Vijay L. Maheshwari
Ravindra H. Patil *Editors*

Natural Products as Enzyme Inhibitors

An Industrial Perspective

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Preface

The history of inventions of enzymes and their applications (enzyme technology) is probably as old as the history of research in modern biochemical sciences. Initially, the term enzyme is coined by Kuhne in 1876 and the commercial uses of it were established after the discovery of uses of enzyme in the removal of stains from cloth in 1913 by Otto Rohm. Enzymes are biological catalysts that serve as a key unit in speeding up all biochemical reactions and are crucial for cellular metabolism in living cells. They are known for their amazing catalytic efficiencies and their high level of specificity for their substrate interactions in all types of biological environments. Therefore, enzymes are currently used in varied applications in many aspects of everyday life including aiding health care, food processing, agriculture, and other industrial applications. However, the kinetic regulation of enzymes is an important criterion when they are used in healthcare, commercial, and industrial processes. The regulation is possible with the help of enzyme-specific inhibitory molecules which may be from living cells (plants, microbes, animals, and algae) or synthetically prepared chemicals.

Enzyme inhibitors are the molecules that can form requisite interaction with the binding site of enzymes to suppress and regulate the rate of reaction of enzymes. These enzyme inhibitors are classified based on their binding potential and mode of action in two types—irreversible enzyme inhibitors and reversible enzyme inhibitors. Currently, the vast majority of enzyme inhibitors are reported to inhibit the various classes of enzymes. These enzyme inhibitors are the focus of the scientific community because they may answer an increasing array of questions in the research area of biological sciences (biochemistry, medicine, physiology, pharmacy, agriculture, ecology) as well as serve as useful tools in the study of enzyme structures and reaction mechanisms and the development of technologies useful in agriculture, food processing, health management, etc. There is tremendous diversity in enzyme inhibitors due to their varying mode of action, target enzymes, and sources (mostly from plants and microbes). While the major constraints found to be associated with the inhibition of enzyme activity are (1) inhibitors have strict enzyme specificity for their interaction with one out of several closely related isoenzymes or enzymes of

different species, (2) some of the enzyme inhibitory molecules form irreversible binding with enzymes and degrade the biochemically important enzymes, (3) some of the inhibitory molecules are associated with their toxicity towards nontarget organisms and mammals, (4) adaptation and advantageous mutations like responses of inhibitor-attached enzymes in living cells, and (5) the degradations of proteinaceous inhibitors due to excessive secretary proteases activities. In light of these facts, it will be really interesting to explore this area and update it. Three major areas of applications of enzymes covered in this book are (1) human health management, (2) agriculture and food processing and research (leading to drug discovery or enzyme activity mechanisms). It contains many interesting articles highlighting the plant-derived inhibitors of serine proteases, pancreatic lipase (PL) inhibitors from indigenous medicinal plants, amylase inhibitors and their applications in agriculture and food processing industries, advances in in silico techniques used in the study of enzyme inhibitors, etc.

Non-small cell lung cancer (NSCLC) is the leading cause of mortality in oncology, and EGFR-TK plays a critical role in this disease. Third-generation drugs (Osimertinib) can overcome the EGFR T790M mutation; a recent C797S mutation makes these agents ineffective against it. To overcome EGFR T790M/C797S resistance, allosteric mutant-selective fourth-generation EGFR inhibitors look to be a promising treatment approach. In *Chap. 1, Ahmad et al.* describe the advantages of blocking allosteric sites in the EGFR-TK receptor domains and compare novel fourth-generation EGFR TKIs for overcoming drug treatment resistance. Protease inhibitors from plants are proteinaceous molecules which form complex with proteases and inhibit their activity. Plants utilize these inhibitors to regulate different physiological processes as well as to protect themselves against insect pests or pathogens. In *Chap. 2, Barbole et al.* survey the different classes of protease inhibitory proteins and peptides from plant origin, with their agronomical and pharmaceutical applications, with emphasis on short peptides. The chapter also highlights various biological and synthetic approaches for production of peptides and methods for improving activity and specificity. *Chapter 3, by Kasar et al.*, systematically reviews different strategies of purification, biochemical characteristics, biological applications of α-AIs, and bottlenecks in commercial utilization of α-AIs. The challenges in the safe marketable utilization of α-AIs are discussed in detail, and alternative approaches and various efficient solutions based on recent advancements in biotechnological research which could be helpful to broaden the scope of α-AIs are also elaborated in detail.

Patil et al. in Chap. 4 highlight the recent developments in the virtual screening of enzyme inhibitors using various docking tools and their significant applications in designing potent inhibitors for the management of various metabolic and infectious diseases. Molecules like acetylcholine, histamine, and gastrin stimulate gastric acid secretions. H^+/K^+-ATPase, also referred to as the proton pump, plays a central role in controlling gastric secretions. The use of synthetic proton pump inhibitors (PPIs) has revolutionized the management of peptic ulcers; nevertheless, there are still various challenges associated with long-term usage that calls for pharmacotherapeutic alternatives. Adinortey and N'guessan present an overview of the structure of H^+/K^+-

ATPase highlighting its central role as one of the most appropriate drug targets necessary for the control of hyperacidity in *Chap. 5*. In addition, the role of plant natural nutraceutical products as inhibitors of H^+/K^+-ATPase is also discussed.

In Chap. 6, Rodrigues Silva et al. highlight an updated overview of advances in protease inhibitors in the field of insect pest control. The chapter also includes some interesting findings on the work carried out in their laboratory particularly on the biochemical mechanisms involved in plant-pest interaction from an enzymatic, proteomic, and molecular biology point of view.

Obesity is increasingly recognized as a global issue, and its prevalence is rising at an alarming rate around the globe. One of the investigated targets for the treatment of obesity is pancreatic lipase (PL) suppression. Orlistat is the only clinically approved drug as a lipase inhibitor and is currently available for long-term obesity treatment. However, various side effects are associated with the long-term usage of Orlistat, which warrants discovery of safe and effective treatment methods for obesity. *Chapter 7 by Patil et al.* thoroughly reviews the importance and PL inhibitory activity of different phytoconstituents from medicinal plants.

Chapter 8 by Shafie et al. presents the most recent data on sources, extraction/identification techniques, and mechanism of action of inhibiting angiotensin-converting enzyme (ACE) inhibitors. The chapter also highlights the commercialization potential and the effectiveness of ACE inhibitors after the oral consumption.

Proteases are the potential targets for the treatment of viral diseases. Since December 2019, the world has observed the emergence of SARS-CoV-2 that resulted in the COVID-19 pandemic and brought the world to a standstill. In silico approaches such as structure and ligand-based virtual screening, docking, and molecular dynamics were extensively used to search for the promising inhibitor of M^{pro} from the existing library of natural molecules. *Chapter 9 by Vadnere and Patil* summarizes the potential inhibitors of M^{pro} from the natural sources such as plant and microorganisms. *Srivastava et al. in Chap. 10* review the recent approaches to telomerase-directed therapy, deliberate the aids, their shortcomings, and speculate on the forthcoming perspective of inhibitors that target telomerase as cancer therapeutics. Modern agriculture practices use of enzyme inhibitors to control pests and to regulate the soil microbial activity. The use of enzyme inhibitors has several drawbacks like sensitivity to temperature, pH, development of resistance and phytotoxicity, etc. Use of nanotechnology in agriculture and human health is well appreciated by the scientific community. *Patil et al. in Chap. 11* highlight the applications of nanomaterial as enzyme inhibitors with its special application as pest control agents and fertilizers additives. Diabetes type 2 has become one of the ten leading causes of death worldwide. Alpha-glucosidase inhibitors are a class of oral medication used in treating diabetes mellitus type 2. However, the side effects of chemical inhibitors of alpha-glucosidase are well known warranting the discovery of new and safer molecules as alpha-glucosidase inhibitors. *Mishra and Bhatnagar in Chap. 12* review the use of plant metabolites as inhibitors of alpha-glucosidase and their potential in the management of type 2 diabetes.

We appreciate the patience and cooperation extended by all the contributors of this book. Thanks are also due to reviewers who took their time to review the

manuscripts in due time. We are also thankful to the entire team of Springer Nature Singapore Pvt. Ltd., Singapore, for giving us this opportunity. We hope that the contents of the book will serve as a useful resource for all those working in the area and will be received with enthusiasm and interest.

Jalgaon, Maharashtra, India — Vijay L. Maheshwari
Shirpur, Maharashtra, India — Ravindra H. Patil
December 2021

Contents

1 **Fourth-Generation Allosteric EGFR Tyrosine Kinase Inhibitors to Combat the Drug Resistance Associated with Non-small Cell Lung Cancer (NSCLC)** 1
Iqrar Ahmad, Rahul Pawara, Asama Pathan, and Harun Patel

2 **Plant Peptides as Protease Inhibitors for Therapeutic and Agricultural Applications** 25
Ranjit S. Barbole, Nidhi Saikhedkar, and Ashok Giri

3 **Bioactive α-Amylase Inhibitors: Sources, Mechanism of Action, Biochemical Characterization, and Applications** 59
Sainath S. Kasar, Vijay L. Maheshwari, and Pankaj K. Pawar

4 **Recent Updates on In Silico Screening of Natural Products as Potential Inhibitors of Enzymes of Biomedical and Pharmaceutical Importance** 105
Mohini Patil, Samadhan Patil, Vijay L. Maheshwari, Laxmikant Zawar, and Ravindra H. Patil

5 **H^+/K^+-ATPase Inhibitors from Plants: A Potential Source for Drug Discovery** 125
Michael Buenor Adinortey and Benoit Banga N'guessan

6 **Use of Protease Inhibitors as a Promising Alternative for Pest Control** 137
Neilier Rodrigues Silva-Junior, Yaremis Beatriz Meriño Cabrera, Rafael de Almeida Barros, and Maria Goreti de Almeida Oliveira

7 **Pancreatic Lipase (PL) Inhibitors from Medicinal Plants and Their Potential Applications in the Management of Obesity** . . . 153
Samadhan Patil, Mohini Patil, Vijay L. Maheshwari, and Ravindra H. Patil

8 Bioactive Peptides and Polysaccharides: Setting a New Trend in Replacing Conventional Angiotensin-Converting Enzyme Inhibitors 169
Muhammad Hakimin Shafie, Pei Gee Yap, and Chee-Yuen Gan

9 Natural Protease Inhibitors and Their Therapeutic Potentials Against SARS-CoV-2 205
Nilesh Chandrabhan Vadnere and Nitinkumar P. Patil

10 Telomerase and its Inhibitor in Cancer Therapeutics: Current Status and Future Prospective 227
Vivek Srivastava, Saleha Siddiqui, Akanksha Dhondiyal, Pakhi Gupta, and Ankush Yadav

11 Metal Nanomaterials as Enzyme Inhibitors and Their Applications in Agriculture and Pharmaceutics 251
Satish V. Patil, Kiran R. Marathe, Hemant P. Borase, and Bhavana V. Mohite

12 α-Glucosidase Inhibitors for Diabetes/Blood Sugar Regulation 269
Aditi Bhatnagar and Abha Mishra

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Chapter 1
Fourth-Generation Allosteric EGFR Tyrosine Kinase Inhibitors to Combat the Drug Resistance Associated with Non-small Cell Lung Cancer (NSCLC)

Iqrar Ahmad, Rahul Pawara, Asama Pathan, and Harun Patel

Abstract Non-small cell lung cancer (NSCLC) is the leading cause of mortality in oncology, and EGFR-TK plays a critical role in this disease. As a result, EGFR-TK is a viable target for therapeutic development in NSCLC. The T790M EGFR TK mutation was resistant to both first-generation and second-generation (selectivity issue) EGFR-TK inhibitors. Although third-generation drugs (Osimertinib) can overcome the EGFR T790M mutation, a recent C797S mutation makes these agents ineffective against it. All of the currently available EGFR kinase inhibitors target the kinase's highly conserved ATP site, underlining the need for therapeutics with a different mechanism of action (allosteric binding). EAI001, EAI045, JBJ-04-125-02, DDC4002 and a series of small compounds (fourth generation) having an affinity for the EGFR allosteric site have been discovered and are currently being investigated. To overcome EGFR T790M/C797S resistance, allosteric mutant-selective fourth-generation EGFR inhibitors look to be a promising treatment approach. This chapter discusses the advantages of blocking allosteric sites in the EGFR-TK receptor domains and compares novel fourth-generation EGFR-TKIs for overcoming drug treatment resistance.

Keywords EGFR · Non-small cell lung cancer · T790M/C797S · Allosteric inhibitors

Abbreviations

Ba/F3 cell	A murine Interleukin-3-dependent Pro-B cell line
C797S	Cystein797 to Serine790
EGFR	Epidermal growth factor receptor
EGFR-TKI	Epidermal growth factor receptor tyrosine kinase inhibitors

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PROTAC	Proteolysis-targeting chimera
PROTACs	Proteolysis-targeting chimeric molecules
RTKs	Receptor tyrosine kinases
T790M-C797S	Threonine 790 methionine-cysteine 797 serine
TKIs	Tyrosine kinase inhibitors
WT EGFR	Wild-type epidermal growth factor receptor

1.1 Introduction

Lung cancer continues to be one of the leading causes of cancer-related death, owing to the ineffectiveness of conventional chemotherapeutics (Sharma et al. 2007). Based on cellular morphology, lung cancer is further divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). According to the American Cancer Society, 85% of lung cancer patients have NSCLC, and 15% have SCLC (Fig. 1.1) (Molina et al. 2008; https://www.cancer.org/cancer/lung-cancer.html). Adenocarcinoma (AC) (40%), squamous cell carcinoma (SQCC) (25%), and large cell carcinoma (LCLC) (15%) are the three sub-types of NSCLC (Molina et al. 2008).

The involvement of the epidermal growth factor receptor (EGFR) in NSCLC is well recognized, and significant therapeutic progress has been made in the treatment of this disease in the last decade (Maulik et al. 2003; Wee and Wang 2017; Patel et al. 2018; Ahmad et al. 2020). The USFDA has approved gefitinib and erlotinib, two small molecule EGFR inhibitors, for the treatment of NSCLC. As first-generation, ATP-competitive, and reversible EGFR inhibitors, gefitinib and erlotinib were found to be efficacious in NSCLC patients with somatic EGFR mutations L858R and delE746 A750, which account for 90% of all EGFR mutations in NSCLC (Vansteenkiste 2004; Kobayashi et al. 2005a, b; Li et al. 2008; Kawahara et al. 2010; Pao et al. 2005; Patel et al. 2019). Unfortunately, the ability of these EGFR inhibitors to effectively treat NSCLC patients is to 10–12 months due to acquired secondary mutations such as the T790M mutation in EGFR, which leads to drug resistance in around half of NSCLC patients (Yun et al. 2008; Murakami et al. 2012). The EGFR T790M mutation recovers EGFR's affinity for ATP similar to wild-type (WT) EGFR and inhibits reversible inhibitors from binding at higher ATP concentrations (Singh et al. 2010; Ramalingam et al. 2012; Song et al. 2016).

The second-generation EGFR inhibitors, such as Afatinib and Dacomitinib, irreversibly inhibit the EGFR-TK by covalently binding with Cys797 residue. These inhibitors have effectively inhibited the activating (Exon 19 deletion or L858R) mutation and T790M mutation (Kobayashi et al. 2005a, b; Balak et al. 2006; Kosaka et al. 2006; Patel et al. 2017, 2020). Because of their low selectivity between the WT EGFR and the T790M EGFR, these inhibitors caused skin rash and diarrhoea due to WT EGFR inhibition (Michalczyk et al. 2008).

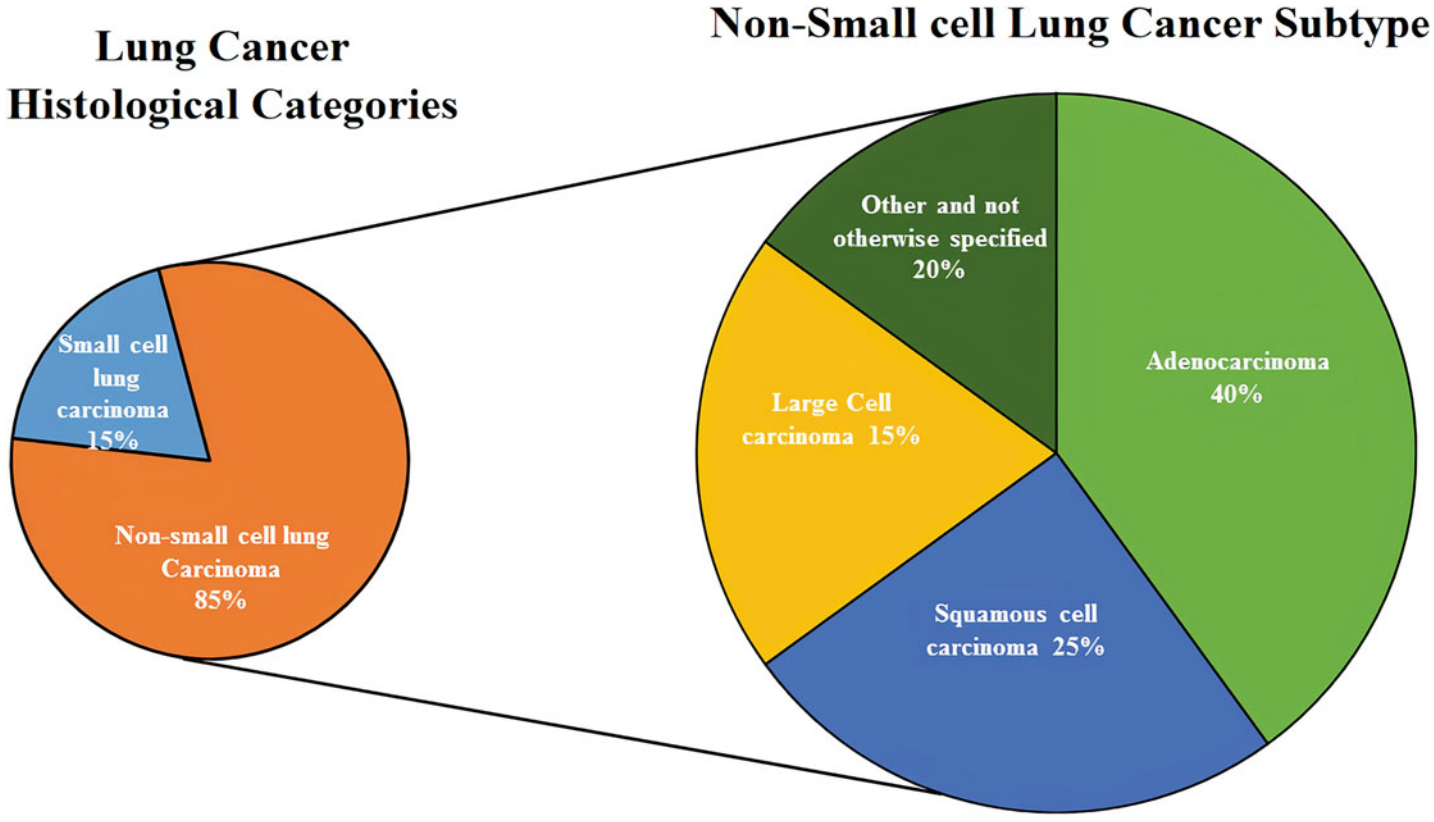


Fig. 1.1 Types of lung cancer

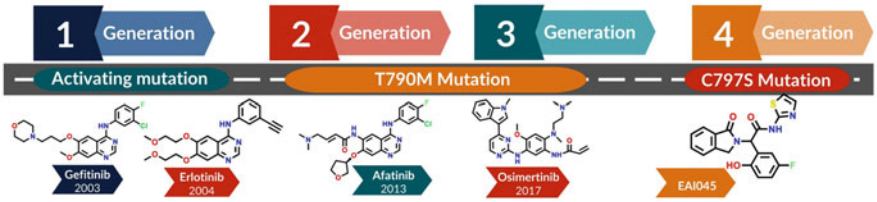


Fig. 1.2 First-, second-, third-, and fourth-generation EGFR-TK inhibitors

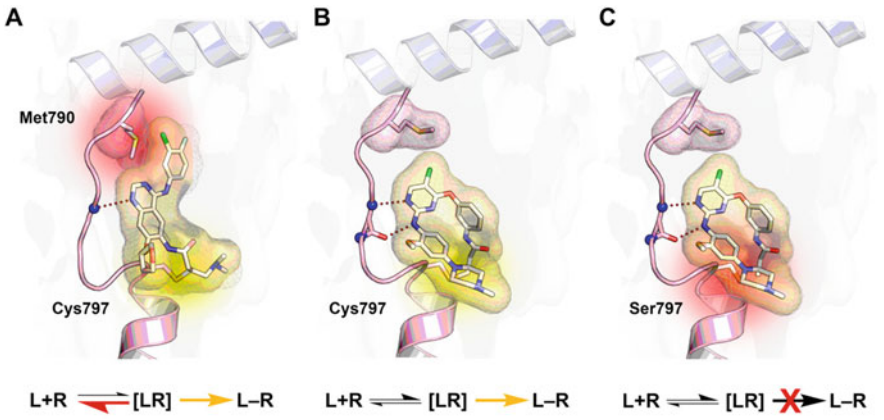


Fig. 1.3 (**a**, **b**) Covalent binding of second- and third-generation EGFR inhibitors with C797 residue of T790M EGFR-TK; (**c**) C797S resistance to the third-generation EGFR inhibitors, rendering them ineffective. (Reprinted (adapted) with permission from ref. Engel et al. 2015. Copyright 2016, American Chemical Society)

Several irreversible third-generation EGFR inhibitors with mutant selectivity have been developed to alleviate the toxicity induced by inhibition of WT EGFR (second-generation EGFR inhibitors), such as WZ4002 (Zhou et al. 2009), Rociletinib (Walter et al. 2013), Osimertinib (Finlay et al. 2014), and Olmutinib (Lee et al. 2014) (Fig. 1.2). Of these inhibitors, AstraZeneca's Osimertinib (Tagrisso™) got FDA approval in 2017 for the treatment of NSCLC patients with the T790M EGFR-TK mutation (AstraZeneca (n.d.); US FDA (n.d.); Ahmad et al. 2020). Despite its good clinical efficacy in treating EGFR mutant NSCLC patients, osimertinib resistance (tertiary mutation C797S) develops after 14–20 months of treatment (Fig. 1.3) (Thress et al. 2015; Grabe et al. 2018; Nie et al. 2018; Du et al. 2021; Patel et al. 2021a, b). However, osimertinib's selectivity for mutant kinases has not yielded the intended results.

In vivo, Osimertinib's binding to non-target EGFR receptors has resulted in serious side effects such as diarrhoea, rash, nausea, decreased appetite, hyperglycaemia, prolongation of the corrected QT interval, pneumonia (Jänne et al. 2015), and Vortex keratopathy (Chia and John 2015), all of which severely

limit Osimertinib's clinical application. As a result, a more effective mutant-selective EGFR L858R/T790M inhibitor that targets both the sensitizing and resistant T790M mutations while sparing the wild type of the receptor is desperately needed.

All currently available EGFR kinase inhibitors target the kinase's highly conserved ATP site, emphasizing the need for inhibitors with a different mechanism of action (Wan et al. 2019). The allosteric site's low sequence homology offers a unique potential for more targeted inhibition with less off-target pharmacology (Wan et al. 2019). Other benefits of allosteric inhibitors over traditional ATP-competitive inhibitors include the ability to overcome mutation-associated drug resistance, particularly mutations in the ATP-binding site, which confer resistance to almost all related ATP-competitive inhibitors (Zhang et al. 2009). Furthermore, allosteric inhibitors may not need to have nanomolar affinity to compete with high intracellular ATP concentrations, making it easier to identify weak binding inhibitors to overcome the drug resistance problem (Engel et al. 2015; Abe et al. 2011). The T790M and C797S mutations do not affect the efficiency of the allosteric inhibitors as the allosteric site is far away from the EGFR ATP-binding site (Tripathi and Biswal 2021). The purpose of this chapter is to provide a complete summary of fourth-generation allosteric EGFR inhibitors and their recent development (Fig. 1.3).

1.2 EGFR Tyrosine Kinase Allosteric Binding Site

For the treatment of NSCLC, EGFR targeting therapeutics are widely accepted as the treatment of choice. When a ligand binds with TK, the receptor undergoes conformational changes, which leads to autophosphorylation of the complex, which commences the further signal transduction cascade (Zang et al. 2017; Maity et al. 2020).

The activation of the cell proliferation cycle in NSCLC is triggered by this cascade, which activates the RAS/RAF/MAPK pathway, which is critical for cell survival regulation and cell proliferation. The cascade triggers the PI3K/AKT/mTOR pathway, which inhibits apoptosis and promotes malignant cell development (Zang et al. 2017; Maity et al. 2020).

The N-lobe, C-lobe, ATP site, hinge region, and allosteric site are the five key sections of the kinase structure (Fig. 1.4). The amino acid hand of N-lobe is short, with five anti-parallel β-sheets and one helix. The ATP-binding site, which is a highly reactive region of the kinase domain, connects the N-lobe to the C-lobe (Zhao et al. 2018; Li and Guo 2021). Binding of the ligand to the N-lobe results in an extension of the α-helix hand. The ligand interaction causes conformational changes in the kinase structure, allowing the ATP site to be activated for autophosphorylation. The C-lobe shrinks towards the N-lobe and the kinase receptor is activated once phosphorylation begins (Mitchell et al. 2018). Due to continual autophosphorylation, the ATP site is a highly reactive and unstable site. Epidermal growth factor (EGF), transforming growth factor (TGF-α), tumour necrosis factor

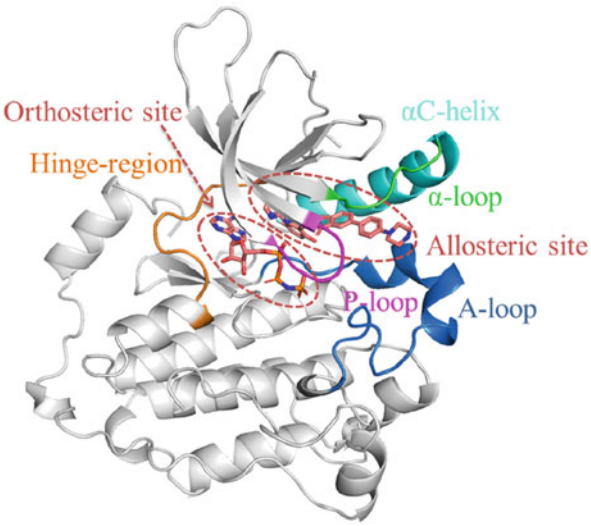


Fig. 1.4 Allosteric binding site of the EGFR Tyrosine Kinase. (Reprinted (adapted) with permission from Li and Guo 2021. Copyright 2021, American Chemical Society)

(TNF), epiregulin, and amphiregulin are some of the major ligands that interact with TK and cause dimerization of the TK complex (Maity et al. 2020).

There are three binding sites on a TK receptor: an ATP-competitive site, an inactive site, and an allosteric site. The majority of available drugs target the ATP-binding site, whereas no drugs bind at the inactive site (Gao et al. 2016; Santarpia et al. 2017; Zhou et al. 2018; Maity et al. 2020; Patel et al. 2021a, b). Targeting the allosteric region results in a stable complex formation with protein, which changes both the effectiveness and binding of the primary ligand. This renders the protein less active to the ligand-complex formation or exhibits the neutral functionality during ligand interaction (Maity et al. 2020). When allosteric site inhibitors bind to the ATP site but not the hinge region, kinase activity is blocked without ATP being displaced. Furthermore, autophosphorylation is blocked, and the conformational change in the kinase domain is also inhibited, making it easier to achieve equilibrium (Purba et al. 2017). As a result, allosteric sites can inhibit or stop the signal induction cascade completely. Thus, inhibiting the allosteric site can help to overcome the problem of "undruggable" resistance (C797S) with minimal adverse effects (Fig. 1.5) (Maity et al. 2020).

1.3 Allosteric Inhibitors

Jia et al. (2016) reported EAI001 (1), a new EGFR allosteric inhibitor hit molecule with a thiazole amide structure, which was identified using high-throughput screening (HTS) from a library of 2.5 million compounds with a specific selectivity for mutant EGFR. **EAI001** (**1**) had an IC_{50} of 24 nm against the L858R/T790M mutant

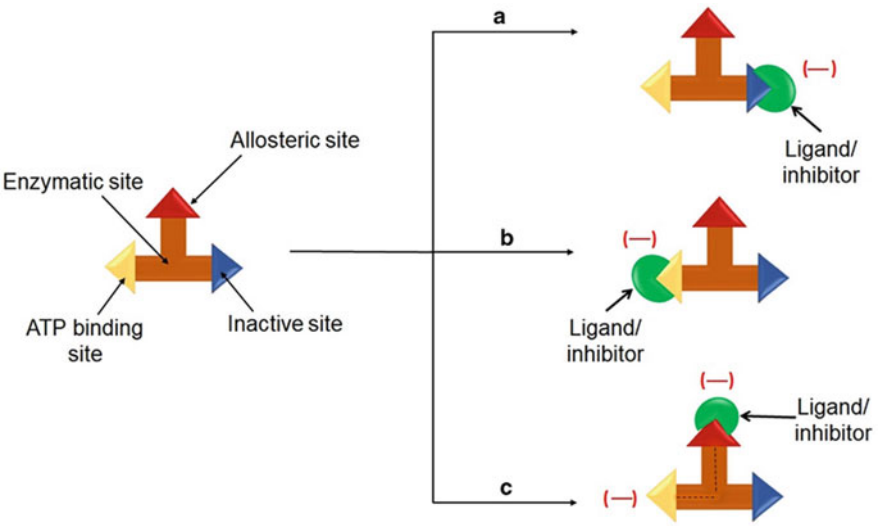


Fig. 1.5 Binding mechanism of the allosteric inhibitors. (Reprinted (adapted) with permission from Maity et al. 2020 under Creative Commons CC BY 4.0. Copyright 2020, Springer)

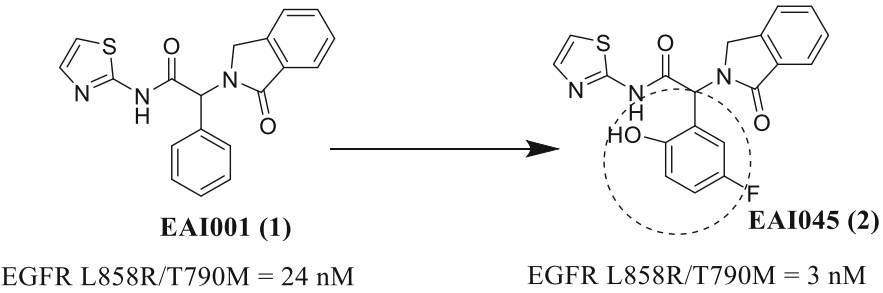


Fig. 1.6 Structural optimization of **EAI045** (**1**) from **EAI001**(**2**)

EGFR kinase, whereas the IC_{50} value for the WT EGFR was more than 50 μM (Fig. 1.6). According to X-ray crystal structure (PDB Id: 5D41), EAI001 binds to the allosteric region of EGFRT790M generated by displacement of the C-helix in the active conformation. EAI001 aminothiazole group makes direct interaction with gatekeeper Met790, while the carboxamide's NH group serves as a hydrogen bond donor with Asp-Phe-Gly (DFG) motif residue Asp855. The direct interaction with the gatekeeper residue and the inability to bind the inactive conformation of WT EGFR explain the selectivity for T790M EGFR. The 1-oxoisoindolinyl reaches to the solvent accessible area, while the phenyl group enters hydrophobic pocket formed by Met766, Leu777, and Phe856 (Fig. 1.7).

Further medicinal chemistry optimization of EAI001 (**1**) at phenyl ring provided a more potent analogue EAI045 that has proven to exhibit high mutant inhibitory

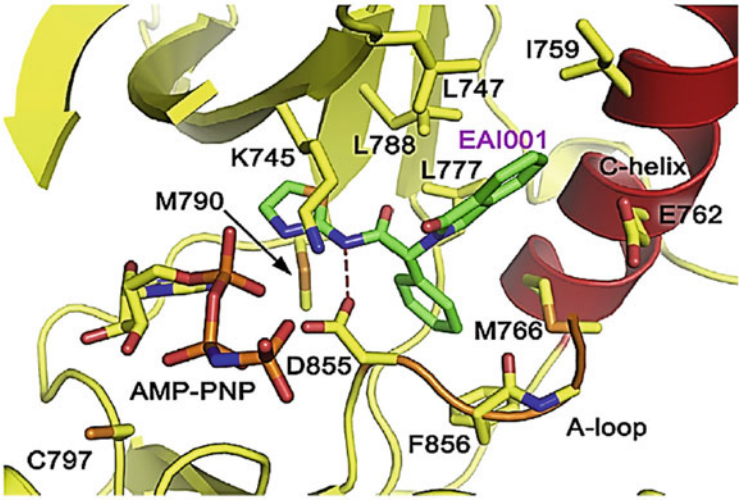


Fig. 1.7 Binding approach of **EAI001 (1)** in EGFR T790M/V948R kinase (PDB code 5D41). (Reprinted (adapted) with permission from Zhao et al. 2018. Copyright 2018, Elsevier)

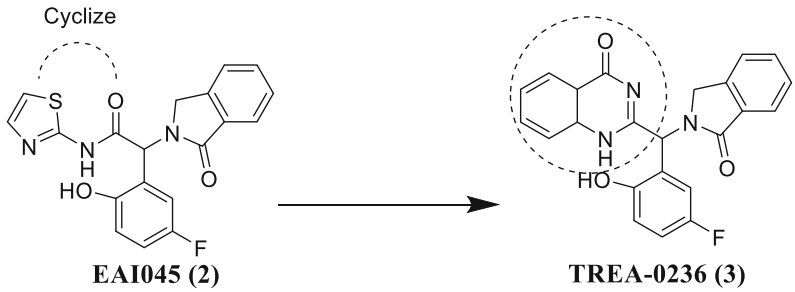


Fig. 1.8 Development of non-peptidic analogue of **EAI001 (1)**

potency (EGFR L858R/T790M IC_{50} = 3 nM) and 1000-fold selectivity (WT EGFR IC_{50} = 4.3 μM). On the other hand, EAI045 partially inhibited EGFR phosphorylation in cells and demonstrated weak antiproliferative activity in L858R/T790M mutant cells. The combination of EAI045 with EGFR dimer-disrupting antibody Cetuximab can stymie EGFR dimerization and cause significant tumour antiproliferative response in both genetically engineered mice carrying the L858R/T790M EGFR mutation and the L858R/T790M/C797S EGFR mutation. This is the first inhibitor reported that can overcome EGFR T790M and EGFR C797S mutations. However, human clinical trials are now required to validate EAI045's clinical efficacy.

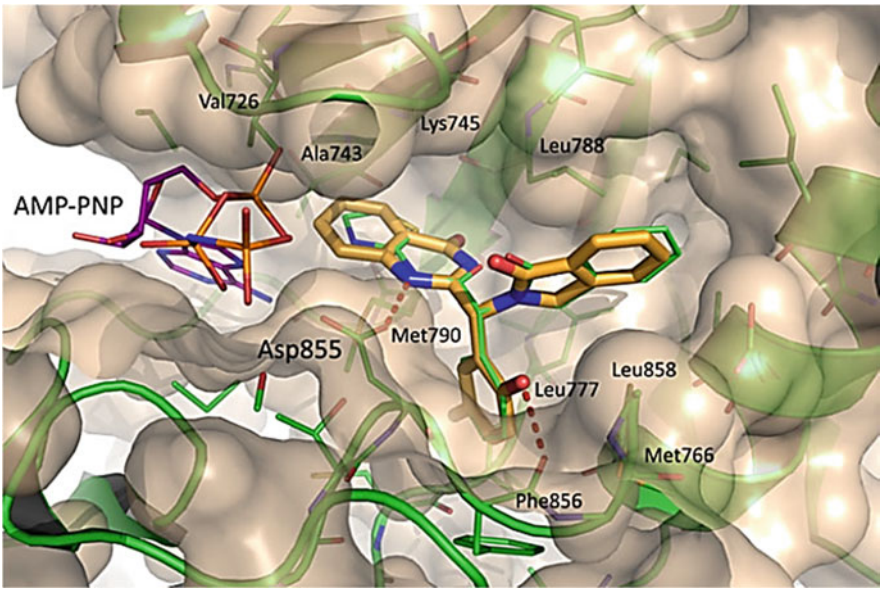
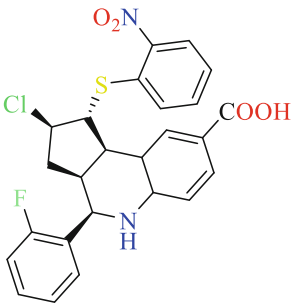


Fig. 1.9 Binding mode of **TREA-0236** (**3**) (*thick yellow sticks*) and **EAI001** (**1**) (*thin green lines*). (Reprinted (adapted) with permission from Lee et al. 2018. Copyright 2018, Korean Chemical Society)

Lee et al. (2018) synthesized TREA-0236 (3), a new EGFR allosteric inhibitor by substituting non-hydrolysable quinazoline-4-one for aminothiazole on EAI045 (Fig. 1.8). EAI045 has a di-peptide core with an aminothiazole at the *N*-terminus; peptides are metabolically unstable, yielding 2-aminothiazole metabolites; these metabolites found promiscuous hitting scaffold, causing methemoglobinemia toxicity; the reactive intermediate formation leads to extensive covalent protein binding, as seen in high-throughput screening (HTS).

To avoid haematological toxicities, they modified the structure of EAI045 (**2**) by cyclization, converting 2-aminothiazole amide to quinazoline-4-one for improved safety and pharmacokinetics. However, TREA-0236 (**3**) (EGFR L858R/T790M/C797S IC_{50} = 5.3 μM) was less potent in contrast to parent EAI045 (EGFR L858R/T790M/C797S IC_{50} = 4 nM) (Fig. 1.8). Further molecular modelling studies revealed that TREA-0236 (**3**) anticipated binding pose is nearly similar to EAI001 (**1**) crystal structure, which is close to the ATP-binding site. Their structure-activity relationship study suggests that the 5-fluoro-2-hydroxylphenyl substitution on the quinazoline-4-one scaffold is required for the EGFR L858R/T790M/C797S mutant activity and when it is replaced with alkyl group the activity of the compound is considerably diminished or even completely inactivated (Fig. 1.9).

Carlino et al. (2018) synthesized and reported SAR of hexahydrocyclopenta[*c*] quinoline derivatives as allosteric inhibitors of cyclin-dependent kinase-2 (CDK2) and EGFR (Fig. 1.10). Among the synthesized compound, **4** was a selective



Compound 4

WT EGFR (10μm)= 0% inhibition
WT EGFR (50μm)= 23% inhibition
L858R/T790M EGFR (10μm)= 28% inhibition
L858R/T790M EGFR (50μm)= 70% inhibition

Fig. 1.10 Chemical structure and reported as percent inhibition at 10 μm and 50 μm of compound **4**

allosteric inhibitor of the double mutant L858R/T790M EGFR with respect to wild-type EGFR/CDK2. Dose–response curves revealed that **4** was able to inhibit the T790M/L858R EGFR with an EC_{50} of 44 ± 4 μM. Compound **4** was further evaluated in the presence of a higher concentration of ATP (1 mM) to demonstrate its allosteric activity on the T790M/L858R EGFR.

The dose–response curves clearly showed that compound **4** retained its inhibitory profile, indicating that it had an allosteric mechanism of action. Induced-fit docking of the compound **4** to the T790M/L858R EGFR-KD crystal structure (PDB ID: 3W2R) revealed that the compound binds to the allosteric pocket by fitting the 2-phenyl-substituted ring into the deeper hydrophobic pocket lined by Leu788, Met766, Met790, and Phe856. The carboxylate group of the hexahydrocyclopenta [*c*]-quinoline forms a salt bridge with the polar heads of Arg858 and Arg748. Particularly, the interaction of compound **4** carboxylate with the primary mutated Arg858 residue may help to explain the compounds' selectivity for the double mutant EGFR over the wild-type EGFR. Molecular dynamics simulations of compound **4** within the T790M/L858R EGFR-KD revealed that the proposed binding mode was stable and that the key interactions within the allosteric pocket were remained throughout the 100 ns simulation time.

Their SAR study suggested that all the compounds in which the carboxylate group was removed or modified were inactive, when tested on the T790M/L858R protein. It was also observed that the various substituents of the 2-phenyl ring, which is suited to the deeper hydrophobic pocket lined by the side chains of Phe856, Leu788, Met766, Met790, Phe723, and Lys745 amino acid residues, played a crucial role in modulating the selectivity for the double mutant EGFR (Fig. 1.11).

Caporuscio et al. (2018) reported a low-cost high-throughput docking protocol for the fast identification of EGFR allosteric inhibitors using the crystal structure of the EGFR in complex with an allosteric inhibitor (PDB code 5D41). Commercially available drugs were employed for virtual screening by high-throughput docking

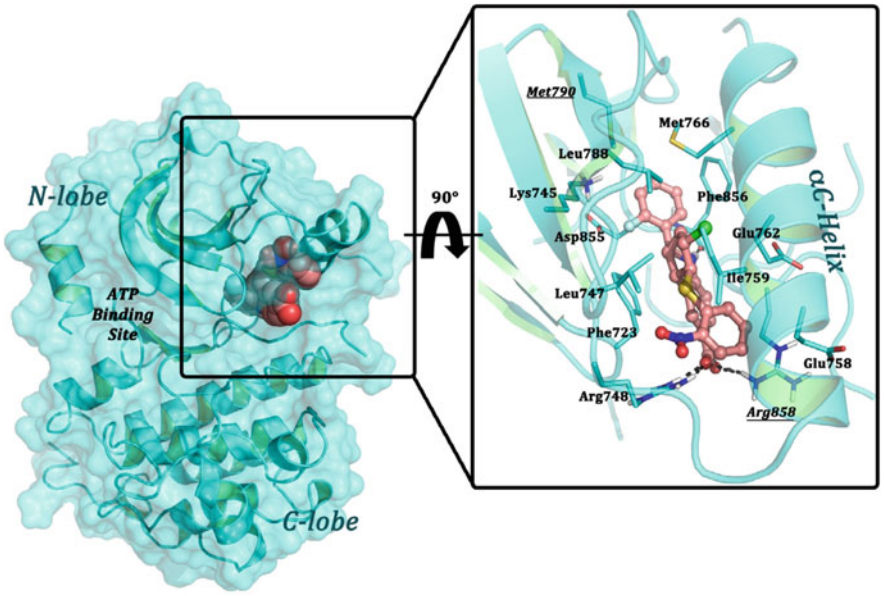
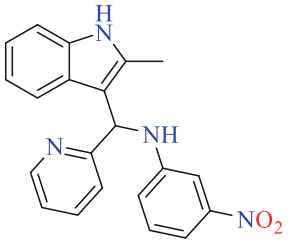


Fig. 1.11 Induced-fit docking binding approach of compound **4** within the cavity of L858R/T790M EGFR structure (PDB ID: 3W2R). (Reprinted (adapted) with permission from Carlino et al. 2018. Copyright 2018, Wiley-VCH Verlag GmbH and Co.)

Fig. 1.12 EGFR inhibition profile of compound **5**



Compound 5

WT EGFR (10μm)=70% inhibition

WT EGFR (50μm)=91% inhibition

L858R/T790M EGFR (10μm)=71% inhibition

L858R/T790M EGFR (50μm)=90% inhibition

using the structures of wild-type and T790M/L858R EGFRs, yielding 92 compounds for biological evaluation. In at least two fixed concentration tests with the same or different protein structures (WT, T790M, T790M/L858R), 9 compounds were discovered whose percentage of inhibition was higher than or about 50% at 50 μM and higher than or about 20% at 10 μM.

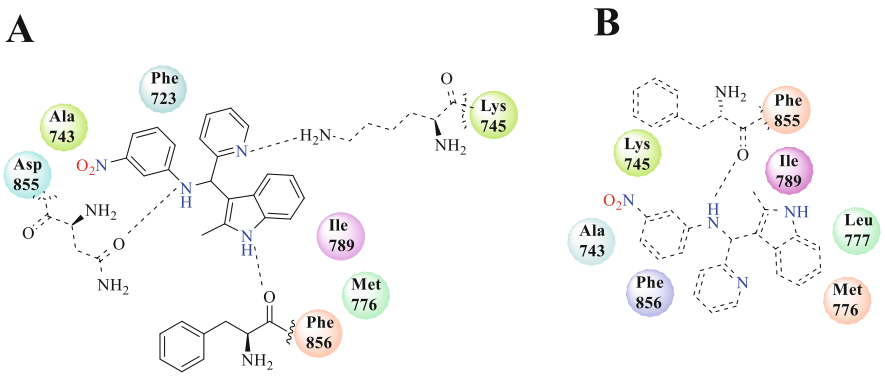


Fig. 1.13 Putative binding modes of compound **5**; (**a**) Binding mode of the (R)-enantiomer of compound **5**; (**b**) Binding mode of the (S)-enantiomer of compound **5**

The dose–response curve of promising compound **5** demonstrated that the compound was able to bind the WT EGFR and T790M/L858R double mutant EGFR with EC_{50} values of 4.7 μM ± 0.7 μM and 6.2 μM ± 0.5 μM, respectively (Fig. 1.12). Importantly, dose–response curve was unaffected by changing concentration of ATP (1 μM and 1000 μM), indicating that the compound inhibited the T790M/L858R EGFR with an allosteric mechanism. Moreover, compound **5** was able to inhibit the growth of non-small cell lung cancer cell lines (H1299, H1650, and H1975) with micromolar potency. Binding mode analysis revealed that compound **5** (R)-enantiomer's pyridine nitrogen forms H-bond with the Lys745 side chain, NH of indole forms hydrogen bonds with the Phe856 carbonyl group, and NH of nitrophenylaniline forms H-bond with the Asp855 carboxyl group. Polar moiety of compound **5** established good van der Waals interaction with Phe723, Ala743, the alkyl chain of Lys745, Leu747, Met766, Leu777, Leu788, Ile789, Met790, and Phe856. The (S)-enantiomer shows a hydrogen bond contact with the carboxyl group of Asp855 through its nitrophenylaniline NH and contacts with the Phe723, Val 726, Ala743, the alkyl chain of Lys745, Leu747, Met766, Leu777, Leu788, Ile789, Met790, and Phe856 (Fig. 1.13).

Surprisingly, the indole and pyridine rings are inverted in relation to the (R)-enantiomer's pose. According to the putative binding modes both enantiomers can form favourable interactions within the allosteric region.

To et al. (2019) and colleagues developed the JBJ-02-112-05 (**6**) mutant-selective allosteric EGFR inhibitor by linking 5-indole substituent on isoindolinone moiety of EAI001 (**1**).

For the EGFR L858R/T790M mutant variant, the compound JBJ-02-112-05 (**6**) exhibited an IC_{50} of 15 nM. Further medicinal chemistry optimization of JBJ-02-112-05 (**6**) by incorporating 2-hydroxy-5-fluorophenyl group of EAI045 (**1**) and phenyl-piperazine on isoindolinone substituents delivers a more potent EGFR allosteric inhibitor, JBJ-04-125-02 (**7**), with an IC_{50} of 0.26 nM for T790M/L858R mutant EGFR (Fig. 1.14). JBJ-04-125-02 (**7**) shown anticancer effect on L858R,

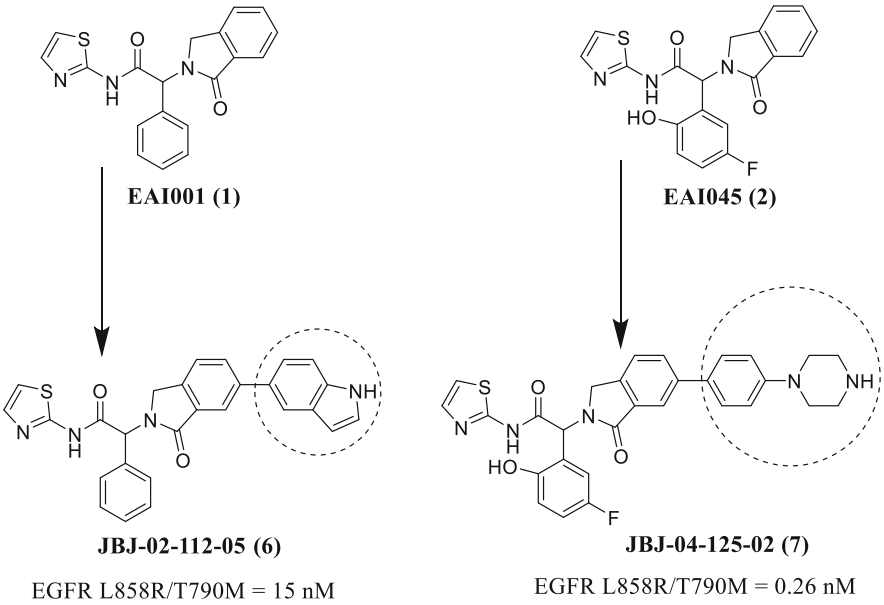


Fig. 1.14 Molecular structures of **JBJ-02-112-05 (6)** and **JBJ-04-125-02 (7)**, functionalized from **EAI001** (**1**) and **EAI054** (**2**)

L858R/T790M, or L858R/T790M/C797S mutations EGFR variant without co-administration of Cetuximab. JBJ-04-125-02 (**5**) has also no affinity for WT EGFR or the Ex19del mutant. Further investigation revealed that osimertinib may increase **JBJ-04-125-02** (**7**) binding affinity to EGFR, resulting in more potent cytotoxic activity. These results suggest that combining a covalent mutant-selective allosteric EGFR-TKI with a third-generation EGFR-TKI could be a useful treatment approach for some lung cancer patients who are resistant to third-generation EGFR-TKIs. On the other hand, both **EAI045** (**2**) and **JBJ-04-125-02** (**7**) are unable to overcome the resistance mediated by the triple mutant EGFR del19/T790M/C797S.

According to crystal structure (PDB code: 6DUK) **JBJ-04-125-02** (**7**) also binds to the allosteric pockets in the C-helix-out conformation of EGFR, like EAI045. **JBJ-04-125-02** (**7**) binding generated a unique A-loop conformation, which appears to be stabilized by a hydrogen bond between the compound's piperazine group and Glu865 in the A-loop (Fig. 1.15).

Dries and colleagues reported a second series of allosteric mutant-selective EGFR inhibitors. They previously reported the discovery of **EAI001** (**1**) through selective screening of mutant L858R/T790M EGFR inhibitors (Fig. 1.16) (De Clercq et al. 2019). The same screen also yielded EAI002 (**8**), which was composed of a 5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4]diazepin-11-one scaffold that selectively inhibited L858R/T790M with a biochemical IC_{50} of 52 nM compared to >1000 nM for WT. Subtle fluorine shift optimization of EAI002 (**8**) resulted in DDC4002 (**9**),

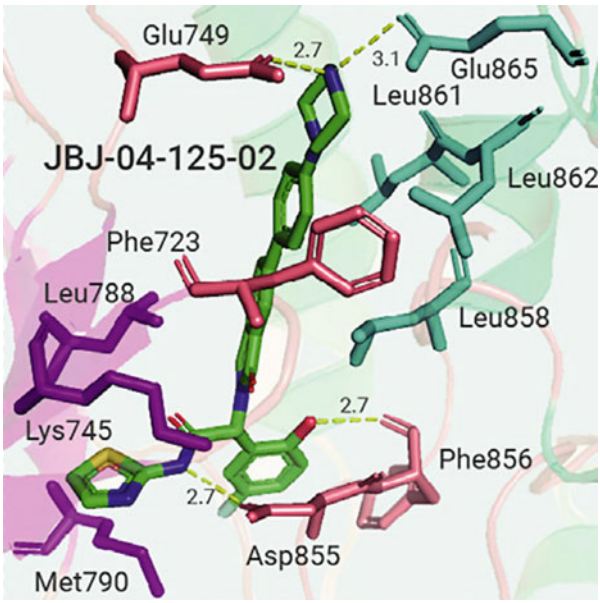


Fig. 1.15 Binding approach of **JBJ-04-125-02** (**7**). (Reprinted (adapted) with permission from Du et al. 2021 under Creative Commons CC BY 4.0. Copyright 2021, Elsevier)

which had mutant-selective nanomolar biochemical IC_{50} values towards the mutant L858R/T790M and L858R/T790M/C797S EGFR variant compared to WT EGFR.

C-2-functionalized EAI045 (**2**) and JBJ-04-125-02 (**7**) led to an increase in biochemical potency; inspired by this observation, the 4-(piperazinyl)-phenyl substituent was linked at C-2 to increase potency (**10**). Furthermore, the SAR study revealed that increasing the flexibility of C-2 site via the Ullmann biaryl ether linkage moderately improved the effectiveness of these inhibitors (**11** and **12**). They discovered that the biochemical potencies of the three compounds (**10–12**) obtained for L858R/T790M and L858R/T790M/C797S were equivalent to EAI045. Compound **12** exhibited the highest potency against EGFR L858R/T790M/C797S in a biochemical assay ($IC_{50} = 13$ nM) and significant antiproliferative action when co-administered with Cetuximab.

Binding analysis showed that the DDC4002 (**9**) diazo ring bends inward towards the C-helix, whereas the 8-fluorobenzene ring interacts in the hydrophobic pocket and the unsubstituted benzene ring accompanies the C-helix outward towards the solvent. A hydrogen bond is observed in between the diazepine NH and the DFG motif residue Asp856 backbone carbonyl. The benzyl substitution propagates to the *N*-lobe, which is wedged between AMP-PNP and the residues L788, K745, and T790M (Fig. 1.17).

Hoffmann La Roche disclosed a series of oxo-indole compounds as potent and selective allosteric EGFR inhibitors in the WO2020002487A1 patent (Duplessis et al. 2020). Among the synthesized compound, **13** and **14** were very effective inhibitors of the EGFR L858R/T790M/C797S kinase, with IC_{50} values of 5 nM

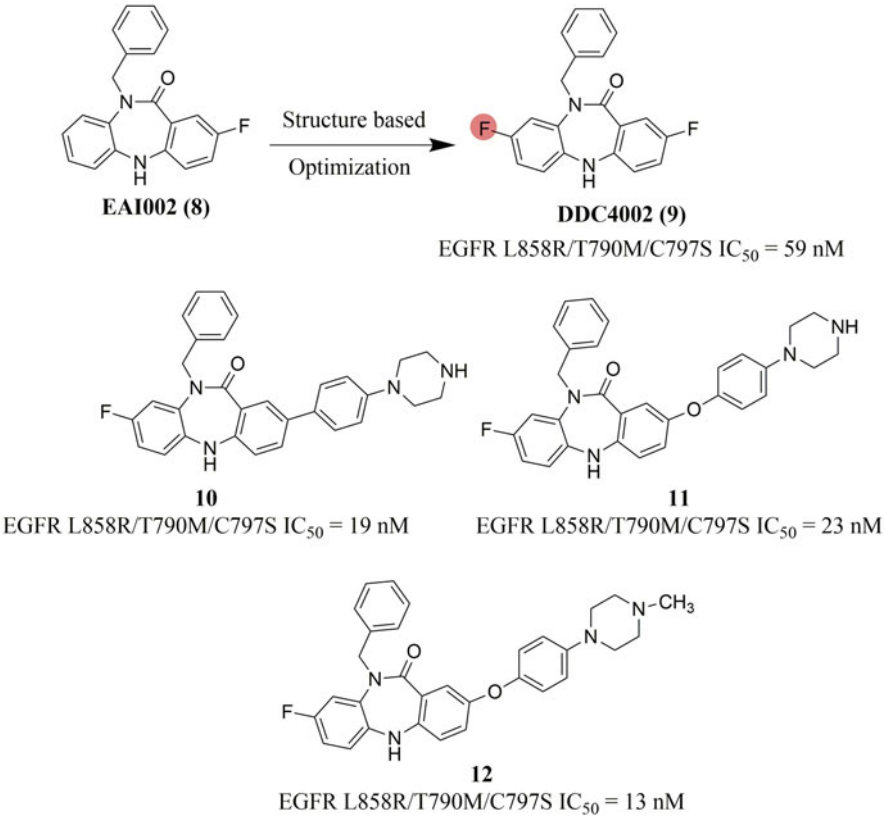


Fig. 1.16 Structure-based optimization of **EAI002 (8)** afforded **DDC4002 (9)** and chemical structure of C-2 functionalized compounds (**10–12**)

and 4 nM, respectively, against the Ba/F3 cell line harbouring EGFR L858R/T790M/C797S mutation (Fig. 1.18).

Jang et al. (2020) discovered mutant-selective allosteric EGFR degraders that are effective against a wide range of drug-resistant mutations. They used EAI001 (**1**) (allosteric inhibitor) as a starting point for their design of a degrader because the binding approach of the first allosteric inhibitor, EAI001 (**1**), revealed that it is in a deep allosteric pocket. They prepared the JBJ-07-149 (**15**) by introducing 1-(pyridin-2-yl) piperazine at the 6-position of isoindolinone, which resulted in the expansion of EAI001 (**1**) to a solvent-exposed area. In vitro catalytic activity of JBJ07–149 in EGFR L858R/T790M found that it inhibited the enzyme with an IC_{50} of 1.1 nM. JBJ07–149 (**15**) had a significant antiproliferative activity in the presence of Cetuximab ($EC_{50} = 0.148$ μM) but was unsuccessful as a single agent in proliferation tests ($EC_{50} = 4.9$ μM). They also developed a bifunctional degrader molecule, DDC-01-163 (**16**), by modifying JBJ-07-149 (**15**) and coupling it to the CRBN ligand pomalidomide via a linker conjugated to the piperazine moiety of JBJ-07-149

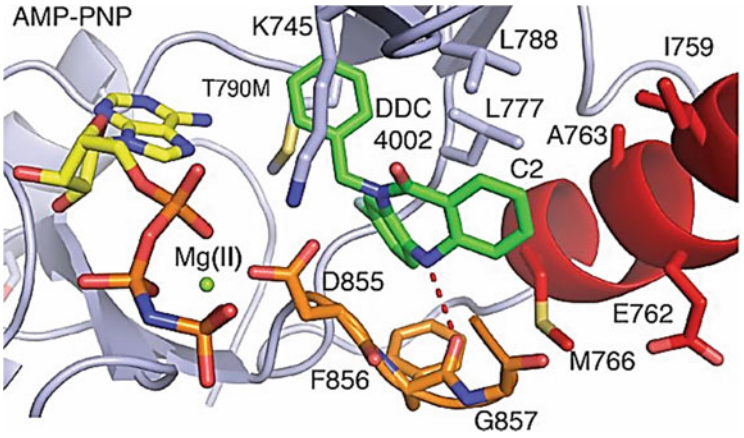
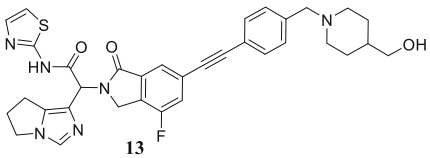
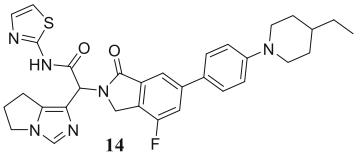


Fig. 1.17 Binding of **DDC4002 (9)** at the allosteric pocket. (Reprinted (adapted) with permission from De Clercq et al. 2019. Copyright 2019, American Chemical Society)



13

EGFR L858R/T790M/C797S IC_{50} = 3 nM



14

EGFR L858R/T790M/C797S IC_{50} = 4 nM

Fig. 1.18 Chemical structure and biological activity of **13** and **14**

(**15**) and orientated towards the solvent-exposed exterior (Fig. 1.19). DDC-01-163 (**16**) demonstrated reasonable potency in an in vitro biochemical experiment against EGFR L858R/T790M, with an IC_{50} value of 45 nM, comparable to its parent allosteric EGFR inhibitor JBJ-07-149 (**15**) (IC_{50} = 1.1 nM). DDC-01-163 (**16**) was further examined for its ability to inhibit mutant cell proliferation in Ba/F3 cell lines that had been stably transfected with either the EGFR L858R/T790M mutant or the wild type of EGFR. DDC-01-163 (**16**), which is 51-fold more powerful than its parent allosteric EGFR inhibitor (0.096 μM vs. 4.9 μM), substantially inhibited the growth of L858R/T790M mutant EGFR Ba/F3 cells (EC_{50} = 0.096 μM) while preserving wild-type EGFR Ba/F3 cells (EC_{50} > 10 μM). They performed competitive in vitro tests in which they treated L858R/T790M Ba/F3 cells with the CRBN ligand pomalidomide or the parent allosteric EGFR inhibitor JBJ-07-149 (**15**) in the presence or absence of 0.1 μM DDC-01-163 (**16**) to further establish the mechanism of action of DDC-01-163 (**16**).

Densitometry examination of western blotting revealed a significant reduction of EGFR protein to 44% in cells treated with DDC-01-163 (**16**) alone. Furthermore, even in the presence of DDC-01-163 (**16**), pomalidomide and JBJ-07-149 (**15**) inhibited the EGFR degradation, suggesting that EGFR degradation needed binding

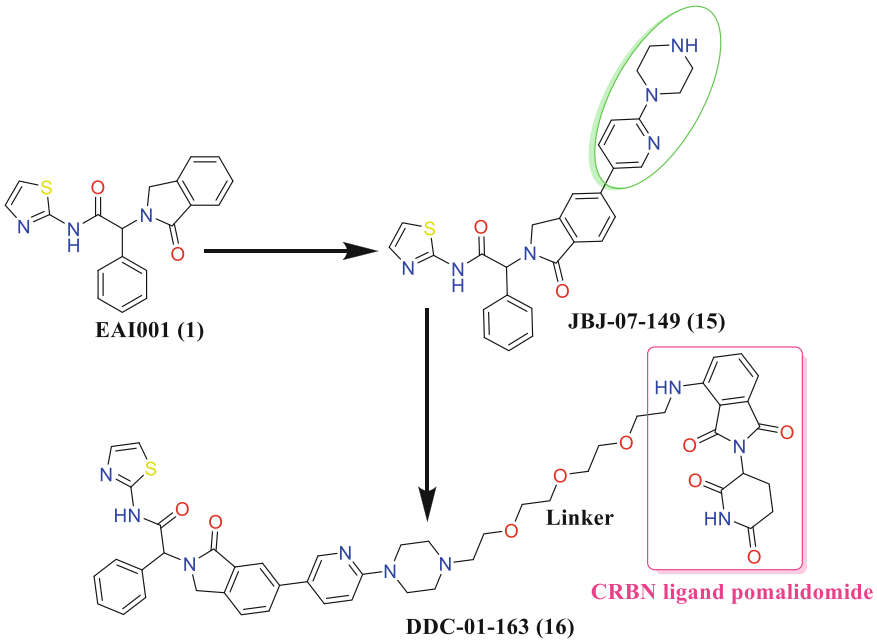


Fig. 1.19 Structure functionalization of EAI001 (**1**) yielded EGFR degrader DDC-01-163 (**16**)

to both EGFR and CRBN and confirmed that DDC-01-163 (**16**) antiproliferative action was substantially dependent on EGFR degradation. The researchers also showed that combining DDC-01-163 (**16**) with osimertinib dramatically shifted the dose–response curve, suggesting that the combination treatment was more effective at reducing cell proliferation than the single-agent treatment. DDC-01-163 (**16**) is a potential allosteric EGFR degrader with selective efficacy against several therapeutically relevant EGFR mutations whether used alone or in conjunction with an ATP site inhibitor.

Patel et al. (2021a, b) reported virtual screening of zinc compound library to search allosteric T790M/C797S EGFR inhibitor. From complete sequential filter of molecular docking, Lipinski's Rule of Five, ADMET prediction, and molecular dynamic simulation (MD) study, they found most promising compound ZINC20531199 (**17**).

Molecular docking study showed that ZINC20531199 binds to the Asp855 amino acid of the DFG motif via the amidal hydrogen of the ligands, which (DFG motif) is critical for regulating kinase activity in T790M/C797S EGFR. In MD study ZINC20531199 (**17**) exhibited the favourable interaction with the Asp855, Phe856, Met790, Leu754, and Lys745. These compounds demonstrated H-bond interactions with the Asp855 and Lys745 residues, which contributed to the ligand's binding affinity. The π–π stacking has been observed between Phe856 and the ZINC20531199 (**17**) phenyl ring, while Met790 and Leu747 interacted with

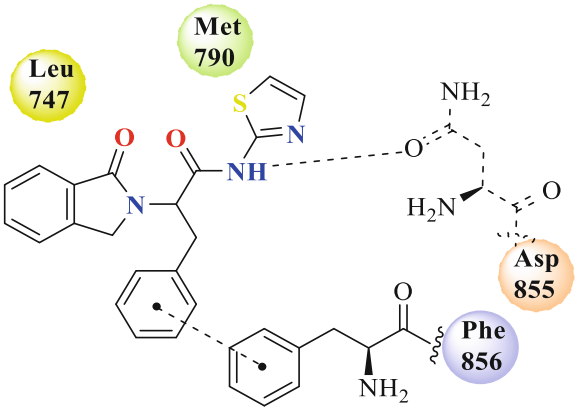


Fig. 1.20 Chemical structure and binding mode of virtual screened allosteric compound ZINC20531199 (**17**)

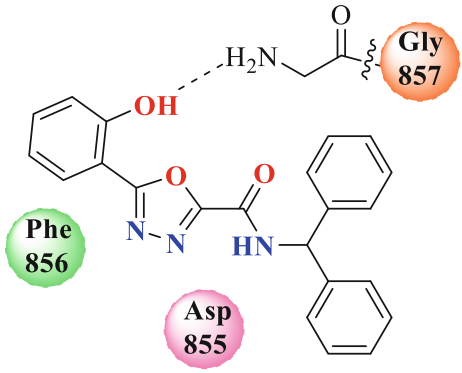


Fig. 1.21 Chemical structure and binding mode of virtual screened allosteric compound **18**

hydrophobic interaction via thiazole ring and isoindoline ring of the ZINC20531199 (**17**). These critical residual interactions with ZINC20531199 (**17**) assisted in the development of its EGFR T790M/C797S allosteric inhibitor (Fig. 1.20).

Karnik et al. (2021) developed EGFR inhibitors that bind allosterically to the C797S mutant EGFR enzyme using a database, library screening, R-group enumeration, and scaffold hopping. They obtained the **18** as promising compound after the virtual screening.

The results of the above experiment showed that the protein-ligand complex was stable for 10 ns in the allosteric pocket of the C797S EGFR tyrosine kinase. The key interactions observed during each simulation frame were Asp 855, Phe 856, and Gly 857, which were found to be stable throughout the simulation process. Other interactions were Leu777, Lys745, and Met 790, which were inconsistent during simulation. Compound **18**, along with Asp855 and other amino acid residues, formed significant hydrogen bonding interactions, which was critical for increasing inhibitory activities (Fig. 1.21).

1.4 Conclusion and Future Perspectives

Resistance to EGFR-TKIs (first to third generation) indicates the need for better tyrosine kinase inhibitors that can stop the activation of the mutant versions of EGFR (T790M and C797S) (Tripathi and Biswal 2021). For treating EGFR mutant NSCLC, targeting the allosteric region of EGFR offers a very promising and selective new treatment approach. Two allosteric EGFR mutant-selective drugs have been recently developed by the researchers [EAI001 (**1**) and EAI045 (**2**)] (Jia et al. 2016). These allosteric inhibitors can overcome the T790M mutation by decreasing the autophosphorylation of the T790M mutant EGFR. However, these allosteric inhibitors are ineffective individually and found to be effective when given in combination with cetuximab (dimerization inhibitors) and exhibited significant antiproliferative activity (Jia et al. 2016; Tripathi and Biswal 2021).

JBJ-04-125-02 (**7**), an allosteric EGFR inhibitor, demonstrated strong antiproliferative and downstream AKT and ERK phosphorylation suppressing effects in Ba/F3 cell lines harbouring L858R, L858R/T790M, or L858R/T790M/C797S mutations, and it has spared the wild-type EGFR Ba/F3 cells, indicating its selectivity towards the mutant form (Lee et al. 2018; Tripathi and Biswal 2021). In comparison to osimertinib, **JBJ-04-125-02** (**7**) demonstrated more significant antiproliferative and tumour regression efficacy at nanomolar range in H1975 cells (L858R/T790M mutant) and tumour xenografts, respectively (To et al. 2019; Tripathi and Biswal 2021). In addition, combining osimertinib with **JBJ-04-125-02** (**7**) slows the emergence of mutant EGFR-mediated resistance in NSCLC (Tripathi and Biswal 2021; To et al. 2019). In an osimertinib-resistant lung cancer cell line (H3255GR), **JBJ-04-125-02** (**7**) (1–10 μM) greatly improves the apoptotic potency and effectiveness of osimertinib (To et al. 2019; Tripathi and Biswal 2021).

DDC-01-163 (**16**), a potential allosteric EGFR degrader that was recently discovered, has shown preferential antiproliferative action ($IC_{50} = 0.096$ μM) against L858R/T790M mutant Ba/F3 cells over wild-type Ba/F3 cells ($IC_{50} > 10$ μM) (Jang et al. 2020). **DDC-01-163** (**16**) ($IC_{50} = 0.096$ μM) was shown to be 51-fold more powerful in cellular testing than its parental allosteric EGFR inhibitor **JBJ-07-149** (**15**) ($IC_{50} > 4.9$ μM), although having 45-fold lower potency in biochemical studies. **DDC-01-163** (**16**) was effective in Ba/F3 cell lines with L858R/T790M/C797S ($IC_{50} = 0.041$ μM) and L858R/T790M/L718Q ($IC_{50} = 0.028$ μM). The same study found that treating L858R/T790M/C797S and L858R/T790M/L718Q EGFR mutant cells with **DDC-01-163** (**16**) (0.1 M) resulted in 74% and 71% degradation, respectively, in L858R/T790M/C797S and L858R/T790M/L718Q EGFR mutant cells (Jang et al. 2020; Tripathi and Biswal 2021). The **DDC-01-163** (**16**) (0.01 μM and 0.1 μM) synergistically improves the antiproliferative and apoptotic efficacy of osimertinib (0.01 μM) (Jang et al. 2020; Tripathi and Biswal 2021). These all results indicate that allosteric inhibitors were more effective when used in combination with other chemotherapeutic agents. Still further research is needed to find out the allosteric inhibitors, which could individually inhibit the T790M/C797S EGFR-TK mutations.

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Conflicts of Interests

There are no conflicting financial interests declared by the authors.

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Chapter 2
Plant Peptides as Protease Inhibitors for Therapeutic and Agricultural Applications

Ranjit S. Barbole, Nidhi Saikhedkar, and Ashok Giri

Abstract Protease inhibitors from plants are proteinaceous molecules which form complex with proteases and inhibit their activity. Plants utilize these inhibitors to regulate different physiological processes as well as to protect themselves against insect pests or pathogens. Plant protease inhibitors have proven to be novel candidates for pest control and also used as drugs targeting proteases associated with diseases. In this book chapter we survey the different classes of protease inhibitory proteins and peptides from plant origin, with their agronomical and pharmaceutical applications, with emphasis on short peptides. We also review the various biological and synthetic approaches for production of peptides, and methods for improving activity and specificity. The overall objective of this book chapter is to enlighten the newly discovered classes of small peptides which can inhibit proteases and can act as a drug delivery vehicle.

Keywords Protease · Protease inhibitors · Peptides · Engineering protease inhibitors

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2.1 Introduction

Plants are a source of numerous phytochemicals (specialized metabolites), which have been used for therapeutic purposes in the traditional and modern medicine (Anand et al. 2019; Carqueijeiro et al. 2020). In addition to their role in plant protection against biotic and abiotic stress, phytochemicals show bioactivity against several clinically important targets (Joshi et al. 2014; Retzl et al. 2020). Phytochemicals are interesting drug leads because of advantages like low toxicity, specificity, and "metabolite-likeness." In contrast to non-natural drugs, phytochemicals can be easily delivered to intracellular targets by the host transport system. Also, phytochemicals serve as natural scaffolds for the production of several drugs. Prominent examples are alkaloids such as vincristine, vinblastine (from *Catharanthus roseus*), and paclitaxel (*Taxus brevifolia*) and lignins such as etoposide and teniposide (*Podophyllum hexandrum*), which are used to treat different types of cancer (Veeresham 2012). Also, the neuroregulatory effects of caffeine and theanine from tea plant are acknowledged all over the world (Owen et al. 2008). Further, phytochemicals have been used as antibacterial, antifungal, antiviral, and antiparasitic molecules.

Unlike the specialized metabolites, which are low molecular weight compounds, phytopeptides are a comparatively less explored class of phytochemicals that are potential lead molecules for drug development (Jacobowitz and Weng 2020). Phytopeptides (2–50 amino acids) play crucial roles in plant physiology such as regulation of plant metabolism, response to abiotic and biotic stress, and defense and signaling during development. Signaling peptides like CLE family (clavata3/embryo surrounding region) are post-translationally modified peptides of 13 amino acids that function in the maintenance of root, shoot, and floral meristems, lateral root emergence, and vascular development (Kiyohara and Sawa 2012; Yamaguchi et al. 2016). GLV (root growth factor/CLE-like/golven) peptides are 10–20 amino acid peptides involved in the cell elongation in growing tissues during root development (Fernandez et al. 2013).

Phytopeptides play protective roles during pathogen infections. For example, stable antimicrobial peptides (SAMPs) from Australian finger lime (*Citrus australasica*) protect the citrus plants from the Citrus Huanglongbing disease, which is a devastating bacterial infection by inhibiting the causative bacteria *Candidatus Liberibacter asiaticus* and activating the host immunity to prevent infection (Huang et al. 2021a, b). Likewise, disulfated pentapeptide phytosulfokine from tomato acts as an immunity regulatory signal to counteract necrotrophic fungi *Botrytis cinerea* (Zhang et al. 2018). A class of plant-based antifungal peptides called defensins (e.g., Rs-AFP2, MsDef, Wasabi defensin) protect against important pathogenic fungi like *Candida buinensis and Fusarium graminearum*. Defensins possess unique disulfide stabilized structure and have been engineered to produce smaller peptides of 15–30 amino acids with efficacy against the fungal diseases (Lacerda et al. 2014).

Several phytopeptides function as protease inhibitors (PIs), which are crucial in modulating the function of endogenous proteases, protection against biotic and abiotic stresses, and storage molecules. PIs are important and well-characterized defense proteins produced by the plants to combat insect attack. Phytopeptides function directly as PIs or play indirect roles as signaling molecules to induce the production of PIs. For example, systemin, an 18-amino acid signaling peptide isolated from tomato leaves, is an integral component of the jasmonic acid signaling pathway, which leads to the synthesis of a series of defense components (Pearce 2011). PIs are found abundantly in the reproductive, storage, and vegetative tissues and are present in different cell organelles such as cytoplasmic matrix, vacuole, cell wall, and nucleus depending on the type of PI. The amount and type of PIs found in a plant differ with respect to the plant variety, developmental stage, tissue type and location, time of harvest, as well as stress factors. Overall, PIs from plants have been classified based on their specificity to different types of proteases, namely cysteine, serine, aspartic and metallo-protease (Birk 2003) (Fig. 2.1).

In addition to defense, PIs are also involved in physiological processes such as growth and development of the plant. PIs are reported to fulfill carbon, nitrogen, and sulfur requirements during the seed germination. For example, Pin-II PIs from *Solanum americanum* are reported to be involved in the development of phloem, sieve tube, and flowers (Xu et al. 2002). Similarly, the serine PI Serpin1 and cysteine PI WSCP (water-soluble chlorophyll-binding protein) show a fine-tuned expression in *Arabidopsis thaliana* and differentially regulate cysteine protease RD21 during plant development (Rustgi et al. 2017). Some PIs are constitutively expressed, whereas others are induced upon wounding or infestation. Several signaling chemicals such as systemins, jasmonic acid, salicylic acid, and abscisic acid regulate the PI levels in response to infection or insect attack. Green and Ryan (1972) first discovered a rapid accumulation of PIs in the leaves of tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) attacked by Colorado potato beetles (Green and Ryan 1972). These PIs were later characterized as the Potato-type inhibitor (Pin-I and -II) family of serine protease inhibitors. These PIs protect the plant from insect infestation by blocking the digestive proteases of the insect pests. For example, co-expression of *Solanum tuberosum* potato type I inhibitor (StPin1A) and *Nicotiana alata* potato type II inhibitor (NaPI) in cotton gave protection against two insect pests, namely *Helicoverpa punctigera* and *H. armigera* (Dunse et al. 2010). Similarly, cysteine PIs (cystatin) from wild diploid potato *Solanum pinnatisectum* helped in the cultivated potato resistance against infestation by the pest *Spodoptera litura* (Zhu et al. 2019b).

In addition to the well-documented roles of large proteins as plant PIs, small peptides from plants also play an important role as protease inhibitors. For example, SFTI-1 (Sunflower trypsin inhibitor-1) is the smallest known naturally occurring trypsin inhibitor found in sunflower which plays a role in plant defense (de Veer et al. 2021). Similarly, many plants produce small peptides called cyclotides that possess a unique cysteine backbone and are known to function as potent inhibitor of proteases (Weidmann and Craik 2016). The majority of the PI peptides are

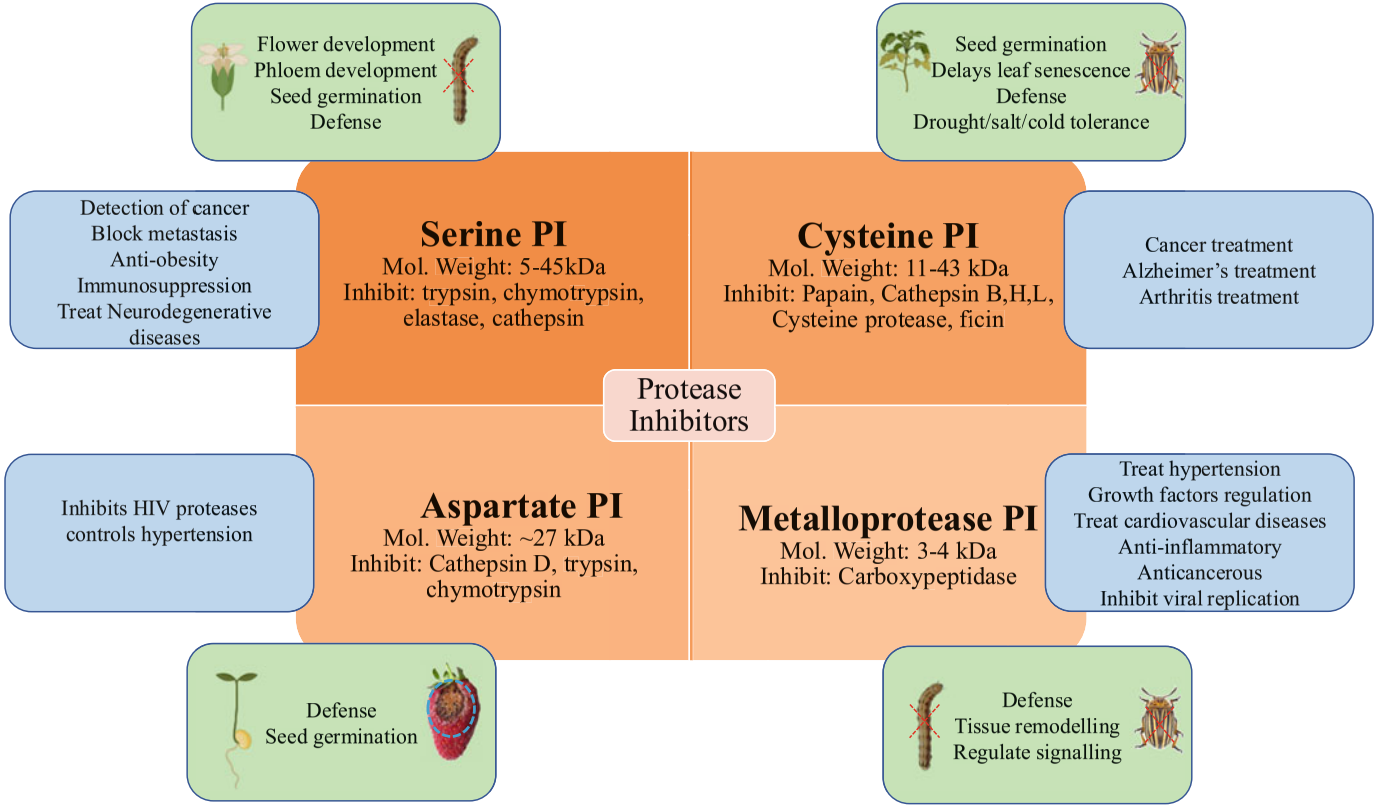

Fig. 2.1 Classification of protease inhibitors. Role of PIs in *in planta* and pharmaceutical applications

characterized by the presence of disulfide stabilized structure (Joshi et al. 2013) which provides excellent thermal and proteolytic stability. Moreover, elucidation of the structure of plant PIs has revealed signature sequences that are essential for the inhibitory activity and stability of PIs (Barrette-Ng et al. 2003). Using the signature sequences, researchers can derive functional smaller peptides based on large PI proteins for pharmaceutical applications.

With the rapid discovery of phytopeptides and their underlying synthesis mechanisms, scientists are unraveling a hidden treasure of molecules for novel applications. Recently these peptides are included in plant specialized metabolites since several small (di, tri, tetra, penta, and up to XX) peptides are being identified with important functions in plants (Jacobowitz and Weng 2020). In this chapter, we discuss the various types of phytopeptides which function as PIs and their use in agriculture and therapeutics. We also describe reports of peptide engineering based on PIs, and the challenges for their higher production, downstream processing, and characterization for the desired applications.

2.2 Plant Protease Inhibitors: Defensive Function

Plants possess several physical and molecular mechanisms to protect themselves against biotic threats such as microbes and insects. PIs play an important role in the plant defense by inactivating the proteases of the attacker during infection or infestation. For example, PIs competitively bind to the catalytic site of the insect proteases and block the proteolysis of food proteins ingested. This causes reduction in the amino acid assimilation in the insect gut which delays their growth and development, leading to reduced fecundity and survival (Mishra et al. 2010). PIs from different plants have been extensively studied at biochemical level, in vitro insect feeding assays and transgenic plants, for their role in insect control strategies.

In addition, PIs have several applications as therapeutics since precise regulation of proteases is essential for several physiological processes like digestion, apoptosis, blood clotting, blood pressure, etc. Owing to their unique structure and target specificity, PIs have been explored as drugs to deal with cancer, cardiovascular diseases, osteoporosis, inflammatory diseases, neurological disorders, and obesity. For example, sunflower trypsin inhibitor-1 (SFTI-1), a 14 amino acid peptide, can block the tumorigenic and metastatic pathways by blocking the Human kallikrein-related peptidase 4 (Swedberg et al. 2009). Also, Pin-II type inhibitors from potato have been used as anti-obesity molecules because they lower the food intake in humans by elevating the level of cholecystokinin (satiety hormone) through a luminal trypsin inhibitory mechanism (Peters et al. 2011). In the following subsections, we describe the structure, function, and applications of various types of plant PIs classified on the basis of their specificity to different types of proteases, namely serine, cysteine, aspartic and metallo-protease (Fig. 2.1).

2.2.1 Serine PIs

Serine PIs are predominant in the plant kingdom. Plant serine PIs are one of the main anti-nutritional components that interfere with the digestive system of herbivorous insects, limiting their growth and development. Insects produce extracellular serine proteases in their digestive tract (mainly in the midgut) to derive nutrients from the ingested plant tissues. Thus, plant serine PIs inhibit these digestive proteases, blocking the assimilation of nutrients by the insects. Serine PIs are further classified into several types on the basis of their mass, cysteine content, structural features, etc. Here, we present a brief overview of these types, namely, Kunitz type, Bowman-Birk, potato type I and II, squash inhibitors, cereal inhibitors, and mustard trypsin inhibitors.

2.2.2 Kunitz-Type Serine PIs: Diverse and Sometimes Can be Multifunctional

Kunitz-type serine PIs are named after M. Kunitz, who first isolated soyabean trypsin inhibitor in 1945 (Kunitz 1945). This type of inhibitors is predominantly found in the leguminous seeds of Fabaceae (Mimosoideae, Caesalpinioideae, and Papilionoideae) plants. The structure of most Kunitz PIs consists of a single polypeptide chain of approximately 20 kDa folded in β-trefoil manner, single reactive loop, and two disulfide bonds stabilizing the structure. The reactive loop protrudes from the protein scaffold and interacts with the active site residues of serine proteases (Oliva et al. 2011). Several studies have shown that one of the disulfide bridges surrounding the reactive loop plays an important role in the interaction of these inhibitors with their target protease(s). Kunitz PIs are known to function in plant defense as well as organ development. Recent evidence suggests that Kunitz-type PIs from *Arabidopsis thaliana* regulate protease activity during flower development. Also, these PIs from *A. thaliana* conferred resistance to spider mites by exhibiting dual inhibition of serine and cysteine proteases upon transient overexpression in *Nicotiana benthamiana* (Arnaiz et al. 2018). Also, isoforms of Kunitz-type PIs from tea plant *Camellia sinensis* show resistance to insect stress as well as pathogen infection (Zhu et al. 2019a).

2.2.3 Bowman-Birk-Type Serine PI: Smaller but Strong

Bowman-Birk PIs (BBIs) are named after D. E. Bowman and Y. Birk, who first characterized a member of this type from soybean (Birk 1985). BBIs are found primarily in seeds of legumes and in cereal grains. These proteins have a molecular mass of 8–10 kDa and contain antiparallel β-sheet core separated by a reactive loop

in each of the domains, making them multifunctional inhibitors. The reactive loop of these PIs consists of highly conserved nine-amino acids motif CTP1SXPPXC (where P1 is the inhibitory active site). BBIs in dicots are double-headed serine protease inhibitors with two functional active sites on opposite sides of the molecule, which interact simultaneously with two proteases and can inhibit trypsin as well as chymotrypsin-like proteases (Grosse-Holz and van der Hoorn 2016). The smallest known bicyclic PI, SFTI-1, also belongs to BBI family. BBIs are involved in regulation of endogenous proteases in plants, as well as proteases present in microbes and insects. *Acacia senegal* BBIs have shown growth inhibitory effects on the *H. armigera* larvae (Babu and Subrahmanyam 2010). Rice plants overexpressing BBI showed resistance towards the fungal pathogen *Magnaporthe grisea*, a causative agent for rice blast (Coca et al. 2006). Being double-headed and bifunctional inhibitors, a scaffold of this family of PIs can be used to generate versatile and potent PIs. BBIs have been extensively studied for drug development to treat many diseases, such as cancer (Gitlin-Domagalska et al. 2020).

2.2.4 Potato Type I and II Serine PIs: Inducible and Multidomain

The PIs of this type are overexpressed upon wounding and infestation and are therefore important plant defense molecules. These are present mainly in Solanaceae family, but few members have been discovered from other plant families. Potato type I (Pin-I) inhibitors follow a systemic route of transmission. These inhibitors are monomeric proteins of ~8 kDa and do not contain disulfide bridges. Pin-I type PIs are exemplified by an 8 kDa chymotrypsin inhibitor from *Amaranthus hypochondriacus* which is effective against the *Prostephanus truncates* insect (Valdes-Rodriguez et al. 1993; Hejgaard et al. 1994).

Wound-inducible potato type II (Pin-II) PIs are multidomain PIs. Initially, these PIs were found only in the Solanaceae family; however extensive studies using expressed sequence tag and genomic databases led to the identification of the Pin-II family in mono- and dicotyledonous plants (Barta et al. 2002). Pin-II type PIs are secreted inhibitors as they contain 25 amino acids, an endoplasmic reticulum signal peptide followed by one or more inhibitor domains. These domains, called the inhibitory repeat domain (IRD), are connected by proteolytically susceptible linker peptides of five amino acids. Mature IRDs are 50–55 amino acids long proteins composed of a core of three antiparallel β-sheets stabilized by disulfide bonds and a flexible loop containing the reactive site. The reactive site is three amino acid loop, called reactive center loop (RCL), which is crucial for protease binding (Saikhedkar et al. 2018; Tamhane et al. 2012) (Fig. 2.2a). Pin-II PIs undergo proteolysis at linker peptide, leading to separation of IRDs into individual inhibitory units (Mishra et al. 2010). Pin-I and Pin-II type PIs show potent inhibitory activity against insect proteases and are used as insect control agents. Transgenic tomato plants

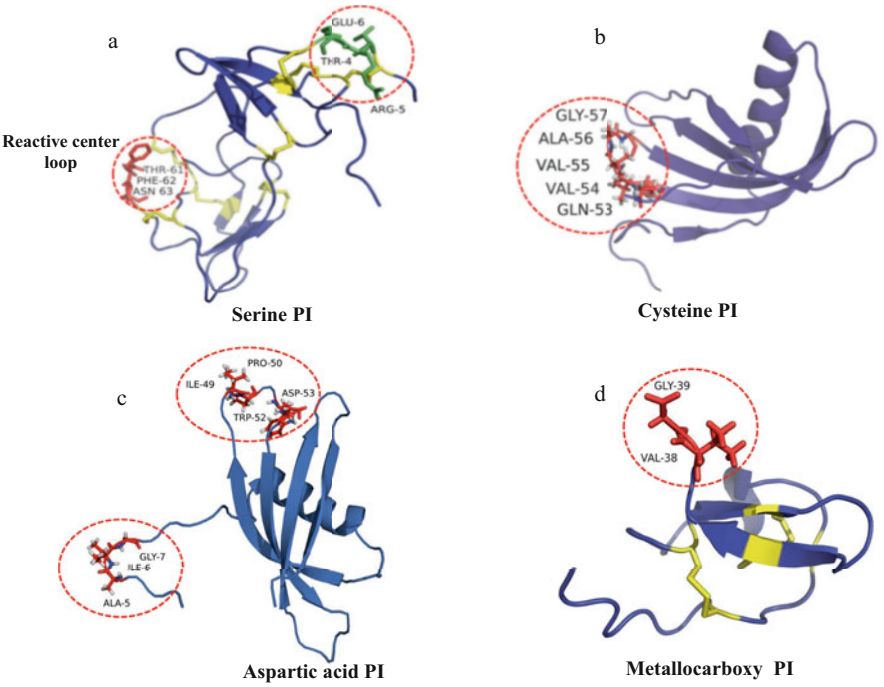

Fig. 2.2 Types of plant protease inhibitors. Representative examples are shown for (**a**) Serine PI—Pin-II type inhibitor from *Solanum lycopersicum*/tomato (PDB ID: 1OYV), (**b**) Cysteine PI—Oryzacystatin from *Oryza sativa*/rice (PDB ID: 1EQK), (**c**) Aspartate PI—Squash Aspartic acid PI from *Cucurbita maxima*/pumpkin (PDB ID: 2KXG), (**d**) Metallocarboxy PI from *Solanum tuberosum* (PDB ID: 1H20). All proteins are shown as *ribbon* representation with disulfide bonds as sticks in *yellow*. Inhibitory loops are shown as sticks and *circled*. (All images are prepared by PyMol software)

overexpressing the Pin-II type PI (CanPI-7) from *Capsicum annuum* showed improved resistance against the pest *Helicoverpa armigera* (Tanpure et al. 2017). The IRD and RCL of Pin-II type PIs are attractive lead molecules for designing proteins to be used in pharmaceutical and agricultural purposes (Saikhedkar et al. 2018, 2019; Meriño-Cabrera et al. 2020).

2.2.5 *Cereal-Type Serine PI: Poaceae Specific and Bifunctional*

Cereal-type serine protease inhibitors are mostly reported and characterized from rice (*Oryza sativa*) and other cereal crops including wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rye (*Secale cereale*), maize (*Zea mays*), ragi (*Eleusine coracana*), etc. (Clemente et al. 2019). Cereal-type serine PIs consist of a single

polypeptide chain with a molecular mass of about ~13 kDa containing five disulfide bonds (John and William 2005). The barley trypsin inhibitor (BTI-CMe) found in barley endosperm shows high specificity towards trypsin-like proteases from *Spodoptera frugiperda*, whereas it was found to be inactive against porcine and human trypsins (Altpeter et al. 1999). Transgenic wheat lines expressing trypsin inhibitor BTI-CMe (*Itr1*) gene showed improved resistance against grain moth *Sitotroga cerealella* (Altpeter et al. 1999). In addition, *BTI-CMe* expressing transgenic indica and japonica rice showed increased mortality to rice weevil (*Sitophilus oryzae*) insects (Alfonso-Rubí et al. 2003).

Several PIs from this type show bifunctional activity. These PIs range from 10 to 15 kDa with several cysteine spanning loops and α-helical segments. They are mainly reported from the endosperm of starch-containing crop plants, such as barley, amaranth, wheat, and maize (Svensson et al. 2004). Bifunctional trypsin/α-amylase inhibitor from pigeonpea (*Cajanus cajan*) showed a negative effect on the growth rate of *Helicoverpa armigera*, demonstrating potential for pest control applications (Gadge et al. 2015).

2.2.6 Squash-Type Serine PIs: Cucurbitaceae Specific, Small, Highly Stable, and Strong

Squash-type serine protease inhibitors are exclusively found in seeds of Cucurbitaceae, e.g., watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), balsam pear (*Momordica charantia*), etc. They are small proteins of 3–5 kDa and crosslinked with three disulfide bridges in a knot-fold topology, which is characteristic of this family (Kojima et al. 1996). Their secondary structure contains α-helical and β-sheet secondary elements and pyroglutamate protecting N-terminal. These small proteins have extraordinarily high affinities for the active site of target serine proteases: trypsin, chymotrypsin, elastase, plasmin, kallikrein (Hatakeyama et al. 1991; Hayashi et al. 1994). The squash inhibitor McoTI-I and II have been extensively studied for the design of novel drug molecules against human plasma kallikrein (Swedberg et al. 2018).

2.2.7 Mustard-Type Serine PI: Cruciferous Specific and Special

Mustard-type trypsin inhibitors are found in Cruciferous plants, which are host of specialist lepidopteran insects like *Plutella xylostella*. These PIs are polypeptides of around 7 kDa and are induced upon insect infestation. The secondary structure of these inhibitors comprises an α-helix, one antiparallel β-sheet joined by two β-strands in a α-hairpin conformation. This structure is stabilized by four disulfide

bonds. Studies have shown that the reactive loop of this type of inhibitor adopts a noncanonical mode of inhibition (John and William 2005). Two trypsin inhibitors (MTI1 and MTI2) have been identified from the seeds of the crucifera mustard (*Sinapis alba*), which show heat stability and potent activity against trypsin (Volpicella et al. 2000).

2.2.8 Cysteine PIs: From Diverse Plant Species with Endogenous Function

Cysteine PIs have been studied from several plants including pineapple, millet, potato, rice, cowpea, apple, to name a few. Cysteine PIs inhibit the proteases belonging to papain and cathepsin superfamily. In Coleopteran and Hemipteran insects, digestion-related proteolytic activities are carried out by extracellular papain-like cysteine proteases whereas, in higher animals, cysteine proteases are not secreted into the intestine but are restricted intracellularly. Most plant cysteine PIs belong to the cystatin superfamily. Plant cystatins are grouped into three subfamilies. Type 1 cystatins are 12–16 kDa single domain proteins, predominantly intracellular proteins without disulfide bonds and associated glycosylations. Type 2 cystatins are ~23 kDa proteins with functional domains in their N- and C-termini which can interact with cysteine proteases of type C13. These are generally secreted proteins with two disulfide bonds in the C-terminal region. Type-3 cystatins contain three tandemly arranged type-2 cystatin like domains N-terminal, which are linked to a vasoactive kinin sequence and a varying C-terminal extension (Dubin 2005). Cystatin "signature motif" QXVXG is directly involved in the inhibitor and target protease interactions. The wedge shape of these PI molecules helps in blocking the active site groove of the cysteine proteases (Mishra et al. 2020) (Fig. 2.2b).

Cystatin (MpCYS4) from apple (*Malus prunifolia*) plant was shown to be induced/activated under drought stress, heat, and abscisic acid treatment. Transgenic expression of *MpCYS4* in *A. thaliana* and apple increased the expression of stress-mediated signal transduction pathways and increases drought resistance (Tan et al. 2017b). Similarly, transgenic tobacco expressing cystatin from rice showed improved resistance towards cold stress (Van Vyver et al. 2003).

2.2.9 Aspartate PIs

Aspartate PIs are reported from sunflower, barley, thistle (*Cynara cardunculus*), and potato tubers. Aspartate PI from potato is a 27 kDa protein with 3 disulfide bonds; however certain aspartate PI does not possess disulfide bond (Fig. 2.2c). These inhibitors show high sequence homology with soyabean trypsin inhibitor and demonstrate inhibition of the aspartate protease, cathepsin D as well as serine proteases,

viz. trypsin and chymotrypsin (Habib and Fazili 2007). Interestingly, it cannot affect the activity of other aspartic proteases like pepsin, cathepsin E, and renin. Aspartate PIs from the seeds of mung bean (*Vigna radiata*) play a role in early germination by regulating endogenous aspartate proteases (Kulkarni and Rao 2009). This aspartate PI protein is a linear peptide of 1660 Da, which shows pH and thermal stability over a wide range. Also, aspartate PI from apple fruits inhibited the growth of the pathogen causative agent of the destructive fungal disease anthracnose (*Colletotrichum acutatum*), highlighting the importance of these PIs in pathogen resistance (Gregori et al. 2010).

2.2.10 Metalloprotease PIs: Rare but Unique

Metalloprotease PIs (MPIs) are small peptide inhibitors of around 3–4 kDa. MPIs are rarely present in plants. They have been reported from Solanaceous plants like tomato and potato (Polya 2003). Potato carboxypeptidase PIs (PCPIs) from tubers are one of the most characterized MPIs. It is a polypeptide of 39 amino acids, with a globular core of 27 residues and three disulfide bonds making a T-knot motif. A five amino acid tail of residues 35–39 protrudes from the C-terminal and forms a stable complex with the carboxypeptidase active site (González et al. 2003) (Fig. 2.2d). Metalloproteases are involved in several physiological processes such as blood coagulation, inflammation, and growth factors regulation, and hence are key targets in the development of therapeutic strategies for several diseases.

Plant MPIs have been used for the inhibition of the metalloproteinase angiotensin-converting enzyme (ACE). Due to the critical role of ACE in the regulation of blood pressure, ACE inhibitors are used to treat hypertension. ACE inhibitors have been isolated and described from plants such as peptides from mung bean and sesame (*Sesamum indicum*) proteins (Srikanth and Chen 2016).

2.3 Short Peptides as PIs

In addition to high molecular weight PIs, several enzyme inhibitory peptides with small molecular weight and range from 3 to 40 amino acids have been reported from plants (de Veer et al. 2021). These peptides are known to function in plant defense as well as other physiological roles. Several of these peptides have unknown activity, while some possess enzyme inhibitory properties, in particular PI activity. The recent discovery of biosynthetic pathways encoding for disulfide stabilized short peptides led to the characterization of several peptides from plants (Saska et al. 2007, Table 2.1). These peptides, called ribosomal synthesized and post-translationally modified peptides (RiPPs), are categorized into two families: cyclotides and orbitides. In the following sections, we describe several types of plant-derived

Table 2.1 Representative prototypic short peptide protease inhibitors from plants

Name	PI class	Size (amino acids)	Source plant	Target proteases	Reference
SFTI-1	BBIs	14	*Helianthus annuus*	Trypsin, matriptase, human kallikrein-related peptidase	Swedberg et al. (2009), Quimbar et al. (2013)
PSI-1.1	Pin-II	55	*Capsicum annuum*	Trypsin, chymotrypsin	Antcheva et al. (1996)
TRE	Pin-II	3	*Solanaceous members*	Trypsin	Saikhedkar et al. (2018)
ATSI	Pin-I	69	*Amaranthus caudatus*	Trypsin	Hejgaard et al. (1994)
MCoTI-II	Squash	34	*Momordica cochinchinensis*	Trypsin	Hernandez et al. (2000)
CMTI-I	Squash	29	*Cucurbita maxima*	Trypsin	Kojima et al. (1996)
LCTI-III	Squash	29	*Luffa aegyptiaca*	Trypsin, plasma kallikrein	Hayashi et al. (1994)
CSTI-IV	Squash	30	*Momordica charantia*	Trypsin, elastase	Hatakeyama et al. (1991)
Tomato MCPI	MCPI	37	*Solanum lycopersicon*	Metallocarboxypeptidase	Martineau et al. (1991)
β-Lybatide	CPI	36	*Lycium barbarum*	Carboxypeptidases	Huang et al. (2021a, b)
AAI	Bifunctional	32	*Amaranthus hypochondriacus*	α-Amylase, trypsin	Chagolla-Lopez et al. (1994)
SOTI I	Bifunctional	35	*Spinacia oleracea*	α-Amylase, trypsin	Retzl et al. (2020)
Jatrophidin I	Orbitide	8	*Jatropha curcas*	Pepsin	Altei et al. (2014)
Cp-thionin	Defensin	47	*Vigna unguiculata*	Trypsin	Melo et al. (2002)
Lyciumin A	Lyciumin	8	*Lycium barbarum*	Angiotensin-converting enzyme (ACE)	Kersten and Weng (2018)

short peptides including cyclotides, orbitides, PawS-derived peptides, lyciumins, and defensins.

2.3.1 Cyclotides

Cyclotides form a major family of cyclic peptides in plants, which are present abundantly in the leaves, stems, and roots of several plant species belonging to Fabaceae, Solanaceae, Rubiaceae, Violaceae, and Cucurbitaceae families (Weidmann and Craik 2016). Owing to their insecticidal properties, cyclotides are thought to play a role in plant defense (Gruber et al. 2007). The first cyclotide Kalata B1 was discovered in the 1960s for use as uterotonic agent in African medicine (Saether et al. 1995). Cyclotides are cyclic peptides of approximately 37 amino acids, with head-to-tail cyclic backbone and the presence of the characteristic "cystine knot" in which two disulfide bonds are threaded by a third disulfide bond (Gould et al. 2011). Cyclotides belong to RiPPs class of peptides which are synthesized in the ribosome as a precursor peptide (Arnison et al. 2012). Then, the enzyme protein disulfide isomerase introduces disulfide bonds to cyclotides in the endoplasmic reticulum. Finally, the N- and C-terminal of cyclotide pro-peptide are sequentially hydrolyzed and cyclized by asparagine-specific asparaginyl endopeptidases in the plant vacuole (Arnison et al. 2012; Du et al. 2020) (Fig. 2.3). The cysteine knot and cyclic backbone confer high stability at extreme pH and temperature; therefore a high amount of cyclotides accumulates in the plants. Hydrophobic amino acids on the surface of cyclotides make them soluble in both organic and aqueous solvents (Colgrave and Craik 2004). With these properties, cyclotides are considered potential molecules for drug design.

Cyclotides are categorized into two major subfamilies: Mobius and bracelet based on the presence or absence of *cis*-Pro residue in loop 5, respectively (Daly et al. 2009; Park et al. 2017). In addition to these two subfamilies, the trypsin inhibitor cyclotides constitute the third family of cyclotides. This subfamily contains MCoTI-I and MCoTI-II squash trypsin inhibitors from *Momordica cochinchinensis* (Hernandez et al. 2000). These cyclotides contain a cyclic backbone and cysteine knot (Fig. 2.3a). These peptides are produced from the precursor protein called two inhibitor peptide topologies (TIPTOP), which consists of a series of cyclic trypsin inhibitors in tandem with an acyclic trypsin inhibitor (Mylne et al. 2012). MCo-TI-II shows high affinity and selectivity towards matriptase, which is a type-II transmembrane serine protease involved in epithelial homeostasis and is implicated in the development and progression of a variety of cancers (Quimbar et al. 2013). Also, MCoTI-II has been used as a template to design inhibitors of several different proteases, for example, human leukocyte elastase and β-tryptase which are targets for treatment of inflammatory disorders (Thongyoo et al. 2009). The insecticidal activity of cyclotides suggests their role in plant defense. Insecticidal cyclotides like Kalata B1 exert severe adverse effects on the growth and survival of the lepidopteran insect *Helicoverpa punctigera* larvae (Jennings et al. 2001). However,the activity of

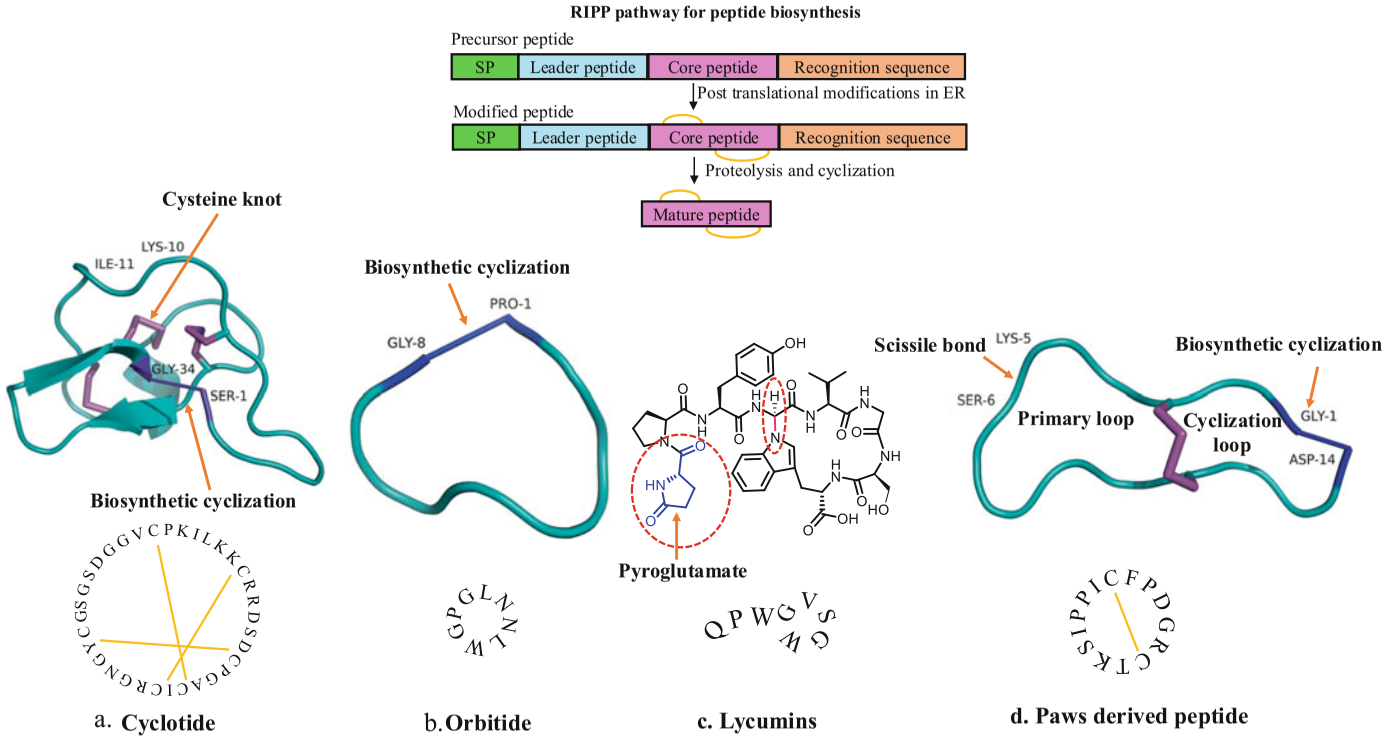

Fig. 2.3 RiPPs and protease inhibitory plant peptides. Biosynthetic pathway for ribosomally synthesized and post-translationally modified peptides (RiPPs), namely cyclotides, orbitides, and lyciumins. Representative examples are shown for (**a**) Cyclotide—McoTI-II which shows trypsin inhibition (PDB ID: 4GUX), (**b**) Orbitide—Jatrophidin I (PDB ID:6dI1), (**c**) Lyciumin (**d**) PawS-derived peptide SFTI-1 (PDB ID: 1SFI). Characteristic features for each type of peptide are highlighted by *arrows*, and simplified 2D structures are shown below each representation. (All images are prepared by PyMol software)

Kalata B1 is not related to enzyme inhibition, it is known to disrupt the midgut membrane of insect (Barbeta et al. 2008).

2.3.2 Orbitides

Orbitides are RiPPs from plants, which contain 5–16 amino acid residues in a backbone cyclized fashion, but are devoid of disulfide bonds (Shim et al. 2015) (Fig. 2.3b). Orbitides are produced by several plant families including Annonaceae, Caryophyllaceae, and Euphorbiaceae. Like cyclotides, orbitides are also processed from precursor proteins via proteolytic cleavage of core peptide from the N-terminal, followed by C-terminal proteolysis and head to tail cyclization (Arnison et al. 2012). Evolidine is the first orbitide isolated from the rainforest tree *Melicope xanthoxyloides* in the 1950s and more than 100 orbitides have been reported so far (Fisher et al. 2020). For example, the segetalins of *Vaccaria hispanica*, linusorbs from flax (*Linum usitatissimum*), curcacyclines from *Jatropha curcas*, orbitides from *Citrus* species, the annomuricatins of *Annona muricata*, and the PawL-derived peptides (PLPs) from Asteraceae family (Morita et al. 1995; Shim et al. 2019). Jatrophidin I, a cyclic octapeptide isolated from *J. curcas*, inhibited the aspartic protease, pepsin, but not serine protease subtilisin, indicating its role as aspartate PI (Altei et al. 2014). Some of these orbitides show anti-inflammatory, antiplatelet, antimalarial, immunosuppressive, and cytotoxic activities (Shim et al. 2019). Further research is required to elucidate the biological activity, biosafety, and further application of orbitides.

2.3.3 Lyciumin

Lyciumin is another class of RiPPs, which have a unique branched cyclic peptide backbone, unlike abundant head-to-tail cyclic peptides. The characteristic structural feature of lyciumins includes an N-terminal pyroglutamate and a macrocyclic linkage between a C-terminal tryptophan-indole nitrogen and a glycine α-carbon (Kersten and Weng 2018) (Fig. 2.3c). Lyciumins are abundantly produced in the roots of the Solanaceous plant Chinese wolfberry (*Lycium barbarum*), which was used to treat hypertension in Chinese medicine (Tan et al. 2017a). These peptides show potent inhibition of angiotensin-converting enzymes, proteases, and renin (Yahara et al. 1989). Genome mining suggests presence of lyciumin chemotypes across diverse flowering plants. This led to the discovery of lyciumin precursor proteins in 21 species which encode a BURP domain and repetitive lyciumin precursor peptide motifs (Kersten and Weng 2018; Jacobowitz and Weng 2020). BURP proteins possess characteristic c terminal conserved domain. These proteins are specifically present in plants, and they are involved in plant development as well

as responses to stress. These proteins possess characteristic c terminal conserved domain (Sun et al. 2019).

2.3.4 SFTI-1-Related Peptides (PawS-Derived Peptides)

Sunflower trypsin inhibitor-1 (SFTI-1) is a peptide of 14 amino acids found in sunflower seeds, with potent inhibitory activity towards trypsin. It is highly similar to PIs from Bowman-Birk type. The structure of SFTI-1 is composed of two loops: the primary loop (Thr4-Ile10), which is crucial for inhibitory activity, and the cyclization loop (Phe12-Arg2), which contains the cyclization site (Gly1-Asp14) (de Veer et al. 2021) (Fig. 2.3d). Despite its small size, SFTI-1 possesses a rigid scaffold of disulfide bond (Cys3-Cys11), prolines at positions 8 and 9 (consecutively in *cis* and *trans* configurations), and a network of intramolecular hydrogen bonds. These features make the SFTI-1 peptide resistant to heat and proteolysis. The biosynthetic production of SFTI-1 is from the precursor protein PawS1 (Preproalbumin with SFTI-1), which is a storage albumin protein (Elliott et al. 2014; Franke et al. 2018; de Veer et al. 2021). Cleavage and cyclization of SFTI-1 is mediated by enzymes from asparaginyl endopeptidase (AEP) family, as seen for RiPPs (Du et al. 2020).

With the discovery of biosynthetic pathway for SFTI-1, 17 peptides were discovered from Asteraceae family, which were derived from storage albumins like PawS1. These peptides were structurally similar to SFTI-1, with head-to-tail cyclic backbone, disulfide bond, and conserved Pro residues; hence this family was called PawS-derived peptides (Elliott et al. 2014; Gitlin-Domagalska et al. 2015; Franke et al. 2018).

In addition to its potent trypsin inhibitory activity, SFTI-1 is reported for its several applications in therapeutics such as the inhibition of human kallikrein-related peptidase 4, which leads to blocking of tumorigenic and metastatic pathways. SFTI-1 also exhibits potent inhibitory activity towards matriptase. Further, SFTI-1 is a popular scaffold for design of novel molecules (Gitlin-Domagalska et al. 2015; Jendrny and Beck-Sickinger 2016).

2.3.5 Defensins

Defensins are small cationic peptides of about 45–54 amino acid residues with conserved cysteine residues that can form three to four disulfide bridges (Wijaya et al. 2000; Stotz et al. 2009). They are found in the cell wall and extracellular space of seeds, xylem, and stomata cells in leaves and the peripheral layers of roots, flowers, and fruits. Such diverse localization of these peptides at the entry point of potential invaders and induced expression upon pathogen infection, mechanical wounding, and some abiotic stresses indicates their significant role in plant

protection. Plant defensins show a broad spectrum of activities ranging from protection against bacteria, fungi, and viruses to inhibition of enzymes, such as amylases and proteases (Janssen et al. 2003). Several defensins from plants such as *Vigna unguiculata, Sorghum bicolor, Capsicum annuum,* and *Tephrosia villosa* are reported to inhibit insect proteases and amylases (Bloch and Richardson 1991). BIα1 and BIα2 defensins isolated from barley also act as α-amylase inhibitors (Zhang et al. 1997). Defensin from seeds of *Cassia fistula* is reported to inhibit trypsin (Wijaya et al. 2000). Similarly, Cp-thionin from cowpea shows potency against trypsin (Melo et al. 2002).

2.4 Synthetic Peptides Engineered from Plant Proteins

Customized PIs for therapeutic applications are gaining interest due to the expanding information about peptide natural products and the establishment of protocols in protein engineering. The protease inhibitory loops are attractive lead for structure-based design of novel molecules for various applications. Generation of tailor-made molecules that mimic the binding and/or functional reactive sites of PIs represents a strategy to alter the functionality of proteins (Mishra et al. 2020). Potential hits of biopesticides and therapeutic drug targets are identified by a computerized approach before initiating extensive experimental protocols (Macalino et al. 2018). Synthetic mimetic peptides designed after in silico studies have proven to be potential candidates for mimicry of protein with diverse chemical modifications (Groß et al. 2016).

The usefulness of engineering peptides is shown by design of peptides based on the Kunitz-type PIs, Trypsin-ILTI from the Fabeaceae plant *Inga laurina*. Implementation of molecular docking studies to calculate binding free energies of each residue around the active site of *Spodoptera cosmioides* trypsin led to identification of 11 amino acid residues that were involved in interaction with Trypsin-ILTI inhibitor. From this interaction, two linear peptides were identified with maximum affinity towards their target proteases. These peptides have features such as stability, ability to inhibit the proteases independent of native protein scaffold, and toxic effects on the *S. cosmioides* trypsin (Meriño-Cabrera et al. 2020).

Several methods have been used for the design of inhibitors based on plant PIs (Fig. 2.4). One of the most popular methods is library screening, which involves substitution of amino acids at various sites in the peptides, generating a library of analogues which are then screened against molecular targets (Fig. 2.4a). Such libraries can be synthesized chemically using peptide synthesis, or genetically using phage display (de Veer et al. 2018). Another approach for peptide design is molecular grafting. Grafting, as the name suggests, involves inserting a peptide epitope with desired activity into a known stable scaffold, such as that of PIs. This generates chimeric molecules which not only possess the activity of the grafted epitope, but also the natural structure and stability of the PI (Mishra et al. 2020). A similar approach is inhibitor loop modification, in which active site loops of PIs are studied independently of the parent protein (Saikhedkar et al. 2018). Libraries of

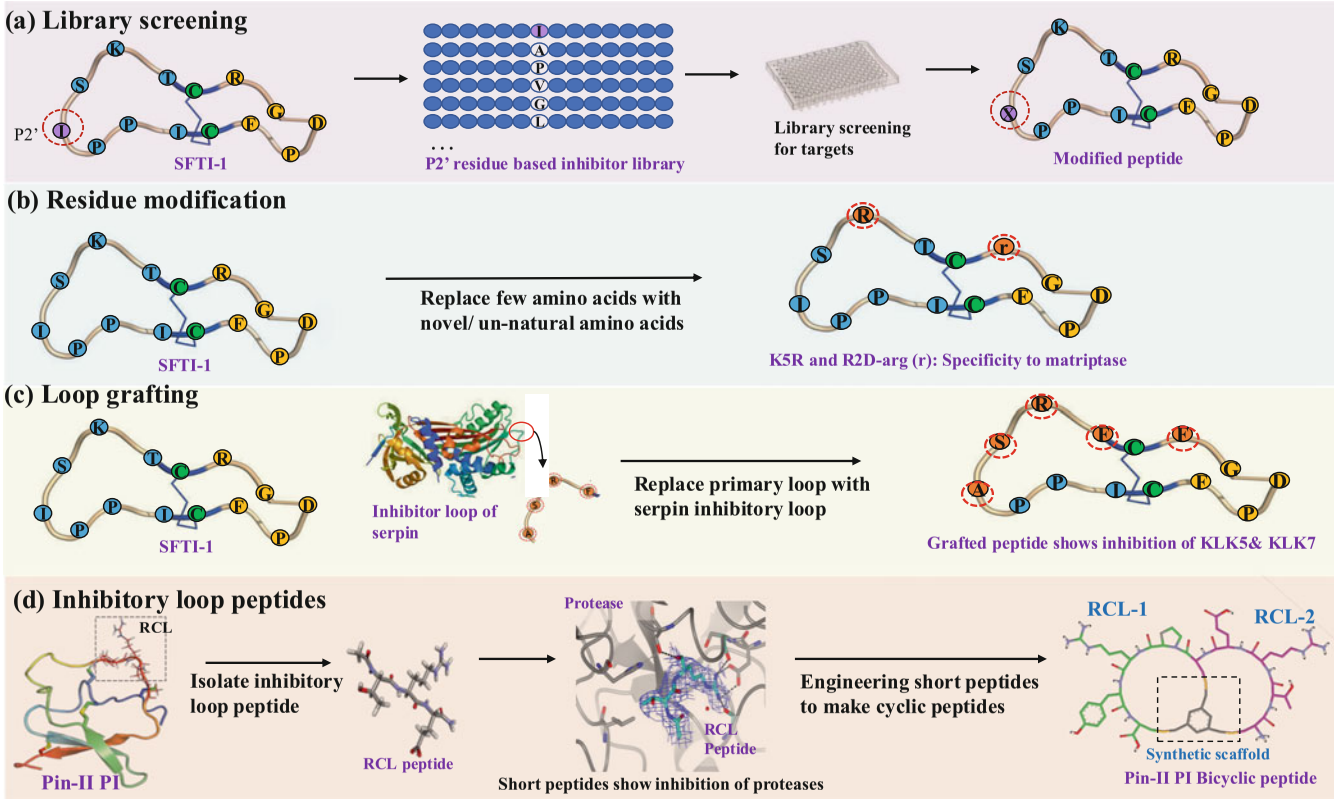

Fig. 2.4 Methods for peptide engineering. Examples are presented for (**a**) Library screening—Engineering of SFTI-1 for P2 Isoleucine residue (de Veer et al. 2018), (**b**) Residue modification—Replacing Lys5 and Arg2 of SFTI-1 with Arg and D-Arg (r) respectively generates matriptase inhibitors (Gitlin-Domagalska et al. 2015), (**c**) Loop grafting—Grafting of serpin reactive loop on primary loop of SFTI-1 yields peptides with specificity to Kallikrein 7 and 5 (Jendrny and Beck-Sickinger 2016), (**d**) Inhibitory loop peptide engineering—Peptides of the reactive center loops (RCL) of Pin-II type PIs show protease inhibition devoid of the parent protein; these peptides are engineered to generate bicyclic peptides (Saikhedkar et al. 2018, 2019)

active site peptides can be screened against target proteases, and the peptides can be modified chemically to generate novel analogues. In the following sections, we demonstrate some examples of peptide engineering using these approaches for plant PIs.

2.4.1 Engineering of Cyclotides

Cyclotides have high chemical as well as thermal stability due to the presence of the unique cystine knot motif. Also, the lack of free N- and C-termini makes them insensitive both to endoproteases and exoproteases. In addition, cyclotides are resistant to the acidic environment present in the stomach, which help to elevate the bioavailability of cyclotide-based drugs (Colgrave and Craik 2004; Wang et al. 2009). Several studies have reported the engineering of the trypsin inhibitor cyclotides McoTI-I and II and their therapeutic applications. The MCoTI-II peptide not only exhibits protease inhibitory activity but also penetrates cells because of the cyclized scaffold, making it suitable as a carrier of epitopes for intracellular targets (Camarero and Campbell 2019). Engineering of McoTI-II peptides has been achieved by inhibitory loop modification to generate the first reported peptide inhibitor against 3C protease of foot-and-mouth disease causing *Aphthovirus* in livestock (Thongyoo et al. 2008). Alterations of loops 1 and 6 in cyclotide MCoTI-II and targeting substitutions in the P1 residue yielded a highly selective and potent inhibitor of this protease (Camarero and Campbell 2019). Similarly, modification of Lys residue in the active loop and truncations in the second loop resulted in MCoTI-II analogues. These analogues showed potent inhibition of serine proteases human leukocyte elastase and β-tryptase, which are important targets in inflammatory diseases (Thongyoo et al. 2009). In another approach, grafting of a preferred cleavage sequence of kallikrein-related peptidase 4 (KLK4) into the loops 1 and 6 of cyclotide MCoTI-II transformed it into a highly potent sub-nanomolar inhibitor with 100,000-fold selectivity compared to related KLKs (Swedberg et al. 2018). Also, the grafted peptides were stable in human serum and nontoxic to human cells. Grafting of inhibitory loops in MCoTI-II scaffold has been shown to impart novel activities to the peptide, including VEGF receptor agonist activity for wound healing and cardiovascular damage and CTLA-4 antagonist activity for cancer immunotherapy (Chan et al. 2016).

2.4.2 SFTI Analogues

The small size, high potent, and stable and minimal scaffold of SFTI-1 offer great potential for design of novel small PIs against physiologically important proteases

(de Veer et al. 2018). SFTI-1 has evolved to optimize binding in trypsin active site cleft; hence, to engineer inhibitors of proteases belonging to the chymotrypsin-like serine proteases (family S1A), only the active site residue of SFTI-1 needs to be changed without altering the scaffold. This phenomenon is exemplified in studies where Lys residue present at the P1 site of SFTI-1 is modified to Phe or Val, which made it a selective inhibitor of chymotrypsin or elastase, respectively (Franke et al. 2018; de Veer et al. 2021). Apart from P1 residue, other residues in SFTI-1 have been studied for their engineering potential using approaches such as Alanine scanning and cellulose-bound peptide arrays. These studies show that several residues are essential for the structure and activity of SFTI-1, while others are amenable to substitutions (Arg2, Thr4, Lys5, Ile7, Ile10, Phe12) (Hilpert et al. 2005; Austin et al. 2010).

Substitutions of proteinogenic or unnatural amino acids in these positions led to the generation of inhibitors for therapeutically relevant proteases. For example, substitution of Ile10 to Arg yielded matriptase inhibitor which is implicated in cancer (Quimbar et al. 2013). Substitution of unnatural amino acid D-Arg in place of Arg2 also generated matriptase-2 inhibitor (Gitlin-Domagalska et al. 2015) (Fig. 2.4b). This design strategy is extended by the cellulose-bound peptide arrays, wherein SFTI-1-based libraries are generated by substituting each of the 14 amino acids and screening them against multiple targets. An example of this strategy is the development of inhibitor of pancreatic elastase by identifying substitutions at P1 (Lys to Leu) and P4 (Thr to Val) (Hilpert et al. 2005).

Another approach to generate libraries based on SFTI-1 is based upon genetically encoded phage display libraries. In this approach, randomized codons are used to introduce mutations to provide a greater diversity of mutations, as compared to chemically synthesized libraries (de Veer et al. 2021). SFTI-1 has also been used as a grafting scaffold. The primary loop, cyclization loop as well as the B-strand of SFTI-1 have been used to graft epitopes specific to other targets, which impart novel activities to SFTI-1 (de Veer et al. 2018) (Fig. 2.4c). For example, six to nine residue epitopes from somatostatin, thrombospondin-1, or pigment epithelium-derived factor were grafted (inserted by replacement of few amino acids) into the primary loop of SFTI-1, which generated analogues with antiangiogenic activity to prevent cancer growth (Chan et al. 2015). Similarly, grafting of the primary loop into the cyclization loop of SFTI-1 generated a bifunctional protease inhibitor (Jaulent and Leatherbarrow 2004).

In another example, reactive loop of serpins was grafted on the SFTI-1 scaffold (Fig. 2.4c). Serpins are large proteins of 330–500 amino acids; however, the reactive center loop of three amino acids is important for the interaction with target proteases. Grafting of serpin reactive loops into SFTI-1 generated inhibitors of kallikrein-related peptidases (KLK7 and KLK5), dysregulation of which is implicated in various cancers (Jendrny and Beck-Sickinger 2016).

2.4.3 BBI Reactive Loop Engineering

Engineering inhibitory loop of BBIs is of significant interest in drug design, as it retains both the activity and structural features of the parent protein. A key understanding of the reactive loop engineering of BBIs lies in the fact that several studies have reported the independent activity of the BBI-trypsin inhibitor loop upon fragmentation of the protein. Nishino et al. first showed in 1975 that a cyclic peptide based on the 9 amino acid antitryptic loop of soyabean BBIs retained the activity of the native inhibitor. Several studies since then have reported cyclic peptides based on the BBIs scaffold (Nishino et al. 1975).

Moreover, Alanine scan (site-directed mutagenesis technique to determine contribution of specific residue to specific peptide features) studies on the P1 residue of BBI inhibitory loop showed that modification of these residues leads to change in the specificity of the peptides. Similar studies on the P2 and P2′ residues containing SCXFSIPPQCY sequence (cyclized via the cysteines) yielded residues that are optimal for protease inhibition. An array of libraries of BBI peptides have been generated in which key positions required for protease inhibition are altered to generate analogues with different biological properties. For example, McBride et al. (2000) synthesized a library of 8000 analogues with variations at the P1/P2/P2′ sites, of which ten peptides showed highly potent inhibition of chymotrypsin (McBride and Leatherbarrow 2001). The X-ray crystallographic studies of a 22 amino acid peptide in complex with trypsin demonstrated that the inhibitory loop peptides follow the same conformation in the catalytic site of trypsin as does the parent PI (McBride et al. 2002). Reviewed by McBride et al. (2002), the three most significant considerations for designing a BBIs inhibitory loop mimic are: the presence of disulfide bond, a cis-Pro in P3 as part of the type VIb β-turn, and a transannular hydrogen bond network involving P2 Thr and P1 Ser in the front side of the loop (McBride et al. 2002).

The 36-amino acid reactive loop from a 76 amino acid BBIs from horsegram (*Dolichos biflorus*) seed was expressed recombinantly in *E.coli*, and shown to possess activity against trypsin and human tryptase (Muricken and Gowda 2011). Another approach used molecular docking and molecular dynamics simulation to study the chymotrypsin inhibition mechanism of 35-amino acid reactive loop of BBIs from soyabean (Fernandez et al. 2007).

2.4.4 Pin-II PI Reactive Loop Engineering

The signature features of Pin-II type PIs are the presence of multiple, IRDs. Each IRD can block the catalytic site of target protease(s) through a tripeptide reactive center loop, RCL. Evolution of this family of PIs has generated diverse sequences of IRDs and RCLs (Mishra et al. 2013). As per the PINIR database (https://pinir.ncl.res.in), there are 65 RCLs present in 415 Pin-II PIs (Yadav et al. 2021). RCLs are

primary interaction sites for target serine proteases, and swapping the RCLs within the two domains of tomato Pin-II type potato inhibitor resulted in changing the specificity of the two domains (Beekwilder et al. 2000). Structural studies of RCL peptides in complex with trypsin have shown that these peptides can inhibit trypsin independent of the native Pin-II type PI parent protein (Saikhedkar et al. 2018). The RCL tripeptides demonstrated both in vitro and in vivo inhibition of midgut serine proteases of the lepidopteran insect, *H. armigera*. These RCLs of Pin-II type PIs can be used to design potent insect control agents; however their stability can be a major hurdle in its application (Saikhedkar et al. 2018). In this direction, Saikhedkar et al. (2019) reported cyclization of linear RCL tripeptides using a synthetic scaffold, TBMB, generating bicyclic peptides (Fig. 2.4d). These peptides lacked free N- and C-termini and showed increased stability and potency towards trypsin. Using molecular dynamics simulations, it was shown that the small molecule scaffold maximized the covalent interactions such as salt bridges and hydrogen bonding between peptide and protease, which resulted in enhanced stability and potency of the bicyclic peptide (Saikhedkar et al. 2019).

2.5 Agricultural and Therapeutic Applications of PI Peptides

2.5.1 Agricultural Applications

Being sessile, plants are presented with a large number of abiotic and biotic stresses such as pests and pathogenic infections. The physiological response of plants to these stresses includes the production of PIs, which target the insect/pathogen proteases. However, in many agricultural scenarios, the physiological responses of the crop plant cultivars do not suffice to protect themselves against biotic stress, which results in reduction of yield. The agriculturists use numerous chemical pesticides to overcome this problem. Prolonged and excessive use of these chemicals leads to the development of resistance against chemical molecules in the target pests due to high selection pressure. Further, it can also exert adverse effects on the environment and human health (Stotz et al. 2009; Janssen et al. 2003). This demands development of safe insecticidal molecules with varied modes of action, which is necessary for long-term control of insect pests, pathogens as well as plant-parasitic nematodes. PIs are potential molecules in this direction, as they specifically target insect proteases with minimal toxic effects to other organisms. For example, transgenic tomato expressing two barley PIs and a cysteine PI showed enhanced defense response against the pest tomato leaf miner (*Tuta absoluta*), but no adverse effects on the hemipteran insect, reuter (*Nesidiocoris tenuis*), which is a predator of the tomato leaf miner (Hamza et al. 2018).

In the integrated insect pest management strategy, it is observed that PIs from non-host plants are more effective than the PIs of the host plants (Harsulkar et al.

1999). Pin-II type PIs from the non-host plant *Capsicum annuum* have potential to protect the host plant tomato against infestation by the lepidopteran insect pest, *H. armigera* (Tamhane et al. 2005; Giri et al. 1998). The formulations developed from IRDs of Pin-II PIs also showed growth retardation of this insect pest (Khandelwal et al. 2015). Also, pyramiding different PI genes is beneficial for transgenic crops. Dunse et al. (2010) reported the co-expression of Pin-I and Pin-II type PIs in cotton, which resulted in overcoming insect resistance towards chymotrypsin inhibitors and increase in yield of cotton bolls (Dunse et al. 2010). Similarly, Outchkourov et al. (2004) developed transgenic potato expressing custom-made multidomain PI, consisting of candidates of cysteine and aspartic proteases inhibitors. This inhibitor cocktail can inhibit a broad spectrum proteases of thrips and is more potent than the individual candidate PIs against Western flower thrips, *Frankliniella occidentalis* (Pergande) (Outchkourov et al. 2004). In another example, ingestion of the cyclotide Kalata B1 showed severe mortality of *H. armigera* larvae by disrupting the midgut epithelial cells (Barbeta et al. 2008).

Plant PIs also show antibacterial and antifungal activities on plant pathogens. Several chymotrypsin inhibitors from the Kunitz family interfered with the development of the potato late blight fungus, *Phytophthora infestans* (Bártová et al. 2019). Similar examples from barley (bifunctional inhibitors of chymotrypsin/subtilisin, amylase/subtilisin) show inhibition of proteases from the fungus *Fusarium culmorum* (Pekkarinen et al. 2007). Given the broad range of activity of plant PIs, novel molecules and formulations can be developed for agricultural applications. In an attempt to design novel inhibitors, computational biology methods like genome mining and molecular modeling are of particular interest. Chemical modification methods such as substitution of specific residues with natural and/or unnatural amino acids, L-to-D heterochiral isomerization, C- and N-terminal modification/capping, cyclization, and formulation into nanoparticles can help in overall improvement of the PIs.

2.5.2 Therapeutic Applications

Use of PIs for therapeutic purposes is advancing due to increasing knowledge of protease-PI interactions and generation of data at genomic and proteomic level. Proteases being critical for major biological processes represent one of the largest classes of therapeutic targets (Hellinger and Gruber 2019).

Plant PIs show inhibitory activity against several therapeutically important proteases like trypsin, kallikrein, human leucocyte elastase, angiotensin-converting enzyme (ACE), matriptase, β-tryptase, etc. Several PIs have undergone preclinical studies by showing in vitro efficacy against disease cell lines (Lau and Dunn 2018). A bifunctional amylase/protease inhibitor from ragi showed cytotoxicity against K562 chronic myeloid leukemia cells (Sen and Dutta 2012) Similarly, EcTI, a Kunitz inhibitor from the Fabeaceae plant, *Enterolobium contortisiliquum,* showed inhibition of trypsin, chymotrypsin, plasma kallikrein, plasmin, and human

neutrophil elastase. EcTI showed cytotoxicity specifically against several human cancer cell lines (Srikanth and Chen 2016; Nakahata et al. 2011). Furthermore, inhibitors from the BBI type of plant PIs have shown promising results in clinical trials for cancer. The role of plant PIs on cancer therapy is reviewed by Srikanth and Chen (2016).

Apart from direct applications in therapeutics, plant PIs also have pharmaceutical applications for the production of vaccines by molecular farming. Several studies exemplify the utilization of PIs to minimize *in planta* proteolysis of the transiently expressed recombinant proteins (Goulet et al. 2010). Co-expression of PIs with the recombinant protein reduces proteolysis, resulting in increased yield. Co-secretion of a BBI-type PI with immunoglobulin complexes increased the antibody production in the roots by inhibiting degradation of the recombinant antibody (Komarnytsky et al. 2006). Similarly, expression of apoplast targeted aspartic and serine PIs from tomato as companion proteins in *Nicotiana benthamiana* leaves resulted in an increase of antibody production yield (Grosse-Holz et al. 2018). These examples demonstrate potential applications of plant PIs in the pharmaceutical industry.

2.6 Synthesis and Production of Plant Peptides

Peptides are small proteins that can be chemically synthesized using peptide synthesis methods. Linear peptides of the reactive site loop of Pin-II type PIs have been synthesized by solid phase peptide synthesis (SPPS), with capped N- and C-termini. However, chemical synthesis of plant PI peptides is difficult, since they possess a unique structural scaffold to retain their biological activity. Synthetic peptide mimics of BBIs were synthesized by SPPS and cyclized by in vitro oxidation (Chen et al. 2020). Similarly, the linear precursors of cyclotides were synthesized by SPPS. Backbone cyclization can be achieved by natural chemical ligation on these precursors containing cysteine at N-terminal and natural chemical ligation of these precursors containing cycteine at Nterminnal and thioester group at C-terminal. Then, disulfide linkages are generated by oxidative folding (Fig. 2.5a). This process of cyclization and folding can also be accomplished in a same reaction by using glutathione (GSH) (Ji et al. 2013).

In addition to chemical cyclization, cyclotides can also be synthesized by a "chemo-enzymatic" approach using ligases similar to the native AEP, which is responsible for *in planta* cyclization of cyclotides (Jackson et al. 2018) (Fig. 2.5b). An interesting approach uses trypsin to mediate the cyclization of linear precursors and has been used to generate SFTI-1 and trypsin inhibitory cyclotides MCoTI-I and II. In this approach, linear precursors with P1 and P1′ residues at the C- and N-termini are synthesized by SPPS and folded by oxidative folding. These precursors are then used as substrate for trypsin-mediated amide bond formation resulting in cyclization (Marx et al. 2003). However, this approach is limited by mutations which can affect the binding of linear precursors to the substrate binding site, thus reducing cyclization efficiency.

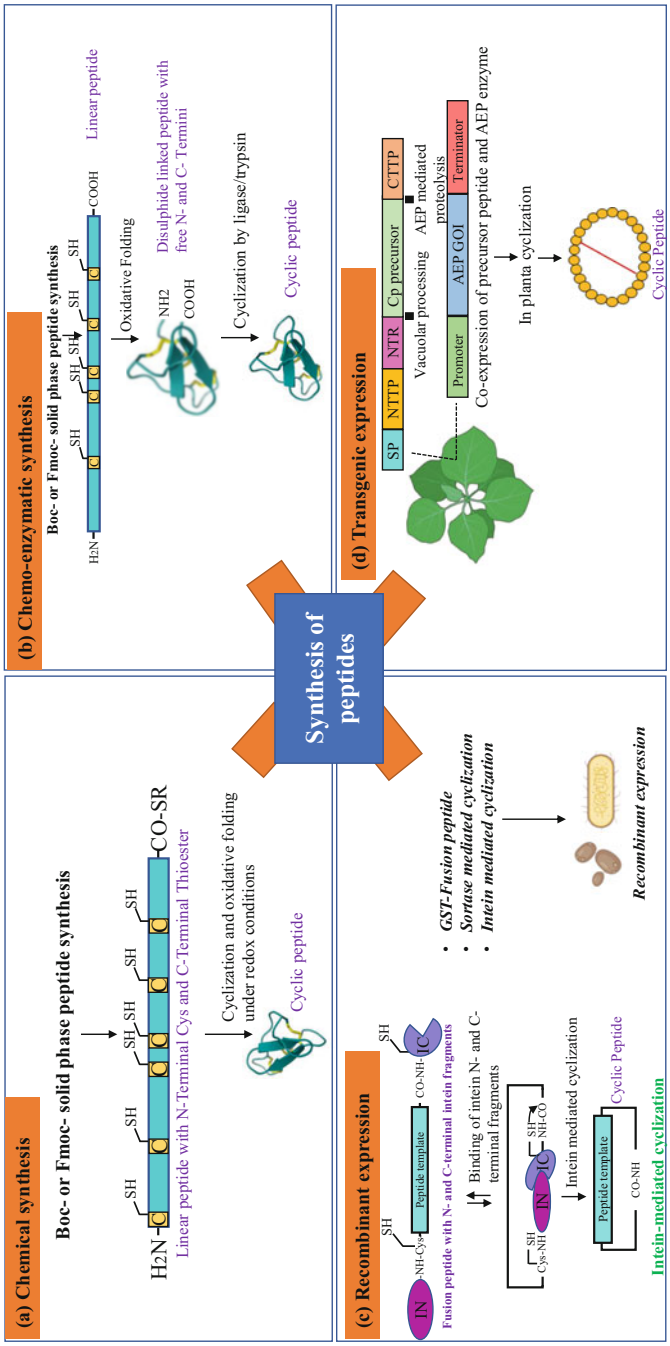

Fig. 2.5 Synthesis and production of peptides. (**a**) Chemical synthesis for linear peptides by F-moc or Boc-chemistry, which can be extended for cyclic peptide synthesis by oxidation of cysteine containing peptides, (**b**) chemo-enzymatic synthesis for enzymatic cyclization of cysteine containing peptides produced by F-moc or Boc-peptide chemistry, (**c**) recombinant expression of peptides can be achieved by fusion with large proteins such as GST, and Sortase-A and Intein-mediated cyclization are used for production of cyclic peptides, (**d**) transgenic expression of cyclic peptides has been achieved for cyclic peptides by co-expression of precursor protein with cyclization enzyme

Furthermore, plant PIs peptides can be produced by recombinant expression using standard microbial expression systems (Austin et al. 2009) (Fig. 2.5c). One approach uses intein-mediated protein splicing to express backbone cyclized polypeptides. This mechanism is similar to RNA splicing, wherein an internal segment of the precursor protein (intein) is removed, resulting in ligation of N- and C-termini of remaining segments (exteins) (Jagadish et al. 2013). The inteins are self-processing domains, which are split into individual N- and C-fragments. These fragments are not active individually, but under appropriate conditions, they bind to form a functional intein splicing domain. By fusion of the N- and C-intein fragments to the linear peptide, the splicing reaction yields a backbone cyclized peptide. Cyclotides, SFTI-1, and defensins have been produced by this method (Camarero and Campbell 2019).

Other enzymes like sortase-A have also been utilized for recombinant cyclization of cyclotide MCoTI-II. The linear precursor of MCoTI-II was expressed in *E. coli* as a fusion protein with glutathione S-transferase (GST), with sortase-A recognition motifs GGG and LPETGG added to the N- and C-termini of the linear peptide. After expression and removal of GST tag, sortase-A catalyzed the cyclization of the linear precursor into the cysteine fold containing cyclotide (Li et al. 2015).

Recent studies have reported the transgenic expression of cyclotides. Since cyclotides accumulate at high levels in plants, this approach leads to high yields of peptides (Fig. 2.5d). Poon et al. (2018) have shown that native and engineered cyclotides can be produced in non-cyclotide producing plants (tobacco, bush bean, lettuce, and canola) by co-expression of the cyclization enzyme AEP with the cyclotide gene (Poon et al. 2018) . Similarly, SFTI-1-based plasmin inhibitor peptide was expressed in *N. benthamiana* by co-expressing AEP enzyme with the peptide gene (Jackson et al. 2019).

2.7 Conclusion and Future Perspectives

The enormous diversity of phytochemicals has always been explored by researchers to develop novel molecules for various applications. The continuous evolution of plants coupled with modern techniques of genome mining has enabled the discovery of previously unknown types of phytochemicals. Plant PIs constitute an important class of defensive proteins, which not only play an important role in plant development, defense, but are also useful for several therapeutic and agricultural applications. Majority of the families of plant PIs are proteins with molecular weight more than 10 kDa. However, recent discovery of plant peptides with protease inhibitory activity has introduced a new class of protease inhibitory phytochemicals. These peptides are small in size and possess high potency and selectivity towards target proteases. Also, the unique structure of these peptides imparts thermal, pH, and proteolytic stability. The small size of peptides allows for intracellular delivery, and the peptide sequence imparts specificity. Several biological activities have been attributed to plant peptides, including antimicrobial and anticancer properties. The

current challenge is, therefore, to explore the previously unknown bioactivities of the plant peptides. Also, small peptides are difficult to identify from sequence databases by mere sequence alignments since these undergo several post-translational modifications. So, for retrieval of phytopeptides from the existing genomic datasets, dedicated databases and in silico tools are needed.

With recent advances in peptide library generation by chemical and genetic methods, it is possible to screen for sequences with potent protease inhibitory potential. Moreover, the versatility of peptides for protein engineering enables scientists to impart novel biological activities to the existing scaffolds. Development of multifunctional inhibitors to target more than one protease is an interesting strategy that can be adapted from plant PIs. Also, phytopeptides can be engineered to target intracellular proteins by utilization of the unique disulfide scaffolds, such as that of cyclotides. These features of plant peptides make them ideal candidates for pharmaceutical applications in the future.

These advantages are complemented by studies demonstrating the recombinant and transgenic expression of plant peptides, which will further advance applied research in this area. Plant PIs have also been used as companion proteins in molecular farming, which help the production of protein epitopes *in planta*. Furthermore, genome mining and computational biology methods are elucidating novel plant peptides and peptide synthesis pathways, further expanding this arena of research. Advancement of characterization techniques like multidimensional chromatography, ultrafiltration, and proteomic analyses has generated a repertoire of peptide sequences. In conclusion, plant peptides are the molecules of the future for pharmaceutical and agricultural applications.

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Chapter 3
Bioactive α-Amylase Inhibitors: Sources, Mechanism of Action, Biochemical Characterization, and Applications

Sainath S. Kasar, Vijay L. Maheshwari, and Pankaj K. Pawar

Abstract In the majority of organisms especially plants, biotic stress induces molecular, cellular, and biochemical changes for the expression of defense-related proteins such as proteinaceous inhibitors of digestive enzymes like amylases, proteinases, and lipases. Such bioactive enzyme inhibitory proteins or peptides are the first line of defense of direct response against infesting pest/pathogens. Among all different enzyme inhibitors, α-amylase inhibitors (α-AIs) are prime and attractive candidates for studies and research due to their potential applications. α-AIs display a great diversity in their structure, expression, inhibition specificity, and effectiveness against digestive enzymes from several sources including mammalians, insects, and microbes. Biological applications of α-AIs primarily include their utilization in (a) pest management; (b) microbial pathogen control (antimicrobial activity); (c) human health care management; and (d) food processing industry.

The current book chapter systematically reviews different strategies of purification, biochemical characteristics, biological applications of α-AIs, and bottlenecks in commercial utilization of α-AIs. The challenges in the safe marketable utilization of α-AIs are discussed in detail and alternative approaches and various efficient solutions based on recent advancements in biotechnological research which could be helpful to broaden the scope of α-AIs are also elaborated in detail.

In our opinion, though, there are bottlenecks in commercial utilization (efficient technology development) of α-AIs in various sectors mentioned above; focused research with meaningful utilization of technological advances will help in materializing commercial utilization of bioactive α-AIs.

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3.1 Introduction

Living creatures of the planet survive only because of the most complicated biochemical enzyme-catalyzed reactions repeatedly happening in their system. The digestive enzymes such as amylases, proteases, and lipases play a crucial role in metabolizing the food material to generate the energy required by the living system, summarized in Fig. 3.1. Among these digestive enzymes, amylases have received special attention because of their appearance in early developmental stages of living systems, like (a) In humans a pregnant woman has the higher secretion of human salivary amylase in the digestive tract as compared to proteases and lipases (Giesbrecht et al. 2013), (b) In pre- and post-harvest insect pest, α-amylases remain

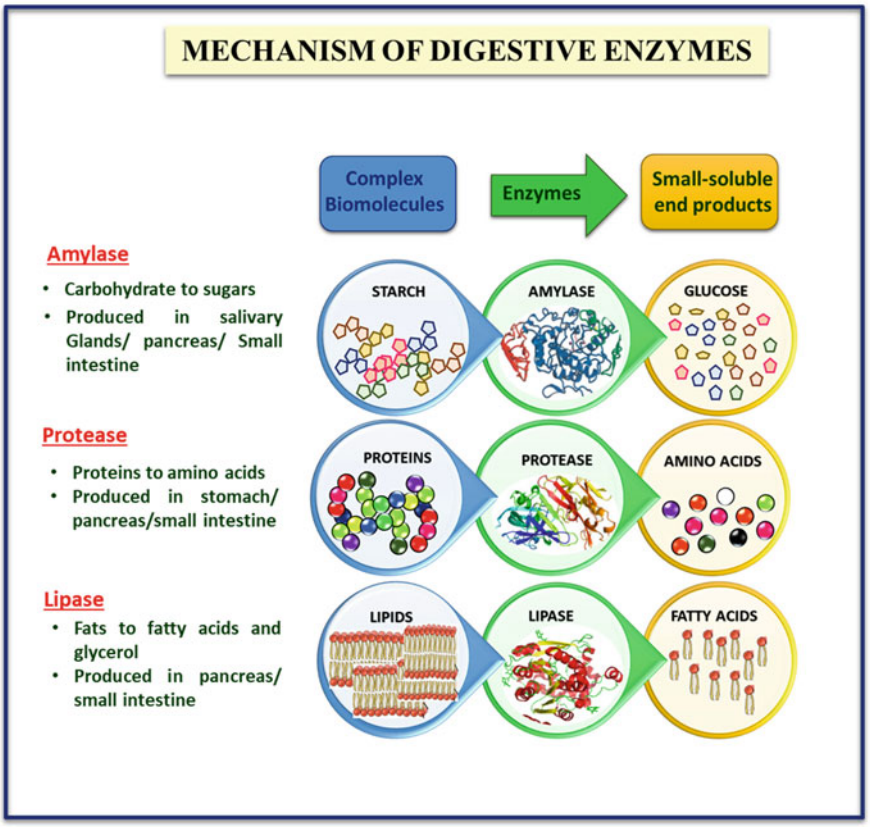

Fig. 3.1 Digestive enzymes and their mode of action.

dominant as they stay active during non-feeding stages like a pupa, adult, and eggs of the insect to digest complex starch from plant-derived food materials into simple sugars for assuring their survival (Kotkar et al. 2009; Kasar et al. 2017), and (c) In seed development, plant hormone gibberellic acid initiates de novo synthesis of endo-amylase in aleurone layer to hydrolyze endospermic starch and supports embryo to meet its nutritional requirements in the form of sugars (Singh and Kayastha 2014).

3.2 Amylases

Digestive enzyme, amylase, is a type of catalytic enzyme from family endo/exo-enzymes that hydrolytically breaks glycosidic bonds of large α-linked polysaccharides such as starch and glycogen yielding to smaller oligo- or monosaccharide for accomplishing energy requirement. Thus, they play a major role in energy generation (Pelegrini et al. 2006; Kaur et al. 2014).

3.2.1 Forms of Amylases

Based on the mechanism of catalysis, amylases can be divided into three types, viz. (a) α-amylases, (b) β-amylases, and (c) γ-amylases. Extracellularly secreted α-amylase (1,4-α-D-glucan glucanohydrolase; glycogenase; EC 3.2.1.1) is an endo-enzyme because it mainly catalyzes the hydrolysis of α-1,4-glucosidic linkage of starch molecule in the central site randomly and releases linear and branched oligosaccharides of various chain lengths (Cherry et al. 2004; Baysal et al. 2008). However, another form of amylase, β-amylase (1,4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase, EC 3.2.1.2) is known as exo-amylases, because it catalyzes the hydrolysis of second α-1,4 glycosidic bonds of starch from the non-reducing end and cleaves short end two glucose units (maltose) at a time from a long chain of polysaccharide. The third form of amylase is γ-amylase (glucan 1,4-α-glucosidase; amyloglucosidase; exo-1,4-α-glucosidase; glucoamylase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase, EC 3.2.1.3). It catalyzes the hydrolysis of last α-1-4-glycosidic linkages at the non-reducing end of amylose and α-1-6-glycosidic linkage of amylopectin, yielding glucose (Saini et al. 2017). Unlike other forms of amylase, γ-amylase is most efficient in acidic environments and has an optimum pH 3. A large number of enzymes are known to hydrolyze starch into different products. However, combined action of these enzymes is required for complete hydrolysis of starch (Saini et al. 2017).

3.2.2 Structural Characteristics of Amylases

The architecture of insect and mammalian α-amylases involves three domains like (a) Catalytic core domain A [comprising a $(\beta/\alpha)_8$ barrel], (b) An extended loop from third β-strand and third α-helix called domain B, and (c) C-terminal distinct globular unit called domain C (Fig. 3.2). Domain A and B are important in the architecture of the three most functional sites of the enzyme such as active site, calcium-binding site, and chloride binding site. Calcium and chloride ions are important in maintaining structural integrity and activation of amylases (Payan 2004). Binding sites of α-amylase serve as an allosteric site due to their proximity to a water molecule, which probably initiates substrate cleavage (Kaur et al. 2014).

3.2.3 Distribution and Significance of Amylases

Amylases are among the most important class of industrial enzymes having approximately 25–30% of the world enzyme market (Rajagopalan and Krishnan 2008; Azad et al. 2009; Deb et al. 2013). As a ubiquitous enzyme, α-amylase is highly produced and widely distributed throughout the living systems—animals, plants, insects, and microorganisms.

Recently, Seetaloo et al. (2019) reviewed that about 58.8% of α-amylases used in the assays were from the mammalian origin (HSA/PPA), 3.2% of microbial amylases used in the industries were from the fungus or the bacterium, a small portion of the amylase, i.e. 2.1% was from plants (wheat), and 35.8% of the α-amylase used

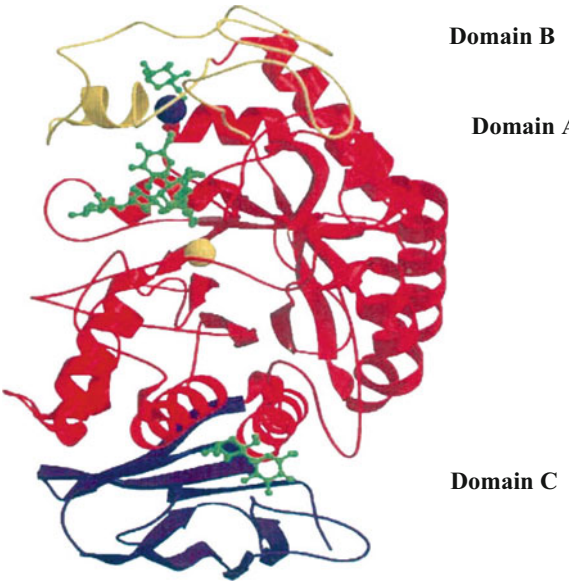

Fig. 3.2 Crystal structure of pancreatic α-amylase (PPA)-substrate complex, where red ribbons indicate domain A, yellow ribbons indicate domain B, purple ribbons indicate domain C, blue sphere indicates calcium ion, and yellow sphere indicates the chloride ion. (Adopted from Payan 2004)

were of unknown source. The use of mammalian enzymes might better reflect the in vivo situation in the human body as compared to microbial or plant enzymes. Over the past few decades, amylases from microorganisms like bacteria, fungi, and viruses are considerably studied due to their easy large-scale production/economically feasible bulk production as compared to amylases from plants and animals (Saini et al. 2017). These microbial amylases have dominated applications in various commercial processes in industries. Due to immense availability and high production at lower optimum temperature, amylases have great commercial value in biotechnological applications ranging from fermentation, textile, paper, leather, biofuel, and detergent industries (Kandra et al. 2002; Rao and Satyanarayana 2007; Noreen et al. 2017). In a given scenario, amylases can be exploited in the field of medicine for the development and designing of therapeutic agents against type II diabetes, obesity, hyperlipidemia, and dental caries (Kandra et al. 2002). Amylases also play an influential role in cell death in the aleurone of wheat genotypes with premature α-amylase activity (Mrva et al. 2006). The activity of α-amylase is influenced directly by substrate, temperature, pH, and concentration of metal ions. Kotkar et al. (2009) reported that amylase activities have special importance in the *H. armigera* life cycle and its midgut can express different isoforms of α-amylases dependent on diet.

3.2.4 Problems Associated with Highly Active Amylases

Carbohydrates, an inevitable constituent of the human diet worldwide, are derived from a variety of crop plants. Along with other living creatures, a number of pre- and post-harvest insect pests satisfy their energy requirement through starchy tissues of crop plants. Highly active amylases in insect midgut cause severe losses to starchy food grains. These herbivorous insect pests are considered to be a major competitor to humans for agricultural resources, contributing nearly 37% to the loss of agricultural produce worldwide. The losses in stored grains by insect pests alone range from 9% to 20% depending upon storage conditions (Pimentel 1991). The damage caused by the insect pests is one of the factors for nutritional and financial insecurity among the vegetarian population and farming community in developing countries. Similarly, higher secretion of α-amylase or highly active expression of carbohydrate hydrolyzing enzymes (isoforms of α-amylase/glucosidase) in human beings is responsible for various metabolic disorders. Such active enzymes frequently hydrolyze the consumed high-calorie starchy food products into simple sugars which elicit distinct, appetite modulatory nutrient signals by various neurotransmitters, gastrointestinal signals mediated by local hormones and sensory inputs or circadian rhythms (Bessesen and Faggioni 1998; Schwartz et al. 1999). The continued excess energy provision in the form of high blood sugars contributes to increased daily caloric/dietary intake relative to energy expenditure (energy loss via metabolic and physical activity). Such unbalancing or fluctuations in body fuel energy (energy intake vs. energy expenditure) results in risk of obesity (weight gain) which invites

many chronic conditions and metabolic disorders such as diabetes, hypertension, hypercholesterolemia, and cardiovascular diseases (Rosin 2008). Likewise, in many industrial processes the balanced activities of amylases are required to avoid pathogenic bacterial and fungal contamination in the final product.

Hence, amylase activity must be precisely regulated, since uncontrolled amylase activities can lead to serious malfunctions. Such problems can be overcome either by degrading enzymes or by using enzyme inhibitors, e.g. α-amylase inhibitor(s). Therefore, identification and characterization of plant-derived primary and secondary molecules which have the potential to inhibit α-amylase enrich our resources to design their use in crop protection and medicines.

3.3 α-Amylase Inhibitors (α-AIs)

Inhibition of α-amylase catalyzed reaction by binding of inhibitory molecules results in prevention of digestion and absorption of dietary starch by the body of the living creature and block energy supplement (Shamki et al. 2012). The term α-AI(s) is defined as the bioactive protein(s) or metabolite(s) or synthetic chemical(s) which have the ability to control α-amylase activity by forming requisite interactions with it. α-AIs are generally known as "starch blockers" because they prevent the degradation of dietary starch and stop its utilization in the body of living organisms as energy source (McEwan et al. 2010). The important characteristic to be a potent amylase inhibitor is that it should be efficient against the target amylase, while it should not interfere with the action of endogenous α-amylases (for example, amylases expressed during seed germination) and mammalian enzymes (Kadziola et al. 1998).

3.4 Sources of α-AIs

α-AIs are found in microorganisms (*Streptomyces* sp., *Actinomycetes* sp.), Brown Algae (*Ecklonia cava*, *Sargassum patens*), and a wide range of food plants (mostly in cereals and legumes) to regulate α-amylase activity (Franco et al. 2002; Sokočević et al. 2011; Senthil et al. 2015).

1. *Microbial α-AIs*

   Microbial α-AIs from *Streptomyces* and *Actinomycetes* sps. Are small proteins with approximately 36–92 amino acid residues (Murao et al. 1980, 1992; Hofmann et al. 1985; Vértesy and Tripier 1985; Katsuyama et al. 1992; Sumitani et al. 1993; Sokočević et al. 2011). They have conserved disulfide topology and their active site mostly contains tryptophan, arginine, and tyrosine (WRY) triad (Chagolla-Lopez et al. 1994; Sun et al. 2015). *Streptomyces* AIs superfamily includes Haim II (Murao et al. 1980), Paim I (Murao et al. 1983), Tendamistat

(Vértesy et al. 1984), Z-2685 (Parvulustat) (Hofmann et al. 1985), AI-3688 (Vértesy and Tripier 1985), AI-409 (Katsuyama et al. 1992), T-76 (Sumitani et al. 1993), and MA-4680 (Ikeda et al. 2003).

2. *Algal α-AIs*

   Marine seaweeds of the three broad groups traditionally known as Chlorophyta (green seaweed), Rhodophyta (red seaweed), and Phaeophyta (brown seaweed) produce compounds with varying bioactivities (Senthil et al. 2013). These algal phyla are known to have α-glucosidase and α-amylase inhibitory potential. Red algae of the family Rhodomelaceae contain bromophenols and 3,4-dihydroxybenzyl which is known for the α-glucosidase inhibitory activity. Similarly, Senthil et al. (2013) reported that red seaweeds, *Chondrococcus hornemanni,* and *Gracilaria gracilis* have maximum α-amylase inhibitory activity without any toxic effects. *Polyopes lancifolia* and *Grateloupia elliptica* were found to produce bromophenol, C_6H_5BrO which have glucosidase and amylase inhibitory capability and it can be used for blood sugar control (Kim et al. 2008, 2010). *Ascophyllum nodosum* red seaweed is reported to have phenolic antioxidant-mediated α-glucosidase and α-amylase inhibitors (Kim et al. 2008). The isolated compound diphlorethohydroxycarmalol from *Ishige okamurae* (brown seaweeds) (Heo et al. 2009) and fucoidan from *Turbinaria ornata* (Lakshmana Senthil et al. 2015) were reported as a potent α-glucosidase and α-amylase inhibitor.

3. *Plants α-AIs*

   α-Amylase inhibitors (AIs) naturally occur in many food plants, particularly abundant in cereals and legumes as part of natural defense mechanism are equally focused candidates for this purpose (Guzman-Partida et al. 2007). α-AIs from cereal plants have inhibitory activity with endogenous amylases due to their role in the metabolic regulation of starch (Weselake et al. 1985; Giri and Kachole 1998). Plant seeds are known to produce a variety of enzyme inhibitors that protect the seed against insect and microbial pathogens. Proteinaceous inhibitors are the best studied of this group (Christou et al. 2006). In particular, the plant-derived purified inhibitor(s) of insect digestive enzymes appear to be a safe, sustainable, and attractive option. The potential of α-AIs as bio-insecticidal molecules has been reviewed previously (Carlini and Grossi-de-Sá 2002). For example, α-AIs of rye, common bean, wheat, and amaranth exert their action by slowing down the digestion of food ingested by insect (Giri and Kachole 1998; Franco et al. 2002; Dias et al. 2005). α-AI of *Hevea brasiliensis* controls the imbibition of reserve starch and responsible for sugars accumulation for seed development (Bunyatang et al. 2016). Reports on α-AIs from plant species for their use to control the post-prandial glucose levels and insect amylase activities are dominant over their utilization from microorganisms for same activities. As far as large-scale production of α-AIs is concerned, use of plants as source material is difficult because they require larger space for cultivation and have complicated extraction procedures. Whereas it is simple to deal with microbial sources at a large scale and inhibitors can be easily recovered after downstream processing (Jayaraj et al. 2013).

3.5 Classification of α-AI

Based on the chemical nature, amylase inhibitor molecules are mainly grouped into two types, viz. (a) non-proteinaceous α-AIs and (b) proteinaceous α-AIs.

3.5.1 Non-proteinaceous α-AI

Non-proteinaceous α-AIs are biochemically active secondary metabolites and carbohydrate-based chemical compounds which inhibit the activity of α-amylase. The inhibitors from this class include various types of organic/chemical cyclic compounds. The cyclic ring from these compounds mimics α-amylase substrates which allow binding of cyclic rings to enzyme active site resulting in α-amylase and α-glucosidase inhibition (Franco et al. 2002).

(a) **Secondary metabolites as α-AI**

Plants and microbes produce secondary metabolites through the activation of many genes with multienzymes pathways (Gatehouse and Gatehouse 1999). A wide array of α-amylase inhibitory phytochemicals and secondary metabolites are isolated from various plants and microbial sources. For example, oleanolic acid and ursolic acid from *Phyllanthus amarus* showed a concentration-dependent inhibition of α-amylases (Ali et al. 2006). Similarly, two hibiscus acid forms are isolated from *Hibiscus sabdariffa* (Hansawasdi et al. 2001) and two α-amylase inhibitory compounds, betulinic acid and 3,5,7,4′-tetrahydroxy flavanone are identified from seed extract of *Syzygium cumini* (Karthic et al. 2008). Organic extract of *Curcuma longa* contains podocarpic acid, curlone, cinnamic acid, 3-cyano-7-hydroxyl-4-mehylcoumarin, and 5-amino-2-hydroxybenzoic acids like phytochemicals which showed α-amylase inhibitory potential (Ponnusamy et al. 2011). Secondary metabolites such as alkaloids, tannins, cardiac glycosides, flavonoids, saponins, and steroids from *Linum usitatissimum*, *Morus alba* and *Ocimum tenuiflorum*, *Urtica dioica* and *Juglans regia* (Sudha et al. 2011; Rahimzadeh et al. 2014) are also reported to have inhibition of α-glucosidase and α-amylase activities.

(b) **Carbohydrate-based inhibitor**

Some carbohydrate-based inhibitory compounds such as adiposin (Namiki et al. 1982), trestatin (Yokose et al. 1983), amylostatin Y (Deshpande et al. 1988), acarviostatins (I03, II03, III03, IV03) (Geng et al. 2008), acarbose (Rockser and Wehmeier 2009), and SF638-1 (Meng et al. 2011) have been isolated from the genus *Streptomyces*. Chemically these α-AIs are pseudo tetrasaccharides. For example, acarbose, a standard α-AI is a pseudo tetrasaccharide and commercially produced using developed strains of *Actinoplanes* and this inhibitor was reported from *Streptomyces glaucescens* (Rockser and Wehmeier 2009). Acarbose contains heterocyclic valienamine ring which plays an important role in the regulation of α-glucosidases, α-amylases,

and other amylolytic enzymes (Bompard-Gilles et al. 1996; Koukiekolo et al. 2001). The insect amylases from *C. chinensis* and *T. castaneum* were reported to be inhibited by acarbose at concentrations ranging from 2 to 40 nM (Channale et al. 2016).

Similarly, cyclodextrins are sugar bound oligosaccharides of varying sizes (α, β, and γ) and their inhibitory activity against porcine pancreatic and human salivary α-amylases is highly dependent on pH, temperature, and substrate concentration (Kim et al. 1999; Qian et al. 2001). Fungal amylases are capable of hydrolyzing α- and β-cyclodextrin (Suetsugu et al. 1974; Kamitori et al. 1999), while, in contrast, γ-cyclodextrin is capable of hydrolyzing human salivary amylase (HSA) and porcine pancreatic α-amylase (PPA) (Arima et al. 1992). In human health management, these α-AIs are attractive candidates for the treatment and diagnostic approaches (O'Donnell et al. 1977; Bischoff 1994). Recently, thiazole based carbohydrazide derivatives have been synthesized in search of the potent antidiabetic agent as α-AI (Khatik et al. 2018). Similarly, fucoidan a compound isolated from brown algae *Sargassum wightii* was reported to inhibit α-glucosidase and amylases (Kim et al. 2014; Senthil et al. 2015).

3.5.2 Proteinaceous α-AIs

Proteinaceous α-AIs are the translated products of multigene family inducible genes, and these genes are distributed over several chromosomes. The expression of these genes results in the translation of varying sizes of α-AIs (monomer, dimmer, and tetramer) with varying amino acids composition (Barber et al. 1989; Poerio et al. 1991). Especially, the plant proteinaceous enzyme inhibitors are part of the reserve storage protein of seeds and have been considered to be a part of the constitutive and inducible array of defense mechanisms against attack by insect and microbial pathogens (Blanco-Labra et al. 1995). Such α-AIs are synthesized with seed storage proteins during grain filling as proteins of the endosperm and the aleuronic layers of the seed (Nielsen et al. 2004). α-AI(s), specifically glycoprotein α-AI(s) are naturally synthesized through multistep signal-dependent biochemical processes with the help of cell organelles such as endoplasmic reticulum (ER) and Golgi apparatus (Robertson and Hill 1989). The synthesis includes four stages, viz. (a) synthesis of α-AI protein by AI gene in the rough endoplasmic reticulum, (b) co-translational removal of signal peptide, (c) post-translational modification of protein through the addition of glycans in the Golgi apparatus which leads to an increase in the molecular weight of the pre-protein, and (d) transportation to protein storage vacuoles for subunit formation (α- and β-chains) of inhibitor in some cases (Pueyo et al. 1993; Maczó et al. 2015). This heterotetrameric $\alpha_2\beta_2$ containing structure is required for establishing tight binding with two molecules of α-amylase (Kasahara et al. 1996). The whole process of α-AI(s) synthesis is diagrammatically represented in Fig. 3.3. Previous studies have showed that the *Phaseolus vulgaris* (common bean) α-AI must

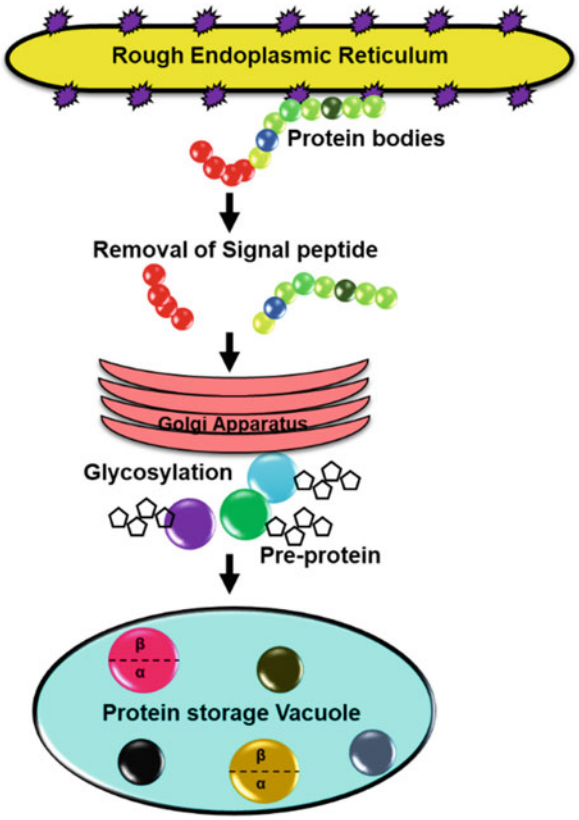

Fig. 3.3 Diagrammatic representation of the synthesis of proteinaceous α-AIs in plants

be proteolytically processed to be a biochemically active molecule (Pueyo et al. 1993; Maczó et al. 2015). Such bioactive inhibitory proteins have balanced amino acid composition so that some members of these proteins contribute major reserves for seedling growth and they act as storage protein rather than defending role by accumulating at a significantly high level in the seed grains (Altenbach et al. 2011). Most α-AI proteins/peptides have specific groups of conserved amino acid sequences, for example, common conserved cysteine residues are C-X_n-C-X_n-C-X_n-CC-X_n-C-X_n-C-X_n-C-X_n-C (Sharma et al. 2012) and they have the ability to slow down α-amylase activity. Generally, based on tertiary structure, molecular weights, amino acid compositions, and specific characterizations the proteinaceous α-AIs are classified into seven different classes like (a) Lectin-like, (b) Knottin-like, (c) Cereal-type, (d) Kunitz-like, (e) Thaumatin-like, (f) γ-purothionin-like, and (g) *Streptomyces* "WRY" motif proteinaceous α-AIs (Franco et al. 2002; Svensson et al. 2004). Each family of α-amylase inhibitors shows particular specificity features (Bonavides et al. 2007). The classes and characteristics of α-AIs are individually summarized in Table 3.1.

Table 3.1 Classes of proteinaceous α-amylase inhibitors (modified from Franco et al. 2002)

Class	Amino acid range	Source	Special characteristics	Structural specifications	References
Lectin like	240–250	Legume plants	1. Stable at wide ranges of pH, concentration, and time.	For example, α-amylase inhibitor from *P. vulgaris* (α-AI1) revealed to have two non-covalently bound glycopeptide subunits, α and β	Hansawasdi et al. (2001), Yamada et al. (2001), Nielsen et al. (2003), Jayaraj et al. (2013)
			2. Affinity to bind glycoproteins.		
			3. Toxic and homologous to Concanavalin A and phytohemagglutinin like lectins/glucanase.		
Knottin-type	30–100	Not specific	1. The high content of proline and a conserved cis-proline and contains a knottin fold.	For example, α-amylase inhibitor from *A. hypocondriacus* seeds (AAI) revealed to have a knottin fold; three antiparallel β strands and a characteristic disulfide topology	Pereira et al. (1999), da Silva et al. (2013), Chagolla-Lopez et al. (1994), Nguyen et al. (2014)
			2. Homologous to as the PI from *Cucurbita maxima*, gurmarin, charybdotoxin, funnel-web spider toxin, and conotoxins.		
Cereal-type	120–160	Cereals	1. Bifunctional α-amylase/trypsin inhibitor.	For example, Ragi bifunctional α-amylase/trypsin inhibitor (RBI), structurally stable monomer of 122 amino acids with 5 disulfide bonds, with a globular fold with four α helix in a simple up-and-down topology and a small antiparallel β sheet	Campos and Richardson (1983), Barber et al. (1986), Strobl et al. (1995), García-Casado et al. (1996), MacGregor et al. (2000), Shewry and Halford (2002), Svensson et al. (2004)
			2. Homologous to lipid transfer protein, seed storage 2S albumin.		
			3. Soluble in chloroform/methanol solvents.		
			4. Cold tolerance and allergens proteins.		
			5. Wide range strong inhibitory potential, with α-amylases of, birds, bacteria, insects and mammals.		

(continued)

Table 3.1 (continued)

Class	Amino acid range	Source	Special characteristics	Structural specifications	References
Kunitz-like	~180	Cereals	1. The inhibitor contains four cystine residues. 2. Bifunctional double-headed inhibitor of α-amylase/subtilisin. 3. Does not interact directly with any catalytic acidic residues of the enzyme.	For example, structure of barley α-amylase/subtilisin inhibitor (BASI), revealed to have two disulfide bonds and a β trefoil topology	Ohtsubo and Richardson (1992), Nielsen et al. (2004), Alves et al. (2009)
Thaumatin-like	>200	Cereals	1. Heavily populated with Arg and Lys residues. 2. Homologous to the sweet protein thaumatin and osmotin and used as antifungal drugs. 3. From the family of pathogenesis-related group 5 (PR-5) proteins.	For example, a bifunctional inhibitor from *Zea mays* is zeamatin, revealed to have total of 13 β strands, 11 of which form a β sandwich at the core of protein. Many of loop extended from core of inhibitor and covered by one or eight sulfide bonds	Vigers et al. (1991), Hejgaard et al. (1991), Schimoler-O'Rourke et al. (2001), Roberts DW and St Leger (2004), Heidari et al. (2005)
γ-Purothionins like	47–50	Cereals	1. Sulfur rich, small, stable, and cationic proteins. 2. Homologous to scorpion toxins proteins. 3. Involved in plant defense mechanisms, such as inhibition of protein synthesis, proteinase inhibition, change of membrane permeability. 4. Strongly inhibit insect, bacterial, and fungal α-amylases.	For example, *Sorghum bicolor*-α-amylase inhibitor SIα-1 revealed to have an α + β sandwich structure that creates an amphipathic molecule. The helix is held in place by two disulfide bridges, which link sequential turns of the helix to β3 strand. Such helix also called cysteine-stabilized helix (CSH) motif	Bloch and Richardson (1991), Thevissen et al. (1996), Melo et al. (1999), Wijaya et al. (2000), Farias et al. (2007)

Lipid transfer type	90–94	Various	1. Antimicrobial peptides. 2. Cluster into two multigenic families, i.e. LTP1 (9 kDa) and LTP2 (7 kDa). 3. Composed of small, basic peptides (isoelectric point (pI) 8–10).	Arranged in a three-dimensional structure formed by a bundle of four to five α-helixes and ahydrophobic cavity for loading a large variety of lipids or hydrophobic molecules but not sterol	Gomar et al. (1998), Kader (1997)
Streptomyces WRY motif containing	Not specific	Microbes	1. Conserved disulfide topology. 2. Cysteine rich motifs present in it 3. Active site mostly contains tryptophan, arginine, and tyrosine (WRY) triad. 4. Specific as a mammalian α-amylase inhibitor.	AAI-CC5 from genus *Streptomyces* contains two rings formed by four cysteine residues at identical position. The "WRY" motif in the middle of the ring was located in a spatially exposed position, which was responsible for the interaction with a-amylase	Sun et al. (2015)

3.6 Mechanism of Interaction of α-AIs with Amylases

The α-amylases from diverse sources contain varying amino acids at the catalytic sites and allosteric sites for the interaction with inhibitory molecules. Enormous post-translational changes are occurring in inhibitors for activation and corresponding interaction with enzymes because α-AIs have interaction specificity with specific amylases only. A number of reports are available suggesting the reaction mechanism involved in the inhibition of α-amylase by α-AIs though the exact mechanism is not yet known (Jayaraj et al. 2013). Some reports in the literature suggest that glycan moieties of glycoprotein α-AI are covalently/non-covalently bound to the enzyme and may play a major role in conformational changes in the enzyme molecules (Shamki et al. 2012).

Mutational studies are advantageous to gain knowledge of inhibitory mechanisms. Mutations were created in a particular inhibitory domain(s) and their effect on inhibitory activity was analyzed (Giri et al. 2016). The molecular insight study proposed that inhibitor–enzyme binding could occur through a variety of factors such as steric factors, electrostatic properties, direct hydrogen bonding or water network, or combinations of the special conformational properties of proline and the disulfide bonding of cysteine (Pereira et al. 1999; Franco et al. 2000; Rodenburg et al. 1995; Svensson et al. 2004). For example, in BASI–AMY 2 interaction study Ser77, Tyr131, Lys140, and Asp150 form different hydrogen bonds with the active site of AMY 2 and create an extended hydrogen bond network (Bønsager et al. 2005). Likewise, the cysteine knot, Cys44-Cys90 and Cys144-Cys148 conserved region with some disulfide bridges are observed to be important in BASI like amylase inhibitor for protein–protein interaction (Nielsen et al. 2004). In the structure of amylase, the amino acid Arg19 of conserved region triad of Trp18-Arg19-Tyr20 and disulfide-stabilized β-turn make the site to interact tendamistat inhibitor which is a responsible factor in inhibition (Vértesy et al. 1984; Jayaraj et al. 2013).

Additionally, non-proteinaceous inhibitors like acarbose, isoacarbose, acarviosine-glucose, hibiscus acid, and cyclodextrins cause inhibition by mimicking as substrate (Cyclic rings) which can fit followed by binding at the catalytic or active site of an enzyme and block its activity. For example, the binding study of acarbose to *C. chinensis* and *T. castaneum* amylases revealed that acarbose mimics the substrate and hydrogen bonding network is involved in requisite interaction (Channale et al. 2016) (Fig. 3.4).

Likewise, at the amino acid triad (i.e. Try59, Asp197, and Glu233) of bean (*P. vulgaris)* α-AI interacted with human pancreatic amylase (HPA) at V-shaped depression (shape of interacting site) binding site. Similarly, α-AI from *P. vulgaris* (α-AI1) interacts with human pancreatic amylase (HPA) at V-shaped depression at amino acids triad (Try59, Asp197, and Glu233). In this complex, two β sheet fold created loops containing Tyr37 and Tyr186 of α-AI1 lie fully in the V-shaped depression of HPA and interact by hydrogen bond network by nucleophile acid catalysis (Payan 2004).

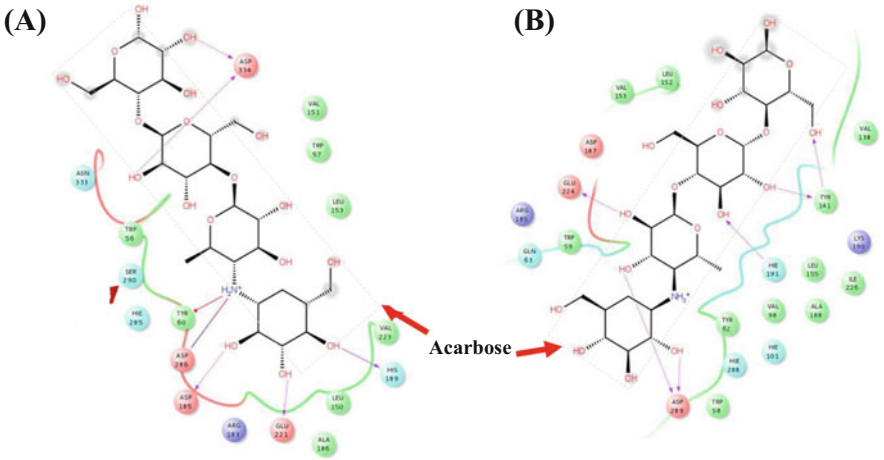

Fig. 3.4 Interaction of acarbose with amylases from *C. Chinensis* and *T. Castaneum.* Hydrogen-bonded interactions with valienamine unit of acarbose are represented using purpledotted arrows: where (**a**) interaction of acarbose with *C. chinensis* amylase and hydrogen bonding network involved, (**b**) interaction of acarbose with *T. castaneum* amylase and hydrogen bonding network involved. (Adapted from Channale et al. 2016)

3.7 Extraction and Purification Strategies for α-AI

Literature survey indicates that there are ample reports available on α-AIs from plants. Extraction is the first step in the purification of proteinaceous α-AIs which includes different conditions of temperature, pH, salts concentration, metal ions, and some chemical agents like zinc hydroxide for selective adsorption of protein. Poly vinyl pyrrolidone (0.1–1.0%) is used for plant protein chelation by precipitating polyphenols (Ambekar et al. 1996; Maczó et al. 2015). Extraction of AI was done, (1) at high temperature (65 °C) to denature interfering enzymes from (a) wheat flour (Chen et al. 2008); (b) Baru seeds (Bonavides et al. 2007); (c) Flamboyant seeds (Alves et al. 2009); and (d) seeds of common beans (Wang et al. 2011), (2) at low temperature (4 °C) from seeds of *W. somnifera* (Kasar et al. 2017), and (3) at high salt concentration (NaCl solution 1%) from *Tendergreen bean* and genetically modified pea (Maczó et al. 2015). Pinto bean α-AI protein extraction was enzyme-assisted, i.e. Protamex (containing both enzyme and substrate) (Ngoh and Gan 2016).

Screening of α-AI proteins for amylase inhibitory activity from various biological materials is an important step after extraction, involving various methods such as ultrafiltration, surface plasmon resonance, cell membrane chromatography, cross-linked enzyme aggregates, functionalized magnetic nanoparticles, etc. (Wang et al. 2011; Li et al. 2014). Magnetic cross-linked enzyme aggregates—amylase particles were used to screen α-AI protein from *Corni fructus* (Liu et al. 2015). Recently, two rapid approaches capable of screening potential α-AIs are reported: (a) UPLC-DAD-

TOF-MS/MS-based metabolomic method to screen α-AI from *Rhodiola rosea* (Ma et al. 2017) and (b) Integrated bioinformatics-phage display approach for screening and identification of α-AI peptides from cumin seed (Siow and Gan 2017).

Purification and isolation of α-AIs from various sources such as plants, animals, and microbes based on bioassay-guided fractionation is a preferred technique. It includes many different methods such as affinity, ion exchange, and gel filtration chromatography, reverse phase HPLC (RP HPLC), two-phase, three-phase solvent system, GELFREE® 8100 System, preparative gel electrophoresis (PGE), and native liquid-phase isoelectric focusing (N-LP-IEF) (Senthil et al. 2015). These methods are used in two- or three-step combinations one after another with slight modification in solvents and temperature to achieve targeted purification. Selective purification of α-AI has been carried out by affinity chromatography which is ligand-based analytical technique involving the use of different matrices such as Red-Sepharose CL-6 (Bonavides et al. 2007), Seralose 6B-Agarose based matrix (Saxena et al. 2010), and Cu^{2+} Hi trap chelating matrix (Iimure et al. 2015).

Ion exchange chromatography separates proteins on the basis of anionic or cationic charges present on resins, viz. DEAE sepharose, DEAE-Cellulose, SP-Sepharose (Alves et al. 2009), DEAE Sephacel (McEwan et al. 2010), DEAE-Sephadex A_{50} (Kumari et al. 2012), etc. Gel filtration involves purification of protein on the basis of sizes and through molecular sieving using resins such as Sephadex-G-50/75/100/200 or Biogel P-50/100 (Kluh et al. 2005). Repetitive fractionation with RP HPLC is a very popular technique documented in many reports by varying column matrices such as polyamide resin 100–200 mesh (Li et al. 2014) and Vydac C-18-TP-522 (Meng et al. 2011; dos Santos et al. 2017). Large-scale α-AI purification is processed with aqueous two-phase systems of polyethylene glycol (PEG)/$(NH_4)_2SO_4$, PEG/dextran and PEG/fructose-1,6-bisphosphate (FBP) (Chen et al. 2008).

Three-phase partitioning (TPP) is time saving purification method in which salting out, isoionic precipitation, cosolvent precipitation, osmolytic and osmotrophic precipitation type of collective operations are included. TPP is performed by addition of a salt-like ammonium sulfate to the crude extract followed by the addition of an organic solvent like *t*-butanol (Wang et al. 2011) and using this method significant separation of ragi (*Eleusine coracana*) bifunctional amylase/protease inhibitor proteins has been reported (Saxena et al. 2010). GELFREE 8100 fractionation system increases α-AI protein recovery by purifying intact proteins over a broad range on the basis of molecular weight and its physicochemical properties (Witkowski and Harkins 2009). N-LP-IEF purification technique is used for α-AI proteins which are insoluble or not separable on gel based IEF media. The separation of α-AI proteins with this technique is based on the native state isoelectric point of protein (pI) (Hey et al. 2008; Gadge et al. 2015). Screening and purification strategies employed for different α-AIs are summarized in Table 3.2.

Table 3.2 Purification strategies and biochemical characteristics of proteinaceous α-amylase inhibitors

Source	Tissue	Purification			Mol. wt.	Source α-amylase used to check activity inhibition	Stability		References
		Method	Fold purification	% Yield			pH	Temp. (°C)	
Achyranthes aspera	Plant seeds	Heat treatment–membrane filtration–DEAE IEC	9.9	–	6	*C. maculatus, T. confusum, H. armigera*	–	30–75	Hivrale et al. (2011)
Colocasia	Plant tubers	Sephadex G-200 GFC–DEAE-Sephadex IEC	17.2	61.6	13.9	*C. chinensis, T. castaneum, C. cephalonica*	6.9	40–70	Kumari et al. (2012)
Carica papaya	Plant seeds	Acid crude–ammonium sulfate precipitation–CM cellulose IEC–RP HPLC	–	–	4.28	*C. maculatus*	–	–	Farias et al. (2007)
Capsicum annuum	Plant seeds	RP HPLC	–	–	9	Mammalian	–	–	Diz et al. (2011)
Coffea canephora	Plant seeds	DEAE IEC–RP HPLC	–	–	9	Mammalian	–	–	Zottich et al. (2011)
D. esculenta	Tubers	DEAE-cellulose IEC–repeated DEAE-cellulose IEC	7.6	49	16	*Coffee bean* and *Sweet potato weevil*	7	40–70	Sasikiran et al. (2004)
Dipteryx alata	Plant seeds	Red-Sepharose CL-6B AC–RP HPLC (Vydac C-18TP 522)	–	–	14.8	*A. grandis, C. maculatus, A. obtectus,* Porcine pancreas	–	–	Bonavides et al. (2007)
Eleusine coracana	Plant seeds	AC seralose 6B-sulphopropyl IEC–Sephadex G-100 GFC	7.0	61	14	Porcine pancreatic, *C. chinensis*	5–8	30–40	Saxena et al. (2010)
Leucas aspera	Whole plant	IEC–FPLC	239	0.049	28	Mammalian, Maize, *B. subtilis, A. oryzae*	4–11	10–70	Meera et al. (2017)

(continued)

Table 3.2 (continued)

Source	Tissue	Purification			Mol. wt.	Source α-amylase used to check activity inhibition	Stability		References
		Method	Fold purification	% Yield			pH	Temp. (°C)	
Mucuna pruriens	Plant seeds	CM cellulose IEC–GFC sephadex G-75	36.6	14.02	25.6	Human salivary α-amylase	6.9	40–65	Bharadwaj et al. (2018)
Pigeon pea	Plant seeds	Native liquid-phase isoelectric focusing (N-LP-IEF)–AFC–preparative electrophoresis	–	–	56	*H. armigera*	4–11	Upto 80	Gadge et al. (2015)
Phaseolus vulgaris	Plant seeds	DEAE-cellulose IEC–GFC on Sephadex G-75	11.8	57.69	30	Mammalian	2–10	20–100	Ali Asgar (2013)
Streptomyces sp. *CC5*	Cells	DEAE-Sephadex IEC–GFC Superdex 75 gel	59.3	5.28	8.21	Human pancreatic amylase, porcine pancreatic amylase	2–10	40–80	Sun et al. (2015)
Triticale seeds	Plant seeds	95% ethanol extraction–GFC Superdex 75–IEC DEAE-cellulose	6.0	4.9	29.2	*Eurygaster integriceps* Human salivary α-amylase	6–10	Upto-98	Mehrabadi et al. (2011)
Triticum aestivum	Plant seeds	DEAE-Sephadex	3.2	–	20.0	Human salivary, porcine pancreatic, chick pea	–	–	Saunders and Lang (1973)
Vigna radiate	Plant seeds	GFC–RP HPLC	750	0.11	27	*C. maculatus*	–	–	Wisessing et al. (2010)
Vigna sublobata	Cotyledon	Sephadex G-50 GFC–C18 column RP HPLC	3.8	55.17	14	Larvae of *C. analis*	–	–	Kokiladevi et al. (2005)
Vigna unguiculata	Plant seeds	SP-sepharose IEC-HPLC	9.3	34.1	25.1	*C. maculatus*, *A. obtectus*, *A. grandis*, *A. oryzae*	–	–	Alves et al. (2009)
Zea mays	Plant seeds	95% ethanol extraction–anionic IEC	13.9	56.2	19.7	*A. obtectus*, Porcine pancreas, Barley alpha-amylase	6–7	50–90	Figueira et al. (2003)

3.8 Biochemical Characteristics of α-AIs

3.8.1 Temperature and pH Stability

Molecular interaction studies have uncovered the knowledge of different types of inhibitors—amylases relationships and their interconnections. The interaction of α-AI with different α-amylases is generally dependent on pH and temperature. Analytical methods such as nuclear magnetic resonance (Senthil et al. 2015), circular dichroism spectra (Sokočević et al. 2011; Gupta et al. 2013), and ultra-performance liquid chromatography (UPLC) (Nguyen et al. 2014) are especially important in detecting the role of pH and temperature on conformational changes in α-AI.

(a) **pH optima of α-AIs**

The inhibitory potential of α-AI can be modulated by pH and the optimum pH value of different α-AI ranges between pH 4 and 11. pH dependent inhibition of α-amylases by α-AI noticeably drops down outside its optimum pH value. Ionizable groups in arginine, lysine, glutamine, and asparagine amino acids of α-AIs help balance of charge present on them, which decide its proper confirmation at a particular pH value. The binding of α-AI-1 isolated from *P. vulgaris* to mammalian α-amylases was dependent on pH, it formed the complex at pH 4.5 or 5.5 but not at pH 6.9 (Kluh et al. 2005). Coleopteran insect gut (e.g. Tenebrionidae, Curculionidae, Cucujidae) contains pH gradient profile (acidic to alkaline pH), so it may cause complex-forming pH shifting (Terra and Ferreira 1994). Especially, acidic pH value improves the inhibitory activity of α-AI through its firm interaction with insect α-amylases. α-AI from *C. esculenta* (CeAI) was observed to have optimal activity at pH 4.0 and 6.0 (McEwan et al. 2010) and α-AI-1 from *P. vulgaris* showed optimal inhibition after its preincubation at acidic pH (Kluh et al. 2005). The highest inhibitory effect of *Triticale* seeds α-AI (TAI) was seen at pH 5.0 (Mehrabadi et al. 2011), while other α-AIs like *D. alata* AI (Sasikiran et al. 2004), α-AI from local Himalayan collections of *Colocasia* (CAI) (Kumari et al. 2012), *Cajanus cajan* α-AI (CcAI) (Gadge et al. 2015), *W. somnifera* AI (WSAI) (Kasar et al. 2017) were found to have significant inhibitory activity at neutral pH (6.9–7.2).

(b) **Thermal stability of α-AIs**

Proteinaceous α-AIs are well known for their thermostable inhibitory nature. Change in temperature changes binding behavior of inhibitor with an enzyme. Whereas CcAI demonstrated its maximum activity up to 60 °C (Gadge et al. 2015). CeAI (McEwan et al. 2010) and CAI (Kumari et al. 2012) inhibitory activities were retained up to 70 °C. Further, temperature increased inhibitory activity loss was observed. α-AIs from *D. alata* and *Wrightia religiosa* (Wr-AI-1) demonstrated high thermal stability by remaining active at 98 and 100 °C, respectively (Sasikiran et al. 2004; Nguyen et al. 2014).

3.8.2 Kinetic Behavior

Kinetic study indicates the behavior of inhibitor with the binding site of an enzyme. Amylase has higher catalytic efficiency with starch than other sugars such as amylose and amylopectin (Kaur et al. 2014). Non-competitive type of inhibition involves the binding of inhibitor at a site other than the active site and brings conformational changes in active site. Competitive inhibition includes the binding of inhibitor at an active site when [I] > [S]. The mixed type of inhibition shows some properties of competitive and some properties of non-competitive inhibition. The interacting affinity of an inhibitor to the enzyme at active or non-active site determines the value of K_i, which is calculated using the Dixon plot (Dixon 1953). Nowadays, analysis of the kinetic study of α-AI with α-amylase involves the use of various computational software to plot graphs (LB, Dixon) and calculate exact values of $K_m/K_i/K_{app}$. Computational software such as Graph Pad Prism 5 for Windows (Graph Pad Software, San Diego, CA, USA), Sigma plot Software (Bengaluru, India), Matlab software are routinely used. Kinetic study of Barley α-amylase/subtilisin inhibitors, viz. sBASI and rBASI with barley α-amylase isozyme 2 (AMY-2) was observed to have variations in K_i values as a function of change in the substrate used for hydrolysis. When insoluble blue starch is hydrolyzed by AMY2, then K_i value was 0.10, 0.06, 0.09 nM for rBASI (intein), His6-rBASI, and sBASI, respectively. Conversely, when amylose DP17 was used as substrate and hydrolyzed by AMY-2, then K_i was 0.10, 0.12, and 0.11 nM for rBASI (intein), His6-rBASI, and sBASI, respectively (Bønsager et al. 2003).

3.8.3 Glycoprotein Nature

Glycosylation of α-amylase inhibitor protein is completed by post-translational modifications which has importance in various biological processes. In case of white kidney bean α-AI, α-chain contained 30% carbohydrate with high mannose-oligosaccharides and β-chain contained 7% sugar moiety mainly composed of xylomannose-type oligosaccharides (Yamaguchi 1993). The glycan moieties of glycoprotein amylase inhibitors help in holding the protomers of alpha-amylase together (Obiro et al. 2008). Glycan moieties added through N-glycosylation to amylase inhibitor glycoproteins do not influence the activity of the inhibitor (Sawada et al. 2002).

Possible amylase inhibitory mechanisms of glycoproteins are (a) glycoprotein α-AI bind at glycan-binding sites or regulatory sites of α-amylase resulting in inhibition and (b) indirect interaction of glycoprotein and glycan receptors on the intestinal/enzyme surface due to which enzyme in the gut get degraded by proteolysis resulting in antinutritional activity (Matsushita et al. 2002; Gadge et al. 2015). Amylase inhibitor gene from *Phaseolus coccineus* requires glycosylation processing for activation of its inhibitory efficiency (Pereira et al. 2006). According to

Santimone et al. (2004), the branched glycans linked to specific amino acids in inhibitor protein create steric conflict between two facing dimers which may result in the avoidance of the formation of tetrameric structures. Phytohemagglutinin (PHA) is a lectin that binds to the glycans on the glycoproteins of the intestinal epithelium of animals and acts as a mitogen. α-AI-1, a dimeric glycoprotein is reported from *P. vulgaris* (Berre-Anton et al. 1997) and a monomeric glycoprotein is reported from Himalayan collections of *Colocasia* (Kumari et al. 2012). Similarly, α-AIs from *Colocasia* tubers (Sharma and Pattabiraman 1980), *D. alata* (Shivaraj et al. 1979), and wheat (Petrucci et al. 1978) have been reported as glycoproteins amylase inhibitors. Recombinant α-amylase/subtilisin inhibitor from *Hevea brasiliensis* is also reported to contain glycosylated loci (Bunyatang et al. 2016).

3.9 Allelic Variants of AIs from the Same Species

A variety of isoforms of AIs may be present in single species of plant or microbes which can be classified on the basis of molecular structure, allelic site/gene encoded site on chromosomes, molecular weight, special characteristics, and interaction specificity. For example, *Triticum aestivum* (Wheat) seeds have various α-AIs as 0.19, 0.28, 0.53 so named on the basis of gel electrophoretic mobility (Goff and Kull 1995). From these, monomeric 0.28 inhibitor proteins are encoded by genes on the short arms of the group 6 chromosomes, while the homodimer 0.19 and 0.53 inhibitors proteins are encoded by genes on the short arms of the group 3 chromosomes. Similarly, three allelic variants of lectin-like α-AI are found in most of several bean varieties named as α-AI-1, α-AI-2, and α-AIL (Lajolo and Finardi Filho 1985; Young et al. 1995). These α-AIs have differential interaction patterns or binding specificity with various α-amylases. α-AI-1 inhibits larval gut α-amylase of adzuki bean weevil and cowpea weevil (*C. maculatus*) as well as mammalian α-amylase (Ishimoto and Kitamura 1989), while α-AI-2 specifically inhibits the larval α-amylase of *Z. subfasciatus* (Minney et al. 1990; Ishimoto et al. 1995). Whereas α-AIL, interestingly, is just an amylase inhibitor like insecticidal protein completely inactive towards all α-amylases tested (Finardi-Filho et al. 1996). The homologous isoforms of α-AI-1, α-AI-2 are α-AI-Pa1and α-AI-Pa2, isolated from tepary bean, also have differential inhibition specificities. α-AI-Pa1 inhibited the α-amylase activity of *C. chinensis* but not porcine pancreatic α-amylase, while α-AI-Pa2 inhibited the α-amylase activity of *Z. subfasciatus* and to *C. chinensis*. Isoforms isolated from *Sorghum bicolor*, namely Slα-l, Slα-2, and Slα-3 have amino acid sequence homology with γ-purothionin like cysteine knot protein (Bloch and Richardson 1991).

3.10 Biological Applications of α-AIs

Plants have the capability to produce certain bioactive enzyme inhibitory proteins/peptides which are useful in designing scientific tools with specialized applications in biotechnology. The specificity of the inhibitor is one of the critical factors that need to be considered while adopting the inhibitor for various applications. The desired characteristics are (a) inhibitor should not adversely affect the own amylases of plants or humans and (b) it should not alter the nutritional value of the crop (Franco et al. 2002; Svensson et al. 2004). There are a lot of reports available on various biological applications of α-AIs (Fig. 3.5). Enzyme inhibitory bioactive molecules could be useful tools for treating metabolic disorders (obesity and diabetes mellitus), in seeds they act as storage or reserve proteins and in another strategy to combat insect pests as they are used to reduce insect growth by interfering with carbohydrate absorption (Yamada et al. 2001).

3.10.1 Pesticidal Applications of α-AIs

In the majority of the plants, biotic stress induces molecular, cellular, and biochemical changes in the expression of defense related bioactive molecules such as proteinaceous inhibitors of enzymes, α-amylases, proteinases, lipases, acetylcholine esterase as well as toxic proteins like phyto lectins, amino acid deaminases, chitinases, oxidases, antimicrobial peptides, and ribosome-inactivating proteins (RIPs) of types 1and 2 (Ryan 1990; Bowles 1990; Peumans and Van Damme 1995; Koiwa et al. 1997; Howe and Jander 2008). Production of such defensive biochemicals is the step carried out followed by surveillance and signal transduction (Dangl and McDowell 2006). In the primary step of plants' surveillance system signals of pest attack are detected which then are transduced via linked signal transduction pathways eventually resulting in the synthesis of induced defensive bioactive molecules (Kaloshian 2004; Smith and Boyko 2007; Chen 2008). A wide array of such defensive compounds concentrates constitutively or after induction in the plants and play a crucial role in the propagation and survival of the plant species by fighting against phytophagous predators and infections caused by viruses, bacteria, fungi, nematodes, etc. (Carlini and Grossi-de-Sá 2002).

A number of studies demonstrated that these metabolites and proteins have crucial functions in the protection of the plant tissue from damage being a part of the natural biochemical defense machinery of plants (Ryan 1990; Schaller and Ryan 1995; Conconi et al. 1996). These proteins are the first line of defense or direct response which leads to food supply limitation, nutrient value reduction, physical structures disruption, preference reduction, and chemical pathways inhibition of stress causing parasites (Chen 2008). Many of insect pests' early developmental stages (larval and/or adult) are dependent on starchy food material to get basic metabolic energy through the digestion of starch by highly active α-amylase from

their gut. When digestive α-amylase is inhibited by inhibitor, insect pest nutrition and dependent metabolism gets hampered due to diminished energy supply (Carlini and Grossi-de-Sá 2002). Thus, α-AIs are more lethal when their efficacy towards the insect pests is compared to harmful effects caused by proteinase inhibitors (Gomes et al. 2005; Bonavides et al. 2007). Such natural resistance by the host plant is an efficient and favorable method for controlling the insect pest and is also harmless to the non-target useful living systems (Andow 2008). Natural resistance develops in plants due to an external physical or chemical stimulation by insects (Smith 1999) and is observed in plant taxa like Brassicaceae, Chenopodiaceae, Leguminosae, Malvaceae, Pinaceae, Salicaceae, and Solanaceae (Smith 1999). α-AI proteins with wide specificity, biochemical stability, and broad-spectrum activities are strongly recommended to be used in insect control strategies (Franco et al. 2002). Inhibition of various insect amylases by different types of plant α-AI reported for their pesticidal applications is summarized in Table 3.1.

(a) **Pesticidal activities of α-AIs on coleopteran stored grain pests**

Bruchid weevils, feeding on stored grains are required to produce a considerable number of α-amylases to digest stored starch or other carbohydrates for energy assimilation. The use of α-AIs is relevant towards the coleopteran seed herbivorous insects because it is not inactivated or digested as insects of coleopteran class have neutral or acidic gut pH (Gatehouse 2008). Amylases of this class of insects are more sensitive for the binding of inhibitor protein. In vivo deleterious effects of AI were observed as far as insect mortality is concerned. *C. maculates* larval mortality was reported in various studies, particularly α-AIs from *D. regia* (Alves et al. 2009), *C. papaya* (Farias et al. 2007) reported to have

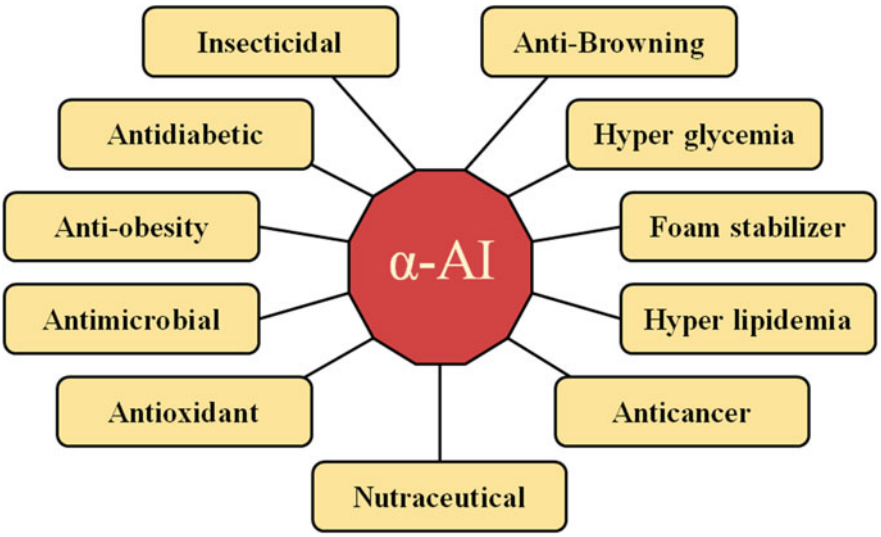

Fig. 3.5 Biological applications of alpha-amylase inhibitors

85% and 50% larval mortality, respectively. Recently, the recombinant α-AI from *A. hypochondriacus* treatment hindered the growth, development, and adult emergence of *C. chinensis* (Bhide et al. 2017). The effect of plant α-AI on the amylase of insects from this class such as *Acanthoscelides obtectus*, *Araecerus fasciculatus*, *Bruchus pisorum*, *C. maculatus*, *C. chinensis, Tenebrio molitor, T. castaneum, Zabrotes subfasciatus,* and *Sitophilus oryzae* was reported extensively in previous studies and is summarized in Table 3.3. The detrimental effect occurred to this class of insects by the AI treatment was mostly measured in the form of fecundity, emergence, developmental time, longevity, larval weight, and reproduction ability.

(b) **Larvicidal effects of α-AIs on lepidopteran pests**

While studying interactions between α-AIs and lepidopteran amylases, researchers found initial upregulation of amylase expression when treated with inhibitor protein. This upregulation upon exposure to inhibitor may be attributed to an adaptive response to overcome the digestive enzyme inhibition observed in *Helicoverpa armigera* (Gadge et al. 2015). Likewise, upregulation pattern was reported by Kotkar et al. (2009), wherein they reported increase in gut α-amylase activity of larvae maintained on inhibitor protein containing pigeon pea as compared to other natural diets. α-AIs effective against lepidopteran insects such as *Helicoverpa armigera, Anthonomus grandis, Mythimna separate, Corcyra cephalonica, Sitotroga cerealella,* etc. have been reported previously (Giri et al. 2016). The effects of inhibition of amylases of this class of insects were evaluated by observing their growth rate, weight loss, larvicidal percentage, survival rate, and effect on next generation insects.

3.10.2 Antimicrobial Activity of α-AIs

Low molecular weight α-AI peptides with antimicrobial action (α-AI AMPs) are effective molecules against a variety of microorganisms including viruses, bacteria, filamentous fungi, and yeast (dos Santos et al. 2017). AMPs synthesized byplants act as key constituents of their innate immunity (Silva et al. 2011) and have been purified from flowers, leaves, fruits, tubers, roots, and especially seeds of various plant species (Pelegrini et al. 2008; Nawrot et al. 2014). Lipid transfer proteins like α-AIs have an antimicrobial role to secure plant from the attack of microbial phytopathogens like *Fusarium solani, Fusarium oxysporum, Pythium aphanidermatum, Sclerotium rolfsii, Pyricularia oryzae, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Pseudomonas syringae, Clavibacter michiganensis* subsp. *sepedonicus, and Ralstonia (Pseudomonas) solanacearum* (Segura et al. 1993; Regente and de la Canal 2000; Wang et al. 2004; Diz et al. 2011). Zottich et al. (2011) reported that the isolated, cysteine rich lipid transfer α-AI from seeds of *Coffea canephora* protects crops from the microbial pathogen, *Candida albicans* attack. AI from *Hevea brasiliensis* (HbASI) was reported to regulate the growth of

Table 3.3 Insecticidal potential of various types of plant-derived proteinaceous α-amylase inhibitors

AI class	Name of AI	Source species	Insect amylase inhibited	References
Cereal-type	0.28, WRP25, WRP26, and WRP27	*Triticum aestivum*	*Sitophilus oryzae, Tribolium castaneum, Tenebrio molitor, Callosobruchus maculatus, Zabrotes subfasciatus*	Feng et al. (1996)
	1, 2, and 3 BIII	*Secale cereale*	*Zabrotes subfasciatus, Acanthoscelides obtectus*	Iulek et al. (2000)
	FMCO11 and FMCO13	*Eleusine coracana*	*Acaeajanata, C. cephalonica, Sitophilus oryzae, Tribolium castaneum*	Sivakumar et al. (2005)
Knottin	α-AIC3, α-AIA11, α-AIG4	*Phaseolus vulgaris*	*Acanthoscelides obtectus*	da Silva et al. (2013)
	AAI	*Amaranthus hypochodracus*	*Prostephanus truncatus*	Chagolla-Lopez et al. (1994)
	Wrightide/ Wr-AI1	*Wrightia religiosa*	*Tenebrio molitor, Tribolium castaneum*	Nguyen et al. (2014)
Kunitz	WASI	*Triticum aestivum,*	*Rhyzopertha dominica*	Mundy et al. (1984)
	BASI	*Hordeum vulgare*	*Tribolium castaneum*	Mundy et al. (1983)
	RASI	*Oryza sativa*	*Tribolium castaneum*	Ohtsubo and Richardson (1992)
	CAI (RSII fraction)	*Vigna unguiculata*	*C. maculatus* larvae	Melo et al. (1999)
Lectin	a-AI1, a-AI2	*Phaseolus vulgaris*	*C. maculatus, C. chinensis, Z. subfasciatus*	Marshall and Lauda (1975), Ho and Whitaker (1993)
Purothionin	SI-α-1, SI-α-2, ST-α-3	*Sorghum bicolor*	*Periplaneta americana, Locusta migratoria*	Bloch and Richardson (1991)
Thaumatin	Zeamatin	*Zea mays*	*Tribolium castaneum, Sitophilus zeamais, Rhyzopertha dominica*	Blanco-Labra and Iturbe-Chinas (1981), Schimoler-O'Rourke et al. (2001)

Phytophthora palmivora when observation was recorded from zoospores germination to mycelium growth (Bunyatang et al. 2016). Heibges et al. (2003) have examined the inhibition of mycelial growth of *Fusarium moniliforme* by different Kunitz group inhibitors and revealed that the growth of mycelium is differentially

affected. Fungal cell wall altering thaumatin-like amylase inhibitor proteins are grouped into pathogen-related proteins 5 (PR-5). For example, α-AI zeamatin is used as antifungal drug which binds to a cell wall containing α-1,3-glucan to make fungal cell wall permeable resulting in cell death (Roberts and Selitrennikoff 1990; Franco et al. 2002; Svensson et al. 2004). Recently, treatment with α-AI flavonoids isolated from *Morus alba* L. leaves suppressed the growth and development of *Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus pumilus* in vitro (Cui et al. 2019). Silver nanoparticles synthesized using extracts of *Pleurotus giganteus* (edible mushroom) having α-AI activity demonstrated antibacterial potential against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* (Debnath et al. 2019).

3.10.3 Human Health Management Related Applications of α-AI

Urbanization and associated changes in life-style are responsible for unwanted calorie accumulation in the body resulting in increased incidents of diseases/disorders such as obesity, diabetes, and other metabolic disorders. α-AI activity against mammalian α-amylases could cause a marked diminution in the accessibility of starch-derived/digested biomolecules (Ali et al. 2006). In the light of these facts, it is relevant to examine α-AIs in the perspective of their pharmaceutical or medicinal applications. α-AIs are practicable in the treatment of several aspects of human health-related diseases such as pancreatic hyperamylasaemia (Turcotte et al. 1994), in the treatment of metabolic disorders like diabetes, obesity, and hyperlipidaemia (Bischoff 1994) and other nutritional and toxicological aspects of food products (Carlini and Grossi-de-Sá 2002). Globally, there is a rampant increase in number of people affected with diabetes and pre-diabetes due to array of clinical factors like aging, smoking, unhealthy diet, hyperlipidaemia, and sedentary life-style (Wild et al. 2004; Rao et al. 2016). Table 3.4 represents the plant α-AI which has dual role as antioxidant and enzyme inhibitory (α-amylase/glucosidase/lipase).

3.10.4 Food Processing Industrial Application of α-AI

Along with rice, maize, and wheat, potato (*Solanum tuberosum* L.) is one of the major carbohydrate rich vegetable food crops globally (Liu et al. 2013). Because of its high carbohydrate content, post-hydrolysis by α-amylase, it serves as the main source of energy for living organisms. However, the released reducing sugars negatively affect potato food processing as they cause browning as well as acrylamide generation during frying (Biedermann-Brem et al. 2003; Kumari et al. 2012).

Table 3.4 Non-proteinaceous α-AIs extracts with the potential of inhibition of α-glucosidase, lipase, and oxidative stress related enzymes

Plant	Used part	Extract	Inhibitory activity (IC 50) (μg/mL)			Antioxidant activity	References
			α-Amylase	α-Glucosidase	Lipase		
Annona muricata	Leaf	Ethanol	25 μg mL	1470	–	28.1%	Justino et al. (2018)
Ascophyllum nodosum	Brown seaweed	Water	240	1340	–	60–70%	Apostolidis et al. (2012)
Chrysanthemum morifolium	Flower	Methanolic	112.5	229.3 μM	161.0 μM	–	Luyen et al. (2013)
Cicer arietinum	Seeds	Boiling water	167	2885	9.74	–	Bhagyawant et al. (2019)
Cissus quadrangulari	Whole plant	50% ethanol	2000	2000	2000	–	Sharp et al. (2007)
Crataeva magna	Leaf	Methanolic	143.5	143.5	–	5.2	Shori (2015)
Dichrostachys glomerata	Fruit	Aqueous	49.73%	48.64		87.58%	Etoundi et al. (2010)
Flacourtia inermis	Fruits	Ethyl acetate	1020 ppm	549 ppm	1290.92	66.20 ppm	Alakolanga et al. (2015)
Glycine max	Seeds	Acetone (30) + acetic acid (40) + water (30)	250	540	190	2978.55 μmol TE/g	Tan et al. (2017)
Macrotyloma uniflorum	Seeds	80% aqueous methanol containing 1% HCL (1:50 w/v)	96.4	63.2	–	22.9 μg/mL	Sreerama et al. (2012)
Nelumbo nucifera	Leaf	70% ethanol	380	2220	1860	–	Liu et al. (2013)
Phaseolus vulgaris	Seeds		670	250	170	5001.38 μmol TE/g	Tan et al. (2017)

(continued)

Table 3.4 (continued)

Plant	Used part	Extract	Inhibitory activity (IC 50) (μg/mL)			Antioxidant activity	References
			α-Amylase	α-Glucosidase	Lipase		
Ribes uva-crispa	Fruit	70% acetone	192	6336	1952	18.56 μmol TE/g	Podsedek et al. (2014)
Smallanthus sonchifolius	Foliar tissue	Methanolic	260	130	–	208	Russo et al. (2015)
Vigna unguiculata	Seeds	Methanolic	159.1	52.8	–	48.2	Sreerama et al. (2012)

Both the factors, in turn, adversely affect the saleability of processed potato products in the consumer market.

Browning of potatoes during processing is caused mainly by the enhanced action of enzymes like polyphenol oxidases, tyrosinases, proteases, and amylases which utilize oxidizing agents, free radicals, tyrosine, peptides, and oligosaccharides, generated during processing, as their substrates (Severini et al. 2003).

Increased enzyme activity leads to increased level of reducing sugars (glucose and fructose) which reacts with free amino acids (asparagine and glutamine) at high temperature (during frying) leading to generation of acrylamide—a potent neurotoxin and carcinogenic compound along with a characteristic roasted flavor and brown color due to Maillard reaction (Whitfield and Mottram 1992; Amrein et al. 2003; Ayvaz and Rodriguez-Saona 2015).

Cold induced generation of reducing sugars during storage leading to browning and generation of acrylamide during frying are the factors of concern in potato processing industry (Amrein et al. 2003). Whereas the first two factors cumulatively affect the organoleptic and physical properties (texture), acrylamide generation is associated with human health concern. One of the strategies to overcome these problem is to make use of compounds of chemical or biological origin which inhibit the activities of enzymes mentioned above. Many chemical inhibitors in various concentrations and combinations have been used and investigated for this purpose (Rocculi et al. 2007). Sulfite, a low cost anti-browning compound was popular in nineteenth century in the potato chips industry (Marshall et al. 2000). However, its use has to be discontinued because of a variety of reasons including reduction in nutritional value, softening, and addition of "off flavors" in potato chips along with detrimental health effects on consumers (Ma et al. 2017).

Regulation of starch degradation and, in turn, sugar mediated browning by using α-amylase inhibitors (α-AIs) can be good alternative, safe, and attractive option. Alpha AIs are reported to be present in many plant species where they play an important role in the regulation of starch hydrolysis. This strategy can be used at industrial scale after proper scale-up studies (Kumari et al. 2012). Inhibition of potato browning will not only improve consumer acceptability but will also lower the risk of generation of acrylamide precursors in fried potato chips because of controlled concentration of reducing sugars (Kasar et al. 2019).

3.11 Limitations of α-AIs to Be Used as Dietary Source Protein for Mammals

The extensive use α-AIs as a potent bioactive molecule in various biological applications is noxious on account of its associated side effects on mammals. Majority of the world's vegetarian population is dependent on plant sources (cereal grains, legumes, and oil seeds) for fulfilling its fundamental nutrient supplement. Some of the cheaper plant proteins are existing in these edible crops, while among

them very few are potentially toxic proteins that may be consumed as food. For example, arcelins from *P. vulgaris* seeds, chitinases, canatoxin, lectin-like inhibitors from common bean *P. vulgaris* seeds (Ishimoto and Kitamura 1989; Huesing et al. 1991; Ishimoto and Chrispeels 1996), glutenins (Shewry and Halford 2002), and modified forms of storage proteins (Sales et al. 2000). The progressive use of such plant proteins as food is accountable for the initiation of many of complex difficulties within vegetarian people and cattle (Carlini and Grossi-de-Sá 2002). Most of plant defense related inhibitory proteins irreversibly block the digestive enzymes of human kinds when consumed and affect the healthy body (Gadge et al. 2015). α-AI protein from most plant seeds is also reported to be associated with allergies and other disorders (Cuccioloni et al. 2016). For example, CM-protein family α-AIs from wheat and rice have been associated with allergies (Svensson et al. 2004). The α-AI peptides or proteins irreversibly bind to the gastrointestinal tract amylases to resist hydrolysis of complex carbohydrates, which lead to indigestion of food and is responsible for malabsorption, diarrhea, abdominal pain, and flatulence (Jayaraj et al. 2013). Among non-gluten proteins, cereal α-amylase/trypsin bifunctional inhibitors (ATIs) are non-conventional gastrointestinal sensitizing agents (Tatham and Shewry 2008). According to Gadge et al. (2015) the bifunctional inhibitory proteins are albumin proteins with the ability to increase the amount of gluten-like immunogenic peptides on account of the inhibition of digestive enzymes resulting in impaired degradation of dietary cereal proteins. In celiac disease, these inhibitors stimulate the release of pro-inflammatory cytokines in monocytes, macrophages, and dendritic cells. α-AI proteins may also prolong inflammation and immune reactions in a number of intestinal and non-intestinal immune disorders.

Such inhibitors also disturb the natural balance of human microbiome of intestine affecting the resulting fermentation process in the gastrointestinal tract leading to impaired digestion. Undigested carbohydrates are associated with side effects such as flatulence, skin allergies, liver failure, and pneumatosis cystoids intestinalis infection (Jayaraj et al. 2013). The amylase/protease inhibitors from wheat are reported to cause respiratory allergy due to their strong affinity towards immunoglobulin E (IgE) from baker's asthma patients (Salcedo et al. 2011; Quirce and Diaz-Perales 2013). Obiro et al. (2008) previously reviewed that lectin-like amylase inhibitors from common beans have hemagglutinin poisoning towards all animals including human being. Acarbose, a synthetic AI which functions similar to that of common bean α-amylase inhibitor (a-AI1) and their prolonged use leads to alteration of colon microbe pathways. Such alteration results in the colonic neoplasia by causing proliferation of rectal upper crypt which is due to disturbances in small chain fatty acids and butyrate production ratio (Holt et al. 1996; Weaver et al. 1997; Wolever and Chiasson 2000).

3.12 Scope of Amylase Inhibitor Research

There is tremendous diversity in α-AIs due to their isolation from various sources (mostly from plants and microbes) and their reported inhibitory pattern of the digestive enzyme (α-amylase) from insect pests, microbes (fungi/bacteria), and mammals (Dias et al. 2005). The major constraints found to be associated with the inhibition of α-amylase activity are (a) inhibitors have strict enzyme specificity for their binding and forming requisite interaction with one out of several closely related isoenzymes or enzymes of different species (Franco et al. 2000); (b) some of the α-amylase inhibitory molecules form irreversible binding with amylases such as human salivary and pancreatic α-amylases (HSA and HPA) and degrade the bound enzyme; (c) vast majority of potent α-AIs from legumes, cereals, and microbes are reported to have toxicity or side effects to non-target pests and human; (d) even potent α-AIs could not completely block α-amylase action from mammals (HSA/HPA) and insects under in vivo conditions; (e) adaptation and advantageous mutations like responses of insect pests to host α-AIs; (f) degradation due to excessive secretary protease activities; and (g) uneven expressions of α-AIs in transgenic systems. In the light of these facts, there is a need to search for alternative α-AIs having high potential and fewer side effects on the environment. To validate various α-amylases for their regulation by α-AIs, it is important to understand their various mechanisms of action, binding specificities, and regulation of expression. Therefore, continued biochemical screening from remarkable natural diversity for the search of α-AI molecules with desirable characteristics will play an important role. It can be done through (a) understanding of the structural basis of α-amylase and α-AI interactions, (b) isolation of α-AIs from natural sources to check their potential and binding specificity with amylases from various sources; (c) enabling specific mutation(s) in existing inhibitor(s) to check its/their stability; and (d) by designing synthetic peptide(s) which will be specific to the α-amylase(s) of the small number of pests.

3.13 Conclusion and Future Perspective of α-AI Research

The research on amylase inhibitors is progressing in the light of its scope and vast biological applications of α-AIs in various fields. There are various starch degradation blocking commercial capsules available in the market to control diabetes and obesity, for example, Carb-Blocker™ by Naturals, White Kidney Bean Extract™ by Arazo Nutrition, Advanced Amylase Inhibitor™ by general sciences. However, some of such marketed products are associated with side effects. Therefore, further investigations are required to remove/minimize its toxicity causing factors and to check their binding specificity and effect on other enzymes/hormones. In concern with the utilization of α-AIs in pest management, there are numerous successful reports available on the potential of α-AI and AI expressing transgenic plants

towards the various food crop infesting insect pests. This research mainly involves the identification of genes responsible for α-AI activity and the development of transgenic plant expressing α-AI. For example, α-AI1 expressing transgenic pea plants demonstrate complete inhibition of α-amylases of *Bruchus pisorum*, *Callosobruchus maculatus,* and *Callosobruchus chinensis* (Shade et al. 1994). Despite of these, their application in the agricultural pest control field is still down hearted as a consequence of their degree of efficiency towards a narrow range of insect pests. Their effectiveness against only target pest is due to the specificity of α-AI for the interaction and this may be the reason for doubts in the commercialization of this technology (Singh et al. 2018).

In light of the challenges in the safe marketable utilization of α-AIs, there are a few alternative approaches and various efficient solutions that come out from recent advancements in biotechnological tools which could be helpful to broaden the scope of α-AIs. Several techniques from recent researches in genetic engineering are gene pyramiding, CRISPR/Cas9 mediated genome editing, RNAi based methods, recombinant α-AI production, transplastomic engineering cross-breeding, etc. Gene pyramiding or gene stacking includes combinations of specified genes or stacking broad-spectrum α-AIs with other insecticidal genes such as cry toxin, chitinase, etc. (Daniell and Dhingra 2002). For example, gene pyramiding of α-AI with degradation-resisting gene such as protease inhibitors is also one of the options at the time of transgenic plant development because it resists premature degradation of other protective inhibitors by proteases (Koundal and Rajendran 2003). This multigene combination concept gained trust in transgenic crops to deal with insect's adaptive responses to single insecticidal genes (Singh et al. 2018). The use of such multigene incorporated transgenic plants is an economically wise approach to conventional insect-resistant strategy in consideration of environmental factors and human health issues. For example, α-AI1 expressing transgenic variety of plant proved to have minimal harmful effects on the nutritional value of pea fed to rats up to 30% of the diet (Pusztai et al. 1995). Cross-breeding of inhibitory gene encoding plant transformants would also be proved as an important strategy for the production of insect-resistant new crops with no health risk to consumers (Schuler et al. 1998). Another wise approach for the future research study is RNAi-mediated gene blocking. In this, instead of silencing a gene sequence or gene family, silencing of master regulators of these genes such as transcription factors will block insect adaptation and counter defense pathways (Singh et al. 2018). The NCBI database provides the gene sequence information of various critical target genes for RNAi-mediated pest control. Very recent technique CRISPR/Cas9 mediated genome editing is contemporary technique of editing genes among the available genome-editing technologies. The recent technology to knockout vital genes by CRISPER/Cas9 from varacious feeding insect pest has provided valuable future prospects in agriculture (Sun et al. 2017). There are recent reports on knockout of vital genes from insect pests such as *Plutella xylostella* (Huang et al. 2016), *Spodoptera litura* (Bi et al. 2016; Koutroumpa et al. 2016), and *Helicoverpa armigera* (Wang et al. 2016). Such recent biotechnological strategies allied with the discovery of novel and potent α-AIs could bring clear benefits to agri business. There is scope for successful

exploitation and implementation of transgenic technology and requires further study for a complete understanding of the regulation of proteinaceous inhibitors in plants and their effect on normal physiological processes.

The amylase inhibitory extracts are available commercially to be utilized as an antidiabetic and anti-obesity drug in the form of Ayurvedic preparations but contaminating antinutritional impurities and other phytochemicals make them inefficient. Therefore, the synthesis of recombinant α-AI through microbial cells is one of the alternative approaches to overcome the disadvantages and associated toxicity. This technique is useful in the production of pure (toxicity free) and functional bioactive α-AI protein by molecular biology techniques for the biotechnological and pharmaceutical applications (Brain-Isasi et al. 2017). Researchers nowadays also tried to lower down toxic effects of AIs by modifying the structures of inhibitors and increasing the steric bulk of the inhibitor (Jayaraj et al. 2013). For this, potent inhibitor protein gene sequence screening and identification along with interactions study of such potential α-AIs and α-amylases using modern bio-informatics tools is an important initial step. For example, the mutagenesis study performed by Wang et al. (2011) proposed that the mutant SA-05 may be used as raw material in commercial preparation of α-AI extracts to control appetite and energy intake. Further research can be initiated for mutant improvement aiming at its potent AI activity trait selection from progenies.

The use of AI in controlling cold induced sweetening in potato can be continued through isolating inhibitor protein responsible gene and incorporating it in potato tubers. Such a new potato tuber variety may prove helpful in increasing the shelf life of potato tubers (Zhang et al. 2014). The researchers continued to find out the exact role and mode of actions of amylase inhibitors in controlling colon cancer. The hypothesis behind this is, AI increases the amount of starch or undigested carbohydrate in the colon which was utilized by colon microbes and caused probiotic effect by producing small chains of fatty acids which could be useful in the control of colon cancer by the apoptotic response to mutated cells (Obiro et al. 2008).

In our opinion, though, there are bottlenecks in commercial utilization (efficient technology development) of α-AIs in various sectors mentioned above, focused research with meaningful utilization of technological advances will help in materializing successful commercial utilization of bioactive α-AIs.

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Chapter 4
Recent Updates on In Silico Screening of Natural Products as Potential Inhibitors of Enzymes of Biomedical and Pharmaceutical Importance

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Abstract Natural products from medicinal plants have been increasingly used in modern medicine due to their safety, efficacy, and lesser toxicity. World over, a large number of natural compounds are evaluated for the desired bioactivity. A wide range of phytoconstituents such as alkaloids, terpenoids, tannins, steroids, etc. have been recognized for their varying biological activities. However, obtaining the natural products with the desired bioactivity is a time-consuming and commercially difficult process. Molecular docking is used for screening known as well as novel drugs to identify novel compounds by predicting their binding mode and affinity. Moreover, in silico molecular docking can be performed to analyze their binding capabilities into the 3D structure of proteins. AutoDock and AutoDockTools are open-source techniques that have been extensively cited in the literature as essential tools in structure-based drug design. These methods are fast enough to permit the virtual screening of ligand libraries containing tens of thousands of compounds. This article highlights the recent developments in the virtual screening of enzyme inhibitors using various docking tools and their significant applications in designing potent inhibitors for the management of various metabolic and infectious diseases.

Keywords Natural products · Virtual screening · Molecular docking · In silico · AutoDock and AutoDockTools

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4.1 Introduction

Natural products have been a valuable source of new drugs. Secondary metabolites are naturally derived substances and/or by-products from plants, animals, and microorganisms (Baker et al. 2000). According to the World Health Organization (WHO) estimates, 80% of the people of developing countries rely on traditional medicines, mostly plant-derived drugs, for their primary health needs. Over 49% of the new medicines registered by the United States Food and Drug Administration (USFDA) are derived from natural products or their derivatives. The Indian subcontinent has been one of the rich hotspots with over 2500 plant species being used in indigenous medicines. The use of medicinal plant resources for health management is an imperative part of tribal culture. It is evident that these people have vast traditional knowledge about medicinal plants and use them for a wide range of health-related applications. In the old traditions, local tribal communities have discovered the medicinal uses of hundreds of plants and plant-based substances. Medicinal plants contain a variety of bioactive constituents known as phytochemicals that make them effective in the treatment of a wide range of diseases (Twilley et al. 2017; Anigboro et al. 2020), and they are becoming more popular as therapeutic agents due to their high safety profile, low cost, and widespread availability (Govindappa 2015; Limanaqi et al. 2020). Natural oxyprenylated ferulic, umbelliferone derivatives and coumarins, flavonoids, glycosides make up a large portion of these natural phytochemicals.

Enzyme inhibitors are mostly plant or microbial bioactive secondary metabolites that bind to enzymes and inhibit/reduce their activity. The use of natural products as enzyme inhibitors is well documented (Rauf and Jehan 2017). The binding of inhibitors to the enzymes alters the conformation of the active site, thereby halting the chemical process. Inhibition of enzymes can be both irreversible and reversible. The inhibitory effects of plant extracts and the isolated compounds on the activity of various enzymes, viz. α-amylase, α-glucosidase, and pancreatic lipase have been studied to discover potential therapeutic candidates. Various reports have demonstrated the in vitro, in vivo, and in silico enzyme inhibitory potential of natural products of plant and microbial origin (Rauf and Jehan 2017).

The discovery of new, safe, and effective enzyme inhibitors is a long and costly process. Conventionally, a trial-and-error method is used in screening vast libraries of chemical constituents against a known enzyme in the hopes of finding some useful lead drugs. Combinatorial chemistry approaches were found effective as they offered rapid and high-throughput screening capabilities to quickly screen these enormous chemical libraries for novel and potent inhibitors.

Molecular docking is important in silico technique normally used to predict the interaction between the receptor and the ligand. This technique is employed in drug discovery because it is inexpensive and time-saving (Miners et al. 2004). For these reasons, the development of in vitro and in silico approaches is important to predict drug interactions, to possibly identify the pharmacophore, to reduce time and costs (FitzGerald et al. 2020). Using in silico docking approach, the most effective

inhibitors of different enzymes can be screened for further investigation to understand the structures of proteins and molecular interaction of ligands to exert their inhibitory activity. Therefore, to understand such possible molecular interactions between the enzyme and inhibitor, a combined in vitro and in silico method is required to screen for bioactive molecules.

Several approaches are available for determining whether a bioactive compound is interacting with a biological macromolecule; for example, kinetic investigations and in silico molecular docking study (Mentes et al. 2018; Moreno et al. 2020). Prediction of chemical compounds' reactivity, chemical hardness, stability, nucleophilic or electrophilic nature, as well as drug-like properties (such as absorption, distribution, metabolism, and excretion) with plausible precision using in silico analytical methods such as semi-empirical quantum mechanics (frontier molecular orbital (FMO) theory calculations) and pre-ADME or adsorption, distribution, metabolism, and excretion cheminformatics tools have also been reported (Bondzic et al. 2020; Siahaan et al. 2021). These methods have been used to investigate molecular interactions between chemical substances or ligands and proteins, including acetylcholinesterase (Bondzic et al. 2020), epoxide hydrolase (Jo et al. 2016), 3-hydroxysteroid dehydrogenase (Oyebamiji et al. 2020), and pancreatic lipase (Hou et al. 2020). The use of molecular docking analysis, as one of the biophysical/biocomputational techniques for probing ligand–protein binding interactions, has gained more recognition and relevance over the years in the fields of biochemistry and biophysics (Almasri 2020).

Molecular Docking for Screening of Enzyme Inhibitors

Molecular docking of protein–ligand interaction is an effective and competent tool for in silico screening of natural products as inhibitors. It plays the most important and ever-increasing role in rational drug design. Molecular docking is a computational program of virtual screening for an appropriate ligand (enzyme inhibitor) that fits both energetically and geometrically the proteins' binding pocket. Consensus molecular docking is one of the approaches to increase detection of real actives within virtual screening campaigns. With molecular docking, it is possible to find the most favorable position, orientation, and conformation for the binding of a molecule to, a protein target, assigning a score that is the estimate of the likelihood of binding of each molecule and conformation (Cavasotto 2015). There are different open-source molecular docking programs, Autodock Vina, Smina, LeDock, and rDock, available for this purpose. Here we propose/present the protocol for Autodock Vina. AutoDock and AutoDockTools are open-source techniques that have been used as essential tools in structure-based drug design. Moreover, these methods are fast enough to permit the virtual screening of ligand (inhibitors) libraries containing thousands of natural compounds. The protocol can be implemented in any virtual screening campaign where proteins (enzymes) are used as molecular targets. In

addition, we introduce a scoring strategy based on the average RMSD value obtained from comparing the different confirmations to predict the best fit.

4.2 Molecular Docking with AutoDock

AutoDock suite is molecular modeling simulation software that attempts to predict the noncovalent binding of the macromolecule (receptor) and ligand (small molecule). It is one of the most accurate and effective tools that is capable of predicting the bound conformations and binding energies of the ligands with macromolecules targets. Furthermore, it is open-source software, and it includes several complementary tools like AutoDock Vina, Autodock, Raccoon2, AutoDockTools, and AutoLigand. AutoDockTool combines precision in identifying the binding pose of a tiny molecule in corresponding to the receptor pocket for computational docking researchers.

There are four basic steps in a typical docking process. First, prepare the ligand and target protein (depending on the nature of the ligand and/or target, this may necessitate more complex processes). Second, prepare to dock and scoring parameters (for Autodock to run, the following files should be created: grid parameter file, map files, docking parameter files). Third, using a graphical interface or a command-line interface terminal to launch the docking program (e.g., AutoDock). The fourth step is to analyze and evaluate the docking data (by comparing docking poses to crystalline ligand). Here we are providing easy step-by-step and practical docking protocols using AutoDock and AutoDockTools. In nutshell, the AutoDock engine uses a proprietary format known as PDBQT. For both ligand and protein, this file contains all information on atom kinds and charges. This file is created when a PDB file (http://www.wwpdb.org/documentation/file-format) is converted. It can be made with AutoDockTools, the preferred graphical user interface for AutoDock. A Grid calculating approach is used to evaluate the binding site's energy (AutoGrid). The ligand's energetics is then compared to values calculated from the interaction terms assigned by the affinity grids computations. In the last step, the ligand is docked utilizing a variety of search techniques. The Lamarckian genetic algorithm (LGA), which is implemented in AutoDock, is one of the most widely used and effective approaches for determining optimal ligand binding conformation with predicted free energies of association.

A landmark enzyme, Pancreatic Lipase (PL) was identified as a major target for binding of inhibitors in the treatment of obesity. Human pancreatic lipase consists of 449 amino acids with a coated catalytic center of the N-terminal domain including Ser-152, his-263, and Asp-176. The catalytic triad (S152–D176–H263) and the lid (peptide stretch C237–C261) are found in the large N-terminal domain (residues 1–335) of 2OXE, which has the typical/-hydrolase fold of lipases and is dominated by a central parallel sheet (Winkler et al. 1990; Van Tilbeurgh et al. 1993). In the present review, PL will be used as a model for docking using AutoDock as it is a promising target for binding of inhibitors in the treatment of obesity.

4.2.1 Computational Tools Required for Docking

4.2.1.1 Starting with a Set of Preliminary and Important Requirements

This would require user-friendly tools such as protein data bank (https://www.rcsb.org/) and Swiss-PdbViever (https://spdbv.vital-it.ch/) in order to understand and deduce the structural alignment, finding the active site, amino acid mutations, H-bond, angles, and distance between the atoms of the protein–ligand complexes. These tools offer a great help that explains all you need to know about manipulating protein–ligand complexes, removing the solvent, fixing structure, and more.

4.2.1.2 Download the PDB (Protein Data Bank) File

The X-ray diffraction crystallographic structure (3D) of human pancreatic lipase-related protein (PDB ID, 2OXE) is downloaded from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (https://www.rcsb.org/structure/2OXE). The current crystal structure has 466 amino acid residues and the monomer has a resolution of 2.8 Å (Fig. 4.2). The 3D structure of the ligand can be obtained from https://pubchem.ncbi.nlm.nih.gov/. The structure of both target protein and corresponding ligand were extracted separately from the original PDB files. Users are encouraged to regenerate the target.pdb and ligand.pdb files using Swiss-PDB viewer http://spdbv.vital-it.ch/.

4.2.1.3 Download the Computational Tools

AutoDock 4.2.6

The latest version of Autodock should be downloaded from http://autodock.scripps.edu/downloads/autodock-registration/autodock-4-2-download-page/. This new version of Autodock runs natively under Windows, see instructions for installation (http://autodock.scripps.edu/downloads/autodock-4-2-x-installation-on-windows). The main files, AutoDock4 and AutoGrid4 are necessary to run the pre-docking (energy maps), docking, and scoring calculation.

MGL/AutoDockTools

AutoDockTools can be downloaded from http://mgltools.scripps.edu/downloads. It is a Graphical User Interface for preparing input, running, and analyzing Autodock dockings (adding atomic charges, fixing bonds, adding hydrogens, preparing the ligand and target in Autodock-compatible PDBQT format, creating grids and docking parameter files, and visualizing interactively docking results).

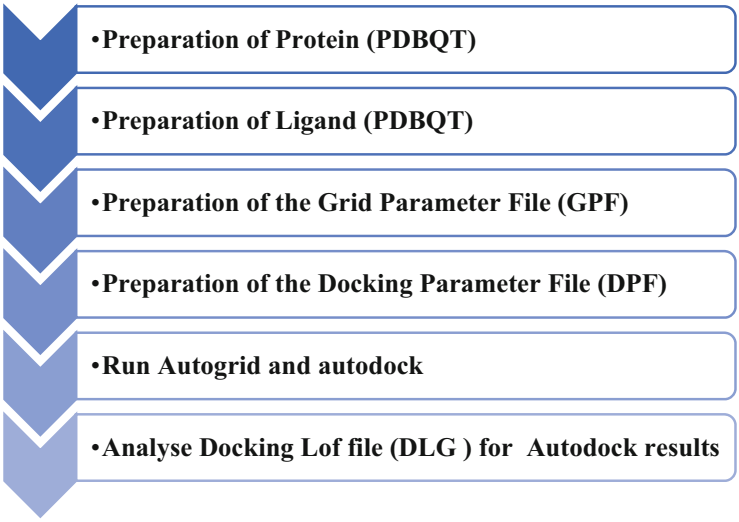

Fig. 4.1 Step-wise docking approach for the molecular docking of protein–ligand interaction

Method-Docking Approach

The following steps are crucial since they specify how to execute AutoGrid and AutoDock and offer accurate docking parameters. The coordinated files and associated information should be created in the PDBQT format, which includes atom/bond types, partial atomic charges, and other information. These data types are prepared typically using AutoDockTools (ADT). The steps in the docking method are represented in Fig. 4.1.

4.2.2 Preparation of Protein for Autodock

The following steps represent the preparation of the crystal structure of PL protein (2OXE Fig. 4.2) PDB file into the PDBQT file format.

- First, open AutoDockTools and MGL tools from desktop or program files. Before preparing protein, make sure that protein.pdb and ligand.pdb files are in the same folder, e.g., Desktop/autodock.
- Click on File > Preferences > Set: change the startup directory > set file path.
- From File menu > open > read molecule > select target protein.pdb from same folder: Desktop/autodock.
- The crystal 3D structure of 2OXE will appear on screen, then Click on Edit > Delete water > Hydrogen > Add (polar Hydrogen's) > Click on Charges

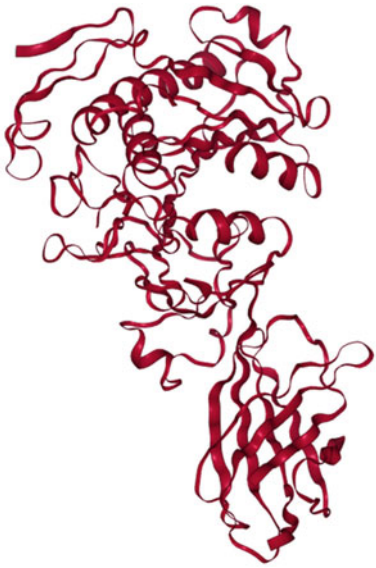

Fig. 4.2 Three-dimensional crystal structure of the generated 2OXE model of pancreatic lipase

> Add Kollman charges > Compute Gasteiger charges. This will add partial charges to protein.

- Click on Grid > Macromolecule > Open: Open and select target protein pdb file, and ADT tool will prompt a new window to save our protein into pdbqt file> save the PDBQT file in the same folder where we created target pdb. This will store the partial charges and Autodock atom types that are compatible with autodock grid computing.

4.2.3 Preparation of Ligand for Autodock

The process of creating ligand PDBQT from a PDB file from the crystal structure of ligand.pdb consists of the following steps.

- Click on Ligand > Input > open: open ligand.pdb file from the same folder, the crystal 3D structure will appear on the screen.
- Click on Edit > Delete water > Hydrogen > Add (polar Hydrogen's) > Click on Charges > Add Kollman charges > Compute Gasteiger charges.
- Click on Ligand > Torsion Tree > Detect Root > Ligand > Torsion Tree > Choose Torsions: decide on the rotatable bonds to be considered and set the number of torsions.
- Select Ligand > Output > Save as PDBQT: save the ligand file with "pdbqt" extension (e.g., ligand.pdbqt). Type in the extension manually and save it in the same folder.

4.2.4 Docking Approach

4.2.4.1 Preparation of Grid Parameter File (GPF)

- For the preparation of the grid parameter file to run with Autogrid4.exe, open the Grid menu in AutoDockTools to prepare the parameters for Autogrid calculations. Autogrid calculates grid maps of interaction energies for various atom types. This is important to calibrate the docking procedure.
- Click on Grid > Set Map Types > Choose Ligand: select the ligand molecule from the list or open the ligand. pdbqt saved previously. Select Grid > Grid Box: Select the number of grid points in *x*, *y*, and *z* directions. Usually, the default 40 is taken. However, we can increase or decrease that number in any direction. From the "Center" menu, select "Center on ligand." Leave other parameters on default.
- The number of grid points in each dimension: only give even numbers (from $2 \times 2 \times 2$ to $126 \times 126 \times 126$). Grid Maps depend on the orientation of the macromolecule.
- Click on File > Close saving current: save the settings before closing. Otherwise, settings will be lost if the program is closed. Select Grid > Output > Save GPF: save our gpf file as docking.gpf. Type in the extension manually and save it in the same directory. This will create one grid map for each atom type in the ligand plus electrostatics and a desolvation map. Check out the content of Grid Parameter File, docking.gpf (by Notepad).

4.2.4.2 Preparation of Docking Parameter File (DPF)

In AutoDockTools, open the docking menu to prepare the docking parameters for Autodock.

- Click on Docking > Macromolecule > Set Rigid Filename: Select target macromolecule protein file (e.g., protein.pdbqt). Again select Docking > Ligand > choose: select ligand in pdbqt format. Leave everything on default and select Accept.
- Select again docking > Search Parameters > Genetic Algorithm: set the number of GA Runs as 10. Leave other parameters on default and select Accept.
- Click on Docking > Docking Parameters: Leave on default parameters. Again click on Docking > Output > Lamarckian GA: save dpf file (e.g., docking.dpf) in the same directory. Type in the extension manually.
- Now we have all the required files for docking; target protein.pdbqt, ligand.pdbqt, docking.gpf, docking.dpf files in the same folder.

4.2.4.3 Running AutoGrid4 and AutoDock4

After downloading and installing AutoDock 4.2.6 from http://autodock.scripps.edu/downloads/autodock-registration/autodock-4-2-download-page/, for Windows users, Start > Run and type "cmd.exe," then type the command: "C:\Program Files\ The Scripps Research Institute\ Autodock\autodock4.exe".

For running Autogrid4, Start > Run and type "cmd.exe," change the working directory to ~Desktop\autodock (using the cd command). Type in the console: autogrid4.exe -p docking.gpf -l docking.glg. For running autodock4, type in the console: autodock4.exe -p docking.dpf -l docking.dlg & . This will take some time depending on your CPU and memory capacity.

All information about the docking runs, the calculated binding energy in Kcal/mol, as well as other data RMSD versus crystal binding pose is contained in the dlg file. Figure 4.3 represents different conformations of protein–ligand interactions.

4.2.4.4 Analyzing Docking Results

Open the analyze menu in AutoDockTools to analyze the docking results. Docking results can be found in the docking.dlg log file in the same directory.

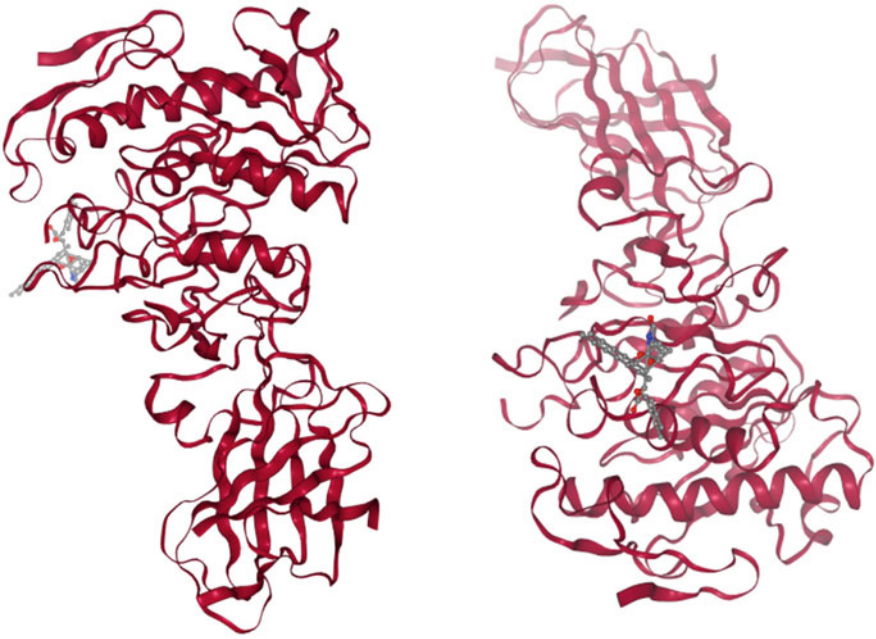

Fig. 4.3 Predicted active sites for binding of compounds (ligands) in the pancreatic lipase enzyme in the molecular docking

- Select the Analyze > Docking> open > Docking.dlg. file> open analyze > select Conformations> play. This will show the conformation from 1 to 10 of the ligand bound to PL protein.
- The best conformation shows binding energy (G) of −10.59 kcal/mol and inhibition constant (K_i) of 17.17 nM (nanomolar) and a RMSD (root-mean-square deviation of atomic positions) from a reference structure of 1.22 Å.
- This demonstrates that Autodock's results are accurate and dependable (in the nanomolar range for a recognized inhibitor). Docking and virtual screening would be extremely useful in the search for novel PL inhibitors.

4.3 Significance of Molecular Docking in Inhibitor Screening for Biomedical Applications

Molecular docking is a widely used platform to understand drug biomolecular interactions that have applications in rational drug design and discovery. It is also used for mechanistic studies binding studies of ligand into the preferred binding site of the target protein/DNA through the noncovalent interactions. The values of the binding energy, free energy, and stability of complexes help predict the binding affinity of the molecule under study. There are numerous examples where the docking approach successfully identifies the target sites for several different receptors/targets. For example, using docking technology, Schames et al. (2004) discovered HIV 1 integrase as a new binding site for drugs treating AIDS. Using AutoDock, this receptor was effectively used for the inhibition of HIV integrase. The most striking discoveries involving docking approaches include the discovery of novel type I TGF-beta receptor kinase inhibitor (Singh et al. 2003), aurora kinase A inhibitor (Park et al. 2018), dopamine D3 receptor for anticancer molecules (Varady et al. 2003), etc. Table 4.1 provides a detailed outlook of various target proteins/receptors that are inhibited using a ligand with its PDB ID, binding sites, software used in the study, and the biological significance of the study.

4.4 Limitations of Molecular Docking

The lack of confidence in scoring functions' ability to provide correct binding energies is a key constraint of molecular docking. This is because some intermolecular interaction terms, such as solvation effect and entropy change, are difficult to anticipate precisely. Furthermore, several intermolecular interactions that have been proved to be significant are rarely taken into account in scoring methods. For example, halogen bonding and guanidine–arginine interactions have been shown to contribute to protein–ligand binding affinity, although they are not taken into account. Secondly, the water molecules that are present in the binding pocket

Table 4.1 Recent studies on screening of protease/enzyme inhibitors using molecular docking and their biological significance

Sr. no	Target enzyme/ protein	Inhibitor/ligand	PDB ID	Binding sites/residues	Biological significance	Software used in the study	References
1.	Acetylcholinesterase inhibitors (AChEIs)	Compound D	NR	Peripheral anionic subsite	In the treatment of Alzheimer's disease	AutoDock Vina 1.1.1	Pablo et al. (2015)
2.	Butyrylcholinesterase	Compound D	NR	Peripheral anionic subsite	In the treatment of Alzheimer's disease	AutoDock Vina 1.1.1	Pablo et al. (2015)
3.	ERK2 (protein kinase)	Hypo1	NA	Glycine rich loop	Inhibition of cell proliferation and oncogenesis	AutoDock	Sofiene et al. (2014)
4.	Glycoprotein receptor-binding domain	Solanine, acetoside, and rutin	6M0J	Arg403 (6.34 Å) and π-π stacking with Tyr505 (4.75 Å)	In the treatment of COVID-19	Schrödinger	Teli et al. (2021)
5.	COVID-19 main protease (Mpro)	Solanine, acetoside, and rutin	6 LU7	Leu141 (1.81 Å), His164 (2.06 Å), and Thr190 (1.69 Å) residues	In the treatment of COVID-19	Schrödinger	Teli et al. (2021)
6.	Hemoglobinase Falcipain-2	Nonpeptidic inhibitors	6JW9	His114 and Thr120	To develop anti-malarial drugs to treat resistant strains of *Plasmodium*	PyRx virtual screening software	Rajguru et al. (2022)
7.	Cyclophilin D	ZINC161011368	1ZKC	π-Sulfur interaction with a methoxy group	For the treatment of acute pancreatitis	Schrodinger	Chen et al. (2022)
8.	Aldehyde dehydrogenases (ALDHs)		1KOL	Thr26, Gly123, Cys145, Glu146, Glu189	In the treatment of cyclophosphamide and cisplatin resistance cancer	Schrodinger	Narendra et al. (2021)
9.	COVID-19 main protease	Bieckol	5R82	Thr24, Thr26, Gly143, and Glu189	In the treatment of COVID-19	MOE dock program	Rauf et al. (2021)
10.	COVID-19 main protease	7-Hydroxyeckol hepta-acetate	5R82	Thr26, Gly143, Cys145, Glu166, Glu189	In the treatment of COVID-19	MOE dock program	Rauf et al. (2021)

(continued)

Table 4.1 (continued)

Sr. no	Target enzyme/ protein	Inhibitor/ligand	PDB ID	Binding sites/residues	Biological significance	Software used in the study	References
11.	COVID-19 main protease	Met165	5R82	π-Sulfur interaction with a methoxy group	In the treatment of COVID-19	MOE dock program	Rauf et al. (2021)
12.	COVID-19 main protease	5-Hydroxy-cystofurano-quinol	5R82	Thr24, Thr26, Ser46	In the treatment of COVID-19	MOE dock program	Rauf et al. (2021)
13.	COVID-19 main protease	Sargaquinoic acid	5R82	Pro168, Gly143, and Gln189	In the treatment of COVID-19	MOE dock program	Rauf et al. (2021)
14.	COVID-19 main protease	Methoxybifurcarenone	5R82	Thr24, Ser46, Met165, Glu166, and Arg188	In the treatment of COVID-19	MOE dock program	Rauf et al. (2021)
15.	COVID-19 main protease	Rutin	5R82	Thr24, Thr26, Ser46	In the treatment of COVID-19	MOE dock program	Rauf et al. (2021)
16.	Monoacylglycerol lipase	Rutin	4CDD		To attenuate the inflammatory response	MOE dock program	
17.	Dipeptidyl-peptidase-4	Butirosin	4CDD	s740(H), Tyr631(H)	In the treatment of type II diabetes mellitus	Schrodinger	Xiong et al. (2021)
18.	Dipeptidyl-peptidase-4	Alogliptin	4CDD	yr631(H), Tyr662(π), Tyr666(π), Glu205 (H),	In the treatment of type II diabetes mellitus	Schrodinger	Xiong et al. (2021)
19.	Dipeptidyl-peptidase-4	Rutin	4CDD	Phe357(π), Tyr585(H), Ser630(H)	In the treatment of type II diabetes mellitus	Schrodinger	Xiong et al. (2021)
20.	Dipeptidyl-peptidase-4	Homatropine	4CDD	Glu206(H), Arg125(H and π-cationic)	In the treatment of type II diabetes mellitus	Schrodinger	Xiong et al. (2021)
21.	Mycobacterial InhA	Enamine 57340	5G0T	Tyr158	Therapeutic applications against TB	GRO-MACS (Groningen Machine for Chemical Simulations) 5.0.2package	Jayaraman et al. (2021)

22.	Mycobacterial InhA	Enamine 57294	5G0T	Phe149 and Tyr158	Therapeutic applications against TB	GRO-MACS (Groningen Machine for Chemical Simulations) 5.0.2package	Jayaraman et al. (2021)
23.	Histone acetyltransferase	Chlorogenic acid (CA)	2RNZ	CA can be buried to the bromine domain of p300	Anti-non-alcoholic fatty liver disease	Autodock, Autogrid, autotors	Xu et al. (2021)
24.	Stearyl coenzyme A desaturase enzyme 1	Stearoyl-coenzyme A	4ZYO	ASN71 ASN144ARG151ASP152ARG184LYS185	To alter the metabolism of tumor cells	Schrödinger software	Huang et al. (2020)
25.	Stearyl coenzyme A desaturase enzyme 1	Aramchol	4ZYO	ASN71ASN144GLH148ARG151ARG21	To alter the metabolism of tumor cells	Schrödinger software	Huang et al. (2020)
26.	Protein tyrosine phosphatase 1B	H1 to H20	1PA1	Arg114andArg222	In the management of diabetes	Schrödinger software	Yang et al. (2020)
27.	Phosphodiesterase 10A	MA8	3HR1	GLN716A	Alleviating the negative and cognitive symptoms of schizophrenia	Autodock Vina 1.1	Mayasah et al. (2018)
28.	Phosphodiesterase 10A	MA98	3HR1	GLN716A	Alleviating the negative and cognitive symptoms of schizophrenia	Autodock Vina 1.1	Mayasah et al. (2018)
29.	Lactate dehydrogenase	Quercetin	1I0Z	Arg111, Gly96	To arrest tumor invasion and metastasis	Autodock Vina 1.1	Li et al. (2021)
30.	Lactate dehydrogenase	Luteolin	1I0Z	Asp51 Thr247	To arrest tumor invasion and metastasis	Autodock Vina 1.1	Li et al. (2021)

(continued)

Table 4.1 (continued)

Sr. no	Target enzyme/ protein	Inhibitor/ligand	PDB ID	Binding sites/residues	Biological significance	Software used in the study	References
31.	Lactate dehydrogenase	Ursolic acid	1I0Z	Arg111, Gly96	To arrest tumor invasion and metastasis	Autodock Vina 1.1	Li et al. (2021)
32.	COVID-19 main protease	Penciclovir	6M0J	HIS 41 LEU141	To develop effective therapeutics to deal with the COVID-19 pandemic	Libdock	Padhi et al. (2021)
33.	COVID-19 main protease	Jasmonic acid	6M0J	MET 165 GLU 16 HIS41	To develop effective therapeutics to deal with the COVID-19 pandemic	Libdock	Padhi et al. (2021)
34.	COVID-19 main protease	Jasmonic acid methyl ester	6M0J	HIS41 MET49 GLY143	To develop effective therapeutics to deal with the COVID-19 pandemic	Libdock	Padhi et al. (2021)
35.	COVID-19 main protease	Putaminoxin B	6M0J	HIS 41 ASN 142 CYS 145	To develop effective therapeutics to deal with the COVID-19 pandemic	Libdock	Padhi et al. (2021)
36.	COVID-19 main protease	Putaminoxin D	6M0J	HIS 41 LEU141 2.20 SER 144 CYS 145	To develop effective therapeutics to deal with the COVID-19 pandemic	Libdock	Padhi et al. (2021)

37.	SARS-CoV-2 3-chymotrypsin-like protease	Chicoric acid	6Y84	CYS^{145} CYS^{148}	Preventive nutraceuticals and/or antiviral drugs in COVID-19	AutoDock Vina in PyRx 0.8	Gyebi et al. (2021)
38.	SARS-CoV-2 3-chymotrypsin-like protease	Rosmarinic acid	6Y84	HIS^{41} LEU^{49}	Preventive nutraceuticals and/or antiviral drugs in COVID-19	AutoDock Vina in PyRx 0.8	Gyebi et al. (2021)
39.	SARS-CoV-2 3-chymotrypsin-like protease	Myricetin	6Y84	CYS^{148} LEU^{49} HIS^{41}	Preventive nutraceuticals and/or antiviral drugs in COVID-19	AutoDock Vina in PyRx 0.8	Gyebi et al. (2021)
40.	SARS-CoV-2 3-chymotrypsin-like protease	Rosmarinic acid	6Y84	GLY^{192} MET^{168}	Preventive nutraceuticals and/or antiviral drugs in COVID-19	AutoDock Vina in PyRx 0.8	Gyebi et al. (2021)
41.	SARS-CoV-2 3-chymotrypsin-like protease	Isorhamnetin	6Y84	MET^{168} CYS^{145}	Preventive nutraceuticals and/or antiviral drugs in COVID-19	AutoDock Vina in PyRx 0.8	Gyebi et al. (2021)
42.	SARS-CoV-2 3-chymotrypsin-like protease	Luteolin	6Y84	SER^{144}, GLU^{166}(2), and GLN^{189}	Preventive nutraceuticals and/or antiviral drugs in COVID-19	AutoDock Vina in PyRx 0.8	Gyebi et al. (2021)

(continued)

Table 4.1 (continued)

Sr. no	Target enzyme/ protein	Inhibitor/ligand	PDB ID	Binding sites/residues	Biological significance	Software used in the study	References
43.	SARS-CoV-2 3-chymotrypsin-like protease	Vernomenin	6Y84	GLY^{143}, SER^{144}, and CYS^{145}	Preventive nutraceuticals and/or antiviral drugs in COVID-19	AutoDock Vina in PyRx 0.8	Gyebi et al. (2021)
44.	Main protease (M^{pro}) of SARS-CoV-2	Kazinol T	6Y7M	GLY^{143}, SER^{144}	Lead compound against the main protease of SARS-CoV-2	AutoDock	Ijaz et al. (2021) and Avwioroko et al. (2020)

create lots of difficulties during the docking process. The major challenges faced in the docking experiments are that of rigid receptors. Most importantly, molecular docking does not provide insights into the spectrum of action against non-target proteins/receptors. One has to depend on animal and human trials to get this information.

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Chapter 5
H^+/K^+-ATPase Inhibitors from Plants: A Potential Source for Drug Discovery

Michael Buenor Adinortey and Benoit Banga N'guessan

Abstract Apart from regulatory biomolecules, namely acetylcholine, histamine, and gastrin that stimulate gastric acid secretions, H^+/K^+-ATPase also referred to as the proton pump plays a central role in controlling gastric secretions. Any disarray in the secretions of these biomolecules can lead to an imbalance between the aggressive secretions and defense/protective factors, thereby causing hyperacidity. Several drug regimens that target these regulatory biomolecules are available, but the most frequently recommended by health practitioners have been synthetic proton pump inhibitors. The use of synthetic proton pump inhibitors (PPIs) has revolutionized the management of peptic ulcers, nevertheless, there are still various challenges associated with long-term usage that calls for pharmacotherapeutic alternatives. This chapter presents an overview of the structure of H^+/K^+-ATPase highlighting its central role as one of the most appropriate drug targets necessary for control of hyperacidity. In addition, the role of plant natural nutraceutical products as inhibitors of H^+/K^+-ATPase is presented. This review also presents evidence that compounds belonging to different classes of natural products make significant contributions to alleviate gastric acid secretion-related diseases, and thus these compounds or their derivatives could be useful "seed" compounds for developing new drugs and nutraceutical supplements for prevention and management of peptic ulcer diseases.

Keywords H^+/K^+-ATPase · Proton pump inhibitors · Peptic ulcer diseases

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5.1 Introduction

In the last three decades, many pieces of research have been carried out to expand our knowledge of the biology of gastric acid secretion and of acid-related disease. Aside from gastric acid secretion's role in the stomach, there are other functions, including gastric motility, excretion of bile salts, and others, which are beyond the scope of this chapter. One of the biological roles of the stomach is the digestion of food. Gastric acid supports digestion by ensuring an optimal pH for lipase and pepsin to catalyze breakdown of food. Gastric acid secretion is influenced by both the central and peripheral nervous systems. The process is regulated by signal transduction biomolecules, namely acetylcholine, gastrin, histamine, and somatostatin (Engevik et al. 2020). The stimulatory effect of acetylcholine and gastrin is intermediated by an upswing in cytosolic calcium, while the effect of histamine is mediated through activation of adenylate cyclase and production of a second messenger called cyclic adenosine monophosphate (cAMP). It is important to note that the foremost stimulus of gastric acid secretion is gastrin, which does not directly stimulate parietal cells, but mobilizes histamine from oxyntic mucosa through enterochromaffin-like cells (Sachs et al. 2007; Schubert and Peura 2008).

The decisive element in gastric acid secretion is the stimulation of protein H^+/K^+-ATPase also known as proton pump which controls the exchange of cytoplasmic H^+ for extracellular K^+. The protons secreted into gastric lumen by the proton pump combine with luminal Cl^- to form hydrochloric acid in the stomach. After adequate gastric acid is secreted, a feedback mechanism is used to stop it. A key inhibitor of gastric acid secretion is the hormone somatostatin. A decline in intragastric pH stimulates the release of somatostatin from antral D cells. It is clear then that somatostatin inhibits not only gastric acid secretion but also slows gastrin release (Wallmark et al. 1985; Engevik et al. 2020). Unreservedly it can be said that histamine, acetylcholine, and gastrin all function through H^+/K^+-ATPase to allow parietal cells to produce HCl as shown in Fig. 5.1. Any disarray in the secretions of these biomolecules [histamine, acetylcholine, and gastrin] can lead to an imbalance between the aggressive secretions (pepsin, gastric secretions) and defense/protective factors (bicarbonates, mucus secretions, mucosal blood flow, cellular regeneration of the epithelial layer, and endogenous protective agents such as prostaglandins and epidermal growth factor) causing hyperacidity (Chung and Shelat 2017). The gastric H^+/K^+-ATPase enzyme is a central regulatory biomolecule that controls gastric secretions and thus it is responsible for the release of H^+ into the lumen of the stomach leading to hyperacidity and gastric ulcerations. Inhibition of gastric H^+/K^+-ATPase is reported to correlate with healing and symptomatic relief in both erosive esophagitis and gastroesophageal reflux disease in patients (Herszényi et al. 2020).

Globally, several synthetic proton pump inhibitors (PPIs) such as Omeprazole, Esomeprazole, Lansoprazole, Rabeprazole, and Pantoprazole are available for managing gastric acid-related diseases. Though PPIs are among the most commonly used and overprescribed medications for PUDs (Spechler 2019), they are also the most effective prophylactic agents (Strand et al. 2017). While the short term side effects of

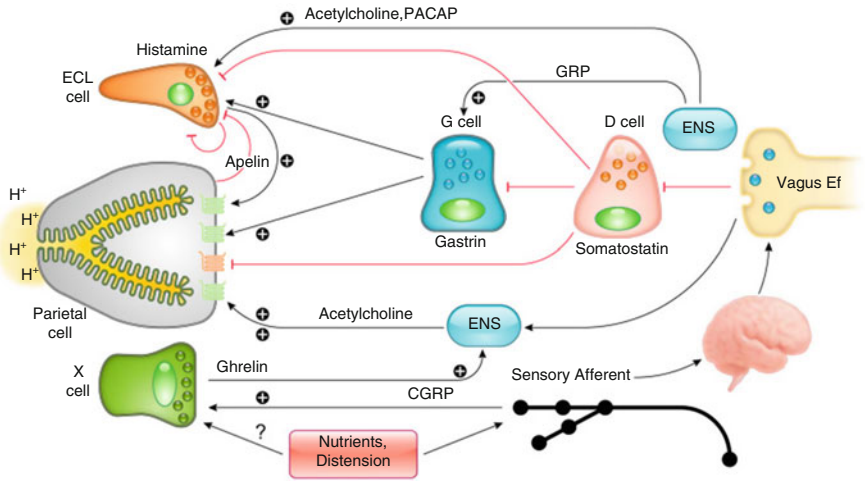


Fig. 5.1 Cellular components that control gastric acid secretions. Numerous cell types regulate gastric acid secretion. Enterochromaffin-like (ECL) cells through histamine and X cells that secrete ghrelin activate parietal cells via paracrine and neural pathways, respectively. (Adapted from Engevik et al. 2020)

PPIs usage such as headache, diarrhea, constipation, and abdominal discomfort are minor and easily managed (Hunt et al. 2015; Maes et al. 2017), systematic and large studies have suggested an association between long-term PPIs usage and several adverse effects such as higher risk of chronic kidney disease (CKD) and dementia, which has been a basis of major concern to patients and physicians (Lazarus et al. 2016; Freedberg et al. 2017; Yu et al. 2017; Moayyedi et al. 2019). This necessitates interest in alternative gastric H^+/K^+ ATPase inhibitors from natural sources which are of nutraceutical value and may be desirable for the prevention of gastric acid-related diseases. It is therefore important to identify plant food components that inhibit gastric H^+/K^+-ATPase.

A potential natural source of H^+/K^+-ATPase inhibitors is provided by the abundance of plants in nature. This chapter presents an insightful overview of the protein structure of H^+/K^+-ATPase highlighting its central role as one of the most appropriate drug targets necessary for the control of hyperacidity. In addition, the impact of different nutraceutical plant products on gastric H^+/K^+-ATPase enzyme is offered as evidence for serious consideration in the drug discovery pipeline globally. Furthermore, selected isolated compounds from plant sources of nutraceutical importance reported to possess H^+/K^+-ATPase inhibitory activity are captured with information on their potency. The pharmaceutical industrial prospects have also been elaborated on.

5.2 Structure of Gastric H^+/K^+-ATPase and Its Role as a Drug Target

The gastric H^+/K^+-ATPase is an enzyme expressed on the apical canalicular membrane of parietal cells. It belongs to P-type ATPase family. This enzyme which is an α,β-heterodimeric protein uses energy derived from ATP hydrolysis to pump intracellular hydrogen ions into the lumen, in exchange for extracellular potassium ions (Abe et al. 2018). Hydrochloric acid is formed through the interaction between Cl^- ions of arterial blood and H^+ from parietal cells.

The α subunit of the enzyme is bonded to the β subunit. The α subunit of a molecular weight (Mwt) 100 kDa contains the catalytic site. It is comprised of ten membrane spanning segments (TM1 to TM10) and three cytosolic domains, namely nucleotide binding-N, phosphorylation-P, and activation-A. The α subunit also has conserved sequences along with the other P_2 type ATPases for the ATP binding site and the phosphorylation site. The phosphorylation site is reported to be at Asp386, which is well conserved in other P-type ATPases (Abe et al. 2018). The β subunit, which has a Mwt of 35 kDa is non-covalently bonded to the α subunit in the region of the sequence Arg898 to Arg922 in the α subunit (Bamberg and Sachs 1994). It contains roughly 290 amino acid residues with a single transmembrane segment that is located at the region near the N-terminus (Shin and Sachs 1994).

Stimulation of gastric acid secretions encompasses the translocation of H^+/K^+-ATPase to the apical membrane of the parietal cell. When the cell is at rest or when unstimulated, H^+/K^+-ATPase is located in vesicles inside the cell. When the cell is stimulated, these vesicles fuse with the plasma membrane, thereby causing an increase in the surface area of the plasma membrane and the number of H^+/K^+-ATPase in the membrane (Abe et al. 2018; Engevik et al. 2020). Implicitly it is clear that gastric H^+/K^+-ATPase enzyme is a key regulatory protein that controls gastric secretions and thus it is responsible for the release of H^+ into the lumen of the stomach leading to hyperacidity and gastric ulcerations. The central role played by the H^+/K^+-ATPase enzyme (proton pump) in gastric acid secretions makes it a key drug target for controlling hyperacidity-related disorders of the stomach.

5.3 Mechanism of Action of H^+/K^+-ATPase Inhibitors

It is worth noting that early treatments of peptic ulcers and hyperacidity-related disorders started with the use of anti-acids, which act to neutralize gastric acid and acetylcholine antagonists. These classes of drugs became outdated in the early1980s by histamine receptor antagonists (H_2RAs) such as cimetidine and ranitidine. By the late 1980s, H^+/K^+ ATPase inhibitors also known as proton pump inhibitors (PPIs) began to emerge. PPIs are prodrugs, which are bioactive only after protonation. They block gastric H^+/K^+-ATPase, inhibiting gastric acid secretions. This outcome enables healing of peptic ulcers, gastroesophageal reflux disease (GERD), Barrett's

esophagus, and Zollinger–Ellison syndrome, as well as other related diseases as part of combination regimens (Sachs et al. 2000). PPIs are a class of drugs that cause a profound and prolong reduction of gastric acid production. They perform this role by irreversibly binding to and inhibiting gastric H^+/K^+-ATPase that resides on the luminal surface of the parietal cell membrane.

In humans, the stomach organ is the only acidic space with a pH lower than 4. With the recognition that the known H^+/K^+-ATPase inhibitors are weak bases with a pK_a between ~4.0 and 5.0, it implied that they would accumulate in the acidic space (Shin et al. 2004). This acid space dependent concentration of the PPIs is the first vital property that controls their therapeutic index, offering a concentration at the luminal surface of H^+/K^+-ATPase pump that is about 1000-fold that in the blood. The second vibrant phase is the low pH dependent conversion from the accumulated prodrug to the activated species that is an extremely reactive cationic thiophilic reagent. This implies that protonation of these compounds is vital for their activation to form disulfides with cysteines of H^+, K^+-ATPase. When the rate of conversion of different compounds was measured as a function of pH, it was uncovered that the pH dependence of activation reflected protonation of the benzimidazole moiety (Shin et al. 2004; Sachs et al. 2007) which explains the different activation rates among synthetic PPIs.

Subsequent to accumulation in the stimulated secretory canaliculus of the parietal cell and binding to the pump, the protonation activates it to form the thiophilic drug that reacts with luminally accessed cysteines on H^+/K^+-ATPase. It implies that the presence of acid secretion is critical for their action. This explains why in prescription, PPIs are given ~30 min before mealtime to ensure that H^+/K^+-ATPase pumps are active during peak concentrations of the PPIs in the blood (Fellenius et al. 1981; Shin et al. 2004). The protonation step results in selective accumulation in the secretory canaliculus of the parietal cell. In acid, there is an acid catalyzed conversion to the sulfenic acid and hence to the sulfenamide. Either of these can inhibit the H, K ATPase although it appears more likely that the sulfenic acid is the primary inhibitor (Sachs et al. 2000).

5.4 Plant Nutraceutical Products as Proton Pump Inhibitors

A nutraceutical product can be defined as a substance that has a physiological benefit or provides protection against chronic diseases (Hay et al. 2019). The use of natural products as nutraceuticals for the prevention of various ailments is as old as human civilization. Generally, natural products are everything produced by life such as wood, bioplastics, cornstarch, milk, and plants extract. Most of these natural products are organic compounds synthesized by a living organism via a process which more or less transforms their biological activities. The chemical constituents of plants that produce certain physiological actions on the human body are known as

phytochemicals. There are thousands of phytochemicals in plants that may not be required as essential nutrients but may enhance the health status of organisms. The important bioactive compounds obtained from plants, called phytochemicals, are terpenes, alkaloids, and phenolics such as tannins and flavonoids (Hay et al. 2019). Extensive use of these natural products as either a spice or food or medicine has several beneficial effects.

The use of plant-based nutraceuticals forms the basis of many modern pharmaceuticals. It is now considered a keystone of health care services all over the world. There is the need to make available evidence-based data on these nutraceuticals in order to promote their development into generally acceptable food supplements or drugs for prevention of diseases including peptic ulcers. Studies on plants used as food and medicine have led to the discovery of proton pump inhibitors from plant sources. Spectrophotometric analytical technique is mostly used to estimate gastric H^+/K^+ ATPase inhibitory activity in vitro and in vivo.

Increased acid secretion in part plays a role in the pathogenesis of gastric ulcers. The unavailability of data on these plant natural products in a one stop-document despite the reported evidence of efficacy necessitates the need to amass information on them and their constituents that possess proton pump inhibitory effects. This section highlights selected medicinal plants used also as food with reported gastric H^+/K^+-ATPase inhibitory activity. A list of selected plants with nutraceutical importance reported to have significant gastric H^+/K^+ ATPase inhibitory activity is shown on Table 5.1. Largely, researchers have reported on the potency of many plant extracts by determining the concentration needed to inhibit 50% (IC_{50}) of the activity of gastric H^+/K^+ ATPase. This section presented IC_{50} data available in μg/mL for easy comparison of their potencies.

There are several mechanisms involved in the inhibition of acid secretion induced by edible natural ingredients. Several studies have shown that some edible natural ingredients prevent the development of gastric ulcers via inhibition of acid secretion in various animal models. Some of these edible plants include *Solanum nigrum* aerial parts, *Arctium lappa* roots, *Garcinia* kola seeds, *Garcinia mangostana* fruit, seeds of *Azadirachta indica*. Hot water extract of *Garcinia mangostana* fruit peel recorded IC_{50} value less than 10 μg/mL compared to its ethanolic extract which showed a higher IC_{50} value of 19.96 μg/mL (Nanjarajurs et al. 2014). The leaf extract of *Carissa carandas* showed potency of 25 μg/mL (Shukla et al. 2016). Plants, namely *Acalypha wilkesiana* (Gupta and Pradeepa 2013), *Arctium Lappa* L. (Dos Santos et al. 2008), *Annona squamosa* (Yadav et al. 2012), *Decalepis hamiltonii* (Wight & Arn.) (Naik et al. 2007), *Delonix* regia (Sachan et al. 2017) *Cecropia glazioui* (Souccar et al. 2008), *Cissus quadrangularis* L. (Yadav et al. 2012), *Pongamia pinnata* (L.) Pierre (Belagihally et al. 2011), and *Tectona grandis* leaves (Lakshmi et al. 2010) exhibited an inhibition on H^+/K^+-ATPase with IC_{50} values ranging from 30 to 70 μg/mL. It is interesting to note that 50% hydroethanolic extract of *Garcinia mangostana* (Nanjarajurs et al. 2014) showed IC_{50} value of 164 μg/mL markedly different from that of 70% hydroethanolic extract. Fruits of *Xylocarpus granatum* plant (Lakshmi et al. 2010), *Hedranthera barteri* (Onasanwo et al. 2011), and *Solanum nigrum* fruits (Jainu and Devi 2006) also showed

Table 5.1 Selected plants with H^+/K^+ ATPase inhibitory activity

Plant name	Family	Part used	Extraction solvent	Study model	IC50 (μg/mL)	References
Scoparia dulcis L.	Scrophulariaceae	Aerial parts	Aqueous	In vivo	500	Mesía-Vela et al. (2007)
Arctium Lappa L.	Asteraceae	Root	Chloroform	In vitro	53	Dos Santos et al. (2008)
Cecropia glazioui	Cecropiaceae	Aerial	*n*-Butanol	In vitro	58.8	Souccar et al. (2008)
Annona squamosa	Annonaceae	Twigs	Ethanol	In vitro	31.43	Yadav et al. (2012)
		Twigs	Chloroform	In vitro	55.98	
		Twigs	Hexane	In vitro	62.24	
Acalypha wilkesiana	Euphorbiaceae	Leaf	Water	In vitro	51.5	Gupta and Pradeepa (2013)
Carissa carandas	Apocynaceae	Leaf	Methanol/acetone	In vitro	25	Shukla et al. (2016)
Cissus quadrangularis L.	Vitaceae	Tubers	Methanol	In vitro	38	Yadav et al. (2012)
Decalepis hamiltonii	Asclepiadaceae	Roots	Aqueous	In vitro	36	Naik et al. (2007)
Delonix regia	Fabaceae	Stem bark	Ethanol	In vitro	68.31	Sachan et al. (2017)
Garcinia kola Heckel.	Guttiferae	Seeds	Petroleum ether	In vitro	43.8	Onasanwo et al. (2011)
Garcinia mangostana	Guttiferae	Fruit peel	Hot water (60 °C)	In vitro	7.6	Nanjarajurs et al. (2014)
			Ethanol (70%)	In vitro	19.96	
			Alcohol (50%)	In vitro	164.58	
Hedranthera barteri	Apocynaceae	Roots	Dichloromethane	In vitro	89.69	Onasanwo et al. (2011)
Pongamia pinnata (L.)	Leguminosae	Seeds	Petroleum ether	In vitro	39.5	Belagihally et al. (2011)
Solanum nigrum	Solanaceae	Aerial parts	Aqueous	In vitro	121.81	Jainu and Devi (2006)
Tectona grandis	Verbenaceae	Leaf	Ethanol/butanol	In vitro	499.36	Lakshmi et al. (2010)
			Butanol		69.03	
Xylocarpus granatum	Meliaceae	Fruit	Chloroform	In vitro	89.37	Lakshmi et al. (2010)

substantial potencies between 71 and 200 μg/mL. Plants that exhibited poor inhibitory activity against gastric H^+/K^+-ATPase with IC_{50} value above 201 μg/mL are *Scoparia dulcis* L. (Mesía-Vela et al. 2007), ethanolic extract of *Tectona grandis* (Lakshmi et al. 2010).

Generally, the plant that showed the highest potency among all available data was hot water *Garcinia mangostana* fruit peel an indication of its potential to be considered in the discovery pipeline. It is interesting to note that most plants that showed potency between 7 and 30 μg/mL were better than the standard drugs used as a positive control in that experiment. This calls for further research into nutraceuticals with evidence-based data on proton pump inhibitory potentials.

5.5 Nutraceuticals Compounds as Proton Pump Inhibitors

Plant medicines that are pure isolated compounds have remained useful and unmatchable sources of molecules for effective prevention and treatment of disease burdens. Plant-derived polyphenols have drawn attention owing to their biological properties, including health-promoting benefits. Most pharmaceutical drugs including opium, aspirin, digitalis, and quinine, which are currently available in orthodox medicine, have a long history of use as herbal medications. Carbenoxolone, the first systemically effective anti-ulcer agent, was isolated from the plant, *Glycyrrhiza glabra*. It is the first plant that proved effective in the treatment of gastric ulcers. Gefarnate, another effective compound isolated from the juice of wild cabbage, was found to be effective against gastric ulcers. It was shown to improve the gastric defensive mechanism, by increasing mucus synthesis in the mucosa through an enhanced synthesis of prostaglandins (Damle 2014).

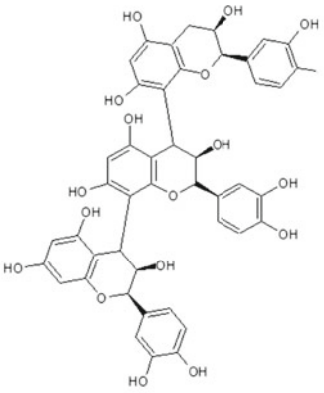
Work on new bioactive compounds from plants has led to the isolation and structure elucidation of a number of new compounds. A list of phytoconstituents having significant gastric H^+/K^+ ATPase inhibitory activity is provided on Table 5.2 and molecular structures of some of these compounds are shown in Fig. 5.2. Among compounds shown on Table 5.2, flavonoids seem to have better gastric H^+/K^+ ATPase inhibitory effects. This could be attributed to the fact that plant-derived phenolic compounds have numerous beneficial properties by virtue of their ability to act as antioxidants. In the gastric H^+/K^+ ATPase, there is a lysine 791 located in the fifth transmembrane segment that replaces a serine present in the Na^+/K^+ ATPase isoforms. This lysine of the H^+/K^+ ATPase seems to characterize the H^+/K^+-enzyme specificity for outward transport of the hydronium ion (Shin et al. 2009). Thus, the potent inhibition of the gastric H^+/K^+ ATPase enzyme might be a result of the interactions that occur between the flavonoids and the lysine residues. Further experiments such as computer-assisted homology modeling, molecular docking, and molecular dynamics simulation could be done to understand the actual mechanisms by which these flavonoids and alkaloidal compounds from a large family of plants are able to inhibit the gastric H^+/K^+ ATPase enzyme. However, the synergistic use of these phytoconstituents could also be explored.

Table 5.2 Selected nutraceutical compounds with H^+/K^+-ATPase inhibitory activities

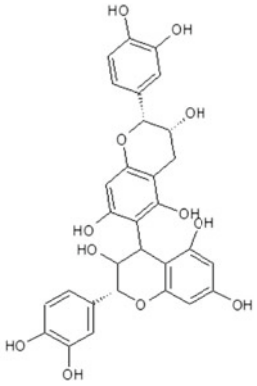
Compound	Plant	Family	IC_{50} (μg/mL)	References
Alkaloid				
Uleine	*Himatanthus lancifolius*	Amaranthaceae	197	Baggio et al. (2005)
Peganine	*Peganum harmala*	Zygophyllaceae	73.47	Singh et al. (2013)
(+)-*O*-methylarmepavine	*Annona squamosa*	Annonaceae	111.83	Yadav et al. (2012)
N-methylcorydaldine	*Annona squamosa*	Annonaceae	60.9	
Isocorydine	*Annona squamosa*	Annonaceae	88.42	
Flavonoids				
Procyanidin B5	*Cecropia glaziovii*	Cecropiaceae	36.9	Souccar et al. (2008)
Procyanidin B3 + cathechin	*Cecropia glaziovii*	Cecropiaceae	34.8	
Procyanidin B2	*Cecropia glaziovii*	Cecropiaceae	23.5	
Epicatechin	*Cecropia glaziovii*	Cecropiaceae	43.8	
Procyanidin C1	*Cecropia glaziovii*	Cecropiaceae	40.3	
Orientin	*Cecropia glaziovii*	Cecropiaceae	31	
Isoorientin	*Cecropia glaziovii*	Cecropiaceae	18.1	
Isovitexin	*Cecropia glaziovii*	Cecropiaceae	25.9	
Verbascoside	*Tectona grandis*	Verbenaceae	60.98	Singh et al. (2010)
Terpenes				
Gedunin	*Xylocarpus granatum*	Meliaceae	56.86	Lakshmi et al. (2010)
Photogedunin	*Xylocarpus granatum*	Meliaceae	66.54	
Azadiradione	*Azadirachta indica seed*	Meliaceae	87.75	Singh et al. 2015)
Anthraquinone				
Chrysophanol	*Rheum emodi*	Polygonaceae	187.13	Mishra (2016)
Emodin	*Rheum emodi*	Polygonaceae	110.30	

5.6 Conclusion

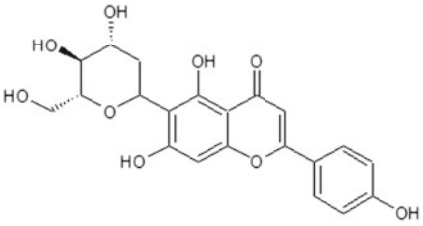
Promising plant species and numerous phytoconstituents as H^+/K^+ ATPase inhibitors are presented in this chapter. The foremost class of natural products widely reported to have H^+/K^+ ATPase inhibition potential is flavonoids. This chapter has collected data to show that edible natural products are effective for the prevention of gastric ulcers induced by hyperacidity. Because these natural products are generally safe and widely available, they could be a promising regime strategy for the prevention and management of hyperacidity-related disorders, particularly for individuals who require long-term usage of PPIs once clinical data is amassed on it.



Procyanidin C1



Procyanidin B5



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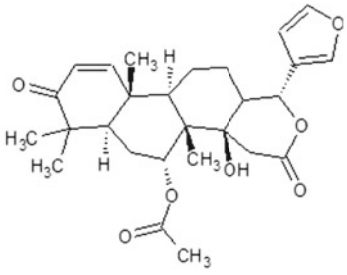
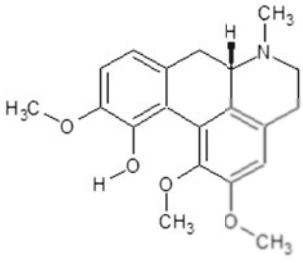


Isocorydine



Gedunin

Fig. 5.2 Molecular structures of some selected compounds

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Chapter 6
Use of Protease Inhibitors as a Promising Alternative for Pest Control

Neilier Rodrigues Silva-Junior, Yaremis Beatriz Meriño Cabrera, Rafael de Almeida Barros, and Maria Goreti de Almeida Oliveira

Abstract Proteases are responsible for several processes essential to life and, controlling their activity is naturally important in many specific metabolic events. When a phytophagous insect feeds, the response machinery of a plant leads to the production of protease inhibitors (PIs), which can occur locally or systemically. Upon reaching the insect's intestine, PIs bind to specific proteases and compromise the insect's digestibility, reducing the absorption of dietary amino acids. The impaired nutritional balance affects the insect's development and can lead to death. In this sense, PIs have gained prominence as alternatives in the control of pest insects, minimizing the toxic effects on other animals and the environment. Conversely, insects express multiple isoforms of important digestive enzymes to circumvent the toxic effect of plant PIs. Our research group is dedicated to understanding the biochemical mechanisms involved in plant–pest interaction from an enzymatic, proteomic, and molecular biology point of view. Because of these efforts, dozens of articles were generated, besides PI patented for use as ecologically correct agricultural defensives. This chapter provides an updated overview of advances in PI research applied to insect pest control.

Keywords Protein · Peptides inhibitors · Plant–pest interaction · Insect pest control

6.1 Introduction

Proteases handle several processes essential to life. This diverse group of enzymes can cleave peptide bonds to finely control protein catabolism, selectively degrade damaged proteins, or promote mass hydrolysis of dietary proteins. Advances in enzymology and proteomics in recent decades have shown that proteases are

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essential not only for providing free amino acids to the cell but also for modulating important processes, such as the removal of specific segments in zymogens (Stroud et al. 1977; Gorelick and Otani 1999; Donepudi and Grütter 2002; Plainkum et al. 2003) and immature proteins (Peng et al. 1989; Muramatsu and Fukazawa 1993; Khan and James 1998) or the removal of a signal peptide when the protein is already in the appropriate cell compartment (Hussain et al. 1982; Novak and Dev 1988; Friedmann et al. 2004; Lemberg 2011).

Proteolytic activity is also linked to the need to control specific metabolic events, such as the final processing of proteins before they play their role in the cell (Lum and Blobel 1997; Guttentag et al. 2003; Grau et al. 2005; Manolaridis et al. 2013), selectively removing proteins when they are no longer useful or recycling amino acids needed to synthesize new polypeptide chains (Ciechanover 1994; Bochtler et al. 1999). Besides intra- and intercellular processes, proteases mediate several molecular interactions that occur between different organisms in a given environment. Intracellular parasites, for example, secrete proteases that help their interactions and survival in the host cell (Alves and Colli 2007; Knox 2007; Laliberté and Carruthers 2008). Likewise, the hydrolysis of plant proteins in the intestine of herbivorous insects are extracellular processes that aim to provide free amino acids that will be absorbed to later make up new proteins (da Silva Júnior et al. 2020).

During the evolutionary process, insects gained complex protease systems, an essential process to get a better nutritional benefit (Silva-Júnior et al. 2021). If, on the one hand, an arsenal of proteases favors herbivory, co-evolution has selected plants with strategies to overcome the adverse effects of insect proteases (Zhu-Salzman and Zeng 2015; Pilon et al. 2017a; Meriño-Cabrera et al. 2018). Plants challenged by a pathogen or predator, for example, produce protease inhibitors (PI) that bind to proteolytic enzymes and prevent or limit their activity (Ryan 1990; Habib and Fazili 2007); this digestive deficiency implies less free amino acids to be absorbed and used as raw material in the synthesis of proteins necessary for the proper development of the insect. It is already well established that a wide variety of organisms use PIs not only to control endogenous proteolytic functions but also to ensure their protection against herbivory or infection. In this sense, PIs act for the complex set of molecular interactions that occur between different organisms in an ecosystem, acting as regulators of proteolytic events. Therefore, it is not surprising that PIs are being proposed as a tool for the control of herbivores and pathogens (Clemente et al. 2019).

Based on this, pest control strategies using PIs were developed to control nematodes (Turrà et al. 2009), viruses (Masoud et al. 1993) bacteria (Mishra et al. 2020), and phytophagous insects (Senthilkumar et al. 2010). The effects of dietary PIs on the fecundity and growth of herbivorous insects have been described for several species (Thomas et al. 1995; Telang et al. 2003; Jamal et al. 2015; Dantzger et al. 2015; Singh et al. 2020), and the implication of extracellular proteases in pathogenic processes has been documented in several cases (Dunaevsky et al. 2005; Armstrong 2006; Santos and Braga-Silva 2012). For this, the use of PIs of protein origin was proposed to be expressed through transgenics to protect plants from agricultural pests (Gatehouse et al. 1993; De Leo et al. 2002; Zhu-Salzman and

Zeng 2015). Today, the expression of PIs by genetically modified plants is a reality in the control of herbivores and plant parasites (Rahbé et al. 2003; Riglietti et al. 2008; Senthilkumar et al. 2010; Khalf et al. 2010). Therefore, this additional protection granted to economically important plants has a powerful appeal from the food, biofuel, textile industry and from the entire production chain that involves them.

6.2 Serine Proteases and Plant Protease Inhibitors

According to the enzymatic classification system created in 1956 (Knight 1962), serine proteases (EC 3.4.21) belong to the class of hydrolases, a sub-class of hydrolases that act on peptide bonds. The family name derives from the nucleophilic Ser residue in the active site of the enzyme, which attacks the peptide carbonyl group of the substrate to form a tetrahedral acyl-enzyme intermediate (Hedstrom 2002; Cox and Nelson 2008). At the end of the peptide bond hydrolysis, the complete organic reaction mechanism of the serine proteases involves the catalytic triad composed of Ser, His, and Asp (Matthews et al. 1967; Blow et al. 1969; Henderson 1971).

Serine proteases are the best studied peptidases and are considered the main responsible for protein digestion in the intestine of important pest insects, such as those belonging to the orders Lepidoptera (Pilon et al. 2017b; Zhao and Ee 2018; Meriño-Cabrera et al. 2018; Zhao et al. 2019; da Silva Júnior et al. 2020) and Coleoptera (Mochizuki 1998; Alarcón et al. 2002; Marshall et al. 2008). At this point, enzymes from the Trypsin-like, Chymotrypsin-like, and Elastase-like families stand out as the main representatives (Kuwar et al. 2015; da Silva Júnior et al. 2020). Because of this importance, several plant serine protease inhibitors have been described and characterized.

Protease inhibitors of protein origin are classified into 99 families according to the homology existing in the amino acid sequence of their representatives, at least in the inhibitory unit. There may also be subfamilies when there is evidence of a very old evolutionary divergence within the family. PIs are also grouped into clans, which represent a group of inhibitors in one or more families that show evidence of an evolutionary relationship from their similar tertiary structures (Rawlings et al. 2018).

Against this background, plant protease inhibitors (PPI) are usually small proteins found in plant storage tissues such as the root, but also in leaves (De Leo et al. 2002). In seeds, tubers, and other plant storage tissues, trypsin inhibitors represent about 10% of the total protein content (Mandal et al. 2002). These high levels of PPI are associated with plant resistance to insects and pathogens (Kim et al. 2009; Dunse et al. 2010). Although high levels of PPI are often found in Leguminosae, Solanaceae, and Gramineae (Brzin and Kidrič 1996; Xu et al. 2001; Sin and Chye 2004), the expression of these PPI depends on the stage of development of the plant, tissue, and presence of stressors, even presenting different isoforms in the same tissue (Sels et al. 2008).

Two very well-studied PPI families are Kunitz and Bowman-Birk. Members of the Kunitz family have in their primary structure some conserved residues, such as the four cysteine residues that form the two intrachain disulfide bonds (Pouvreau et al. 2003), besides being monomeric proteins containing from 150 to 200 amino acid residues and approximately 20 kDa (Norioka et al. 1988). Each molecule contains a unique binding site that interacts strongly with the protease against which the inhibitor is targeted (Salier 1990).

On the other hand, Bowman-Birk PPIs are polypeptide chains of approximately 8 kDa that can form oligomers, ranging from 54 to 133 amino acid residues (Birk 1985; Kennedy 1998). A Bowman–Birk basic unit contains a high proportion of cysteine residues and forms several intrachain disulfide bonds, leading to a rigidly folded conformation (Losso 2008). The monomeric unit contains two binding loops with reactive sites on the enzyme. Therefore, each inhibitor can inhibit up to two proteases with different inhibitory specificities (Qi et al. 2005).

Although PPIs are very well documented as plant defensive compounds, the damage caused to insects and pathogens goes far beyond just decreasing the activity of digestive proteases. The molecular mechanisms are not fully known, but metabolome, transcriptome, proteome, and histology studies have shown several effects on the physiology of insects subjected to PPI (Valueva and Mosolov 2004; Bayés et al. 2006; Quilis et al. 2007; Bobbarala 2009; Sabotič and Kos 2012; Radanovic and Anauate 2013; Quilis et al. 2014; Shao et al. 2016; Cingel et al. 2017; Shamsi et al. 2018).

6.3 Contributions in the Field from Our Research Group

The use of PIs as an agricultural defensive was suggested as far back as 1947, when Mikel and Standish (1947) observed that a soy-based diet limited the development of some insects. Just 25 years later, Green and Ryan (1972) demonstrated that damage to nightshade leaves induced PI synthesis, suggesting the protective role of this compound. In the following decades, the economic and environmental importance of developing alternative strategies for the ecologically correct control of agricultural pests increased interest in the development of IP for this purpose. However, the biochemical mechanisms involved in the interaction between insect physiology and PIs were not well known.

Given this scenario, our research group focused on understanding the biochemical mechanisms involved in the plant–pest interaction from the point of view of biochemistry and molecular biology, exploring techniques of enzymology, proteomics, metabolomics, and transcriptomics. The interaction between soybean (*Glycine max*) and soybean caterpillar (*Anticarsia gemmatalis*) was used as a model to validate the biochemical mechanism of plant response via the Lipoxygenase pathway (Fig. 6.1). Soy was chosen for its importance in agricultural production in Brazil, and *A. gemmatalis* was chosen because, besides being a key soybean pest, it is an insect that is easy to breed and presents a good yield of biological material for

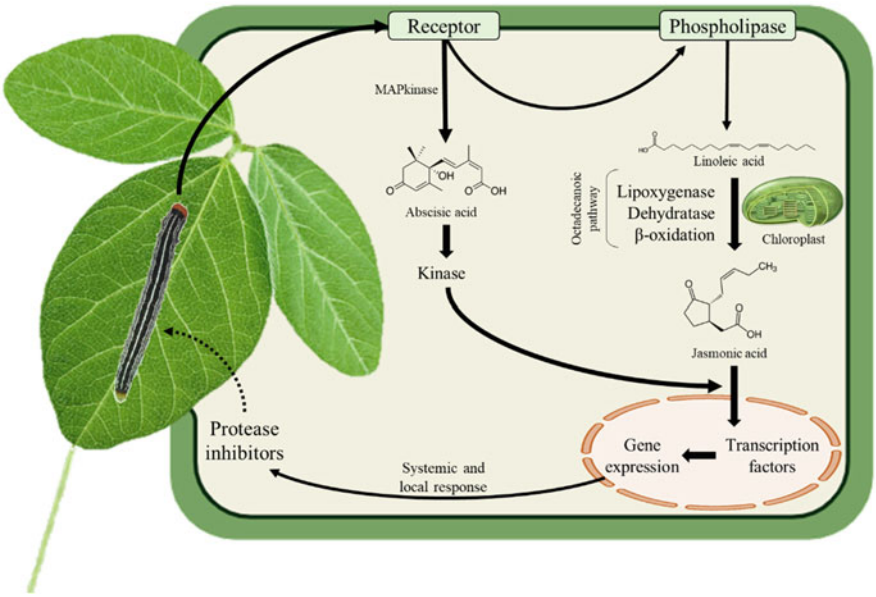

Fig. 6.1 Lipoxygenase pathway. The mechanical damage caused by the biting insect activates a cascade of intracellular messengers and leads to the formation of jasmonic acid. This plant hormone activates transcription factors that end with the production of protease inhibitors, compromising the insect's digestibility

studies involving biochemical analyses that require purification and characterization of the enzymes involved in the insect–plant interaction process.

The study of plant–insect interaction requires a thorough understanding of the arthropod and the plant under properly controlled conditions. In this sense, the determination of the enzymatic profile in the different larval instars of *A. gemmatalis* was an important step to determine the proteases responsible for the digestive process of the caterpillar throughout its development. For this, da Silva Júnior et al. (2020) showed that the proteolytic profile in the intestine of *A. gemmatalis* changes during larval development, with a predominance of cysteine protease activity in the third instar and serine protease in the fourth and fifth instars, suggesting modulation in gene expression accompanied by different nutrient demand throughout this internship. Previous studies involving Lepidoptera focused on the physiology and biochemistry of the insect only in the fifth instar, as this is the moment of the greatest voracity of the caterpillar. However, some studies show changes in the morphological profile in the intestine of some insects during development (Chougule et al. 2005; Kipgen and Aggarwal 2014; Zhao et al. 2019). This dataset suggests that both cysteine and serine proteases are important targets for the development of PIs, aiming to compromise larval development in different instars. Insects express an arsenal of isoforms for digestive enzymes, having as an important consequence the attempt to circumvent the negative effects of PIs (Kotkar et al.

2009; Lomate and Hivrale 2011; Crava et al. 2013). Therefore, knowledge of the primary and tertiary structures of proteins is of fundamental importance. Thinking about that, Silva-Júnior et al. (2021) described the proteomic profile of the intestine of *A. gemmatalis*, showing a large number of functional enzymes, their sequences and post-translational modifications (PTM) through proteomics techniques. The description of a proteomic profile of enzymes is a challenge because of the low concentrations of these hydrolases concerning other abundant proteins present in the sample. However, the conciliation of several proteomic methodologies allowed for high coverage of the intestinal proteome of the caterpillar as shown in the workflow in Fig. 6.2. Furthermore, research by our group showed the energies and points of interaction between enzymes and inhibitors by molecular docking are important information for the rational design of PIs (Meriño-Cabrera et al. 2019, 2020; de Almeida Barros et al. 2021; Silva-Júnior et al. 2021).

A deeper knowledge of the enzymatic kinetics of digestive proteases from *A. gemmatalis* allows a better understanding of the active centers, the mechanisms of reaction of these enzymes, and the PI that need to be applied as inhibitors of the complex arsenal of digestive proteases of the insect. In this sense, trypsins bound to the intestinal membrane of the soybean caterpillar were partially purified and identified by mass spectrometry (Reis et al. 2012). In addition, our research group also evaluated the contribution of endosymbiotic bacteria in the production of proteolytic enzymes in the intestine of *A. gemmatalis* (Pilon et al. 2013). The main trypsins of these bacterial isolates were purified and kinetically characterized (Pilon et al. 2017b) and this dataset allowed us to infer that endosymbiont bacteria synthesize trypsin, contributing to the insect's digestibility.

These works developed by our group brought a look towards the insect and its intestinal enzymology of *A. gemmatalis* under normal conditions, that is, free from PI treatments. However, the development of PIs, peptides, or organic peptide mimetics with inhibitory activity requires a thorough understanding of the enzyme-inhibitor complex. Inhibition kinetic studies are tools for understanding the multi-mechanistic enzyme system. Thus, we performed the kinetic characterization of trypsin-like inhibition of the insect against natural soybean PI (SKTI and SBBI) and synthetic PI (Benzamidine and Berenil) to understand the inhibition from the physiological structure/function point of view (Patarroyo-Vargas et al. 2020; Silva-junior and de Almeida Oliveira 2021). Our study showed, for the first time, the adaptation of trypsin-like enzymes in the intestine of *A. gemmatalis* against different inhibitors. The effect of PI was also evidenced when the caterpillar was challenged with Benzamidine (Pilon et al. 2018), Berenyl (Moreira et al. 2011; Paixão et al. 2013), synthetic peptides (Patarroyo-Vargas et al. 2018; de Oliveira et al. 2020; de Almeida Barros et al. 2021), SKTI, SBBI (Mendonça et al. 2020), ILTI and ApTI (Meriño-Cabrera et al. 2020).

If, on the one hand, the in-depth study of the pest insect is important, it is necessary to understand the physiology of the target plant and its response systems against the herbivore. With this in mind, we performed biological assays associated with metabolome analysis in two soybean genotypes contrasting for herbivory resistance in response to *A. gemmatalis* (Gomez et al. 2018). This approach allowed

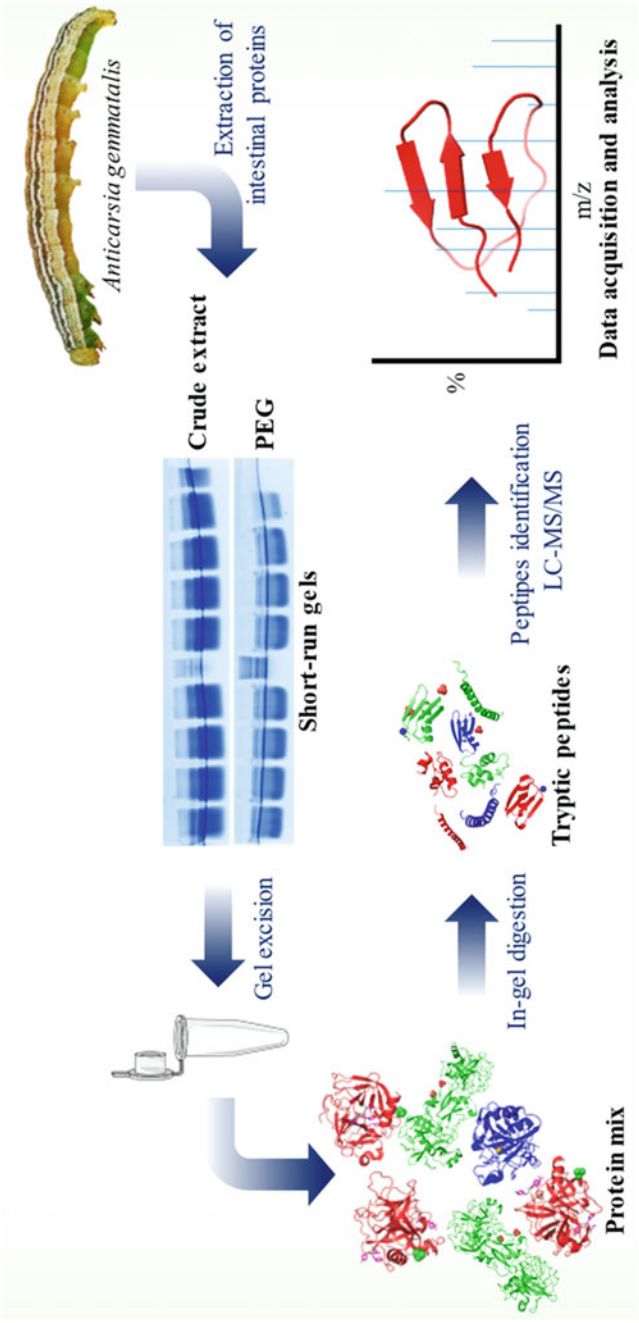

Fig. 6.2 The intestine extracted from *Anticarsia gemmatalis* was fractionated with polyethylene glycol to remove abundant proteins. The crude extract and the result of PEG precipitation were submitted to short-run SDS-PAGE. The proteins present in the gel were identified by mass spectrometry

showing flavonoid profiles from soybean leaf extract and efficiently identifying some new compounds related to resistance. With the metabolic profiles, it was possible to reconstruct the biosynthetic pathways of flavonoids, revealing upregulated glycoconjugate flavonoids in the resistant soybean genotype. These differences in abundance between genotypes suggest they handle resistance to herbivory in these varieties and open the door to a vast field of investigation aimed at increasing soybean resistance against insects. Still from the perspective of how the plant perceives and reacts to damage caused by the herbivore, we show that the response to flavonoids also occurs when the plant suffers artificial mechanical damage (da Silva Júnior et al. 2021). In addition, the deletion of genes in soybean seeds that code for proteins important to plant defense, such as the lipoxygenase enzyme and PI SKTI, does not interfere with the plant's ability to respond to wounds through the lipoxygenase pathway (da Silva Fortunato et al. 2007). These results have industrial and practical appeal since these proteins are undesirable in the seed, but fundamental in the plant's defense against agricultural pests.

Given the reality of climate change, it is important to foresee how the plant–insect interaction responds to environmental variations. Faustino et al. (2021) showed that soybean subjected to drought reduces herbivory and survival of *A. gemmatalis*. The group relied on gene expression, enzymatic kinetics, and metabolomic analysis to conclude that the drought signal alone is not enough to promote increased resistance to insect attack.

These results generated by our research group in the last decade made it possible to identify the target enzymes and map the active sites, allowing the development of potent peptide PIs to be sprayed, used as models for mimetic peptide production, or even as a model for transgenics in the agricultural pest control. We have developed promising protein inhibitors for agricultural pest control. Part of these contributions is compiled in Fig. 6.3.

6.4 Final Considerations

The agricultural ex vivo application of protease inhibitors is still limited because of the large molecular size, which turns them unstable in the environment. We believe that smaller scaffold peptides designed according to the active site of important digestive proteases and reactive domains of protease inhibitors could overcome this bottleneck. Besides that, novel designed peptides have an advantage over vegetable PIs that are not having co-evolved with insects, which might avoid adaptations. To counteract the complex set of proteases that insects possess in their midgut, exposing them to PIs for different classes of proteases could overcome the adaptative mechanisms more efficiently. Despite all the bottlenecks, PIs could be useful in integrated pest management as an alternative/supplementary approach if well explored.

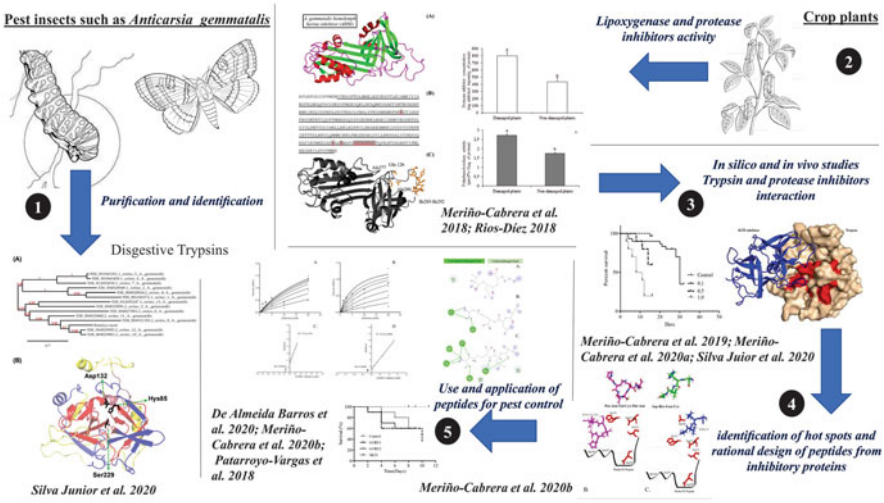

Fig. 6.3 Some researches carried out in our group are characterized by the study of the insect–plant interaction. *Anticarsia gemmatalis* represents the main object of study of our papers. And the plants are crops such as soybeans, tomatoes, and coffee, but mainly soybeans have been used because it is the host plant of *A. gemmatalis*. In insect pests, the activity of enzymes from the digestive tract of caterpillars has been evaluated and characterized, mainly trypsins, enzymes that catalyze the degradation of proteins, the activity has been determined in crude extracts from the intestine and in samples enriched as trypsin isoforms from affinity purification, as well as two-dimensional electrophoresis associated with mass spectrometry (**1**). In parallel, in the host plants of these pest insects, a metabolic defense pathway has been evaluated, which is the lipoxygenase pathway that activates the expression of protease inhibitors, as well as the purification and stability analysis of these inhibitors (**2**). From the results of several studies of insect–plant interaction, we started to evaluate the interaction of protein inhibitors and trypsins through in silico studies (protein modeling, protein–protein docking, and simulations by molecular dynamics) that allow predicting the inhibitory effect and its capacity of trypsin binding complementing the experimental study (in vitro and in vivo) (**3**). From these studies and analyzes of a region known as interface (protein–protein interaction site) several peptides (patent deposits) have been proposed with bioinsecticide application on *Anticarsia gemmatalis* and *Spodoptera cosmioides* (**4**) corroborated from in vitro and in vivo tests (**5**)

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Chapter 7
Pancreatic Lipase (PL) Inhibitors from Medicinal Plants and Their Potential Applications in the Management of Obesity

Samadhan Patil, Mohini Patil, Vijay L. Maheshwari, and Ravindra H. Patil

Abstract Obesity is increasingly recognized as a global issue, and its prevalence is rising at an alarming rate around the globe. Obese people are more likely to develop a variety of metabolic illnesses, and metabolic syndrome is frequently related to obesity. Obesity is more common as lipid homeostasis becomes disrupted as a result of genetic, environmental, and lifestyle factors. One of the investigated targets for the treatment of obesity is pancreatic lipase (PL) suppression. Orlistat is the only clinically approved drug as a lipase inhibitor and is currently available for long-term obesity treatment. However, various side effects are associated with the long-term usage of Orlistat in obesity management. Hence, it is important to find comparatively safe and effective treatment methods for obesity. Due to the high structural diversity and wide range of biological activities, natural products are the major area of focus for researchers to find new and safe PL inhibitors from natural sources. The present chapter discusses the PL inhibitory activity of different phytoconstituents of medicinal plants and highlights their PL inhibitory potential.

Keywords Obesity · Pancreatic lipase · Medicinal plants · Phytoconstituents · Orlistat

7.1 Introduction

Obesity is one of the chronic metabolic diseases with increased morbidity and premature mortality all over the world. Obesity is nothing but a disease caused by an unnecessary accumulation of fats and their improper storage in the body, which has a significant impact on human metabolic health (Sukhdev and Singh 2013). An energy imbalance between calories consumed and calories spent is the principal

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cause of obesity. The facts and statistics of WHO say that 1.9 billion adults worldwide were obese and 39% of adults aged over 18 years were overweight in 2016. Over 38 million children under the age of 5 were overweight or obese in 2019. Obesity is one of the risk factors for the diseases like cardiovascular disease, hypertension, hyperlipidemia, diabetes, and even cancer and it may emerge as a chronic disease (Francisco et al. 2016). Moreover, the obese population is relatively at higher risk of type 2 diabetes and gallbladder disease than people of normal weight (Jiang et al. 2016). Therefore, preventing and treating obesity is critical for lowering the prevalence and mortality of chronic metabolic diseases. One of the recent reports says that susceptibility of acute respiratory distress syndrome (ARDS), the prime cause of COVID-19 mortality, is significantly higher among individuals with obesity (Barry et al. 2020). The meta-analysis and prospective cohort studies say that obese children and adolescents were five times more likely to be obese as adults than non-obese children and adolescents (Simmonds et al. 2016). To predict future obesity in adolescence and adulthood the childhood obesity needs to be evaluated. A well-planned dietary regulation, exercise, lifestyle change are key interventions used to manage obesity. In addition to these, short-term treatment with anti-obesity drugs and surgeries like liposuction are also advised.

7.1.1 Mechanism of Obesity and Pancreatic Lipases

Obesity has now been recognized as a high risk for metabolic disorders such as type 2 diabetes, non-alcoholic fatty liver disease, and cardiovascular disease as a global pandemic due to the popularity of bad eating and living practices (Fan et al. 2021). Lipids build up in the liver and white adipose tissue, causing non-alcoholic fatty liver and white adipose tissue hypertrophy (Cuspidi et al. 2014). Obesity and cardiovascular disease are linked to an increased rate of death due to lipotoxicity, insulin resistance, hyperinsulinemia, central fat deposition, dyslipidemia, hypertension, and atherosclerotic cardiovascular disease (Pierre et al. 2013). Insulin resistance is linked to an increase in fatness, particularly in visceral and ectopic fat depots. The free fatty acids (FFA) produced by lipases are a crucial link between obesity and insulin resistance, as increased FFA in obesity leads to an increase in plasma FFA, which enhances insulin resistance in both non-diabetic and diabetic patients (Golay and Ybarra 2005). The tri-acylgycerides absorbed into the human body are first hydrolyzed by lipases into monoglyceride, glyceryl ester, and free fatty acid (Fig. 7.1). As a result, the metabolism of FFA plays a major role in the development of obesity.

7.1.2 Pancreatic Lipases

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) belong to the class of hydrolases which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids.

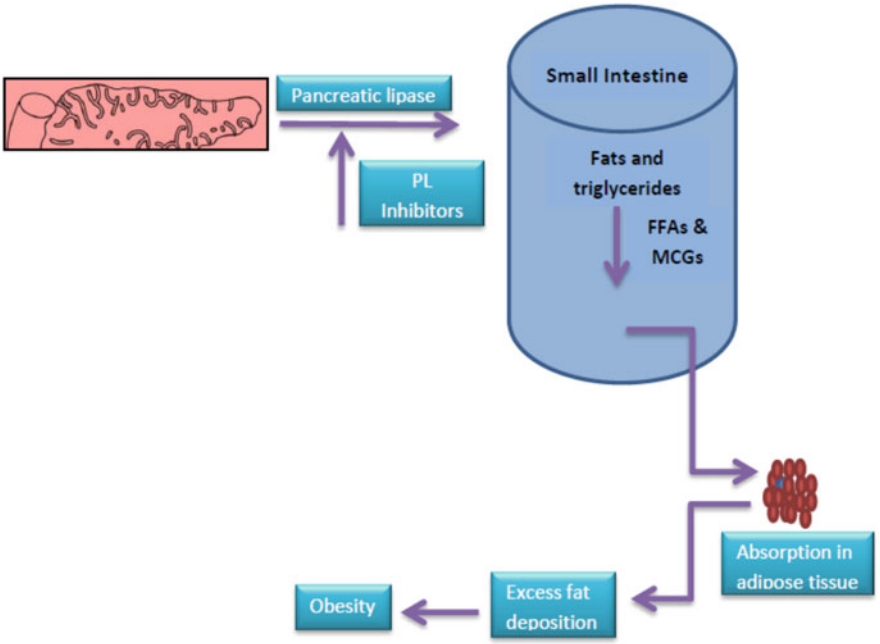

Fig. 7.1 Metabolism of lipids (in brief) and action of pancreatic lipase (*FFA* free fatty acids, *MCG* monoacylglycerols)

Lipases are categorized into neutral esterase, amidase, and phospholipase. There are various neutral esterases, i.e., triacylglycerol esterase, pancreatic lipase, gastric lipase, fatty triglyceride lipase, hormone-sensitive lipase, carboxylesterase, hepatic lipase, lipid-protein lipase, lysosomal acid lipase, monoacylglycerol lipase, diacylglycerol lipase, cholesterol lipolytic enzyme, etc. Amidase is mainly fatty acid amide hydrolase. Phospholipase mainly has phospholipase A2, phospholipase C, phospholipase D (Nomura and Casida 2016).

7.1.3 Mechanism of Action of PL Inhibitors

The PL inhibitors act on lipases by combining to the active site of the enzyme which reduces the catalytic activity and ultimately reduces the hydrolysis of lipids such as triglycerides and, in turn, accumulation of fats into the adipose tissues (Juana et al. 2008). Orlistat is a strong and selective inhibitor of several lipase enzymes that are involved in fat metabolism. It works in the GI tract by binding covalently to the serine residues on the active sites of both gastric and pancreatic lipase (Fig. 7.2). When orlistat is taken with fat-containing foods, it partially inhibits triglyceride hydrolysis. This reduces the absorption of monoacylglycerides and free fatty acids,

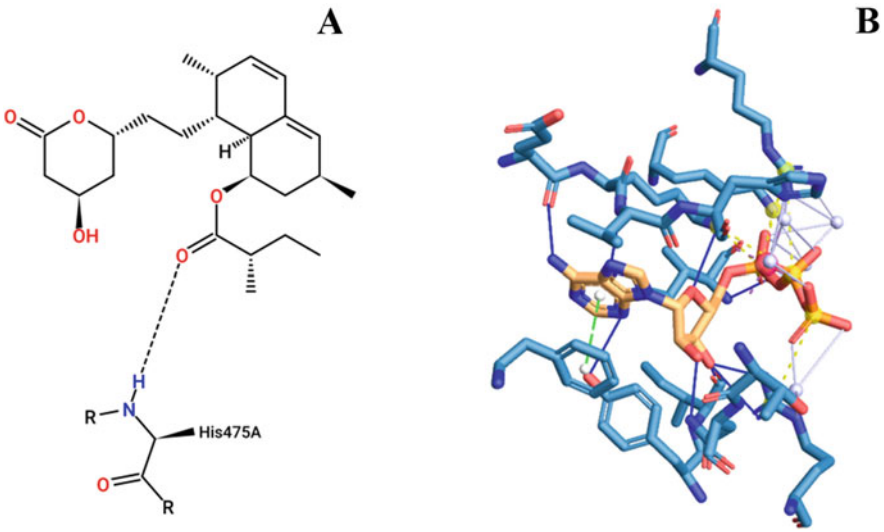

Fig. 7.2 2D interaction of PL inhibitor Orlistat and PL (**a**), 3D interaction of PL inhibitor and PL (**b**). (The images are prepared using online program—Protein-Ligand Interaction Profiler)

resulting in preventing obesity. Orlistat works by inhibiting lipase in the intestines on a local level. Orlistat's action does not require systemic absorption. It reduces dietary fat uptake by about 30% when taken at the prescribed dose. Once the PL inhibitors act, they are excreted from the human body and does not cause long-term effect in the human body. The mechanism of treating obesity is categorized into the peripheral inhibition of lipase and central appetite suppression.

The inhibitors interact with the inner cleft and are buried deeper within the cleft, as indicated by interaction sites (Fig. 7.3a–d; shown in pink). These locations of contacts are close to the crucial Ser169 residue, implying competing binding mechanisms (Chen et al. 2012). PL inhibitors engage to the binding site at a specific binding energy, which results in a stable association. To understand the binding mode of the ligands to the active site of PL, in silico docking studies are conducted using programs such as Glide, Auto dock, Schrodinger, etc. The binding strength and binding energy are compared with that of the standard drug such as orlistat. The ligands that exhibit good interactions with desired glide scores illustrate their binding ability to the target side of the protein.

7.2 PL Inhibitors and Anti-obesity Regime

Orlistat, phentermine/topiramate, lorcaserin, naltrexone/bupropion, and liraglutide are all licensed for the treatment of obesity, but little is known about their risk factors and outcomes. Appetite suppressants such as sibutramine and fenfluramine are also

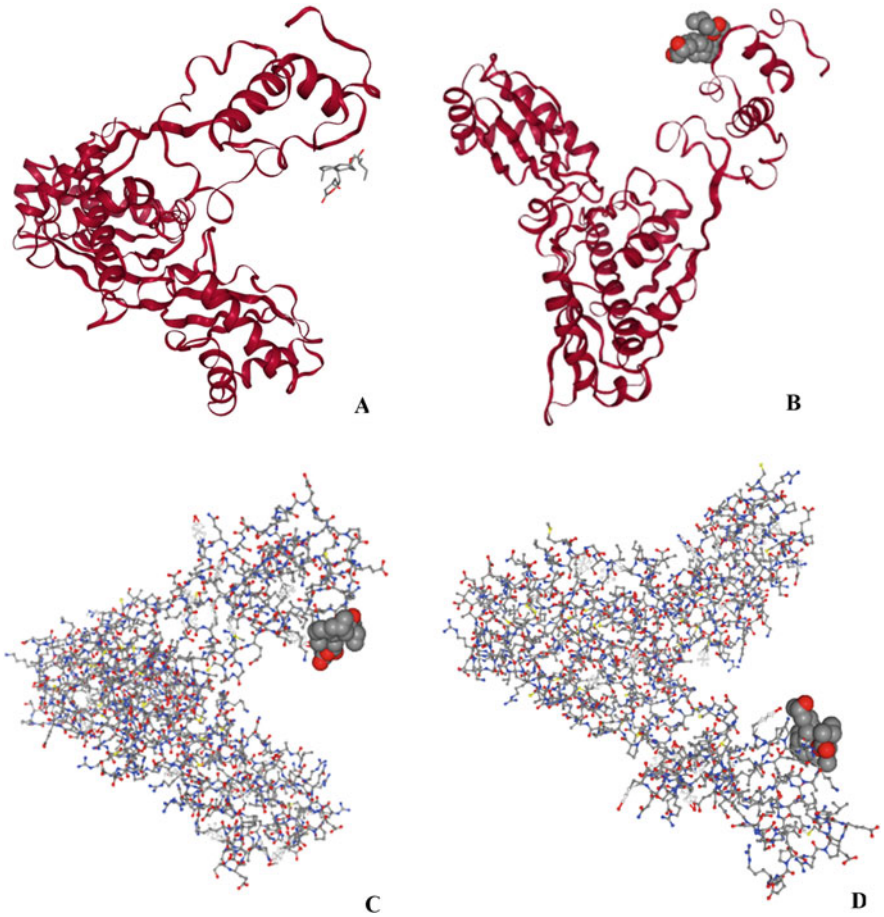

Fig. 7.3 Docking poses of PL inhibitors (ligands) to the binding sites of the protein (Cartoon model (**a**, **b**), Ball and stick model (**c**, **d**)). (The images are prepared using online program—Protein-Ligand Interaction Profiler)

used. Patients with a body mass index (BMI) more than or equal to 30 kg/m^2 and those with cardiovascular risk factors were given sibutramine for the treatment of obesity, weight loss, and weight maintenance (Talaulikar 2020). However, because of the potential of major cardiovascular issues in some individuals, sibutramine is no longer advised in clinical practice, severely limiting its clinical applications. Therefore, the development of peripheral lipase inhibitors is a major area of focus—as a proven and relatively safe drug therapy for obese patients.

PL inhibition is the most generally studied mechanism for the identification of potential anti-obesity agents. Various physiological roles of PL inhibitors have been shown in animals and clinical trials to improve lipolysis in obese patients. They are majorly responsible for fatty acid absorption inhibition leading to reduced fatty acid

accumulation in the body. Orlistat reduces fat absorption by 30%, resulting in weight loss, lowers LDL cholesterol, and also leads to decreased incidences of type 2 diabetes. However, it does not lead to significant improvement in HDL and triacylglycerides levels (Padwal Raj 2007). The adverse effects include hypoglycemia, headache, upper respiratory infection, fatty and oily stool, fecal urgency, and fecal incontinence, spotting from the rectum, abdominal pain or discomfort, flatus with discharge, flatulence, liquid stools, oily evacuation, and increased defecation (Giuseppe and Pamela 2012). Another drug, Sibutramine, is a monoamine-reuptake inhibitor. The randomized double-blind, placebo-controlled weight-loss trials by Padwal et al. (2003a, b) showed a significant reduction in body weight with the treatment of sibutramine in obese patients. While there is no significant effect on concentrations of LDL cholesterol and glycemic control and has contradictory effects on concentrations of triglyceride and HDL cholesterol (McNulty et al. 2003; Sanchez et al. 2004). Sibutramine was associated with the potential increase in the blood pressure and pulse rate and leads to major concern about the cardiovascular toxic effect.

7.3 Natural Products as PL Inhibitors

Plants are the rich source of secondary metabolites/natural products, with notable pharmacological activities and therefore widely popular as alternative medicine. The significant role of natural products (NPs) in drug discovery is based on their embedded biosynthetic molecular recognition. Despite the crucial role of NPs in drug discovery, their use over the past two decades has decreased in the pharmaceutical industry (Newman and Cragg 2012). This decline is mainly due to the non-availability of the plant material, time, cost of isolating the bioactive materials, and identifying active NPs from plant extracts (Pascolutti and Quinn 2014). Nowadays, the process of drug discovery involves approaches such as genomics, proteomics, and bioinformatics. Technologies like combinatorial chemistry, high-throughput screening (HTS), virtual screening, de novo design, in vitro and in silico ADME/T screening, and structure-based drug designing are used for drug discovery. Plant-based natural product inhibitors are more significant than chemically produced medications in terms of safety, thus screening of novel PL inhibitors from different plants sources with fewer side effects is a major area for research.

Alkaloids are the class of nitrogen-containing compounds mostly found in medicinal plants and are well known for their various pharmacological activities. Stephalagine, an isoquinoline skeleton-derivatived alkaloid component of *Annona crassiflora* fruit has been reported as a potential PL inhibitor. Stephalagine showed high PL inhibitory activity with IC_{50} of 8.35 μg/mL along with low cytotoxicity, suggesting it to be a potential anti-obesity agent (Mariana et al. 2017a). Sridhar et al. (2017) identified the potent PL inhibitor—Conophylline alkaloid (IC_{50} of 3.31 μM) from methanolic extract of *Tabernaemontana divaricata* leaves by exhibiting a competitive reversible inhibition. The conophylline provides adequate information

of the ligand's binding site with a K_i value of 15.4 μM and exhibited the potential of bis-indole scaffold-based alkaloids from the leaves of *T. divaricata* for PL inhibition (Sridhar et al. 2017). Saponins such as Platycodin, Scabiosaponins, Sessiloside, chiisanoside, Chikusetsusaponins, Escins have been evaluated for their PL inhibitory potential (Kiyofumi et al. 2004). Similarly, other phytoconstituents such as polyphenols, terpenes are widely studied for their PL inhibitory potential. Conversely, metabolites such as Lipstatin (Hochuli et al. 1987), panclins, and Valilactone (Mutoh et al. 1994) have been isolated from various microorganisms and evaluated for their Pl inhibitory potential. A detailed outlook of NPs with Pl inhibitory potential is provided in Table 7.1.

Terpenoids, a structurally diverse class of natural products derived from the 5-carbon compound isoprene, and the isoprene polymers, are found in many medicinal plants. In one of our previous studies, we extracted a pancreatic lipase inhibitor from the root of *Calotropis procera* performed kinetic analysis to explore its hypolipidaemic activity. The results showed that the purified diterpenoid fraction has the strongest PL inhibitory activity (IC_{50} of 9.47 μg/mL). In the kinetic analysis, the isolated diterpenoid fraction was found increase the K_m value while V_{max} remained unchanged suggesting competitive type of inhibition (Patil et al. 2015). The in vitro PL inhibition of the Coumaroyl triterpene and Ursolic acids, isolated from the *Actinidia arguta* showed IC_{50} of 14.95 μM and IC_{50} of 15.83, respectively (Jang et al. 2008).

Polyphenols are another most studied natural products for pancreatic lipase inhibition. Mechanistically they bind to the enzyme pancreatic lipase by polyvalent site present in them. The anti-obesity potential of the black chokeberry fruits was determined by its ability to strongly inhibit PL leading to reduced dietary fats absorption from the intestinal tract. The results indicate that the polyphenol-rich extracts containing the procyanidins and anthocyanins are responsible for the inhibition of PL (Dorota et al. 2018).

7.4 Contributions in the Field from Our Research Group

Plants and microorganisms are recognized as cheap and diverse source of bioactive metabolites. Among microorganisms, secondary metabolites from endophytic fungi are largely novel, unexplored, and unexploited. Moreover, these metabolites are complementary to synthetic or combinatorial libraries and these substances can be used as lead compounds for the design of synthetic drugs having new targets of action. So, bioactive metabolites from endophytes have the potential as a sustainable solution for modern biomedical problems. Further, endophytic fungi are ubiquitous and extremely diverse and every plant examined to date harbors at least one or more species of endophytic fungus. It appears that there are enormous possibilities for exploiting endophytic fungi for the production of a range of bioactive metabolites important for human health. In the last 5 years, over 300 endophytes with the potential to synthesize metabolites with therapeutic values have been isolated and

Table 7.1 Pancreatic lipase inhibitors from natural sources and their phytocompounds

Source/origin	Plant part used	Phytocompounds	IC_{50} value/ mechanism	References
Curcuma amada	Rhizome	Labdane diterpenes and drimane sesquiterpene	Inhibits α-glucosidase and porcine pancreatic lipase	Yoshioka et al. (2019)
Justicia carnea	Leaves		Mixed inhibition pattern	Anigboro et al. (2021)
		Campesterol	K_i 107.69 μg/mL	
		Stigmasterol	K_i 398.00 μg/mL	
Trigonella foenumgraceum	Leaves	Polyphenols	Major inhibition of PL	Neda and Ghasem (2021)
St. John's Wort	NA	Hypericin and pseudohypericin	Mixed inhibition	Hou et al. (2020)
			$IC_{50} > 1$ μM	
Fructus Psoraleae	Not specified	Isobavachalcone	K_i 1.61 μmol/L	Dong et al. (2020)
		Bavachalcone	K_i 3.77 μmol/L	
		Corylifol	K_i 10.16 μmol/L	
Populus alba	Whole plant	*p*-Hydroxybenzoic acid	IC_{50} 68.5 μM	Marwa et al. (2019)
		Trans *p*-Coumaric acid	IC_{50} 112.2 μM	
		Trans-Cinnamic acid	IC_{50} 98.7 μM	
		(+)-Ampelopsin	IC_{50} 46.2 μM	
		(+)-Taxifolin	IC_{50} 23.2 μM	
		(+)-Aromadendrin	IC_{50} 173.1 μM	
		Salicin	IC_{50} 193.7 μM	
		Quercetin-3-*O*-rutinoside (rutin)	IC_{50} 152.3 μM	
		Isorhamnetin-3-*O*-rutinoside (narcissin)	IC_{50} 122.4 μM	
Trigonella foenum-graecum	Seeds	Vicenin-1	IC_{50} 207 μg/mL	Fernando et al. (2019)
		Isoschaftoside	IC_{50} 303 μg/mL	
		Schaftoside	IC_{50} 107 μg/mL	
Cortex Mori Radicis	NA	Sanggenone C	IC_{50} 3.0 μM; K_i 1.07 μM	Hou et al. (2018)
		Sanggenone D	IC_{50} 0.77 μM; K_i 0.43 μM	
		Kuwanon C	IC_{50} 4.47 μM; K_i 1.67 μM	
		Kuwanon G	IC_{50} 4.85 μM; K_i 3.50 μM	
Annona crassiflora Mart	Fruit peel	Stephalagin	IC_{50} 8.35 μg/mL	Mariana et al. (2017b)
Santalum acuminatum	Fruit	Cyanidin-3-glucoside	IC_{50} 0.6 mg/mL	Sakulnarmrat et al. (2014)
Ligustrum purpurascens	Leaves	Acteoside	Non-competitive inhibition	Xuli et al. (2014)

(continued)

Table 7.1 (continued)

Source/origin	Plant part used	Phytocompounds	IC_{50} value/ mechanism	References
Aronia melanocarpa	Not specified	Methanolic polyphenol extract	IC_{50} 83.45 mg/mL	Paulina et al. (2014)
		Cyanidin-3-glucoside	IC_{50} 1.17 mg/mL	
Jalapeno pepper	Not specified	Polyphenols	Competitive inhibition	Martinez-Gonzalez et al. (2017)
		Caffeic acid	IC_{50} 401.5 μM	
		p-Coumaric acid	IC_{50} 170.2 μM	
		Quercetin	IC_{50} 6.1 μM	
		Capsaicin	K_i 50.7 μM	
Camellia Sinensis	Leaves	Theaflavin	IC_{50} 0.679 μg/mL	Yuda et al. (2012)
		Theaflavin-3-*O*-gallate	IC_{50} 0.368 μg/mL	
		Theaflavin 3,3′-di-Ogallate	IC_{50} 0.316 μg/mL	
		Theaflavin 3′-*O*-gallate	IC_{50} 0.320 μg/mL	
		(−)-Epigallocatechin	IC_{50} 39.19 μg/mL	
		(−)-Epigallocatechin gallate	IC_{50} 0.081 μg/mL	
		(−)-Epicatechin gallate	IC_{50} 0.081 μg/mL	
Intsia palembanica	Wood	3,7,3,5-tetra hydroxy flavone	IC_{50} 885.6 μM	Batubara et al. (2014)
		Ampelopsin	IC_{50} 36.1 μM	
		Myricetin	IC_{50} 375.5 μM	
		Fustin	IC_{50} 13.7 μM	
		Quercetin	IC_{50} 421.5 μM	
		(−)-robidanol	IC_{50} 100.2 μM	
Nelumbo nucifera	Leaves	Quercetin-3-*O*-β-D-rabinopyranosyl-(1 → 2)β-D-galactopyranoside	IC_{50} 66.86 μM	Tao et al. (2013)
		Quercetin-3-*O*-β-D-glucuronide	IC_{50} 135.01 μM	
		Kaempferol-3-*O*-β-D-glucuronide	IC_{50} 94.0 μM	
Undaria pinnatifida	Whole part	Fucoxanthin	IC_{50} 660 nM	Matsumoto et al. (2010)
		Fucoxanthinol (764 nM)	IC_{50} 764 nM	
Cassia auriculata	Aerial parts	Kaempferol-3-*O*-rutinoside	2.9 μM	Habtemariam (2013)
Eremochloa ophiuroides	Leaves	Luteolin 6-C-b-D-boivinopyranoside	IC_{50} 50.5 μM	Lee et al. (2010)
		Orientin	IC_{50} 31.6 μM	
		Isoorientin	IC_{50} 44.6 μM	
		Isoorientin2-*O*-α-L-rhamnoside	IC_{50} 25.9 μM	
		Derhamnosylmaysin	IC_{50} 18.5 μM	

(continued)

Table 7.1 (continued)

Source/origin	Plant part used	Phytocompounds	IC_{50} value/ mechanism	References
Glycyrrhiza glabra	Roots	Isoliquiritigenin	IC_{50} 7.3 μM	Birari et al. (2011)
		Liquiritigenin	IC_{50} 35.5 μM	
		Isoliquiritoside	IC_{50} 14.9 μM	
		Isoononin	IC_{50} 37.6 μM	
Alpinia galanga	Rhizome	Galagine	Direct PL inhibition	Shivkumar and Alagawadi (2013)
Filipendula kamtschatica	Aerial part	3-*O*-caffeoyl-4-*O*-galloyl-L-threonic acid	IC_{50} 26 μM	Kato et al. (2012)
Cassia siamea	Roots	Cassiamin A	IC_{50} 41.8 μM	Kumar et al. (2013)
Chrysanthemum morifolium	Lowers	10α-hydroxy-1α,4-α-endoperoxy-guaia-2-en-12,6α-olide	IC_{50} 161 μM	Luyen et al. (2013)
Zingiber officinale	Rhizome	Polyphenols	IC_{50} 6.16 mg/mL	Sakulnarmrat et al. (2015)
Tribulus terrestris and Cicer arietinum	Leaves	Saponin	Direct PL inhibition	Ercan and EI (2016)
Salacia reticulata	Leaves	Epigallo catechin gallate	IC_{50} 10.9 μM	Koga et al. (2013)

NA not applicable

successfully cultivated in the laboratory (Gunatilaka 2006). Endophytes which produce the metabolites identical to their host plant and mimic the host chemistry have also been isolated; for example, paclitaxel (Taxol®), camptothecin and its structural analogs (Puri et al. 2005; Kusari et al. 2009), jasmonic acid, ginkgolide (Cui et al. 2012) azadirachtin (Kusari and Spiteller 2012), etc. However, the isolation of promising endophytic microorganisms capable of producing bioactive metabolite (s) from the huge plant biodiversity is always challenging. To identify the extent of a plant's endophytic species diversity, successful isolation of endophytes is necessary.

The authors' group is working on PL inhibitors from microbial and plant sources for the last 10 years. The authors could successfully isolate and characterize several endophytic fungi from the indigenous medicinal plants. The isolated endophytic fungi when cultivated in the laboratory produced the metabolites that effectively inhibited PL. Under the extensive screening program, the isolation and PL inhibitory potential of 18 endophytic fungi were isolated from the various parts of 6 indigenous medicinal plants. The bioassay-based method was designed to screen the PL inhibitory potential of the isolated endophytic fungi. The PL inhibitory potential of the ethyl acetate extract of the test endophytes was screened for pancreatic lipase inhibitory activity by the chromogenic olive oil plate method. This method is based on the change in the color of media due to acid production as a result of enzyme activity (Patil and Patil 2019).

In another study, the porcine pancreatic lipase (PPL) inhibitory potential of a phenolic fraction from the fermented extract of endophytic *Diaporthe arengae* was evaluated. The isolated phenolic fraction was able to competitively inhibit the PPL with initial K_m and V_{max} of 3.83 ± 0.94 μM and 11.54 ± 0.54 μmol/min, respectively, and the results were comparable to those obtained with standard, Orlistat. The inhibition constant (K_i) for the phenol-rich fraction was recorded as 13.47 ± 5.01 μg/mL, compared to 10.65 ± 3.81 μM obtained for Orlistat. It was proved that the phenol-rich fraction from endophytic *D. arengae* was able to inhibit PPL competitively like Orlistat (Patil et al. 2017).

Our recent work demonstrated the macromolecular interaction and inhibitory effect of pentacyclic triterpenoids (PTT) on PL. It was observed that PTTs from endophytic *Colletotrichum gigasporum* isolated from the Indian medicinal plant—*Withania somnifera* were found to show significant inhibitory activity against PL with IC_{50} of 16.62 ± 1.43 μg/mL. The bioactive compound was isolated through bioassay-guided isolation and it showed a dose-dependent inhibition against porcine PL and the results were comparable with the standard (Orlistat). The inhibition kinetic data showed a gradual increase in K_m (app) with increasing PTT concentration indicated a competitive type of inhibition. The animal studies in Wistar rats at the oral dose (100 mg/kg body weight) of PTT significantly decreased ($p < 0.05$) incremental plasma triglyceride levels as in the tested group of animals. Triglyceride absorption was downregulated as compared to normal animals. The isolated PTT was identified as lupeol based on chromatographic and spectral data. The endophytic isolate was identified as *Colletotrichum gigasporum* based on morphology and ITS gene sequencing. This study for the first time established that PTT had the potential to be used as a natural PL inhibitor in the treatment of obesity and the isolated endophyte can be a valuable bioresource for it (Patil et al. 2021).

7.5 Conclusion

The global burden of obesity is rapidly increasing. Obesity-related metabolic diseases are a significant economic and social burden that continuously increase the cost of healthcare. Exploring the natural products from plants and microorganisms is the central aspect of drug discovery studies. The World Health Organization (WHO) estimates that 80% of the people of the developing countries rely on traditional medicines, mostly plant-derived drugs, for their primary health needs. Over 49% of the new medicines registered by the United States Food and Drug Administration (USFDA) are derived from natural products or their derivatives. Moreover, plant secondary metabolites have featured significantly in the treatment of many diseases and providing base substances for the development of more efficient drugs. It can be concluded that obtaining bioactive compounds from natural sources is relatively cheaper and environment-friendly. Appropriate scale-up and process development are however necessary for the commercial exploration of the PL inhibitors from the natural sources.

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Chapter 8
Bioactive Peptides and Polysaccharides: Setting a New Trend in Replacing Conventional Angiotensin-Converting Enzyme Inhibitors

Muhammad Hakimin Shafie, Pei Gee Yap, and Chee-Yuen Gan

Abstract Angiotensin-converting enzyme (ACE) inhibitors are used to control blood pressure or to prevent congestive heart failure by dilating blood vessel. These inhibitors are also applied to treat patients with kidney injury or diabetic nephropathy. Due to the side effects of the synthetic drugs, such as dizziness, hyperkalemia, angioedema, dysgeusia and renal impairment, there is an increasing trend in using natural products, especially bioactive peptides and polysaccharides, that can be extracted from various plants, animals or microorganisms, in which much lower cytotoxicity was detected. In this chapter, we have gathered the most recent data and reviewed it for these two active components in the aspect of sources, extraction/identification techniques and mechanism in inhibiting ACE, as well as the commercialization potential including the effectiveness after the oral consumption.

Keywords Angiotensin-converting enzyme · Hypertension · Inhibitor · Peptide · Polysaccharide

8.1 Introduction

Hypertension or high blood pressure (defined as systolic blood pressure of >140 mm Hg, whereas diastolic blood pressure of >90 mm Hg) is an essential risk factor for cardiovascular diseases, such as heart attack or failure and stroke (Iwaniak et al. 2014). There are many ways for hypertension management and treatment, such as medication, exercise, stress reduction and diet. Of all choices, partaking angiotensin-converting enzyme (ACE) inhibitor is one of the most popular options. ACE is a well-known zinc metallopeptidase that contributes to the high blood pressure by

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converting the decapeptide angiotensin I into octapeptide angiotensin II (i.e. a strong vasoconstrictor) as well as deactivating bradykinin (a vasodilatory hormone) (Priyanto et al. 2015). Therefore, researchers suggested that inhibiting ACE activity is an effective strategy in treating hypertension patients. The ACE inhibitors function by interrupting the renin–angiotensin–aldosterone system in our body. By inhibiting the production of angiotensin II, the dilation of blood vessels is induced because less angiotensin II is available to induce the muscles contraction in blood vessels and without the angiotensin II, the release of aldosterone could not be stimulated. Therefore, water and sodium reabsorption in the blood vessels, which increases blood volume and pressure, could be avoided. In addition, ACE inhibitors could also interrupt the bradykinin from breaking down, hence allowing blood vessels to easily expand with the presence of high concentration of the bradykinin.

Food-derived components are generally considered as safe for consumption compared to synthetic drugs, which could cause side effects, such as dizziness, hyperkalemia, angioedema, dysgeusia and renal impairment; therefore, there is an increasing trend in using natural products as ACE inhibitors, especially bioactive peptides and polysaccharides, that are extracted from various plants, animals or microorganisms, in which much lower cytotoxicity was detected. In general, these peptides and polysaccharides were proposed to be able to compete with the substrate for the catalytic sites and substrate binding sites of ACE and/or bind to the ACE and alter the ACE conformation and thereby disabling the interaction between ACE and angiotensin I (Ngoh et al. 2016).

The sources of the polysaccharides and their extraction/identification techniques will be discussed in the following sections. The ACE inhibitory mechanism as well as the considerations in choosing ACE inhibitory polysaccharides/peptides were also included in this chapter.

8.2 Sources of ACE Inhibitory Polysaccharides and Peptides

Due to the unavoidable side effects of synthetic drugs, natural sources of ACE inhibitory agent are explored, in particular from plant, animal and microbial origins. This is an on-going process and has attracted involvements of many researchers.

8.2.1 Polysaccharides

Polysaccharides are complex macromolecules, made up of more than ten monosaccharides joined together by α- or β-glycosidic bond or both (Wang et al. 2021). They can be classified into two groups: homopolysaccharides (e.g. starch and cellulose) and heteropolysaccharides (e.g. hemicellulose and pectin) (Yarley et al.

2021). The main function of these polysaccharides is to store energy as well as to support the rigidity of plant structure. Other than that, polysaccharides are involved in different biological processes, particularly cell–cell communication and cellular immunity. Most of these structural polysaccharides are present in middle lamella and primary cell wall rather than a secondary cell wall.

In the recent years, polysaccharides, which are capable of exhibiting therapeutic effects for the treatment of diseases, have been widely employed in pharmaceutical and nutraceutical industries due to their health benefit apart from being functional food ingredients (Guo et al. 2021). These physiological effects of polysaccharides from various plant sources have been extensively documented. For example, antitumor (Liu et al. 2012), antibacterial (Shanmugam et al. 2016), antidiabetic (Tan and Gan 2016), antiviral (Feng et al. 2017), antioxidant (Jiang et al. 2019), anticoagulant (Liu et al. 2019b), immunoregulatory activity (Lin et al. 2020), hepatoprotective activity (Duan et al. 2020) and antihyperlipidemic (Jiang et al. 2020). Investigation on the ACE inhibitory activity was also carried out. Sila et al. (2014) have proven that polysaccharides from the agro-industrial by-products (e.g. pistachio and bitter almond) could be a promising source of ACE inhibitory agents. Based on the literature, only 14 sources of ACE inhibitory polysaccharides have been reported, as shown in Table 8.1.

As referring to Table 8.1, most of the anti-ACE polysaccharides are derived from plants. It can be seen that the polysaccharides from watermelon rind had higher ACE inhibitory activity with low IC_{50} value of 0.21 mg/mL compared to other plant-based polysaccharides (0.4–2.81 mg/mL). To our surprise, the discovery of ACE inhibitory polysaccharides from animals is also on the rise, especially from marine origins, such as *Loligo vulgaris* skin (Abdelmalek et al. 2015), *Amphioctopus neglectus* (Chakraborty et al. 2020) and common smooth hound (Abdelhedi et al. 2016). The literature showed that the polysaccharides from *L. vulgaris* skin exhibited a higher ACE inhibitory activity with IC_{50} value of 0.14 mg/mL compared to other sources. As compared to plant polysaccharides, these marine based polysaccharides gave a higher ACE activity in general. These polysaccharides are now on high industrial demand due to their multifunctional properties. In addition, they are also known as non-toxic and biocompatible components. Exploration of this valuable component by researchers has therefore increased.

8.2.2 Peptides

Since the first isolation of ACE inhibitory peptide mixture from snake venom in 1965 (Ferreira 1965), research focusing on peptide discovery has grown by leaps and bounds. To date, approximately 1050 novel ACE inhibitory peptides have been deposited to the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep, accessed on 16th June 2021) and there are more undeposited sequences to be explored. For the past decade, ACE inhibitory peptides have been identified from different sources such as animals, plants, algae, fungi and yeast (Table 8.2). Animals

Table 8.1 The ACE inhibitory polysaccharides

Sources	Extraction technique	ACE inhibition	References
Momordica charantia	Citric acid extraction using incubator shaker at pH 2, 80 °C and 2 h	94.1% at 10 mg/mL of sample concentration	Tan and Gan (2016)
Bitter almond	Extracted twice with twenty volumes of deionized water at 90 °C for 4 h with stirring	79.5% at 5 mg/mL of sample concentration (IC_{50} = 2.81 mg/mL)	Sila et al. (2014)
Pistachio		81.8% at 5 mg/mL of sample concentration (IC_{50} = 2.59 mg/mL)	
Konjac	Extracted with distilled water at 80 °C for three times (each for 2 h)	88.04% at 1 mg/mL of sample concentration (IC_{50} = 0.791 mg/mL)	Song et al. (2018)
Gastrodia elata Blume	Extracted with distilled water at 60 °C for three times (each for 2 h)	74.4% at 1 mg/mL of sample concentration (IC_{50} = 0.66 mg/mL)	Zhu et al. (2019)
Watermelon rind	Water extraction using water bath at 60 °C for 80 min	93.93% at 1 mg/ml of sample concentration (IC_{50} = 0.21 mg/mL)	Romdhane et al. (2017)
Chickpea flours	Water extraction at 90 °C for 4 h with stirring	87.83% at 1 mg/mL of sample concentration (IC_{50} = ~0.4 mg/mL)	Ghribi et al. (2015)
Loligo vulgaris skin	Extraction using 250 mL sodium acetate (0.1 M), EDTA (5 mM) and cysteine (5 mM) pH = 6. Alcalase®. The mixture was kept for 24 h at 50 °C	86.3% at 1 mg/mL of sample concentration (IC_{50} = 0.14 mg/mL)	Abdelmalek et al. (2015)
Amphioctopus marginatus	Extracted with deionized water (600 mL × 2) at 95 °C for about 4 h	IC_{50} = 1.02 mg/mL	Chakraborty et al. (2020)
Uroteuthis duvaucelii		IC_{50} = 0.43 mg/mL	
Amphioctopus neglectus		IC_{50} = 0.49 mg/mL	
Sepiella inermis		IC_{50} = 0.61 mg/mL	
Common smooth hound	Extracted using sodium acetate buffer (0.1 M, pH 6) containing 5 mM EDTA and 5 mM cysteine. Following endogenous enzymes inactivation (20 min at 95 °C), the pH was adjusted to 10.0 and addition of an alkaline protease (Purafect®) for proteolysis (incubated for 24 h at 60 °C). Precipitated using cetylpyridinium chloride	IC_{50} = 1.04 mg/mL	Abdelhedi et al. (2016)
Common smooth hound	Extracted using sodium acetate buffer (0.1 M, pH 6) containing 5 mM EDTA and 5 mM cysteine. Following endogenous enzymes inactivation (20 min at 95 °C), the pH was adjusted to 10.0 and addition of an alkaline protease (Purafect®) for proteolysis (incubated for 24 h at 60 °C). Precipitated using absolute ethanol	IC_{50} = 0.75 mg/mL	

Table 8.2 Anti-ACE peptides reported since year 2010

Source	Extraction technique	Sequence	IC_{50}	Inhibition mechanism	Interaction with human ACE (PDB ID = 1O86/1O8A)				References
					H-bond	HI	π-in	Cod-bond	
Camellia (*Camellia oleifera*) cake glutelin-2	Enzymatic hydrolysis (alcalase and trypsin)	GYGYNY	384 μM	Uncompetitive	S526, S461, R468, D415, P407, P519, V518, A356	–	–	–	Zheng et al. (2021)
Distilled spent grain	Ultrasonic-assisted water extraction	PR	50.0 ± 3.16 μM	Competitive	D415,E383, E384, K511, Y520, Q281	–	–	–	Wei et al. (2021)
		DR	110.4 ± 6.43 μM	–	–	–	–	–	
		LP	117.8 ± 1.80 μM	–	–	–	–	–	
		NGGPPT	307.4 ± 12.42 μM	–	–	–	–	–	
Rape (*Brassica napus*) bee pollen	Enzymatic hydrolysis (alcalase)	PY	75.5 ± 2.34 μM	–	–	–	–	–	Zhu et al. (2021)
		SPAPGH	81.4 ± 2.76 μM	–	–	–	–	–	
		HPVTGL	81.9 ± 8 μM	Competitive	N277, Q281, D377, A354, H383	–	–	–	
Wakame (*Undaria pinnatifida*)	Magnetic affinity purification	KNFL	225.87 μM	Non-competitive	E162, Q281, H353, E384, S222, T226, E225, D218	–	–	–	Feng et al. (2021)
Black cumin (*Nigella sativa*) seed	Enzymatic hydrolysis (chymotrypsin)	VTPVGVPKW	1.8 ± 0.09 μM	Non-competitive	N66, R124, S516	–	Y62	–	Sutopo et al. (2020)

(continued)

Table 8.2 (continued)

Source	Extraction technique	Sequence	IC_{50}	Inhibition mechanism	Interaction with human ACE (PDB ID = 1O86/1O8A)				References
					H-bond	HI	π-in	Cod-bond	
Defatted lemon basil (*Ocimum citriodorum*) seeds	Enzymatic hydrolysis (alcalase)	GPAGPAGL	0.013 ± 0.001 mM	Non-competitive	N27, A317, Y480, H344, S316	–	–	–	Kheeree et al. (2020)
		LGRNLPPI	0.124 ± 0.02 mM and	Non-competitive	A315, N242, K468, R479, H344, H348	–	W318, H371	–	
Gac (*Momordica cochinchinensis*) seed	Enzymatic hydrolysis (SGD)	ALVY	7.03 ± 0.09 μM	Competitive	H353, H513, R522, Y523	–	–	–	Ngamsuk et al. (2020)
		LLAPHY	15.91 ± 0.52 μM	–	R124, H353, Y523, S213	–	–	–	
		LLVY	87.76 ± 3.37 μM	–	A356, Y523	–	–	–	
		LSTSTDVR	>166.67 μM	–	E384	–	–	–	
Jujuba (*Zizyphus jujuba*) fruit	Enzymatic hydrolysis (trypsin and papain)	IER	0.144 mM	Competitive	Q281, K511, Y520, H513	–	–	–	Memarpoor-Yazdi et al. (2020)
		IGK	0.228 mM	Competitive	–	–	–	–	
Longan (*Dimocarpus longan*) seed	Enzymatic hydrolysis (pepsin and pancreatin)	ETSGMKPTEL	2.15 ± 0.016 mM	Non-competitive	Q541, N543, Q489, N543, S189	–	–	–	Nuchpraphapha et al. (2020)
		ISSMGILVCL	3.88 ± 0.004 mM	Non-competitive	N543, S189, D193, R196, N192	–	–	–	

Microalgae (*Isochrysis zhanjiangensis*)	Enzymatic hydrolysis (SGD)	FEIHCC	61.38 μM	Non-competitive	D121, R522, H387, R402, Y360, K118	–	–	–	Chen et al. (2020b)
Sesame (*Sesamum indicum*)	Enzymatic hydrolysis (SGD)	GHIITVAR	3.60 ± 0.10 μM	Competitive	Zn^{2+}, A354, Y523, Q281, H353, Y520, K511, E162, H383, A356, R522, E123, D377, E376	–	–	–	Wang et al. (2020)
		IGGIGTVPVGR	6.97 ± 0.18 μM	–	–	–	–	–	
		FMPGVPGPIQR	11.08 ± 0.15 μM	–	–	–	–	–	
		PNYHPSPR	18.98 ± 0.26 μM	–	–	–	–	–	
		AFPAGAAHW	29.00 ± 0.20 μM	–	–	–	–	–	
		HIGNILSL	36.69 ± 0.33 μM	–	–	–	–	–	
		MPGVPGPIQR	54.79 ± 0.37 μM	–	–	–	–	–	
		AGALGDSVTVTR	68.49 ± 0.14 μM	–	Zn^{2+}, A356, R522, E123, D377, E376, E162, A354, S516, E403, M223, F570, H513, H353, Y523,	–	–	–	

(continued)

Table 8.2 (continued)

Source	Extraction technique	Sequence	IC_{50}	Inhibition mechanism	Interaction with human ACE (PDB ID = 1O86/1O8A)				References
					H-bond	HI	π-in	Cod-bond	
		HIITLGR	74.65 ± 0.13 μM	–	Zn^{2+}, A354, H353, E411, C370, D377, E162, A356, R522, E123, E376, K511, E162,	–	–	–	
		LAGNPAGR	148.41 ± 0.35 μM	–	–	–	–	–	
		INTLSGR	149.63 ± 0.33 μM	–	–	–	–	–	
Shiitake mushroom (*Lentinula edodes*)	Enzymatic hydrolysis (alcalase)	KIGSRSRFDVT	37.14 μM	Non-competitive	R479, N31, A315, E345, K468, Y477, H344	–	V475, W318, F414, D376	–	Paisansak et al. (2020)
Tilapia (*Oreochromis niloticus*) skin gelatin	Enzyme hydrolysis (properase E)	LSGYGP	2.577 μM	Mixed non-competitive	N66, E143, Y523, Q281,	–	V380, H383	–	Chen et al. (2020a, b, c)
Big-belly seahorse (*Hippocampus abdominalis*)	Enzymatic hydrolysis (alcalase)	IGTGIPGIW	<164.2 μM	Competitive	H513, Y523, E123, R124, R522, S516	–	–	–	Kim et al. (2019)
		GIIGPSGSP	<0.191 μM	Competitive	E376, N277, E384, Y360, E403, R402,	–	–	–	
		QIGFIW	<196.5 μM	Non-competitive	R522, E403	–	–	–	

Coconut cake albumin	Enzymatic hydrolysis (sequential alcalase, flavourzyme, pepsin and trypsin)	KAQYPYV	37.06 μM	Mixed inhibition	–	–	–	–	Zheng et al. (2019)
		KILIYG	53.31 μM	Mixed inhibition	–	–	–	–	
		KIIIYN	58.72 μM	Mixed inhibition	–	–	–	–	
Corn silk	Boiling water extraction	SKFDNLYGCR	81.71 ± 1.06 μM	Competitive	N227, Q281, T282, T302, H353, N374, H513, S516, S517, Y523	–	–	–	Li et al. (2019)
Ginko (*Ginkgo biloba*) seed	Enzymatic hydrolysis (alcalase, dispase, trypsin and flavourzyme)	RVFDGAV	1.006 mM	Competitive	H387, A354, Y523, H353, E162	–	–	–	Ma et al. (2019)
		RADFY	1.35 mM	Competitive	H383, H387, E411, Y523, E384, N281, H353, K511, H513, Y520				
		TNLDWY	1.932 mM	Non-competitive	A 354, Y523, H353, E384, E411, H513	–	–	–	
Horse gram flour	Enzymatic hydrolysis (alcalase)	TVGMTAKF	30.3 μM	Competitive	–	–	–	–	Bhaskar et al. (2019)
		QLLLQQ	75.0 μM	Competitive	–	–	–	–	

(continued)

Table 8.2 (continued)

Source	Extraction technique	Sequence	IC_{50}	Inhibition mechanism	Interaction with human ACE (PDB ID = 1O86/1O8A)				References
					H-bond	HI	π-in	Cod-bond	
Pearl oyster (*Pinctada fucata*) meat	Enzymatic hydrolysis (alkaline protease)	GWA	109.25 ± 1.45 μM	Competitive	A354, E384	–	–	–	Liu et al. (2019a, b, c)
		HLHT	458.06 ± 3.24 μM	Non-competitive	A356	–	–	–	
Sicklepod (*Senna obtusifolia*) seeds	Enzymatic hydrolysis (thermolysin)	FHAPWK	16.83 ± 0.90 μM	Competitive	–	–	–	–	Shih et al. (2019)
Black clam (*Cyclina sinensis*)	Enzymatic hydrolysis (trypsin)	WPMGF	0.789 mM	Competitive	Zn^{2+}, N31, E104, V312, S316, A317, F352, H371, F473, V479, Y484	H314, A315, E345, H348, H474, R483	–	–	Yu et al. (2018)
Bovine casein	Enzymatic hydrolysis (pepsin and trypsin)	YQKFPQYLQY	11.068 μM	Competitive	–	–	–	–	Xue et al. (2018)
Clam (*Ruditapes philippinarum*)	Microbial fermentation (*Bacillus natto*)	VISDEDGVTH	8.16 μM	Competitive	–	–	–	–	Chen et al. (2018)
Hazelnut (*Corylus heterophylla*)	Enzymatic hydrolysis (alcalase)	YLVR	15.42 μM	Non-competitive	E384, A354	–	H383, Y523	–	Liu et al. (2018a)
		AVKVL	73.06 μM	Non-competitive	D415, S526, E411	–	–	–	
		TLVGR	249.3 μM	Non-competitive	D415, K454	–	–	–	

Jellyfish (*Rhopilema esculentum*) gonad	Enzymatic hydrolysis (neutrase)	SY	1164.79 μM	Non-competitive	Zn^{2+}, A354	F391, A365	F391, R522, F391, R522	–	Zhang et al. 2018
Lizard fish (*Saurida elongata*)	Magnetic affinity purification	GMKCAF	45.7 ± 1.1 μM	Non-competitive	T135, T266, N249, L339, E340	–	–	–	Lan et al. (2015, 2018)
Ostrich (*Struthio camelus*) egg white ovalbumin	Alkaline hydrolysis	YV	213.2 μM	Competitive	Y523, A354, A356, E384, H353	Y253,	–	–	Khueychai et al. (2018)
Red algae (*Gracilariopsis lemaneiformis*)	Enzyme hydrolysis (trypsin)	FQIN[M(O)]CILR	9.64 ± 0.36 μM	Non-competitive	N277, Q281, K511, Y523, S517, S516, E123, H353, E376.	–	–	–	Deng et al. 2018
		TGAPCR	23.94 ± 0.82 μM	Non-competitive	K511, Q281, E376, E384, H353, Y394, R402	–	–	–	
Sea grape (*Caulerpa lentillifera*)	Enzymatic hydrolysis (thermolysin)	FDGIP	58.89 ± 0.68 μM	Competitive	E411, Y523, V 518, H353, H513	–	–	–	Joel et al. (2018)
		AIDPVRA	65.76 ± 0.92 μM	Competitive	A354, S517, S516, Q281, E162, Y520, R522	–	H353	–	

(continued)

Table 8.2 (continued)

Source	Extraction technique	Sequence	IC_{50}	Inhibition mechanism	Interaction with human ACE (PDB ID = 1O86/1O8A)				References
					H-bond	HI	π-in	Cod-bond	
Walnut	Enzymatic hydrolysis (pepsin)	EPNGLLLPQY	233.178 μM	Mixed inhibition	N66, K118, D121, E123, H353, A354, S355, H387, S516, R522	Y62, A63, M223, A354, Y360, H383	–	–	Wang et al. (2018)
Buffalo (*Bubalus bubalis*) colostrum whey	Enzymatic hydrolysis (pepsin and pancreatin)	IQKVAGTW	300 ± 2 μM	Competitive	Y62, E123, D121, A354, E384, Y523, A356	–	Y523	Zn^{2+}	Ashok and Aparna (2017)
Potato tuber patatin	In silico hydrolysis (ficain)	PRY	95.33 μM	Competitive	R124, D358, R522, E123, Y523	–	–	–	Fu et al. (2016a, 2017)
	(papain/ficain)	WG	112.34 μM	Non-competitive	Zn^{2+}, R124, E123, E411. Y523	–	–	–	
Qula casein from yak (*Bos grunniens*) milk	Enzymatic hydrolysis (alcalase)	KYIPIQ	7.28 μM	Competitive	E376, E162, D415, A354, H383, E384, Y523, R522, A356, E411	–	–	–	Lin et al. (2017)
	(trypsin)	LPLPLL	10.46 μM	Competitive	E411, R522, R124, S516	–	–	–	
	(proteinase K)	PFPGPIPN	12.79 μM	Non-competitive	E411, Y523, R522, Y360, S517	–	–	–	

Scorpion (*Hemiscorpius lepturus*) venom	Chromatographic purification	YLYELAR	9.37 μM	Competitive	Zn^{2+}, E411, N66, N522, A354, E411, Y523	–	–	–	Setayesh-Mehr and Asoodeh (2017)
		AFPYYGHHLG	17.22 μM	Competitive	Zn^{2+}, R522, H410, T92	–	–	–	
Sipuncula (*Phascolosoma esculenta*)	Enzymatic hydrolysis (pepsin and trypsin)	GNGSGYVSR	29 μM	Non-competitive	D358, R522, M223, S517, Y523, E384	K118, A405, N406, F570, P407, E403, Y62	–	–	Guo et al. (2017a)
		YASGR	184 μM	Non-competitive	R522, A354, H353	P519, P407, V518, Y523, W357, F512	–	–	
		RYDF	235 μM	Non-competitive	G404, A356	–	R522	–	
Bovine collagen	Enzymatic hydrolysis (alcalase and papain)	GPRGF	200.91 μM	Non-competitive	E123, E411, L129, S516	–	–	–	Fu et al. (2016a, b)
		VGPV	405.12 μM	Non-competitive	Y62, R124, Y360, S517	–	–	–	
Cupuassu (*Theobroma grandiflorum*)	Enzymatic hydrolysis (subtilisin)	GSGKHVSP	3.11 ± 0.4 μM	Mixed inhibition	–	–	–	–	da Cruz et al. 2016
		MVVDKLF	59.34 ± 4.1 μM	Competitive	–	–	–	–	
		MEKHS	63 ± 7.6 μM	Non-competitive	–	–	–	–	
		FLEK	79.5 ± 6.0 μM	Non-competitive	–	–	–	–	
		LDNK	131 ± 15.0 μM	Competitive	–	–	–	–	

(continued)

Table 8.2 (continued)

Source	Extraction technique	Sequence	IC_{50}	Inhibition mechanism	Interaction with human ACE (PDB ID = 1O86/1O8A)				References
					H-bond	HI	π-in	Cod-bond	
Flounder fish (*Paralichthys olivaceus*) muscle	Enzymatic hydrolysis (pepsin)	MEVFVP	79 μM	Competitive	E411, R522, N66	–	Y523	Zn^{2+}	Ko et al. (2016)
		VSQLTR	105 μM	Non-competitive	E131, E202, K210	–	–	–	
Mushroom (*Tricholoma matsutake*) fruiting body	Water extraction	WALKGYK	0.40 μM	Non-competitive	–	–	–	–	
Sea cucumber (*Stichopus horrens*)	Enzymatic hydrolysis (alcalase)	EVSQGRP	0.05 ± 0.00 mM	Mixed inhibition	–	–	–	–	Forghani et al. (2016)
		CRQNTLGHNTQTSIAQ	0.08 ± 0.00 mM	Uncompetitive	–	–	–	–	
		VSRHFASYAN	0.21 ± 0.00 mM	Mixed inhibition	–	–	–	–	
		SAAVGSP	1.71 ± 0.10 mM	Mixed inhibition	–	–	–	–	
Bitter melon (*Momordica charantia*) seed	Enzymatic hydrolysis (thermolysin)	VSGAGRY	8.64 ± 0.60 μM	Competitive	E162, H353, A354, E384, D377, D453, C370, C352, A356, D358, Y360	–	–	–	Priyanto et al. (2015)
		VDSDVVKG	13.30 ± 0.62 μM	–	–	–	–	–	
Chebulic myrobalan (*Terminalia chebula*) fruit	Enzymatic hydrolysis (pepsin)	DENSKF	100 μM	Non-competitive	N66, E123, Y360, S516	W357, Y360		–	Sornwatana et al. (2015)

Silkworm pupa (*Bombyx mori*)	Enzymatic hydrolysis (SGD)	ASL	102.15 μM	Competitive	K453, D415, H383, V380, V379, A354, H353, Q281	–	–	–	Wu et al. (2015)
Fertilized egg	Chromatographic purification	HLFGPPGKKDPV	125 μM	Non-competitive	N66, K118, R124, Y394, E403, R522	–	–	Zn^{2+}	Duan et al. 2014
		VGVIKAVDKKAG GAGKVT	>250 μM	–	–	–	–	–	
Mushroom (*Agaricus bisporus*)	Protein precipitation (ammonium sulfate)	AHEPVK	63 μM	Competitive	–	–	–	–	Lau et al. (2014)
		RIGLF	116 μM	Competitive	–	–	–	–	
		PSSNK	129 μM	Non-competitive	–	–	–	–	
Chinese soft-shelled turtle (*Pelodiscus sinensis*) egg white	Enzymatic hydrolysis (thermolysin)	IVRDPNGMGAW	4.39 ± 0.31 μM	Competitive	A354, Y523, H513, H353, Y135, R124	–	–	–	Rawendra et al. (2013)
Bovine Achilles tendon collagen	Enzymatic hydrolysis (bacterial collagenase)	AKGANGAPGIAGAPGF PGARGPSGPQGPSGPP	51.10 ± 1.145 μM	Competitive	G200, H383, Y523	–	–	–	Banerjee and Shanthi (2012)
		PAGNPGADGQPGAK GANGAP	79.85 ± 0.1813 μM	Competitive	Y520, N281, H353, E384, Y523	–	–	–	
Grass carp	Enzymatic hydrolysis (alcalase)	VAP	18.6 μM	Competitive	–	–	–	–	Chen et al. 2012)

(continued)

Table 8.2 (continued)

Source	Extraction technique	Sequence	IC_{50}	Inhibition mechanism	Interaction with human ACE (PDB ID = 1O86/1O8A)				References
					H-bond	HI	π-in	Cod-bond	
Salmon by-product	Enzymatic hydrolysis (alcalase)	FNVPLYE	7.72 μM	Mixed inhibition	–	–	–	–	Ahn et al. (2012)
		VWDPPKFD	9.10 μM	Non-competitive	–	–	–	–	
		FEDYVPLSCF	10.77 μM	Mixed inhibition	–	–	–	–	
Yeast (*Saccharomyces cerevisiae*)	Enzymatic hydrolysis (lywallzyme and protease)	TPTQQS	111.7 μM	Non-competitive	Y62, N66, N70, A356, D358, K368, H387, E411, Y523	–	–	–	Ni et al. (2012)
Peanut (*Arachis hypogaea*) arachin	Enzymatic hydrolysis (SGD and pancreatin)	NAQRP	32 ± 2 μM	Competitive	K511, E384, H353	F457, F512, F527, V518	–	Zn^{2+}	Jimsheena and Gowda (2011)
		LRW	71 ± 4 μM	–	–	–	–	–	
		IETWNPNNQ	72 ± 5 μM	–	–	–	–	–	
		FW	97 ± 6 μM	–	–	–	–	–	
		NLAG	332 ± 14 μM	–	–	–	–	–	
Cuttlefish (*Sepia officinalis*) muscle	Enzymatic hydrolysis (proteases from *B. mojavensis* A21)	AHSY	11.6 μM	Non-competitive	–	–	–	–	Balti et al. (2010)

Note: *H-bond*, hydrogen bond; *HI*, hydrophobic interaction; *π-in*, π-interaction; *Cod-bond*, coordinate bond; –, not determined; *SGD*, simulated gastrointestinal digestion using pepsin, trypsin and chymotrypsin

and plants represented the greatest source of antihypertensive peptides which could be due to their relative ease of accessibility. Moreover, some of them were common food source and known to house peptides with a broad bioactivity spectrum. In particular, eggs, milk, grain and seed proteins were extensively explored and reported with antihypertensive, antidiabetic, anti-cancer, antioxidant, antimicrobial and cholesterol-lowering peptides (Marambe and Wanasundara 2012; Park and Nam 2015; Liu et al. 2018a, b; Liu et al. 2019a, b, c), suggesting the potential of food-derived peptides as value-added nutraceuticals. Notably, the discovery of ACE inhibitory peptides from marine animals was on the rise. Potent anti-ACE peptides were discovered from grass carp, salmon, sea horse and cuttlefish (Table 8.2). This could be ascribed to the successful approval of a bonito hydrolysate with antihypertensive effect as Foods for Specified Health Use (FOSHU) by the Ministry of Health and Welfare in Japan (Hayes and Tiwari 2015). The bonito hydrolysate produced using thermolysin contained the active pentapeptide LKPNM which had significantly reduced the systolic blood pressure in mildly hypertensive subjects without rebound effect throughout the 10-week clinical trial (Fujita et al. 2001). It was then marketed as functional ingredient or health supplement in powder, capsule or tablet form with the market names of Peptide ACE 3000 and Peptide Tea in Japan, Vasotensin® in the USA and PeptACE™ and Levenorm® in Canada (Hayes and Tiwari 2015). This further advocated the investigation of marine species as peptide-based antihypertensive drugs. Conversely, relatively few anti-ACE peptides were reported from algae, fungi and yeast sources compared to animals and plants. These sources may constitute another natural protein reservoir for the discovery of novel antihypertensive peptides (Huang et al. 2021; Jiang et al. 2021; Zhou et al. 2020).

8.3 Extraction and Identification of ACE Inhibitory Polysaccharides and Peptides

Extraction process plays an essential role in the ACE inhibitory activity of the extracted components. The extraction techniques and/or parameters could affect the composition, structure and the functional groups in the polysaccharides or peptides, thus influencing the interaction with ACE.

8.3.1 Polysaccharides

Generally, a number of techniques can be applied for extracting polysaccharides from plant materials. Extraction of polysaccharides typically involves two steps. The first step is the extraction of polysaccharides by dissolving them in extraction solvent. Subsequently, the precipitation of soluble polysaccharides using alcohol is carried out (Methacanon et al. 2014). Hot acidified water (pH 1.0–3.5) is the most

frequently used extraction medium for extraction of polysaccharides. As stated by Yuliarti et al. (2015), the extraction using acid as an extraction solvent has been used commercially in the industry due to its high extraction efficiency. This can be explained by acidic condition that is not only able to solubilize water-soluble polysaccharides, it is also capable of dissolving protopectin through partial hydrolysis. However, there are many other approaches. Figure 8.1 summarizes the currently reported extraction techniques used for polysaccharides extraction (i.e. hot water, acid-base, enzyme-assisted, successive cold aqueous, ultrasound-assisted, microwave-assisted and subcritical water extraction). The advantages and drawbacks of different extraction techniques that can influence extraction yield/quality, chemical structure of polysaccharide and their biological activities are also presented in the same figure.

Table 8.2 shows that water, citric acid or sodium acetate buffer was used to extract the ACE inhibitory polysaccharides. Therefore, it was suggested that these aqueous extraction techniques were the most preferred approaches for this purpose. Although this is the most conventional and easiest extraction technique in isolating water-soluble polysaccharides from plant tissues, this technique is not recommendable from the industrial perspective due to the high temperature and long extraction period (i.e. high energy cost) required. Apart from that, water-based extraction is unable to extract water-insoluble polysaccharides, such as protopectin, which is strongly bound to the cell wall, resulting in reduction of extraction yield. However, researchers opted for this technique. Further study using advanced techniques as elaborated in Fig. 8.1 should be considered.

The extraction conditions, such as temperature and time, are varied with the type of polysaccharide sources. Commonly, temperature range of 50–100 °C and period

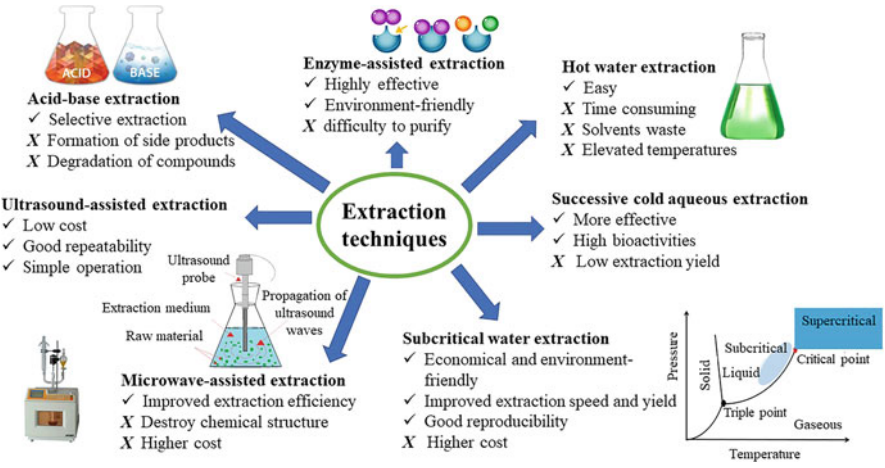

Fig. 8.1 The advantages and drawbacks of different extraction techniques. Note: √—advantage; *X*—drawback. (The information is adapted from Seidel (2012), Roselló-Soto et al. (2016), Zhang and Wang (2016) and Gong et al. (2020))

of 0.5–10 h are applied. Notably, the extraction of polysaccharides at low temperature (50 °C) obtained high ACE inhibitory polysaccharide compared with extraction at temperature higher than 50 °C (Table 8.1). This could be attributed to the structure of extracted polysaccharides that are responsible for the interaction with ACE. As mentioned by Yang and Yang (2020), the extraction temperature influenced the structure of polysaccharides that were isolated from *Eucheuma*. In addition, Chen et al. (2016) also reported that the cold-water extract of *Pleurotus citrinopileatus* showed a stronger ACE inhibitory activity than the hot water extract. As stated by Chen et al. (2020c), the extraction at high temperature could hydrolyze the linear and branching structures of the polysaccharides. This occurrence might reduce the interaction with ACE due to reduction of steric hindrances. Depending on the characteristics of the polysaccharide, the extraction parameters should be carefully well-thought-out.

8.3.2 Peptides

ACE inhibitory peptides could be extracted from protein sources using different methods such as enzymatic hydrolysis, alkaline hydrolysis, protein precipitation, water extraction, chromatographic purification and fermentation. Based on Table 8.2, enzymatic hydrolysis of proteins was the most preferred technique used to produce ACE inhibitory peptides. This could be attributed to a relatively short reaction time, mild reagent and ease of scalability, predictability and effluent discard without the requirement of additional equipment compared to the aforementioned extraction techniques.

Alcalase was the most frequently used enzyme for protein hydrolysis (Table 8.2). The choice of enzyme selected for hydrolysis was highly dependent on its cleavage preference of peptide bond. Alcalase preferably cleaves at the C-terminal of hydrophobic residues such as glutamic acid, methionine, leucine, tyrosine, lysine and glutamine, resulting in hydrophobic peptides (Adamson and Reynolds 1996). Since the presence of hydrophobic and aromatic residues was among the key features of potent ACE inhibitory peptides, protein hydrolysis using Alcalase could be a wise choice. This is evidenced by the Alcalase-treated Qula casein and salmon by-product which gave rise to the peptides KYIPIQ and FNVPLYE with low IC_{50} values of 7.28 μM and 7.72 μM, respectively. Most peptides identified from Alcalase hydrolysate also contained the branch-chained amino acids (BCAA) isoleucine, leucine and valine (Table 8.2), which was ascribed with strong anti-ACE property (Aluko 2015). Apart from the choice of enzyme, pH, temperature, substrate-to-enzyme (S/E) ratio and hydrolysis time were crucial factors to produce ACE inhibitory peptides. Different enzymes function optimally at different pH and temperatures, whereas S/E ratio and time could be experimentally monitored. These factors collectively affect the degree of hydrolysis (DH), amino acid composition of hydrolysate and ultimately the ACE inhibitory activity of peptides. A high DH indicates more release of peptides from the protein molecule, yet this does not always correlate with high ACE

inhibitory activity (Baharuddin et al. 2016; Gao et al. 2019). Potent ACE inhibitory peptides could be cleaved into smaller peptide fragments or free amino acids and lose bioactivity at higher DH. Hence optimization of enzymatic hydrolysis conditions is usually conducted to ensure the production of hydrolysate with strong ACE inhibitory activity.

Another advantage of enzymatic hydrolysis is the permission of combined enzyme treatment where enzymes could be added either sequentially or simultaneously to improve the biological activities of resultant peptides. For instance, Zheng et al. (2019) hydrolyzed coconut cake albumin by adding Alcalase, flavourzyme, pepsin and trypsin in a sequential manner. Firstly, Alcalase cleaves and releases peptides with hydrophobic or aromatic side chains. Then, flavourzyme containing a mixture of endopeptidase and exopeptidase less specifically cleaves a large number of peptide bonds to yield small molecular weight peptides. Lastly, pepsin and trypsin produce peptides with high resistance against gastrointestinal digestion. This is because the ability of peptide to survive the proteolytic actions of digestive enzymes would ultimately impact its in vivo efficacy after oral consumption (Yap and Gan 2020), hence peptide production through simulated gastrointestinal digestion (SGD) comprising of pepsin, trypsin and chymotrypsin was commonly reported (Table 8.2). Three potent ACE inhibitory peptides KAQYPYV, KIIIYN and KILIYG with IC_{50} values of 37.06, 53.31 and 58.72 μM were identified from the hydrolysate. Upon SGD, peptide KAQYPYV maintained its IC_{50}, whereas the IC_{50} of KIIIYN and KILIYG significantly decreased, implying the advantages of multiple enzyme treatments to improve the peptide bioavailability and ACE inhibitory activity.

Moreover, the integration of bioinformatics technology in the conventional enzymatic hydrolysis approach has become mainstream in peptide discovery. A typical bioinformatics-integrated workflow may involve in silico screening for potential biologically active peptide followed by molecular docking. This workflow had greatly minimized the time and cost in trial-and-error and improved the reliability of findings. In particular, molecular docking plays a crucial role in the prediction of ACE-peptide binding interaction and elucidation of mechanism of action of the selected peptide at molecular level (Tao et al. 2020). Generally, molecular docking could be executed using different modelling approaches, namely the template-based, global and local docking methods (Ciemny et al. 2018) as summarized in Table 8.3. Template-based method is also known as comparative or homology modelling where it performs similarity-based docking using highly homologous protein-peptide complex template from the Protein Data Bank (PDB) as scaffold. This is because homologous proteins tend to evolve from a common ancestor and shares similar sequences, structures and biological functions (Szilagyi and Zhang 2014). Although structure conservation could improve docking, the quality of modelling is dependent on the availability of homologous template and its resolution. The global docking method, or commonly known as blind docking or ab initio docking, refers to the docking of a ligand/peptide to the whole protein structure without any prior knowledge on the location of binding sites. Vast computational resources are required for the exhaustive global search and sampling. The local docking method,

Table 8.3 List of molecular docking software or servers for structure–activity relationship analysis

Molecular docking approach	Server	Server address	Features	References
Template-based docking	Galaxypepdock	http://galaxy.seoklab.org/pepdock	• Superpositions query protein and peptide with experimentally solved, similar protein–peptide complex structures from the PDB • Accepted protein size: ≤900 residues • Accepted peptide size: ≤30 residues • Flexible refinement of protein–peptide complex is supported	Lee et al. (2015)
	PRISM 2.0	http://cosbi.ku.edu.tr/prism/	• Searches for holding protein interface templates from PDB and matches query peptide with every possible interaction based on the templates • Protein hot spots prediction is supported	Baspinar et al. (2014)
Global docking	PepSite2	http://pepsite2.russelllab.org	• The preferred binding environment for each peptide residue is computed into spatial position-specific scoring matrix (S-PSSM) and scanned across protein surface for identification of potential binding sites • Accepted peptide size: ≤10 residues	Trabuco et al. (2012)
	CABS-dock	http://biocomp.chem.uw.edu.pl/CABSdock	• Random 3D structure generation of peptide based on the input sequence followed by docking of peptide onto the surface of protein • Accepted protein size: ≤500 residues • Accepted peptide size: 4–30 residues • Flexible docking (i.e. peptide is fully flexible peptide, whereas only small fluctuation is allowed on protein receptor backbone)	Kurcinski et al. (2015)

(continued)

Table 8.3 (continued)

Molecular docking approach	Server	Server address	Features	References
	pepATTRACT	https://bioserv.rpbs.univ-paris-diderot.fr/services/pepATTRACT/	• Automatic generation of peptide structure followed by rigid-body docking using the ATTRACT engine • Local docking is supported • Accepted peptide size: ≤20 residues • Rigid-body docking	Schindler et al. (2015)
	HPEPDOCK	http://huanglab.phys.hust.edu.cn/hpepdock/	• Hierarchical peptide docking by first modelling the receptor structure followed by peptide docking • Local docking is supported • Accepted protein size: ≤2000 residues • Accepted peptide size: No limit but ≤30 residues is recommended for higher prediction accuracy • Flexible peptide docking	Zhou et al. (2018)
	ClusPro	https://peptidock.cluspro.org/nousername.php	• Motif-based prediction of peptide structure followed by docking to protein receptor structure based on the fast Fourier transform (FFT) correlation approach • Rigid-body docking	Porter et al. (2017)
	ZDock	http://zdock.umassmed.edu/	• Employs FFT algorithm to search for grid-based shape complementarity (GSC), desolvation and electrostatics along the grid points covering the surface of protein receptor for potential peptide binding sites • Local docking is supported by manually selecting the contacting and blocking residues • Rigid-body docking	Pierce et al. (2014)
Local docking	HADDOCK	https://bianca.science.uu.nl/haddock2.4/	• Information-driven docking driven by ambiguous interaction restraints (AIRs) defined by users • Flexible refinement of model • Rigid-body docking by allowing flexibility for both "active" protein side chains and peptide structures	van Zundert et al. (2016)

(continued)

Table 8.3 (continued)

Molecular docking approach	Server	Server address	Features	References
	PEP-FOLD3	https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/	• Translation and rotation of peptide as a rigid block within the predefined protein binding sites • De novo peptide structure prediction is supported • Rigid-body docking	Lamiable et al. (2016)

in contrast to global docking, searches for peptide binding conformations at predefined protein hotspots. Predetermined hotspots using mutagenesis experiments or bioinformatics prediction could greatly reduce the resources required for exhaustive global search and help to filter out wrong predictions for higher accuracy results. Nonetheless, each docking approach comes with advantages and disadvantages. Thorough understanding on the characteristics of the enzyme and peptide would be the key to select the most suitable molecular docking approach to aid the elucidation of enzyme–peptide interactions at the microcosmic level.

8.4 Inhibition Mechanism of ACE Inhibitory Polysaccharides and Peptides

According to Belovic et al. (2013), this enzyme is accountable for two body systems, namely rennin–angiotensin (RAS) and kinin–kallikrein (KKS). In RAS, ACE cleaves decapeptide angiotensin I (i.e. Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to octapeptide angiotensin II, a potent vasoconstrictor by removing two amino acids (His-Leu). Conversely, ACE inactivates bradykinin, a vasodilator (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) by cleaving C-terminal dipeptide of Phe-Arg in KKS, which can further increase the chances of high blood pressure. Polysaccharides or peptides can inhibit ACE using different mechanism of actions depending on how they interact with the enzyme. To elucidate the mode of inhibition, it is crucial to understand the structure and catalytic mechanism of ACE. ACE is a zinc metallopeptidase structurally divided into two subdomains by a central groove which houses the enzyme active site (Natesh et al. 2003). The active site is deeply buried in the groove and is accessible through an entrance channel of approximately 3 Å diameter, which may restrict the access of large molecules. The catalytic site is characterized by a HEXXH zinc-binding motif where a zinc ion is tetrahedrally coordinated to the ligands H383, H387, E411 and a water molecule. It is surrounded by three subsites namely S_1, S_2 and S_1'. Subsite S_1 contains A354, E384 and Y523, subsite S_2 contains N281, H353, K511, H513 and Y520, whereas subsite S_1' contains E162. These hotspot residues function to stabilize the enzyme structure

upon substrate binding. During catalysis, the zinc-bound water molecule acts as a nucleophile to attack the scissile carbonyl carbon of substrate. The substrate carbonyl oxygen then replaces the water as the fourth ligand of zinc. The neighbouring E384 acts as an acid catalyst to transfer proton to the leaving amide nitrogen group to complete the catalysis and release of product molecule (Zhang et al. 2013).

8.4.1 Polysaccharides

Interaction between polysaccharides and enzymes was well elaborated by Wingender et al. (1999) and Shukla et al. (2021). The polysaccharide as the extracellular polymeric substances (known as EPS) could form a biofilm matrix together with other soluble microbial products. These polysaccharides were reported to consist of different charged and a polar group (e.g. $–COO^-$, $–OH^-$ and hydrophobic regions), whereas the enzyme consists of opposite charged groups, such as $NH4^+$ as well as aromatic and aliphatic amino acids. These polysaccharides could interlink with enzyme using different forces. Zhang et al. (2019) reported that the polysaccharide–ACE interaction can made up by an average over a large number of different intermolecular forces arising between the various segments and side chains, including electrostatic forces, hydrogen bonding, hydrophobic interactions, ion-bridging and Van der Waals interactions depending on their functional group and environmental condition. The functional groups found at the end of polymeric chains determine the interaction of polysaccharides with other molecules and the anion groups (COO^-) in polysaccharide could interact with cation groups ($NH4^+$) of ACE. This scenario causes steric hindrances for ACE as the enzyme is occupied by polysaccharide instead of its substrate.

Apart from that, the polysaccharides also can bind with metal ions that are required for activation of certain enzymes. As mentioned by Panyayai et al. (2018), the active site of ACE contains Zn^{2+}, which is tetrahedrally coordinated with ACE residues H383, H387 and E411. It was suggested that the anion groups in polysaccharides could interact with the cation Zn^{2+} and histidine residues, which ultimately disrupts the coordinate complex of ACE. Thus, the conformation of ACE is altered and preventing the substrate to be bound. Other than that, Tan and Gan (2016) stated that the acidic properties of polysaccharides could create an acidic microenvironment around ACE when it is bound, which is not an optimum condition for ACE because its optimum pH is pH 8.3, and therefore, ACE could not be functioning or losing its activity.

8.4.2 Peptides

Referring to Table 8.2, most peptides appeared to be competitive or non-competitive inhibitors of ACE. Competitive inhibitor peptides compete with substrates for the

enzyme active site and are characterized by an increase in K_M with a constant V_{max} through kinetic studies. These peptides tend to interact with the zinc ion or hotspot residues to interfere enzyme activity. For example, the competitive peptide GHIITVAR isolated from sesame hydrolysate ($IC_{50} = 3.60 \pm 0.10$ μM) formed hydrogen bonds with zinc ion and its ligand H383 to distort the catalytically essential tetrahedral coordinate complex and prevent proton transfer to cease substrate catalysis in the active site. This peptide was also hydrogen bonded to the stabilizing residues E162, Q281, H353, K511, Y520 and Y523 to further suppress substrate binding. Conversely, non-competitive inhibitor peptides bind equally to the enzyme regardless of the presence of substrate and are characterized by a reduced V_{max} with a constant K_M. The peptide may also reversibly bind to non-catalytic site to either induce conformational change or form dead-end complex with the enzyme to prevent product formation. This is exemplified by the wakame-derived non-competitive peptide KNFL ($IC_{50} = 225.87$ μM) which showed similar binding affinities towards the active site hotspots E162, Q281, H353 and E384 as well as non-active site residues D218, S222, T226 and E225.

Generally, the ACE inhibitory activity of peptide is strongly related to its structural properties such as peptide length, amino acid composition and sequence (Aluko 2015; Daskaya-Dikmen et al. 2017). Based on literature search, the favourable structural features of anti-ACE peptides were summarized in Table 8.1. Although short peptides with molecular weight <3 kDa may have more ready access to the narrow active site of ACE, a shorter peptide did not guarantee stronger ACE inhibitory activity. Referring to Table 8.2, the bovine Achilles tendon collagen derived 20-mer peptide PAGNPGADGQPGAKGANGAP recorded a lower IC_{50} value (79.85 ± 0.18 μM) than the ostrich ovalbumin dipeptide YV (213.2 μM). This implied that peptide length was not the main determinant of ACE inhibitory activity. Regarding the amino acid composition of peptide, the presence of charged and hydrophobic amino acids may inhibit ACE through Zn^{2+} chelation or disrupt the active site stability. For instance, the negatively charged glutamic acid of flounder fish muscle-derived peptide MEVFVP formed coordinate bond with Zn^{2+}, whereas the aromatic tryptophan of buffalo colostrum derived peptide IQKVAGTW formed π–π stacking interaction with the stabilizing residue Y523. These interactions had negatively affected the Zn^{2+}-mediated substrate catalysis in ACE. For amino acid sequence, different types of reactive amino acids were favoured at the N- and C-terminals of peptides with different lengths. Based on Table 8.4, dipeptides featuring BCAA and positively charged residues at the N-terminal and hydrophobic or aromatic residues at the C-terminal could positively enhance the peptide ability to interact and inhibit ACE. The distilled spent grain-derived dipeptide LP fulfilled these criteria and recorded an IC_{50} of 117.8 ± 1.80 μM. Tripeptides, however, tend to possess hydrophobic or aromatic and BCAA at the N- and C-terminal, respectively. Readers may refer to Panyayai et al. (2018) for more detailed tripeptide rule which associated the relationship between amino acid properties and their positional preferences in tripeptide sequences. For tetrapeptides and long-chained peptides, different amino acids were favoured at the peptide terminals yet the exact properties contributing to ACE inhibition remained inconclusive. Overall, hydrophobic and

Table 8.4 General structural characteristics of ACE inhibitory peptides

Structural characteristics	Description	References
(a) Peptide length		
• 2–20 amino acids (<3 kDa)	• ACE active site is accessible via a channel of approximately 3 Å diameter hence long peptides may not pass through easily	Natesh et al. (2003), Aluko (2015), Daskaya-Dikmen et al. (2017)
(b) Amino acid composition		
• Charge		
(i) Negatively charged amino acid: D and E.	• Negatively charged side chain may chelate Zn^{2+} in the ACE active site	Ko et al. (2016)
(ii) Positively charged: H, K, R	• Positively charged guanidine and ε-amine groups of R and K may attract and form new bond with the Zn-bound water molecule to distort the catalytically essential tetrahedral coordinate complex	FitzGerald et al. (2004), Aluko (2015)
• Hydrophobic		
(i) BCAA: I, L, V	• Side chains of BCAA may form a hydrophobic fence to shield one side of the coordination plane from water to stabilize the Zn-peptide chelate complex	Bal et al. (1996)
(ii) Aromatic amino acid: F, W, Y	• Aromatic ring structure may interact with Zn^{2+} through cation-π interaction to distort the catalytically essential tetrahedral coordinate complex or interact with aromatic hotspots of ACE through π–π interaction	Hu et al. (1995), Ashok and Aparna (2017)
(c) Amino acid sequence		
• Dipeptide: $N_1 - C_1$	• N_1: BCAA, positively charged residues • C_1: Hydrophobic/aromatic residues (F, W, Y) and P	Wu et al. (2006a)
• Tripeptide: $N_1 - X - C_1$	• N_1: Hydrophobic/aromatic residues (P, F, W)	Wu et al. (2006a)

(continued)

Table 8.4 (continued)

Structural characteristics	Description	References
• Tripeptide: $N_1 - X - C_1$	• X: Positively charged residues (K, R) • C_1: Hydrophobic residues (BCAA)	Panyayai et al. (2018)
• Tetrapeptide: $N_1 - N_2 - C_2 - C_1$	• N_1: V, I, M • N_2: R, H, W, F • C_2: F • C_1: Y, P, F	Wu et al. (2006b)
• Long-chained peptide: $N_1 - N_2 - N_3 - N_4 - X_n - C_4 - C_3 - C_2 - C_1$	• Tetrapeptide residues from C-terminal played more prominent role in ACE binding • C_1: Y, C • C_2: H, W, M • C_3: I, L, V, M • C_4: W	Wu et al. (2006b)

charged amino acids seemed favourable in di-, tri-, tetrapeptides and longer chained peptides. The presence of proline may also contribute to ACE inhibition. This is because the clinically approved ACE inhibitor drug lisinopril is a dipeptide sequenced KP with IC_{50} of 1.2 nM (Patchett et al. 1980).

8.5 Consideration in Choosing ACE Inhibitory Polysaccharides/Peptides

The findings have shown a substantial evidence to prove the effectiveness of the components. However, whether peptides and polysaccharides are the right ACE inhibitor is commonly asked question. Pharmacokinetic and pharmacodynamic aspects of this antihypertensive products are required for further investigation. Among the considerations are oral administration effect and their absorption in the gastrointestinal system (also known as intestinal permeability). Peptides could be small and easily absorbed in the system, depending on their peptide length; however, long peptides tend to be digested by gastrointestinal enzymes, such as pepsin, trypsin and chymotrypsin, and ultimately lose their activity (Yap and Gan 2020). Conversely, polysaccharides are macromolecules which are usually large in molecular size; therefore, their permeability could be limited. Since drug molecules usually have multiple interactions with the biological membranes, predicting their rate and extent of intestinal absorption as well as their bioavailability are essential (Dahlgren and Lennernäs 2019). Apart from that, their half-life, in which how long is the duration of the antihypertensive effect, is also crucial, as the dosage adjustment is required. It should be noted that the antihypertensive efficacy of these components is different. It could be due to the mechanism of the component itself, or the protocol of

the experiment used by the researchers. In addition, not all the synthetic drugs could reach the peak ratio more than 50–60%, as proposed by the United States Food and Drug Administration (Leonetti and Cuspidi 1996). Will ACE inhibitory peptides/polysaccharides have the same issue? In addition, the cytotoxicity and allergenicity of polysaccharides/peptides are another concern. To the best of our knowledge, not many clinical studies were conducted and this is a hot topic of on-going studies. It is expected that more research could be performed in order to understand the long-term effect and other secondary health benefits that come along with these natural ACE inhibitors.

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Chapter 9
Natural Protease Inhibitors and Their Therapeutic Potentials Against SARS-CoV-2

Nilesh Chandrabhan Vadnere and Nitinkumar P. Patil

Abstract Proteases play a key role in the pathogenesis of RNA viruses. They modify viral polypeptides by proteolytic cleavage post translation. Proteases are the potential targets for the treatment of viral diseases. Since December 2019, the world has observed the emergence of SARS-CoV-2 that resulted in Covid-19 pandemic and brought the world to a stand-still. It exposed the limitations of medical facilities and medicines to treat Covid-19. The search for vaccines and drugs against SARS-CoV-2 became the major task of the scientific community. The thrust area of research was the search for an inhibitor of protease M^{pro} (also known as the main protease) of SARS-CoV-2. The search for new molecules and their in vitro trials is time consuming. Therefore, the in silico approaches such as structure and ligand-based virtual screening, docking and molecular dynamics were extensively used to search for the promising inhibitor of M^{pro} from the existing library of natural molecules.

The present review summarizes the potential inhibitors of M^{pro} from the natural sources such as plants, metabolites from microorganisms including marine algae.

Keywords SARS CoV-2 M^{pro} · Natural protease inhibitors · Docking · Molecular dynamics

9.1 Introduction

Proteases are proteolytic enzymes that irreversibly break the peptide bond between amino acid residues of their substrate protein. Interchangeably, proteases are also referred to as peptidases or proteinases. Proteases are proteinic in nature. Specific reactive amino acid residues are present in the catalytic pocket of proteases. Based

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Table 9.1 Classification of proteases

	Class	Group used for catalytic activity	Mechanism	First described in
1	Aspartic protease	Carboxylic group of aspartate	Water molecule is activated that nucleophilically attacks the peptide bond	1993
2	Cysteine protease	–SH group of cysteine	Acts as a nucleophile to attack the peptide bond	1993
3	Serine protease	–OH group of serine	Acts as a nucleophile to attack the peptide bond	1993
4	Metalloprotease	Metal ions	Water molecule is activated that nucleophilically attacks the peptide bond	1993
5	Threonine protease	–OH group of serine (secondary alcohol)	Acts as a nucleophile to attack the peptide bond	1997
6	Glutamic protease	Carboxylic group of glutamate	Water molecule is activated that nucleophilically attacks the peptide bond	2004
7	Asparagine protease	Asparagine	Uses asparagine residue as a nucleophile (Rawlings et al. 2011)	2010

on these residues, proteases are classified into seven classes (Table 9.1) (Oda 2012; Rascon and McKerrow 2013) (MEROPS—the peptidase database (http://merops.sanger.ac.uk/)).

Many proteases are involved in pathogenesis by different microorganisms such as protozoa, bacteria, fungi, and viruses where proteases act as virulence factors (Haq et al. 2010). Proteases are also involved in emphysema and asthma, Alzheimer's and other neurodegenerative, cancer and osteoporosis, rheumatoid arthritis, and cardiovascular disorders.

Since the action of protease is irreversible, it needs to be controlled to avoid pathophysiological conditions. Protease inhibitors (PIs) are the substances that bind to proteases and inhibit their activity (Marathe et al. 2019). Enzyme inhibitors have largely been used for determining the structure of the enzyme and its mechanism of action (Bode and Huber 1992). Now they are finding application in pharmacology. In recent years, protease inhibitors have emerged as a major approach to diagnose and treat diseases and disorders (Karthik et al. 2014). There are two main challenges in using protease inhibitors for treatment: (1) correct identification of target protease and (2) use of target-specific protease inhibitor. Proteases are a family of closely related proteolytic enzymes; hence, a less specific protein may inhibit the non-target protease, leading to side effects (Deu et al. 2013). This problem demands the continuous search for highly specific protease inhibitors. The protease inhibitors are classified as natural and synthetic inhibitors. Natural protease inhibitors are usually proteins or peptides, whereas synthetic protease inhibitors, which are also referred to as small molecule inhibitors (SMIs), are usually reagents used in the

laboratory for the characterization of enzymes or drugs used in the treatment of some viral diseases (Rawlings et al. 2008).

A series of cases of severe acute respiratory disease caused by novel coronavirus was reported from Wuhan, China, in December 2019. The disease was named as Covid-19, and the virus was named as Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2, 2019-nCoV) (Yuki et al. 2020). Due to the high rate of infectivity, SARS-CoV-2 infection spread alarmingly over 216 countries and territories (Ghosh et al. 2021). In March 2020, World Health Organization (WHO) declared Covid-19 a pandemic.

The search for antiviral drugs and vaccines was initiated at the warfront. Several drugs such as lopinavir, ritonavir, hydroxychloroquine, azithromycin, chloroquine, remdesivir, etc., and some vaccines have been approved for emergency use. As these drugs were recommended from the experience of their use in the treatment of SARS, MERS, and influenza virus, they are useful, but their efficacy against SARS-CoV-2 needs to be confirmed. Mutations in the viral genome might affect the efficacy of vaccines. Therefore, there is a need to develop new vaccines and antiviral drugs against Covid-19 (Tang et al. 2020; Mittal et al. 2021).

In this review, we have attempted to illuminate the role of natural protease inhibitors as a therapeutic agent in the treatment of SARS-CoV-2 and other patho-physiological conditions.

9.2 Viral Proteases and Their Role in the Pathogenesis

Proteases are commonly produced by viruses and are involved in the activation of some of the key viral proteins of the viral replication machinery. Most of the viral proteases are also known for their ability to cleave host proteins to decrease host immunity and benefit viral replication. Papain-like proteases and chymotrypsin-like cysteine proteases are two proteases involved in the activation of SARS-CoV-2 replication machinery and initiation of its pathogenesis.

Viral protease that activates several polyproteins of the virus by proteolytic cleavage during posttranslational modification has been conventional target for antiviral drug discovery (Fischer et al. 2020). Phylogenetic studies conducted by Wu et al. (2020) revealed that the complete genome of SARS-CoV-2 contains 29,903 nucleotides and has 89.1% similarity with genus *Betacoronavirus*, subgenus *Sarbecovirus*. The order of genes (5′ to 3′) as revealed by sequence alignment was replicase ORF1ab, spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Wu et al. 2020). The two big open reading frames (ORFs) rep1a and rep1b are in the replicase gene that expresses two overlapping polyproteins, pp1a and pp1ab (Jin et al. 2020) non-structural proteins (NSPs) in pp1a are 1–11 and in pp1ab are 1–16. Following the extension of pp1a into pp1b, nsp11 from pp1a becomes nsp12. These polypeptides undergo posttranslational modification by extensive proteolysis into individual NSPs such as RNA-dependent RNA polymerase (RdRp) and helicase. These NSPs form viral replication and transcription complex

(V'kovski et al. 2021; Yuhang and Matthew Grunewald 2020; Zumla et al. 2016). The virus-encoded papain-like proteases cleave the N-proximal region of pp1a/pp1ab, while the coronavirus main protease (M^{pro}), also known as 3C-like protease, is a chymotrypsin-like cysteine protease that processes the C-proximal region ($3CL^{pro}$) (Hegyi and Ziebuhr 2002). M^{pro} is essential for viral replication and infection, and in humans, there are no closely comparable homologs, making it a potential target for antiviral therapy (Jin et al. 2020; Pillaiyar et al. 2016).

9.3 Natural Inhibitors of M^{pro} Proteins

M^{pro} is a component of the viral polyprotein, and it must be activated by autocleavage from an inactive precursor before it may cleave the polyprotein at other cleavage sites.

Being a protease, M^{pro} binds specifically to polypeptide. Peptide inhibitors derived from these polypeptide substrates have the potential to inhibit M^{pro} activity (Li and Kang 2020). Substrate polypeptide can be converted into inhibitors by (1) mutation to improve its binding affinity, (2) variation in length to improve its potency, and (3) attachment of warhead to the peptide. Dipeptide inhibitors have low molecular weight and high ligand effectiveness. The side chains of amino acids of dipeptide can be modified to make it an effective medicine (Li et al. 2017). A peptidomimetic is a short peptide-like chain that resembles a peptide. They are usually created by manipulating an existing peptide or by creating comparable systems that mimic peptides (Marshall and Ballante 2017; Citarella et al. 2021). N3 is a broad spectrum peptidic inhibitor that irreversibly and effectively inhibits M^{pro} of SARS-CoV-2 (EC50 = 16.77 μM) by binding to the active site and formation of the covalent bond between its warhead and the Cys145 (Li and Kang 2020). Peptidomimetic compound α-Acyloxymethylketone warheads with six-membered Lactam P1 glutamine has been reported to exhibit SARS-CoV-2 M^{pro} inhibition. It has low cytotoxicity and good plasma and glutathione stability. This finding demonstrated the potential of substituted warheads as M^{pro} inhibitors (Bai et al. 2021). Another peptide inhibitor, 11r, has been reported to inhibit M^{pro} with EC50 in micromoles. Peptide inhibitor aldehyde GC373 and bisulfite adduct GC376 have also been reported to be effective inhibitors of M^{pro} of SARS-CoV-2 with and IC50 value of 0.19 and 0.40 μM by covalently attaching to Cys145 as a hemithioacetal (Vuong et al. 2020). Myricetin is a flavonoid found in many food sources including bayberry. It has therapeutic values for the treatment of different types of tumors, inflammatory diseases, atherosclerosis, thrombosis, cerebral ischemia, diabetes, Alzheimer's disease, and pathogenic microbial infections (Song et al. 2021). The use of pyrogallol group as a warhead with myricetin has been reported as an effective covalent inhibitor of the SARS-CoV-2 M^{pro}. The Pyrogallol group works by covalently modifying the catalytic cysteine as an electrophile (Su et al. 2021). The natural inhibitors are obtained from different plant and animal sources.

9.3.1 Plant-Based Inhibitors of M^{pro}

Plant chemicals are excellent for discovering medicinal components of interest and are economical to generate fast. The potent plant compounds have been reported to inhibit M^{pro} of SARS-CoV-2 (Khaerunnisa et al. 2020).

For example, *Androghraphis paniculata* is an Acanthaceae family plant. Commonly cultivated in Southern and South-eastern Asia, it is also known as creat or green chiretta. According to Traded Medicinal Plant Database, the parts of this plant are traditionally used for the treatment of cold and other viral respiratory infections (Yarnell 2018). It has been reported to be a promising inhibitor of M^{pro} of SARS-CoV-2 (Vellingiri et al. 2020). *Androghraphis paniculata* is known to contain a range of flavonoid and their derivatives. An extensive study on isolation and characterization of flavonoids from *Andrographis paniculata* is carried out by Chen et al. (2014b). Because of its anticancer potential, Andrographolide, a labdane diterpenoid extracted from this plant has been reported to have antiviral properties against many viral infections (Gupta et al. 2017; Latif and Wang 2020). Molecular docking studies on Andrographolide by Enmozhi et al. (2021) revealed the significant potential of andrographolide to dock in the binding site of M^{pro} of SARS-CoV-2. The docking analysis generated significant negative free energy change (−3.094357 kcal/mol) or docking score suggesting high affinity of Andrographolide to binding pocket (Enmozhi et al. 2021). The docking score is also significant when compared with the synthetic inhibitors disulfiram (−46.16 kcal/mol), tideglusib (−61.79 kcal/mol), and shikonin (−17.35 kcal/mol) (Jin et al. 2020).

A similar study was conducted by Sukardiman et al. (2020) using 45 phytoconstituents of *Androghraphis paniculata* and synthetic drugs as the internal control. It was found that two *Androghraphis paniculata* ligands; GAD and DGE, have low free energy change and overlap binding interaction on docking site to 6 LU7 pockets of M^{pro} compared to Indnavir and REM (anti-HIV drug) and GAD (Andrographolide glycoside form) had lower free energy change than its aglycone. This study revealed that *Androghraphis paniculata* is a potential 6 LU7 SARS-CoV-2 M^{pro} inhibitor (Sukardiman et al. 2020). Other medicinal plants such as *Eurycoma harmandiana* and *Sophora flavescens* have also been reported as a potential source for inhibition of M^{pro} (Verma et al. 2021).

9.3.2 Phytochemicals with M^{pro} Inhibitory Activity

Phytochemicals are the metabolites of plants. They offer characteristic aroma, color, and flavor to the plant parts. Many phytochromes are produced by plants in response to stress. Traditionally, medicinal plant extracts have been used in the treatment of diseases. With the advancement of technology, many phytochemicals have been characterized. Anthocyanidins, flavonoids, and isoflavones are some of the broad groups of phytochemicals. Many phytochemicals are the choice of bioactive

molecules over synthetic molecules due to their health benefits. They are used in natural or derived form for their effective use in the treatment of various diseases and disorders. An in silico analysis has revealed many phytochemicals as potential candidate of M^{pro} inhibitor.

9.3.2.1 Flavonoids

Rehman et al. (2020) used molecular docking and simulation approach to identify some of the lead molecules from nature as inhibitors of M^{pro} and reported three flavonoids with potential to treat Covid-19, viz.: (1) Kaempferol, (2) Quercetin, and (3) Rutin. Kaempferol, a major flavonoid aglycone found in beans, bee pollen, broccoli, cabbage, capers, cauliflower, chia seeds, chives, cumin, moringa leaves, endive, fennel, garlic, etc., is reported to have antimicrobial, anti-inflammatory, antioxidant, antitumor, cardioprotective, neuroprotective, and antidiabetic activities, and used in cancer chemotherapy (Imran et al. 2019). Quercetin is a natural flavonoid found abundantly in vegetables and fruits that have therapeutic potential for the prevention and treatment of cardiovascular disease, cancer, and neurodegenerative disease (Muhammet et al. 2016). Rutin is a flavonol glycoside found in many plants, including buckwheat, tobacco, forsythia, hydrangea, viola, etc. It has been used therapeutically to decrease capillary fragility (NCBI 2021a). These flavonoids were able to interact with residues (His41 and Cys145) in the binding pocket of the active site of M^{pro} (Rehman et al. 2020).

9.3.2.2 Coumarin

Antiviral coumarin phytochemicals have been reported as potential inhibitors of M^{pro} by in silico approaches (Abdizadeh et al. 2021). Coumarin, which has aromatic and fragrant characteristics, is found in vegetables, spices, fruits, and medicinal plants and in high concentrations in some cinnamon (Loncar et al. 2020).

9.3.2.3 Alkaloids and Terpenoids

Gyebi et al. (2021) explored alkaloids and terpenoids derived from some African plants as potential inhibitors of M^{pro} using in silico approach. They reported two alkaloids (10-hydroxyusambarensine and cryptoquindoline) and two terpenoids (6-oxoisoiguesterin and 22-hydroxyhopan-3-one) as a potential drug as they bind favorably to catalytic diad of M^{pro} and are non-toxic (Gyebi et al. 2021). 10-Hydroxuusamarensine has been reported as an antimalarial bisindole alkaloid from roots of *Strychnos usambarensis* (NCBI 2021b), whereas cryptoquindoline is an alkaloid isolated from *Cryptolepis sanguinolenta*, with antifungal and antibacterial activities (Cimanga et al. 1998). 6-Oxoisoiguesterin is a bisnorterpenes isolated from *Salacia madagascariensis* (Celastraceae). Phytochemicals such as

bavachinin, psoralidin, betulinic acid, curcumin, and hinokinin have been reported, encouraging anti-M^{pro} activity. These phytochemicals were isolated from traditional medicinal plants (Mandal et al. 2021). Bavachin is known for its anti-asthma activity, and it is isolated from the Chinese herb Fructus Psoraleae (Chen et al. 2014a, b, c). Psoralidin is a prenylated coumestan, isolated from the seed of *Psoralea corylifolia* which is known to induce apoptosis in prostate cancer cells (Kumar et al. 2009). Beutilinic acid is a triterpene derivative of botulin, isolated from the bark of Betula alba, with anti-inflammatory, anti-HIV, and antineoplastic activity (NCBI 2021a). Curcumin is the primary bioactive substance in turmeric. It is a natural polyphenol derived from the rhizome of the Curcuma longa. It has been reported to have anti-inflammatory activity and is effective in the treatment of chronic pain and depression (NCBI 2021a, b, c, d) Hnokinin is a lignan isolated from the plant. It is reported to have anti-inflammatory and antimicrobial activity (Marcotullio et al. 2014).

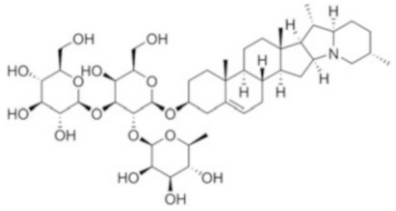
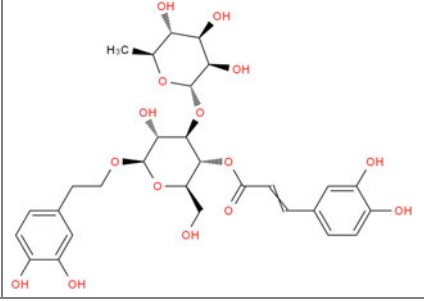
Jade et al. (2021) explored natural compounds from the Natural Product Activity and Species Source (NPASS) database and Maybridge database using in silico virtual high-throughput screening and reported six compounds Bemcentinib, Clofazimine, Abivertinib, Dasabuvir, MFCD00832476, and Leuconicine F as an inhibitor of M^{pro}.

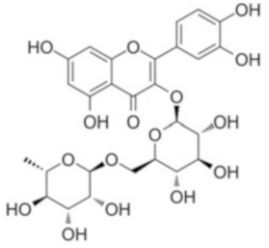
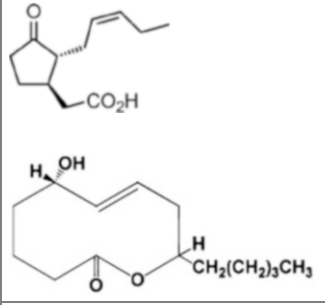
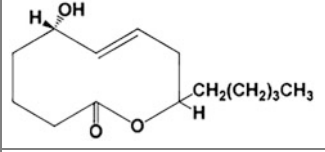
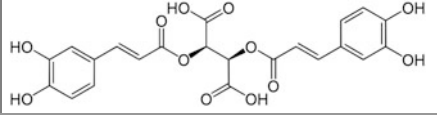
9.3.3 *Microbial Products as Inhibitors of M^{pro}*

The Seaweed Metabolite Database (SWMD) is a catalog of known compounds and their biological applications as reported in the literature and is beneficial in the development of drugs from marine resources (Davis and Vasanthi 2011). Muteeb et al. (2020) explored 1110 unique metabolites of marine algae from SWMD against M^{pro} of SARS-CoV-2 using a computational approach. High-Throughput Virtual Screening (HTVS), Standard Precision Docking helped to shortlist nine compounds based on binding energy (<-6 kcal/mole) and were further subjected for extra-precision docking to conclude that RC002, GA004, and GA006 are the potent inhibitors of M^{pro} of SARS-CoV-2. There ADMET analysis revealed that RC002 has properties of the potential drug since it passed different medicinal chemistry filters viz. Lipinski's, Veber's, PAINS, and Brenk's filters (Lipinski et al. 2012; Veber et al. 2002). RC002 is callophysin A, a cytotoxic tetrahydro-β-carboline, isolated from the methanol extract of the red alga *Callophycus oppositifolius* (Ovenden et al. 2011). Callophycin A has been reported as a potential chemopreventive and anticancer agent (Shen et al. 2011). Callophysin A binds to the active site of M^{pro} of SARS-CoV-2 through the salt bridges with Cys145, hydrogen bonding with Glu166 and His164, hydrophobic interactions with His41, and van der Waals' interaction with other amino acids (Muteeb et al. 2020).

In comparison to plant-derived products, natural compounds generated from microbial sources are favored for their chemical diversity (Table 9.2). According to Patridge et al. (2016), about 53% of FDA-approved natural product-based drugs

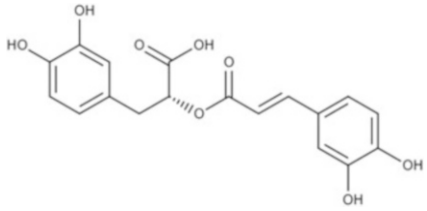
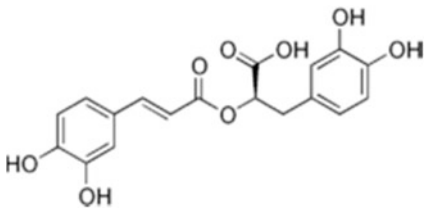
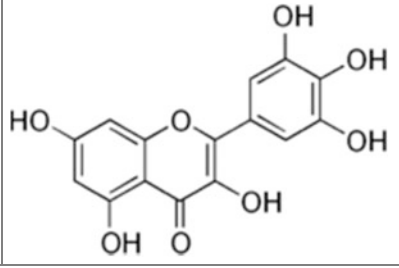
Table 9.2 Potential SARS-CoV-2 protease inhibitors/ligands and their sources

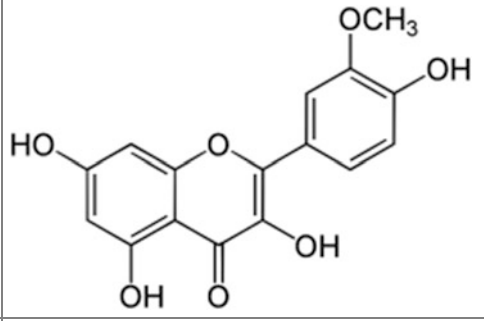
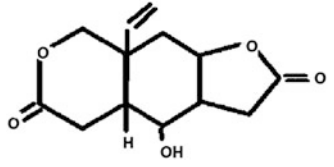
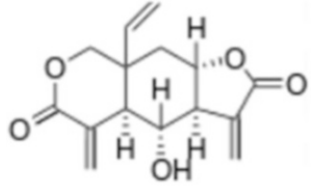
Sr. no.	Inhibitor/ligands	Source		Reference
Plants				
1	Solanine	*Solanum tuberosum, Solanum lycopersicum, solanum melongena*		Teli et al. (2021)
2	Acetoside	*Kabasura kudineer*		Teli et al. (2021)

3	Rutin	*Fagopyrum tataricum, Styphnolobium japonicum, and eucalyptus*		Teli et al. (2021)
4	Jasmonic acid, Jasmonic acid methyl ester	*Jasminum grandiflorum* L.		Padhi et al. (2021)
5	Putaminoxin B	*Phoma putaminum*		Padhi et al. (2021)
6	Putaminoxin D	*Phoma putaminum*		Padhi et al. (2021)

(continued)

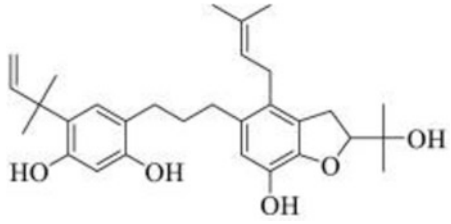
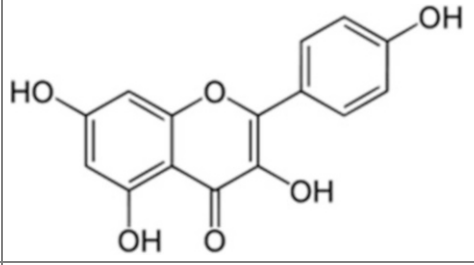
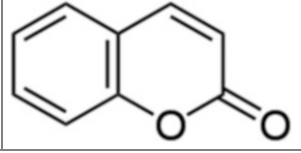
Table 9.2 (continued)

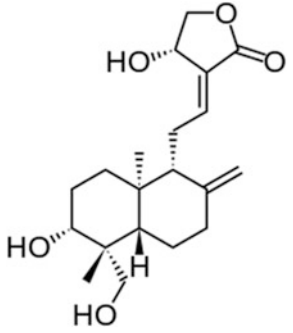
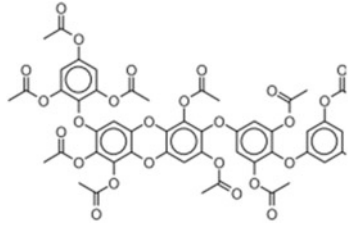
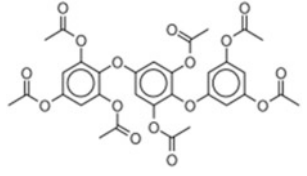
Sr. no.	Inhibitor/ligands	Source		Reference
7	Chicoric acid	*Cichorium intybus, Echinacea purpurea*		Gyebi et al. (2021)
8	Rosmarinic acid	*Rosmarinus officinalis, Perilla frutescens*		Gyebi et al. (2021)
9	Myricetin	*Rosa damascene, Pistacia lentiscus, Camellia sinensis*		Gyebi et al. (2021)

10	Isorhamnetin	*Fruits of Hippophae rhamnoides* L., leaves of *Ginkgo biloba* L.		Gyebi et al. (2021)
11	Luteolin	Vegetables and fruits, e.g., celery, parsley, broccoli, onion leaves, carrots, peppers, cabbages, apple skins, and flowers of chrysanthemum		Gyebi et al. (2021)
12	Vernomenin	*Vernonia amygdalina*		Gyebi et al. (2021)

(continued)

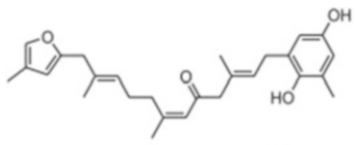
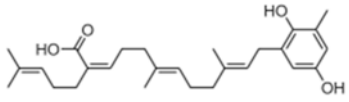
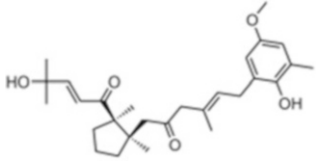
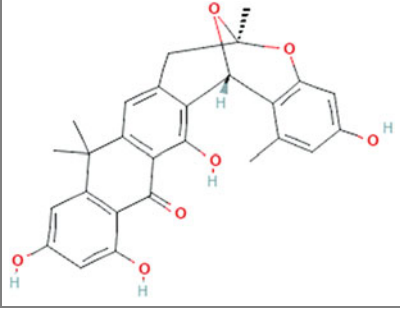
Table 9.2 (continued)

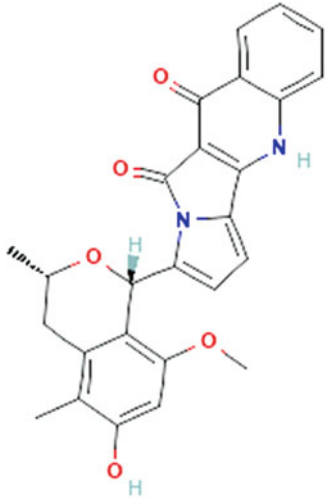
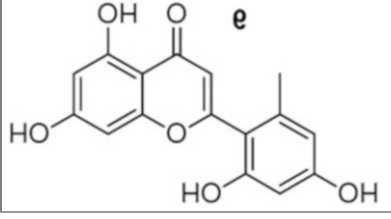
Sr. no.	Inhibitor/ligands	Source		Reference
13	Kazinol T	*Broussonetia kazinoki*		Muhammad et al. (2021)
14	Kaempferol	Beans, bee pollen, broccoli, cabbage		Rehman et al. (2020)
15	Coumarin	Cinnamon		Abdijadeh et al. (2021)

16	Andrographolide	*Androghraphis paniculata*		Enmozhi et al. (2021)
Microorganisms (including marine algae)				
17	Bieckol	*Ecklonia cava*		Rauf et al. (2020)
18	7-Hydroxyeckol hepta-acetate	*Ascophyllum nodosum*		Rauf et al. (2020)

(continued)

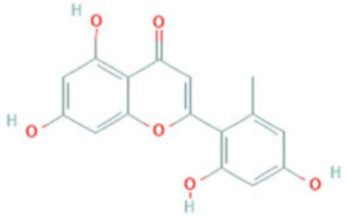
Table 9.2 (continued)

Sr. no.	Inhibitor/ligands	Source		Reference
19	5-Hydroxy-cystofurano-quinol	Brown algae		Rauf et al. (2020)
20	Sargaquinoic acid	*Sargassum sagamianum*		Rauf et al. (2020)
21	Methoxybifurcarenone	*Cystoseira tamariscifolia*		Rauf et al. (2020)
22	Ivermectin Anthrabenzoxocinone	*Streptomyces avermitilis*		Leon caly et al. (2020)

23	Citriquinochroman	*Penicillium citrinum*		Sayed et al. (2020)
24	Pityriacitrin	*Malassezia furfur*		Sayed et al. (2020)

(continued)

Table 9.2 (continued)

Sr. no.	Inhibitor/ligands	Source		Reference
25	Penimethavone A	*Penicillium chrysogenum*		Sayed et al. (2020)

are produced by microorganisms, and most of them are antiviral. Ivermectin, a semisynthetic pentacyclic 16-membered lactone developed from the soil bacteria *Streptomyces avermitilis*, has been shown to suppress SARS-CoV-2 replication in vitro. The approval of ivermectin for the treatment of Covid-19 by FDA (Caly et al. 2020) stimulated many researchers to explore microbial products against SARS-CoV-2 using in silico ligand techniques such as structure and ligand-based virtual screening, docking, and molecular dynamics. Molecular docking is an effective in silico screening tool that plays an important role in drug design. It is used to identify key interactions of ligands within the active site of a target protein and assigns a predicted binding affinity (Ngwa et al. 2020). Sayed et al. (2020) explored the in silico techniques to evaluate the potential of 24,581 compounds from different microbial sources, retrieved from the Natural Products Atlas database. They selected only drug-like molecules using Lipinski's rules. Further selection was done based on their ability to show good binding affinities toward the molecular dynamic simulation (MDS)-derived enzyme's conformers. Final MDS experiments on the ligand–protein complexes with 12 compounds resulted in the selection of six compounds with high potential as anti-SARS-CoV-2 drug candidates (Sayed et al. 2020). These compounds include Citriquinochroman ($\Delta G = -12.4$ kcal/ mol), Holyrine B ($\Delta G = -12.3$ kcal/ mol), Proximicin C ($\Delta G = -12.2$ kcal/ mol), Pityriacitrin B ($\Delta G = -12.2$ kcal/ mol), Anthrabenzoxocinone ($\Delta G = -11$ kcal/ mol), and Penimethavone A ($\Delta G = -10.8$ kcal/ mol).

Citriquinochroman is N-containing polyketide. It was isolated from the endophytic fungus *Penicillium citrinum* and was reported to have moderate anticancer activity (El-Neketi et al. 2013). It showed the least binding energy with perfect fitting inside the active site in the crystallized form of M^{pro}. Holyrine B is an indolocarbazole class of alkaloid. It was isolated from a marine-derived actinomycete (Williams et al. 1999), and it was the second-best M^{pro} scoring ligand after citriquinochroman. Proximicin C is aminofuran antibiotic. It was isolated from the marine actinomycete *Verrucosispora* strain MG-37 (Fiedler et al. 2008). Pityriacitrin is an ultraviolet-absorbing indole alkaloid from the yeast *Malassezia furfur* (Mayser et al. 2002).

Anthrabenzoxocinone is a hexacyclic aromatic compound. It was isolated from a *Streptomyces* sp. (Chen et al. 2014c), and penimethavone A is a flavone that possesses a rare unique methyl group in ring B. It was isolated from *Penicillium chrysogenum*, a fungus cultured from a gorgonian *Carijoa* sp. collected from the South China Sea (Hou et al. 2016).

9.4 Conclusion

The pandemic of Covid-19 has not only created health problems in the affected persons but also a severe economic and psychological impact on the entire world. The effective treatment against SARS-CoV-2 has not yet been developed. The protease inhibitors are the first potential antiviral drug reported against SARS-CoV-2. The present review summarizes the potential inhibitors of M^{pro}

from natural sources such as plant metabolites, metabolites from a marine alga, microbial metabolites, and inhibitors derived from natural metabolites. The protease inhibitors described in the current review can serve as prophylactics to prevent the clinical manifestations and disease progression especially in those who come in contact with COVID-19 patients. The potent plant compounds have been reported to inhibit M^{pro} of SARS-CoV-2. Medicinal plants such as *Eurycoma harmandiana* and *Sophora flavescens* have also been reported as a potential source for inhibition of M^{pro} (Verma et al. 2021). Callophycin A from marine sources has been reported as a potential chemopreventive and anticancer agent. The important prerequisite for the development of a new molecule as an inhibitor of protease is mainly based upon a clear understanding of catalytic activity and molecular structure. The thrust area of research is the development of different combinations of protease inhibitors with its effector molecule which is time-consuming in vitro trials.

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Chapter 10
Telomerase and its Inhibitor in Cancer Therapeutics: Current Status and Future Prospective

Vivek Srivastava, Saleha Siddiqui, Akanksha Dhondiyal, Pakhi Gupta, and Ankush Yadav

Abstract Telomerase is an extremely specific reverse transcriptase (RT) that involves maintaining telomeric length by duplicating TTAGGG nucleotide DNA sequences on the human chromosomes at the 3′ terminal position. It functions as a shielding effect from degradation and loss of gene sequences. In humans, the ribonucleoprotein complex consists of a catalytic subunit: hTERT (human telomerase reverse transcriptase) and RNA subunit: hTR (human telomerase RNA). It is expressed in embryonic cells and suppressed during maturity. The enzyme is reactivated in around 85–90% of solid tumors. These findings mark it a likely drug target that could be established for therapeutic purposes with negligible effects. Herewith, we assess recent approaches to telomerase-directed therapy, deliberate the aids, their shortcomings, and speculate on the forthcoming perspective of inhibitors that target telomerase as cancer therapeutics.

Keywords Telomerase · Telomerase activity · Cancer · Telomerase RNA · Inhibitors

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10.1 Introduction

The nucleus of a eukaryotic cell, as seen under the microscope, which is about to divide, appears as double-sausage-like structures called chromosomes. At the end of these double-sausage-like structures, we see molecular probes labeled for visualization of the DNA, called telomeric DNA, which has many tandem copies of short oligonucleotides sequences. They could vary from a few base pairs to a few thousand base pairs. These G-rich telomeric DNA sequences at the 3′OH are the unit of telomeres that function as a cap to the end of chromosomes. The sequence is T_xG_y on one strand and A_xC_y on the other strand, where x and y can range from 1 to 4. The TG strand stretches longer than its complementary strand, leaving the strand rich in "G," as a single strand of DNA, hanging up to a few hundred nucleotides towards the 3′OH end. These help in stabilizing the chromosome. The human telomeric region consists of tandem arrays of non-coding hexameric repeat sequences, 5-′-(TTAGGG)n-3′, which is followed by a G-rich single-stranded that overhang (150–200 nucleotides long) at 3′OH terminally (Fig. 10.1). The exact sequence of the telomeric repeat can vary from species to species (Blackburn and Szostak 1984).

Their size ranges from 15 to 20 kbp to sometimes 50 kbp in chronic disease or tumors. Telomeres get reduced with each cell cycle. They are just like the plastic end of the shoelaces. Just like the plastic ends prevent the threads from segregating and protect the shoelace from ruining telomeres, it helps maintain chromosome integrity and provides a buffer of expandable DNA (Dunn et al. 1984).

There can be two types of end in DNA: end of chromosomal DNA and DNA breaks. These DNA breaks can be repaired via different types of DNA repair mechanisms found in cells. One of the aspects of telomere is to function as caps to the tips of chromosomes to prevent telomeres from undergoing DNA reactions which could be one of the consequences of broken DNA ends. If these breaks at the end are not repaired, DNA can undergo degradation. Therefore, it is clear that the

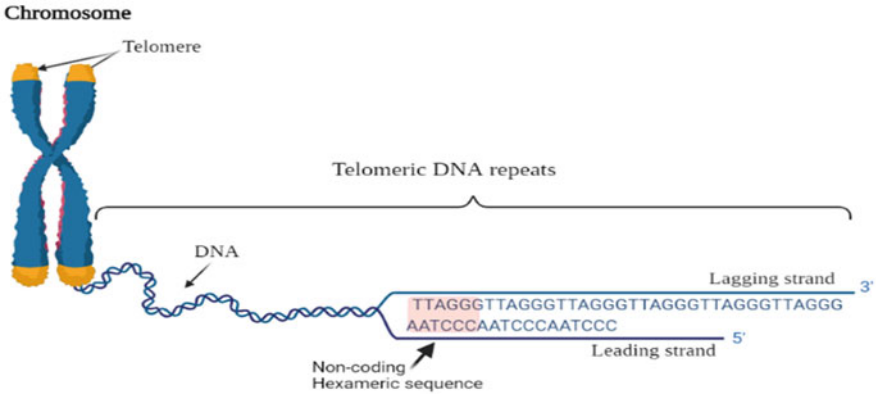


Fig. 10.1 Representing human chromosome and telomeric repeats at its end. (Source: Figure generated at BioRender (https://biorender.com/)

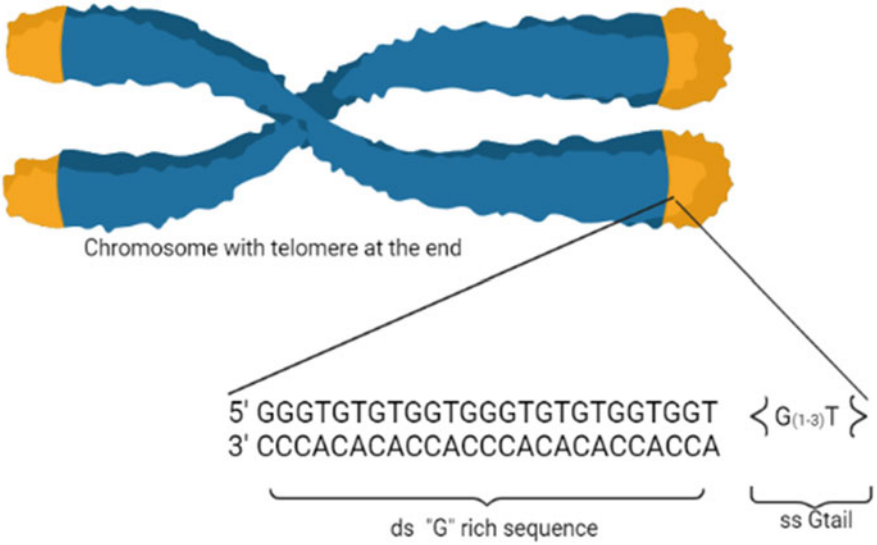


Fig. 10.2 Telomeric DNA sequence at the end of *Tetrahymena* chromosome representing the ds strand and the hanging G-tail. (Source: Figure generated at BioRender (https://biorender.com/)

telomeres prevent DNA from undergoing degradation. These DNA sequences at the tip of chromosomes are repetitive and similar; for example, TTAGGG is one of the short sequences repeated over again in human chromosomes. These repeats are called telomeric simple tandem repeats.

Another feature of telomeric DNA in chromosomal DNA is that it is single-stranded, unlike the remaining. The orientation of telomeric DNA is 5′–3′ towards the end, as shown in Fig. 10.2 (Jafri et al. 2016).

These telomeric sequences are repetitive non-coding DNA sequences. These sequences attract specific proteins like the shelterin protein that can bind to the double- and single-stranded portions of telomeric DNA repeats (Herbert 2011). The end of the telomere is protected by a group of proteins present in variable amounts known as the Shelterin complex, and it forms a T-loop (telomeric loop) and a small D-loop (displacement loop). The 3′ G-rich DNA sequence, which is overhanging, assists telomeric DNA in constituting a more advanced structure. The 3′ single-strand, which is overhanging, folds backward and occupies the homologous double-stranded TTAGGG region, forming a telomeric loop (t-loop). T-loop prevents the end of telomeres from being acknowledged as breakpoints by the DNA repair mechanism. The Shelterin complex comprises three core shelterin subunits, namely telomeric repeat binding factor (TRF1 and TRF2), human protection of telomeres 1 (POT1), TERF1-interacting nuclear factor 2 (TIN2), tripeptidyl peptidase 1 (TPP1), and repressor/activation protein 1 (RAP1) (van Steensel and de Lange 1997). Each subunit of shelterin separately consists of the following:

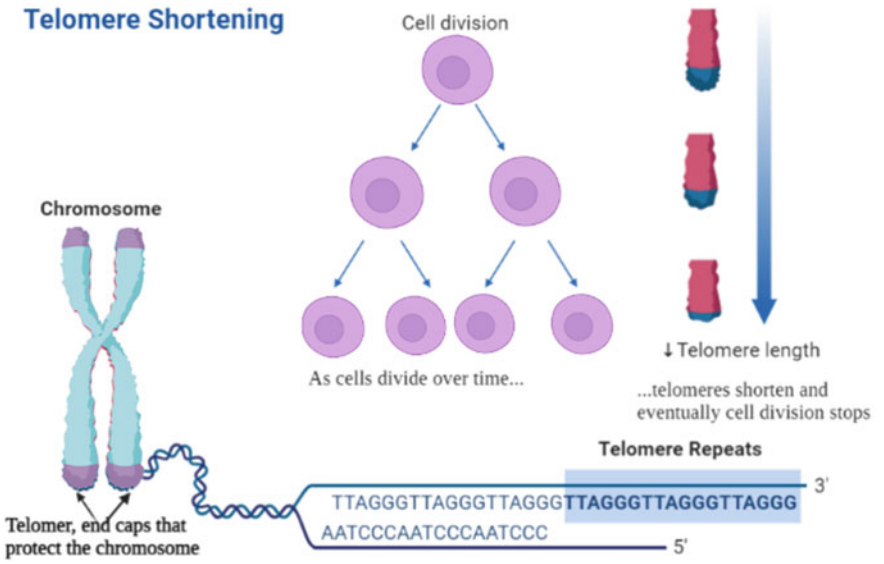


Fig. 10.3 Representing shortening of telomerase with every cell division. (Source: Figure generated at BioRender, (https://biorender.com/)

TRF1 (Telomeric Repeat Binding Factor 1): It is a dimeric protein of identical subunits that controls telomeric DNA replication by binding to the ds (double-stranded) TTAGGG domain of telomere. It decreases with aging in humans and mice (Takai et al. 2010).

TRF2 (Telomeric Repeat Binding Factor 2): It is anatomically related to TRF1, which is necessary for t-loop formation. It binds to the ds TTAGGG region of the telomere, overexpression of TRF2 results in shortening the telomere. Loss of TRF2 results in loss of t-loop, activating p53, and ATM-arbitrated apoptosis (Palm and de Lange 2008).

POT1 (Protection of Telomeres 1): It consists of OB folds (oligonucleotide/oligo-saccharide binding). The OB folds increase POT1's affinity for the single-stranded TTAGGG region of the telomere. POT1 assists in forming a telomere stabilizing d-loop. It also prohibits ss-DNA degradation by nucleases and shields the 3′G overhang and subdues ATR-intervened DNA repair (Liu et al. 2004).

RAP1 (Repressor/Activator Protein 1): It inhibits DNA repair and is a stabilizing protein associated with TRF2. Its main function is to regulate transcription and influence NF-kB signaling (Celli and de Lange 2005).

TIN2: It binds to TRF1, TRF2, and TPP1-POT1 complexes, stabilizes them, and bridges complexes linked to double-stranded DNA and single-stranded DNA TTAGGG region of the telomere. It also promotes glycolysis (Takai et al. 2003). Each time the cell divides, it results in the shortening of the telomere (Fig. 10.3). Cell division leads to growth arrest (replicative senescence) and gradually apoptosis. It has been reported that replicative senescence occurs

when one or more critically short telomeres trigger a DNA damage response regulated by p53. This growth arrest stage can be bypassed temporarily when p53 and RB are disabled. Nevertheless, till this time, the telomere is excessively shortened and leads to multiple chromosome end fusion that ultimately leads to loss of cell viability (Fig. 10.3) (Fagagna et al. 2003; Smith et al. 1998; Lee et al. 2006).

When there is an alteration in genomic stability, telomere maintenance is disrupted, resulting in end-to-end chromosome fusion or ends being represented as a double-stranded break. Many studies have suggested that cells respond to telomere dysfunction by undergoing apoptosis, genome instability, or senescence. There could be different reasons for telomere dysfunction, such as shortening the length of telomeric DNA, which can be caused due to malfunctioning of an enzyme called telomerase malfunctioning, and telomere dysfunction that can be caused when the proteins cannot bind to telomeric DNA. Molecular interruptions dismantle this binding of protein to telomeric DNA. Both can cause slowing of cell division and loss of cell renewal capacity leading to genomic instability. Telomere dysfunction can be sensed because they have regulatory action against the malfunctioning of DNA. End-to-end fusion of telomeres between two chromosomes can happen if the protein attached to telomeric DNA repeats is disrupted. This fusion of two chromosomes will lead to two centromeres in a chromosome. When it divides during cell division, the chromosomes will be ripped apart, leading to genetic instability (Galati et al. 2013). The machinery of DNA replication has high fidelity. However, DNA replication machinery cannot copy the end of linear DNA (in eukaryotic chromosomal DNA) that can be demonstrated. Every time DNA replicates as the cell divides, the daughter DNA gets shorter and shorter after every replication, concluding that the DNA ends cannot be replicated without compensatory loss. This compensatory loss after every replication can lead to senescence of the cell.

In the early 1970s, James Watson and Olovnikov, after seeing the machinery of DNA replication, concluded that senescence of cells after multiple cell division is due to loss of terminal DNA. Loss of terminal DNA during cell division is the reason that human cells cannot proliferate indefinitely in culture (Corey 2009). It is, therefore, essential to prevent the shortening of telomeric DNA, which will further prevent cell death (Osterhage and Friedman 2009).

10.2 Telomerase: The Anti-Aging Enzyme

In the late 1970 and early 1980s, telomerase was observed in a ciliated protozoa *Tetrahymena*, which has many small minichromosomes that help in the molecular analysis of telomeric DNA. Telomeric repetitive sequences in *Tetrahymena* (GGGGTT) were heterogeneous in different chromosomes, which means some mini chromosomes had 20 repeats of this sequence, some had 50, some had 200, and so on. At the same time, these were supposedly expected to be homogenous

in a population of *Tetrahymena*. This led to a question on the replication of telomeric DNA (Blackburn 2010). Another observation was made in a single-celled parasitic organism that causes sleeping sickness called *Trypanosoma*. It was found that telomeric DNA gradually got longer (Hayflick 1998; Blackburn and Challoner 1984). In another experiment, yeast telomeric (TG (1–3) repeats) DNA was grafted on *Tetrahymena* (TTGGGG) telomeric DNA repeats, and the linear plasmid formed was introduced into a yeast cell. It was observed that the ends of the DNA were maintained in the yeast cell as linear minichromosomes after replication. This observation was contrary to the standard known model of DNA replication or DNA recombination. All these observations suggested that a cell was capable of adding telomeric sequences (Shampay et al. 1984).

In her experiment, Barbara McClintock, a geneticist working on maize, noted that a mutant maize stock lost the capacity found in normal wild-type maize. If the chromosome breaks by radiation or mechanical rupture in wild-type maize, the broken ends of chromosomes can be healed to make a normal stable telomere. Nevertheless, she discovered this capacity of maintaining stable telomere had been lost in a mutant stock (McClintock 1941). All of these were directed towards a question then raised "if there was an enzyme that could extend the telomeric DNA sequence?" Elisabeth Blackburn and Carol Greider, in the early to mid-1980s, designed an experimental system with a high number of minichromosomes and, therefore, a high number of telomeres to study the presence of enzymes that can extend or maintain telomeres. They choose single-celled ciliated protozoan *Tetrahymena thermophila*. "G"-rich strand of telomeric DNA found at the tip of chromosomes was taken as an oligomer with a free 3′OH end. It was mixed with extract of *Tetrahymena* cell S-100 at a developmental stage at which telomeric sequence of DNA was added to freshly broken ends of chromosome because that would be very likely for an enzyme (if present) to show high activity than when present in the normal state. Mg^{2+} was added, followed by nucleotide triphosphate precursor (radiolabeled dGTP and TPP) (Fig. 10.4). This reaction mixture studied under autoradiograph found that the DNA sequence at the end of the telomere was added to the oligomer.

This experiment confirmed the assumed hypothesis that the given sets of newly added telomeric repeats were added due to the presence of an enzyme, telomerase (Greider and Blackburn 1985). Telomerase is a cellular ribonucleoprotein DNA polymerase enzyme. It is responsible for the extension and maintenance of the telomere and adds the TTAGGG sequence to the 3′ end of the chromosome. Telomerase activity is necessary to overcome the shortening of telomere and increase the ability of the cell to divide limitlessly. Telomerase is a unique and interesting enzyme because it has a DNA polymerase and RNA sequence that acts as a template for synthesizing telomeric repeats of DNA. Part of the RNA sequence hybridizes with the single-stranded overhanging DNA sequence (Sedivy 2007).

Telomerase has an associated RNA with a nucleotide sequence complementary to the telomeric repeat sequence. Using complementary RNA as a template adds the nucleotides and extends the 3′ overhang telomeric DNA strand. A matching or complementary synthesis of strand can be done by the standard DNA machinery,

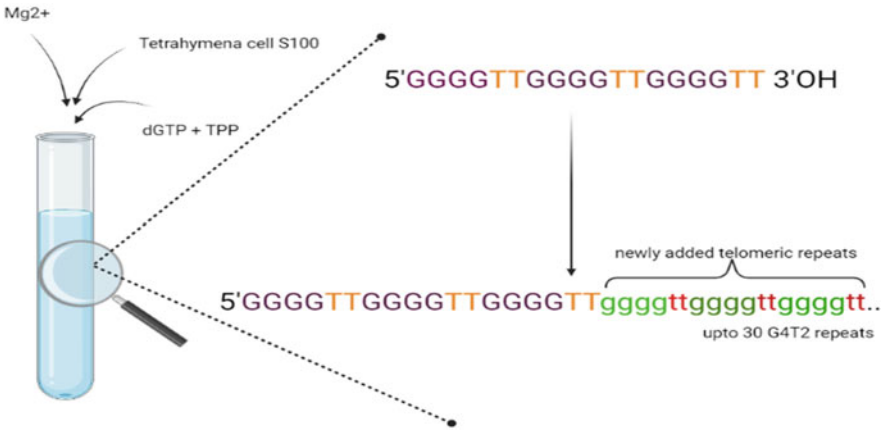


Fig. 10.4 Schematic representation of experiment carried out by Blackburn and Greider, using *Tetrahymena* cells which led to the discovery of telomerase. (Source: Figure generated at BioRender, (https://biorender.com/)

which uses an RNA primer and DNA polymerase, producing ds DNA when the overhang is extended long enough. These repeats were added to the DNA strand corresponding to the sequence at the 3′OH end of a chromosome and found that the complementary strand was not competent to add telomeric repeats (Bodnar et al. 1998).

In another experiment, yeast telomeric repeat with different sequences (TGTGTTGGTGGGT ...) was used as a primer oligonucleotide to *Tetrahymena* extract in a similar reaction mixture. It was found that the repeats added by telomerase (enzyme) maintained the alignment of the addition of a new sequence of telomeric repeats to maintain the set of G4T2 depending on the 3′OH of the primer (Greider and Blackburn 1985).

This concludes that there could be a template within the telomerase enzyme complementary to the oligomer that maintains the G_4T_2 sequence when *Tetrahymena* cells are added to yeast telomeric oligomer repeats (Dunn et al. 1984) (Fig. 10.5). Later, a built-in template was found to be present in the telomerase complex, which was made up of RNA. This template is a short portion of RNA (human telomerase RNA) with other subunits like a protein called human telomerase reverse transcriptase (hTERT) that has reverse transcriptase activity. The telomerase enzyme complex consists of two significant subunits: a catalytic subunit, hTERT, which has reverse transcriptase activity, and a structural RNA component, hTERC or hTR.

This provides a template of 11 bp to encode telomeric repeats that are to be added to the chromosome. The expression of the human telomerase RNA gene (hTERC) occurs in both normal and cancerous cells, while hTERT expression is limited to a cancerous cell. Telomerase activity and hTERT mRNA expression are linked to human cancers. Experiments have shown that hTERC levels are unregulated in the early neoplastic stages, which further increases during the progression of tumors.

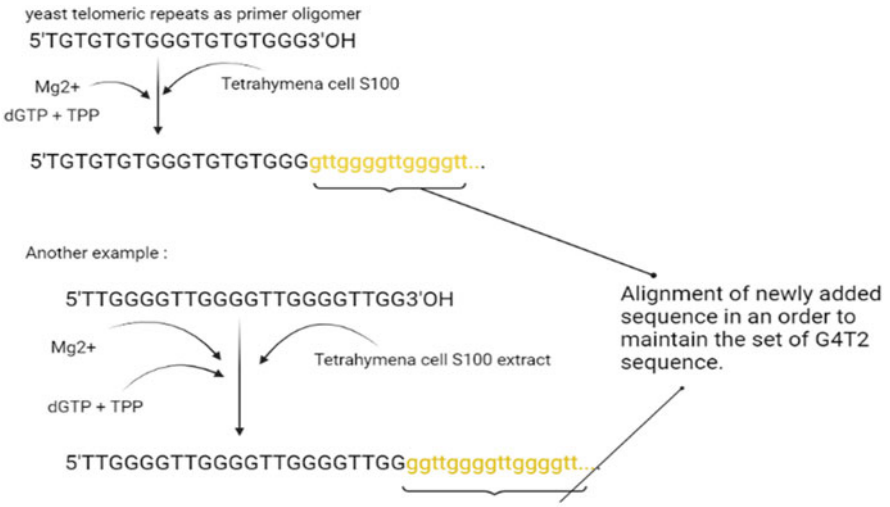


Fig. 10.5 The experimental system shows how the newly added sequences are aligned to maintain the G_4T_2 sequence due to the template RNA component in telomerase. (Source: Figure generated at BioRender, (https://biorender.com/)

Whereas regulation of telomerase activity is primarily by hTERT and is detected only in the late stage of tumors. This indicates that the regulation of telomerase activity is different from the expression of its RNA component. Initial upregulation of telomerase RNA is responsible for the hyper-proliferation of cancer cells. Therefore, telomerase activation confirms the unlimited proliferative capacity of the emerging and evolving cancer cells. However, telomerase activity is not correlated with tumor invasion. Telomerase activity is a decent marker for detecting gastric carcinoma (Blackburn 2001; de Lange 2005).

10.3 Mechanism of Action of Telomerase

Telomerase takes a single-strand rich in G sequence and aligns the 3′OH end of the ss-strand by Watson-Crick base pairing rule onto the RNA template sequence present in the telomerase enzyme. Once aligned, it polymerizes complementary nucleotides, extending the telomeric DNA. This proves that telomerase is a unique polymerase, and it copies RNA to DNA hence also a reverse transcriptase. It has an intrinsic RNA component that helps synthesize short repeats on the telomeric end of chromosomes (Fig. 10.6). *Tetrahymena,* when grown in the culture they keep on propagating. In other terms, they are effectively immortal. One of the reasons could be the presence of telomerase in high amounts. By manipulating telomerase in *Tetrahymena* (where it was initially discovered), it can lead cells towards senescence. Manipulation can also be done by changing a few nucleotides of its RNA

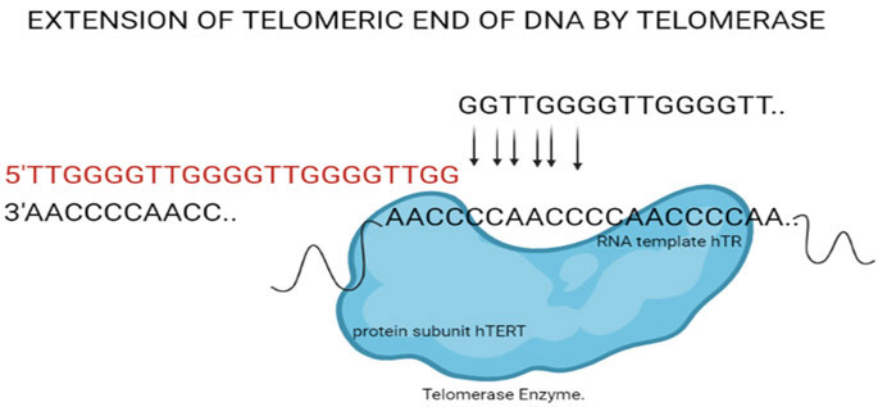


Fig. 10.6 Representation of how the RNA component of telomerase adds a new sequence to the telomeric DNA end in telomeres of *Tetrahymena* chromosomes. (Source: Figure generated at BioRender, (https://biorender.com/)

template subunit. When this manipulation is done in *Tetrahymena*, it becomes mortal. Concluding, telomerase maintains the end of chromosomes allowing cell division to progress and preventing cell death. Telomerase replenishes telomeres. Telomerase remains active during fetal development in the human cell. It also remains active during the proliferative phase of germ cells, stem cells, and lymphocytes, activated in response to a pathogen. Its activity in the cancer cell and tumors are extremely high, and hence, they maintain cell division and cell proliferation which causes tumors to grow. Epithelial cells, fibroblasts, endothelial cells, and somatic cells have low levels of it. Telomerase upregulation is a feature of the majority of cancers (Shampay et al. 1984).

Diagnosis of various cancers can be made by examining the level of telomerase activity in different stages of cancer, which will help us further study the differentiation and metastasis of these cells. High telomerase activity is expressed in patients with ductal carcinoma and small-cell cancer of the lungs. If the expression of hTERT mRNA is higher in any cell, its telomerase activity will be high. However, in some cases, protein and hTERT mRNA are highly expressed without telomerase activity, such as in ductal carcinoma cells.

Telomerase activity is high in cancer cells known for proliferating continuously and differentiating because of genetic and epigenetic changes. Replenishment of this enzyme keeps shortening of telomeres away, and hence, the cell divides and becomes immortal. Human cells can have either high telomerase or ALT (alternative lengthening of telomeres) mechanism (Hanahan and Weinberg 2000).

Alternative lengthening of telomeres (ALT) mechanism is also one of the causes of 10–15% of tumors. ALT uses the homologous DNA recombination method to maintain the length of telomeres. This mechanism is based on the loss of DAXX and ATRX, which help in chromatin remodeling. Due to the loss of these factors, there is a reduction in telomeric chromatin compaction. This further leads to alteration in

telomeric DNA sequence, which activates the DDR pathway specific to the telomere. This pathway stimulates the synthesis of DNA sequences at the end of the telomere. Flynn and his colleagues discovered that the ALT mechanism could be disrupted by protein kinase ATR inhibition in all cells that have the ALT mechanism. That will result in the death of the cell. Hence, ATR inhibitors can be considered therapy to all the tumors resulting from the ALT mechanism (Flynn et al. 2015).

Cells multiply, and their telomeres will subsequently become shorter in the absence of telomerase. To counteract the shortening of telomeres, eventually, cells will cease to divide. Cells respond to short telomeres, also called senescence response, in which cells will not replicate DNA anymore, which ultimately will lead to cell death. One can propose that apoptosis can be induced by malfunctioning telomerase. Without it, human cells can lose telomeric DNA. Human telomeres are made up of thousands of copies of telomeric repeats. So it would take a lot of cell cycle of cell division before the cell dies. If telomerase catalytic function is inhibited, it will not carry out DNA polymerase reaction by reverse transcriptase, causing the cell to die eventually. Hence, the proliferation of tumor cells can be stopped. When the catalytic function is inhibited, the ribonucleoprotein level of the enzyme "telomerase" is kept high because the purpose is not to deplete the cell of the enzyme but to render the enzyme inactive. By knocking down telomerase RNA, there was inhibition of cancer cells. Human telomerase can be knocked down using RNA-hairpin-SiRNA (Short interfering RNA). Two strands of RNA complementary to target RNA cause the breaking down of target RNA (Blackburn et al. 2006).

Telomerase knocked down in cancerous cells causes a reduction in metastasis by downregulating the cell cycle and tumor progression genes. It also downregulates glucose metabolism as cancer cells have high glucose metabolism. The replicative capacity of fibroblasts cells can be predicted by studying their telomeric length. Loss of telomeric DNA repeats induces signals that regulate cell division and apoptosis of the cells.

10.4 Telomerase: A Critical Hallmark of Cancer

Cancer is one of the most aggressive pathological disorders in humans all over the world, some of its types are curable, and some have lasted for a long time. Cancer could be considered an age-related disorder. Many reasons and factors provide a favorable environment for cancer cells to develop and spread. Moreover, this enzyme is one of those factors which are responsible for causing cancer in humans. Telomere is the non-coding repetitive sequence, present at the end tip of chromosomes, maintains stability, and protects human chromosomes (just like the plastic thing, present at the end tip of shoelaces). However, every successive cell division in the body system would shorten the telomere length and, this process limits the proliferative ability of cells to a certain number of cell divisions by inducing cellular senescence or apoptosis.

So, the mechanism of maintaining the length of chromosomes (telomere length) ever after the cell division is done by telomerase which adds the guanine-rich repeated nucleotide sequence at the end of the chromosome to regain its actual length after the successive cell division. Telomerase is predominantly seen in human cancer cells, about 85–90%, not in the normal cell of humans. The re-expression and reactivation of the telomerase enzyme are responsible for uncontrolled growth of the cell, survival of tumors, and tumorigenesis. Therefore, telomerase is also known as "immortal enzyme''(Bourgoin 2012).

10.5 Identification of Cancer Cells

Since cancer cells contain telomerase enzymes in very high concentrations, it is used as a key to identifying these abnormal cells. Based on the level of telomerase enzyme in the cells, the behavior of tumor cells (benign or metastatic) could also be determined easily. According to molecular data, very high telomerase activity is found in breast, gastrointestinal, and colorectal cancer patients. Only 15% of cancers do not express telomerase enzymes. Therefore, nowadays, telomerase is one of the recommended diagnostic biomarkers for different types of cancer treatment and diagnosis (Sarvesvaran 1999; Nakanishi et al. 2002; Oztas et al. 2016). Scientists have been investigating telomerase as a predominant tool for cancer diagnosis and treatment. It is a reliable marker for developing new cancer therapies; hence, deactivation, destabilization, and suppression are key to curing cancer. What are the factors that activate the telomerase enzyme so many times in abnormal cells? What genes or what specific nucleotide sequence is responsible for it being investigated, enhancing information for anti-cancer drug design? Strategies that inhibit telomerase's action or function in the cancer cell include many drugs, SMI (small molecular inhibitors), vaccines, etc. Telomerase inhibitors as anti-cancer agents could be one of the most reasonable and reliable strategies (Huang et al. 2013).

10.6 Telomerase Inhibitors

Eukaryotic telomerase contains reverse transcriptase components (hTERT), a catalytic protein subunit, and RNA components (hTR), essential for adding repetitive sequences at the ends. Researchers have telomerase inhibitors that contain RNA and RNA binding protein parts for better binding affinity (Kazemi-Lomedasht et al. 2013). Telomerase inhibitors are derived from natural sources as well as synthetically generated. Moreover, some inhibitors contain modified oligonucleotides. Telomerase binding agents like G4 ligands (quadruplex ligands) and ALT cells play a primary role in inhibitions. Stabilizations by G4 ligands and deactivation of telomerase or telomerase gene suppression are new opportunities to target cancer cells and tumors. Usage of synthetic inhibitors against telomerase shows some side effects and

complications in cancer patients. On the other hand, natural inhibitors against telomerase have very few or no side effects. Natural inhibitors, taken from the diet, are better suppressors and healers and are safe to consume than synthetic inhibitors (Chen et al. 2011; Badrzadeh et al. 2014; Zhang and Wang 2017).

10.6.1 AZT: Inhibitor of Reverse Transcriptase

2′-Azido-2′ 3′-dideoxythymidine (AZT), an analog of thymidine, has the ability to inhibit reverse transcriptase. It is an antiviral agent that is also used to treat HIV AIDS, as it inhibits the replication of HIV by blocking its reverse transcriptase. When it is phosphorylated by thymidine kinase, it forms AZT-TP (Falchetti et al. 2004), which can be incorporated at the place of thymidine in DNA. It has a low affinity towards DNA polymerase alpha, beta, gamma but a high affinity towards reverse transcriptase (Faraj et al. 2000). AZT integrates with CHO DNA cell line (most commonly used mammalian cell line, derived from Chinese hamster ovary) with the help of immune fluorescence tagged antibodies produced against AZT. After incorporation, followed by separating telomeric DNA from genomic DNA via methods like restriction digestion and size fractionation of DNA to quantitatively compare the incorporation of (H^3)-AZT in telomeric and non-telomeric regions of CHO cell lines (Olivero and Poirier 1993), it was found that (H^3)-AZT integration was three times more in the telomeric region than in the non-telomeric region (Gomez et al. 1995). Cells of dermal fibroblasts of mice have long telomeric repeats, but they do not have telomerase, and it was seen that they did not incorporate (H^3)-AZT in their telomeric region.

Incorporation of (H^3)-AZT in the telomeric region of CHO cell lines with telomerase, while it did not get incorporated in cells of dermal fibroblasts of a mouse that did not have telomerase, showed it regulates AZT incorporation. Later it was experimentally proven that AZT either inhibits telomerase or shortens its length. Strahl and Blackburn in 1996 found shortening of telomerase in B cell line JY616 and T cell line JukratE61 when passed through 100 micromolar AZT, which confirms that AZT inhibits telomerase (Strahl and Blackburn 1996). Yegorov et al., in 1996, confirmed induction of apoptosis in the culture of immortal fibroblast of a mouse with the help of AZT. This process was reversible as after removing inhibitors (AZT), the cell entered the cell cycle again. They concluded that AZT blocks telomerase function in mouse cells (Yegorov et al. 1996).

Later on, it was found that AZT inhibited the progression of all tumor cell lines. For example, Multani in 1998 conducted an experiment using fluorescence in situ hybridization, discovered a reduction in telomeric length in murine melanoma (K-1275 clone X-21) and human breast cancer cell line (MCF-7) when treated with AZT. Human endometrial carcinoma cells (HEC-1) showed similar results. The reduction in length of telomeres can be seen in HeLa cells after exposure to AZT for an extended period without senescence. Concluding, telomeric inhibition by AZT depends on the concentration of AZT and duration of exposure. Sometimes

AZT exposure might not lead to senescence or cell death because AZT-resistant phenotype could have been developed. The number of AZT treatment passages is insufficient for a cell to go to senescence, or a different mechanism called alternative lengthening of telomeres is compensating for the telomeric loss (Multani et al. 1998).

Telomerase subunit hTERT and c-Myc's activity reduced on treatment with AZT followed by alteration in hTR, Mad1, hTEP1. AZT also causes a reduction in checkpoint kinases (Chk1) and (Chk2) and an increase in phosphorylated Chk1 (Ser345) and Chk2 (Thr68). Telomerase inhibition will not affect humans until telomeres reach a critical size, causing the cell to die. This means during telomerase inhibition, tumor cells will continue to grow for some divisions until telomeres inside these cells reach a critical size leading to cell death (Gomez et al. 2012; Hájek et al. 2005).

10.6.2 Natural Telomerase Inhibitors (NTI)

Natural telomerase inhibitors (NTI) are derived from natural sources such as plant material and include secondary metabolites like alkaloids, polyphenols, triterpenes, xanthones, indol-3-carbinol, telomestatin, gingerol, etc. (Ganesan and Xu 2017). The alkaloid inhibitors include Boldine (natural aporphine alkaloids) and Berberine (isoquinoline quaternary alkaloid). Another category of natural telomerase inhibitors is polyphenol inhibitors such as curcumin, quercetin, and resveratrol (Chen et al. 2011). Table 10.1 summarizes naturally derived telomerase inhibitors with structures, properties, probable mode of action.

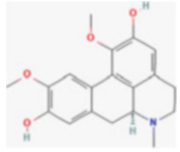
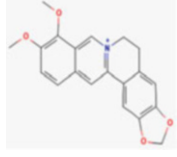
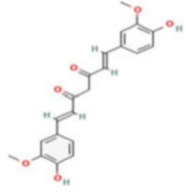
10.6.2.1 Inhibitors Targeting hTERT

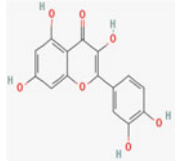
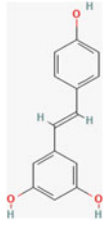
2-((E)-3-naphthalen-2-yl-but-2-enoylamino)-benzoic acid (BIBR1532) is a small synthetic non-nucleic and non-peptide compound, which links to the hTERT in its active site, competitively inhibiting telomerase. It is the most promising hTERT inhibitor among the inhibitors developed to date. BIBR1532 binds to the reverse transcriptase active site of hTERT non-covalently and inhibits telomerase, which reduces the number of added TTAGGG repeats. BIBR1532 does not cause chain termination. It can act as translocating enzyme-DNA-substrate complex or favoring the DNA substrate disjunction from the enzyme during the copy of the template (Pascolo et al. 2002).

10.6.2.2 hTERT Immunotherapy

The property of cancerous cells to overexpress their efficient, functional telomerase, and specific epitopes against hTERT helps in targeting the telomerase. Therefore, they can be eliminated by immune system stimulation with specific vaccines.

Table 10.1 Natural telomerase inhibitors, properties, structure, and mode of action

Inhibitors	Derived from	Properties	Mode of action	Structure	Reference
Boldine (an alkaloid)	Boldo plants (*Peumus boldus*)	Anti-inflammatory and antioxidant properties It was found to inhibit telomerase function using (q-TRAP quantitative real-time telomerase repeat amplification protocol). It also reduces cell viability and cell proliferation in T24 cells Majorly used to treat breast cancer (MCF-7) and MDA-MB-231 cells and embryogenic kidney (HEK293)	Plays a role in changing the splicing variant of hTERT Blocks synthesis of prostaglandin by inhibiting cyclooxygenase activity and reduces the effect of inflammatory cytokines and cell proliferation		Kazemi Noureini and Tanavar (2015)
Berberine (an isoquinoline quaternary alkaloid)	Root, stem, bark of European barberry (*Berberis vulgaris*), and turmeric (*Curcuma longa*)	They are used for the treatment of cancer, diabetes, heart failure, high blood pressure, *H. pyloric* infection It can also bind with the G-quadruplex structure of DNA	It inhibits telomerase activity by stabilizing G-quadruplex It also inhibits the AMPK pathway Responsible for weakening the strength of cancer cells		Wu et al. (1999)
Curcumin (a polyphenolic)	Turmeric plants (*Curcuma longa* L.)	It has various antitumor, anticarcinogenic, and antiinflammatory properties It is considered ineffective in brain tumor cells and binds to the cell surface to provoke apoptosis It plays a vital role in cell viability	It was found to inhibit telomerase activity in MCF-7 breast cancer cells Curcumin increases the production of ROS and inhibits special protein 1 binding affinity, followed by downregulation of hTERT		Chakraborty et al. (2007); Ramachandran et al. 2002; Lee and Chung 2010)

		It is highly recommended in adjunct cancer therapy			
Quercetin (a polyphenolic)	Flavonoid (secondary metabolites found in plants)	It is considered a primary chemoprevention agent in several cancers such as prostate, breast, lungs, colon, etc. It has anti-inflammatory, free radical oxidant, and antitumor activities	Inhibitor of PI3K and NF-K3 It also inhibits cell proliferation and cell transduction in cancer cells		Choi et al. (2001), Kuo et al. (2004; Ayşe et al. 2011)
Resveratrol (a phenolic phytoalexin)	Found in various plants and in the skin of fruit like peanuts, grapes, etc.	It has chemopreventive potential Its anticancer property shows direct inhibitory action as well as cell proliferation, apoptosis, and signal transduction Pterostilbene reduces the catalytic action of telomerase It is most effective in reducing cell viability	Telomerase inhibition activity and downregulation of hTERT protein expression in cancer cell lines Treatment with pterostilbene (as an analog of resveratrol) decreased telomerase activity and protein expression in lung cancer line H460 It also inhibits the cell proliferation of HT-29 and WiDr cell line and downregulation of telomerase activity in a dose-dependent manner Inhibits promoter activity of hTERT and prevents cell proliferation in colon cancer		Chen et al. (2017)

Targeting telomerase for immunotherapy stimulates immune cells against tumors expressing hTERT peptides as surface antigens. As a result, telomerase-specific $CD8^+$ cytotoxic T-lymphocyte amplification is regulated to target and exterminate telomerase-positive tumor cells. Numerous vaccine strategies have been developed and used in recent times, including hTERT-specific immune responses (Ellingsen et al. 2021). Some of them include GV1001, Vx-001, 1540. Tumor-associated antigens are self-proteins in humans, and their precise T-cells are tolerated. Self-tolerance is commonly regulated against "dominant" (high affinity for HLA) but not against "cryptic" (low affinity for HLA) peptides. Hence, the easiest way to evade this self-tolerance is to use cryptic peptides as in Vx-001. In cancer immunotherapy, overwhelming this tumor-specific self-tolerance is a primary goal (Jafri et al. 2016; Gellert et al. 2005; Greider and Blackburn 1985; Incles et al. 2003).

10.6.2.3 Antisense against hTR and hTERT

The catalytic activity of telomerase needs an intrinsic RNA template. The antisense regulated against the template region of hTR (5′-CUAACCCUAA) tends to be both selective and potent. In 1994, it was demonstrated that telomerase activity could be inhibited by treating cells with appropriate antisense molecules. However, the problem that arises is the stability and uptake of antisense when attempting treatment with oligonucleotides. Adding the 2′-*O*-(2-methoxyethyl) (2′-MEO) group to the antisense RNA molecule targets the 11 base pair template region inhibiting the telomerase. The antisense molecules against both hTR and hTERT form a duplex with the targeted mRNA and prevent translation of the target protein (Mender et al. 2015a, b).

10.6.3 Altering Telomerase Activity to Induce Telomeric Dysfunction, which Causes Cancer Cell Death

A long log period to observe telomerase-induced cell death by anti-telomerase-directed therapy is a significant challenge we are facing today. It requires a series of cell cycles and cell division to induce relevant effects in therapeutically reducing tumors. In this phase of treatment, most of the tumor cells will grow, further requiring other treatments for a clinical outcome. Natural telomerase inhibition therapy is given to patients with other toxicities, such as hematological toxicities, which require drug holidays. Stopping treatment for a few days will reverse the benefit of therapy. Therefore, it is desirable to use therapeutic agents that act fast, which can inhibit telomerase activity.

One of the methods of doing so is not directly targeting telomerase; instead can be done by introducing modified nucleoside into the cell, which might help incorporate telomerase into telomeric DNA. When this nucleotide is incorporated into the

telomere, it will not bind to the Shelterin protein effectively, leading to the rapid death of the cell.

Mender and colleagues proved that a telomerase-positive cell was introduced to a nucleoside analogous to 6′-thioguanine (6′-thio-2′deoxygaunosine). It got incorporated into telomeres, resulting in telomeric dysfunction as the telomeric-associated DNA damage signal was activated and led to cell death. This treatment to cell lines showed a significant reduction in tumor growth rate. Hence, it was concluded that telomere disruption mediated by telomerase could be a window to effective cancer treatment (Mender et al. 2015a, b; Corey 2002).

10.6.4 Antagonist Template to hTR (RNA Template of Telomerase)

It delivers oligonucleotides complementary to the template RNA component (hTR) of telomerase into a tumor cell. It has its advantages and disadvantages. One of the significant disadvantages is that inhibiting telomerase takes longer to show telomere's shortening effect in tumor cells. Oligonucleotides targeting the RNA component of telomerase serve as a classic enzyme to inhibit telomerase activity. In some experiments, the antagonistic oligonucleotides administered into tumor cells showed a reduction in telomerase activity and telomerase activity, which leads to telomere shortening and decreases in cell proliferation, and on the other hand, it increases apoptosis rate (Shay and Wright 2002).

It was observed that when this antagonist template was removed from the culture, the telomerase activity of cells was regained and entered the cell cycle again, growing their telomeres back. This observation revealed that the action mechanism is based on competitive inhibition of the enzyme telomerase (Corey 2002; Herbert et al. 1999; Lee et al. 2001).

10.6.5 Combination Therapies

A pitfall of the telomerase inhibitor is the lag time, and it typically requires shortening the telomere length and the cell to respond by arresting its growth, which we can overcome by the combination of inhibitors of telomerase with existing chemotherapeutics. When combined with telomerase inhibitors via antisense, DNA damaging drugs like doxorubicin and daunorubicin create a knockout hTR or inactivate the hTERT, respectively, increasing the sensitivity of cells towards DNA interacting drugs. Because telomerase has a role in DNA repair and replication, if components of telomerase are knocked out, it will render the cell's ability to protect it from DNA damage (Nakajima et al. 2003; Ishibashi and Lippard 1998).

10.6.6 Cisplatin in Cancer Therapy

Cisplatin, or cis-diamminedicholoroplatinum II, is a well-known platinum-based chemotherapeutic drug/agent. It has been introduced to treat human cancer such as testicular, ovarian, breast, brain, lungs, liver, neck, etc. M. Peyron first synthesized it in 1844. Also, its first FDA-approved platinum component for cancer treatment was in 1978. For the first time, Burger et al. showed that cisplatin minimized telomerase activity in tumor cells like testicular tumor cells (Burger et al. 1997; Greider 1998). Molecular results showed that cisplatin also affects the gene transcription and reduction in the expression of hTR in testicular tumors. Cisplatin is a crucial ingredient in the systematic treatment of germ cell cancer. Ishibashi and Lippard (1998) first discovered the loss of telomere in Hela cells after treating it with cisplatin.

10.6.7 Mode of Action

Cisplatin is a chemotherapeutic drug that can direct interactions with DNA and shows crosslinking with purine bases in the ds DNA. It inhibits DNA synthesis, has cytotoxic activities, causes DNA damage, and promotes apoptosis in abnormal cells. Cisplatin can also reduce telomerase activity and the length of telomere in the tumor cell. It could also induce apoptosis and arrest the cell cycle at specific stages, and it could induce oxidative stress, which leads to the production of reactive oxygen species (ROS). It could also induce p53 tumor suppressor signal transduction and downregulation of proto-oncogenes. High doses of cisplatin have various taxological effects such as hepatotoxicity, nephrotoxicity, cardiotoxicity, and other organ toxicity. Cisplatin is an antitumor chemotherapeutic agent/drug used in combination with other drugs such as paclitaxel, doxorubicin, UFT, gemcitabine, osthol, etc. According to several studies at the molecular level, combining cisplatin with antitumor other medicines is the best therapeutic approach to reduce cisplatin's toxicity or toxic effect and reduce the inflammatory effects in cancer patients (Nakamura et al. 1997).

10.6.8 Cisplatin and Primary Hepatocellular Carcinoma (PHCC)

A new strategy has been introduced to treat the solid tumor, primary hepatocellular carcinoma (PHCC). PHCC is one of the most aggressive and primary types of liver cancer, which causes several deaths per year worldwide.

Cisplatin affects the telomerase activity in hepatic cells because PHCC has a high concentration of telomerase enzymes, leading to unlimited liver cell proliferation.

Telomerase activity in liver cells is clinically analyzed by PCR based on TRAP (telomeric repeat amplification protocol), and the rate of apoptosis and cell cycle is analyzed by flow cytometry (Zhang et al. 2002; Andreassen and Margolis 1994).

Inhibition activity of telomerase is based on the dose and time of cisplatin. BEL-7404 human hepatoma cells inhibited by 12 different concentrations of cisplatin (concentration range: 0.8–50 μm) in 72 h. During the treatment with cisplatin, it causes no change in the gene transcription as well as expression of hTR, hTERT, or TP1 MRNA in BEL-7404 human hepatoma cells. Cisplatin's treatment also arrests the cell cycle at the G1/M phase in BEL-7404 human hepatoma cells (Zhang et al. 2000).

10.6.9 Cisplatin and Lung Cancer

Lung cancer is also an excellent example in which cisplatin is a key antitumor chemotherapeutic drug used. Currently, platinum-based treatment plays a significant role in small cell lung cancer (SCLCs). In non-small-cell lung cancer (NSCLCs), cisplatin chemotherapy is used in the second and third stages at the primary or first stage of NSCLCs followed by routine surgery only (Dasari and Bernard Tchounwou 2014).

10.7 Conclusion

Cancer remains the primary cause of death regardless of notable advancement in the understanding of its molecular mechanism and the development of various treatments options. It has been years since the discovery of telomerase and its association with human tumor cells. A large number of inhibitors and drugs have been discovered or designed to date. Telomerase-based drugs can affect in a novel way, which layout new options for cancer therapy. On the other hand, telomerase inhibitors directly limit or stop the growth of human tumors and may act in a symbiotic manner with subsist therapeutic methods and amplify their effectiveness. Telomerase is the diagnostic and therapeutic biomarker as they are present in almost all cancer cells and are absent in most somatic cells. To explore the functions of telomerase in tumors and foreshadow the possibilities, it is being considered a future therapeutic target. Therefore, the discovery and design of new validated drugs towards telomerase is the priority.

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Chapter 11
Metal Nanomaterials as Enzyme Inhibitors and Their Applications in Agriculture and Pharmaceutics

Satish V. Patil, Kiran R. Marathe, Hemant P. Borase, and Bhavana V. Mohite

Abstract As enzymes play a vital role in all biological systems, their regulation is also an important mechanism to drive various systems. In modern agriculture practices, the use of enzyme inhibitors to control pests and to regulate soil microbial activity is becoming an essential practice. The synthetic and natural organic chemicals are already documented for their use in pest control and fertilizers as a protectant. But these chemicals have reported some drawbacks like sensitivity to physical factors like temperature, pH, development of resistance and phytotoxicity, etc. The current review focuses on the potential and the use of metal nanomaterials as enzyme inhibitors in important agriculture practices. Various nanomaterials like lead, copper, gold, etc. were reported for their enzyme inhibition potential such as proteases, ureases, nitrate reductase, acetylcholine esterases, etc. Their potential with some conjugates as important agrochemicals such as pest control agents, fertilizers additives is briefly described in this review which will induce researchers for designing future agro formulations.

Keywords Enzyme inhibitors · Pests · Nanomaterials · Fertilizer additives · Agriculture practices

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11.1 Introduction

Enzymes are the biocatalyst of the living system. After nucleic acid, enzymes are considered to be the most vital biomolecules. Around 5000 biochemical reactions are carried out by enzymes (Ahmed et al. 2016). The biological functions of enzymes are diverse ranging from metabolism, nerve impulse transmission, control of biological activities such as replication, translation, organ specialization, blood coagulation, breathing, reproduction, generation of resistance, and diseases to name a few (Katsimpouras and Stephanopoulos 2021; Gomez-Fabra Gala and Vögtle 2021).

Owing to the essential role of enzymes not only in humans but also in microbes and insects, a class of molecules known as "enzyme inhibitors" are researched as a promising drug candidate in diabetes, Alzheimer's, and cancer and as a biocidal agent to kill plant pathogens and pests (Chen et al. 2017). Enzyme inhibitors act on enzymes because of the association of enzymes with life-supporting processes and diseases such as metabolic, neurological, cardiovascular, etc. Inhibition of enzymes can be lethal to pathogens, and therefore, enzyme inhibitors can be used as pesticides (Copland 2005). Enzyme inhibitor binds to an enzyme's active site and partially or completely inhibits its activity. However, they need not cover the complete binding site.

A variety of molecules such as paracetamol, rivastigmine, mupirocin, fosinopril, ritonavir, atorvastatin inhibit target enzymes such as acetylcholinesterase, cyclooxygenase, HIV protease, HMG-CoA reductase and, therefore, are used in the treatment of bacterial and viral infections, pain, cancer, hypertension, hypercholesteremia, etc. (Copeland et al. 2007; Ouertani et al. 2019). However, most of the currently available enzyme inhibitors are organic and have several drawbacks such as instability, degradation, and low catalytic activity (Cha et al. 2015; Ouertani et al. 2019). Moreover, organic enzyme inhibitors work on relatively few major types of inhibitory mechanisms such as competitive, irreversible, or allosteric inhibition with several limitations (Ouertani et al. 2019). There is a need to find enzyme inhibitors having a broad inhibition spectrum.

Global pharma-biotech-agro industries such as Ranbaxy Laboratories, AstraZeneca, Merck, Pfizer, Roche are investing a big amount of money in enzyme inhibitor research (Cision 2017). The global market for enzyme inhibitors was estimated to be more than US$ 95 billion in 2006 and is predicted to grow at an average rate of around 8% per annum (BCC Research 2018). Hence, there is a plenty of space to find versatile and robust enzyme inhibitors with unconventional chemical structures, desirable features, degradation resistance, and diverse inhibitory effects.

11.2 Potential of a Metal Nanomaterial as an Enzyme Inhibitor

According to the national nanotechnology initiative (United States), nanotechnology is science, engineering, and technology conducted at the nanoscale (1–100 nm) and utilizing products obtained from this unique phenomenon in diverse fields from chemistry and physics to medicine and engineering (NNI 2020). Pieces of evidence of the use of nanomaterials by humans trace back to the fourth century AD in the time of Greek and Democritus and can be found in historical objects like the Lycurgus cup and medieval church windows (Bayda et al. 2020). Nanotechnology is holding great economical potential as evidenced by its tremendous industrial applications and incorporation in day-to-day consumer goods (Janković and Plata 2019). Nanoforms of silver, gold, copper, zinc, and other metals are successfully applied in the pharmaceutical and agriculture industry (Khan et al. 2019; Singh et al. 2021).

There are two approaches for the synthesis of metal nanoparticles, viz. top-down and bottom-up. Extensive research was done on chemical, physical, and biological methods of nanoparticles synthesis, but the growing demand for nanomaterials is fulfilled by chemical and physical methods despite growing toxicity issues. On the other hand, biological methods utilizing microbes and plants present a promising approach but are still not feasible for large-scale nano synthesis (Patil et al. 2016; Borase et al. 2014, 2021; Ahmed Mohamed et al. 2020).

Pharmaceutics and agriculture are expected to gain tremendous benefits from nanotechnology interventions (Singh et al. 2021; Contera et al. 2020). Current agricultural practices in most of the world rely on high doses of agrochemicals such as fertilizers, pesticides that adversely affect soil rhizospheric microbiome, causing water pollution and biomagnification, affecting food quality and supply (Singh et al. 2021).

Plant growth is largely affected by various biotic and abiotic factors such as disease-causing pathogens, genetic traits, moisture availability, and soil fertility (Lahiani et al. 2013). Nanoscience is an innovative platform that involves the development of approaches to a range of inexpensive nanotech applications for enhanced seed germination, plant growth, development, and acclimation to environments. In this regard, an extensive number of studies have shown that the application of nanomaterials has positive effects on germination as well as plant growth and development. Likely, the application of multiwalled carbon nanotubes (MWCNTs) positively influences seed germination of different crop species including tomato, corn, soybean, barley, wheat, maize, peanut, and garlic (Khodakovskaya and Biris 2010; Srivastava and Rao 2014). Nanoparticles as enzyme inhibitors are promising due to several properties such as the high surface area to mass ratio, nano size, different shape, chemical functionalization, resistance to degradation in environmental conditions, etc. (Maccormack et al. 2012; Chen et al. 2017). Nanoparticles are expected to act as broad-spectrum enzyme inhibitors due to the above characteristics (Benelli 2018).

11.3 Nanoparticles Inhibiting Vital Enzymes (Some Examples)

Most of the commercially available antibiotics and agrochemicals act on pathogens and insects by targeting important enzymes. However, due to the widespread issue of resistance emergence, nanoparticles offer a promising alternative to the traditional arsenal of enzyme inhibitors (Ahmed et al. 2016; Ali et al. 2018). Nanoparticles have useful properties including shape, size tenability, binding of multiple ligands on the surface, and diverse enzyme inhibitory strategies.

Penicillin inhibits enzyme transpeptidase, which is essential for bacterial cell wall synthesis. Thus, the β-lactam-mediated inhibition of transpeptidation leads to cell lysis. However, after the introduction of penicillin, within a few years, various bacterial strains started showing drug resistance by naturally producing an enzyme, penicillinase (Drawz et al. 2014).

Two-dimensional molybdenum disulfide (2D-MoS_2) nanomaterials were reported to inhibit the β-lactamase enzyme. The negatively charged ligand functionalized MoS_2 materials exhibit electrostatic and other non-covalent interactions between enzyme and inhibitor leading to competitive inhibition of the latter (Ali et al. 2018).

Trypsin (223 amino acid residue protein) is another important target enzyme involved in human diseases and also helps insects to neutralize biocidal agrochemicals (Schnebli and Braun 1986). It was observed that absorption of trypsin on TiO_2 NPs decreased the β sheet content. TiO_2 NP–trypsin interaction altered the secondary structure due to electrostatic force, van der Waals force, and hydrogen bonding (Wang et al. 2011). In another study, gold nanoparticles were also found to inhibit trypsin. Electrostatic and hydrophobic interactions, along with covalent interactions between Cys-58, 42, Lys-60, and gold nanoparticles, might be involved in the binding and inhibition process (Zhang et al. 2014).

Urease (nickel-containing metalloenzymes) is an important factor in peptic ulcers, and commercially available urease inhibitors have multiple side effects such as antibiotic resistance (Naz et al. 2019). Silver nanoparticles functionalized with N-substituted methyl 5-acetamido-β-resorcylate (AgL) with an average size of 20 nm were found to be stable at variable temperatures and pH. AgL showed enhanced urease inhibition as compared to the standard drug (thiourea), N-substituted methyl 5-acetamido-β-resorcylate, and silver nanoparticles. Interestingly, AgL is inactive against other metalloenzymes like xanthine oxidase and carbonic anhydrase II as well as for non-metallic enzymes such as α-chymotrypsin and acetylcholinesterase. Therefore, AgL is very selective to inhibit urease only (Benelli 2018).

Silver nanoparticles fabricated using *Cassia fistula* fruit pulp extract inhibits the fourth instar larvae of *Aedes albopictus* and *Culex pipienspallens* (Coquilett) with a substantial decrease of acetylcholine esterase and α- and ß-carboxylesterase activities. Similar to the above study, silver nanoparticles prepared using salicylic acid and

3,5-dinitrosalicylic acid inhibit *Ae. albopictus*, with the decrease of total proteins, esterase, acetylcholine esterase, and phosphatase enzymes.

Multivalent nanoconstructs are another new advanced technology involving the conjugation of multiple copies of enzyme inhibitors on nanosurface to improve inhibitory potency and selectivity. Nanometer-sized fluorescent hybrid silica (NFHS) particles (size 150 nm) loaded with fluorescent and sulfonamide carbonic anhydrase inhibit carbonic anhydrase activity several-fold because of higher silica nanoparticles adsorption on the border of carbonic anhydrase (Touisni et al. 2015).

Detoxification enzymes (GST, Catalase, SOD) of insects act as the first line of defense against chemical-induced stress and hence are an attractive target for designing insecticidal and pesticide formulations. Several other enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) serve an important role in protein and carbohydrate metabolism (Borase et al. 2021). Insects characteristically lack important detoxification enzymes glutathione peroxidase and catalase known to have little affinity with hydrogen peroxide (H_2O_2). Hence, ascorbate peroxidase (APOX) plays a major role in the clearance of (H_2O_2). Beetles species *Blapspolychresta* were treated with 26.27 ± 4.43 nm Nickel (II) oxide nanoparticles (NiO NPs) at sub-lethal concentrations of 0.02 mg/g. NiO NPs were found to cause a significant decrease in the activity of APOX as compared to the untreated group (El-Ashram et al. 2021).

Borase et al. (2019, 2020, 2021) evaluated the effects of the most widely used metal nanoparticles (silver nanoparticles (20 and 40 nm), gold nanoparticles (30 nm), and zinc oxide particles (250 and 500 nm)) on important enzymes of aquatic organism *Moina macrocopa.* Interestingly all nanoparticles under investigation inhibit key enzymes including acetylcholinesterase and digestive enzymes (trypsin, amylase) and β-galactosidase. It was found that the size of nanoparticles is a crucial factor during their interaction with enzymes along with the resistance/sensitivity of the organism. Although *M. macrocopa* is an aquatic organism but finding from the above studies can be a reference for developing enzyme inhibitors for pharma and agriculture sectors.

11.4 Future of Nanoparticles as an Enzyme Inhibitor

Although several studies put forward the superiority of metal nanoparticles as a new class of enzyme inhibitor (BCC Research 2018), more research is needed to study the complete life cycle of nanomaterials including nonspecific interactions with other proteins normally present in biological fluid. Toxicity and accumulation in non-target host and environmental consequences are also unanswered questions.

11.4.1 Nanoparticles as Protease Inhibitors in Pest Management

Nanoparticles have various applications in different fields of agriculture like pest management, vector-pest management, herbicide delivery, and nanosensors for pest detection (Scrinis and Lyons 2007; Rahman et al. 2009). The role of nanoparticles as a protease inhibitor, their mechanism of action in insects, and their possible applications in agriculture are summarized below.

Proteases or peptidases are hydrolytic enzymes that selectively catalyze the cleavage of peptide bonds in proteins. Peptidases participate in various cellular physiological processes and irreversible proteolytic reactions whose control is essential for cell functions. The proteolytic activity of proteases is controlled by regulating secretions, specific degradation, and also by inhibition. Several natural specific and selective protease inhibitors are now known as major regulating proteins to control proteolytic activity in all life forms (Umezawa 1982). These characteristics make protease inhibitors good diagnostic and therapeutic tools for the treatment of various microbial (hepatitis, herpes, AIDS, aspergillosis) and mortal (arthritis, muscular dystrophy, malaria, cancer, obesity), neurodegenerative, and cardiovascular diseases (Karthik et al. 2014). Until now, several protease inhibitors are identified from plants, animals, and microbes for each mechanistic class of proteases, e.g., serine, cysteine, aspartyl, and metalloproteases (Lorito et al. 1994; Joshi et al. 1999; Bijina et al. 2011). Although the role of protease inhibitors have been identified, they are not widely used in agriculture due to ability of insects to produce insensitive proteases or degradation of inhibitors to neutralize their effects. Hence, there is the necessity to look for alternative solutions to overcome protease inhibitor resistance developed by insects. With the emergence of widespread antibiotic, enzyme, and insecticides resistance, a better alternative is the use of different metal nanoparticles. Nanoparticles possess several advantages over conventional protease inhibitors in respect of their stability, reproducibility, and reactivity with other chemicals (Friedman et al. 1993). The different biologically synthesized metal nanoparticles, their size, applications, and enzyme inhibition pattern are summarized in Table 11.1.

The biosynthesized chromium nanoparticles (Cr_2O_3 NPs) from *Hyphaene thebaica* showed antiviral activity against the poliovirus by inhibiting the protein kinase enzymes (Khalil et al. 2018). Similarly, Iqbal et al. (2020) reported the Cr_2O_3 NPs from *Rhamnus virgata* leaves extract showed the inhibition of protein kinase and α-amylase enzymes with biopotential against the fungi and bacterial species. The silver, aluminum oxide, zinc oxide, and titanium dioxide NPs have been successfully used as insecticides to manage different pests, most of these NPs showed detrimental effects on treated insects by inhibiting the gut proteases enzymes (Table 11.1). Of these, silver nanoparticles are the most studied and utilized nanoparticles for biosystem due to their strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities (Becker et al. 2005). Feng et al. (2000) reported that silver ions interact strongly with the thiol groups of vital enzymes, causing their inactivation. Patil et al. (2016) observed the

Table 11.1 Some examples of metal nanoparticles as an enzyme inhibitor

Sr. No.	Metal nanoparticles	Size (nm)	Shape	Target enzyme	Major finding	Reference
1	Gold nanoparticles (AuNPs)	20–50	Triangular, hexagonal	Protease	Serum protease of *A. aegypt*, *H. armigera, C. maculatus* inhibited due to multiple bonding between enzyme and AuNPs	Patil et al. (2016)
2	Zinc oxide nanoparticles (ZnO NPs)	20	Pyramids, plates, and spheres	β-Galactosidase	Shape-dependent denaturation, electrostatic attraction	Cha et al. (2015)
3	Silica nanoparticles	4, 20 and 100	Spherical	Chicken egg lysozyme	Loss in alpha-helix content. Strong adsorption for large NPs	Vertegel et al. (2004)
4	Copper oxide nanoparticles	<50	Spherical	Nitrate reductase and nitric oxide reductase	Inhibition of electron transport chain	Zhao et al. (2020)
5	Silver nanoparticles (AgNPs)	10	–	Urease, acid phosphatase, arylsulfatase, β-glucosidase	AgNPs negatively affect soil exoenzyme activities, with the urease activity especially sensitive to AgNPs due to non-competitive inhibitor by blocking sulfhydryl groups	Shin et al. (2012)
6	Silver nanoparticles (AgNPs)	20	Spherical	Catalase (CAT) and superoxide dismutase	Disturbing α-helicalsecondary structure of enzymes	Liu et al. (2020)
7	Silver-moxifloxacin nanoparticles	50–60	Spherical	Urease	250 times better compared to moxifloxacin	Nisar et al. (2015)
8	Two-dimensional molybdenumdisulfide (2D-MoS2) nanomaterials	–		β-Lactamase	Electrostatic, non-covalent interactions and steric obstruction. Useful in β-lactamase resistance	Ali et al. (2018)

(continued)

Table 11.1 (continued)

Sr. No.	Metal nanoparticles	Size (nm)	Shape	Target enzyme	Major finding	Reference
9	Lead oxide (PbO) nanoparticles	27		Acetylcholinesterase	Potential use in the treatment of Alzheimer's	Khalil et al. (2018)
10	Gold nanoparticles (AuNPs)	5–70	Triangular	Cytochrome P450	AuNPs likely block the substrate pocket on the CYP surface	Ye et al. (2014)

protease-inhibiting properties of latex fabricated AuNPs, particularly with regard to trypsin when mixed with insect serum. Protease activity was found to be inhibited by 66% in the serum of *A. aegypti* mosquito larvae and pests including *Helicoverpa armigera*, *C. maculatus, C. chinensis,* and *M. hirsutus*. Kantrao et al. (2017) also reported the inhibition of gut protease activity of *H. armigera* due to biosynthesized AgNPs. Raj et al. (2017) reported that increased oral dosages of AgNPs in the larval stage of *Drosophila melanogaster* alter the protein, carbohydrate, and lipid levels by impairing the metabolic activity, which may be due to the inhibition of gut proteases. Similarly, the silver nanoparticles lower the activity of copper-dependent superoxide dismutase and tyrosinase enzymes in *Drosophila melanogaster* which is involved in the antioxidant activity and pigment production, respectively (Posgai et al. 2011; Armstrong et al. 2013; Ávalos et al. 2015). The biosynthesized AgNPs using the fruits of *Cassia fistula* showed a significant decrease in the activity of AChE, a-and b-carboxylesterases, phosphatase enzymes, and total proteins in *Aedes albopictus* and *Culex pipienspallens* larvae and pupa (Fouad et al. 2018). Yasur and Rani (2015) also reported the insecticidal activity of AgNPs against the *Spodoptera litura* F. and *Achaea janata* L. due to the accumulation of nanoparticles in the gut of larvae which decreases larval and pupal weight. Also, the conjugated silica nanoparticles with plant protease inhibitors showed excellent larvicidal activity against the *Helicoverpa armigera* by inhibiting gut proteinase activity (Bapat et al. 2020; Khandelwal et al. 2015). Marathe et al. (2021) also investigated the mode of action of AgNPs and suggested that antifungal activity could be due to inhibition of ergosterol biosynthesis leading to severe damage to the cell membrane. A summary of the effects of different metal nanoparticles on various pests is presented in Table 11.2.

11.4.2 Nano Metals as Urease Inhibitors

Urease is the nickel metalloenzyme produced by various microbes such as bacteria and fungi, which are the major component of soil. They are the key component of urea loss by gaseous ammonia and other minerals of nitrogen. Although very efficient urea inhibitors are available, they have some negative impacts on crop health, i.e., phytotoxicity and get rapidly hydrolyzed by the other soil microbes. They also lose their urease inhibiting potential by various environmental factors. Leaf scorches and necrosis of leaf margin are common examples of phytotoxicity of these synthetic inhibitors. It was also proved that N-(n-butyl) thiophosphoric triamide (NBPT), taken by peas and spinach roots and translocated to leaves, also inhibits endogenous urease of roots and leaves plants or crops (Artola et al. 2011). The glutamate synthetase and amino acid levels were found to be reduced by NBPT (Cruchaga et al. 2011).

These drawbacks led the scientific community to find other nonorganic urease inhibitors. Various metals have already been identified to possess potent urease inhibition potential. Some already identified metals (silver and mercury) forming insoluble sulfides act as potent urease inhibitors by reacting with sulfhydryl groups.

Table 11.2 A summary of effect of metal nanoparticles against different pest

Sr. No.	Nanoparticles	Size (nm)	Application	Mechanism of action	References
1	Cr_2O_3-NPs	25–38	Antiviral, antioxidant	Inhibits the protein kinases in polio virus	Khalil et al. (2018)
2	Cr_2O_3-NPs	28	Antibacterial, antioxidant, antifungal	Inhibition of protein kinase and alpha amylases	Iqbal et al. (2020)
3	AgNPs synthesized with Ficus extracts	20	Insecticidal activity against *H. armigera*	Inhibits larval gut proteases of *H. armigera*	Kantrao et al. (2017)
4	AgNPs	20–100	Insecticidal activity against *Drosophila melanogaster*	Altered the protein, carbohydrate, lipid metabolism	Raj et al. (2017)
5	AgNPs uncoated and polysaccharide-coated	10 and 60	Insecticidal activity against *Drosophila melanogaster*	Depigmentation by inhibiting the tyrosinase enzymes	Posgai et al. (2011)
6	AgNPs	10–50	Insecticidal activity against *Drosophila melanogaster*	Depigmentation, impaired movement, compromised fertility	Armstrong et al. (2013)
7	AgNPs	4.7	Insecticidal activity against *Drosophila melanogaster*	Depigmentation, impaired movement, compromised fertility	Ávalos et al. (2015)
8	AgNPs PVP coated	–	Insecticidal activity against *Spodoptera litura* F. and *Achaea janata* L.	Larval and pupal weight decreased due to the accumulation of nanoparticles in gut	Yasur and Rani (2015)
9	AgNPs synthesized with *Cassia fistula* fruit	148–900	*Aedes albopictus* and *Culex pipienspallens* larvae and pupa	Inhibition of acetylcholinesterase and a- and b-carboxyl esterases activity	Fouad et al. (2018)
10	AgNPs synthesized with *Streptomyces* spp.	20–40	Antifungal activity against *fusarium verticilloides*	Inhibition of ergosterol biosynthesis and membrane damage	Marathe et al. (2021)
11	AuNPs synthesized with plant latex *J. gossypifolia*	20	Insecticidal activity against *H. armigera and A. aegypti*	Inhibition of larval gut protease	Patil et al. (2016)
11	SiNPs conjugated with soyabean trypsin inhibitor (SiNPs-STI)	20–100	Insecticidal activity against *H. armigera*	Inhibition of gut proteinase activity (HGP)	Bapat et al. 2020)

(continued)

Table 11.2 (continued)

Sr. No.	Nanoparticles	Size (nm)	Application	Mechanism of action	References
12	Silica nanoparticles conjugated with plant protease inhibitors	240	Insecticidal activity against *H. armigera*	Inhibition of gut proteinase activity	Khandelwal et al. (2015)
13	Copper nanoparticles (CuNPs)	10–70	Insecticidal activity against *Tribolium castaneu*	Body deformation throughout the life cycle of insect	El-Saadony et al. (2020)
14	Copper oxide nanoparticles (CuO NPs)	10–70	Insecticidal activity against *Spodoptera littoralis*	Reduced pupation and adult emergence, adult malformation, adult fecundity, and egg hatchability	Shaker et al. (2016)
15	Zinc nanoparticles (ZnO NPs)	25–50	Insecticidal activity against *Spodoptera frugiperda*	Effect on fecundity, fertility and longevity of insect	Pittarate et al. (2021)
16	ZnO nanoparticles (ZnO NPs)	50–60	Insecticidal activity against *Aedes aegypti*	Programmed cell death (apoptosis)	Banumathi et al. (2017) Gunathilaka et al. (2021)
17	Silica nanoparticles (SiO_2 NPs)	50–150	Insecticidal activity against *Bombus terrestris*	Midgut epithelial injury	Mommaerts et al. (2012)
18	Graphene oxide nanoparticles	1–10	Insecticidal activity against *Acheta domesticus*	Increased enzymatic activity of catalase and glutathione peroxidases, as well as heat shock protein (HSP 70) and total antioxidant capacity level	Dziewięcka et al. (2016)
19	TiO_2 nanoparticles	<100	Insecticidal activity against *Bombyx mori*	Upregulation of signaling pathway and downregulation of development and molting period	Li et al. (2014)
20	Alumina nanoparticle	30–60	Insecticidal activity against *Sitophilus oryzae* (L.)	Binding to beetle cuticle resulting in insect dehydration	Stadler et al. (2017)

The general potential of metal ions as urease inhibitors is in the order of $Ag^{2+} > Hg^{2+}$ $Cu^{2+} > Ni^{2+} > CO^{2+} > Fe^{2+} > Mn^{2+}$ (Upadhyay 2012). These data may lead to the use of the above metals/non-metals as urease inhibitors. Although no commercial use of nanomaterial as urease inhibitor for soil application is available as yet, but

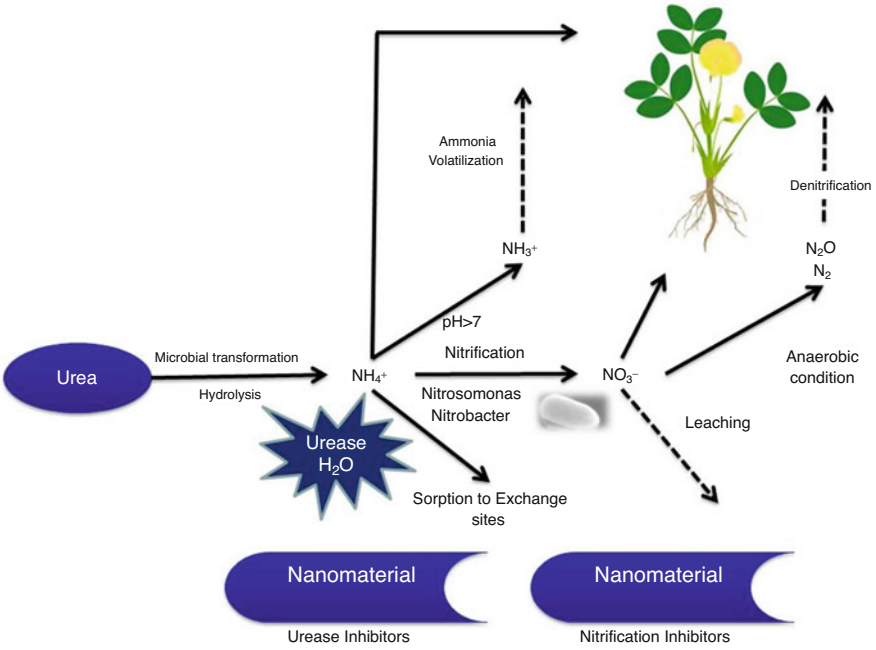

Fig. 11.1 Possible mechanism of urease regulations by metal nanomaterials

various reports of these metal nanomaterials as urease inhibitors make them potential candidates as the future component of nitrogenous fertilizers for controlled release of nitrogen, i.e., ammonia as per the requirement of the crop by controlling urease activity (Fig. 11.1).

Some reports on the use of nanometals as urease inhibitors also indicate their future potential. It was documented that PVP(polyvinyl alcohol)-based silver nanoparticles (AgNPs) having 15 nm size inhibiting the ammonia-oxidizing capacity of soil bacteria after direct application in soil (Huang et al. 2018). Similarly, Vandevoort and Arai (2018) and Simonin et al. (2018) found that copper nanometals, less than 50 nm in size, significantly decreased conversions of gaseous ammonia in nitrogen cycling.

Size-dependent reduction in ammonia oxidation potential of N*itrosomonas eutropea* by zero-valent silver polyvinyl alcohol (PVA) composite was also reported (Yan et al. 2020). Yuan et al. (2013) revealed that Na_2 ATP-doped silver nanoparticle composite retains 58.2% AgNPs after 24 h while PVA-coated AgNPs remain or retain 9.9%; hence, the latter is more efficient in inhibiting NH_3 oxidation. It was also found that this reduction may be due to cell wall damage and disintegration of nuclei. Silver nanomaterial with 20 nm size functionalized with N-substituted methyl acetoamide B-resocyclate was found to be more stable at the wide range of pH, temperature, and salt concentration with significantly increased urease inhibition. It was also proved that Ag-5-Amino-ß-resorcylic acid hydrochloride dihydrate

(AR) had significantly greater urease inhibition than the thiourea. The observed inhibitory activity of the latter was recorded at 11–18 times lower concentration than the former. Similarly, silver nanoconjugates of 5-amino-beta-resorcylic acid hydrochloride dihydrate were found to possess significant in vitro inhibitory potential against important enzymes like urease, xanthin oxidases, cholinesterase, and chymotrypsin which are targeted in various nitrogen fertilizer protection and agricultural pest management (Naz et al. 2014).

Zheng et al. (2017) reported that AgNPs inhibit the nitrification rate which was found to be inversely related to the concentration of AgNPs. Borase et al. (2015) reported significant urease inhibition by plant latex-mediated AgNPs of 21 nm size. Similarly, Jadon et al. (2018) advocated urea coated with 4% neem, 4% pine oleoresins, 35% rock phosphate, and 2% nano zinc particles (ZnO) for reducing the ammonia volatilization by 27%, 41%, 26% and 35% respectively.

11.5 Conclusion

The present literature points to the enzyme inhibitory and entomologic properties of metal nanoparticles. Especially, the silver nanoparticles showed the maximum inhibitory activity against the gut proteases, acetylcholine esterase, and phosphatases. This review suggests the possible use of metal nanoparticles as a biocontrol agent in agriculture, which can withstand alkaline conditions of an insect's gut. Moreover, with the commencement of insecticides and antibiotic resistance, nanoparticle-based enzyme inhibitory strategies could open up a new tool in therapeutics and agriculture as nano-insecticides in pest management and revolutionize insect and phytopathogen control which can benefit humans in the long run.

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Chapter 12
α-Glucosidase Inhibitors for Diabetes/Blood Sugar Regulation

Aditi Bhatnagar and Abha Mishra

Abstract According to a recent analysis, nearly 450 million people get affected by type 2 diabetes, which corresponds to 6.28% of our population. Type 2 diabetes has become one of the ten leading causes of death worldwide. This disease reports over 10 lakh deaths per year, with a current widespread reporting rate of 6000 cases per 100,000; the rate is reckoned to increase to 7100 patients per 100,000 by the end of 2030 (Yang et al. 2018). Diabetes mellitus requires immediate attention because of its increasing burden globally, especially in developed nations, such as America and Europe. Alpha-glucosidase inhibitors are a class of oral medication used in treating type 2 diabetes mellitus. They can be administered alone or in combination therapy with other antidiabetic medications. They are used for patients with low blood sugar tolerance and delayed type 2 diabetes. They are convenient for patients at risk of hypoglycemia or lactic acidosis. Thus, they are not suitable candidates which are on other antidiabetic drugs such as sulfonylureas and biguanides. Alpha-glucosidase inhibitors can also be referred to as starch blockers that help to reduce postprandial blood sugar levels. These enzyme inhibitors do not directly affect insulin secretion or sensitivity, unlike other antidiabetic drugs. Instead, they work by delaying the digestion of sugars found in starchy foods by raising post-meal levels of GLP-1 and lowering HbA1c levels. Alpha-glucosidase inhibitors like acarbose and migitol have shown an increase in expectancy of life in patients with type 2 diabetes mellitus and have proven reduced risk of cardiovascular incidents in patients with reduced glucose tolerance. It has also proven to be effective in stabilizing carotid plaques, lowering hyperglycemia, and can very well counter oxidative stress and endothelial dysfunction.

Keywords Diabetes · Enzyme inhibitors · Alpha-amylases · Alpha-glucosidase · Acarbose

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12.1 Introduction

Diabetes mellitus or type 2 diabetes is the most familiar form of diabetes found all over the world. It is a metabolic disorder that stems from a person being obese or leading a sedentary lifestyle; WHO stated that the number of people suffering from this disease would nearly double in the next 10 years (Saeedi et al. 2019). It is characterized by hyperglycemia, resistance to insulin, abnormal pancreatic insulin secretions, and increased hepatic glycation. Blood sugar is the primary source from which our body derives energy which originates from the food we take. After the glucose levels rise in the blood, the pancreas produces insulin, a hormone that aids in absorbing glucose into cells for energy production. The problem arises when our body does not produce enough or any insulin. It results in the lingering of glucose in our blood for a long time leading to the onset of health issues (Lo Piparo et al. 2008). There are mainly three types of diabetes diagnosed in the population. Type 1 diabetes, commonly known as juvenile diabetes, occurs when our body's ability to manufacture insulin fails (Jasem et al. 2019). Patients with type 1 diabetes are insulin-dependent, so to stay alive, they must be administered with synthetic insulin daily. Type 1 diabetes often occurs when our immune system fails to distinguish our pancreatic beta cells. In this reaction, the body targets and kills its insulin-secreting beta cells in the pancreas (Quandt et al. 2020). It is usually diagnosed early in children and adolescent people. The patient's cells do not get enough glucose to function correctly, leading to fatigue and other issues (Katsarou et al. 2017). Type 2 diabetes has a direct effect on how the body takes up insulin. While the body is still making insulin, unlike type I, the cells stop responding to it as effectively as they used to (Xu et al. 2018). Therefore, this leads to the pancreas producing an elevated amount of insulin to replenish the body's requirements. It is often nearly impossible to maintain fluctuating demand; with an insufficient amount of insulin in the body, diabetes develops. Over the long haul, the beta cells become harmed and may quit delivering insulin out and out. Similar to type 1 diabetes, type 2 can lead to high blood sugar levels and keep the cells from getting the required energy. Type 2 diabetes might result from hereditary qualities and family ancestry. Way of life factors, like weight, absence of activity, and horrible eating routine likewise assume a part. Treatment regularly includes expanding activity levels, working on eating, and taking some professionally prescribed prescriptions. A specialist might have the option to distinguish type 2 diabetes right on time, in a prediabetes phase. An individual with prediabetes might have the option to forestall or postpone the beginning of the condition by changing their eating regimen and exercise schedule (Carbone et al. 2019). Yet another type of diabetes develops in a woman during their pregnancy, known as gestational diabetes. The symptoms of this type of diabetes mostly go away after the delivery of the child (Plows et al. 2018). As a matter of fact, for these women, there is a greater chance of being diagnosed with type 2 diabetes later in life if one has already encountered gestational diabetes. A less common type

of diabetes is the inherited form of the disease known as monogenic and diabetes-related to cystic fibrosis (Hangül et al. 2020). Diabetes can be of different types, for example, insulin-dependent (type 1), non-insulin-dependent (Type 2), gestational and cystic fibrosis diabetes (Jain et al. 2021). All these types of diabetes are targeted with the use of mono or combination therapy of several drugs. These drugs include chemical moieties and also various digestive enzyme inhibitors. Bioactive metabolites derived from plants have proven to be an excellent source of enzyme inhibitors that could target diabetes with low toxicity and negative effects. New inhibitors with higher efficacy and fewer side effects should be focused as a reliable source for antidiabetic medication.

12.2 Digestive Enzymes Targeted for Diabetes

Breakdown of food begins in the oral cavity. The first interaction of food takes place with saliva which helps in the formation of the bolus and eases mastication. Afterward, as the food bolus takes the course and moves down the esophagus and reaches the stomach via peristalsis, the enzymes in saliva already start hydrolyzing the food to give energy packets. The process continues many new enzymes act on bolus to release energy until all the digestion has been completed and nutrient assimilation has occurred. Few enzymes have a direct link in food digestion and the onset of diabetes. They can be noted as mentioned further.

12.2.1 Alpha-Amylase

Alpha-amylases (E.C.3.2.1.1) are enzymes that catalyze the breaking of the interior α-1,4-glycosidic linkages in carbohydrates, hydrolysis of starch into smaller-atomic weight particles like glucose, maltose, and maltotriose units (Kandra 2003; Rajagopalan and Krishnan 2008). These catalysts are amidst the principle modern proteins involving around one-fourth of the world's chemical industry (Gupta et al. 2003; Rajagopalan and Krishnan 2008). These enzymes are also obtained from few of the parasites, yeasts, and microorganisms (Reddy et al. 2003). Nonetheless, enzymes from fungi and bacteria are utilized in mechanical areas. The fundamental approach in the assimilation of carbohydrates begins in the oral cavity with the discharge of salivary amylase that results in the absorption of carbs into more modest oligosaccharides (Lee et al. 2001). Proficient discharge of amylases by the salivary organs decreases the responsibility on the digestive tract. α-Amylase is the significant stomach-related compound in saliva. The productivity of rumination is important for salivary amylase to infiltrate the food bolus (Mikawlrawng 2016).

Notwithstanding the less time of digestion of carbs in the mouth, the salivary processing of sugars is consequential because it advances after the food has reached the stomach (Fried et al. 1987). Gastric juices in the stomach due to their lower pH

start inactivating α-amylase. As the food enters the stomach, other enzymes start breaking it into smaller entities, and salivary amylase has only half an hour to process carbs inside the stomach before it finally gets deactivated (Ansari et al. 2020). Alpha-amylase works best at a somewhat basic pH. The starch in tubers or bread might be processed to the degree of up to three fourth by salivary alpha-amylase before the enzyme is inactivated by acidic pH in the stomach (Rosenblum et al. 1988).

12.2.2 Alpha-Glucosidase

Alpha-glucosidase (EC 3.2.1.20) is an exoenzyme acting in a way like that of glucoamylase on di- and oligo-saccharides and aryl glucosides. It yields glucose. All plants contain α-glucosidase as an endocellular chemical, and it dwells in developed and non-sprouted cereals. The unbiased α-glucosidase from porcine serum showed up very substrate explicit.

It hydrolyzes oligosaccharides, phenyl α-maltoside, nigerose, solvent starch, amylose, amylopectin, and β-limit dextrins. Isomaltose and phenyl α-glucoside could be difficult to hydrolyze (Koyama et al. 2000) Alpha-glucosidases catalyzes the hydrolysis of ends, non-reducing alpha-1,4-connected glucose formed from aryl or alkyl-glucosides, disaccharides, and oligosaccharides. Alpha-glucosidases are habitually called maltases, albeit some of them might have feeble action on maltose (Zhang et al. 2006). Insect alpha-glucosidases turn out as dissolvable structures in the midgut lumen or are captured in the midgut glycocalyx (Terra and Ferreira 1994). They are additionally bound to microvillar layers, to lipoprotein layers ensheathing the midgut cell microvillar layers in many hemipterans (Silva et al. 2004) or to the altered perimicrovillar layers of aphid midgut cells. The last two-layer bound a-glucosidases, just as the dissolvable compound from honey bee midguts (Nishimoto et al. 2001).

12.2.3 Insulin

Individuals with type 1 diabetes and specific individuals with type 2 diabetes might have to administer insulin, so their blood sugar levels do not reach excessively high (Biniek and Johnson 2019). There is a varied length of acting insulins short, average, middle, and long. Many of these insulins are accessible, and most of them congregate by how long their impact undergoes. Specific individuals would utilize a long-acting insulin infusion to keep up with reliably low glucose levels. Patients might take shorter-acting insulin or a combination of them. Whatever the sort, an individual should keep their blood sugar levels in check utilizing a fingerstick glucometer. An individual with type 1 diabetes will then, depending on the observed glucose level, would administer the required amount of insulin (Bari et al. 2020; Biniek and

Johnson 2019). Self-checking is the primary way an individual can discover their glucose levels. Expecting the level from any actual side effects that happen might be risky except if an individual suspect's incredibly low sugar levels and thinks they need a fast portion of glucose.

Insulin begins acting by limiting to the glycoprotein receptor present on the outer layer of the cell surface. On this receptor, an alpha-subunit is present, which ties the chemical, and another beta-subunit, an insulin-invigorated, tyrosine-distinct protein kinase acts on it. Commencement of this kinase is greatly known to create a sign that at last outcomes in insulin's activity on blood sugar, lipid, and protein digestion (Stadtmauer 1983). The development effects of insulin seem to happen past the actuation of receptors for the group of related insulin-like development factors. There are several reasons for receptors to not work altogether. Genetics and acquired traits get results in some anomalies in the number of insulin receptors, receptor kinase movement, and the changes in post-receptor steps in insulin activities happen in illness leads to prompting tissue protection from insulin action (Pearson et al. 2003).

12.3 Oral Agents for Diabetes

Drugs used to treat non-insulin-dependent diabetes are purposed to address at the minimum one of these metabolic anomalies: insulin resistance, insulin deficiency, and increased hepatic glucose output (Sudnikovich et al. 2007). Presently, five hypoglycemic specialists are accessible, among them many of which show extraordinary pharmacologic properties. The drug is classified into sulfonylureas, thiazolidinediones, meglitinides, biguanides, and alpha-glucosidase inhibitors (Table 12.1) (Preiser et al. 2020). Treatment with the solo oral specialist is attempted in patients in whom diet and physical fitness do not give satisfactory blood sugar control (Carbone et al. 2019). While picking a special class of drug, it is reasonable to think about both patient history and medication-directed attributes. If sufficient blood sugar control is not achieved using a solo oral agent, a blend of specialists with varied acting components might be used that have added material remedial influence. It may result in better control of hyperglycemia (Perreault et al. 2019).

The management of type 2 diabetes mellitus in the last few years has been done through natural sources in mainly two significant ways: first includes scavenging of free radicals and the other inhibiting critical enzymes utilized in carbohydrate metabolism. *Alpha*-glucosidase and *alpha*-amylase are the most discussed enzymes in this case. Diabetes, known to be a metabolic disorder, has been linked with an accumulation of ROS in the body and its inability to defend itself by antioxidants (Oboh et al. 2013). Furthermore, the build-up of reactive oxygen species leads to oxidative stress that has been implicated as the cause of diabetogenic relay. Therefore, foods rich in antioxidants have an exceptional dietary interference in the treatment of type 2 diabetes. Ghasemi-Dehnoo et al. (2020) *Alpha*-glucosidase and

Table 12.1 Different types of agents used to treat diabetes

S. no.	Class of drug	Mechanism of action	Examples
1	Insulin (different acting length)	Increase glucose absorption	Regular insulin, Humulin.
2	Biguanides	Lowers hepatic glycation	Metformin
3	Sulfonylureas	Increases insulin secretion	Glyburide, tolazamide, glibenclamide
4	Meglitinides	Increases insulin secretion after a meal	Repaglinide, nateglimide
5	Thiazolidinediones	Increases insulin sensitivity	Pioglitazone
6	Bile acid sequestrants	Works in combination therapy	Colesevelam
7	Alpha-glucosidase inhibitors	Slows down the absorption of carbs	Acarbose, migitol
8	DPP-4 inhibitors	Increases insulin production, decreases glucose production by the liver	Sitagliptin, saxagliptin
9	SGLT-2 inhibitors	Prevents reabsorption of glucose from kidneys	Canagliflozin

alpha-amylase are two main enzymes that are therapeutic targets in controlling diabetes. In the digestion process, these enzymes are utilized in the hydrolysis of carbohydrates to simple sugars, thereby increasing glucose in the blood. However, the inhibition of *alpha*-glucosidase and *alpha*-amylase activities delays glucose absorption which moderates "post-prandial blood sugar elevation" (Takahashi et al. 2018). Rules for commencement of antidiabetes treatment with an oral specialist in place of insulin are bantered among medical professionals. However, this choice is ought to be made mutually by the doctor and patient to acquire the best results. The reformist nature of the beta-cell causes imperfection in type 2 diabetes, present oral treatments might not forestall a possible decrease in glycemic control, and almost certainly, numerous patients will, at last, require insulin treatment (Koyama et al. 2000). Once the choice is made to start treatment with an oral antidiabetic agent, it is judicious to think about understanding important parameters such as age, weight, level of glycemic control, and drugs with special qualities like relative potencies, term of activity, incidental effect profiles, and cost is usually able to come up as the most suitable choice. This illustrates a sensible stepwise methodology for starting oral treatment in patients with type 2 diabetes and is predictable with the proposals advanced by a few master boards and diabetes subspecialists (Hanefeld and Schaper 2008).

12.4 Alpha-Glucosidase Inhibitors for Diabetes

These are oral agents that lessen gastrointestinal glucose assimilation by deferring sugar absorption. Featuring acarbose, miglitol, and voglibose seriously restrain α-glucosidases, an influential group of gastrointestinal proteins that partake in sugar absorption. Natural isolates from several plant and fungal species are being investigated for the presence of novel alpha-glucosidase inhibitors (Table 12.2). It is realized that they decline post-prandial raised blood glucose levels and raised insulin, hence further developing the insulin cell affectability and limiting β-cells stress (van de Laar 2008). Furthermore, these mixtures benefit from not initiating hypoglycemia and having an agreeable security profile, regardless of specific gastrointestinal unfavorable impacts. They are relatively safer to use and might not bring about troublesome long-haul consistency to this remedial alternative.

In vitro AGIs have been found in nitrogen-containing heterocyclic compounds without glycosyl, such as triazole, quinazoline, imidazole, thiazole, and pyrazole. Furthermore, various natural and synthetic flavonoids, namely luteolin, anthocyanins, naringenin and baicalein, coumarin, and chromones, as well as their derivatives, have been identified as strong AGIs. Due to their crucial pharmacological properties, triazole and quinazoline compounds have gotten a lot of attention. Alpha-glucosidase inhibitory activity has been demonstrated in carbazole-linked triazole compounds. Substituted quinazolines have also been shown to exhibit antihyperglycemic and antiglucosidase inhibitory properties. Unlike other oral antidiabetic drugs, AGIs have a limited effect on the intestine instead of impacting metabolic processes throughout the body. As a consequence, extensive research into the role of AGIs in the treatment of pre-diabetic disorders such as impaired glucose tolerance and impaired fasting glucose has been conducted.

12.5 Mechanism of Action of Alpha-Glucosidase Inhibitors

Alpha-glucosidase inhibitors hinder the ingestion of sugars from the brush borders of the small intestines system. They seriously hinder proteins that hydrolyze complex nonabsorbable sugars into basic absorbable smaller forms of carbohydrates (Ansari et al. 2020). These chemicals incorporate glucoamylase, maltase, sucrase, and isomaltase. Figure 12.1 shows the mechanism of action of alpha-glucosidase inhibitors where they block the enzyme and stop the breakdown of glucose (Umamaheswari and Sridevi Sangeetha 2019). By postponing carb retention, they diminish the ascent in postprandial blood sugar fixations by around 3 mmol/L. Among alpha-glucosidase inhibitors; acarbose is the main ordinarily utilized medication of this class and also the most broadly concentrated one. Others incorporate miglitol and voglibose. Acarbose represses alpha-amylase, sucrase, dextranase, and maltase, and it is best known against glucoamylase (Furman 2017). Lactase is not influenced by it, which is a beta-glucosidase (Yee et al. 1996). FDA does not support

Table 12.2 Natural compounds as an alpha-glucosidase inhibitor

S. no.	Natural alpha-glucosidase inhibitor	Class	Source	Reference
1	Taxumariene F	Diterpenoid	*Taxus mairei*	Chen et al. (2020)
2	Akebonoic acid	Triterpenoid	*Akebia trifoliata*	Dirir et al. (2021)
3	Curcumin	Beta-diketone	*Curcuma longa*	Nabavi et al. (2015)
4	Berberine	Alkaloid	*Berberis* sp.	Pan et al. (2003)
5	Fisetin	Flavonoid	Fruits and vegetable	Yue et al. (2018)
6	Rhaponticin	Polyphenol	Rhei Rhizoma	Choi et al. (2006)
7	Catechin	Flavonoid	Various plants	Matsui et al. (2007)
8	Quercetin	Flavonoid	Various plants	Oboh and Ademosun (2015)
9	Morusin	Flavonoid	*Morus alba*	Jang et al. (2015)
10	Myricetin	Flavonoid	*Syzygium*	Kang et al. (2015)
11	Psoralidin	Coumestans	*Psoralea corylifolia*	Wang et al. (2013)
12	Acarbose	Pseudotetrasaccharide	*Actinoplanes*	Song et al. (2005)
13	Antroquinonol	Meroterpenoid	*Fungus*	Jhong and Chia (2015)
14	Docosanol	Aliphatic alcohol	*Toona sinensis*	Jhong and Chia (2015)
15	Eupafolin	Flavonoid	*Artemisia sinensis*	Jhong and Chia (2015)
16	Actinodaphnine	Alkaloid	*Cinnamomum insularimontanum*	Riyaphan et al. (2017)
17	Tetracosanol	Fatty alcohol	*Polyalthia longifolia*	Riyaphan et al. (2017)
18	Rutin	Flavonoid	*Carpobrotus edulis*	Oboh and Ademosun (2015)
19	Luteolin	Flavonoid	*Reseda luteola*	Oboh and Ademosun (2015)

acarbose and voglibose in the USA because they are ineffectively assimilated from the gut. They are known to show low bioavailability and are mostly discharged in the stool. Miglitol, then again, is not ingested from the intestines totally and is

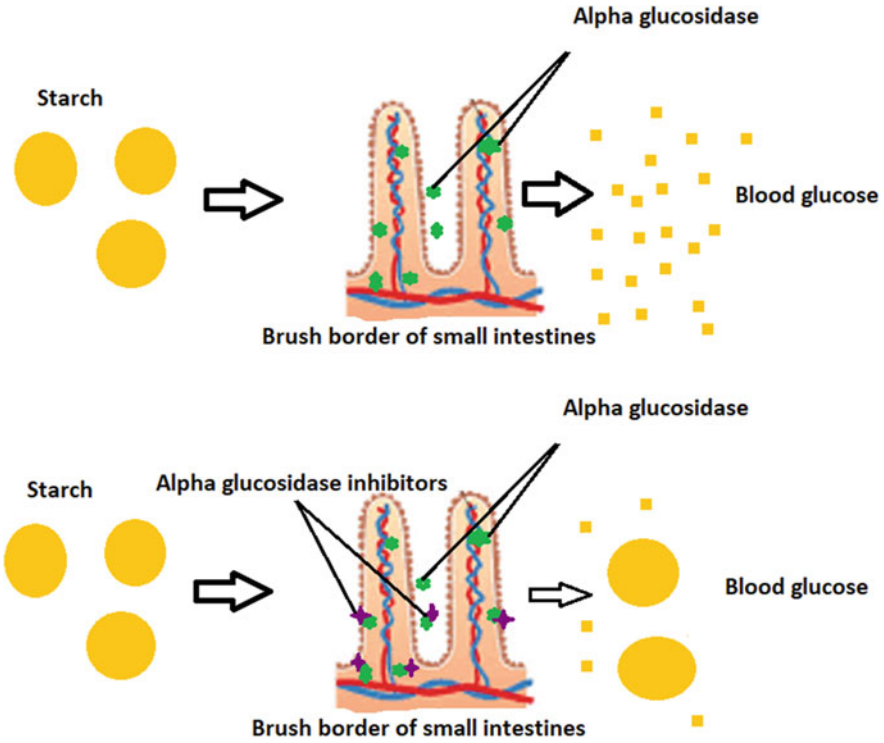

Fig. 12.1 Mechanism of action of alpha-glucosidase inhibitors. (Source: Umamaheswari and Sridevi Sangeetha 2019)

discharged through the kidney. Acarbose is the only one that goes through digestion in the colon, while others have no metabolites (Baxter et al. 2019).

Alpha-glucosidase inhibitors are not recommended for patients with an inflammatory bowel disease, for example, ulcerative colitis or Crohn's disease, or people with some obstruction in the intestines. Any disorder of the intestines would lead to normal to severe side effects if prescribed an alpha-glucosidase inhibitor without studying the patient's medical history (Hedrington and Davis 2019). In conditions like diabetic ketoacidosis, in which fat is utilized instead of carbs as a source of energy administration, this class of oral agents can cause further damage (Tseng 2021). People with an ulcer, liver cirrhosis, and pregnant woman should not take this drug.

12.6 Alpha-Glucosidase Inhibitors in the Market

The evolution of the alpha-glucosidase inhibitor, mainly acarbose, gives another methodology in the regulation of diabetes. By reversible and aggressive hindrance of gastrointestinal alpha-glucosidases, acarbose defers sugar assimilation. It draws out the available starch hydrolyzing time and in this manner lowers the pace of glucose ingestion by competing for active sites (Gao et al. 2018). After the drug is taken, the postprandial ascent in the blood glucose portion is conditionally diminished, and glucose-actuated insulin emission is weakened. Due to decreased postprandial hyperinsulinemia and hyperglycemia by acarbose, the fatty materials take up into fat tissue, hepatic lipogenesis, and fatty oil content is diminished (Baxter et al. 2019). In this way, acarbose treatment straightens postprandial glycemia because of the essential and auxiliary pharmacodynamic impacts and enhances the metabolic state of cells overall. In patients with diabetes, acarbose diminished urinary glucose levels, the blood glucose region under the curve forestalled the decline in skeletal muscle GLUT4 glucose carriers. As a result of the decreased mean blood glucose under the curve, the measure of protein nonenzymatically glycated was lowered, just like the development of advanced glycation final results. The anticipation of cellar layer glycation and thickening in different tissues demonstrated that the acarbose treatment of diabetic patients delivered valuable results against improving nephropathy, neuropathy, and retinopathy. Accordingly, the alpha-glucosidase inhibitor acarbose may postpone or conceivably forestall the advancement of diabetic intricacy (Baxter et al. 2019; Hanefeld and Schaper 2008; Lee et al. 2001). The worldwide alpha-glucosidase inhibitors market enlisted an income of approximately 4000 USD million. It is relied upon to develop at a CAGR of 2% during the conjecture time of 2019–2024. The development of alpha-glucosidase inhibitors (AGIs) is moderate, as it is thought about generally as second-line monotherapy treatment of medications or given in blend with other first-line treatment drugs. The essential driver for the development is generally in more carb utilization locales. China and India hold the most noteworthy market esteem because of their intense usage of sugars, trailed by North America (Xu et al. 2018). Acarbose is the most broadly recommended medication of the other two medications as of now accessible. Acarbose is the most generally endorsed of the three as of now. Other accessible drugs, Miglitol and Voglibose, are also given and utilized to administer hyperglycemia for quite a long time. Acarbose acts non-systemically to dial back sugar processing and lessens significant degrees of postprandial plasma glucose—a fundamental indication in the time course of type 2 diabetes mellitus and a fundamental objective for accomplishing glycemic control. Patients take these specialists toward the start of every fundamental dinner. Alpha-glucosidase inhibitors do not cause hypoglycemic occasions or other hazardous occasions, even at excesses, and cause no increase in weight. Regardless of various investigations showing practical impacts of acarbose as a first-line, second-line, and third-line treatment alternative, recommendation of this medication fluctuates worldwide because of an insight that adequacy is restricted in some ethnic and local gatherings.

12.7 Side Effects Caused by Alpha-Glucosidase Inhibitors

In any case, alpha-glucosidase inhibitors often cause diarrhea and tooting when utilized at the suggested dosages, which is 100 mg acarbose multiple times every day. Since these impacts are portion-related, it is encouraged to begin the treatment with low dosages and slowly increment accordingly. The result of diarrhea might be valuable in a patient distracted with bowel obstruction. Treatment should be started with the least viable portion and titrated gradually over periods to about a month. Patients ought to be told to take this prescription with food. For most significant adequacy, the dietary carb admission ought to surpass 50%. Although hypoglycemia is not commonly connected with single-drug therapy with alpha-glucosidase inhibitors, it can be blended with different medications. It is significant, along these lines, to illuminate patients with the conventional treatment for hypoglycemia and not only for devouring glucose. Alpha-amylases are supposed to disintegrate starch into disaccharides, followed by isomaltase, particularly alpha-glucosidase, to deliver glucose. This way, the presence of alpha-glucosidase solid inhibitory action shows up more significant in checking the arrival of smaller sugar from disaccharides in the small intestines than alpha-amylase restraint. Notwithstanding, moderate α-amylase restraint with powerful α-glucosidase inhibitory movement might offer a better restorative methodology that could lull the accessibility of dietary starch substrate for glucose creation in the gut. Food-grade phenolic inhibitors from edible plant extricate are conceivably more secure and may, consequently, be a favored option for adjustment of carb processing and control of glycemic file of food items (Gao et al. 2018). Work carried out in our laboratory focuses on repurposing traditional medicine for the treatment of diabetes. Natural metabolites from plants are screened by using several high-resolution spectrometry techniques. The probable compounds are then tested in vitro for their therapeutic potential. Presently, the research is being conducted for novel compounds as alpha-amylase and alpha-glucosidase inhibitors found in *Azadirachta indica, Berberis aristata, Leptadenia reticulata, and Solanum virginianum*. Traditionally, these plant species are found effective in various diseases such as diabetes, wound healing, inflammation related to skin, cough, dehydration, tuberculosis, hematopoiesis, chickenpox, and respiratory disorder. They have a huge amount of phytochemicals such as cardiac glycosides, flavonoids, alkaloids, sterols and/or triterpenes, carbohydrates, and coumarins, saponins, catechol, and pyrogallol tannins. We are directing our work on in vitro and in silico studies of plant-based biochemicals for the natural treatment of diabetes. Alpha-amylase, alpha-glucosidase, DPP4, aldolase reductase, PPAR-gamma enzymes are being targeted to validate antidiabetic potential by performing enzyme kinetics and mode of inhibition studies. IC50 value, molecular docking, and molecular dynamics simulation are further utilized for concluding effectivity similar or equal to existing enzyme inhibitors.

12.8 Conclusion

Natural products from plants or mixtures from other regular sources can be utilized as medications or could fill in as lead compounds to blend bioactive mixtures. Likewise, from a portion of the lead constituents from plants, new analogs with more prominent manufactured openness could be inferred. In the early improvement of current medication, organically dynamic mixtures from higher plants were imperative in giving prescriptions to battle pain and sicknesses. The vast majority of these were winnowed from plants generally utilized for that reason in one culture or another. One such compound is alpha-glucosidase that was discovered in several plant species. Traditional medications and homegrown cures make use of such naturally available phytochemicals were used to treat diabetes. In the quest for additional robust and more secure antidiabetic specialists from traditional sources, medicinal plants offer the main wellspring of lead compounds. These might fill in as industrially critical elements themselves or may give lead constructions to the improvement of changed subordinates having improved movement and diminished harmfulness. Accordingly, more exploration is expected to investigate the vast swath of customary wellsprings of the medications, particularly plants, to support humanity.

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