Chapter 12
Peptidic Inhibitors of Serine Proteinases of Plant Origin

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Abstract Serine proteinases play important roles in many physiological processes and in consequence, when unbalanced, are responsible for numerous severe diseases. The most predominant mechanism of their control is the ubiquitous presence of their inhibitors. On the basis of their inhibition mode, serine proteinase inhibitors are classified into canonical (standard mechanism) inhibitors, serpins and non-canonical inhibitors. The best studied are inhibitors assigned to the first group. At least 18 canonical inhibitor families have been recognized. Inhibitors isolated from the seeds of Cucurbitaceae are small (around 30 amino acid residues), containing three disulfide brides. Some of them are characterized by a cyclic polypeptide backbone. Head-to-tail cyclization is also present in the smallest (14 amino acid residues) trypsin inhibitor isolated from sunflower seeds. All these potent inhibitors display well-defined, rigid structures and, unlike most proteins, are also resistant to denaturizing agents. Modifications introduced into the molecules of these low-molecular-mass inhibitors are well tolerated, retaining their tertiary structure and inhibitory activity. They are able to cross cell membranes and are the first examples of cyclic cell-penetrating peptides. Recent results obtained on plant peptidic inhibitors and discussed in this mini-review have proved that they are promising molecules for drug design.

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E. F. Fang and T. B. Ng (eds.), *Antitumor Potential and Other Emerging Medicinal Properties of Natural Compounds*, DOI: 10.1007/978-94-007-6214-5_12,

12.1 Introduction

Proteolysis is probably the most widespread mechanism of biological regulation. By controlling protein synthesis, turnover and activity, it is involved in fundamental physiological processes including cell differentiation, growth and signaling, apoptosis, fertilization, blood coagulation, immune response and digestion. Yet, uncontrolled proteolysis can be harmful for organisms, causing amongst others such diseases as emphysema, inflammation, cancer, neurodegenerative, immunological, and cardiovascular disorders. Proteolytic enzymes can be divided into at least seven families: serine, aspartic, cysteine, glutamic, metallo, asparagine and threonine proteases. Serine proteases (SPs) are among the most widely studied proteins; they are widespread in nature and are involved in numerous and diverse physiological and pathological processes. Over 82 thousand sequences of SPs have been collected in the MEROPS database (http://merops.sanger.ac.uk/), whereas the second largest family is the family of metalloproteinases (over 75 thousands). SPs originate from extracellular matrix, mast cells, cytotoxic cells, and neutrophils. Some of the most studied SPs are those involved in blood coagulation. There are several mechanisms found in mammalian systems that strictly control the activities of proteases. Like other proteins, their ribosomal synthesis can be controlled at several stages, followed in many cases by post-translational modifications. Compartmentalization and the manner in which proteinases are released in the pericellular environment are additional important mechanisms for the regulation of their activity. Release and folding of the active enzyme from its inactive precursor is one of the predominant methods of protease control. A second method is effected by the ubiquitous presence of protease inhibitors. Naturally occurring inhibitors are proteins or peptides except in the case of non-proteinaceous ones isolated from microorganisms.

According to recent data brought together in the MEROPS database almost 21 thousand inhibitor amino acid sequences have been identified. Based on their sequence homology, they have been grouped into 91 families and further assigned to 36 clans according to their similar tertiary structures. There are also a large number of small-molecule inhibitors, both naturally occurring and synthetic. These peptidomiometics, or simply small organic molecules, are outside the scope of this mini-review. Since more than one third of identified proteases are serine proteinases, it is no surprise that their inhibitors (SPIs) also represent the largest group. They are found in all living organisms. In fact, the first described were trypsin inhibitors. In 1936 Kunitz and Northrop isolated and crystallized a bovine pancreatic trypsin inhibitor (BPTI). Later, Kunitz identified another, named STI, from soybean. In the meantime, another inhibitor, the Bowman-Birk inhibitor (BBI), was identified from the same source and characterized. These proteins inhibited trypsin and at neutral pH formed stable complexes which were dissociated at low pH. Progress in bioanalytical techniques has confirmed earlier suggestions that these inhibitors interact with enzymes in a substrate-like manner. They are probably the best studied proteins and their complexes with cognate enzymes are among the best known examples of protein—protein interactions. This was

also one of the reasons that knowledge about inhibitors of other proteolytic enzymes was, until the last decade, significantly less well advanced.

12.2 Inhibition Mode of Serine Proteinase Inhibitors

On the basis of their mechanism of action, SPIs are classified as: canonical (standard mechanism), serpins and non-canonical. Except for the last group, SPIs interact with target enzymes in a substrate-like manner. Canonical inhibitors are small proteins or peptides with molecular weights varying from 1.5 to 2.1 kD, corresponding to 14−200 amino acid residues. They are characterized by rather compact, thermally resistant structures, and although they possess different folding motives, they are usually stabilized by several disulfide bridges. These native structures are also stable in denaturizing agents. A common structural element in canonical inhibitors is a short fragment comprising less than 10 amino acid residues which is directly involved in interaction with cognate enzymes. This exposed fragment, named the "binding loop" or the "primary binding segment", has an extended conformation with a similar anti-parallel β-strand. Bode and Huber [1] proposed to call it "canonical". Some inhibitors contain a single binding loop, some others consist of multiple domains where such elements are located. Thus, such inhibitors interact with proteinases with stoichiometry other than 1:1. Alternatively, they are able to inhibit more than one enzyme simultaneously. It transpires that all standard mechanism inhibitors exhibit this canonical conformation of binding loop, and are therefore named "canonical inhibitors". This mode of inhibition was studied in detail by the group of Laskowski [2]. Upon enzyme—inhibitor interaction, a stable complex C is formed rapidly and then (unlike in substrates) very slowly dissociated into a free enzyme and modified inhibitor. As indicated by X-ray data, the majority of contacts with enzymes are made by the inhibitor binging loop [1] that fit into the active site of a cognate enzyme. The intermolecular contact area is relatively small, restricted to 6−9 nm^2. In the middle of this loop is located a peptide bond P_1-P_1' (reactive site). According to the Schechter and Berger nomenclature [3], P_1-P_1' is the scissile peptide bond and both amino acid residues P_1 and P_1' interact with the corresponding enzyme—S_1 and S_1'- subsites, respectively. The hyper-exposed amino acid residue in position P_1 interacts with the S_1 cavity of the enzyme accounting for the largest part (up to 50 %) of the total association energy released during complex formation with the target enzyme. Therefore, P_1 residue determines inhibitor specificity and is often referred to as the primary specificity residue. As in substrates, the reactive site P_1-P_1' is selectively cleaved by proteinase yielded modified inhibitor. After sufficient incubation time, an equilibrium between intact and modified inhibitor is formed. The determined values of the hydrolysis constant ($K_{hyd} = [I]/[I^*]$) for most canonical inhibitors are, in neutral pH, close to unity. It is worth mentioning that in a number of 3D structures of proteinase-inhibitor complexes determined by X-ray crystallography the reactive site P_1-P_1' remains intact. The mechanism

of inhibition of canonical inhibitors with cognate enzymes is shown in Fig. 12.1. One of the most eminent researchers in the field of serine proteinase inhibitors was undoubtedly Michael Laskowski Jr. who devoted his whole scientific life to the elucidation of this mode of inhibition. In recognition of his contribution, MEROPS suggests the adoption of the new name, the "Laskowski mechanism", for what has been accepted as the standard mechanism.

The acronym "Serpin" (serine proteinase inhibitors) was introduced in the mid 1980s [4] to describe a superfamily of relatively large proteins (45–55 kD); some of them are serine and cysteine protease inhibitors, others play non-inhibitory functions. Despite a low sequence homology, they adopt a similar metastable conformation comprising three β-sheets, eight to nine α-helical segments and an exposed binding loop, also called the "reactive center loop" (RCL). This large family (I4) consists of over 2500 members, found in all kingdoms of organisms including 44 in humans. Serpins interact with target proteinases based on a unique mechanism that is described as the suicide substrate-like inhibition. Inhibition by serpins is irreversible. Proteinase is inactivated, kinetically trapped in the complex and can be cleared from the circulation by binding to members of the low density lipoprotein receptor family. Irreversibility is also reinforced by the induced proteolytic susceptibility of proteases in complex with serpins.

The first non-canonical inhibitor, hirudin, was isolated from the saliva of the medicinal leech in the 1950s and a decade later its primary structure was determined. This polypeptide, consisting of 65 amino acid residues, displays anticoagulant activity and is a very potent and specific thrombin inhibitor. Hirudin and its variants isolated from leech species share a common 3D structure, *N*-terminal globular domain cross-linked by three disulfide bridges and an acidic *C*-terminal fragment, which displays flexible high conformational freedom. Both structural elements are directly involved in the interaction with proteinase. Negatively charged *C*-terminal segment directs hirudin towards a positively charged thrombin exosite I (fibrinogen recognition exosite), followed by their tight binging. This induces conformational changes in the enzyme allowing the interaction of *N*-terminal tripeptide of the inhibitor with the thrombin active site. In contrast to canonical inhibitors, this fragment does not form an anti-parallel but rather a parallel β-sheet with side chains of a thrombin Ser^{24}—Gly^{219} segment and a negatively

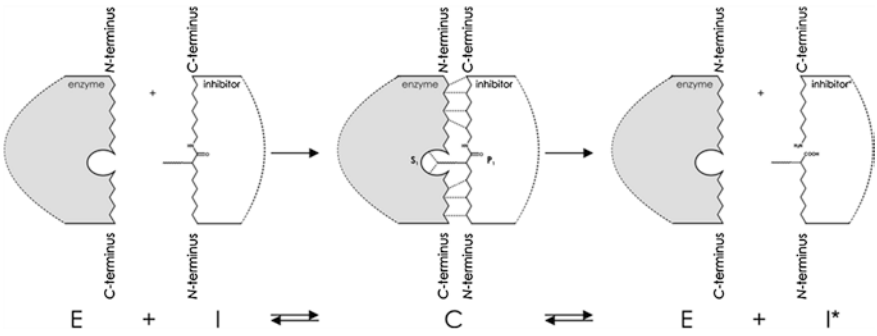

Fig. 12.1 Enzyme—inhibitor interaction according to Laskowski Jr

charged side chain of Asp^{189} located in the enzyme S_1 subsite does not interact with the inhibitor. Another thrombin inhibitor isolated from leeches (*Hirudinaria manillensis*)—named haemadin, although it does not share sequence homology with hirudin, displays a similar tertiary structure and similar mode of thrombin inhibition. This was the reason that hirudin and haemadin were assigned to the same family (I14). Nevertheless, the analysis of its complex with thrombin revealed several differences. One is the direct contact of the positively charged guanidine group of inhibitor's Arg2 with carboxylate of enzyme's Asp189. This resembles the P_1—S_1 interaction of canonical inhibitors. The second is the interaction of the *C*-terminal segment not with exosite I but with exosite II. Several Cys-rich inhibitors including antistatin, therostatin, tessulin, bdellastatin, guamerin and the most potent thrombin inhibitor theromin were assigned to this family (I15) and this is collectively called the antistatin family. These inhibitors display a common folding motif consisting of several repeats of *N*- and *C*-domains. On the basis of limited structural data, it is postulated that inhibitors of the I15 family interact with target enzymes in a canonical (substrate-like) manner with P_1 position located in the *C*-domain, while (in at least some inhibitors) the highly acidic *N*-domain may interact with thrombin exosites. A more detailed mechanism of interaction between non-canonical inhibitors and proteinases has been presented in a recently published review [5].

12.3 Classification of the Canonical Serine Proteinase Inhibitors

Extensive studies on the isolation, characterization and determination of the 3D structures of inhibitors obeying a common, so-called standard mechanism (later named canonical) led to the conclusion that not all inhibitors are homologous. They were found in almost all tissues where they were looked for—animal, plant and microbial. Laskowski and Kato proposed grouping them into families. Originally, nine families were recognized, but information on three of them was rather marginal. Subsequently, with progress in the identification and characterization of new canonical inhibitors more members of each family were found and, in addition, more families were added to the list. Finally, based on results obtained over the following two decades, Laskowski Jr's group [2] recognized at least 16 different families (Table 12.1). More recently, Rawlings et al. [6] proposed a new classification system for the protease inhibitors that allows them to be added to the MEROPS database. They introduced the term "inhibitor unit" which is a segment of the inhibitor containing a single reactive site. An inhibitor containing a single inhibitor unit is named a simple inhibitor, whereas one containing multiple units is termed a compound inhibitor. Based on sequence homology, inhibitors were grouped into 48 families (currently, there are 79 distinct families). In addition, inhibitors which display the same type of protein fold were assigned to the same clan. To date 39 clans have been recognized, and this means that some clans contain representatives of more than one family. In the case of the canonical inhibitors of serine proteinases 18 families have been recognized and grouped in

Table 12.1 Families and clans of canonical serine proteinase inhibitors

Family				Number	
According to Laskowski [2]	According to MEROPS	Clan	Representative	Entries/ sequences	PDB structures
Kazal	I1	IA	OMTKY	1,413	13
Kunitz (BPTI)	I2	IB	BPTI	1,448	16
Kunitz (STI), Arrowhead	I3	IC	STI	231	10
Squash	I7	IE	CMTI-I	32	4
Ascaris	I8	IE	BTI	397	5
Marinostatin	I10	Unassigned	Marinostatin	23	0
Ecotin	I11	IN	Ecotin	107	1
BBI	I12	IF	BBI, SFTI-1	138	11
Potato I	I13	IG	PPI	131	3
Antistasin	I15	IM	Antistasin	36	3
SSI	I16	IY	S–SI	69	1
Chelonianin	I17	IP	SLPI	443	3
Rapeseed	I18	JD	MTI	18	2
Grasshopper	I19	IW	LMPI	14	2
Potato II	I20	JO	PPI-II	57	5
Bombyx subtilisin inhibitor	I40	Unassigned	BSI	1	0

OMTKY turkey ovomucoid trypsin inhibitor; *BPTI* bovine pancreatic trypsin inhibitor; *STI* soybean trypsin inhibitor; *CMTI-I Cucurbita maxima* trypsin inhibitor; *BTI* barley trypsin inhibitor; *BBI* Bowman-Birk inhibitor, *SFTI-1* Sunflower trypsin inhibitor-1; *S–SI* S*treptomyces* subtlisin inhibitor, *SLPI* secretory leukocyte peptidase inhibitor; *MTI* mustard trypsin inhibitor-2; *LMPI Locusta migratoria* trypsin inhibitor; *PPI* potato peptidase inhibitor II; *BSI* Bombyx subtilisin inhibitor; *VTI Veronica* trypsin inhibitor

14 clans. Table 12.1 presents a summary of the Laskowski and MEROPS classifications of these inhibitors. The size of these proteins varies from 14 to 200 amino acid residues. As shown in Table 12.1, canonical inhibitors are widely distributed in the plant kingdom. To date, at least eight families have been classified as being of plant origin. They are especially ubiquitous in tissues responsible for storage (seeds and drupes). It has been proved that the biosynthesis of inhibitors in plants is stimulated by infection or damage of vegetative tissue. Thus, they are important components of plant defense systems targeting exogenous proteinases.

12.4 Squash Family of Serine Proteinase Inhibitors

The first members of this family were isolated from squash (*Cucurbita maxima*) seeds and characterized at the beginning of the 1980s independently by Polanowski et al. from University of Wroclaw (Poland) and Hojima et al. from NIH, Bethesda

(USA). These polypeptides inhibiting several serine proteinases (such as bovine β-trypsin, human Hageman factor XIIa and kallikreins) were named ITD I, ITD III and PHFI, respectively. It turned out that ITD III is identical to PHFI. Subsequently, more homologues inhibitors were isolated from *Cucurbitaceae*, and in 1985 Wieczorek et al. [7] postulated that these low-molecular mass inhibitors could be assigned to a new family of serine proteinase inhibitors (I7). In addition, the Polish group introduced a new nomenclature using the scientific names of the plant which is consequently applied to all members of the squash family. Thus, the acronym ITD was replaced by CMTI (*Cucurbita maxima* trypsin inhibitor). To date over 40 inhibitors assigned to this family have been identified in seeds of Cucurbitaceae. Their amino acid sequences, sources and years of discovery are summarized in Table 12.2. A unique feature of the discussed family of inhibitors is its unusually small size, compact structure and high resistance to denaturizing conditions. Squash inhibitors consisting of 28–34 amino acid residues and their structure are stabilized by three disulfide bridges. Two disulfide bridges form a ring that is threaded by a third one. Such a structural motive is called the cystine knot (CK) [8]. Interestingly, two members of the discussed family called MCoTI-I and MCoTI-II isolated from *Momordica cochinchinensis* possess an additional cyclic element—head-to-tail cyclization and are the first macrocyclic inhibitors of the squash family [9]. At present over 160 such cyclic peptides found in nature are collectively named cyclotides [10]. They display a characteristic folding path where the cystine knot is embedded within a macrocyclic backbone, defining a motif referred to as a cyclic cystine knot (CCK). The topology of disulfide bridges, cystine knots and CCK motives is illustrated in Fig. 12.2.

12.4.1 Cystine Knot Inhibitors

In squash inhibitors, the P_1-P_1' corresponds to positions 5 and 6 in CMTI inhibitors. In substrate specificity P_1 position Arg or Lys is present; therefore, they are

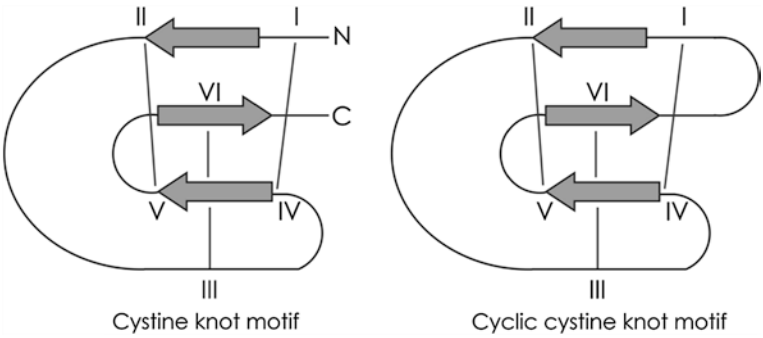

Fig. 12.2 The cystine knot motifs

Table 12.2 Amino acid sequences of squash family inhibitors

Sequence	Name	Source	Year of discovery							
RV	CPRILME	CKKDSD	CLAE	CV	CLEH	GY	CG	CMTI-I	*Cucurbita maxima*	1980, 1983
RV	CPRILMK	CKKDSD	CLAE	CV	CLEH	GY	CG	CMTI-III	*Cucurbita maxima*	1980, 1983
HEERV	CPRILMK	CKKDSD	CLAE	CV	CLEH	GY	CG	CMTI-IV	*Cucurbita maxima*	1980, 1983
HEERV	CPKILME	CKKDSD	CLAE	CI	CLEH	GY	CG	CPTI-III	*Cucurbita pepo*	1983
RV	CPKILME	CKKDSD	CLAE	CI	CLEH	GY	CG	CPTI-II	*Cucurbita pepo*	1983
MV	CPKILMK	CKHDSD	CLLD	CV	CLEDI	GY	CGVS	CSTI-IIb	*Cucumis sativus*	1983
MM	CPRILMK	CKHDSD	CLPG	CV	CLEHI	EY	CG	CSTI-IV	*Cucumis sativus*	1983
GI	CPRILME	CKRDSD	CLAQ	CV	CKRQ	GY	CG	MRTI-I	*Momordica repens*	1984
AI	CPRILME	CKRDSD	CLAQ	CV	CKRQ	GY	CG	MRTI-III	*Momordica repens*	1984
	CPRILMP	CKVNDD	CLRG	CK	CLSN	GY	CG	TTI I	*Trichosanthes kirilowii*	1984
	CPRILMP	CQVNDD	CLRG	CK	CLSN	GY	CG	TTI II	*Trichosanthes kirilowii*	1984
RV	CPKILMK	CKKDSD	CLAE	CI	CLEH	GY	CG	CPGTI-I	*Cucurbita pepo var Giromontia*	1985
GRR	CPRIYME	CKRDAD	CLAD	CV	CLQH	GI	CG	CVTI-I	*Citrullus vulgaris*	1987
<ERG	CPRILMR	CKRDSD	CLAG	CV	CQKN	GY	CG	BDTI-I	*Bryonia dioica*	1987
RG	CPRILMR	CKRDSD	CLAG	CV	CQKN	GY	CG	BDTI-II	*Bryonia dioica*	1987
G	CPRILMR	CKQDSD	CLAG	CV	CGPN	GF	CGSP	EETI-II	*Ecballium elaterium*	1989
ERR	CPRILKQ	CKRDSD	CPGE	CI	CMAH	GF	CG	MCTI-I	*Momordica charantia*	1989
RI	CPRIWME	CKRDSD	CMAQ	CI	CVD	GH	CG	MCTI-II	*Momordica charantia*	1989
RI	CPLIWME	CKRDSD	CLAQ	CI	CVD	GH	CG	MCEI-I	*Momordica charantia*	1989
RI	CPRILME	CSSDSD	CLAE	CI	CLEQ	GF	CG	LCTI-1	*Luffa cylindrical*	1990
RI	CPRILME	CSSDSD	CLAE	CI	CLEQD	GF	CG	LCTI-2	*Luffa cylindrical*	1990
RS	CPRIWME	CTRDSD	CMAK	CI	CVA	GH	CG	MCTI-A	*Momordica charantia*	1992
M	CPKILMK	CKQDSD	CLLD	CV	CLKE	GF	CG	CMCTI-I	*Cucumis melo*	1992
RM	CPKILMK	CKQDSD	CLLD	CV	CLKE	GF	CG	CMCTI-II	*Cucumis melo*	1992
<ERM	CPKILMK	CKQDSD	CLLD	CV	CLKE	GF	CG	CMCTI-III	*Cucumis melo*	1992
<ERR	CPRIYME	CKHDSD	CLAD	CV	CLEH	GI	CGG	LLTI-I	*Langeria leucantha*	1992
RR	CPRIYME	CKHDSD	CLAD	CV	CLEH	GI	CG	LLTI-II	*Langeria leucantha*	1992
ERR	CPRIYME	CKHDSD	CLAD	CV	CLEH	GI	CG	LLTI-III	*Langeria leucantha*	1992
R	CPRIYME	CKHDSD	CLAD	CV	CLPQ	GI	CG	BHIT-I	*Benicasa hispida*	1992

Table 12.2 (continued)

Sequence	Name	Source	Year of discovery							
RR	CPRIYME	CKHDSD	CLAD	CV	CLPQ	GI	CG	BHIT-III	*Benicasa hispida*	1992
QRM	CPKILMK	CKQDSD	CLLD	CV	CLKE	GF	CG	CMTI-II	*Cucumis melo*	1992, 1995
VG	CPRILMK	CKTDDD	CLLG	CK	CLSN	GY	CG	HMTI-I	*Hami melon*	1993
I	CPRILMP	CSSDSD	CLAE	CI	CLEN	GF	CG	TGTI-I	*Luffa cylindrical*	1993
MASVAESSGVVEVIELISDGGNDLPRKIMSGRHGGI	CPRILMP	CKTDDD	CMLD	CR	CLSN	G		TGTI-II	*Luffa cylindrical*	1993
MAAFVESARAGAGAEVIQLVSDGVNEYSEKMME GVVA	CPRILMP	CKVNDD	CLRG	CK	CLS			TTII	*Trichosanthes kirilowii*	1994
RI	CPRILME	CSYDSD	CFGE	CI	CLPS	GY	CG	LCTI II	*Luffa cylindrical*	1994
RI	CPRILME	CSSDSD	CLAE	CI	CLEN	GF	CG	LCTI III	*Luffa cylindrical*	1994
RM	CPRILMK	CKQDSD	CTTD	CV	CKKE	GF	CG	CMeTI-A	*Cucumis melo*	1995
VG	CPRILMK	CKTDRD	CLTG	CT	CKRN	GY	CG	CMeTI-B	*Cucumis melo*	1995
ERG	CPRILKQ	CKQDSD	CPGE	CI	CMAH	GF	CG	MCTI-III	*Momordica charantia*	1995
ERG	CPLILKQ	CKQDSD	CPGE	CI	CVD	GF	CG	MCEI-II	*Momordica charantia*	1995
EERG	CPLILKQ	CKQDSD	CPGE	CI	CVD	GF	CG	MCEI-III	*Momordica charantia*	1995
EEERG	CPLILKQ	CKQDSD	CPGE	CI	CVD	GF	CG	MCEI-IV	*Momordica charantia*	1995
KEEQRV	CPRILMR	CKRDSD	CLAQ	CT	CQQS	GF	CG	ELTI-I	*Echinocystis lobata*	1996
RV	CPRILMR	CKRDSD	CLAQ	CT	CQQS	GF	CG	ELTI-II	*Echinocystis lobata*	1996
I	CPRILME	CSHDSD	CFGE	CI	CLSS	GY	CG	LA I	*Luffa acutangula*	1996
IR	CPRIYME	CSHDSD	CLGE	CI	CLES	GF	CG	LA II	*Luffa acutangula*	1996
RV	CPRILMR	CKRDSD	CLAE	CT	CQGS	GY	CG	SATI-I	*Sicyos australis*	1996
GRI	CPRILMR	CKRDSD	CLAE	CT	CQS	GY	CG	SATI-II	*Sicyos australis*	1996
<ERGRI	CPRILMR	CKRDSD	CLAE	CT	CQS	GY	CG	SATI-III	*Sicyos australis*	1996
&SGSDGGV	CPKILQR	CRRDSD	CPGA	CI	CRGN	GY	CG&	MCoTI-I	*Momordica cochinchinensis*	2000
&SGSDGGV	CPKILKK	CRRDSD	CPGA	CI	CRGN	GY	CG&	MCoTI-II	*Momordica cochinchinensis*	2000
<ERA	CPRILKK	CRRDSD	CPGE	CI	CKEN	GY	CG	MCoTI-III	*Momordica cochinchinensis*	2000
EDRK	CPKILMR	CKRDSD	CLAK	CT	CQES	GY	CG	SETI-IIa	*Sechium edule*	2006
EEDRK	CPKILMR	CKRDSD	CLAK	CT	CQES	GY	CG	SETI-IIb	*Sechium edule*	2006
	CPKILMK	CKLDTD	CFPT	CT	CRPS	GF	CG	SETI-V	*Sechium edule*	2006

(continued)

Table 12.2 (continued)

Sequence	Name	Source	Year of discovery							
I	CPRILME	CKADSD	CLAQ	CI	CEES	GF	CG	CyPTI I	*Cyclanthera pedata*	2006
I	CPRILME	CKADSD	CLAQ	CI	CQES	GF	CG	CyPTI II	*Cyclanthera pedata*	2006
RI	CPRILME	CKADSD	CLAQ	CI	CEES	GF	CG	CyPTI III	*Cyclanthera pedata*	2006
RI	CPRILME	CKADSD	CLAQ	CI	CQEN	GF	CG	CyPTI IV	*Cyclanthera pedata*	2006
RI	CPRILME	CKADSD	CLAQ	CI	CQES	GF	CG	CyPTI V	*Cyclanthera pedata*	2006
ARI	CPRILMK	CKKDSD	CLAE	CI	CEEH	GF	CG	CyPTI VI	*Cyclanthera pedata*	2006
ARI	CPRILMK	CKKDSD	CLAE	CI	CQEH	GF	CG	CyPTI VII	*Cyclanthera pedata*	2006

potent inhibitors of trypsin and other serine proteinases with a negatively charged S_1 subside. The exceptions are MCEI inhibitors with Leu5 which therefore inhibit elastase. Also, P_1' position is conservative and is occupied in all in squash inhibitors by Ile residue.

The unusually small size of squash inhibitors as compared with other *proteinaceous* serine proteinase inhibitors makes them readily accessible by means of chemical synthesis. In contrast to biological methods of synthesis where introduction into the protein molecule other than by proteinogenic amino acids is rather difficult, chemical methods allow practically all modifications to be achieved. CMTI-III and -I, the first members of the family were synthesized by us in the mid 1980s [11]. This definitely confirmed the correctness of their sequences. A few years later, the French group isolated, synthesized and determined solution structure [12] homologues EETI-II from seeds of *Ecballium elaterium*. The authors showed that deletion of its *C*-terminal dipeptide Ser^{29}-Pro^{30} retained its inhibitory activity. Both CMTI-III and EETI-II (shortened 28 amino acid residue variant) were used for structure- activity-relationships. Since the first synthesis, we have synthesized over 60 analogues of CMTI/EETI analogues. The obvious modification introduced by several groups into the squash inhibitors was the substitution of Arg5 by aromatic or aliphatic amino acid residues. This yielded analogues with specific chymotrypsin and elastase inhibitory activity, respectively. On the other hand, we have shown that the requirements for P_1 position are very strict. The introduction of Arg or Lys derivatives in this position dramatically lowered trypsin inhibitory activity. It is worth mentioning that derivatives differ from proteiongenic amino acids by one methylene group in the side chains (L-homoarginine or L-homolysine) introduced in the P_1 position reducing the affinity of such analogues towards trypsin by 2–3 fold. We were also able to considerably simplify the structure of CMTI. The analogue with a truncated *N*-terminal dipeptide, and *C*-terminal Gly and contains 7 amino acid residues replaced by Gly or Pro residues retaining high inhibitory activity. All three disulfide bridges are important in maintaining the tertiary structure of squash inhibitors. On the other hand, we have recently shown [13] the substitution of each disulfide bridge by diselenide yielded potent trypsin inhibitors and more redox-resistant EETI-II analogues. Le-Nguyen et al. [14] reported the synthesis of hybrids of EETI-II and carboxypeptidase inhibitors. The rationale of their work was the fact that both peptides display the same fold as the cystine knot motive. It turned out that such an analogue inhibited both proteases used. Analogues of EETI-II were also synthesized by Hilpert et al. [15]. They replaced an *N*-terminal heptapeptide in EETI-II by an optimized binding loop of the turkey ovomucoid inhibitor (OMTKY3). The hybrid inhibitor HEI-TOE I retained inhibitory activity against porcine pancreatic elastase with considerably higher proteolytic stability. Inhibitors of this family were also successfully obtained using biological methods. Sato et al. [16] obtained recombinants of CMTI-II by expression of its gene in silkworms, whereas Grzesiak et al. [17] expressed series of CMTI-I analogues modified within a binding loop in *E. coli*. Since plant inhibitors play an important role in defense systems against insects or micro-organism predators, Milner et al. [18] fused CMTI-1 to the coat

protein of potato potyvirus Y and bacterial β-glucuronidase in order to check whether such an insert could protect a protein against proteolysis. They showed that inhibitory activity was retained when the inhibitor was fused to the first protein only. All the above mentioned synthetic studies on squash inhibitors proved that their analogues with possible therapeutic applications can be accessible for both chemists and biochemists.

12.4.2 Cyclic Cystine Knot Inhibitors

It has already been mentioned that MCoTI-I/II are members of a family of miniproteins named cyclotides. Peptides displaying the CCK motive possess a diverse range of biological activities including antimicrobial, insecticidal and pesticidal activity. Cyclotides represent attractive scaffolds for the design of peptide-based drugs. This was the main reason that MCoTI-I/II became the most studied inhibitors of the squash family. Both were isolated from the seeds of the squash *Momordica cochinchinensis* in 2000, and in the following year the tertiary structure of MCoTI-II was determined. It was independently shown by two groups that its structure is similar to that of the other members of the squash family. Cysteine-rich proteins are attractive models to study oxidative folding pathways. Bearing in mind that the discussed inhibitors contain 6 Cys residues, 15 different sets of disulfide bridges can be formed. It turned out that the pathways of oxidation folding of the reduced precursors of cyclic and acyclic squash inhibitors (EETI-II and MCoTI-II were studied) are similar, yielding the native species. In both cases, only one major intermediate with two native disulfide bridges was present [19].

In contrast to other inhibitors of the discussed family, MCoTIs contain macrocycle formed by a polypeptide chain. Therefore, chemical synthesis of such inhibitors requires an additional step. One of the options is generation of a thioester at the *C*-terminus of the fully protected precursor followed by removal of the side chain protecting groups and a ring-closing macrolactam formation by native chemical ligation. Oxidative refolding of homodetic peptides gives the desired inhibitor [20]. MCoTIs were also biosynthesized in *E. coli* cells using a biomimetic approach that involves the use of a modified protein splicing unit in combination with an in-cell intramolecular native chemical ligation reaction [21]. Briefly, a MCoTI-I linear precursor was inserted into a modified intein yielding Met-Cys dipeptide at the *N*-terminus. After the enzymatic removal of Met and generation of thiester at the *C*-terminus of the MCoTI precursor, native chemical ligation was used to accomplish the main chain cyclization. In the final step, oxidative folding produced MCoTI. Finally, the inhibitor was purified on HPLC or using trypsin-immobilized Sepharose beads. A biochemical approach was applied to obtain the library of MCoTI-I analogues and affinity on trypsin-immobilized Sepharose beads was utilized to select active ones. Leatherbarrow's group [22] reported on modification (including deletion of fragment 3–6) within the binding loop of MCoTI-II that gave potent inhibitors of physiologically important tryptase

and human leukocyte elastase (HLE). Substitution of Lys10 located in substrate specificity P_1 position by Arg increased inhibitory activity towards the first enzyme, whereas the introduction in this position of Val or Ala gave analogues possessing potent inhibitory activity against HLE. Moreover, considering the fact that a shortened analogue (28 amino acid residues) preserved tryptase inhibitory activity without significantly altering the three-dimensional structure, this proved the impressive adaptability of this cyclotide scaffold. These results correlate well with high proteolytic stability in the stomach and in plasma of cyclic hybrid of MCoTI-II(6-21) and EETI-II(16-28) named McoEeTI [23]. It was pointed out that this inhibitor can be considered a novel pharmacophoric carrier for oral peptide drug delivery.

Perhaps the most promising feature of MCoTIs is their membrane permeability. MCoTI-II biotin labeled at all three Lys residues was internalized into macrophages RAW264.7 and breast cancer MCF-7 cells at a non-toxic concentration [24]. This was the first reported cyclic cell-penetrating peptide. In this first report Craik's group has shown that translocation into cells is mediated through macropinocytosis. By contrast, another cyclotide—kalata B1 analogue (with Thr16 replaced by the biotin labeled Lys)—is not only significantly more toxic but is also not able to cross the cell membrane. This finding opens a new possibility to use MCoTI-II as a carrier of bioactive peptides to intracellular targets. The above mentioned internalization of MCoTI-II was analyzed in fixed cells. Recently, this process was studied on MCoTI-I in live HeLa cells by Camarero's group [25]. Since this inhibitor contains one Lys residue only (corresponding to inhibitor P_1 position), its acylation by AlexaFluor488 N-hydroxysuccinimide yielded a mono-labeled analogue. With the use of various endocytic markers and inhibitors, the authors proved the uptake of AF488-MCoTI-I in HeLa cells following multiple endocytic pathways. Moreover, a significant amount of MCoTI-I was found in lysosomes, and also in late endosomes or other types of acidic organelles. These results were confirmed very recently by Craik's group who re-evaluated their studies; this time on live cells [26]. They observed that Alexa-labeled MCoT-II and also kalata B1 analogue (Thr20 replaced by labeled Lys) were present inside macrophages and breast cancer cells. The first peptide penetrated cells mainly by macropinocytosis, whereas the second did through direct membrane interaction. Interestingly, uptake in MCF-7 cells of the mono-labeled analogue of MCoT-II (at Lys10 only with the remaining two replaced by Ala) was increased by 20 % as compared with the native sequence.

12.5 Sunflower Trypsin Inhibitor 1 (SFTI-1)

Since its discovery in 1999, trypsin inhibitor isolated from sunflower seeds (SFTI-1) has become the object of study for several groups. To date, SFTI-1 is the smallest naturally occurring circular proteinase inhibitor. SFTI-1 is a member of the Bowman-Birk group of inhibitors (I12) retaining the nonapepide binding loop of

other inhibitors of this family, spanning the disulfide bridge. It consists of 14 amino acid residues and, in addition to the circular polypeptide chain, contains the second cycle—the disulfide bridge formed by Cys residues. The P_1-P_1' reactive site is located at Lys^5-Ser^6. Despite the fact that this is much smaller than other BBIs which are composed of 60–90 amino acids, SFTI-1 is the most potent trypsin inhibitor of this family. The elimination of one of the cycles retained inhibitory activity and the three dimensional structures of such analogues. Structure-activity-relationships studies on SFTI-1 have shown that substitution in the P_1 position caused dramatic changes in specificity, opening the possibility to design inhibitors of physiologically important proteinases. Several groups have shown that modifications introduced within the SFTI-1 reactive site and its vicinity led to potent physiologically important enzymes including: chymotrypsin, human neutrophil elastase, cathepsin G, matriptase, β-tryptase, proteinase K, and human kallikrein-related peptidase 4. Nevertheless, none of the analogues displaced inhibitory activity higher than the wild inhibitor. This might indicate that the structure of SFTI-1 is already optimized by nature. Most of the results obtained for SFTI-1 in the first decade of its study are already summarized in review papers [27, 28]; therefore, here we wish to discuss only those published recently. Pereira et al. [29] proposed the use of SFTI-1 analogues with different specificity immobilized on Sepharose as tools to identify proteinases in biological samples. Authors covalently attached SFTI-1 analogues through hydrazone bonds formed by the hydrazine group of Sepharose and the aldehyde moiety of a side chain of *N*-terminal amino acid of the inhibitor. The aldehyde group was introduced by the oxidation of the hydroxyl group of additional Ser coupled to *N*-termini of inhibitors. Such affinity resins display high capacity and stability under experimental conditions. An interesting issue discussed in recent papers was the cyclization mechanism of SFTI-1. Craik's group [30] determined that in the case of SFTI-1, the ratio between intact and modified inhibitors reached 9:1, regardless of whether or not intact or modified inhibitor was incubated with trypsin. More recently, the same group studied cyclization and degradation of series SFTI-1 analogues with cleaved peptides bonds at Arg2, Lys5 [31]. In the case of substrates, proteolysis of a single peptide bond usually gives two products, whereas in these cyclic inhibitors a single product is formed and re-synthesis of cleaved bonds is observed. Interestingly, when an analogue lacking a disulfide bridge was incubated with an enzyme, two peptide bonds were cleaved (after Arg2 and Lys5), tripeptide Arg-Abu-Lys was removed and peptide bond Arg^2-Ser^6 was formed yielding a 11 amino acid residue cyclic analogue. This corresponds with our results on proteolysis of double-sequence of SFTI-1 analogues. Following this mechanism, incubation of these analogues with trypsin or chymotrypsin yielded monocyclic SFTI-1 or [Phe^5]SFTI-1, depending on the amino acid residue present in P_1 position. We proposed to name this re-arrangement of the peptide chain peptide splicing [32].

Recently, Cascales et al. [26] reported that fluorescent Alexa-labeled SFTI-1 penetrates MCF-7 cells and is located in endosomal compartments. The cellular uptake of SFTI-1 is comparable with that determined for Alexa-labeled MCoTI-II, but in contrast to the latter inhibitor, no affinity of SFTI-1 to phospholipids was observed. Taking into consideration that all three cyclic peptides tested (MCoTI,

kalata B1 and SFTI-1) enter cells, the authors proposed that they constitute a new family of cyclic cell-penetrating peptides. Moreover, in our recent work with the help of new fluorescent labels, we have shown that also monocyclic SFTI-1 analogues (with disulfide bridge only) are able to enter cancer cells. The work is in progress, and only preliminary results have been published [28].

12.6 Biosynthesis of Plant Cyclic Peptides

Cyclic peptides discussed herein were found in the plant kingdom. All are composed of proteinogenic amino acids. Their precursors are synthesized on ribosomes that undergo post-translational processing to generate cyclized inhibitors. It was observed that Asp or Asn is a conservative *C*-terminal residue present in cyclotides (including MCoTIs) and also in SFTI-1 precursors. This led to the suggestion that an asparaginyl endoprotease is involved in head-to tail-cyclization in plants. According to the proposed mechanism, in the first step a peptide bond after Asp (or Asn) is cleaved, followed by the formation of the peptide bond with *N*-terminal residue. This mechanism is also supported by experiments showing in vitro cyclization of linear precursors. Although other cyclization mechanisms are not excluded (e.g. the peptide splicing discussed above) the one mediated by asparaginyl endoprotease seems to be the most accepted [33].

12.7 Conclusions and Future Perspectives

Inhibitors of serine proteinase isolated from the seeds of *Cucurbitaceae* and sunflowers are a very exciting group of peptides. They are the smallest naturally occurring proteinase inhibitors composed of proteinogenic amino acids. They display well-defined, compact structures stabilized by disulfide bridges. Some of them are characterized by a head-to-tail cyclic backbone. These cyclic peptides are able to cross cell membranes. Unlike most peptides discussed here, low-molecular plant inhibitors are exceptionally resistant to denaturizing conditions and proteolysis. The observed rearrangement of the polypeptide chain (peptide splicing) upon incubation of some SFTI-1 analogues with serine proteinases is another feature worth considering for further studies. It might be possible to utilize such compounds for transfer and release inside the cell of certain peptidic or peptidomimetic drugs. All the information discussed above makes these plant serine proteinase inhibitors valuable scaffolds for designing compounds with potential commercial possibilities for medical and agrochemical applications. The size of these inhibitors makes them readily accessible by chemical synthesis. In contrast to biological methods, the possible modifications introduced to the chemically synthesized compounds are almost unlimited.

Acknowledgments This work was supported by the National Science Center under No. UMO-2011/01/B/ST5/03772.

References

1. Bode W, Huber R (1992) Natural protein proteinase inhibitors and their interaction with proteinases. Eur J Biochem 204:433–451
2. Laskowski M Jr (2000) Qasim MA, What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes? BBA 1477:324–337
3. Schechter I, Berger A (1967) On the size of the active site in proteases. I. Papain. Biochem Bophys Res Commun 27:157–162
4. Carrell RW, Travis J (1985) Trends α1-antitrypsin and the serpins: Variation and countervariation. Biochem Sci 10:20–24
5. Corral-Rodriguez MA, Macedo-Ribeiro S, Pereira PJB, Fuentes-Prior P (2010) Leech-derived thrombin inhibitors: from structures to mechanisms to clinical applications. J Med Chem 53:3847–3861
6. Rowlings ND, Tolle DP, Barret AJ (2004) Evolutionary families of peptidase inhibitors. Biochem J 378:705–716
7. Wieczorek M, Otlewski J, Cook J, Parks K, Leluk J, Wilimowska-Pelc A et al (1985) The squash family of serine proteinase inhibitors: amino acid sequences and association equilibrium constants of inhibitors from squash, summer squash, zucchini, and cucumber seeds. Biochem Biophys Res Co 126:646–652
8. Pallahgy PK, Norton RS, Nielsen KJ, Craik DJ (1994) A common structural motif incorporating a cystine knot and a triple-stranded beta-sheet in toxic and inhibitory polypeptides. Protein Sci 3:1833–1839
9. Hernandez J-F, Gagnon J, Chiche L, Nguyen TM, Andrieu J-P, Heitz A et al (2000) Squash trypsin inhibitors from *Momordica cochinchinensis* exhibit an atypical macrocyclic structure. Biochemistry 39:5722–5730
10. Ireland DC, Clark RJ, Daly NL, Craik DJ (2010) Isolation, sequencing, and structure-activity relationships of cyclotides. J Nat Prod 73:1610–1622
11. Kupryszewski G, Ragnarsson U, Rolka K, Wilusz T (1985) Synthesis of trypsin inhibitor CMTI III from squash seeds (*Cucurbita maxima*). Experientia 41:1422–1423
12. Favel A, Mattras H, Coletti-Previero MA, Zwilling R, Robinson EA, Castro B (1989) Protease inhibitors from *Ecballium elaterium* seeds. Int J Pept Protein Res 33:202–208
13. Walewska A, Jaśkiewicz A, Bulaj G, Rolka K (2011) Selenopeptide analogs of EETI-II retain potent trypsin inhibitory activities. Chem Biol Drug Des 77:93–97
14. Le-Nguyen D, Mattras H, Coletti-Previero MA, Castro B (1989) Design and chemical synthesis of a 32 residues chimeric microprotein inhibiting both trypsin and carboxypeptidase A. Biochem Biophys Res Commun 162:1425–1430
15. Hilpert K, Wessner H, Schneider-Mergener J, Welfle K, Misselwitz R, Welfle H et al (2003) Design and characterization of a hybrid miniprotein that specifically inhibits porcine pancreatic elastase. J Biol Chem 278:24986–24993
16. Sato S, Kamei K, Taniguchi M, Sato H, Takano R, Mori H et al (2000) Cloning and expression of the Momordica charantia trypsin inhibitor II gene in silkworm by using a baculovirus vector. Biosci Biotechnol Biochem 64:393–398
17. Grzesiak A, Buczek O, Petry I, Szewczuk Z, Otlewski J (2000) Inhibition of serine proteinases from human blood clotting system by squash inhibitor mutants. Biochim Biophys Acta 1478:318–324
18. Milner M, Chroboczek J, Zagorski-Ostoja W (2007) Engineered resistance against proteinases. Acta Biochim Pol 54:523–536
19. Cemazar M, Daly NL, Haggblad S, Lo LP, Yulyaningsih E, Craik DJ (2006) Knots in rings. The circular knotted protein Momordica cochinchinensis trypsin inhibitor-II folds via a stable two-disulfide intermediate. J Biol Chem 281:8224–8232
20. Thongyoo P, Tate EW, Leatherbarrow RJ (2006) Total synthesis of the macrocyclic cysteine knot microprotein MCoTI-II. Chem Commun 27:2848–2850

21. Austin J, Wang W, Puttamadappa S, Shekhtman A, Camarero JA (2009) Biosynthesis and biological screening of a genetically-encoded library based on the cyclotide MCoTI-I. ChemBioChem 10:663–670
22. Thongyoo P, Bonomelli C, Leatherbarrow RJ, Tate EW (2009) Potent Inhibitors of β-Tryptase and Human Leukocyte Elastase Based on the MCoTI-II Scaffold. J Med Chem 52:6197–00
23. Werle M, Kafedjiiski K, Kolmar H, Bernkop-Schnurch A (2007) Evaluation and improvement of the properties of the novel cystine-knot microprotein McoEeTI for oral administration. Int J Pharm 332:72–79
24. Greenwood KP, Daly NL, Brown DL, Stow JL, Craik DJ (2007) The cyclic cystine knot miniprotein MCoTI-II is internalized into cells by macropinocytosis. Int J Biochem Cell B 39:2252–2264
25. Contreras J, Elnagar AZO, Hamm-Alvarez SF, Camarero JA (2011) Cellular uptake of cyclotide MCoTI-I follows multiple endocytic pathways. J Control Relase 155:134–143
26. Cascales L, Henriques ST, Kerr MC, Huang Y-H, Sweet MJ, Daly NL et al (2011) Identification and characterization of a new family of cell-penetrating peptides. J Biol Chem 286:36932–36943
27. Korsinczky MLJ, Schirra HJ, Craik DJ (2004) Sunflower trypsin inhibitor-1. Curr Protein Pept Sci 5:351–364
28. Lesner A, Łęgowska A, Wysocka M, Rolka K (2011) Sunflower trypsin inhibitor 1 as a molecular scaffold for drug discovery. Curr Pharm Design 38:4308–4317
29. Pereira HJV, Salgado MCO, Oliveira OB (2009) Immobilized analogues of sunflower trypsin inhibitor-1 constitute a versatile group of affinity sorbents for selective isolation of serine proteases. J Chrom B 877:2039–2044
30. Marx UC, Korsinczky MJL, Schirra HJ, Jones A, Condie B, Otvos L et al (2003) Enzymatic cyclization of potent Bowman-Birk protease inhibitor, sunflower trypsin inhibitor-1, and solution structure of an acyclic precursor peptide. J Biol Chem 278:21782–21789
31. Colgrave ML, Korsinczky MJL, Clark RJ, Foley F, Craik DJ (2010) Sunflower trypsin inhibitor-1 proteolytic studies on a trypsin inhibitor peptide and its analogs. Biopolymers 94:665–672
32. Łęgowska A, Lesner A, Bulak E, Jaśkiewicz A, Sieradzan A, Cydzik M et al (2010) Inhibitory activity of double-sequence analogues of trypsin inhibitor SFTI-1 from sunflower seeds: an example of peptide splicing. FEBS J 277:2351–2359
33. Gillon AD, Saska I, Jennings CV, Guarino RF, Craik DJ, Anderson MA (2008) Biosynthesis of circular proteins in plants. Plant J 53:505–515

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