Protein Hydrolysates as Biostimulants of Plant Growth and Development

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Abstract

For the actual climate crisis, resilient agriculture is required to guarantee access to enough food, in quality and quantity, for a growing population. To face these challenges, innovative agricultural practices under organic or biological concepts for a sustainable crop production are required. To achieve the goal of sustainable agriculture, it is necessary to incorporate new models, agricultural supplies, and biotechnologies to enhance crop productivity. Plant biostimulants emerge as an innovative option to conventional chemical plant nutrition schemes. Active molecules in these compounds trigger complex physiological and metabolic responses in plants, enhancing plant performance and stress adaptation traits that ultimately result in an increased yield. Biostimulants based on protein hydrolysates (PH) are particularly relevant in the concept of plant stimulation. PH-based biostimulants are produced from different protein by-products and wastes by enzymatic processing, and the mixture of oligopeptides released during these proteolytic events is the main active compound associated with the stimulatory effects observed in different crops. This chapter describes the fundamentals in the technologies used in PH-based biostimulant productions, enzymatic processing, and recent advances in biostimulant research and development, as

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well as the incorporation of new phenomics and transcriptomic technologies to elucidate the mode of action of these biostimulants in a concept of rational design.

Keywords

Bioactive peptides · Proteolysis · Phenomics · Rational design · Ecofriendly · Sustainability

6.1 Introduction

Since the past decade, serious questions are being raised about the overuse of agrochemicals in agricultural systems and their adverse effects on the environment and soil biochemistry. An increase in soil salinization, toxicity, and loss of soil microbial diversity are the most common problems around this production concept that ultimately result in poor crop productivity (Ganguly et al. 2021). This concern has led to an increasing interest in new agroecological alternatives for crop nutrition and management, especially under a climate change scenario. As a result of different research efforts, plant biostimulants emerge as an innovative option to chemicals in agriculture; additionally, it has been proven that the use of these compounds also promotes interesting changes in crop physiology, enhancing environmental resilience, yield, and quality (du Jardin 2015; Colla et al. 2017). Biostimulants differ in their chemical nature, stimulatory mechanism, and efficiency; particularly protein hydrolysates (PH) are considered one of the most complex in composition, as well as in the mode of action, triggering intricate plant responses at the cellular level. Conceptually, PH-based biostimulants must be produced from any protein source (food, waste, or by-product); however, some technical issues arise during its manufacturing, characterization, and testing (Moreno-Hernández et al. 2020). Given the increasing use of PH-based biostimulants in food production, regulation of the market is necessary to provide accurate pieces of evidence of experimental biostimulants and their primary function (Ricci et al. 2019; EBIC 2021). Recently, numerous PHs are recognized as plant biostimulants by improving specific traits in crops, and others are under continuous evaluation by phenomics and omic-based approaches. This chapter highlights some fundamentals involved in the science of PH-based biostimulants, focusing on the technology for its production and evaluation for agricultural purposes.

6.2 Proteins from By-Products for Hydrolysates Production

By-products represent an excellent source of valuable compounds for organic agriculture. Some of these resources are especially rich in protein content that might be recovered, isolated, and bioconverted into add-value products. Annually, large volumes of waste effluents and solid by-products are produced worldwide, only 54 billion pounds correspond animal-derived, and similar volume to

cereal-processing waste, under-utilized foods, and unoptimal edible horticultural fruits (one-third of food for human consumption), unfortunately only a small part of these resources are properly exploited, and transformed into commodities (Martínez-Alvarez et al. 2015; FAO 2021). Table 6.1 indicates the most representative by-products produced by agriculture, livestock, and seafood industries as well as its protein component and summarizes protein recovery principles applied for waste processing. Solid or effluent by-products from vegetable or animal sources are complex matrices with a differentiated composition, not only in protein content but also other macromolecules, including other aggregated proteins, fatty acids, starch, fibers, gums, and polyphenols, rendering difficult its extraction and utilization for PH production. Protein solubility is a key feature for successful protein recovery in general; highly soluble proteins are extracted-solubilized easily by washing process or maceration with low ionic strength solutions (<0.05 M NaCl, pH 4.5–5.5) that include sarcoplasmic fraction in minced meal and green leaves proteins (chloroplastic, cytosolic protein) accounting high recovery yields (70–80%) by combining centrifugation process (Kim et al. 2005; Tamayo Tenorio et al. 2016). Similar approaches have been used in aqueous two-phase partitioning (ATP) for a proper resolution of whole blood proteins (hemoglobin, plasmin, albumin) employing polyethylene glycol/sal combination, recovering around 85% of albumin from blood suspension in the aqueous phase (Rito-Palomares et al. 1998). In contrast, extraction of stromal, myofibrillar, and structural proteins requires the combination of several extractions and fractionation principles. In meat wastes, both terrestrial and aquatic organisms, myofibrillar contractile proteins (myosin-actin complex) represent over 70% (w/w) of total protein content, its separation requires consecutive washing protocols with concentrated chaotropic salt solutions (0.3–0.6 M NaCl or KCl) improving protein extractability in different meat systems (Dara et al. 2021).

Particularly, fish processing waste represents an important source of proteins with biotechnological potential, typically 60–70% of the fish weight is discarded in the form of frames, heads, tails, and guts, representing a suitable resource for myofibrillar, collagen, and elastin protein isolated manufacturing. The pH-shifts process improves the separation of myofibrillar proteins from collagen-enriched structures, by employing isoelectric solubilization/precipitation (ISP). ISP-disruption induces selective solubilization in conditions away from isoelectric point (pI) of proteins, and precipitation near to protein pI (pH 5.5 for myofibrillar proteins) increasing over 90% the concentration of crude hydrolyzable protein (Matak et al. 2015). After separation of collagen-containing tissues (bones, skin, cartilage) from other proteins, insoluble collagen might be extracted by a combination of acid-saline-enzymatic conditions to increase collagen solubilization, the amount of protein recovered vary according to cross-linking grade in collagen molecule, source, and method for processing. Generally, higher collagen yields (80-84%) are obtained by pepsin-solubilized methods in comparison with acid-assisted extraction (Ahmed et al. 2020). Solubility problems are observed in keratin, which is the main component in chicken feathers and horn wastes. Sinkiewicz et al. (2017) describe a method for the preparation of soluble feather keratin, coupling ether pre-treatment (defatting),

Table 6.1 Proteins from by-products and extraction-recovery approaches

Industry	By-products source	Proteins components	Extraction, concentration, and recovery process	References
Agrifoods	Alfalfa Legume seeds Meals Processing wastes Soybean paste Wet/dry-milling	Chloroplastic green proteins Cytoplasmic Germins Globulins Prolamins	Alcoholic-solubilization Centrifugation Filtering Hydrolysis-assisted extraction Hydrothermal extraction pH-shifts Surfactant solubilization Ultrasonic-assisted extraction	Tamayo Tenorio et al. (2016); Tapia-Hernández et al. (2019); Rahman et al. (2020); Rico et al. (2020)
Livestock	Blood Feathers Feet Gut Heads Hooves Leather Wastewater Whey	Albumin Collagen Gelatin Hemoglobin Immunoglobulins Keratins Peptides Whey proteins	Centrifugation Dialysis Extraction by reducing agents Hydrothermal alkaline extraction Hydrothermal hydrolysis Microwave irradiation Superheat process Two/three-phase partitioning Ultrafiltration	Rito-Palomares et al. (1998); Sinkiewicz et al. (2017); Chilakamarry et al. (2021)
Seafood	Carcass Fishmeal Gut Heat Skin Tails Wastewater	Actin Collagen Elastin Myoglobin Myosin Sarcoplasmic Tropomyosin	Acid solubilization Chitonsan flocculation Electro-flocculation Extrusion-hydro-extraction Floating Freeze-drying Isoelectric solubilization/precipitation	Ahmed et al. (2020); Liu et al. (2020); Dara et al. (2021); Venugopal and Sasidharan (2021)

(continued)

Table 6.1 (continued)

Industry	By-products source	Proteins components	Extraction, concentration, and recovery process	References
			Salting in/out Sedimentation Ultra-nano filtration/ fractionation Ultrasound	

and chemical alkaline-hydrolysis reaction in presence of reducing agents (2-mercaptoethanol, sodium *m*-bisulfite, sodium bisulfite, or dithiothreitol) obtaining over 80% of keratin yield. Microbial fermentation, microwave irradiation, and superheat processing have been also discussed in detail for keratin extraction from different natural resources (Chilakamarry et al. 2021).

Agrofood wastes offer extraordinary potential as sources of protein substrates in PH manufacturing. Waste from wet-milling, soy paste, and legumes are excellent sources of albumins, globulins, glutenins, and prolamin proteins, the last one represents around 80% of crude protein content. Prolamins extraction protocols include alcoholic solubilization of cereal meal in 70% ethanol aqueous solution in continuous stirring system, after centrifugation the supernatant containing prolamins is recovered and concentrated by lyophilization. This approach is employed practically for different cereal meals, including barley, sorghum, wheat, and corn, with minor modifications to obtain between 60 and 80% of prolamin protein (Tapia-Hernández et al. 2019). Recently, ultrasonic and hydrothermal processing have been proposed to improve protein/peptide extraction from soy, legume, algal material as an alternative to chemical-based processes (Rahman et al. 2020; Rico et al. 2020).

6.3 Fundamentals in Protein Hydrolysates Production

Protein hydrolysates (PH) are considered as mixtures of polypeptides, oligopeptides, and amino acids released by partial hydrolysis of proteins (Schaafsma 2009). Due to the importance of peptides and amino acids as basic building blocks of proteins and their multiple physiological functions in the plant, the selection of protein source is a key factor to obtain PH-based biostimulant with attractive functions (Popko et al. 2018; Moreno-Hernández et al. 2020). In addition, since amino acid synthesis is a highly energy-consuming process, its presence in PH allows plants to save energy and increase the metabolic rate or *de-novo* reconstruction (Popko et al. 2014).

6.3.1 Amino Acid Content and Profile Analysis

Amino acids are one of the main bioactive ingredients in PHs applied as biostimulants, the accurate determination of its content is important. Generally, protein substrates are hydrolyzed at acidic conditions to their constituent amino acids and consequently are separated by chromatographic techniques, mainly Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). Once the amino acids are separated, its detection and quantification involve the reaction of the amine portion with a derivatizing reagent (Klampfl 2005). The selection of hydrolysis conditions of samples and the kind of derivatization (pre- or post-column) is an important aspect to be considered to obtain reliable results of amino acid composition. For instance, the most common method for protein hydrolysates involves the usage of strong monoprotic acid at high concentration (HCl 6 M) in combination with high temperature (110 °C) under vacuum for 20–24 h; however, this method might lead to loss of Ser, Thr, and Tyr (Jajić et al. 2013). Regarding derivatization reagents, the most common is *o*-phthaldialdehyde (OPA), which reacts with primary amines producing a fluorophore that is able to be excited at 302–395 nm and detected at 420–650 nm (Roth and Hampaĭ 1973). However, the OPA reagent is only suitable to detect primary amines, therefore, other reagents such as 9-Fluorenylmethyl chloroformate (FMOC) is utilized for the derivatization of secondary amino acids, including Hyp and Pro (Turnell and Cooper 1982). The main issue of derivatives reagents like OPA is their limited stability and so must be prepared routinely before each run to avoid interferences (Halket et al. 2005). More recently, the application of high-performance liquid chromatography (HPLC), coupled with tandem mass spectrometry (LC–MS/MS) using hydrophilic columns, offers a suitable method that doesn't need the usage of unstable derivatives reagents and has been used for successful resolution of amino acids profile for animal and plant protein matrices (Kambhampati et al. 2019).

Amino acids are crucial for plant biochemistry, participating as anti-stress (Hyp, Pro) and chelating agents (Cys, Glu, Gly, His, Lys), as well as stimulating chlorophyll synthesis (Ala, Lys, Ser), seed germination (Asp, Glu, Lys, Met, Phe, Thr), and signaling process in hormone metabolism. (Ala, Pro) (Paleckiene et al. 2007; Popko et al. 2018); however, some consideration must be taken into account during protein source processing in order to guarantee amino acid integrity and functionality. In practice, chemical (strong acids or alkalis) and enzymatic methods are used for hydrolysate production, and both strongly affect the amino acidic composition of the final product (Colla et al. 2015). For instance, acid hydrolysis is a low-cost process, but causes the destruction of Trp and a partial loss of Met. Alkaline hydrolysis (using NaOH or KOH) has the advantage of low cost and full recovery of Trp, but can generate the loss of most amino acids (Hou et al. 2017). Also, chemical hydrolysis combining high temperatures (121–137 °C) and acid or alkaline treatment, is considered a drastic process that results in the conversion from L-forms to D-form of amino acids, and the hydrolyzed product is composed by free amino acids and to a lesser extent by soluble peptides, limiting their metabolism and causing other toxic effects in plants (Cerdán et al. 2008). On the other hand, enzymatic hydrolysis can be

performed under mild conditions with precise control of the degree of hydrolysis, minimizing side reactions and the presence of toxic chemicals in the products; thus, the final PH contains higher peptides: free amino acids ratio, and proportion of L-amino acids (proteinogenics) in comparison with those obtained by chemical hydrolysis (Colla et al. 2017; Álvarez-Viñas et al. 2020).

Related to the protein source (animal or vegetal) and amino acid composition of PH-based biostimulants, it has been observed that animal protein hydrolysates from collagen possess elevated concentrations of Gly and Pro, whereas in legume derived PH, Asp and Glu acids are predominant (Ertani et al. 2014; Colla et al. 2015, 2017). In this sense, comparing several protein sources applied as plant biostimulants, it has been observed that Gly concentration of fish and chicken hydrolysates per 100 g of protein is higher than that of alfalfa but less than that of animal gelatin, although this parameter must be strongly influenced by the source (Table 6.2). Legume-derived PH biostimulant Trainer® (Italpollina S.P.A., Italy) comprises mainly amino acids and soluble peptides (75% of free amino acids and peptides), 22% of carbohydrates, and 3% of mineral nutrients (Di Mola et al. 2020; Lucini et al. 2020). The foliar application of Trainer® in spinach and lamb's lettuce (sprayed four times at 21, 27, 33, and 39 days after sowing, at a concentration of 4 mL/L), induced a major improved N uptake/use efficiency compared to untreated plants (Di Mola et al. 2020). Feather keratins have low bio-availability, and it is deficient in amino acids His, Lys, Met, and Trp (Callegaro et al. 2019), while collagen has high assimilation by its significant amounts of Gly, Pro, and Lys (Colla et al. 2015) with distinctive assimilation/functions in plant metabolism and development. The deficiency of some amino acids could limit the biostimulant activity for some PH.

6.3.2 Hydrolysis Degree

Protein hydrolysates are composed of a complex mixture of free amino acids and peptides of different chain lengths. Generally, it is assumed that the degree which indicates that a protein substrate has been hydrolyzed is proportional to the number of peptide bonds broken and, consequently, to the average size and molecular mass of the peptides present. In this regard, the number of peptide bonds broken as a proportion of the total number of peptide bonds present is defined as the percentage degree of hydrolysis (DH) (Rutherfurd 2010).

Protein enzymatic hydrolysis is frequently preferred over chemical hydrolysis for several reasons. For instance, it preserves the nutritional quality of the amino acids better than chemical hydrolysis. In addition, the choice of the enzyme allows the control of protein breakdown at desired DH and drives the hydrolysis toward the desired hydrolysis products (Friedman 1996; Wouters et al. 2016). Although, a high percentage of PHs used as biostimulants are produced by chemical hydrolysis of proteins from animal origin and their production processes are considered harmful to the environment. In this sense, due to enzymatic hydrolysis being ecologically safe, this kind of PH is gaining acceptance for organic agriculture, being the preferred

Table 6.2 Amino acids and the general composition of PH-based biostimulants from different sources

Source	Hydrolysis principle	General composition Amino acid content (five major concentrations, %)		References
Vegetal				
Sunflower seed meal (by-product from biodiesel process); liquid product	Enzymatic hydrolysis (Alcalase-Flavourzyme)	Glu (14.3); Arg (11.8); Leu (8.7); Val (5.9); Asp (5.5)	Protein content: 67.7% Organic matter: 75.8% Majority of peptides comprised low MW (<25 kDa)	Ugolini et al. (2015)
Alfalfa protein hydrolysate; liquid formulation Trainer®; commercial liquid product, derived from legume seeds	Not declared Enzymatic hydrolysis (Enzyme not declared)	Asp (0.99); Glu (0.74); Ala (0.41); Gly (0.36); Val (0.33) Glu (5.4); Asp (3.3.); Leu (2.4); Lys (1.9); Ser (1.7)	Organic matter: 23% Free amino acids: 1.5% Total amino acids: 5.1% Organic nitrogen: 5% Organic carbon: 19% Free amino acids and soluble peptides: 31%	Soppelsa et al. (2018) Paul et al. (2019a)
Chickpea; liquid formulation	Enzymatic hydrolysis (proteases and cellulases)	Arg (0.22); Glu (0.18); Pro (0.17); Leu (0.14); Met (0.12)	Total carbon: 23. 6% Total nitrogen: 2.7% Organic nitrogen: 5.1%	Ertani et al. (2019)
Animal				
Gelatin capsules	Not reported	Gly (27.2); Pro (15.5); Hyp (13.3); Glu (11.6); Ala (11.3)	MW: 40% of peptides around 50–150 kDa	Wilson et al. (2018)
Chicken feathers; liquid formulation of PH (Amino-Hort)	Acid hydrolysis (H_2SO_4/ H_3PO_4)	Glu (23); Pro (20); Gly (19); Asp and Leu (17)	Not reported	Popko et al. (2018)
Porcine by-products; micro granular form (Pepton 85/16®)	Enzymatic hydrolysis (Enzyme not declared)	Leu (10.9); Asp (9.9); Glu (7.2); Lys (7.2); Ala (6.9)	Average MW distribution around 2–3 kDa; 66% of peptides are considered short-chain (with less than 50 amino acids per chain) and 16% are considered long-chain peptides (>50 amino acids)	Casadesús et al. (2020)
Chicken feathers; sprayed PH	Alkaline hydrolysis (KOH)	Pro (13.1); Glu (8.63); Leu (6.86); Gly (6.65); Val (5.40)	Total protein 72.8% Total N: 11.7%	Ebru and Atici (2019)

(continued)

Table 6.2 (continued)

Source	Hydrolysis principle	General composition Amino acid content (five major concentrations, %)		References
Fish by-products (heads and tails); sprayed PH	Enzymatic hydrolysis (Alcalase)	Glu (22.72); Gly (15.79); Ser (14.45); Val (7.42); Leu (7.02)	Total organic matter: 87.2%	Al-Malieky and Jerry (2019)
Chicken feathers; sprayed PH	CO_2-assisted pressure hydrolysis	Ile + Leu (25.9); Gly (24); Val (18.1); Phe (10.1); Lys (8.0)	2.81 g/L of peptides and 0.039 g/L of free amino acids	Schmidt et al. (2020)

option for farmers and informed consumers (Bradshaw et al. 2012; Colla et al. 2015; Caruso et al. 2019; Madende and Hayes 2020).

Several methods exist to determine DH during protein hydrolysis, but there is no standard technique to accomplish reliable results for samples that have been produced by chemical or enzymatic hydrolysis (Spellman et al. 2003). For instance, DH can be measured by determining the amount of nitrogen released during hydrolysis, which becomes soluble in the presence of a precipitating agent such as trichloroacetic acid (Hung et al. 1984). Another approach to determine DH is by quantification of the free amino groups released during hydrolysis using compounds that react specifically with amino groups such as trinitrobenzene-sulphonic acid (TNBS) and *o*-phthaldialdehyde (OPA); several modifications of these techniques exist (Polychroniadou 1988; Caer and Colas 1993; Nielsen et al. 2001). Another technique used to measure DH is the pH-stat method, having the advantage of monitoring the hydrolytic process in real-time, taking advantage of the dissociation of protons from the free amino groups that occurs when hydrolysis is carried out at neutral or alkaline conditions (Adler-Nissen 1986).

Since hydrolysis on protein structure causes a decrease of molecular weight (MW) and also increases the number of ionizable groups and the accessibility of hydrophobic regions in the protein structure (Panyam and Kilara 1996) the biostimulant effect of PH can be affected. For example, Lucini et al. (2020) analyzed the effect of peptide fractions on the performance of a legume-derived PH biostimulant in tomato; interestingly, the smallest (MW <1 kDa) peptides showed the most active stimulatory activity.

6.4 Protein Hydrolysate Production

The production of protein hydrolysates has been increased in the last three decades (CAGR of 6.5% and a market size value of $844.2 m by 2019), and are specially used as additives in food products and feed for animals. This process converts raw

agricultural materials or pure proteins into value-added products for use in several agro-industries However, recently, its uses as plant biostimulants (PB) in agricultural practices has gained relevance for improving nutrition, quality, yield, and abiotic tolerance in different crops (Colla et al. 2015).

PH-based biostimulants can be manufactured from agro-industrial by-products or by using pure proteins. The use of isolated proteins results in better quality products; however, it increases the costs of production. Therefore, the use of protein-rich by-products is more attractive. Protein sources are pre-treated by either heating them with acid, fermented with specific microorganisms, applying separation procedures (e.g., pressing, defatting, sedimentation, centrifugation, filtration, etc.), or adding enzymes to remove undesirable material, as was discussed in previous sections. PH is a complex mixture of polypeptides of different sizes and free amino acids, and their composition and properties are highly variable depending on the protein source, type of hydrolytic method, degree of hydrolysis, fractionation, etc. (Moreno-Hernández et al. 2020). Protein hydrolysate production by enzymatic methods has been the preferred process (around 70% of the PH is produced by this procedure) due to its higher efficiency than acid and alkaline treatments since these last can destroy essential amino acids such as lysine, serine, arginine, and threonine (Fiormarket 2020). On the contrary, enzymatic methods are eco-friendly and hydrolytic-conditions controllable, which yields products with characteristics and quality reproducibles.

6.4.1 Protein Substrates Treatment

Most commercial protein hydrolysate-based biostimulants are plant protein-derived (P-PH); however, animal protein-derived (A-PH) have also gained acceptance due to their satisfactory results and their lower cost (Lucini et al. 2020). Several plants (e.g., legume seeds, alfalfa hay, corn wet-milling, and vegetable by-products) and animal sources (e.g., leather by-products, collagen, blood meal, fish by-products, chicken feathers, and milk proteins), have been used for this purpose. Protein-rich plant material for PH production can be used either minimal processed (raw) or pre-treated to concentrate the protein before its proteolytic enzymatic processing. For example, sunflower defatted seed meal (SDSM) (a by-product from oil production), is concentrated by a sedimentation/flotation fractionation procedure. If required, a further alkaline extraction followed by precipitation at the isoelectric point (pH 4.3) is used to obtain a protein isolate (PI) (Ugolini et al. 2015).

A legume-derived PH-PB, known as Trainer®, is a commercial product manufactured by Italpollina (Rivoli Veronese, Italy) and has become the focus of several studies due to its high activity as a plant biostimulant. It contains 27–31% of amino acids and soluble peptides and has been obtained through a process of enzymatic hydrolysis of proteins derived from legume seed flour, followed by separation of insoluble residual compounds by centrifugation and concentration to obtain a product with a final acid pH (Colla et al. 2015; Lucini et al. 2020).

Proteins from animal sources (e.g., leather by-products, collagen, blood meal, fish by-products, chicken feathers, and milk proteins) have been also used for PH production. Collagen, elastin, and keratins are the prevalent fibrous proteins found in animal by-products generated from meat production. It is estimated that approx. five million tons chicken feather are generated worldwide by the poultry industry, representing an attractive source of protein (approx. 90% of keratin) to convert into PH-PB. However, due to the insolubility and hydrolysis resistance efficient hydrothermal, chemical, biological, or enzymatic processes are required (Callegaro et al. 2019). The use of microorganisms with high keratinolytic activity has been one of the preferred processes for keratin feather hydrolysis. *Bacillus licheniformis, B. subtilis, B. pumilus,* and *B. cereus* are among the most effective feather-degrading microorganisms. However, other bacteria genera such as *Chryseobacterium, Serratia,* and *Stenotrophomonas,* and the fungi *Chrysosporium spp.* and *Aspergillus spp*. have been also considered (Callegaro et al. 2019; Gurav et al. 2020).

Feather microbial fermentation (whole or milled) is usually produced through submerged cultivations with mesophilic (5–20 g feathers/L, 30–40 °C, 24–96 h) or thermophilic bacteria (30–50 g milled feathers /L, 45–50 °C, pH 10.0, 48 h). The hydrothermal and enzymatic process, alone or combined, has been also used for this purpose (Callegaro et al. 2019).

Bryndina et al. (2019) describe a procedure to hydrolyze non-ground pen keratin by applying a pre-treatment with sodium sulfide, urea, sodium thioglycolate, or sodium tetraborate (0.3% by weight) using a ratio of 1:20 (solid: liquid) and pressure of 0.15 MPa for 2 h. Then a protease preparation from *Str. chromogenes* s.g. 0832 at a concentration of 3 U/g of protein was used and the enzymatic hydrolysis was carried out for 6 h with continuous stirring, at 40 °C, pH 8. The degree of hydrolysis (DH) after enzymatic treatment was higher in pen keratin pre-treated with sodium tetraborate (DH 80%), followed by sodium thioglycolate (DH 50%) (Bryndina et al. 2019).

A recent strategy for whey valorization has been the production of PH-based biostimulant. A fermentation process using *Lactobacillus ramnosus* (considered as a plant growth-promoting bacterium, PGPB) under controlled conditions (pH 5.5, 37 °C and agitation at 300 rpm with 0.1% of protease added as inductor), was developed. Lactic acid, peptides, and free amino acids and the biomass of *Lactobacillus rhamnosus* were fractionated with a triple system membrane device (MMS AG membrane System) using a 0.2-μm PVDF membrane to separate *L. rhamnosus* biomass (microfiltration) and a 200-Da MW cut-off TFM membrane to separate the protein hydrolysate (nanofiltration), and the lactic acid recovered by distillation. *L. rhamonsus* presented biocontrol activity against some phytopathogenic microorganisms and the PH and the lactic acid were used as soil biostimulant which induced microbial activity and had a modifying effect on microbial biodiversity, favoring the growth of plant growth-promoting bacterial (Caballero et al. 2020).

6.4.2 Enzymatic Proteolysis Performance

To maximize bioactive peptide/oligopeptide proportion and yield, proteolytic enzymes (proteases) must be added at specific ratios and under controlled conditions. Enzymatic hydrolysates have been developed by utilization of pure proteases, enzymatic mixtures, and raw aqueous preparations extracted from food or by-products themselves (Salazar-Leyva et al. 2017). However, proteolytic enzymes such as Alcalase, Flavourzye, Neutrase, Pepsin, and Protamex are used frequently in both commercial and experimental PH manufacture (Mazorra-Manzano et al. 2018). The selection of proteolytic enzyme is based on operative conditions and specificity. Generally, fermentation-produced enzymes by specific microbial strains (i.e., *Bacillus subtilis*, *Bacillus lichenifromis, Aspergillus flavus Asperguillus niger*) show thermostability in comparison with animal proteases (pepsin, chymotrypsin, trypsin) improving hydrolysis rates (dos Santos Aguilar and Sato 2018). The chemical nature of active sites in protease structure provides remarkable insights into its proteolysis mechanism (enzyme-substrate interaction), pH conditions, and specificity. Specificity parameter determines the positions of clave sites in which the enzyme catalyzes peptide bond break, as well as reveals the nature of amino and carboxylic terminal groups of the released peptides. For instance, flavourzyme ad alcalase preferentially hydrolyses peptide bonds between aromatic residues (Phe, Trp, and Tyr), while papain preferentially cleavages peptide bonds containing large hydrophobic side chains (Tavano 2013). It has been observed that acceptable DH (20–50%) percentage is obtained by using 1–5% w/v of E/S ratio respect protein basis. Higher doses of proteases, especially in their purified forms, induce excessive proteolysis, increasing the content of free amino acids, and might reduce bioactivity by overhydrolysis of oligopeptides. Actually, there are a lot of proteases from animal, microbial, and plant sources employed in the production of a protein hydrolysate, and extensive literature have been generated around protein hydrolysates manufacture and characterizations, as well as a hydrolysis optimization parameter, protease selection, enzyme/substrate ratio, pH, temperature, DH, additives, etc. (dos Santos Aguilar and Sato 2018; Mazorra-Manzano et al. 2018; Etemadian et al. 2021). Most of these enzymes are actually explored in the production of PH-based biostimulant at experimental and commercial levels (Moreno-Hernández et al. 2020). The releases of peptides and amino acids will depend on the extent of hydrolysis and appropriated protease selection, since the specificity of the protease, protein substrate digestibility, DH, among other factors, determining the final characteristics of PH.

6.5 Effects of PH-Based Biostimulant on Crops' Traits

Agro-industry and farmers search for products that promote plant growth, productivity, and quality crop. Biostimulant are other than fertilizer, induce growth and resistance of biotic and abiotic stress; many biostimulants are made from diverse agro and seafood sources, these have macromolecules or chemicals substances, that

can modify physiological processes of plants that enhance nutrient uptake, resistance to biotic or abiotic stresses, and remarkable improvement on crop yield and quality (Xu and Geelen 2018; Ricci et al. 2019; Shukla et al. 2019). Additionally, the fertilizers supplemented with biostimulants (protein hydrolysate, chitosan, algal extract, humic acid, or microorganism) might reduce the drawbacks of agrochemicals fertilizers or pesticides (Drobek et al. 2019; Aktsoglou et al. 2021).

6.5.1 Foliar and Radicular Administration of Hydrolysates

Crops exhibit different physiological responses to the application of PH, which seems to be affected by the protein source and amino acid composition of hydrolysates, application mode, and dosage used (Aktsoglou et al. 2021). PH as liquid, soluble powder, and granular forms promotes macro and micronutrient assimilation when these compounds are applied in a foliar spray and radicular manner (Fig. 6.1), stimulating plant metabolism with a potential effect on the quality and yield, in many crops (Calvo et al. 2014; Nardi et al. 2016; Colla et al. 2017). Amino acid composition is a key feature in the stability of PH employed in crop nutritional programs. It has been proposed that PH incorporated into fertigation solutions requires a high balance of hydrophilic/hydrophobic oligopeptides (with high solubility) to prevent insoluble aggregates or undesirable interactions with other nutrients, especially mineral, due to chelating properties of some amino acids (Schiavon et al. 2008; Ertani et al. 2013b). Moreover, radicular plant systems, root secretions (hydrolytic enzymes), soil biochemistry, and root absorption coefficient are some critical aspects for optimal utilization of peptides through the root system (Moreno-Hernández et al. 2020). While leaves' porosity (stomata activity) is critical for proper peptide or amino acid acquisitions during the foliar application, other factors involved are peptide size/sequence, relative humidity, temperature, evaporation parameters, and leaf area to improve diffusion (Koukounararas et al. 2013). For instance, Sestili et al. (2018) showed that the application of PH is more effective in improving plant growth and total N uptake than foliar sprays. This is because free amino acids in PHs have been reported to activate nitrate transporters.

Amino acids in PH represent an important source of nitrogen that could be equally effective such as inorganic nitrogen fertilizers when they are used as a nutrient hydroponic solution (Aktsoglou et al. 2021). The hydroponic cultivation of peppermint and spearmint has not affected plant growth either positively or adversely by the addition of PH Amino16® (Evyp LLP, Greece) in the nutrient solution and was attributed to either the increased root growth on or to the low rate of PHs applied (lower than 0.5%) (Aktsoglou et al. 2021). Pieces of evidence suggest that amino acids and small peptides derivated from PH, are uptake and translocated by amino acid transport proteins involved in phloem loading and unloading, xylem-phloem transfer, import into seed, and intracellular transport in plants from leaves or root tissues (Yang et al. 2020). Foliar application of PH can increase amino acid and peptide availability for plant uptake by reducing the competition with a microorganism (Colla et al. 2015). Glu is rapidly absorbed by creeping bentgrass foliage and

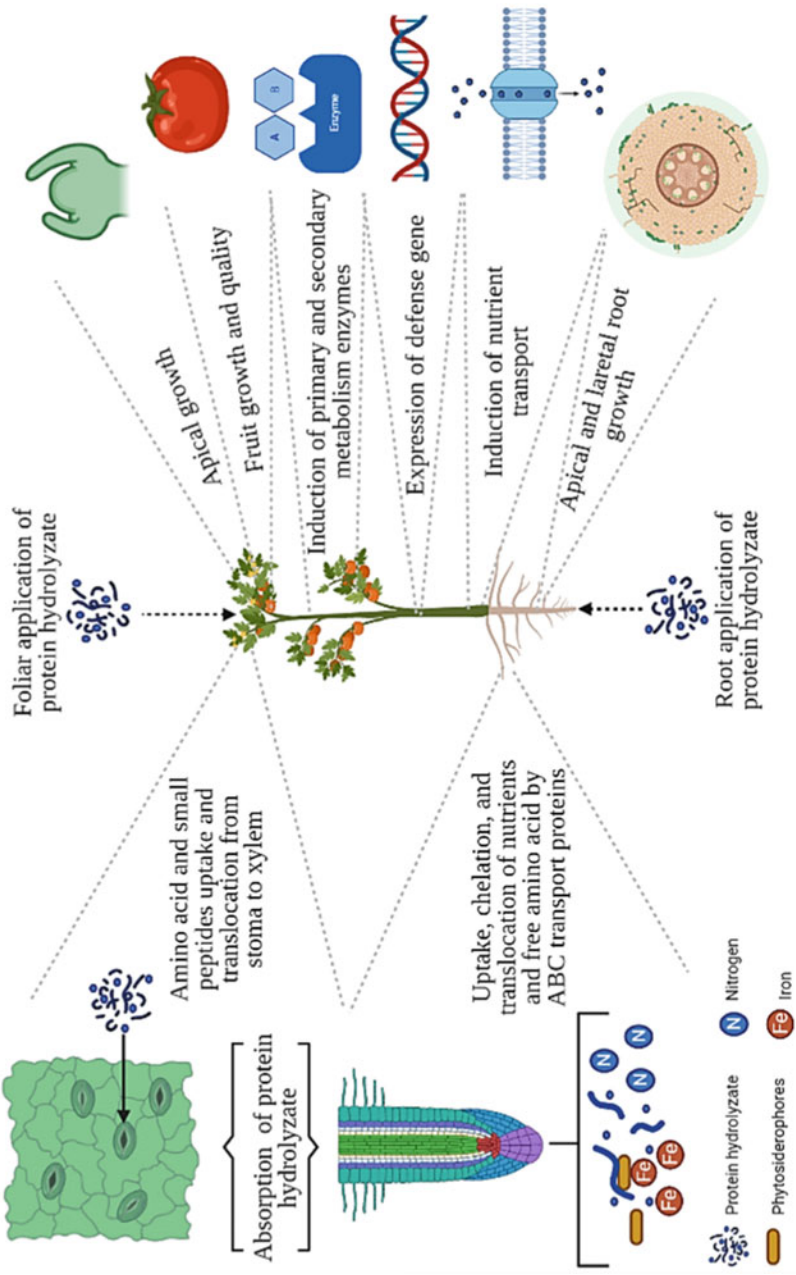

Fig. 6.1 Schematic view of the effect of foliar and root application of protein hydrolysate effects on tomato plants. (Created with BioRender.com)

directly utilized as a precursor to synthesize gamma-aminobutyric acid and proline, two important metabolites with well-known roles in plant stress adaptation (Rouphael and Colla 2020). Tryptophan is considered a fundamental amino acid for the synthesis of indoleacetic acid (IAA), a hormone with important functions on plant growth. However, its activity can be affected when it is applied separately. In a study, $_{L}$-methionine stimulated lettuce growth parameters; however, distinct effects have been observed when L-Gly and L-Trp were applied radicular on butterhead lettuce hydroponically grown (Rouphael and Colla 2020). Paul et al. (2019a) suggest that foliar application of PH reach mesophyll cells by absorption trough cuticle, epidermal cells, and stomata, while in drench or hydroponically application, the absorption occurs through root epidermal cells via ABC membrane transport and gets redistributed through the xylem. Most PH-based biostimulant induces positive physiological effects as growth and development; moreover, it enhances uptake nutrient from soil or microorganism of the rhizosphere; however, to perform the desired effect, PH must be able to penetrate the plant tissue at low dosages, depending species, cultivars, and vegetative stage, but also depend on environmental condition, stomata, and cuticle that act as a barrier (Pecha et al. 2012).

6.5.2 Effects in Crop Growth and Quality

Biostimulants have significant effects on many crop traits related to productivity and quality, including root architecture, change in endogen phytohormone levels, photosynthetic rate, increased pigment content, protein, phenolic contents, stimulate the growth, antioxidant activity, and enhance macro and microelements in vegetal tissues (Yakhin et al. 2017; Drobek et al. 2019; Ertani et al. 2019; Ambrosini et al. 2021). Table 6.3 includes a description of the most applied PH-based biostimulants, their intended use, and primary functions.

PH-based biostimulants probably contain molecules that display phytohormone-like activities as has been proposed in a recent revision (Moreno-Hernández et al. 2020). PH-containing peptides might act as auxin-like and gibberellin-like elicitors, triggering signaling as naturally occurring peptides in plants and promoting vegetative plant growth, and early maturation of fruits (Drobek et al. 2019), effects triggered by some endogenous regulatory signaling peptides and protein-like hormones (e.g., phytosulfokine) influencing productive traits such as fruit maturation, root length, and thickness of stem, or inducing primary and secondary metabolism biosynthesis through the activation of multiple signaling pathways that involve second messengers that stimulate enzymes of the nitrate assimilation pathway, like nitrate reductase and glutamine synthetase which catalyze a rate-limiting step in nitrogen assimilation (Ertani et al. 2013a). In this context, Ertani et al. (2019) reported indole-3-acetic acid (IAA)-like and gibberellin (GA)-like activities of PH, obtained from *Cicer arietinum* L. and *Spirulina platensis*, and that they induced plant growth and accumulation of N-compounds (proteins, chlorophylls, and phenols) on hydroponically *Zea mays* L. culture; furthermore, PH from *C. arietinum* and *S. platensis* increased the activity of two enzymes (peroxidase and esterase)

Table 6.3 Commercial PH-based biostimulants' functionality and target crops

Trademark	Purpose	Intended use	Source
AminoHort®	Micronutrient deficiency corrector Nutrient uptake enhancer	Fruit trees, grapevine, greenhouse vegetables, industrial crops	USAGRO (2021)
AminoPrim®	Stress modulator Metabolic regulator Increase stress tolerance and plant recovery Improve yield quality and quantity	Flax, fruit trees, olive trees, fruit bushes, berries, grapevine, citrus, coffee, vegetables, ornamentals, lawns, plant nurseries	INTERMAG (2021)
Brown's Fish Hydrolysate®	Nutrient uptake regulator	Broccoli, ornamentals, grasses, oats	BrownsFish Genesis (2021)
Hydrostim®	Growth promoter Anti-stress regulator	Citrus, greenhouse vegetables, kiwifruit, olive, strawberry, grape	Hydro Fert (2021)
Ilsadrip Forte N9®	Growth promoter Radicular stimulant	Banana, fruit trees, horticultural crops, mango, vid, wheat	Ilsagroup (2021)
Pepton 85/16®	Growth promoter Phytohormonal-like action Photosynthetic activity enhancer Increase nutrient uptake, yield, and quality	Carrot, chili, citrus, industrial crops, lettuce, onion, ornamentals, potato, rice, tomato, vid, watermelon	FEMSSA (2021)
PROTIFERT LMW®	Nutrient uptake enhancer metabolic stimulant	Citrus, fruit trees, pineapple, melon, onion, ornamentals, peanut, rice, tomato, carrot, broccoli, sugar cane	SICIT (2021)
Siapton®	Growth promoter Anti-stress regulator (salinity, drought, low temperatures, transplant shock) Increase micronutrient intake and pollen germination	All crops	Isagro (2021)
Sinergon 3000®	Promotes vegetative and productive growth Plant recovery from environmental and physiological stress Increases fruit size and development of new vegetal tissues (buds, sprouts) and on fruit swelling	Non declared by the manufacturer	Cifo Srl (2021)

(continued)

Table 6.3 (continued)

Trademark	Purpose	Intended use	Source
StresSal®	Osmotic regulator for saline stress	Citrus, fruit trees, greenhouse vegetables, olive, ornamentals, strawberry	Bioiberica (2021a)
Terra-Sorb® Radicular	Growth promoter Improves nutrient absorption	Leafy vegetables, citrus, fruit trees, tomato, olive, spinach, strawberries, vid, industrial crops	Bioiberica (2021b)
Trainer SP®	Anti-stress regulator Growth and yield stimulator Fruit color and sugar content, fruit caliber, homogeneity enhancer	Non declared by the manufacturer	ITALPOLLINA (2021)

related with plant growth and differentiation of organogenesis; in the same way, Casadesús et al. 2020 reported hormonal signaling for improving root growth in tomato (*Solanum lycopersicum*, var. Ailsa Craig) plants, mediated by chorismate-derived hormones, in particular by salicylic acid.

Recently, Ceccarelli et al. (2021) reported foliar application of vegetal-derived PH on tomato cutting-promoted rooting and biomass density, length, and the number of lateral root branching, with promoting plant growth and development owing to stimulation of auxins (particularly precursors as 4-(indol-3-yl) butanoate and tryptamine), cytokinin, and gibberellin biosynthesis or IAA precursors. The foliar application (spraying) of animal-derived PH (from fish by-products) on lettuce, showed significant effects on the total number and area of leaves, carbohydrate, proline, shoot-fresh weight of plants, dry matter, total soluble solids, and total yield (Al-Malieky and Jerry 2019). In celery, Plant-PH Tyson® obtained from soy protein extract, Trainer® (legume-derived PH), and animal-PH Aswell® (bovine epithelium hydrolysate) improved plant growth and nutritional balance in both foliar and radicular applications (Consentino et al. 2020). Recently, the biostimulant activity of five plant-derived PH on tomatoes was evaluated for their ability to promote rooting in tomato cuttings following quick dipping. All PHs increased root length (45–93%) and root number (37–56%) (Ceccarelli et al. 2021).

Since, in addition to its biostimulant activity, PH also mediates plant adaptation to several stress conditions, including mineral depletions, cold, thermal, drought, and saline. StresSal® and Trainer® have been employed as osmoregulator in both fruit trees (persimmon) and horticultural crops (lettuce), to avoid the negative impact of saline stress (Visconti et al. 2015; Luziatelli et al. 2019). Experimental blood-derived PH and commercial Amino 16®, displayed protective effect in lettuce cultivated under extreme climate conditions, hydrolysates showed thermo-protective functionality toward warm and chilling temperatures, respectively (Polo et al. 2006; Tsouvaltzis et al. 2014). Some studies suggest that the accumulation of glycine betaine and proline is associated with increased stress tolerance, and the exogenous

application of protein-based compounds in maize, barley, soybean, alfalfa, and rice has been highly correlated (Ahmad et al. 2013). Ambrosini et al. (2021) evaluated on hydroponic culture the capacity of a commercial PH derived from bovine collagen to mitigate Fe deficiency stress in roots of *Zea mays*, and observed that PH exhibited an increased growth and absorption by chelation of Fe area of the roots compared with control treatment; these studies show how PH have a positive effect by foliar or hydroponic culture; in another way, PH drench application on *Solanum lycopersicum* L. plants enhance transpiration rate and transpiration use efficiency with a positive impact on the biomass and metabolic profile (Paul et al. 2019a). Protective effects in some PH-based biostimulants have been attributed to proline or proline-precursors compounds (glutamate an/or ornithine) in hydrolysates, due their osmolyte and chemical chaperone roles under various stressful sceneries during plant development.

A clear mechanism for peptides found in PH-PB remains uncertain, but most pieces of evidence suggest that PH does not only provide nutrients directly to plants but these compounds also stimulate plant nutrient acquisition processes and is an alternative to diminishing chemical fertilizers. Protein hydrolysates induce a positive effect on plant crops, containing signaling peptides and free amino acid that enhance germination, seedling growth, fruits, and vegetable quality as well as crop productivity (Rouphael and Colla 2020). Table 6.4 shows some examples of PH whose potential as plant biostimulants has been tested for many plant traits such as morphophysiological parameters (stem, leaves number, foliar area), flowering time, fruit set-filling, crop productivity, and nutrient use efficiency. Until now, clear identification and characterization of the peptides (or amino acids) in PH related to the PB activity and associated mechanism of action has not been determined. Therefore, an important characteristic to be included in PH-PB characterization could be the peptidic size fraction, in addition to the common parameter considered such as DH, chemical composition, and amino acids content (Moreno-Hernández et al. 2020).

6.6 Approaches to Elucidate PH Mode of Action

High crop productivity is the ultimate goal of agricultural systems that employ PH-based biostimulants in their production practices. Farmer decision about the application of a particular biostimulant must be supported by accurate information about biostimulant quality and safety, effectivity, and also by the mode of action of the active ingredients or any other parameter about primary-secondary function (EBIC 2021). A collection of evidence about PH function as plant biostimulant has been recovered experimentally under different production systems and are based on the agronomic parameter; however, the trend around these biostimulants is turned into a more multidisciplinary approach, to validate a new generation of PH-based biostimulants employing precision testing technologies and genomic-based tools.

Table 6.4 Effect of PH-based biostimulant on crop traits under different growth systems

Crop	Biostimulant	Application mode	Enhanced traits	Reference
Growth chamber				
Lettuce	Alfalfa hydrolysate	Radicular	Primary-secondary growth/development Root architecture, branching, and root tip density Root physiology by increase in nutrient uptakes Root system length, mass, surface area	Ertani et al. (2009)
	Hemoglobin hydrolysate	Radicular	Induce root stress responses to thermal stress Root dynamic/phenology maintaining primary specific growth rate, mass and surface area Leaves biomass and yield	Polo et al. (2006)
Maize	Connective-tissue hydrolysate	Radicular	Stimulated secondary root growth/development Stem length, size, and mass Root physiology by increase in nutrient uptakes	Ertani et al. (2009)
	Cow connective tissue	Radicular	Enhance root physiology stress response reactive species Stem length and biomass	Ambrosini et al. (2021)
Tomato	Skin fish hydrolysate	Radicular	Seedling vigor, germination rate Leaves dynamic, chlorophyll synthesis Photosynthetic rate Stimulated secondary root growth/development	Horii et al. (2007)
	Legume-derived hydrolysate	Foliar/Radicular	Root length, mass, surface area, hair density	Ceccarelli et al. (2021)
Greenhouse				
Chickpea	Chicken feathers hydrolysate	Radicular	Germination rate, seedling vigor, transplant adaptation Stimulated secondary root growth/development Root dynamic/phenology maintaining primary specific growth rate, mass, and surface area	Paul et al. (2013)

(continued)

Table 6.4 (continued)

Crop	Biostimulant	Application mode	Enhanced traits	Reference
Cucumber Broccoli	Gelatin hydrolysate	Radicular	Early hypocotyl emergency (seed priming) Seedling length, mass, stem length and diameter Shoot mass, dry weight Root dynamic uptake nutrient	Wilson et al. (2018)
Lettuce	Legume seeds hydrolysate	Foliar and radicular	Increase stem growth rate protein and chlorophyll production Root physiology, stress response to ROS indicators Osmoregulation un saline conditions	Lucini et al. (2015)
Lettuce	Trainer®	Foliar	Enhanced plant growth, productivity Uptake nutrients Osmoregulation to salt stress	Luziatelli et al. (2019)
	Amino 16®	Foliar and radicular	Nutrient uptake Leaves morphology uniformity, yield Leaves chemistry quality, secondary compounds (antioxidant)	Tsouvaltzis et al. (2014)
Maize	Alfalfa hydrolysate	Radicular	Leaves length, foliar area, mass Grain yield Root dynamic, macronutrients content	Schiavon et al. (2008)
	Sicit2000®	Radicular	Root growth, length, and surface area Uptake nutrients, micronutrients content Stress response indicators	Santi et al. (2017)
Pepper	Alfalfa hydrolysate	Foliar	Foliar fresh weight Fruit set, filling Number of fruit per plant Root chemistry secondary compounds Fruit nutraceutical quality, yield	Ertani et al. (2014)
Snapdragon	Hydrostim®	Foliar/ radicular	Leaves photosynthetic rate Transpiration rate, conductance Root chemistry nutrient uptake and root nitrogen content, photosynthetic rate, transpiration rate, and stomatal conductance	Cristiano et al. (2018)

(continued)

Table 6.4 (continued)

Crop	Biostimulant	Application mode	Enhanced traits	Reference
			Primary/secondary growth/ development Branching, root tip density, branching intensity	
Strawberry	Hemoglobin hydrolysate	Radicular	Root length, mass, surface area Increases biomass production and yield Flowering time Fruit set and filling	Marfa et al. (2008)
Tomato	Trainer®	Radicular	Root dynamic, phenology, chemistry Root anatomy and architecture, branching density Root dry weight	Sestili et al. (2018)
Field trial				
Apple	Alfalfa protein hydrolysate	Foliar	Fruit quality trait (color index, sugar content) Fruit nutraceuticals (anthocyanin content) Biotic post-harvest resistance	Soppelsa et al. (2018)
Banana	Chicken feathers hydrolysate	Foliar/ radicular	Leaves photosynthetic rate and chlorophyll content Flowering time Fruit set, filling, and yield Fruit quality (antioxidant and nutraceutical)	Gurav and Jadhav (2013)
Celery	PHs from soy extract and bovine animal epithelium	Foliar	Whole plant length, weight Nutrient uptake Yield	Consentino et al. (2020)
Grapevine	Carob germ hydrolysate	Radicular	Fruit quality traits, nutraceutical value Plant growth/development	Parrado et al. (2007)
Lettuce	Fish hydrolysate	Radicular	Leaves number Stem diameter, shoot fresh and dry weight Quality trait succulence and nutraceutical	Xu and Mou (2017)
Maize	Chicken feathers hydrolysate	Foliar	Root surface area, nutrient uptake Grain yield	Tejada et al. (2018)
Persimmon	StresSal®	Radicular	Plant adaptation Stress response indicator ROS Osmoregulation	Visconti et al. (2015)

(continued)

Table 6.4 (continued)

Crop	Biostimulant	Application mode	Enhanced traits	Reference
Soybean	Terra-sorb® complex	Foliar	Pods numbers Seed yield Seed quality (phenolic, flavonoid) Oil content	Kocira (2019)
Tomato	Pepton 85/16®	Foliar/ radicular	Plant height Stem diameter Flowering time Fruit set and yield	Polo and Mata (2018)
Wheat	Terra-sorb® complex	Foliar	Leaves area, mass, photosynthetic rate Seed sugar content, yield	Martinez-Esteso et al. (2016)

6.6.1 High-Throughput Phenotyping Characterization

Biostimulants induce significant changes in crop development at different physiological levels. Most results on biostimulant efficiency have been based on agronomical traits (germinations, adaptation, flowering time, fruit set, and yield), monitoring phenotypical changes to identify biostimulant candidates, and also provide clues about its modes of action. However, conventional phenotyping protocols for reporting these traits might prove time-consuming, laborious, with low reproducibility, and strong subjectivity. Additionally, some of these methods are destructive and unsuitable for large-scale probes. This has driven the development of new tools for the automatic management of crops, and the continuous monitoring of plants treated with biostimulants. The concept of high-throughput phenotyping (HTP) in agriculture emerged with the necessity of high-precision systems for data recovery in agronomy; these powerful robotic tools can analyze broad scenarios and their influences on plant traits, also known as plant phenomics. In the field of biostimulants, HTP has been used to evaluate the influence of active components in biostimulants on physiological plant traits quantitatively and enables to compare dynamic plant-environment responses in a real-time manner (Dalal et al. 2019).

During the development of new biostimulants, HTP platforms have provided an accurate assessment of different active products contained in protein hydrolysates. An interesting feature of this platform is that a set of biostimulants can be assayed in a wide range of conditions, including water stress, nutrient deficiency, temperature stress conditions (heat/cold), and light intensity, in a continuous lab-field-lab cycle (Rouphael et al. 2018). In a drought model, this technology was used to analyze the morphophysiological traits of Trainer®-treated (spray or substrate drench) tomato plants, under semi-controlled greenhouse conditions. By employing imaging sensors, visible red, green, blue images for digital biomass increase, and fluorometers to report photosynthetic performance, HTP identified the best-performing plants as an effect of biostimulant applications. Moreover, analysis of

spectral data revealed the most active photosynthetic tissues and their correlation with biomass accumulation, and the metabolomics profiling of stimulated plants, annotated over 1900 compounds associated with ROS signaling, sterols, carotenoids, membrane lipids, phytohormones, polyamines, and chlorophyll-related molecules (Paul et al. 2019a, b). Similar approaches have been applied to understand the role of biostimulants on peppers' productivity and survival under drought conditions (Dalal et al. 2019). Recent advances in imaging acquisition technology as spectrograph (hyperspectral analysis) offers new opportunities in the field of biostimulant research by analysis of whole plants; till date, around 16 crops, including barley, maize, potato, grapevines, wheat, oak, and peppers have been analyzed with this technology (Mishra et al. 2020). Although these studies do not contemplate the use of biostimulants, the tests evidence the efficacy of these systems for the high-throughput phenotyping in a variety of conditions.

6.6.2 Metabolomic Analysis

Plant metabolite profiling is an emerging field to describe cellular plant response to a wide range of biotic or abiotic conditions. The metabolomic approach seeks to establish a relationship between cellular metabolites and a specific variation factor, as well as its influence on particular traits shown by plants (Schauer and Fernie 2006). In the field of plant sciences, metabolomics is a powerful tool to integrate new metabolic pathways to omic-data, for high-throughput analysis, combining analytical techniques like Liquid/Gas Chromatography-MS/MS systems to explore primary/secondary plant metabolites in many economically important crops like maize, rice, tomato (Sharma et al. 2021). Metabolomics analyses have been applied to profile the metabolite change of plant to HP-based biostimulants.

Plant crops treated with protein hydrolysates derivatives from animal, plant, or algae sources induce growth and development of fruits, leaf, roots, and phytochemicals metabolites—in tomato, the application of PH-enhanced root and quality of fruits with increased diameter, weight, and volume—these effects were reported in diverse crops such as, kiwifruit, papaya, banana, passionfruit, and vegetables such as lettuce and pepper (Rodrigues et al. 2020; Ceccarelli et al. 2021). Furthermore, several studies show that application of PHs stimulate secondary metabolites with antioxidant activity such as carotenoids, polyphenols, and flavonoids, as well as defense metabolites like alkaloids, salicylic acid, jasmonates, and ethylene, as well as phytoalexins as indole-3-carboxyl and psoralen (Casadesús et al. 2020; Lucini et al. 2020; Ambrosini et al. 2021), thus promoting crop productivity. The benefit of PH is not only over crops but also has beneficial effects on the microbiome of the rhizosphere, improving physiological and development processes in plants, favoring greater nutrient and water uptake as well as enhanced resistance against biotic and abiotic stress. PH also promotes nitrogen assimilation via coordinate regulation of carbon and nitrogen metabolism, by inducing activity enzymes as nitrate reductase, nitrite reductase, glutamine synthase, glutamate synthase, and aspartate aminotransferase (Ertani et al. 2009, 2019; Sestili et al.

2018; Paul et al. 2019a), and carbon metabolism as malate dehydrogenase, isocitrate dehydrogenase, and citrate synthase (Ertani et al. 2013a), or esterase and peroxidase enzymes that have a role in meristematic growth that induces vegetative development (Ertani et al. 2019), as well HP drench, foliar, or hydroponic application induce high-affinity nitrate transporters belonging to NRT2 family: NRT2.1 and NRT2.3, that play a key role in the coordination of root development, acting on lateral root initiation and nitrate uptake and long-distance transport system from root to shoot (Sestili et al. 2018; Paul et al. 2019a), as well as iron transporters (ZmTOM1 and ZmIRT1) involved in phytosiderophores and FeII assimilation (Ambrosini et al. 2021), the PH application favoring minerals absorption and transport, known as nutrient acquisition response (Rouphael et al. 2021).

Metabolomic data of four plant-derived PH indicate a reprogramming of phytohormones profile by modulating gibberellin and cytokinin biosynthesis with a lower effect on auxins and brassinosteroids biosynthesis. The hierarchical cluster analysis (HCA) revealed that metabolomic profiles in roots were significantly influenced by PHs foliar application, in a PH-dependent specific manner, providing evidence that such hormone-like activity of PHs depends on the protein source (Ceccarelli et al. 2021).

6.6.3 Differential Gene Expression Analysis

In the last decade, improvements in Next-Generation Sequencing (NGS) technology have led to a substantial increase of data about plant genetics, providing relevant knowledge about genome functionality. This information is fundamental in the construction of transcriptomes, employing RNA-seq analysis. Due to the transcription process is the very first genome response to biotic and abiotic stimulus; this provides a complete scenario about changes in expression patterns, genomic information flux, and gene network enrichment in plant cells for a particular time-lapse. Fundamentally, RNA-seq reflects the total RNA identities produced by a single cell or tissue that has been successfully sequenced and mapped to an annotated genome or curated transcriptome dataset. Coupled with bioinformatics exploration, RNA-seq tells us about a group of genes and gene networks significantly upregulated or downregulated as a response to certain factors (Differential Gene Expression Analysis; DGEA) like variations in nutritional status, phenological stage, light/dark cycle, environmental conditions, and also biostimulant application.

Advances in NGS have reduced the cost of sequencing services and allowed access to this technology by biostimulant developers. This approach has gained relevance in the field of biostimulant research, providing not only accurate evidence about the effects of these compounds in crop development but also recovering robust data to elucidate the mode of action of biostimulant at the molecular level. Although NGS is relatively time-consuming and costly in comparison with non-omic approaches (limiting its application to some horticultural crops), the number of available genomes in databases is growing faster than ever, and this is impacting the number of scientific reports documenting biostimulant uses. Transcriptomic-

wide identification of DEG has been used to explore the mechanistic process of plant growth-promoting bacteria (González-Morales et al. 2021), humic substances (Galambos et al. 2020), and PH-based biostimulants (Trevisan et al. 2017). For instance, DGEA has been used to understand the influence of PH-based biostimulants at the transcriptional level in crops like cucumber, maize, soybean, and tomato (Table 6.5). Wilson et al. (2015) evidenced the complexity of transcriptional regulation on two-week-old cucumber seedling by hydrolyzed collagen (gelatin) capsules, employing the RNA-seq data to coexpress gene network construction. In this spot trial controlled conditions, gelatin biostimulant induced 620 differentially expressed genes (DEGs), grouped in five modules by Weighted Gene Coexpression Network Analysis (WGCNA) with interconnected hub networks. An upregulation of phloem amino acid, N-transporter genes (amino acid transporter, amino acid permease, ammonium transporter), and nitrogen metabolism, as well as detoxifying mechanism (Glutathione S-transferase), was evidenced, indicating its relevance at first-level of plant response during the early stage of biostimulation process (Wilson et al. 2015). Other reports have evidenced new target molecules in tomato seedlings exposed to alfalfa hydrolysates, inducing overexpression of stress-related genes as phytohormone modulation, antioxidant-related genes, phenylpropanoid pathways, detoxification process, and MAPK signaling pathway in crosstalk between biotic and abiotic stress responses (Ertani et al. 2017). A recent study reveals insights on the mechanistic action of PH-stimulant by combining transcriptomic and quantitative proteomics approaches. By examining maize seedlings exposed to commercial PH-stimulant under chamber controlled conditions, Ebinezer et al. (2020) identified 608 DEGs and 242 differentially abundant proteins (DAP) at a high dosage of biostimulant. The bioinformatics to construct Gene Ontology Enrichment (GO terms), clustered DEG and DAP into 20 categories associated with stimulus responses, including osmotic, salt, hormone regulation, brassinosteroid metabolic, and biosynthesis, phenylpropanoid, lignin, siderophore, and homeostasis maintenance. Pathway Enrichment Analysis discriminates significant metabolic pathways impacted by PH, mainly metabolic pathways, biosynthesis of secondary metabolites, glutathione metabolism, and amino acid metabolism, all with up/down-regulated genes under essay conditions (Ebinezer et al. 2020).

These studies evidence all possible target molecules in broad metabolic pathways for biostimulant design; however, this scenario evokes a major challenge in the development of active molecules as plant biostimulants. Information about the size, sequences, and functionality of peptides are characteristics that need to be included in PH characterization to associate their putative role as plant biostimulants (Lucini et al. 2020; Moreno-Hernández et al. 2020). Until now, a clear identification of peptides found in PH (with diverse composition, characteristics, and properties) responsible for the plant biostimulation activity has not been completed and established. Figure 6.2 proposes an interdisciplinary approach to biostimulant rational design to provide accurately integrated evidence on the effectivity of plant stimulation phenom. To accelerate the re-investigation on the first and new generation of plant biostimulant based on protein hydrolysates.

Table 6.5 Transcriptional changes in crops exposed to PH-based biostimulant

Crop tissue	Biostimulant	Experimental conditions	Change in expression pattern	References
Cucumber leaves	Collagen hydrolysate	Seeds planted with gelatin capsules (radicular) in spot trial under controlled greenhouse conditions. Temperature 24/21 °C, 14/10 h photoperiod	620 DEGs respect control conditions KEGG: Cell wall degradation, photosynthesis, hormone metabolism, abiotic stress response, signaling, plant development, transcription factors, amino acid transporter, and metabolism	Wilson et al. (2015)
Maize seedling lateral roots	APR®	Seedlings treated by irrigation with two increased concentrations of APR under pot trial essay, grown in a climatic chamber to maintain standard conditions, 70/90% relative humidity, 14 h day/10 h night cycle, and 280 µmol m^{-2} s^{-1} photon flux density	608 DEGs in response to the high concentration of APR KEGG: Biosynthesis of secondary metabolites, metabolic pathways, phenylpropanoid biosynthesis, monoterpenoid biosynthesis, glutathione metabolism, cysteine, and methionine metabolism	Ebinezer et al. (2020)
Soybean cotyledons	KIEM®	Seed soaked with biostimulant solution for a complete distribution on the seed surface. Primed seeds incubated under heat stress (35 °C), 24–48 h until germination	Biostimulant-treated seeds showed 879 DEGs after 24 h of incubation at 35 °C, 93% of genes were downregulated GO: stimuli and chemical response, hormone stimuli response, programmed cell death, oxidative stress response, carbon metabolism, transferase activity, cell wall organization or biogenesis	Campobenedetto et al. (2020)

(continued)

Table 6.5 (continued)

Crop tissue	Biostimulant	Experimental conditions	Change in expression pattern	References
Tomato seedling leaves and roots	Alfalfa hydrolysate	Tomato seed germinated on agar medium under grow chamber conditions, 70/85% relative humidity, 2621 °C air temperature, 14 h day/10 h night cycle, and 280 µmol m^{-2} s^{-1} photon flux density. Seedling in spot trial treated by fertigation with hydrolysate	2988 DEGs (1938 leaves, 1054 roots) GO: organic substances metabolic process, primary metabolic process, cellular metabolic process, nitrogen compounds metabolic process, response to stress, catabolic process, cellular component biogenesis, response to abiotic stimulus	Ertani et al. (2017)

Differentially expressed genes (DEGs), Kyoto Encyclopedia of Gene and Genomes (KEGG) Pathway, Gene Ontology (GO) terms

6.7 Conclusions

Emerging agriculture is necessary to meet the millennium goal of food security. Biostimulants are a strategic issue to break with limitations of traditional crop production practices and overcome the challenges of the climate crisis. PH-based biostimulants offer broad opportunities for the development of efficient and eco-friendly systems for food production. These compounds can be produced from a variety of protein substrates from food wastes or by-products discarded by industries, promoting revalorization of these residues and mitigating their environmental impacts. The collective efforts on biostimulant development and research have to lead to the identification of functional PH, experimentally and commercially probed, with agricultural applications. Many of these bioactive PH with attractive improvements on crop vegetative growth, plant nutrition, stress adaptation, yield, and quality.

Advances in high-throughput phenotyping and genomics have increased our understanding of the mode of action of some PH-based biostimulants at the physiological and molecular levels. Nonetheless, only a few studies report the evaluation of biostimulants under these approaches, partially due to the availability of reference genomes and transcriptomes, as well as the inherent limitations on the technology transferences process. To face the challenges of PH-based biostimulant production, the structure-function relationship of peptides released during the hydrolytic process must be conducted, to link specific peptide sequences to particular bioactivity detected in plants. Coupled to phenomic-metabolomic-transcriptomic studies, structural data will lead us to a rational design for “ad-hock” production of biostimulants

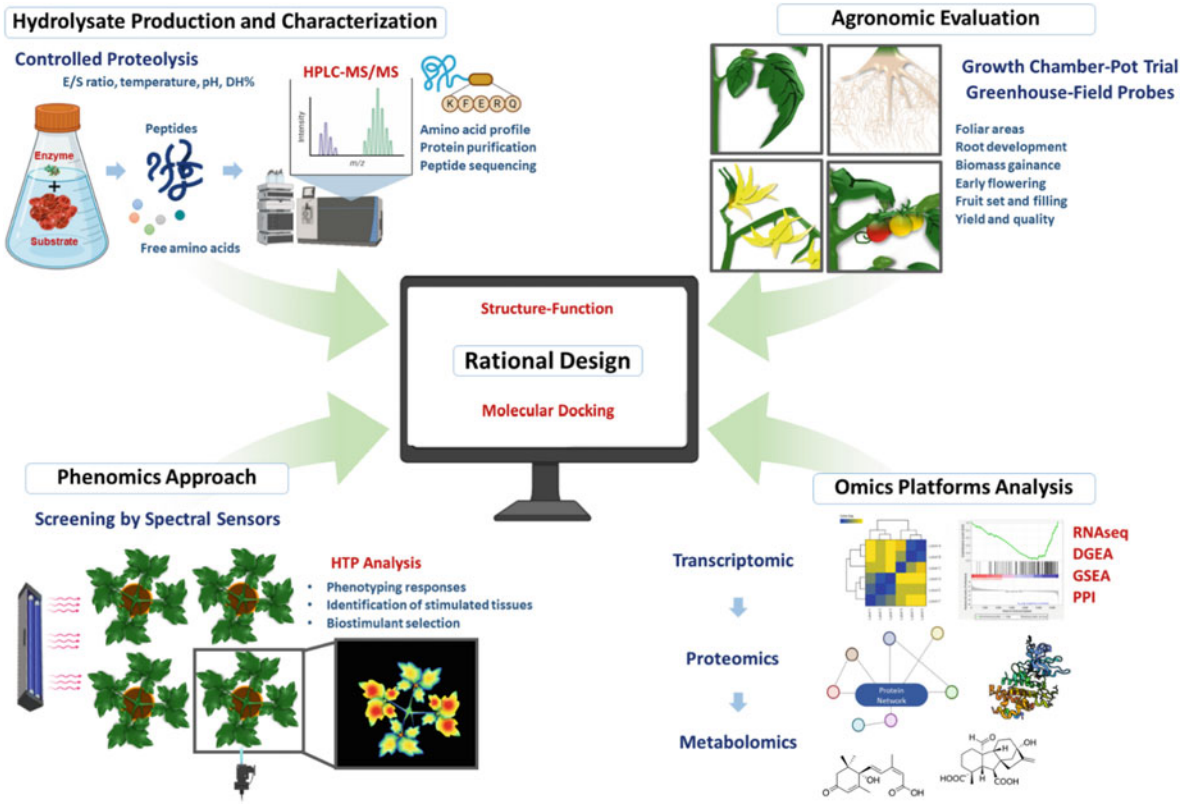

Fig. 6.2 Rational design for PH-based biostimulant production. Schematic representation of multidisciplinary approach for "ad-hoc" design bioactive peptide for plants. *DGEA* Differential Gene Expression Analysis, *DH%* hydrolysis degree, *E/S* enzyme-substrate ratio, *GSEA* Gene Set Enrichment Analysis, *HPLC-MS/MS* High-Performance Liquid Chromatography-Mass Spectrometry, *HTP* High-Throughput Phenotyping, *PPI* Protein-Protein Interaction, *RNAseq* RNA-sequencing, (RNAseq)

based on proteins, by developing specific peptide mixtures, biologically or synthetically produced, to improve target traits for a particular crop, and finally migrate toward more sustainable agriculture.

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