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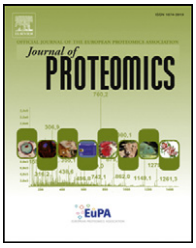

Review

An overview of proteomics approaches applied to biopharmaceuticals and cyclotides research☆

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ABSTRACT

The evolution in proteomics approaches is notable, including quantitative proteomics and strategies for elucidation of post-translational modifications. Faster and more accurate mass spectrometers as well as cleverer bioinformatics tolls are making the difference in such advancement. Among the wide range of research in plant proteomics, biopharmaceutical production using plants as "biofactories" and the screening of new activities of new molecules, in this case, peptides, are quite important regarding translational proteomics. The present review is focused on "recombinant proteins and bioactive peptides", with biopharmaceuticals and cyclotides chosen as examples. Their application and challenges are focused on a "translational proteomics" point of view, in order to exemplify some new areas of research based on proteomics strategies.

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Contents

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1. Introduction

Considering the composition, the wide structural diversity and the consequent multitude of physical–chemical properties and functions of proteins, their study may be considered one of the most challenging tasks in analytical chemistry. Nevertheless notorious advances in proteomics approaches have been achieved [1]. The search for novel plant proteins or peptides associated to biochemical event(s) involved in disease or promoting resistance to conditions like stress or predators are examples of such studies [2–4]. The necessity to explore deeper and deeper in a particular proteome forced researchers to overcome the limits of pre-fractionation techniques, the robustness of mass spectrometers and the scope of bioinformatics softwares, as the complexity of the sample to be analyzed imposed different strategies for success [1,5]. To achieve a high number of identified proteins and eventually quantifying them depends on a range of equally important steps including protein preparation/extraction, sample pre-fractionation, protein digestion, data acquisition, and data analysis.

Protein extraction might involve previous organelle fractionation, membrane separation, or immunoprecipitation, among other approaches, in order to obtain the desired proteins sample, in sufficient amount for analysis [6,7]. Gel-based or multidimensional chromatography, at protein or peptide level, respectively, is mostly used for sample pre-fractionation [8].

Peptidases employed in protein digestion may influence the choice of fragmentation method. Trypsin, for instance, is not a good choice in electron-transfer dissociation (ETD), in which longer peptides, with higher charge states, are intended to be analyzed preserving post-translational modifications (PTMs) during the fragmentation step [9].

Quantitative proteomics represents one of the most studied approaches of proteomics research, encompassing tasks related to the identification of proteins involved in a given biological process and, eventually, the quantification of such proteins in one or more situations, uncovering differences in protein abundance among defined proteomes or conditions [10]. Several approaches for quantitative proteomics are nowadays available and were extensively reviewed, addressing their bottlenecks, challenges, advantages, and disadvantages [4,11–13]. In the case of label-free quantification, the abundance of one particular protein may be estimated based on the abundance of peptides derived from that particular protein, detected by any of the several liquid chromatography-tandem mass spectrometry analyses (LC-MS/MS). For spectral-counting, the signal intensities or peak areas at the MS^1 level, or the frequency of MS/MS scans attributed to peptides belonging to a particular protein, are considered [4,10,13].

Label-free approaches have been successfully used to compare biochemical events in different plants and other biological systems. In a study performed by Shen and collaborators [14], maize etiolated seedlings were analyzed using a label-free quantitative proteomics approach based on nano-UPLC-MS^E. About 400 proteins were identified and quantified yielding important data for further approaches on C4 plants like maize, sugarcane, millet, and sorghum, and providing a good example of the use of a nano-UPLC-MS^E label-free method in plant research [14]. In a more detailed and extended work performed by Ferro and collaborators, a total of 1323 non-redundant proteins from three plastid sub-compartments (envelope, thylakoids and stroma) were identified and quantified based on label-free spectral counting. The author's choice of sample pre-fractionation and combination of two high-resolution mass spectrometers to collect the data resulted in such high number of quantified proteins [15].

Protein post-translational modifications (PTMs) are another important field in proteomics. PTM analyses depend on the intended purpose of the study. The type of PTM to be addressed and the necessity of qualitative or quantitative data will define how the sample should be prepared, a critical step for a successful result [16]. Among the most studied PTMs are acetylation, acylation, glycosylation, methylation, oxidation, phosphorylation and ubiquitylation [17]. The amount of data already acquired in PTM analyses made available to researchers the use of prediction tools to identify possible modification sites and databases containing information about modified proteins [18].

2. Translational plant proteomics

In a recent publication, Agrawal and collaborators [19] proposed as a definition of translational plant proteomics "the application and outcomes of any discovery or technological development in plant proteomics to solve issues related but not limited to the recreational and economic values of plants, food security and safety, energy sustainability, and human health". Here we aimed to address the independent topics of "translational plant proteomics" where it relates to "recombinant proteins and bioactive peptides", focusing on biopharmaceuticals (for recombinant proteins) and cyclotides (for bioactive peptides), as examples of technological developments in plant proteomics translated into beneficial applications in several fields.

2.1. *Plant transformation for biopharmaceuticals*

The formidable advances of recombinant protein technologies brought new expression systems for proteins used as pharmaceutical products (named biopharmaceuticals from now on) [12,20]. In the meantime, the growing demand of new biopharmaceuticals for use in a large variety of diseases imposed to researchers and industries the need to develop new production strategies, aimed to minimize costs while improving yield and quality of final products. The challenge is to achieve a simple and inexpensive system able to produce high amounts of purified recombinant proteins [12,21]. In this scenario, plants have emerged as an efficient system to express biopharmaceuticals in high quantity and quality, reducing downstream process costs [20,22,23].

In general, plants are advantageous when compared to cultured cell systems or fermentation processes [20,24] because they are cost-effective and production-efficient systems [25]. The technologies for harvesting and processing in large scale are well known for a great number of plants [25,26] and, additionally, costs can be substantially reduced in the post harvesting phase in which the expressed protein could be accumulated and stored in seeds, maintaining sufficient

stability for reasonable long periods of time [23,25–27], even years when the protein is accumulated in cereals [28].

Plants expressing biopharmaceuticals could be grown in contained greenhouses, under restrict control, or in open fields, with appropriate biological containment features such as maternal inheritance or male sterility, in order to restrict foreign gene dispersal [23]. Compared to mammalian and other animal cell systems, plant or microbial production systems are much easier to scale up [29]. However, it is not unusual that recombinant proteins tend to precipitate or assemble in an incorrect fold when expressed in bacteria such as *Escherichia coli* [29,30].

Among plants considered for large scale production of biopharmaceuticals are major crops like tomato, potato, rice, maize, canola, cotton and soybean. Mainly because of some special, technical characteristics, additional plants like sweet pepper, carnation and carrot, besides the model 'lab' plants *Arabidopsis thaliana* and tobacco, are also being considered for the production of proteins of therapeutical importance [29,31–33].

As pointed out by several reviewers and original articles, the challenge to successfully produce biopharmaceuticals through genetic engineering is still at the "molecular biology" step: most studies are focused on developing better strategies for gene expression, increasing yield and promoting the correct assembly of the final recombinant proteins [23,24,26,30,34].

The regeneration and propagation of stably transformed, high yielding plants are fundamental steps in molecular farming. In *stable plant genetic transformation*, a transgene is inserted into the host genome [20], and the information is passed to subsequent generations in a new genetic locus [28]. In this case, the proteins of interest will be expressed in *stably transformed plants*, in which the transgene is integrated into nuclear, mitochondrial or plastid DNA [20,24,29,30]. A variety of transformation methods are available for plant transgenesis [20]. The infiltration by *Agrobacterium tumefaciens* is widely used for dicots such as tobacco and pea [20,35] and monocots like rice, while biolistics is more often used for monocots such as maize and wheat [20,27,36].

Many authors have pointed out that *stable plastid transformation* offers advantages when compared to nuclear transformation [30]. It was shown that large amounts of proteins could be expressed in chloroplasts and other plastids basically due to the high copy number of plastid genomes (plastomes) in each plant cell and the very effective translational machinery of these organelles [24,37]. Another important advantage of transplastomic plants is that the insertion of transgenes into plastomes would eliminate the high variation of expression among independent transgenic lines [23,30,38]. Since plastids are of maternal inheritance (in most plants), this feature is also pointed out as a relevant advantage of transplastomics. Plastids are also considered versatile because proteins from a wide range of molecular weights could be expressed in the organelle, where chaperones help to assemble proteins into the correct folding [23,30,38–40], usually leading to the production of high yields of foreign proteins [24]. Additionally, similar to the organization of genes in bacterial chromosomes, multiple transgenes could be organized in operons to be expressed in plastids from a single transformation event, therefore regulated by a single promoter. This facilitates the expression of proteins consisting of multiple subunits or multicomponent vaccines [24].

Unfortunately, plastid transformation is feasible to a limited number of plant species including rice, tomato and *A. thaliana*. Major difficulties are the isolation of homoplastomic cells and derived plants, i.e., the regeneration step associated to plant transformation [41–43]. The yield of recombinant protein production in plants is another important challenge in molecular farming and several approaches have been tested to overcome this issue, including the use of stronger or more specific promoters, increasing protein stability and driving different subcellular targeting [27].

In contrast to stably transformed plants, *transient gene expression systems* are being developed not only to test genes of interest in plants, but also to produce recombinant proteins in large scale. Transient transgene expression can be achieved by agroinfiltration of plant tissues or by viral vectors [20,30]. In *Agrobacterium* infiltration, the transgene is transferred from bacteria to a large number of hosts' cells [44]. Via viral infection, tobacco mosaic virus (TMV) or potato virus X (PVX) are widely used to deliver the gene(s) of interest into host plant cells, without integration into the genome. Plant viruses do not integrate the viral DNA into the host genome and hence the transgene is not passed through out the germ line [28]. The development of plant viral vector systems for transient gene expression has enabled the rapid expression of proteins and at higher levels, even when compared to the stably transformed plants [23]. The protein of interest will be transiently expressed in plant tissues [30]. These systems have been often used to test the function and compatibility of a gene expression construct before advancing to a large-scale production in a stable system [28].

2.2. *Biopharmaceuticals*

The most promising (plant) biopharmaceutical products for veterinary or human medical purposes are antigenic proteins to be used as vaccines and, in fact, vaccine antigens are among the biopharmaceuticals most studied for expression in plants [24]. One important advantage of vaccine production in plants is the possibility of direct oral delivery to patients. Crops like tomato, carrots, potatoes, and lettuce would be eligible vaccine vectors and their direct use could considerably reduce post harvesting processing of the antigenic proteins [23,24,26,34]. Additionally, these plants are easily produced in large scale even under confinement [30].

A vaccine for amebiasis, consisting of a surface antigen of *Entamoeba histolytica* expressed in tobacco chloroplasts was successfully tested in mice by subcutaneous injection. The antigen yield was about 24 mg per plant, and it was estimated that about 29 million doses of vaccine could be produced per acre of the transgenic plant in the field [24].

Although several biopharmaceuticals are being produced in plants and evaluated in clinical trials, few in fact were approved for human or animal therapy [22]. Additionally, the number of publications on highly purified recombinant proteins produced in plants is still limited [23,24]. Genetics, crop management, disease and pest control are well developed for plants such as tomato, soybean, rice, carrot, potato, maize and alfalfa, making them more suitable candidates for biopharmaceutical

production [25]. Several plant-derived vaccine antigens, antibodies, therapeutical and nutraceutical proteins as well as non pharmaceutical proteins are already in clinical trials or in the market. For further information please refer to [30].

Besides vaccine proteins like hepatitis B, cholera, non-Hodgkin's lymphoma, and feline parvovirus, other biopharmaceuticals produced in plants include antibodies for cold, dental caries, therapies for side effects of cancer, and therapeutic human proteins for cystic fibrosis, blood clotting, diabetes and cardiovascular disorders [30].

Human insulin for the treatment of type I diabetes was successfully expressed in *A. thaliana* by targeting the molecule to seed oil bodies. The fusion of proteins of interest with the highly hydrophobic oleosin protein has allowed the isolation of many recombinant proteins by a simple solvent extraction procedure [31,45].

Monoclonal antibodies are increasingly being used in therapy of rheumatoid arthritis, cancer, cardiovascular diseases and autoimmune disorders. They are considered one of the fastest growing classes of novel therapeutics [25]. Recently, Komarova and collaborators [46] expressed in tobacco leaves the humanized monoclonal antibody 'trastuzumab' for treating breast tumors. The antibody encoding genes (light and heavy chains) were cloned under the control of the cauliflower mosaic virus 35S promoter in virus-based vectors, and the protein was expressed in *Nicotiana benthamiana* leaves, with high yield and expression levels [46].

There is a high demand for collagen for pharmaceutical and cosmetic industries and alternative processes for collagen production are required. Type I collagen has also been object of study in molecular farming approaches. Back in 2000, Ruggiero and collaborators [47] were able to express the molecule in tobacco leaves in an average yield of 10 mg of protein per 100 g of powdered plant. It was also reported that, although tobacco does not contain the correct machinery for successful assembly of the collagen molecule, the triple helical molecules were correctly assembled [47]. PTMs essential for the pro-collagen-I assembly include proline and lysine hydroxylation and both intra- and inter-chain disulfide bond formation [47,48]. Following this previous work, in which the authors identified the lack of hydroxyproline residues in the recombinant collagen molecule, they successfully expressed in tobacco the human homotrimeric collagen I by transient expression using *A. tumefaciens* as vector [49]. The hydroxyproline levels thus obtained were quite similar to that observed in recombinant type I collagen produced in *Saccharomyces cerevisiae* [49]. Stein and collaborators (2009) [48] also reported the expression of the human type I collagen in tobacco plants but, in this case, co-expressed with the key enzymes proly-l-4-hydroxilase and lysyl hydroxylase 3, which are essential for PTMs of the molecule [48].

The final price of a biopharmaceutical product will be a combination of several key issues such as set up, scale up and maintenance costs, length of production cycle, biomass yields, edibility, costs of downstream processing, storage, distribution and containment. The costs can be dramatically reduced depending on the delivery route of the final product. An intravenous injected biopharmaceutical, for instance, would require a higher degree of purification when compared to a plant like carrot used to deliver a vaccine and that could be eaten raw [28].

2.3. *PTMs in biopharmaceutical research*

Protein PTMs have been largely studied under both proteomics and classical biochemistry approaches. PTMs are considered critically relevant to protein functions and may be responsible for sub-cellular localization, turnover and interaction with other proteins and/or molecules within a system [18,50]. They influence protein's life cycle, folding and, finally, biological function. Protein PTMs can be described as any change in polypeptide chains by the addition or removal of chemical moieties, oxidation, reduction, covalent cross-linking or photolytic processing occurring after peptide chain synthesis [51]. Another important characteristic of PTMs is their reversibility. PTMs are basically divided into two main groups: covalent cleavage of side chains and covalent attachment of chemical groups [18]. About 40 % of proteins employed in therapeutics are N-glycosylated [24,52] and, in plants, the process is quite well characterized under proteomics and molecular biology approaches [16,53], being similar to other eukaryotic organisms in terms of frequency of glycosylation [54]. Even thought PTMs are known to be evolutionarily conserved, there are plant specific PTMs that should be taken into consideration when proteins are to be expressed in plant systems through genetic engineering [53].

Regarding transplastomic plants, the lack of glycosylation processes in plastids is a limiting disadvantage of this system for protein expression [24], although artificial or synthetic glycosylation could be performed after protein purification.

The N-glycosylation takes place when target proteins are linked to complex oligosaccharides chains (N-glycans) which are covalently attached to the amide nitrogen of the side chain of an asparagine residue [53,55]. In O-glycosylations, the carbohydrate is linked to the hydroxyl group of serine, threonine, hydroxylysine or hydroxyproline residues of the protein chain [55]. Several biopharmaceuticals are N-glycosylated [53,56]. Therefore the production of these molecules in heterologous systems must consider this type of PTMs and plants are more suitable systems when compared to organisms that are unable to perform these reactions, such as *E. coli* and other bacteria. However, plants are unable to reproduce the patterns of human-type glycosylations. Thus plant-type glycosylation is considered the major limitation for their use as biopharmaceutical biofactories [53,57,58].

One important aspect to be taken into consideration when using plant-made biopharmaceuticals is the possibility of allergic reactions in final users (animals or humans). For instance, the presence of α-1,3-fucose and β-1,2-xylose in plant-made biopharmaceuticals has been suggested as the cause of undesirable immune or allergic responses [53,59].

There are about 300 known forms of PTMs, being phosphorylation, glycosylation and ubiquitination the most studied [53]. Proteomics studies of PTMs are not simple since the versatility and stoichiometry of PTMs require specific conditions for protein/peptide enrichment and data collection in high resolution mass spectrometers [16,18,50]. The traditional methods for PTM analyses are based on immunological approaches like precipitation with specific antibodies, modified gel stains for two dimensional SDS-PAGE, or selective MS scan strategies [17]. Regardless the approach, large amounts of protein samples are

needed since the modifications to be studied may only occur in a small fraction of a particular protein or proteins [50], requiring additional enrichment steps, either at protein or peptide levels. For example, immobilized metal affinity chromatography (IMAC) using Fe^{+3} or Ga^{+3} loaded resins, or metal oxide affinity chromatography (MOAC) using titanium and zirconium dioxide columns, are widely used for phosphopeptide enrichment [51,53,60].

2.4. *Proteomics approaches for biopharmaceuticals*

Proteomics and mass spectrometric approaches can be used to address several important concerns in a biopharmaceutical production pipeline such as the amount of the biopharmaceutical produced (expressed) and extracted from the plant; the integrity of the biopharmaceutical (correct folding, PTMs); the stability from harvesting up to final delivery. However, as pointed by several studies in these fields, determining whether the protein has acquired correct folding and the protein quantification might be the most challenging steps.

To extend and purify the biopharmaceutical from the host plant, a number of protein purification techniques might be required, such as affinity, gel filtration, ion exchange, hydrophobic interaction, reverse phase chromatographies, among others [61]. During the purification workflow, some strategies can be aggressive as to compromise the correct folding and function of the biopharmaceutical [61,62]. Non-chromatographic procedures could be an alternative specially in the preparative phase, in which the desired protein is being "concentrated" in one particular fraction for further polishing [62]. Aqueous-two phase processes can be applied to antibodies preparation, using either polymer-salt or polymer-polymer fractionation. In case of polymer-salt, the two phases are composed of a PEG-rich phase and a salt rich phase. Concentration of the antibody in the PEG-phase can be achieved by decreasing the molecular weight of PEG, or increasing the salt concentration in the salt-phase. The methodology is extensively discussed in Azevedo and co-workers review [62].

Other aspects such as oligomeric state, post-translational modification and protein-protein or protein-ligand interaction of the biopharmaceutical are essential for the desired biological activity [63]. The biological efficiency, clearance, safety and immunogenicity of biopharmaceuticals are strictly connected to their structure [64].

To address the previous important aspects, structural mass spectrometry has gained space in biopharmaceutical drug discovery and characterization [64]. Among others, hydrogen/deuterium exchange method (HDX) combined to mass spectrometry (HDX-MS) is being used for biopharmaceutical characterization. HDX is based on a chemical process in which "labile hydrogens within a protein are exchanged with hydrogen from bulk water" [65]. The backbone amide hydrogens offers exchange rates that can be measured in a timescale way [65,66], which can be followed by mass spectrometry (direct infusion, coupled to a LC system, with or without fragmentation). This approach provides important information regarding the protein dynamics in global, peptide and amino-acid levels [66–68]. The HDX rate is directly related to the protein exposure to the solvent and also on inter and intra-molecular hydrogen bonding. By monitoring these "exposure rate", HDX-MS information can be related to the protein structure [64]. Theoretically, the use of HDX can be applied to almost any protein as most proteins have a hydrophobic region with protected amide hydrogens atoms, which are exposed to solvent upon unfolding and thus accessible to exchange [69].

When compared to different approaches to study protein folding, such as X-ray crystallography and nuclear magnetic resonance, HDX can be considered simpler regarding sample preparation [64]; however, data analysis might be exhaustive and some wrong conclusions can be achieved by simply calculating the centroid mass of the target peptide [66]. HDX-MS methods are being also used to investigate protein conformation and interaction with their target receptors [64–66]. This methodology is a powerful way to study the relationship between the protein and its function [65].

HDX-MS approaches have been successfully used in epitope mapping (by characterizing the interaction of antigen and antibody). The peptides involved in the antigen-antibody interaction display a lower HDX rate, when compared to the same peptides in the free form of the antigen of the antibody as reported by Zhang and collaborators [64]. The epitope binding sites obtained by HDX-MS were consistent with the previous sites previously identified by other methods, such as mutagenesis, molecular modeling and electron microscopy [70]. The later incorporation of ECD/ETD to MS-HDX for fragmentation of labeled peptides extended the power of this technique, enabling the measures of HDX rates at amino acid level [64,70].

In another study performed by Nakazawa and co-workers [71], the authors compared two different insulin types from commercial sources (regular insulin and fast-acting insulin). When used, insulin must act in a fast way (decrease high levels of sugar in the blood). Oligomerization of the peptide causes delay in absorption resulting in consecutive delay in the desired effect. The fast acting insulin studied by the authors contains an inversion of two aminoacids positions: the proline B28 and lysine at B29 are inverted [71]. Hydrogen/deuterium exchange reactivity monitored by mass spectrometry showed that the self associated regular insulin peptide is stable as compared to the fast acting insulin which incorporated deuterium in a much faster way. The authors concluded that hexamers formed by the fast acting insulin are not stable as human insulin and that the method was efficient to map two important regions in the insulin whole molecule that are important in the formation of hexamers and dimers. In this case, the study was performed using a nanoACQUITY UPLC™ system coupled to a Xevo G2 Q-TOF ™ mass spectrometer, and the peptides were in line digested with pepsin [71].

An HDX-MS approach applied to the biopharmaceutical interferon to study the alkylation of this protein when it is subjected to degradation [72]. Interferon has a strong tendency of aggregation which cause loss of activity, and undesired immunogenic responses. The data provided by HDX study comparing regular interferon and alkylated interferon allowed the conformational properties of intact and alkylated interferon to be compared directly [72]. The points addressed in the previous section are summarized in Fig. 1. The points addressed in the previous section are summarized in Fig. 1.

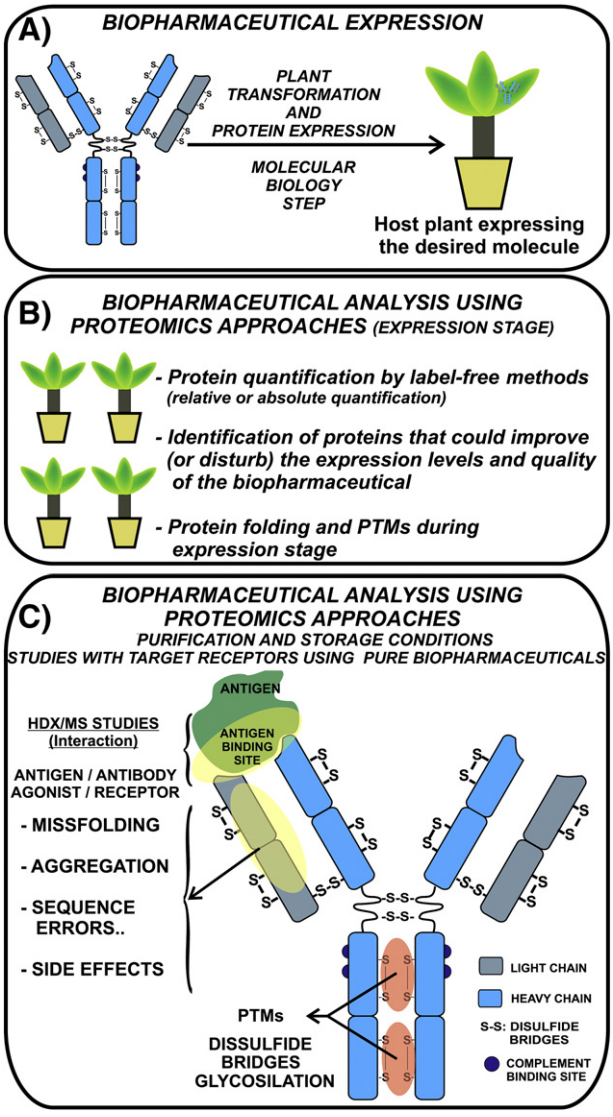

Fig. 1 – Proteomics strategies to improve biopharmaceutical development. In A, plant transformation approaches aimed to obtain the desired biopharmaceutical in sufficient amounts and correctly folded. In B, once the biopharmaceutical is being expressed, it must be purified from the host plant. In this case, proteomics strategies can help to ensure that: the folding was not lost in this process, the PTMs are correct, the stability of the molecule up to use. In C, the final product must be tested for its desired activity. Proteomics approaches can be used for studies on protein-protein interaction, folding and stability issues. In this figure an antibody is being used as example for biopharmaceutical.

2.5. *Cyclotides as examples for bioactive peptides*

Among several classes of bioactive peptides naturally produced by plants, cyclotides are quite unique. They are small molecules composed of 28 up to 37 amino acid residues and are rich in cysteines. As the name suggests, cyclotides contain a head-to-tail cyclized backbone and are stabilized by three disulfide bonds arranged as a cyclic cysteine knot [73–75]. Their knotted structure makes cyclotides very stable molecules, resistant to heat and to hydrolysis by most peptidases as well as to chaotropes (both widely used in sample preparation for MS analyses) [76–78].

The first cyclotide was discovered in a tea made from the plant *Oldenlandi aaffinis* (Rubiaceae) that women in Africa use to accelerate childbirth [74,79]. Later, other biological activities of cyclotides were discovered [78]. Cyclotides are today known to be present in the Violaceae, Rubiaceae, Curcubitaceae and Fabeaceae families [80,81].

Cyclotides present uterotonic activity, anti-human immunodeficiency virus (HIV) activity, neurotensin antagonism, haemolytic properties, insecticidal and anti-helmintic activities [73–75,80]. Anti-HIV, insecticidal and haemolytic properties of cyclotides are the most relevant and perhaps promising activities [74]. The anti-HIV and hemolytic activities of cyclotides seem to be directly related to the cyclic backbone of the peptides, since synthetic linear peptides were inactive against HIV infection [74] and unable to reduce hemolysis [74,80,81].

The insecticidal activity of these peptides is not related to inhibition of insect's digestive peptidases [82–84]. It involves midgut membrane disruption [85], probably by pore formation in the host membrane [86], which might also contribute to their toxic effects in snails and nematodes [74]. Their function in the source plants probably related to plant defense is not completely clear yet [74,78,87].

The biosynthesis of cyclotides, which are gene-encoded products generated via processing of a precursor protein synthesized ribosomally, is a complex series of events [88]. Structurally, the cyclotide precursor protein can be divided into: an endoplasmatic reticulum signal sequence, the Pro-region, the highly conserved N-terminal repeat region (small but significant variation among species can be detected), the cyclotide domain and the C-terminal tail, usually short (Fig. 2) [88,89]. In a recent work performed by Conlan and colleagues using GFP tagged constructs, the authors pointed out that excision from precursor, cyclization and deposition of cyclotides happen the plant vacuoles [90]. The vacuolar enzyme asparaginyl endopeptidase might play an important role in this process [90]. The processing site is thought to be at the C-terminal side of a highly conserved Asn (or Asp) residue [88] in the C-terminal portion of the cyclotide sequence. MALDI-TOF was employed to analyze the cyclic and linear forms of the cyclotides expressed in the study [90].

The cyclization process is probably even more complicated than originally thought. Three peptides motifs (Gly-Leu-Pro or Ser-Leu-Pro or Ala-Leu-Pro), present in loop 6 of the formed cyclotide, seem to be essential for the cyclization process [81]. On the other hand, among new cyclotides discovered in the Fabaceae family, cyclization target motifs lack "susceptible" sites for the activity of the enzyme asparaginyl endopeptidase, previously implicated in cyclotide processing [78,81].

The primary methods for discovery and characterization of the new cyclotides are via classic isolation, purification and structural characterization, especially by nuclear magnetic resonance [88]. At a first sight, cyclotides seem to be good subjects for peptide sequencing using MS/MS. However, their structural features impose additional challenges. Besides the presence of three disulfide bridges, they have few positively charged amino acid residues and as such enzymatic cleavage may produce undesirable (i.e., too small) fragments not suitable

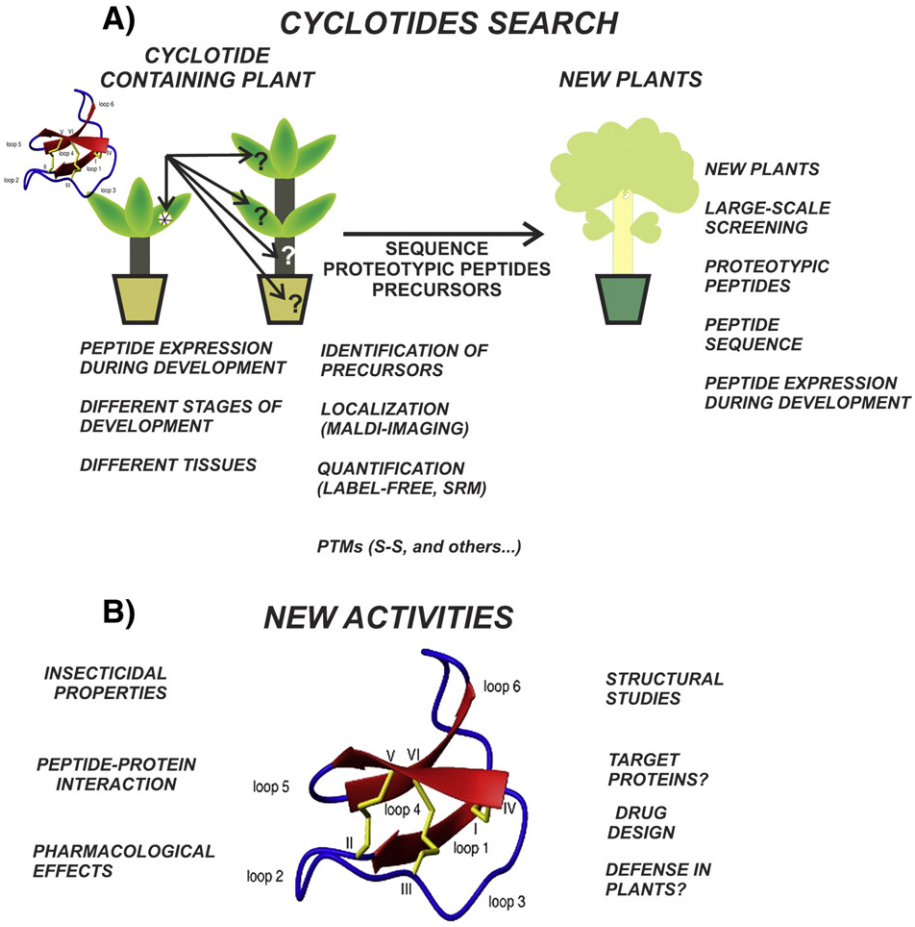

Fig. 2 – Proteomics strategies for cyclotides research. **In A: new cyclotides are being discovered. In this case, information obtained from the already discovered peptides can be useful for large-scale screening for cyclotides in new plants. Some strategies are presented. In B: the cyclotides present a wide range of activities that can be fully studied using several proteomics approaches. Some of these activities are presented. Cyclotide used in the figure was extracted from http://www.cyclotide.com/structures.html.**

for MS/MS [91]. Standards methods for analysis of protein or large peptides such as gel electrophoresis or immunoblotting are not suitable for cyclotides because of their compact nature [73].

According to Ovesen and collaborators (2011), a better understanding of the synthesis of cyclotides and their role in plant defense would be possible if these peptides could be quantified in different plant tissues [73]. For that end, relative quantification or absolute quantification using MS approaches can be used.

Cyclotides are structurally related but lack sequence similarity. Although cyclotides are found in only a limited number of plant families, large screening for these peptides have not been made so far, and the search for proteotypic peptides (peptide uniquely associated with the protein of interest [92]), might be helpful in this issue. Some cyclotide sequences are deposited in a database available on line, the CyBase16, found at http://www.cybase.au [88,93].

Cyclotides have been studied by MS approaches. Sze and collaborators applied partial acid hydrolysis to determine the cyclic structure and disulfide linkages of a cyclotide mixture [94]. The hydrolyzed peptide mixture was analyzed by LC-MS/MS using CAD as fragmentation. The method proved efficient for high-throughput cyclotide disulfide bond characterization based on flash partial acid hydrolysis, LC-MS/MS and a software used for disulfide bond assignment [94].

Cyclotide sequencing by proteomics approaches was considered slow by Colgrave and collaborators, especially due the challenges in the chromatographic procedures and the multiple chemical and enzymatic derivatization steps required to make cyclotides analyzable by mass spectrometry with sequencing purposes [95]. The authors developed an iterative proteomics workflow with a differential data acquisition mode in the software used for search. This strategy allowed identification of known cyclotides, cyclotide analogs, and most important, to identify new plant species containing cyclotide-related sequences [95].

Cyclotides were reported by Poth and collaborators in the Solanaceae family, which includes important crop species, such as potato and tomato [89]. In the study a cyclotide encoding gene from *Petunia* was described to encode the shortest known cyclotide precursors, quite distinct from previously known precursors [89]. MALDI-imaging of a paradermal leaf section of a *Petunia* leaf detected a large amount of peaks in the range

of *m/z* 3000–3600. Leaf extracts analyzed by LC-MS gave a peak of *m/z* 3069 named Phyb, which after sequencing was shown to be a cyclotide, which was also detected by MALDI-imaging [89].

The potential applications of the cyclotides are diverse, and one challenge to plant molecular biologists is to express these peptides in the circular form. As cyclotides biosynthesis are not yet fully understood, proteomics approaches can be used as a powerful tool aimed to elucidate these pathways. Screening for cyclotides in new plant species can be done with proteotypic peptides using the selected reaction monitoring, and searching for precursor molecules. Proteomics approaches can help also to "customize" new activities of these molecules, for instance, cyclotides can serve as templates for drug design, including anti-HIV, antimicrobial, antitumor, and neurotensin antagonism activities [76]. Recently, Poth and collaborators reported the presence of a cyclotide from *Clitoria ternatea* (Fabaceae family), with an unusual biosynthetic origin when compared to previous described cyclotides. In this case, the cyclotide domain is embedded within an albumin precursor. Among other experiments, leaf extracts were digested with one or three proteolytic enzymes and analyzed by LC-MS/MS. The MS/MS spectra were searched against a custom-built database of cyclotides for correct sequencing of the new discovered peptide [96].

Fig. 2 summarizes the points addressed in this section.

3. Concluding remarks

Biopharmaceuticals and cyclotides are promising candidates for translational proteomics studies. There are undoubtedly important challenging steps to overcome such as the expression of biopharmaceuticals in considerable amounts and correct folding, or obtention of purified biologically active cyclotides.

Proteomics approaches can be applied to understand the folding, stability, interaction with target molecule(s) and side effects of biopharmaceuticals. In case of plant biopharmaceuticals, the challenge is yet at the molecular biology step.

Cyclotides from new plant sources and with new activities are being discovered at a considerable rate. Proteomics approaches (fragmentation strategies and MALDI-imaging, for example) can be applied to study the sequence and structural characteristic of the cyclotide and its localization in the plant, respectively. Quantitative proteomics approaches (spectral count, SRM), have been applied to understand the wide range of activities of these peptides.

In our point of view, plant biopharmaceuticals (example of recombinant proteins) and cyclotides (example of plant bioactive peptides) are strong candidates for a wide range of translational plant proteomics studies, in several fields. These studies will improve the quality of new medicines available in the future and perhaps help to create new drugs based on the structure and activity of the powerful cyclotides.

Conflict of interest

The authors declare no conflicts of interest.

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REFERENCES

[1] Zhou H, Ning Z, Starr E, Abu-Farha M, Figeys D. Advancements in top-down proteomics. Anal Chem 2012;84:720–34.
[2] Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature 2003;422:198–207.
[3] Altelaar AF, Heck AJ. Trends in ultrasensitive proteomics. Curr Opin Chem Biol 2012;16:206–13.
[4] Elliott MH, Smith DS, Parker CE, Borchers C. Current trends in quantitative proteomics. J Mass Spectrom 2009;44:1637–60.
[5] Yates JR, Ruse CI, Nakorchevsky A. Proteomics by mass spectrometry: approaches, advances, and applications. Annu Rev Biomed Eng 2009;11:49–79.
[6] Brunet S, Thibault P, Gagnon E, Kearney P, Bergeron JJ, Desjardins M. Organelle proteomics: looking at less to see more. Trends Cell Biol 2003;13:629–38.
[7] Agrawal GK, Bourguignon J, Rolland N, Ephritikhine G, Ferro M, Jaquinod M, Alexiou KG, Chardot T, Chakraborty N, Jolivet P, Doonan JH, Rakwal R. Plant organelle proteomics: collaborating for optimal cell function. Mass Spectrom Rev 2010;30:772–853.
[8] Spross J, Brauch S, Mandel F, Wagner M, Buckenmaier S, Westermann B, Sinz A. Multidimensional nano-HPLC coupled with tandem mass spectrometry for analyzing biotinylated proteins. Anal Bioanal Chem 2012;405(7):2163–73.
[9] Wiesner J, Premsler T, Sickmann A. Application of electron transfer dissociation (ETD) for the analysis of posttranslational modifications. Proteomics 2008;8:4466–83.
[10] Oeljeklaus S, Meyer HE, Warscheid B. Advancements in plant proteomics using quantitative mass spectrometry. J Proteomics 2009;72:545–54.
[11] Gao BB, Stuart L, Feener EP. Label-free quantitative analysis of one-dimensional PAGE LC/MS/MS proteome: application on angiotensin II-stimulated smooth muscle cells secretome. Mol Cell Proteomics 2008;7:2399–409.
[12] Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. Quantitative mass spectrometry in proteomics: a critical review. Anal Bioanal Chem 2007;389:1017–31.
[13] Lundgren DH, Hwang SI, Wu L, Han DK. Role of spectral counting in quantitative proteomics. Expert Rev Proteomics 2010;7:39–53.
[14] Shen Z, Li P, Ni RJ, Ritchie M, Yang CP, Liu GF, Ma W, Liu GJ, Ma L, Li SJ, Wei ZG, Wang HX, Wang BC. Label-free quantitative proteomics analysis of etiolated maize seedling leaves during greening. Mol Cell Proteomics 2009;8:2443–60.
[15] Ferro M, Brugiere S, Salvi D, Seigneurin-Berny D, Court M, Moyet L, Ramus C, Miras S, Mellal M, Le GS, Kieffer-Jaquinod S, Bruley C, Garin J, Joyard J, Masselon C, Rolland N. AT_CHLORO: a comprehensive chloroplast proteome database with subplastidiallocalization and curated information on envelope proteins. Mol Cell Proteomics 2010;9:1063–84.
[16] Reinders J, Sickmann A. Modificomics: posttranslational modifications beyond protein phosphorylation and glycosylation. Biomol Eng 2007;24:169–77.

[17] Hoffman MD, Sniatynski MJ, Kast J. Current approaches for global post-translational modification discovery and mass spectrometric analysis. Anal Chim Acta 2008;627:50–61.
[18] Kamath KS, Vasavada MS, Srivastava S. Proteomic databases and tools to decipher post-translational modifications. J Proteomics 2011;75:127–44.
[19] Agrawal GK, Pedreschi R, Barkla BJ, Bindschedler LV, Cramer R, Sarkar A, Renaut J, Job D, Rakwal R. Translational plant proteomics: a perspective. J Proteomics 2012;75:4588–601.
[20] Schillberg S, Twyman RM, Fischer R. Opportunities for recombinant antigen and antibody expression in transgenic plants-technology assessment. Vaccine 2005;23:1764–9.
[21] Ma JK, Drake PM, Christou P. The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 2003;4:794–805.
[22] Faye L, Gomord V. Success stories in molecular farming — a brief overview. Plant Biotechnol J 2010;8:525–8.
[23] Daniell H, Singh ND, Mason H, Streatfield SJ. Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci 2009;14:669–79.
[24] Daniell H. Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. Biotechnol J 2006;1:1071–9.
[25] Peterson RKD, Arntzen CJ. On risk and plant-based biopharmaceuticals. Trends Biotechnol 2004;22:64–6.
[26] Daniell H, Streatfield SJ, Wycoff K. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 2001;6:219.
[27] Fischer R, Stoger E, Schillberg S, Christou P, Twyman RM. Plant-based production of biopharmaceuticals. Curr Opin Plant Biol 2004;7:152–8.
[28] Schillberg S, Fischer R, Emans N. Molecular farming of recombinant antibodies in plants. Cell Mol Life Sci 2003;60: 433–45.
[29] Ahmad P, Ashraf M, Younis M, Hu X, Kumar A, Akram NA, Al-Qurainy F. Role of transgenic plants in agriculture and biopharming. Biotechnol Adv 2012;30:524–40.
[30] Obembe OO, Popoola JO, Leelavathi S, Reddy SV. Advances in plant molecular farming. Biotechnol Adv 2011;29:210–22.
[31] Nykiforuk CL, Boothe JG, Murray EW, Keon RG, Goren HJ, Markley NA, Moloney MM. Transgenic expression and recovery of biologically active recombinant human insulin from *Arabidopsis thaliana* seeds. Plant Biotechnol J 2006;4:77–85.
[32] James C. A global overview of biotech (GM) crops: adoption, impact and future prospects. GM Crops 2010;1:8–12.
[33] Ashraf M, Akram NA. Improving salinity tolerance of plants through conventional breeding and genetic engineering: an analytical comparison. Biotechnol Adv 2009;27:744–52.
[34] Shinmyo A, Kato K. Molecular farming: production of drugs and vaccines in higher plants. J Antibiot 2010;63:431–3.
[35] Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT. A simple and general method for transferring genes into plants. Science 1985;227:1229–31.
[36] Lico Chiara, Desiderio Angiola, Banchieri Selene, Benvenuto Eugenio. Plants as biofactories: production of pharmaceutical recombinant, proteins; 2005 577–93.
[37] Bock R. Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering and molecular farming. Curr Opin Biotechnol 2007;18:100–6.
[38] Verma D, Daniell H. Chloroplast vector systems for biotechnology applications. Plant Physiol 2007;145:1129–43.
[39] DeGray G, Rajasekaran K, Smith F, Sanford J, Daniell H. Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiol 2001;127:852–62.
[40] McBride KE, Svab Z, Schaaf DJ, Hogan PS, Stalker DM, Maliga P. Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Biotechnology (N Y) 1995;13:362–5.
[41] Gao M, Li Y, Xue X, Wang X, Long J. Stable plastid transformation for high-level recombinant protein expression: promises and challenges. J Biomed Biotechnol 2012;2012:158232.
[42] Scotti N, Cardi T. Plastid transformation as an expression tool for plant-derived biopharmaceuticals. Methods Mol Biol 2012;847:451–66.
[43] Scotti N, Rigano MM, Cardi T. Production of foreign proteins using plastid transformation. Biotechnol Adv 2012;30:387–97.
[44] Simmons CW, VanderGheynst JS, Upadhyaya SK. A model of *Agrobacterium tumefaciens* vacuum infiltration into harvested leaf tissue and subsequent in planta transgene transient expression. Biotechnol Bioeng 2009;102:965–70.
[45] Avesani L, Bortesi L, Santi L, Falorni A, Pezzotti M. Plant-made pharmaceuticals for the prevention and treatment of autoimmune diseases: where are we? Expert Rev Vaccines 2010;9:957–69.
[46] Komarova TV, Kosorukov VS, Frolova OY, Petrunia IV, Skrypnik KA, Gleba YY, Dorokhov YL. Plant-made trastuzumab (herceptin) inhibits HER2/Neu + cell proliferation and retards tumor growth. PLoS One 2011;6:1–7.
[47] Ruggiero F, Exposito JY, Bournat P, Gruber V, Perret S, Comte J, Olagnier B, Garrone R, Theisen M. Triple helix assembly and processing of human collagen produced in transgenic tobacco plants. FEBS Lett 2000;469:132–6.
[48] Stein H, Wilensky M, Tsafrir Y, Rosenthal M, Amir R, Avraham T, Ofir K, Dgany O, Yayon A, Shoseyov O. Production of bioactive, post-translationally modified, heterotrimeric, human recombinant type-I collagen in transgenic tobacco. Biomacromolecules 2009;10:2640–5.
[49] Merle C, Perret S, Lacour T, Jonval V, Hudaverdian S, Garrone R, Ruggiero F, Theisen M. Hydroxylated human homotrimeric collagen I in *Agrobacterium tumefaciens*-mediated transient expression and in transgenic tobacco plant. FEBS Lett 2002;515:114–8.
[50] Mann M, Jensen ON. Proteomic analysis of post-translational modifications. Nat Biotechnol 2003;21:255–61.
[51] Remmerie N, De Vijlder T, Laukens K, Dang TH, Lemire F, Mertens I, Valkenborg D, Blust R, Witters E. Next generation functional proteomics in non-model plants: a survey on techniques and applications for the analysis of protein complexes and post-translational modifications. Phytochemistry 2011;72:1192–218.
[52] Higgins E. Carbohydrate analysis throughout the development of a protein therapeutic. Glycoconj J 2010;27:211–25.
[53] Webster DE, Thomas MC. Post-translational modification of plant-made foreign proteins; glycosylation and beyond. Biotechnol Adv 2012;30:410–8.
[54] Seo J, Lee KJ. Post-translational modifications and their biological functions: proteomic analysis and systematic approaches. J Biochem Mol Biol 2004;37:35–44.
[55] Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye L. Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 2010;8: 564–87.
[56] Tekoah Y, Ko K, Koprowski H, Harvey DJ, Wormald MR, Dwek RA, Rudd PM. Controlled glycosylation of therapeutic antibodies in plants. Arch Biochem Biophys 2004;426:266–78.
[57] Faye L, Boulaflous A, Benchabane M, Gomord V, Michaud D. Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming. Vaccine 2005;23:1770–8.
[58] Gomord V, Faye L. Posttranslational modification of therapeutic proteins in plants. Curr Opin Plant Biol 2004;7: 171–81.
[59] Bosch D, Schots A. Plant glycans: friend or foe in vaccine development? Expert Rev Vaccines 2010;9:835–42.
[60] Salih E. Phosphoproteomics by mass spectrometry and classical protein chemistry approaches. Mass Spectrom Rev 2005;24:828–46.

[61] Guiochon G, Beaver LA. Separation science is the key to successful biopharmaceuticals. J Chromatogr A 2011;1218:8836–58.
[62] Azevedo AM, Rosa PAJ, Ferreira IF, Aires-Barros MR. Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing. Trends Biotechnol 2009;27:240–7.
[63] Oliva AM, Fariña JB, Llabrés M. New trends in analysis of biopharmaceutical products. Curr Pharm Anal 2007;3:230–48.
[64] Mo J, Tymiak AA, Chen G. Structural mass spectrometry in biologics discovery: advances and future trends. Drug Discov Today 2012;17:1323–30.
[65] Burns KM, Rey M, Baker CA, Schriemer DC. Platform dependencies in bottom-up hydrogen/deuterium exchange mass spectrometry. Mol Cell Proteomics 2013;12:539–48.
[66] Zhang J, Ramachandran P, Kumar R, Gross ML. H/D exchange centroid monitoring is insufficient to show differences in the behavior of protein states. J Am Soc Mass Spectrom 2013;24:450–3.
[67] Hamuro Y, Coales SJ, Morrow JA, Molnar KS, Tuske SJ, Southern MR, Griffin PR. Hydrogen/deuterium-exchange (H/D-Ex) of PPARgamma LBD in the presence of various modulators. Protein Sci 2006;15:1883–92.
[68] Englander SW, Kallenbach NR. Hydrogen exchange and structural dynamics of proteins and nucleic acids. Q Rev Biophys 1983;16:521–655.
[69] Liyanage R, Devarapalli N, Pyland DB, Puckett LM, Phan NH, Starch JA, Okimoto MR, Gidden J, Stites WE, Lay J. Theory of the protein equilibrium population snapshot by H/D exchange electrospray ionization mass spectrometry (PEPS-HDX-ESI-MS) method used to obtain protein folding energies/rates and selected supporting experimental evidence. Int J Mass Spectrom 2012;330–332:63–70.
[70] Zhang Q, Willison LN, Tripathi P, Sathe SK, Roux KH, Emmett MR, Blakney GT, Zhang HM, Marshall AG. Epitope mapping of a 95 kDa antigen in complex with antibody by solution-phase amide backbone hydrogen/deuterium exchange monitored by Fourier transform ion cyclotron resonance mass spectrometry. Anal Chem 2011;83:7129–36.
[71] Nakazawa S, Ahn J, Hashii N, Hirose K, Kawasaki N. Analysis of the local dynamics of human insulin and a rapid-acting insulin analog by hydrogen/deuterium exchange mass spectrometry. Biochim Biophys Acta Protein Proteomics 2012;1834:1210–4.
[72] Kaltashov IA, Bobst CE, Abzalimov RR, Berkowitz SA, Houde D. Conformation and dynamics of biopharmaceuticals: transition of mass spectrometry-based tools from academe to industry. J Am Soc Mass Spectrom 2010;21:323–37.
[73] Ovesen RG, Göransson U, Hansen SH, Nielsen J, Hansen HCB. A liquid chromatography-electrospray ionization-mass spectrometry method for quantification of cyclotides in plants avoiding sorption during sample preparation. J Chromatogr A 2011;1218:7964–70.
[74] Craik DJ. Discovery and applications of the plant cyclotides. Toxicon 2010;56:1092–102.
[75] Craik DJ, Daly NL, Bond T, Waine C. Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. J Mol Biol 1999;294:1327–36.
[76] Henriques ST, Craik DJ. Cyclotides as templates in drug design. Drug Discov Today 2010;15:57–64.
[77] Craik DJ, Daly NL, Mulvenna J, Plan MR, Trabi M. Discovery, structure and biological activities of the cyclotides. Curr Protein Pept Sci 2004;5:297–315.
[78] Craik DJ, Conibear AC. The chemistry of cyclotides. J Org Chem 2011;76:4805–17.
[79] Gran L. On the effect of a polypeptide isolated from "Kalata-Kalata" (*Oldenlandia affinis* DC) on the oestrogen dominated uterus. Acta Pharmacol Toxicol (Copenh) 1973;33:400–8.
[80] Hashempour H, Koehbach J, Daly NL, Ghassempour A, Gruber CW. Characterizing circular peptides in mixtures: sequence fragment assembly of cyclotides from a violet plant by MALDI-TOF/TOF mass spectrometry. Amino Acids 2012;44:581–95.
[81] Poth AG, Colgrave ML, Philip R, Kerenga B, Daly NL, Anderson MA, Craik DJ. Discovery of cyclotides in the *Fabaceae* plant family provides new insights into the cyclization, evolution, and distribution of circular proteins. ACS Chem Biol 2011;6:345–55.
[82] Chagolla-Lopez A, Blanco-Labra A, Patthy A, Sanchez R, Pongor S. A novel alpha-amylase inhibitor from amaranth (*Amaranthus hypocondriacus*) seeds. J Biol Chem 1994;269:23675–80.
[83] Gruber CW, Cemazar M, Anderson MA, Craik DJ. Insecticidal plant cyclotides and related cystine knot toxins. Toxicon 2007;49:561–75.
[84] Pinto MF, Fensterseifer IC, Migliolo L, Sousa DA, de CG, Arboleda-Valencia JW, Colgrave ML, Craik DJ, Magalhaes BS, Dias SC, Franco OL. Identification and structural characterization of novel cyclotide with activity against an insect pest of sugar cane. J Biol Chem 2012;287:134–47.
[85] Barbeta BL, Marshall AT, Gillon AD, Craik DJ, Anderson MA. Plant cyclotides disrupt epithelial cells in the midgut of *lepidopteran larvae*. Proc Natl Acad Sci U S A 2008;105:1221–5.
[86] Nourse A, Trabi M, Daly NL, Craik DJ. A comparison of the self-association behavior of the plant cyclotides kalata B1 and kalata B2 via analytical ultracentrifugation. J Biol Chem 2004;279:562–70.
[87] Jagadish K, Camarero JA. Cyclotides, a promising molecular scaffold for peptide-based therapeutics. Biopolymers 2010;94:611–6.
[88] Ireland DC, Clark RJ, Daly NL, Craik DJ. Isolation, sequencing, and structure — Activity relationships of cyclotides. J Nat Prod 2010;73:1610–22.
[89] Poth AG, Mylne JS, Grassl J, Lyons RE, Millar AH, Colgrave ML, Craik DJ. Cyclotides associate with leaf vasculature and are the products of a novel precursor in petunia (*Solanaceae*). J Biol Chem 2012;287:27033–46.
[90] Conlan BF, Gillon AD, Barbeta BL, Anderson MA. Subcellular targeting and biosynthesis of cyclotides in plant cells. Am J Bot 2011;98:2018–26.
[91] Göransson U, Broussalis AM, Claeson P. Expression of Viola cyclotides by liquid chromatography-mass spectrometry and tandem mass spectrometry sequencing of intercysteine loops after introduction of charges and cleavage sites by aminoethylation. Anal Biochem 2003;318:107–17.
[92] Selevsek N, Matondo M, Carbayo MS, Aebersold R, Domon B. Systematic quantification of peptides/proteins in urine using selected reaction monitoring. Proteomics 2011;11:1135–47.
[93] Wang CK, Kaas Q, Chiche L, Craik DJ. CyBase: a database of cyclic protein sequences and structures, with applications in protein discovery and engineering. Nucleic Acids Res 2008;36:D206–10.
[94] Sze SK, Wang W, Meng W, Yuan R, Guo T, Zhu Y, Tam JP. Elucidating the structure of cyclotides by partial acid hydrolysis and LC-MS/MS analysis. Anal Chem 2009;81:1079–88.
[95] Colgrave ML, Poth AG, Kaas Q, Craik DJ. A new "era" for cyclotide sequencing. Biopolymers 2010;94:592–601.
[96] Poth AG, Colgrave ML, Lyons RE, Daly NL, Craik DJ. Discovery of an unusual biosynthetic origin for circular proteins in legumes. Proc Natl Acad Sci U S A 2011;108:10127–32.