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Review

Proteomic approaches to study structure, functions and toxicity of legume seeds lectins. Perspectives for the assessment of food quality and safety

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ABSTRACT

Lectins are a structurally diverse class of (glyco)proteins which bind mono- and oligosaccharides with high specificity and in a reversible way. For many years, the unique sugar binding properties of plant lectins have been exploited for the development of biochemical tools for glycoprotein isolation and characterisation, and the use of lectins as a glycoprofiling tool has became much more sophisticated with the advent of lectin microarrays, in which a panel of lectins are immobilized on a single chip for glycomic analysis. Among the numerous lectins studied so far, those from legumes represent the largest family. They can be present at relatively high amounts depending on genetic as well as environmental factors, and are accumulated especially in the seeds. For this reason, some lectins as the phytohemagglutinin from the common bean *Phaseolus vulgaris* constitute a possible risk, since consumption of raw or incorrectly processed beans has been shown to cause outbreaks of gastroenteritis, nausea and diarrhoea. On the other hand, for these anti-nutritional properties, bean extracts enriched in lectins or in lectin-related amylase inhibitors are also finding a growing use as active ingredients of "weight-blockers" in dietetic preparations for obesity treatment. Current methods to determine the lectin levels in foods are based on immunoenzymatic or toxicity tests, which are largely aspecific. Very recently, the availability of proteomic methodologies has allowed to start development and validation of sensitive and specific assays for detecting trace amounts of harmful lectins in either raw or processed foods. In this review, the main aspects of current and perspective applications of mass spectrometry and proteomic technologies to the structural characterisation of legumes are presented, with focus on issues related to detection, identification, and quantification of phytohemagglutinins relevant for their biochemical, immunological and toxicological aspects.

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Contents

1. Plant lectins

The term "lectins" identifies proteins of non-immune origin which reversibly bind carbohydrates of glycoproteins, glycolipids, or polysaccharides with high affinity [1]. Because of their binding specificity, they play specific roles as mediators of cell recognition in a variety of biological processes, ranging from the symbiosis between nitrogen fixing bacteria and legumes to the adhesion of bacteria to host tissues and of leukocytes to endothelial cells. According to several studies, lectins have evolved through gene duplication and divergence, a process in which the carbohydrate-binding domains of lectins have become incorporated into families of proteins whose individual members play important roles in plant defense [2].

Lectins are generally named according to the plant they are extracted from. Of the many plant lectins that have been characterised extensively, most are secretory proteins, which means they enter the secretory system and subsequently accumulate either in vacuoles or in the cell wall and intercellular spaces. For example, the lectins concanavalin A, soybean agglutinin, phytohemagglutinin, pea lectin, and favin are all present at quite high levels and accumulate in vacuoles in the cotyledons (8–10% of total protein), and at lower levels in the embryonic axes of the seeds. These lectins are synthesized during seed development together with the more abundant seed storage proteins (see [3] for a discussion of plant lectins).

Despite their distinct sugar specificities, extensive homology in primary structure of lectins, also from unrelated taxonomic families, demonstrates a close evolutionary relationship. In addition, one plant species may contain structurally related lectin proteins with different biological properties. For example, the castor bean expresses two distinct but structurally related lectins with different biological properties: ricin and *Ricinus communis* agglutinin (RCA). Ricin is highly cytotoxic but is a weak agglutinin, whereas RCA is weakly cytotoxic but a strong agglutinin.

2. The use of lectins in proteomic research: from affinity chromatography to lectin microarrays and lectinomics

The different and high specificity of sugar-binding properties of plant lectins have made these molecules useful probes for glycan detection in carbohydrates and glycoproteins for many years, providing the basis for either biochemical analysis or for development of diagnostics tools in histology, blotting and biosensor applications [4,5]. Lectins are used as "glycan deciphers" to interpret the carbohydrate structure in living organs and cells [6,7]. Lectin affinity column chromatography, in which lectins are immobilized to agarose or other separation matrices, has been widely used as an efficient technique to fractionate not only N-glycosylated but also O-glycosylated peptides [8]. On this basis, the combination of mass spectrometry (MS) analysis with lectin chromatography has allowed the structural definition of highly complex glycoprotein systems, overcoming the limitations due to the glycan heterogeneity. In this integrated approach, the lectin-binding specificity provides the crucial, although partial, structural information to appropriately direct the fine structural MS analysis. The sequential use of different sets of available lectins may provide additional information to support glycopeptide MS analysis. Recently, the lectin affinity preparation of glycopeptides with *Sambucus nigra* agglutinin and concanavalin A provided the glycan structure outlines for the sialyl linkages and the core structure of N-glycans, soon confirmed by MS investigation [9]. On the other hand, the capability of the up-to-date mass spectrometric techniques in glycan profiling, especially when coupled with high-resolution separation techniques such as HPLC or capillary electrophoresis [10] allows for employing set of lectins for the capture of the entire glycoprotein panels and subsequent release of carbohydrates for analysis (Fig. 1). In this direction, multidimensional lectin affinity chromatography, based on a sequential approach [11], or multi-lectin affinity chromatography, in which a number of immobilized lectins are pooled in the stationary phase [12,13], has been developed for glycoproteome purposes. Glycoproteins selectively enriched by using the lectins can be separated by mono- or two-dimensional electrophoresis and identified with mass spectrometry based techniques. Because of the sensitivity of the MS techniques, the amount of glycoprotein sample required for multistage MS analysis is generally very low (about 1 nmol), also allowing to perform the glycoprotein enrichment at a microscale [14].

The electrophoresis-free proteomic approach usually addressed to the profiling of N-glycoproteins with the use of lectins is schematised in Fig. 1: the steps (Fig. 1, route 1) include lectin column-mediated affinity capture of glycoproteins, proteolytic digestion and PNGase F hydrolysis of glycans also mediating incorporation of ^{18}O as stable isotope label at

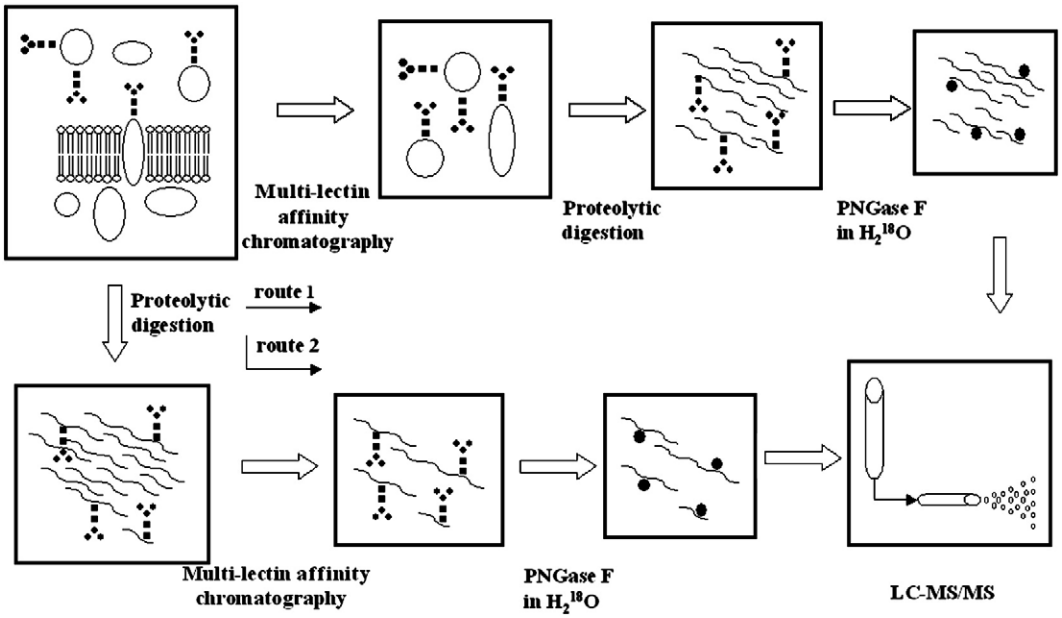

Fig. 1 – Schematic illustration of the proteomic-based strategy for profiling N-glycoproteins with the use of lectins.

the linker Asn, and sequencing of the ^{18}O-tagged peptides by LC-MS/MS. Alternatively, lectins can be used in the fishing of glycopeptides arising from a proteolytic digest; the isolated glycopeptides are then de-glycosylated with PNGase F in $H_2^{18}O$ and sequenced by mass spectrometry (Fig. 1, route 2). Even though in principle this latter strategy would allow to arrive to LC-MS/MS analysis with a highly enriched mixture of glycopeptides, the first route is preferable because of the greater specificity of lectins toward glycoproteins with respect to glycopeptides.

These features have also driven the most recent developments in modern glycomics [15], founding what has been very recently defined as "lectinomics" [16], emerging as a versatile biotechnology for understanding the complexity of living systems. The trend is reflected in the frequent use of lectins in glycoprotein enrichment and glycan profiling, so that modern glycoanalysis has clearly become a multi-methodological task. Methods in this field have now focused on the lectin microarrays, a kind of glycol-catch microarray in which a variety of lectins are bound to a solid support (lectin chip) and used for the rapid survey of diverse glycosylation features of glycoproteins [17], as schematised in Fig. 2. Typically, biological samples are labelled with a fluorescent tag and hybridised to the array. Analysis of the resulting pattern of spots then yields information about the carbohydrate/glycoprotein composition of the sample on the basis of the binding specificities of the lectins utilised. These arrays are capable of evaluating the glycosylation of small samples of glycoproteins (1 μg with conventional microarray scanners [5], picogram quantities with evanescent-wave based scanners [18]) and readily distinguishing differences in glycosylation.

The use of lectins in glycan profiling provides considerable advantages, such as discrimination between the isomers on the basis of biochemical rather than physiochemical properties [7]. Furthermore, lectin microarrays facilitate the extraction of glycan structure information about glycoproteins in a high-throughput and sensitive way, which is then validated by high resolution MS techniques (Fig. 2). For the advantages in terms of accurateness and convenience, their use has already

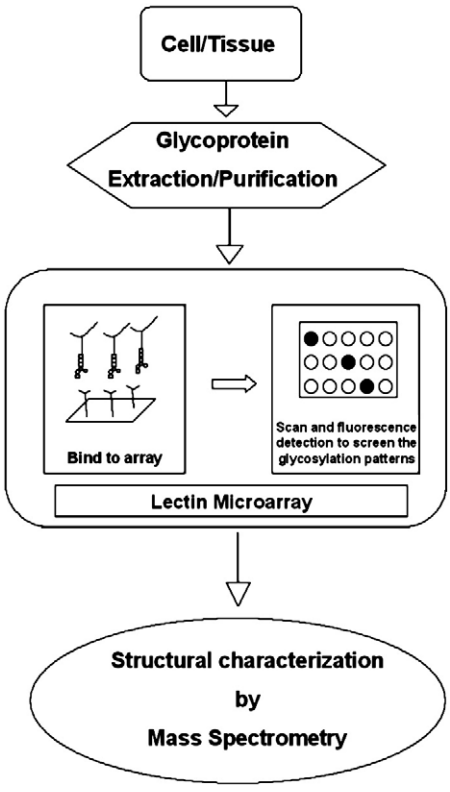

Fig. 2 – Lectin microarray technology. Fluorescently labelled samples (cells, tissues, foods) are hybridzed to the microarray. The resulting pattern is diagnostic of the glycosylation type and structure of the sample. Structural confirmation is obtained by mass spectrometry.

greatly contributed to the development of cancer-related glycomics research [6,19–21] as in the case of the profiling of markers in colorectal [22] and pancreatic cancer [23]. At present, a limitation to the use of lectin microarrays is the overlap in the affinity of the various lectins and glycans, making it difficult to describe the sequence and the construction of the N-glycans. Furthermore, although the analytical range of lectins is potentially enormous, only about 70 lectins are commercially available, most from natural sources, which can introduce some variability in their binding affinity dependent on cultivars and in the method of purification. Therefore the lectin microarrays need to be further optimised and their validity more extensively assessed from the viewpoint of disease-related glycomics. This also requires the careful characterisation of the structural and functional properties of the lectins from the various sources to enrich the set of available probes. A 'look-up' table detailing lectin specificity in the recognition patterns, in addition to strategies for generating lectins from enzymes, has been recently reported [24].

3. The lectins of common bean *Phaseolus vulgaris*

Among the numerous lectins studied so far, those of the legumes occupy a special position, since they represent the largest family of these proteins, and their biological properties have been extensively investigated (Table 1). Their ease of isolation has also facilitated structural studies focused on protein–carbohydrate interactions. Lectins from the species of the *Vicia* genus, *P. vulgaris* (common bean), *Glycine max* (soybean), *Pisum sativum* (pea), *Lens culinaria* (lentil), *Vicia faba*, *Lathyrus odoratus* and from several species of the *Erythrina* genus have been characterised in the last years [25,26]. Legume lectins show an extensive homology in their primary structures, although they possess different carbohydrate specificities, the latter being determined by a narrow stretch of residues belonging to six peptide strands, primarily those that are part of loop D of the amino acid sequence [27–29]. A prerequisite for understanding the structural basis of their diverse specificities toward carbohydrates is the knowledge of their amino acid sequence [4,27]. All legume lectins examined so far exhibit a truncated C-terminus, as the result of post-translational proteolysis which may affect their biological activity [27,28]. A further source of microheterogeneity comes from the N-linked oligosaccharides, which may vary in the number of monosaccharide residues [29].

Similarly to the other plant proteins, the N-glycosylation of lectins begins in the endoplasmatic reticulum (ER) with the transfer of the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ to an Asn residue in the consensus triplet Asn-Xaa-Ser/Thr (where Xaa is any amino acid but Pro). This precursor is then modified during the transfer of the glycoprotein by glycosidases and glycosyl-transferases located in the ER and in the Golgi apparatus. If the glycan side chain is accessible to the processing enzymes, these can convert the precursor to high-mannose-type N-glycans spanning $Man_{5-9}GlcNAc_2$ and then to complex-type glycan [30]. These complex oligosaccharides can be further modified by the elimination of terminal residues, resulting in the formation of truncated oligosaccharide structures, called paucimannosidic-type N-glycans, having only an α(1,3)-fucose and/or β(1,2)-xylose residue linked to the core $Man_3GlcNAc_2$. The glycoproteins present usually a constitutive microheterogeneity that occurs also in the PHA [30,31].

In the seeds of the common bean *P. vulgaris*, the protein fraction which has sugar binding property and hemagglutinating ability [1] is called phytohemagglutinin (PHA). It consists of five different tetramers formed by two polypeptide chains (PHA-E and PHA-L) in all possible combinations (L_4, L_3E, L_2E_2, LE_3, E_4). PHA-E and PHA-L are responsible respectively for the erythroagglutinating and leukoagglutinating properties [32]. Mixed tetramers can bind to both types of blood cells. The polypeptides PHA-E and PHA-L both recognize terminal galactose residues on complex glycans of mammalian glycoproteins. PHA-E e PHA-L are encoded by two tandemly linked genes, *dlec1* e *dlec2* respectively, probably deriving from a common ancestral gene [33], which present 90% homology at the nucleotide level and 82% at the protein level [34]. The biosynthesis, post-translational modifications, and transport to the vacuole of PHA have been studied: PHA-L and PHA-E have a similar carbohydrate and amino acid composition, but a different binding specificity for complex-type N-glycans. Several studies correlate the different specificity of the two isolectins to a difference in amino acid sequence of the loop B [34].

In addition to lectins, legume seeds contain other proteins that show sequence homology to the lectins but do not bind carbohydrates. In the common bean several of these lectin-like proteins have been identified, and have been shown to function as defensive proteins. They include inhibitors of insect gut α-amylases (αAI) [35], of which five variants have been sequenced so far [36], and a small group of proteins termed arcelins, that are only found in some Mexican wild bean varieties. It is unknown if a natural ligand exists for arcelin but the crystal structures of arcelin 1 and 5 from *P. vulgaris* indicate that it is unlikely that they are capable of carbohydrate binding. Arcelin and αAI can be considered as truncated forms of PHA, in which, respectively, one and two loops that play a sugar binding role are missing, abolishing the sugar binding properties. The major interest in these proteins is that they can be used to produce transgenic plants resistant to insect [37]. αAI is synthesized on the rough endoplasmic reticulum, glycosylated, and then transported to the protein storage vacuoles, where it undergoes proteolytic processing to polypeptides of *M*, 15,000 to 18,000 Da [38,39]. It was found that αAI isoform variants differed significantly in their glycosylation patterns, despite their high sequence homology [40].

4. Occurrence of *P. vulgaris* phytohemagglutinin in foods and in food ingredients

Ingestion of raw beans has long been known to be toxic to a variety of animals. The toxicity of purified PHA toward mammals [41,42] and birds [43] has been also demonstrated in feeding trials. The concentration of lectins in beans depends on

Table 1 – Lectin content in some legume seeds (raw material, g/kg) and some related properties and biological effects.

Species (common name)	Lectin concentration in raw material (in seeds, g/kg)	Toxicity and biological effects
Arachis hypogaea (peanut)	0.2–2	Slight oral toxicity; reactivity toward neuraminidase-treated cells; heat-unstable, harmful in raw and processed foods
Glycine max (soybean)	0.2–3	Slight oral toxicity; reacts with type A cells; low heat-stable, harmful in raw foods only
Lens culinaris (lentil)	0.1–1	Slight oral toxicity, heat-unstable, harmful in raw foods only; reacts with red cells from a number of animal species
Phaseolus coccineus (runner bean)	1–10	Antifungal activity towards some plant pathogenic fungi; antineoplastic activity; high oral toxicity, moderate heat-stability, harmful in raw foods and possible harmful in processed foods
Phaseolus lunatus (lima bean)	1–10	High oral toxicity; specificity for A cells ($A_1 > A_2$); moderate heat-stability, harmful in raw foods and possibly in processed foods
Phaseolus acutifolius (tepary bean)	1–10	High oral toxicity, moderate heat-stability, harmful in raw foods and possibly in processed foods
Phaseolus vulgaris (kidney bean)	1–10	High oral toxicity; PHA-E is responsible for erythroagglutinating activity; PHA-L is responsible for leucoagglutinating properties. unstable, harmful in raw foods and possibly in processed foods Heat-unstable, harmful in raw foods and possibly in processed foods
Pisum sativum (pea)	0.2–2	Slight oral toxicity; reacts with *A. neslundi*, *A. viscosus*, *S. mutans*; heat-unstable, possibly harmful in raw foods
Vicia faba (fava bean)	0.1–1	Slight oral toxicity; reacts well with rabbit and guinea pig erythrocytes; heat-unstable, possibly harmful in raw foods
Erythrina costaricensis (Poró Cimarrón)	~1	Agglutinates human and rabbit erythrocytes
Robinia pseudoacacia (Black locust)	~3	Two lectins purified from *Robinia pseudoacacia* seeds, RPA 1 and RPA 3 exert a mitogenic effect on human lymphocytes; both RPA 1 and RPA 3 agglutinate human erythrocytes
Sophora japonica (Japanese pagoda tree; scholar's Tree)	~1.7	Reacts with human erythrocytes, agglutinates type B better than type A cells and is relatively inactive toward type O erythrocytes
Wistaria sinensis (Chinese wisteria)	~1.6	A 2-acetamido-2-deoxy-D-galactose-binding agglutinin from *Wistaria sinensis* seeds agglutinates several vertebrate (including human) erythrocytes
Vicia cracca (tufted vetch; bird Vetch)	1.5–14	Reacts specifically with human blood group A erythrocytes
Cytisus scoparius (scoth broom)	~0.82	Mitogenic towards human lymphocytes treated with neuraminidase
Lotus tetragonolobus (winged pea)	~0.65	Recognizes blood group O(H) specifically
Laburnum alpinum (alpine golden chain tree; scotch laburnum)	~0.55	LAA-I is an anti-H(O) lectin
Bauhinia purpurea (butterfly tree)	~0.28	*Bauhinia purpurea* agglutinin (BPA) agglutinates human asialo erythrocytes regardless of their ABO and MN blood group type
Ulex europaeus (gorse)	0.09–0.16	UEA-I lectin recognizes blood group O(H) specifically. In the neuropathology it has been used as a marker of small dorsal root ganglionic neurons; it also recognizes skeletal muscle capillaries or endothelial cells
Canavalia ensiformis (jack bean)	~21	Positive effect of heat on product nutritive value; peritoneal and oral toxicity
Griffonia simplicifolia (griffonia)	3–7	Proved insecticidal activity for GS-II (*Griffonia simplicifolia* lectin II): when included in cowpea weevil (*Callosobruchus maculates*) delayed development of cowpea bruchid was observed. Isolectin-IB4 is known to bind to microglia, group B erythrocytes, perivascular cells, and endothelial cells
Dolichos biflorus (horse gram)	~1.1	Positive effect of heat on product nutritive value; oral toxicity; specificity for type A cells
Caragana arborescens (Siberian peashrub)	~0.35	Agglutinates mouse fibroblasts

Data are based on a compilation of data from different articles (cited in Supplementary information), as well as on unpublished data from authors' own research.

both genetic and environmental factors [41,44,45]. PHA binds to the intestinal mucosa of rats, resulting in the appearance of lesions, disruptions, and abnormal development of the microvilli (see [46], for a review). When PHA is added to the diet of experimental animals, it inhibits the absorption of nutrients across the intestinal wall and greatly increases the bacterial colonization of the small intestine. Gatehouse et al. [47] showed that impure PHA was more effective than pure PHA in arresting larval development, probably because of contamination by αAI-derived polypeptides.

In humans, the consumption of raw kidney beans has been shown to cause gastroenteritis, nausea and diarrhoea [48,49]

and several severe but non-fatal poisoning outbreaks caused by ingestion of abnormal PHA concentrations present in foods have been reported during years [50,51]. The PHA hemoagglutinating activity derives from its interactions with specific carbohydrate residues on the cell membrane structure [52]. A 2 kDa glycopeptide with potent phytohemagglutinin-receptor site activity has been isolated from the human erythrocyte membrane [53]. The involvement of dietary lectins, including PHA, in the etiology and development of several pathologies such as insulin-dependent diabetes, rheumatoid arthritis, IgA nephropaty, peptic ulcer, for which evidence has been accumulating in the last several years, has been reviewed by several authors [50,54].

On the other hand, commercial protein concentrates of common beans, the so-called "starch-blockers" are more frequently used as dietary supplements to control body overweight in therapy obesity [55], notwithstanding the absence of reliable scientific evidence for their efficacy [56–58]. The preparations are known to contain high levels of αAI, which hinders digestion of complex carbohydrates, thereby promoting weight loss. However, residual PHA activity may be also present, contributing to the blocker activity, but also inducing adverse effects. The increasing success of these supplements among patients and consumers call the need for appropriate methods to determine both the αAI and lectin levels, which are currently based on colorimetric assays or high performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD) analysis for αAI [55] and toxicity tests for PHA lectins [59] which are of necessity aspecific.

Significant variations in the content of toxins and antinutrients, as lectins, have been observed in plants, due to environmental influences, such as drought or salinity stress [60,61] (Table 1). The normal range of lectin levels in *P. vulgaris* is 1–10 g/kg (and up to 5% seed dry weight), which also implies a risk for health in processed food [44]. Some bean cultivars have not a detectable level of lectins in seeds, a trait inherited as a recessive allele [41,44,45]. The hemoagglutinating activity of bean lectins is progressively reduced with heat treatment, and thermal degradation is favoured if bean seeds are presoaked [49]. The most common method of destruction is in boiling water at atmospheric pressure or in autoclave [62–68]. Extrusion is considered the best method to abolish αAI and PHA activity [69] but, on the other side, bean PHA were shown to resist to even drastic microwave treatment [70]. Considering the possible risk for health in processed food, it is important to introduce efficient heat-based or other kinds of treatments in the processing of bean derived food as well as techniques to ensure the absence of toxicity in these products. The development of specific and sensitive methods needs to rely on the lectin structural and functional definition, which can now be achieved by integrated proteomic and glycomic methodologies.

5. Extraction, isolation and structural characterisation of legume lectins

Legumes are of enormous importance to human nutrition and are crucial crops for sustainable agriculture. Therefore, scientific research has been particularly active in the genomic and, recently, proteomic studies of leguminosae. Two model species, *Lotus japonicas* and *Medicago truncatula*, have initially been the focus of genome sequencing and programs of functional genomics. Recently the complete genome sequencing of the *P. vulgaris* has been announced [71]. The soybean genome characterisation is also rapidly evolving. By identifying the lectin genes it is possible to deduce amino acid sequences and to perform comparative and phylogenetic studies. Gene isolation/characterisation and definition of the amino acid sequence at the protein level has been also accomplished for lectins from seeds of pea (*P. sativum*, L.) and chickpea (*Cicer arietinum*, L.) [72]. The isolation of gene nucleotide sequences has allowed, by multiple alignment, to establish species relationships among lectins in the *Lens* genus [73].

The cloning of the legume lectin genes not only provides a basis for further investigation of structure, expression and regulation mechanism, but also enables to test its potential role in controlling pests and fungal diseases by transferring the gene into plants in the future. Proteomics is emerging as a powerful tool to complement genomic data and investigate functional and dynamic aspects of legume storage proteins and, thus, the number of systematic proteomic focused analysis for the leguminosae seeds is steadily increasing. According to a recent survey, 210 sequences of legume lectins are known, which are all homologues. Furthermore, by now, the three-dimensional structure of more than 35 legume lectins has been solved by high-resolution X-ray crystallography, either in free form or as complex with a variety of carbohydrate ligands [74].

Nevertheless, the recovery of lectins from crude leguminous extracts is a challenging task and represents a particularly interesting system with which to evaluate the potential of novel "multifunctional" purification technologies. In recent years there has been considerable interest in designing new methods to capture target products directly from crude unclarified process liquors and thereby fuse the three classical steps—clarification, concentration, and initial purification—into one unit operation. At the present time the so-called "expanded bed adsorption" (EBA) is probably the benchmark technique in this field [75]. Lectin extraction from aqueous solution by reverse micelles is another process that has been successfully applied to isolate plant lectins [76]. It utilises basic techniques such as classical liquid–liquid extraction, and hence has also the potential for industrial application. This process can be used to separate proteins which have been solubilized in organic solvents using surfactants, without affecting their functional properties.

For routine analyses, however, legume seed storage proteins are usually suspended in appropriate aqueous buffers; solutions are clarified by centrifugation and the proteins in the supernatant are then precipitated by ammonium sulphate (Fig. 3). Lectins can be purified by means of electrophoresis and reversed phase HPLC, or also by size exclusion and ionic exchange chromatography; these latter two techniques also preserve the tertiary and quaternary structure of the lectins, allowing to assay their biological properties (agglutinating profiles).

Taking advantage of the specific affinity for carbohydrates (or glycoproteins), plant lectins are more effectively isolated

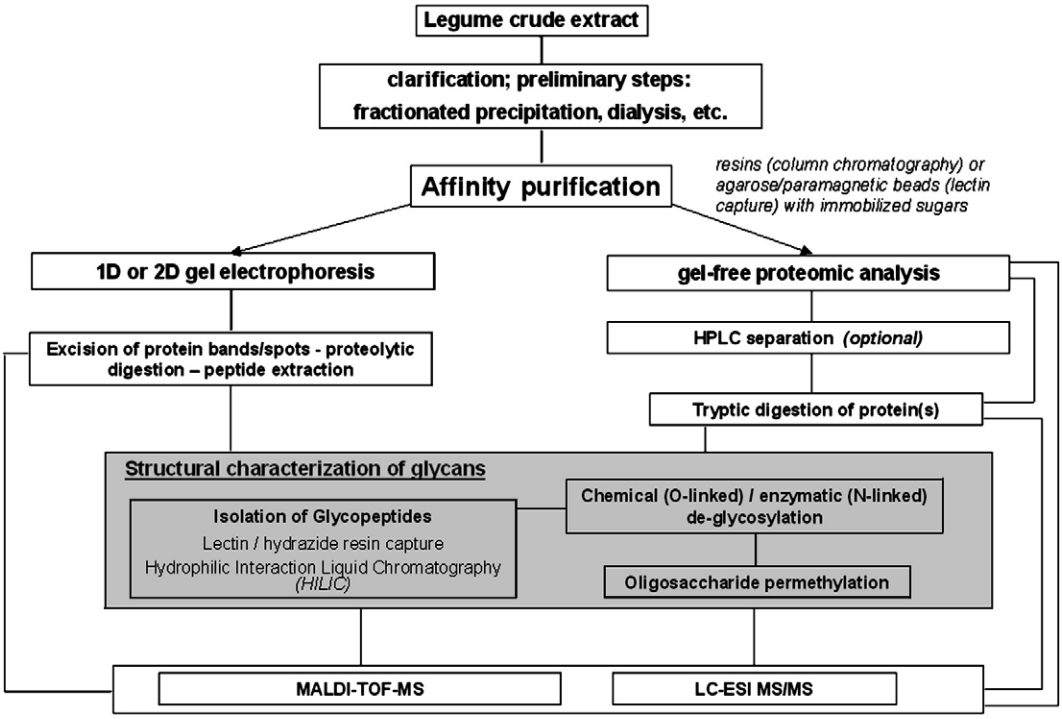

Fig. 3 – The lectin analysis workflow illustrating strategies for purification and structural characterisation of legume lectins.

using sugar-derived or sugar-immobilized stationary phases. Lectins with binding specificities for beta-D-galactosides, alpha-D-mannosides, alpha-L-fucosides, alpha-D-glucosides and for N-acetyl-D-glucosamine and N-acetyl-D-galactosamine have been isolated on agarose or sephadex resins with the proper carbohydrates immobilized as affinity ligands [77,78] (Fig. 3).

"Fishing" of lectins in crude extracts can be carried out utilising immobilized sugars on either agarose or paramagnetic beads. A number of activated polystyrene or agarose and functionalized paramagnetic beads are now available to immobilize the opportune sugar ligand and to capture the target lectin. Sugar chain immobilized on gold nano-particles can also be purchased. After binding to the support, lectins are eluted by competitive displacement from the column or from the beads with an elution buffer containing high concentration of the haptene saccharide. Some plant lectins, also including concanavalin A, have been successfully isolated by affinity using the inexpensive porcine plasma, rich in glycoproteins, immobilized on agarose beads [79].

When the affinity supports are applied to carbohydrate-containing legume extracts, the sugar-linked adsorbents could not supply sufficient competition to dissolved sugars to selectively bind lectins. The dextran-linked iron oxide paramagnetic beads have revealed themselves very efficient in high-gradient magnetic fishing experiments when other carbohydrates occur. In fact, adsorbents derivatized with dextran chains of 90–120 glucose residues have been successful in specifically capturing concanavalin A and *Lens culinaris* agglutinin from crude legume extracts [80].

After isolation, mass spectrometric sequence confirmation or structural characterisation of lectins can be carried out according to the gel electrophoresis based or to electrophoresis-free proteomic strategies (Fig. 3). In the case of glycosylated lectins, identification of glycosylation site(s) can be accomplished as illustrated in Fig. 1, following the general scheme: 1. proteolytic digestion; 2. capture of the glycopeptides choosing among several possible strategies including hydrophilic interaction chromatography (HILIC), affinity enrichment by lectins and hydrazide resin glycol-capture [81]; 3. chemical or enzymatic de-glycosylation (by peptide N-glycosidase F in the case of the N-glycosylation), preferably in $H_2^{18}O$; 4. LC-ESI MS/MS sequencing of deglycosylated peptides for identification of the linker amino acid. Characterisation of sugar moiety can be performed after oligosaccharide isolation and mass spectrometric analysis of native or derivatized glycans.

6. Strategies to assess structure–activity relationship in the food PHA lectin

PHA is a glycoprotein having one paucimannosidic-type N-glycan $Man_3XylFuc\text{-}GlcNAc_2$ on Asn-60 and one high-mannose-type $Man_nGlcNAc_2$ oligosaccharide on Asn-12 (Fig. 4) [30,31]. The presence of a high-mannose-type side chain results from the low accessibility of the oligosaccharide chain N-linked to Asn-12 on mature PHA to Golgi glycosidases and glycosyl-transferases [82]. HPAEC-PAD [30] or MALDI-TOF-MS [31] studies of the N-glycosylation patterns of purified isolectins E and L have demonstrated a large heterogeneity of the high mannose-type N-glycans ranging from $Man_6GlcNAc_2$ to $Man_9GlcNAc_2$. The PHA glycosylation heterogeneity has been studied by various MS approaches also in recombinant proteins expressed in various organisms including transgenic tobacco [83], in the methylotrophic yeast *Pichia pastoris* [84], and in *E. coli* [40].

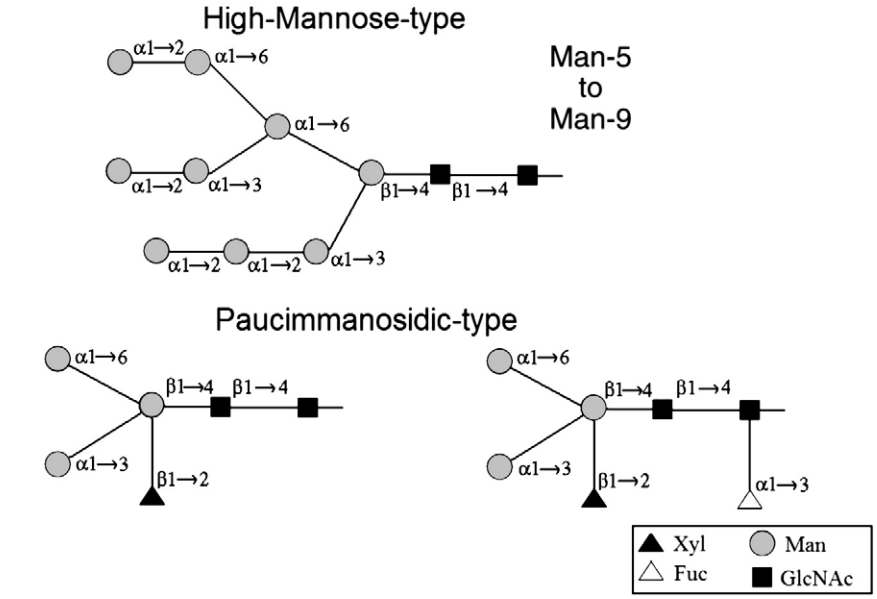

Fig. 4 – Structure of high-mannose and paucimannosidic N-glycans found in common bean phytohemagglutinin. The abbreviation systems applied herein names the terminal residues starting with the residue of the 6-linked antenna and proceeding counterclockwise.

These issues may be fundamental in the case of lectins considering that the N-glycosylation is often essential for stability, folding and biological activity of glycoproteins [85]. The proteomic and glycomic approach to study PHA (Fig. 3) starts from the development of methods for efficient extraction and purification of the lectin from bean flour, which is subsequently analysed with the aid of high resolution chromatographic and/or electrophoretic techniques in combination with mass spectrometry for structural and functional characterisation. Fig. 5 shows the MALDI-TOF spectrum of *P. vulgaris* PHA with molecular masses (M_r) of about 30 kDa [86] showing a series of components due to the microheterogeneity of the oligosaccharide moiety usually observed in the analysis of N-glycosylated proteins [87].

This indication can be confirmed by analysis of the oligosaccharide moieties, accomplished by analysis of the sugars released by hydrolysis with N-glycosidase F enzyme. In this approach, permethylated sugar samples released by endoglycosydase action are analysed by MALDI-TOF-MS which showed the presence of the expected two types of oligosaccharide moiety, one $Man_3XylFucGlcNAc_2$ formerly linked to Asn-60 and one high-mannose-type $Man_{6-9}GlcNAc_2$ (Fig. 6) oligosaccharide formerly linked to Asn-12.

7. Proteomic approach to quantitative analysis of PHA in raw and processed foods

Integrated proteomic and glycomic strategies are able to define the structural changes (proteolysis, oxidation, sugar changes) which can occur in raw and in industrially treated

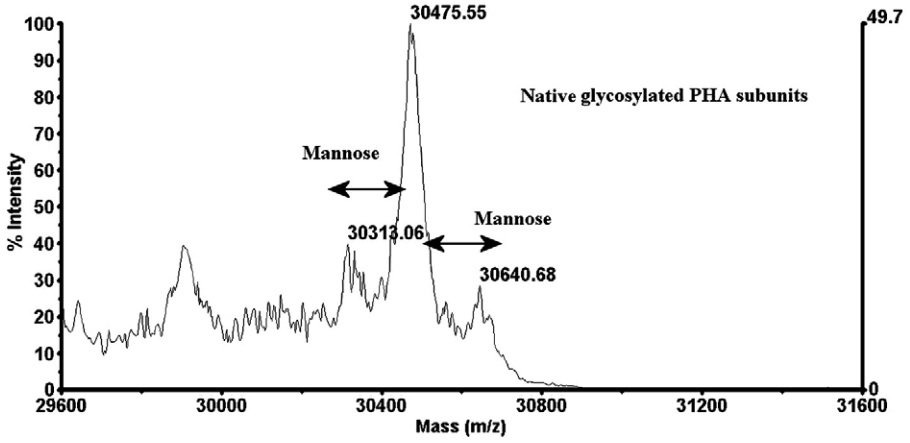

Fig. 5 – MALDI-TOF-MS spectra of bean PHA lectin. In the spectrum a series of components at 30 kD molecular mass (M_r) were present, differing by multiples of 162 Da (corresponding to a different number of mannose units) typically observed in the analysis of N-linked glycoproteins.

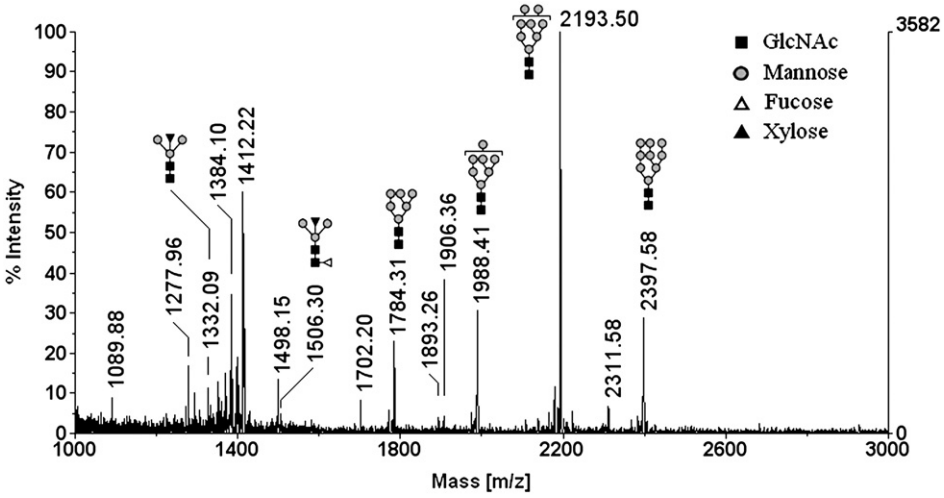

Fig. 6 – The N-linked oligosaccharides released by PNGase treatment of isolated PHA were permethylated and analysed by MALDI-TOF-MS. The two expected types of oligosaccharide moiety, one $Man_3XylFucGlcNAc_2$ formerly linked to Asn-60 and one high-mannose-type $Man_{6-9}GlcNAc_2$ were detected. The structures of N-glycans are depicted on top of the peaks.

products. In this way, they constitute the basis to allow for either the structural or quantitative analysis of PHAs in different origin samples.

An illustrative example is the control of bean flour deriving from the industrial dry thermal treatment normally carried out to inactivate lectins. If the thermal treatment is inefficient, residual lectin activity can survive, with moderate risks for consumers' health, as shown by several reports of outbreaks [42–45]. Available assays to reveal PHA contamination in food are based on immunological tests or on the measurement of hemoagglutinating activity on hemolysate sample. These analytical methods are sensitive, but very time-consuming and need to be performed by experienced personnel in specific facilities. Furthermore, because of the not rare occurrence of false positive results arising from non-specific response, a non-toxicological method to confirm results is desirable. Mass spectrometry is the only alternative method presently available to detect with high specificity PHA and related lectins in legumes, flours and in food samples. The direct observation by MALDI-TOF-MS can be used for semiquantitative measurement of lectins in food after appropriate isolation.

Because of the complexity of composition of the flour samples, which prevents direct mass spectrometry analysis, a procedure for lectin purification has to be introduced, based on extraction of seed proteins followed by affinity column purification which utilises the sugar binding properties of PHA lectins themselves. In this case, the affinity approach which normally uses lectins to purify glycoproteins is reversed to use a standard glycoprotein, α_1-acid glycoprotein, immobilised on an affinity resin [86]. This procedure isolates the undenatured lectins still present in the samples as distinct from the lectins denatured by industrial heat treatment. With this assumption, and with the efficient step of column washing to eliminate aspecifically bound proteins, this analytical method can isolate lectins in native form, and therefore potentially toxic, still present in the samples.

Analysis of PHA fraction purified from a bean flour sample positive to lectin toxicity tests, as a consequence of an

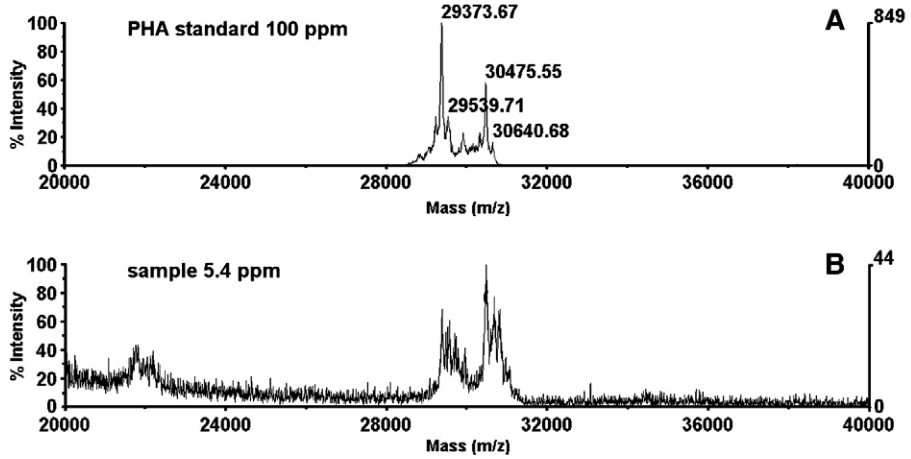

Fig. 7 – MALDI-TOF-MS spectra of PHA lectins isolated from a contaminated commercial bean flour sample (B) in comparison with standard PHA (A). Peak area intensity was used for semi-quantitative determination.

inefficient thermal treatment during the industrial process, confirms the presence of the native PHA subunits, whose presence is confirmed by MS analysis (Fig. 7). A step forward will be the scaling-down of this procedure to an automated miniaturized scale through immobilization of the glycoprotein on a solid support to obtain an affinity sensor capable of detecting low-ppm amounts of native lectins in legumes and in the derived foods. This approach reverses the idea of "glycan-capture lectin array" to a "lectin-capture glycan array", as it uses an immobilized glycoprotein to detect and quantify PHA lectins.

8. Perspectives

Genes coding plant proteins are part of multigene families which evolved rapidly, among related plant species and even within the same species. This occurrence and post-translational events such as glycosylation, to a subsequent step, make plant proteins extremely heterogeneous. Also because of variability caused by genotype, growing conditions, and technological processes, lectins are among the most complex protein networks in nature. For these reasons, in spite of the large number of studies dedicated to characterising their structure and functionality, important gaps of knowledge have to be filled by future research. As shown, mass spectrometry, which was unused until a few years ago for the investigation on plant proteins, has rapidly evolved into a comprehensive proteomic approach able to characterise the complete protein pattern in plant seeds, in order to detect differences in their composition among varieties and species, or in differently processed food products. The proteomic approach is already giving us a strong contribution to determine the structural features of lectins, and the structure–function relationship of specific lectin components, which will turn to be of economic, social and scientific value. In fact, as a first direct consequence, we are assisting to the development of novel microarrays-based techniques offering high-throughput of detection and showing promise as efficient tools to decipher the enormous complexity of the glycocode which influences the physiological and/or pathological status of cells. This will be of help, for instance, in the identification of biomarkers for early and specific diagnosis of several types of cancer and of genetic/autoimmune disorders.

As a further, not secondary issue, proteomic protocols developed for characterisation of lectins from bean flour samples and for quantitative evaluations by combination of affinity chromatography, HPLC, MALDI-TOF-MS and ESI-Q-TOF-MS/MS analysis, result in a high-sensitivity detection and characterisation of the structural features of legume PHAs, including glycosylation. This can be important considering that the N-glycosylation is often essential for stability, folding and biological activity of glycoproteins.

The application of the novel MS methodologies (mainly but not only *nano*LC/ESI-MS/MS, MALDI-TOF-MS, and MALDI-TOF/TOF-MS) to the characterisation of the harmful lectins in foods and in dietary preparations would help to improve the quality and safety of products and therefore to minimise the risk of intoxications. However, due to the high complexity and cost of the instrumentation, a wide spreading of the MS approach for routine measurement in foods cannot be foreseen yet. Rather, these studies envisage the development of analytical tools which can simply and efficiently detect lectin contaminants in foods and to standardise the quantitative detection of trace amounts through the settlement of specific "food-based" protocols, to ultimately arrive to a quality control system for foods, designed either as kits similarly to glucose or pregnancy tests available to industry, or to develop glycan-based sensors for specific detection. These last applications are meant to be broadly used either by food producers and industries or by consumers, restaurants, refectories which will be able to efficiently control their own foods. Also the advances and future developments of protein array technology, which will be largely driven by MS-derived structural information, will ensure more rapid and accurate detection of food safety for the consumer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2009.02.001.

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