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Analysis of isolectins on non-porous particles and monolithic polystyrene-divinylbenzene based stationary phases and electrospray ionization mass spectrometry

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In memoriam of University Professor Dr. Werner Lindinger.

Abstract

This paper reports on the use of non-porous particular octadecylated and monolithic polystyrene-divinylbenzene (PS-DVB) based stationary phases coupled to mass spectrometry (MS) for the fast separation and characterization of important plant isolectins. Optimization of the mobile phase allowed the analysis of a mixture consisting of lentil, wheat germ lectins and concanavalin A within 10 min using both column technologies. The monolithic PS-DVB phase showed higher efficiency for the separation of the wheat germ lectin isoforms and the concanavalin A proteolytic fragments. Using UV-detection at 214 nm multiple peaks were observed corresponding to fragments or isoforms of each individual lectin. Due to the higher efficiency and the lower flow rate (3 μL/min) the monolithic capillary column was coupled to mass spectrometry (MS) via an electrospray ionization (ESI) interface. MS detection allowed the selective identification of three wheat germ and two lentil isolectins. In case of concanavalin A two proteolytic fragments were identified, which are generated during post-translational processing of the protein. Finally, the established LC-ESI-MS method was used for the identification of the lentil isolectin in a complex lentil extract. (Int J Mass Spectrom 223–224 (2003) 519–526)

Keywords: Lectins; Column liquid chromatography; Electrospray ionization (ESI) mass spectrometry; Non-porous and monolithic polystyrene-divinylbenzene; Isoforms

1. Introduction

Lectins are carbohydrate binding proteins, which have been isolated from microorganisms, plant seeds, roots and bark as well as mammalian cell membranes [1]. Plant lectins are involved in the attachment of nitrogen fixing bacteria to legumes and the protection against pathogens [2]. These proteins have proven to be valuable tools for several applications, e.g., blood grouping and mitogenic stimulation of lymphocytes, isolation of glycoproteins and characterization of their carbohydrate structure [3,4]. Lectins can occur in several isoforms, which may originate from genetic polymorphisms resulting in a slightly different amino acid sequence and 3D-structure or may be generated during posttranslational processing [1,5]. Although

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closely related, these isoforms can differ in their carbohydrate specificity as well as in other biological functions [6,7]. Isoelectric focusing, chromatofocusing or hydrophobic interaction chromatography are most commonly used for their separation. However the elucidation of the structural differences of the isoforms by protein chemical methods may be laborious [8,9]. Reversed-phase liquid chromatography (RP-LC) has emerged as one of the predominant separation techniques for proteins. The suitability of non-porous, micropellicular polystyrene-divinylbenzene (PS-DVB) stationary phases for the fast and efficient separation of proteins in general has been demonstrated [10]. Advantages are favorable mass transfer properties, the stability at strong alkaline or acidic pH and at elevated temperatures. For the separation of lectins only a fraction of analysis time compared to silica-C8 (300 Å, 7 μm) based stationary phases [11] is required. Polymer based monolithic columns are prepared by in situ polymerization of monomers and porogens within a tube. They have been used for the separation of peptides and proteins [12–14].

UV detection often offers insufficient information for the structural identification and characterization of proteins. Mass spectrometry (MS), which allows accurate molecular weight determination, is therefore the preferred detection method for these biomolecules. PS-DVB based capillary columns have been successfully hyphenated to electrospray mass spectrometry and their suitability for the separation of standard proteins and peptides has been shown [15,16].

The aim of this work is to develop a rapid method for the separation and characterization of phytochemically related plant isolectins. Isolectins of three different plants species are separated in this study: lentil (*Lens culinaris*), wheat germ (*Triticum vulgaris*) and jack bean (*Canavalia ensiformis*). The isoforms are of proteolytic (lentil) and genetic (wheat germ) origin and share high sequence homology. The suitability of PS-DVB micropellicular and monolithic columns in combination with electrospray mass spectrometry for isolectin separation and characterization is compared. Finally, the method is used for the identification of a plant lectin in a complex biological mixture.

2. Experimental

2.1. Materials and reagents

Acetonitrile (gradient grade), acetic acid (analytical-reagent-grade), divinylbenzene (synthesis grade), formic acid (analytical-reagent-grade), ammonium sulfate (analytical-reagent-grade), styrene (synthesis grade) and trifluoroacetic acid (analytical-reagent-grade) were purchased from Merck (Darmstadt, Germany). Styrene and divinylbenzene were distilled before use. Azobisisobutyronitrile (synthesis grade), decanol (synthesis grade) were purchased from Fluka (Buchs, Switzerland). For preparation of all aqueous solutions, water purified by a NanoPure-unit (Epure, Barnstead, Boston, MA, USA) was used. Concanavalin A, lectins from lentil and wheat germ were from Sigma (Deisenhofen, Germany). For the analysis of lectins in biological samples lentils were purchased at a local market.

2.2. Biological sample–ammonium sulfate precipitation of lentil proteins

Precipitation was carried out in accordance to [8]. Lentils (250 g) obtained from a local food store were soaked overnight at 4 °C in 1.25 L of 0.9% NaCl. The mixture was homogenized with an Ultra-Turrax T25 (IKA, Staufen, Germany), left at room temperature for 30 min, and centrifuged. The supernatant was fractionated by addition of solid ammonium sulfate to obtain the material between one- and two-thirds of saturation. The precipitate was dissolved in Tris buffer (0.05 M, pH 8.1) and dialyzed against the buffer. The solution was centrifuged and passed through a 0.2 μm membrane filter.

2.3. Preparation of packed and monolithic columns

Octadecylated PS-DVB particles (PS-DVB-C18) were synthesized according to the literature [17,18]. The PS-DVB-C18 stationary phase (2.1 μm, 50 mm × 4.6 mm i.d.) has been commercialized as DNASep by Transgenomic Inc. (Santa Clara, CA, USA).

Polyimide-coated fused silica capillary tubing of 350 μm o.d. and 200 μm i.d. was obtained from Polymicro Technologies (Phoenix, AZ, USA). A 1 m piece of fused silica capillary tubing was salaried with 3-(trimethoxysilyl)propyl methacrylate according to the procedure published in [19,20] to ensure immobilization of the monolith at the capillary wall. A 300 mm piece of the silanized capillary was filled, using a plastic syringe, with a mixture comprising 100 μL of styrene, 100 μL of divinylbenzene, 280 μL of decanol, 20 μL of dioxane, and 5 mg of azobisisobutyronitrile. After the polymerization at 70 °C for 24 h, the capillary was extensively flushed with acetonitrile at a flow rate of 5.0 μL/min and finally cut into 70 mm long pieces.

2.4. High-performance liquid chromatography (HPLC)

The HPLC-system used for analyses using particular PS/DVB consisted of a low pressure gradient pump (model 616, Waters, Milford, MA, USA) connected to a controller (model 600S, Waters), an autosampler (model 717 plus, Waters), a photo diode array detector (model 996, Waters) with a 10 mm pathlength flowcell, a fluorescence detector (model 474, Waters) with a 16 μL flowcell and a helium degassing system. Data were recorded on a PC, using the manufacturers software package (Millenium^{32}, Version 3.05.01, Waters).

The μ-LC-system used for analyses using monolithic PS/DVB consisted of a low-pressure gradient micropump (model Rheos 2000, Flux, Karlskoga, Sweden), a degasser (ERC-3415α, Ercatech, Alteglofsheim, Germany), a microinjector (model 7520, Rheodyne, Cotati, CA, USA) with a 0.5 μL rotor connected to a UV-Vis detector (model 345, Varian, Darmstadt, Germany) with a bubble cell.

2.5. High-performance liquid chromatography (LC) coupled to electrospray ionization (ESI) mass spectrometry (LC-ESI-MS)

Mass spectrometry was performed on a Finnigan MAT LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization source. The monolithic capillary column was connected inline to the spray capillary (150 μm o.d., 75 μm i.d., Polymicro Technologies, Phoenix, AZ) by means of a connector. A voltage of 4.5 kV was applied and a temperature of 190 °C was used to heat the capillary. Nitrogen was used as sheath gas for pneumatically assisted ESI. The mass spectra were recorded on a personal computer equipped with the LCQ Xcalibur software version 1.2 (Finnigan). Cytochrome C was used as tuning substance. For protein identification and sequence comparisons the Molecular Biology Server of the Swiss Institute of Bioinformatics (Genève, Switzerland) was used.

3. Results and discussion

3.1. Separation of lectin isoforms by liquid chromatography and UV detection

For the analysis of concanavalin A, lectin isoforms from lentil and wheat germ in biological samples of complex origin, a non-porous PS-DVB-C18 stationary phase (2.1 μm, 50 mm × 4.6 mm i.d.) allowed short analysis time and high resolution as well as coupling to MS. Separations with a water–acetonitrile gradient containing 0.1% TFA gave promising results, alternative acid additives were evaluated and their concentration optimized. Using acetic acid at its optimum concentration regarding resolution of 1%, the concanavalin A corresponding peak disappeared. Changing to 0.1% TFA or 0.5% formic acid improved selectivity, 0.5% formic acid showing 34% higher reproducibility of peak areas. The peak width at half height increased slightly with 4.9% in TFA. Further optimization of the gradient showed the determination of concanavalin A, lentil and wheat germ lectins within 10 min (Fig. 1), which means a reduction of analysis time by a factor of six compared to silica-C8 (300 Å, 7 μm) based stationary phases [11]. For UV detection, a wavelength of 280 nm was used due to the self-absorption of formic acid at 214 nm. Subsequently, investigation of the amount of injected

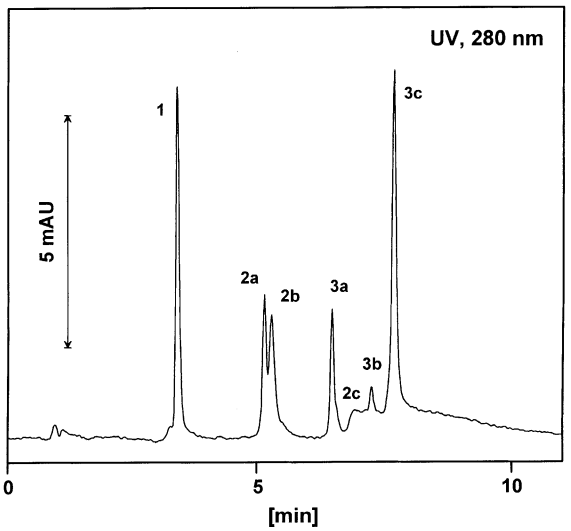

Fig. 1. LC-UV of a lectin standard mixture using non-porous PS-DVB-C18 (2.1 μm, 50 mm × 4.6 mm i.d.); mobile phase, (A) 0.5% formic acid in water; (B) acetonitrile, 0.5% formic acid; gradient, 0 min, 90% A; 0.5 min, 77% A, 2.5 min, 77% A; 6 min, 50% A; flow rate, 1.0 mL/min; detection, UV, 280 nm; temperature, 25.0 °C; sample volume, 20 μL. Peak 1, wheat germ lectin; peak 2a, lentil isolectin B-α subunit; peak 2b, lentil isolectin A-α subunit; peak 3a, concanavalin A; peak 2c, lentil isolectins A+B-β subunit; peak 3b and 3c, concanavalin A.

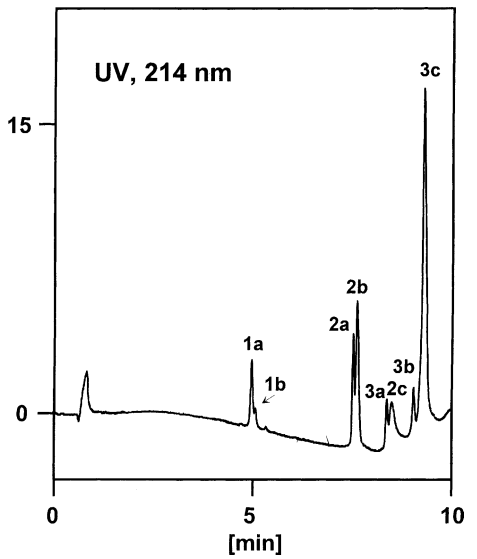

Fig. 2. LC-UV of a lectin mixture using monolithic PS-DVB (7 cm × 200 μm i.d.); mobile phase: (A) 0.1% aqueous TFA, (B) 0.1% TFA in acetonitrile; gradient, 0 min, 90% A; 1 min, 70% A; 10 min, 0% A; flow rate, 2.3 μL/min; detection, UV, 214 nm; sample size, 0.5 μL. Peak 1a, wheat germ isolectin 2 and 3; peak 1b, wheat germ isolectin 1; peak 2a, lentil isolectin B-α subunit; peak 2b, lentil isolectin A-α subunit; peak 3a, concanavalin A, proteolytic fragment 1–118; peak 2c, lentil isolectins A+B-β subunit; peak 3b, concanavalin A, proteolytic fragment 119–237; peak 3c, concanavalin A.

analytes regarding resolution, showed its optimum at 380 ng. Furthermore, UV-detection allowed the determination of seven lectins originating from wheat germ (one peak) and lentil (three peaks) as well as concanavalin A (three peaks). The third broad peak was observed after injecting a higher amount of lentil lectines (peak 2c, Fig. 1).

In order to get a highly efficient and hyphenated separation and MS technology, monolithic stationary phases based on PS-DVB were applied. In contrast to the non-porous particular column, the highest separation efficiency was observed using 0.1% TFA instead of formic acid as mobile phase additive. Generally, using the monolithic phase injected amounts of analytes are 10–40-fold lower than in the case of the particular PS-DVB column and due to the low flow rate of 3 μL/min no splitting for the hyphenation with MS is necessary. For the wheat germ lectin, the change from PS-DVB-C18 particles to the monolithic phase allowed the partial resolution of two peaks, which can be deduced from Fig. 2 (peak 1a and 1b). Injection of the lentil lectin resulted in three peaks using both stationary phases (Fig. 1; peaks 2a, 2b, 2c). In Fig. 2 peaks 3a, 3b and 3c belong to concanavalin A.

3.2. Separation of lectin isoforms by μ-liquid chromatography and ESI mass spectrometric detection

The utility of LC separation using non-porous PS-DVB as stationary phase for a more specific and selective identification of lectins was greatly enhanced by direct coupling to MS via an electrospray ionization interface (ESI), especially for the determination of isoforms in samples with coeluting peaks. Under these ionization conditions, a series of multiply charged ions is observed, which can efficiently be deconvoluted into one peak in a real mass scale,

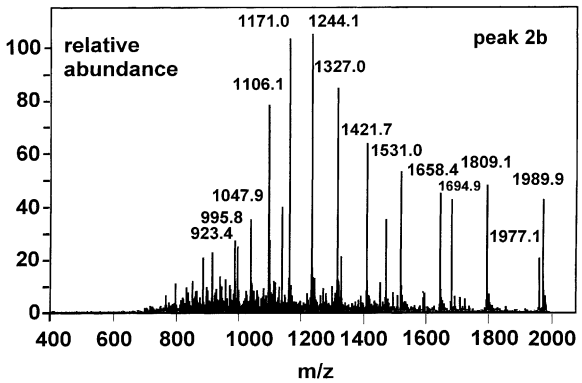

Fig. 3. Mass spectrum of lentil isolectin A-β subunit (peak 2b, Fig. 2); full scan, *m/z* 500–2000 units in 1.5 s.

corresponding to the average mass of the lectin. Since multiple peaks with the same mass spectrum were observed upon injection of each individual lectin corresponding to fragments or isoforms, advanced mass spectrometric analyses had to be carried out (Fig. 3).

Fig. 4a depicts an LC-MS chromatogram of the wheat germ lectin. Since TFA as mobile phase additive caused signal suppression, it was substituted by 0.3% formic acid, which resulted in improved detection sensitivity. In accordance with Fig. 2, two partially resolved peaks are observed in the chromatogram. The mass spectrum of the first peak deconvoluted to two masses, 17,090 and 17,175; the second peak to 17,081 (Table 1). Sequences of three wheat germ isolectins have been elucidated, which share 95–97% of sequence homology. Isolectin 1 and 2 deviate at five, isolectin 3 deviates from isolectin 1 at eight and from isolectin 2 at seven sequence positions. Average molecular masses of 17,081, 17,090 and 17,175 Da can be calculated from the published cDNA sequences of wheat germ isolectins 1, 2 and 3, respectively [21]. These masses agree well with the most intense ions detected. Fig. 4b and c illustrate the extracted mass chromatograms from the total ion current (TIC) (Fig. 4a) for the 10+ charged ions of isolectin 1 (Fig. 4b–*m/z*, 1709.1) and isolectin 2 (Fig. 4c–*m/z*, 1710.1). Surprisingly, isolectin 2 is partially separable from isolectin 1 but not from isolectin 3, although isolectin 1 and isolectin 2 share a higher sequence homology.

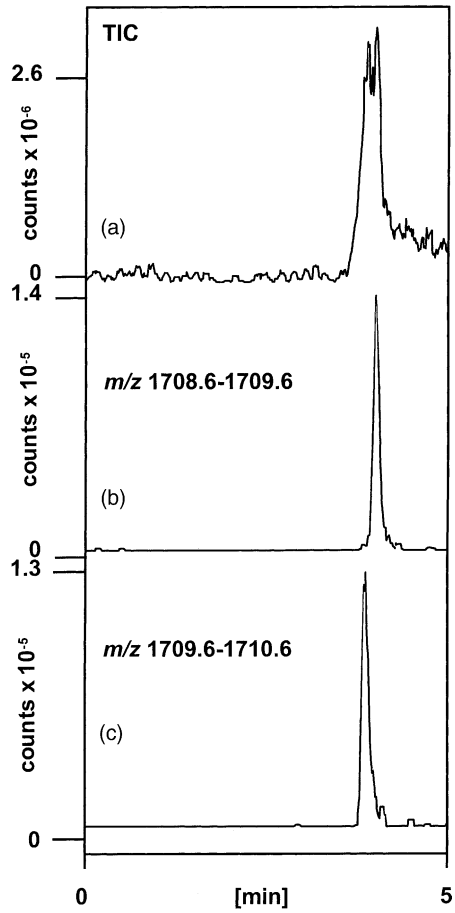

Fig. 4. LC-ESI-MS of wheat germ isolectins. (a) Total ion current (TIC); (b) extracted ion trace for wheat germ isolectin 1; (c) extracted ion trace for wheat germ isolectin 2. Column: PS-DVB monolith (7 cm × 200 μm i.d.); mobile phase: (A) 0.3% formic acid, water; (B) 0.3% formic acid, acetonitrile; gradient, 0 min, 90% A; 1 min, 70% A; 14 min, 0% A; flow rate: 3 μL/min; scan, 500–2000 units in 1.5 s.

Lentil contains two electrophoretically distinguishable isolectins (lentil isolectin A and B) [8]. In isoelectric focusing, isolectins A and B can be separated into two distinct bands of pI 8.5 and 9.0, respectively. Both isolectins are composed of two polypeptide chains: a N-terminal β chain (MW 19,891 Da) and a smaller C-terminal α chain (5878 Da; Table 1). Three peaks are observed in the LC-MS chromatogram (Fig. 5a; peak 2a, 2b, 2c). The mass spectrum of the peak, which eluted first (peak 2a), deconvoluted to two masses

Table 1
Masses of lectins after deconvolution measured by liquid chromatography and electrospray ionization mass spectrometric detection

Lectin	Theoretical mass[a]	Measured mass[b]
Wheat germ		
Isolectin 1	17,081	17,081
Isolectin 2	17,090	17,090
Isolectin 3	17,175	17,175
Lentil isolectins A+B-β subunit	19,891	19,890
Lentil isolectin A-α subunit	5621	5621
	5533	5534
	5445	5446
Lentil isolectin B-α subunit	5878	5878
	5750	5750
Concanavalin A	25,598	25,598
Fragment 1–118	12,939	12,939
Fragment 119–237	12,678	12,678

[a] The sequence data used for the mass calculations were from the following references: wheat germ lectin [21], lentil lectin α subunit [24], lentil lectin β subunit [22] and concanavalin A [23].
[b] Calculated by the deconvolution program from a series of multiple-charged ions.

(5878; 5750), the second peak (peak 2b) to three (5621; 5534; 5446). The deconvoluted mass spectrum of the third peak (peak 2c) revealed one single mass of 19,890. The masses detected in the first two peaks agree with the theoretical average molecular weight of the α subunit (amino acids 1–54) and its C-terminal truncations 1–53, 1–52, 1–51, 1–50. The mass in the third peak corresponds to the β subunit (theoretical MW: 19,891 Da). These C-terminal truncations of the α subunit cause the separation of two isoforms in isoelectric focusing. From the sequences of the intact α subunit and fragment 1–53 a pI of 5.37 can be calculated (isolectin B-α subunit). Cleavage of K-53 causes the pI to shift to a lower value of 4.98 (isolectin A-α subunit). Similarly, in reversed phase chromatography, the intact α subunit and fragment 1–53 (isolectin B-α subunit) elute earlier than the fragments 1–52, 1–51 and 1–50 (isolectin A-α subunit), which lack the positively charged lysine residue at position 53. The separation of the lentil lectin into three peaks facilitated the interpretation and deconvolution of the mass spectra significantly. Fig. 5b–d depict the extracted mass chromatograms from the total ion current in Fig. 5a for the 5+ charged ions of isolectin B-α subunit (*m/z* 1176.3), isolectin A-α subunit (*m/z* 1107.8) and the 12+ charged ion of the β subunit (*m/z* 1658.5). If the lentil lectin is injected directly into the mass spectrometer without previous separation, the spectra of the five α-subunit fragments and the β-subunit overlap.

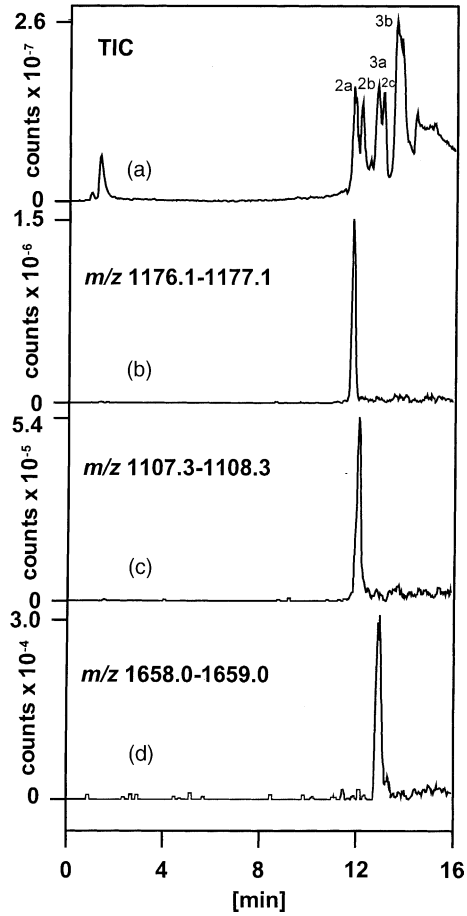

Fig. 5. LC-ESI-MS of lentil isolectins and concanavalin A. (a) TIC; (b) extracted ion trace for lentil isolectin B-α subunit; (c) extracted ion trace for lentil isolectin A-α subunit; (d) extracted ion trace for lentil isolectin A+B-β subunit; peak 2a, lentil isolectin B-α subunit; peak 2b, lentil isolectin A-α subunit; peak 3a, concanavalin A, proteolytic fragment 1–118; peak 2c, lentil isolectins A+B-β subunit; peak 3b, concanavalin A, proteolytic fragment 119–237; conditions as in Fig. 4.

Concanavalin A is expressed as a precursor and processed proteolytically to produce two chains of 12,939 and 12,678 Da. These chains are subsequently transposed and religated by formation of a new peptide bond. The mature protein has a molecular weight of 25,598 Da (Table 1). Fig. 5a shows the LC-MS separation of concanavalin A (peak 3a and 3b). Three peaks have been observed in the LC-UV chromatogram (Fig. 2, peaks 3a, 3b, 3c). Two of them overlap in the LC-MS chromatogram (Fig. 5a). The spectrum of the earlier eluting peak deconvoluted to a single mass of 12,939, the second peak to the two masses 12,678 and 25,598. The two ions of approximately 12 kDa represent the fragments 1–118 and 119–237 of concanavalin A, generated during the proteolytic processing of the lectin. The mass 25,598 corresponds to the intact, mature concanavalin A. No further proteolytic fragments or isoforms of concanavalin A were observed.

3.3. Isolectin analysis in complex matrices applying LC-ESI-MS

The optimized LC system and the employment of MS detection was evaluated for the identification of lentil isolectins in a complex lentil extract pretreated

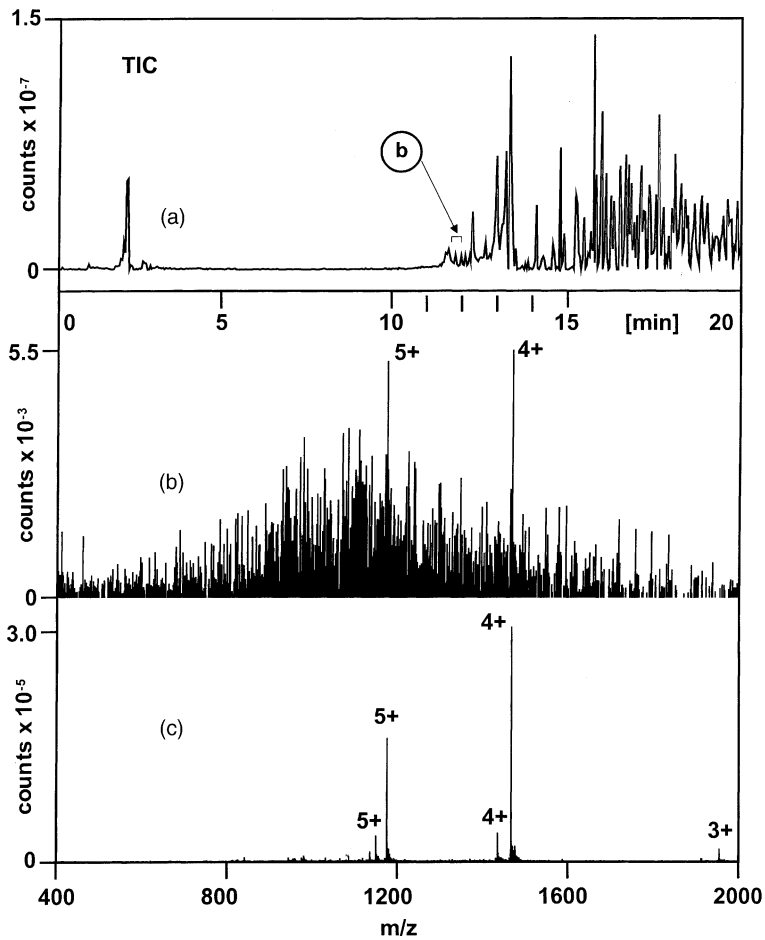

Fig. 6. Separation and ESI-MS detection of proteins extracted from lentils. (a) TIC; (b) average of nine scans at 11.7–12.0 min; (c) average of 14 scans at 11.6–12.1 min of peak 2a, Fig. 5. Conditions as in Fig. 4.

as described in chapter 2.2. Fig. 6a shows the reconstructed ion chromatogram of a pretreated lentil extract. Although the chromatogram is very complex and separation is incomplete, extraction of mass spectra from selected sections of the chromatogram enabled the identification of the α-subunit of lentil isolectin B. Averaging nine scans at 11.7–12.0 min yielded the mass spectrum depicted in Fig. 6b, which was comparable to the mass spectrum of the standard lentil B-α subunit (see Fig. 5, peak 2a) received by averaging 14 scans at 11.6–12.1 min (Fig. 6c). Fig. 6b also demonstrates the limits of LC as separation method for highly complex samples. In order to enable a more efficient analysis of lectin isoforms in complex matrices more selective sample preparation methods or multidimensional separations are necessary.

4. Conclusions

Finally, this elaborated LC-ESI-MS method using monolithic PS-DVB stationary phase allows further investigations of lectin isoforms in a wide plant variety such as e.g., food and feed samples. Liquid chromatography in combination with MS is a powerful tool for the identification of isoforms with multiple and heterogeneous subunits.

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