Solid-phase extraction method for the isolation of plant thionins from European mistletoe, wheat and barley using zirconium silicate embedded in poly(styrene-co-divinylbenzene) hol...

ARTICLE *in* ANALYTICAL AND BIOANALYTICAL CHEMISTRY · JULY 2013

Impact Factor: 3.44 · DOI: 10.1007/s00216-013-7202-9 · Source: PubMed

Anal Bioanal Chem
DOI 10.1007/s00216-013-7202-9

RESEARCH PAPER

Solid-phase extraction method for the isolation of plant thionins from European mistletoe, wheat and barley using zirconium silicate embedded in poly (styrene-*co*-divinylbenzene) hollow-monoliths

Shah Hussain · Yüksel Güzel · Stefan A. Schönbichler · Matthias Rainer · Christian W. Huck · Günther K. Bonn

Received: 25 April 2013 / Revised: 24 June 2013 / Accepted: 1 July 2013

Abstract Thionins are cysteine-rich, biologically active small (~5 kDa) and basic proteins occurring ubiquitously in the plant kingdom. This study describes an efficient solid-phase extraction (SPE) method for the selective isolation of these pharmacologically active proteins. Hollow-monolithic extraction tips based on poly(styrene-*co*-divinylbenzene) with embedded zirconium silicate nano-powder were designed, which showed an excellent selectivity for sulphur-rich proteins owing to strong co-ordination between zirconium and the sulphur atoms from the thiol-group of cysteine. The sorbent provides a combination of strong hydrophobic and electrostatic interactions which may help in targeted separation of certain classes of proteins in a complex mixture based upon the binding strength of different proteins. European mistletoe, wheat and barley samples were used for selective isolation of viscotoxins, purothionins and hordothionins, respectively. The enriched fractions were subjected to analysis by matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometer to prove the selectivity of the SPE method towards thionins. For peptide mass-fingerprint analysis, tryptic digests of SPE eluates were examined. Reversed-phase high-performance liquid chromatography hyphenated to diode-array detection was employed for the purification of individual isoforms. The developed method was found to be highly specific for the isolation and purification of thionins.

Keywords Solid-phase extraction · Zirconium silicate · European mistletoe · Thionins · Viscotoxins · Purothionins and hordothionins

S. Hussain · Y. Güzel · S. A. Schönbichler · M. Rainer · C. W. Huck (✉) · G. K. Bonn
Institute of Analytical Chemistry and Radiochemistry, CCB-Center for Chemistry and Biomedicine, Leopold-Franzens University, Innrain 80/82, 6020 Innsbruck, Austria
e-mail: christian.w.huck@uibk.ac.at

Abbreviations

ACN	Acetonitrile
AIBN	2,2′-Azobis-2-methylpropionitrile
BHK	Baby hamster kidney
CN	Co-ordination number
DAD	Diode-array detection
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DVB	Divinylbenzene
FA	Formic acid
HCCA	α-Cyano-4-hydroxycinnamic acid
HPLC	High-performance liquid chromatography
IAA	Iodoacetamide
m/z	Mass-to-charge
MALDI	Matrix-assisted laser desorption/ionisation
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
nOGP	*n*-Octyl β-D-glucopyranoside
PBS	Phosphate buffer saline
RPM	Revolutions per minute
SA	Sinapinic acid
SEM	Scanning electron microscopy
SPE	Solid-phase extraction
STY	Styrene
TFA	Trifluoroacetic acid
TOF	Time-of-flight

Introduction

Thionins (derived from the Greek word for sulphur) are small basic sulphur-rich proteins (~5 kDa) with a polypeptide chain of 45 to 48 amino acids and 3 to 4 disulfide bridges. Thionins are usually found in the

Published online: 17 July 2013

endosperms of Gramineae, e.g. wheat and barley, as well as leaves and stems of different plants (mistletoe and *Pyrularia pubera*) and rosids. They are divided into two groups namely α/β-thionins and γ-thionins (plant defensins). α/β-Thionins can be sub-divided into the types I, II, III, IV and V [1, 2].

Type I thionins are present in the endosperm of grains such as wheat and barley [1, 3] (α-, β-purothionins and hordothionins, respectively). They are highly basic and consist of 45 amino acids, 8 of which are cysteines. α/β-Purothionins are antibacterial [4]; they show in vitro protein synthesis inhibition in cell-free systems derived from wheat germ or rabbit reticulocytes [5]. Furthermore, they inhibited the synthesis of macromolecules in BHK cells in vivo [6]; they showed toxicity to some yeast strains [7] and insect larvae [8]. α/β-Hordothionins are reported to be active against plant pathogenic bacteria [9]. Highly purified α- and β-hordothionins are active in promoting the inhibition of endogenous mRNA translation in cell-free systems derived from rabbit reticulocytes, *Artemia* embryos or mouse liver. Inhibition of endogenous translation in a cell-free system from rat brain was also observed [10]. α-Hordothionins are reported to be antifungal agents through rupturing the fungal membrane triggered by increased calcium ion uptake and permeabilisation of the fungal membrane [11]. Franky et al. proved a synergistic enhancement of the antifungal activity of purothionins and hordothionins by 2S albumins and trypsin inhibitors [12]. Type II thionins were isolated from leaves and nuts of the parasitic plant *P. pubera* [13] and from the leaves of barley (*Hordeum vulgare*) [14]. They are slightly less basic than type I thionins and consist of 46 to 47 amino acids. Both types I and II thionins have four disulfide bonds.

Type III thionins (viscotoxins) have been extracted from leaves and stems of mistletoe species such as *Viscum album L.* [15]. They consist of 45 to 46 amino acids, contain three disulfide bonds and are as basic as type II thionins. The European mistletoe (*Viscum album L.*) is a semi-parasite growing on coniferous and leafy trees. Mistletoe extracts are used as complementary medicine for cancer therapy [16].

To date, seven different viscotoxin isoforms have been reported, namely A1, A2, A3, B, B2, 1-PS and C1 [17–19]. There are appreciable differences in toxicity between the diverse viscotoxins despite their sequence similarities. Viscotoxin A3 is the most cytotoxic whereas B is the least cytotoxic [20]. The overall shape of viscotoxins is very similar to that found for the other members of the thionin family comprising two antiparallel alpha helices and a short beta sheet [17, 21]. Their biological activity is related to plant defense against pathogens [22]. Viscotoxins enhance the natural killer cell-mediated killing of tumor cells [23]. Furthermore, they exert a strong immunomodulatory effect on human granulocytes [24, 25], induce apoptosis in human lymphocytes [26] and, in addition, they form complexes with negatively charged DNA [27]. Antifungal effects of viscotoxin A3 were reported by Giudici et al. [28]. Type IV thionins, which consist of 46 amino acids with three disulfide bonds, are found in seeds of Abyssinian cabbage [29, 30]. Type V thionins are neutral thionins present in some grains like wheat without toxic activities [31].

Solid-phase extraction (SPE) is an indispensable tool in many areas of research. It is a rapid and effective way to clean up and pre-concentrate the analytes of interest for subsequent instrumental analysis. SPE was proven to be very useful for isolating polypeptides from complex biological tissues [32] and from food samples [33]. Herraiz et al. evaluated different SPE sorbents based upon non-polar and ionic interactions using small synthetic peptides and casein enzymatic hydrolysates [34]. Different SPE sorbents containing cyanopropyl, ethyl, cyclohexyl, phenyl, octyl or octadecyl functional groups can be employed providing a wide range of possibilities for pre-concentration of peptides. The sorbent must be selected with respect to the polarity, hydrophobicity and length of the peptide [35].

The aim of this study was to design a highly selective sorbent for plant thionins. Hollow-monolithic extraction tips based on poly(styrene-*co*-divinylbenzene) (poly(STY-*co*-DVB)) with embedded zirconium silicate nano-powder were synthesised, which demonstrated an excellent selectivity for sulphur-rich proteins. To investigate the basic principle of binding between the monolithic sorbent and the thionins, additionally, the pure zirconium-free hydrophobic sorbent (poly(STY-*co*-DVB) and the pure zirconium silicate nano-powder were tested.

To the author's knowledge, for the first time, a selective SPE method for thionins (of mistletoe, wheat and barley) was developed and optimised using a poly(STY-*co*-DVB) hollow-monolithic extraction column containing zirconium silicate nano-powder. The sorbent offers a combination of hydrophobic and electrostatic interactions and demonstrated highest selectivity for plant thionins.

Materials and methods

Chemicals and reagents

Acetonitrile (ACN) high-performance liquid chromatography (HPLC)-grade, methanol ultra liquid chromatography mass spectrometry (LC-MS) grade and water HPLC-grade were purchased from Carl Roth Gmbh+Co. KG (Karlsruhe, Germany). Formic acid (FA) was received from Merck KGaA (Darmstadt, Germany). *n*-Octyl β-D-glucopyranoside (nOGP, 98 %), iodoacetamide (IAA, ≥98.0 %), sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (HCCA), divinylbenzene (DVB), styrene (STY), 2,2′-azobis-2-methylpropionitrile (AIBN),

decanol and zirconium (IV) silicate nano-powder (100 nm, 98.5 % trace meals basis) were purchased from Sigma Aldrich (St. Louis, USA) and phosphate buffer saline (PBS, 0.01 M Na_2HPO_4/NaH_2PO_4 and 0.15 M NaCl, pH=7.4) PhyNexus, Inc., San Jose, CA (US). Trifluoroacetic acid (TFA, for protein sequence analysis), dithiothreitol (DTT, ≥99.0 %) and ammonium bicarbonate (ultra, ≥99.5 %) were purchased from Fluka (Buchs, Switzerland). Trypsin (sequencing grade modified) was obtained from Promega Biosciences (San Luis Obistpo, CA, USA). The peptide calibration standard (bradykinin 1–7, angiotensin I, angiotensin II, substance P, bombesin, renin substrates, ACTH clip 1–17, ACTH clip 18–39, somatostatin) and protein calibration standards (insulin, ubiquitin I, cytochrome C, myoglobin (mass range, ~5–17.5 kDa) were from Bruker Daltonics Care (Bremen, Germany). *V. album* L. (European mistletoe) was received by a local pharmacy. Wheat flour and barley seeds were purchased from a local market in Innsbruck.

Preparation of extracts

Mistletoe and barley seeds were milled with a Retsch ZM 200 mill (Retsch, Hann, Germany) to a particle size less than 0.5 mm. Then, 200 mg of mistletoe powder, wheat flour and barley flour were separately extracted in 5 ml PBS for 60 min in ultra-sound. After extraction, samples were centrifuged, and the supernatants were stored at −20 °C.

Synthesis of poly(STY-*co*-DVB) embedded zirconium silicate hollow-monolith

The polymerisation mixture contained 50 μl of distilled DVB (cross-linker), 100 μl of STY, 4 mg AIBN (radical initiator) and 150 μl decanol (porogen). After thorough mixing, the mixture was transferred into a vial containing 90 mg ultra-sonicated zirconium silicate nano-powder and vortexed for 2 min. Twenty microlitres of this mixture was taken into 200-μl pipette tips and dispensed off. Subsequently, the tips were placed at 75 °C into an oven for 16 h. The total mass of dried packing in filled monolithic tips was 4.02±0.11 mg; the total amount of zirconium silicate in the extraction tip was 2.70±0.11 mg, and the height of the extraction tip was 1.60±0.1 cm. The monolithic extraction tips were used only for one extraction.

Synthesis of poly(STY-co-DVB) powder

The 2.5 ml of DVB, 120 ml of ACN and 62.5 mg AIBN were added into a round-bottom flask equipped with a magnetic stirrer, a condenser, a nitrogen inlet and a thermometer. The mixture was first purged with nitrogen for 10 min under stirring. With continuous stirring and nitrogen purge, the mixture was heated at 60 °C in an oil bath for 1 h. Then, 2.5 ml of styrene and 100 mg AIBN were added, and the mixture was heated at 70 °C in an oil bath for 16 h with continuous stirring and nitrogen purge. Finally, the mixture was cooled to room temperature, filtered and washed thoroughly in a sintered-glass filter with ACN and methanol. The obtained beads were dried under vacuum at room temperature for 4 h.

Scanning electron microscopic analysis of hollow-monolith

Scanning electron microscopy (SEM) was carried out by Electron Micro Probe (JEOL 8100, Japan). Before examination under the scanning electron microscope, the sorbent was sputtered with gold. SEM pictures were taken with an acceleration voltage of 15 kV and currents of 5–10 nA.

SPE method

The hollow-monolithic extraction tip columns were activated by washing with 20 μl methanol (aspirating and expelling twice). Equilibration was performed in the same manner using water. Twenty microlitres of sample was loaded onto the monolithic tip by aspirating and dispensing several times. Washing was done three times with deionised water in case of mistletoe and with 30 % FA in methanol/water (1/1, *v/v*) for wheat and barley samples. Elution was performed by 2 % TFA in ACN/water (1/1, *v/v*).

Protein digestion

For protein digestion, the method of Güzel et al. was used with slight modifications [36]. SPE eluents (from mistletoe, wheat and barley containing viscotoxins, purothionins and hordothionins, respectively) were evaporated (to dryness) in an Eppendorf Concentrator 5301 (Hamburg, Germany). Reconstitution was achieved in 40 μl of 0.5 M ammonium bicarbonate (pH<8.0), 8 μl 40 mM nOGP and 8 μl 45 mM DTT. The samples were placed on a thermomixer (Eppendorf AG) for 30 min at 37 °C and 900 rpm in order to perform denaturation. After cooling down to room temperature, the denaturised proteins were alkylated by incubating them 30 min under light exclusion by adding 8 μl 100 mM IAA to each fraction. In a next step, 8 μl of 0.1 μg/μl trypsin solution (diluted in 50 mM acetic-acid buffer pH 3.0) were added to the samples and placed on a thermomixer for enzymatic digestion at 37 °C for 16 h. In order to stop the digestion process, 5 μl of 5 % TFA solution (pH<3.0) were added. Afterwards, the digested protein solution was spotted on a stainless steel target followed by the addition of saturated HCCA solution (HCCA in ACN/water (1/1, *v/v*) containing 0.1 % TFA)) for further matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometer (MALDI/TOF MS) analysis.

MALDI/TOF MS and MS/MS analysis including database search

For MALDI/TOF MS measurements, 1 μl of sample was spotted on a stainless steel target (Bruker Daltonics GmbH, Bremen, Germany) followed by the addition of 1 μl of saturated SA solution (SA in ACN/water (1/1, *v/v*) containing 0.1 % TFA) for protein analysis or 1 μl of saturated HCCA solution in case of peptide analysis. All measurements were recorded on an Ultraflex I (Bruker Daltonics, Germany) MALDI/TOF MS in linear and reflectron mode. An external calibration was performed by spotting 0.5 μl of protein or peptide calibration standard (Bruker Daltonics, Bremen, Germany). All mass spectra were recorded by summing 500 laser shots. Laser power was adjusted between 50 % and 70 % of its maximal intensity, using a 337 nm laser at 50 Hz. The Flex Analysis version 2.4 and BioTools 3.0 software packages provided by the manufacturer were used for data processing. Database searching analysis was performed with Mascot software (http://matrixscience.com) and SwissProt as database. For peptide mass-fingerprint database searching analysis, the parameters were set as following: C-carbamidomethyl (fixed modification), M-oxidation, mass value (monoisotopic), peptide mass tolerance (200–300 ppm), mass tolerance (0.6 Da), missed cleavage (1 to 3) and taxonomy (other green plants).

HPLC-DAD

A Shimadzu (Tokyo, Japan) high-performance liquid chromatography hyphenated to diode-array detection (HPLC-DAD) was used for the analysis comprising an online degasser unit (DGU-14A), two solvent delivery pumps (LC-10ADvp), an auto-injector (SIL-10ADvp), column oven (CTO-10Avp), system controller (SCL-10Avp) and a diode-array detector (SPD-M10 Avp). System control and data analysis were performed by using the manufacturer's software packages (LC-MS Solution, version 3 and LCMS-post run, version 3-H2).

A Hypercrab S graphitised carbon HPLC-column (100×4.6 mm, 250 Å, 7 μm particle size; Shandon Scientific Ltd., Astmoor, Great Britain) was used for chromatographic separations. The mobile phase was a combination of water containing 0.1 % TFA (A) and ACN (B).

Following gradient was used: (minute per %B) 0/25, 20/35, 21/60, 30/65, 31/95, 40/95, 42/5 and 47/5. Flow rate was 1 ml/min, and the injection volume was 20 μl.

Identification of HPLC-DAD peaks

The peaks corresponding to the major isoforms were assigned after manual collections of fractions. The fractions were dried in a concentrator and reconstituted in 5 μl 2 % TFA in ACN/water (1/1, *v/v*) for further MALDI/TOF MS analysis.

Investigation of binding mechanism

To elaborate the binding mechanism between the monolithic sorbent and the sulphur-rich thionins, wheat purothionins were investigated exclusively for the retention behavior on poly(STY-*co*-DVB) (1:1) polymer powder (see section on "Synthesis of poly(STY-*co*-DVB) powder") and the zirconium silicate nano-powder. One hundred milligrams of each sorbent was taken separately into a 1.5-ml vial, activated with 500 μl methanol and equilibrated with 500 μl water. One hundred microlitres of wheat extract was loaded in each vial and vortexed for 2 min. Washing was performed three times with 500 μl 30 % FA in methanol/water (1/1, *v/v*). Elution was performed by using 2 % TFA in ACN/water (1/1, *v/v*). The supernatants from the individual steps were subjected to MALDI/TOF MS analysis.

Results and discussion

Isolation of thionins from complex samples is often complicated and needs many purification steps. This cumbersome procedure can be considerably simplified by a prior enrichment of the thionins using a selective SPE resin. The aim of this study was to design a sorbent which possesses a high selectivity to sulphur rich proteins. Xu et al. observed an increased binding of cysteine containing peptides by incorporating gold nanoparticles in the stationary phase [37]. This characteristic property of thiol-groups to coordinate with gold metal atoms is now also proved for zirconium atoms, and their ability to enrich and isolate thionins was demonstrated. Mistletoe, wheat and barley extracts were loaded on the sorbent and enriched in a pipette tip. Then, the selectively retained thionins were subjected to instrumental analysis.

Figure 1 displays the SEM micrographs of the poly(STY-*co*-DVB) embedded zirconium silicate sorbent before activation. Figure 1 (a–f) depicts the micrographs recorded at different resolutions. The embedded nano-particles (<100 nm) are clearly visible below the resolution power of 30 μm. Figure 1b shows the thickness of the polymer bed (~30 μm). MALDI/TOF MS measurements were carried out to prove the selectivity of the SPE method towards the enrichment of thionins. In Fig. 2, the selective isolation of viscotoxins from mistletoe sample is demonstrated, and the mass spectrum of the mistletoe extract (prior SPE) (A), the wash from the sorbent (B) and the eluted thionins (C) is depicted. Non-specifically bound proteins were easily washed away with water. No signal from the interested viscotoxins was observed in the mass spectra obtained from the washing fractions, ensuring a strong binding of the

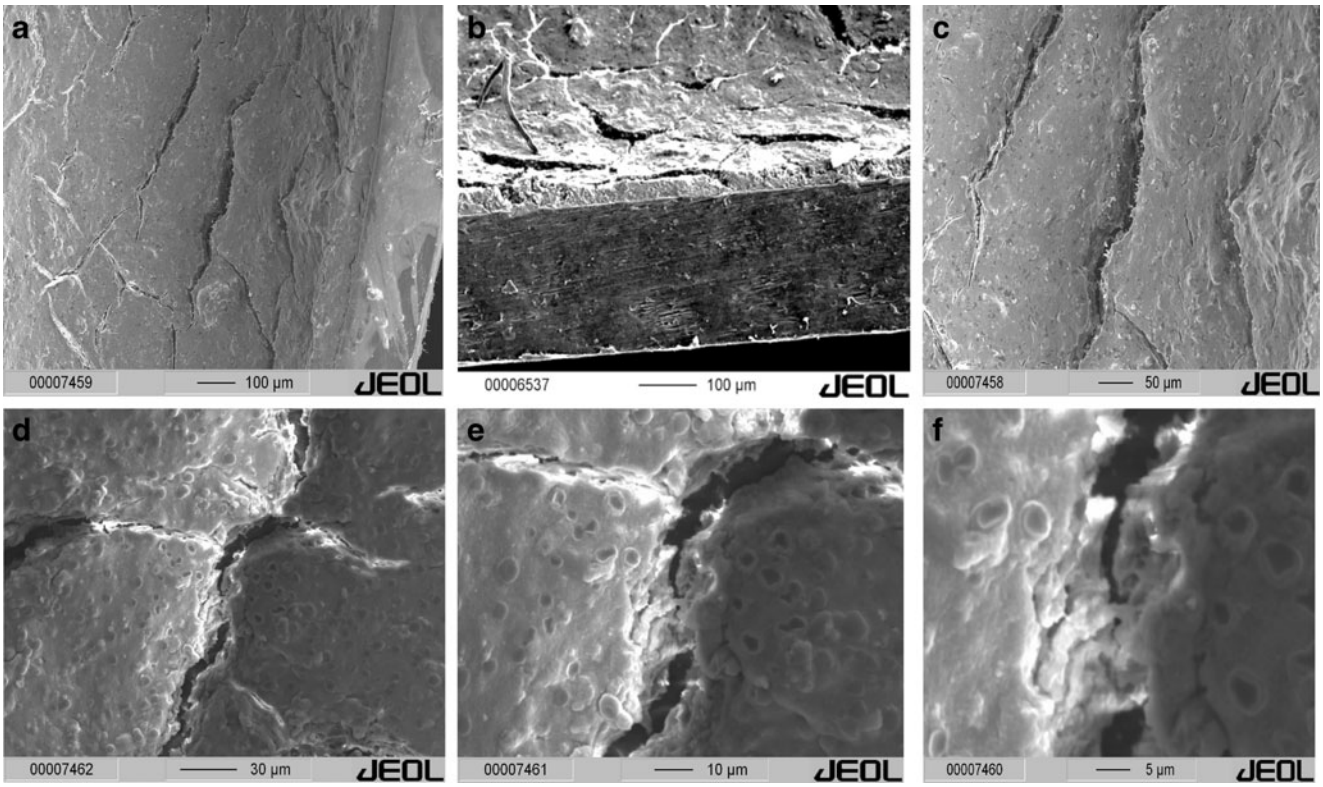

Fig. 1 SEM micrographs of monolithic poly(STY-*co*-DVB) embedded zirconium silicate nano-powder

viscotoxins to the sorbent. The viscotoxins which could be detected in the eluted fraction are listed in Table 1 with their amino acid sequences [38–43]. The reported molecular masses are based upon their known amino acid sequences. Protein Information Resource software http://pir.georgetown.edu/pirwww/search/comp_mw.shtml was used for the determination of the exact mass.

Figure 3 shows the MALDI/TOF MS spectra of a wheat sample. Wheat has a diverse range of proteins which makes the isolation of thionins extremely difficult without a prior enrichment step. The presented hollow-monolithic extraction tip exhibited a strong interaction for wheat purothionins which allowed the removal of the weaker bound non-specific proteins by using 30 % FA in methanol/water (1/1, *v/v*). Mass spectrum A reveals the entire range of proteins up to 14 kDa in wheat flour extract before subjecting to SPE. Spectrum B represents proteins removed by the washing step. Again, no known purothionin signal could be observed in the washing fraction. Lastly, spectrum C depicts the eluted purothionins from the sorbent. The purothionins detected in the eluate are listed in Table 2, including their known amino acid sequences [44, 45].

Figure 4 unveils the efficiency of the previously mentioned protocol. Figure 4 (a, b and c) presents the mass spectra obtained from the barley extract, the wash and the eluate, respectively. The barley sample displayed an almost identical diversity of proteins as described for the wheat sample. The washing step allowed the removal of most undesired proteins. The hordothionins detected in eluate are reported together with their known amino acid sequences [46, 47] in Table 3.

Confirmation of thionin identity by protein digestion

Furthermore, a tryptic digest was performed with the SPE eluates as described in "Protein digestion." The eluted proteins with the most intensive signals were confirmed through their peptide mass-fingerprint. The minor constituents could not be confirmed. Table 4 describes the results of the peptide mass-fingerprint analysis of the enriched and digested mistletoe, wheat and barley proteins using Mascot as search engine. Viscotoxin A2, A3 and B exhibited the sequence coverage of 65, 20 and 27 %, respectively, obtained from mistletoe extract. α_1-, α_2-, and β-purothionins revealed sequence coverage of 12, 39 and 37 %, respectively. α-Hordothionin disclosed sequence coverage of 47 % while 21 % could be achieved for β-hordothionin from barley.

HPLC-DAD separation of isolated thionins

After the selective isolation of thionins from their respective samples, the purification of individual isoforms was carried out by HPLC-DAD; the chromatograms are shown in Fig. 5. The fractions were collected manually and measured off-line with

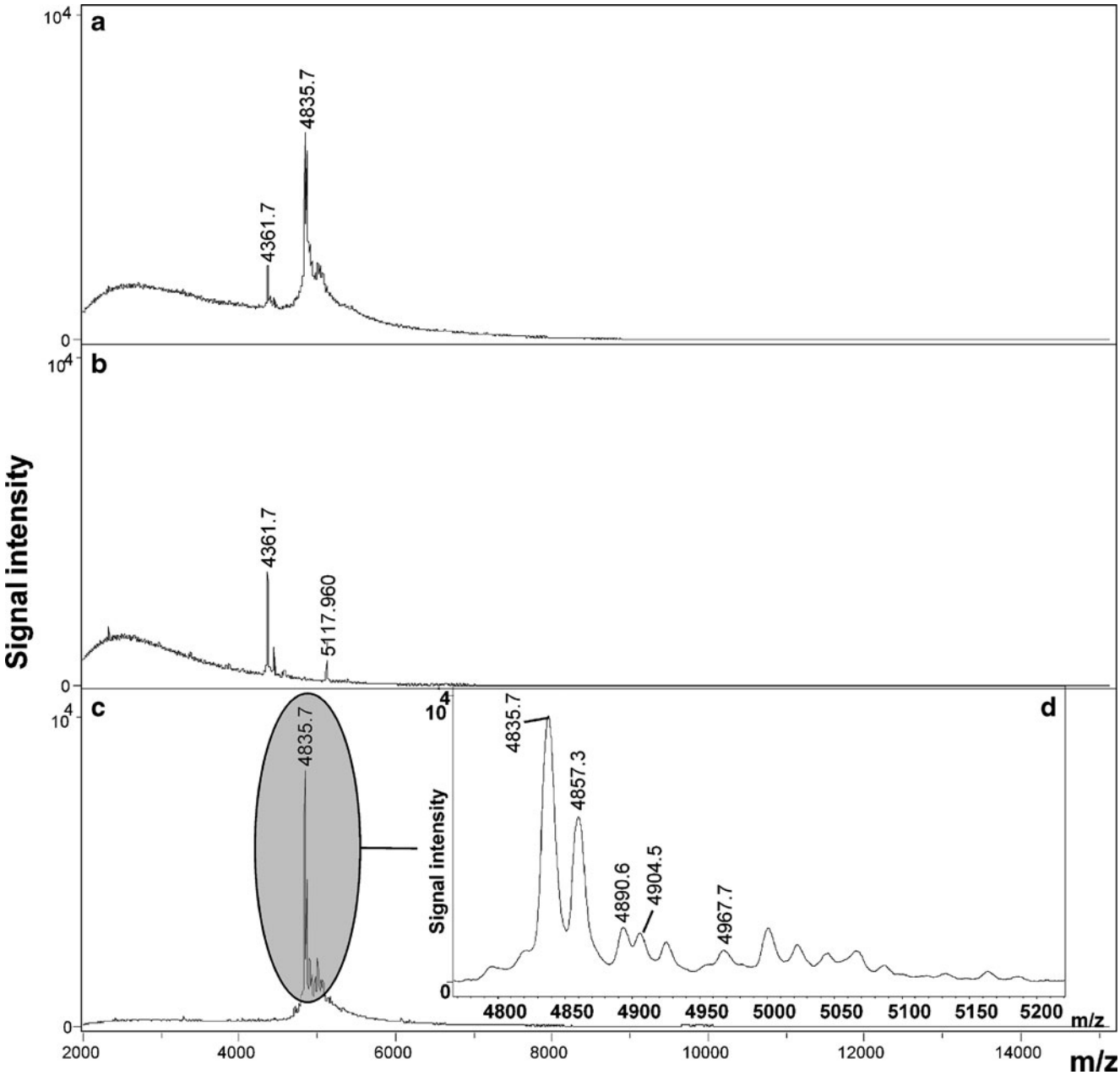

Fig. 2 MALDI/TOF MS analysis of the mistletoe sample. **a** Mass spectrum of mistletoe extract before SPE, **b** wash from the sorbent and **c** eluate strongly retained viscotoxins inset **d** is the expanded view of retained viscotoxins

Table 1 Detectable viscotoxins and their reported amino acid sequences from the SPE eluate from European mistletoe

Viscotoxins	Amino acid sequence	Theoretical mass (Da)	Observed mass (Da)
A3	KSCCPNTTGRNIYNACRLTGAPRPT CAKLSGCKIISGSTCPSYPDK [38]	4,835.53	4,835.7
A2	KSCCPNTTGRNIYNTCRFGGGSRE VCASLSGCKIISASTCPSYPDK [39]	4,834.42	4,835.7
A1	KSCCPSTTGRNIYNTCRLTGSSRETC AKLSGCKIISASTCPSNYPK [40]	4,889.53	4,890.6
B	KSCCPNTTGRNIYNTCRLGGGSRERCASLSGCKIISASTCPSYPDK [41]	4,857.45	4,857.3
1-PS	KSCCPBTTGRBIYBTCRFGGGSRZV CARIS GCKIISASTCPSYPBK [42]	4,904.02	4,904.5
B2	KSCCKNTTGRNIYNTCRFAGGSRER CAKLSGCKIISASTCPSDYPK [43]	4,977.65/4,967[a]	4,967.7

[a] Kong et al. reported viscotoxin B2 to show the molecular mass at 4,967 Da in MALDI/TOF MS [43]

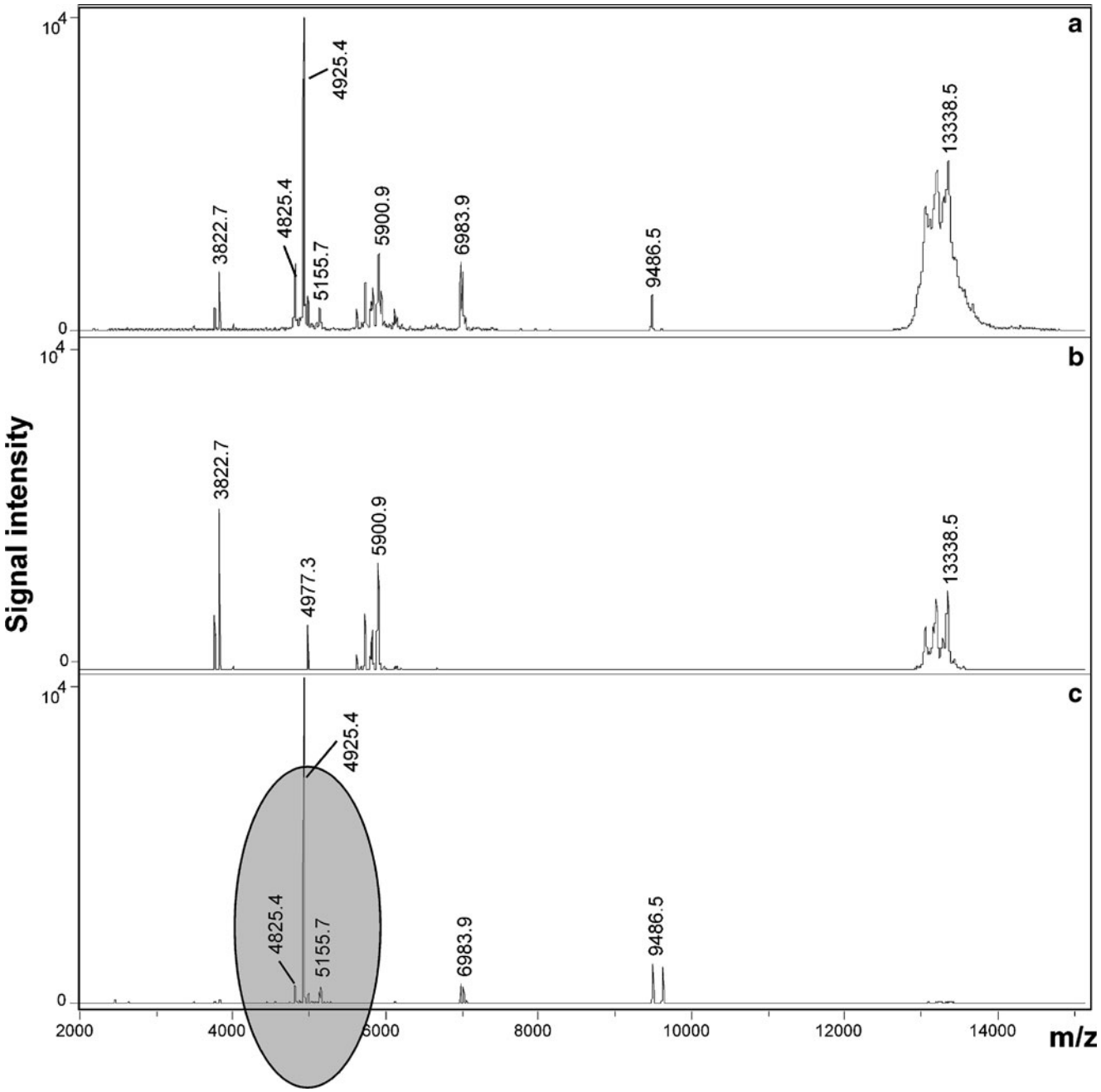

Fig. 3 MALDI/TOF MS analysis of wheat flour. **a** Mass spectrum of wheat extract prior to SPE, **b** wash from the sorbent and **c** eluate strongly retained purothionins

MALDI/TOF MS. In Fig. 5a, the chromatogram of the enriched viscotoxins are shown. For peaks 1 (retention time (RT), 27.57 min), 2 (RT, 30.13 min) and 3 (RT, 33.13 min), the molecular masses of 4,834, 4,835 and 4,857 Da were found,

Table 2 Detectable purothionins and their reported amino acid sequences from the SPE eluate of wheat flour

Purothionins	Amino acid sequence	Theoretical mass	Observed mass (Da)
α_1-	KSCCRSTLGRNCYNLCRARGAQKLCAGVCRCKISSGLSCPKGFPK [44]	4,825.70	4,825.4
α_2-	KSCCRTTLGRNCYNLCRSRGAQKLCSTVCRCKLTSGLSCPKGFPK [44]	4,929.82	4,925.4
β-	KSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK [45]	4,926.81	4,925.4

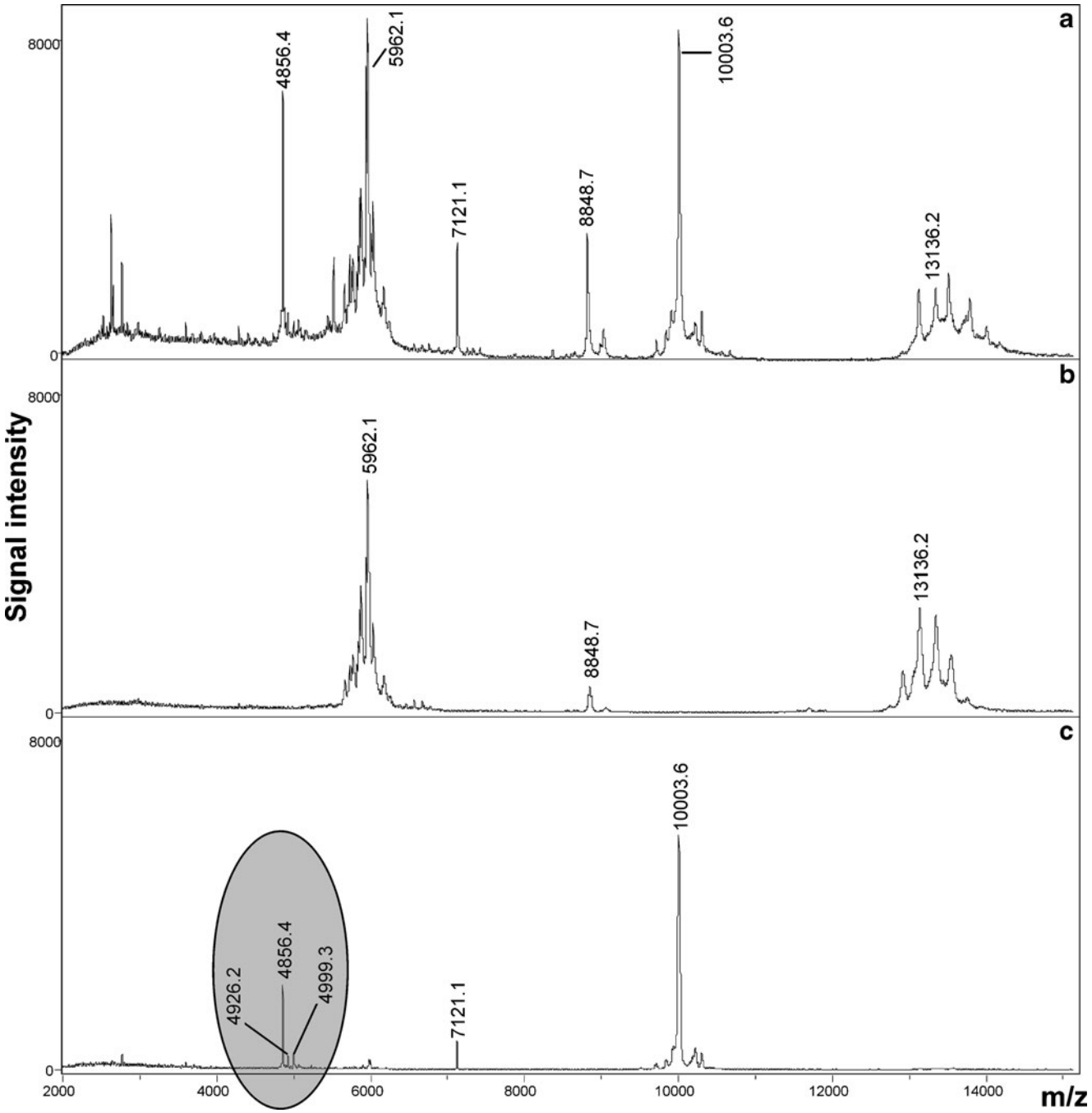

Fig. 4 MALDI/TOF MS analysis of barley. **a** Mass spectrum of barley extract before subjecting to SPE, **b** wash from the sorbent and **c** eluate strongly retained hordothionins

and they could be assigned as viscotoxin A2, A3 and B, respectively. Figure 5b depicts the HPLC-DAD chromatogram of purothionins after enrichment from a wheat sample extract. Peaks 1 (RT, 27.57 min) and 2 (RT, 33.57 min) were identified

Table 3 Detectable hordothionins and their reported amino acid sequences from the SPE eluate of barley

Hordothionins	Amino acid sequence	Theoretical mass (Da)	Observed mass (Da)
α-	KSCCRSTLGRNCYNLCRVRGAQKL CAGVCRCKLTSTGSCPKGFPK [46]	4,855.75	4,856.4
β-	KSCCRSTLGRNCYNLCRVRGAQKL CANACRCKLTSGLKCPSSFPK [47]	4,926.81	4,926.2

Table 4 Identification of thionins from peptide mass-fingerprints

	Seq. coverage [%]	Mascot search score	Average error [ppm]
Mistletoe			
Viscotoxin-A2	65	37	258
Viscotoxin-A3	20	35	215
Viscotoxin-B	27	40	64
Wheat			
α_1-Purothionin	12	21	126
α_2-Purothionin	39	34	177
β-Purothionin	37	44	87
Barley			
α-Hordothionin	47	39	155
β-Hordothionin	21	28	133

as α_1- and β-purothionins exhibiting molecular masses of 4,834 and 4,925 Da, respectively. In Fig. 5c, the chromatogram of isolated hordothionins from barley is depicted. Peaks 1 (RT, 27.57 min) and 2 (RT, 33.57 min) could be identified as α- and β-hordothions with the molecular masses of 4,856 and 4,926 Da. The similar RTs displayed by the different isoforms of thionins could be ascribed to the amino acid sequence similarities of the isoforms. Sequence similarities between viscotoxins and other related α- and β-thionins along with disulphide bridge arrangements were described comprehensively by Orru et al. [40]. All these proteins consist of 45 or 46 amino acids and share a high degree of sequence homology. The RT of viscotoxin A2, α_1-purothionin and α-hordothion was observed to be 27.57 min. α_1-Purothionin and α-hordothionin in their respective polypeptide chains share the similar amino acid residues at 40 positions while, for viscotoxin A2 (depicting similar amino acids residues at 22 positions to α_1-purothionin and α-hordothion), the resemblance of RT cannot be explained so convincingly. Likewise, the RTs displayed by viscotoxin B, β-purothionin and β-hordothionin were 33.13, 33.57 and 33.57 min, respectively. Again, β-purothionin and β-hordothionin only differ in five amino acid residues in their polypeptide chain; hence, a very similar retention time was observed. The similar RT shown by viscotoxin B which shares 22 amino acids residues to β-purothionin and β-hordothionin cannot be explained so persuasively.

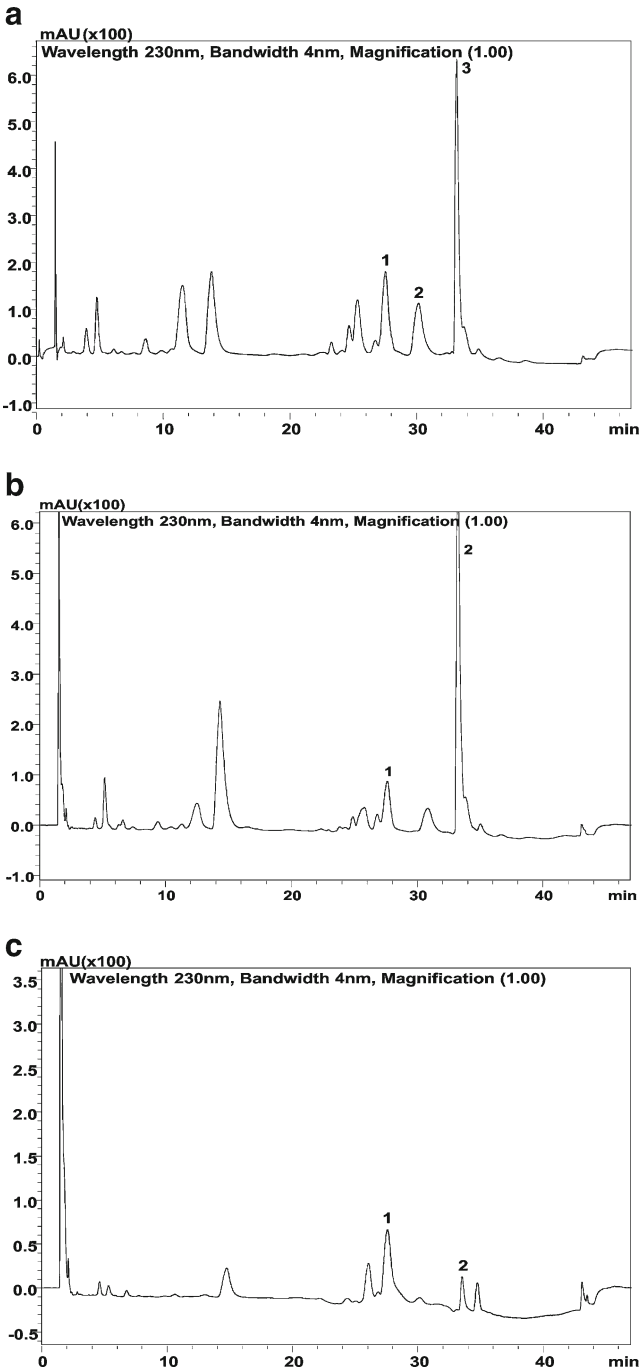

Fig. 5 HPLC-DAD purification of individual isoforms of thionins. **a** Chromatogram from mistletoe eluate: *1* viscotoxin A2, *2* viscotoxin A3 and *3* viscotoxin B. **b** Chromatogram from wheat eluate: *1* α_1-purothionin, *2* β-purothionin. **c** Chromatogram from barley eluate: *1* α-hordothionin, *2* β-hordothionin

Proposed binding mechanism

The involved binding mechanism for the strong interaction between thionins and poly(STY-*co*-DVB) embedded zirconium silicate nano-powder can be explained by the experiment explained in "Investigation of binding mechanism." As the presented monolithic sorbent combines the characteristics of both materials, the organic polymer and the inorganic particles; it was important to investigate the binding behavior of both materials individually in order to obtain better understanding of the actual retention mechanism. Figure 6 displays the MALDI/TOF MS analysis of poly(STY-*co*-DVB) powder for wheat extract including the

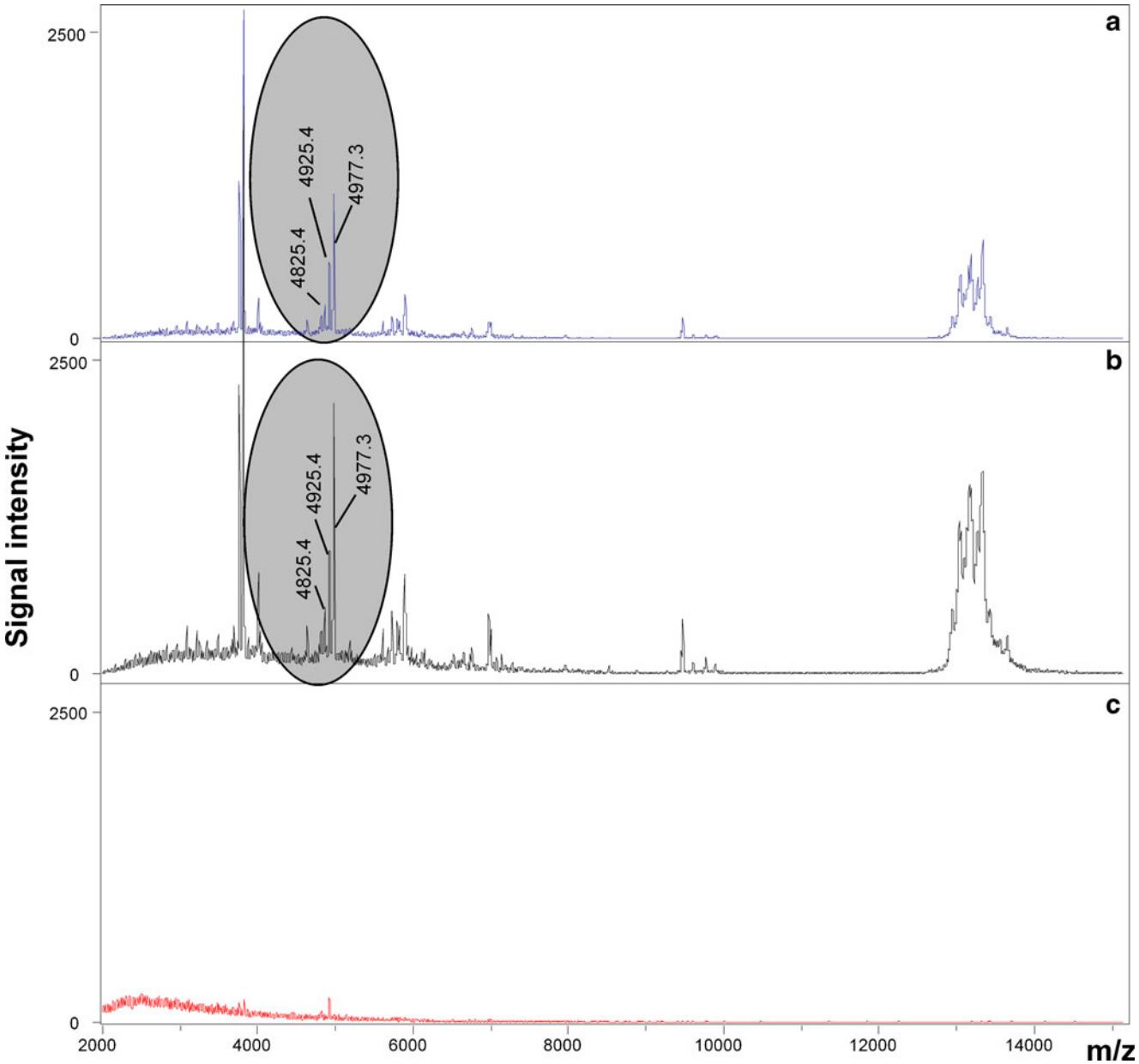

Fig. 6 MALDI/TOF MS analysis of wheat extract evaluated on poly(STY-*co*-DVB) polymer powder sorbent. **a** and **b** are the wash fraction 1 and wash fraction 2 from the sorbent showing no specific retention for thionins. **c** Eluate from the same sorbent showing nothing retained specifically

washing fraction 1, washing fraction 2 (A, B) (two washing fractions are presented in Fig. 6 to ascertain a non-selective binding of hydrophobic stationary phase to thionins) and the eluate (C) from the hydrophobic sorbent. The washing solution (methanol/water (1/1, *v/v*) containing 30 % FA) could remove all the purothionins from the hydrophobic sorbent, and no signal could be seen in the eluate. When the same sample was subjected to SPE analysis on the zirconium silicate nano-powder (predominantly electrostatic) sorbent, an increased binding to purothionins was observed in contrast to the hydrophobic poly(STY-*co*-DVB) resin. Figure 7 displays the MALDI/TOF MS spectra of the fractions of washing (A) and elution (B) for the zirconium silicate nano-powder material, respectively.

Based on these results, it can be concluded that the main binding interaction between thionins and the developed sorbent is determined by the coordination between zirconium and the thiol-groups of the cysteine rich proteins. In general, the presented resin offers strong hydrophobic and electrostatic interaction sites which synergistically accounts for a very strong binding of thionins by allowing stringent washing steps in order to remove unspecific constituents. Zirconium has a CN of 8 in zirconium silicate. Four of the coordination sites are occupied by oxygen atoms [48], and the

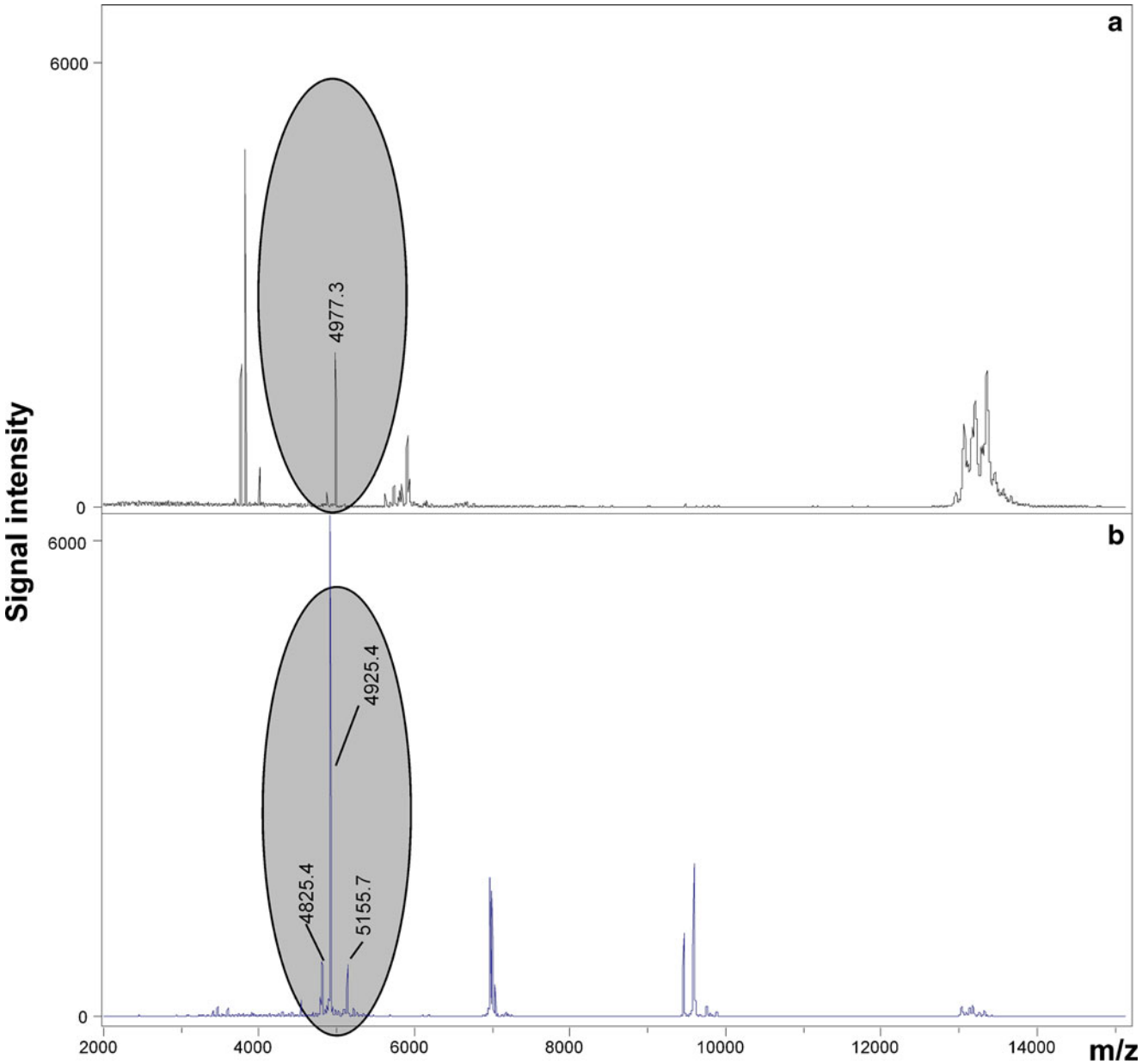

Fig. 7 MALDI/TOF MS analysis of wheat extract evaluated on zirconium silicate nano-powder sorbent. **a** is the wash from the sorbent; **b** eluate from the same sorbent showing strongly retained purothionins

remaining four are assumed to be surrounded by water molecules (after equilibration of the sorbent). These coordination sites surrounded by water molecules are proposed to be exchanged by an electron pair from sulphur atoms of thiol-groups for each coordination site causing strong retention of thionins.

Conclusion

Zirconium silicate embedded in poly(STY-*co*-DVB) hollow-monoliths is suitable for the purification and enrichment of thionins in extracts obtained from mistletoe, wheat and barley. Strong coordination between zirconium and the sulphur atoms of thiol-groups causes a strong binding of thionins to the developed material. This approach reduces the complexity of protein extracts enormously, and therefore, it offers a simpler chromatographic separation of individual isoforms. The SPE method may be suitable for screening of novel isoforms of thionins from still uninvestigated samples.

Acknowledgement Special thanks to the Higher Education Commission of Pakistan and OeAD for Author's PhD scholarship. The authors also want to thank Eurasia-Pacific Uninet (EPU) (Salzburg, Austria), the Ministry for Science and Research and the Ministry for Health, Family and Youth (Vienna, Austria) (Novel analytical tools for quality control of immunomodulatory, anti-inflammatory and neuroprotective

agents in traditional Chinese medicine). The authors also would like to acknowledge Prof. Dr. Richard Tessadri from Faculty of Geo- and Atmospheric Sciences, Institute of Mineralogy and Petrography, University of Innsbruck, for SEM analysis.

References

1. Stec B (2006) Plant thionins—the structural perspective. Cell Mol Life Sci 63(12):1370–1385
2. Bohlmann H, Apel K (1991) Thionins. Annu Rev Plant Biol 42(1):227–240
3. Egorov TA, Odintsova TI, Pukhalsky VA, Grishin EV (2005) Diversity of wheat anti-microbial peptides. Peptides 26(11):2064–2073
4. De Caleya RF, Gonzalez-Pascual B, García-Olmedo F, Carbonero P (1972) Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro. Appl Microbiol 23(5):998–1000
5. Garcia-Olmedo F, Carbonero P, Hernandez-Lucas C, Paz-Ares J, Ponz F, Vicente O, Sierra JM (1983) Inhibition of eukaryotic cell-free protein synthesis by thionins from wheat endosperm. Biochim Biophys Acta 740(1):52–56
6. Carrasco L, Vazquez D, Hernandez-Lucas C, Carbonero P, Garcia O (1981) Thionins: plant peptides that modify membrane permeability in cultured mammalian cells. Eur J Biochem 116(1):185
7. Hernandez-Lucas C, De Caleya RF, Carbonero P (1974) Inhibition of brewer's yeasts by wheat purothionins. Appl Microbiol 28(2):165–168
8. Jones BL, Lookhart GL, Johnson DE (1985) Improved separation and toxicity analysis methods for purothionins. Cereal Chem 62(5):327–331
9. Florack DE, Visser B, De Vries PM, Van Vuurde J, Stiekema W (1993) Analysis of the toxicity of purothionins and hordothionins for plant pathogenic bacteria. Eur J Plant Pathol 99(5):259–268
10. Mendez E, Moreno A, Colilla F, Pelaez F, Limas GG, Mendez R, Soriano F, Salinas M, Haro C (2005) Primary structure and inhibition of protein synthesis in eukaryotic cell–free system of a novel thionin, γ–hordothionin, from barley endosperm. Eur J Biochem 194(2):533–539
11. Thevissen K, Ghazi A, De Samblanx GW, Brownlee C, Osborn RW, Broekaert WF (1996) Fungal membrane responses induced by plant defensins and thionins. J Biol Chem 271(25):15018–15025
12. Terras FR, Schoofs HM, Thevissen K, Osborn RW, Vanderleyden J, Cammue BP, Broekaert WF (1993) Synergistic enhancement of the antifungal activity of wheat and barley thionins by radish and oilseed rape 2S albumins and by barley trypsin inhibitors. Plant Physiol 103(4):1311–1319
13. Vernon LP (1992) Pyrularia thionin: physical properties, biological responses and comparison to other thionins and cardiotoxin. Toxin Rev 11(3):169–191
14. Bohlmann H, Apel K (1987) Isolation and characterization of cDNAs coding for leaf-specific thionins closely related to the endosperm-specific hordothionin of barley (*Hordeum vulgare* L.). Mol Gen Genet 207(2):446–454
15. Samuelsson G, Pettersson B (1970) Separation of viscotoxins from the European mistletoe, *Viscum album* L. (Loranthaceae) by chromatography on sulfoethyl sephadex. Acta Chem Scand 24:2751–2756
16. Kienle GS, Kiene H (2010) Review article: influence of *Viscum album* L (European mistletoe) extracts on quality of life in cancer patients: a systematic review of controlled clinical studies. Integr Cancer Ther 9(2):142–157
17. Romagnoli S, Fogolari F, Catalano M, Zetta L, Schaller G, Urech K, Giannattasio M, Ragona L, Molinari H (2003) NMR solution structure of viscotoxin C1 from *Viscum album* species *Coloratum ohwi*: toward a structure-function analysis of viscotoxins. Biochem 42(43):12503–12510
18. Bhave M, Methuku DR (2011) Small cysteine-rich proteins from plants: a rich resource of antimicrobial agents. Science against microbial pathogens: communicating current research and technological advances, Ed A Méndez-Vilas:1074–1083
19. Pal A, Debreczeni JE, Sevvana M, Gruene T, Kahle B, Zeeck A, Sheldrick GM (2008) Structures of viscotoxins A1 and B2 from European mistletoe solved using native data alone. Acta Crystallogr D: Biol Crystallogr 64(9):985–992
20. Büssing A, Schaller G, Pfüller U (1998) Generation of reactive oxygen intermediates (ROI) by the thionins from *Viscum album* L. Anticancer Res 18(6A):4291–4296
21. Romagnoli S, Ugolini R, Fogolari F, Schaller G, Urech K, Giannattasio M, Ragona L, Molinari H (2000) NMR structural determination of viscotoxin A3 from *Viscum album* L. Biochem J 350(2):569–577
22. Holtorf S, Ludwig-Müller J, Apel K, Bohlmann H (1998) High-level expression of a viscotoxin in *Arabidopsis thaliana* gives enhanced resistance against *Plasmodiophora brassicae*. Plant Mol Biol 36(5):673–680
23. Tabiasco J, Pont F, Fournié JJ, Vercellone A (2002) Mistletoe viscotoxins increase natural killer cell-mediated cytotoxicity. Eur J Biochem 269(10):2591–2600
24. Stein GM, Schaller G, Pfüller U, Wagner M, Wagner B, Schietzel M, Büssing A (1999) Characterisation of granulocyte stimulation by thionins from European mistletoe and from wheat. Biochim Biophys Acta 1426(1):80–90
25. Stein G, Schaller G, Pfüller U, Schietzel M, Büssing A (1999) Thionins from *Viscum album* L: influence of the viscotoxins on the activation of granulocytes. Anticancer Res 19(2A):1037–1042
26. Büssing A, Stein GM, Wagner M, Wagner B, Schaller G, Pfüller U, Schietzel M (1999) Accidental cell death and generation of reactive oxygen intermediates in human lymphocytes induced by thionins from *Viscum album* L. Eur J Biochem 262(1):79–87
27. Woynarowski JM, Konopa J (1980) Interaction between DNA and viscotoxins. Cytotoxic basic polypeptides from *Viscum album* L. Hoppe Seylers Z Physiol Chem 361(2):1535–1546
28. Giudici M, Antonio Poveda J, Molina ML, de la Canal L, González–Ros JM, Pfüller K, Pfüller U, Villalaín J (2006) Antifungal effects and mechanism of action of viscotoxin A3. FEBS J 273(1):72–83
29. VanEtten C, Nielsen H, Peters J (1965) A crystalline polypeptide from the seed of *Crambe abyssinica*. Phytochemistry 4(3):467–473
30. Schrader-Fischer G, Apel K (1994) Organ-specific expression of highly divergent thionin variants that are distinct from the seed-specific crambin in the crucifer *Crambe abyssinica*. Mol Gen Genet 245(3):380–389
31. Castagnaro A, Maraña C, Carbonero P, García-Olmedo F (1992) Extreme divergence of a novel wheat thionin generated by a mutational burst specifically affecting the mature protein domain of the precursor. J Mol Biol 224(4):1003–1009
32. Bennett H, Hudson AM, Kelly L, McMartin C, Purdon G (1978) A rapid method, using octadecasilyl-silica, for the extraction of certain peptides from tissues. Biochem J 175(3):1139
33. Voirin A, Letavernier J-F, Sebille B (1991) Improvement of extraction and concentration of milk peptides with solid-phase cartridges for analysis by high-performance liquid chromatography. J Chromatogr A 553:155–164
34. Herraiz T, Casal V (1995) Evaluation of solid-phase extraction procedures in peptide analysis. J Chromatogr A 708(2):209–221
35. Herraiz T (1997) Sample preparation and reversed phase-high performance liquid chromatography analysis of food-derived peptides. Anal Chim Acta 352(1):119–139
36. Güzel Y, Rainer M, Mirza MR, Bonn GK (2012) Highly efficient precipitation of phosphoproteins using trivalent europium, terbium, and erbium ions. Anal Bioanal Chem 403(5):1323–1331

37. Xu Y, Cao Q, Svec F, Fréchet JM (2010) Porous polymer monolithic column with surface-bound gold nanoparticles for the capture and separation of cysteine-containing peptides. Anal Chem 82(8):3352–3358
38. Samuelsson G, Pettersson B (1971) The disulfide bonds of viscotoxin A3 from the European mistletoe (*Viscum album* L., Loranthaceae). Acta Chem Scand 25(6)
39. Olson T, Samuelsson G (1972) The amino acid sequence of viscotoxin A2 from the European mistletoe (*Viscum album* L., Loranthaceae). Acta Chem Scand 26:585–595
40. Orrù S, Scaloni A, Giannattasio M, Urech K, Pucci P, Schaller G (1997) Amino acid sequence, SS bridge arrangement and distribution in plant tissues of thionins from *Viscum album*. Biol Chem 378(9):989–996
41. Samuelsson G, Pettersson BM (1971) The amino acid sequence of viscotoxin B from the European mistletoe (*Viscum album* L, Loranthaceae). Eur J Biochem 21(1):86–89
42. Samuelsson G, Jayawardene AL (1974) Isolation and characterization of viscotoxin 1-Ps from *Viscum album* L. ssp. *austriacum* (Wiesb.) Vollmann, growing on *Pinus silvestris*. Acta Pharm Suec 11(2):175
43. Kong JL, Du XB, Fan CX, Cao Y, Jiang H, Xu JF, Zheng XJ (2004) Purification and primary structure determination of a novel polypeptide isolated from mistletoe *Viscum coloratum*. Chin Chem Lett 15(11):1311–1314
44. Jones B, Mak A (1977) Amino acid sequences of the two alpha-purothionins of hexaploid wheat. Cereal Chem 54
45. Stec B, Rao U, Teeter MM (1995) Refinement of purothionins reveals solute particles important for lattice formation and toxicity. Part 2: structure of-purothionin at 1.7 A resolution. Acta Crystallogr D: Biol Crystallogr 51(6):914–924
46. Lecomte JT, Jones BL, Llinas M (1982) Proton NMR studies of barley and wheat thionins: structural homology with crambin. Biochem 21(20):4843–4849
47. Hernández-Lucas C, Royo J, Paz-Ares J, Ponz F, García-Olmedo F, Carbonero P (1986) Polyadenylation site heterogeneity in mRNA encoding the precursor of the barley toxin β-hordothionin. FEBS Lett 200(1):103–106
48. Jameson JR, Harrison W, Griffin P (2001) Tight-binding model and electronic structure of tetrahedral zirconium silicate. J Appl Phys 90(9):4570–4577