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Characterisation of the rubber elongation factor from ammoniated latex by electrophoresis and mass spectrometry

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Abstract

Rubber elongation factor (REF) is considered as one of the major allergens present in latex. An extraction and purification protocol for preparation of REF standards has been modified. A protein fraction was extracted from ammoniated latex sap and purified by gel filtration chromatography. The purified and concentrated proteins were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis into two major bands. These bands were further characterised by matrix-assisted laser desorption/ionisation time-of-flight and nano–electrospray ionization mass spectrometry. REF and a truncated form could be ascertained by the mass and fragmentation pattern of the tryptic peptides. In the faster migrating band an additional peptide could be identified. This peptide is also present in *Hevb3* and a M_r 27 000 latex allergen. Our findings indicate that conventional REF preparations as standards may contain additional allergenic proteins. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Rubber elongation factor; Latex; *Hevea brasiliensis*; Allergens; Peptides; Proteins

1. Introduction

In the past two decades the immediate type of latex allergy has become a serious problem for an increasing number of individuals. At high risk for this type I allergy against latex are patients with spina bifida, patients who have undergone a number of surgeries in the genitourinary tract and individuals who are frequently exposed to latex products [1–7]. Three to ten percent of health care workers are allergic against components of different latex products [6,8,9]. Yassin et al. [10] have predicted an even higher prevalence of IgE-mediated latex allergy of approximately 17%. The symptoms range from a minor skin rash to anaphylactic reactions causing the death of a patient [11,12]. According to the significance of this problem, a number of allergens present in latex were identified in the last few years. The main allergens in latex products such as gloves are no longer the additives required for production. Proteins originating from the rubber tree *Hevea brasiliensis* are considered as potential allergens. Two proteins are regarded as the most crucial allergens in latex products; namely the rubber elongation factor Hev b1 (REF) [13–19] and *Hevein* [20–25]. REF is a highly hydrophobic M_r 14 600 protein located on the surface of the rubber particles. *Hevein* is the M_r 4700 subunit of Prohevein, a wound-induced protein in the laticifers of the rubber tree.

Purified REF is frequently used in various test

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systems for diagnosis of latex allergy. Common to many protocols for preparation of an extract representing all soluble proteins from ammoniated latex sap is a batch extraction with phosphate-buffered saline (PBS) or Tris buffer. REF, as a highly hydrophobic protein, is usually isolated from this protein extract by an additional extraction with an sodium dodecylsulfate (SDS)-buffer followed by gel filtration [15,18] or SDS–polyacrylamide gel electrophoresis (PAGE) combined with electroelution [13].

Our interest lies in the area of identification of allergenic proteins present in latex gloves. Latex itself is a common and rich source to extract this proteins as reference for structural and biological studies. For our investigations, the extraction of REF was performed as previously described by Czuppon et al. [15] while the purification step was modified. The dialysis step was omitted and an additional gel filtration with a Sephadex G 25 column combined with a discontinuous ultradiafiltration to concentrate the diluted eluates was added. Further purification was achieved by gel filtration with a Superdex 75 column as described by Czuppon et al. [15]. The eluates was lyophilized.

The conventional separation on SDS–PAGE in combination with the mass spectrometric methods of nano–electrospray ionization (nano–ESI) or matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry introduces a new dimension for identification of these proteinogenic allergens. The separated proteins can be directly eluted from the gel [26] or can be measured after in-gel digestion with endoproteases [26,27]. These methods offer the possibility of peptide mapping as well as the characterization and identification of these molecules by aligning the sequence of the peptides to the sequences of known proteins.

Proteins present in ammoniated latex sap have been extracted and purified by our modified procedure. The proteins have been partially characterized either directly from bands in SDS–electrophoresis gels by MALDI–TOF-MS or after tryptic in-gel digestion by nano–ESI-MS.

2. Material and methods

All chemicals and buffer ingredients used for extraction, purification, electrophoresis and sample preparation for mass spectrometry were purchased from Merck (Vienna, Austria), Sigma–Aldrich (Vienna, Austria) or J.T. Baker (Vienna, Austria). Water was prepared using an Elgastat system (Elga, High Wycombe, UK).

α-Cyano-4-hydroxycinnamic acid (CHCA) was obtained from Sigma (Sigma–Aldrich, Vienna, Austria), sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was purchased from Fluka (Sigma–Aldrich, Vienna, Austria) and was used without further purification. Dithiothreitol (DTT) for reduction of proteins before in-gel digestion was purchased from Serva (Novex, San Diego, CA, USA).

2.1. Extraction procedure

The procedure described by Czuppon et al. [15] had been slightly modified for extraction of REF.

Briefly, 120 g ammoniated (0.7%) latex sap was diluted 1:2 with 50 m*M* Tris buffer, pH 8.0, containing 0.01% of Triton X-100. The homogenized suspension was centrifuged for 45 min at 41 400 *g* and 10°C. The aqueous, yellow intermediate phase, the C-serum of the latex sap, was discarded. The creamy supernatant was supplemented with Tris buffer to 120 g and resuspended. After a second centrifugation of the sap, the C-serum was discarded and the latex cream was added with 2% sodium dodecylsulfate to the original mass. The suspension was stirred for 30 min at room temperature. The rubber particles were removed from the protein solution by three centrifugation steps for 15 min at 41 400 *g*. The supernatant was stored at 4°C overnight and before the following steps of purification it was filtered through a Millex GV 0.22 μm polyvinylidene difluoride (PVDF) filter (Millipore, Bedford, MA, USA).

2.2. Chromatographic separation and concentration

The high content of SDS in the extract was removed by desalting. 50 ml of the filtered extract were loaded onto a Sephadex G 25 column (XK 50, 200×50 mm, Pharmacia, Uppsala, Sweden). A 0.1 *M* ammonium acetate buffer, pH 6.8, was selected for elution. The column was connected to a Prosys

workstation (Biosepra, Marlborough, USA) equipped with a 280 nm flow UV monitor and operated at 15.3 cm/h. The eluate was concentrated by discontinuous tangential ultradiafiltration in a Pellicon XL, Biomax 8 system (Millipore) using a polyethersulfone-based membrane (*NWGG* 8 from Millipore) with an M_r cut off of 8000. The ultradiafiltration was operated with a feed pressure of 2.5 to 3 bar and a retentate pressure of 1 to 1.5 bar. The concentrated protein solution was loaded onto a Superdex 75 HiLoad column (600×26 mm, Pharmacia) and was eluted with a 0.1 *M* ammonium acetate buffer, pH 6.8, with a flow-rate of 56.6 cm/h. The eluates were lyophilized and stored at −20°C.

2.3. Electrophoresis

The lyophilized sample was dissolved in reducing sample buffer for electrophoresis (0.06 *M* Tris–HCl, pH 6.8; 10% (v/v) glycerol; 2% SDS; 0.0025% bromphenol blue; 5% 2-mercaptoethanol) and boiled for 10 min. SDS–electrophoresis was carried out according to a modified Laemmli procedure described by Necina et al. [28]. The separated proteins were visualized either by Silver [29] or Coomassie Brilliant Blue staining.

2.4. Matrix-assisted laser desorption/ionization mass spectrometry

The mass spectrometer used in this work was a Kratos Kompact MALDI IV–time-of-flight instrument (Shimadzu Kratos Analytical, Manchester, UK) equipped with a standard nitrogen laser (337 nm). The spectra were recorded in linear mode, with 20 kV extraction voltage and in order to obtain better resolution the time delayed extraction technique was applied. The samples were prepared either with α-cyano-4-hydroxycinnamic acid [saturated solution in acetonitrile–0.1% trifluoroacetic acid (TFA) (70:30 v/v)] and with the so called sandwich technique or with sinapic acid (saturated solution in acetonitrile–0.1% TFA, 50:50, v/v) and with the dry droplet technique [30]. Two microliters of analyte and matrix solution were typically mixed in an Eppendorf tube and 0.5–1 μl of the mixture was placed on the sample holder and allowed to dry at room temperature. One microliter of saturated CHCA solution in acetone was placed first as a thin layer onto the target at the samples analyzed with this matrix. In order to remove salts and contaminants, a washing step with ice-cold water was applied. Each spectrum was produced by accumulating data from 50 to 100 consecutive laser shots.

2.5. Recovery of the proteins from the SDS–PAGE gel for the MALDI–TOF-MS measurements

The proteins were eluted from the SDS–PAGE gel according to the method of Ehring et al. [26]. The Coomassie stained bands were excised, cut in ca. 1 mm×1 mm cubes and completely destained. The elution was performed with 30–40 μl of the solvent mixture described by Ehring et al. [26]. The supernatant was mixed 1:1 with the matrices for the MALDI measurements. One band was eluted from a Coomassie stained SDS–PAGE gel for each experiment.

2.6. Nano–electrospray ionization mass spectrometry

The nano–ESI-MS spectra were obtained with an ion trap mass spectrometer (LCQ, Finnigan MAT San Jose, CA, USA). The spray voltage was set to 0.8 kV and the capillary temperature was set to 200°C. The spectra were acquired with the automatic gain control (AGC) on, the MS^n target was set to 2.000e7 for the collision induced dissociation (CID) measurements. He was used as collision gas. The mass isolation window was set to four mass units and the arbitrary relative collision energy was set to 30–35% for the MS/MS measurements. The standard electrospray source of the LCQ was replaced with the nanospray unit of Protana. The nano–ESI fused-silica needles (''long'' and ''medium'' type) were also supplied by Protana. The calibration was performed externally with polyethylene glycol. Monoisotopic mass values were reported in this protocol.

2.7. Recovery of the proteins from the SDS–PAGE gel for analysis with nano–ESI-MS

The bands of interest were excised, cut into small cubes and washed three times with water for 5 min. The gel pieces were shrunk with acetonitrile and

dried in a Speed Vac concentrator (Eppendorf, Hamburg, Germany). Sulfhydryl groups of the proteins were reduced with 10 m*M* DTT in 50 m*M* NH_4HCO_3 at 56°C for 45 min and carboxymethylated with a 55 m*M* iodacetamide solution in 50 m*M* NH_4HCO_3 buffer at room temperature in the dark for 30 min. The gel pieces were washed twice with 50 m*M* NH_4HCO_3, shrunk with acetonitrile and dried in a Speed Vac concentrator. The samples were swollen in 50 m*M* NH_4HCO_3 containing 12.5 ng trypsin (sequencing grade, modified, Promega Madison, WI, USA) at 4°C. The supernatant was removed after 20 min and gel pieces were incubated at 37°C overnight.

The supernatants of the gel pieces were used after purification and concentration for nano–ESI-MS measurements and the rest of the peptides were in two steps (with 50% methanol in 5% formic acid and with 80% methanol in 10% formic acid) extracted. These extracts were dried in a Speed Vac concentrator, solubilised in 20 μl 5% formic acid and after purification and concentration, measured with nano–ESI. The purification and concentration of the peptide mixture after digestion was performed with the non-coated fused-silica needles from Protana and filled with Poros 10 R2 (Perseptive Biosystems, Framingham, MA, USA) material according to the instructions of Protana. The tryptic peptides were eluted directly into the coated nanospray needles with 1.2 μl methanol–5% formic acid (50:50 v/v) mixture. A second elution step was applied with 1.2 μl methanol–10% formic acid (80:20 v/v) to recover hydrophobic peptides.

Doubly or triply protonated peptides were chosen for the MS–MS experiments as precursor ions. Peptide sequencing was performed in the MS–MS mode optimizing the collision energy for each case.

Fragment ions were labelled according to the nomenclature proposed by Roepstorff et al. [31]. Fragment ion spectra were interpreted either with the aid of the Sequest software (ThermoQuest, Finnigan San Jose, CA, USA) or with the Mascot search program using the NR database (NCBI Resources, NIH, Bethesda, MD, USA).

3. Results and discussion

The goal of this work was to extract and characterize the latex allergen REF from ammoniated latex sap as a standard protein for further investigations. We used the same extraction procedure described previously by Czuppon et al. [15]. The subsequent purification method of the extracted proteins was

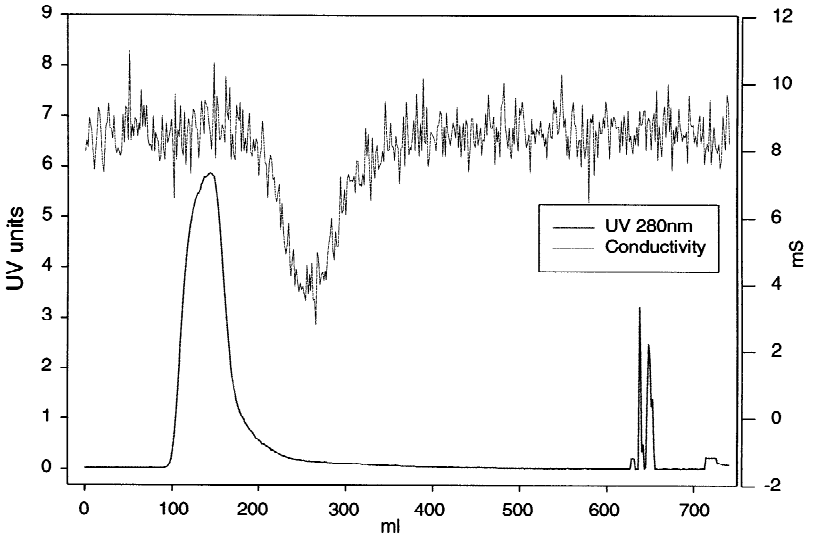

Fig. 1. Desalting of latex extract by gel filtration on a Sephadex G 25 column; 50 ml were loaded at a flow-rate of 15.3 cm/h. As mobile phase a 0.1 *M* ammonium acetate, pH 6.8, was used.

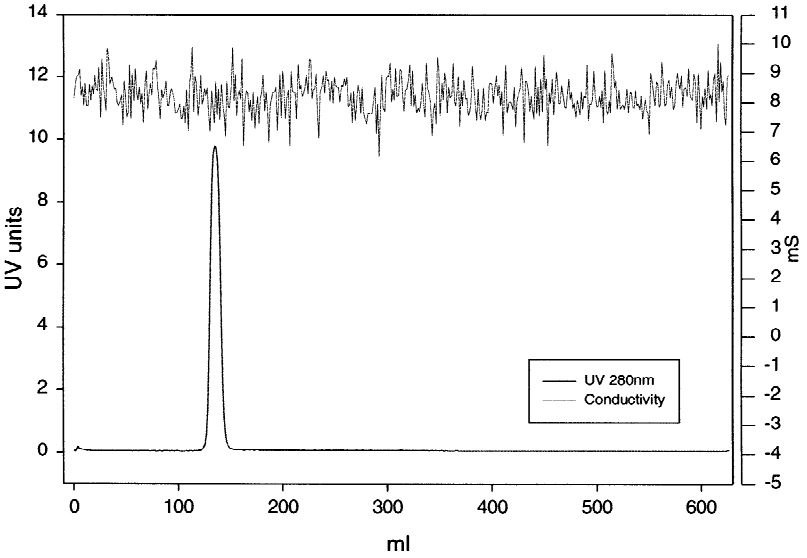

Fig. 2. Gel filtration of concentrated latex extract on a Superdex 75 column; 10 ml were loaded at a flow-rate of 56.6 cm/h. As mobile phase a 0.1 *M* ammonium acetate, pH 6.8, was used.

slightly modified. Instead of the dialysis step, which was applied to reduce the high content of SDS in the extract, we introduced a desalting step by gel filtration on a Sephadex G 25 column. The chromatogram of the gel filtration is shown in Fig. 1. The following lyophilization step to concentrate the diluted extract before the purification of the latex proteins was replaced by a discontinuous ultradiafiltration. The advantage of the chosen method compared to the lyophilisation is the further reduction of the salt content. Reconstitution of such a lyophilizate may be very difficult due to low solubility of the obtained

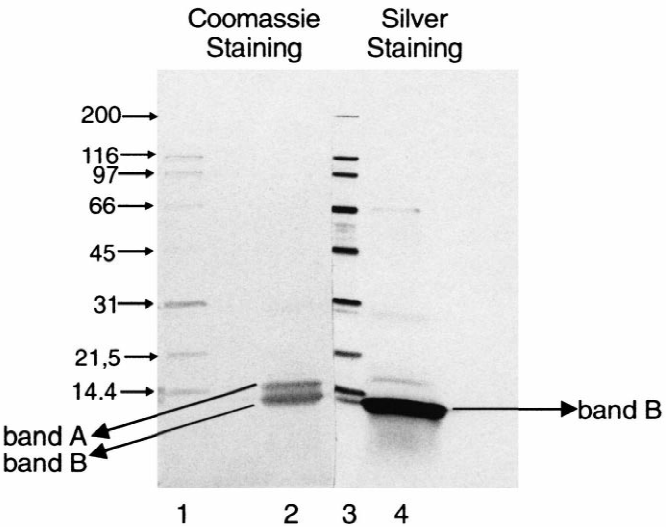

Fig. 3. SDS–PAGE on a 4–20% gradient polyacrylamide gel of purified latex extract using a modified Laemmli procedure. Lanes 1,3= molecular mass marker; 2,4= purified latex extract.

dry matter. The applied membrane with an M_r cut off of 8000 retains the desired proteins, while SDS can pass through.

As the final step we loaded the concentrated extract onto a Superdex 75 column to separate REF from other components. The chromatogram of this separation is shown in Fig. 2. Only one peak could be eluted. As shown later the peak contained more than one component, but the resolving power of the gel filtration is too low for further resolution. Low molecular mass contaminants have been already removed by Sephadex G 25. The eluate was collected, lyophilized and further analyzed. The lyophilized product was reconstituted in reducing sample buffer for electrophoresis, separated by SDS–PAGE and proteins were visualized with Silver or Coomassie Brilliant Blue staining. The developed gels are shown in Fig. 3. The silver stained gel shows only one band below the M_r 14 400 marker which was denoted as band B, while the Coomassie stained gel shows two bands, one above and one below the M_r 14 400 marker. The band above the M_r 14 400 marker protein was denoted as band A. In the silver stained gel proteins with M_r approximately 66 000, 30 000 and 18 000 are also visible.

3.1. Determination of the molecular mass of the proteins by MALDI–TOF

The purified and lyophilized extract was further characterized by MALDI–TOF-MS mass spectrometry. The extracted proteins turned out to be extremely hydrophobic and could be solubilised only in a 2% SDS solution.

The high SDS content in the sample prevented the detection of the protein with sinapic acid as matrix.

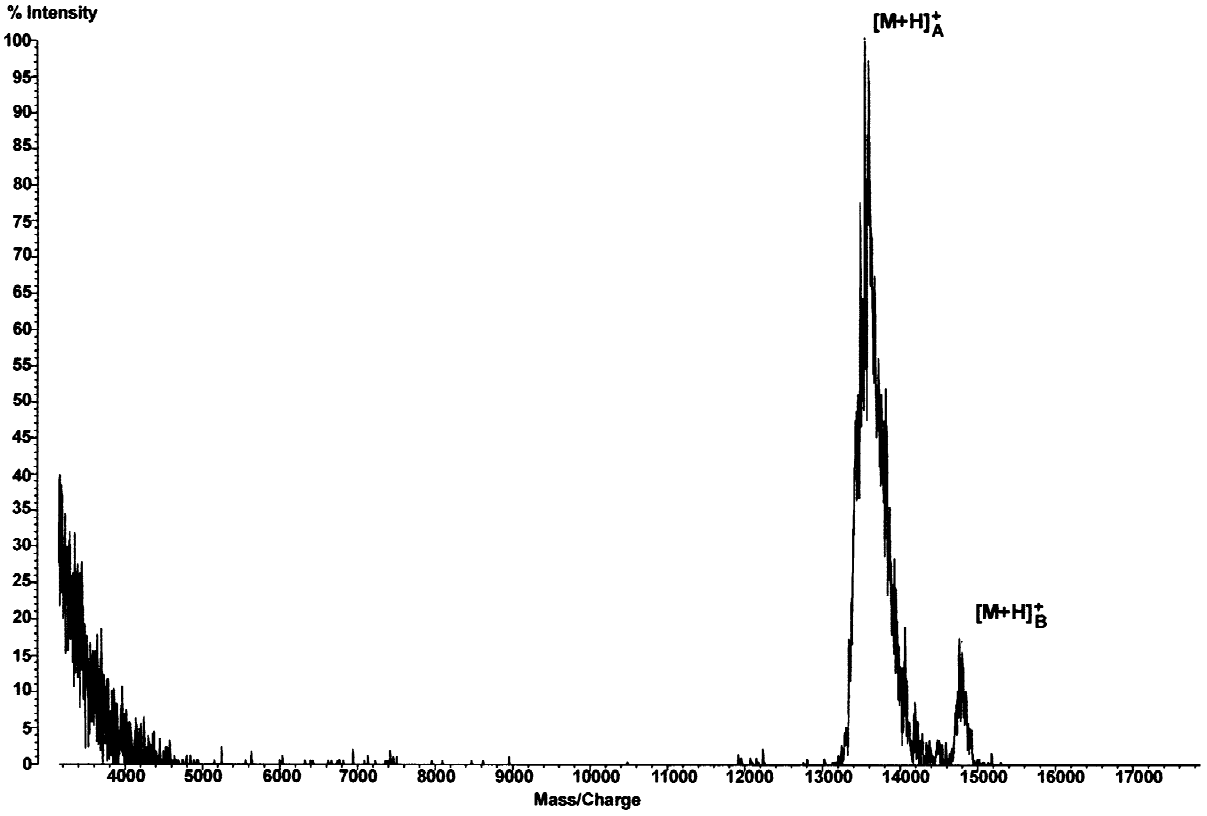

Fig. 4. Positive ion mass spectrum of the purified latex extract recorded by MALDI–TOF-MS (linear mode). The samples were prepared with CHCA matrix using the sandwich method. The spectrum is smoothed by the Savitzky-Golay algorithm.

2,5-dihydroxybenzoic acid (DHB) has been described as a matrix tolerating a high content of SDS [26]. However we observed inhomogeneous crystallization with only a few crystals developed on the proper part of the sample target. Therefore we chose CHCA and the sandwich method as described by Kussmann et al. [30] to analyze this sample. The advantage of this sample preparation is, that the salt content of the analyte can be reduced with an additional washing step. The disadvantage of this matrix is, that it is a so-called hot matrix supporting metastable decay resulting in peak broadening. This effect can be clearly seen in Fig. 4, but we were still able to deduce an average molecular mass. Under these conditions we detected two proteins denoted as A and B in Fig. 4 with a molecular mass of 13 500±200 for protein A and 14 700±200 for protein B.

In SDS–PAGE we found a double band migrating similarly to the M_r 14 400 marker protein. A few minor proteins were found in the M_r range of 66 000 to 18 000 (see Fig. 3). The double bands migrating with the M_r 14 400 marker protein were individually excised from a Coomassie stained gel and further treated for MALDI–TOF-MS analysis as described in the experimental section. We detected one peak in the band A with a molecular mass of 14 700±50 (Fig. 5). The eluate of the band B contained at least three other proteins with very similar molecular mass between M_r 13 300–13 700 (Fig. 6). Due to the obtained accuracy of the protein masses for the intact protein postranslatorial modifications of REF could not be deduced. Acetylation of the N-terminus would result in a mass difference of 42 mass units while formylation caused by the extraction procedure in a mass change of 28 mass units.

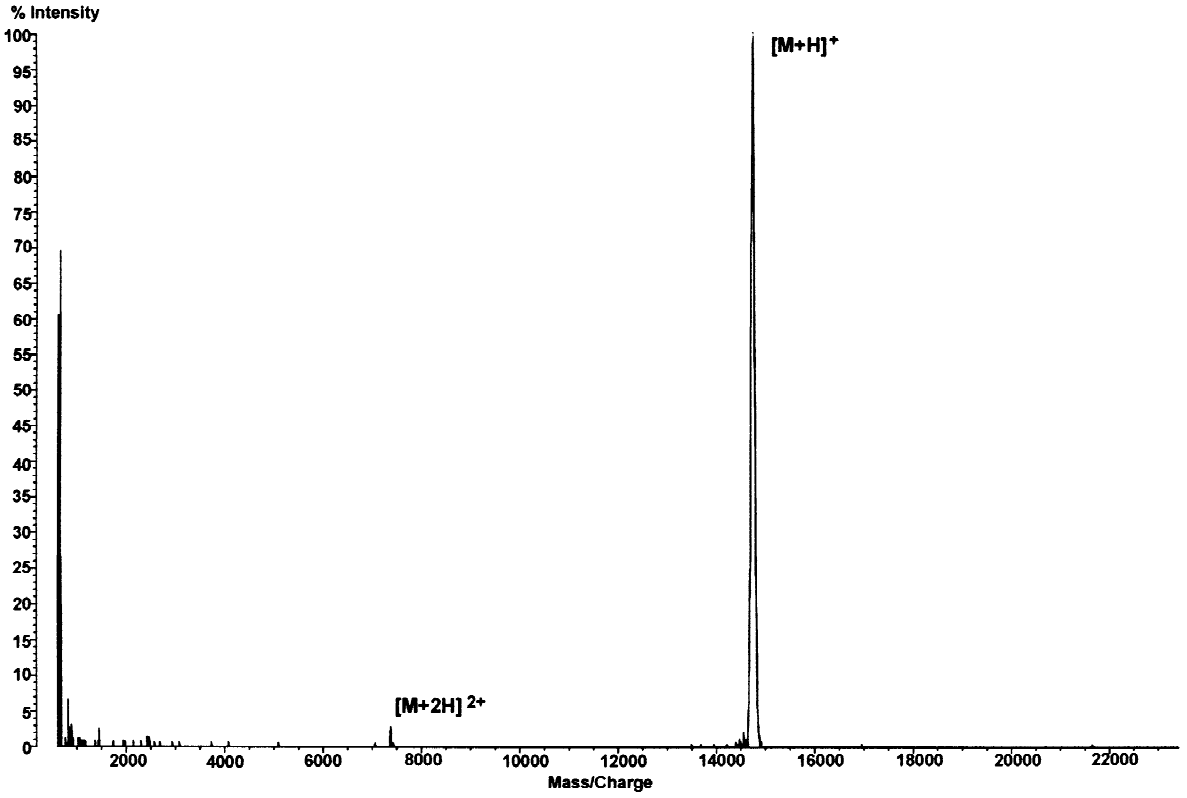

Fig. 5. Positive ion mass spectrum of the protein eluted from band A recorded by MALDI–TOF-MS (linear mode). The samples were prepared with sinapic acid matrix using the dry droplet method. The spectrum is smoothed by the Savitzky-Golay algorithm.

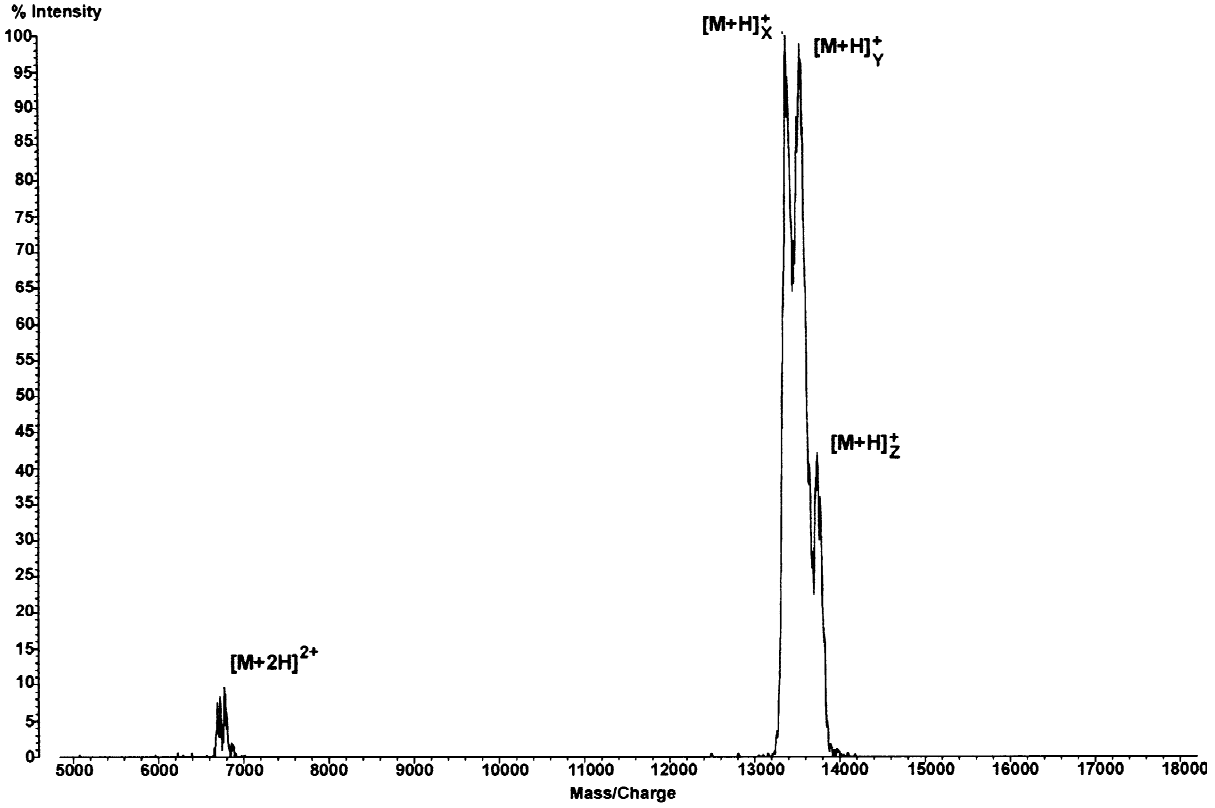

Fig. 6. Positive ion mass spectrum of the proteins eluted from band B recorded by MALDI–TOF-MS (linear mode). The samples were prepared with sinapic acid matrix using the dry droplet method. The spectrum is smoothed by the Savitzky-Golay algorithm.

3.2. Characterisation of the extracted proteins by nano–electrospray mass spectrometry

One Coomassie stained band was used to characterize the proteins in the band A and one silver stained band was used to characterize the proteins in the band B. In contrast to a previous report of Gharahdagi et al. [27] it is not essential to remove the silver from the gel pieces prior to in-gel tryptic digestion. Band A and B were excised immediately after staining and the Coomassie stained band A was completely destained with methanol–5% formic acid (1:1, v/v) and further treated as described in the experimental section. After extraction of the tryptic peptides from the gel pieces of the band A, we could detect several peptide species (Fig. 7), that could be assigned to REF according to their fragmentation pattern and molecular mass (Table 1) their fragment spectra are shown in Fig. 8a–f. Since the peptides were fragmented by an ion trap instrument, there could only be b and y type fragment ions recovered [31]. The peptides identified in this measurements overlap 31% of the sequence of REF. Additional peptides not originating from REF and keratin, a common contamination of protein samples [32,33], could be detected.

Interestingly, the results of the MALDI–TOF-MS measurements showed that band B does not contain the protein with the molecular mass of 14 700, however, the spectra obtained from band A and B by nano–ESI-MS experiments were very similar (Figs. 7 and 9). The identified peptides in band B overlap with 61% of the sequence of the REF. Additionally unknown peptides are present in this sample and could not assigned to a protein sequence available in the database used for the search.

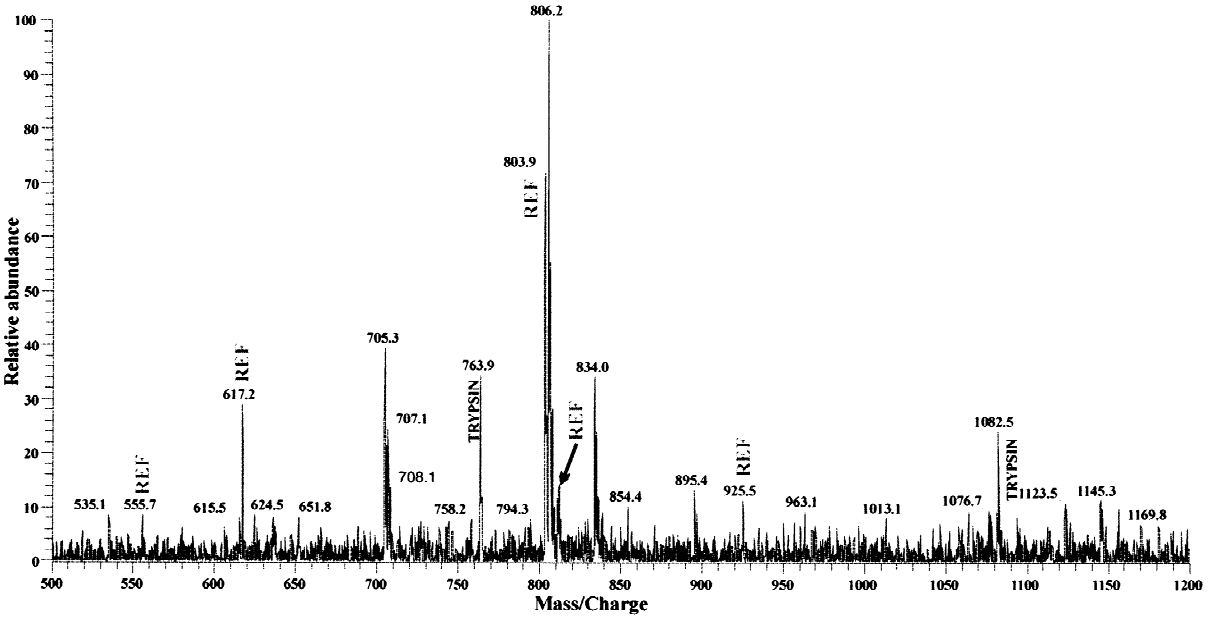

Fig. 7. Positive ion mass spectrum of the tryptic peptide digest of band A recorded by nano–ESI-MS; the identified peptides are marked. Peaks marked with ''REF'' originate from rubber elongation factor and peaks labelled with ''TRYPSIN'' originate from the cleavage enzyme trypsin.

The tryptic peptides in band B which were assigned to REF according to their mass and their fragmentation pattern are listed in Table 2 and their fragment spectra are shown in Fig. 10a–f. One peptide (m/z 879) shows a mass difference of two mass units compared to the theoretical molecular mass of this peptide, but according to its fragmentation pattern this peptide can be also assigned to REF (Table 2). The two asparagine residues in this peptides might be deamidated, since deamidation of a asparagine or glutamine results in a gain of 0.98 mass units. Unfortunately the evidence of this modification could not be confirmed, since it is not possible to analyze this low mass fragment by an ion-trap instrument. A peptide from the N-terminus could never be recovered from band A and B. According to the molecular mass complete REF must be present in Band A. In band B only a truncated form can be present, since a full length molecule could not be detected.

In the extract of the peptides from band B we also found a peptide (m/z 846), that according to its fragment ions could not be originate from REF (Table 3). The corresponding fragment spectrum is

Table 1
Tryptic peptides assigned to REF determined by nano–ESI-MS obtained from band A

Amino acid residues	m/z	Molecular mass [a]	No. [b]	Sequence[a]
67–76	555	1109.6	0	FSYIPNGALK
40–57	617	1849.0	1	DKSGPLQPGVDIIEGPVK
117–137	742	2223.2	2	SLASSLPGQTKILAKVFYGE N
42–57	803	1605.9	0	SGPLQPGVDIIEGPVK
77–91	812	1621.9	0	FVDSTVVASVTIIDR
40–57	925	1849.0	1	DKSGPLQPGVDIIEGPVK

[a] according to NR database
[b] number of missed cleavages

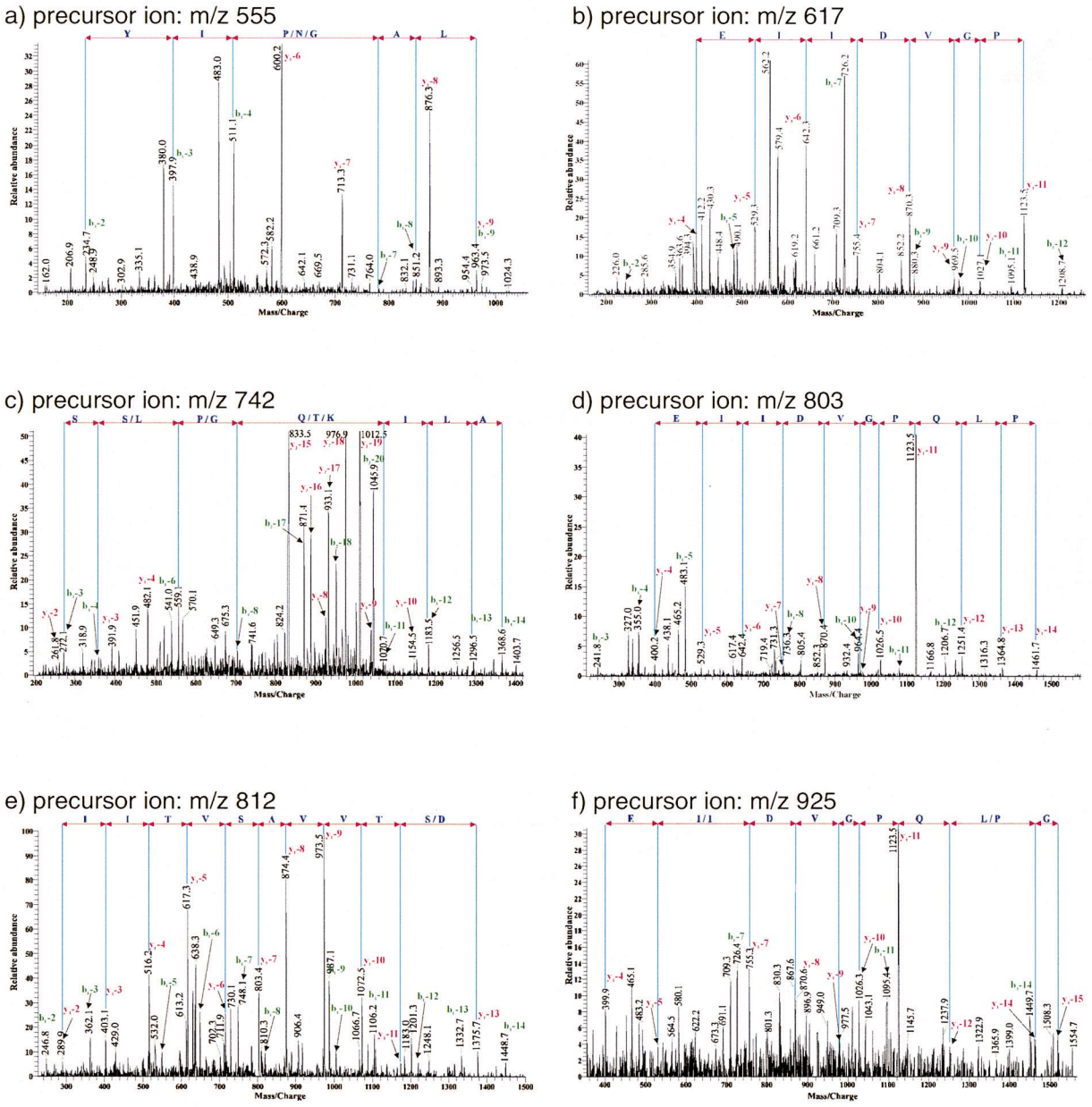

Fig. 8. (a–f): Sequencing of the tryptic peptides of band A. 15–20 consecutive scans were accumulated for each fragment spectrum. The precursor ions of spectra shown in (b) and (c) are triply protonated, while all other ions are doubly charged. The charge of the fragment ions is indicated as subscript of the fragment ion type (only type y and type b ions were detected) followed by the a number indicating the position of the fragmentation site.

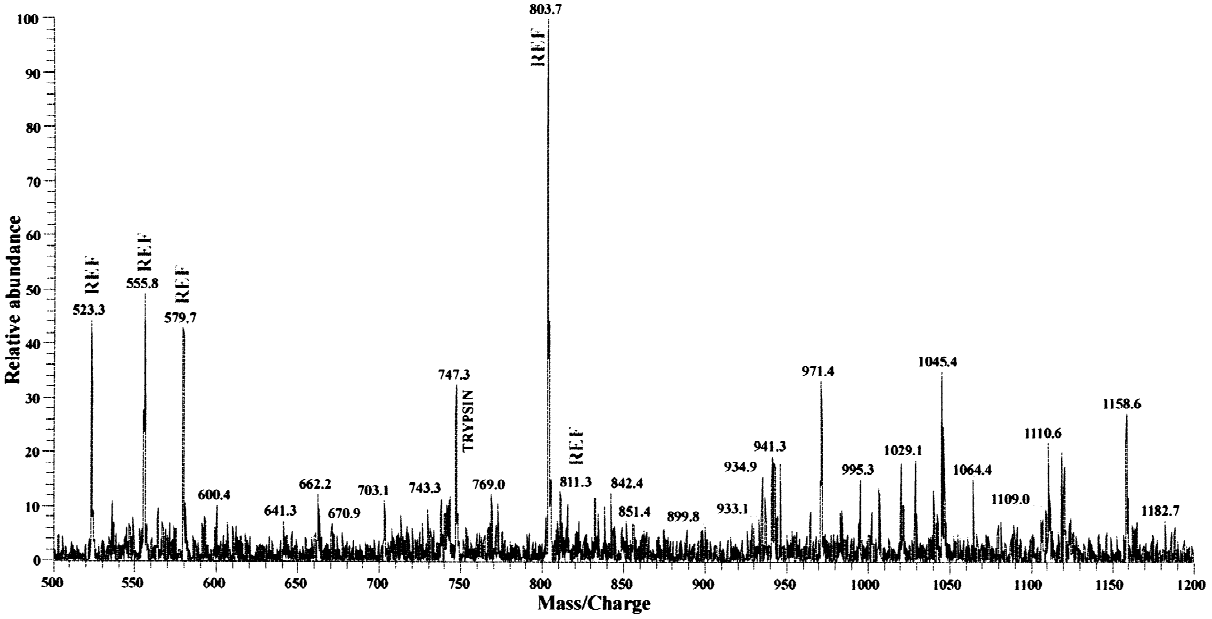

Fig. 9. Positive ion mass spectrum of the tryptic peptide digest of band B recorded by nano–ESI mass spectrometry; the identified peptides are marked. Peaks marked with ''REF'' originate from rubber elongation factor and peaks labelled with ''TRYPSIN'' originate from the cleavage enzyme trypsin.

shown in Fig. 11. After performing a BLAST search with this sequence, we found two proteins, namely an M_r 27 000 latex allergen and Hevb 3, that contained this structure. Both of them have higher molecular mass, but it is still possible, that processed forms of these molecules co-migrated with the other proteins in band B. We assumed that the proteinaceous compounds extracted from band B were a

Table 2
Tryptic peptides assigned to REF determined by nano–ESI-MS obtained from band B

Amino acid residues	m/z	Molecular mass[a]	No. [b]	Sequence[a]
58–66	523	1045.6	0	NVAVPLYNR
67–76	555	1109.6	0	FSYIPNGALK
99–109	579	1158.6	0	DASIQVVSAIR
42–57	803	1605.9	0	SGPLQPGVDIIEGPVK
77–91	811	1621.9	0	FVDSTVVASVTIIDR
42–66	879	2632.4	1	SGPLQPGVDIIEGPVKNVAV PLYNR

[a] According to NR database.
[b] Number of missed cleavages.

Table 3
Tryptic peptides assigned to hevb 3 or an M_r 27 000 latex allergen determined by nano–ESI-MS obtained from band B

Amino acid residues	m/z	Molecular mass[a]	No.[b]	Sequence[a]
54–68	846	1692.0	0	TVVTPVYYIPLEAVK

[a] According to NR database.
[b] Number of missed cleavages.

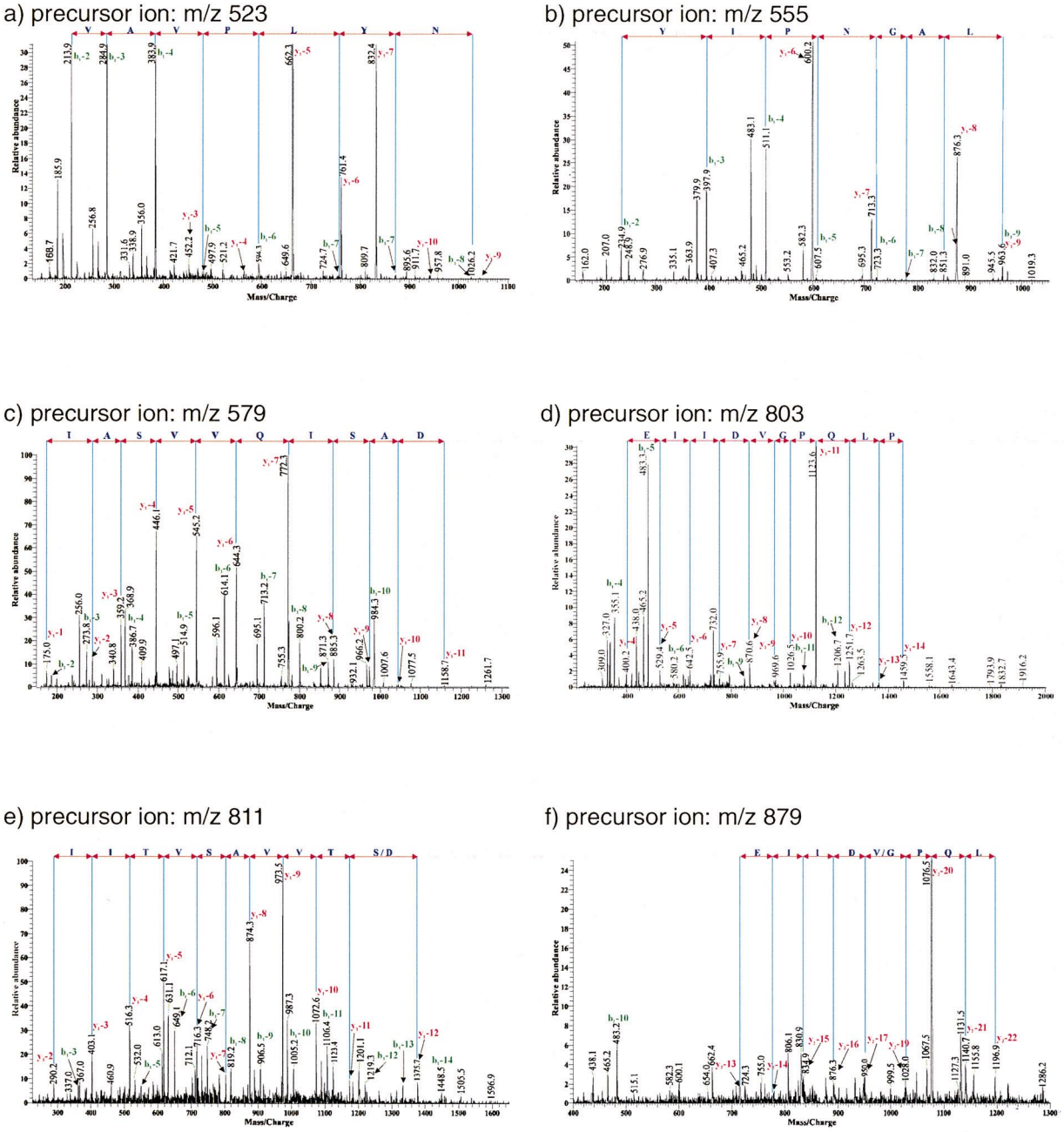

Fig. 10. (a–f): Sequencing of the tryptic peptides of band B. 15–20 consecutive scans were accumulated for each fragment spectrum. The precursor ions of spectrum shown in (f) is triply protonated, while all other ions are doubly charged. The charge of the fragment ions is indicated as subscript of the fragment ion type (only type y and type b ions were detected) followed by the a number indicating the position of the fragmentation site.

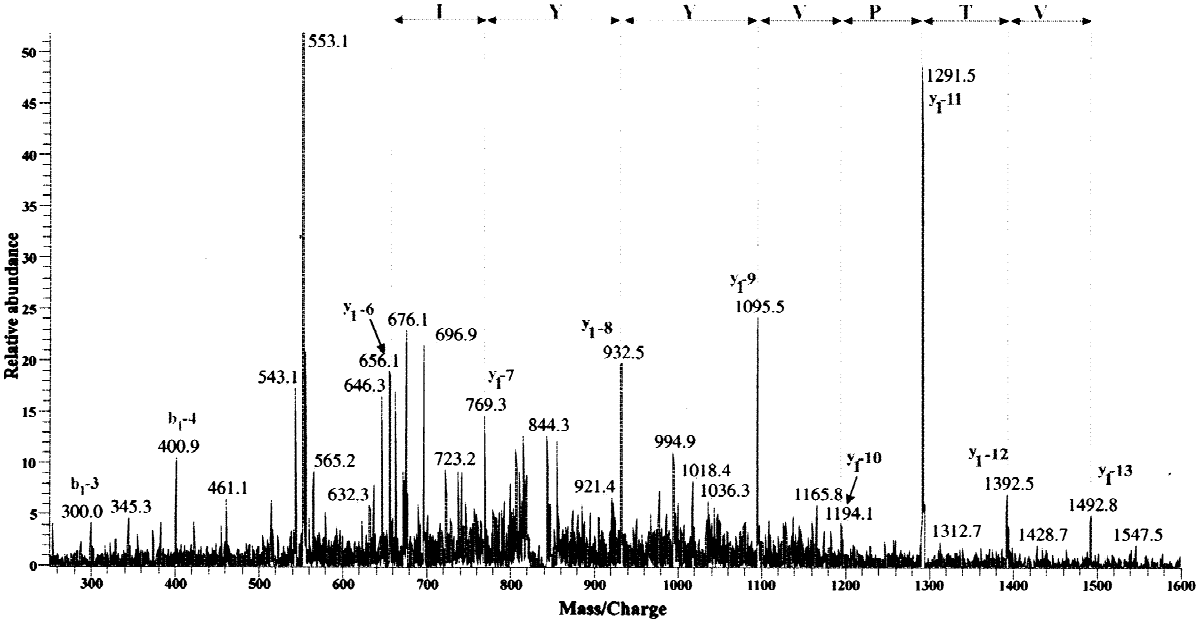

Fig. 11. Sequencing of the triply protonated peptide m/z 846 from band B. 15–20 consecutive scans were accumulated for the fragment spectrum. The charge of the fragment ions is indicated as subscript of the fragment ion type (only type y and type b ions were detected) followed by the a number indicating the position of the fragmentation site.

mixture of proteins with similar molecular masses. The results of the MALDI measurements are supported by the results obtained by nano–ESI-MS.

4. Conclusion

Combining all methods we detected at least two proteins, that had been extracted with the described extraction procedure. According to the results of the nano–ESI-MS analysis of the tryptic peptides, the protein with M_r 14 700 could be identified as REF. The proteins in band B showed in the nano–ESI-MS measurements high sequence homology with REF.

In addition to these results a two dimensional gel electrophoresis of the reconstituted product was carried out, which showed a pattern of several proteins with low molecular mass in the extracted product (results are not shown).

These results show that allergy test using REF preparations obtained by conventional methods must be very carefully evaluated, since the extracted compound seems to be a mixture of different proteins or peptides and not a pure REF at all.

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