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Determination of Staphylococcal Enterotoxin B by on-line (micro) liquid chromatography–electrospray mass spectrometry

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Abstract

The use of (micro) liquid chromatography electrospray mass spectrometry (LC–ES-MS) was investigated as potential technique for the determination of the high-molecular-mass protein toxin Staphylococcal Enterotoxin B (SEB). The molecular mass was determined (28 366.3±1.1) by flow injection analysis and micro-LC–MS with a TSK-gel Phenyl-5PW column packing. Both methods allowed molecular-mass determination of SEB at levels down to 3 pmol/ml. Additional evidence of identification was obtained by detecting the presence of a disulfide bridge by the addition of 2-mercaptoethanol and by tryptic digestion. Collision induced dissociation spectra were recorded from the major tryptic fragments resulting in a sufficient number of sequence ions to allow for the determination of the amino acid sequence. On the analysis of the tryptic digests with C_{18} reversed-phase columns the use of micro-LC–MS–MS resulted in an 30–40-fold increase in sensitivity as compared with conventional-size LC–MS–MS.

Keywords: Mass spectrometry; Electrospray ionization; Staphylococcal enterotoxin B

1. Introduction

New developments in biochemistry and biotechnology create opportunities to produce extremely toxic substances on a scale that has never before been possible. These toxic substances can be used in military attacks or terrorist actions and may constitute a serious threat [1]. The substances of interest in this context are poisonous toxins, or bioregulators which influence different physiological and biochemical processes in the body. Toxins can be classified according to their physiological action such as toxins affecting the nervous system (neurotoxins) and toxins affecting the stomach and intestines (enterotoxins). They can also be grouped on the basis of size, large toxins are generally proteins, whereas the smaller ones are peptides or rather complicated organic molecules e.g., paralytic shellfish poisons. Toxins can be extremely poisonous, e.g., the *Botulinum A* toxin, produced by the bacterium *Clostridium botulinum* is one of the most poisonous substances known to human (minimum lethal dose (human), ca. 1 ng/kg) [2]. Due to the wide spectrum present in nature and the possibility of large scale production of "new" toxin producing micro-organisms by gene manipulation they may not only be difficult to detect but may also cause toxicity symptoms that are difficult to diagnose. In disarmament and other contexts toxins and bioregulators are regarded as possible biological warfare agents (BWA) or chemical warfare agents (CWA). Hence, these agents are sometimes also referred to as mid-

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spectrum agents. In 1971 the Conference of the Committee on Disarmament (CCD), completed its work on the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological and Toxin Weapons [3]. This convention entered into force in 1975. Two toxins, i.e., the paralytic shellfish poison saxitoxin and the plant toxin ricin isolated from castor beans, are placed as examples on the schedule list of the Chemical Weapon Convention [4]. This Convention, which prohibits the development, production, stockpiling and use of chemical weapons, is in its preparatory phase. In view of both Conventions, analytical methods have to be developed to prove alleged exposure to toxins in case of an incident.

Reflecting their position between BWA and CWA, methods for the analysis of toxins may be based on either a biological approach using immunological techniques or a chemical approach using spectrometric techniques. At present mass spectrometry (MS) is the most suitable chemical analytical technique due to its sensitivity and selectivity and the possibility of combining it with liquid chromatography (LC) or capillary electrophoresis (CE). In the past years LC–MS interfaces, such as thermospray, and particle beam (PB) have enabled MS detection and determination of relatively small molecules (M_r<500) on a more or less routine basis. In recent years, we have used thermospray LC–MS for the identification of the paralytic shellfish poison saxitoxin [5] and for the identification of a number of (bio) degradation products of CW agents [6–8]. Recently, we have developed an eluent-(droplet)jet interface to couple micro-LC with electron impact MS [9].

These interfaces, however, are unsuited for the identification of relatively large biomolecules, e.g., peptides and proteins. In this field more promising results were obtained with continuous-flow fast atom bombardment [10–12], but its use with routine LC separations of biomolecules, is limited to relative small peptides (M_r<2000) [13]. A real breakthrough was obtained by the work of Fenn and co-workers [14–16]. They were the first to describe the analysis of samples containing polymers, peptides and proteins with molecular masss beyond 20 000 using an electrospray (ES) interface to affect ES ionization at atmospheric pressure. In the gas-phase multiple charged molecules can be formed from highly charged liquid droplets produced. This multiple charging phenomenon allows the use of conventional mass spectrometers of limited mass range (m/z 1 to ≤4000) such as quadrupole instruments. Later on Henion and co-workers [17–19] introduced a modification of the ES method which they called ion spray (IS), where the nebulization of the effluent is assisted by a turbulent gas (N_2) flow. Both techniques produce the same type of mass spectra and are compatible with LC and CE separation methods [20–22]. An important feature of ES or IS is that essentially no fragmentation accompanies ionization of peptides or proteins. Molecular mass determination will be uncompromised by fragment ions, and for molecules that show an ability for multiple charging, precision is enhanced by the possibility for multiple mass measurements from a single mass spectrum. Due to the fact that ES and IS are very gentle ionization processes and generally yield no fragment ions, the MS spectra provide no additional structural information. However, with the use of cone voltage fragmentation and collision-induced dissociation (CID) in tandem mass spectrometry (MS–MS) sufficient structural information can be required from ES-derived ions [23]. Today ES-MS(MS) using flow injection analysis or in combination with LC or CE is widely used for the determination of peptides and proteins including toxins and bioregulators. The combination of LC–ES-MS(MS) allows for the analysis of high-molecular-mass protein-like toxins either by the accurate determination of the molecular mass or by the analysis of the amino acid sequence of fragments of the toxin obtained after enzymatic or chemical cleavage. Staphylococcal Enterotoxin B (SEB) produced by various strains of *Staphylococcus Aureus* is considered as a potential toxin threat [24]. The ingestion of food containing *Staphylococci* bacteria may lead to severe food-poisoning. Due to the fact that SEB is commercially available and not as extremely toxic as for instance *Botulinum* toxin, it may be considered as a good test substance for the development of LC–ES-MS(MS) procedures directed to the unequivocal identification of high-molecular mass toxins. In this paper we report the results of the determination of SEB by either molec-

ular mass determination and amino acid sequence analysis based on the well-known tryptic cleavage, which has been described for SEB [25].

2. Experimental

2.1. Materials

Analytical-grade acetonitrile, formic acid and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). Analytical-reagent grade ammonium acetate, ammonium formate, trifluoroacetic acid (TFA), sodium phosphate and ammonium bicarbonate (NH_4HCO_3) were obtained from Aldrich-Chemie (Steinheim, Germany). Sodium dodecyl sulfate (SDS) 99% was obtained from Janssen Chimica (Geel, Belgium). Throughout the study, deionized water (Milli-Q water Purification System, Millipore, Milford, MA, USA) was used. All solvents and solutions were filtered prior to use through 0.45-μm pore size filter discs from Millipore. SEB mixed with sodium phosphate was obtained from Sigma (St. Louis, MO, USA; cat. No. S4881) and Makor Chemicals (Jerusalem, Israel; cat. No. 0734). 2-Mercaptoethanol was purchased from Fluka (Buchs, Switzerland). Immobilized TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone) trypsine gel was obtained from Pierce (Rockford, IL, USA). For mass axis calibration myoglobin (horse heart) was supplied by Sigma and poly(ethylene glycol)s (PEG-400 and PEG-1000) were from Aldrich-Chemie. The column packing material LiChrosorb RP-18 and the conventional-size LC column packed with TSKgel Phenyl-5PW were obtained from Merck and TosoHaas (Stuttgart, Germany), respectively. The fused-silica and PEEK tubing were obtained from Composite Metal Services (Hallow, UK) and Jour Research (Onsala, Sweden), respectively.

2.2. Sample preparation

2.2.1. Dialysis

For the dialysis an amount of 1 mg of SEB was dissolved in 1 ml water. Approximately 150 μl of this solution was diluted to 1 ml with water. Dialysis was performed against 0.2% formic acid during 24 h using Slide-A-Lyzer Dialysis Cassettes (Pierce) with a M_r 10 000 cut-off membrane and 0.5–3.0 ml capacity. After dialysis the resulting volume was approximately 1.5 ml.

2.2.2. Disulfide bond cleavage

Aliquots (100 μl) of a 3 nmol dialysed SEB solution were incubated in closed vials under nitrogen atmosphere with 5 μl 10% 2-mercaptoethanol for 20 h at ambient temperature and for 0.5, 1 and 2 h at 60°C. Before ES-MS analysis the samples were diluted 1:20 with water–acetonitrile (1:1) containing 0.2% formic acid.

2.2.3. Tryptic cleavage

A 250-μl amount of the untreated SEB solution (concentration 30 nmol/ml was added to 1.5 ml water. Immobilized TPCK-treated trypsin [0.25 ml gel; suspended in aqueous NH_4HCO_3 (0.5 ml; 0.1 *M*; pH 8)], which had been washed with aqueous NH_4HCO_3 (5×4 ml; 0.1 *M*; pH 8.0) was added. The mixture was incubated at 37°C for 6, 20, 24, 48 and 60 h in a shaking device. The immobilized trypsin was removed by filtration through a 0.45-μm Millex-HV_{13} filter (Millipore).

2.3. Liquid chromatography (LC)

2.3.1. Conventional-size LC

For the analysis of the tryptic fragments of SEB an amount of 40 μl of the digested solution was injected using a Valco injection valve (VICI, Schenkon, Switzerland) on a laboratory-made LC column (250 mm×5 mm I.D.) packed with 5 μm Lichrosorb RP-18 (Merck). Gradient elution was performed using the following eluent compositions: eluent (A), water–acetonitrile (98:2) containing 0.5% formic acid and (B), water–acetonitrile (30:70) containing 0.5% formic acid. A linear gradient was used from eluent (A) to eluent (B) in 30 min. The flow through the column was 1 ml/min, and was regulated by two Waters solvent delivery systems (Model 590 and Model 510) in combination with a Model M680 solvent programmer (Waters Corporation, Milford,

MA, USA). At the end of the column approximately 5% of the flow was directed towards the ES inlet probe by means of a laboratory-made splitter.

2.3.2. Microcolumn liquid chromatography (micro-LC)

Under identical LC-conditions as described in Section 2.3.1 micro-LC was performed by replacing the conventional-size LC column by a laboratory-made micro-LC column (600 mm×0.3 mm I.D., fused-silica tubing, packed with 5 μm Lichrosorb RP-18) and using a Valco sample injection valve (VICI) provided with a 10-μl loop. The ml/min flow-rate delivered by the above-mentioned solvent delivery systems was converted to a μl/min flow-rate using an Acurate Model IC-70-CAP microflow processor with a split ratio of ca. 1:70 (LC Packings, Zurich, Switzerland).

For the micro-LC separation of intact SEB, PEEK tubing (400 mm×0.5 mm I.D.) was packed with TSKgel Phenyl-5PW. Gradient elution was performed using the following eluent compositions: eluent (A), water–acetonitrile (9:1); eluent (B), water–acetonitrile (2:8) both containing 0.2% TFA. A linear gradient was used from eluent (A) to eluent (B) in 25 min.

2.4. Capillary electrophoresis

Capillary electrophoresis (CE) was applied to check the quality of the SEB standard solutions, the recovery of SEB after dialysis and the conversion of SEB after the tryptic digests. The CE instrument used was a Prince (Prince Technologies, Emmen, The Netherlands) equipped with a fused-silica column (Composite Metal Services, UK) of 84 cm L_t×50 μm I.D. and 52.5 cm L_d connected with a UV–Vis Detector (Prince Technologies). For the separation of SEB the following conditions were used: buffer; 150 m*M* sodium phosphate pH 2.4; voltage, 25 kV; current, 71 μA; oven temperature, 30°C; UV wavelength, 200 nm; injection, hydrodynamic, 25 mbar for 0.2 min (5.5 nl). The concentration of the SEB solution was 3 nmol/ml in 0.15 *M* NaCl in water.

2.5. Mass spectrometry

2.5.1. Flow injection

Positive ES mass spectra were obtained on a VG Quattro II triple quadrupole instrument provided with comprehensive VG software for handling biopolymers (Biolynx, Max Ent) (Fisons Instruments, Altrincham, UK). Samples were directly injected by means of a Waters Model 590 solvent delivery system and a Rheodyne injector (Cotati, CA, USA) with a 10-μl sample loop and using fused-silica capillaries (300 mm×0.1 mm I.D.) supplied by Polymicro Technologies (Phoenix, AZ, USA). For the determination of the molecular mass of SEB, 100 μl of the solution obtained after dialysis was diluted to 1 ml with water–acetonitrile (50:50) containing 0.2% formic acid. A mixture of water–acetonitrile (50:50) was used as eluent at a flow-rate of 10 μl/min. The following MS conditions were applied: mass range, m/z 500–2000; scan time, 10 s; ES capillary tip voltage, 3.8 kV; cone voltage, 25 V; source temperature, 80°C. The detection limit of SEB was determined with a cone voltage of 35 V and a reduced scan rate of m/z 770–940 in 3 s. The SEB samples treated with 2-mercaptoethanol were analysed with a scan range of m/z 700–900 in 10 s.

2.5.2. LC–MS

The following MS conditions were applied for the above-mentioned conventional-size LC and micro-LC analysis of the tryptic fragments of SEB: mass range, m/z 300–1200; scan time, 3 s; ES capillary tip voltage, 3.8 kV; cone voltage, 35 V; source temperature, 100°C (conventional LC) and 80°C (micro-LC). The same MS conditions were applied when using the micro-LC TSKgel Phenyl-5PW column with exception to the mass range m/z 800–930 (2 s).

2.5.3. LC–MS–MS

Collision-induced dissociation (CID) MS–MS experiments were carried out with Argon as collision gas and a gas cell pressure of approximately 0.5 Pa. The cone voltage and the collision energy were dependent on the charge state and the mass of the precursor ion and varied from 35 to 50 V and 20 to 25 V, respectively. Product ion spectra were obtained

by scanning the second quadrupole from m/z 20 to just above the expected molecular mass of the tryptic fragments and the scan time was set between 3 and 4 s.

3. Results and discussion

3.1. Analysis of intact SEB

3.1.1. Flow-injection analysis

The most useful mode of operation for analysis of intact peptides and proteins is the direct injection of small volumes of analyte solution into a carrier solvent that continuously flows into the ES source. Due to the fact that sodium ions strongly interfere during positive ion ES-MS analysis, these cations were first removed from the SEB stock solution by means of dialysis against formic acid (0.2%, v/v). Attempts to dialyse the SEB solution against water were unsuccessful, possibly due to the fact that the protein precipitates after removal of sodium phosphate. After dialysis the sample purity was checked using CE separation and UV detection. In Fig. 1 the electropherogram of intact SEB is depicted showing one major peak of SEB at 10.8 min and a smaller peak at 11.1 min indicating the presence of an impurity. For the molecular mass determination flow injections of 10-μl amounts of the sample containing approximately 30 pmol of SEB/ml were directly introduced into the ES-MS. The result of the determination of the molecular mass of SEB is presented in Fig. 2A. The ES spectrum was obtained after averaging the recorded spectra during the elution time of SEB (approximately 1.5 min) followed by smoothing and subtracting as determined by the software of the VG Quattro II system. Under the MS conditions used the maximum of the envelope of the protonated species was at 36 charges. Ions corresponding with 43 positive charges were detected, which equals the number of basic amino acids (Lys, Arg, His). The reconstructed spectrum using the MaxEnt option of the VG Quattro II software is presented in Fig. 2B. From the multiple charged ions an average molecular mass of 28 366.3±1.1 was calculated, which agrees quite well with the molecular mass of 28 366.1 calculated from the amino acid sequence published by Jones and Khan [26]. The obtained accuracy is enough to decide that the amino acid composition published by Huang and Bergdoll [27] in 1970 was incorrect. A second smaller peak was detected at m/z 28 400.1 (Fig. 2B), possibly corresponding to the impurity found by CE analysis.

The system proved to be linear within the investigated range of 0.1–10 ppm. The limit of detection obtained with 1 μl injection volumes was about 3 femtomol SEB corresponding to a concentration of 0.1 μg/ml. However, the accuracy of the mass measurement was reduced leading to a molecular mass of 28 366±5.

In order to collect additional structure information the presence of the disulfide bridge in the SEB molecule was determined by using the method of Svoboda et al. [28]. They developed a simple and rapid method for counting the number of internal disulfide bridges in a protein by incubation with 2-mercaptoethanol and subsequent ES-MS analysis. As a result of the nucleophilic attack on the S–S bond by 2-mercaptoethanol a mixture of proteins is formed with mass increments of 78 per disulfide bond. The dialysed SEB sample was treated with 2-mercaptoethanol at ambient temperature for 20 h. The reaction appeared to proceed unsatisfactorily at room temperature and was repeated at 60°C for 0.5, 1 and 2 h. The best results were obtained after 1 h incubation at 60°C as presented in Fig. 3 showing the mass spectrum with the reconstructed molecular mass of reduced SEB (28 367.7) and M_r 28 444.6 which is in accordance with the structure of SEB in which the two cysteine amino acids are linked together through a disulfide bridge.

3.1.2. Micro-LC–ES-MS

For the separation of intact SEB the use of TSK-gel type column material (TSK-gel Phenyl-5PW) was investigated. This packing material consists of TSK-gel G5000PW applied with phenyl groups with an average pore size as large as 1000 Å suitable for the separation of macromolecular proteins. With this type of column protein separations based on hydrophobic interaction are usually carried out with phosphate buffers or salt (NaCl) gradients. These type of

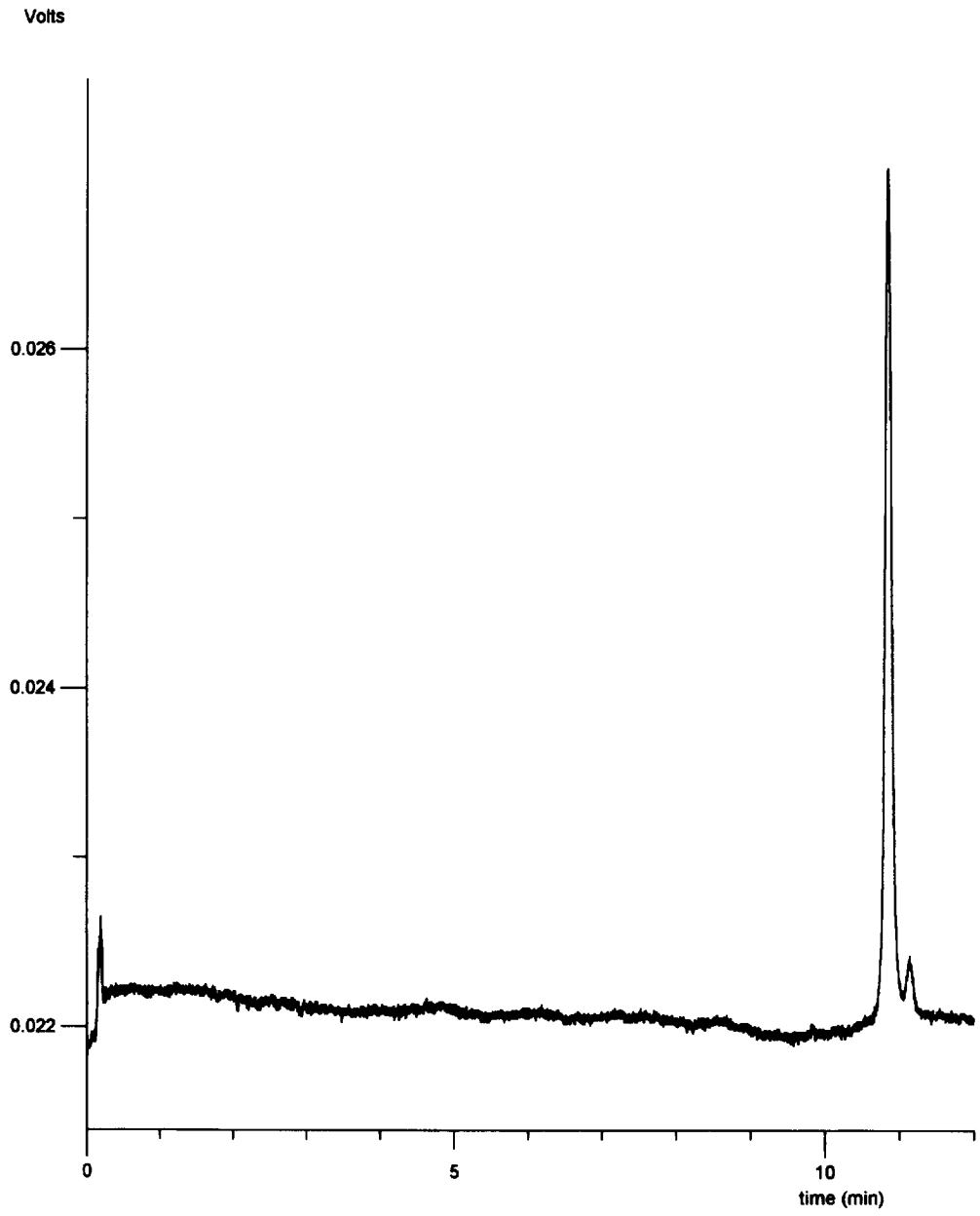

Fig. 1. Electropherogram of intact SEB. System Prince equipped with a fused-silica column 84 cm L_t×50 μm I.D. and 52.5 cm L_d connected with a UV–Vis detector. Conditions: buffer, 150 m*M* sodium phosphate, pH 2.4; voltage, 25 kV; current, 71 μA; oven temperature, 30°C; UV wavelength, 200 nm; injection, hydrodynamic, 25 mbar×0.2 min (5.5 nl). The concentration of the SEB solution was 3 nmol/ml in 0.15 *M* NaCl in Milli-Q water.

eluents, however, are not applicable in combination with ES-MS. Therefore, several gradients were investigated using water, methanol or acetonitrile in the presence of small amounts of either formic acid or TFA in order to obtain a sufficient retention and acceptable peak shape of SEB. Initial experiments were carried out using a commercial available conventional-size TSK-gel Phenyl-5PW LC column and

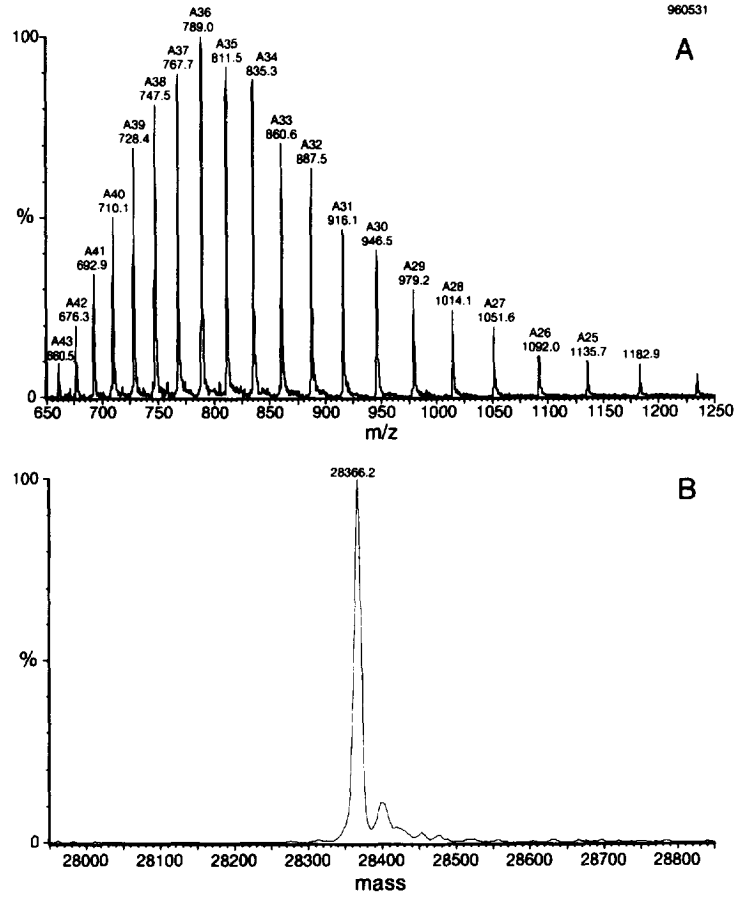

Fig. 2. ES-MS of SEB. Conditions: mass range, m/z 500–2000; scan time, 10 s; ES capillary tip voltage, 3.8 kV; cone voltage, 25 V; source temperature, 80°C. (A) Ion abundance profile; (B) reconstructed spectrum using MaxEnt.

UV detection. Later on this column was emptied and the packing material was repacked into 0.5 mm I.D. PEEK tubing using a water–NaCl–sodium dodecyl sulfate (SDS) mixture according to a recently developed procedure by Zimina et al. [29]. The best chromatographic results were obtained using water–acetonitrile gradients with addition of 0.2% TFA (see Fig. 4). By means of flow injection it was found that the use of TFA results in a 4-fold reduced sensitivity as compared with formic acid. However, to obtain a good peak shape and retention for intact SEB, TFA had to be used instead of formic acid. Applying the micro-LC-system in combination with ES-MS under above-mentioned conditions ca. 3 pmol/ml of intact SEB was detectable by injecting a volume of 1 μl.

Signal reduction on spraying TFA containing eluents has been recognized by others [30,31]. Recently, Apffel et al. [32] described a method for recovering the sensitivity of peptide mapping using post-column addition of mixtures of propionic acid and 2-propanol [32]. The addition of this mixture may, however, lead to an increase of the background.

3.2. Analysis of tryptic fragments of SEB

Generally, tryptic digestion of a protein generates a complex mixture encompassing the expected peptides but may also contain peptides possessing uncleaved bonds, autodigestion products i.e., peptides formed by the proteolytic agent acting on itself and peptides resulting from contaminating protease proteins in the trypsin used, e.g., chymotrypsin. For

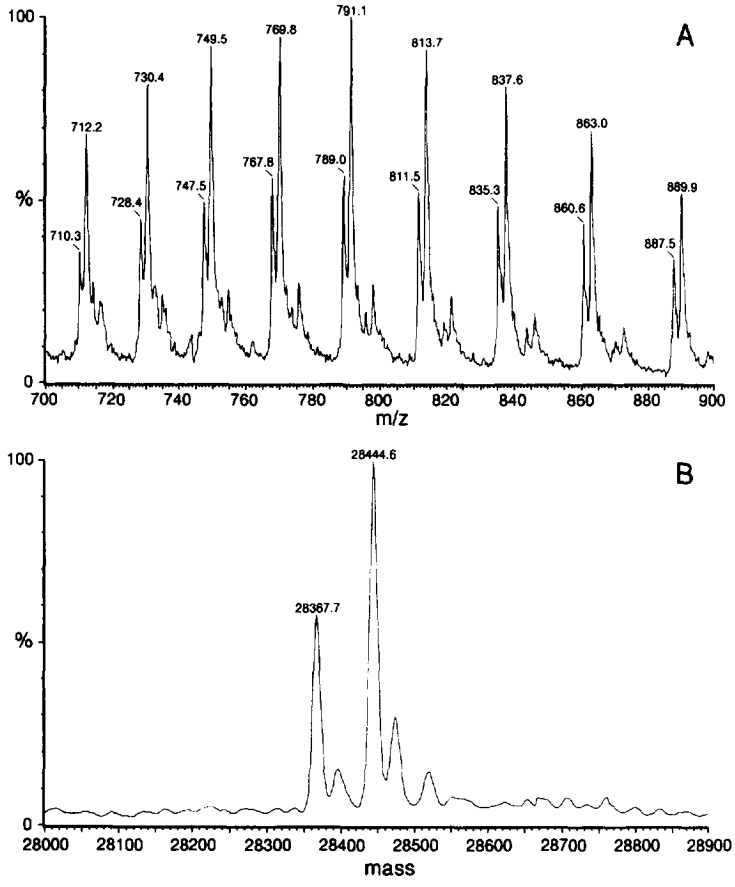

Fig. 3. ES-MS of SEB after reaction with 2-mercaptoethanol: mass range, *m/z* 770–940; scan time, 10 s; ES capillary tip voltage, 3.8 kV; cone voltage, 25 V; source temperature, 80°C. (A) Ion abundance profile; (B) reconstructed spectrum using MaxEnt.

SEB a number of 37 tryptic fragments can be expected based on the number of Lys and Arg residues in the protein of which seven are identical Lys fragments (T14, T17, T23, T24, T28, T36 and T37) and two identical Asn–Lys (T9 and T27) fragments.

3.2.1. CE–UV and LC–MS

The samples were investigated by CE–UV and LC–MS after digestion periods of 6, 20, 24, 48 and 60 h. The LC separation was performed on a C_{18} reversed-phase LC system, gradient elution turned out to be necessarily. The use of CE–UV enables to estimate the percentage of the tryptic digestion just by following the decrease of the SEB peak in time. Moreover, CE provides a high-resolution separation tool to obtain a sufficient resolution of the resulting tryptic fragments. After incubation of 6 h only a small part (<20%) was digested. From the results of Huang and Bergdoll [25], it is known that SEB is difficult to digest. To reduce the incubation time and to prevent the formation of a number of by-products, they used SEB oxidized with performic acid for the tryptic digest. The presence of by-products hampers the application of different separation methods including the classical procedure of peptide sequencing. However, this problem is less important when using ES-MS, therefore, we decided to work with unmodified SEB. As an example Fig. 5 shows the tryptic map obtained with CE after 20 h of incubation of SEB at 37°C. From the CE analysis it was calculated that at least 75% of SEB was converted.

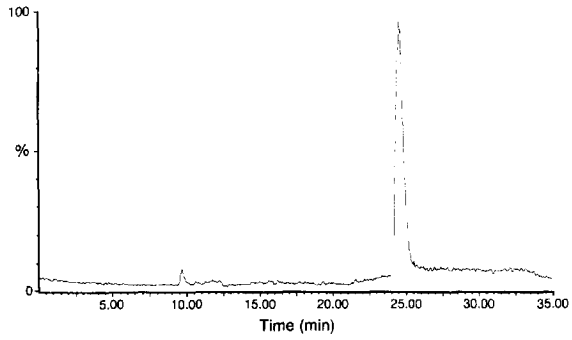

Fig. 4. Micro-LC–ES-MS chromatogram of intact SEB. Column (400 mm×0.5 mm I.D., packed with 10 μm TSK-gel Phenyl-5PW. Conditions: eluent (A), water–acetonitrile (90:10) and (B), water–acetonitrile (20:80) both containing 0.2% TFA, gradient linear from eluent (A) to eluent (B) in 25 min; flow-rate, 3 μl/min. MS conditions: mass range, m/z 800–930; scan time, 2 s; ES capillary tip voltage, 3.6 kV; cone voltage, 35 V; source temperature, 100°C. Injection, 10 μl containing 30 pmol of SEB in 0.1 *M* ammonium acetate.

The same sample was investigated by LC–MS. Despite the fact that the use of TFA resulted in somewhat sharper LC-peaks for some fragments, formic acid was added to the eluent because of the about 5-fold increased sensitivity as compared to TFA.

Due to the limited scan range of m/z 300–1200 only those tryptic fragments giving ions in that mass range are detected. Based on the observed MH^+, MH_2^{2+} or MH_3^{3+} ions in the ES mass spectra of the peptides, a number of tryptic fragments of SEB could be retrieved. The mass chromatograms of 16 fragments with a molecular mass above m/z 400 are presented in Fig. 6 in order of their LC-elution.

For several fragments the mass spectra gave some additional information on the amino acid sequence. As examples fragments, T33, T21 and T7 showed in addition to the protonated species also some characteristic ions. In the spectrum of fragment T33 a loss of Met (m/z 448.6) and Met–Val (m/z 349.5) from the MH^+ (m/z 579.6) was found. In the spectrum of fragment T21 both the MH^+ (m/z 694.5) and MH_2^{2+} (m/z 348) ions were present, whereas m/z 595.4 and m/z 448.5 correspond with the loss of Val and Val–Phe from the MH^+, respectively. The ES spectrum of fragment T7 produced in addition to a strong MH_2^{2+} ion at m/z 476.1, a weak MH^+ ion at m/z 950.6, which fragmented towards m/z 837.6 by the loss of Leu.

3.2.2. LC–MS–MS

In order to obtain a more substantial identification of the tryptic fragments several digests (6, 20, 48, 60 h) were investigated by LC–MS–MS. The doubly charged molecular ions were preferably selected as precursor ions because the total fragmentation yields from the doubly charged precursors are appreciably higher than those from their singly charged species. The nomenclature of the sequence ions was adopted from Roepstorff and Fohlmann [33]. As examples the MS–MS spectra of the fragments T4, T7, T22, T25, T29, T30, are shown in Fig. 7. The summarized MS–MS results leading to molecular masses, y"-, a- and b- mode ion series including some i-ions of amino acids corresponding with the predicted sequence of the tryptic fragments are given in Table 1. This table covers the most relevant tryptic fragments with the exception of the low molecular-mass fragments (M_r<400) which were excluded, and the disulfide linked T13/T18 fragment (M_r 3992) and T15 (1272) which were not found. A part of the T13 fragment (Tyr–Val–Asp–Val–Phe) resulting from chymotryptic cleavage, was found in the 6, 20, 48 and 60 h digests with increasing concentration levels over time. Chymotrypsin is known to cleave mainly at the carboxyl side of Trp, Tyr and Phe residues. Also due to the residual chymotryptic activity, the T5 fragment was found only in the 6 h and 20 h samples, but not in the samples obtained after 48 h and 60 h digest. From the T5 fragment the following chymotryptic fragments were identified: Ser–Ile–Asp–Gln–Phe–Leu–Tyr–Phe–Asp–Leu–Ile–Tyr, Ser–Ile–Asp–Gln–Phe–Leu–Tyr–Phe, Ser–Ile–Asp–Gln–Phe and Leu–Tyr–Phe–Asp–Leu–Ile–Tyr and Asp–Leu–Ile–Tyr. Generally, the chymotryptic activity increased after more then 20 h digestion. After 60 h the tryptic fragments T25, T29 and T35 were completely disappeared as a result of the long incubation time which largely exceeds the recommended 2–18 h.

3.2.3. Micro-LC–ES-MS

The 20-h tryptic digest sample was also injected on a laboratory-made micro-LC column packed with LiChrosorb RP18 (600 mm×0.32 mm I.D.) (see Fig. 8). The eluent composition was the same as used for

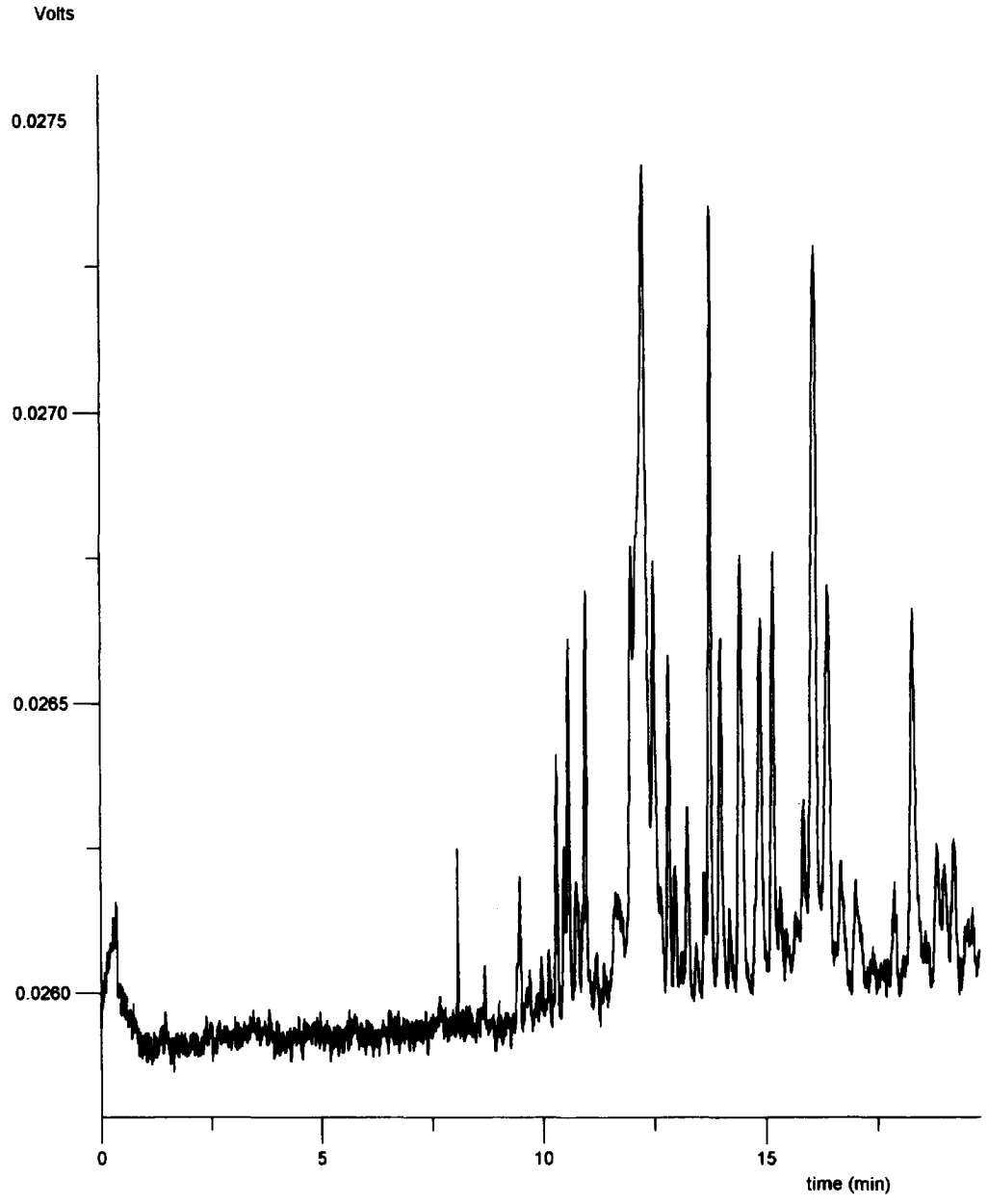

Fig. 5. Electropherogram of a tryptic digest after 20 h incubation. System Prince equipped with a fused-silica column 84 cm L_t×50 μm I.D. and 52.5 cm L_d connected with a UV–Vis detector. Conditions: buffer; 150 m*M* sodium phosphate, pH 2.4; voltage, 25 kV; current, 71 μA; oven temperature, 30°C; UV wavelength, 200 nm; injection, hydrodynamic, 25 mbar×0.2 min (5.5 nl).

the conventional-size LC separation but using a longer eluent programme. The most important advantage of micro-LC proved to be the increased sensitivity – as compared with the conventional-LC system an about 30–40-fold increase in sensitivity was measured applying the same injection volume. In Table 2 the increase in response using micro-LC vs. conventional LC is summarized for some tryptic

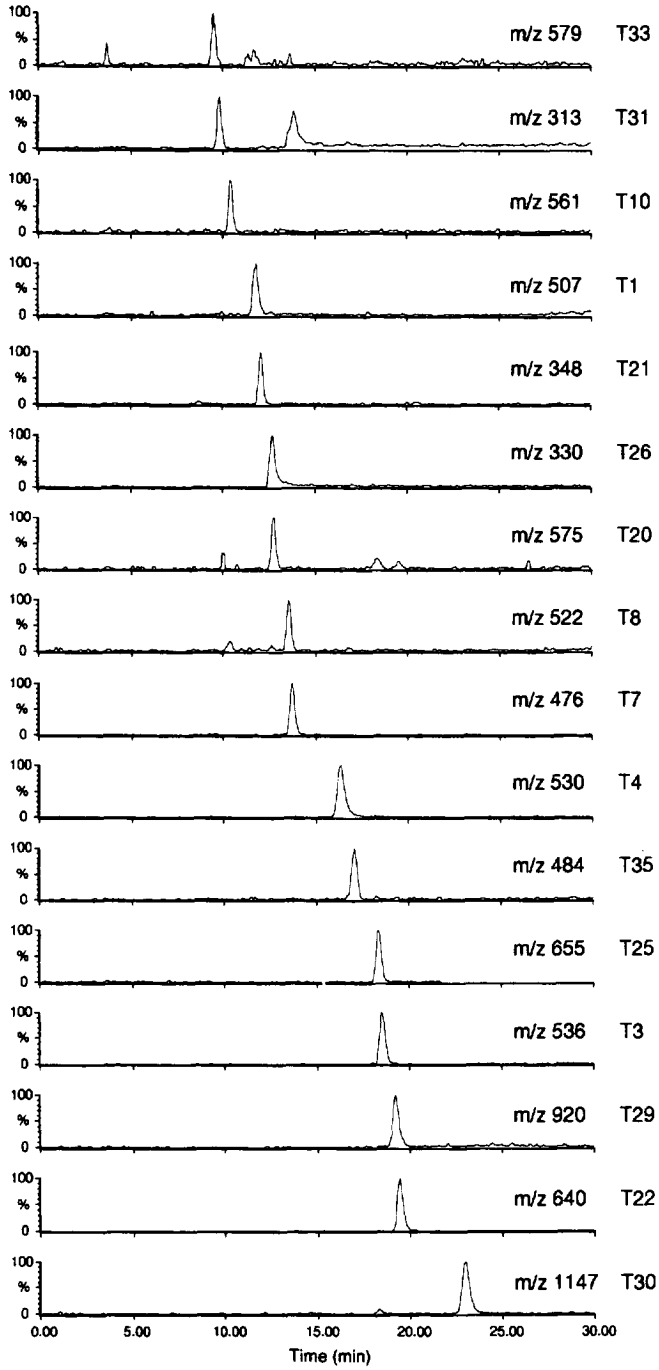

Fig. 6. Mass chromatograms of the tryptic fragments. Column (250 mm×5 mm I.D., packed with 5 μm Lichrosorb RP-18. LC conditions: eluent (A), water–acetonitrile (30:70) containing 0.5% formic acid and (B), water–acetonitrile (98:2) containing 0.5% formic acid, gradient linear from eluent (A) to eluent (B) in 30 mins; flow-rate, 1 ml/min. Post column split: 5%, a 50 μl/min flow was directed towards the ES inlet probe. MS conditions: mass range, *m/z* 300–1200; scan time, 3 s; ES capillary tip voltage, 3.6 kV; cone voltage, 35 V; source temperature, 120°C.

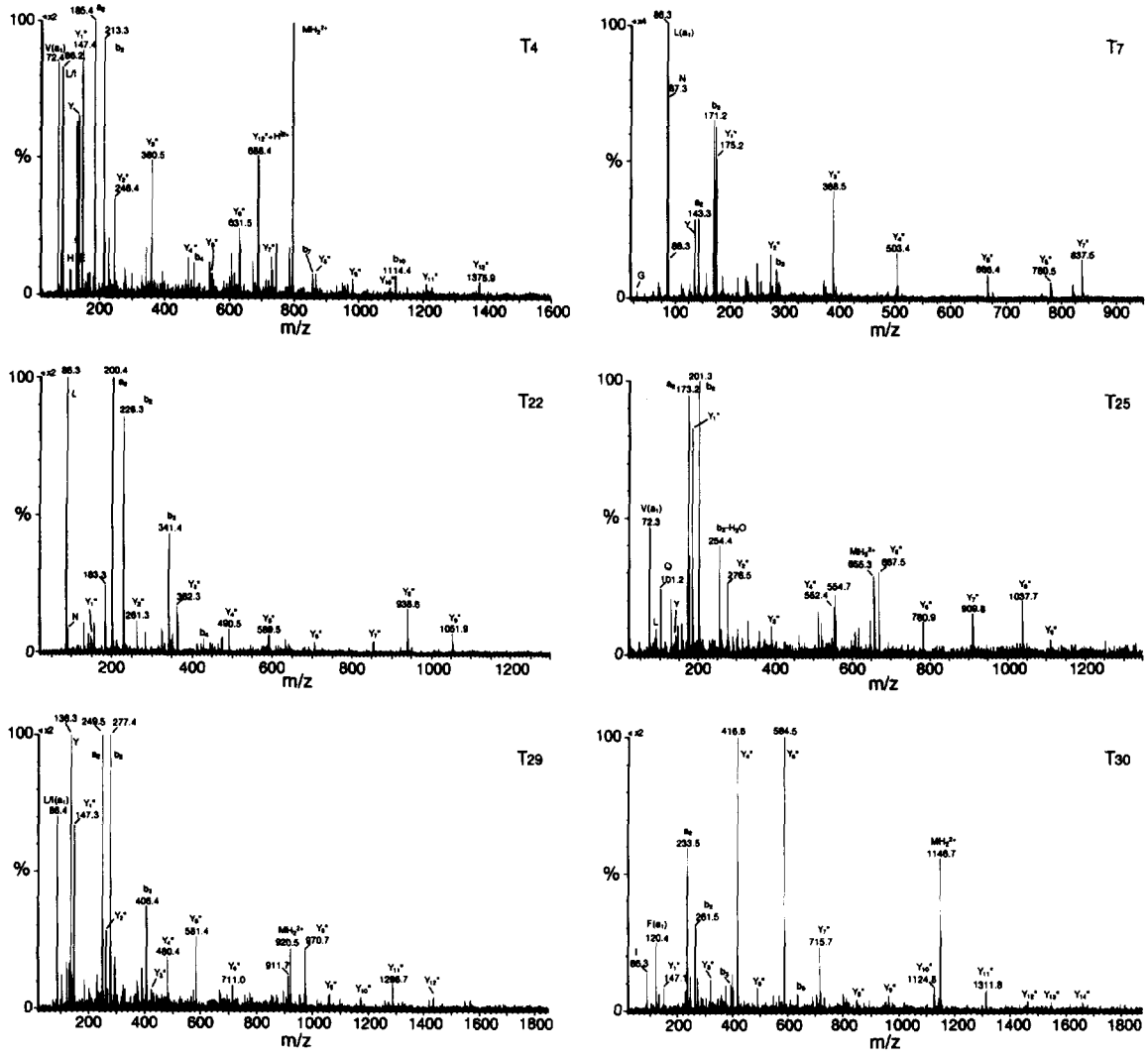

Fig. 7. MS–MS spectra of fragments T4, T7, T22, T25, T29 and T30.

fragments after correction for the injected volume. Because of the different flow-rates between the LC-columns used, it should be emphasized that the sensitivity also depends on the optimisation of the ES-MS source conditions.

Another advantage is the improved separation efficiency. A comparison of the quality of a micro-LC column of 250 mm length with a conventional column with an identical packing and equal length would produce a comparable chromatographical efficiency. The improved permeability (about 1.5 to 2 times) of the micro-LC column enables us, however, to use the larger 600 mm length in relation to the 250 mm length conventional LC column, which ultimately results in an improvement in resolution.

4. Conclusions

ES-MS turned out to be an accurate technique for the mass determination of the high-molecular-mass protein toxin SEB. The presence of a disulfide bridge in SEB could adequately be proven by addition of 2-mercaptoethanol. On-line micro-LC–MS with TSK

Table 1
Observed and theoretical monoisotopic mass values and identified MS–MS fragments of tryptic peptides of SEB

No.	Peptide Sequence	Mass		Identified fragments		
		Observed	Theoretical	y''	a, b	i
T1	ESQPDPKPDELHK	1518.8	1518.7	y''_1–y''_3, y''_6, y''_8, y''_{10}	a_2, a_3, b_2, b_3	E, Q/K, P, D, L, H
T3	FTGLMENMK	1069.6	1069.5	y''_1–y''_8	a_1, a_2, b_2, b_3	F, T, L, M
T4	VLYDDNHVSAINVK	1586.0	1585.8	y''_1–y''_{12}	a_1, a_2, b_2, b_4–b_7	V, L/I, Y, N, H
T7	LGNYDNVR	949.4	949.5	y''_1–y''_7	a_1–a_3, b_2, b_3	L, G, N, Y, D
T8	VEFK	521.4	521.3	y''_1–y''_3	a_1, a_2, b_2, b_3	V, E, F, K
T10	DLADK	560.3	560.3	y''_1–y''_4	a_2, b_2–b_4	K
T20	SITVR	574.4	574.3	y''_1–y''_3	a_1, a_2, b_2–b_4	V
T21	VFEDGK	693.4	693.3	y''_1–y''_5	a_1, a_2, b_2–b_4	V, F, E, K
T22	NLLSFDVQTNK	1278.0	1277.7	y''_1–y''_9	a_1, a_2, b_2–b_4	N, L, D
T25	VTAQELDYLTR	1308.1	1307.7	y''_1–y''_9	a_1, a_2, b_2	V, Q, Y, L
T26	HYLVK	658.4	658.4	y''_1, y''_2	a_1, a_2, b_1–b_3	H, Y, L, V, K
T29	LYEFNNSPYETGYIK	1837.2	1836.9	y''_1–y''_6, y''_8–y''_{12}	a_1, a_2, b_2, b_3	L/I, Y, E, F, N
T30	FIENENSFWYDMMPAPGDK	2290.6	2290.0	y''_1–y''_{14}	a_1, a_2, b_2, b_3, b_5	F, I, E, M
T31	FDQSK	623.5	623.3	y''_1–y''_4	a_1–a_3, b_2–b_4	F, Q/K
T33	MVDSK	578.4	578.3	y''_1–y''_4	a_1, a_2, b_2–b_4	M, V, K
T35	IEVYLTTK	965.5	965.5	y''_1–y''_7	a_1, a_2, b_2	I/L, E, Y

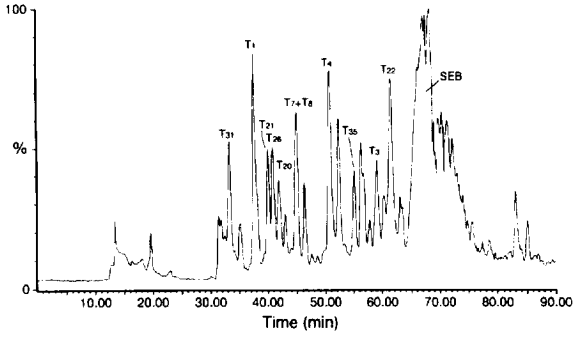

Fig. 8. Micro-LC–ES-MS chromatogram of a 20-h tryptic digest sample. Column (600 mm×0.32 mm I.D., packed with 5 μm Lichrosorb RP-18. Conditions: eluent (A), water–acetonitrile (30:70) containing 0.5% formic acid and (B), water–acetonitrile (98:2) containing 0.5% formic acid, gradient linear from eluent (A) to eluent (B) in 90 min; flow-rate, 3 μl/min. MS conditions: mass range, *m/z* 300–1200; scan time, 3 s; ES capillary tip voltage, 3.6 kV; cone voltage, 35 V; source temperature, 120°C. Injection, 10 μl.

gel Phenyl-5PW column packing allowed for the determination of SEB at levels down to 3 pmol/ml SEB was successfully cleaved by tryptic digestion and by analysing the tryptic fragments by LC–ES-MS–MS amino acid sequence information could be obtained. The use of micro-LC results in an increased sensitivity in sample limited cases. Both the analysis of intact SEB and its tryptic fragments provide sufficient information to unequivocally identify the SEB toxin by mass spectral techniques.

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Table 2
Comparison of the response between micro-LC and conventional-size LC

Tryptic fragment	Micro-LC	Conventional-LC	Gain factor
T4	39 141.606	5025.025	31
T7	23 540.952	3104.928	30
T20	9882.899	1115.795	35
T21	14 705.069	1108.553	49
T31	7841.501	941.927	33

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