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Reversed-phase high-performance liquid chromatography combined with tandem mass spectrometry in studies of a substance P-converting enzyme from human cerebrospinal fluid

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Abstract

The application of reversed-phase HPLC in combination with micro-electrospray mass spectrometry to study a substance P (SP)-hydrolysing endoprotease in human cerebrospinal fluid (hCSF) is reported. The enzyme was partially purified from the hCSF specimens by ion-exchange chromatography and molecular sieving. During the purification procedure the enzyme activity was monitored by measuring the formation of the SP-fragment 1–7 from SP by radioimmunoassay. Regarding its behaviour upon molecular sieve chromatography, the enzyme was suggested to be associated with an apparent molecular mass of around $100 \cdot 10^3$. In subsequent experiments using the partially purified endopeptidase, the hydrolysis of SP was demonstrated by HPLC. The reaction product mixture was resolved in several components including the N-terminal fragments 1–8, 1–7 and 1–6 and the C-terminal fragment 8–11. The identity of these fragments were confirmed by tandem mass spectrometry. It was concluded that the present SP-degrading enzyme is different from those previously identified and purified from hCSF. The applied techniques were proven to be highly efficient for the recovery and identification of the released peptide products.

Keywords: Enzymes; Endoproteases; Peptides; Substance P; Electrospray; Mass spectrometry

1. Introduction

The access of sensitive high-resolution techniques for separation and identification of peptide structures is essential in many areas of biomedical research. In particular, this is of importance in studies of peptides participating in neurotransmission or neuromodulatory processes. The tissue concentrations of these compounds are very low and very sensitive techniques are required for their detection. Also in studies of proteolytic enzymes acting on neuroactive peptides the availability of highly efficient techniques are necessary in order to identify the released products and settle the actual cleavage sites.

Current research on neuropeptide converting or degrading enzymes often uses HPLC [1–3] and electrophoresis [4,5] for the separation of reaction mixtures and identification of peptide products. To improve the accuracy of these techniques they are frequently combined with immunological techniques. For instance, radioimmunoassay (RIA) in conjunc-

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tion with HPLC was applied to identify the enkephalin hexapeptide product following conversion of the opioid peptide dynorphin by a specific endoprotease [6].

However, although the RIA technique is simple to use it is connected with some limitations. One of these results from cross-reaction with similar structures. Another difficulty is that the RIA does not reveal the entire structure of the analyzed peptide. In recent years these problems have been overcome by the introduction of mass spectrometry in peptide research. This technique has been shown to be powerful for structure elucidation of isolated peptides [7,8] or proteolytic products [6,9]. In this work HPLC was applied along with a recently developed micro-electrospray mass spectrometry (micro-ES-MS) to study a substance P degrading enzyme.

Substance P (SP, Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2) is an undecapeptide with a proposed function in neurotransmission and neuromodulation [10]. It has a widespread distribution in the central nervous system (CNS) but is also found in peripheral tissues [11]. SP is believed to have a function in pain transmission and is postulated to facilitate the nociceptive impulses in peripheral sensory afferents. The levels of SP are controlled by proteolytic enzymes. Several proteases known to hydrolyze SP have been described [12]. An endopeptidase has been previously reported (substance P endopeptidase, SPE) in human cerebrospinal fluid (hCSF) [13] with high specificity towards the undecapeptide. The bioactive SP fragments 1–8 (SP_{1-8}) and 1–7 (SP_{1-7}) were identified as major products formed by this enzyme. The enzyme was characterized as a protein with a molecular mass of $40 \cdot 10^3$. It has an optimum for its activity at neutral pH and is dependent on thiol groups and metal ions. The activity of SPE was found to be affected under certain chronic pain conditions but also in morphine tolerant rats (for review see [14]). In addition to SPE there are several other enzymes present in CSF which potently act on SP. For instance, Kato et al. [15] reported a post-proline cleaving dipeptidyl amino-peptidase capable of releasing the SP fragments 3–11 and 5–11, respectively, from the parent peptide. Furthermore, both angiotensin converting enzyme (ACE) and enkephalinase (NEP or 24.11) with an endopeptidase action on SP, have been found in hCSF [12,16]. Therefore, in order to study the metabolism of SP in CSF the availability of efficient techniques for discriminating its fragments is demanded.

In the present work, an SP_{1-7}-generating activity of a new enzyme isolated from hCSF which appears different from SPE was focused upon. The enzyme was partially purified from large amounts of hCSF and was further studied with regard to its cleavage specificity towards SP. For that purpose reversed-phase HPLC [17] and micro-ES-MS were applied. The recently developed micro-ES-MS technique allows analysis and identification of peptide structures in the attomole to femtomole per microliter range [18,19]. Combination of these two techniques allowed SP_{1-8}, SP_{1-7}, SP_{1-6} and SP_{8-11} to be distinguished as major products formed in the enzyme reaction. The presence of the SP_{1-6} fragment indicates that the activity of this enzyme is different from the previously described SPE enzyme activity.

2. Experimental

2.1. Chemicals

Peptides used in this study were purchased from Sigma (St. Louis, MO, USA) except for the N-terminally extended Tyr–SP_{1-7} used for radiolabelling, which was prepared by Dr. G. Lindeberg (Department of Medical Immunology, Uppsala University, Uppsala, Sweden). Iodination of SP_{1-7} was performed according to the chloramine-T procedure as described below. The protease inhibitors amastatin, phosphoramidon and captopril were obtained from Sigma. All other chemicals and solvents were of analytical-reagent grade and purchased from various commercial sources.

2.2. Cerebrospinal fluid

The hCSF was collected by lumbar puncture from patients undergoing investigation for suspected but not confirmed increased intracranial pressure. To obtain a large volume ($\geq$1000 ml) of hCSF for subsequent analysis, samples from a number of different patients were pooled.

2.3. Purification procedure for the hCSF enzyme

The pooled hCSF (1240 ml from 20 different subjects) was applied on an anion-exchange column (DEAE-Sepharose CL-6B, 16 cm×3.2 cm, Pharmacia, Uppsala, Sweden). The column was equilibrated with 0.01 *M* sodium phosphate buffer pH 7.4, containing 0.15 *M* NaCl and 5% ethylene glycol. After washing with three volumes of the same buffer, the column was eluted with 0.01 *M* sodium phosphate buffer pH 7.4, containing 0.5 *M* NaCl and 5% ethylene glycol. By increasing elution time, the eluate was divided into four different fractions, fraction 1 (35 ml), fraction 2 (30 ml), fraction 3 (30 ml) and fraction 4 (27 ml). The absorbance at 280 nm was measured and the approximate protein content in the different fractions were calculated. The two fractions with the highest amount of protein, fractions 3 and 4, were pooled. This material, which also contained high enzyme activity was further fractionated on a gel filtration column (Sephadex G-100, 86 cm×5 cm, Pharmacia). The column was equilibrated with and eluted with 0.04 *M* NH_4HCO_3 containing 5% ethylene glycol. A flow-rate of 60 ml h^{-1} was maintained and fractions of 10 ml were collected and assayed for SP-degrading enzyme using the enzyme assays, based on RIA and SMART system (Pharmacia) as described below. In order to record the distribution of proteins, the UV absorbance at 230 nm was measured. The active fractions were pooled and used in further experiments.

2.4. Enzyme assay

The SP-degrading enzyme was measured in terms of activity. The enzyme activity was monitored by measuring the formation of the fragment SP_{1-7}, from substance P (SP_{1-11}), using a radioimmunoassay (RIA) specific for the product, or a micropreparative chromatography system to identify the product. To avoid interference by other enzymes present in the hCSF, the protease inhibitors amastatin, captopril and phosphoramidon, were added to the incubation mixture. The inhibitors were preincubated with the chromatographic fraction (20 μl), for 15 min at 37°C before the substrate was added. The final concentrations of the inhibitors were 20 μ*M* for amastatin and phosphoramidon and 10 μ*M* for captopril. The substrate was added to give a final concentration of 1.6 μ*M* for assays using the RIA procedure and 40 μ*M* for using the SMART system to detect the products. The reaction mixtures were buffered at pH 7.4 with 50 m*M* sodium phosphate buffer. The incubations were performed at 37°C for 2 h during the purification of the enzyme and for 24 h in the hydrolysis experiments. In experiments using the RIA for product detection, the reactions were terminated by boiling at 100°C for 5 min. The volume was doubled with methanol–0.2 *M* HCl (1:1) and the amount of SP_{1-7} was measured directly by RIA. For product analysis by HPLC the reactions were terminated by freezing at −70°C.

2.5. Radioimmunoassay

A 25 μl aliquot of sample or standard, 100 μl of antisera and 100 μl of iodinated peptide (~4500 cpm) were incubated in triplicate overnight at 4°C. The antisera and the labelled peptide were diluted in assay buffer containing 0.1% gelatin, 0.1% bovine serum albumin, 0.8% NaCl and 0.93% EDTA in 50 m*M* sodium phosphate (pH 7.4). The bound and free peptides were separated by adding 200 μl dextran-coated charcoal. The charcoal suspension contained 750 mg active charcoal and 75 mg dextran T 70 dissolved in 200 ml 50 m*M* sodium phosphate buffer, pH 7.4. After 10 min of incubation, the mixture was centrifuged for 1 min at 9000 *g* in a Beckman Microfuge (Fullerton, CA, USA). A sample (300 μl) of the supernatant was removed and the radioactivity was counted for 4 min in a gamma counter (1470 Wizard, Wallac, Turku, Finland). The detection limit of the RIA was 3.1 fmol/tube and 50% of tracer inhibition was obtained at 17.7 fmol/tube.

2.6. Generation of antibodies

The antiserum for SP_{1-7} was raised in rabbits. SP_{1-7} (1 mg, i.e., 1.1 μmol) and thyroglobulin (5 mg, i.e., 7.0 nmol) were dissolved in 300 μl of 100 m*M* sodium phosphate buffer, pH 7.4 and cooled to 0°C. A 25% aqueous solution of glutaraldehyde was diluted with ice-cold distilled water (1:100, v/v) and 180 μl of this solution (i.e., 4.8 μmol glutaraldehyde) was added dropwise to the SP_{1-7}–thyro-

globulin solution. The reaction mixture was stirred for 30 min at 0°C followed by 24 h at room temperature. By this coupling procedure, the N-terminus of SP_{1-7} was linked to the carrier protein.

After extensive dialysis against 0.9% NaCl, the peptide–thyroglobulin conjugate was ready for injection into rabbits. A 500 μl aliquot of the conjugate, corresponding to 100 μg (i.e., 0.11 μmol) of SP_{1-7}, was emulsified with an equal volume of Freund's complete adjuvant. The animals were injected intracutaneously on their backs at multiple sites. Booster doses in volumes of 250 μl, corresponding to 50 μg (i.e., 0.055 μmol) of SP_{1-7}, were emulsified with an equal volume of Freund's incomplete adjuvant and injected subcutaneously at intervals of 3–4 weeks. The cross-reactivity of the SP_{1-7} antiserum with other SP fragments and related peptides is shown in Table 1. In the RIA the antiserum was used in a final dilution of 1:150 000.

Table 1
Cross reactivity of the substance P_{1-7} antiserum (89-2D) with various substance P (SP) fragments and other related peptides

Peptide	Cross-reactivity (%)
SP_{1-8}	2
SP_{5-11}	5
SP	1.7
β-Endorphin	<1
β-Neurokinin	10.2
a-Neurokinin	13.3

2.7. Labelling of peptide

Tyr–SP_{1-7} was iodinated by the chloramine-T method as follows. The peptide (4 μg), 8 μl sodium phosphate buffer (0.2 *M*), pH 7.4, 0.5 mCi $Na^{125}I$ and 2 μl chloramine-T solution (0.2 mg ml^{-1}) were mixed, and after 1 min the reaction was terminated by adding 200 μl 15% acetonitrile (ACN). For purification of the labelled peptide, a reversed-phase HPLC column (Hichrome C_{18}, 20 cm×4 cm) was used. The HPLC column was eluted with a gradient of ACN (15–45%) containing 0.04% trifluoroacetic acid (TFA).

2.8. Micropreparative HPLC

The micropreparative chromatography SMART system [17] is equipped with built-in detector cells for UV (μPeak Monitor) with variable-wavelength detection. In the present experiments the wavelength was set to 214 nm. The column used was a μRPC C_2/C_{18}, SC 2.1/10 column (particle size 3 μm, 120 Å; 100×2.1 mm). Elution was performed with 0.13% TFA and 60% ACN containing 0.13% TFA at a flow-rate of 240 μl min^{-1}. The gradient was 0–25% for 8 min, 25–75% for 30 min and 75–100% for an additional 5 min. Fractions of 240 μl were collected and dried in a Savant Speed Vac Concentrator (Savant, Hicksville, NY, USA).

2.9. Micro-electrospray mass spectrometry

Electrospray (ES) ionization was performed on a Finnigan TSQ70 triple quadrupole mass spectrometer updated with TSQ700 software (San Jose, CA, USA) that had been fitted with a modified micro-ES source (Vestec Products, PerSeptive Biosystems, Boston, MA, USA) [18,19]. Microspray needles were constructed from fused-silica capillary with I.D. of 50 μm and O.D. of 220 μm. The opposite end of the needle was attached to a stainless-steel zero dead volume fitting with a 0.02 in. (1 in.=2.54 cm) through-hole and the high-voltage connection was made through this union. Specific source conditions were: needle voltage, 3.5 kV; spray current, 0.05–0.2 μA; nozzle voltage, 250 V; repeller voltage, 10 V. The mass spectrometer was scanned from 200–800 mass-to-charge (*m/z*) units in 1 s and all data were acquired in the profile mode. In general, mass measurement accuracies were ±0.4 *m/z* units or less.

Dried samples from the micropreparative HPLC were dissolved in 50 μl of 50% methanol–0.25% acetic acid and analyzed by continuous infusion at a flow-rate of 820 nl min^{-1} using a syringe pump (Stoelting, model 200, Wood Dale, IL, USA).

To provide structure verification of the peptides produced in the enzyme hydrolyses, tandem mass spectrometry (MS–MS) was employed. The first quadrupole of the tandem mass spectrometer was set to transmit the parent ion with an acceptance window of about ±1.5 *m/z* units. The parent ion was dissociated by collision with Xe gas (1.0–1.5 mTorr; 1 Torr=133.322 Pa) in the second quadrupole region and the third quadrupole was scanned over a mass

range from 200–800 m/z units for the appropriate product ions. Mass measurement accuracy for the product ions was typically ±0.2 m/z units.

3. Results

After ion-exchange chromatography and gel filtration chromatography of hCSF, the activity of SP-degrading enzyme were recovered in three different molecular mass regions. One active fraction appeared in the void volume, a second emerged ahead of the albumin peak, whereas a third fraction containing SP-degrading activity eluted in conformity with SPE. In this study the enzyme activity eluting ahead of albumin, fractions 70–76 (Fig. 1), was chosen for further experiments. From calibration runs with standard proteins an apparent molecular mass of around $100 \cdot 10^3$ was calculated for this material. The fractions were pooled and used in another enzyme assay, where reaction mixtures were analyzed with micropreparative HPLC. The major fragments released from SP by the present enzyme co-eluted with synthetic SP_{1-7} and SP_{1-6} (Fig. 2). Minor fragments eluting in conformity with synthetic SP_{1-8} and SP_{8-11} were also observed. In the subsequent micro-ES-MS–MS analysis of the collected RP-HPLC fractions, the identity of the SP metabolites SP_{1-8}, SP_{1-7}, SP_{1-6} and SP_{8-11} was confirmed.

Mass spectra obtained from SP and various synthetic fragments showed either singly or doubly charged protonated molecular species with no or little fragmentation. The N-terminal fragments 1–8, 1–7 and 1–6 showed doubly charged $[M+2H]^{2+}$ species, whereas the C-terminal fragment 8–11 was recorded as singly charged $[M+H]^+$ ions. The collision activated dissociation (CID) analysis of the fractions collected after RP-HPLC confirmed the identity of the SP-metabolites SP_{1-8}, SP_{1-7}, SP_{1-6} and SP_{8-11}. Fig. 3a shows the tandem mass spectrum of SP_{1-8} (m/z 524.3). The CID process resulted primarily in the formation of singly and doubly charged A and B product ions. Fig. 3b–d show the tandem mass spectra for SP_{1-7} (m/z 451.0), SP_{1-6} (m/z 377.6) and SP_{8-11} (m/z 466.3), where similar spectra were obtained with mainly A and B product ions.

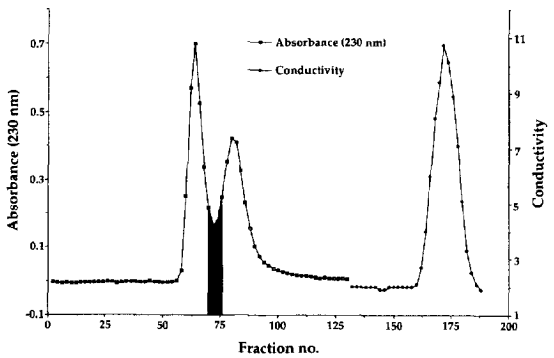

Fig. 1. Protein distribution and conductivity profile after gel filtration chromatography of SP-degrading enzyme from hCSF. Fractions indicating a high activity of SP-degrading enzyme (dark field in figure) were pooled and used for further experiments.

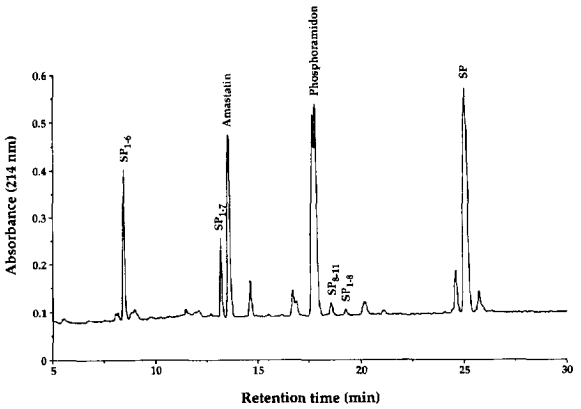

Fig. 2. Reversed-phase chromatography on SMART system of a reaction mixture containing the protease inhibitors amastatin and phosphoramidon, after 24 h incubation of SP with partially purified enzyme.

4. Discussion

In this work a substance P-degrading enzyme was recovered from hCSF, yielding the SP fragments SP_{1-7} and SP_{1-6} as major products. Following gel filtration of CSF material, three different fractions containing enzyme activity capable of releasing SP_{1-7} from SP were observed. According to their behaviour upon gel filtration chromatography these fractions appeared to represent proteins of different molecular sizes. One of these fractions eluting from the gel filtration column as a protein of molecular size around $40 \cdot 10^3$ mass units was the same as the

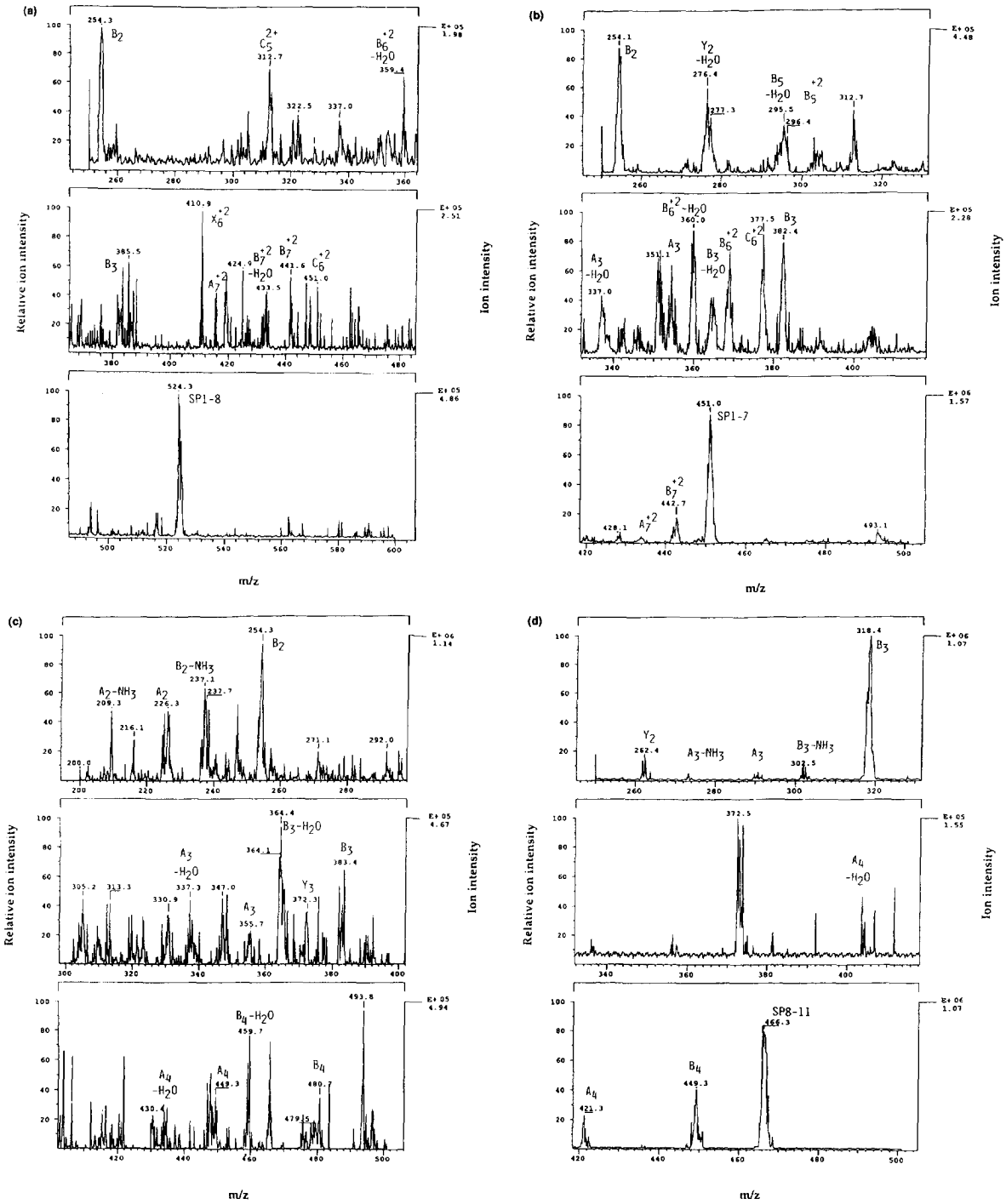

Fig. 3. (a) Continuous infusion of RP-HPLC fraction dissolved in 50 μl 50% methanol–0.25% acetic acid at 820 nl min^{-1} into the micro-electrospray source. The MS–MS spectrum (a) shows the parent ion m/z 524.3 for SP_{1-8} (the doubly-charged ion for SP_{1-8}) and doubly- and singly-charged product ions. The spectrum was obtained from an average of 20 scans (nomenclature according to [21]). (b)–(d) show similar MS–MS spectra of SP_{1-7}, SP_{1-6} and SP_{8-11} respectively.

previously described SPE [13], whereas the other active fractions behaved as proteins of higher molecular sizes. The enzyme eluting in between the void volume (see darkened area in Fig. 1) and albumin was taken for further studies. In conformity with SPE this enzyme showed high potency of releasing both SP_{1-8} and SP_{1-7} from the parent compound. However, in contrast to SPE the present activity also released SP_{1-6} as a major product (see Fig. 2). Whether this enzyme represents a prestage, aggregate or dimer form of SPE is not clear from this study but its pronounced ability to release the SP_{1-6} fragment suggests that this fraction represents a separate enzyme. Its resistance towards the protease inhibitors phosphoramidon and captopril suggest that the enzyme is not identical to NEP or ACE, respectively. Earlier studies have demonstrated the presence of a similar enzyme in the human brain [20].

The micropreparatory HPLC technique used in this study for product separation, is a powerful technique for analysis of peptide digests. The resolved fragments could easily be taken for further studies by MS–MS. The technique also allowed the identification of some protease inhibitors added to the reaction mixture.

Tandem MS capabilities are important in measuring peptides because of the highly structure specific nature of the analysis. The MS–MS spectrum obtained, for example that of SP_{1-7} (Fig. 3b), in this study yielded several product ions that could only originate from the parent molecule m/z 451.0, the doubly charged molecular species of SP_{1-7}. The same product ions produced from other peptides or proteins having a different molecular weight will not be recorded in this analysis, i.e., this analysis does not only identify the mass-to-charge of the measured molecule, but also verifies the primary structure by identifying several sequence-specific fragments. The micro-ES-MS–MS analysis provides an assay specificity of high confidence, with the molecular mass acceptance window of ± 1.5 m/z units and a product ion accuracy of ± 0.2 m/z units. Since other peptides containing the SP_{1-8}, SP_{1-7}, SP_{1-6} or SP_{8-11} sequence have different molecular masses, they are not recorded in this assay.

In conclusion, this work provides evidence for the presence of a SP-degrading enzyme in hCSF capable of releasing the N-terminal fragment SP_{1-7} and SP_{1-6} as major products. With regard to its cleavage specificity, its inhibitory profile and its apparent molecular size, this enzyme appears different from those previously identified in hCSF. The study also confirms the usefulness of RP-HPLC combined with micro-ES-MS for separation and structure identification of proteolytic products.

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