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Purification of substance P endopeptidase (SPE) activity in human spinal cord and subsequent comparative studies with SPE in cerebrospinal fluid and with chymotrypsin

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An enzyme activity capable of hydrolysing the neuroactive undecapeptide substance P (SP) between its Phe^7-Phe^8 residues was purified from the membrane-bound fraction of human spinal cords. The enzyme preparation yielded was compared with a previously described SP-hydrolysing enzyme from human cerebrospinal fluid (CSF) with regard to inhibition profile, protein chemical properties and kinetics. In addition, the results were compared with those of bovine pancreatic chymotrypsin (a serine protease that cleaves the carboxy-terminal side preferentially at hydrophobic amino acids). The SP peptidase activity was extracted from human spinal cords with 1% Triton X-100 in 20 mM Tris–HCl pH 7.8. After ion exchange chromatography (DEAE–Sepharose) where the enzyme activity was separated from other proteins by gradient elution, the pooled enzyme fraction was further purified by molecular sieving (Sephadex G-50). The enzyme activity was finally recovered by HPLC molecular sieving (Superdex® 75 HR 10/30) using a new preparative system, ÄKTA™-purifier, controlled by UNICORN® software version 2.20. © 1998 John Wiley & Sons, Ltd.

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Introduction

Substance P (SP) is a neuroactive peptide which is involved in a wide range of biological functions (Pernow, 1983). It comprises 11 amino acids (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) with its C-terminal amidated. The attention concerning substance P as a neurotransmitter/neuromodulator has particularly been focused on its role in pain transmission, since substance P-like immunoreactivity and peptide release after noxious stimuli as well as the capability to initiate slow depolarization have all localized SP transmission to primary afferent terminals or the superficial region of the dorsal horn of the spinal cord (Otsuka and Yoshioka, 1993). SP is synthesized as part of larger precursor proteins. Following release, SP is likely to be inactivated by peptidases. There are several enzymes that have the capacity to hydrolyse SP, such as dipeptidyl-aminopeptidase IV (DAP-IV) (Kato *et al.,* 1978), angiotensin-converting enzyme (ACE) (Skidgel *et al.,* 1984, 1987) and neutral endopeptidase (NEP) (Oblin *et al.,* 1989; Katayama *et al.,* 1991), although these are known to have a rather broad specificity and thus markedly hydrolyse other peptides. However, peptidases with their specificity restricted to the SP structure have been reported, such as the membrane-bound enzyme isolated from human brain, reported by Lee *et al.* (1981). The enzyme cleaved the SP at its Gln^6-Phe^7, Phe^7-Phe^8 and Phe^8-Gly^9 bonds and was classified as a neutral metallo-endopeptidase with a molecular weight of 40–50 kDa. This enzyme was also reported in rat spinal cord as a substance P-degrading enzyme (SPDE) with a molecular weight of 70 kDa (Probert and Hanley, 1987). Another enzyme with high specificity towards SP was found in human CSF (Nyberg *et al.,* 1984); it cleaved SP between the Phe^7-Phe^8 and Phe^8-Gly^9 residues. This enzyme was named substance P endopeptidase (SPE) and was described as a metal-dependent thiol-sensitive endoprotease. SPE has been characterized in rat spinal cord (Karlsson *et al.,* 1997), where it strongly resembled the above-mentioned CSF enzyme in respect of substrate and cleavage specificity, sensitivity towards group-specific inhibitors and high affinity for the substrate (K_m 5 μM). It is interesting to note that the fragments released by SPE are associated with biological activity (Hasenöhrl *et al.,* 1990; Hall and Stewart, 1991; Larson and Sun, 1993), which suggests that it is operating as a converting enzyme and not solely is an inactivating activity. In the present paper we describe the purification of an SP-hydrolysing activity in human spinal cord capable of

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releasing the SP (1–7) fragment from the parent peptide. This was compared with SPE in human CSF and with chymotrypsin, which in some aspects shares the features of SPE.

Experimental

Materials

Human brain tissues and cerebrospinal fluid were removed at autopsy (15–48 h *post mortem*) and kept frozen at −70 °C until processed. Chymotrypsin and aprotinin were commercially purchased from Boehringer-Mannheim Biochemicals (Mannheim, Germany). Substance P, PHMB, pepstatin A, phosphoramidon and captopril came from Sigma Chemical Co. (St Louis, MO, USA). GR-82334, L-703,606, WIN 51,708 and GR-73632 came from RBI Research Biochemicals International (Natick, MA, USA). All other chemicals and reagents were of analytical reagent grade and purchased from various commercial sources.

Purification of SPE from human spinal cords

All procedures were carried out at 5 °C. Frozen spinal cords (24 g) were thawed and homogenized in 20 mM Tris–HCl buffer pH 7.8 (10 ml g^{-1}) for 1 min. The homogenate was extracted for 2 h during continuous stirring. A subsequent centrifugation was performed in a Beckman J-21 centrifuge at 20 000 *g* for 20 min. The supernatant was collected and the pellet was resuspended in the same Tris buffer as before. The suspension was stirred for 2 h and then centrifuged at 20 000 *g* for 20 min. The resulting pellet was again resuspended, this time with 1% Triton X-100 in 20 mM Tris–HCl buffer pH 7.8. The suspension was stirred for 4 h before additional centrifugation at 20 000 *g* for 20 min. The supernatant was collected and the pellet was discarded. The Triton X-100-containing extract was applied directly on a DEAE–Sepharose CL-6B column (50×250 mm^2) and eluted with a linear gradient of NaCl (0–0.5 M) in 20 mM Tris–HCl buffer pH 7.8 with 0.1% Triton X-100. Fractions of 10 ml were collected at a flow rate of 2.5 ml min^{-1} and assayed for protein content and enzyme activity. The active fractions were pooled and subjected to molecular sieving on a Sephadex G-50 column (5×90 cm^2). Elution was carried out with 0.04 M NH_4HCO_3 at a flow rate of 1 ml min^{-1}. Fractions of 10 ml were collected and those holding activity were pooled and concentrated by lyophilization. The protein was resuspended in 50 mM Na phosphate pH 7.8 and run on HPLC molecular sieving using a Superdex® Peptide HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden). Elution was performed with 0.04 M NH_4HCO_3 at a flow rate of 0.70 ml min^{-1}, collecting fractions of 1.40 ml. Those holding activity were pooled and regarded as the spinal cord enzyme preparation. To manage the last purification step, a new HPLC system was used, ÄKTA™ purifier (Pharmacia Biotech, Uppsala, Sweden). ÄKTA™-purifier is an automated HPLC system scaled for preparative purification and is equipped with a binary high-performance gradient pump with two switch valves. Flow rates can be up to 10 ml min^{-1} and pressures up to 25 MPa. A triple-wavelength UV-vis monitor is used on-line with a conductivity/pH monitor for detection.

Purification of SPE from human cerebrospinal fluid

The procedure was carried out as described previously (Nyberg *et al.*, 1984). Briefly, the CSF enzyme was purified by direct subjection of human CSF to ion exchange chromatography (DEAE–Sepharose) followed by molecular sieving (Sephadex G-100).

Enzyme assay

Enzyme activity was monitored by measuring the formation of SP (1–7) using substance P as substrate (Persson *et al.*, 1992). A radioimmunoassay (RIA) specific for SP (1–7) was utilized for this purpose. Substrate (0.05 μg) was incubated with 30 μl of enzyme and a cocktail of phosphoramidon/captopril (to a concentration of 15 μM) in a final volume of 50 μl at 37 °C for 30 min. The reaction was terminated by adding 1.0 ml of ice-cold methanol–0.1 M HCl (1:1) followed by centrifugation (Eppendorf centrifuge 5417, rotor F45-24-11) at 18 000 *g* for 10 min and evaporation of the supernatant. The RIA was carried out in triplicate and was conducted as described in a previous paper (Persson *et al.*, 1992). Briefly, the antibody was raised in rabbits against the peptide–thyroglobulin conjugate and the iodinated peptide was used as tracer. The ^{125}I-labelled Tyr^7-SP (1–7) and the sample or unlabelled SP (1–7) (standard) were thus incubated with specific antibody against SP (1–7) in 1 ml Eppendorf tubes. The tubes were stored at 5 °C until expected equilibrium had been established (>8 h). Then separation of bound peptide from unbound peptide was done based on the charcoal adsorption technique as described by Persson *et al.*, (1992). The quantity of SP (1–7) was determined by measuring the radioactivity in a gamma counter. The detection limit of the RIA and the cross-reactivity of SP (1–7)-related peptides in the RIA were as described by Persson *et al.*, (1992).

Results

The Triton X-100-soluble fraction ('membrane-bound') of the spinal cord extract was subjected to ion exchange chromatography, which resulted in the recovery of an endopeptidase activity capable of releasing the SP (1–7) fragment from SP. The enzyme activity obtained from this step was purified from contaminants, since over 90% of the protein was removed by this procedure (Table 1). The protease activity was eluted in an area corresponding to 170 ml in the NaCl range 0.33–0.46 M of the gradient. This activity was further purified by molecular sieving on a Sephadex G-50 column, which yielded an activity over 15 times purified from the previous step on protein basis (Table 1). The pooled active fraction from the Sephadex G-50 column was concentrated and applied to the new HPLC system. ÄKTA™-purifier (Pharmacia Biotech), for HPLC molecular sieving. The peak associated with the enzyme activity was considered as the spinal cord preparation. The

Table 1. Purification and recovery of substance P endopeptidase activity from human spinal cord (24 g)

Step	Total protein (mg)	Total activity (pmol min^{-1})	Specific activity (pmol min^{-1} mg^{-1})	Factor
Extract	852	–	–	–
DEAE–Sepharose	80	227	2.83	1
Sephadex G-50	5.2	73.9	14.2	5
Superdex® 75	1.05	151	143	51

Table 2. Effects of various agents on enzyme activity

Agent	Conc. (mM)	SPE human spinal cord (%control)	SPE human cerebrospinal fluid (%control)	Chymotrypsin bovine pancrease (%control)
GR-82334	0.2	42	74	41
L-703,606	0.2	81	53	38
WIN 51,708	0.2	19	37	19
GR-73632	0.2	44	44	28
Aprotinin	1	51	51	25
PHMB	1	16	54	30
Pepstatin A	1	18	86	33
EDTA	5	52	59	40

Abbreviations used are: EDTA, ethylenediamine tetraacetic acid; PHMB, *p*-hydroxymercuribenzoate.

results from this step and the overall purification are shown in Table 1. The specific activity in the crude extract was difficult to estimate owing to the many contaminating protease activities present; however, the ion exchange step performed on the extract did probably at least purify the specific activity of the extract to a factor of 10 in magnitude, giving the overall purification factor to be above 500. In order to characterize the enzymes' substrate recognition sites, four NKl-receptor agonists/antagonists were used: GR-82334, L-703,606, WIN 51,708 and GR-73632 (RBI Research Biochemicals International, Natick, MA, USA). The compounds were incubated together with substrate to test the sensitivity of the enzyme activity. Interestingly, the two SPE preparations differed in their inhibition profile to these agents (Table 2). The peptidergic NKl-receptor agonist GR-73632 potently decreased the SP turnover rate for all three enzymes, while the cerebrospinal fluid SPE activity appeared more insensitive than both the spinal cord SPE and chymotrypsin for the peptide NKl-receptor antagonist GR-82334 (Table 2). The non-peptide (androstanol derivative) NKl-receptor antagonist WIN 51,708 seemed to be robust at inhibiting the peptidase activity of all three enzymes, with a striking similarity between the spinal cord SPE and chymotrypsin. The non-peptidergic NKl-receptor antagonist L-703,606 appeared to be the least potent of the four NKl-receptor substances to inhibit the SPE activity. However, since chymotrypsin was inhibited, this compound distinguishes chymotrypsin from the SPE preparations. The three enzymes were also tested for other agents (Table 2). The inhibition profiles of the peptidases for these agents were similar as far as the metal dependence is concerned, showing moderate but clear inhibition by the chelating agent EDTA. The sensitivity towards thiol-specific inhibitors/activators seemed to be slightly less pronounced for the cerebrospinal fluid SPE preparation. To verify the cleavage of SP by the three enzymes, RP-HPLC separation was conducted as described by Karlsson *et al.* (1997). The major cleavage sites determined were at the Phe^7-Phe^8 bond and at the Phe^8-Gly^9 bond. This observation distinguishes SPE from the cathepsins, which cleave SP exclusively at the Phe^7-Phe^8 bond (Benuck *et al.*, 1977).

Discussion

In the present study we have partially purified an enzyme acting on substance P from the membrane-bound fraction of human spinal cord. A substance P endopeptidase activity was also recovered from human cerebrospinal fluid by the method described previously (Nyberg *et al.*, 1984). Both enzymes were capable of hydrolysing the undecapeptide at the Phe^7-Phe^8 bond. With regard to their molecular sizes and inhibition profile, the two purified enzymes differed from each other. The most obvious divergence between the two SPE activities is reflected by the difference in sensitivity to thiol-blocking agents and pepstatin A. The spinal cord SPE seemed much more sensitive than the CSF enzyme towards PHMB. Regarding the NKl-receptor agonists/antagonists, GR-82334 had a less potent inhibitory action on the CSF SPE, while the opposite was seen for L-703,606 which is a non-peptide compound. The non-peptidergic NKl-receptor compound WIN 51,708 potently inhibits all three enzymes, chymotrypsin included. The tachykinin C-terminal-mimicking compound GR-73632 gives rise to a clear inhibition of the enzyme activity for both the SPE preparations and chymotrypsin, suggesting that the C-terminal sequence which is common for all tachykinins (Phe-X-Gly-Leu-Met-NH_2) is important for recognition. It is true that SPE shares some features with, for instance, cathepsin D, but as previously shown (Karlsson *et al.*, 1997), several properties, such as molecular weight, cleavage specificity, kinetics and stability at neutral pH, are distinct from those of the cathepsins. At neutral pH in this study, K_m was determined by means of SP turnover to be 2, 5 and 12 μM for the spinal cord peptidase, the CSF enzyme and chymotrypsin respectively. This high affinity for the substrate is of the same order of magnitude as previously reported for an SPE-like activity (Nyberg *et al.*, 1984; Karlsson *et al.*, 1997). Another substance P-degrading enzyme (designated SPDE), which resembles the present preparations, is a human brain endopeptidase (Lee *et al.*, 1981), which also has been reported to be present in rat spinal cord (Probert and Hanley, 1987). The present SPE may have a regulatory function on the substance P level in the spinal cord area and thereby control the action of the peptide in, for instance, pain-processing pathways. Previous studies have shown that the activity of an SPE-like enzyme in rat CSF is affected during chronic pain condition (Persson *et al.*, 1995). In human CSF the activity of SPE was seen to be affected in certain states of chronic pain (Lindh *et al.*, 1996). Also during conditions of opioid tolerance the activity of SPE in rat CSF seemed to be affected (Persson *et al.*,

1995). It is clear that both enzymes purified in this study could be of importance for the control of the substance P level in the spinal cord and thus affect the pain signal in this area.

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