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CHARACTERIZATION OF SUBSTANCE P(1-7) AND (1-8) GENERATING ENZYME IN HUMAN CEREBROSPINAL FLUID

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A substance P-hydrolyzing endopeptidase has been purified from a large quantity of human cerebrospinal fluid by ion exchange chromatography (DEAE-Sepharose CL-6B) and molecular sieving (Sephadex G-100 and Sephacryl S-200). The purification was monitored by measuring the conversion of synthetic substance P using a radioimmunoassay specific for its (1-7) fragment. The enzyme has an apparent molecular weight of 43,000. It cleaves predominantly at the Phe^{7}-Phe^{8} and Phe^{8}-Gly^{9} bonds but gives no or negligible conversion of the other tachykinins, neuromedin K and L (substance K).

In view of its proposed function as a neurotransmitter and its wide-spread occurrence within the central nervous system the inactivation mechanism of substance P (SP; Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) is of interest. Several brain enzymes are capable of hydrolyzing the undecapeptide (1-6). Prolyl endopeptidases sequentially cleaving substance P at Pro^{2}-Lys^{3} and Pro^{4}-Gln^{5} have been purified from rabbit (1) and human brain (2). A post-proline cleaving enzyme (PPCE) from bovine brain known to catalyze the deamidation of thyroliberin has been shown to hydrolyze substance P at Pro^{4}-Gln^{5} (3). Benuck and Marks reported a partially purified cytosolic enzyme from rat brain, which cleaves the peptide at the Gln^{6}-Phe^{7}, Phe^{7}-Phe^{8} and Gly^{9}-Leu^{10} bonds (4). A membrane bound enzyme with similar specificity has also been purified from human brain (5). Cleavage of substance P at Phe^{7}-Phe^{8} has also been observed with endopeptidases from calf brain (6) and bovine hypothalamus (7).

Several studies of substance P-like immunoactivity in human cerebrospinal fluid (CSF) (8-10) and plasma (11) in neurologic and psychiatric patients have been reported. In a recent paper we described an electrophoretic characterization of substance P-like activity elevated in CSF of psychiatric patients (10). One component reacted in a radioimmunoassay specific for the substance P(1-7) fragment. It appeared to us that enzymatic conversion of SP might occur in CSF. Pilot experiments confirmed this hypothesis. The present report

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describes the purification and characterization of an SP(1-7) and (1-8) generating enzyme in human CSF.

MATERIALS AND METHODS

Materials. The CSF was obtained by lumbar puncture from neurologic patients undergoing investigation for suspected increased intracranial pressure. All samples used in the study had essentially normal protein content. The chromatographic media (DEAE-Sepharose CL-6B, Sephadex G-100 and Sephacryl S-200) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All reference peptides were obtained from Peninsula Laboratories, San Carlos, CA, USA, except for the substance P fragments (1-6), (1-7), (8-11) and (1-8), which were prepared by Dr. J.M. Stewart, Department of Biochemistry, University of Colorado, Denver, CO, USA. Phosphoramidon and dithiothreitol (DTT) were from Sigma Chemical Company and captopril from Squibb & Sons Inc, Princeton, N.J.. All other chemicals and solvents were of reagent grade from usual sources.

Enzyme purification. The CSF material (about 900 ml collected from 20 different subjects) was thawed and directly applied to a DEAE-Sepharose CL-6B column (3.2 cm x 10 cm), previously equilibrated with "artificial CSF"(Na^+ 151.1mM; K^+ 2.6mM; Mg^{2+} 0.9mM; Ca^{2+} 1.3mM; Cl^- 122.7mM; HCO_3^- 21.0mM; HPO_4^{2-} 2.5mM, adjusted to pH 7.4). After washing with two volumes of artificial CSF the enzyme, initially adsorbed to the column, was eluted with 20 mM Tris-HCl, pH 7.8, containing 0.5 M KCl. The eluate which was received in a volume of 50 ml was subsequently chromatographed on a Sephadex G-100 column (5 cm x 90 cm) using 0.04 M NH_4HCO_3 as eluent buffer. Fractions of 20 ml were collected at a flow rate of 80 ml/h and aliquots were withdrawn and analyzed for enzyme activity. The active fractions were pooled and lyophilized. Further purification was achieved by fractionation on Sephacryl S-200. The column (2 cm x 60 cm) was equilibrated with 0.04 M NH_4HCO_3 and operated at a flow rate of 20 ml/h. Fractions of 4 ml were collected and analyzed as before. Both columns used for molecular sieving were calibrated with proteins of known molecular weight.

Enzyme assay. All incubations were performed in Eppendorf tubes at 37 °C. During the purification procedure and in routine experiments, as well, the enzyme activity was monitored by RIA measurements of SP(1-7) formation from synthetic SP. Lyophilized aliquots of the enzyme fractions were redissolved and incubated with 40 pmole substance P in 50 ul of 20 mM Tris-HCl buffer, pH 7.8, for different time periods. The reaction was terminated by boiling for 1-2 minutes before the addition of 1 ml methanol and removal of protein by centrifugation (Beckman Microfuge B, 1 min). The sample was subsequently evaporated (Savant Vac centrifuge) and analyzed by radioimmunoassay.

Product identification by HPLC. The HPLC experiments were performed in a Hewlett and Packard 1084B liquid chromatograph with a Waters uBondapak C_{18} column (4.5 x 250 mm). Products of substance P hydrolysis were resolved with a 40 min linear gradient of methanol (10-40%) in 0.04% trifluoroacetic acid (TFA) followed by isocratic elution for 10 minutes with 40% methanol/0.04% TFA. Fractions of 1 ml were collected at a flow rate of 1 ml/min, taken to dryness and analyzed by radioimmunoassay.

The HPLC system was also used to study the substance P hydrolysis at nmole levels in the absence and presence of enzyme inhibitors. In these experiments the incubation conditions were those described above but using 3-10 nmole substrate in each vial. Products were identified by uv-recording at 220 nm using a Varian UV-50 detector. Amino acid analysis was performed on a LKB Alpha Plus analyzer following hydrolysis in 6 M HCl at 110° for 24 h.

Radioimmunoassay. Radioimmunoassay procedures for SP and SP(1-7) were conducted as described previously (10). The I^{125}-labelled Tyr^8-SP analogue and ^3H-labelled SP(1-7) were used as tracers, respectively. Antisera had been

raised against the peptide-thyroglobulin conjugates in rabbits. The SP antiserum crossreacted with the SP(5-11) fragment to about 60% and with the N-terminal fragments SP(1-7) and (1-8) less than 0.1%. Crossreaction of the SP(1-7) antiserum for the SP(1-8) fragment was about 1%. Its crossreactivity with SP and other SP fragments was less than 0.2%.

RESULTS

The major part of the SP(1-7) generating activity present in crude CSF was initially adsorbed to the DEAE-Sepharose column. In the subsequent Sephadex G-100 fractionation the enzyme activity was recovered in regions representing different molecular sizes. Two principal peaks eluted ahead of and later than human serum albumin (MW 69,000). In order to obtain an improved resolution of these fractions further separation was performed on a Sephacryl S-200 column (Fig. 1). As shown in the figure a major peak of substance P(1-7) generating activity appeared close to the region of low molecular weight proteins, whereas three minor peaks were observed in regions corresponding to proteins of higher molecular weight. A similar elution profile was obtained when the fractions were analyzed for SP degrading activity. Calibration of the Sephacryl gel column with protein standards allowed the estimation of approximate molecular weights of the active fractions. Thus, the enzyme activity eluted as

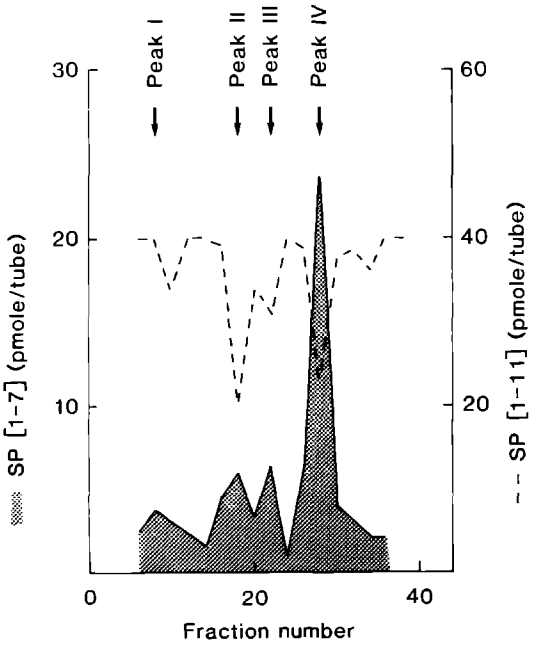

Fig. 1 Elution profile of substance P(1-7) generating activity after Sephacryl S-200 chromatography.The sample originated from 100 ml CSF and was collected from the Sephadex G-100 column. Aliquots of 0.2 ml were lyophilized and assayed for enzyme activity. The formation of SP(1-7) from 40 pmole SP after 10 min incubation at 37°C was determined by radioimmunoassay. Levels of unaltered SP are also inserted.

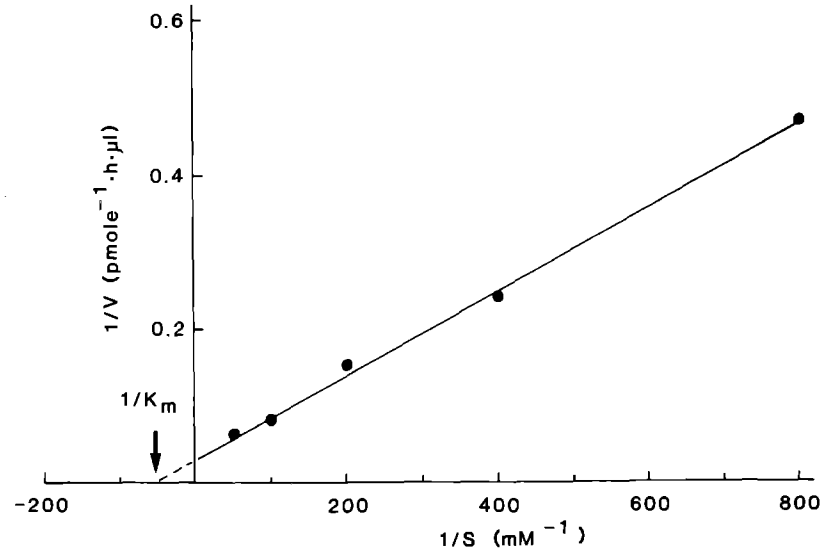

Fig. 2 Lineweaver-Burk plot for the substance P(1-7) generating activity in human CSF.The rate of SP(1-7) formation was measured with 5 concentrations of substance P (1-20 uM). The peptide was incubated at 37°C for 15 min. with 0.5 ug purified enzyme and the extent of SP(1-7) formation was determined by radioimmunoassay.

proteins with apparent MW of > 160,000 (peak I), 140,000 (peak II) 90,000 (peak III) and 43,000 (peak IV). Formation of the SP(1-7) fragment was verified with HPLC analysis. The protein yield in peak IV was about 1 ug/ml CSF corresponding to a 700-fold purification in terms of protein content. The specific activity of peak IV was 3.7 nmol SP(1-7) min^{-1} $(mg\ protein)^{-1}$.

Fig. 2 illustrates the Lineweaver-Burk plot for the hydrolysis of substance P by the purified CSF enzyme (Peak IV, Fig. 1). The K_m value calculated by linear regression is about 19 uM. The peak IV activity was selected for further characterization. In the following experiments degradations were run with larger amount of substrate, and the products quantified by analytical HPLC. The fragments SP(1-7), SP(8-11), SP(1-8) and SP(9-11) were identified as the major products (Fig. 3). No or negligible conversion of SP to its (1-6) fragment was observed. A quantitative study of the fragmentation indicated that SP(1-7), SP(1-8) and SP(8-11) were generated in approximately equimolar concentrations (Table I). The presence of these fragments was confirmed by amino acid analysis.

The substrate specificity of the enzymatic activity was investigated. The N-terminal (3-11) and (5-11) SP-fragments generated the SP(8-11) sequence with somewhat reduced rate when compared to that observed with parent substance, whereas no conversion was observed for the (1-7) and (1-8) fragments. The

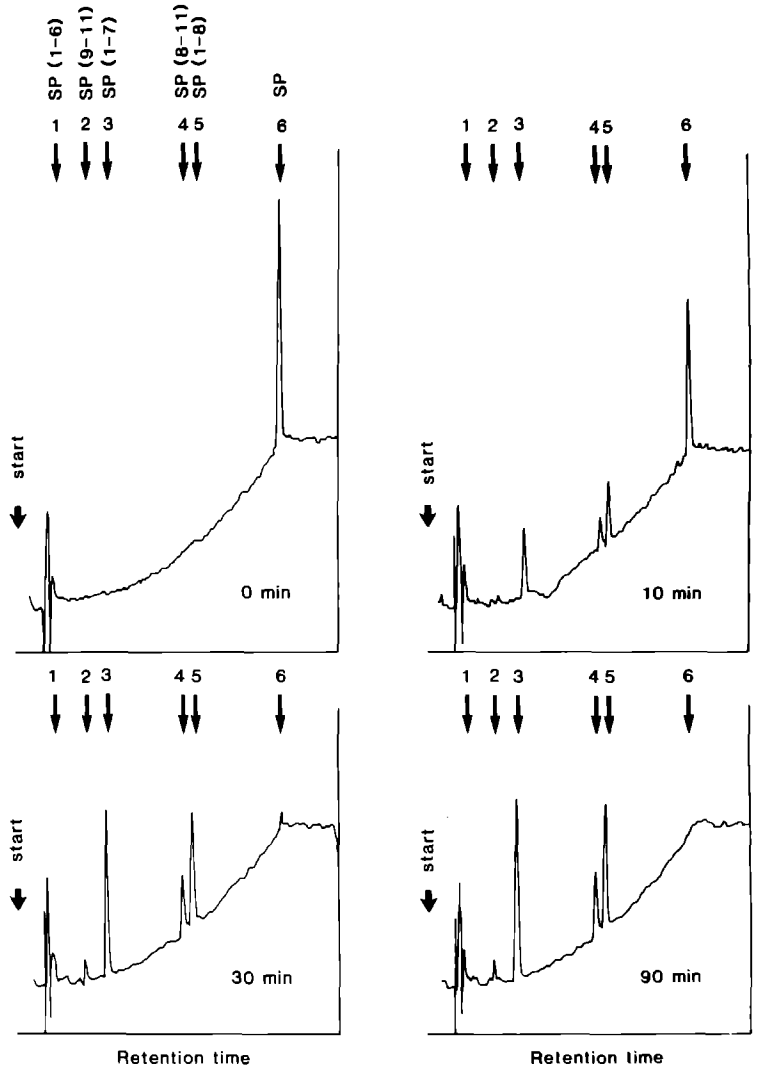

Fig. 3 HPLC analysis of enzyme digests of substance P collected after increasing incubation time. Degradation was performed in 50 ul at 37°C with about 6 nmoles SP and 5 ug of the purified enzyme and incubation mixtures were removed at different times and analyzed by HPLC. Products were eluted from the column by a linear gradient of methanol (10-40%) containing 0.04% TFA followed by isocratic elution with 40% methanol/0.04% TFA.

naturally occurring substance P related peptides, neuromedin K and neuromedin L, remained unaltered and similar results were obtained for two other phenylalanine-containing peptides, [Leu]-enkephalin and β-casomorphin (not shown). The effects of various peptidase inhibitors on the conversion of substance P to its (1-7) fragment have also been studied. As shown in Table II the addition of captopril (inhibitor of angiotensin converting enzyme) and phosphoramidon (inhibitor of enkephalinase) did not inhibit the formation of the SP fragments. EDTA and dithiothreitol gave partial inhibition.

TABLE 1. Time-course of substance P conversion by the purified CSF enzyme

Time (min)	SP	SP(1-7)	SP(8-11)	SP(1-8)	Total	Molar ratio SP(1-7)/SP(1-8)
			(nanomoles)			
0	4.99	0	0	0	4.99	-
10	2.53	1.22	1.24	0.95	4.70	1.28
30	0.27	2.70	2.64	1.93	4.90	1.40
90	0	3.14	3.42	2.18	5.32	1.44

The degradation experiments were identical with those described in Fig. 3. The reaction mixtures were separated by HPLC and products quantified by calibration runs of the authentic peptides at different concentrations. "Total" represents the sum of SP, SP(1-7) and SP(1-8).

DISCUSSION

The purification procedure described in this report yielded an enzyme fraction (peak IV, Fig. 1) with properties similar to those of the substance P hydrolyzing endopeptidase reported by Lee et al. (5). Thus, the CSF enzyme was capable of hydrolyzing the neuropeptide at Phe^{7}-Phe^{8} and Phe^{8}-Gly^{9}. It was inhibited by EDTA and dithiothreitol but retained its activity in the presence of captopril and phosphoramidon. Modifications at the N-terminal region of substance P appeared to reduce the rate of conversion in agreement with findings reported for the brain enzyme. The estimated K_m and molecular weight of peak IV are also in agreement with those observed for the brain enzyme (5). The molecular heterogeneity of the CSF enzyme observed in the gel filtration experiments (Fig. 1) probably results from a progressive aggregation during the purification procedure. This would also be compatible with the apparent molecular weights being approximate multiples of 43,000.

Clearly differentiating enzymatic activity from CSF and brain, however, is the absence of formation of SP(1-6) with the CSF enzyme. The fragment was not

TABLE II. Effects of peptidase inhibitors on the conversion of substance P by the enzyme purified from human cerebrospinal fluid

Inhibitor	SP	SP(1-7)	SP(8-11)	SP(1-8)
		(nanomoles)		
None	< 0.2	1.35	1.35	1.14
Captopril (10 uM)	< 0.2	1.40	1.31	0.91
Phosphoramidon (1 mM)	< 0.2	1.33	1.48	1.03
EDTA (5mM)	1.58	0.66	0.64	0.27
Dithiothreitol (5 mM)	1.04	0.83	0.90	0.58

Incubations were performed at 37°C for 15 min. as described in the text with 3 nmoles SP and 5 ug purified enzyme in each vial. HPLC separated products and unaltered substrate were quantified by calibration runs with the peptide standards at various concentrations.

detected by HPLC analysis whereas on the other hand the (1-7) and (1-8) fragments were recovered in stoichiometric amounts (Table I). A possible explanation for the difference is an impurity in the activity described by Lee et al. (5). Actually, Fig 6 of their paper indicates that after a final purification step using electrophoresis, the formation of SP(1-6) is reduced relative to SP(1-7) and (1-8).

The presence of considerable quantities of neuropeptide degradation enzymes in human CSF has several implications. It is certainly going to influence the levels of SP measured in CSF (8-11). The CSF used here was from patients with suspected and in some cases verified neurologic disease. It remains to be studied whether differences exist in various clinical states and whether such differences have pathogenetic consequences.

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