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Purification and Characterisation of a Membrane-Bound Substance-P-Degrading Enzyme from Human Brain

Chi-Ming LEE, Bengt E. B. SANDBERG, Michael R. HANLEY, and Leslie L. IVERSEN

Medical Research Council Neurochemical Pharmacology Unit, Cambridge

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A membrane-bound enzyme which degrades substance P (an undecapeptide) has been purified from human brain. The properties of this enzyme suggest that it may be involved in the physiological inactivation of the peptide by neural tissues. Enzyme activity was extracted from a membrane fraction of human diencephalon with a non-ionic detergent, Brij 35, and activity was monitored by measuring the disappearance of added substance P using radioimmunoassay, bioassay or radiochemical assay. The enzyme was purified about 1000-fold by chromatography on DEAE-cellulose, hydroxyapatite and Sephadex gel filtration columns. To identify the cleavage sites in substance P, the peptide was incubated with the purified enzyme and the breakdown products were separated by reverse-phase high-performance liquid chromatography and identified by amino acid analysis. The results suggested that the enzyme preparation was functionally homogeneous and it cleaved substance P between Gln^6-Phe^7, Phe^7-Phe^8 and Phe^8-Gly^9, with no exopeptidase action. The enzyme had a pH optimum in the range 7–9 and was strongly inhibited by metal-chelating agents, but not affected by most other peptidase inhibitors; it can thus be classified as a neutral metallo-endopeptidase. The enzyme was thermolabile and had a molecular weight of 40000–50000 as estimated by gel filtration, density-gradient ultracentrifugation and sodium dodecylsulphate gel electrophoresis. The highly purified substance-P-degrading enzyme could be distinguished from previously described peptidases for which substance P is a substrate. An important feature was that substance P was the preferred substrate among various other neuropeptides tested.

Substance P, an undecapeptide (Fig. 1), is a putative neurotransmitter [1,2]. Its depolarizing action on neurons [3,4], axonal transport [5], vesicular localisation [6] and Ca^{2+}-dependent release from brain slices [7] and nerve endings [8,9] all support this hypothesis.

The mechanism involved in the inactivation of substance P after its synaptic release is unknown, although a number of enzymes capable of degrading this peptide have been reported to exist in brain [10–12]. These include both cytosolic [12–15] and membrane-bound [13,14,16–18] substance-P-degrading enzyme activities. A cytosolic enzyme has been partially purified from rat brain and shown to release phenylalanine and leucine preferentially from substance P, suggesting cleavage at two or more internal peptide bonds (Gln^6-Phe^7 or Phe^7-Phe^8 and Gly^9-Leu^{10}) [19]. Akopyan and coworkers [20] purified a neutral endopeptidase from bovine hypothalamus which degraded luteinizing-hormone-releasing hormone (luliberin) and somatostatin and also cleaved substance P, probably between Gln^6-Phe^7 and Phe^7-Phe^8. In addition, a prolyl endopeptidase has been purified from rabbit brain and shown to cleave substance P between Pro^2-Lys^3 and Pro^4-Gln^5, although this enzyme hydrolyses other proline-containing peptides such as angiotensin II, bradykinin and neurotensin more rapidly than substance P [21]. A thyrotropin-releasing hormone (thyroliberin) deamidating enzyme purified from bovine brain also hydrolyses prolyl bonds in luliberin, angiotensin II, neurotensin and substance P [22,23]. A dipeptidylaminopeptidase B purified from human brain also liberated N-terminal Arg-Pro and subsequently Lys-Pro from substance P [24]. Furthermore, purified lysosomal cathepsin D from calf brain cleaves substance P between Phe^7-Phe^8 [25].

Although the importance of the aforementioned cytosolic and lysosomal enzymes in the regulation of concentration of substance P in the brain is unknown, their intracellular localization makes it unlikely that they can participate directly in inactivating extracellular substance P in the synaptic cleft. These enzymes would require an uptake of substance P into the cell before they could act on it, and to date there is no evidence for an active re-uptake process for substance P [7,26,27]. By analogy with acetylcholinesterase, a plasma membrane enzyme would be more likely to be involved in inactivating substance P at the synapse. We now report the first purification and partial characterisation of a membrane-bound substance-P-inactivating enzyme from human brain.

Abbreviations. Substance P, the undecapeptide Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-$MetNH_2$; [^{3}H]substance P, [*Phe*^{8}-^{3}H]substance P; Ala-Ala-Phe-Nan, alanyl-alanyl-phenylalanyl *p*-nitroanilide; Fao-Gly-$LeuNH_2$, 3-(2-furylacryloyl)-glycyl-L-leucine amide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

Enzymes. Substance-P-degrading enzyme (EC 3.4.24.–); angiotensin-converting enzyme or dipeptidyl carboxypeptidase (EC 3.4.15.1); α-chymotrypsin (EC 3.4.21.1); thermolysin (EC 3.4.24.4).

Trivial Names. Luliberin, luteinizing-hormone-releasing hormone; thyroliberin, thyrotropin-releasing hormone.

Arg^1-Pro^2-Lys^3-Pro^4-Gln^5-Gln^6-Phe^7-Phe^8-Gly^9-Leu^{10}-$MetNH_2^{11}$

Fig. 1. *Chemical structure of substance P*

MATERIALS AND METHODS

Materials

Substance P, [Tyr^8]substance P, substance P free acid, the shorter fragments of substance P, eledoisin, eledoisin-related peptide, physalaemin, bombesin, luliberin, thyroliberin, angiotensin I, bradykinin, vasopressin and neurotensin were obtained from Peninsula Laboratories, Inc. (San Carlos, CA, USA). Substance P and related peptides were analysed by high-performance liquid chromatography and were found to be >95% pure. Somatostatin, 3-(2-furylacryloyl)-glycyl-L-leucine amide (Fao-Gly-LeuNH_2) and alanyl-alanyl-phenylalanine *p*-nitroanilide (Ala-Ala-Phe-Nan) were purchased from Bachem (Bubendorf, Switzerland). α-Chymotrypsin (three-times crystallised) and thermolysin were from Worthington Biochemical Corp. (Freehold, NJ, USA) and Sigma Chemical Co. (Poole, Dorset, UK), respectively. Synthetic analogues of substance P were prepared in this laboratory by solid-phase synthesis [28]. Phyllomedusin was a gift from Dr R. de Castiglione (Farmitalia Carlo Erba). Teprotide (2-D-methyl-3-mercapto-propanoyl-L-proline) and captopril (<Gln-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) were gifts from Dr Z. P. Horovitz (Squibb Institute, NJ, USA). The enkephalin-related dipeptides, tripeptides and tetrapeptides were kindly supplied by Dr S. Wilkinson (Wellcome Laboratories). Brij 35 (polyoxyethylene dodecyl ether) was a product of BDH Chemicals (Poole). Ultrapure sucrose for ultracentrifugation was obtained from Schwarz/Mann (Orangeburg, NY, USA). DEAE-cellulose was purchased from Merck and hydroxyapatite from Bio-Rad. Sephadex G-100 and Sephadex G-200 were obtained from Pharmacia (Uppsala, Sweden). Far-ultraviolet grade acetonitrile was supplied by Fisons (Loughborough, Leics, UK). [*Phe*^{8}-^{3}H]Substance P was prepared and characterised as previously reported [29]. The labelled peptide was at least 90% pure and was identical to substance P in biological activity and chromatographic behaviour. Pepstatin, diisopropylfluorophosphate and phosphoramidon were gifts from Dr A. J. Barrett (Strangeways Research Laboratory, Cambridge). Benzamidine was obtained from Calbiochem-Behring Corp. (Bishops Stortford, Herts, UK). Trasylol and bacitracin were purchased from Sigma Chemical Co. (Poole, UK).

Measurement of Substance-P-Degrading Activity

Radioimmunoassay. Throughout the purification procedures, the activity of substance-P-degrading enzyme was monitored by measuring the rate of disappearance of added substance P by radioimmunoassay [14]. All enzyme assays were performed in duplicate at 37 °C unless otherwise indicated. Enzyme preparations were incubated in 50 mM potassium phosphate buffer (pH 7.5) in a final volume of 400 µl. The reaction was started by adding synthetic substance P at a final concentration of 0.2 µM and stopped at appropriate time intervals by placing the tubes in a boiling water bath for 10 min. The denatured proteins in the reaction mixture were sedimented by centrifugation at 3000 × *g* for 10 min. Duplicate aliquots (10–20 µl) of the clear supernatant were then assayed for substance P immunoreactivity, using a C-terminus sequence-directed guinea pig antiserum and in some cases also an N-terminus sequence-directed rabbit antiserum [30].

Bioassay. An alternative and complementary procedure for monitoring the activity of substance-P-degrading enzyme involved measuring the remaining substance P after enzymatic digestion by bioassay, using an isolated guinea pig ileum preparation. A modified Krebs bicarbonate solution (127 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4 and 10 mM glucose) containing 1 µM atropine sulphate, 10 µM tryptamine hydrochloride and 1 µM mepyramine maleate was used. A segment (1–1.5 cm) of the distal ileum was suspended in a 10-ml organ bath (B. Braun, Melsungen, FRG) which was maintained at 30 °C and gassed with a mixture of O_2/CO_2 (95:5). After an equilibration period of 45 min, the contractions induced by substance P (applied at 3 min intervals, contact time less than 40 s) were recorded isotonically under a resting load of 0.5–0.8 g, using an Ealing isotonic transducer.

Radiochemical Assay. After the cleavage pattern of substance P by the purified substance-P-degrading enzyme had been established, a radiochemical method was developed for assaying the purified enzyme. Aliquots of the purified enzyme were incubated with 380 nM [*Phe*^{8}-^{3}H]substance P (spec. act. 23 Ci/mmol) at 37 °C for 10–30 min with various test substances at the indicated concentrations. Substance P and its degradation products were separated by thin-layer chromatography using silica gel plates (LQD, Quantum Industries) and developed in ethyl acetate/pyridine/acetic acid/water (5:5:1:3, v/v) for 2.5 h at room temperature. After drying, the separation was checked with a thin-layer chromatography radiochromatogram scanner (Berthold, FRG) and then the plates were sprayed with 0.1% fluorescamine in acetone and the positions of markers visualised under ultraviolet light. R_F values of markers were Phe = 0.74, substance P = 0.51, fragment 1–8 = 0.32, 8–11 = 0.87 and 7–11 = 0.96. The radioactive peak corresponding to unchanged [^{3}H]substance P was scraped off and measured by liquid scintillation spectrometry, after addition of 4 ml ethoxyethanol and 10 ml toluene scintillant. Enzyme activity was calculated as the rate of disappearance of unchanged [^{3}H]substance P.

Purification of Particulate Substance-P-Degrading Enzyme from Human Diencephalon

Solubilization. Frozen diencephalon was pooled from seven post-mortem human brains obtained at autopsy from patients dying without psychiatric or neurological illness (at Addenbrooke's Hospital, Cambridge), with post-mortem delays of 24–48 h, using standard procedures described elsewhere [31]. The frozen tissue was cut into small pieces and homogenised in 2.5 l 20 mM Tris/HCl buffer, pH 7.8, in a Waring blender for 10 min at 4 °C and all subsequent steps were carried out at this temperature. After sieving through two layers of nylon cloth (300-µm mesh), the homogenate was centrifuged at 27000 × *g* for 90 min in a Sorval RC-5 centrifuge. The precipitate was re-homogenised in 2.5 l of the same buffer (Polytron, setting 5) and centrifuged again at 27000 × *g* for 90 min. The final pellet was resuspended (Polytron, setting 5) in 2.7 l 10 mM potassium phosphate buffer, pH 6.5. The non-ionic detergent Brij 35 was added to a final concentration of 0.1% (w/v) (buffer A) and the suspension was stirred for 18 h. The detergent-treated preparation was centrifuged at 27000 × *g* for 2 h, and the pellet was re-suspended (Polytron, setting 5) in 500 ml of buffer A. Following centrifugation again at 27000 × *g* for 2 h, the two supernatant fractions were combined for further purification.

First DEAE-cellulose Chromatography. The solubilized enzyme preparation was applied to a DEAE-cellulose column

(4.5 × 55 cm) equilibrated with buffer A. After application of the sample, the column was washed with 4 l of buffer B (10 mM potassium phosphate buffer, pH 6.5 containing 0.005% Brij 35) and the enzyme was eluted with buffer B containing 0.3 M KCl at a flow rate of 60 ml/h; 15-ml fractions were collected. The fractions containing substance-P-degrading activity were pooled and dialyzed overnight against three changes of 5 l of buffer C (1 mM potassium phosphate buffer, pH 7.5 containing 0.005% Brij 35).

Hydroxyapatite Column. The dialyzed eluate (500 ml) from the first DEAE-cellulose column was applied to an hydroxyapatite column (4.5 × 24 cm) equilibrated with buffer C. After sample application, the column was washed with 4 l of buffer C followed by 2 l of 20 mM potassium phosphate buffer, pH 7.5, containing 0.005% Brij 35 (buffer D), and the enzyme was eluted by gently dispersing the column material in two 300-ml portions of buffer C containing 200 mM potassium phosphate. After stirring for 30 min the slurry was filtered and the pooled filtrates were concentrated to 150 ml by vacuum dialysis. The concentrated filtrate was then dialysed against three changes of 5 l of buffer A.

Second DEAE-cellulose Chromatography. The dialysed sample from the hydroxyapatite column was applied to a DEAE-cellulose column (4.5 × 55 cm) equilibrated in buffer A as before. After sample application, the column was washed with 2 l of buffer B and the enzyme was eluted with buffer B containing 0.25 M KCl at a flow rate of 60 ml/h; 15-ml fractions were collected. The fractions containing substance-P-degrading activity were pooled, concentrated to 20 ml by ultrafiltration using an Amicon cell with a PM10 Diaflo membrane. The concentrated sample was dialysed overnight against three changes of 5 l of buffer D.

Sephadex G-200 Chromatography. The dialysed sample from the second DEAE-cellulose column (20 ml) was applied to a Sephadex G-200 column (2.5 × 80 cm) equilibrated with buffer D. The column was eluted at a flow rate of 17.1 ml/h and 10-ml fractions were collected. The fractions containing substance-P-degrading activity were combined, and concentrated to 10 ml by ultrafiltration.

Sephadex G-100 Chromatography. The concentrated sample from the previous step was applied to a Sephadex G-100 column (2.5 × 80 cm) equilibrated with buffer D. The column was eluted at a flow rate of 17.1 ml/h and 10-ml fractions were collected. The fractions containing substance-P-degrading activity were combined, concentrated by ultrafiltration and stored at −20 °C. The subsequent characterisation of the enzyme reported in this paper was performed with this preparation, which contained 0.23 mg protein/ml.

Characterisation of the Partially Purified Substance-P-Degrading Enzyme

Identification of Cleavage Sites in Substance P. To identify the enzymatically-sensitive sites in substance P, 200 nmol of the synthetic peptide was incubated with 10 µl of the enzyme preparation in 50 mM potassium phosphate buffer, pH 7.5, in a final volume of 400 µl for 20 − 90 min. The reaction was stopped by boiling for 10 min. The breakdown products in a sample (300 − 350 µl) of the digest were separated by reverse-phase high-performance liquid chromatography using a µBondapak C_{18} column (0.39 × 30 cm). The column was equilibrated with acetonitrile/50 mM phosphoric acid, pH 1.9 (5:95, v/v). All buffers were filtered and degassed before use. After sample application, the peptides were eluted with a 20-min linear gradient of acetonitrile/50 mM phosphoric acid, pH 1.9, from 5:95 (v/v) to 65:35 (v/v) at a flow rate of 2 ml/min at room temperature. The absorbance at 220 nm was monitored with a model 450 ultraviolet detector (Waters Associates). Fractions containing peaks of ultraviolet-absorbing material were collected, dried and analysed for their amino acid composition following hydrolysis in 5.7 M HCl containing 2% thioglycolic acid at 110 °C for 18 h, using a Beckman model 119CL analyser. When [^{3}H]substance P digests were analysed, eluates were collected in 0.2-min fractions and the radioactivity in each fraction measured by liquid scintillation counting.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in a Shandon apparatus according to the procedure of Sargent and George [32]. Cylindrical gels (6.5-cm length, 7.5% acrylamide, 0.2% bisacrylamide) were cast in glass tubes (8-mm internal diameter) using chemical polymerization. Glycine/Tris buffer, pH 8.6, was used and the gels were pre-electrophoresed at 2 mA/tube for 2 h to remove unpolymerized acrylamide and excess oxidant before the sample run. Samples (20 − 50 µg protein) were applied in 20% sucrose and electrophoresis was carried out at 2 mA/tube until the bromophenol blue marker was about 5 mm from the bottom of the gel. Proteins were stained with Coomassie blue in isopropanol, according to the procedure of Fairbanks et al. [33]. Sodium dodecyl sulphate electrophoresis was performed in the presence of 0.1% sodium dodecyl sulphate according to the method described by Weber and Osborn [34]. Samples were incubated at 37 °C for 2 h in 10 mM Tris/HCl buffer (pH 7.5) containing 2.5% sodium dodecyl sulphate and 1% 2-mercaptoethanol before electrophoresis.

Molecular Weight Estimations. Gel filtration on Sephadex G-100 was performed according to the method of Andrews [35] using bovine serum albumin, ovalbumin and myoglobin as molecular weight markers. Ultracentrifugation was performed according to the method of Martin and Ames [36] using myoglobin, horseradish peroxidase, and bovine serum albumin as molecular weight markers.

Effects of Inhibitors and Peptides on [^{3}H]Substance P Degradation. To screen for potential inhibitors and substrates of substance-P-degrading enzyme, radiochemical assays were used where the substrate concentration (380 nM) was very much lower than the K_m of the enzyme for substance P. This condition allowed a sensitive detection of inhibition, although it did not give any information on the mechanism involved. For inhibitor studies, the enzyme was pre-incubated at 37 °C for 20 min with various peptidase inhibitors before the addition of [^{3}H]substance P. In screening potential peptide substrates, a 3-min pre-incubation at 0 °C was used before initiating the reaction.

Chromogenic Substrates. To screen for possible chromogenic substrates of the enzyme, a chymotrypsin substrate (Ala-Ala-Phe-Nan) and a thermolysin substrate (Fao-Gly-LeuNH_2) were tested, using the respective purified enzymes as controls. For the chymotrypsin substrate test, the reaction mixture contained 1 mM Ala-Ala-Phe-Nan in 29 mM potassium phosphate buffer, pH 7.5 and 2 µg/ml of α-chymotrypsin or substance-P-degrading enzyme in a total volume of 3 ml. The hydrolysis of the substrate was followed by the increase in absorbance at 405 nm. For the thermolysin substrate test, the reaction mixture contained 1 mM Fao-Gly-LeuNH_2 in 50 mM Tris/HCl buffer, pH 7.5, and 2 µg/ml of thermolysin or substance-P-degrading enzyme in a total volume of 3 ml. The hydrolysis of the dipeptide was followed by the decrease in absorbance at 345 nm.

Identification of Sites of Cleavage of [^{3}H]Substance P in Rat Brain Synaptic Membranes

A subcellular fraction enriched in synaptic membranes was prepared according to method B of Wang and Mahler [37]. The washed membrane pellet was resuspended by a teflon/glass homogeniser in 20 mM Hepes (pH 7.4), containing 0.5% bovine serum albumin, to a final concentration of 10 mg membrane protein/ml; 100 µl of this suspension was added to 400 µl of the same buffer containing [^{3}H]Substance P (final concentration 250 nM), and incubated for 20 min at 20 °C. Digestion was terminated by the addition of 100 µl of 6 M HCl, and centrifugation at 4000 × *g* for 10 min. 100 µl of the supernatant were analysed by reverse-phase high-performance liquid chromatography on a µBondapak column (0.39 × 30 cm) equilibrated with acetonitrile/10 mM ammonium acetate, pH 4.0 (5:95, v/v). The breakdown products were separated using a linear gradient of acetonitrile/10 mM ammonium acetate, pH 4.0 from 5:95 (v/v) to 65:35 (v/v) at a flow rate of 2 ml/min at room temperature. The elution position of substance P was determined by calibration runs under identical conditions using 10–100 µg of synthetic substance P and ultraviolet detection at 220 nm. Fractions were taken every 0.2 min during separation of the radiolabelled products of the digestion, and counted by liquid scintillation spectrometry using 4 ml of ethoxyethanol and 10 ml of toluene scintillant.

Other Assays

Angiotensin-converting enzyme was measured fluorimetrically using the method of Yang and Neff [38] and enkephalin-degrading enzyme activity was measured by radioimmunoassay as described by Arregui et al. [39]. Protein determinations were performed according to the procedure of Lowry et al. [40] using bovine serum albumin as the standard.

RESULTS

Solubilization

In preliminary experiments we found that in homogenates of human diencephalon more than two thirds of the total substance-P-degrading activity was present in the 100000 × *g* supernatant fraction, and the remaining activity was particulate (Table 1) and could not be released by repeated washing of the pellet with hypotonic buffer. As in the rat brain [14], most of the particulate degrading activity was sedimented by centrifugation at 27000 × *g* for 90 min. Thus, the 27000 × *g* particulate fraction was chosen as the starting material for the present purification. Treatment of this particulate fraction with the non-ionic detergent Brij 35 solubilised more than 50% of the membrane-bound substance-P-degrading activity together with only about 10% of total membrane protein, resulting in an about sixfold increase in specific activity (Tables 1 and 2). Only negligible amounts of substance-P-degrading activity remained in the detergent-extracted particulate material, suggesting that a partial loss of activity occurred during the extraction procedure.

Purification of Substance-P-Degrading Enzyme

The results are summarised in Table 2. The solubilised enzyme was adsorbed onto DEAE-cellulose at pH 6.5 and no substance-P-degrading enzyme was eluted on washing with low-ionic-strength buffer. When the ionic strength of the elution buffer was increased with 0.3 M KCl, a broad band of substance-P-degrading activity emerged from the column (Fig. 2). The pooled enzyme-containing fractions (fractions 33–60) were found to also contain both angiotensin-converting and enkephalin-degrading activities. The results of a preliminary experiment with a smaller quantity of solubilized substance-P-degrading enzyme on DEAE-cellulose chromatography indicated that the three activities represented different enzymes which were separable (Fig. 3). Indeed, during the present purification, the bulk of enkephalin-degrading and angiotensin-converting activities were separated from substance-P-degrading enzyme during the hydroxyapatite step, where the latter enzyme could only be eluted with a high concentration of potassium phosphate. The remaining angiotensin-converting enzyme was separated from substance-P-degrading enzyme during the second DEAE-cellulose column step, when the latter enzyme was eluted with

Table 1. *Membrane-bound and cytosolic substance-P-degrading activities from human diencephalon*
Pooled diencephalic tissue from a single human brain (approximately 200 g wet weight) was homogenised in 20 mM Tris/HCl buffer, pH 7.8. The crude homogenate was spun at 100000 × *g* for 1 h and the substance-P-degrading activity in the supernatant and in the precipitate was measured by radioimmunoassay as described in Materials and Methods. The specific activity was expressed as nmol substance P degraded · h^{-1} · (mg protein)$^{-1}$. Values are means of triplicate determinations

Fraction	Protein	Substance-P-degrading activity	
		total	specific
	mg	nmol · h^{-1} (%)	nmol · h^{-1} · (mg protein)$^{-1}$
Homogenate	15600	9610 (100)	0.62
100000 × *g* supernatant	5800	7500 (78)	1.29
100000 × *g* pellet	10620	3250 (34)	0.31
0.1% Brij 35 solubilised	1150	1770 (18)	1.54

Table 2. *Purification of membrane-bound substance-P-degrading enzyme from human brain*
Pooled diencephalic tissue from seven brains was homogenised in 20 mM Tris/HCl buffer, pH 7.8. The crude homogenate was spun at 27000 × *g* for 90 min and the membrane fraction was used as the starting material for enzyme purification as described in Materials and Methods

Stage	Protein	Specific activity	Yield	Purification
	mg	nmol · h^{-1} · (mg protein)$^{-1}$	%	-fold
27000 × *g* pellet	111510	0.6	100	1
0.1% Brij 35 solubilised	11605	3.4	59.5	6
1st DEAE-cellulose	1422	12.0	25.4	20
Hydroxyapatite	265	34.2	13.5	57
2nd DEAE-cellulose	17.4	297.3	7.7	496
Sephadex G-200	8.0	326.3	3.9	544
Sephadex G-100	2.3	626.0	2.2	1040

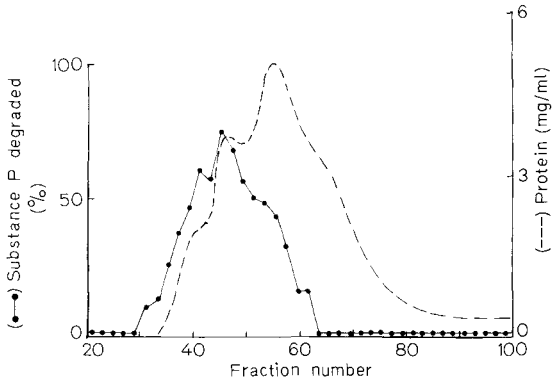

Fig. 2. *Elution profile of substance-P-degrading enzyme from the first DEAE-cellulose column.* The enzyme activity was solubilized with 0.1% Brij 35 from a washed 37000 × *g* particulate fraction of human diencephalon, and applied to the column (4.5 × 55 cm). After washing with 4 l of 10 mM potassium phosphate buffer, pH 6.5, containing 0.005% Brij 35, the enzyme was eluted with 0.3 M KCl in the same buffer; 15-ml fractions were collected and 20-µl aliquots of each fraction were incubated with 0.2 µM substance P in a final volume of 400 µl 50 mM potassium phosphate buffer, pH 7.5 for 10 min at 37 °C; the amount of substance P remaining was measured by radioimmunoassay of duplicate samples

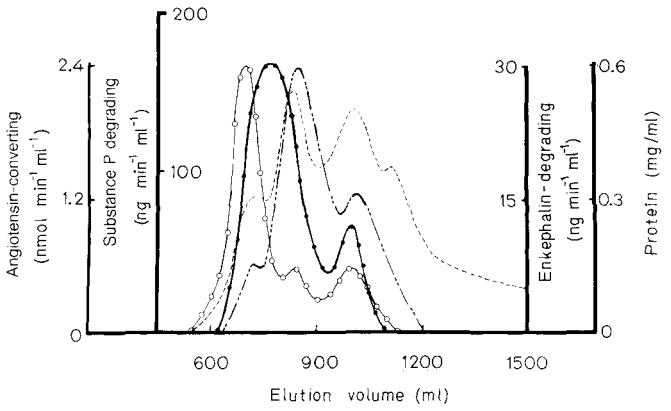

Fig. 3. *Elution profiles of angiotensin-converting (○——○) substance-P-degrading (●——●) and enkephalin-degrading (----) activities from a DEAE-cellulose column.* Enzyme activities were solubilised with 0.1% Brij 35 from a washed 100000 × *g* particulate fraction of human diencephalon. After sample application, the column (4.4 × 53 cm) was washed with 4 l of 10 mM potassium phosphate buffer, pH 6.4 containing 0.005% Brij 35; enzymes were eluted with 0.3 M KCl in the same buffer. 15-ml fractions were collected and enzyme activities were measured as described in Materials and Methods

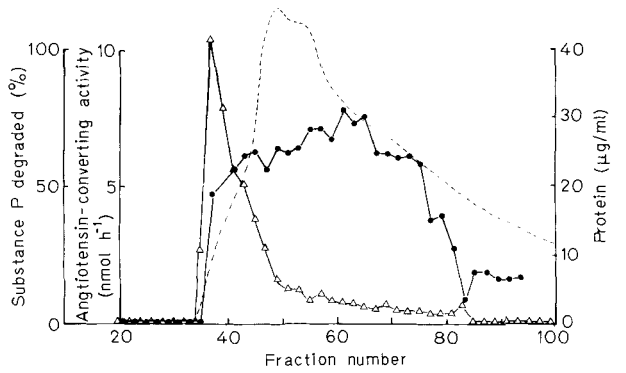

Fig. 4. *Elution profiles of angiotensin-converting (△——△) and substance-P-degrading (●——●) activities from a second DEAE-cellulose column after the hydroxyapatite step.* The enzymes were eluted from the column (4.5 × 55 cm) with 0.25 M KCl in 10 mM potassium phosphate buffer, pH 6.5, containing 0.005% Brij 35. 15-ml fractions were collected and enzyme activities in 50-µl aliquots of each fraction were determined as described in Materials and Methods. The incubation times for angiotensin-converting, substance-P-degrading and enkephalin-degrading activities were 60, 10 and 30 min respectively. No enkephalin-degrading activity was detectable in any of the eluates

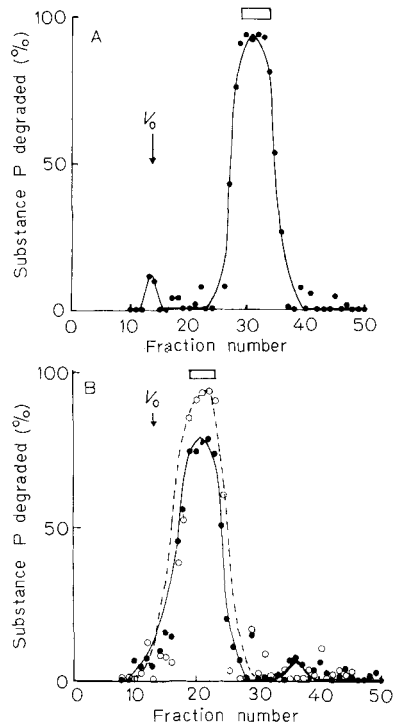

Fig. 5. *Elution profile of substance-P-degrading activity from (A) a Sephadex G-200 column and a subsequent (B) Sephadex G-100 column.* 10-ml fractions were collected from each column (2.5 × 80 cm) and 50 µl aliquots of each fraction were incubated with 0.2 µM substance P for 15 min at 37 °C. The amount of substance P remaining was measured by the C-terminus-directed IgG (●——●) and the N-terminus-directed antisera (○——○) radioimmunoassay. V_0 was determined with blue dextran 2000. The open bar indicates the active fractions which were pooled for further study

0.25 M KCl and again angiotensin-converting enzyme emerged before it. No enkephalin-degrading enzyme was detectable in any of the eluate fractions from the second DEAE-cellulose column. Fractions 50–80, containing substance-P-degrading enzyme, were pooled (Fig. 4) and subjected to gel filtration chromatography. The enzyme gave a symmetrical peak on both Sephadex G-200 and Sephadex G-100 columns (Fig. 5 A, B) with a further twofold purification.

The substance-P-degrading activity was enriched 1040-fold over that in the original crude particulate fraction, with a recovery of 2%. This preparation was then used for the subsequent characterisation of the enzyme. It showed about a 50% decrease in activity after 6-months storage at −20 °C. This deterioration was more pronounced upon repeated freezing and thawing. The presence of a low concentration of detergent was necessary to stabilize the enzyme.

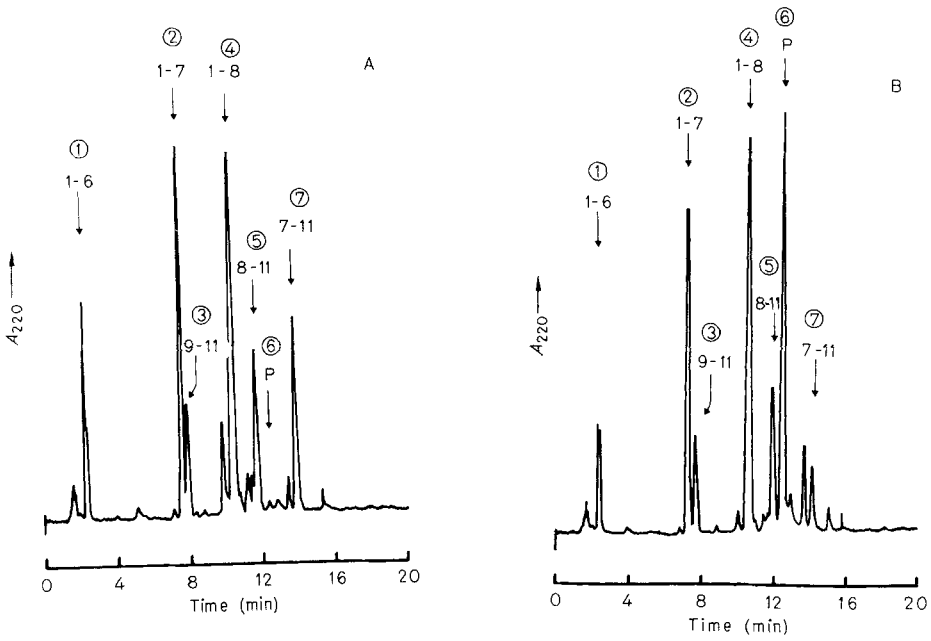

Fig. 6. *Reverse-phase high-performance liquid chromatography analysis of substance-P-degrading enzyme digest of substance P.* Substance P (200 nmol) was incubated with substance-P-degrading enzyme which had not (A) or had (B) been electrophoretically purified. After 90 min (A) or 7 h (B), the reaction was terminated by boiling and the reaction mixture applied to a μBondapak C_{18} column. Elution was carried out at room temperature with a 20-min linear gradient of 5–65% acetonitrile in 50 mM H_3PO_4, pH 1.9, at a flow rate of 2 ml/min. The absorbance at 220 nm was monitored, using a detector setting of 1.0 full scale

Table 3. *Amino acid composition of purified degradation products of substance P*
10 μl of substance-P-degrading enzyme was incubated at 37 °C with 0.5 mM substance P in 50 mM potassium phosphate buffer, pH 7.5, for 90 min and a part of the digest was analysed by reverse-phase high-performance liquid chromatography. Peptide-containing peaks were numbered in the order of increasing retention time as illustrated in Fig. 6A. Glutamine was determined as glutamic acid, methionine amide as methionine. Total recovery amounted to 92%

Chromatography peak	Fragment	Yield	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	$MetNH_2$
			mol/mol peptide										
1.	1–6	39.8	0.98	1.03	0.89	1.03	0.99	0.99	–	–	–	–	–
7.	7–11	30.8	–	–	–	–	–	–	0.98	0.98	1.06	1.01	0.98
2.	1–7	50.2	0.94	0.99	0.86	0.99	1.07	1.07	0.93	–	–	–	–
5.	8–11	48.8	–	–	–	–	–	–	–	1.16	0.99	0.96	0.89
4.	1–8	44.5	0.96	0.97	0.95	0.97	1.08	1.08	1.00	1.00	–	–	–
3.	9–11	55.3	–	–	–	–	–	–	–	–	1.04	1.00	0.96
6.	Complete	5.3											

CHARACTERISATION OF THE PARTIALLY PURIFIED SUBSTANCE-P-DEGRADING ENZYME

Cleavage of Substance P

When the substance-P-degrading activity of eluate fractions from the final gel filtration step on Sephadex G-100 was monitored by radioimmunoassay, it was noted that in the most active fractions the N-terminus-directed assay registered more extensive degradation of substance P than that monitored by the C-terminus-directed immunoassay (Fig. 5 B). Since the C-terminus-directed assay showed a complete cross-reactivity with all the biologically active C-terminal fragments of substance P down to the pentapeptide [30], we reasoned that there might be an accumulation of such C-terminal fragments during the digestion of substance P by the purified enzyme. Indeed, after incubation of substance P with the partially purified enzyme preparation, seven major peptide-containing peaks could be separated by high-performance liquid chromatography (Fig. 6A). The peptide materials in peaks 1–5 showed no substance-P-like immunoreactivity, while peak 6, which co-eluted with standard substance P (retention time 12.4 min), cross-reacted to the same extent with both the C-terminus and the N-terminus-directed antisera. On the other hand, peak 7 cross-reacted only with the C-terminus-directed antiserum but not with the N-terminus-directed one. The retention times for the synthetic C-terminal tripeptide, tetrapeptide and pentapeptide of substance P were 7.9, 11.7 and 13.9 min respectively, which compared very closely to those of peak 3 (8.0 min), 5 (11.7 min) and 7 (13.9 min). The identities of the degradation products were confirmed by amino acid analyses (Table 3). Since the cleavage pattern did not change even on prolonged incubation and the molar ratios of the peptide fragments (1–6:7–11 = 1.3, 1–7:8–11 = 1.0 and 1–8:9–11 = 0.8) were close to 1, it is unlikely that the shorter fragments could have resulted from secondary cleavages. It is, therefore, suggested that the enzyme cleaved

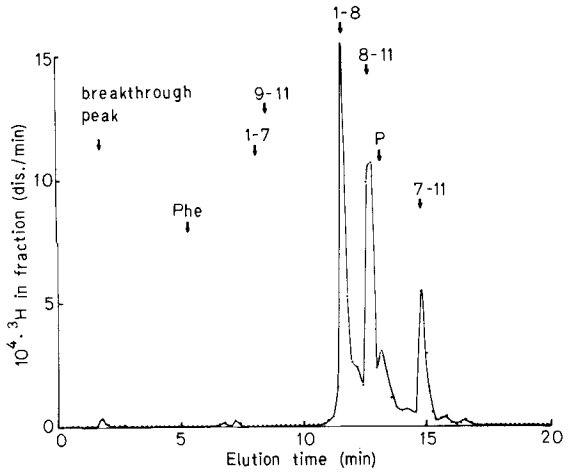

Fig. 7. *High-performance liquid-chromatography radiochromatogram of substance-P-degrading enzyme digest of [^{3}H]substance P.* [^{3}H]Substance P (75 pmol) was incubated at 37 °C with 2 µl of substance-P-degrading enzyme in a final volume of 200 µl for 20 min and 50 µl of the incubation mixture was applied to a µBondapak C_{18} column. Elution was carried out at room temperature with a 20-min linear gradient of 5–65% acetonitrile in 50 mM H_3PO_4, pH 1.9, at a flow rate of 2 ml/min. 0.2-min fractions were collected and radioactivity measured by liquid scintillation counting. Recovery of tritium from the column was 106%. Arrows indicate the elution positions for standard markers

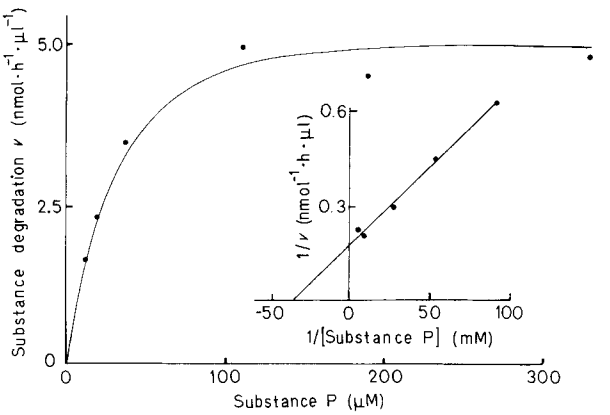

Fig. 8. *Kinetic analysis of the rate of degradation of substance P by substance-P-degrading enzyme.* Mixtures of labelled and unlabelled substance P were incubated at 37 °C with 2 µl (0.46 µg protein) substance-P-degrading enzyme and the extent of degradation of [^{3}H]substance P was analysed by thin-layer chromatography as described in Materials and Methods. Insert: Lineweaver-Burk plot of the velocity data. Each point is the mean of duplicate determinations in three or four separate experiments

Table 4. *A comparison of the ratio of cleavages on substance P by substance-P-degrading enzyme at different substrate concentrations*
75 pmol of [^{3}H]substance P was diluted with appropriate amounts of the unlabelled peptide to give the required final concentrations, and the mixtures were incubated with 2 µl of substance-P-degrading enzyme at 37 °C for 10–90 min. The digests were analysed by high-performance liquid chromatography as described in Materials and Methods. The ratio of the radioactivity in the peaks corresponding to fragments 1–8, 8–11 and 7–11 was used to calculate the ratio of different cleavages, using that of fragment 7–11 as the reference

Substance P concn	Ratio of cleavages		
	Gln^6-Phe^7	Phe^7-Phe^8	Phe^8-Gly^9
µM			
0.38	1	2.88	2.46
11.1	1	2.75	2.50
93.0	1	2.57	2.46
371	1	2.62	2.51

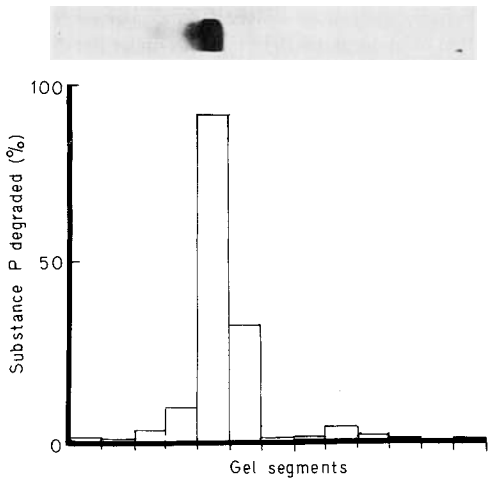

Fig. 9. *Electrophoretic profile of substance-P-degrading enzyme.* Electrophoresis was performed as described in Materials and Methods. The gel was sliced into 13 segments. Each segment was extracted with 500 µl of Tris/glycine buffer (pH 8.6) containing 0.005% Brij 35 for 24–48 h at 4 °C. 50 µl of the extracts was incubated with 0.2 µM substance P at 37 °C for 1 h and the amount of substance P remaining was measured with duplicate radioimmunoassay. Protein staining of a gel run in parallel (illustrated above) was performed with Coomassie blue

substance P between Gln^6-Phe^7, Phe^7-Phe^8 and Phe^8-Gly^9 by an endopeptidase action.

When [^{3}H]substance P was digested with the enzyme and the degradation products analysed by high-performance liquid chromatography, there was an accumulation of ^{3}H-labelled peptide fragments 1–8, 8–11 and 7–11 as expected (Fig. 7). The absence of free [^{3}H]phenylalanine again argued strongly against any secondary exopeptidase action. Further analysis indicated that very similar cleavage patterns were obtained over a wide range of [^{3}H]substance P concentrations (0.38–371 µM) which was reflected by the very constant ratio of the Gln^6-Phe^7, Phe^7-Phe^8 and Phe^8-Gly^9 cleavages (Table 4).

For measurements of K_m and V, 75 pmol of [^{3}H]substance P was mixed with appropriate amounts of unlabelled substance P to give final concentrations of 0.38–330 µM, and then incubated with 2 µl of substance-P-degrading enzyme at 37 °C for various lengths of time. The digests were then analysed by thin-layer chromatography as described in Materials and Methods. The rate of degradation was found to increase with increasing concentration of substance P up to about 100 µM. A Lineweaver-Burk plot of these results indicated an apparent Michaelis constant (K_m) of 29 µM and a maximum rate of degradation (V) of 25.7 µmol · h^{-1} · (mg protein)$^{-1}$ was obtained (Fig. 8).

Purity

When the enzyme was electrophoresed under non-denaturing conditions, one major and two minor protein bands

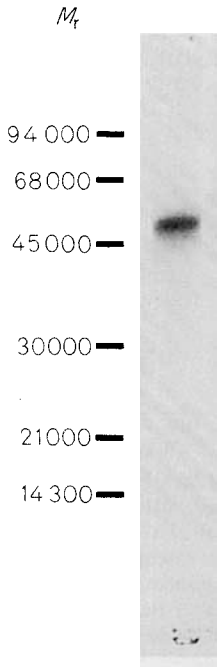

Fig. 10. *Molecular weight estimation of electrophoretically purified substance-P-degrading enzyme on sodium dodecyl sulphate gel electrophoresis.* The migration of molecular weight markers under the same experimental conditions is indicated. Molecular weight markers used were lysozyme (14300) soya bean trypsin inhibitor (21000), carbonic anhydrase (30000), ovalbumin (45000), bovine serum albumin (68000) and phosphorylase B (94000)

were observed. When the gel was sliced into 4-mm segments and extracted for enzyme activity (buffer D, 24 h at 4 °C), the majority of substance-P-degrading enzyme activity was associated with the major band of protein (Fig. 9), thus the preparation appeared to be highly pure. Furthermore, the enzyme extracted from the major protein peak on the polyacrylamide gel displayed an identical cleavage pattern with substance P to that previously observed with the partially purified substance-P-degrading enzyme before electrophoresis (Fig. 6B).

There was no evidence to suggest that the enzyme was a glycoprotein, since it was not stained by Alcian blue [41] nor by periodate/Schiff reagent [33] after electrophoresis, and it was not retained on columns of agarose–lentil-lectin or agarose–concanavalin-A (P. L. Biochemicals Inc., Milwaukee, WI). When the electrophoretically purified substance-P-degrading enzyme was analysed by a second electrophoretic procedure, using dissociating conditions with sodium dodecyl sulphate, a single protein band was observed, suggesting that the enzyme consists of a single polypeptide chain with a molecular weight of about 52000 (Fig. 10).

Molecular Weight Estimations

Gel filtration on Sephadex G-100 indicated a molecular weight of 36000, and the enzyme co-migrated with horseradish peroxidase (M_r = 40000) on sucrose gradient ultracentrifugation.

Effect of Peptidase Inhibitors

The effects of various peptidase inhibitors on the activity of substance-P-degrading enzyme are summarised in Table 5.

Table 5. *Effect of peptidase inhibitors on the degradation of [^{3}H]substance P by the degrading enzyme purified from human brain*
The inhibitory spectrum of the inhibitors was adopted partly from Barrett [45]. 2 µl of substance-P-degrading enzyme was preincubated with various inhibitors at the indicated concentrations. Reaction was started by the addition of [^{3}H]substance P (380 nM) and terminated after 10 min by boiling. Boiled enzyme was used as the enzyme blank. Unchanged [^{3}H]substance P was separated from labelled degradation products by thin-layer chromatography and quantified by liquid scintillation counting. Each value of is the mean of three experiments (S.E. always less than 15%)

Inhibitor	Concentration	Inhibition	Inhibitory spectrum
	mM	%	
1,10-Phenanthroline	1	100	metallo-endopeptidases
	0.3	82	
	0.1	60	
	0.03	39	
	0.01	7	
EDTA	5	100	
	1	80	
	0.2	34	
	0.02	17	
EGTA	1	54	
Dithiothreitol	1	69	
	0.1	7	
Phosphoramidon	1	0	
	0.5	0	
Iodoacetate	2	0	thiol-endo-peptidases
N-Ethylmaleimide	2.5	1	
p-Chloromercuribenzoate	1	0	
p-Chloromercuriphenyl-sulphonate	1	56	
	µg/ml		
Pepstatin	1	0	carboxyl-endo-peptidase
	mM		
Benzamidine	10	21	serine-endo-peptidases
	1	0	
Diisopropylfluoro-phosphate	1	5	
Captopril	1	3	angiotensin-converting enzyme
Teprotide	2.7	58	
	1.4	46	
	0.5	30	
	units/ml		
Trasylol	5000	21	kallikrein
Bacitracin	1	3	unknown

The enzyme was strongly inhibited by 1,10-phenanthroline, EDTA, EGTA and dithiothreitol. Among the chelating agents, 1,10-phenanthroline (IC_{50} = 50 µM) was the most effective inhibitor, followed by EDTA (IC_{50} = 350 µM) and dithiothreitol (IC_{50} = 500 µM). Phosphoramidon, an effective inhibitor of thermolysin, did not inhibit substance-P-degrading enzyme even at 1 mM concentration. The serine protease inhibitors such as benzamidine (1 mM) and diisopropylfluorophosphate (1 mM) had no inhibitory action on the enzyme. Among the thiol protease inhibitors tested,

Table 6. *Effect of peptides on the degradation of substance P by the degrading enzyme from human brain*
2 µl of substance-P-degrading enzyme was preincubated for 3 min at 0 °C with various peptides at the indicated concentrations. Reaction was started by the addition of [^{3}H]substance P to give a final concentration of 380 nM and terminated after 20 min by boiling. Boiled enzyme was used as enzyme blank. Unchanged [^{3}H]substance P was separated from labelled degradation products by thin-layer chromatography and quantified by liquid scintillation counting. Each value is the mean of three experiments (S.E. less than 15% in all cases). IC_{50} values (i.e. concentration necessary to give 50% inhibition), where given, were estimated from the inhibition curves obtained by varying the concentrations of the tested peptides

Peptide	Concn	Inhibition	IC_{50}
	µM	%	µM
Substance P	370	95	34
Its free acid	370	98	–
Fragments: 2–11	420	92	–
3–11	460	100	–
4–11	520	57	–
7–11	820	31	–
8–11	1070	2	–
1– 9	450	32	–
[Tyr^8]Substance P	370	90	–
[Tyr(Me)^8]Substance P	313	80	–
[*p*IPhe^8]Substance P[a]	290	92	–
[Tyr(Me)^7, MeGly^9]Substance P	310	85	–
[MePhe^8, MeGly^9]Substance P	360	71	–
<Glu-Gln-Phe-MePhe-MeGly-Leu-MetNH_2	550	3	–
Phyllomedusin	420	65	240
Physalaemin	400	50	400
Eledoisin	420	26	850
Eledoisin-related peptide	710	22	–
Somatostatin	305	34	750
Bombesin	310	30	–
Luliberin	420	22	–
Thyroliberin	1380	1	–
Angiotensin I	390	17	–
Bradykinin	470	35	–
Vasopressin	460	6	–
Neurotensin	300	0	–
Phe-Gly	2500	0	–
Gly-Leu	2500	1	–
Tyr-Gly-Gly	1600	3	–
Tyr-Gly-Gly-Phe	1250	4	–
Gly-Gly-Phe-Leu	1250	2	–

[a] Substance P with a *p*-iodo group on Phe^8.

iodoacetate (2 mM) and *N*-ethylmaleimide (2.5 mM) had no effect on substance-P-degrading enzyme, while *p*-chloromercuriphenyl sulphonate was a weak inhibitor of the enzyme with an IC_{50} of about 1 mM. Pepstatin (1 µg/ml), a carboxyl-endopeptidase inhibitor and bacitracin (1 mM), a peptidase inhibitor of unknown specificity, were both without effect on the enzyme. Trasylol, a well known kallikrein inhibitor, exerted only a weak (21%) inhibitory action on the enzyme when tested at a very high concentration (5000 units/ml). Of the two angiotensin-converting enzyme inhibitors tested, captopril had no effect when tested at 1 mM concentration. On the other hand, teprotide was a weak inhibitor of the enzyme (IC_{50} = 1.8 mM).

The effects of the enzyme inhibitors were assessed by assaying substance-P-degrading activity by measurements of the rate of disappearance of [^{3}H]substance P. In all experiments, however, the three major radiolabelled cleavage products were also separated by thin-layer chromatography and separately measured. These results showed that all of the effective inhibitors of substance-P-degrading enzyme acted equally at each of the three cleavage sites, maintaining a constant ratio among the labelled degradation products. This further supports the conclusion that substance-P-degrading enzyme alone is responsible for the three cleavages of substance P observed.

Substrate Specificity

The results of screening for potential substrates of substance-P-degrading enzyme are summarised in Table 6. Besides substance P, its free acid and the fragments 2–11 and 3–11 were all very effective in inhibiting the breakdown of [^{3}H]substance P, suggesting that the C-terminal amide and the N-terminal Arg-Pro were not essential for the enzyme to recognise its substate. Among other fragments of substance P, fragments 1–9, 4–11 and 7–11 were considerably less effective and 8–11 was ineffective in inhibiting the enzyme. Of the substance P analogues with modifications in positions 7, 8 or 9, most were as potent as substance P in preventing the enzyme from acting on [^{3}H]substance P, except for the fragment 5–11, analogue, <Glu-Gln-Phe-MePhe-MeGly-Leu-MetNH_2 which was not degraded (see the following paper [28]) and failed to inhibit degradation of [^{3}H]substance P.

Among naturally occurring peptides related to substance P, phyllomedusin, physalaemin and eledoisin were all weakly inhibitory, with IC_{50} values of 240, 400 and 850 µM respectively, indicating that they were much less favourable substrates than substance P (IC_{50} = 34 µM) for this enzyme. Indeed, when 0.5 mM of substance P, physalaemin (<Glu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-MetNH_2) and eledoisin (<Glu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2) were incubated with 50 µl of the electrophoretically purified enzyme for 7 h, the extent of degradation of substance P (72%) was greater than that of physalaemin (50%) or eledoisin (12%). Also, unlike substance P, physalaemin was predominantly (about 90%) cleaved between Tyr^8-Gly^9 (Fig. 11 A) and eledoisin was only cleaved between Ala^6-Phe^7 (Fig. 11 B). The eledoisin-related peptide (Lys-Phe-Ile-Gly-Leu-MetNH_2) was even less potent than eledoisin, similar to the situation in substance P were shortening the peptide led to a decrease in inhibitory potency. Many other neuropeptides, including somatostatin, bombesin, luliberin, bradykinin and angiotensin I were found to be weakly inhibitory, and others such as thyroliberin, neurotensin and vasopressin had little or no effect on the degradation of [^{3}H]substance P.

Small peptides such as Phe-Gly, Gly-Leu, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe and Gly-Gly-Phe-Leu and large proteins such as bovine serum albumin (1 mg/ml), haemoglobin (1 mg/ml) and insulin (0.5 mg/ml) were all without effect on substance-P-degrading enzyme. These findings suggested that the enzyme was unlikely to represent a non-specific protease, and indeed substance P was found to be the preferred substrate among the many peptides tested.

In contrast to α-chymotrypsin and thermolysin, substance-P-degrading enzyme did not hydrolyse the chromogenic substrates Ala-Ala-Phe-Nan and Fao-Gly-LeuNH_2.

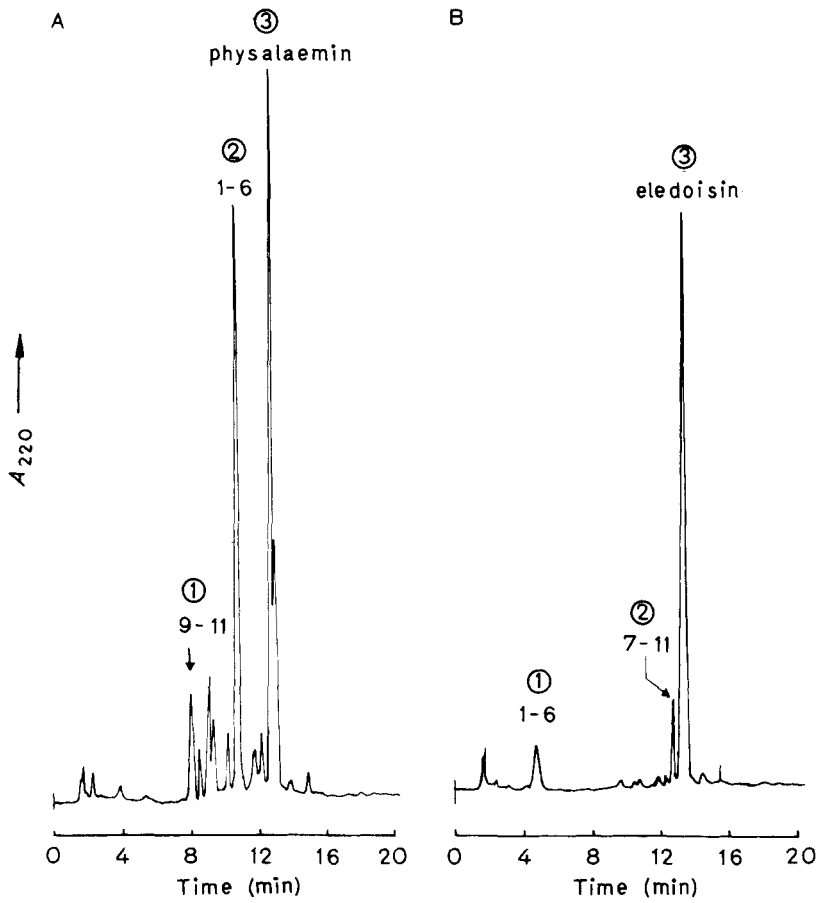

Fig. 11. *Reverse-phase high-performance liquid chromatography of substance-P-degrading digest of physalaemin (A) and eledoisin (B).* Physalaemin (0.5 mM) and eledoisin (0.5 mM) were incubated with the electrophoretically purified substance-P-degrading enzyme for 7 h at 37 °C. The reaction was stopped by boiling and the reaction mixture applied to a μBondapak C_{18} column. Elution was carried out as described under Fig. 6 and the absorbance at 220 nm was monitored

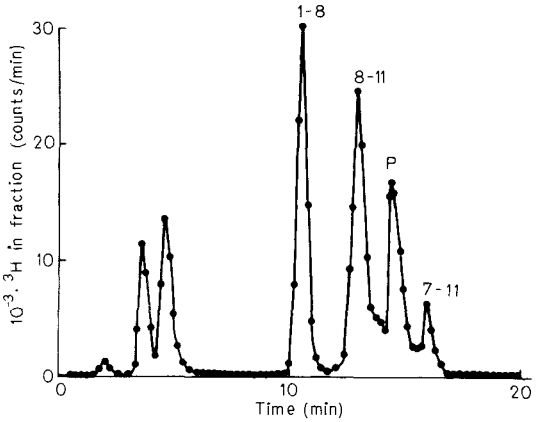

Fig. 12. *High-performance liquid chromatography analysis of digestion products of [^{3}H]substance P after incubation with rat brain synaptic membranes.* Conditions are described in Materials and Methods. Recovery of the applied radioactivity was quantitative (102%). The results were replicated in duplicate analyses from two separate experiments. Retention times of early peaks are consistent with their tentative assignment to free [^{3}H]phenylalanine (at 3.6 min) and phenylalanyl-[^{3}H]phenylalanine (at 4.6 min)

pH and Temperature Dependence

Substance-P-degrading enzyme has a broad pH optimum in the range 7–9. Very low activity was observed at pH values below 5 or above 10. Both N-terminus and C-terminus-directed radioimmunoassays yielded very similar pH-dependence profiles, with the N-terminus assay registering more extensive degradation throughout, suggesting that the Gln^6-Phe^7 cleavage occurred at the different pH values tested.

The enzyme was thermolabile and a 10-min preincubation at 50 °C and 80 °C resulted in a loss of 50% and 90% of activity respectively. The temperature optimum of the enzyme was in the range 35–40 °C.

Cleavage of [^{3}H]Substance P by Rat Brain Membranes

Fig. 12 shows the cleavage pattern obtained using [^{3}H]substance P as a substrate for digestion by a rat brain synaptic membrane preparation. The cleavage pattern was very similar to that observed after incubation of [^{3}H]substance P with the enzyme preparation from human brain (cf. Fig. 7). [^{3}H]Substance P was identified at a retention time of 14.6 min. Based upon their retention times, the peaks at 10.6 min, 13.0 min, and 16.0 min were assigned to the ^{3}H-labelled peptide fragments 1–8, 8–11 and 7–11 respectively. Early peaks at 3.6 min and 4.6 min were assigned to the products of secondary cleavages.

DISCUSSION

As previously reported in the rat brain [14], the bulk of substance-P-degrading activity in human brain homogenates (over 70%) was recovered in the soluble fraction (Table 1). This predominance of soluble over membrane-bound peptide-

degrading activities has also been observed for the degradation of other neuropeptides, such as enkephalin [42], neurotensin [43], and luliberin [44]. However, as outlined in the introduction, a high degrading capacity may not be as important a criterion as accessibility, specificity and affinity, in determining the importance of an enzyme in the physiological inactivation of its substrate. For instance, Malfroy et al. [42] have described a membrane-bound enkephalin-degrading enzyme that has a higher affinity and higher degree of specificity towards enkephalin than the soluble enzyme, and they suggested that the membrane-bound enkephalin-degrading enzyme may be more important in regulating enkephalinergic transmission. A plasma membrane peptidase would be in a better position than a cytosolic enzyme in regulating the level of substance P at the synapse. We have now purified a membrane-bound substance-P-degrading enzyme from human brain to near homogeneity and carried out a detailed assessment of its affinity and specificity towards substance P.

Although the substance-P-degrading enzyme preparation cleaved substance P in three different places, namely, between Gln^6-Phe^7, Phe^7-Phe^8 and Phe^8-Gly^9, it was functionally homogeneous, as reflected by the monophasic double-reciprocal plot (Fig. 8), and the constant ratio observed among the cleavages (Table 4) over a wide range of substrate concentrations. The conclusion that a single enzyme was responsible for all three cleavages was further supported by the single band of enzyme activity obtained on gel electrophoresis (Fig. 9), by the finding that the cleavage pattern of substance P by the enzyme before and after separation by electrophoresis was identical (Fig. 6 A, B), and by the parallel inhibition of all three cleavages by a variety of peptidase inhibitors. From the kinetic studies, the enzyme had a K_m of 29 μM towards substance P. This compares quite favourably with most other mammalian tissue peptidases, which have K_m values in the range of 10 – 100 μM for their substrates [45].

The observation that substance-P-degrading enzyme cleaved substance P by an endopeptidase action is interesting, since substance P, in contrast to peptides possessing free amino and/or free carboxyl-terminal groups which are potential substrates for exopeptidases, has a penultimate proline at its N terminus and a C-terminal amide, which makes it resistant to the classical aminopeptidases and carboxypeptidases [46, 47]. Although the C-terminal amide does not exclude the possibility of an action by carboxyamide peptidases which are known to inactivate oxytocin and vasopressin by releasing C-terminal dipeptide amides [48], our results do not suggest that such an enzyme action occurs with substance P.

Detailed structure-activity relationship studies of analogues of substance P have indicated the importance of its C-terminal pentapeptide structure and the integrity of its C-terminal amide for the expression of many of its biological actions [49]. Although C-terminal deamidating enzymes are known to occur and may be responsible for inactivating thyrotropin-releasing hormone [12], there is no evidence that substance P is inactivated by deamidation [19, 30]. More recent studies on a thyroliberin-deamidating enzyme purified from rat [50] and bovine [22] brain have shown that this enzyme can also cleave other neuropeptidades such as luliberin, angiotensin I and neurotensin on the carboxyl side of their proline residues, and hence is referred to as a 'post-proline cleaving enzyme'. The bovine brain post-proline cleaving enzyme was weakly active on substance P, cleaving the peptide to an N-terminal tetrapeptide and a C-terminal heptapeptide [23]. This enzyme, like the prolyl endopeptidase purified by Orlowski et al. [21], however, degrades but does not inactivate substance P, as the C-terminal fragments produced by these enzymes remain biologically very active. Such an enzyme could play a role in substance P inactivation only if it acted in concert with other enzymes such as aminopeptidases. On the other hand, the substance-P-degrading enzyme that we have isolated from human brain could undoubtedly inactive substance P since any one of the three cleavages it catalyses would give rise to biologically inactive fragments.

The substrate specificity of substance-P-degrading enzyme as revealed by the characteristic cleavage pattern has some features in common with those of chymotrypsin and thermolysin. Chymotrypsin has a preference for hydrolysing peptide bonds on the carboxyl side of hydrophobic amino acid residues and thermolysin has a preference for hydrolysing peptide bonds on the amino side of hydrophobic amino acid residues [51]. However, the fact that substance-P-degrading enzyme did not act on the chromogenic substrates of chymotrypsin and thermolysin suggest that it is different from these enzymes. Unlike chymotrypsin and other serine proteases, substance-P-degrading enzyme was not inhibited by the specific inhibitor diisopropylfluorophosphate (Table 5). Furthermore, we have found that α-chymotrypsin cleaved substance P between Phe^7-Phe^8 and Phe^8-Gly^9 bonds with a preference for the Phe^7-Phe^8 cleavage (70%) and did not cleave the Gln^6-Phe^7 bond at all (unpublished work). The thermolabile nature of substance-P-degrading enzyme also distinguishes it from thermolysin, which can withstand heating at 80 °C for up to an hour, and phosphoramidon, which is a very potent inhibitor of thermolysin, had no inhibitory action on substance-P-degrading enzyme even at 1 mM concentration (Table 5). We found that thermolysin cleaved substance P predominantly (90%) between Phe^7-Phe^8 and Gly^9-Leu^{10} and to a slight extent (10%) between Gln^6-Phe^7 and Phe^8-Gly^9 (unpublished work).

Since substance-P-degrading enzyme was active in the pH range 7 – 9 and was strongly inhibited by metal-chelating agents (Table 5), while most inhibitors of thiol and carboxy endopeptidases were without effect, it can be classified as a neutral metalloenzyme. Although angiotensin-converting enzyme and enkephalin-degrading enzyme are two other well characterized neutral metalloenzymes found in mammalian brains [37, 52], they were separated from substance-P-degrading enzyme by the present purification procedure. Furthermore, unlike angiotensin-converting enzyme and enkephalin-degrading enzyme, substance-P-degrading enzyme was not inhibited by captopril and was only weakly inhibited by teprotide (IC_{50} = 1.8 mM). The tetrapeptides Tyr-Gly-Gly-Phe and Gly-Gly-Phe-Leu, which are very effective in inhibiting the degradation of [^{3}H]Leu^5-enkephalin by enkephalin-degrading enzyme (IC_{50} = 0.1 mM) [52], were ineffective on substance-P-degrading enzyme.

The neutral pH optimum and the lack of effect of pepstatin (Table 5) distinguish substance-P-degrading enzyme from cathepsin D [25] and from the hypothalamic acid proteinase studied by Akopyan et al. [53].

Although the substance-P-degrading enzyme purified from human brain is a neutral endopeptidase with a molecular weight very similar to that reported for a soluble neutral endopeptidase (M_r = 40000) purified from bovine hypothalamus [20], the particulate human brain enzyme is clearly distinguishable from the cytosolic bovine enzyme. The soluble enzyme can be classified as a thiol enzyme as it is strongly

inhibited by *p*-chloromercuribenzoate and iodoacetate while its action is potentiated by dithiothreitol. It is also strongly inhibited by Zn^{2+} and Co^{2+} but not affected by the presence of EDTA even at 1 mM concentration. These data, together with our earlier finding on the crude rat brain enzyme preparation that the neutral substance-P-degrading enzyme activity in the cytosol differed from that in the membrane by differences in sensitivity to teprotide, p-chloromercuribenzoate and bacitracin inhibition, are consistent with the suggestion that the membrane-bound and cytosolic substance-P-degrading enzyme may represent different enzymes [14]. On the other hand, bacitracin which has been demonstrated to inhibit partially the degradation of substance P *in vivo* [54] and *in vitro* [13,14], was found to be without effect on purified substance-P-degrading enzyme (Table 5). Therefore, great care must be exercised in comparing pharmacological data from whole animal and crude enzyme preparations with purified enzyme studies, since in the former case one may be dealing with multiple enzyme systems having different sensitivities to different protease inhibitors.

Unlike other reported enzymes that degrade substance P, substance-P-degrading enzyme prefers substance P as its substrate among the many other neuropeptides tested (Table 6). Although the enzyme did not require the C-terminal amide and the N-terminal Arg and Lys for recognising substance P as its substrate, the relatively weak inhibitory potencies of its peptide fragments 1–9 and 4–11 in blocking the degradation of [^{3}H]substance P appear to reflect a need for some structural integrity in the substrate molecule for the enzyme to be fully effective. Among the naturally occurring peptides related to substance P, physalaemin and eledoisin were poor substrates compared with substance P and were hydrolysed only slowly by the electrophoretically purified enzyme. Moreover the cleavage patterns for these peptides by substance-P-degrading enzyme were somewhat different from that of substance P. This suggests that the behaviour of this enzyme towards different substrates may be similar to that observed in papain, where the enzyme has a large active site which contains various 'subsites' and it is the interactions between the amino acid residues on the substrate with these subsites that determines the specificity of the enzyme. In the case of papain, its active site can accommodate up to seven amino acid residues, indicating that the enzyme is capable of 'recognizing' a large portion of a peptide chain and this may be an explanation for 'unexpected' cleavages observed in proteins [55]. This may also explain the generally wide range of substrates that can be utilized by most peptidases. One would not expect even a regulatory peptidase to be completely specific for only one peptide substrate. It is the relative affinity towards different substrates that determines the specificity and the role of such enzymes under physiological conditions.

To summarise, we present here the first report on the purification to near homogeneity of a membrane-bound peptidase from human diencephalon, which has a high specificity and affinity towards substance P. It is a thermolabile, neutral metallo-endopeptidase with a molecular weight of 40000–50000. The enzyme inactivates substance P by cleaving the peptide between Gln^6-Phe^7, Phe^7-Phe^8 and Phe^8-Gly^9. In view of its high selectivity towards substance P, it is probably the best candidate to date as a peptidase likely to be important in the synaptic inactivation of substance P.

An important point is the relationship between cleavage sites of human-brain substance-P-degrading enzyme and those observed in animal-brain preparations. The close similarities between the cleavage patterns of [^{3}H]substance P by the purified enzyme from human brain and a synaptic membrane preparation from rat brain (Fig. 7 and 12) indicates the presence of a substance-P-degrading enzyme as the major peptidase responsible for degrading substance P in rat brain membranes, in a preparation known to be enriched in receptor binding sites for this peptide [29] (and unpublished work). This result also shows that substance-P-degrading enzyme is an excellent model for the identification of sensitive sites in substance P that must be protected for studies *in vitro* with this peptide or its synthetic analogues.

Since substance P is rapidly degraded *in vivo* and *in vitro*, it would be very useful to have enzymatically stable and bioactive analogues of substance P with which to extend studies on the functional role of substance P. Therefore, using the purified enzyme as a tool and knowing the enzymatically sensitive sites in the sequence of substance P, we attempted to design synthetic analogues of this peptide that may be stabilised against enzymatic digestion in the brain; the results of this study are reported in the following paper [28].

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C.-M. Lee, B. E. B. Sandberg, M. R. Hanley, and L. L. Iversen,
Neurochemical Pharmacology Unit of the Medical Research Council, Postgraduate Medical School, University of Cambridge, Hills Road, Cambridge, Great Britain, CB2 2QH