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ION-EXCHANGE CHROMATOGRAPHIC ASSAY OF PEPTIDASES ACTING ON THE C-TERMINAL HEXAPEPTIDE SEQUENCE OF SUBSTANCE P

RALPH LAUFER*, URI WORMSER and ZVI SELINGER
Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904 (Israel)
MICHAEL CHOREV
Department of Pharmaceutical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904 (Israel)
and
CHAIM GILON
Department of Organic Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904 (Israel)
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SUMMARY

A rapid and sensitive assay for peptidases acting on the C-terminal hexapeptide sequence of the neuropeptide substance P is described. The radiolabelled substrate, N^{α}-[^{125}I]desaminotyrosyl-substance P (6–11) is easily prepared by coupling commercially available radioiodinated Bolton–Hunter reagent with substance P (6–11). Peptidase activity is determined by quantitative separation of the degradation products from the intact substrate on small QAE-Sephadex columns. The assay has been used to measure degradation of the substrate by rat parotid and diencephalon slices. The peptidase activity in the latter system was inhibited by substance P and substance P fragments and was sensitive to metal chelators and thiol reagents.

INTRODUCTION

Substance P, a putative neurotransmitter[1], is rapidly inactivated *in vivo* and *in vitro* by the action of proteolytic enzymes present both in the central nervous system and in the periphery[2–7]. A membrane-bound substance P-degrading enzyme that seems to be involved in the physiological inactivation of the peptide has been purified from human brain[8]. This enzyme cleaves peptide bonds in the C-terminal part of the substance P molecule, leading to the formation of biologically inactive fragments.

Degradation of substance P has been previously measured by bioassay[4,6,7] radioimmunoassay[5] and electrophoretic and chromatographic procedures[4,8,9]. However, none of these techniques could be easily used as a routine assay of substance P hydrolysis, as they are either laborious and time consuming or underestimate the extent of cleavage of the substance P molecule. In this paper we describe a rapid and simple method for the assay of enzymes acting on the C-terminal part of substance P. The substrate, N^{α}[^{125}I]desaminotyrosyl-substance P (6–11) ([^{125}I]BH-SP$_{6-11}$)[1], a

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radioiodinated, biologically active analogue of the substance P C-terminal heptapeptide, is easily prepared by coupling substance P (6-11) (SP_{6-11})* with commercially available radioiodinated Bolton–Hunter reagent. Radiolabelled degradation products of the substrate are isolated by ion-exchange chromatography on small QAE-Sephadex columns, which can be regenerated and reused many times.

EXPERIMENTAL

Materials

Substance P, HEPES, gelatin, imidazole, dithiothreitol, phenylmethyl sulphonyl fluoride, *p*-chloromercuribenzoate, *p*-chloromercuriphenylsulphonate, *β*-hydroxybutyrate and glucose were obtained from Sigma (St. Louis, MO, U.S.A.). *o*-Phenanthroline and isobutyl chloroformate were purchased from Fluka (Buchs, Switzerland), hydroxyphenylpropionic acid and N-methylmorpholine from Aldrich (Milwaukee, WI, U.S.A.) and phosphoramidon and bestatin from the Peptide Institute (Osaka, Japan). QAE-Sephadex A-25 was obtained from Pharmacia (Uppsala, Sweden) and LiChroprep RP-18 from Merck (Darmstadt, F.R.G.). Monoiodinated ^{125}I-labelled Bolton–Hunter reagent (2000 Ci/mmol) was purchased from New England Nuclear. Captopril was kindly supplied by Dr. M. A. Ondetti (Squibb Institute, NJ, U.S.A.). SP_{4-11} and SP_{5-11} were generous gifts from Dr. S. Blumberg (Weizmann Institute of Science, Rehovot, Israel). The other substance P fragments were synthesized by the excess mixed anhydride method[10]. All solvents were distilled before use.

Synthesis of the non-radiolabelled substrate

3-(3-Iodo-4-hydroxyphenyl)propionic acid was prepared according to Michelot *et al.*[11] and purified by low-pressure liquid chromatography on a 100 × 1 cm I.D. column filled with LiChroprep RP-18 and eluted with methanol–0.05% trifluoroacetic acid (40:60, v/v) at a flow rate of 3–5 ml/min and a pressure of 100 psi. The product had m.p. 110–112°C (lit.[11], 112–113°C). Analysis: calculated for $C_9H_9O_3I$, C 36.97, H 3.08, I 43.49; found, C 36.72, H 3.23, I 43.10%.

3-(3-Iodo-4-hydroxyphenyl)propionic acid (23 mg) was dissolved in 0.3 ml of dimethylformamide and the solution was cooled to −18°C. N-Methylmorpholine (6 μl) was added, followed by isobutyl chloroformate (9 μl). After 2 min, a cold solution of HCl–SP_{6-11} (35 mg) in dimethylformamide (0.7 ml) was added, followed by N-methylmorpholine (8 μl). Water was added 90 min later and the product was collected by centrifugation and repeatedly washed with water. A 50-mg amount of crude product was obtained, part of which was purified by preparative HPLC on a Spectra-Physics LiChrosorb 10 RP-18 column (25 × 0.9 cm I.D.), eluted isocratically with methanol–0.05% trifluoroacetic acid (65:35, v/v) at a flow-rate of 3.7 ml/min and 40°C.

Amino acid analysis, performed on a LKB-4400 analyser, gave the following values: Glu, 1.00; Phe, 2.00; Gly, 0.95; Leu, 1.00; Met, 1.03.

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* Abbreviations used: IBH-SP_{6-11}, N$^{\alpha}$(iododesaminotyrosyl)-substance P (6–11) hexapeptide; SP_{6-11}, substance P (6–11) hexapeptide; Bolton–Hunter reagent, N-hydroxysuccinimidyl 3-(4-hydroxyphenyl)propionate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

[^{127}I]BH-SP$_{6-11}$ is a full agonist, one third as potent as substance P in the isolated guinea-pig ileum assay.

Synthesis of the radiolabelled substrate

The vial containing monoiodinated Bolton–Hunter reagent (1 mCi) was placed on ice and benzene was removed under a stream of dry nitrogen. A cooled solution of HCl–SP$_{6-11}$ (40 μg) in dimethylformamide, containing 10% of triethylamine (20 μl), was added, followed by a solution of imidazole (34 ng) in dimethylformamide (10 μl). After 19 h at 4°C, the raction was stopped by addition of 0.5 ml of 20 m*M* ammonium formate. Aliquots of 200 μl of the mixture were applied to a 0.6-ml column of LiChroprep RP-18 packed in a 2-ml plastic syringe. The column was washed with 60 ml of methanol–20 m*M* ammonium acetate (20:80, v/v). Fast elution was achieved by application of a light pressure of compressed air. [^{125}I]BH-SP$_{6-11}$ was eluted with four 1-ml portions of absolute methanol; 44% of the radioactivity had been incorporated into the product. HPLC analysis showed one major radioactive peak co-eluting with standard [^{127}I]BH-SP$_{6-11}$, thus establishing the identity of the radiolabelled compound (Fig. 1).

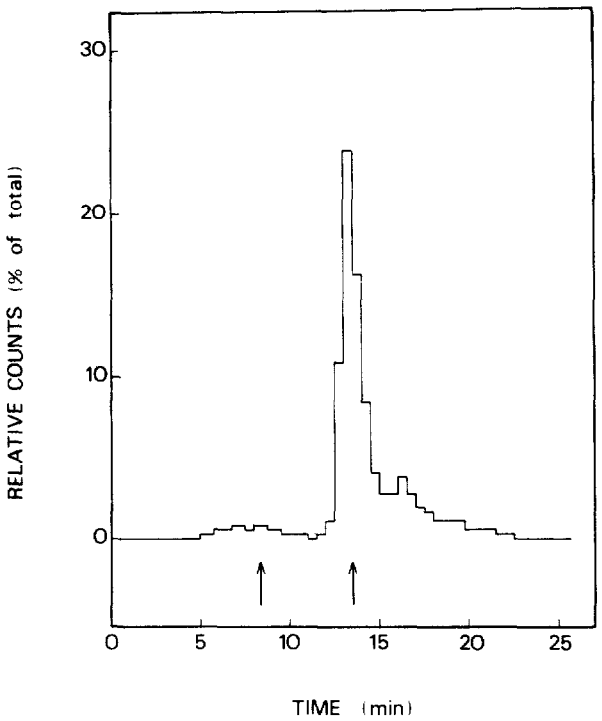

Fig. 1. An aliquot of the tracer (47,000 cpm) was injected on to a Whatman Partisil 10 ODS-3 (250 × 4.2 mm I.D.) column. The column was eluted at 40°C with methanol–0.05% trifluoroacetic acid (60:40, v/v) at a flow-rate of 1 ml/min. Fractions of the eluate (0.5 ml) were collected and counted. The recovery of applied radioactivity was 90%. Results are expressed as the percentage of counts in each fraction relative to the total radioactivity. Arrows from left to right indicate the retention times of the oxidation product of [^{125}I]BH-SP$_{6-11}$ (obtained by treatment of the tracer with H_2O_2 at 40°C for 45 min) and of standard [^{127}I]BH-SP$_{6-11}$.

[^{125}I]BH-SP$_{6-11}$ was stored in absolute methanol at −20°C, and was stable for at least 2 months.

Preparation of slices from rat parotid and diencephalon

Rat parotid slices were prepared as previously described[7]. Slices from rat diencephalon were obtained by cross-chopping sections of the diencephalon from male albino rats at 200-μm intervals with a McIlwain tissue chopper. The slices were suspended in oxygenated Krebs-Ringer–Hepes buffer (118 m*M* NaCl, 2.9 m*M* KCl, 2.5 m*M* $CaCl_2$, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, 25 m*M* HEPES, 5 m*M* β-hydroxybutyrate and 4 m*M* glucose), incubated at 30°C in a shaking water-bath, washed three times for 15 min with fresh buffer and finally suspended in the same buffer (2–4 mg/ml of protein).

Degradation assay

Incubation of [^{125}I]BH-SP$_{6-11}$ with slices from rat diencephalon or parotid. The assay was performed in duplicate glass vials containing, in a final volume of 0.25 ml (diencephalon system) or 1 ml (parotid system), Krebs–Ringer–HEPES buffer, slices (diencephalon, 0.2–0.4 mg of protein; parotid, 10–15 mg of protein), gelatin (0.25 mg/ml), [^{125}I]BH-SP$_{6-11}$ (200,000–300,000 cpm/ml), [^{127}I]BH-SP$_{6-11}$ (0.1 μ*M*) and various additions as indicated under the separate experiments. The assay systems were pre-incubated at 30°C in a shaking water-bath for 10 min before initiation of the reaction by addition of IBH-SP$_{6-11}$. The reaction was terminated after 20 min (unless indicated otherwise) by placing the reaction vials in an ice-bath or by boiling for 10 min.

Isolation of degradation products. Aliquots of 50–125 μl of the incubation medium were applied to columns of QAE-Sephadex A-25 (0.8 ml) packed in 2-ml plastic syringes. The columns were first washed with 10 ml of absolute methanol and then eluted with 10 ml of 1 *N* NaCl directly into plastic vials which were counted in a Packard auto-gamma counter. Fast flow-rates through the columns were achieved by application of a light pressure of compressed air. The columns were regenerated by washing with 4 ml of 1 *N* NaCl, followed by 8 ml of water. The recovery of total radioactivity was quantitative. The same columns were reused many times.

Calculation of results. As the assay was performed at a substrate concentration well below the K_m of the proteolytic acitivity of the slice systems, the degradation reaction followed pseudo-first-order kinetics (see Results). In experiments with inhibitors, the rate constants in the presence and absence of inhibitor (k_I and k_0, respectively) were derived by dividing the observed first-order rate constant by the protein content of the slices. The percentage of inhibition by a given compound was calculated as 100 $(1 - k_I/k_0)$. IC_{50} is the concentration of inhibitor which reduces the degradation rate constant by 50%. Apparent K_m and V_{max} of the proteolytic activity of rat diencephalon slices were determined under conditions of initial velocity (less than 15% degradation of the substrate).

Protein determination. Slices were digested in 1% SDS, 1 *N* NaOH and the protein content was determined by a modification of the method of Lowry *et al.*[12] using bovine serum albumin as the standard.

Isolated guinea-pig ileum assay. This assay was performed as previously described[7].

High-performance liquid chromatography (HPLC). HPLC was performed using a Spectra-Physics SP 8000B instrument, equipped with a variable-wavelength UV monitor.

RESULTS

Separation of the substrate from its degradation products

Owing to its lack of charge at neutral pH, [^{125}I]SP_{6-11} is not retained by the anion exchanger QAE-Sephadex, and about 95% of the radioactivity applied to a 0.8-ml QAE-Sephadex column is eluted by absolute methanol. Only about 5% of the label remains on the column and is eluted with 1 *N* NaCl (Fig. 2). Degradation of [^{125}I]SP_{6-11} by proteolytic enzymes results in the formation of radiolabelled fragments bearing a free carboxyl group, which are adsorbed on QAE-Sephadex at neutral pH. After incubation of [^{125}I]BH-SP_{6-11} with slices from rat diencephalon, part of the label is retained by the resin and can be eluted with 1 *N* NaCl (Fig. 2).

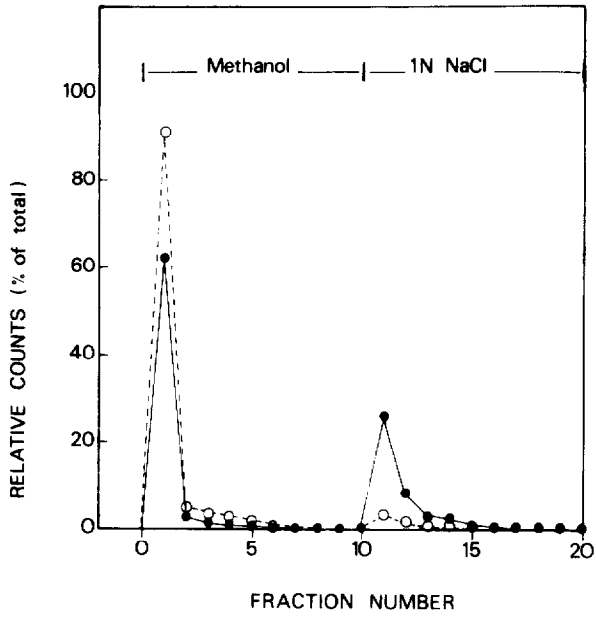

Fig. 2. Elution pattern of [^{125}I]BH-SP_{6-11} and its radiolabelled degradation products from a QAE-Sephadex column. [^{125}I]BH-SP_{6-11} (60,000 cpm) was incubated with (●) or without (○) slices from rat diencephalon as described under Experimental. After 20 min, 125 μl of the incubation medium were applied to a 0.8-ml QAE-Sephadex A-25 column. The column was washed with 2-ml fractions of absolute methanol and then with 1 *N* NaCl. Fractions of 4 ml were collected and counted. The recovery of applied radioactivity was quantitative.

HPLC analysis of the QAE-Sephadex column eluate fractions showed that the radioactivity eluting in the methanol fraction is associated solely with the substrate [^{125}I]BP-SP_{6-11} (Fig. 3A). The 1 *N* NaCl fraction does not contain intact substrate, but only its breakdown products; these more hydrophilic fragments are well separated by HPLC from standard [^{127}I]BH-SP_{6-11} (Fig. 3B). In another HPLC system

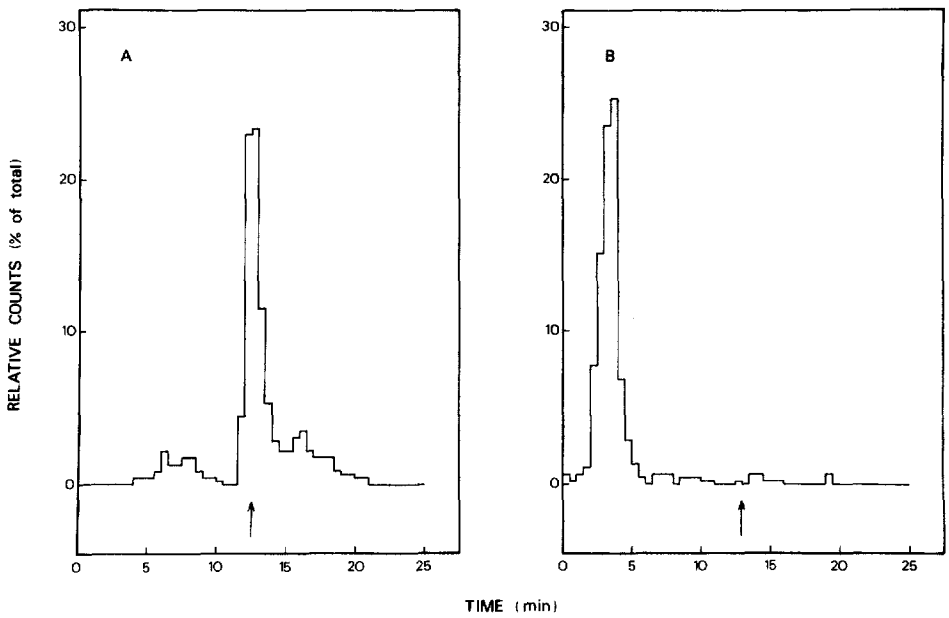

Fig. 3. HPLC analysis of eluate fractions from QAE-Sephadex columns. [^{125}I]BH-SP$_{6-11}$ was incubated with or without rat diencephalon slices and separated from its breakdown products as described under Experimental. Samples of the methanol and 1 *N* NaCl fractions collected from the QAE-Sephadex column were analysed by HPLC as described in Fig. 1. A, Methanol fraction; 4800 cpm were injected together with 20 nmol of carrier [^{127}I]BH-SP$_{6-11}$. The recovery of applied radioactivity was 93%. A Blank system (incubation without slices) gave the same HPLC elution pattern (not shown). B, 1 *N* NaCl fraction; 3700 cpm were injected and 97% of the radioactivity was recovered. Arrows indicate the retention time of standard [^{127}I]BH-SP$_{6-11}$.

they can be resolved into several peaks, none of which co-elutes with standard iodohydroxyphenylpropionic acid (Fig. 4). It can be concluded that the substrate is degraded by cleavages at several internal peptide bonds, and that chromatography on small QAE-Sephadex columns separates [^{125}I]BH-SP$_{6-11}$ from its radiolabelled fragments.

Degradation of [^{125}I]BH-SP$_{6-11}$ by slices of rat diencephalon and rat parotid

We applied the assay method to measure degradation of the substrate by rat diencephalon and parotid slices, two systems in which we have recently characterized substance P-degrading activities[7]. The degradation of [^{125}I]BH-SP$_{6-11}$ was assayed at a substrate concentration of 0.1 μM, which is well below the apparent K_m of the proteolytic activity (see below), and therefore the appearance of breakdown products with time followed pseudo-first-order kinetics (Fig. 5).

The rate of degradation in the rat diencephalon slice system increased linearly with increasing protein content of the slices over a range of 50–500 μg of protein per slice system. Kinetic constants of the proteolytic activity of rat diencephalon slices were determined under conditions of initial velocity (Fig. 6). The apparent K_m was found to be 30 μM and V_{max} was 250 pmol/min/mg of protein.

Substance P inhibited the degradation with an IC_{50} of 100 μM. The C-terminal

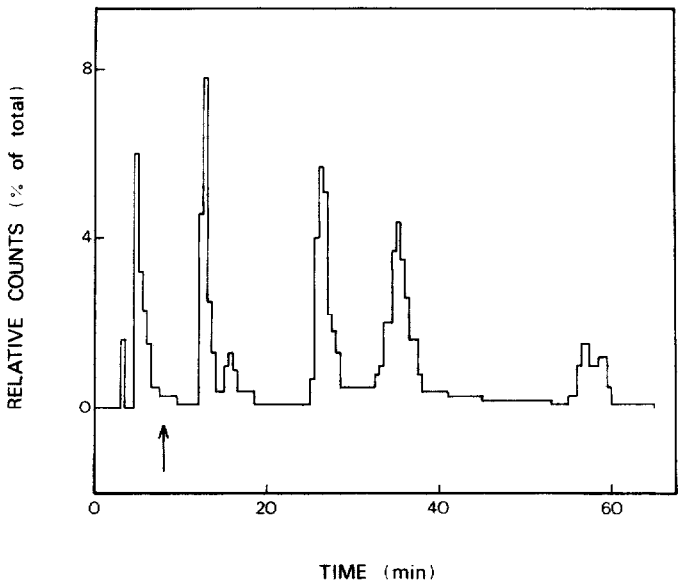

Fig. 4. HPLC analysis of degradation products of [^{125}I]BH-SP$_{6-11}$ formed in the rat diencephalon slice system. A sample (2300 cpm) of the 1 *N* NaCl fraction (see legend to Fig. 3) was analysed by HPLC. The column was eluted with methanol–0.05% trifluoroacetic acid (45:55, v/v) for 50 min, followed by a 10-min linear gradient up to 100% of methanol. The arrow indicates the retention time of standard 3-(3-iodo-4-hydroxyphenylpropionic acid).

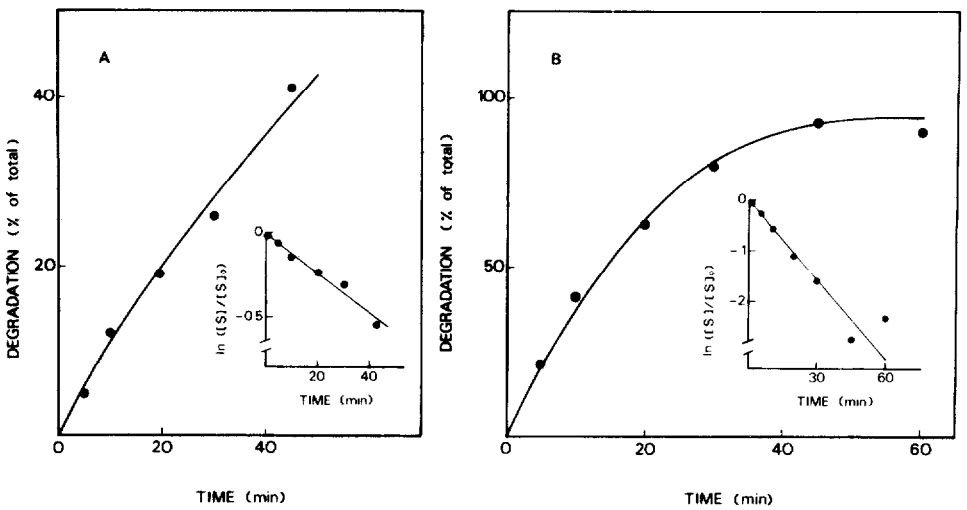

Fig. 5. Time course of degradation of [^{125}I]BH-SP$_{6-11}$ in the rat diencephalon and parotid slice systems. [^{125}I]BH-SP$_{6-11}$ was incubated with rat diencephalon (A) or parotid (B) slices and after various incubation periods the extent of degradation was determined as described under Experimental. The results are from experiments that were repeated at least twice, and are corrected for the blank value obtained at zero time, and for slight variations in the protein content of diencephalon slice systems (*ca.* 0.3 mg of protein per system). Inserts: semi-logarithmic plots of the degradation time course data; [S] and [S]$_0$ represent the substrate concentrations at the indicated time and at zero time, respectively.

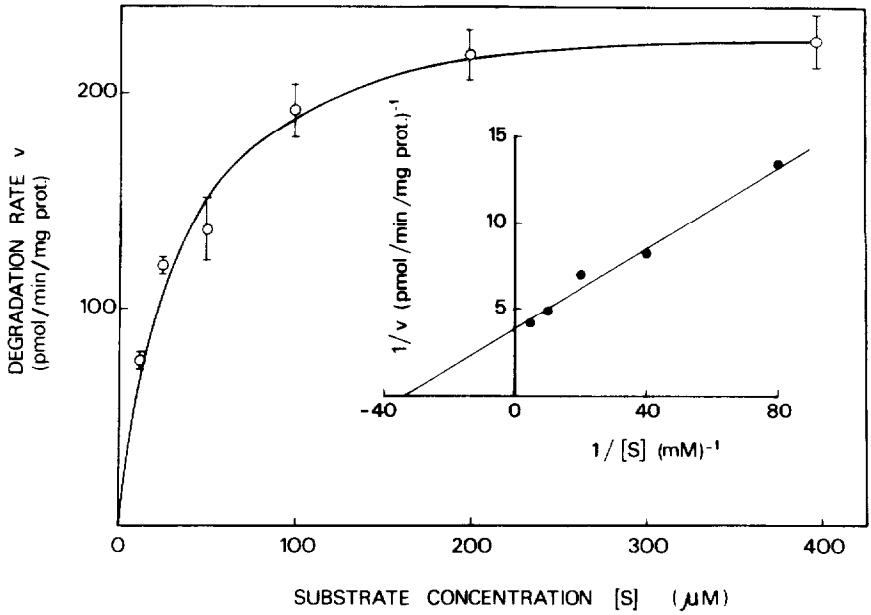

Fig. 6. Kinetic analysis of [^{125}I]BH-SP$_{6-11}$ degradation in the rat diencephalon slice system. Degradation of [^{125}I]BH-SP$_{6-11}$ was assayed in the presence of increasing concentrations of unlabelled [^{127}I]BH-SP$_{6-11}$. Insert: double reciprocal plot of the kinetic data, from which the apparent K_m (30 μM) and V_{max} (250 pmol/min/mg of protein) were calculated. The results represent mean values of two separate experiments, each performed in duplicate.

tetra- and pentapeptides are less effective inhibitors of the degrading activity than the C-terminal hexa-, hepta- and octapeptides and substance P itself. At a concentration of 400 μM, substance P, SP$_{4-11}$, SP$_{5-11}$, SP$_{6-11}$, SP$_{7-11}$ and SP$_{8-11}$ reduced the degradation rate by 65, 75, 66, 77, 51 and 38%, respectively*. Recognition of the substrate by the proteolytic enzymes in rat diencephalon slices therefore seems to involve the phenylalanine residues of the substrate molecule.

The proteolytic activity was strongly inhibited by both metal chelating agents (*o*-phenanthroline, dithiothreitol) and thiol reagents (*p*-chloromercuribenzoate, *p*-chloromercuriphenylsulphonate). The serine protease inhibitor phenylmethylsulphonyl fluoride, the angiotensin-converting enzyme inhibitor captopril[13], the enkephalinase inhibitor phosphoramidon[14] and the aminopeptidase inhibitor bestatin[15] had little or no effect.

DISCUSSION

C-Terminal fragments of substance P as short as the pentapeptide retain most of the biological activities of the full-length peptide[16]. Endopeptidases that inactivate substance P must therefore cleave peptide bonds in the C-terminal part of the mol-

* The data are mean values from at least two separate experiments, each performed in duplicate; standard error less than 15%.

ecule[8]. The present method for the assay of C-terminal substance P degradation offers a convenient alternative to existing procedures because of its ease and rapidity. Two major factors contribute to the simplicity of the assay.

First, the substrate, [^{125}I]BH-SP$_{6-11}$, a radioiodinated, biologically active analogue of the substance P C-terminal heptapeptide, is easily prepared by coupling commercially available radioiodinated Bolton–Hunter reagent to SP$_{6-11}$. It contains no free amino or carboxyl groups and is therefore stable to most exopeptidases. As the radiolabel is incorporated at the N-terminus of the molecule, hydrolysis of the substrate by endopeptidases results in the formation of radiolabelled fragments bearing a free carboxyl group, which can be quantitatively separated from the uncharged substrate by QAE-Sephadex chromatography. This principle may be used in the design of rapid degradation assays for other neuropeptides lacking a free carboxy terminus, such as luteinizing hormone-releasing hormone, vasopressin, oxytocin and others.

Second, the use of many small QAE-Sephadex columns, which are rapidly eluted by application of a light pressure of compressed air, permits the processing of a large number of samples in a short time. A typical experiment with forty slice systems can be performed in a single afternoon, the most laborious part being the preparation of the slices. An additional advantage of the chromatographic procedure is that the columns are readily regenerated and can be reused many times.

We have used this method to assay degradation of the substrate by rat parotid and diencephalon slices. In the latter system, [^{125}I]BH-SP$_{6-11}$ is cleaved by endopeptidases into several as yet unidentified products. The degradation was effectively inhibited by substance P and its C-terminal fragments, indicating that the enzymes cleaving the substrate are also acting on substance P. The breakdown was inhibited by metal chelating agents and thiol agents, but it was not significantly affected by inhibitors of serine proteases, aminopeptidases, angiotensin-converting enzyme and enkephalinase. These characteristics are similar to those of the substance P-degrading enzyme described by Lee *et al.*[8] and the enzymes degrading [pyroglutamyl^{6}]SP$_{6-11}$ in rat hypothalamic slices[7], both of which act on the C-terminal part of the substance P molecule. We hope that the assay will prove valuable for monitoring the purification of substance P-degrading enzymes and for screening potential inhibitors of these enzymes.

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