A peptidase that cleaved neurotensin at the Pro^{10-13}-Tyr^{11} peptide bond, leading to the formation of neurotensin-(1-10) and neurotensin-(11-13), was purified nearly to homogeneity from rat brain synaptic membranes. The enzyme appeared to be monomeric with a molecular weight of about 70,000-75,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and high pressure liquid chromatography filtration. Isoelectrofocusing indicated a pI of 5.9-6. The purified peptidase could be classified as a neutral metallopeptidase with respect to its sensitivity to pH and metal chelators. Thiol-blocking agents and acidic and serine protease inhibitors had no effect.

Several authors have shown that neurotensin was destroyed after exposure to brain tissues (6) or homogenates (7). We have recently proposed a model for the inactivation of neurotensin by highly purified rat brain synaptic membranes (8-10) that primary inactivating cleavages occurred at the Arg^{2}-Arg^{5}, Pro^{10}-Tyr^{11}, and Tyr^{11}-Ile^{12} bonds, leading to the formation of the biologically inactive fragments neurotensin-(1-8), neurotensin-(1-10), neurotensin-(1-11), neurotensin-(11-13), and neurotensin-(9-13). We have clearly demonstrated that a recently purified rat brain soluble metalloendopeptidase (11) and endopeptidase 24-11 were totally responsible for the primary cleavages at the Arg^{2}-Arg^{5} and Tyr^{11}-Ile^{12} peptide bonds, respectively (8, 9). Although endopeptidase 24-11 was also shown to participate to the cleavage at the Pro^{10-11} bond, we established that the major contribution at this site was due to a peptidase not identified but clearly distinct from proline endopeptidase (10). Several data suggest that cleavage of the Pro^{10-11} bond is a key event in the physiological process that leads to neurotensin inactivation. Thus, several authors have described the enhanced potency in the central nervous system, in vivo, of neurotensin analogues in which the tyrosyl residue in position 11 was substituted by a D-amino acid (12-14). Furthermore, we demonstrated that these modified analogues were totally resistant to degradation to brain tissues in vitro and in vivo (15). Here we report on the purification and characterization from rat brain synaptic membranes of the as yet unidentified peptidase activity that cleaves neurotensin at the Pro^{10-11} bond.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Purification of the Peptidase—Table I shows that the purification procedure led to a 541-fold enrichment of the peptidase with an overall yield of 15%. Chromatography of the Triton X-100-solubilized material on DEAE-Trisacryl resin indicated that the peptidase was quantitatively retained on the column and eluted as a single peak of neurotensin-(1-10)-generating activity by increasing the ionic strength (Fig. 1). At this step, the pooled fractions kept for further purification were totally resolved from aminopeptidase M, proline endopeptidase, and post-proline dipeptidylaminopeptidase as illustrated in Fig. 1 by the profiles of Leu-7AMC, Z-Gly-Pro-7AMC, and Gly-Pro-7AMC-hydrolyzing activities, respectively.

1 Portions of this paper (including "Experimental Procedures") are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-604, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: Z, benzoyloxycarbonyl; 7AMC, 4-methyl coumarinyl-7-amide; MES, 2-(N-morpholino)ethanesulfonic acid; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; HPLC, high pressure liquid chromatography; CPE, carboxyphenethyl; pAB, para-aminobenzoate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
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Table I

Purification of the neurotensin-degrading metallopeptidase

Activity was isolated from the homogenate (540 mg of protein) of purified rat brain synaptic membranes. Neurotensin-(1-10)-generating activity was detected by HPLC as described under "Experimental Procedures," and the amount of neurotensin-(1-10) was estimated from the absorbance of known quantities of synthetic neurotensin-(1-10).

| Step                      | Protein | Specific activity | Recovery | Purification factor |
|---------------------------|---------|-------------------|----------|---------------------|
| Homogenate                | 540     | 2.51              | 100      | 1                   |
| Triton X-100-solubilized fraction | 457    | 3                 | 100      | 1.2                 |
| Pooled DEAE fractions     | 4.5     | 212               | 71       | 84                  |
| Dialyzed pooled DEAE fractions | 3.5   | 234               | 60       | 93                  |
| Pooled hydroxylapatite fractions | 0.15  | 1560              | 15       | 541                 |

Fig. 1. DEAE chromatography of Triton X-100-solubilized rat brain synaptic membranes. Solubilized material was loaded on a DEAE-Trisacryl column previously equilibrated with 20 mM Tris/HCl, pH 7.5. The resin was washed with the same buffer until the absorbance (Abs) returned to baseline; then activity was eluted with a linear gradient of NaCl as described under "Experimental Procedures." All fractions were tested for neurotensin degradation and fluorimetrically assayed for aminopeptidase M (U), basic aminopeptidase (W), post-proline dipeptidylaminopeptidase (-), and proline endopeptidase (-) activities. The bar represents the pooled fractions of neurotensin-(1-10)-generating activity kept for further purification.

Characterization of the Peptidase - The routine assay described under "Experimental Procedures" was used to further characterize the purified peptidase. The enzyme activity was maximal between pH 7 and 8 (not shown). An Eadie plot for the hydrolysis of [3H]neurotensin by the peptidase is presented in Fig. 7. This plot was linear as expected for a pure enzyme and gave a \( K_m \) value of 2.6 \( \mu \text{M} \).

The effect of various inhibitors is summarized in Table II. The peptidase was very sensitive to metal chelators and was fully inhibited by 1 mM \( \alpha \)-phenanthroline. The serine protease inhibitors diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and benzamidine were ineffective as well as the thiol-blocking agent iodoacetamide and the acidic protease inhibitor pepstatin. Furthermore, \( \beta \)-mercaptoethanol had no effect on [3H]neurotensin-degrading activity. Several specific inhibitors of purified peptidases were tested against the enzyme. Captopril, the potent angiotensin-converting enzyme inhibitor (23), and thiorphan, the endopeptidase 24-11 inhibitor (24), were found totally inactive at micromolar concentrations. Furthermore, the activity was totally insensitive to 10 \( \mu \text{M} \) of the aminopeptidase inhibitor bestatin (25) and was not characterized by a pI of 5.9-6 and a \( M_r \) of 72,000, but also revealed a few minor faint spots of lower \( M_r \) and slightly different pI (Fig. 6).

Molecular Weight, Purity, and Functional Properties - HPLC filtration of the purified peptidase allowed us to determine a molecular weight of about 75,000 for the native enzyme (Fig. 4). This value was in good agreement with that deduced from one-dimensional polyacrylamide gel electrophoresis (PAGE) in dissociating conditions that revealed a single band with an apparent \( M_r \) of 72,000 (Fig. 5). Fig. 6 shows a two-dimensional SDS-PAGE analysis of a higher amount of post-hydroxylapatite proteins. The pattern confirmed the major spot characterized by a pI of 5.9-6 and a \( M_r \) of 72,000, but also revealed a few minor faint spots of lower \( M_r \) and slightly different pI (Fig. 6).

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FIG. 2. Hydroxylapatite of the pooled active fractions from DEAE chromatography. Pooled fractions from DEAE chromatography were dialyzed against 1 mM KH₂PO₄, pH 7.4, and loaded onto a 10 × 60-mm hydroxylapatite column previously equilibrated with the same buffer. Washing and elution procedures were carried out as described under "Experimental Procedures." All fractions were tested for neurotensin degradation and basic aminopeptidase activity (M). At this step, neurotensin-(1-10)-generating activity was free of other neurotensin-degrading peptidases and eluted as a single peak of absorbance. The bar represents the pool of active fractions kept for the characterization of the peptidase.

FIG. 3. HPLC of neurotensin hydrolysis by pooled active fractions from hydroxylapatite. Neurotensin (2 nmol) was incubated for 30 min at 37 °C with 90 ng of the purified enzyme. Degradation products were separated by HPLC as described under "Experimental Procedures" except that the gradient of acetonitrile was linear. Three products eluting with the retention times of neurotensin-(1-10), neurotensin-(11-13), and neurotensin were detected indicating a single cleavage of the neurotensin molecule.

FIG. 4. HPLC filtration of the active fractions from hydroxylapatite. An aliquot of posthydroxylapatite pool was concentrated on ultrafiltration cell, injected onto two connected columns, and eluted as described under "Experimental Procedures." All fractions were assayed for neurotensin degradation. Total (V₁) and void (V₀) volumes were determined by injecting 10 nmol of neurotensin-(11-13) and dextran blue, respectively. Molecular weight of the native enzyme was deduced from a standard curve established from the retention times of various markers of known molecular weights.

affected by a concentration of 1 μM Z-Pro-Proinal, a specific inhibitor of proline endopeptidase (26). Finally, CPE-Ala-Ala-Phe-pAB, an inhibitor recently developed against a rat brain soluble metalloendopeptidase that cleaves neurotensin at the Arg⁸-Arg⁹ peptide bond produced a 30% inhibition at 10 μM, a concentration 10-fold higher than the Kᵢ for its enzyme.

The relative abilities of neurotensin partial sequences to inhibit [³H]neurotensin degradation are illustrated in Table III. Neurotensin-(9-13) was the shortest C-terminal fragment with full inhibitory potency (compare compounds 2-5). Neurotensin-(10-13) and neurotensin-(11-13) were about 13-15-fold less active than neurotensin, while neurotensin-(12-13) was unable to inhibit [³H]neurotensin degradation. On the
FIG. 5. One-dimensional SDS-PAGE of purified enzyme. Aliquots of homogenate (100 μg) and posthydroxylapatite pool (7μg) were evaporated and resuspended in 100 μl of sodium phosphate buffer (pH 7.5) containing 2% of sodium dodecyl sulfate and 5% of β-mercaptoethanol. Boiled samples were then electrophoresed in 8% acrylamide gel slabs and the proteins revealed as described under “Experimental Procedures.” Lane a, homogenate; lane c, posthydroxylapatite pool; lanes b and d, standards.

FIG. 6. Two-dimensional SDS-PAGE of purified enzyme. Hydroxylapatite proteins (30 μg) were loaded in 4% acrylamide gels containing 2% ampholine, pH 5-7, and 8 M urea. Isolelectric focusing (IEF) was carried out as described under “Experimental Procedures.” Drawn circles indicate the migrating pattern of standard proteins (bovine serum albumin (a), ovalbumin (b), and carbonic anhydrase (c)) run in parallel gels in the same conditions. The second dimension in sodium dodecyl sulfate was performed in slab gels with 10% acrylamide.

other hand, shortening the C-terminal end of the neurotensin molecule strongly decreased the potency of the analogues (see sequences 9-12, Table III). Thus, neurotensin-(1-12) was 4-fold less potent than neurotensin while further removal of C-terminal amino acids (neurotensin-(1-11) and neurotensin-(1-10) led to about 30-fold less potent compounds. Neurotensin-(1-8) was unable to inhibit [3H]neurotensin degradation.

In order to assess the specificity of the peptidase for neurotensin, a series of analogues with structural or conformational modifications were tested as competitors of [3H]neurotensin degradation. Table IV indicates that tyrosine in position 11 could be replaced by an aromatic residue (Trp, Phe) without alteration of the IC₅₀ (compare 1, 11, and 12), whereas substitution by the corresponding D-amino acid strongly affected the inhibitory potencies (compare analogues 1, 11, and 12 to 13-16). Investigations at the sites 8 and 9 clearly indicated that the charge of the amino acid was not an important requirement for the expression of the activity...
TABLE III
Effect of neurotensin partial sequences on [3H]neurotensin degradation

[3H]Neurotensin was incubated as described under “Experimental Procedures” with 90 ng of peptidase, for 30 min at 37°C in the absence (control) or in the presence of 7-9 concentrations of various neurotensin partial sequences. Activity was monitored by the two-step chromatography on SPC25 Sephadex (see “Experimental Procedures”). IC50 values correspond to the concentration of peptide that half-inhibited control [3H]neurotensin degradation. Data are the mean values of two or three determinations obtained with two distinct preparations of posthydroxylapatite activity.

| No. | Sequence | IC50 (µM) |
|-----|----------|-----------|
| 1   | Neurotensin | 2.6       |
| 2   | Neurotensin-(4-13) | 4.5       |
| 3   | Neurotensin-(6-13) | 4         |
| 4   | Neurotensin-(8-13) | 1.5       |
| 5   | Neurotensin-(9-13) | 2.8       |
| 6   | Neurotensin-(10-13) | 35       |
| 7   | Neurotensin-(11-13) | 40       |
| 8   | Neurotensin-(12-13) | >1000    |
| 9   | Neurotensin-(1-12) | 11       |
| 10  | Neurotensin-(1-11) | 80       |
| 11  | Neurotensin-(1-10) | 70       |
| 12  | Neurotensin-(1-8) | >100     |

TABLE IV
Effect of modified neurotensin analogues on [3H]neurotensin degradation

[3H]Neurotensin was incubated as described under “Experimental Procedures” with 90 ng of peptidase, for 30 min at 37°C, in the absence (control) or in the presence of 7 increasing concentrations of various neurotensin partial sequences. Activity was monitored by the two-step chromatography on SPC25 Sephadex (see “Experimental Procedures”). IC50 values correspond to the concentration of analogue that half-inhibited control [3H]neurotensin degradation. Data are the mean values of two or three determinations obtained with two distinct preparations of posthydroxylapatite activity.

| No. | Analogue       | IC50 (µM) |
|-----|----------------|-----------|
| 1   | Neurotensin    | 2.6       |
| 2   | Acetyl[Ala'3]neurotensin-(8-13) | 1         |
| 3   | [D-Phe'6]neurotensin-(8-13) | 2.5       |
| 4   | [Lys'5]neurotensin-(8-13) | 2         |
| 5   | [D-Arg'9]neurotensin-(8-13) | 4         |
| 6   | [Ala'2]neurotensin-(8-13) | 2         |
| 7   | [D-Trp'11]neurotensin | 2.7       |
| 8   | [D-Tyr'11]neurotensin-(8-13) | 15        |
| 9   | [D-Phe'11]neurotensin-(8-13) | 37        |
| 10  | [D-Leu'11]neurotensin-(8-13) | 59        |
| 11  | [Ala'12]neurotensin-(8-13) | >100      |
| 12  | Acetyl[Phe'2]neurotensin-(8-13) | 5         |
| 13  | [Ala'12]neurotensin-(8-13) | 1         |

TABLE V
Effect of natural peptides on [3H]neurotensin degradation

[3H]Neurotensin was incubated as described under “Experimental Procedures” with 90 ng of peptidase, for 30 min at 37°C, in the absence (control) or in the presence of 7-9 concentrations of various natural peptides. Activity was monitored by the two-step chromatography on SPC25 Sephadex (see “Experimental Procedures”). IC50 values correspond to the concentration of peptide that half-inhibited control [3H]neurotensin degradation. Data are the mean values of two or three determinations obtained with two distinct preparations of posthydroxylapatite activity.

| No. | Peptide          | IC50 (µM) |
|-----|------------------|-----------|
| 1   | Neurotensin      | 2.6       |
| 2   | Neuremedin N     | 2.5       |
| 3   | Xeopsin          | 3         |
| 4   | Angiotensin I    | 4         |
| 5   | Lys'5-Asn'6-neurotensin-(8-13) | 11       |
| 6   | Substance P      | 25        |
| 7   | α-MSH            | 60        |
| 8   | Somatostatin     | 70        |
| 9   | Tryptsin         | 80        |
| 10  | Bombesin         | 80        |
| 11  | Met-enkephalin   | >100      |
| 12  | [Arg'3]vasopressin | >100     |
| 13  | Oxytocin         | >100      |
| 14  | TRH              | >100      |
| 15  | LHRH             | >100      |
| 16  | Eledoisin        | >100      |
| 17  | Physalaemin      | >100      |
| 18  | VIP              | >100      |
| 19  | GIP              | >100      |
| 20  | Gastrin          | >100      |

since citrullines could efficiently replace arginyl 8 and 9 residues (analogues 4 and 7). A basic lysyl residue in position 9 also led to a fully potent analogue (compound 6). By contrast [D-Arg'9]neurotensin, [D-Arg'9]neurotensin, and [D-Arg'9]neurotensin clearly demonstrated a stereospecificity of the peptidase for the residues in positions 8 and 9 (compounds 5, 8, and 9). Finally, neither acetylation of neurotensin-(8-13) (compound 2) nor substitution of Ile'6 and Leu'13 by alanyl residues induced significant changes in the inhibitory potencies (compounds 17 and 19).

The specificity of the peptidase toward biologically active peptides is illustrated in Table V. Angiotensin I and the neurotensin-related peptides, neumedin N (27) and xenopsin (28), were the only natural peptides with full inhibitory potency. The chicken neurotensin variant Lys'5-Asn'6-neurotensin-(8-13) (29) and substance P were, respectively, 4- and 10-fold less potent than neurotensin. HPLC of the peptides 2-6 after prolonged exposure to the enzyme clearly showed a marked decrease of intact peptide and, therefore, indicated that they were substrates of the peptidase (not shown). The sites of cleavages of the neurotensin-related peptides were deduced from the retention times of the degradation-products after HPLC separation. Table VI indicates that hydrolysis by the peptidase always occurred at the Pro-Tyr (neurotensin, neuremedin N, and Lys'5-Asn'6-neurotensin-(8-13)), or Pro-Trp (xenopsin) peptidyl bonds. The other natural peptides (7-20) displayed very little, if any, ability to inhibit [3H] neurotensin degradation. None of them was found to behave as a substrate of the enzyme (not shown).

DISCUSSION
The present paper reports on the purification and characterization of rat brain synaptic membranes of a neurotensin-degrading metallopeptidase that cleaves neurotensin at the Pro10-Tyr11 peptidyl bond, yielding the biologically inactive fragments neurotensin-(1-10) and neurotensin-(11-13).

The purification procedure leads to a 541-fold enrichment of the peptidase with a yield of 15% and affords a nearly homogeneous protein as judged by one- and two-dimensional SDS-PAGE. The apparent molecular weight of the native enzyme as determined by HPLC filtration is 75,000. This value in close agreement with that deduced from SDS-PAGE analysis (72,000). These results indicate that the peptidase is a monomer.

In order to facilitate the characterization of the purified peptidase, it was necessary to set up a suitable routine assay.
of neurotensin degradation. Tritiated neurotensin was used as substrate. We showed previously (9) that radioactivity was equally distributed on both tyrosyl residues (positions 3 and 11 of the neurotensin molecule). Therefore, the degradation products generated by the purified peptidase, i.e. neurotensin-(1-10) and neurotensin-(11-13), contain equal amounts of radioactivity. Based on the fact that neurotensin-(11-13) has no net positive charge whereas neurotensin-(1-10) and neurotensin are positively charged at neutral pH, it was possible to separate [3H]neurotensin-(11-13) from [3H]neurotensin-(1-10) and [3H]neurotensin. Using this procedure, several properties of the purified peptidase were investigated.

The enzyme is optimally active in the pH range between 7 and 8 and is readily inhibited by metal chelators such as EDTA and o-phenanthroline. By contrast, the peptidase is totally insensitive to thiol-blocking agents, acidic and serine protease inhibitors (Table II), and can be classified as a neutral metallopeptidase.

The peptidase displays a rather high affinity for neurotensin (Km = 2.6 μM, Fig. 7). From the data obtained with a series of neurotensin partial sequences, it is clear that the pentapeptide neurotensin-(9-13) is the shortest fragment with full [3H]neurotensin degradation inhibitory potency (Table III). Shortening the C-terminal end of the neurotensin molecule (neurotensin-(1-12), neurotensin-(1-11), neurotensin-(1-10), and neurotensin-(1-8)) gradually reduces the potency of neurotensin (Table III). This may indicate that a C-terminal extension of at least three amino acids from the scissile Pro''-Tyr" bond is essential to maintain the sequence fully active. If this requirement is respected, the peptidase is not sensitive to the replacement of the amino acids in positions 12 (Ile) and 13 (Leu) by less hydrophobic residues (Table II), and can be classified as a neutral metallopeptidase.

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(115,300) and dissociated in two subunits under denaturing conditions (33, 34). Postproline dipeptidyl aminopeptidase (dipeptidyl peptidase IV) (EC 3.4.14.5) has a molecular weight in the neighborhood of 200,000, and a free N-terminal amino acid is a crucial requirement for the expression of the activity (35).

Finally, three other peptidases have been isolated from mammalian brains or pituitaries and were shown to cleave neurotensin. However, their sites of cleavage on the neurotensin molecule together with their sensitivity to inhibitors make easy their distinction from the present enzyme. Indeed angiotensin-converting enzyme releases the C-terminal dipeptide of neurotensin (cleavage at the Tyr11-Ile12 bond) (30) and is totally inhibited by a concentration of 10^{-5} M of its specific inhibitor captopril (23), a dose totally ineffective on the neurotensin-inactivating mechanism involving the Pro10-Tyr11 peptide bonds. The enzyme also hydrolyzes other peptides such as luteinizing hormone releasing hormone (38) that was not found to behave as a substrate for the purified peptidase described here (Table V).

With respect to its original properties including both specificity and sensitivity to inhibitors, it seems reasonable to assume that we have isolated and characterized a novel rat brain synaptic peptidase that could be named neurotensin-degrading neutral metallopeptidase. The physiological importance of this enzyme in the inactivation of neurotensin remains to be established. However, it is of interest to underline the fact that the neurotensin analogues modified in position 11 by substitution with a d-amino acid [(D-Tyr^{11})]neurotensin, [D-Phe^{11}]neurotensin] are resistant to degradation by the peptidase as well as other peptides which fact that they totally resist degradation in vivo, after stereotaxic intracerebroventricular injection in the rat (15). The fact that these analogues were found to be more potent than neurotensin in eliciting central effects following intracerebroventricular injection in the rat (12-14), although they are very poor agonists of neurotensin receptors in vitro (39), supports the hypothesis of a physiological neurotensin-inactivating mechanism involving the Pro^{10}-Tyr^{11} peptidyl bond. These data should provide a basis for a strategy consisting of the development of both neurotensin analogues resistant to degradation and inhibitors of the neurotensin-degrading neutral metallopeptidase in order to assess its physiological contribution in the inactivation of neurotensin in the rat central nervous system.

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A Neurotensin-degrading Metallopeptidase from Rat Brain

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Supplementary material:

PURIFICATION AND CHARACTERIZATION OF A NOVEL NEUROTENSIN DEGRADING PEPTIDASE FROM RAT BRAIN SYNAPTIC MEMBRANES.

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EXPERIMENTAL PROCEDURES

Materials: 4-Methyl-coumaril-7-carboxyl, 7-carboxyamide (7-SCAM), Arg-7AM, Leu-7AM, 2-Arg-7AM, Gly-Pro-7AM, Glu-Pro-7AM, Arg-Pro-7AM, amine, benzyl, ethylamine, histamine, HCl, arginine, histidine, phospho-CoA, and arginine (Cala. 4.9 PAM were kindly provided by Dr. G. Will and M. Sandberg (Department of Pharmacology, Mount Sinai School of Medicine, New York, Tohoku University, Japan, respectively). Neurotensin and all other natural peptides were from Peninsula Laboratories (CA, USA). Pre-pro-proline (N-benzyloxycarbonyl-valyl-lysyl-Pro) and CPE-Ala-Ala-Phe-DAB (carboxyphenyl-alanine-alanine-phenylalanine-para-aminobenzoate) were generously provided by Dr. J. M. Wilson and M. Orlowski (Department of Pharmacology, University of Pennsylvania, Philadelphia, PA). All other inhibitors were from Sigma Chemical Co. (St. Louis, MO). [2-14C]-arginine, [14C]-neurotensin, [7-14C]-neurotensin, [2-14C]-neurotensin were kindly provided by Dr. H. Vierke (Department of Physiology and Pharmacology, Faculty of Medicine, Umeå, Sweden). All other neuropeptides and analogues were synthesized and kindly provided by Dr. S. Gruber and J. Van Vliet Woudtoorn (Faculty of Medicine, Utrecht, Netherlands). Tritiated neurotensin (510-critter, 510-MN) was from New England Nuclear (Boston, MA).

Preparation of rat brain synaptic membranes. Male Sprague-Dawley rats (200-250 g) were decapitated. Male rats were removed and synaptic membranes were prepared following the procedure of Jones and Matus (1966). The resulting pellets were stored at -80°C.

Purification of the enzyme

Preparation and stabilization. Synaptic membranes (30 mg of proteins) corresponding to 30 rat brains were resuspended in 30 ml of ice-cold tris(hydroxymethyl)amino-methane (Tris)-buffered saline (206/9) and centrifuged at 170,000 g for 30 min. The supernatant was discarded, and the resulting pellet was washed twice following the same procedure. The final pellet was resuspended in 10 ml of 0.1 M tris(hydroxymethyl)amino-methane (Tris)-buffered saline (206/9) containing 1% Triton X-100 and gently stirred for 60 min. The suspension was then centrifuged at 12,000 g for 10 min, and the supernatant kept for further purification. All steps were carried out at 4°C unless otherwise indicated.

Ion exchange chromatography on CM Sepharose. The supernatant was applied to a CM Sepharose column (2.5 x 50 cm) and eluted with 0.01 M Tris(hydroxymethyl)amino-methane (Tris)-buffered saline (206/9) containing 0.01% Triton X-100 at a flow rate of 2.5 ml/min. Ten fractions of 2 ml each were collected.

Hydrophobic interaction. Activities were loaded at a flow rate of 10 ml/min onto a hydrophobic interaction column (2.5 x 50 cm) previously equilibrated with 0.01 M Tris(hydroxymethyl)amino-methane-Tris-buffered saline containing 0.01% Triton X-100 at a flow rate of 2.5 ml/min. The column was then washed with the same buffer and activity was eluted with a linear gradient established between 200 ml of 0.01 M Tris(hydroxymethyl)amino-methane-Tris-buffered saline containing 0.01% Triton X-100 and 200 ml of the same buffer containing 0.02 M trifluoroacetic acid (TFA) at a flow rate of 2.5 ml/min. Ten fractions of 2 ml each were collected and assayed for neurotensin-degrading activity.

FRACTIONS OF THE CM-Sephase were applied onto a TFF-10 cation-exchange column (2.5 x 50 cm) at a flow rate of 2.5 ml/min. Ten fractions of 2 ml each were collected and assayed for neurotensin-degrading activity.

Results

One dimensiontral sodium dodecyl sulfate (SDS) gel electrophoresis. All buffers were prepared in Tris(hydroxymethyl)amino-methane-Tris-buffered saline containing 0.01% Triton X-100 at a flow rate of 2.5 ml/min. Ten fractions of 2 ml each were collected and assayed for neurotensin-degrading activity.

2-Dimensional PAGE. All buffers were prepared in Tris(hydroxymethyl)amino-methane-Tris-buffered saline containing 0.01% Triton X-100 at a flow rate of 2.5 ml/min. Ten fractions of 2 ml each were collected and assayed for neurotensin-degrading activity.

Characterization of the peptide

Kinetics of 4.41 degradation. The kinetic complexity of the degradation was determined by the presence of 4.41 peptides in the presence of 50% of all the selected fractions. In a final volume of 200 ml of 0.1 M tris(hydroxymethyl)amino-methane-Tris-buffered saline containing 0.01% Triton X-100 at a flow rate of 2.5 ml/min. Ten fractions of 2 ml each were collected and assayed for neurotensin-degrading activity.

Pharmacological assays. Neurotensin-degrading activity was detected by incubating at 37°C for 10 min. A 1:10 dilution of the enzyme in the presence of 50% of all the selected fractions. In a final volume of 200 ml of 0.1 M tris(hydroxymethyl)amino-methane-Tris-buffered saline containing 0.01% Triton X-100 at a flow rate of 2.5 ml/min. Ten fractions of 2 ml each were collected and assayed for neurotensin-degrading activity.

Denaturation of proteins. Denaturation of proteins was determined according to the Procedure (1982) with glycine as a standard.

Effect of pH. Neurotensin was incubated at pH 4.41 above as described above. The buffer consisted of a mixture of 10 mM sodium acetate, MES, TRIS and CAPS adjusted to the desired pH. The degradation was then monitored as described following the above procedure.