Proc. Natl. Acad. Sci. USA
Vol. 78, No. 11, pp. 6623–6627, November 1981
Biochemistry

Purification of a membrane-bound metalloendopeptidase from porcine kidney that degrades peptide hormones

(octyl β-glucoside/affinity chromatography/gel chromatography/hydroxamic acid inhibitors/fluorescent substrates)

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Communicated by P. Roy Vagelos, June 29, 1981

ABSTRACT **A porcine kidney microsomal metalloendopeptidase has been enriched 3900-fold. Gel filtration on a calibrated Toyo-Soda G-3000 SW column indicated an approximate molecular weight for the endopeptidase of 88,000 ± 2000. The purified enzyme is inhibited by a number of synthetic inhibitors of thermolysin. The endopeptidase hydrolyzes the succinyl (Suc)-containing fluorogenic peptide substrate Suc-Ala-Ala-Phe-(7-amino-4-methylcoumarin) at the Ala-Phe position with a K_m of 2.0×10^{-4} M. The endopeptidase also hydrolyzes a variety of peptides including corticotropin, substance *P*, angiotensin I and II, neurotensin, somatostatin, bradykinin, and the renin tetradecapeptide substrate. The endopeptidase hydrolyzes both [Leu]- and [Met]enkephalin at the Gly-Phe bond.**

We have reported that octyl β-D-glucopyranoside (OcβGlc*p*)-solubilized rough microsomes of dog pancreas and various other membrane preparations (1–4) contain a metalloendopeptidase activity. The enzyme is inhibited by 1,10-phenanthroline, phosphoramidon, and a number of synthetic inhibitors of thermolysin (5) when assayed against fluorogenic peptide substrates. Additionally, these solubilized membranes have been shown to correctly process a variety of precursor proteins such as preplacental lactogen (2, 4) and pre-pro-albumin (4) to the stable intracellular form of the protein.

The biological role of this enzyme is presently unknown; however, one possible role might be to modify *in vivo* biological activity of various peptide hormones by enzymatic degradation. For example, somatostatin (6), [Leu]enkephalin, [Met]enkephalin (7), and bradykinin (8) analogs resistant to proteolysis have been shown to be "longer acting" at lower concentration in various *in vivo* assays. Additionally, insulin binding to adipocyte membranes (9–11) has been reported to activate a membrane-bound protease to produce a peptide that has biological activity. Thus, study of membrane proteases and peptidases has deservedly attracted the attention of an increasing number of investigators.

In this report we describe a purification scheme and partial characterization of a metalloendopeptidase isolated from porcine kidney microsomes.

MATERIALS AND METHODS

Chymostatin and phosphoramidon were provided by the U.S.–Japan Cooperative Cancer Research Program. Bestatin was obtained from H. Umezawa, (Institute of Microbial Chemistry, Tokyo, Japan). The Toyo-Soda TSK G-3000 SW column was purchased from Varian.

Pancreatic Membrane Extract. Microsomes were prepared from frozen porcine kidney as described for dog pancreas (2) and suspended in 50 mM 2-{[tris(hydroxymethyl)methyl]amino}-ethanesulfonate (Tes) buffer (pH 7.5); OcβGlc*p* was added to a final concentration of 50 mM. After centrifugation at 100,000 × *g* for 1 hr at 4°C, the resulting supernatant fluid ("OcβGlc*p* extract"; 29 mg of protein per ml) was aspirated and stored at −20°C.

Sephacryl S-300 Chromatography. To a 1.5 × 100 cm column of Sephacryl S-300 previously equilibrated with 50 mM Tes/50 mM OBG, pH 7.5, 150 mg of OcβGlc*p* extract was applied at a flow rate of 2.0 ml/min.

Concanavalin A-Sepharose 4B. A concanavalin A-Sepharose 4B column (5 × 25 cm) was equilibrated with 50 mM Tes/50 mM OcβGlc*p*, pH 7.5. The pooled endopeptidase active fraction obtained from Sephacryl S-300 was applied to the concanavalin A-Sepharose 4B column at a flow rate of 1.0 ml/min. Endopeptidase activity was eluted from the column with 50 mM Tes/50 mM OcβGlc*p*/1.0 M NaCl/1.0 M methyl α-D-mannopyranoside, pH 7.5.

HONHCOCH($CH_2C_6H_5$)CO-Ala-Gly-Affi-Gel Affinity Chromatography. The affinity adsorbent was prepared as described by Nishino and Powers (5). The adsorbent was packed in a column (0.9 × 20 cm) and equilibrated with 50 mM Tes/50 mM OcβGlc*p*, pH 7.5. The pooled endopeptidase-enriched activity obtained from the concanavalin A-Sepharose 4B column was applied directly to the affinity column. The bound protein was eluted with 0.1 M Tris·HCl/0.1 M $CaCl_2$, pH 9.0, with a flow rate of 1.0 ml/min.

TSK G-3000 SW Gel Permeation Chromatography. The molecular weight for the partially purified enzyme was determined by high-performance liquid chromatography (HPLC) (12) on a Toyo-Soda TSK G-3000 SW column (0.75 × 50 cm) in 50 mM Tes/0.5 M KCl, pH 7.5, with a flow rate of 1.0 ml/min and a pressure of approximately 400 psi (1 psi = 6895 Pa). It was absolutely necessary to include high salt in the eluting buffer (>0.3 M KCl or NaCl), otherwise the standard proteins elute from the column with a much shorter retention time.

Indirect Coupled Enzyme Assay. The peptide substrates were prepared as described by Zimmerman *et al.* (13–15). Activities against these substrates (0.2 mM) were measured by the use of two methods. Endopeptidase activity was determined at 25°C as described (1) in 50 mM Tes/5% (vol/vol) dimethyl sulfoxide, pH 7.5, in a final volume of 0.5 ml containing leucine aminopeptidase (5 μg/ml), the succinyl (Suc)-containing fluorogenic peptide substrate Suc-Ala-Ala-Phe-AMeCou (7-amino-4-methylcoumarin) (0.2 mM), zinc acetate (50 μM), and calcium

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Abbreviations: OcβGlc*p*, octyl β-D-glucopyranoside; AMeCou, 7-amino-4-methylcoumarin; Tes, 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonate; HPLC, high-performance liquid chromatography; Suc, succinyl; IC_{50}, concentration that inhibits 50% of the maximal enzyme activity.

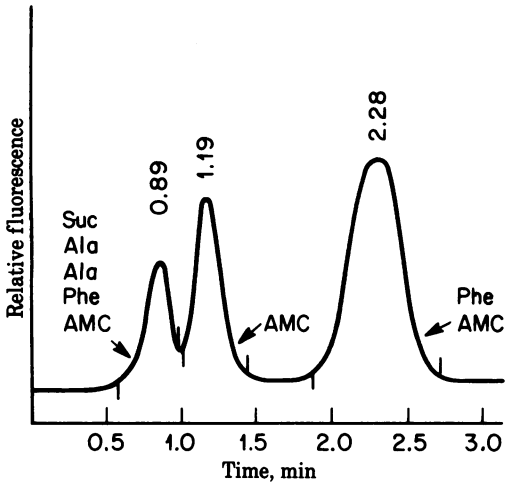

FIG. 1. HPLC retention times for cleavage products of Suc-Ala-Ala-Phe-AMeCou (here shown as AMC) on C_{18} μ-Bondpak. The values printed above each peak are the retention times for the various compounds. The limits of detection were 0.5 μM for Phe-AMeCou and 1.0 nM for AMeCou.

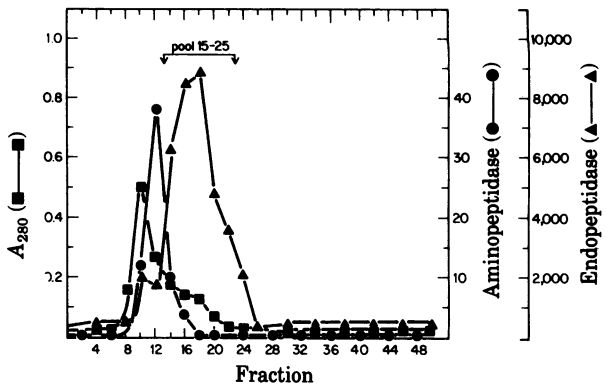

FIG. 2. Gel permeation chromatography of OcβGlc*p* extract with a column (1.5 × 100 cm) of Sephacryl S-300. ■——■, Protein (absorbance at 280 nm); ●——●, aminopeptidase activity assayed with Phe-AMeCou (0.2 mM); ▲——▲, endopeptidase activity.

chloride (10 mM). Fluorescence of the 7-amino-4-methylcoumarin (AMeCou) produced was determined as described (15). One unit of enzyme is equal to 10 nmol of AMeCou liberated per min in this assay.

HPLC Identification of Endopeptidase Products. Endopeptidase activity was determined in 50 mM Tes/5% dimethy sulfoxide containing bestatin (40 μg/ml), Suc-Ala-Ala-Phe-AMeCou (0.2 mM), zinc acetate (50 μM), and calcium chloride (10 mM), pH 7.5, in a final volume of 1.0 ml at 25°C for 30 min. Reaction products were extracted with an equal volume of water-saturated toluene after addition of 50 μl of 2 M NaCl and were quantitated by use of Waters HPLC system equipped with an automatic sample injector (WISP, model 710A), Data Module, and a C_{18} μ-Bondpak reverse-phase column (Waters) with methanol/H_2O, 95:5 (vol/vol). The flow rate was 2.5 ml/min.

RESULTS

An automated rapid HPLC assay for endopeptidase activity that greatly facilitated purification studies was developed. The fluorescent reaction products were extracted with toluene and separated by reverse-phase chromatography within 3 min (Fig. 1) and the data were quantitated by a Waters Data Module.

Purification of Endopeptidase. OcβGlc*p* quantitatively solubilized the endopeptidase activity from porcine kidney microsomes (Table 1) into a 100,000 × *g* supernatant fraction. An enrichment of endopeptidase activity relative to protein concentration was achieved by passing the OcβGlc*p* extract through a Sephacryl S-300 column (Fig. 2). If the eluting buffer for the Sephacryl S-300 contained 0.1 M NaCl, the endopeptidase migrated further into the gel and eluted in later fractions with a large amount of the protein comigrating with the endopeptidase. The pooled endopeptidase-enriched fraction obtained from the Sephacryl S-300 column was next applied to a concanavalin A-Sepharose 4B column (Fig. 3). The endopeptidase bound to this column when the OcβGlc*p* concentration was 50 mM or higher and could only be eluted by high levels of methyl α-D-mannopyranoside (1.0 M). Sodium chloride (1 M) helped prevent the endopeptidase from trailing. Next, the endopeptidase-enriched fraction obtained from this column in 50 mM Tes/50 mM OcβGlc*p*/1.0 M methyl α-D-mannopyranoside/1.0 M NaCl, pH 7.5, was applied to the hydroxamic acid Affi-Gel affinity column (Fig. 4). The endopeptidase activity was eluted from the column with 0.1 M Tris/0.1 M $CaCl_2$, pH 9.1. This column resolves the contaminating aminopeptidase activity from the endopeptidase. This affinity column has been shown previously by Nishino and Powers to bind the zinc metalloendopeptidase, thermolysin, and *Pseudomonas aeruginosa* elastase (5). We were unable to concentrate the affinity chromatography-purified material either by ultrafiltration or lyophilization without complete loss of enzyme activity. Therefore, an estimation of purity could not be determined because the affinity chromatography-purified material was too dilute. The final preparation does not possess any trypsin-like, chymotrypsin-like, aminopeptidase-like, or elastase-like activities when assayed with the appropriate synthetic peptide fluorogenic substrates (13–15).

The results in Table 1 show that during purification the endopeptidase activity recovered is 27-fold higher after Sephacryl S-300 chromatography than in the microsomal homogenate. Mixing of homogenate and Sephacryl S-300-purified material did not result in the loss of any enzyme activity. Likewise, addition of boiled microsomal homogenate to the Sephacryl S-300 enzyme preparation did not result in any inhibition of enzyme

Table 1. Purification of endopeptidase from porcine kidney microsomes

Fraction or step	Total activity*	Total protein, mg	Volume, ml	Specific activity†	Enrichment	% recovered
Microsome homogenate	1,500	144	5	10.4	0	100
100,000 × *g* supernatant	6,500	68	5	96	9	430
Sephacryl S-300	40,800	22	40	1,790	171	2700
Con A-Sepharose	15,400	0.4	110	38,500	3698	1026
Affinity column	13,108	<0.3	80	43,693	4197	874

Con A, concanavalin A.
* Activity is in mol × 10^{-8} of AMC liberated per min in the coupled enzyme assay with Suc-Ala-Ala-Phe-AMeCou as substrate.
† Specific activity is in mol × 10^{-8} of AMeCou liberated per min per mg of protein.

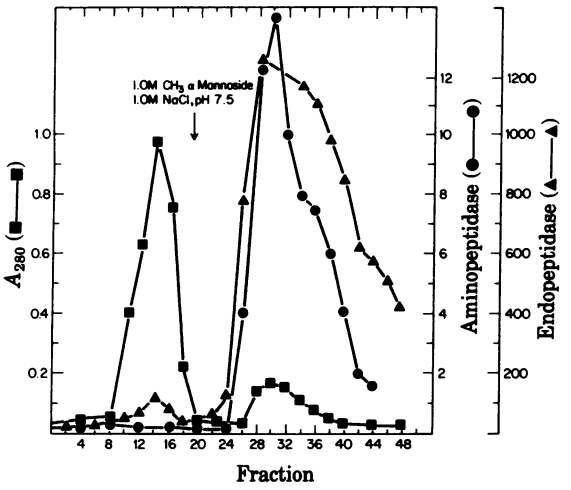

FIG. 3. Chromatography of endopeptidase-enriched fraction (60 ml) from concanavalin A-Sepharose 4B column on 2-(*N*-hydroxycarboxamido) 3-phenylpropanoyl-L-alanylglycine-Affi-Gel 101; elution (arrow) was with 0.1 M Tris/0.1 M $CaCl_2$, pH 9.1.

activity. These tentative data suggest but by no means prove either the presence of an inhibitor–enzyme complex in the microsomal homogenate or perhaps the activation of a precursor form of the enzyme.

The effect of varying pH upon the hydrolysis of Suc-Ala-Ala-Phe-AMeCou showed that the enzyme had a pH optimum of 7.0–7.5 in Tes buffer (data not shown). A linear reciprocal plot of activity vs. Suc-Ala-Ala-Phe-AMeCou concentrations indicated typical Michaelis–Menten kinetics for endopeptidase with an apparent K_m of 2.0×10^{-4} M (data not shown). The enzyme was maximally active with 50 μM Zn^{2+} and 10 mM Ca^{2+}. Zinc was inhibitory at higher concentrations (data not shown).

Molecular Weight Determination of Endopeptidase. Kato *et al.* (12) have recently described a variety of commercially available HPLC gel permeation columns on which high resolution of proteins is obtainable in aqueous buffered systems. Fig. 5 shows the calibration curve of the G-3000 SW column for a variety of globular proteins. Chymotrypsinogen is the only protein to date which we have run that elutes from the G-3000 SW column with an improper retention time and does not plot on the line in Fig. 5. The molecular weight of the endopeptidase was estimated on this column as 88,000 ± 2000. The same calibration curve for the standard proteins was obtained in the absence of Oc*β*Glc*p*.

Synthetic Substrate Specificity of the Endopeptidase. The endopeptidase hydrolyzes a variety of synthetic fluorogenic

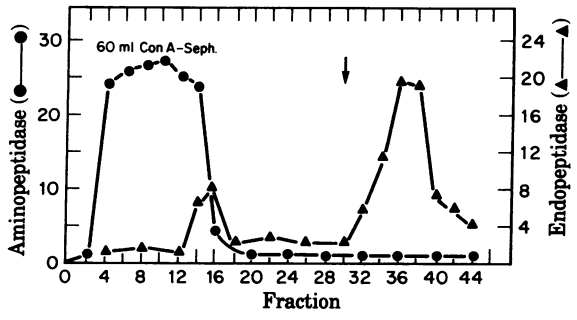

FIG. 4. Affinity chromatography of endopeptidase-enriched fraction (60 ml) from concanavalin A-Sepharose 4B column on 2-(*N*-hydroxycarboxamido)3-phenylpropanoyl-L-alanylglycine-Affi-Gel 101; elution (arrow) was with 0.1 M Tris/0.1 M $CaCl_2$, pH 9.1.

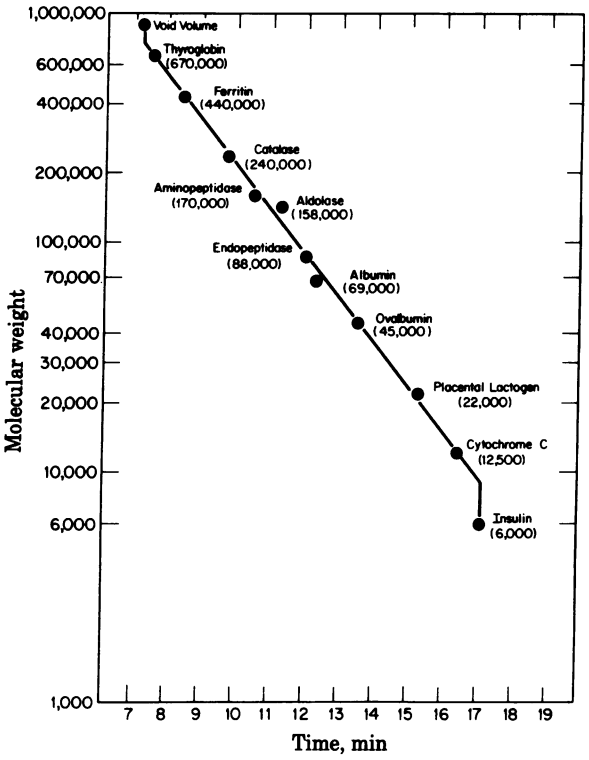

FIG. 5. Molecular weight determination of endopeptidase by analytical high-performance gel filtration chromatography on a Toyo-Soda TSK G-3000 SW column (0.75 × 50 cm).

peptide substrates (Table 2). No activity could be detected when phosphoramidon (0.1 μg/ml) was included in the assay. The site of cleavage for each substrate was determined by thin-layer chromatographic identification (data not shown) of the reaction products and the indirect coupled assay (Table 2). The best substrates were Suc-Ala-Ala-Phe-AMeCou, Suc-Ala-Ala-Pro-Ala-Phe-AMeCou, and Suc-Ala-Gly-Met-AMeCou. The enzyme prefers small aliphatic amino acids such as glycine and alanine at the P_1 position and aromatic (phenylalanine) or long-chain aliphatic amino acids (methionine) at the P_1' position.

Hydrolysis of Various Peptides by Endopeptidase. Endopeptidase hydrolysis of Suc-Ala-Ala-Phe-AMC is inhibited by a variety of polypeptides; the concentrations that inhibit maximal response by 50% (IC_{50}) are presented in Table 3. Bovine serum albumin (50 μg/ml), oxytocin (50 μg/ml), proinsulin (50 μg/ml), insulin (50 μg/ml), eledoisin (50 μg/ml) and des-octa-insulin (50 μg/ml) did not compete with the hydrolysis of Suc-

Table 2. Synthetic substrate specificity of endopeptidase

Substrate	Activity*
Suc-Ala-Phe↓Ala-AMeCou	80
Suc-Ala-Ile↓Ala-AMeCou	53
Suc-Ala-Ala↓Ala-AMeCou	233
Suc-Ala-Ala↓Phe-AMeCou	1053
Suc-Ala-Gly↓Met-AMeCou	733
Suc-Ala-Ala-Pro-Ala↓Phe-AMeCou	1000
Suc-Ala-Phe-Ala↓Ala-AMeCou	80
Suc-Ala-Ala-AMeCou	0

Arrows indicate sites of endopeptidase cleavage.

* Activity is in nmol of AMeCou liberated per min per ml. Incubations were performed in 1.0 ml of Tes buffer (pH 7.5) containing Suc-Ala-Ala-Phe-AMeCou (0.2 mM), leucine aminopeptidase (5 μg/ml), and endopeptidase (2 units). Enzyme activity was monitored by measuring the release of AMeCou in a spectrofluorometer with an excitation of 383 nm and emission at 455 nm.

Table 3. Effect of various peptides on endopeptidase activity

Peptide	IC_{50},* μM
Substance *P*	1.5
Renin substrate	3.7
Angiotensin II	4.2
Corticotropin	1.9
Somatostatin	5.8
Angiotensin I	6.6
Neurotensin	7.1
Bradykinin	10
[Met]Enkephalin	14
[Leu]Enkephalin	28

* Activity was determined as described in Table 2.

Ala-Ala-Phe-AMeCou. Fig. 6 shows the disappearance of substance *P* and neurotensin upon incubation with endopeptidase. The disappearance of both substance *P* and neurotensin was time dependent and could be blocked by phosphoramidon (0.1 μg/ml). HPLC assay experiments with the other peptides in Table 3 gave results similar to neurotensin and substance *P*, demonstrating that each peptide is in fact a substrate. Cleavage of [Leu]enkephalin by endopeptidase occurred at the Gly-Phe position as determined by chromatography on a Supelco C_8 column by cochromatography of the reaction products with Tyr-Gly-Gly and Phe-Leu standards (Fig. 7). The disappearance of [Leu]enkephalin was time dependent and could be blocked by phosphoramidon. Cleavage of [Met]enkephalin by endopeptidase was also at the Gly-Phe position.

Effect of Various Inhibitors on Endopeptidase Activity. Table 4 shows that the porcine endopeptidase was inhibited by phosphoramidon, $HONHCOCH(CH_2CH(CH_3)CO\text{-}Ala\text{-}Gly\text{-}NH_2$, $HONHCOCH(CH_2C_6H_5)$ CO-Ala-Gly-NH_2, $HSCH_2\text{-}CH(CH_2C_6H_5)$-Ala-Gly-$NH_2$, and Z-Gly-Leu-NHOH but not by $HONHCOCH_2$-CO-Ala-Gly-NH_2. 1,10-phenanthroline inhibited the endopeptidase with an IC_{50} of 5.0×10^{-4} M. These findings are similar to those observed for the dog enzyme (1). Captopril is an extremely poor inhibitor (IC_{50}, $>10^{-4}$ M) of porcine endopeptidase activity.

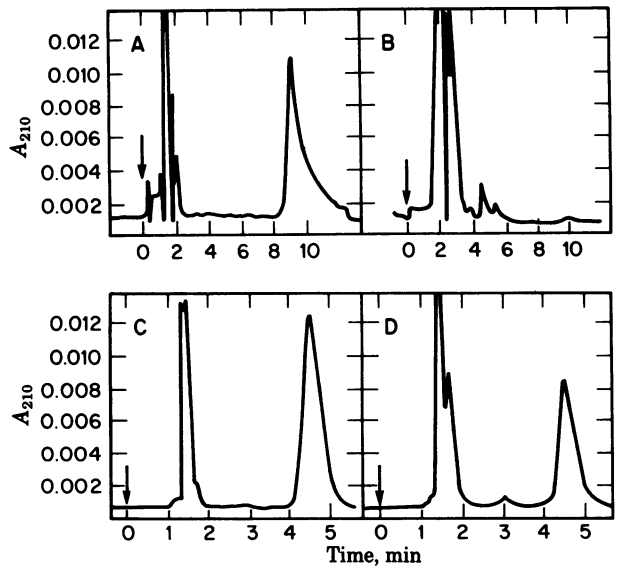

FIG. 6. (*A*) HPLC of substance *P* (retention time, 9 min) on a C_{18} μ-Bondapak column with acetonitrile/triethanolamine phosphate buffer, 23:77 (vol/vol), pH 3.5. The flow rate was 2.0 ml/min. Substance *P* (5 μg/ml), endopeptidase (2 units), and Tes buffer (pH 7.5) at 0-time incubation. (*B*) Digestion of substance *P* by endopeptidase after a 2-hr incubation at 22°C. (*C*) HPLC of neurotensin (retention time, 4.5 min) on a fatty acid column (Waters Associates) with acetonitrile/triethanolamine phosphate buffer, 20:80 (vol/vol), pH 3.5. The flow rate was 2.0 ml/min. Neurotensin (5 μg/ml), endopeptidase (2 units), and Tes buffer, pH 7.5 at 0-time incubation. (*D*) Digestion of neurotensin after a 2-hr incubation at 22°C. Neurotensin was completely digested after a 16-hr incubation.

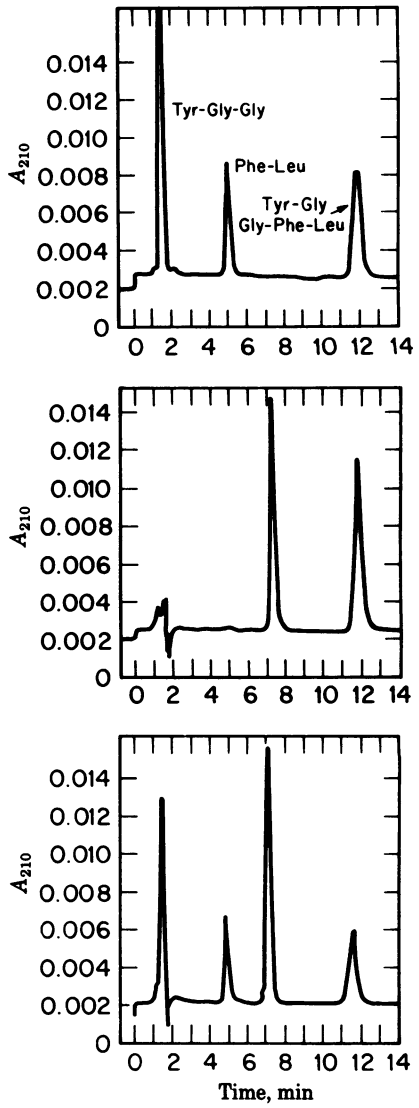

FIG. 7. (*A*) HPLC of [Leu]enkephalin, Tyr-Gly-Gly and Phe-Leu on LC 8 column (Supelco) with acetonitrile/triethanolamine phosphate buffer, 19:81 (vol/vol), pH 3.5. The flow rate was 2.0 ml/min. (*B*) [Leu]enkephalin (2 μg/ml), endopeptidase (2 units), bestatin (1 μg; retention time, 7 min) and Tes buffer (pH 7.5) at 0 time. (*C*) Digestion of [Leu]enkephalin by endopeptidase after a 30-min incubation at 22°C.

DISCUSSION

In this communication we have described a purification scheme and partial characterization of a zinc metalloendopeptidase isolated from porcine kidney microsomes. The synthetic substrate specificity of the enzyme appears to be virtually identical to that of the enzyme solubilized from membranes of the dog pancreas (1). The endopeptidase prefers small aliphatic amino acids such as glycine and alanine at the P_1 position and aromatic (phenylalanine) or long-chain aliphatic amino acids (methionine) at the P_1' position.

The endopeptidase is inhibited by phosphoramidon, a competitive inhibitor of thermolysin-like proteases. Additionally

Table 4. Effect of various inhibitors on endopeptidase activity

Inhibitor	IC_{50},* nM
Phosphoramidon	94
$HONHCOCH(CH_2CH)(CH_3)_2CO$-Ala-Gly-$NH_2$	49
$HONHCOCH(CH_2C_6H_5)CO$-Ala-Gly-NH_2	149
$HSCH_2CH(CH_2C_6H_5)$-Ala-Gly-NH_2	673
Z-Gly-Leu-NHOH	5,300
$HONHCOCH_2CO$-Ala-Gly-NH_2	30,000

* Activity was determined as described in Table 2.

the endopeptidase is inhibited by 1,10-phenanthroline and a variety of hydroxamic acid inhibitors that have been shown by Nishino and Powers (5) to be potent inhibitors of thermolysin and *Pseudomonas aeruginosa* elastase, both zinc metalloendopeptidase. This inhibitor data strongly suggests that the porcine endopeptidase is a zinc enzyme.

The endopeptidase hydrolyzes several peptide hormones, including substance P, angiotensin I and II, somatostatin, neurotensin, bradykinin, [Met]-, and [Leu]enkephalin. The endopeptidase hydrolyzes [Met]- and [Leu]enkephalin at the Gly-Phe bond.

We have found similar endopeptidase activity associated with rat parotid gland membranes, purified rough and smooth endoplasmic membranes from rat liver, rat lacrimal gland membranes, murine macrophages, porcine brain, spleen, and liver microsomes (1). Additionally endopeptidase activity has been detected in hamster eggs and cells of the cumulus oophorus (16). Kerr and Kenny (17, 18) have described the enrichment and characterization of an endopeptidase activity from rabbit kidney brush border, which has a molecular weight of 93,000 and requires zinc and calcium ions for maximum activity. Verandani and Shroyer (19) also have described the enrichment of a neutral proteinase from rat kidney which hydrolyzes several polypeptide hormones.

Recent results (to be reported in detail elsewhere) from our laboratories have shown that mouse brain striatal microsomal preparations contain an endopeptidase activity that cleaves the Gly-Phe bond of [Leu]- or [Met]enkephalin and the Ala-Phe bond in our standard endopeptidase substrate, Suc-Ala-Ala-Phe-AMeCou. Together with evidence reported earlier (1), these results suggest that this ubiquitous membrane endopeptidase may, in the brain, be an enkephalinase similar to the enzyme described by Benuck and Marks (20) and Gorenstein and Snyder (21).

We thank Dr. James C. Powers, Department of Chemistry, Georgia Institute of Technology, Atlanta, Georgia, for supplying the thiol and various hydroxamic acid inhibitors and Donna Sloan for typing the manuscript. A.W.S. is an Established Investigator of the American Heart Association.

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