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Purification and Characterization of a Cathepsin D Protease from Bovine Chromaffin Granules†

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ABSTRACT: Purification and potential tachykinin and enkephalin precursor cleaving enzymes from bovine chromaffin granules was undertaken using as substrates the model precursors ^{35}S-(Met)-β-preprotachykinin [^{35}S-(Met)-β-PPT] and ^{35}S-(Met)-preproenkephalin [^{35}S-(Met)-PPE]. Purification by concanavalin A-Sepharose, Sephacryl S200, and chromatofocusing resulted in a chromaffin granule aspartyl protease (CGAP) that preferred the tachykinin over the enkephalin precursor. CGAP was composed of 47-, 30-, and 16.5-kDa polypeptides migrating as a single band in a nondenaturing electrophoretic gel system, and coeluting with an apparent molecular mass of 45–55 kDa by size-exclusion chromatography. These results suggest that two forms exist: a single 47-kDa polypeptide and a complex of 30+16.5-kDa-associated subunits. CGAP was optimally active at pH 5.0–5.5, indicating that it would be active within the acidic intragranular environment. Cleavage at basic residues was suggested by HPLC and HVE identification of ^{35}S-(Met)-NKA-Gly-Lys as the major acid-soluble product generated from ^{35}S-(Met)-β-PPT. Neuropeptide K was cleaved at a Lys-Arg basic residue site, as determined by identification of proteolytic products by microsequencing and amino acid composition analyses. Structural studies showed that the three CGAP polypeptides were similar to bovine cathepsin D in NH_2-terminal sequences and amino acid compositions, indicating that CGAP appears to be a cathepsin D-related protease or cathepsin D itself. The 47- and 16.5-kDa polypeptides of CGAP possessed identical NH_2-terminal sequences, suggesting that the 16.5-kDa polypeptide may be derived from the 47-kDa form by proteolysis. CGAP resembled cathepsin D by cleaving at hydrophobic residues, as shown by CGAP cleavage of neuropeptide K between Leu-Tyr and Phe-Val residues. Processing of proendothelin to endothelin, present in chromaffin granules, requires processing at both hydrophobic and paired basic residues, which would be compatible with CGAP's cleavage site specificity. In addition, CGAP's cathepsin D-like cleavage specificity for hydrophobic residues suggests that it may also be involved in degrading precursor segments that are not part of the active peptide sequences. In summary, CGAP shows substrate selectivity, and cleaves at paired basic residues and at hydrophobic residues. These properties may be compatible with possible participation of CGAP in cleaving some peptide precursors.

Peptide hormones and neurotransmitters are synthesized as protein precursors that require proteolytic processing to form the smaller active neuropeptides (Docherty & Steiner, 1982). Most precursors require endoproteolytic processing at paired basic residue sites (Lys-Arg, Arg-Lys, Lys-Lys, or Arg-Arg), and some also require processing at monobasic arginine sites. Several putative processing enzymes cleaving at such dibasic residues have been identified. These include the yeast Kex2 gene product required for processing the yeast pro-α-mating factor (Julius et al., 1984; Fuller et al., 1989), homologous Kex2-related human and mouse furin genes (Bresnahan et al., 1990; Hatsuzawa et al., 1990), and PC1, PC2, and PC3

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Kex2-related prohormone convertases (Smeekens & Steiner, 1990; Smeekens et al., 1991; Seidah et al., 1991; Benjannet et al., 1991). These Kex2-related proteases, members of the subtilisin family, cleave at Lys-Arg and Arg-Arg dibasic sites. Two Ca^{2+}-dependent endoproteases involved in proinsulin processing possess the same cleavage specificity as the yeast Kex2 protease (Davidson et al., 1988). Also, evidence for aspartyl proteases in processing proopiomelanocortin (Loh et al., 1985) and provasopressin (Parish et al., 1986) has been demonstrated.

While these endoproteases cleave at dibasic sites, they have not been shown to cleave at monobasic sites. We recently purified from bovine adrenal medullary chromaffin granules a novel "prohormone thiol protease" (PTP) that processes the enkephalin precursor at both dibasic (Krieger & Hook, 1991) and monobasic sites (Krieger et al., 1992). In addition to PTP's cleavage site specificities, its distinct biochemical properties distinguish it from other thiol proteases.

Bovine chromaffin granules contain, in addition to enkephalins, several other neuropeptides including substance P (Saria et al., 1980), neuropeptide Y (Waschek et al., 1987), galanin (Rokaeus et al., 1986), somatostatin (Lundberg et al., 1979), and endothelin (Sawamura et al., 1990) which are synthesized as precursors that require proteolytic processing. The localization of precursors, intermediates, and peptide products within secretory vesicles (Gainer et al., 1985; Liston et al., 1983; Spruce et al., 1988) suggests that many processing steps occur in secretory vesicles. Thus, chromaffin granules should possess some of the endoprotease(s) responsible for processing neuropeptide precursors.

In this study, purification of tachykinin and enkephalin precursor cleaving activities from chromaffin granules was undertaken to find if these precursors may be cleaved by similar or different proteases. Proteolytic activity was monitored by cleavage of tachykinin and enkephalin precursors in the form of ^{35}S-(Met)-β-preprotachykinin [^{35}S-(Met)-β-PPT][1] and ^{35}S-(Met)-preproenkephalin [^{35}S-(Met)-PPE]. Purification resulted in the isolation of a distinct chromaffin granule aspartyl protease (CGAP) that preferred the tachykinin over the enkephalin precursor. Characterization of CGAP with respect to pH optimum, cleavage sites, NH_2-terminal microsequencing, and amino acid composition is described.

Experimental Procedures

Materials

SP6 RNA polymerase, Riboprobe in vitro transcription system, RNasin, RQ1 DNase, and wheat germ cell-free translation system were from Promega. [^{35}S]Methionine (1000 Ci/mmol) was obtained from New England Nuclear. Iodo-[2-^{14}C]acetic acid (50 mCi/mmol) was from Amersham. Iodoacetic acid, PMSF, EDTA, *p*-(hydroxymercuri)benzoate (PHMB), soybean trypsin inhibitor, and methyl α-D-mannopyranoside were from Sigma. Pepstatin A, leupeptin, and chymostatin were from Boehringer. DTT, α_1-antichymotrypsin, and α_1-antitrypsin were from Calbiochem. Concanavalin A–Sepharose, Sephacryl S200, Sephacryl S300, Sephadex G-75, Polybuffer Exchanger 94, and Polybuffers were from Pharmacia. Pepstatin A–agarose was from Pierce. Standard peptides used in HPLC and HVE were purchased from Peninsula Laboratories.

Methods

In Vitro Synthesis of ^{35}S-(Met)-β-PPT and ^{35}S-(Met)-PPE. ^{35}S-(Met)-β-PPT and ^{35}S-(Met)-PPE were synthesized by in vitro transcription and translation of the rat PPE (Yoshikawa et al., 1984) and human β-PPT (Harmar et al., 1986) cDNAs as previously described (Hook et al., 1989). Human β-PPT and rat PPE should be suitable for assay of putative processing activity in bovine adrenal medulla, since the basic and monobasic cleavage sites within the two precursors from different species are identical.

For precursor synthesis, β-PPT and PPE cDNAs in the plasmid pSP65 (Promega-Biotech), containing the SP6 promoter, were linearized with restriction enzymes, and mRNAs were synthesized by in vitro transcription with SP6 RNA polymerase according to the manufacturer's protocol (Promega Biotech). RNAs were precipitated in 70% EtOH/0.45 M sodium acetate, pH 6.0, at −20 °C, collected by centrifugation (20 min at 20000*g*), dried in vacuo, and resuspended in 30 μL of H_2O for protein translation. Cell-free protein translation (using wheat germ extract from Promega according to the manufacturer's protocol) with [^{35}S]methionine synthesized ^{35}S-(Met)-β-PPT and ^{35}S-(Met)-PPE with specific activities of 4000 and 11 000 Ci/mmol, respectively, based on TCA-precipitable radioactivity. The lower [^{35}S]Met incorporation into the 15 000-dalton β-tachykinin precursor compared to the 31 000-dalton enkephalin precursor is expected since β-PPT contains 4 methionine residues (Harmar et al., 1986) and PPE contains 11 methionine residues (Yoshikawa et al., 1984). After the ^{35}S-(Met)-β-PPT translation mixture was heated in 1% SDS at 60 °C for 15 min, free [^{35}S]Met was removed by Sephadex G-25 gel filtration (PD-10 column) with 10 mM Tris-HCl, pH 7.5, and 0.1% SDS as elution buffer. The ^{35}S-(Met)-PPE translation mixture contained minor low molecular weight proteins; therefore, ^{35}S-(Met)-PPE was purified by SDS–PAGE and electroelution, with removal of residual [^{35}S]Met by gel filtration.

Assay of ^{35}S-(Met)-β-PPT and ^{35}S-(Met)-PPE Cleaving Activities. Proteolytic activities in lysed chromaffin granules and in column fractions (5-μL aliquots) were assayed (total volume 15 μL) in 100 mM citric acid/NaOH, pH 5.0, 15 mM DTT, and 15 000 cpm of ^{35}S-(Met)-β-PPT or 40 000 cpm of ^{35}S-(Met)-PPE, resulting in substrate concentrations of 100–150 pM, depending on the specific activity of [^{35}S]Met at the time of assay. After incubation at 37 °C for 1 h, 50 μL of bovine serum albumin (10 mg/mL) and 600 μL of ice-cold 11% trichloroacetic acid (TCA) were added with vortexing. Samples were centrifuged at 17000*g* for 5 min, and radioactivity in the supernatant fraction was determined. Assays were also assessed by autoradiography of SDS–PAGE gels as described previously (Hook et al., 1989). SDS at final concentrations of 0.01–0.005% (from the precursor preparations) had no effect on enzyme activities.

Preparation of Purified Chromaffin Granules and Soluble Extract. Chromaffin granules were purified from fresh bovine adrenal medulla (Biological Research and Delivery Service, Gaithersburg, MD) by a discontinuous sucrose density gradient as previously described (Hook & Eiden, 1984; Smith & Winkler, 1966). Briefly, adrenal medulla homogenate in 0.32 M sucrose was centrifuged at 1500 rpm in a GSA rotor, the resultant supernatant was centrifuged at 8800 rpm in a GSA rotor to obtain a pellet of enriched chromaffin granules, and

[1] Abbreviations: CGAP, chromaffin granule aspartyl protease; DTT, dithiothreitol; PPT, preprotachykinin; PPE, preproenkephalin; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; HVE, high-voltage electrophoresis; NKA, neurokinin A; NPK, neuropeptide K; PHMB, *p*-(hydroxymercuri)benzoate; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

granules were washed 3 times in 0.32 M sucrose. This enriched fraction contained only 5% of total tissue lysosomal acid phosphatase (Hook & Eiden, 1984). Further purification of enriched chromaffin granules was achieved on a discontinuous sucrose density gradient (10 mL of the enriched fraction layered over 25 mL of 1.6 M sucrose) by centrifugation in an SW28 rotor at 25000 rpm for 120 min at 4 °C, to obtain a pellet of purified chromaffin granules.

Chromaffin granules and lysosomes were effectively separated by the 1.6/0.32 M discontinuous sucrose gradient since analysis by a linear sucrose gradient shows that chromaffin granule fractions, containing (Met)enkephalin and catecholamines, were obtained at 1.7–2.0 M sucrose whereas lysosomes detected by acid phosphatase were obtained at 1.1–1.6 M sucrose (Hook & Eiden, 1984). This procedure, utilizing a discontinuous sucrose gradient, results in a purified preparation of chromaffin granules containing only 0.5% of total tissue lysosomal acid phosphatase, indicating successful removal of lysosomes (Hook & Eiden, 1984). In some experiments (Figure 9), the purified granules were further purified on a second identical sucrose gradient to obtain a highly purified granule fraction containing only 0.05% of the total tissue lysosomal acid phosphatase (Hook & Eiden, 1984).

A soluble chromaffin granule extract was prepared by lysing purified granules from 160 adrenal medulla in 48 mL of 15 mM KCl and freeze–thawing 2 times. This granule lysate was centrifuged at 47000*g* for 30 min, and the supernatant fraction was collected and kept on ice. The sediment was resuspended in 48 mL of 100 mM citric acid/NaOH, pH 6.0, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.5 M NaCl with a ground-glass homogenizer (Kontes) and centrifuged for 30 min at 47000*g*, and the resultant supernatant fraction was combined with the first 15 mM KCl supernatant fraction. The pooled soluble supernatant fraction was subjected to ultracentrifugation at 110000*g* for 1 h, and the resulting supernatant fraction was taken as the soluble granule extract. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Concanavalin A–Sepharose Chromatography. The soluble granule extract was applied onto a concanavalin A–Sepharose column (1.5 × 20 cm) equilibrated with 100 mM citric acid/NaOH, pH 6.0, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.5 M NaCl (equilibration buffer) at 30 mL/h. The column was washed with the equilibration buffer and was eluted with 0.2 M methyl α-D-mannopyranoside in equilibration buffer at 15 mL/h. Fractions of 3.9 mL were collected and examined for ^{35}S-(Met)-β-PPT cleaving activity, ^{35}S-(Met)-PPE cleaving activity, and absorbance at 280 nm.

Sephacryl S200 Chromatography. The concanavalin A-bound fractions were concentrated to 2.0 mL in a 50-mL Amicon ultrafiltration cell equipped with a YM-5 membrane. The concentrate was diluted to 50 mL with 50 mM β,β-dimethylglutaric acid/NaOH, pH 6.2, and 1 mM EDTA and was then concentrated to 2.0 mL. This dilution/concentration cycle was repeated once more. The resulting concentrate was loaded onto a Sephacryl S200 (1.5 × 160 cm) column equilibrated with 50 mM β,β-dimethylglutaric acid/NaOH, pH 6.2, and 1 mM EDTA (S200 equilibration buffer). The column was eluted with the equilibration buffer at 14 mL/h, and fractions of 2.9 mL were assayed for ^{35}S-(Met)-β-PPT and ^{35}S-(Met)-PPE cleaving activities.

Chromatofocusing of Sephacryl S200 Peak II. Peak II from the Sephacryl S200 column was concentrated to 1.0 mL in a 10-mL Amicon ultrafiltration cell equipped with a YM-5 membrane. The concentrate was taken up with 4.0 mL of chromatofocusing elution buffer, pH 5.0 (prepared by combining 35 mL of Polybuffer 74 plus 15 mL of Polybuffer 96 with H_2O to a total volume of 500 mL, with adjustment of the pH to 5.00 with 2 N acetic acid). The sample was loaded onto a Polybuffer Exchanger 94 column (0.9 × 10 cm) equilibrated with 25 mM Tris/acetic acid, pH 8.3, and was eluted with chromatofocusing elution buffer at 10 mL/h, and fractions of 1.9 mL were assayed for ^{35}S-(Met)-β-PPT cleaving activity.

Denaturing and Native Gel Electrophoresis of Purified Enzyme. Denaturing gel electrophoresis of 2.5 μg of purified chromaffin granule aspartyl protease (CGAP) on 12.5% polyacrylamide SDS–PAGE gels was performed by the method of Laemmli (1970), and proteins were visualized by silver staining according to the manufacturer's protocol (Bio-Rad). Native (nondenaturing) gel electrophoresis was performed by a modification of the method of Reisfeld (Reisfeld et al., 1962). The enzyme (2 μg) in 37.5 mM acetic acid/KOH, pH 4.3, 1 mM pepstatin A, and 25% glycerol was electrophoresed in a native gel system consisting of a 4.75% polyacrylamide stacking gel buffered with 60 mM acetic acid/KOH, pH 5.5, a 10% polyacrylamide resolving gel buffered with 750 mM acetic acid/KOH, pH 4.3, and electrophoresis buffer of 0.14 M acetic acid/0.35 M β-alanine, pH 4.5. Proteins were visualized by silver staining.

pH Optimum. Purified enzyme (50 ng) was incubated (20-μL total volume) in 88 mM citric acid/NaOH, pH 3.0–7.0, with 40000 cpm of ^{35}S-(Met)-β-PPT at 37 °C for 2 h. TCA-soluble radioactivity was determined in 10 μL of the assay, and the other 10 μL was analyzed by autoradiography of SDS–PAGE gels.

Protease Inhibitors. Purified enzyme (50 ng) and inhibitors were preincubated in 133 mM citric acid/NaOH, pH 5.0, in a total volume of 15 μL for 30 min on ice. Assays were initiated by adding 5 μL of ^{35}S-(Met)-β-PPT (40000 cpm) and were incubated for 2 h at 37 °C. The assays were split into 2 × 10 μL aliquots for determination of TCA-soluble radioactivity.

Analysis of TCA-Soluble Products by HPLC and HVE. ^{35}S-(Met)-β-PPT (240000 cpm) was incubated with enzyme in 100 mM citric acid/NaOH, pH 5.0 (120-μL total volume), for 2 h at 37 °C. After addition of 10 μg of bovine serum albumin and 15 μL of 100% TCA (w/v) with vortexing, samples were centrifuged for 10 min at 17000*g*, and the supernatant was extracted with 0.5 mL of diethyl ether to remove TCA. This sample was subjected to chromatography on HPLC and HVE.

HPLC was performed on a C18 reverse-phase column (Beckman) utilizing a Waters Model 721 system controller with data Module 730 and WISP Model 710B. Chromatography was performed with buffer A as 0.1% TFA and buffer B as 60% acetonitrile in 0.1% TFA. Elution of peptides was achieved with a linear gradient from 10% to 80% buffer B with a flow rate of 1 mL/min, and fractions of 0.5 min were collected. Radioactivity in 50-μL aliquots from each fraction was determined. Peptide standards were included in each run and were detected at 210 nm.

For HVE analysis, appropriate HPLC fractions were lyophilized and resuspended in 10 μL of 1 N acetic acid, and subjected to HVE (200 V) on cellulose TLC plates (Merck) in a solvent system of 2/8/90 formic acid/acetic acid/water. Radioactive spots were detected by autoradiography with a 1–3-day exposure to Kodak XOMATIC-AR film. Standard peptides were visualized by staining with fluorescamine as described previously (Hook & Loh, 1984).

Cleavage of Neuropeptide K (NPK). NPK (0.5 mg) was incubated with the enzyme (2 μg) in 100 mM citric acid/NaOH, pH 5.0 (total volume of 200 μL), for 6 h at 37 °C, and peptide products were separated by HPLC as described above.

Carboxymethylation of Aspartyl Protease for Microsequencing. Purified CGAP (400 μg) was concentrated to 1 mL by ultrafiltration, desalted on Sephadex G75 equilibrated with 25 mM pyridine/acetic acid, pH 5.8, and lyophilized. The enzyme was denatured in 8 M urea, 1 mM DTT, and 100 mM Tris-HCl, pH 8.1 (denaturation buffer), at 37 °C for 30 min. For carboxymethylation, 100 μL of 5 mM [^{14}C]iodoacetate (specific activity adjusted to 20 000 cpm/nmol with cold iodoacetic acid) in denaturation buffer was added, and incubation at 37 °C was continued for 1 h in the dark. The reaction was quenched by adding 10 μL of β-mercaptoethanol, and excess [^{14}C]iodoacetate was removed by gel filtration (PD-10 column) with 25 mM pyridine/acetic acid, pH 5.8. ^{14}C-Proteins were lyophilized for microsequencing and amino acid analyses.

Microsequencing and Amino Acid Analyses. The three polypeptide chains of CGAP were separated by SDS–PAGE gels and electroblotted onto poly(vinylidene difluoride) membranes (Matsudiara, 1987). For neuropeptide K-derived peptides, HPLC-purified peptides were absorbed onto a precycled Biobrene-impregnated glass fiber disk. Microsequencing of polypeptides was performed by Dr. Liane Mende-Mueller (Medical College of Wisconsin) on an Applied Biosystems Model 477A pulsed liquid-phase protein sequencer. For determination of amino acid composition (by Dr. Liane Mende-Mueller), peptides were hydrolyzed in 6 N HCl/10 mM β-mercaptoethanol at 110 °C for 24 h. Identification of amino acids was performed on a Beckman Model 6300 amino acid analyzer after ion-exchange chromatography and postcolumn reaction with ninhydrin.

CGAP Antibody Production and Western Blots. CGAP antiserum was produced by immunizing (subcutaneous injection) Balb-c male mice with 15 μg of purified CGAP in a 300-μL emulsion consisting of a 1/1 ratio of PBS (phosphate-buffered saline)/Freund's complete adjuvant. Three boosts at 7, 28, and 48 days after the first immunization each consisted of injecting 12 μg of CGAP (subcutaneously) in 300 μL of emulsion composed of a 1/1 ratio of PBS/Freund's incomplete adjuvant, and antiserum was collected 1 week after the last injection.

Western blots of CGAP were performed by SDS–PAGE gel electrophoresis of purified chromaffin granules followed by electrophoretic transfer to nitrocellulose membranes. Membranes were blocked for 90 min in buffer A that was composed of phosphate-buffered saline with 0.1% bovine serum albumin, 1% gelatin, and 0.05% Tween 20. Membranes were then incubated with anti-CGAP serum (1/500 final dilution in buffer A) for 3 h, and anti-CGAP immunoreactivity was detected with alkaline phosphatase conjugated anti-mouse (1/500 final dilution in buffer A) using BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium) for development, essentially according to the Bio-Rad immunoblot kit protocol.

Results

Purification. Most (90–95%) of the chromaffin granule ^{35}S-(Met)-β-PPT and ^{35}S-(Met)-PPE cleaving activities were in the soluble granule fraction; therefore, this fraction was used for further purification. Concanavalin A–Sepharose chromatography resulted in binding of the majority (60–70%) of ^{35}S-(Met)-β-PPT and ^{35}S-(Met)-PPE cleaving activities which were eluted with methyl α-D-mannopyranoside (Figure 1a), indicating the glycoprotein nature of the enzyme(s). The unbound activity when reapplied to the column became bound and was eluted with methyl α-D-mannopyranoside. This step resulted in a 17-fold purification with a recovery of 60% of the ^{35}S-(Met)-β-PPT cleaving activity.

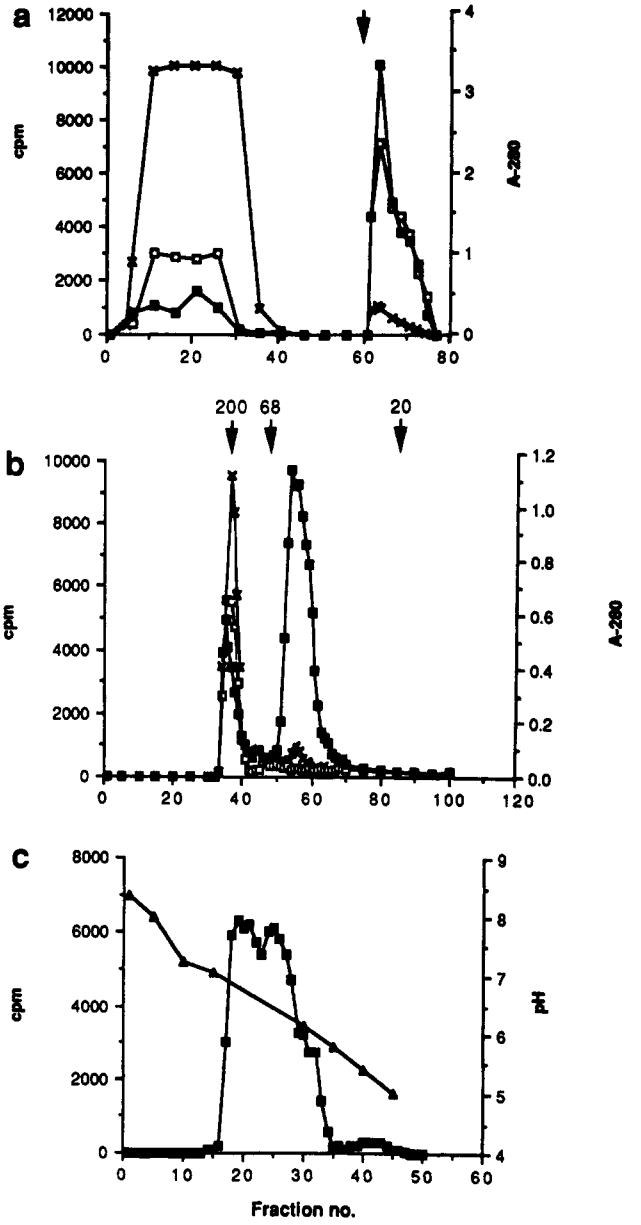


FIGURE 1: Purification of chromaffin granule aspartyl protease (CGAP). (a) Concanavalin A–Sepharose chromatography of soluble chromaffin granule extract. Elution of bound proteins with methyl α-D-mannopyranoside is indicated by the arrow. ^{35}S-(Met)-β-PPT activity (■) and ^{35}S-(Met)-PPE cleaving activity (□) in column fractions were determined as cpm of TCA-soluble radioactivity generated per 5-μL aliquot; the relative absorbance at 280 nM (×) was measured in each column fraction (panels a–c). (b) Sephacryl S200 chromatography. The concanavalin A-bound activity was fractionated by gel filtration on Sephacryl S200. Molecular mass markers of 200, 68, and 20 kDa are indicated by arrows. (c) Chromatofocusing. Peak II from the Sephacryl S200 column was subjected to chromatofocusing. The pH (△) of column fractions was determined.

Sephacryl S200 chromatography of the concanavalin A-bound activity resulted in two peaks of ^{35}S-(Met)-β-PPT cleaving activity (Figure 1b). Peak I, eluting at the void volume, possessed both ^{35}S-(Met)-β-PPT and ^{35}S-(Met)-PPE cleaving activities. Peak II, eluting with an apparent molecular

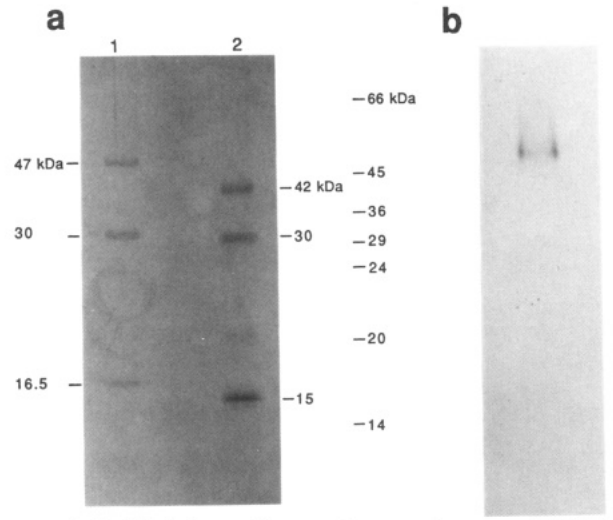


FIGURE 2: Purified chromaffin granule aspartyl protease (CGAP). (a) Denaturing gel electrophoresis of purified CGAP (2.5 μg) on 12.5% SDS–polyacrylamide gels in the presence (lane 1) or absence (lane 2) of β-mercaptoethanol. Polypeptides were visualized by silver staining. (b) Nondenaturing gel electrophoresis of purified CGAP (2 μg).

mass of 45–55 kDa, cleaved only ^{35}S-(Met)-β-PPT and not ^{35}S-(Met)-PPE. Peak II represented 80–90% of the total ^{35}S-(Met)-PPT cleaving activity eluted from the S200 column. Examination of the cleavage of ^{35}S-(Met)-PPE by SDS–PAGE gels and autoradiography confirmed that peak II enzyme did not cleave ^{35}S-(Met)-PPE (data not shown). Peak II represented a 10-fold purification with a 50% yield of ^{35}S-(Met)-β-PPT cleaving activity. This study continued with the purification of peak II; purification and characterization of peak I are described in a separate report (Krieger & Hook, 1991).

Purification of peak II by chromatofocusing (Figure 1c) resulted in elution of activity between pH 6.8 and 6.0. Analysis of this peak by SDS–PAGE under reducing conditions revealed the presence of three polypeptide bands with apparent molecular masses of 47, 30, and 16.5 kDa (Figure 2a). Electrophoretic mobilities under nonreducing conditions resulted in polypeptides with apparent molecular masses of 42, 30, and 15 kDa. These changes in mobilities suggest that the 42- and 15-kDa bands possess disulfide bonds.

Electrophoresis under nondenaturing conditions resulted in a single band (Figure 2b), suggesting that the three polypeptides exist as a complex of subunits. Coelution of the three polypeptides by gel filtration with an apparent molecular mass of 45–55 kDa suggested the possibility that two forms—a single polypeptide of 47 kDa and a 30+16.5-kDa complex—may exist. It is predicted that a single form composed of three subunits (47, 30, and 16.5 kDa) would have a larger apparent molecular mass (90–100 kDa) by gel filtration, which was not observed. Thus, the data suggest that the purified enzyme exists as a single peptide of 47 kDa and as a 30+16.5-kDa complex.

Binding of all proteolytic activity and the three polypeptides to pepstatin A–agarose indicated that all forms possessed a pepstatin A binding site that is characteristic of aspartyl proteases (data not shown). Attempts to separate the two hypothesized forms by S-Sepharose Fast Flow (cation exchanger), Sephadex G-75, Sephacryl S300, wheat germ lectin–Sepharose, and a variety of chromatofocusing conditions were not successful, since all of these chromatographic methods yielded a single peak of enzymatic activity consisting of the same three-band pattern on SDS–PAGE gels.

Table I: Purification of Chromaffin Granule Aspartyl Protease[a]

step	total protein (mg)	total act. (units)	sp act.	yield of total act. (%)	x-fold purification
lysate	251	281	1.12	100	1
extract	202	305	1.51	109	1.4
Con A bound	6.5	171	26.3	61	24
Sephacryl S200 peak II	0.346	81	234	29	209
chromatofocusing	0.101	52	515	18	459

[a] One unit of activity is 1 pmol of TCA-soluble [^{35}S]methionine/h. Specific activity is units per milligram of protein.

Table II: Effect of Protease Inhibitors on Purified ^{35}S-(Met)-PPT Cleaving Activity[a]

inhibitor	concn	% control act.
none		100
pepstatin A	150 nM	2.1
PHMB	105 μM	97
iodoacetate	2.5 mM	107
DTT	2.5 mM	129
PMSF	250 μM	114
α_1-antichymotrypsin	0.25 mg/mL	102
α_1-antitrypsin	0.25 mg/mL	125
soybean trypsin inhibitor	1.25 mg/mL	101
	0.125 mg/mL	109
EDTA	2.5 mM	133
leupeptin	250 μM	106
	25 μM	103
chymostatin	63 μM	99
	6.3 μM	102

[a] The effects of inhibitors are expressed as the percent control activity, averaged from three experiments.

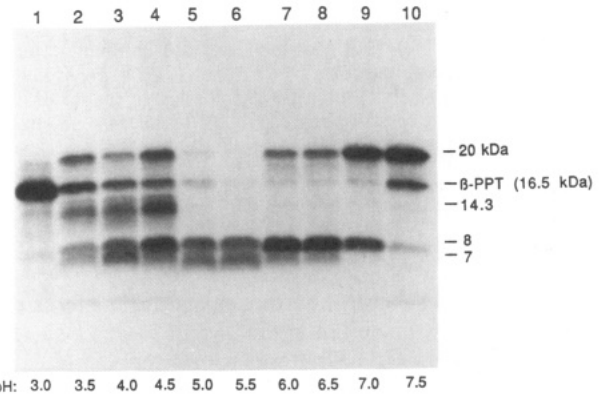


FIGURE 3: Effect of pH on cleavage of ^{35}S-(Met)-β-PPT. CGAP (converting less than 20% of substrate to TCA-soluble products) was incubated at pH 3, 4, 5, 6, and 7 with ^{35}S-(Met)-β-PPT, and cleavage of the tachykinin precursor was assessed by SDS–polyacrylamide gel electrophoresis and autoradiography.

The 3-step purification procedure (Table I) yielded 101 μg of CGAP from 440 g of adrenal medulla (160 glands) with an overall yield of 18% of the total granular ^{35}S-(Met)-β-PPT cleaving activity. The enzyme was stable when stored at −70 °C but was sensitive to repetitive freezing and thawing. The enzyme was also unstable above pH 7.0.

pH Optimum. Cleavage of ^{35}S-(Met)-β-PPT was optimal at pH 5.0–5.5 (Figure 3). No cleavage occurred at pH 3.0, and some cleavage occurred at pH 7.5. Different levels of products of 20, 14.3, 8, and 7 kDa were detected at the various pHs. The slower mobility of the 20-kDa band compared to ^{35}S-(Met)-β-PPT is likely to be due to anomalous electrophoretic mobility of some PPT-related peptides, which has been demonstrated for β-protachykinin compared to β-PPT (McDonald et al., 1988). Production of TCA-soluble radioactivity was also optimal at pH 5.0–5.5 with no activity at pH

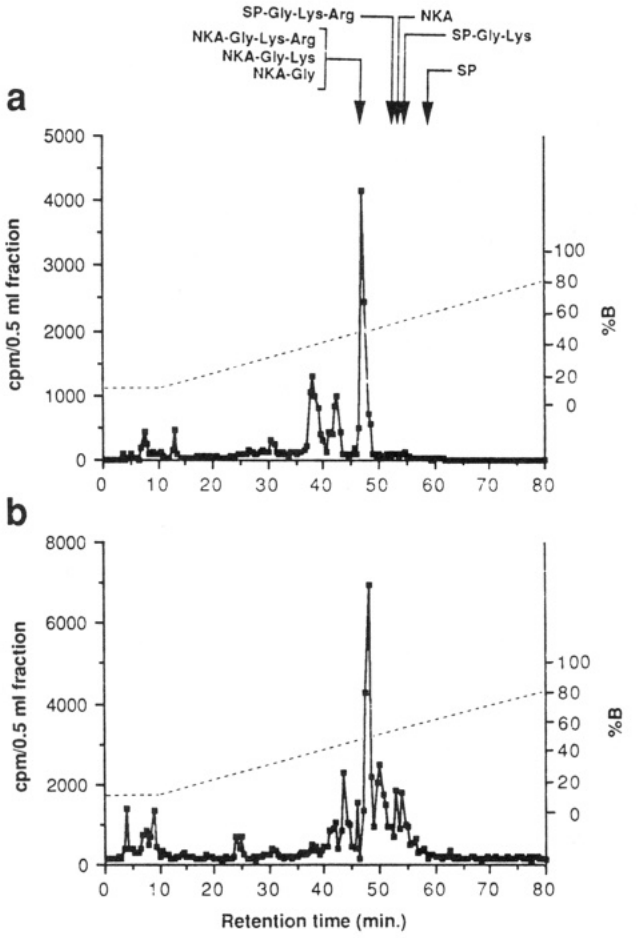


FIGURE 4: HPLC of [^{35}S]Met-labeled TCA-soluble products. TCA-soluble cleavage products of ^{35}S-(Met)-β-PPT generated by CGAP (panel a) and by chromaffin granule lysate (panel b) were chromatographed by reverse-phase HPLC (C18 column) in a buffer system of 0.1% TFA. ^{35}S-Peptides and standard peptides (indicated by arrows) were eluted with an acetonitrile gradient (buffer B = 60% acetonitrile in 0.1% TFA). Fractions of 0.5 mL (equivalent to 0.5 min) were collected, and the radioactivity in a 50-μL aliquot of each fraction was counted and plotted.

3.0 or 7.0 (data not shown). Further enzyme characterization was conducted at the optimum pH of 5.0.

Protease Inhibitors. CGAP was completely inhibited by pepstatin A (Table II), an aspartyl protease inhibitor. Inhibitors of other mechanistic classes of proteases had no effects. Thiol protease inhibitors (PHMB and iodoacetate), serine protease inhibitors (PMSF, soybean trypsin inhibitor, and α_1-antitrypsin), and a metalloprotease inhibitor (EDTA) had no effects. Leupeptin and chymostatin had no effects. ^{35}S-(Met)-β-PPT cleaving activity in lysed granules was also inhibited only by pepstatin A (data not shown), indicating that CGAP is the main activity that cleaves the tachykinin precursor in these vesicles.

Chromatography of TCA-Soluble Products by HPLC and HVE. HPLC of TCA-soluble products generated from ^{35}S-(Met)-β-PPT by CGAP or by granule lysate (Figure 4) showed a main radioactive peak comigrating with NKA-Gly-Lys-Arg, NKA-Gly-Lys, and NKA-Gly standards which coelute. To resolve these NKA-related peptides, further HVE analysis of this HPLC peak produced by CGAP (Figure 5) showed a single spot that comigrated with NKA-Gly-Lys, suggesting cleavage of the tachykinin precursor at Lys-Arg pairs. HVE of the main HPLC peak generated by lysed granules also showed a spot comigrating with NKA-Gly-Lys, showing that CGAP represents the major chromaffin granule

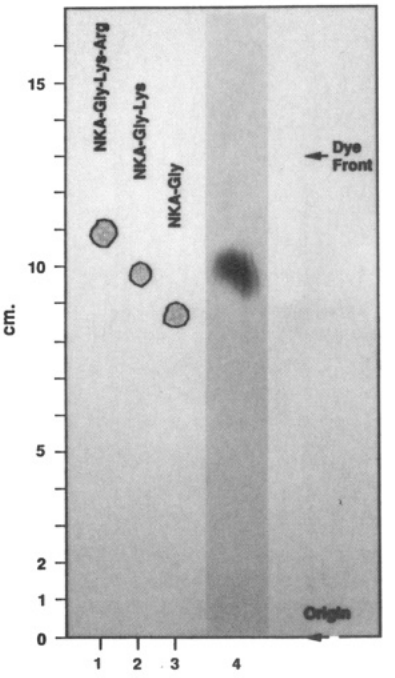


FIGURE 5: HVE of [^{35}S]Met-labeled peptide(s). High-voltage electrophoresis was performed on the main radioactive HPLC peak (Figure 4, 47–48-min retention time) generated by CGAP. Lanes 1, 2, and 3 represent standard migration positions of NKA-Gly-Lys-Arg, NKA-Gly-Lys, and NKA-Gly, respectively, as detected with fluorescamine. HVE of the major ^{35}S-(Met)-β-PPT peptide product, shown in lane 4, was detected by autoradiography of the TLC plate.

^{35}S-(Met)-β-PPT cleaving activity.

HPLC also showed that CGAP produced minor peaks at 38- and 42.5-min retention times and the granule lysate generated peaks at 44, 46, 53, and 54 min (Figure 4). Since the granules contain another protease capable of cleaving ^{35}S-(Met)-β-PPT, detected as peak I (Figure 1, Sephacryl S200 column), the production of additional peaks by the granule lysate is expected.

In control experiments, when oxidized forms of standard tachykinin peptides were analyzed by HPLC, the Met-sulfoxide forms of SP-Gly-, SP-Gly-Lys, and SP-Gly-Lys-Arg coeluted with the main radioactive peak generated from ^{35}S-(Met)-β-PPT (data not shown). However, on HVE, the Met-sulfoxide forms of the SP-related peptides did not comigrate with the radioactive spot. These results confirm the chromatographic identity of the main acid-soluble radioactive product as ^{35}S-(Met)-NKA-Gly-Lys.

Analysis of NPK Cleavage Products. The low mass of the [^{35}S]Met-labeled peptide precludes use of microsequencing for peptide identification and determination of cleavage sites. However, neuropeptide K (NPK), a segment of the β-tachykinin precursor containing neurokinin A, could be used as substrate at quantities that allow microsequencing and amino acid analyses of products. After proteolysis of NPK with CGAP, peptide products were isolated by HPLC (Figure 6), and peaks 1–3 were subjected to microsequencing and amino acid analysis. Microsequencing of two peptides in HPLC peaks 1 and 2 was apparent, and amino acid compositions were in agreement. The microsequencing results (Figure 7) show that NPK was cleaved at the COOH-terminal side of Lys-Arg, and between Leu-Lys, Leu-Tyr, and Phe-Val sites.

NH_2-Terminal Sequences and Amino Acid Compositions of Purified CGAP. The three polypeptides of the purified CGAP were subjected to NH_2-terminal microsequencing (Figure 8). The 47- and 16.5-kDa polypeptides possessed identical NH_2-terminal sequences, suggesting that the 16.5-

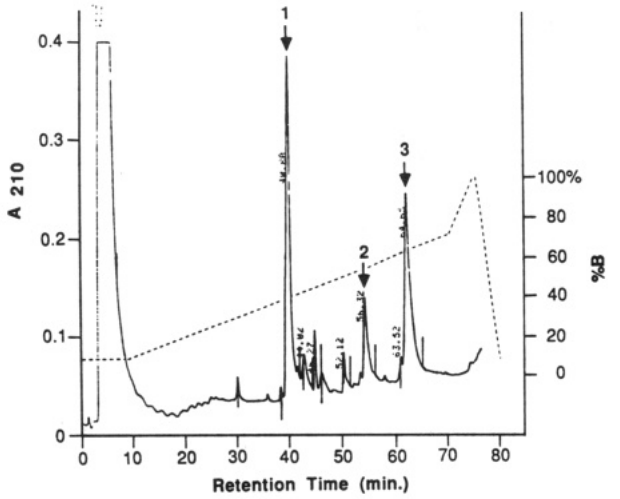
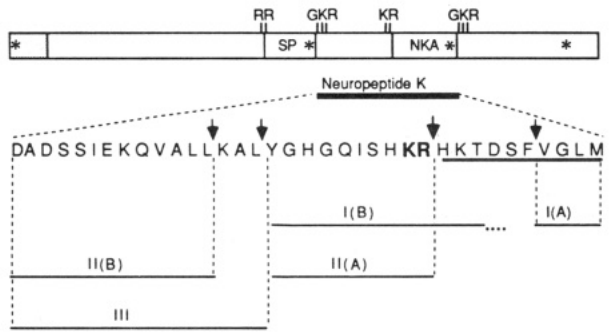


FIGURE 6: HPLC of neuropeptide K (NPK) cleavage by CGAP. Cleavage products of NPK were chromatographed by reverse-phase HPLC (C18 column) in a buffer system of 0.1% TFA with an acetonitrile gradient (buffer B = 60% acetonitrile in 0.1% TFA). Peptides were detected by the relative absorbance at 210 nm. Standard neuropeptide K had a retention time of 71 min. HPLC of purified CGAP alone resulted in a peak (detected at 210 nm) at 3–10-min retention time.

a



b

Peak I: (A) Val-Gly-Leu-Met-NH_2
(B) Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr....

Peak II: (A) Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg
(B) Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu

Peak III: (A) Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu

FIGURE 7: Products of neuropeptide K cleavage by chromaffin granule aspartyl protease. (a) The segment of β-preprotachykinin (Harmar et al., 1986) corresponding to neuropeptide K (NPK) is illustrated. SP represents substance P that possesses the sequences Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2. NKA represents neurokinin A that possesses the sequence His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2. Asterisks indicate the positions of methionine residues. Arrows indicate CGAP-catalyzed cleavage sites within the sequence of NPK, as deduced from the illustrated peptide products. (b) Amino acid sequences of the peptides obtained from HPLC peaks 1–3 (Figure 6) determined by microsequencing and amino acid composition. Periods at the COOH-terminus of peptide I(B) represent incomplete sequencing.

kDa polypeptide may be a proteolytic product of the 47-kDa protein. The sequence of the NH_2-terminal 19 residues of the 47-, 30-, and 16.5-kDa peptides possessed 100%, 90%, and 100% homology to bovine spleen cathepsin D (Yonezawa et al., 1988), respectively. CGAP, however, lacks the Ser-Pro residues that are present at the NH_2 terminus of the 30-kDa polypeptide of bovine spleen cathepsin D.

47 kDa: Bovine CGAP GPIPELLKNYMDAQYYGEI
Bovine Cathepsin D GPIPELLKNYMDAQYYGEI

30 kDa: Bovine CGAP GPVTVQRQTFGEAIKEPGV
Bovine Cathepsin D SPGPVTVQRQTFGEAIKEPGV

16.5 kDa: Bovine CGAP GPIPELLKNYMDAQYYGEI
Bovine Cathepsin D GPIPELLKNYMDAQYYGEI

FIGURE 8: NH_2-terminal sequences of bovine CGAP and bovine spleen cathepsin D. Microsequencing results of the 47-, 30-, and 16.5-kDa polypeptide subunits of CGAP and bovine cathepsin D (Yonezawa et al., 1988) are shown. The NH_2-terminal sequences of the 45- and 15-kDa polypeptides of bovine spleen cathepsin D are from Tang (1979). Our sequence determination of the 30-kDa peptide of bovine cathepsin D is identical with that previously reported (Yonezawa et al., 1988) except for a Gly residue at position 4 from the NH_2-terminus.

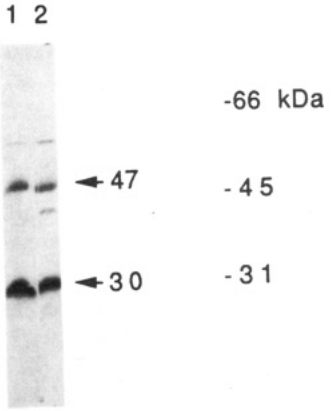


FIGURE 9: Western blot of CGAP in chromaffin granules purified through one and two discontinuous sucrose gradients. Immunoblots of CGAP in one- and two-gradient-purified chromaffin granules (60 μg of protein per lane of gel), prepared as described under Methods, are shown in lanes 1 and 2, respectively.

Table III: Amino Acid Composition (Mole Percent) of CGAP and Bovine Spleen Cathepsin D

	CGAP			cathepsin D[a]		
amino acid	47 kDa	30 kDa	16.5 kDa	47 kDa	30 kDa	16.5 kDa
Cm-Cys	1.9	1.5	0.0	1.8	1.8	2.1
Asx	10.6	9.5	12.2	9.7	9.2	11.6
Thr	5.7	4.7	7.3	5.5	5.0	7.4
Ser	6.0	4.0	10.1	6.9	5.9	10.5
Glx	9.2	10.8	8.5	8.8	9.5	6.3
Pro	6.5	6.2	9.4	6.7	6.5	7.4
Gly	9.7	10.0	12.4	10.9	11.2	9.5
Ala	5.4	6.4	2.8	5.3	5.9	3.2
Val	9.8	10.6	5.8	8.5	9.2	6.3
Met	2.4	3.0	1.0	2.5	3.0	1.1
Ile	5.5	5.1	6.1	5.3	5.0	6.3
Leu	9.5	9.9	8.0	8.5	8.9	7.4
Tyr	4.4	3.3	5.8	4.8	4.1	7.4
Phe	3.9	4.8	2.2	4.4	4.4	4.2
His	0.9	0.7	2.4	1.8	1.5	3.2
Lys	5.2	5.1	4.9	4.8	4.7	5.3
Arg	3.4	4.4	1.3	3.5	4.1	1.1
Trp	ND	ND	ND	NI	NI	NI

[a] Data from Takahashi and Tang (1981). ND = not determined due to unstability under acid hydrolysis. NI = not included for purposes of comparison. The 0.0 value for Cm-Cys of the 16.5-kDa polypeptide of CGAP may be due to incomplete carboxymethylation.

Amino acid composition data (Table III) showed that the mole percents for all residues of CGAP and bovine spleen cathepsin D were virtually identical, with the exception of hisitidine which was 50% lower in the 30- and 47-kDa polypeptides of CGAP compared to bovine cathepsin D.

CGAP Immunoreactivity in Highly Purified Chromaffin

Granules. Anti-CGAP Western blots of chromaffin granules (Figure 9) detected the 47- and 30-kDa bands of CGAP, but the 16.5-kDa polypeptide of CGAP was not detected since it was present at a much lower level of protein than the other two bands. To further test that the purified CGAP was associated with chromaffin granules, the amount of CGAP immunoreactivity was compared in granules prepared through one and two sucrose gradients, which result in purified granules containing 0.5% and 0.05% of total tissue lysosomal acid phosphatase, respectively (Hook & Eiden, 1984). Thus, if CGAP is associated with chromaffin granules, it should copurify with granules in the second gradient. If, however, CGAP is of lysosomal origin, then CGAP would not remain with two gradient-purified granules, since CGAP would be removed with lysosomes. A similar level of immunoreactive CGAP was detected in granules (60 μg of granule protein) obtained through one and two sucrose gradients (Figure 9). This finding shows that CGAP in the second gradient copurified with granules (not lysosomes), providing further evidence and support for the hypothesis that CGAP appears to be associated with chromaffin granules.

Discussion

Purification of potential tachykinin and enkephalin precursor cleaving enzymes from bovine chromaffin granules resulted in the isolation of an aspartyl protease that shows precursor selectivity and resembles cathepsin D. The chromaffin granule aspartyl protease (CGAP) cleaved the tachykinin but not the enkephalin precursor in the in vitro assay. Activity was optimal near the intragranular pH of 5.5–5.8 (Pollard et al., 1979). Apparent HPLC and HVE chromatographic identification of ^{35}S-NKA-Gly-Lys as the major acid-soluble product generated from ^{35}S-(Met)-β-PPT and cleavage of neuropeptide K at Lys-Arg suggest that some cleavage occurred at paired basic residues. Interestingly, like cathepsin D, CGAP also cleaved at hydrophobic residues. CGAP's similar NH_2-terminal sequences and amino acid composition confirmed its resemblance to cathepsin D. Overall, CGAP's acidic pH optimum near the intragranular pH and cleavage specificity for dibasic and hydrophobic residues suggest that CGAP may possibly be involved in cleaving some peptide precursors. In addition, CGAP's cathepsin D-like properties suggest that it could also be involved in degrading precursor segments that are not part of the active peptide.

Purified CGAP consisted of 47-, 30-, and 16.5-kDa polypeptides (on SDS–PAGE gels) which comigrated in a native gel system and coeluted at 45–55 kDa by gel filtration, suggesting that CGAP exists as a single 47-kDa form and as a complex of 30+16.5-kDa subunits. This structure is similar to the 45-, 30-, and 15-kDa polypeptides of cathepsin D that exist in a single-chain form (45 kDa) and a heavy plus light chain (30+15 kDa) form (Tang, 1979; Takahashi & Tang, 1981; Shewale & Tang, 1984).

Structural analyses of CGAP showed that the NH_2-terminal sequences and amino acid compositions of the three polypeptides were virtually identical to bovine cathepsin D (Yonezawa et al., 1988). Like cathepsin D, the 47- and 16.5-kDa polypeptides of CGAP possess identical NH_2-terminal amino acid sequences, indicating that proteolysis of the 47-kDa polypeptide could result in the 30- and 16.5-kDa peptides. Cloning of human cathepsin D (Faust et al., 1985) shows that a single cDNA encodes the 45-, 30-, and 15-kDa polypeptides.

Some minor differences between CGAP and cathepsin D were observed. First, the 30-kDa polypeptide of CGAP lacks the NH_2-terminal Ser-Pro residues present in the 30-kDa peptide of bovine spleen cathepsin D. It is possible that aminopeptidase activity has removed the Ser-Pro residues from the CGAP 30-kDa peptide. Aminopeptidase activity has been detected in chromaffin granules that removes NH_2-terminal Lys and Arg from Lys-Arg-(Met)enkephalin (Hook & Eiden, 1984); it is not known, however, if this activity or other unidentified aminopeptidases possess specificity for Ser or Pro residues. It is also possible that endoproteolytic processing of the 47-kDa CGAP polypeptide may differ from cathepsin D processing. Differential processing of cathepsin D's from bovine, porcine, and rat species (Yonezawa et al., 1988) has been observed. A second difference between CGAP and cathepsin D lies in their amino acid compositions, as the histidine content of the 47- and 30-kDa polypeptides of CGAP was 50% of the values obtained for the corresponding bovine cathepsin D subunits.

The major similarities and minor differences between CGAP and cathepsin D lead to the hypothesis that CGAP is cathepsin D or a "cathepsin D-like" protease. Like cathepsin D, CGAP cleaved at hydrophobic residues; CGAP also cleaved at a Lys-Arg-paired basic residue site. Precursor cleavage at hydrophobic and basic residues is required in the processing of some precursors such as preproendothelin (Yanagisawa et al., 1988). The endothelin vasoconstrictor peptides, present in chromaffin granules (Sawamura et al., 1990), are synthesized by proteolytic processing of proendothelin at dibasic residues followed by cleavage at a hydrophobic Trp-Val site. CGAP cleavage at dibasic and hydrophobic residues would be consistent with a possible role for CGAP in proendothelin processing.

Cleavage at cathepsin D-like hydrophobic sites also suggests that CGAP could be involved in degrading precursor segments that do not include the active peptide, as demonstrated by CGAP cleavage at Leu-Lys and Leu-Tyr hydrophobic sites within a segment of the tachykinin precursor that does not include the sequences of substance P and substance K. The conditions or factors that influence CGAP's choice for dibasic or hydrophobic cleavage sites are not known, and should be the subject of future investigations.

Further evidence for association of CGAP with chromaffin granules was provided by Western blot analyses of CGAP in granules purified through one and two 1.6 M/0.32 M discontinuous sucrose gradients, which provide purified granules containing only 0.5% and 0.05% of the total tissue lysosomal acid phosphatase, respectively (Hook & Eiden, 1984). The similar levels of CGAP immunoreactivity in granules isolated through one- and two-gradient purified granules suggest that CGAP copurified with the chromaffin granules and not with lysosomes. In addition, preliminary immunoelectron microscopy experiments show localization of CGAP in chromaffin granules. Future electron microscopy studies showing CGAP colocalization with peptide precursor(s) in chromaffin granules will be required to support the hypothesized role of CGAP in cleaving peptide precursors.

Precedence for the localization of cathepsin D in renin secretory granules of juxtaglomerular epithelioid cells has been demonstrated by electron microscopic immunohistochemistry (Taugner et al., 1986). It is also interesting to note that cathepsin D mRNA is expressed in rat adrenal and submaxillary gland at a 5–10-fold higher level than in liver, pituitary, kidney, and pancreas (Faust et al., 1985). These findings suggest a special function for cathepsin D in tissues possessing high secretory functions.

In addition to CGAP, chromaffin granules possess a novel thiol protease that is involved in enkephalin and tachykinin precursor processing (Krieger & Hook, 1991). This 33-kDa

protease is represented by peak I in the Sephacryl S200 column from this study. It converted ^{35}S-(Met)-PPE to multiple intermediates that contain the precursor's NH_2-terminal segment; proenkephalin in vivo is processed similarly (Patey et al., 1984; Birch et al., 1987). (Met)enkephalin, the final product of proenkephalin processing, was generated by cleavage of peptide F at Lys-Arg and Lys-Lys sites. The thiol protease's appropriate vesicular localization, acidic pH optimum near the intragranular pH, proteolytic products, and cleavage site specificity support its role in neuropeptide precursor processing.

Chromaffin granules also possess serine proteases that may be involved in peptide precursor processing. A serine protease with a basic pH optimum of pH 8.5–8.7 has been purified from chromaffin granule membranes (Shen et al., 1989). More recently, Kex2-related proteases (Christie et al., 1991), members of the subtilisin protease family, have been identified in chromaffin granules.

It appears that several proteases of different mechanistic classes—thiol, aspartyl, and serine proteases—may be involved in processing adrenal medullary neuropeptides. It will be important to determine the relative activities of each protease in processing peptide precursors.

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