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Characteristics of the Chromaffin Granule Aspartic Proteinase Involved in Proenkephalin Processing

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Abstract: Proteolytic processing of neuropeptide precursors is required for production of active neurotransmitters and hormones. In this study, a chromaffin granule (CG) aspartic proteinase of 70 kDa was found to contribute to enkephalin precursor cleaving activity, as assayed with recombinant ([^{35}S]Met)preproenkephalin. The 70-kDa CG aspartic proteinase was purified by concanavalin A-Sepharose, Sephacryl S-200, and pepstatin A agarose affinity chromatography. The proteinase showed optimal activity at pH 5.5. It was potently inhibited by pepstatin A, a selective aspartic proteinase inhibitor, but not by inhibitors of serine, cysteine, or metalloproteinases. Lack of inhibition by Val-D-Leu-Pro-Phe-Val-D-Leu—an inhibitor of pepsin, cathepsin D, and cathepsin E—distinguishes the CG aspartic proteinase from classical members of the aspartic proteinase family. The CG aspartic proteinase cleaved recombinant proenkephalin between the Lys^{172}–Arg^{173} pair located at the COOH-terminus of (Met)enkephalin-Arg^{6}-Gly^{7}-Leu^{8}, as assessed by peptide microsequencing. The importance of full-length prohormone as substrate was demonstrated by the enzyme's ability to hydrolyze ^{35}S-labeled proenkephalin and proopiomelanocortin and its inability to cleave tri- and tetrapeptide substrates containing dibasic or monobasic cleavage sites. In this study, results provide evidence for the role of an aspartic proteinase in proenkephalin and prohormone processing. **Key Words:** Chromaffin granule aspartic proteinase—Proenkephalin processing—Prohormone processing.
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Conversion of many neuropeptide precursors into active peptide hormones or neurotransmitters requires proteolytic cleavage of the precursors at paired basic residue sites (Lys–Arg, Arg–Arg, Lys–Lys, and Arg–Lys) (Docherty and Steiner, 1982; Hook et al., 1994). Secretory vesicles from bovine adrenal medulla, known as chromaffin granules (CGs), contain several neuropeptides—including enkephalins (Udenfriend and Kilpatrick, 1983), neuropeptide Y (Carmichael et al., 1990), somatostatin (Lundberg et al., 1979), and others—that are generated by proteolytic processing of the respective precursors. Hence, CGs should contain the relevant neuropeptide precursor processing enzymes.

Recent studies suggest the involvement of subtilisin, cysteine, and aspartic proteinases in prohormone processing (Steiner et al., 1992; Loh et al., 1993; Hook et al., 1994). CGs and other neuroendocrine secretory vesicles contain the subtilisin-related proprotein convertases PC1/3 and PC2 (Christie et al., 1991; Azaryan and Hook, 1992; Kirchmair et al., 1992; Azaryan et al., 1995), which were cloned (reviewed by Seidah et al., 1991; Steiner et al., 1992; Smeekens, 1993) based on active site homology to the yeast *Kex2* gene product. Evidence supporting the role of these PC proteases in prohormone processing is based on studies of recombinant PC enzymes coexpressed with prohormone cDNAs in eukaryotic cell lines (Benjannet et al., 1991; Seidah et al., 1991; Thomas et al., 1991; Smeekens et al., 1992) and by in vitro processing studies with recombinant PC enzymes (Shennan et al., 1991; Jean et al., 1993; Rufaut et al., 1993; Zhou and Lindberg, 1993). Endogenous PC1/3 and PC2 activities have been characterized in CGs (Azaryan and Hook, 1992; Azaryan et al., 1995), as well as in secretory vesicles of insulinoma cells (Bennett et al., 1992), in intermediate pituitary (Estivariz et al., 1992), and in pancreatic islets of anglerfish (Mackin et al., 1991).

The cysteine protease prohormone thiol protease (PTP) has been demonstrated as an important candidate proenkephalin (PE) processing enzyme in CGs (Krieger and Hook, 1991; Krieger et al., 1992; Azar-

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Abbreviations used: Boc, *N-tert*-butoxycarbonyl; CG, chromaffin granule; DTT, dithiothreitol; MCA, methylcoumarinamide; PC, proprotein convertase; PCE, proopiomelanocortin converting enzyme; PCR, polymerase chain reaction; PE, proenkephalin; POMC, proopiomelanocortin; PPE, preproenkephalin; PTP, prohormone thiol protease; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

yan and Hook, 1994*a,b*). PTP cleaves paired basic and monobasic sites to generate the final peptide product (Met)enkephalin (Krieger et al., 1992; Azaryan and Hook, 1994*a*). It is important that PTP is distinguished from cysteine cathepsins B, L, and H based on PTP binding to concanavalin A (cathepsin B does not bind to concanavalin A) (Krieger and Hook, 1991), selectivity for peptide–methylcoumarinamide (MCA) substrates, cleavage specificity, potency of active site-directed protease inhibitors, and differences in characteristic proteolytic activity (Azaryan and Hook, 1994*b*). It is hypothesized that PTP represents a novel cysteine protease.

Several studies also provide evidence for the involvement of aspartic proteinases in processing pro-opiomelanocortin (POMC) and provasopressin in pituitary (Loh et al., 1985, 1993; Parish et al., 1986), as well as processing of the yeast pro-α-mating factor (Egel-Mittani et al., 1990; Azaryan et al., 1993). In this report we describe the characterization of the purified CG aspartic proteinase with respect to its role in PE processing. The properties of the CG aspartic proteinase are compared with those of pituitary POMC converting enzyme (PCE) (Loh et al., 1985; Parish et al., 1986) and yeast (YAP3) aspartic proteinases (Egel-Mittani et al., 1990; Azaryan et al., 1993). Results suggest the CG aspartic proteinase as a candidate prohormone processing enzyme.

EXPERIMENTAL PROCEDURES

Assay of enzymatic activity with recombinant enkephalin precursor generated by in vitro transcription and translation

([^{35}S]Met)preproenkephalin [([^{35}S]Met)-PPE] was used as the routine model substrate for assay of enkephalin precursor cleaving activity in CGs, as described previously (Hook et al., 1990; Krieger and Hook, 1991). ([^{35}S]Met)-PPE was synthesized from the rat PPE cDNA in the pSP65 vector (Promega) by in vitro transcription and translation as described previously (Hook et al., 1990; Krieger and Hook, 1991). Activity was assayed by incubating enzyme and ([^{35}S]Met)-PPE (40,000–50,000 cpm) for 2 h at 37°C, and production of ^{35}S-peptide products was quantified by measuring the production of trichloroacetic (TCA)-soluble radioactivity according to the method of Krieger and Hook (1991).

Purification of aspartic proteinase from CGs

The soluble fraction of the CGs (from 650 adrenal medullae) was subjected to chromatography on concanavalin A-Sepharose and Sephacryl S-200 gel filtration as previously described (Krieger and Hook, 1991). The Sephacryl S-200 fractions corresponding to 70 kDa in molecular size were pooled and further purified by affinity chromatography on pepstatin A-agarose. After the pH of the 70-kDa fraction was adjusted to 4.5, it was rocked with pepstatin A-agarose (5 ml; Pierce) in 50 m*M* sodium citrate buffer (pH 4.5) and 0.2 *M* NaCl for 4 h at 4°C. The mixture was packed into a column, the resin was washed with 50 m*M* sodium citrate (pH 4.5), and bound proteins were eluted with 0.1 *M* Tris-HCl (pH 8.5) and 0.2 *M* NaCl. The pH of eluted fractions was immediately adjusted to 6.0. The pepstatin A-bound fractions were dialyzed against 50 m*M* sodium citrate (pH 6.0) and concentrated to 5 ml on a YM-5 Amicon filter. The homogeneity of the purified enzyme was assessed by SDS-PAGE as described previously (Krieger and Hook, 1991).

pH optimum and effect of protease inhibitors

Purified enzyme was assayed at pH values between 3.0 and 8.5 with 0.1 *M* sodium citrate (pH 3.0–6.0) and 0.1 *M* Tris-HCl (pH 6.5-8.5) using ([^{35}S]Met)-PPE as substrate, as described above. Enzyme and protease inhibitors were incubated for 30 min at room temperature in 0.1 *M* sodium citrate (pH 5.5) and 1 m*M* DTT, and reactions were initiated by adding ([^{35}S]Met)-PPE (40,000–50,000 cpm/assay). After incubation at 37°C for 2 h, enzymatic activity was determined by measuring production of TCA-soluble radioactivity, as described previously (Hook et al., 1990; Krieger and Hook, 1991).

CG aspartic proteinase and peptide-MCA substrates

The ability of the CG aspartic proteinase to hydrolyze peptide–MCA substrates containing basic residues was performed as described (Azaryan and Hook, 1992, 1994*a,b*). Enzyme was incubated with 100 μM peptide–MCA substrates (from Peninsula Laboratories, except pGly-Arg-Thr-Lys-Arg-MCA and Arg-Gln-Arg-Arg-MCA, which were from Bachem) at 37°C for 18 h in 0.1 *M* sodium citrate (pH 5.5) containing 1 m*M* DTT in 160 μl. Measurement of the hydrolysis of peptide–MCA substrates to generate 7-amino-4-methylcoumarin was performed as previously described (Azaryan and Hook, 1992, 1994*a,b*).

CG aspartic proteinase and ([^{35}S]Met)-PE and ([^{35}S]Met)-POMC substrates

To test the ability of the CG aspartic proteinase to hydrolyze authentic prohormones, ([^{35}S]Met)-PE and ([^{35}S]-Met)-POMC were produced by in vitro transcription and translation. The rat PE (Yoshikawa et al., 1984) and porcine POMC (Boileau et al., 1983), with signal sequences deleted, were subcloned in the pSP65 vector (Promega). Signal sequences of the preprohormones were deleted by polymerase chain reaction (PCR) deletion mutagenesis (Higuchi, 1990). For construction of PE/pSP65, PCR primers were 5′-TTTAGGTGACAC-3′, 5′-GGCTGCAGTCCATGAGGCTG-3′, 5′-GTCTTCCAGCT-3′, and 5′-CAGCCTCATGGACTGCAGCC-3′; the PCR-amplified 681-bp fragment was digested with *Eco*RI and *Stu*I to generate a 626-bp fragment that was ligated into *Eco*RI- and *Stu*I-digested PPE/pSP65 plasmid. Positive colonies were screened by restriction enzyme digestion (*Eco*RI and *Stu*I) of isolated plasmids. For construction of the POMC/pSP65 plasmid, the prePOMC cDNA (*Eco*RI/*Pst*I fragment) was subcloned into *Eco*RI and *Pst*I sites of the pSP65 vector. PCR deletion mutagenesis of the prePOMC/pSP65 construct used the primers 5′-GTTGCGCGTCG-3′, 5′-CGTGGGAGATGTGGTGCTTGGAG-3′, 5′-CTCCAAGCACCACATCTCCCACGCAGGCTGAGG-3′, and 5′-TTTGAATTCAGAGGGAAGAGCAAGAGGGGAAG-3′ to generate a 262-bp fragment. This 262-bp fragment was digested with *Eco*RI and *Bgl*II and ligated into *Eco*RI- and *Bgl*II-digested prePOMC/pSP65 to generate the POMC/pSP65 construct. After transformation into competent DH5α *Escherichia coli* and analysis of isolated plasmids by restriction enzyme digests, deletion of the signal sequences was confirmed by DNA sequencing of PE/pSP65 and POMC/pSP65 constructs by the dideoxy chain

terminating method (Sanger et al., 1977) using the Applied Biosystems automated sequencer, as previously described (Hwang et al., 1994). In vitro transcription and translation were performed as described previously (Hook et al., 1990; Krieger and Hook, 1991) to generate ([^{35}S]Met)-PE and ([^{35}S]Met)-POMC.

CG aspartic proteinase (1.25 μg) was incubated with ^{35}S-labeled PE (83,000 cpm per assay) and POMC (47,000 cpm per assay). To obtain equimolar concentrations of ^{35}S-PE and ^{35}S-POMC in the assay, total cpm of ^{35}S-PE or ^{35}S-POMC in the reactions was adjusted to account for the fact that PE contains nine methionine residues and POMC contains five methionine residues. After incubation of enzyme and ^{35}S-prohormones in 0.1 *M* sodium citrate (pH 5.5), 1 m*M* DTT, 1 m*M* EDTA, and 10 m*M* 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (total reaction volume was 20 μl) at 37°C for 3 h, the production of TCA-soluble radioactivity was determined as previously described (Krieger and Hook, 1991).

Expression of recombinant PE in *E. coli* and purification

Expression of recombinant PE in *E. coli* used the T7 expression system (Studier et al., 1990). The rat PE cDNA (Yoshikawa et al., 1984) was subcloned into *Nde*I and *Bam*HI sites of the pET3c expression vector by ligating (T4 DNA ligase) a 51-bp *NdeI*/*Hpa*II fragment and an *Hpa*II/*Bam*HI PE DNA fragment of ~714 bp. The 51-bp DNA was generated by annealing two complementary oligonucleotides (kinased by T4 polynucleotide kinase) consisting of 5′-TATGGACTGCAGCCAGGACTGCGCTAAATGCAGC-TACCGCCTGGTACGTGG-3′ and 5′-CGGGACGTAC-CAGGCGGTAGCTGCATTTAGCGCAGTCCTGGCTG-CAGTCCA-3′. After transformation of the PE/pET3c plasmid into DH5α cells, restriction enzyme digests of plasmid DNA confirmed colonies containing the PE/pET3c plasmid. The PE sequence in the pET3c vector was confirmed by DNA sequencing by the dideoxy chain terminating method (Sanger et al., 1977).

The PE/pET3c expression vector was transformed into BL21(DE3) cells grown in M9ZB media with 100 μg/ml of ampicillin as described by Studier et al. (1990). Cells at a density of A_{600} = 0.1 were incubated with rifampicin at 100 μg/ml for 2 h, and then PE expression was induced with isopropyl β-D-thiogalactopyranoside (0.2 m*M*) for 30 min at 37°C. Cells were pelleted by centrifugation (2,600 *g* at 4°C for 20 min).

Purification of recombinant PE was achieved by diethylaminoethyl-Sepharose ion exchange chromatography, preparative SDS-PAGE, and reverse-phase HPLC. Cells were resuspended in 50 m*M* Tris-HCl (pH 7.5) and 6.0 *M* urea (buffer B), rocked at 4°C for 1 h, and centrifuged for 30 min at 27,000 *g* at 4°C, and the supernatant contained PE. Soluble PE was chromatographed on a diethylaminoethyl-Sepharose column (1.5 × 35 cm) eluted with a 0–500 m*M* NaCl gradient in buffer B. PE in column fractions was monitored by anti-PE immunoblots [PE-18 antibody at 1:1,000 (Spruce et al., 1988)], performed as described previously (Hook et al., 1990; Schiller et al., 1995). PE was then subjected to preparative SDS-PAGE using the Bio-Rad model 491 preparative electrophoresis cell (37-mm cell), followed by dialysis against 0.25 m*M* Tris-HCl (pH 7.5) and lyophilization. Reverse-phase HPLC of PE was conducted with a C8 column (4.6 × 250 mm; Vydac) with a precolumn (4.6 × 20 mm; NEST Group), using a Waters HPLC system.

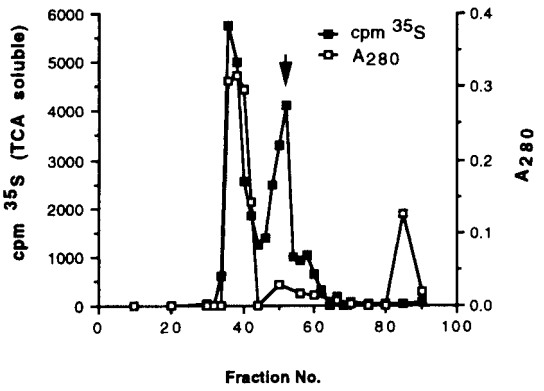

FIG. 1. Sephacryl S-200 chromatography. The concanavalin A bound activity was subjected to gel filtration on Sephacryl S-200. ([^{35}S]Met)-PPE cleaving activity (■) expressed as total TCA-soluble radioactivity generated (by a 5-μl aliquot) and absorbance at 280 nm (□) were determined in all column fractions.

Elution of PE used a 10–80% acetonitrile gradient in 0.1% trifluoroacetic acid, with detection of proteins at 215 nm. PE fractions were lyophilized and resuspended in 5 m*M* citrate–NaOH, pH 6.0. Peptide microsequencing and amino acid composition analysis of purified PE, performed as described previously (Krieger and Hook, 1991; Krieger et al., 1992), confirmed that authentic PE was obtained (Schiller et al., 1995) based on the known PE primary sequence deduced from the preproenkephalin cDNA (Yoshikawa et al., 1984).

CG aspartic proteinase and recombinant PE

Purified CG aspartic proteinase (2 μg) and recombinant PE (8 μg) were incubated for 0.5–4 h at 37°C in 0.1 *M* sodium citrate buffer (pH 5.5), 1 m*M* DTT, 1 m*M* EDTA, and 10 m*M* CHAPS (volume, 30 μl). Reaction mixtures were subjected to SDS-PAGE and electrophoretically transferred to PVDF membranes with amido black staining of peptide bands, which were subjected to peptide microsequencing as described previously (Krieger and Hook, 1991; Krieger et al., 1992).

RESULTS

Purification of the CG aspartic proteinase

The soluble CG extract, containing ~90% of total granule enkephalin precursor cleaving activity (Krieger and Hook, 1991), was used as starting material. Chromatography on concanavalin A-Sepharose was an effective step that resulted in a 32-fold purification of enkephalin precursor cleaving activity with nearly complete recovery of activity (83%) (Krieger and Hook, 1991). Subsequent gel filtration on Sephacryl S-200 resolved two peaks of activity (Fig. 1). The first peak eluting at the void volume has been identified as the cysteine protease PTP, which participates in PE processing (Krieger and Hook, 1991; Krieger et al., 1992). The second peak eluting at ~70 kDa was inhibited by 75% with pepstatin A (1 μ*M*), a selective inhibitor of aspartic proteinases. Chromatofocusing of the 70-kDa fraction from the Sephacryl S-200 column indicated that the enkephalin precursor cleaving activ-

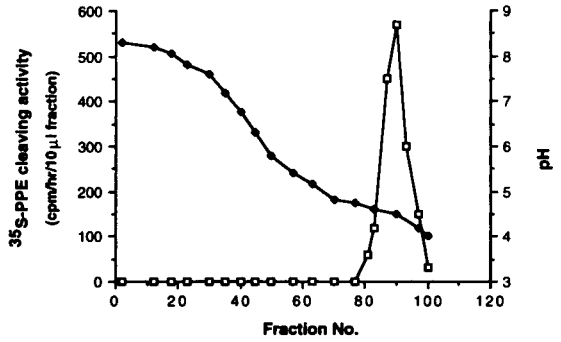

FIG. 2. Chromatofocusing of enkephalin precursor cleaving activity. The 70-kDa fraction from the Sephacryl S-200 column was subjected to chromatofocusing; enkephalin-precursor cleaving activity (□) and the pH (♦) were determined in column fractions.

ity elutes at pH 4.5–4.0 (Fig. 2). These results indicated that the 70-kDa fraction from the Sephacryl S-200 column contained enkephalin precursor cleaving activity with an acidic pI of 4.4.

To isolate the aspartic proteinase from the 70-kDa peak, pepstatin A affinity chromatography was used as the next purification step. Pepstatin A-sensitive enkephalin precursor cleaving activity was bound and eluted from the pepstatin A-agarose resin (Fig. 3). SDS-PAGE analysis showed the enzyme as a single 70-kDa band (Fig. 4), indicating its purification to apparent homogeneity. The pepstatin A step provided a 5.7-fold increase in specific activity, and total activity eluted from the pepstatin A column represented ~10% of total CG enkephalin precursor cleaving activity (Table 1). The yield of the CG aspartic proteinase was 0.25 mg from 650 adrenal medullae.

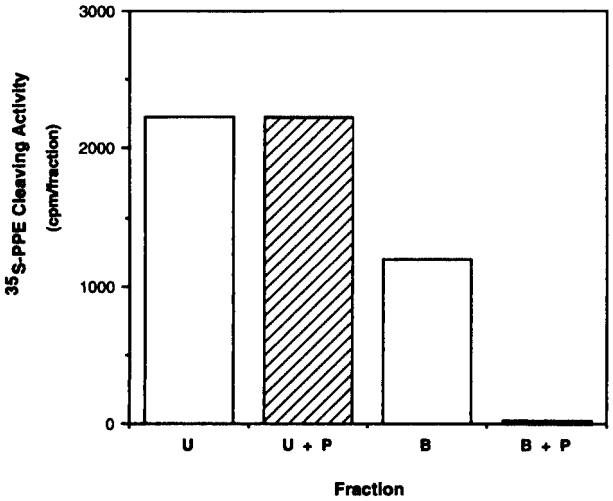

FIG. 3. Pepstatin A chromatography. The 70-kDa fraction eluted from the Sephacryl S-200 column (25 ml) was applied to pepstatin A-agarose column. The bound enzyme was then eluted with 0.1 *M* Tris-HCl (pH 8.5) containing 0.2 *M* NaCl. Unbound (U) and bound (B) materials were collected, pooled as U and B fractions, and assayed with ([^{35}S]Met)-PPE in the absence or presence of 10 μM pepstatin A (P).

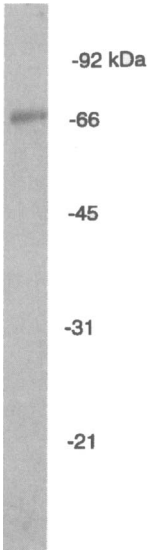

FIG. 4. SDS-PAGE of purified CG aspartic proteinase. Amido black staining, as described in Experimental Procedures, of the CG aspartyl protease enzyme (1.5 μg) is shown on SDS-PAGE (12% polyacrylamide).

Effect of pH and protease inhibitors

The CG aspartic proteinase demonstrated pH-dependent activity with a pH optimum of 5.5 (Fig. 5). Therefore, further studies were conducted at 5.5. Protease inhibitor studies (Table 2) showed that the CG aspartic proteinase was potently blocked by 1–10 μM pepstatin A, a selective aspartic proteinase inhibitor. The enzyme was not inhibited by serine (diisopropyl fluorophosphate or phenylmethylsulfonyl fluoride), cysteine (E-64c), or metallo- (EDTA) protease inhibitors. Partial inhibition by the thiol-reactive agent *p*-chloromercuribenzoic acid and twofold stimulation by the reducing agent DTT suggested functional cysteine(s) in the vicinity of the enzyme's active site. Alternatively, DTT reduction of cysteine residues of PE may affect its susceptibility to proteolytic processing. It is interesting that Val-D-Leu-Pro-Phe-Val-D-Leu, which inhibits the classical aspartic proteinases pepsin, cathepsin D, and cathepsin E (Pohl et al., 1984), had little effect. These results suggest that the CG aspartic proteinase may be a distinct member of the aspartic proteinase family compared with classically known aspartic proteinases.

Comparison of peptide–MCA and prohormone—PE and POMC—substrates for the CG aspartic proteinase

CG aspartic proteinase cleavage of peptide–MCA substrates containing paired and single basic residues was assessed. Peptide–MCA substrates tested were Boc-Arg-Val-Arg-Arg-MCA, pGlu-Arg-Thr-Lys-Arg-MCA, Arg-Gln-Arg-Arg-MCA, Boc-Gly-Lys-Arg-MCA, Boc-Gly-Arg-Arg-MCA, Boc-Gln-Arg-Arg-MCA, Boc-Glu-Lys-Lys-MCA, Boc-Gln-Gly-Arg-MCA, Boc-Val-Leu-Lys-MCA, and Boc-Phe-Val-Arg-MCA. Even after prolonged (18-h) incubation,

TABLE 1. *Purification of the CG aspartic proteinase*

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield of activity (%)
CG lysate	788	1,004	0.78		100
Concanavalin A	652	26	25	32	83
Sephacryl S-200	196	4	49	63	25
Pepstatin A-agarose	70	0.25	280	358	9

Enkephalin precursor cleaving activity was assayed with ([^{35}S]Met)-PPE in 0.1 *M* sodium citrate (pH 5.0), 1 m*M* DTT, 1 m*M* EDTA, and 10 m*M* CHAPS. Total activities of the pools at each step were measured in the linear range (<20% conversion) of the enkephalin precursor cleaving assay. One unit of activity equals 1 pmol of TCA-soluble [^{35}S]methionine generated/h.

none of the peptide–MCA substrates were cleaved. These results indicate that tri- or tetrapeptides are not appropriate substrates.

To test whether the enzyme recognizes full-length prohormone, recombinant ([^{35}S]Met)-PE and ([^{35}S]-Met)-POMC were generated by in vitro transcription and translation. Indeed, the CG aspartic proteinase readily cleaved recombinant ([^{35}S]Met)-PE and ([^{35}S]Met)-POMC (Table 3), indicating recognition of authentic prohormones.

CG aspartic proteinase processing of recombinant PE expressed in *E. coli*

To examine CG aspartic proteinase cleavage of PE near in vivo concentrations of PE at 10^{-5}–10^{-4} *M* (Ungar and Phillips, 1983) and to generate products in adequate amounts for analysis by peptide microsequencing, high-level expression of milligrams of PE was achieved in *E. coli*. The PE was purified from *E. coli* by diethylaminoethyl-Sepharose, preparative SDS-PAGE, and reverse-phase HPLC (as described in Materials and Methods) (Schiller et al., 1995).

The purified PE (10^{-5} *M*) was incubated with CG aspartic proteinase in a time course study (Fig. 6). PE was nearly completely hydrolyzed after a 1-h incubation with the enzyme, with simultaneous formation of 22-, 14-, and 13-kDa products. The time course of PE processing (Fig. 7) indicates that PE was cleaved at a rate of 0.12 mmol/h/mg. Peptide microsequencing of these products (Table 4) indicated 22- and 13-kDa PE products that contain the NH_2-terminus of PE. It is important that the 14- and 13-kDa products that possess the NH_2-terminal peptide sequence of RSPQLE, as determined by peptide microsequencing (Table 4), indicated cleavage of PE between Lys^{172}–Arg^{173} located at the COOH-terminus of (Met)enkephalin-Arg^6-Gly^7-Leu^8 within PE (Fig. 8). Cleavage of the Lys^{172}–Arg^{173} paired basic residue site of PE by the CG aspartic proteinase is compatible with the typical cleavage specificity of prohormone processing enzymes.

The predicted PE products generated by the CG aspartic proteinase (illustrated in Fig. 8) are based on analyses of PE products by peptide microsequencing (Table 4), their molecular size by SDS-PAGE (Fig. 6), and the theoretical molecular weights of PE fragments based on their known primary sequence (Yoshi-

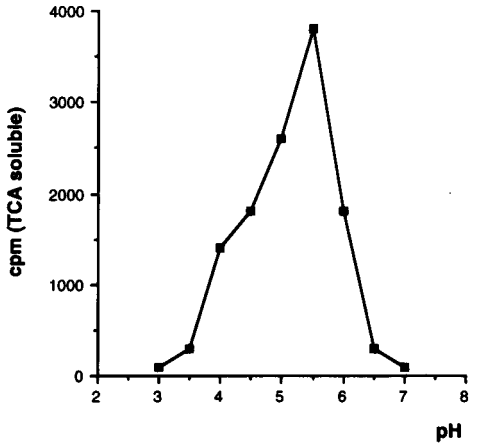

FIG. 5. pH dependence of the CG aspartic proteinase. Purified aspartic proteinase (1.25 μg) was assayed with ([^{35}S]Met)-PPE as substrate (as described in Experimental Procedures) at the indicated pH values.

TABLE 2. *Effect of protease inhibitors on CG aspartic proteinase*

Inhibitor	Final concentration	Residual enzymatic activity (%)
None		100
Pepstatin A	1.0 μ*M*	12
	10 μ*M*	5
	100 μ*M*	0
Leupeptin	1.0 m*M*	97
DFP	1.5 m*M*	100
PMSF	0.25 m*M*	100
E-64c	0.1 m*M*	96
p-CMB	1.0 m*M*	70
DTT	1.0 m*M*	214
EDTA	1.0 m*M*	106
Val-D-Leu-Pro-Phe-Val-D-Leu	0.1 m*M*	80

Enkephalin precursor cleaving activity was assayed in 0.1 *M* sodium citrate (pH 5.5) and 1 m*M* DTT, with 1 μg of CG aspartic proteinase per assay. DFP, diisopropyl fluorophosphate; *p*-CMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethylsulfonyl fluoride.

TABLE 3. *CG aspartic proteinase with PE and POMC prohormones*

Substrate	Activity (TCA-soluble cpm/h)
^{35}S-PE	1209
^{35}S-POMC	1716

CG aspartic proteinase (1.25 μg) hydrolysis of ^{35}S-PE and ^{35}S-POMC was conducted in 0.1 *M* sodium citrate (pH 5.5), 1 m*M* DTT, 1 m*M* EDTA, and 10 m*M* CHAPS.

kawa et al., 1984). The production of 22- and 13-kDa fragments containing the NH_2-terminal segment of PE is consistent with the presence in vivo of multiple high-molecular-mass (8–27 kDa) enkephalin-containing fragments possessing the NH_2-terminus of PE (Liston et al., 1984; Spruce et al., 1988; Hook et al., 1990). Furthermore, the 14- and 13-kDa products that are generated by cleavage between Lys^{172} and Arg^{173} are consistent as possible intermediates that contain the final products peptide I (residues 174–212 of PE), peptide E (residues 188–212), and peptide B (residues 215–245). The CG aspartic proteinase generates PE products that are similar to in vivo PE products in bovine adrenal medulla (Liston et al., 1984; Spruce et al., 1988; Hook et al., 1990).

DISCUSSION

Purification and characterization of the CG aspartic proteinase demonstrate the role of this enzyme as a candidate PE processing enzyme. The CG aspartic proteinase was purified from the soluble extract of granules to apparent homogeneity by concanavalin A, Sephacryl S-200, and pepstatin A affinity chromatography. The enzyme was demonstrated as a 70-kDa glycoprotein that was completely inhibited by the aspartic proteinase inhibitor pepstatin A but was not inhibited

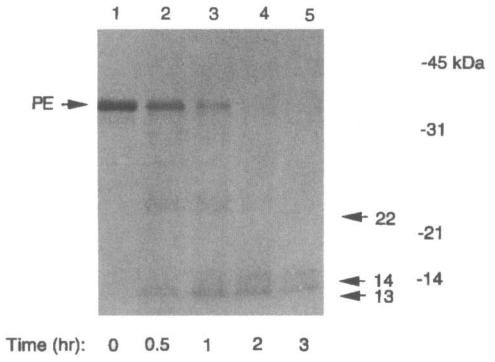

FIG. 6. PE processing by CG aspartic proteinase. PE was digested with CG aspartic proteinase and analyzed by SDS-PAGE. PE (8 μg) was incubated at 37°C with CG aspartic proteinase (2 μg) for 0, 0.5, 1, 2, and 4 h (lanes 1–5, respectively). The products were analyzed by SDS-PAGE (12% polyacrylamide) and amido black staining on PVDF membranes.

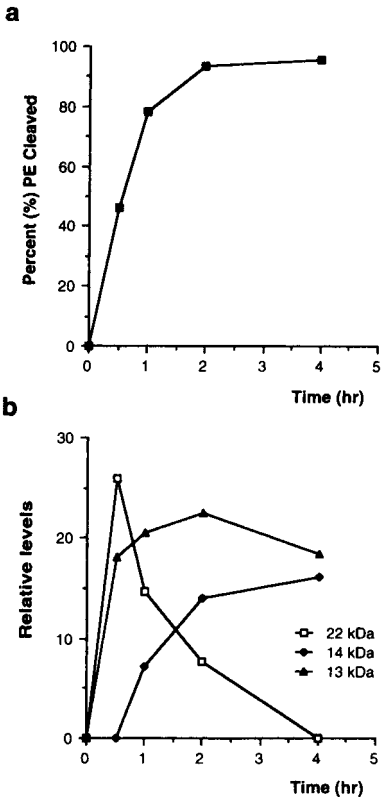

FIG. 7. Densitometry of PE cleaved and products formed. **a:** Time course of PE cleaved. Densitometry of SDS-PAGE gels (from Fig. 6) was used to quantify relative levels of PE cleaved, with the amount of uncleaved PE defined as 100% PE. **b:** Time course of formation of 22-, 14-, and 13-kDa products. Relative levels of product bands, expressed as percentages of PE content in the reaction (100%) converted to the indicated band (quantified by densitometry of SDS-PAGE gels from Fig. 6), were used to plot the relative amount of PE cleaved and relative amounts of 22-, 14-, and 13-kDa products formed.

by thiol, serine, or metalloprotease inhibitors. Activation by DTT and inhibition by the thiol-blocking reagent *p*-chloromercuribenzoic acid indicate involvement of reduced cysteine(s) of the enzyme or precursor for activity. The enzyme's pH optimum of 5.5 is consistent with the known intragranular pH of 5.5–5.8 (Pollard et al., 1978), indicating that the enzyme would be active in vivo. It is important that cleavage of recombinant PE by the CG aspartic proteinase between the Lys^{172} and Arg^{173} of this dibasic processing site, as analyzed by peptide microsequencing, was indicative of the appropriate cleavage specificity expected of a prohormone processing enzyme. These properties of the CG aspartic proteinase support its role as a candidate PE processing enzyme.

Of particular interest was the finding that the CG aspartic proteinase cleaved full-length PE at a paired basic residue Lys–Arg site but did not cleave tri- or tetrapeptides containing dibasic or monobasic cleavage sites. These results indicate that substrate binding requires enzyme recognition of prohormone domains

TABLE 4. *Microsequencing of PE products generated by CG aspartic proteinase*

PE-derived fragment	Sequence determined	Region of PE
22 kDa	M D X S Q D X A K X	NH_2-terminus of PE
		↓
14 kDa	R S P Q L E	KR site at COOH-terminus of ME-RGL
		↓
13 kDa	R S P Q L	KR site at COOH-terminus of ME-RGL
	M D X S Q D X A K X	NH_2-terminus of PE

The X indicates amino acid positions in the sequence that could not be determined. Peptides were not alkylated to derivatize cysteine residues before sequence determination, and underivatized cysteines are not stable to the sequencing chemistry. The known NH_2-terminal sequence of PE is MDCSQDCAKC. (Met)enkephalin-Arg-Gly-Leu, ME-RGL. The 13-kDa band represents two sequences.

other than the cleavage site alone. This property of the CG aspartic proteinase contrasts with the candidate prohormone processing enzymes PC1/3 and PC2 (Shennan et al., 1991; Jean et al., 1993; Rufaut et al., 1993; Zhou and Lindberg, 1993), as well as the CG cysteine protease known as PTP (Azaryan and Hook, 1994*a,b*), which readily cleave both peptide-MCA and prohormone substrates.

The CG aspartic proteinase resembles the bovine pituitary aspartic proteinase PCE in secretory vesicles that cleaves POMC and provasopressin (Loh et al., 1985; Parish et al., 1986). The CG aspartic proteinase is also similar to the yeast YAP3 protease involved in pro-α-mating factor processing (Egel-Mittani et al., 1990; Azaryan et al., 1993). These prohormone processing aspartic proteinases possess the selectivity for cleavage at paired basic residues that is predicted for putative prohormone processing enzymes. Both CG and pituitary aspartic proteinases are unable to hydrolyze the peptide Boc-Arg-Val-Arg-Arg-MCA but readily hydrolyze the full-length prohormones PE and POMC. The CG, pituitary, and yeast aspartic proteinases are glycoproteins with molecular sizes of ~70 kDa and are optimally active at acidic pHs of 4.5–5.5. They are inhibited by pepstatin A, a specific aspartic proteinase inhibitor, but are not sensitive to serine, thiol, or metalloproteinase inhibitors. The CG aspartic proteinase in this study appears to be the CG counterpart of pituitary secretory vesicle PCE.

These prohormone processing aspartic proteinases that cleave at basic residues differ in cleavage specificity from classical aspartic proteinases such as pepsin, renin, cathepsin D, and cathepsin E. These classical aspartic proteinases possess specificity for hydrophobic residues (Dunn et al., 1986; Azaryan et al., 1987; Azaryan and Galoyan, 1988) and are potently inhibited

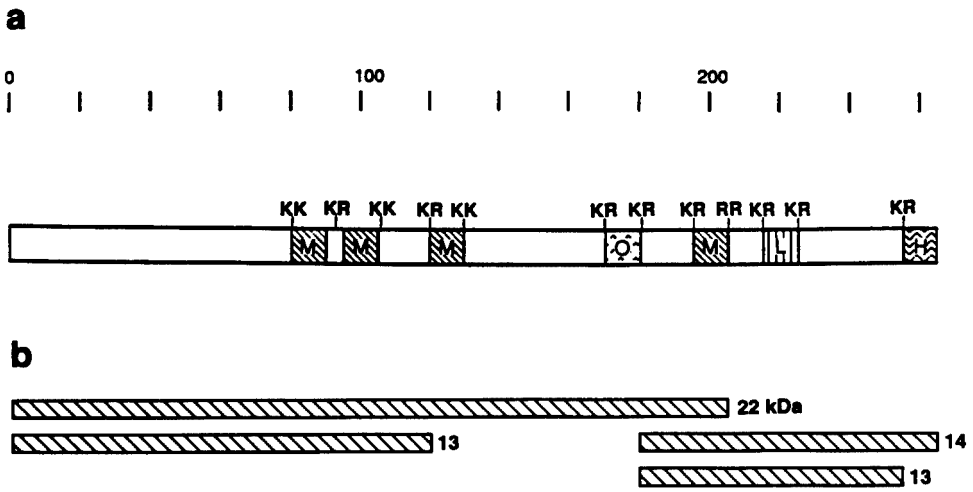

FIG. 8. Predicted PE products generated by CG aspartic proteinase. **a:** PE structure. PE is illustrated showing the active opioid peptides (Met)enkephalin (M), (Met)enkephalin-Arg^6-Gly^7-Leu^8 (O), (Leu)enkephalin (L), and (Met)enkephalin-Arg^6-Phe^7 (H). **b:** Predicted PE products. The predicted PE products generated by the CG aspartic proteinase were identified based on NH_2-terminal peptide microsequencing of PE products (Table 4), molecular size of products as assessed by SDS-PAGE (Fig. 6), and theoretical molecular sizes of PE fragments from the known primary sequence (Yoshikawa et al., 1984).

by the peptide inhibitor Val-D-Leu-Pro-Phe-Val-D-Leu with K_i values of 10^{-8}–10^{-6} M (Pohl et al., 1984). Val-D-Leu-Pro-Phe-Val-D-Leu, which resembles cathepsin D peptide substrates containing hydrophobic residue cleavage sites (Lin and Williams, 1979), would be predicted to have little effect on the CG aspartic proteinase that cleaves at a dibasic Lys–Arg site. Indeed, the CG aspartic proteinase was minimally affected by high concentrations (0.1 mM) of Val-D-Leu-Pro-Phe-Val-D-Leu. It will be of interest in future studies to determine whether the prohormone processing aspartic proteinases represent novel members of the family of aspartic proteinases.

It is important to consider that enkephalin precursor cleaving activity in CGs has been resolved into three classes of proteases: (a) the 70-kDa CG aspartic proteinase, (b) the novel cysteine protease known as PTP (Krieger and Hook, 1991), and (c) the subtilisin-related PC1/3 and PC2 (Azaryan et al., 1995). At the Sephacryl S-200 step of the purification scheme (Fig. 1), the major peak eluting at the void volume has been purified and characterized as PTP (Krieger and Hook, 1991). The second 70-kDa peak contains the aspartic proteinase isolated in this study. In addition, the 70-kDa peak also contains PC2 and lower levels of PC1/3, which have been characterized (Azaryan et al., 1995). These studies indicate that the total enkephalin precursor cleaving activity in chromaffin granules is represented as 60% PTP, 20% PC1/3 and PC2, and 10% CG aspartic proteinase activities that are present in the soluble component of the granule. The membrane component contains ~10% of granule enkephalin precursor cleaving activity (Krieger and Hook, 1991). It will be important to define the specific role of each of these classes of prohormone processing proteases in converting PE to active opioid peptides. Furthermore, investigation of the role of the different classes of processing enzymes in the biosynthesis of different peptide hormones can indicate common or specific prohormone processing mechanisms.

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