Characterization of *Aplysia* carboxypeptidase E

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Abstract Carboxypeptidase E (CPE) is involved in the biosynthesis of peptide hormones and neurotransmitters. To determine whether a recently reported *Aplysia californica* cDNA encodes a CPE-like enzyme, this cDNA was expressed in the baculovirus system. The *Aplysia* CPE is optimal at pH 5.5–6.5 and is inhibited by chelating agents and by the sulfhydryl reagent *p*-chloromercuriphenyl sulfonate. The effect of divalent cations and active site-directed inhibitors on enzyme activity are generally similar for *Aplysia* and rat CPE. Western blot analysis using antisera to the N- and C-terminal regions of the *Aplysia* CPE show that the *Aplysia* CPE is present in atrial glands and ovotestis. This *Aplysia* CPE is purified on a *p*-aminobenzoyl–Arg Sepharose affinity column under conditions that selectively purify rat CPE. Taken together, these results suggest that the previously cloned cDNA represents a CPE-like enzyme that is expressed in *Aplysia* tissue.

Key words: Peptide processing; Carboxypeptidase E; Carboxypeptidase H; Carboxypeptidase D; Enkephalin convertase

1. Introduction

In mammals, carboxypeptidase E (CPE; EC3.4.17.10) functions in the processing of peptide hormones and neurotransmitters [1,2]. CPE functions after an endopeptidase has initially cleaved the peptide precursor at the C-terminal side of a basic residue within a particular cleavage site. Following the endopeptidase, CPE then removes the Lys or Arg residues remaining on the C-terminus of the peptide intermediate. CPE was initially found to be associated with enkephalin in the bovine adrenal medulla [3] and has been found in numerous peptide-producing tissues [4]. Strong evidence that CPE is physiologically involved with peptide biosynthesis comes from studies on the Cpe^{fat}/Cpe^{fat} mice, which lack functional CPE activity due to a point mutation in the coding region of the *CPE* gene [5]. These mice have reduced levels of a variety of peptide hormones and neurotransmitters [5–7], supporting the proposal that CPE is the major peptide-processing carboxypeptidase in mouse neuroendocrine tissues. However, since correctly processed peptides are present in Cpe^{fat}/Cpe^{fat} mice [5–7], it is likely that another carboxypeptidase also is involved in peptide processing. A candidate enzyme, metallocarboxypeptidase D (CPD), has been recently identified [8].

The amino acid sequence of CPE, deduced from the nucleotide sequence, is highly conserved among human, bovine, rat, and mouse, with over 90% amino acid identity among all four species [5,9–11]. Anglerfish CPE also shows a high degree of conservation, with approximately 80% amino acid identity between the fish sequence and each of the mammalian sequences [12]. Recently, cDNAs have been obtained for several *Aplysia* metallocarboxypeptidases [13]. Three of these are isoforms that differ in only two amino acids near the N-terminus of the protein. All three isoforms have highest amino acid sequence similarity to CPE, and have been designated CPE-1, -2, and -3 [13]. However, this similarity with mammalian CPE (50% amino acid identity) is not much higher than the similarity to other mammalian metallocarboxypeptidases such as carboxypeptidase N (41%), and carboxypeptidase M (35%). A distinct *Aplysia* cDNA clone appears to encode a homolog of CPD, with approximately 50% amino acid identity among the *Aplysia* clone and rat CPD [13].

The purpose of the present study was to investigate whether the *Aplysia* CPE-1 cDNA clone encodes a CPE-like enzyme despite the modest amino acid sequence similarity among the *Aplysia* CPE-1 and the mammalian CPEs. Another purpose was to examine whether *Aplysia* contain both CPE- and CPD-like enzymes. A previous report on a CPE-like enzyme in *Aplysia* [14] used a purification scheme that would isolate both CPE and CPD together [8], and so it was not clear whether the reported properties reflected one or more enzymes. Our finding that *Aplysia* CPE-1 expressed in baculovirus has properties similar to those of mammalian CPE confirms that this *Aplysia* cDNA encodes a CPE-like enzyme. Furthermore, both CPE- and CPD-like enzymes are detected in *Aplysia* tissues, suggesting that despite the modest conservation of amino acids the enzymatic properties have been highly conserved through evolution.

2. Materials and methods

2.1. Expression of CPE-1 in baculovirus

The open reading frame of the 561-residue preproCPE-1 (Clone V, accession number U37755) was amplified by PCR using sense and antisense primers (GIBCO BRL) corresponding to the initiation Met and stop codon regions. The sense primer (CAGAATTC4T-GAGGGATGGATACT) corresponds to nucleotides 96–111, and contains an *Eco*RI site (underlined). The antisense primer (CAGG-TACCGAGTTACTTGGAC) corresponds to nucleotides 1779–1794, and contains a *Kpn*I site (underlined). PCR amplification was performed using eLONGase (GIBCO BRL), which contains the proofreading enzyme *Pyrococcus* GB-D polymerase along with *Taq* polymerase. Hot start PCR reactions were performed for 35 cycles, each cycle consisting of 94°C for 30 s, 55°C for 30 s, and 68°C for 2 min. PCR products were fractionated on 2% NuSieve/1% SeaPlaque agarose (FMC), excised from the gel, and purified using the SpinBind DNA method (FMC). After digestion with *Eco*RI and *Kpn*I, the DNA was subcloned into the *Eco*RI/*Kpn*I sites of the baculovirus expression vector pAcSG2 (Pharmingen). Sequence analysis was performed to confirm the identity of the insert.

The *Aplysia* CPE cDNA in the baculovirus expression vector (5 μg) was combined with 0.25 μg of Baculogold viral DNA (Pharmingen) and used to transfect 10^6 Sf9 cells using the standard calcium phosphate procedure recommended by Pharmingen. The Sf9 cells were

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grown in 4 ml of Sf900-II serum-free media (GIBCO BRL) in a 25 cm^2 flask. Five days after the transfection, the medium was removed and a 0.1 ml of aliquot was used to infect another 25 cm^2 flask containing 10^6 Sf9 cells in 4 ml of Sf900-II serum-free media. This second infection was harvested after 3 days and 1 ml of the medium was used to infect 10^8 cells in 50 ml of media, growing in a 500 ml shaker flask. Medium from this third infection was subsequently used for large-scale preparation of infected cells. Typically, 10 ml of the medium from the third infection was used to infect 2×10^9 cells growing in 1 l of Sf900-II media in shaker flasks. Baculovirus expressing rat CPE was generated by a similar protocol and has been described elsewhere [15].

2.2. Purification of CPE and CPD from baculovirus and tissues

Adult *Aplysia californica* were purchased from Alacrity Marine Biological Services (Redondo Beach, CA). Tissues were dissected and stored at −70°C until analysis. Tissues were homogenized (Brinkmann Polytron) in 5–10 vol. of 50 mM NaAc, pH 5.5, containing 1 M NaCl and 1% Triton X-100. The homogenate was centrifuged at $50\,000\times g$ for 20 min at 4°C. The supernatant was loaded onto a *p*-aminobenzoyl–Arg Sepharose affinity column [8]. The column was washed with 20 mM NaAc, pH 5.5, containing 1 M NaCl and 1% Triton X-100 followed by 10 mM NaAc, pH 5.5. CPE was eluted with 50 mM Tris, pH 8.0, containing 50 mM NaCl and 0.01% Triton X-100. CPD was subsequently eluted with 25 mM arginine in the same Tris buffer. CPE was purified from the media of baculovirus-infected Sf9 cells using a similar affinity column procedure. For this purification, the medium was adjusted to pH 5.5 with NaAc buffer, and then applied to a 20 ml *p*-aminobenzoyl–Arg Sepharose column as described above.

2.3. Enzyme assay

Carboxypeptidase assays were performed with the substrate dansyl–Phe–Ala–Arg, as described [16]. Typically, 200 μM substrate and enzyme were combined in a final volume of 250 μl of 100 mM NaAc, pH 5.5. The reaction mixture was incubated at 37°C for 10 min to 5 h, depending on the required sensitivity, and the reaction was terminated by the addition of 100 μl of 0.5 M HCl followed by 2 ml of chloroform. The tubes were mixed, centrifuged at $300\times g$ for 5 min, and the fluorescence in the lower chloroform phase was determined (excitation, 350 nm; emission, 500 nm). To measure the pH optima, the reactions were performed in 0.1 M Tris acetate at the indicated pH (measured at 37°C). To measure the effect of inhibitors and ions, the purified enzyme was pre-incubated with various compounds for 1 h at 4°C and then assayed as described above. For all measurements the assay was performed in duplicate, with variations typically less than 10%.

2.4. Antisera generation

Peptide synthesis and antisera production were performed by Quality Controlled Biochemicals (Hopkinton, MA). Two peptides were synthesized corresponding to the N- and C-terminal regions of *Aplysia* preproCPE-1 [13]. The N-terminal peptide (acetyl-CPEVTRIYNL-SEPSVEKR-amide) corresponds to residues 64–81 and the C-terminal peptide (acetyl-CLKELNPDQMREVLERLP-amide) corresponds to residues 528–544. Peptides were purified by reverse-phase HPLC, analyzed by mass spectrometry, and conjugated to keyhole limpet hemocyanin for immunization of New Zealand white rabbits. Each of the two rabbits were immunized with protein conjugates of both N- and C-terminal peptides. The antiserum to rat CPD was raised against the 180 kDa form of purified rat brain CPD [17].

2.5. Polyacrylamide gel electrophoresis and Western blot analysis

Samples were fractionated on a denaturing 10% polyacrylamide gel and transferred to nitrocellulose. The blots were probed with a 1:500 dilution of rabbit polyclonal antisera produced against the peptides corresponding to the N- and C-terminal regions of *Aplysia* CPE, or a 1:1000 dilution of antiserum directed against CPD. The blots were incubated for 1 h with the primary antisera, washed several times with Tris buffer saline containing 0.1% Tween-20, and then incubated for 1 h with a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG. After this incubation, the blot was further washed with Tris buffer saline, and the antiserum was detected using the enhanced chemiluminescence method (Amersham).

3. Results

Upon expression of *Aplysia* CPE-1 in baculovirus, the standard carboxypeptidase assay (at pH 5.5) resulted in 440 nmol of product per minute per 10^6 cells, whereas cells infected with a control virus showed only 2 nmol/min per 10^6 cells. The amount of carboxypeptidase activity produced with the *Aplysia* CPE-1 construct in baculovirus is comparable to the amount of carboxypeptidase activity produced with rat CPE, which typically produces 200–500 nmol/min per 10^6 cells. To assess whether the *Aplysia* CPE-1 represents an enzyme with properties similar to those of mammalian CPE, the baculovirus-expressed protein was purified using a method previously developed for the purification of mammalian CPE [18]. The *Aplysia* CPE was purified by this procedure, indicating a similarity to mammalian CPE.

Baculovirus-produced *Aplysia* CPE-1 has a pH optimum in the 5.5–6.5 range, which is slightly different from the more acidic pH optimum of rat CPE (Fig. 1). The biggest difference in the effect of pH on the *Aplysia* and rat enzymes is in the 6–7 range; at pH 6.5, the *Aplysia* enzyme has approximately 70% of its maximal activity, but the rat enzyme has only 20% of the maximal activity. This difference in pH optima between the two enzymes was observed in 3–4 separate experiments.

A variety of inhibitors were used to compare the *Aplysia* and rat enzymes. Both enzymes are substantially inhibited by the chelating agents 1,10-phenanthroline and EDTA (Table

Table 1
Effect of inhibitors on *Aplysia* and rat CPE

Compound	Conc.	% Control	
		Aplysia	Rat
1,10-phenanthroline	1 mM	<1	<1
EDTA	1 mM	<1	<1
EGTA	1 mM	25	24
Phenylmethylsulfonyl fluoride	1 mM	105	103
E-64	1 mM	101	99
Iodoacetamide	1 mM	91	79
PCMPS	0.1 mM	<1	<1
PCMPS	0.001 mM	57	37
Leupeptin	1 mM	84	91
Benzamidine	1 mM	100	81
Benzylsuccinic acid	1 mM	105	101
Tosylphenylalanylchloromethyl ketone	0.1 mM	84	113
Tosyllysylchloromethyl ketone	0.1 mM	109	91

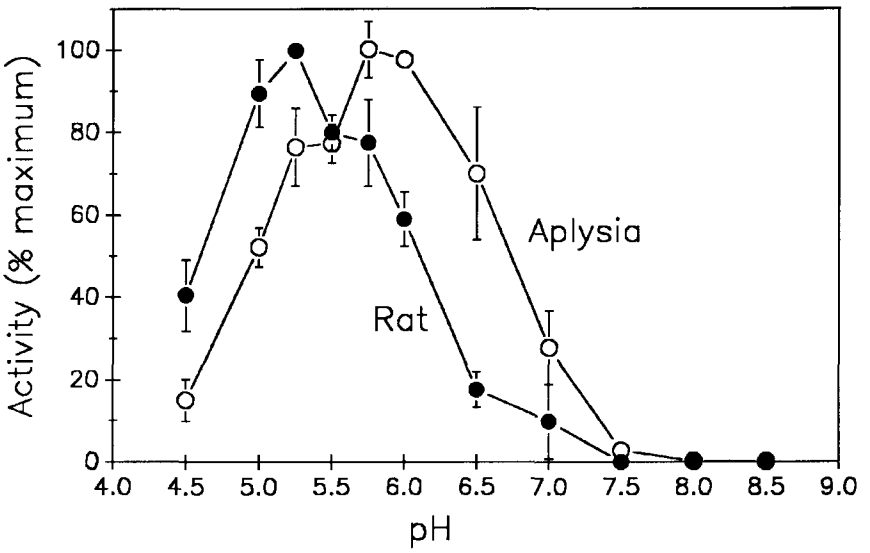

Fig. 1. Effect of pH on CPE purified from baculovirus-infected Sf9 cell media. Enzyme was assayed with dansyl–Phe–Ala–Arg in Tris acetate buffer at the indicated pH, as described in Section 2. Error bars show range of duplicate determinations.

1). Inhibitors of serine proteases (phenylmethylsulfonyl fluoride) and aspartyl proteases (leupeptin) do not affect either *Aplysia* or rat CPE. Also, some reagents that interact with thiol groups (E-64 and iodoacetamide) do not substantially inhibit either enzyme. However, a property of CPE that is unique among metallocarboxypeptidases is the sensitivity to low concentrations of *p*-chloromercuriphenyl sulfonate (PCMPS). Both the *Aplysia* and rat enzymes are substantially inhibited by 100 μM PCMPS, and are partially inhibited by 1 μM of this reagent. Neither enzyme is substantially influenced by inhibitors of carboxypeptidase A (benzylsuccinic acid), chymotrypsin (tosylphenylalanylchloromethyl ketone) or trypsin (tosyllysylchloromethyl ketone).

Mammalian CPE, like other metalloenzymes, is influenced by a variety of divalent cations. Although *Aplysia* and rat CPE are both activated by Co^{2+} and inhibited by Cd^{2+}, the two enzymes differ in the magnitude of the response to the cation (Table 2). Ni^{2+} also differentially affects the two enzymes, with no substantial effect on the rat enzyme, and moderate inhibition of the *Aplysia* enzyme. Both enzymes are substantially inhibited by 1 mM Cu^{2+} and 1 μM Hg^{2+} (Table 2), which may reflect their interaction with a thiol group on the CPE rather than an interaction in a metal binding site.

To further explore the similarity between the *Aplysia* and rat enzymes, the inhibitory potency of a variety of active site-directed compounds was investigated. Of the three active site-directed inhibitors tested, guanidinoethylmercaptosuccinic acid (GEMSA) was the most potent inhibitor of either *Aplysia* or rat CPE (Fig. 2). Another arginine-based inhibitor, 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA) also potently inhibits both enzymes. Aminopropylmercaptosuccinic acid (APMSA), which is based on a lysine, is several orders of magnitude less potent as a CPE-inhibitor than the two arginine-based inhibitors (Fig. 2). This preferred affinity for C-terminal arginine residues over C-terminal lysine residues was confirmed by examining the ability of benzoyl–Gly–Arg (Hipp–Arg) and benzoyl–Gly–Lys (Hipp–Lys) to inhibit the cleavage of dansyl–Phe–Ala–Arg by CPE (Fig. 2). Hipp–Arg is approximately an order of magnitude more potent than the Hipp–Lys. None of the compounds examined showed a large difference between rat and *Aplysia* CPE, suggesting that the active site has been conserved.

Taken together, the properties of the baculovirus-expressed *Aplysia* CPE-1 are more similar to those of rat CPE than to a previously reported *Aplysia* CPE activity [14]. However, this previous report used a purification method that does not separate CPE from CPD. Thus, if *Aplysia* contain a CPD-like enzyme, the previous report would have represented a mixture of these two enzymes. To investigate this further, and to see if *Aplysia* express a protein that corresponds to the cDNA clone expressed in baculovirus, the purification of CPE was performed with several *Aplysia* tissues. Both atrial gland and ovotestis contain a protein of approximately 70 kDa that binds to the *p*-aminobenzoyl–Arg Sepharose column at pH 5.5 and elutes when the pH is raised to 8.0. This protein is recognized by two separate antisera directed against the N- and C-terminal regions of the *Aplysia* CPE cDNA (Fig. 3). These antisera also recognize the baculovirus-produced *Aplysia* CPE (lane 2), but not the rat CPE (lane 1, Fig. 3). After elution of the affinity columns with the pH 8 buffer, the columns were eluted with Arg in the pH 8 buffer; this has previously been shown to elute bovine and rat CPD from the affinity column [8,17]. The second affinity column eluate contains much less of the 70 kDa protein than the first elute

Table 2
Effect of divalent cations on *Aplysia* and rat CPE

Compound	Conc.	% Control	
		Aplysia	Rat
$CoCl_2$	1 mM	160	360
$ZnCl_2$	1 mM	50	49
$CaCl_2$	1 mM	96	80
$MgCl_2$	1 mM	100	89
$MnCl_2$	1 mM	89	87
$CdCl_2$	1 mM	7	25
$CuSO_4$	1 mM	1	<1
$HgCl_2$	0.001 mM	<1	<1
$NiCl_2$	1 mM	38	96

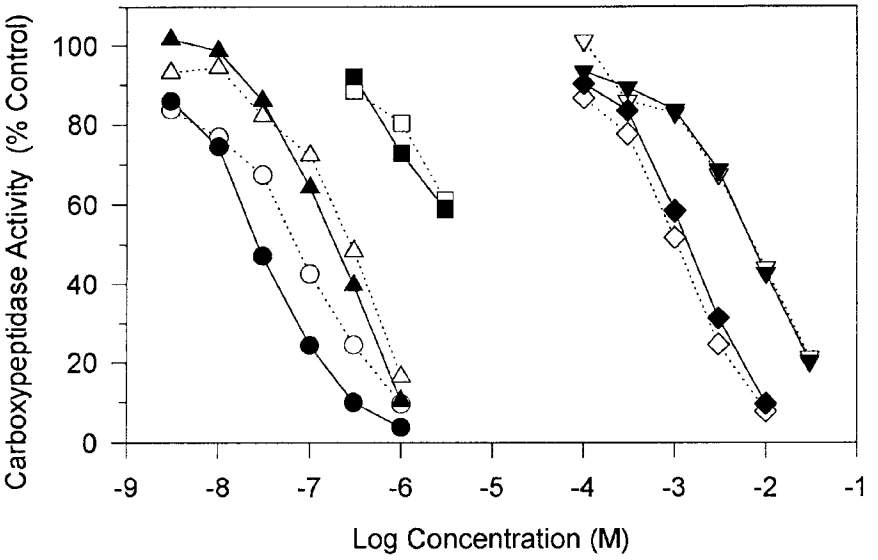

Fig. 2. Effect of active site-directed inhibitors and peptides on CPE activity. The CPE was purified from the media of baculovirus-infected Sf9 cells, and assayed using dansyl–Phe–Ala–Arg. Solid lines = *Aplysia* CPE. Dashed lines = rat CPE. ○, GEMSA; △, MGTA; □, APMSA; ◇, Hipp–Arg; ▽, Hipp–Lys.

fraction (Fig. 3). Interestingly, an antiserum to rat CPD recognizes a protein of 180 kDa in the second eluate of the ovotestis, but does not crossreact with baculovirus-produced rat or *Aplysia* CPE (Fig. 3).

The carboxypeptidase activity in the two affinity column eluates were compared with representative inhibitors. The activity in the first affinity column eluate is strongly inhibited by 1 mM $CdCl_2$, as is the baculovirus-expressed *Aplysia* CPE (Table 3). Both the baculovirus-expressed *Aplysia* CPE and the enzyme present in the first affinity column eluate are partially inhibited by 1 mM Hipp–Arg, 10 mM Hipp–Lys, or 1 μM PCMPS. The degree of inhibition of the material in the first eluate is generally similar to that of the baculovirus-expressed *Aplysia* CPE and distinct from that in the second elute. In contrast, the properties of the material in the second affinity column eluate are different from those of the baculovirus-produced CPE (Table 3).

4. Discussion

The major findings of the present study are that the cDNA previously identified as *Aplysia* CPE-1 encodes an enzyme with properties similar to those of mammalian CPE, despite modest amino acid sequence similarity. An enzyme with similar properties is detected in *Aplysia* tissues. Specifically, the atrial gland and ovotestis contain a protein that co-purifies with *Aplysia* CPE, has the same apparent molecular mass and enzyme properties, and is recognized by antisera directed against two distinct regions of the protein predicted from the cDNA clone.

The properties of *Aplysia* CPE-1 and rat CPE are more similar to each other than towards other mammalian carboxypeptidases that have a similar level of amino acid identity [9,13,19,26]. For example, CPE, M, and N all have approximately 45–50% amino acid identity with each other but show large differences in their inhibition by the active site-directed compounds used in this study. Thus, the overall level of amino acid sequence similarity does not correlate with the apparent conservation of the active site, based on the potencies of active site-directed inhibitors. The sensitivity of CPE to low concentrations of PCMPS also distinguishes this enzyme from CPM and CPN; this property is shared by *Aplysia* and mammalian CPE. Another major difference between mammalian CPE and most other known carboxypeptidases is the pH optima; mammalian CPE is maximally active at pH 5–5.5, whereas all other metallocarboxypeptidases (except CPD) are maximally active at neutral pH. The *Aplysia* enzyme is maximal at pH 6–6.5 (Fig. 1), which is intermediate between that of mammalian CPE and the majority of other metallocarboxypeptidases. The pH optimum of mammalian CPE corresponds to the internal pH of regulated pathway secretory vesicles [20,21]. The internal pH of *Aplysia* secretory vesicles is unknown, and it is possible that the pH optimum of the *Aplysia* CPE reflects the internal pH of these vesicles.

In addition to detecting a CPE-like enzyme in *Aplysia* tissues, a second CPD-like activity was detected (Table 3). The

Table 3
Effect of various compounds (% control) on CPE activity purified from *Aplysia* tissues or from baculovirus expression of CPE-1

Compound	Conc.	Bac virus	Affinity column elute 1		Affinity column elute 2	
			Atrial gland	Ovotestis	Atrial gland	Ovotestis
$CdCl_2$	1 mM	7%	7%	11%	44%	44%
Hipp–Arg	1 mM	58%	59%	50%	30%	32%
Hipp–Lys	10 mM	43%	40%	43%	57%	70%
PCMPS	1 μM	56%	59%	72%	89%	90%

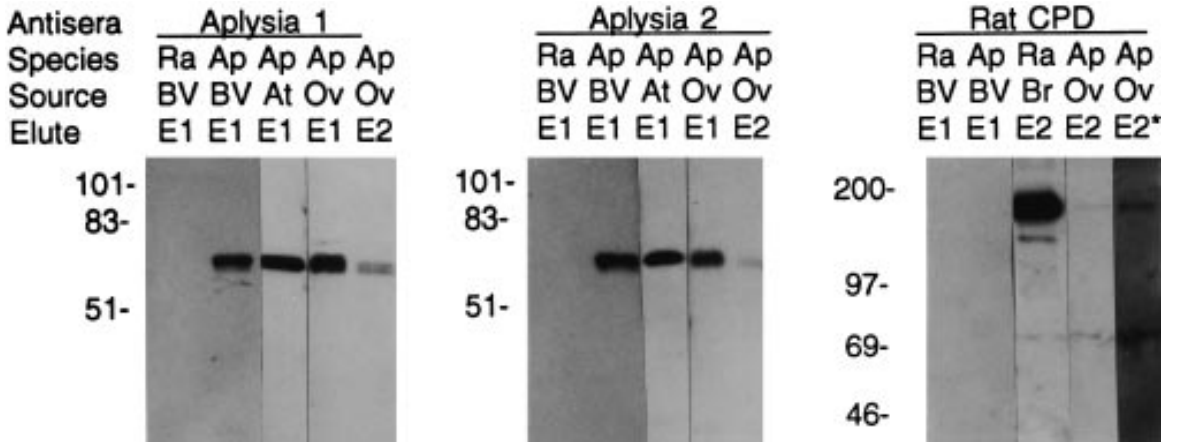

Fig. 3. Western blot analysis of CPE- and CPD-like enzymes. Protein was purified from the media of baculovirus-infected cells (BV) infected with rat or *Aplysia* (Ap) CPE, from *Aplysia* atrial gland (At) or ovotestis (Ov), or from rat brain (Br), as indicated. Material present in the elute 1 (E1) or elute 2 (E2) fractions were fractionated on a denaturing 10% polyacrylamide gel, transferred to nitrocellulose, and probed with two separate antisera raised against the N- and C-terminal regions of the *Aplysia* CPE (*Aplysia* 1 and 2), or to rat brain CPD, as indicated. The positions and sizes of prestained molecular mass markers (BioRad) are indicated. Two exposures of the *Aplysia* ovotestis E2 probed with antiserum to rat CPD are shown (*, longer exposure).

primary criteria that distinguishes mammalian CPD from CPE is the ability of CPD to bind to the Arg-containing substrate affinity column at both pH 5.5 and 8.0, whereas CPE binds only at pH 5.5 and not at pH 8 [8]. The finding that *Aplysia* tissues contain both CPE and CPD-like enzymes is consistent with the recent identification of a partial cDNA clone encoding a protein with homology to CPD [13]. CPD-like cDNAs have been identified in rat [22], duck [23], and *Drosophila* [24], and so it is likely that this protein would also be expressed in *Aplysia*.

The properties of the *Aplysia* CPE are generally similar to those described in a previous report, even though this previous characterized material presumably represented a mixture of CPE and CPD-like enzymes due to the purification scheme used [14]. The major differences between the *Aplysia* CPE produced in the baculovirus and the previously reported activity are the sensitivity to $CdCl_2$ and the relative inhibitory potency of Hipp–Arg versus Hipp–Lys. The previous report found that 0.1 mM $CdCl_2$ was much less effective as an inhibitor of *Aplysia* CPE than of CPE from mammalian species [14]. However, in the present study the baculovirus-expressed *Aplysia* CPE is more sensitive to this ion than the rat CPE (Table 2). Also, the previous study found that Hipp–Lys was approximately 50-fold less potent at blocking substrate cleavage than Hipp–Arg, whereas the present study shows this difference to be only 5–10-fold (Fig. 2 and Table 3). Both differences could be due to the presence of CPD in the previous preparation of CPE: the *Aplysia* CPD is less sensitive to both $CdCl_2$ and to Hipp–Lys than CPE (Table 3). In addition to CPE and CPD, there are several other known mammalian metallocarboxypeptidases, and it is possible that one or more of these were also present in the previous preparation. An advantage of the present study is the use of cloned cDNAs for expression in the baculovirus system. This ensures the analysis of a single enzyme since wild type baculovirus does not show any significant amount of enzyme activity after purification on the affinity columns.

In addition to playing a role in the processing of peptide hormones, mammalian CPE has also been proposed to function in the sorting of prohormones [15]. Recent evidence on the interaction of mammalian CPE and proopiomelanocortin supports this role for CPE [25]. It is possible that *Aplysia* CPE performs a similar function; the binding of proopiomelanocortin to its receptor (presumably CPE) is blocked by thiol reagents [25], and both rat and *Aplysia* CPE are potently inhibited by the thiol reagent PCMPS (Table 1).

Mutations within human CPE may contribute to obesity, as recently found in the Cpe^{fat}/Cpe^{fat} mouse [5]. Currently, efforts are underway to identify mutations within human CPE, and several variants have been found (unpublished data). For example, residue 323 of CPE appears as either a Pro or a Leu in the human population. Since the *Aplysia* CPE contains an Ala in the corresponding position [13], it is unlikely that the Pro versus Leu in human CPE would substantially alter the enzymatic properties. In contrast, a variation within a residue that is conserved between human and *Aplysia* CPE would be more likely to alter the enzymatic properties. The previously published *Aplysia* CPE sequence, together with the findings from the present study that this protein represents a CPE-like enzyme, will help evaluate the importance of individual residues in the overall structure/function relationship of human CPE.

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