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A PUTATIVE PROCESSING ENZYME FROM APLYSIA THAT CLEAVES DYNORPHIN A AT THE SINGLE ARGININE RESIDUE

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Summary: A peptidase activity cleaving at single arginine residues has been detected in extracts of the atrial gland of Aplysia Californica. The enzyme assay consisted of incubation of enzyme with the mammalian opioid peptide dynorphin A and detection by specific radioimmunoassay of dynorphin(1-8), a single arginine cleavage product. The peptidase activity was characterized following chromatography on DEAE-cellulose. Activity was abolished by a thiol-directed inhibitor and chelators and activated by dithiothreitol and cobalt chloride. The pH optimum was 6.2 in phosphate buffer. Analysis of the products of two substrates suggested that cleavage was occurring on the amino side of the arginine residue.

Most secretory peptides are synthesized as biologically inert precursors requiring specific enzyme cleavage for activation. Paired basic residues are favored sites at which the processing enzymes are known to cleave (1). Considerable evidence suggests that single arginine residues also serve as processing recognition sites for the production of secretory peptides found in the nervous system. Such a cleavage is required for the release of dynorphin(1-8) [dyn(1-8)] (2-4) and dynorphin B [dyn B] (5-7) from pro-dynorphin. Preliminary studies have demonstrated this enzyme activity in mammalian brain (8-10).

Biologically active peptides resulting from single arginine cleavage have also been demonstrated in invertebrate systems. Scheller et al. (11) found that production of secretory peptides A and B in the atrial gland of Aplysia, (Aplysia Californica), occurs via this mechanism. Processing of

Abbreviations: Dyn, Dynorphin peptides; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N, N, N',N'-tetraacetic acid; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone.

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NH_2-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-COOH

Dynorphin A

NH_2-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-COOH

Dynorphin(1-8)

Figure 1: Amino acid sequences of dyn A and dyn(1-8) and illustration of the single arginine processing site at which dyn A is cleaved to form dyn(1-8).

atrial gland peptide precursors shows other similarities to mammalian systems (11) such as cleavages at paired basic residues and carboxy-terminal amidation suggesting that the enzymes involved have similar properties to their mammalian counterparts. Since the atrial gland of Aplysia contains very high concentrations of processed peptide products (12), we presumed that this tissue would be a rich source of processing enzymes.

We have used the mammalian opioid peptide dyn A (13,14) as a substrate to demonstrate an enzyme activity in atrial glands that cleaves at single arginine residues. The cleavage product, dyn(1-8) (Fig. 1), was detected by a specific RIA that does not crossreact with the parent substrate. This paper describes the initial characterization of the enzyme activity.

MATERIALS AND METHODS

Enzyme extraction

All procedures were carried out at 4°C. Atrial glands, previously frozen and stored at -70°C, were finely minced and suspended in 0.01 M sodium phosphate buffer, 0.1 M sodium chloride, pH 7. Homogenization in a Brinkmann Polytron (setting 3) for 30 seconds was followed by low speed centrifugation at 1,000 x g for 10 minutes, reextraction of the pellet and recentrifugation. The combined supernatants were brought to a final volume of 7.5 x original tissue weight and centrifuged for 20 minutes at 27,000 x g.

Enzyme assay

The production of immunoreactive N-acetyl dyn(1-8) from N-acetyl dyn A was used to determine single arginine cleaving activity. Dyn A was protected by acetylation at primary amino groups against some nonspecific degradation Acetylation involved treatment of dyn A with an 50-fold excess of acetic anhydride in 0.1 M phosphate buffer at pH 7.3, followed by base treatment to hydrolyze O-acetyl esters of the peptide.

Enzyme was incubated for 1-2 hours at 37°C in 250 µl of 0.1 M sodium phosphate buffer, pH 6.0 containing 80 pmoles of N-acetyl dyn A. After boiling for 5 minutes and centrifuging, duplicate 50 µl supernatant aliquots were assayed for immunoreactive dyn(1-8) as previously described (2).

The antiserum used in the RIA is C-terminally directed (2). The RIA to dyn(1-8) had less than 0.001 percent crossreactivity with dyn A and it recognized acetylated dyn(1-8) and nonacetylated dyn(1-8) equally well.

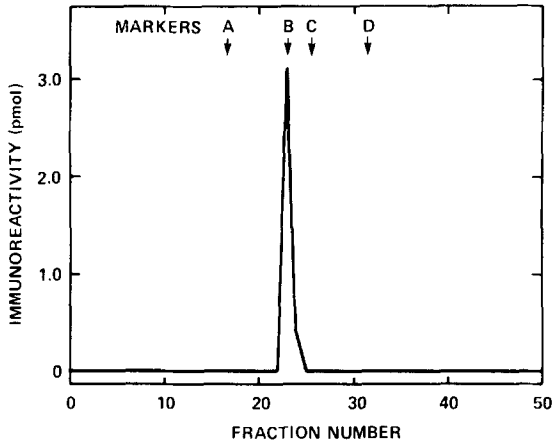

Figure 2: Reversed-phase HPLC analysis of immunoreactive N-acetyl dyn(1-8) produced from the incubation of N-acetyl dyn A with the 27,000xg supernatant fraction of atrial gland extract. Eighty pmoles of N-acetyl dyn A and 14 µg of enzyme were used. Incubation was carried out for 80 minutes at 37°C, terminated by boiling and supernatant applied to the HPLC column. The HPLC separation was performed on a reversed phase, P-18 Altex Ultrasphere ODS column (250 mm x 4.6 mm, particle size 5 µm). A linear gradient of acetonitrile in 0.05 M monosodium phosphate, 5 percent methanol, and phosphoric acid, (1 ml/l) pH 2.7 was generated. The acetonitrile concentration was brought to 27 percent in 5 minutes followed by isocratic elution. The flow rate was 1.25 ml/minute and 1 minute fractions were collected. Aliquots of each fraction were evaporated to dryness and residues were assayed for dyn(1-8) immunoreactivity. Immunoreactivity per fraction is shown. Markers were (A) N-acetyl dyn(1-9) (B) N-acetyl dyn(1-8) (C) N-acetyl dyn(1-13) and (D) N-acetyl dyn A.

RESULTS

Incubation of N-acetyl dyn A with the 27,000 x g supernatant fraction of atrial gland extract produced N-acetyl dyn(1-8), the identity of which was confirmed by reversed phase HPLC (Figure 2). Preboiling of the atrial gland extract abolished this activity.

DEAE-cellulose chromatography of the extract is shown in Figure 3. Single arginine cleaving activity eluted as a single major peak from the column in an approximately 60 percent yield. This partially purified preparation was used to further characterize the activity.

Figure 4 shows that N-acetyl dyn(1-8) production was, after an initial lag, linear with time and with concentration of enzyme. Addition of reducing agents, the weak chelator glycine, or bovine serum albumin did not abolish this lag. Using high levels of enzyme, over 30 percent conversion of substrate to product was demonstrated. The immunoreactive N-acetyl dyn(1-8)

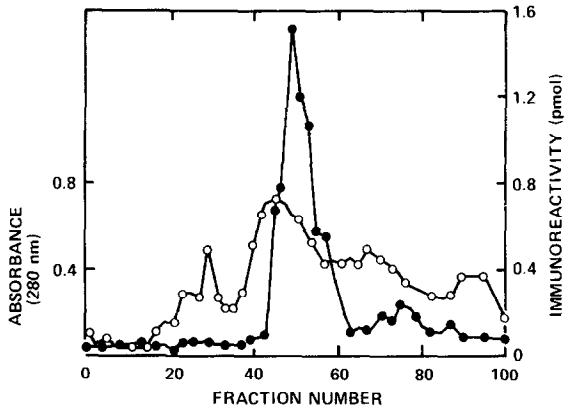

Figure 3: DEAE-cellulose chromatography of 27,000xg supernatant fraction. The column (2.5 x 10 cm) was equilibrated with 0.01 M phosphate buffer, pH 7 and loaded with 87 ml of supernatant derived from 15 grams of atrial tissue. Washing was carried out with 200 ml of the equilibration buffer before elution with a linear salt gradient consisting of 300 ml of 0.01 M phosphate buffer, pH 7.0 and 300 ml of 0.01 M phosphate buffer, 0.3 M sodium chloride, pH 7.0. After application of the gradient five ml fractions were collected and assayed for absorbance at 280 nm and for enzyme activity. o-o-o, absorbance at 280 nm; •-•-•, enzyme activity.

was stable to further degradation since recovery of 90-100 percent of N-acetyl dyn(1-8) subsequent to a two-hour incubation with enzyme was obtained. This suggested that leu-enkephalin was not generated by the

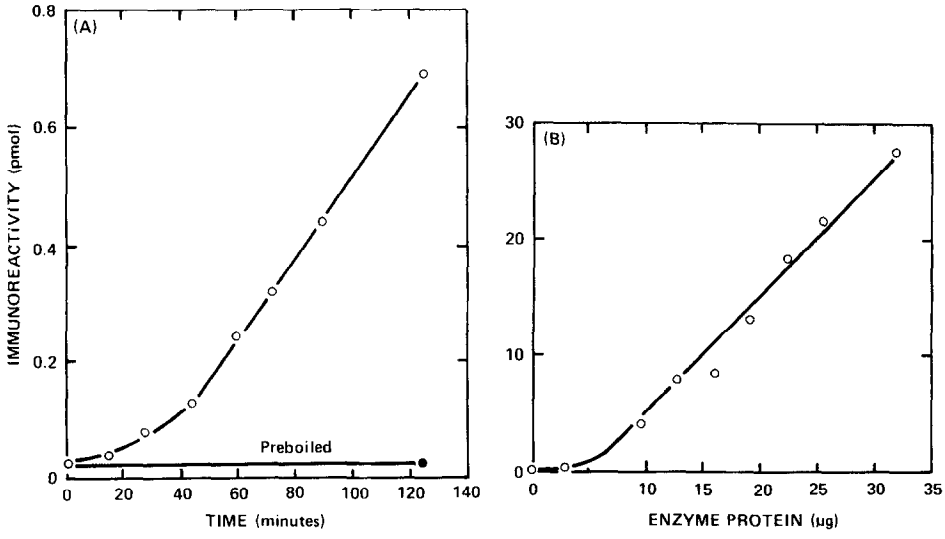

Figure 4: The variation of single arginine cleaving activity with time (A) and with enzyme protein (B). Immunoreactivity of N-acetyl dyn(1-8) was measured. In (A) 7.0 µg of DEAE-cellulose purified enzyme were used for each assay. In (B) reactions were carried out for 130 minutes. All other conditions were as described in Methods. Protein assays were performed as described by Lowry et al. (17)

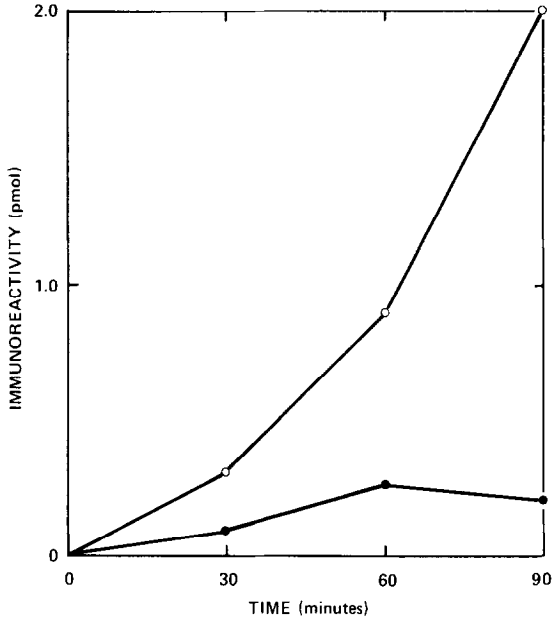

Figure 5: The production of immunoreactive N-acetyl dyn(1-8) as a function of time and substrate. Incubations were carried out with 15 μg of DEAE-cellulose purified enzyme as described in Methods either N-acetyl dyn A (o-o-o) or N-acetyl dyn(1-9) (●-●-●) as substrate.

enzyme activity and that the paired arginine residues following the leu-enkephalin sequence are not cleavage sites for this enzyme. The enzyme exhibited a pH optimum of 6.2 in phosphate buffer (data not shown).

To determine if the activity proceeded via a two-step mechanism, i.e. trypsin-like cleavage followed by carboxypeptidase B-like activity, we used N-acetyl dyn(1-9) as substrate. Incubation of N-acetyl dyn(1-9) with the enzyme resulted in only neglible production of N-acetyl dyn(1-8) (Figure 5).

The inhibitor profile of the activity is shown in Table 1. Soybean and ovomucoid trypsin inhibitors did not affect the enzyme activity. The chelating agents EDTA and O-phenanthroline were powerful inhibitors as was p-chloromercuriphenyl sulfonic acid. The enzyme activity was insensitive to low concentrations of EGTA.

Addition of 0.01 mM to 1 mM dithiothreitol yielded up to 10-fold stimulation of activity depending on the enzyme preparation. At higher concentrations inhibition was observed, presumably by interference with

Table 1. Effect of inhibitors on enzyme activity

INHIBITOR	FINAL CONCENTRATION	PERCENT OF CONTROL
Soybean trypsin inhibitor	100 μg/ml	117
Ovomucoid trypsin inhibitor	100 μg/ml	96
O-phenanthroline	0.1 mM	3
EDTA	0.1 mM	3
EGTA	0.5 mM	73
EGTA	0.1 mM	100
p-Chloromercuriphenylsulfonic acid	0.1 mM	6
Phenylmethylsulfonyl fluoride	0.5 mM	73
TLCK	0.2 mM	59
Leupeptin	1.0 mM	60
Chloroquin	0.1 mM	86
$MgSO_4$	0.3 mM	107
$ZnCl_2$	0.3 mM	21
$MnCl_2$	0.3 mM	74
$CaCl_2$	0.3 mM	106
$CoCl_2$	0.3 mM	219

Enzyme was preincubated with or without inhibitors for thirty minutes at room temperature before addition of N-acetyl dyn A and determination of enzyme activity. Data are mean values of 2-5 determinations. Organic chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

binding of metal ligand. Enzyme that had lost activity after prolonged storage at -70°C was not reactivated by the addition of dithiothreitol.

Zinc chloride was a strong inhibitor of enzyme activity (possibly via protein aggregation), manganese chloride a moderate inhibitor and calcium chloride did not affect the activity. Cobalt chloride at 0.3 mM concentration produced a 2-fold activation of enzyme activity. After EDTA treatment, manganese chloride and zinc chloride partially restored activity while cobalt chloride both restored activity and activated the enzyme (Table 2).

Table 2. Reactivation of enzyme activity by metal ions after pretreatment of enzyme with EDTA

Addition	Percent of Activity
Control	100
0.1 mM EDTA	6
+ 0.3 mM $CuCl_2$	9
+ 0.3 mM $FeCl_2$	7
+ 0.3 mM $ZnCl_2$	31
+ 0.3 mM $CoCl_2$	227
+ 0.3 mM $MnCl_2$	60
+ 0.3 mM $MgCl_2$	6
+ 0.3 mM $CaCl_2$	6

Enzyme was preincubated with or without 0.1 mM EDTA for 15 minutes at room temperature, before metals were added and mixtures allowed an additional 30 minutes of incubation. Activity was measured following addition of N-acetyl dyn A.

DISCUSSION

We have demonstrated the existence of an enzyme activity from Aplysia that cleaves dyn A at a single arginine site to form dyn(1-8). Inhibitor studies suggest the enzyme is a metallo-enzyme, possibly containing cobalt, with essential sulfhydryl residue(s). The lability of the enzyme to EDTA differentiates it from the cysteine proteinase, cathepsin B (15). The partially purified preparation hydrolyzes dyn (A) to dyn(1-8) at a considerably faster rate than it hydrolyzes dyn(1-9) to dyn(1-8). This indicates that cleavage of dyn A may be occurring directly on the amino side of the arginine residue at position 9 (Figure 1) and that N-acetyl dyn(1-9) is not an intermediate in a two-step cleavage reaction. This differs from the mechanism of trypsin-like activity followed by carboxypeptidase-like hydrolysis proposed for cleavage at dibasic residues (1). Mizono et al, have described an enzyme preparation from bovine adrenal chromaffin granules that cleaves at double basic amino acid sites but lacks carboxypeptidase activity. They suggest a one-step mechanism involving cleavage by the enzyme at both sides of consecutive basic residues (16). A similar mechanism at the single arginine may be operative here. Alternatively, it is possible that there is tight coupling between a trypsin-like enzyme and a carboxypeptidase B-like enzyme in the preparation. Purification of the enzyme is necessary to elucidate the nature of the metal ligand, the substrate specificity, and the mechanism of action of the enzyme. This work suggests that the atrial gland of Aplysia may be a useful tissue for studying enzymes involved in posttranslational processing of secretory peptides.

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