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Characterisation and partial purification of a novel prohormone processing enzyme from ovine adrenal medulla

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An enzymatic activity has been identified which is capable of generating a product chromatographically identical with adrenorphin from the model substrate BAM12P. This enzyme was purified by gel filtration and ion-exchange chromatography and characterised as having a molecular mass between 30 and 45 kDa and an acidic p*I*. The enzyme is active at the acid pH expected in the secretory vesicle interior and is inhibited by EDTA, suggesting that it is a metalloprotease. This activity could not be mimicked by incubation with lysosomal fractions and it meets the criteria to be considered as a possible prohormone processing enzyme.

Prohormone processing; Adrenorphin; Secretory vesicle purification

1. INTRODUCTION

Active peptide hormones are released from their precursors by endoproteolytic cleavage at highly specific sites. The commonest of these is cleavage at pairs of basic residues such as lysine and arginine [1,2]. However another common class of processing sites are known to be at single arginine residues, adjacent to a proline [3]. It is thought that this type of cleavage might be important in tissue-specific cleavage patterns [3].

Adrenorphin, a proenkephalin fragment, is derived by cleavage on the amino side of an arginine-proline sequence [4]. Adrenorphin is a highly active mu agonist [5] and is of interest since the ratio of this peptide to other proenkephalin fragments varies from one brain region to another [6] indicating tissue-specific processing.

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Abbreviations: BAM12P, bovine adrenal medullary dodecapeptide; HPLC, high-pressure liquid chromatography; Mes, morpholinoethanosulphonic acid; PAM, peptidyl-glycine α-amidating mono-oxygenase

The purification of an endopeptidase responsible for the generation of adrenorphin was undertaken, using the ovine adrenal medulla as a source. Adrenorphin is known to be located in the adrenal medulla of all species so far investigated [6]. Secretory vesicles (also known as chromaffin granules) were isolated as a preliminary purification step, since it is known that prohormone processing occurs within the secretory vesicle [1]. It is particularly important that lysosomes be eliminated at this stage so that lysosomal proteases do not interfere with, or become confused with, the prohormone processing activity.

2. MATERIALS AND METHODS

2.1. *Subcellular fractionation*

Ovine adrenals were collected on ice and the medullas were dissected out and homogenised in isotonic buffer (10 mM Hepes, 0.25 M sucrose, pH 7.4). A vesicle pellet was isolated by differential centrifugation as previously described [7]. This was applied onto an isoosmolar Metrizamide gradient [8] with steps of 0.15, 0.28 and 0.45 M Metrizamide (see fig.1). Fractions were collected from the bottom of the tube with a peristaltic pump. The vesicle enriched fractions from this gradient (fractions 6 and 7 in fig.1) were then applied to a sucrose gradient with a 1.8 M sucrose cushion. The vesicles were collected as a pellet after centrifugation. Lysosome fractions were collected

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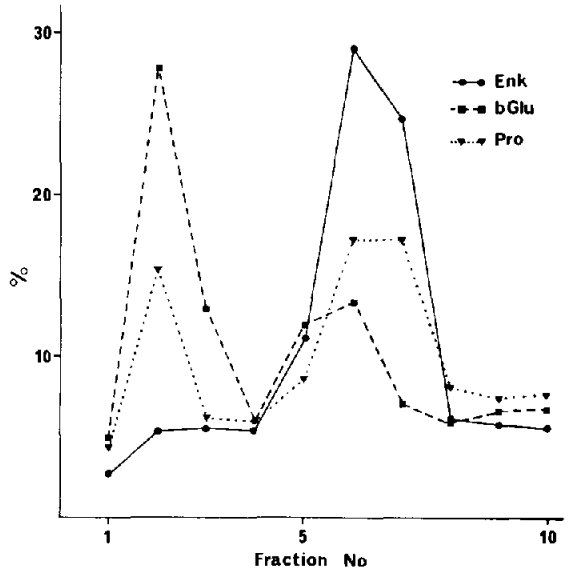

Fig.1. Purification of secretory vesicles on a Metrizamide gradient with steps of 0.45 M (1 ml), 0.28 M (5 ml) and 0.15 M (2 ml). 1 ml fractions were collected from the bottom of the tube (therefore fraction 1 is the densest). The percentage distribution is shown within the gradient for protein (▼), β-glucuronidase as a lysosomal marker (■) and enkephalins and other opiate peptides detected by radioreceptor assay (●) as a marker for vesicles.

both from the Metrizamide gradient (fraction 2) and from the sucrose gradient (at the interface with the 1.8 M cushion).

The vesicles were lysed by freezing and thawing in 10 mM Hepes, pH 7.4, and the vesicle contents separated from the membranes by centrifugation at 10000 × *g* for 15 min.

Table 1

Purification of secretory vesicles

Fraction	% recovery of vesicles[a]	% recovery of lysosomes[a]	Purification[b]
S1[c]	78.0 ± 6.0	49.9 ± 6.7	1.6 ± 0.3
P1	14.9 ± 2.7	38.0 ± 2.7	0.4 ± 0.08
S2	38.0 ± 3.2	23.6 ± 3.8	1.7 ± 0.3
P2	33.6 ± 2.6	23.3 ± 5.6	1.6 ± 0.5
Band from Gradient 1	20.0 ± 2.5	4.2 ± 1.2	5.1 ± 0.9
Sucrose pellet	4.7 ± 1.1	0.08 ± 0.03	54 ± 8

[a] Calculated as a percentage of the amount in the homogenate (n = 5)

[b] The ratio of the two markers in a fraction divided by the same ratio in the homogenate

[c] P1, S1, P2, S2 refer to the differential centrifugation fractions

2.2. *Assays for sub-cellular fractions*

β-Glucuronidase was assayed as a lysosomal marker by incubation with 4-methyl umbelliferol β-glucuronide and detection of the fluorescent leaving group [8]. Opiate peptides were assayed as a vesicle marker, using a radioreceptor assay [7]. Protein was assayed by the Lowry method [7].

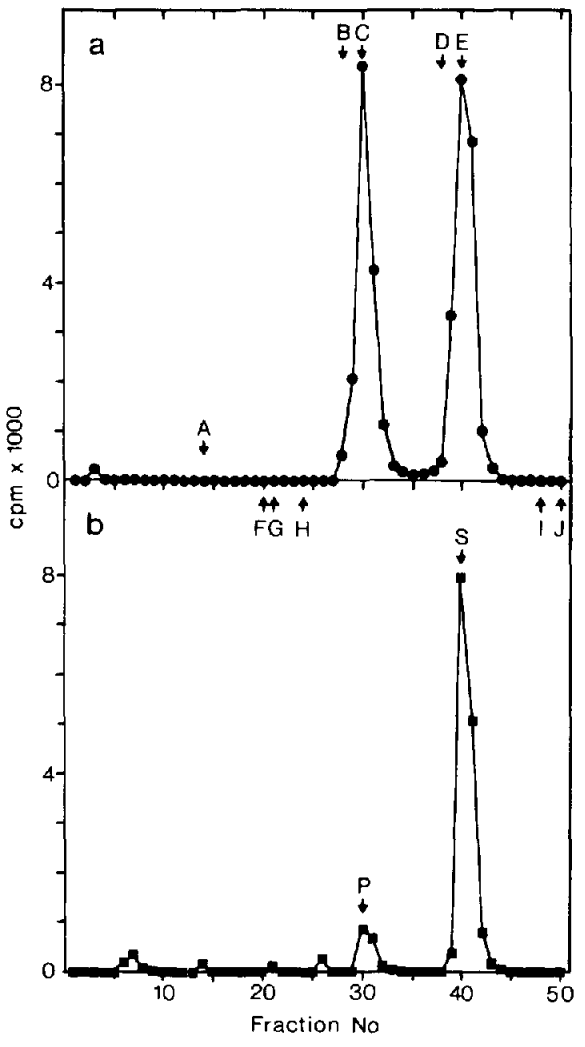

Fig.2. Reverse-phase HPLC separation of peptides. After an initial 30 min of 100% buffer A, a linear gradient was run up to 40% buffer B in 30 min. Panel a shows a separation of [^{125}I]adrenorphin and [^{125}I]BAM12P. Panel b shows the separation of peptides after incubation of [^{125}I]BAM12P with fraction 11 from gel filtration. The position of the following markers is shown: A, Met-enkephalin; B, adrenorphin and adrenorphin Gly^9; C, [^{125}I]adrenorphin and [^{125}I]adrenorphin Gly^9; D, BAM12P; E, [^{125}I]BAM12P; F, dynorphin(1–6); G, dynorphin(1–7); H, Leu-enkephalin; I, dynorphin(1–9); J, dynorphin(1–8); P, expected product, [^{125}I]adrenorphin; S, substrate, [^{125}I]BAM12P.

2.3. *Enzyme activity incubations*

Fractions were incubated for 2 h at 37°C with 20000 cpm of [^{125}I]BAM12P (iodinated by the Chloramine T method and purified by HPLC) in 0.1 M Mes, pH 6. Inhibitors were dissolved in the same buffer. Phosphate and Tris were used as the buffers to determine pH optimum (5–7 and 6–8, respectively). After incubation, the reaction was stopped by addition of HCl to 0.1 M and an aliquot applied to an LKB Spherisorb ODS reverse-phase column. Buffers were 20% and 80% acetonitrile with 0.1% TFA.

Gel filtration was conducted on an LKB TSK G3000SW column in 0.1 M ammonium formate and ion exchange on an LKB TSK DEAE 5PW column with a gradient from 0 to 0.5 M NaCl in 10 mM Mes, pH 6.

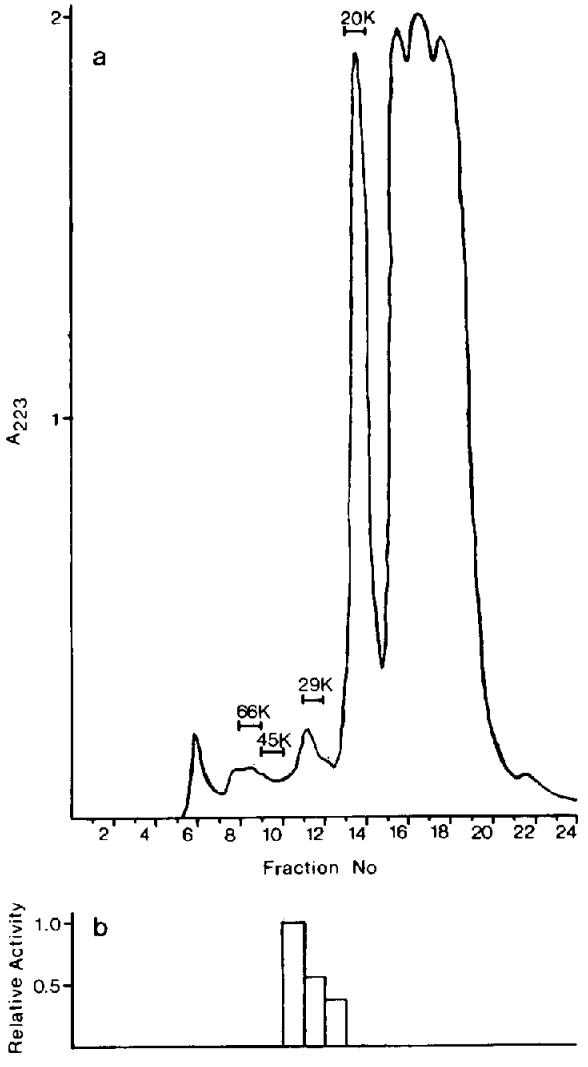

Fig.3. Gel filtration of vesicle soluble fraction. The flow rate was 0.2 ml/min and 1 ml fractions were collected. Panel a, absorbance at 223 nm. The position of molecular mass markers from a parallel run is shown: 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 29 kDa, carbonic anhydrase; 20 kDa, trypsin inhibitor. Panel b shows incubations of the fractions with [^{125}I]BAM12P. The amount of product generated by fraction 11 is set as a relative activity of 1.0.

3. RESULTS

Density-gradient centrifugation on a Metrizamide density gradient yielded a partially pure vesicle preparation (fig.1). Further purification on a sucrose cushion gave a purification of vesicles over lysosomes of 54-fold compared with the initial homogenate (see table 1).

Elution positions for the model substrate BAM12P and the expected product, adrenorphin, were established on HPLC (fig.2). Secretory vesicles were lysed and the membrane and soluble fractions incubated. Only the soluble fraction generated any product.

The soluble fraction from the vesicles was further purified by gel filtration (fig.3). The peak of

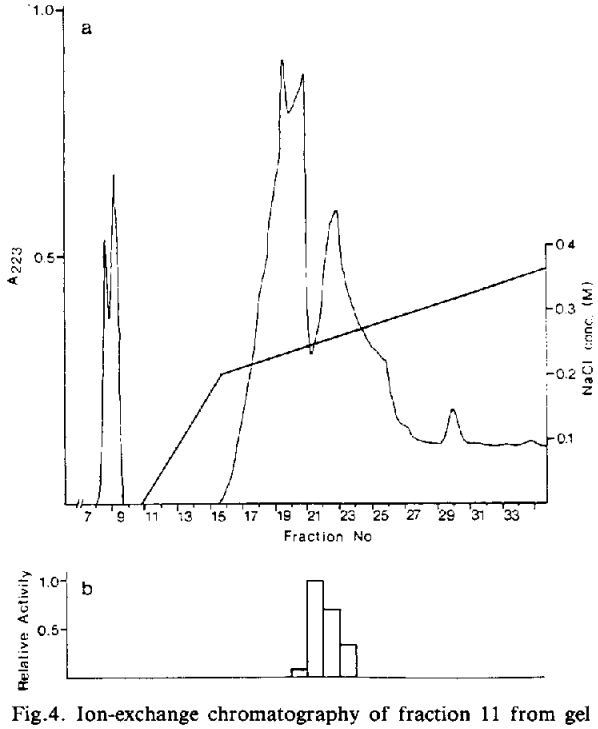

Fig.4. Ion-exchange chromatography of fraction 11 from gel filtration. The flow rate was 1 ml/min and 1 ml fractions were collected. Panel a shows absorbance at 223 nm. Panel b shows the amount of product generated (setting fraction 21 as 1.0, see fig.3b).

Table 2

Effect of inhibitors on enzyme activity

Inhibitor[a]	Conc.	% of control[b]
PMSF	1.0 mM	51
TLCK	1.0 mM	34
PCMB	1.0 mM	23
Aprotinin	0.1 mg/ml	30
DTT	0.01 mM	92
DTT	0.1 mM	46
DTT	1.0 mM	37
EDTA	1.0 mM	1
EDTA and Co^{2+}	1.0 mM + 1.5 mM	41
EDTA and Mn^{2+}	1.0 mM + 1.5 mM	44
EDTA and Cu^{2+}	1.0 mM + 1.5 mM	1
EDTA and Ca^{2+}	1.0 mM + 1.5 mM	1

[a] PMSF, phenylmethylsulphonyl fluoride; TLCK, tosyl-L-lysine-chloromethyl ketone; PCMB, *p*-chloromercuribenzoic acid; DTT, dithiothreitol

[b] These values are the amount of product detected on HPLC as a percentage of that seen in a parallel incubation using the same enzyme preparation without inhibitors

activity emerged in fraction 11, corresponding to a molecular mass of 45 kDa, predicted from a parallel run with standards. The soluble fraction from the lysosomal band, purified by Metrizamide density gradient and sucrose cushion, was also subjected to gel filtration. Aliquots from this separation were then incubated with substrate. The size of the aliquots was calculated to contain the same amount of β-glucuronidase activity (the marker enzyme for lysosomes) as was found as a contaminant in the vesicle preparation. These incubations did not generate any product, neither did incubations with ten times larger amounts.

Fraction 11 from gel filtration was further purified by ion-exchange chromatography (fig.4). The enzyme had a pH optimum of 7.8 but retained 30% of its maximal activity at pH 6, and was most strongly inhibited by EDTA and DTT (table 2).

4. DISCUSSION

The vesicle purification protocol yielded such a high level of purification because of the successive isoosmolar and then hyperosmolar gradients. A previous study had revealed that ovine medullary vesicles shrink and become denser in hyperosmotic media to a greater extent than lysosomes [7]. Therefore this method first removes the densest lysosomes by isoosmolar separation and then increases the density of the vesicles in the second gradient so that they pellet leaving the remaining lysosomes behind.

Cleavage of BAM12P might be expected to occur between glycine and arginine in the sequence Val-Gly-Arg-Pro. An amidating enzyme could then convert the Val-Gly C-terminus to valine amide [9], although the levels of amidating enzyme are reported to be low in the adrenal [10]. The activity in this study generates a substance which cochromatographs with adrenorphin. This may be adrenorphin generated directly or by a copurifying amidating enzyme; however, the HPLC conditions chosen do not separate adrenorphin from adrenorphin Gly[9] and the latter seems the most likely product. It is clear that the product has an intact Tyr since it is radioactive with iodine-125 and is larger than Met-enkephalin (from the available markers). Since the activity bound to an anion-exchange column at pH 6 it can be deduced to have a p*I* of less than 6. The inhibition by EDTA indicates a metalloprotease and that by DTT suggests the involvement of thiol groups. The partial inhibition by PMSF, TLCK and aprotinin, although at relatively high doses, presents a more complicated picture and will be investigated further with a full dose-response profile.

Although crude lysosome preparations might be expected to cleave the substrate at multiple sites it remains possible that the isolation procedure selects a single lysosomal protease which has this activity. Therefore lysosomal material was subjected to the same purification and incubation protocol as the vesicle material. However, no adrenorphin-like product was detected, strongly suggesting that this enzyme is vesicular rather than lysosomal.

This activity does not generate significant amounts of Met-enkephalin under these conditions, indicating that it is different from the enzymes described which cleave at pairs of basic residues in the adrenal [11]. This activity also differs from prolyl endopeptidase, which is fully active in the presence of 1 mM EDTA [12]. This activity is similar to one described in the atrial gland of *Aplysia californica* [13] which cleaved dynorphin A at the amino side of arginine-proline sequences and was inhibited by EDTA. It may be that there is an evolutionary relationship between

the *Aplysia* prohormone processing enzyme and the ovine activity.

Further purification of this activity is now being undertaken in order to raise antibodies against the pure protein and study its distribution and regulation.

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