# An aminopeptidase activity in bovine pituitary secretory vesicles that cleaves the N-terminal arginine from $\beta$-lipotropin$_{60-65}$

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Secretory vesicles isolated from the neural and intermediate lobes of the bovine pituitary contained a membrane-bound aminopeptidase activity which cleaved arginine from $\beta$-LPH$_{60-65}$ (Arg-Tyr-Gly-Gly-Phe-Met) and Arg-MCA. Neither methionine enkephalin (Tyr-Gly-Gly-Phe-Met) nor Substance P, which has an N-terminal arginine followed by a proline, could serve as substrates for this aminopeptidase activity; nor could cathepsin B-like or chymotrypsin-like enzyme activities be detected in the vesicle preparations. Maximal enzyme activity was at pH 6.0, and the activity was inhibited by EDTA, stimulated by $Co^{2+}$ and $Zn^{2+}$, but was unaffected by leupeptin, pepstatin A, phenylmethylsulfonyl fluoride and *p*-chloromercuribenzenesulfonate, suggesting that the enzyme is a metalloaminopeptidase. The presence of this aminopeptidase activity in secretory vesicles suggests that it may be involved in peptide prohormone processing.

*Aminopeptidase* *Prohormone processing (converting) enzyme* *Secretory vesicle* *Neurohypophysis* *Intermediate lobe*

## 1. INTRODUCTION

The conversion of peptide prohormones to biologically active peptides requires a sequence of enzymatic post-translational processing steps which appear to occur within secretory vesicles [1,2]. The first cleavages often occur at sites in the prohormone which contain pairs of basic amino acids (e.g., Lys-Arg) and prohormone converting enzyme activities which cleave at these sites have been found in intermediate [3] and neural [3,4] lobe secretory vesicles in the pituitary. The traditional view has been that following this cleavage, a carboxypeptidase-B-like enzyme was necessary to remove the carboxyl-terminal basic amino acids from the resultant peptides [5], and such enzymes have been reported in secretory vesicles from the pituitary neural and intermediate lobes [6,7]. Recent studies in our laboratory suggest that the initial cleavage at Lys-Arg sites in pro-opiomelanocortin and pro-oxytocin occurs principally between the two basic amino acids (in preparation), therefore also requiring an intravesicular aminopeptidase to remove the arginine from the amino terminus of an excised peptide. Here, we report the preliminary characterization of such an aminopeptidase activity in secretory vesicles isolated from the intermediate and neural lobes of the bovine pituitary.

## 2. MATERIALS AND METHODS

Previously described methods were used to prepare pure secretory vesicles from the neural lobes (i.e., neurosecretory vesicles, NSVs [8]) and the intermediate lobes (ILSVs [9]) of bovine pituitaries. To prepare secretory vesicle membrane (NSV-M or ILSV-M) and soluble (NSV-S or ILSV-S) fractions, isolated vesicles were diluted 10-fold with ice-cold distilled water, lysed by 3 cycles of freeze-thawing,

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and centrifuged for 1 h at $100\,000 \times g_{max}$ on a Beckman air-driven ultracentrifuge. Protein concentrations were measured as in [10] or by the fluorescamine method [11].

The aminopeptidase activities in the above fractions were measured by high-pressure liquid chromatography (HPLC) and fluorometric assay. The HPLC procedure is illustrated in fig.1. Between 20 and 25 $\mu$g protein from a given vesicle fraction was incubated in 60 $\mu$l incubation buffer (see below) containing 0.63 mM $\beta$-LPH$_{60-65}$ (Arg-Tyr-Gly-Gly-Phe-Met) as the substrate. The $\beta$-LPH$_{60-65}$ was obtained from Peninsula Laboratories (Belmont, CA) as was the HPLC standard for the product, met-enkephalin (Tyr-Gly-Gly-Phe-Met). Incubations were for 1 h at 37°C, which was found to be in the linear range of the assay (not shown). Following the incubation, the incubates were acidified to pH 1 with HCl, and centrifuged for 30 min at $100\,000 \times g_{max}$ in a Beckman airfuge. The supernatants were injected onto a Hi-pore, RP-304 Reverse-Phase Column (Biorad Laboratories, Richmond, CA) connected to a Beckman model 332 HPLC system. The eluant was isocratic 0.1% trifluoracetic acid (TFA), 30% methanol, and absorbance was monitored at 280 nm. The retention times for the $\beta$-LPH$_{60-65}$ substrate and the met-enkephalin product, in this system, were 8.4 and 9.4 min, respectively (see arrows in fig.1). In most of the incubations, which were conducted at pH 6, either of two buffers were used, 0.1 M 2-[*N*-morpholino]ethanesulfonic acid (Mes) or 0.1 M sodium phosphate. No significant differences in activity were found between these buffers. Other buffers used were: pH 2–4.5, 0.1 M sodium citrate; pH 4.5–5.5, 0.1 M sodium acetate; pH 5.5–7.5, 0.1 M sodium phosphate; pH 7.5–9.0, Tris–HCl. Quantitative measurements of the peptides (in $\mu$gs) were made from HPLC peak heights (at 280 nm absorbance) compared to standard curves of peak heights *vs* peptide quantity for both $\beta$-LPH$_{60-65}$ and met-enkephalin. Kinetic constants for both the HPLC and fluorogenic assays (below) were calculated from Lineweaver–Burk plots by conventional procedures. Incubations of vesicle extracts with substance P and met-enkephalin as potential substrates were also as described above, except that the substance P HPLC assay used an isocratic 0.1% TFA, 45% methanol eluant. The retention times for substance P and its potential aminopeptidase product desArg$^1$ substance P were 7.5 and 8.8 min, respectively. The substance P HPLC assay was monitored at 212 nm absorbance.

Incubations and buffers for the fluorometric assays were identical to those described above. The fluorogenic substrates used in this study, Arg-

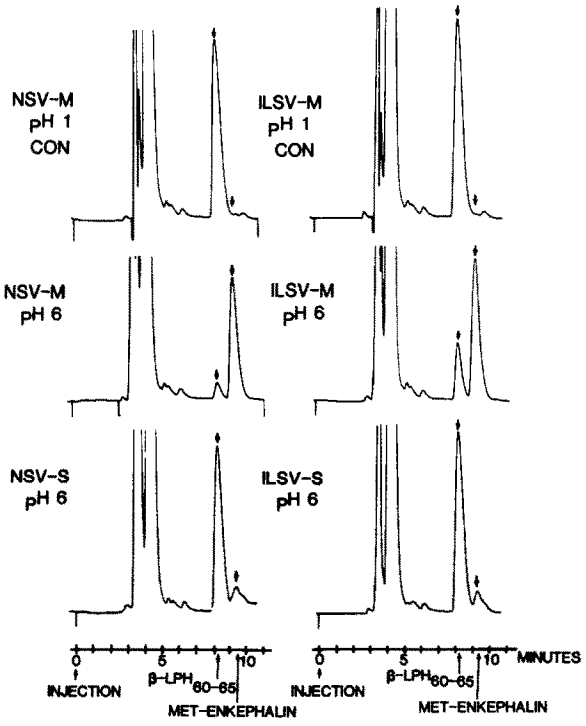


Fig.1. Aminopeptidase activity in secretory granules (vesicles) purified from neural lobe (NSV, left) and intermediate lobe (ILSV, right) of the bovine pituitary. Soluble or membrane fractions from the isolated vesicles (22 $\mu$g protein) were incubated with buffers containing $\beta$-LPH$_{60-65}$ (0.63 mM) for 1 h at 37°C. The incubates were then acidified to pH 1 with HCl, centrifuged for 30 min at $100\,000 \times g_{max}$, and the supernatants analysed by HPLC (see section 2). HPLC absorbance patterns, recorded at 280 nm, are shown together with the retention times of the $\beta$-LPH$_{60-65}$ substrate (arrow) and methionine enkephalin product (arrow). Left: top, control incubation with neurosecretory vesicle membranes at pH 1 (NSV-M, pH 1); middle, NSV-M incubation at pH 6; bottom, NSV-S (soluble) fraction incubated at pH 6. Right: incubations with membranes (ILSV-M) or soluble (ILSV-S) fractions from intermediate lobe vesicles. Note substantial generations of met enkephalin from $\beta$-LPH$_{60-65}$ at pH 6 with NSV-M and ILSV-M, but significantly lower activity at this pH using the soluble fractions.

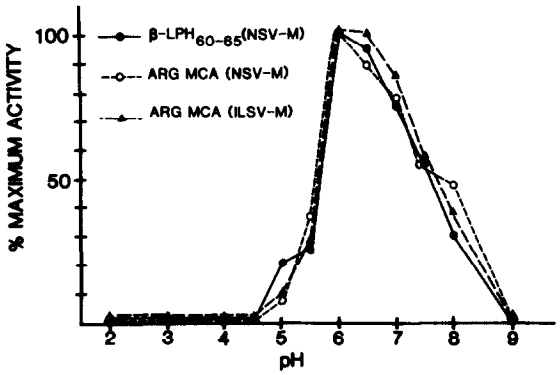


Fig.2. pH dependency of aminopeptidase activity in NSV-M and ILSV-M using the $\beta$-LPH$_{60-65}$ substrate (for NSV-M) and the fluorogenic Arg-MCA substrate (for NSV-M and ILSV-M). The maximum activity in both preparations was at pH 6.0.

MCA and Leu-MCA (aminopeptidase substrates), Lys-Ala-MCA (diaminopeptidase II substrate), Succ-Leu-Leu-Val-Tyr-MCA (chymotrypsin substrate), and Z-Arg-Arg-MCA (cathepsin B substrate), and the AMC standard were obtained from Peninsula Laboratories. The MCA-substrates and standard AMC were dissolved in dimethyl sulfoxide (DMSO) as stock solutions, and were employed using conventional procedures [12]. The fluorescence of the samples was measured using an excitation wavelength of 383 nm and an emission wavelength of 455 nm, on an Aminco SPF125 spectrofluorometer (American Instrument Co., Silver Spring, MD). The protease inhibitors, leupeptin, and pepstatin A were obtained from Peninsula Laboratories, and the PCMS (*p*-chloromercuribenzenesulfonate), PMSF (phenylmethylsulfonylfluoride) and EDTA were obtained from Sigma (St. Louis, MO).

## 3. RESULTS

Fig.1 illustrates the HPLC assays of $\beta$-LPH$_{60-65}$ conversion to met-enkephalin by the aminopeptidase activity in NSVs and ILSVs. Over 95% of the aminopeptidase activity at pH 6 was found associated with the membrane fractions, i.e., the NSV-M (middle left trace) and ILSV-M (middle right trace) as opposed to the respective soluble fractions (NSV-S and ILSV-S). The pH range of the aminopeptidase activity is shown in fig.2. Two assays of the aminopeptidase activity in the NSV-M fraction, the conversion of $\beta$-LPH$_{60-65}$ to met-enkephalin (HPLC assay) and a fluorometric assay using the aminopeptidase substrate Arg-MCA,

Table 1

Kinetic constants of aminopeptidase activity in NSV-M[a]

| Substrate | | $K_m$ ($\times 10^{-6}$ M) | $V_{max}$ ($\mu$mol/$\mu$g protein per h) |
|---|---|---|---|
| A. | Peptide substrates | | |
| | $\beta$-LPH$_{60-65}$ | 195.0 | 0.005 |
| | Methionine enkephalin | [b] | |
| | Substance P | [b] | |
| B. | MCA substrates | | |
| | Arg-MCA | 13.8 | 0.003 |
| | Leu-MCA | 27.0 | 0.008 |
| | Lys-Ala-MCA | 220.0 | 0.008 |
| | Z-Arg-Arg-MCA | [b] | |
| | Succ-Leu-Leu-Val-Tyr-MCA | [b] | |

[a] The incubations in these studies were done at 37°C for 1 h, pH 6.0. The peptide substrates were assayed by HPLC and the fluorogenic (MCA) substrates by spectrofluorometry (see section 2). Concentration of substrates used in these kinetic studies ranged from 10 $\mu$M to 0.5 mM

[b] No activity detected

show a broad pH curve with a maximum at pH 6.0, and with half-maximum activities ranging from pH 5.5–7.5. Essentially identical data were obtained with ILSV-M fractions using the Arg-MCA assay (fig.2).

Kinetic constants for the aminopeptidase activity in the NSV-M preparations were determined from conventional Lineweaver–Burk plots (not shown). Table 1 illustrates these values for the aminopeptidase substrates, $\beta$-LPH$_{60-65}$, and Arg-MCA shown in fig.2, as well as for a number of other substrates. All incubations were performed at pH 6 and 37°C. Significant enzyme activities were detected using the $\beta$-LPH$_{60-65}$, Arg-MCA, and Leu-MCA aminopeptidase substrates, and the diaminopeptidase substrate, Lys-Ala-MCA. Although the $V_{max}$ values were comparable between these substrates, ranging from 0.003–0.008 $\mu$mol/$\mu$g protein per h, the $K_m$ values varied considerably from 13.8 $\mu$M for Arg-MCA to 220 $\mu$M for Lys-Ala-MCA. It is notable that neither methionine-enkephalin with an amino terminus tyrosine nor substance P (which has an Arg at its N-terminus, followed by a proline) could serve as substrates for the vesicle enzyme(s) (table 1A). In the latter experiments, incubations were carried out for as long as 5–12 h at 37°C with no evidence of degradation of the met-enkephalin or substance P substrates. The lack of activity on the Z-Arg-Arg-MCA substrate (table 1B) suggests that this vesicle membrane activity is not due to an Arg-specific endopeptidase (e.g., cathepsin B). In addition, no chymotrypsin-like activity could be detected in the NSV-M fraction using the Succ-Leu-Leu-Val-Tyr-MCA substrate (table 1B).

Tests of various inhibitors on the NSV-M aminopeptidase activity indicate that it is a metalloprotease (table 2). EDTA (0.5 mM) inhibited its activity, and $Co^{2+}$ and $Zn^{2+}$ strongly stimulated its activity. $Cu^{2+}$ strongly inhibited the activity,

Table 2

Effects on ions and inhibitors on NSV-M aminopeptidase activity[a]

| Inhibitor (mM) | Control activity (%) | |
|---|---|---|
| | $\beta$-LPH$_{60-65}$ substrate | Arg-MCA substrate |
| A. Ions | | |
| EDTA (0.5) | 15 | 18 |
| $Co^{+}$ (0.5) | 177 | 121 |
| $Cu^{2+}$ (0.5) | 16 | 7 |
| $Zn^{2+}$ (0.5) | 197 | 150 |
| $Mg^{2+}$ (0.5) | 90 | 106 |
| $Ca^{2+}$ (0.5) | 85 | 88 |
| B. Recovery from partial EDTA inhibition | | |
| EDTA (0.1) | 63 | 60 |
| EDTA (0.1) + $Co^{2+}$ (0.5) | 106 | 103 |
| EDTA (0.1) + $Cu^{2+}$ (0.5) | 42 | 57 |
| EDTA (0.1) + $Zn^{2+}$ (0.5) | 118 | 98 |
| EDTA (0.1) + $Mg^{2+}$ (0.5) | 54 | 55 |
| EDTA (0.1) + $Ca^{2+}$ (0.5) | 55 | 66 |
| C. Protease inhibitors | | |
| Leupeptin (0.5) | 96 | 100 |
| Pepstatin A (0.5) | 88 | 101 |
| PMSF (1) | 109 | 99 |
| PCMS (1) | 110 | 102 |

[a] Assays were done using $\beta$-LPH$_{60-65}$ (0.6 mM) and Arg-MCA (0.5 mM) substrates as described in section 2

whereas $Ca^{2+}$ and $Mg^{2+}$ appeared to have no significant effect (table 2A for both $\beta$-$LPH_{60-65}$ and Arg-MCA substrates). Partial inhibition of the NSV-M enzyme activity (60–63%) by 0.1 mM EDTA is reversed only by $Co^{2+}$ and $Zn^{2+}$ (table 2B). Leupeptin, pepstatin A, PMSF, and PCMS at 0.5–1.0 mM concentrations were without effect on the NSV-M aminopeptidase activity, indicating that it is not a cysteinyl, aspartyl, or serine protease (table 2C).

## 4. DISCUSSION

We report here the presence of an aminopeptidase activity in secretory vesicles, isolated from the neural and intermediate lobes of the bovine pituitary, which specifically cleaves Arg from the $NH_2$ terminus of $\beta$-$LPH_{60-65}$. This enzyme activity is membrane bound, maximally active at acid pH values (i.e., pH 6.0), and appears to be a metalloprotease since EDTA inhibits its activity, whereas serine (i.e., PMSF), cysteinyl (PMCS), and aspartyl (pepstatin A) protease inhibitors do not affect its activity (table 2). The acidic pH optimum (fig.2) is consistent with its putative role as a peptide processing enzyme in secretory vesicles which have an internal pH of 5.1–5.5 [1,2].

It is of significance that of the peptide substrates tested (table 1A), only the amino-terminal arginine in $\beta$-$LPH_{60-65}$ could serve as a substrate (fig.1). The product of this reaction, methionine enkephalin, could not be degraded by this enzyme activity. Therefore, this enzyme activity differs from the membrane-associated enkephalin-degrading aminopeptidases which have been reported in a variety of other tissue preparations [13–16]. This intravesicular aminopeptidase resembles aminopeptidase-B in that it appears to cleave amino-terminal arginine [17–19], but cannot function if the second amino acid in the peptide is a proline [17], as is the case in substance P (table 1A). However, since aminopeptidase-B is a neutral enzyme (pH optimum 7.2) and a serine protease [21–23], the intravesicular aminopeptidase activity we have described clearly is different. Nor does it correspond in its characteristics to leucine aminopeptidase, aminopeptidase-M or dipeptidyl-aminopeptidases I–IV [17,20]. Table 2 and fig.2 indicate that the enzyme activities measured by the $\beta$-$LPH_{60-65}$ and Arg-MCA assays have similar characteristics, and suggest that both assays are measuring the same enzyme. Efforts are now under way in our laboratory to purify these aminopeptidase(s) and fully characterize them.

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