SPECIFIC CLEAVAGE OF A SINGLE PEPTIDE BOND (RESIDUES 77–78) IN β-LIPOTROPIN BY A PITUITARY ENDOPEPTIDASE

László GRÁF and Ágnes KENESSEY
Research Institute for Pharmaceutical Chemistry, Budapest 1325, P.O. Box 82, Hungary

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1. Introduction

β-LPH of adenohypophysis is a lipolytic polypeptide consisting of 91 amino acid residues [1–4]. Its structure contains the complete sequence of pituitary β-MSH [1] and brain methionine-enkephalin [5] between residues 41–58 (or 37–58) and 61–65, respectively. Based on these structural relationships, it has been suggested that β-LPH may be the biological precursor of both β-MSH [6–8] and methionine-enkephalin [5,9,8]. This view is supported by the occurrence of several 'intermediate-like' lipotropin fragments in the hypophysis or in the hypothalamus*, comprising residues 1–58 [6,10], residues 61–76* [11], residues 61–87 [12] and residues 61–91 [12–14] of the structure, respectively. On the other hand, no direct evidence has been obtained so far on the conversion of β-LPH to any of the above fragments by pituitary enzymes, either in vitro, or in vivo. The preliminary results of our search for such converting enzymes are described in this paper.

2. Experimental

2.1. *Incubation of β-LPH with pituitary homogenate*

Anterior lobes were separated from freshly removed porcine pituitary glands, thoroughly washed with cold physiological saline solution, and homogenized in 2 volumes of a solution of 2% (w/v) NaCl containing 1 mM EDTA and 15 mM HCl in an Ultraturrax homogenizer (Janke-Kunkel KG) at 4°C for 30 s. The homogenate was stored at −20°C for later use.

Porcine β-LPH was prepared as described previously [15].

The homogenate (50 mg of glands per ml of incubation mixture) was incubated with porcine β-LPH (1 mg/ml) in 0.05 M ammonium acetate of pH 6.5 in the presence of 10 mM DTT and 10 mM EDTA at 37°C for 5 h.

Incubation in the presence of soy bean trypsin inhibitor (REANAL Fine Chemicals; 25 mg/ml of incubation mixture) or *p*-chloromercuribenzoate (Fluka, 3.6 mg/ml of incubation mixture) was also carried out. Samples were lyophilized for gel electrophoresis.

2.2. *Peptide analytical procedures*

Homogeneity of the lipotropin fragments was examined by disc electrophoresis in polyacrylamide gel (8%) at pH 9.0 [16], high-voltage paper electrophoresis at pH 6.5, and NH_2-terminal residue analysis [17]. Carboxypeptidase A (Worthington) digestion was carried out in 0.1 M Tris buffer of pH 7.4 with an enzyme to peptide ratio of 1 : 10 (w/w) at 37°C for 5 h. Tryptic hydrolysis was performed in 0.05 M ammonium acetate of pH 7.4 with an enzyme to peptide ratio of 1 : 50 (w/w) at 37°C for 2 h. Paper electrophoresis of the tryptic digest was accomplished in a two-dimensional system, first at pH 6.5 (pyridine/acetic acid/water, 100 : 3 : 900, by volume; 50 V/cm; 50 min) and then at pH 2.0 (formic acid/acetic acid/

Abbreviations: LPH, lipotropic hormone (lipotropin); MSH, melanocyte stimulating hormone, EDTA, ethylene diamine tetraacetic acid; DTT, dithiothreitol.

* LPH-(61–76)-peptide was isolated from porcine neurohypophysis-hypothalamus [11].

water, 20 : 80 : 900, by volume; 50 V/cm; 40 min). The map was developed with 0.1% ninhydrin solution in acetone. As the peptide spots became slightly visible they were cut out from the paper and eluted with 0.5 M ammonium hydroxide. The dried eluates were hydrolyzed for amino acid analysis. Amino acid analysis of the acid (6 M HCl, 110°C, 24 h) and carboxypeptidase A hydrolysates were carried out in a JEOL (JLC-5AH) automatic analyzer.

3. Results

3.1. *Action of pituitary homogenate on β-LPH*

Conversion of β-LPH brought about by the enzymes) of the pituitary homogenate was followed by gel electrophoresis (fig.1).

Figure 1a shows the electrophoretic pattern of the homogenate incubated in the absence of β-LPH. By adding 1 volume acetone to this sample the electro-

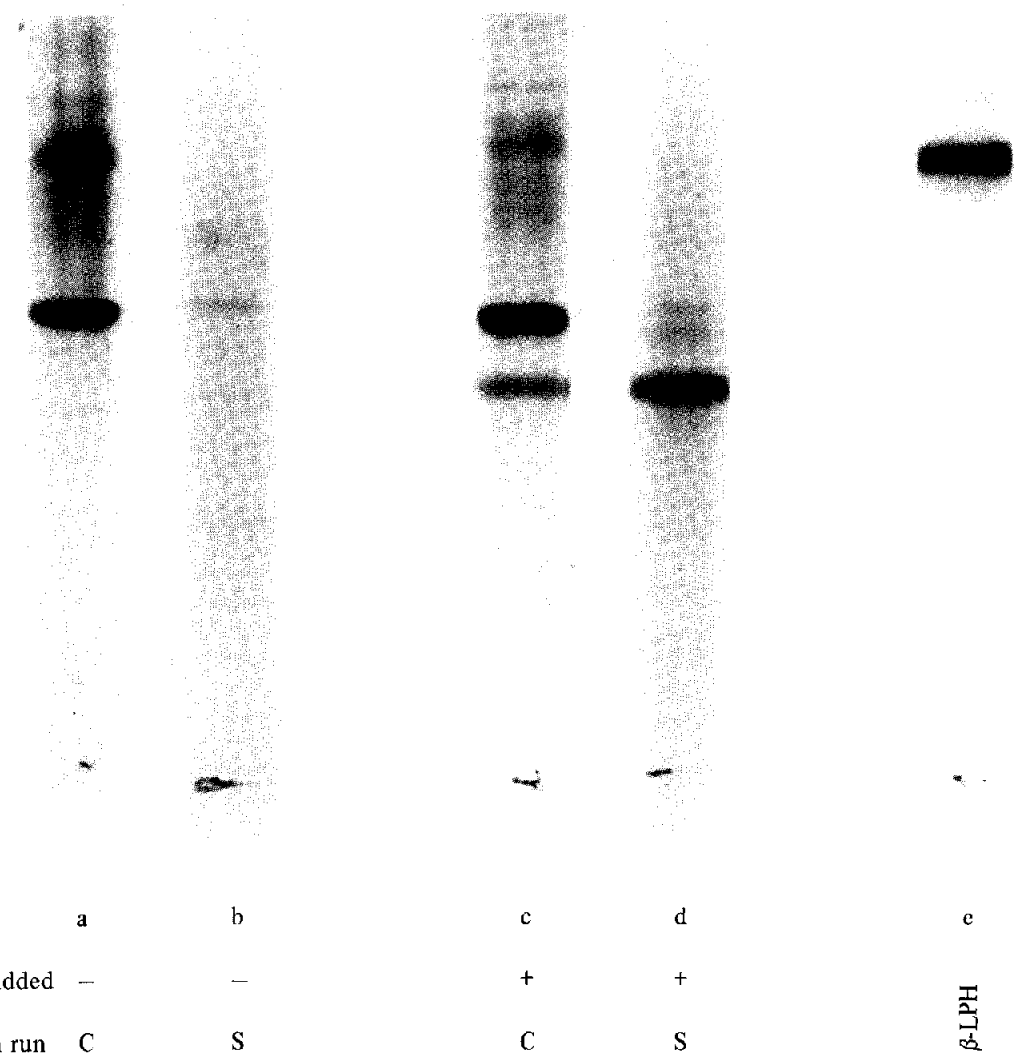

Fig.1. Incubation of porcine β-LPH with a homogenate of porcine pituitary glands. Incubated homogenate not containing exogenous β-LPH (a,b) and β-LPH alone (e) were run as controls. The complete (C) samples (a,c) and the supernatant fractions (S) obtained in 50% acetone (b,d) were subjected to electrophoresis. Samples contained 100 μg of β-LPH.

phoretic components of the pituitary homogenate were almost completely precipitated (fig.1b). On the incubation of β-LPH with the homogenate (fig.1c,d), a new electrophoretic component appeared. Since the incubation of the homogenate alone did not result in the formation of any electrophoretically detectable peptides in the 50% acetone supernatant fraction (fig.1b), the new component seen in fig.1d originated from β-LPH.

DTT and EDTA appeared to activate the β-LPH converting enzyme(s), whereas *p*-chloromercuribenzoate blocked the conversion of β-LPH by the pituitary homogenate in the absence of DTT and EDTA. Soy bean trypsin inhibitor did not affect this process.

3.2. *Isolation and identification of the cleavage products of β-LPH*

30 mg of porcine β-LPH was incubated with a pituitary homogenate under the conditions described

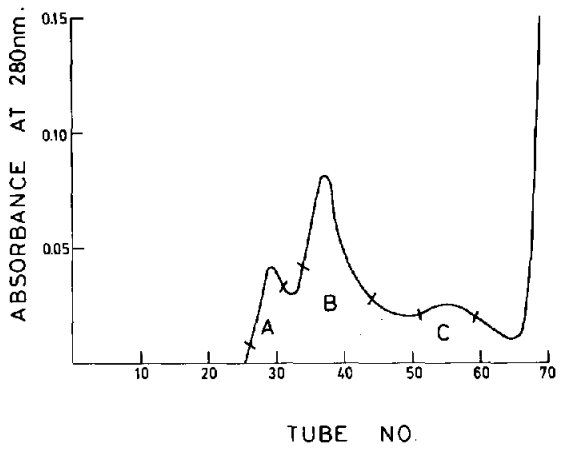

Fig.2. Chromatography of the 50% acetone supernatant fraction (41 mg) obtained from the incubation mixture of β-LPH and pituitary homogenate on a Bio-Gel P-6 column in 0.5 M acetic acid. Column size, 135 × 2 cm; tube volume, 5 ml; flow rate, 40 ml/h. The yields of fractions A, B and C were 6.6 mg, 17.2 mg and 7.5 mg, respectively.

Table 1
Amino acid composition[a] and terminal residues of components B and C isolated from fractions B and C of fig.2, respectively

Amino acid	B		C	
	exp.	1–77[b]	exp.	78–91[b]
Lys	6.1	6	3.9	4
His	1.1	1	1.0	1
Arg	3.7	4	–	–
Asp	3.1	3	2.0	2
Thr	3.2	3	–	–
Ser	2.9	3	0.2	–
Glu	12.8	13	1.1	1
Pro	8.6	9	–	–
Gly	7.3	7	1.2	1
Ala	12.5	13	2.0	2
Val	2.0	2	0.6	1
Met	1.6	2	–	–
Ile	–	–	0.6	1
Leu	5.0	5	–	–
Tyr	2.7	3	–	–
Phe	2.0	2	0.5	1
NH_2-terminal res.[c]	Glu		Phe, Lys	
COOH-terminal res.[d]	Leu (Thr, Val)		Gln (Gly, Lys)	

[a] Molar ratio
[b] From the amino acid sequence [2]
[c] Determined by the dansyl-method [17]
[d] Residues identified in the carboxypeptidase A digest by amino acid analysis.

in section 2.1. One volume acetone was added to the incubation mixture, and the supernatant fraction obtained by centrifugation was evaporated and lyophilized. This material (41 mg) was fractionated by chromatography on a Bio-Gel P-6 column (fig.2). Fraction A was shown to contain some larger proteins of the pituitary homogenate, whereas fraction B was composed of the newly formed electrophoretic component (see fig.1d). This fragment, designated as component B, was further purified by re-chromatography on the same column. From fraction C (fig.2) a peptide with an R_F value of 0.8 relative to lysine was isolated by high voltage paper electrophoresis at pH 6.5, and designated as component C.

The purified peptides were subjected to amino acid and terminal residue analyses, and the results are summarized in table 1. Comparison of the tryptic peptide maps of component B (fig.3a) and β-LPH (fig.4 in [18]) revealed that all the tryptic fragments of component B are present in the tryptic digest of β-LPH, except fragment n_x (fig.3a). By the amino

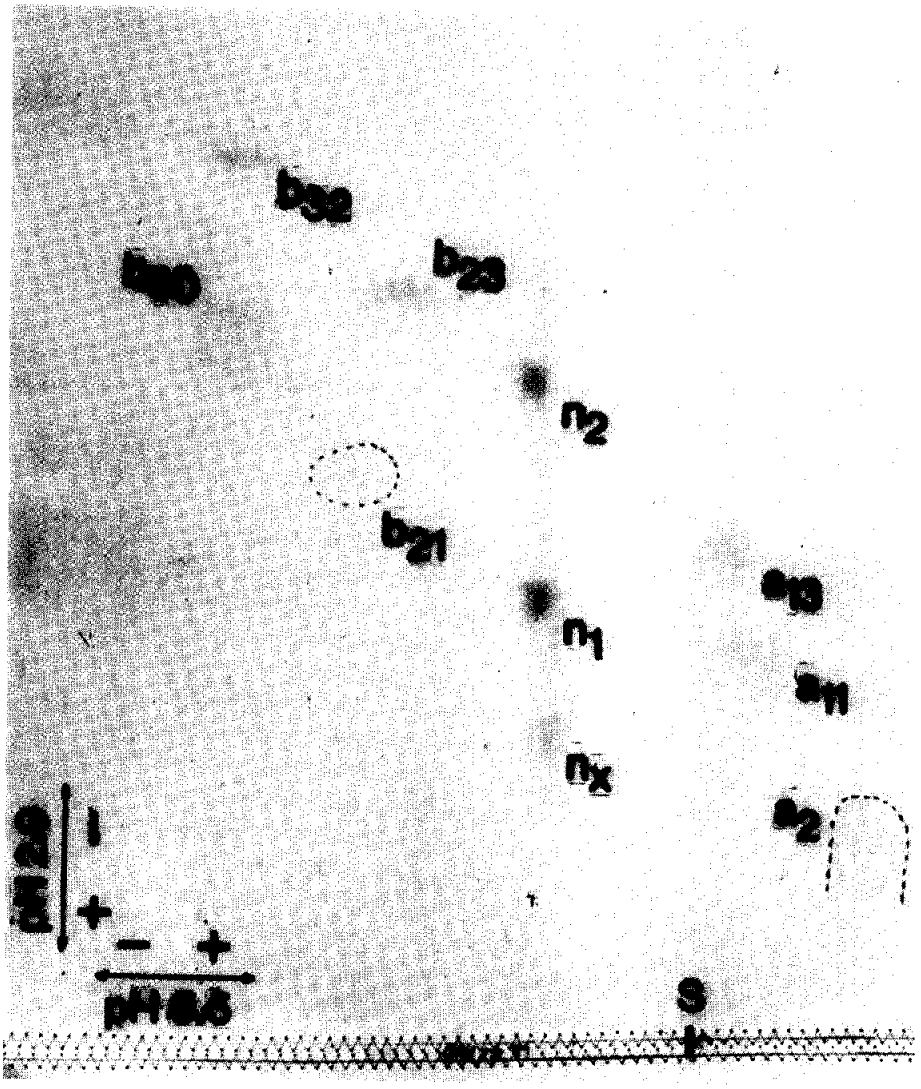

Fig.3a

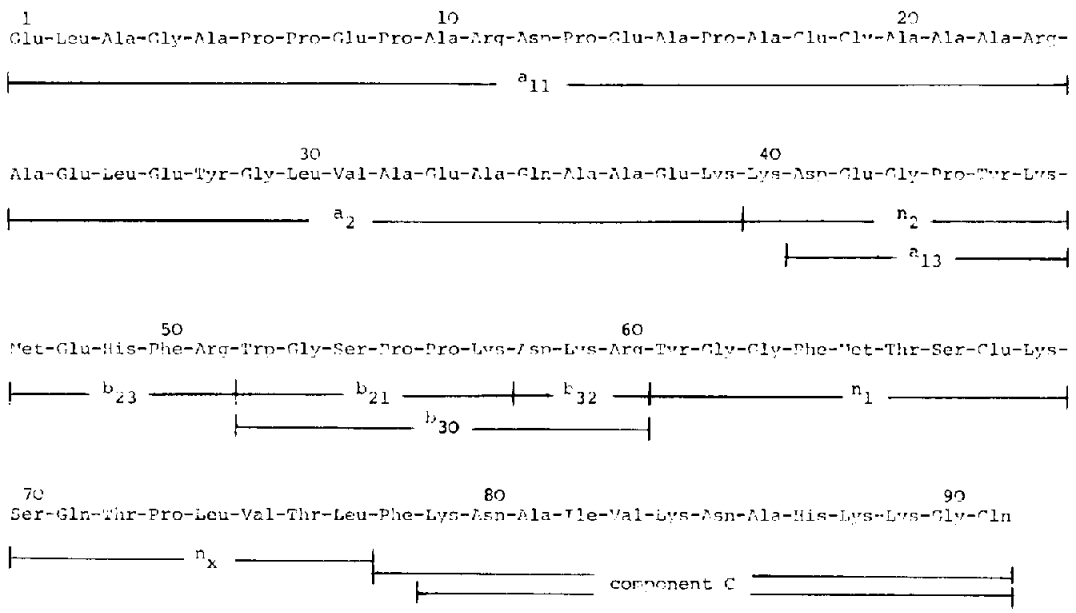

Fig.3. (a) Two-dimensional paper electrophoresis of a tryptic digest of component B recovered from fraction B of fig.2. (b) The amino acid sequence of porcine β-LPH [2]. The tryptic peptides of component B (fig.3a) were positioned by amino acid composition. The designation of these fragments is the same as in fig.4 of [18]. Component C isolated from fraction C of fig.2 is also marked in the figure.

acid content of these fragments (fig.3a) they could be located in the primary structure of β-LPH between residues 1–77 as shown in fig.3b**.

4. Discussion

Our results gave evidence that the action of a crude pituitary homogenate on porcine β-LPH at pH 6.5 is restricted to the cleavage of a single peptide bond, Leu–Phe at sequence positions 77–78, of the hormone, and to a partial removal of Phe-78 from the newly formed COOH-terminal lipotropin fragment (fig.3b).

The substrate specificity, SH-dependence and pH optimum of the β-LPH converting pituitary endopeptidase strongly suggest it to be a papain-like enzyme, presumably identical with or similar to lysosomal cathepsin B1 isolated from bovine spleen [19]. It is notable that the cathepsin B1 preparation of McDonald et al. [20] contained an SH-activated aminopeptidase, termed leucine naphthylamidase. Such an enzyme may be responsible for the cleavage of the NH_2-terminal Phe residue of component C (fig.3b).

The biological significance of the cleavage of β-LPH at Leu–Phe (positions 77–78) by this pituitary endopeptidase is not clear at this stage. It may be recalled however, that a neurohypophyseal-hypothalamic peptide with in vitro opiate agonist activity consists of residues 61–76 of the β-LPH structure [11]. One is tempted to speculate that one of the enzymes involved in the conversion of β-LPH to LPH-(61–76)-peptide is identical with the SH-dependent pituitary endopeptidase described in this paper≠. In view of

** In a preliminary experiment fragment n_x was overlooked in the tryptic peptide map of component B, and the component was erroneously identified as residues 1–69 of the β-LPH structure [9].

≠ Action of a carboxypeptidase, such as cathepsin B2 [21], on LPH-(61–77)-peptides might give rise to fragments with Thr-76 as COOH-terminal residue.

our recent observation that residues 80–91 are essential for the in vivo analgesic effect of LPH-(61–91)-peptide [22], the cathepsin B1-like endopeptidase may serve to inactivate the excess amount of LPH-(61–91)-peptide in the pituitary and/or in the brain.

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