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Action of cathepsin D on human β-lipotropin: A possible source of human "β-melanotropin"

(γ-lipotropin/β-endorphin/γ-endorphin)

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ABSTRACT **Highly purified calf brain cathepsin D (EC 3.4.23.5) selectively splits the Leu^{77}-Phe^{78} and Ala^{36}-Ala^{37} peptide bonds of human β-lipotropin. It is suggested that the formation of human "β-melanotropin" from γ-lipotropin, and that of γ-endorphin from β-endorphin, is due to the action of cathepsin D during isolation procedures.**

Since the discovery of β-lipotropin (β-LPH) and its isolation from ovine pituitaries (1), our knowledge of the chemistry of lipotropins has steadily grown. The complete amino acid sequences (91 amino acids) of ovine (2–4), porcine (5), human (6), and bovine (7) β-LPHs have been elucidated. With respect to the biological significance of β-LPH, it is now generally accepted that it serves as a biological precursor of both β-melanotropin (β-MSH) and β-endorphin, two peptides with different biological functions (for review see refs. 8 and 9). In fact, the conversion of endogenously labeled β-LPH into β-endorphin has been clearly demonstrated in both mouse pituitary tumor cell cultures (10, 11) and rat intermediate pituitary cell suspensions (11, 12). To our knowledge, however, no comparable biosynthetic evidence for the generation of β-MSH from β-LPH has yet been reported.

Scott and Lowry (13) initially proposed that the intermediate lobe of the pituitary might be the exclusive site of β-MSH biosynthesis. This suggestion was based on the failure of these authors to identify β-MSH in the human pituitary, which lacks a discrete intermediate lobe. Scott and Lowry (13) have suggested that human "β-MSH" originally isolated (14) and subsequently identified as a 22-residue peptide containing four more amino acids than certain other species homologs (8, 15), might have been artifactually formed from human β-LPH during the extraction procedure.

The present paper provides evidence for the proposition that lysosomal cathepsin D (EC 3.4.23.5) is one of the proteinases involved in the generation of human "β-MSH" from human β-LPH.

EXPERIMENTAL

Isolation of cathepsin D from bovine brain was carried out by a modification of the procedure of Benuck *et al.* (16). The brains were removed within 1 hr of killing and homogenized in 2 vol of 0.05 M sodium citrate, pH 2.7, in an Ultraturrax homogenizer (Janke–Kunkel KG) at 4°C for 40 s. The homogenate was centrifuged at 25,000 × *g* for 20 min at 4°C and the supernatant fraction was stored at −20°C for later purification.

Pepstatin-Sepharose was prepared as follows: 0.7 mg of pepstatin (The Protein Research Foundation, Osaka, Japan), 0.5 g of AH-Sepharose 4B (Pharmacia), and 16.5 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide meto-*p*-toluenesulfonate (Fluka, Buchs, Switzerland) were mildly agitated in 10 ml of dimethylformamide/water, 1:3 (vol/vol), pH 6, for 16 hr at room temperature. The product was filtered and washed with 50-ml portions of dimethylformamide/water, 1:1 (vol/vol); dimethylformamide/dioxane, 1:1 (vol/vol); 4 M urea; and 0.1 M sodium citrate, pH 3.9/1 M NaCl in this sequence. The pepstatin-Sepharose column (5 cm × 0.7 cm) was prepared and washed alternately with 0.1 M sodium bicarbonate, pH 7.6/1 M NaCl and 0.1 M sodium citrate, pH 3.9/1 M NaCl, equilibrated with the latter solution, and subsequently loaded with 25 ml of the brain extract previously dialyzed against the pH 3.9 solution for 10 hr. After the column was washed with the latter solution, cathepsin D was eluted with 0.1 M sodium bicarbonate, pH 7.6/1 M NaCl, and the pH of the enzyme solution was adjusted to about 4 by addition of 1 M sodium citrate, pH 3.9. Enzyme solutions were kept frozen for later use.

Cathepsin D was assayed on porcine β-LPH (β_p-LPH) as described (17). β_p-LPH and human β-LPHs (β_h-LPHs) were prepared according to Gráf and Cseh (18) and Cseh *et al.* (19, 20), respectively.

Digestion of β-LPH with purified cathepsin D was performed in 0.1 M ammonium formate, pH 4, at a ratio of 1 to 1000 (wt/wt) of enzyme to peptide at 37°C. Digestion was terminated by addition of 1 μM pepstatin to the incubation mixture.

The β_h-LPH fragments were separated by column chromatography on Sephadex G-50, by ion exchange chromatography on CM-cellulose (CM11; Whatman), and by preparative paper electrophoresis at pH 5.0 [pyridine/acetic acid/water, 10:10:1000 (vol/vol); 1500 V; 1.5 hr]. Fractions obtained from the columns or eluted from the paper electrophoretogram were lyophilized and examined for homogeneity by gel electrophoresis at pH 4.0 (21) and by amino-terminal residue analysis (22). Sequence analysis was performed by the dansyl-Edman procedure (23). Digestion with carboxypeptidase Y (Pierce) was carried out with an enzyme-to-substrate ratio of 1:100 (wt/wt) in 0.1 M pyridine acetate, pH 5.5, at 37°C for 2 hr. The amino

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Abbreviations: LPH, lipotropin; MSH, melanotropin; β_p-LPH, porcine β-LPH; β_h-LPH, human β-LPH.

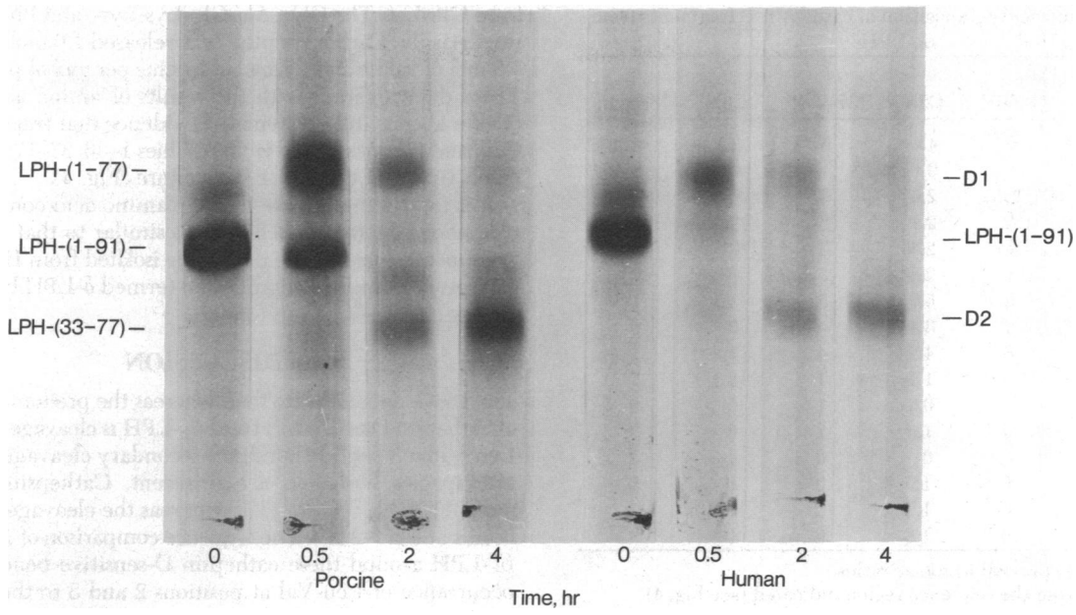

FIG. 1. Time-course of digestion of β_p-LPH and β_h-LPH by cathepsin D. Gel electrophoresis was carried out at pH 4.0; samples contained 100 μg of β-LPH.

acid composition of the acid (6 M HCl, 110°C, 24 hr) and carboxypeptidase Y hydrolysates were determined in a JEOL (JLC-5AH) automatic analyzer.

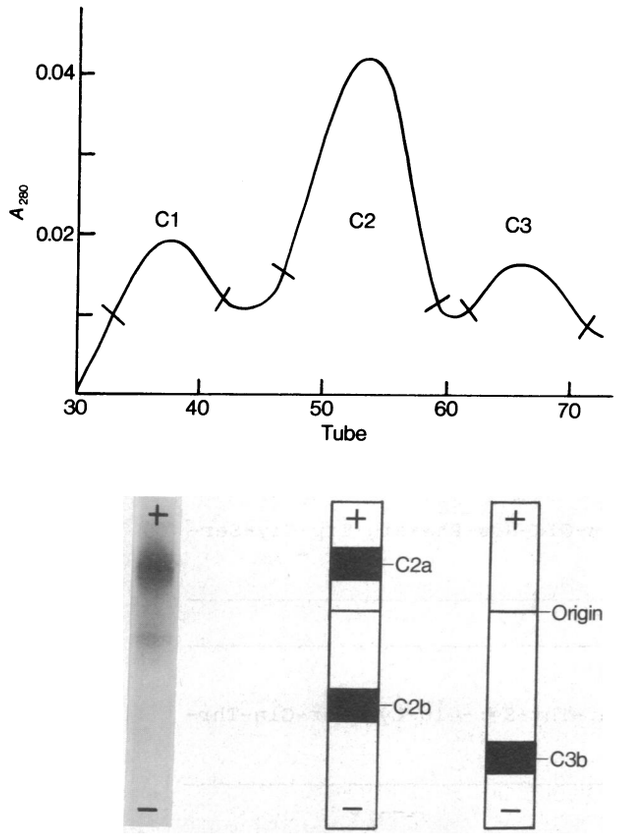

FIG. 2. Chromatography of a 4-hr cathepsin D digest of β_h-LPH (16.0 mg) on a Sephadex G-50 column (120 × 1.5 cm) in 0.05 M ammonium bicarbonate, pH 8.0. Flow rate, 30 ml/hr; tube volume, 2 ml. The yields of lyophilized fractions C1, C2, and C3 were 4.4 mg, 7.6 mg, and 2.0 mg, respectively. The gel electrophoretograms of fractions C1 and separations of fractions C2 and C3 by paper electrophoresis at pH 5.0 are illustrated.

RESULTS

Gel electrophoresis at pH 4.0 revealed a similar pattern of conversions of β_p-LPH and β_h-LPH by the action of highly purified cathepsin D (Fig. 1). The two electrophoretic components generated from β_p-LPH have been identified previously as fragments containing residues 1–77 and 33–77 (17). In order to prepare and characterize the cathepsin D fragments of β_h-LPH, a 4-hr digest of this polypeptide was subjected to gel filtration on Sephadex G-50, as shown in Fig. 2. Gel electrophoresis of fraction C1 showed that it was composed of component D1 and a relatively small amount of undigested β_h-LPH (see Fig. 1). The amino acid and amino-terminal residue analyses of this fraction (data not shown) indicated that component D1 represented the amino-terminal portion, residues 1–77, of the β_h-LPH structure.

Whereas fraction C3 was shown by paper electrophoresis to be homogeneous at pH 5.0, fraction C2 could be resolved into two peptides, C2a and C2b, by the same method (Fig. 2). These

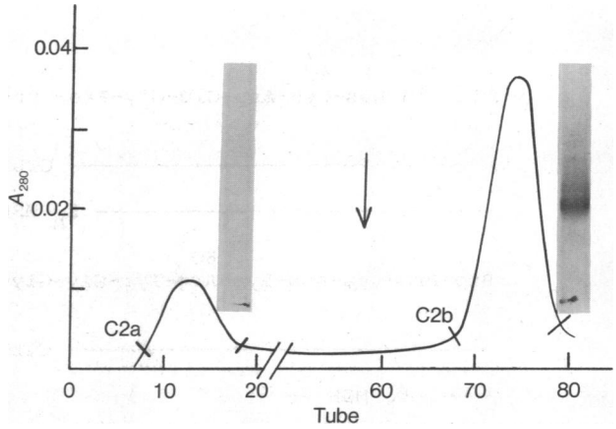

FIG. 3. Chromatography of fraction C2 (7.0 mg) on a CM-cellulose column (10 × 1.5 cm) equilibrated with 0.01 M ammonium acetate, pH 4.6 Flow rate, 30 ml/hr; tube volume, 2 ml. Elution was followed with 0.2 M ammonium acetate, pH 6.9, at tube 58. Fractions were lyophilized, and the yield of C2a and C2b was 1.9 mg and 3.6 mg, respectively. Gel electrophoretograms of the fractions are shown.

Table 1. Amino acid composition of cathepsin D fragments from β_h-LPH

Amino acid	C2a	(1–36)*	C2b	(37–77)*	C3b	(78–91)*
Lys	0	0	4.4	5	3.7	4
His	0.8	1	0.8	1	0	0
Arg	1.7	2	2.6	3	0	0
Asp	6.0	6	2.1	2	2.0	2
Thr	0.8	1	2.8	3	0	0
Ser	0.9	1	2.8	3	0	0
Glu	5.1	5	5.0	5	1.1	1
Pro	1.6	2	3.4	4	0	0
Gly	6.8	6	4.2	4	1.2	1
Ala	3.7	5	1.1	1	2.1	2
Val	0.7	1	0.7	1	0	0
Met	0	0	1.5	2	0	0
Ile	0	0	0	0	1.3	2
Leu	4.9	6	1.9	2	0	0
Tyr	0	0	1.9	2	0.9	1
Phe	0	0	1.8	2	0.9	1

Results are expressed as molar ratios.
* Calculated from the sequence region indicated (see Fig. 4).

peptides were prepared by CM-cellulose column chromatography, as illustrated in Fig. 3. As revealed by gel electrophoresis, C2b was identical to component D2 in the electrophoretic pattern of the unseparated cathepsin D digest of β_h-LPH (Fig. 1), whereas peptide C2a did not yield a visible band in the gel.

The amino-terminal sequences of fragments C2a, C2b, and C3b were determined by the dansyl-Edman method, and found to be Glu-Leu-Thr-Gly-, Ala-Glu-Lys-Lys-, and Phe-Lys-Asx-, respectively. Carboxypeptidase Y released 1.0 mol of alanine, 1.0 mol of valine and 1.2 mol of leucine per mol of peptide C2a. These data together with the results of amino acid analysis (Table 1) constitute convincing evidence that fragments C2a, C2b, and C3b originated from residues 1–36, 37–77, and 78–91, respectively, of the β_h-LPH structure (Fig. 4).

It is interesting to note that the amino acid composition of fragment C2b (residues 37–77) is similar to that of an MSH immunoreactive peptide previously isolated from HCl/acetone extracts of human pituitaries and termed δ-LPH by Scott and Lowry (13).

DISCUSSION

The above data indicate that, whereas the predominant action of cathepsin D on β_p-LPH and β_h-LPH is cleavage of the same Leu^{77}-Phe^{78} peptide bond, the secondary cleavage sites of the two species homologs are different. Cathepsin D attacks β_p-LPH at Ala^{32}-Glu^{33} (17), whereas the cleavage of β_h-LPH occurs at Ala^{36}-Ala^{37}. The sequence comparison of β_p-LPH and β_h-LPH around these cathepsin D-sensitive bonds show the occurrence of Leu-Val at positions 2 and 3 to the left of the bonds cleaved in each sequence, suggesting that the enzyme has a preference for these hydrophobic residues rather than for those forming the bond split itself.

Because Ala^{37} is the amino-terminal residue of a MSH-like peptide isolated from human pituitary extracts (14, 15) and long accepted as being β_h-MSH [LPH-(37–58)], it is quite probable that this peptide might have been artifactually formed from human γ-LPH [LPH-(1–58)], as is γ-endorphin from β-endorphin (17), by cathepsin D action during the extraction procedure. In this context it may be recalled that the first ex-

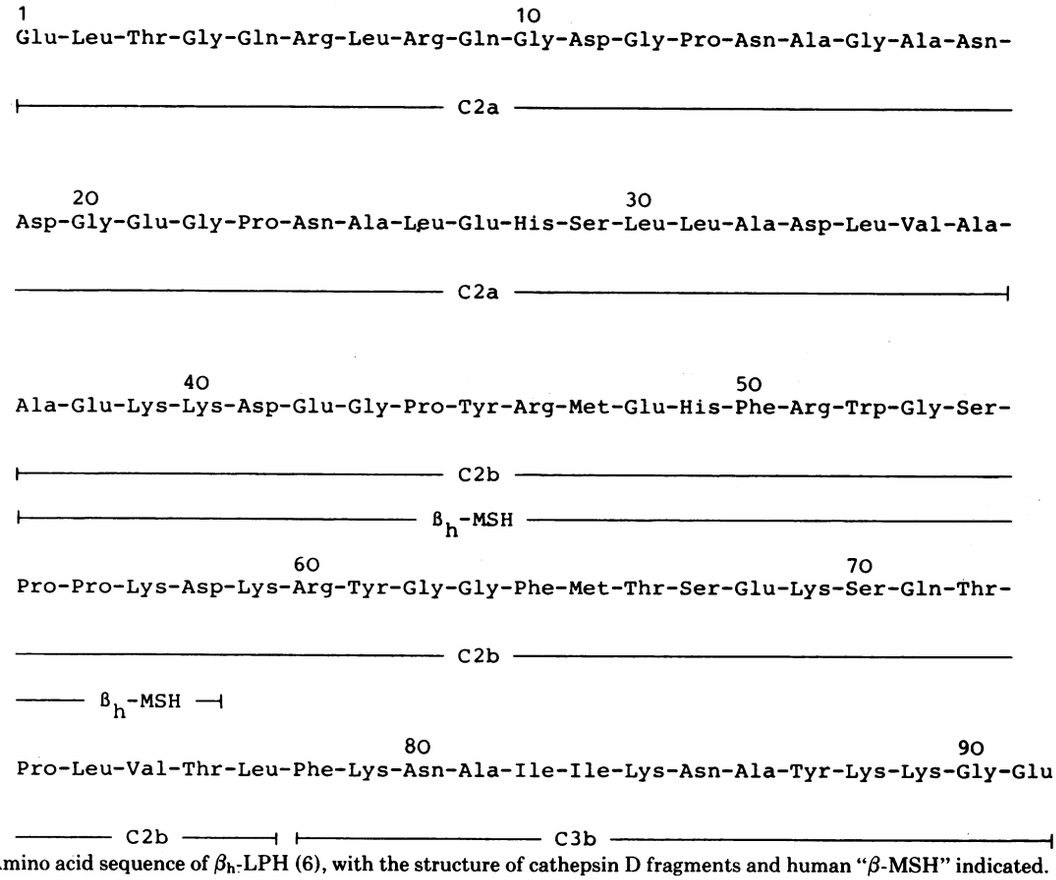

FIG. 4. Amino acid sequence of β_h-LPH (6), with the structure of cathepsin D fragments and human "β-MSH" indicated.

traction step of the human "β-MSH" isolation procedure was performed at pH 5.5 (14), a pH close to the pH optimum of cathepsin D (17, 24).

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