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High molecular weight aspartic endopeptidase generates a coronaro-constrictory peptide from the β-chain of hemoglobin

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Studying the influence of brain cathepsin D (EC 3.4.23.5) and high molecular weight (HMW) aspartic endopeptidase (EC 3.4.23.-) on the processing of hypothalamic calmodulin-binding coronaro-constrictory peptide factors from the β-chain of globin it was found that only HMW aspartic endopeptidase generates the fragment 31–40 of the β-chain of bovine hemoglobin (Hb) by cleavage of the Leu^{30}-Leu^{31} and Phe^{40}-Phe^{41} bonds. Digestion of the β-chain of globin was performed at 37°C at an enzyme/substrate ratio of 1:80 at pH 3.5 using different times of incubation (from 4 h to 10 h). The resulting peptides were separated by reversed-phase high-performance liquid chromatography (HPLC) and then identified by amino acid analysis and Edman degradation. The differences in specificity and activity of these two brain aspartic proteinases could be explained by their different structural features. Our finding provides evidence for a different biological function of these two enzymes. Data obtained give us reason to suppose that HMW aspartic proteinase probably can participate in the processing of the coronaro-constrictory peptide in vivo by limited proteolysis of Hb or Hb-like protein.

Hypothalamus; Coronaro-constrictory peptide; Hemorphin; Aspartic endopeptidase; HPLC; Amino acid sequence

1. INTRODUCTION

Recently it has been found that 5 calmodulin-binding coronaro-constrictory peptide factors (PF_{1-5}) isolated from bovine hypothalamus represent the fragments 33–37, 33–38, 32–38, 32–39, 31–40 of the β-chain of Hb [1,2].

Earlier it has been established that these peptides play an important role in the regulation of some Ca^{2+}, calmodulin (CaM)-dependent enzymes activity, such as myosin light chain kinase (MLCK), 3′,5′ cAMP phosphodiesterase and calcineurin [3,4]. It was shown that PF_{1-5} enhanced the contraction in rabbit aortic strips in a K^+-depolarized mixture (approximately to 20–25%) and stimulated platelet aggregation induced by 5 μM ADP (approximately to 25–30%) too. Stimulation of these processes accounts for MLCK activation by the CaM-PF complex. The K_d value for the CaM-PF complex (2–10 nM) has been determined [5].

It is known that fragments 34–37 and 34–38 of the β-chain of Hb (so-called hemorphins) obtained by enzymatic treatment of the β-chain of Hb have opioid activity [6]. Two opioid peptides corresponding to the sequence 31–40 and 32–40 of the β-chain of bovine Hb were isolated from bovine Hb peptic hydrolysate [7]. Recently an opioid peptide called LVV-hemorphin-6, corresponding to the sequence at position 32–40 of the β-chain of human Hb was reported to inhibit the angiotensin converting enzyme activity (ACE) [8]. This peptide was originally isolated from human pituitary gland. These data support our finding since it is very likely, that LVV-hemorphin-6 corresponding to the sequence 32–40 of the β-chain of human Hb, which contains the coronaro-constrictory peptide sequences, can compete with angiotensin II (a strong vasoconstrictor agent) by inhibition of ACE activity.

The peptide designed LVV-hemorphin-7 identical to the sequence 32–41 of the β-chain of human Hb has been isolated from cerebrospinal fluid (CSF) of patients with cerebrovascular bleeding [9].

It can be noticed that all above mentioned biologically active peptides, whatever their source, originated from the same region of the β-chain of Hb (residue 32–41 of human and residue 31–40 of bovine Hb).

These data suggest that Hb or a Hb-like protein is a precursor of biologically active peptides which can be formed in the organism during physiological or pathophysiological processes as a result of limited proteolysis. Therefore the study of the processing of coronaro- constrictory peptides from the β-chain of Hb under the influence of two intracellular aspartic endopeptidases (cathepsin D and HMW aspartic proteinase) seemed to be advisible. We decided to use these enzymes since it is known that cathepsin D, the main intracellular aspar-

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tic proteinase, plays an important role in the breakdown of proteins in normal cells and also in the pathological processes of tissue breakdown [10]. Its action can lead to the formation of physiologically active peptides from their precursors by limited proteolysis [11,12].

HMW aspartic proteinase (M_r about 90 kDa) with properties similar to cathepsin D (substrate specificity, sensitivity to pepstatin, pH activity profile) has been classified by one group as a cathepsin E-like enzyme, by others as a dimer or precursor of cathepsin D and also as a cathepsin D-like enzyme [13–16].

It was shown that in contrast to cathepsin D HMW aspartic proteinase from bovine brain generates a cardioactive neurohormone 'C' from its protein precursor [17].

In this work, using HPLC, amino acid analysis and Edman degradation for the identification of the products of enzymatic hydrolysis, we have found that HMW aspartic proteinase (but not cathepsin D) generates the coronaro-constrictory peptide (or LVV-hemorphin-7) which corresponds to the sequence at position 31–40 of the β-chain of bovine Hb (residue 32–41 of the β-chain of human Hb).

2. MATERIALS AND METHODS

Cathepsin D and HMW aspartic proteinase were purified from bovine brain cortex to apparent homogeneity by a procedure involving ammonium sulfate precipitation (30–70%), gel-filtration on Sephadex G-100 and affinity chromatography on pepstatin-Agarose [17]. All procedures were carried out at 4°C. Endopeptidases activity during purification was assayed at pH 3.2 with pyridoxal-Hb as substrate [18]. A typical enzyme assay was performed in 0.6 ml of reaction mixture, containing 0.05 M citrate buffer, pH 3.2, 100 μg of pyridoxal-Hb and various amounts of cathepsin D or HMW aspartic proteinase. After incubation at 37°C for 1 h with stirring, the reaction was stopped by the addition of 0.15 ml of 30% trichloroacetic acid. After centrifugation the pH of the supernatant was adjusted to 5.5 with 1 M sodium citrate buffer, pH 7,1 and the fluorescence was measured at 410 nm (excitation at 330 nm) on a fluorescence spectrophotometer (F-2000, Hitachi, Ltd., Tokyo, Japan). As controls the substrate and the enzyme were incubated separately. One unit of enzyme activity is defined as the amount of enzyme releasing phosphopyridoxyl peptide with fluorescence equal to that of 1 nmol of pyridoxamine under the assay conditions.

Digestion of the β-chain of globin was performed at 37°C at an enzyme/substrate ratio of 1:80 (w/w) at 37°C, at pH 3.5 using different times of incubation (from 4 h to 10 h). For the assay, 0.1 ml of reaction mixture, containing 0.05 M citrate buffer, pH 3.5, 20 μg of the β-chain of globin and 0.25 μg enzyme was used. The reaction was stopped by the addition of 0.025 ml of 30% formic acid. The peptide mixture was separated by reversed-phase HPLC on LiChrospher RP-18 (4 mm × 125 mm, 5 μm) column with a 60 min linear gradient of 0–60% acetonitrile containing 0.1% trifluoroacetic acid; flow rate 1 ml/min, absorbance was measured at 206 nm.

Amino acid analysis was done by the pre-column *o*-phthaldialdehyde derivatization method [19]. Amino acid sequence analysis was performed on a 2020 gas-phase sequencer equipped with an on-line PTH Analyser System Gold (Both Beckman, Fullerton, CA) using the conditions recommended by the manufacturer.

Solid phase peptide synthesis was carried out on a synthesizer (Applied Biosystems Model 430 A) using the Fmoc procedure [20].

3. RESULTS AND DISCUSSION

The influence of brain cathepsin D and HMW-aspartic proteinase on the β-chain of bovine globin was studied at 37°C at pH 3.5 at an enzyme/substrate ratio of 1:80. Variation of the incubation time (from 4 h to 10 h) did not change the set of major peptides in the mixture as judged from respective chromatographic profiles. Figs. 1 and 2a demonstrate the HPLC profile of the β-chain of globin digestions by cathepsin D and HMW aspartic proteinase, respectively. As can be noticed from these figures the influence of cathepsin D and HMW aspartic proteinase on the β-chain of globin is different. This difference can be explained by the cleavage of susceptible bonds at different sites of the β-chain of globin.

The resulting peptides were separated by reversed-phase HPLC and characterized by amino acid analysis. Subsequently the fractions which had amino acid composition similar to fragment 31–40 of the β-chain of globin were analysed by N-terminal sequence analysis. Data obtained revealed that only HMW aspartic proteinase (but not cathepsin D) generates the fragment 31–40 of the β-chain of globin (Fig. 2a, fraction 4) by cleavage of the Leu^{30}–Leu^{31} and Phe^{40}–Phe^{41} bonds of the β-chain of bovine globin (Fig. 3). It should be noticed that the synthetic peptide (Fig. 2b), corresponding to the sequence at position 31–40 of the β-chain of bovine globin had the same retention time as fraction 4 (Fig. 2a) using the same HPLC systems as used during the purification of the product of enzymatic digestion.

It is known that in some cases cerebral cathepsin D and HMW aspartic proteinases show almost identical substrate specificity. It was shown that these two enzymes cleaved the Leu^{10}–Leu^{11} bond in the tetradecapeptide renin substrate and Phe^{7}–Phe^{8} in substance P [22–23]. However, in the case of hydrolysis of the β-

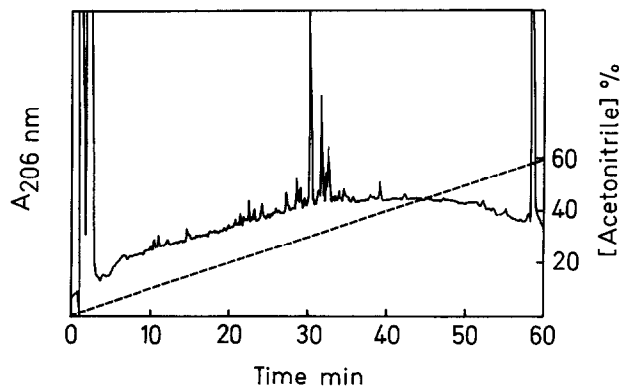

Fig. 1. HPLC profile of the β-chain of globin digestion by bovine brain cathepsin D. Separation was done on a LiChrospher RP-18 (4 mm × 125 mm, 5 μm) column with a 60 min linear gradient of 0–60% acetonitrile, containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The absorbance was measured at 206 nm.

chain of bovine globin these two aspartic endopeptidases have differences in their specificities. As mentioned above HMW aspartic proteinase in contrast to cathepsin D also generates the cardioactive neurohormone 'C' from its protein precursor [17]. These findings provide evidence for a different biological function of two brain aspartic endopeptidases. Brain HMW aspartic proteinase shares a number of properties with cathepsin D (sensitivity to pepstatin, substrate specificity, pH activity profile) and shows partial immunological identity; HMW aspartic proteinase has a specific activity 7–10 times lower than that of cathepsin D [24]. Until the present time it is not clear, whether the HMW aspartic proteinase is a dimer or a precursor of cathepsin D or an individual enzyme. The differences in specificity and activity of these two enzymes may be explained by their different structural features.

Thus, data obtained give us reason to suppose that Hb itself or a Hb-like protein is the precursor of the coronaro-constrictory peptide (or LVV-hemorphin-7) which can be formed in the organism during physiological or pathophysiological processes as a result of limited proteolysis of this protein by HMW aspartic proteinase. Furthermore it can be speculated that this peptide can be metabolized into shorter fragments (like PF_{1-4}, hemorphin-4, hemorphin-5 etc.) by involving the cascade system of different types of intracellular proteinases, such as metalloendopeptidase (enkephalinase or EC 3.4.24.11) [25], cathepsin H (endoaminopeptidase

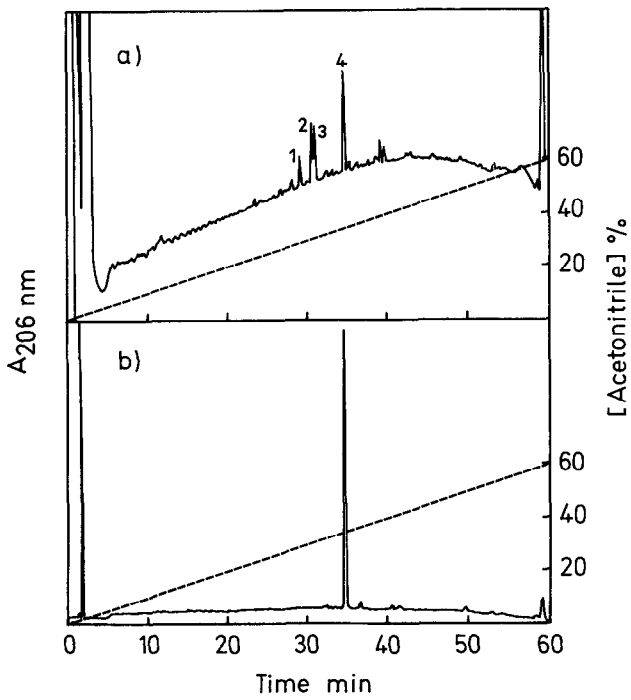

Fig. 2. HPLC profile of the digestion mixture of the β-chain of globin by bovine brain HMW aspartic proteinase (a) and of the synthetic peptide (Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) (same conditions as in Fig. 1).

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1   Met-Leu-Thr-Ala-Glu-Glu-Lys-Ala-Ala-Val-Thr-Ala-Phe-Trp-
15  Gly-Lys-Val-Lys-Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-
29  Arg-Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-Phe-Glu-
43  Ser-Phe-Gly-Asp-Leu-Ser-Thr-Ala-Asp-Ala-Val-Met-Asn-Asn-
57  Pro-Lys-Val-Lys-Ala-His-Gly-Lys-Lys-Val-Leu-Asp-Ser-Phe-
71  Ser-Asn-Gly-Met-Lys-His-Leu-Asp-Asp-Leu-Lys-Gly-Thr-Phe-
85  Ala-Ala-Leu-Ser-Glu-Leu-His-Cys-Asp-Lys-Leu-His-Val-Asp-
99  Pro-Glu-Asn-Phe-Lys-Leu-Leu-Gly-Asn-Val-Leu-Val-Val-Val-
113 Leu-Ala-Arg-Asn-Phe-Gly-Lys-Glu-Phe-Thr-Pro-Val-Leu-Gln-
127 Ala-Asp-Phe-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-
141 Ala-His-Arg-Tyr-His
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Fig. 3. Complete amino acid sequence of the β-chain of bovine hemoglobin [21]. The coronaro-constrictory peptide derived from the β-chain of globin under the influence of bovine brain HMW aspartic proteinase by the cleavage of the Leu^{30}–Leu^{31} and Phe^{40}–Phe^{41} bonds is boxed.

EC 3.4.22.-) [26] or cathepsin B (EC 3.4.22.1) taking into account its dipeptidyl carboxypeptidase properties [27].

Until present PF_{1-5}, LVV-hemorphin-6 and LVV-hemorphin-7 were isolated only from bovine and porcine hypothalamus [1,28], human pituitary glands [8] and CSF of patients with cerebrovascular bleedings [9]. Therefore we cannot exclude the possibility of the existence of a Hb-like protein in nervous tissue which can be a precursor of these peptides. In the same time since erythrocyte membranes contain the HMW aspartic proteinase [16], it seems more likely that in some physiological or pathological processes this enzyme can participate in processing of the above mentioned biologically active peptide from Hb. It is known that in certain areas of the brain (hypothalamus, pituitary etc.) endothelial cells do not form tight junctions and allow a free exchange of molecules between the blood and adjacent neurons [29]. Taking into consideration this fact, one can suppose that the decapeptide Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe derived from Hb by HMW aspartic proteinase can enter the hypothalamus and participate in the regulation of some metabolic processes of nervous tissue.

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