

LIMITED TRYPTIC DIGESTION OF BOVINE EYE LENS LEUCINE AMINOPEPTIDASE

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1. Introduction

Leucine aminopeptidase (EC 3.4.11.1.) is an exopeptidase catalyzing the hydrolysis of amino acid residues from the N-terminus of polypeptide chains, and has been obtained in crystalline form from bovine eye lens tissue [1]. Bovine lens leucine aminopeptidase (LAP) is an enzyme of mol. wt 326 000 consisting of 6 identical subunits of mol. wt 54 000 [2,3]. The crystalline enzyme contains 2 Zn^{2+} /subunit, one of which can be easily replaced by Mg^{2+} or Mn^{2+} , with a concomitant enhancement of activity [4]. The properties of bovine lens leucine aminopeptidase have been reviewed [5]. At present little information is available concerning the structure of LAP. Electron microscopy studies of LAP in solution revealed that the 6 subunits are arranged at the vertices of a distorted triangular prism [6], and a preliminary X-ray study showed that the enzyme crystallizes in the hexagonal space group $P6_322$ with unit cell dimensions $a = 132 \text{ \AA}$ and $c = 122 \text{ \AA}$ [7]. However, since studies of the structure of LAP as well as an elucidation of the exact mechanism of action are hampered by the fact that the primary structure of LAP is still unknown, we have undertaken a study of the amino acid sequence of this enzyme. In the course of this study we performed short proteolytic digestions with several enzymes. This should, theoretically, yield distinct fragments, because exposed parts of the molecule should be more susceptible to attack by proteolytic enzymes than buried regions.

Here we show that limited digestion of leucine aminopeptidase by trypsin results in a splitting of only one specific bond. It is demonstrated that despite the cleavage of this bond, the leucine amino-

peptidase aggregate remains intact and retains all of its catalytic properties.

2. Materials and methods

Leucine aminopeptidase was isolated from calf lens tissue by the method in [1]. The resulting crystals were dissolved in 0.1 M Tris-HCl buffer (pH 8.0) at $\sim 5 \text{ mg/ml}$. In some cases a further purification was performed by gel-filtration on a column ($120 \times 2.0 \text{ cm}$) of Ultrogel AcA 34 (LKB), eluted with 0.1 M Tris-HCl buffer (pH 8.0) at 18 ml/h flowrate.

Protein concentrations were determined by the standard method [8]. Limited digestion of native LAP by trypsin (Worthington TRTPCK) was performed at 37°C in 0.1 M Tris-HCl buffer (pH 8.0) at 5 mg protein/ml using 5% (w/w) of enzyme. Digestion was stopped after varying periods of time by the addition of soybean trypsin inhibitor (Sigma).

Limited digestion of native LAP by chymotrypsin (Worthington CBI), plasmin (Sigma) and thrombin (Sigma) was performed under the same conditions as for trypsin. Digestions were stopped by lyophilization.

SDS-polyacrylamide gel electrophoresis was performed according to [9] with significant modifications [10]. The samples were heated for 2 min at 100°C in sample buffer containing SDS and β -mercaptoethanol before application to the gel.

Activity was measured spectrometrically [11] in a Hitachi spectrophotometer using L-leucinamide (Serva) as substrate. Prior to activity measurement, the enzyme was activated for 2–3 h at 40°C in 22 mM Tris-HCl buffer (pH 8.5) containing 1 mM $MnCl_2$.

Enzyme in the activation mixture was at ~ 0.1 mg/ml. The incubation mixture contained 1.0 ml L-leucinamide (0.125 M, brought to pH 8.5), 0.1 ml 0.125 M MgCl_2 , 1.2 ml H_2O , 0.1 ml 0.5 M Tris-HCl buffer (pH 8.5) and 0.1 ml activated enzyme solution. Activity was measured at 238 nm at 25°C against a control consisting of 2.0 ml of L-leucine (0.0625 M, brought to pH 8.5), 0.1 ml 0.125 M MgCl_2 , 0.2 ml H_2O , 0.1 ml 0.5 M Tris-HCl buffer (pH 8.5) and the same quantity of activated enzyme solution. Activity was expressed as mmol L-leucinamide hydrolyze $\cdot \text{min}^{-1} \cdot \text{mg enzyme}^{-1}$.

Kinetic parameters were determined using a series of 6 substrate concentrations ranging from 20–100 mM. Analyses at each concentration were in triplicate. Plots of $1/V$ versus $1/S$ were linear over the range examined, and standard errors for K_m and V_{\max} determined by least square regression were within 5% of the values given in the results. The apparent Michaelis constant, K_m , is given in mM. $K_{\text{cat}} = V_{\max}/E$, where V_{\max} represents the maximal velocity in $\text{mmol} \cdot \text{min}^{-1} \cdot \text{mg enzyme}^{-1}$, and E represents the total amount (in mmol) of leucine aminopeptidase present in the assay.

Chromatography was performed on a column

(120 \times 2.0 cm) of Ultrogel AcA 34 (LKB), eluted with 0.1 M Tris-HCl buffer (pH 8.0) at 18 ml/h flow-rate. Sedimentation studies were performed at 20°C in a Beckman Spinco model E analytical ultracentrifuge at 64 000 rev./min, using Schlieren optics.

Purification of the fragments obtained by limited tryptic digestion of native LAP was achieved by chromatography on Sephadex G-100 fine. About 100 mg of lyophilized digestion mixture was dissolved in 10% acetic acid containing 6 M urea, brought onto the column (200 \times 3.5 cm) and eluted with the same buffer at 10 ml/h flowrate. The pooled fractions were desalted on a column of Sephadex G-25 coarse (30 \times 4.0 cm) in 10% acetic acid.

N-terminal amino acid residues were determined according to [12].

3. Results

Digestion products of native leucine aminopeptidase, incubated with trypsin for varying periods of time, were analyzed by SDS-gel electrophoresis (fig.1A). Under the conditions of gel electrophoresis, native

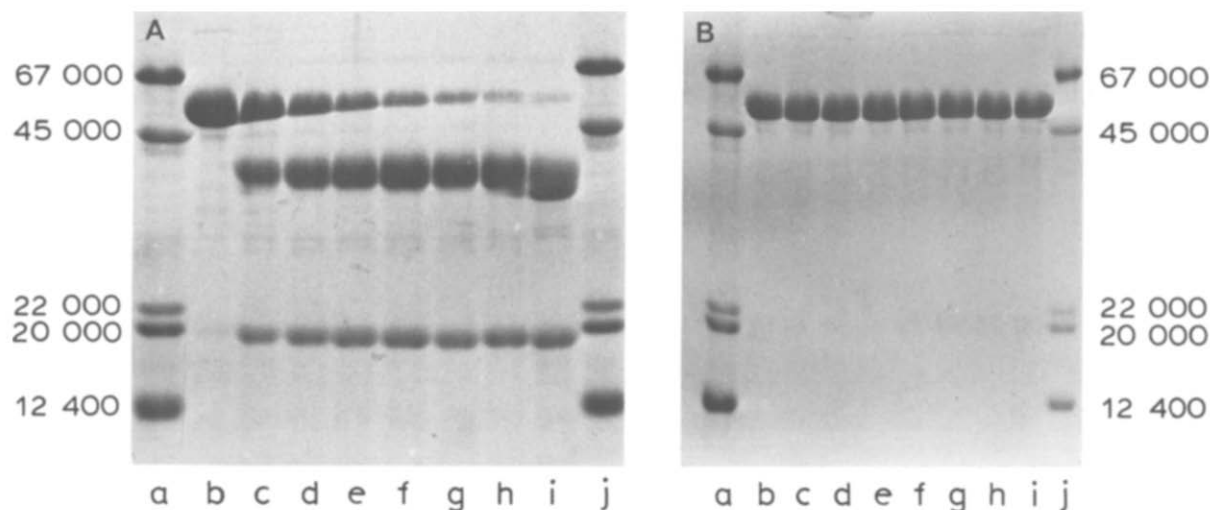


Fig.1. SDS-polyacrylamide gel electrophoresis of native LAP after limited digestion. Native LAP in Tris-HCl buffer was incubated with 0.5% (w/w) of trypsin (A) or chymotrypsin, plasmin and thrombin (B). Samples were taken from the digestion mixture before addition of enzyme (b), and after digestion times of 2 min (c), 5 min (d), 10 min (e), 20 min (f), 30 min (g), 60 min (h) and 180 min (i). (a) and (j) are marker proteins (mol. wt $\times 10^3$): bovine serum albumin (67); ovalbumin (45); calf α -crystallin B chain (app. 22); calf α -crystallin A chain (app. 20) and cytochrome c (12.4). Since the results for chymotrypsin plasmin and thrombin are essentially the same, only one pattern is shown.

LAP is dissociated into its subunits of mol. wt 54 000 (b). After < 2 min, two digestion products of apparent mol. wt 37 000 and 17 000 appeared, with a concomitant decrease of LAP-subunits (c). This continued until nearly all of the LAP-subunit disappeared after 3 h digestion (b-i). The same two digestion products were found using other enzyme/substrate ratios (0.05–1% (w/w) of trypsin). When native LAP was first activated by incubation with 1 mM MnCl_2 for 2 h at 40°C, or inactivated by incubation with 10 mM EDTA, and then incubated with trypsin for varying periods of time, essentially the same SDS-gel electrophoretic pattern as shown in fig.1A was obtained. Figure 1B shows the SDS-gel electrophoretic analysis of the digestion of native LAP by chymotrypsin, plasmin and thrombin. In all 3 cases no digestion products appeared, even after an incubation time of 3 h.

The samples from the digestion mixture of trypsin-treated LAP were also assayed for hydrolytic activity against L-leucinamide (fig.2). It appeared that even after 1 h digestion, when most of the LAP-subunit was split into two fragments, the hydrolytic activity remained virtually constant. When LAP, digested by trypsin for 0.5 or 3 h, was chromatographed on Ultrogel AcA 34, it eluted in a homogeneous peak at

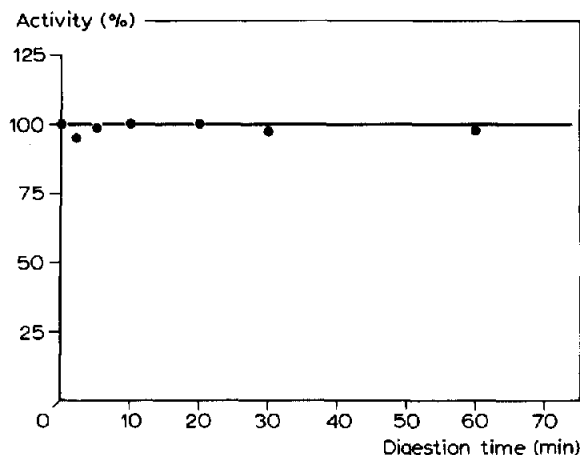


Fig.2. Assay of hydrolytic activity of native LAP against L-leucinamide after different digestion times by trypsin. Native LAP in Tris-HCl buffer was incubated with 0.5% (w/w) of trypsin. Samples were taken from the digestion mixture after varying periods of time and analyzed for hydrolytic activity. Values are expressed as percentages of the activity of native LAP before addition of trypsin.

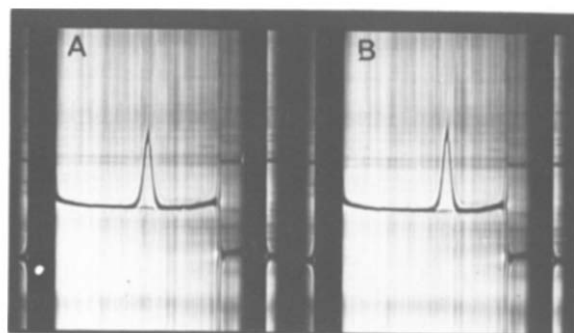


Fig.3. Sedimentation analysis of native LAP after limited tryptic digestion. (A) undigested, (B) digested with trypsin for 60 min.

exactly the same position as native LAP, reflecting a mol. wt ~300 000.

No change was observed in the sedimentation behavior of native LAP after incubation with trypsin, since both native LAP and LAP treated for 1 h with trypsin, revealed a sharp, homogeneous peak in the ultracentrifuge (fig.3). The calculated sedimentation constants were 10.85 ± 0.06 and 10.79 ± 0.05 S, respectively, in 0.1 M Tris-HCl buffer (pH 8.0).

Kinetic parameters for the hydrolysis of L-leucinamide by trypsin-digested LAP were determined by means of a Lineweaver-Burk plot, and were compared with those of native LAP (fig.4). In both cases the

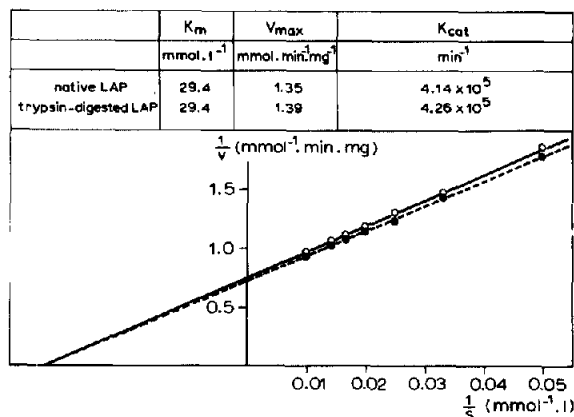


Fig.4. Lineweaver-Burk plot of the hydrolytic activity of native LAP and of trypsin-digested LAP. Native LAP (○—○) and LAP which had been treated with 0.5% trypsin for 60 min (●—●) were activated by 1 mM MnCl_2 and assayed for hydrolytic activity against different concentrations of L-leucinamide.

K_m -value was determined to be 29.4 mM. The V_{max} -values differed only slightly: 1.35 and 1.39 mmol/min . mg enzyme, for native and for trypsin-digested LAP, respectively. The corresponding K_{cat} -values were 4.14 and $4.26 \times 10^5 \text{ min}^{-1}$.

When the activation of leucine aminopeptidase by Mn^{2+} was investigated, it appeared that native LAP was optimally activated by 1 mM Mn^{2+} after ~2 h incubation. On the other hand, when LAP was digested by trypsin for 1 h, then activated by 1 mM Mn^{2+} , this activation was already optimal after < 0.5 h (fig.5).

The purification of the fragments obtained by tryptic digestion of native LAP was achieved by chromatography of a 2 h digestion mixture on Sephadex G-100 fine under dissociating conditions (fig.6). Peak III and IV yielded the mol. wt 37 000 and the mol. wt 17 000 fragments, respectively, in a pure form. Determination of the N-terminal amino acid residue yielded threonine for native LAP, lysine for the mol. wt 37 000 fragment and threonine for the mol. wt 17 000 fragment.

4. Discussion

Trypsin is able to split a very specific bond in

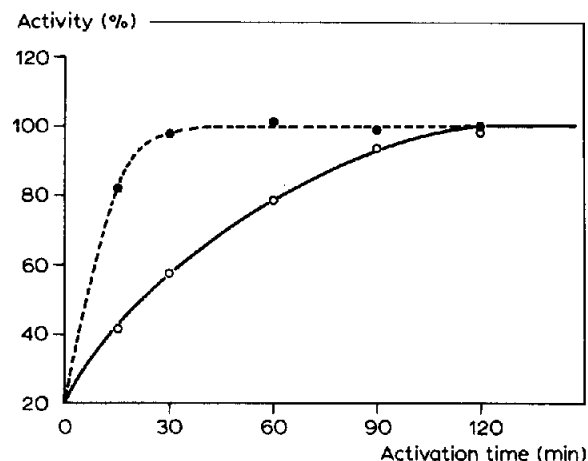


Fig.5. Activation of native LAP and of trypsin-digested LAP by Mn^{2+} . Native LAP (○—○) and LAP which had been treated with 0.5% trypsin for 60 min (●- -●) were incubated at 40°C in 22 mM Tris-HCl buffer (pH 8.5) containing 1 mM MnCl_2 . After varying periods of time samples of 100 μl were withdrawn and assayed for activity against L-leucinamide. Activities are expressed as % of the optimal value.

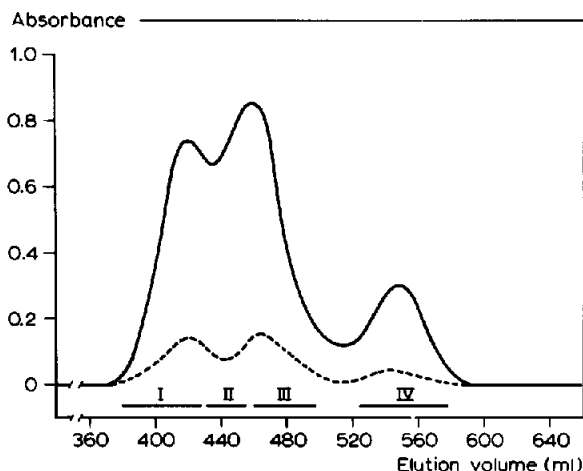


Fig.6. Gel-filtration of trypsin-digested LAP on Sephadex G-100. Native LAP (100 mg) in Tris-HCl buffer was incubated with trypsin (0.5%), w/w, for 60 min. The lyophilized digestion mixture was dissolved in 10% acetic acid containing 6 M urea, brought onto the column and eluted with the same buffer. Absorbance was measured at 280 nm (---) and at 206 nm (—).

native leucine aminopeptidase, as shown by the appearance under dissociating conditions of two fragments. These fragments are not further digested by trypsin. Other enzymes like chymotrypsin, plasmin and thrombin fail to produce a cleavage in native LAP. The splitting of the bond by trypsin is independent of the presence or the nature of the activating metal ion, since the same digestion pattern is obtained with native LAP, Mn^{2+} -activated LAP and inactivated LAP. The fact that the splitting by trypsin of this very susceptible bond is complete after 2 h, indicates that this bond is split in all 6 subunits. This means that this bond, in all 6 subunits, must necessarily be located at the surface of the molecule.

However, although trypsin actually produces a cleavage in the LAP-molecule, the catalytic properties of LAP are not changed. This may have two causes: either the LAP-aggregate remains intact, and therefore retains its activity, or, when the structure of LAP is changed or the aggregate falls apart, the catalytic activity is retained in one or both the fragments. The chromatographic and sedimentation behaviour of trypsin-digested LAP as compared to that of native LAP, clearly points to the first possibility. Also the

fact that prolonged dialysis could not remove the fragments from the aggregate, and that this could only be achieved by dissociation in 6 M urea, sustains the idea that digestion of native LAP by trypsin does not result in dissociation of the LAP-aggregate. To investigate whether the cleavage by trypsin of this specific bond in LAP has any effect on the active site, we determined the K_m and V_{max} values for native and for trypsin-digested LAP after activation by Mn^{2+} . The K_m -values are identical in both cases, indicating that the substrate binding is not influenced by the splitting of the bond. The V_{max} -values differ only slightly. However, the difference between the two values lies well within the standard error of 5% inherent in this method. We therefore can conclude that digestion of LAP by trypsin neither has an effect on substrate binding, nor does it affect the actual catalytic step.

It has already been stated that native leucine aminopeptidase possesses two nonequivalent metal-binding sites/subunit [16]. The Zn^{2+} in the 'structural site' can be exchanged only with extreme difficulty, indicating that it might be buried in the enzyme. The Zn^{2+} in the 'activation site' can easily be replaced, for instance by Mn^{2+} , and this site is probably located near the surface of the molecule. The fact, however, that the nature of the metal ion present in the activation site affects activity, indicates that it is located near the active site. Our results concerning the limited digestion of LAP by trypsin, support this hypothesis. Thus, the splitting of a bond at the surface of the molecule has no effect at all on the structural site that is believed to be buried in the molecule, but it does affect the activation by Mn^{2+} . A possible explanation is, that the splitting of this bond in some way facilitates the exchange of a Zn^{2+} in the activation site by a Mn^{2+} . However, the binding of the metal in the activation site itself is not influenced.

Determination of the N-terminal residues of the fragments indicates that the mol. wt 17 000 fragment represents the N-terminal part, and the mol. wt 37 000 fragment the C-terminal part of the LAP-subunit. From our sequence results (L. v. L.-K. et al., in preparation) we can conclude that it is an Arg-Lys bond that has

been split by trypsin. No S-S bridges are present between the two fragments, as can be concluded from the fact that both fragments can be separated in urea without prior reduction. Further investigation of the position of the tryptic cleavage point in the primary structure of leucine aminopeptidase is in progress.

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