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# Purification and Partial Characterization of Leucine Aminopeptidase from *Actinomyces viscosus*

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**Summary**

Leucine aminopeptidase was purified from cell-free extracts of *Actinomyces viscosus* ATCC 19246 and some properties were investigated. The enzyme had a molecular weight of 65,000 and its isoelectric point was 4. 0. Optimum pH was found at 7. 0. The enzyme was quite labile over 40℃. It was sensitive to inhibition by diisopropylfluorophosphate, phenylmethane sulfonylfluoride, or tosyl-L-lysine chloromethyl ketone. Inhibition by urea was also obvious and this inhibition was found to be irreversible. $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ or $Co^{2+}$ had no no effect on the activity. Leucine-*p*-nitroanilide was the most suitable substrate among the tested synthetic substrates.

## INTRODUCTION

Proteolytic enzyme of oral bacteria is important as a periodontpathic virulence factor (Laughon et al., 1982a, 1982b), but little information for the production and properties of this enzyme is available, except for *Bacteroides melaninogesicus* and *B. gingivalis*. For these species, several report on isolation, characterization, and pathogesic importance of protease has been discussed (Fujimura and Nakamura, 1981 ; Carlsson et al., 1984 ; Sundqvist et al., 1984 ; Yoshimura et al., 1984).

*A. viscosus* has been implicated as the causative agent of gingivitis (Jordan and Hammond, 1972 ; Loesche and Syed, 1978) and periodontitis (Williams et al., 1976 ; Jordan et al., 1972). As pathogesic factors, possible functions of extracellular polysaccharide (Rosan and Hammond, 1974) and chemotactic factors for polymorphonuclear leukocytes (PMNs) (Taichman et al., 1966 ; Engel et al., 1976) produced by this organism have been noticed. But enzymatic aspects of a pathogenic factor of *A. viscosus* were not frequently considered. We decided, therefore, to survey enzymes which may respond directly to tissue damage in this species. Although unsuccesful in finding such enzymes in this study, we detected leucine aminopeptidase (LAP) activity, which is a protease-related enzyme. In this report we describe some properties of this enzyme and its purification.

## MATERIALS AND METHODS

*Bacterial strain and cultivation conditions*

*A. viscosus* ATCC 19246 was used for producer of LAP. The strain was inoculated into a

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medium consisting of 3.7% brain heart infusion (Difco Laboratories, Detroit, USA) and 0.2% yeast extract (Difco). Cultivation was carried out with shaking in atmosphere at 37℃ for two days. After growth, the cells were collected by centrifugation at 10,000 x*g* for 15 min.

*Assay of enzyme activity*

LAP activity was assayed colorimetrically using a chromogenic substrate, leucin-*p*-nitroanilide (Leucine-PNA) according to Appel (1974). Reaction mixture consisted of 0.8 ml of 1 mM leucine-PNA (first dissolved as 100 mM solution in dimethylformamide) in 0.05 M Tris-maleate buffer (pH 7.0), 0.1 ml of enzyme preparation, and 0.1 ml of 0.05 M Tris-maleate buffer (pH 7.0). After incubation of the reaction mixture at 37℃ for 30 min, reaction was stopped by the addition of 0.2 ml of 5 N acetic acid and then liberated *p*-nitroaniline was assayed by absorbance at 410 nm. One unit of activity was defined as the amount of enzyme which liberated 1 $\mu$mol *p*-nitroaniline per min under these conditions.

Hydrolyzing activity of LAP toward other synthetic substrates was examined by the same methods.

Proteolytic activity against casein, bovine serum albumin, hemoglobin, gelatin, and fibrinogen was examined using a method described by us (Fujimura and Nakamura, 1981).

*Determination of optimum pH*

Determination of optimum pH for the enzyme activity was carried out according to our previous report (Fujimura et al., 1983). Reaction was performed at various pH values using different buffers; sodium acetate buffer (pH 5.0 to 5.5), potassium phosphate buffer (pH 6.0 to 7.0), Tris-maleate buffer (pH 7.5 to 8.5), and carbonate-bicarbonate buffer (pH 9.0 to 11.0). Each buffer was used at a final concentration of 0.1 M.

*Analytical methods*

To test the purity of samples, sodium dodecyl sulfate polyacrylamide disc gel electrophoresis was carried out using 7.5% gel (Weber and Osborn, 1969).

The molecular weight of LAP was estimated by gel filtration method (Andrews, 1964) using a Sephadex G-200 column (2.6 by 100 cm). Standard proteins were aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome C.

Determination of pI value was done with isoelectric focusing according to the method of Vesterberg et al.(1967). The purified LAP was dialyzed against 1% glycine solution overnight before application to an isoelectric focusing column. Ampholite was used at a concentration of 1% (v/v). pH range was 3 to 6. Focusing was done at 600 V for 48 h. The column was maintained at about 4℃ with a circulating bath.

## RESULTS

*Purification*

Cells harvested from a 5 liter culture were washed twice with 0.05 M Tris-hydrochloride buffer (pH 8.0) and the cells were disrupted by ultrasonication at 9 KHz for 20 min. The sonicate was clarified by centrifugation at 100,000 x*g* for 30 min. Ammonium sulfate was added to the crude extracts to give 30% saturation and the resulting precipitate was removed by centrifugation at 10,000x*g* for 20 min. The concentration of ammonium sulfate of the supernatant solution was raised to 70% saturation and stirred for 1 h. The precipitate was collected by centrifugation and dissolved in 0.05 M Tris-hydrochloride buffer (pH 8.0) and dialyzed against this buffer overnight and applied to a column (2.6×40 cm) of DEAE cellulose, previously equilibrated with 0.05 M Tris-hydrochloride

buffer (pH 8.0). After washing of this column with the same buffer until absorbance of the eluates at 280 nm became less than 0.1, the proteins were eluted with a linear gradient of NaCl concentration, produced by mixing 700 ml of the buffer and the same volume of the buffer containing 0.6 M NaCl. Flow rate was 40 ml per hour. LAP activity emerged at eluate containing about 0.3 M NaCl. The active fractions were collected and dialyzed against 0.05 M Tris-hydrochloride buffer (pH 8.0) containing 0.15 M NaCl. The dialyzed material was chromatographed repeatedly on a column (1.5×30 cm) of DEAE cellulose, equilibrated using dialysis buffered saline with a linear gradient concentration of NaCl from 0.15 M to 0.5 M (300 ml each side). The fractien containing LAP activity was pooled and concentrated with a rotary evaporator at 30℃ *in vacuo*, followed by dialysis against 0.05 M Tris-hydrochloride buffer (pH 7.2) containing 0.15 M NaCl. This sample was further purified by gel fitration on a Sephadex G-100 column (2.6×100 cm) at a flow rate of 25 ml per hour. The gel filtration of the active fraction from the column was repeated once more using this Sephadex G-100 column. In the second gel filtration, two UV-absorbing peaks emerged and the elution position of the LAP activity rightly coincided to a later protein peak. LAP was purified by these procedures about 150 fold by comparing specific activity, and thd recovery rate was 8%.

When purity of the active fraction obtained from the second Sephadex G-100 chromatography was examined on disc electrophoresis, a single stained protein band was observed (Fig. 1).

*Molecular weight*

The molecular weight was calculated to be 65,000 from Sephadex G-200 gel filtration.

*pI value*

The purified enzyme preparation was electrophoresed on isoelectric focusing, and activity was focused at a pH value of 4.0.

*Optimum pH*

The effect of pH on LAP activity was examined in a series of buffers for different pH value. As shown in Fig. 2, the optimum pH for LAP is pH 7.0. No significant activity was observed pH values below pH 5.0 or over pH 9.5.

*Thermostability*

When the enzyme was incubated at various temperatures for 5 min at pH 7.0, the activity decreased rapidly over 40℃ (Fig. 3). The same inactivation pattern was obtained in the presence of $Ca^{2+}$ or $Mg^{2+}$ at concentrations from 1 mM to 10 mM. No significant loss of activity was observed on storage at −40℃ for one month.

*Effect of inhibitors on the activity*

Effect of inhibitors on LAP activity is presented in Table 1. The results indicate that the enzyme is sensitive to diisopropylfluorophosphate (DFP) or phenylmethane sulfonylfluoride (PMSF). These findings suggest that LAP is a serine enzyme. The activity was inhibited significantly by tosyl-L-lysine chloromethyl ketone (TLCK), an inhibitor of trypsin, however, soybean trypsin inhibitor gave no effect. Chymotrypsin inhibitor such as tosyl-L-phenylalanine chloromethyl ketone (TPCK) and chymostatin also had no substantial effect. Urea strongly inhibited the activity and the activity did not reappear by removal of denaturant by dialysis. Therefore, the inhibition by urea may attributed to an irreversible change of enzyme molecules. A similar effect was found with guanidine-HCl.

*Effect of divalent ions*

The enzyme activity was not enhanced with $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, or $Co^{2+}$ at concentrations of

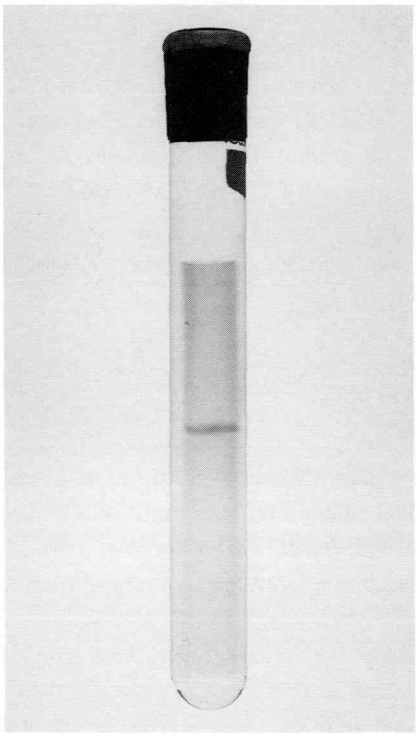

Fig. 1. Polyacrylamide disc gel electrophoresis of the purified LAP in the presence of sodium dodecylsulfate. Migration is from top to bottom. Gel was stained with Coomassie Brilliant Blue.

Table 1. Effect of various inhibitors on the activity of LAP

| Inhibitor | Concentration | Residual activity (%) |
|---|---|---|
| none | - | 100 |
| DFP | 10 mM | 7 |
| DFP | 1 mM | 56 |
| PMSF | 5 mM | 1 |
| TLCK | 5 mM | 29 |
| TPCK | 5 mM | 81 |
| soybean trypsin inhibitor | 1 mg/ml | 100 |
| chymostatin | 0.1 mM | 87 |
| mercaptoethanol | 10 mM | 100 |
| dithiothreitol | 10 mM | 100 |
| EDTA | 10 mM | 100 |
| urea | 2 M | 2 |
| urea | 1 M | 10 |
| urea | 0.5 M | 44 |

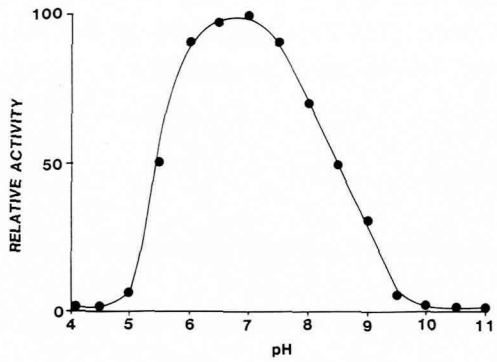


Fig. 2. Effect of pH on the activity of LAP.

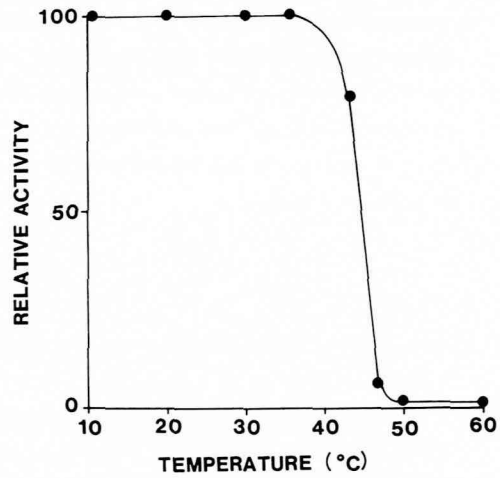


Fig.3. Stability of LAP at different temperature.

1 mM, 5 mM, and 10 mM. $Zn^{2+}$ at 0.1 mM inhibited 98% of the activity (Table 1).

*Substrate specificity*

Results of the hydrolyzing activity of LAP against various synthetic substrates are as follows: Hydrolysis rate by LAP of leucine-PNA is supposed to be 100%, that of proline-PNA was 15%. However, no detectable hydrolysis was demonstrated with arginine-PNA, glycine-PNA, cystine-PNA, lysine-PNA, *N*-acetyl-alanine-PNA, benzoyl-tyrosine-PNA, benzoyl-arginine-PNA, and glutaryl-phenylalanine-PNA.

Proteolytic activity against casein, bovine serum albumin, hemoglobin, gelatin, and fibrinogen was not detected,

## DISCUSSION

LAP of *A. viscosus* was purified from cell extracts by the sequential procedures of fractionation with ammonium sulfate precipitation, ionexchange chromatography, and gel filtration. Molecular weight (65,000) seems to be higher than other microbial aminopeptidase from *Bacillus subtilis* (Wagner et al., 1979), *Aeromonas proteolytica* (Prescott et al., 1970), *Bacillus licheniformis* (Rodriguez-Absi and Prescott, 1978), and *Streptomyces griseus* (Vosbeck et al., 1973). These ranged from about 30,000 to 47,000.

The enzyme was found to be sensitive to DEP and PMSF, indicating serine residue is important to the activity.

It was demostrated that activity of microbial aminopeptidase was enhanced by $Co^{2+}$ (Rodriguez-Absi and Prescott, 1978; Wagner et al., 1979; Trumbly and Bradley. 1973), but our results revealed that *A. viscosus* LAP was not stimulated by $Co^{2+}$, as $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$.

As illustrated in Fig. 3, the inactivation curve obtained by heating for 5 min at various tempratures demostrates that the enzyme stability decreased, rapidly over 40℃. Other bacterial aminopeptidase is reported to be more stable; for example, *Bacillus licheniformis* aminopeptidase was quite stable even when it was heated at 50℃ for 150 min (Rodriguez-Absi and Prescott, 1978).

Substrate specificity of LAP was rather strict among the synthetic substrates examined: only proline-PNA was hydrolyzed weakly other than leucine-PNA.

Production of LAP was confirmed not only in strain ATCC 19246, but also in all four stock strains of *A. viscosus* in our laboratory (data not show). This indicates that production of LAP may not be unique to *A. viscosus*.

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