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The Occurrence of Amino Acid Naphthylamidase Distinct from Leucine Aminopeptidase in *Serratia indica*

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An enzyme which hydrolyzes amino acid naphthylamides, an arylamidase, has been found in cell extracts of *Serratia indica*. The cells also contain a leucine aminopeptidase. The latter enzyme is distinct from the arylamidase, which is without activity against L-leucine amide. A separate esterase hydrolyzing β-naphthylacetate has also been demonstrated in the extract.

The arylamidase activity is highest during the late exponential phase of cultures grown in complex peptone media, but the enzyme is also produced by cells grown in a synthetic salt medium containing glycerol.

The optimal pH for the hydrolysis of L-leucyl-β-naphthylamide is between 6 and 7. The enzyme requires divalent metal ions and is not very stable. Attempts to purify the enzyme have been hindered by activity losses.

The inadequacy of chromogenic naphthylamides as substrates for the assay of peptidase activity of bacteria is discussed.

Enzymes capable of hydrolyzing simple peptides are widely distributed in nature. Enzymes of the leucine aminopeptidase type (EC No. 3.4.1.1) have been found in a variety of animal tissues and fluids, plants, and microorganisms.[1] As part of a research program concerned with the formation and properties of various proteolytic enzymes in bacteria we have studied the peptidase activity of bacteria belonging to the genus *Serratia*. Most strains of the genus are strongly pigmented, and most of them produce an extracellular proteinase in addition to cell bound aminopeptidases when grown in media containing proteins or peptides.[2]

All leucine aminopeptidases hydrolyze L-leucinamide, which can be used to assay the enzyme according to the titrimetric method originally described by Grassman and Heyde.[3] About ten years ago, spectrophotometric assay methods involving chromogenic substrates (*e.g.* L-leucyl-β-naphthylamide) were introduced.[4–7] Because they are simple and sensitive, the spectrophotometric assays are used routinely today. Since purified leucine aminopeptidase is known to hydrolyze amino acid naphthylamides, these substrates are often

used to assay the enzyme in crude cell extracts. However, in their review on leucine aminopeptidase, Smith and Hill [1] point out that enzymes other than leucine aminopeptidase might also hydrolyze these chromogenic substrates.

The present report shows that bacteria of the genus *Serratia* contain an enzyme which hydrolyzes L-leucyl-β-naphthylamide (LNA), but which exhibits no activity toward L-leucinamide. However, the bacteria also contain a "true" leucine aminopeptidase that hydrolyzes L-leucinamide and a number of simple peptides. This latter enzyme can be separated from the former. The cells also produce an enzyme that hydrolyzes β-naphthylacetate.

R−CO−NH_2	R−CO−NH−naphthyl	CH_3−CO−O−naphthyl
Leucinamide	Leucyl-β-naphthylamide	β-Naphthylacetate

R−COOH = leucine. The arrows indicate the position of hydrolysis.

G. G. Glenner (see Ref. 11) has suggested the name amino acid naphthylamidases or, more generally, arylamidases, for enzymes that hydrolyze substrates of the type studied in this investigation. This nomenclature will be adopted here, since the natural substrates of these enzymes are still unknown.

MATERIAL AND METHODS

Bacterial cultures and cell disintegration. The taxonomy of the genus *Serratia* is currently under debate, and the suggestion has been made [8] that most strains which have been given species names are actually variants of the type species *Serratia marcescens*. The studies described here were performed with a strain, designated *Serratia indica*, which was obtained from the Department of Bacteriology, Karolinska Institutet, Stockholm. The strain conforms closely with the description given in the seventh edition of Bergey's Manual.

Cultures were grown at 30° either in Fernbach flasks incubated on a shaking machine or in a 10-liter flask provided with vortex aeration. Growth was followed by measuring the absorbance of culture samples in an Eppendorf photometer at 546 mμ under conditions where the absorbance was directly proportional to the cell density.

The bacteria were harvested by centrifugation and were washed with 0.05−0.1 M Tris adjusted to pH 8.0 with HCl. The washed cells were resuspended in the same buffer. The cells were then disintegrated with a Raytheon 250 W, 10 kc Sonic Oscillator at 5−10°. Cells from one-liter cultures were usually suspended in 10−20 ml of buffer and sonicated for 15 min. Microscopic examinations showed that almost all the cells were disintegrated under these conditions. Cell debris was removed by centrifugation at 10 000 *g* for 20 min. The resulting clear solution will be referred to as "cell free sonicate".

Enzyme activity measurements. Arylamidase activity was determined according to Goldbarg and Rutenburg [7] using 3×10^{-4} M LNA in Tris-HCl buffer, pH 8.0. One unit of arylamidase is defined as that amount of enzyme which hydrolyzes one μmole of LNA per min at 25°. Leucine aminopeptidase was determined by the titrimetric method of Grassmann and Heyde.[3] Esterase activity was determined according to Nachlas *et al.*[9] using β-naphthylacetate in Veronal buffer, pH 7.4, as substrate.

Chromatographic experiments. Amino acids and peptides were analyzed by ascending chromatography on 0.25 mm layers of Merck Kiselgel G. The solvent systems used were propanol-water, 70:30, and butanol-pyridine-acetic acid-water, 30:24:6:20.

Fractionation experiments. Gel filtration was carried out at + 4° on columns of Sephadex G 100 (Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M Tris-HCl buffer, pH 8.0. Zone electrophoresis was performed on a vertical column packed with cellulose powder according to the technique described by Porath.[10]

RESULTS

1. *Influence of culture conditions on the arylamidase activity of* Serratia. The arylamidase activity of *Serratia* cells was released rapidly by sonication. When cell free sonicates were centrifuged for 90 min at 105 000 *g* all of the enzyme activity remained in the supernatant, indicating that the arylamidase was completely solubilized.

Preliminary experiments indicated that the enzyme activity varied with the composition of the culture medium and the physiological age of the cells at the time of harvest. Fig. 1 shows a plot of the enzyme activity of cell samples

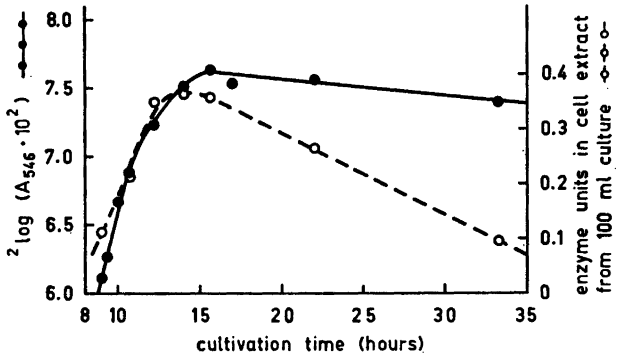

Fig. 1. Growth of *Serratia indica* in 1 % Bacto Casitone and arylamidase content of cell extracts as a function of cultivation time. Aliquots of the culture were removed at different times, the cells disintegrated and their arylamidase activity was determined.

removed from a culture grown in a rich peptone medium. The specific activity was highest late in the exponential phase of growth and decreased to less than one third of the maximal value when the incubation was continued beyond this stage. Apparently, the decline was partly due to autolysis, since substantial amounts of soluble enzyme were present in the media of old cultures. Cells grown in rich peptone media showed a specific arylamidase activity 2—3 times higher than that of cells grown in a synthetic salt medium with 0.5 % glycerol as the carbon source. At any rate, the presence of amino acids or peptides is not required for the formation of the enzyme.

2. *Fractionation experiments.* Attempts to purify the arylamidase of cell free sonicates by ammonium sulfate precipitation failed because most of the activity was lost. Gel filtration was more successful, but even here only 60—70 % of the original activity was recovered. Figs. 2 and 3 illustrate gel filtration experiments in which the effluent fractions were assayed for arylamidase activity, leucine aminopeptidase activity, and activity toward β-naphthyl acetate. The peak which elutes at the void volume of the column contains only a few per cent of the total arylamidase activity recovered, most of which is contained in the peak which elutes at about 1.7 void volumes. However, the first peak contains an enzyme that hydrolyzes leucinamide. Thin layer

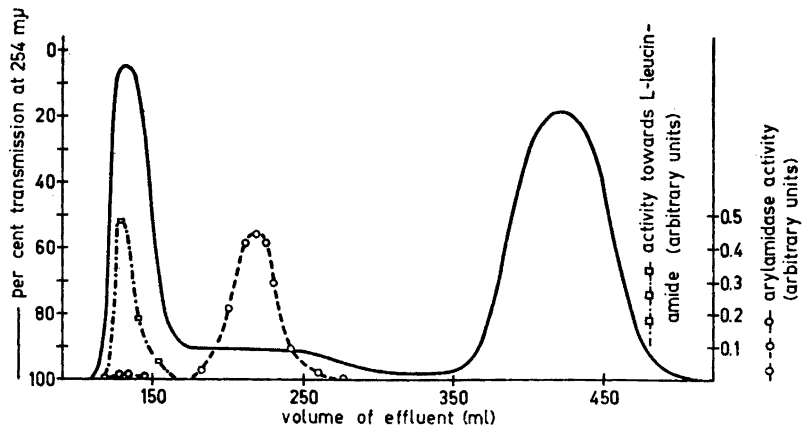

Fig. 2. Gel filtration of a cell free sonicate from *Serratia indica*. Sephadex G 100, equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, 5×10^{-3} M in Mg^{2+}, was used in a column, 3.5 cm in diameter and 48 cm long. The void volume was 125 ml. The UV-curve was obtained with the aid of a LKB Uvicord at 254 mμ.

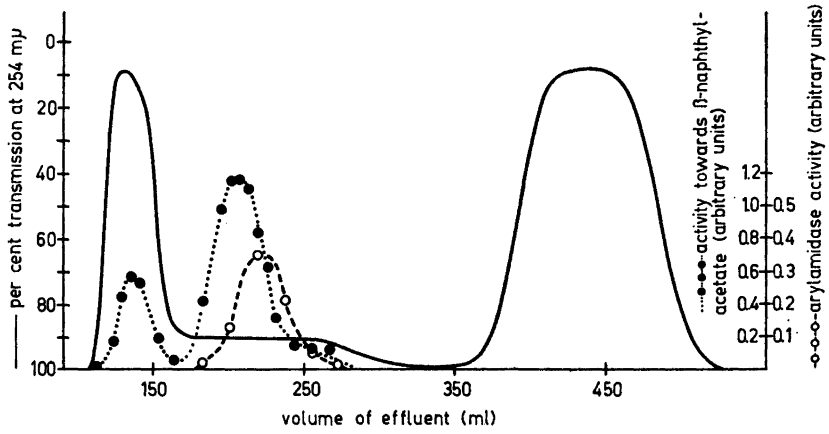

Fig. 3. Gel filtration of a cell free sonicate from *Serratia indica*. The same conditions were used as described in the legend to Fig. 2.

chromatography showed that this peak also hydrolyzed L-leucyl-glycine, glycyl-L-leucine and poly-L-lysine to free amino acids. Activity toward β-naphthyl acetate was observed in two peaks, one at the void volume and the other near, but distinct from, the arylamidase peak.

A number of zone electrophoresis experiments were performed both with crude cell extracts and with pooled material from gel filtration experiments. Although the recovery of arylamidase activity was only about 20 % the enzyme

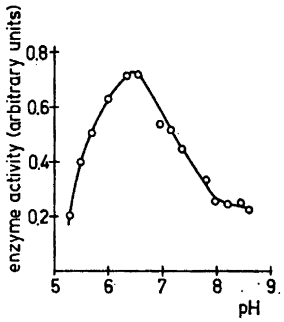

Fig. 4. pH dependence for hydrolysis of LNA by partially purified arylamidase from *Serratia indica*.

moved toward the anode in 0.1 M Tris-HCl buffer, pH 8.0, and large amounts of impurities were removed.

3. *Some properties of the partly purified arylamidase.* When either the crude cell free sonicate or the pooled fractions from gel filtration experiments were stored at 4° about half the enzyme activity disappeared within four days, even in the presence of compounds which prevented bacterial growth. At −26°, frozen solutions of the enzyme could be stored for longer periods without loss in activity. Outside the pH range 6.5—8.5 the enzyme was very unstable.

Fig. 4 illustrates that the pH optimum for the hydrolysis of LNA is between 6 and 7. The addition of 5×10^{-3} M Mg^{2+} to either cell free extracts or gel filtered enzyme solutions increased the arylamidase activity by 10—50 %. Dialysis against buffers containing 10^{-2} M EDTA caused a complete loss of activity, but attempts to reactivate the enzyme by the addition of Mg^{2+} or other divalent metal ions were unsuccessful.

Glycyl-β-naphthylamide and DL-alanyl-β-naphthylamide were also tried as substrates for the *Serratia* enzyme. At substrate concentrations of 3×10^{-4} M both compounds were hydrolyzed at rates which were even higher than that for LNA. No separation of these three different activities was observed in gel filtration or electrophoresis experiments, indicating that they are due to a single enzyme.

DISCUSSION

One purpose of this paper is to demonstrate the danger of using the chromogenic amino acid naphthylamides as substrates for the assay of aminopeptidase activity of bacteria. *Serratia* is a highly proteolytic organism and contains enzymes capable of hydrolyzing various peptides. However, our results demonstrate clearly that the cells also contain a different enzyme which cleaves several arylamides but which is inactive against L-leucinamide, the classical substrate for the determination of leucine aminopeptidase. The trace of arylamidase activity which we have observed in gel filtration fractions containing "true" leucine aminopeptidase probably indicate that this enzyme can hydrolyze LNA very slowly. At any rate the LNA assay is certainly not suitable for the determination of leucine aminopeptidase in *Serratia*

extracts or fractions thereof. Patterson *et al.*[11] have reported that mouse ascites carsinoma cells contain several enzyme components which hydrolyze LNA without cleaving L-leucinamide.

Arylamidases appear to be widespread, but their natural substrates are unknown and their functions in cell metabolism are obscure. Their instability makes them difficult to purify, but a careful study of their metal ion requirements for stability and activity might lead to a solution of this problem. The enzyme studied in this investigation seems similar to the yeast arylamidase on which one of us[12] has reported earlier. However, the two enzymes have markedly different pH optima for the hydrolysis of LNA, and the relative rates at which they hydrolyze different amino acid naphthylamides also differ.

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