

# Amino Acid- $\beta$ -Naphthylamide Hydrolysis by *Pseudomonas aeruginosa* Arylamidase

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The intracellular and constitutive arylamidase from *Pseudomonas aeruginosa* was purified 528-fold by salt fractionation, ion-exchange chromatography, gel filtration, and adsorption chromatography. This enzyme hydrolyzed basic and neutral *N*-terminal amino acid residues from amino- $\beta$ -naphthylamides, dipeptide- $\beta$ -naphthylamides, and a variety of polypeptides. Only those substrates having an L-amino acid with an unsubstituted  $\alpha$ -amino group as the *N*-terminal residue were susceptible to enzymatic hydrolysis. The molecular weight was estimated to be 71,000 daltons. The lowest  $K_m$  values were associated with substrates having neutral or basic amino acid residues with large side chains with no substitution or branching on the  $\beta$  carbon atom.

The utilization of amino acid- $\beta$ -naphthylamides by bacteria has been a subject of investigation in our laboratories and those of other investigators. These amino acid derivatives are hydrolyzed by arylamidases (or naphthylamidases), peptidase-like enzymes, to yield an amino acid and  $\beta$ -naphthylamine ( $\beta$ NA). In a recent paper (21), we reported that an arylamidase from *Pseudomonas aeruginosa* was intracellular and constitutive, whereas the transport mechanism for amino acid- $\beta$ NA was derepressible. *P. aeruginosa* cells grown on a minimal medium produced the enzyme intracellularly but lacked the ability to take up exogenously supplied amino acid- $\beta$ NA. Brief incubation of these cells with certain amino acids converted the cells to ones having the ability to take up the substrate. During this incubation period, protein synthesis essential for amino acid- $\beta$ NA transport occurred. The transport process was energy dependent, and the amino acid moiety of the amino acid- $\beta$ NA was incorporated into bacterial protein.

Other reports from our laboratory have been concerned with the purification and properties of arylamidases from the gram-negative coccus, *Neisseria catarrhalis*. Behal and Cox reported that an unsubstituted amino group on the *N*-terminal L- $\alpha$ -amino residue was required for susceptibility to enzymatic hydrolysis (5). Cox and Behal subsequently found that arylamidase hydrolyzed dipeptide- $\beta$ NA and certain polypeptides in a stepwise manner, beginning with the *N*-terminal residue, whereas those substrates having a penultimate residue of the D-configuration were

not hydrolyzed (11, 12). Arylamidases were initially considered to be characteristic of gram-negative bacteria, but subsequent studies have shown them to be present in gram-positive bacteria as well (6, 20).

Aubert and Millet reported that L-leucyl- $\beta$ NA hydrolase in *Bacillus megaterium* is more repressed during growth than during sporulation (2). Westly et al. have proposed the utilization of substrate specificity patterns as a classification tool for microorganisms (24). Similarly, Muftic has suggested a similar proposition for aid in differentiating mycobacteria (20). Burton et al. found that pathogenic and apathogenic strains of leptospirae could be differentiated since significantly higher levels of naphthylamidase activity were found in the pathogenic strains (10). The arylamidase activity in *Escherichia coli* has been located in the soluble fraction after cellular disruption (23).

This report is concerned with our further investigation into amino acid- $\beta$ NA metabolism in *P. aeruginosa* and describes the purification and some properties of the constitutive arylamidase of this bacterium.

## MATERIALS AND METHODS

**Organism.** *P. aeruginosa* cells were grown and harvested as previously described (21).

**Substrates.** Amino acid- $\beta$ NA, dipeptide- $\beta$ NA, and peptides were secured from Mann Research Laboratories, Inc., New York, N.Y., and from International Chemical and Nuclear Co., Burbank, Calif. The specific rotations of the substrates used were in agreement with literature values. All other chemicals used were of the highest commercial grade available.

**Protein determination.** Protein was determined by the method of Lowry et al. (17). Protein concentration of individual fractions from column chromatography was estimated by measuring the absorbance at a wavelength of 280 nm.

**Polyacrylamide gel electrophoresis.** The alternate method of Davis for layering samples in 40% sucrose was utilized (13). The buffer pH was 8.9, and the final acrylamide concentration was 7%. Protein was detected by staining with amido black in 7% acetic acid. Enzyme activity was detected by incubating the electrophoresed gel at 37 C with a solution of 3 mM L-arginyl- $\beta$ NA containing 0.2 mg of Fast Blue B per ml. A red band appeared at the site of  $\beta$ NA liberation. The gels were stored in 7% acetic acid.

**Paper chromatography.** Chromatography was performed at 27 C for 15 hr on Whatman no. 3MM filter paper. The ascending technique was utilized with a solvent system composed of *n*-butanol-glacial acetic acid-water (4:1:5, v/v). Free  $\beta$ NA was observed by viewing the chromatograms under ultraviolet light. The amino acids and dipeptides were detected by spraying the chromatograms with a solution of 0.25% ninhydrin in acetone and subsequently heating the chromatograms at 100 C for 10 min.

**Molecular weight estimation.** The molecular weight of the enzyme was estimated by a modification of the gel filtration method of Andrews (1). A Pharmacia K25/45 column, equipped with upward-flow adapters, was packed with Sephadex G-200 gel equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1 mole of sodium chloride and 0.1 mole of boric acid per liter. The elution rate of the column was stabilized by a metering pump, and each fraction contained 2 ml of eluant. The elution volume of Blue Dextran, approximately 80 ml, was used as the void volume of the column. Several pure proteins of known molecular weight were employed as references for preparing a standard curve.

**Arylamidase assay.** The rate of  $\beta$ NA release from amino acid- $\beta$ NA was determined fluorometrically. Although unreacted substrate does not fluoresce appreciably, free  $\beta$ NA does fluoresce intensely at 410 nm when excited at 335 nm. The 4.0-ml reaction mixture contained 40  $\mu$ moles of tris(hydroxymethyl)amino-methane-maleate buffer, 4.0  $\mu$ moles of amino acid- $\beta$ NA (or dipeptide- $\beta$ NA), and enzyme. The pH of the reaction mixture was varied as necessary. The rate of arylamidase-catalyzed liberation of  $\beta$ NA at 37 C was determined directly with a Beckman 77204 Ratio Fluorometer and a strip chart recorder. A unit of arylamidase activity is defined as that amount of enzyme that hydrolyzes 1.0  $\mu$ mole of substrate per min under these conditions. In some cases, a previously described colorimetric method (3) was employed instead.

## RESULTS

**Enzyme purification.** A 100-g (wet weight) batch of cells was washed in 0.005 M potassium phosphate buffer, pH 6.8, and then suspended in 100 ml of the same buffer. The cells were then ruptured by ultrasonic treatment (Branson S125 Sonifier) at 4 C. The resulting cell-free extract was clarified by centrifugation at 40,000  $\times$  g.

Solid ammonium sulfate was added slowly with stirring to the clarified extraction until 30% of the saturation (168 g/liter) was reached. After standing for 8 hr at 4 C, the precipitate was sedimented and the supernatant fluid was brought to 70% saturation (254 g/liter). After centrifugation, the precipitate was resuspended in a small volume of 0.005 M potassium phosphate buffer, pH 8.6, and was dialyzed against 20 volumes of the same buffer for 12 hr. The dialyzing buffer was changed each 3 hr. L-Arginyl- $\beta$ NA was used as the substrate to detect enzymatically active fractions throughout the purification procedure.

The dialyzed fraction, containing 1.8 g of protein, was applied to a reverse-flow column (2.5 by 100 cm; Pharmacia K25/100) packed with Sephadex G-200 gel equilibrated at 4 C with a 0.005 M potassium phosphate-0.10 M sodium chloride buffer at pH 8.6. The flow rate was 0.3 to 0.5 ml/min. Enzymatically active fractions were pooled and concentrated by direct-pressure dialysis and dialyzed against 0.005 M potassium phosphate buffer, pH 8.6, to prepare the sample for ion-exchange chromatography.

The pooled Sephadex column fractions, containing 200 mg of protein, were applied to a diethylaminoethyl cellulose ion-exchange column (2.5 by 50 cm) which had been previously equilibrated with 0.005 M potassium phosphate buffer, pH 8.6. The column was eluted with 1,200 ml of a potassium phosphate-sodium chloride gradient system. Initial sodium chloride molarity, potassium phosphate molarity, and pH were 0.0, 0.005, and 8.6, respectively; the respective limiting values were 0.4, 0.01, and 8.6. The column had a flow rate of 1 ml/min. Enzyme fractions were pooled, concentrated by pressure dialysis to a volume of 5 ml, and dialyzed against 0.001 M potassium phosphate buffer, pH 7.0.

The sample was then applied to a column (0.5 by 40 cm) of  $\text{Ca}_3(\text{PO}_4)_2$  (Hypatite C, Clarkson Chemical Co., Inc., Williamsport, Pa.) which had been equilibrated with 0.001 M potassium phosphate buffer. The sample was eluted with a potassium phosphate buffer gradient system; initial and limiting phosphate molarities were 0.005 and 0.05, respectively. The enzymatically active fractions were pooled, concentrated, and subsequently applied to a second hypatite column and eluted with a stepwise gradient consisting of the following molarities of potassium phosphate: 0.005, 0.01, 0.015, 0.02, 0.025, and 0.03. The pH was 7.0 throughout this procedure.

The data for the purification procedure are shown in Table 1. An overall 528-fold purification with a yield of 12% was obtained. The chromatographic elution profiles from gel filtration, ion-exchange, and hydroxylapatite chromatography are illustrated (Fig. 1).

TABLE 1. Purification of arylamidase

Step	Total units	Protein (mg)	Specific activity <sup>a</sup>	Purification
Cell-free extract	8.5	7,712	$1.1 \times 10^{-3}$	1
Ammonium sulfate ppt (30-70% saturation)	7.9	1,968	$4.0 \times 10^{-3}$	3.5
Gel filtration	7.1	228	$31 \times 10^{-3}$	28
DEAE <sup>b</sup> cellulose chromatography	4.6	58	$80 \times 10^{-3}$	73
First hydroxylapatite chromatography	2.4	5	$479 \times 10^{-3}$	435
Second hydroxylapatite chromatography	0.99	1.7	$581 \times 10^{-3}$	528

<sup>a</sup> Specific activity is expressed as units per milligram of protein.

<sup>b</sup> Diethylaminoethyl.

Acrylamide gel electrophoresis of the material from the final step in the purification scheme produced a single enzymatically active band. The results of gel electrophoresis of the purified enzyme are shown on Fig. 2.

**Molecular weight estimation.** Several proteins of known molecular weight (human gamma globulin, 160,000; bovine serum albumin, 67,000; ovalbumin, 45,000; bovine chymotrypsinogen, 25,000; sperm whale myoglobin, 17,800; and cytochrome *c*, 12,400) were applied to and eluted from a Sephadex G-200 gel column as previously described above (Fig. 3).

When *P. aeruginosa* arylamidase was chromatographed, an elution volume to void volume ratio of 1.86 was obtained. This corresponded to a molecular weight of 71,000 daltons.

**Substrate specificity.** The relative rates of hydrolysis of some  $\beta$ NA derivatives by arylamidase were determined (Table 2) under conditions previously established in studies in *P. aeruginosa* (21) and *N. catarrhalis* (5, 11). Certain basic and neutral amino acid- $\beta$ -naphthylamines were hydrolyzed most readily, with L-arginyl- $\beta$ NA and L-alanyl- $\beta$ NA being most rapidly hydrolyzed. The requirement for a primary amino group in the  $\alpha$  position was indicated by the fact that  $\gamma$ -glutamyl- $\beta$ NA and  $\beta$ -alanyl- $\beta$ NA were totally resistant to arylamidase-catalyzed hydrolysis. Substitution of the  $\alpha$ -amino group (e.g., *N*-acetyl-L-alanyl- $\beta$ NA) also rendered the substrates totally resistant to hydrolysis. Furthermore, the length and branching of the carbon chain (e.g., L-leucyl- $\beta$ NA versus L-isoleucyl-, L-valyl-, or glycyl- $\beta$ NA) greatly altered the susceptibility to enzymatic hydrolysis.

**Hydrolysis of peptide- $\beta$ NA and peptides.** The enzyme catalyzed release of  $\beta$ NA from the four optical isomers of alanyl-alanyl- $\beta$ NA was determined by removal of samples of the reaction mixtures at intervals during the course of the incubation period. These samples were chromatographed to identify the reaction products. The hydrolytic products of L-alanyl-L-alanyl- $\beta$ NA detected were alanine,  $\beta$ NA, and some unreacted substrate. Analysis of the reaction products of

the sample containing L-alanyl-D-alanyl- $\beta$ NA showed that, even after 17 hr of incubation, only alanine and alanyl- $\beta$ NA were present. The isomers, D-alanyl-D-alanyl- $\beta$ NA and D-alanyl-L-alanyl- $\beta$ NA, were not susceptible to hydrolysis even though incubated for 18 hr with enzyme (Fig. 4).

Catalytic activity upon short peptides was investigated, as we thought that they were more closely related to the natural substrate of this enzyme (Fig. 5). Complete hydrolysis of L-alanyl-L-alanyl-L-alanine to its component residues occurred. L-Alanyl-L-alanyl-D-alanine was cleaved to form alanine and alanyl-alanine. However, L-alanyl-D-alanyl-L-alanine was completely resistant to hydrolysis. Therefore, total hydrolysis occurred only for those tripeptides composed entirely of L-amino acid residues.

A comparison of the rate of  $\beta$ NA release from the substrates, L-alanyl- $\beta$ NA and L-alanyl-L-alanyl- $\beta$ NA, was made (Fig. 6). The rate of  $\beta$ NA release from L-alanyl- $\beta$ NA was linear. However, with L-alanyl-L-alanyl- $\beta$ NA, there was a lag period prior to reaching a linear release of  $\beta$ NA. Samples of these reaction mixtures, taken at various time intervals, were chromatographed to study the reaction products. In either sample, the only products detected were alanine and  $\beta$ NA. Even a sample taken after 6 min of incubation from the L-alanyl-L-alanyl- $\beta$ NA reaction mixture showed no trace of the dipeptide, alanyl-alanine.

**Effect of substrate concentration on reaction velocity.** The effect of substrate concentration on the rate of arylamidase-catalyzed hydrolysis was determined for the amino acid- $\beta$ NA listed in Table 3. L-Alanyl- $\beta$ NA had the highest  $V_{max}$  value which slightly exceeded that of L-arginyl- $\beta$ NA. The lower  $V_{max}$  values were obtained with those substrates with  $\beta$ -branched R groups or when an R group was absent. The smaller  $K_m$  values were associated with the substrates containing amino acid residues with basic or non-polar R groups.

**Enzyme properties.** The pH optimum for the enzyme was 7.9, which is somewhat higher than

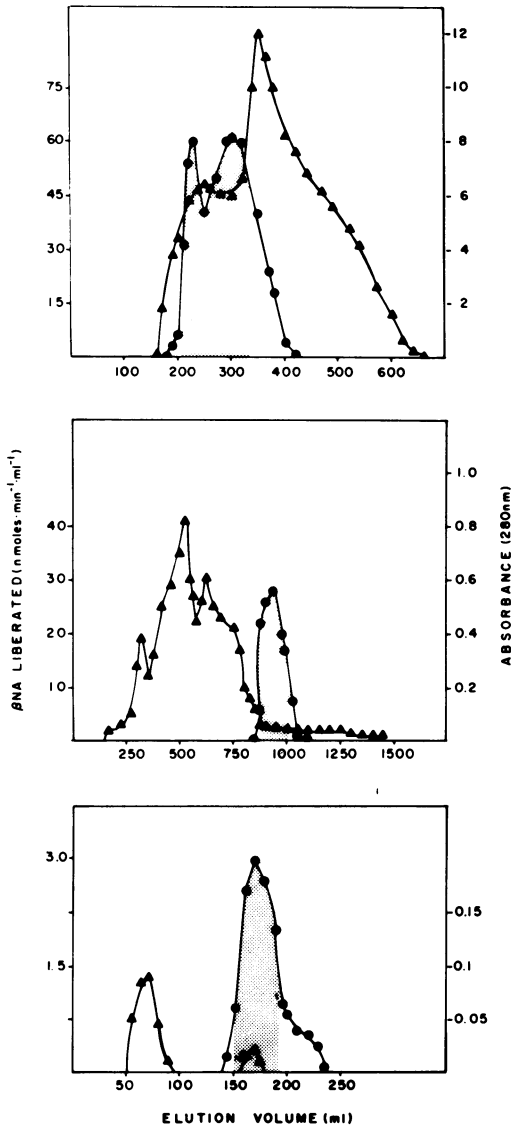


FIG. 1. Column chromatographic elution profiles. Top, gel filtration; middle, ion-exchange chromatography; and bottom, hydroxylapatite chromatography. Protein ( $\blacktriangle$ ); enzyme activity ( $\bullet$ ). The column fractions were tested for enzyme activity with the substrate, L-arginyl- $\beta$ -NA. The enzyme profiles were obtained by using the colorimetric assay (3).

the values observed for arylamidases from *N. catarrhalis* (5, 11) and *Sarcina lutea* (4). Incubation of the enzyme at pH values from 5.5 to 9.5 did not irreversibly inactivate the enzyme, since adjustment to pH 7.9, after preincubation at the other values, resulted in activity levels similar to that of the enzyme preincubated and assayed at the pH optimum (Fig. 7).

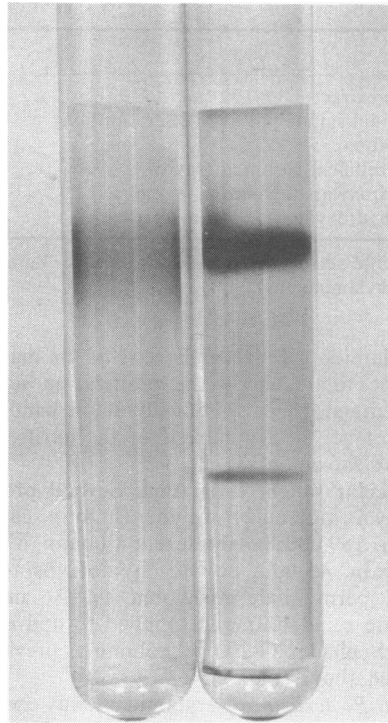


FIG. 2. Polyacrylamide gel electrophoresis of purified arylamidase at pH 8.9. Gel on the right is stained for protein; gel on the left is stained for enzyme activity. The lower band on the right gel is due to the tracker dye. The proteins migrated from the top toward the bottom of the gel.

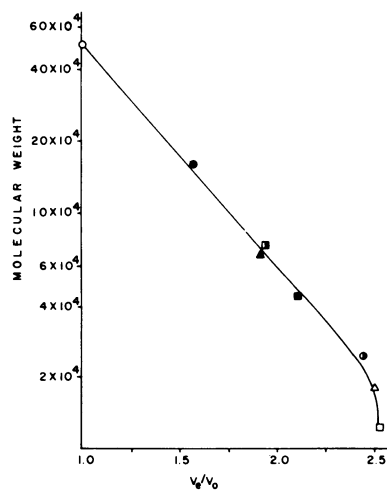


FIG. 3. Gel filtration molecular weight determination. Standard reference materials were blue dextran ( $\circ$ ); gamma globulin ( $\bullet$ ); bovine serum albumin ( $\blacktriangle$ ); ovalbumin ( $\blacksquare$ ); chymotrypsinogen ( $\blacklozenge$ ); myoglobin ( $\circ$ ); cytochrome c ( $\square$ ); arylamidase ( $\blacksquare$ ).

TABLE 2. *Hydrolytic activity of arylamidase on various  $\beta$ NA derivatives<sup>a</sup>*

Amino acid- $\beta$ NA	Relative rate of hydrolysis <sup>b</sup>
L-Arginine .....	100
L-Alanine .....	91
L-Lysine .....	31
L-Methionine .....	24
L-Leucine .....	22
L- $\alpha$ -Glutamate .....	7
L-Isoleucine .....	2
L-Phenylalanine .....	1
L-Histidine .....	1
L-Glycine .....	1

<sup>a</sup> The quantity of enzyme used in the assays was adjusted to liberate 1 nmole of  $\beta$ NA per ml per min from the substrate, L-arginyl- $\beta$ NA. Conditions of the fluorometric assay are presented in Materials and Methods.

<sup>b</sup> The arbitrary value, 100, was assigned to the rate obtained with L-arginyl- $\beta$ NA, the substrate most rapidly hydrolyzed. The following amino acid derivatives of  $\beta$ NA were not susceptible to hydrolysis: L- $\alpha$ -aspartate, L- $\gamma$ -glutamate, L-proline, L-serine,  $\beta$ -alanine, L-threonine, L-valine, N-acetyl-alanine, D-alanyl-L-alanine, D-alanyl-L-alanine, and D-alanine.

The effect of several divalent cations upon the rate of enzymatic hydrolysis of L-arginyl- $\beta$ NA was investigated by comparison of duplicate samples of the enzyme, one dialyzed in 0.05 M potassium phosphate buffer (pH 7.9) and the other in 0.1 M ethylenediaminetetraacetate (EDTA), pH 7.9, followed by dialysis in the phosphate buffer. Comparison of the activity of these samples with undialyzed control samples showed that EDTA treatment caused a loss of approximately 60% of the activity. Moreover, after EDTA treatment, arylamidase was stimulated by most of the metal ions tested (Table 4). Maximum stimulation was provided by Mn<sup>2+</sup> and Mg<sup>2+</sup>. The enzymatic activity of the buffer-dialyzed sample (not treated with EDTA) was not stimulated by any of the metal ions. A strong inhibitory effect following the addition of Cu<sup>2+</sup> was noted in both samples. Increasing the concentration of the metals up to 1 mM resulted in a loss of activity similar to that observed in the untreated enzyme sample.

DISCUSSION

There have been few reports on microbial arylamidases, and the role(s) of these enzymes in bacterial physiology has not been established. There are, however, many reports on animal and human arylamidases for which physiological roles have been suggested (14, 16, 18, 19). Felgenhauer and Glenner (15) proposed that the rat kidney enzyme functions as an angiotensinase or

an aminopolypeptidase and that the hydrolysis of amino acid- $\beta$ NA is fortuitous. Hopsu-Havu and Sarino (16) suggested that the aryl moiety of these substrates is not a significant factor in the formation of enzyme-substrate complexes. However, Behal and Cox (5) observed that the rate of enzymatic action upon amino acid-*p*-nitroanilides was much lower than that for corresponding  $\beta$ NA derivatives, thereby indicating that the aryl group in the substrate is also of importance.

An understanding of the *in vivo* function of arylamidase in microorganisms is an objective of the investigations in our laboratory. Our previous report on this enzyme in *P. aeruginosa* suggested a function other than involvement in protein synthesis for cellular multiplication (21). To study further the hydrolysis and metabolism of amino acid- $\beta$ NA in *P. aeruginosa*, an investigation of some of the characteristics of arylamidase was indicated.

Polyacrylamide gel electrophoresis of the final

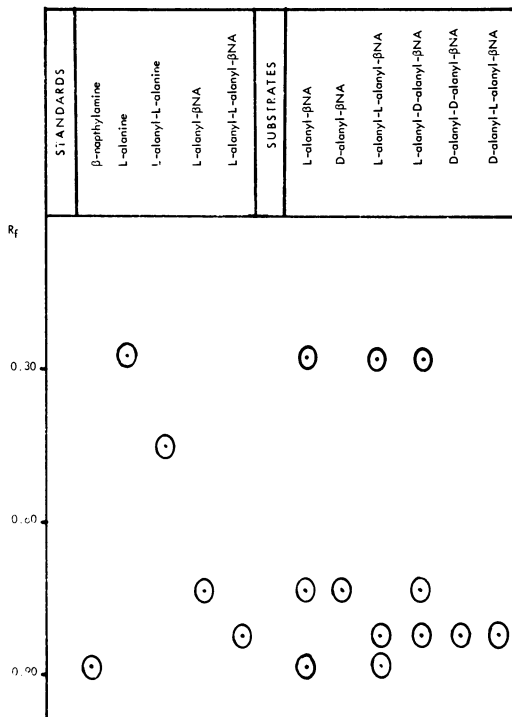


FIG. 4. *Composite chromatogram showing the products of arylamidase-catalyzed hydrolysis of the optical isomers of L-alanyl- $\beta$ NA and L-alanyl-L-alanyl- $\beta$ NA. The assay mixture contained: substrate, 2  $\mu$ moles; enzyme (amount required to completely hydrolyze 2  $\mu$ moles of L-alanyl-L-alanyl- $\beta$ NA in 1 hr), 0.1 ml; and 0.05 M phosphate buffer (pH 7.9) to a final volume of 1.5 ml. The assay mixtures were inactivated by boiling at various time intervals during the incubation period.*

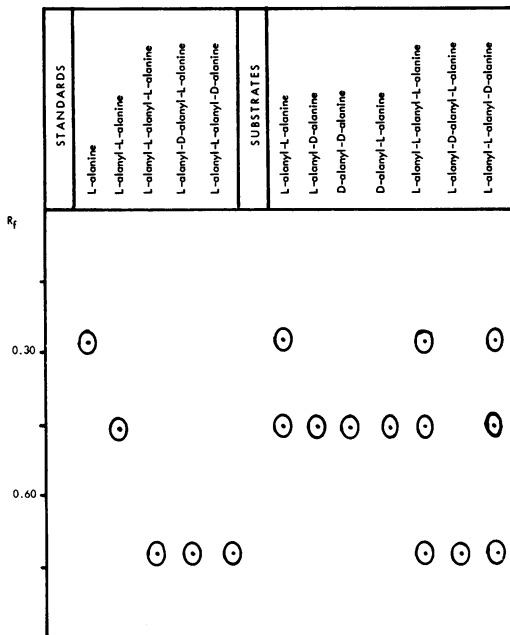


FIG. 5. Composite chromatogram showing the products of arylamidase-catalyzed hydrolysis of alanine peptides. The assay mixtures contained: substrate, 2  $\mu$ moles; enzyme (amount required to completely hydrolyze 2  $\mu$ moles of L-alanyl-L-alanine in 1 hr), 0.1 ml; and 0.05 M phosphate buffer (pH 7.9) to a final volume of 1.5 ml. The assay mixtures were then incubated at 37 C. Samples from each assay mixture were inactivated by boiling at various time intervals during the incubation period.

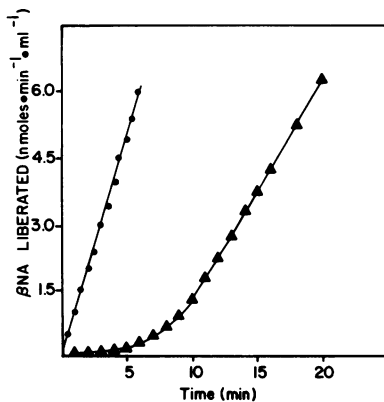


FIG. 6. Hydrolysis of L-alanyl- $\beta$ NA and L-alanyl-L-alanyl- $\beta$ NA by arylamidase. Each reaction mixture contained 1.5  $\mu$ moles of substrate. The enzyme concentration was adjusted to liberate 1  $\mu$ mole of  $\beta$ NA per min from L-alanyl- $\beta$ NA. The substrates were: L-alanyl- $\beta$ NA ( $\bullet$ ); L-alanyl-L-alanyl- $\beta$ NA ( $\blacktriangle$ ). The liberation of  $\beta$ NA at pH 7.9 was determined fluorometrically.

TABLE 3. Effect of substrate concentration on reaction velocity<sup>a</sup>

Substrate	$K_m$ (M)	$V_{max}$ (moles $\times 10^{-1}$ $\times \text{min}^{-1}$ )
L-Alanyl- $\beta$ NA . . . .	$11.7 \times 10^{-4}$	$22.2 \times 10^{-5}$
L-Arginyl- $\beta$ NA . . .	$5.0 \times 10^{-4}$	$15.4 \times 10^{-5}$
L-Lysyl- $\beta$ NA . . . .	$5.3 \times 10^{-4}$	$7.1 \times 10^{-5}$
L-Leucyl- $\beta$ NA . . . .	$5.3 \times 10^{-4}$	$6.9 \times 10^{-5}$
L-Isoleucyl- $\beta$ NA . .	$6.7 \times 10^{-4}$	$1.9 \times 10^{-5}$
Glycyl- $\beta$ NA . . . . .	$20.6 \times 10^{-4}$	$0.54 \times 10^{-5}$

<sup>a</sup> The quantity of enzyme used in the assays was adjusted to liberate 1 nmole of  $\beta$ NA per ml per min from the substrate, L-arginyl- $\beta$ NA. Conditions of the fluorometric assay are presented in Materials and Methods.

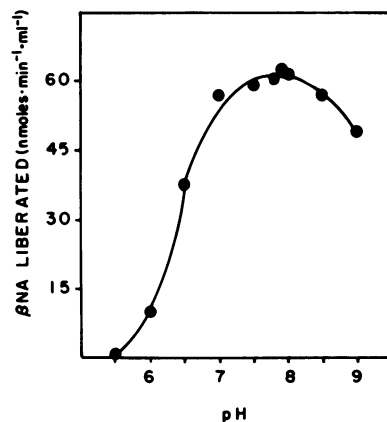


FIG. 7. Effect of pH on arylamidase activity. Enzyme activity was determined at the indicated pH on assay mixtures containing 1 mM L-arginyl- $\beta$ NA. The incubation period was for 30 min at 37 C. The colorimetric assay (3) was used.

preparation from the purification procedure indicated the presence of one enzymatically active protein band. An estimated molecular weight of approximately 71,000 daltons was determined which is nearly one-half of the value reported for the enzymes from *N. catarrhalis* (5, 11). However, it is quite close to the estimates made for arylamidases from *S. lutea* (4) and ox brain (9).

The substrate specificity study (Table 2) indicated that the N-terminal amino acid residue of the substrate must have an  $\alpha$ -amino group. This amino group must also be of the L-configuration and unsubstituted. Those  $\beta$ NA substrates having straight-chain or  $\gamma$ -branched R groups were more susceptible to hydrolytic attack than the ones with  $\beta$ -branched or acidic R groups on the amino acid residue.

Although the highest  $V_{max}$  value was observed with the substrate L-alanyl- $\beta$ NA, the lower  $K_m$

values were associated with substrates containing large R groups on the amino acid residue. Thus, the size of the R groups is an important factor in enzyme-substrate complex formation. That the R group does not interact solely by hydrophobic bonding was indicated by the similar  $K_m$  values of L-leucyl- and L-arginyl- $\beta$ NA. The lower  $V_{max}$  values observed with L-isoleucyl- and glycyl- $\beta$ NA showed that  $\beta$ -branching or the absence of an R group altered the substrate susceptibility to arylamidase hydrolysis.

In studies conducted on bacterial arylamidases, there has been no evidence presented for a metal ion dependency for activity; this is in direct contrast to studies on arylamidases derived from animal tissues (7-9, 22). In the investigation of *N. catarrhalis* arylamidase (11), Cox and Behal observed some stimulation of enzyme activity after EDTA treatment. After treatment of the *P. aeruginosa* enzyme with EDTA, 60% of the enzyme activity was lost. However, after divalent cation replacement, we observed a marked increase in activity which exceeded that recorded with the untreated enzyme. This stimulatory effect has also been observed with bovine brain arylamidase (19). The effect did not appear to be metallo-specific, as each of the metal ions tested produced the stimulation in activity, with the only exception being cupric ion. These results may indicate that the native metal ions are very firmly bound, and only after EDTA treatment does replacement with a more effective metal ion occur. The effect produced by the metal may be one of inducing a conformational change rather than the direct participation in the reaction at the catalytic site of the enzyme.

The activity of this naphthylamidase on the various optical isomers of alanyl-alanyl- $\beta$ NA and alanine peptides demonstrated the absolute requirement for the L-configuration in the N-terminal residue. Single sequential hydrolysis of amino acid residues from the substrates was indicated by the following. (i) Hydrolysis of L-alanyl-D-alanyl- $\beta$ NA yielded the reaction products alanine and alanyl- $\beta$ NA; (ii) hydrolysis of L-alanyl-L-alanyl-D-alanine yielded the reaction products alanine and alanyl-alanine; and (iii) L-alanyl- $\beta$ NA was rapidly hydrolyzed to yield  $\beta$ NA; whereas, in the hydrolysis of L-alanyl-L-alanyl- $\beta$ NA, a lag period prior to the appearance of  $\beta$ NA was noted and there was no release of the intact dipeptide, alanyl-alanine.

Although L-alanyl-L-alanyl-L-alanine was hydrolyzed, L-alanyl-D-alanyl-L-alanine was not hydrolyzed; this may be the result of steric hindrance of the ionized carboxyl group of the latter. That the hindrance may be an ionic effect rather than simply spatial is indicated by the fact that the N-terminal residue of L-alanyl-D-alanyl-

TABLE 4. Effect of divalent cations on enzyme activity<sup>a</sup>

Enzyme	Reagent (10 <sup>-4</sup> M)	$\beta$ NA liberated (units)
Control <sup>b</sup>		50
Dialyzed <sup>c</sup>		50
Dialyzed <sup>c</sup>	FeCl <sub>2</sub>	20
Dialyzed <sup>c</sup>	CoCl <sub>2</sub>	18
Dialyzed <sup>c</sup>	MgCl <sub>2</sub>	28
Dialyzed <sup>c</sup>	MnCl <sub>2</sub>	30
Dialyzed <sup>c</sup>	CuCl <sub>2</sub>	6
EDTA dialyzed <sup>d</sup>		20
EDTA dialyzed <sup>d</sup>	FeCl <sub>2</sub>	100
EDTA dialyzed <sup>d</sup>	CoCl <sub>2</sub>	137
EDTA dialyzed <sup>d</sup>	MgCl <sub>2</sub>	198
EDTA dialyzed <sup>d</sup>	MnCl <sub>2</sub>	205
EDTA dialyzed <sup>d</sup>	CuCl <sub>2</sub>	7

<sup>a</sup> The quantity of enzyme used in the assays was adjusted to liberate 1 nmole of  $\beta$ NA per ml per min from the substrate, L-arginyl- $\beta$ NA. Ten  $\mu$ liters of metal ion solution was preincubated with 90  $\mu$ liters of enzyme solution for 30 min at 37 C. The control sample was preincubated with 10  $\mu$ liters of water for 30 min at 37 C. After preincubation, these mixtures were assayed fluorometrically for activity as described in Materials and Methods.

<sup>b</sup> The original untreated enzyme preparation.

<sup>c</sup> Enzyme dialyzed against 0.05 M phosphate buffer (pH 7.9) for 12 hr.

<sup>d</sup> Enzyme dialyzed against 0.1 M EDTA solution, pH 7.9, for 6 hr and then dialyzed against 0.05 M phosphate buffer (pH 7.9) for 6 hr.

$\beta$ NA is hydrolyzed, even though the penultimate residue is of the D configuration.

Thus, this enzyme does not cleave dipeptide- $\beta$ NA to yield the products dipeptide and  $\beta$ NA, as has been reported for arylamidase of the pituitary (14). Therefore, there is a similarity in mechanism of action between this enzyme and the arylamidases of *N. catarrhalis* (5, 11) and human liver (8).

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