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A DIMERIC, EXTRACELLULAR, HEAT-STABLE AMINOPEPTIDASE PRODUCED BY A MARINE PSEUDOMONAD

JOSEPH R. MERKEL, CLARENCE C. LEE * and THOMAS S. FREUND **

Department of Chemistry and Center for Marine and Environmental Studies, Seeley G. Mudd Building 6, Lehigh University, Bethlehem, PA 18015 (U.S.A.)

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An extracellular aminopeptidase (*Alteromonas* aminopeptidase III) of a marine pseudomonad, designated *Alteromonas* B-207, was purified to homogeneity. The enzyme was found to have a native molecular weight of 63 000 by exclusion chromatography and a subunit weight of 33 000 by SDS-polyacrylamide gel electrophoresis. Cross-linking with dimethyladipimidate prevented dissociation of the dimer. The enzyme has a pH optimum of 9.3, a temperature optimum of 70°C and is stable at 60°C for approx. 1 h. It has high specificity for L-leucyl-β-naphthylamide and, of the peptides tested, it shows a preference for L-Leu, Gly and L-Pro over L-α-Asp and L-Lys. The enzyme is inhibited by 1,10-phenanthroline, added to the enzyme-substrate reaction mixture, and by EDTA when the enzyme is dialyzed against 10^{-3} M soln. Activity can be partially restored to EDTA-dialyzed enzyme by removal of the EDTA and incubation of the enzyme with Zn^{2+}. Atomic absorption data also implicate zinc as a required metal. Sulfhydryl- and serine- inhibitors have no effect on the enzyme.

Introduction

Prescott and Wilkes [1] isolated a heat-stable aminopeptidase from a marine bacterium which was characterized by Merkel et al. [2] as *Aeromonas proteolytica*. *Aeromonas* aminopeptidase withstands 70°C for periods of up to 60 min. This characteristic was employed to facilitate separation of the aminopeptidase from several endopeptidases that are elaborated by *A. proteolytica* [3].

The ease with which *Aeromonas* aminopeptidase could be purified spurred our search for additional marine isolates possessing aminopeptidases which differ from *Aeromonas* aminopeptidase, whose specificity is similar to leucine aminopeptidase [4]. Preliminary studies on the distribution and nature of marine proteolytic bacteria revealed that the majority of inshore bacterial isolates possess proteolytic activity, and most of these produce an aminopeptidase(s) [5–7].

The current study describes the isolation and characterization of a heat-stable aminopeptidase produced by a marine pseudomonad which we have designated as *Alteromonas* B-207 * (ATCC 33 524). During the course of our studies on this marine bacterium and its proteolytic enzymes it became evident that the organism produced several aminopeptidases as well as several endopeptidases. A heat-

* Present address: Dept. of Biological Sciences, Princeton University, Princeton, NJ 08540, U.S.A.
** Present address: School of Dentistry, Fairleigh Dickinson University, Hackensack, NJ 07601, U.S.A.

* With the exception of the low content of cytosine and guanosine in its DNA, the characteristics of the organism fit those of the genus *Pseudomonas*. Baumann et al. [8] suggested that this type of organism should be classified as a species of *Alteromonas* [9]. Details of the isolation, cultivation, and characterization can be obtained from the senior author. A culture has been deposited with the American Type Culture Collection (ATCC 33 524).

stable aminopeptidase (aminopeptidase III) was released into the medium during the early phases of growth, and two intracellular aminopeptidases (aminopeptidase I and II) were demonstrated by disruption of cells and by autolysis of older cell cultures. The intracellular aminopeptidases and their location in the bacterial cell is the subject of another publication.

Materials and Methods

Aminopeptidase III production. Extracellular enzyme production was accomplished in 7.5 l fermentors (New Brunswick Sc. Co.) at 23°C using 5 l of a medium composed of 1% hydrolyzed casein (NZ-amine, Type HD, Sheffield Chem. Co.) and 3% Rila Marine Mix adjusted to pH 7.2 before autoclaving. The sterile fermentor medium was inoculated with 50 ml of a 21-h shaker culture of *Alteromonas* B-207, and an aerobic growth (stirring rate of 600 rev./min and 7 l air/min) was allowed to proceed for 20–21 h after which the culture medium was collected by centrifugation. The culture supernatant was used as the source of crude aminopeptidase III.

Isolation and purification of aminopeptidase III. The supernatant culture fluid was precipitated at 4°C with 2.8 kg $(NH_4)_2SO_4$ (70% saturated). The protein precipitate was collected by centrifugation, redissolved in a minimum of cold 0.02 M Tris-HCl buffer, pH 8.0, and dialyzed at 4°C for 24–36 h against three changes of the buffer. All Tris-HCl buffers used in these studies, with the exception of trace metal studies, were supplemented with $2 \cdot 10^{-5}$ M $CaCl_2$, $MgCl_2$ and $ZnCl_2$.

500 g semi-dry (Buchner filtered) DEAE-cellulose that had been equilibrated in 0.02 M Tris-HCl buffer (pH 8.0) were stirred into the dialyzed enzyme solution (560 ml in the particular purification described below). Stirring was continued at 4°C for 20 min after which the suspension was filtered and the DEAE-cellulose-enzyme material was washed once with 1 l 0.02 M Tris-HCl (pH 8.0)/0.4 M NaCl. The filtrate and washings were pooled, precipitated with $(NH_4)_2SO_4$ (70% satd.) redissolved and thoroughly dialyzed against 0.05 M phosphate buffer (pH 8.0). The dialyzed enzyme solution was next heated at 60°C for 20 min, cooled in an ice bath, concentrated by ultrafiltration (Amicon Diaflo apparatus with a UM-10 filter) and the concentrated enzyme was applied to a Sephadex G-200 column (1.8 X 112 cm). Active fractions were pooled and chromatographed on a 1.6 X 11.5 cm DEAE-cellulose column equilibrated with 0.02 M Tris-HCl (pH 8.0). Aminopeptidase III activity was eluted with a linear NaCl gradient (0.1–0.35 M) in 0.02 M Tris-HCl (pH 8.0).

Assays. Protein concentrations were determined by the method of Lowry et al. [10].

Endopeptidase activity was measured by Prescott and Wilms' [11] modification of Anson's [12] procedure utilizing urea-denatured hemoglobin substrate. The extent of proteolysis was determined by reading the absorbance of trichloroacetic acid-soluble degradation products at 280 nm. 1 unit of endopeptidase activity is arbitrarily defined as the amount of enzyme that produces an absorbance change of one at 280 nm in 1 min of reaction at 37°C, pH 8.0.

Standard assays of aminopeptidase activity reported in this paper employed L-leucyl-*p*-nitroanilide in 0.02 M Tris-HCl (pH 8.0) as the substrate in the procedure of Tuppy et al. [13]. The molar extinction coefficient at 405 nm for *p*-nitroanilide was taken as 9620 $l \cdot cm^{-1} \cdot mol^{-1}$ [13]. 1 aminopeptidase unit is defined as the amount which release 1 μmol *p*-nitroaniline from L-leucyl-*p*-nitroanilide in 1 min at 37°C, pH 8.0.

Molecular weight and subunit determinations. The gel filtration method of Andrews [14] was employed using a 1.8 X 112 cm Sephadex G-200 column and eluting with 0.02 M Tris-HCl (pH 8.0). Subunit molecular weights were estimated by SDS-(7.5%) polyacrylamide gel electrophoresis according to the procedure of Weber et al. [15]. The subunit cross-linking method of Davies and Stark [16] was used to verify the subunit structure. Homogeneity of purified enzymes was verified by polyacrylamide gel electrophoresis according to the method of Ornstein [17] and Davis [18] as modified by Clarke [19].

Effect of pH on enzyme activity. Pure aminopeptidase III was assayed at 0.02 μg/ml with 1 mM L-leucyl-*p*-nitroanilide in 0.02 M Tris-HCl over a range of pH values.

Temperature effects. A temperature profile at an enzyme concentration of 0.038 μg/ml was constructed using 1 mM L-leucyl-*p*-nitroanilide as the substrate and assaying over a range of 23–85°C. Heat

stability of aminopeptidase III was tested by incubating a 0.18 μg/ml solution of the enzyme in 0.02 M Tris-HCl (pH 8.0) without substrate at 40, 50, 60 and 70°C. Samples were removed at various times and the surviving aminopeptidase was immediately assayed at 37°C.

Inhibitor studies. 0.5 ml enzyme solution (0.35 μg/ml) was added to 0.5 ml of each of the following potential inhibitors: 2 mM iodoacetate, 1 mM EDTA, 10 mM diisopropylfluorophosphate, 2 mM 1-acetylimidazole, 1 mM acrylonitrile, 2 mM 1,10-phenanthroline, 2 mM *p*-chloromercuribenzoate and 1 mM diethylprocarbonate. The enzyme and inhibitor solutions were incubated at 25°C for 30 min and the surviving aminopeptidase was assayed.

Metal ion reactivation of EDTA-treated enzyme. An enzyme solution (0.176 μg/ml) was dialyzed for 12 h at 4°C against 0.02 M Tris-HCl (pH 8.0) made 1 mM in EDTA. EDTA was removed by dialysis at 4°C for 6 h against 0.02 M Tris-HCl (pH 8.0) prepared with deionized water. 0.9 ml of dialyzed enzyme solution was added to 0.1 ml of each of the following 10 mM metal ion solutions: $CaCl_2$, $CoCl_2$, $FeSO_4$, $MnSO_4 \cdot H_2O$, $MgCl_2$, $NiCl_2$ and $ZnCl_2$. A control received 0.1 ml deionized water in place of the metal ion solution. The mixtures were incubated at room temperature for 5 min and assayed.

Atomic absorption. Zinc content of purified aminopeptidase III was determined on a Jarrell-Ash atomic absorption spectrometer Model 813 at a protein concentration of 0.17 g/l according to the procedure given in the Jarrell-Ash Manual.

Specificity. The procedure of Goldbarg and Rutenberg [20] was used to determine aminopeptidase III specificity for a variety (see Results) of amino acid-β-naphthylamides. Hydrolysis of 1 mM naphthylamides in 0.02 M Tris-HCl (pH 8.0) was initiated with enzyme at a final concentration of 0.04 μg/ml. 1 unit of aminopeptidase is defined as the amount of enzyme that released 1 μmol β-naphthylamine/min at 37°C.

Specificity for the following peptides was determined by mixing 0.1 ml pure aminopeptidase III (28 μg/ml) with 0.9 ml of the following 1.1 mM substrate solutions in 0.05 M phosphate buffer (pH 8.0): L-α-Asp-L-Phe-L-Ala-OCH_3, Gly-L-Leu-Gly-Gly, L-Lys-L-Leu, L-Pro-L-Val-L-α-Asp and D-leucyl-*p*-nitroanilide. After 30 min of incubation at 37°C, 0.5 ml formic acid was added to the reaction mixture which were then lyophilized. The enzyme hydrolyzates were redissolved in 20 μl distilled H_2O and the hydrolysis products were determined by thin layer chromatography. The purified aminopeptidase III used in these specificity studies failed to show any endopeptidase activity when incubated at a concentration of 80 μg/ml with denatured hemoglobin over a 10 h period, and the synthetic peptide Z-Phe-Tyr-NH_2 for approx. 4 h (23°C) at a final enzyme concentration of 15 μg/ml. Hemoglobin digestion was monitored by following 280 nm absorbance and the hydrolysis of Z-Phe-Tyr-NH_2 was followed by thin layer chromatography.

Kinetic constants. 1 ml 0.4 μg/ml aminopeptidase III was mixed with 1-ml amounts of 0.05 M Tris-HCl (pH 8.0) containing various concentrations of L-leucyl-*p*-nitroanilide in a cuvette with a 1 cm light path, and incubated at 37°C in a Perkin-Elmer Hitachi, Model 200, recording spectrophotometer. The data were used to construct Lineweaver-Burk plots from which K_m and V were obtained.

Results

Enzyme production and purification. Maximum aminopeptidase activity was detected in the culture fluid of *Alteromonas* B-207 after 20–24 h incubation. A typical purification procedure is summarized in Table I. Final traces of endopeptidase activity were removed on a DEAE-cellulose column (step 7, Table I). 400 μg purified aminopeptidase III obtained from this purification procedure had a specific activity of 60 units/mg which amounted to a 350-fold purification and showed no signs of endopeptidase activity when tested with denatured hemoglobin or Z-Phe-Tyr-NH_2 as substrates.

This preparation was homogeneous by analytical polyacrylamide gel electrophoresis, SDS-polyacrylamide gel electrophoresis and Sephadex G-200 gel filtration (Fig. 1). The specific activity was constant at any point on the protein peak shown in Fig. 1.

Molecular weight and subunit structure. Aminopeptidase III moelcular weight was estimated by gel filtration and SDS-polyacrylamide gel electrophoresis. A molecular weight of 63 000 was calculated from th gel filtration data, and 33 000 by SDS-polyacrylamide gel electrophoresis.

TABLE I

ISOLATION AND PURIFICATION OF *ALTEROMONAS* B-207 AMINOPEPTIDASE III

Isolation steps	Volume (ml)	Total protein (mg)	Total endopeptidase activity (U) [a]	Total aminopeptidase activity (U) [a]	Specific aminopeptidase activity (U/mg)
1. Culture fluid	5 000	–	13 700	140	–
2. $(NH_4)_2SO_4$ precipitate; dialyzed [b]	560	638	6 470	110	0.17
3. DEAE-cellulose batch extraction	950	408	2 470	78	0.19
4. $(NH_4)_2SO_4$ precipitate; dialyzed [b]	68	236	1 360	48	0.20
5. Heat treatment: Diaflo concn. [b]	9	23	175	39	1.70
6. Sephadex G-200	53	5.7	168	36	6.31
7. DEAE chromatography	74	0.4	0	24	60.0

[a] Units (defined in Materials and Methods).
[b] Composite steps.

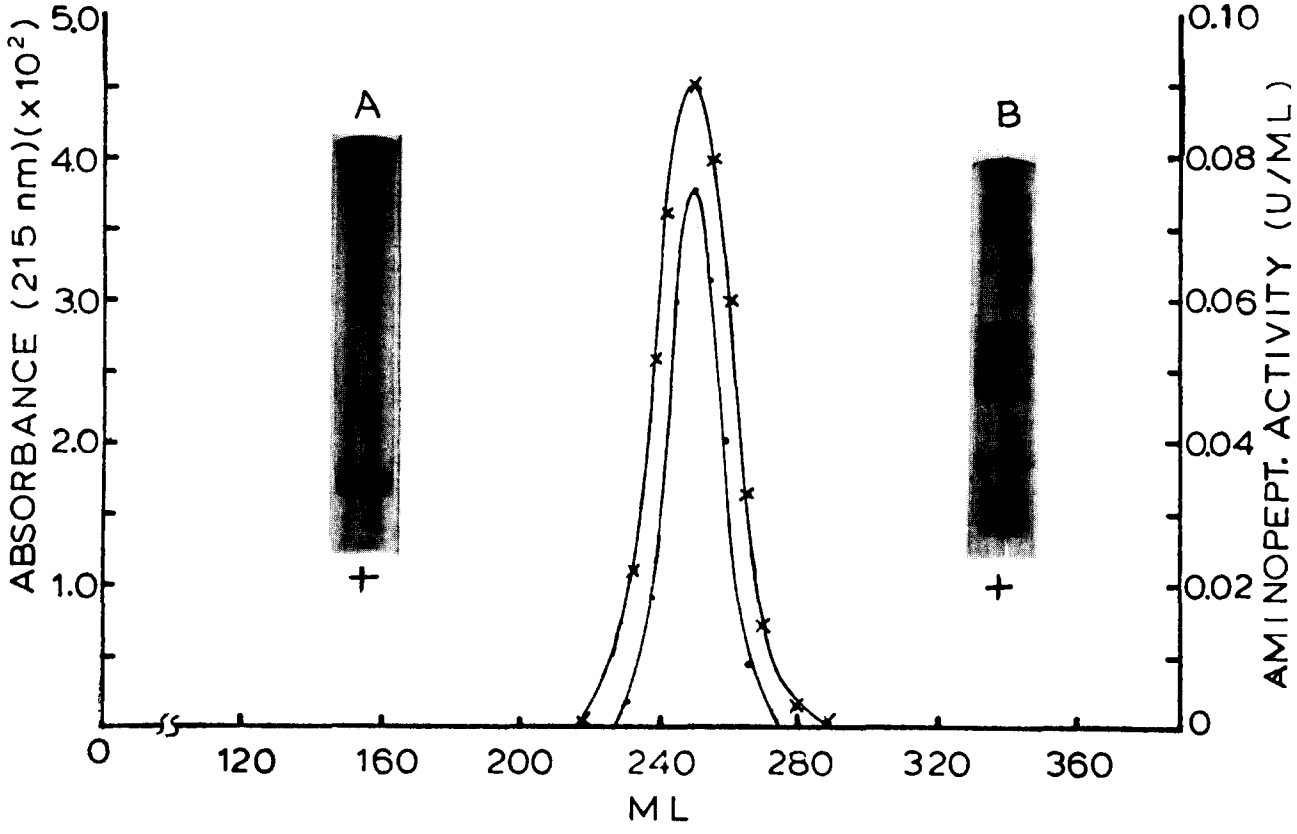

Fig. 1. Homogeneity and subunit composition of *Alteromonas* B-207 aminopeptidase III. Sephadex G-200 elution pattern obtained after DEAE-cellulose chromatography (step 7, Table I). An 0.8 ml solution containing 40 μg purified aminopeptidase III was chromatographed. X———X, represents aminopeptidase activity and ·———·, represents protein absorbance at 215 nm. Insert A: (7.5%) polyacrylamide gel electrophoresis of the above enzyme preparation. Insert B: SDS-(7.5%)polyacrylamide gel electrophoresis of the same enzyme preparation.

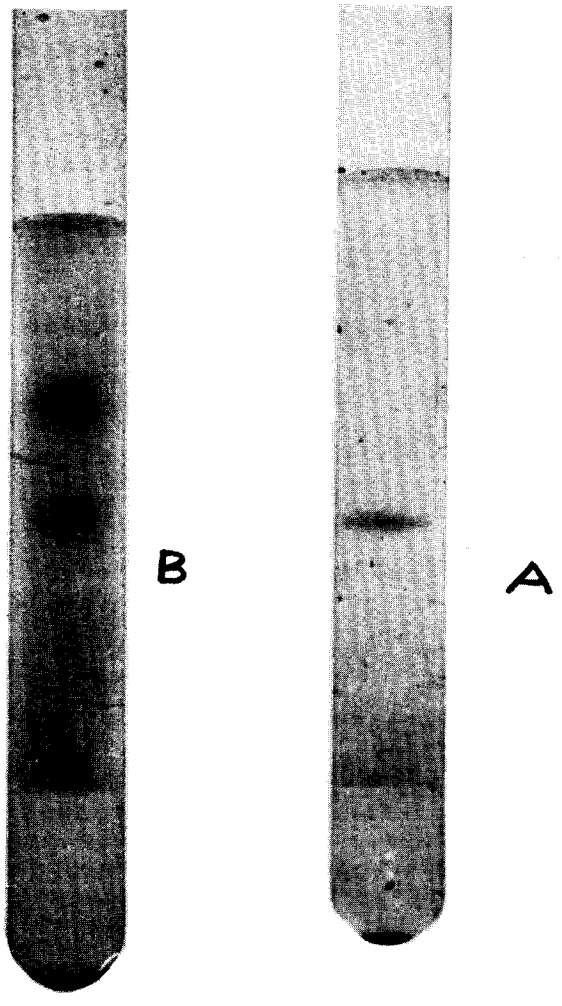

Fig. 2. SDS-(7.5%)polyacrylamide gel electrophoresis of *Alteromonas* B-207 aminopeptidase III. A, before and B, after treatment with dimethyladipimidate.

The above results indicated that aminopeptidase III is a dimeric protein. Direct proof of its being a dimer was accomplished using the cross-linking reagent dimethyladipimidate. When the purified aminopeptidase was treated with dimethyladipimidate two slightly diffused protein bands instead of a single sharp band appeared on the electrophoresis gels (Fig. 2). The electrophoretic mobility of the lower band corresponds to a molecular weight of about 36 000 and the upper band corresponds to 66 000.

Temperature optimum. Aminopeptidase III was slightly activated by incubation at 40°C over a period of 60 min, it was stable at 50°C for 60 min and approx. 50% of the activity remained after heating the enzyme at 70°C for 60 min. Optimum activity with L-leucyl-*p*-nitroanilide as the substrate occurred at 70°C, where its activity was 6-fold greater than at 37°C.

pH optimum and stability. The enzyme activity was optimum at pH 9.3, and appeared to be most stable at pH 8.0 in phosphate buffer. About 20% of its activity was lost when stored at 4°C for 12 h in Tris-HCl buffers ranging from pH 7.0–8.7.

Enzyme inhibitors. Iodoacetamide inhibited aminopeptidase activity, but none of the other inhibitors of sulfhydryl enzymes or the inhibitor of serine proteases showed any appreciable inhibition (Table II). 1,10-Phenanthroline inhibited the enzyme

TABLE II

INHIBITION OF *ALTEROMONAS* B-207 AMINOPEPTIDASE III

Compounds tested	Final concentration (mM)	Percent aminopeptidase activity [a]
Control	0	100
EDTA	0.5	100
8-Hydroxyquinoline	0.5	98
1,10-Phenathroline	1.0	0
p-Chloromercuribenzoate	1.0	100
1-Acetylimidazole	1.0	97
Diethylprocarbonate	0.5	86
Acrylonitrile	0.5	98
Diisopropylfluorophosphate	5.0	83
Iodoacetate	1.0	2

[a] Average results from two experiments are listed.

but 5 mM EDTA had no effect when added to the enzyme 30 min before adding substrate. However, dialysis of the enzyme against 1 mM EDTA destroyed the aminopeptidase activity. Approx. 45% of the activity could be restored by incubating the enzyme in 1 mM $ZnCl_2$.

Atomic absorption analysis. There was just enough pure enzyme remaining after specificity studies to obtain one measurement of its zinc content. This was done on 0.5 ml of an 0.17 mg/ml (dry wt.) sample of protein which had been thoroughly dialyzed against glass-distilled water. Assuming a 33 000 subunit weight, this amounted to a protein concentration of $5.1 \cdot 10^{-6}$ M. Zinc concentration in the same sample was determined from its absorption spectrum to be $6.1 \cdot 10^{-6}$ M. Verification of what appears to be a 1 : 1 ratio must await the purification of additional enzyme.

Peptide specificity. Table III lists the relative activities of aminopeptidase III against various L-amino acid-β-naphthylamides. Aminopeptidase III exhibited high specificity for L-Leu and slightly lower specificity for L-Phe and L-Val residues when they were combined with β-naphthylamines. The specificity of *Alteromonas* B-207 aminopeptidase III for various peptides is demonstrated in Table IV. The enzyme released N-terminal Gly, L-Leu and L-Pro much faster than N-terminal L-α-Asp or L-Lys. It did not hydrolyze D-leucyl-*p*-nitroanilide.

TABLE III

SPECIFICITY OF *ALTEROMONAS* B-207 AMINOPEPTIDASE III FOR VARIOUS L-AMINO ACID-β-NAPHTHYLAMIDES

Substrates (amino β-naphthylamide)	μmol β-naphthylamine released $min^{-1} \cdot ml^{-1}$	% relative activity [a]
L-Ala-	0.002	0.4
L-α-Asp-	0.004	0.8
L-γ-Glu-	0.001	0.2
L-Leu-	0.540	100
L-Orn-	0.004	0.8
L-Phe-	0.042	8.4
L-Ser-	0.003	0.6
L-Tyr-	0.002	0.4
L-Val-	0.031	5.6

[a] The highest activity was arbitrarily chosen as 100%.

TABLE IV

SPECIFICITY OF *ALTEROMONAS* B-207 AMINOPEPTIDEASE III FOR VARIOUS PEPTIDES AND A SYNTHETIC SUBSTRATE

The hydrolysis of these substrates were carried out at 37°C for 30 min as given in the *Specificity Studies* section. An enzyme to substrate ratio of 1 : 25 200 was used in each case.

↓ The bonds are readily cleaved. ↓ *Alteromonas* B-207 aminopeptidase III has less specificity for these bonds.

Sites of cleavage
L-α-Asp-L-Phe-L-Ala-OCH_3 (↓ a)
Gly-L-Leu-Gly-Gly (↓)
L-Lys-L-Leu (↓)
L-Pro-L-Val-L-α-Asp (↓)

[a] Hydrolytic removal of L-α-Asp was apparently rate controlling but L-Phe appeared in the hydrolyzate in amounts equal to L-Asp indicating that as L-Asp was removed, Phe was released.

Michaelis-Menten kinetics. K_m and V values of 0.20 mM L-leucyl-*p*-nitroanilide and $2.9 \cdot 10^{-2}$ units $\cdot$ min^{-1}, respectively, were obtained. A k_{cat} value of 8 650 min^{-1} was obtained with L-leucyl-*p*-nitroanilide as the substrate.

Discussion

Alteromonas B-207 produces several aminopeptidases and endopeptidases when cultured in laboratory media. We have taken advantage of the heat stability of an extracellular aminopeptidase (aminopeptidase III) and fairly rapid DEAE-cellulose batch extraction to eliminate the majority of the other proteases early in the purification procedure, so that hydrolytic destruction of aminopeptidase III was reduced.

Alteromonas B-207 aminopeptidase III appears to be composed of two subunits, each with a molecular weight of about 33 000. This is in contrast to other reported extracellular aminopeptidases of bacteria which appear to be monomeric [21].

From our limited inhibitor studies it appears that aminopeptidase III is a metalloenzyme requiring Zn^{2+} at its active site. This supposition is based on limited atomic absorption data and on the ability of Zn^{2+} to restore a large amount of the enzyme activity lost during dialysis against 10^{-3} M EDTA. Inhibition by iodoacetate and not by other sulfhydryl agents may implicate a sensitive histidyl at the active site. The enzyme shows a decided preference for L-leucine in the form of its naphthylamide, i.e., activity against this substrate is 10-fold greater than that observed with PheBNA and 100-fold greater than that obtained using other amino acid-β-naphthylamides (Table III). Leucine, glycine and proline are readily cleaved from the N-terminal position of tri- and tetra-peptides. However, charged amino acids were only slowly removed from the peptides.

Alteromonas aminopeptidase III is similat to the aminopeptidase produced by another marine bacterium, *A. proteolytica* [22] in several aspects. Both enzymes are specific for L-leucine and stable at 60–70°C for 1 h. The molecular weight of *Aeromonas* aminopeptidase, 29 000, is comparable to the molecular weight of one of *Alteromonas* aminopeptidase subunits.

In the course of the above studies we detected two intracellular aminopeptidases. The later two enzymes are the subject of another report.

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