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SELECTIVE RELEASE AND PURIFICATION OF TWO PERIPLASMIC *ALTEROMONAS* B-207 AMINOPEPTIDASES

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Two periplasmic aminopeptidases were selectively released from *Alteromonas* B-207 when its outer membrane and peptidoglycan layer were systematically removed. Neither enzyme was detected in cytoplasmic materials. The first enzyme (aminopeptidase II) was isolated and purified 160-fold from the supernatant of osmotically shocked cells. The second enzyme (aminopeptidase I) was obtained from the peptidoglycan fraction of lysozyme-treated mureinoplasts and purified 15-fold. The two enzymes are distinguished by molecular weights, subunit structures, temperature profiles, pH optima, effects of EDTA and substrate specificities. Aminopeptidase I has a molecular weight of approx. 450 000 and appears to be a tetramer; while aminopeptidase II is a stable monomer with a molecular weight of 81 000–88 000. Aminopeptidase II has higher pH and temperature optima than does aminopeptidase I. Aminopeptidase II has broader peptide specificity than aminopeptidase I. This is particularly evident when amino acid β-naphthylamides are used as substrates. Aminopeptidase I shows its greatest activity against L-α-Asp-β-naphthylamide and L-Ala-β-naphthylamide, whereas aminopeptidase II shows a decided preference for L-Leu-β-naphthylamide. Aminopeptidase I appears to be more sensitive to EDTA than aminopeptidase II, but both enzymes apparently require Zn^{2+}.

Introduction

While attempting to separate an extracellular aminopeptidase from endopeptidases which are produced by a marine species designated *Alteromonas* B-207, ATCC 33 524 [1], we discovered two additional aminopeptidases. One of the new aminopeptidases was detected in culture fluids taken from stationary phase cultures and was apparently released by cell autolysis [2]. The enzyme is unstable above 60°C and could not be isolated by the procedure used to obtain *Alteromonas* aminopeptidase III [1]. Since it is larger and migrates more slowly during gel electrophoresis than aminopeptidase III, we refer to it as *Alteromonas* aminopeptidase II.

A second intracellular enzyme was detected in cell-free fluids prepared from freeze-thawed *Alteromonas* B-207 cells. We referred to it as *Alteromonas* aminopeptidase I because it is even larger than aminopeptidase II.

Preliminary to determining the physiological role of these intracellular aminopeptidases and their possible relationship to the extracellular aminopeptidase III, the enzymes were selectively released, isolated and partially characterized.

Materials and Methods

All experiments were performed at 4°C unless otherwise noted. For details of the enzyme charac-

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terizations and assays the reader should refer to the preceding publication by Merkel et al. [1].

Isolation of crude Alteromonas B-207 aminopeptidase I and II. Approx. 100 g wet *Alteromonas* B-207 cells obtained from 12-h cultures were osmotically shocked by the procedure of Costerton et al. [3]. The shock fluid was centrifuged at $10000 \times g$ for 40 min and the supernatant was saved as the starting material for the purification of aminopeptidase II. The pellet (mureinoplasts) was gently resuspended in 1 l 0.5 M sucrose prepared in 0.05 M Tris-HCl (pH 7.0) containing 10 mg lysozyme (Worthington Biochem Corp.), incubated on a rotary shaker at 25°C for 30 min, centrifuged at $10000 \times g$ for 40 min, and the supernatant was saved as crude aminopeptidase I. Except for metal ion studies, Tris-HCl buffer routinely contained $2 \cdot 10^{-5}$ M $CaCl_2$, $MgCl_2$ and $ZnCl_2$.

Purification of aminopeptidase I. Crude aminopeptidase I was extracted with 350 g filter-dried DEAE-cellulose which was equilibrated with 0.02 M Tris-HCl (pH 6.7). Aminopeptidase I was eluted with 1.1 l 0.02 M Tris-HCl (pH 6.7)/0.4 M NaCl. The precipitate forming at 85% satd. $(NH_4)_2SO_4$ was collected by centrifugation, dissolved in a minimum amount of 0.02 M Tris-HCl (pH 6.7), dialyzed against $4.0 \cdot 10^{-3}$ M Tris-HCl (pH 6.7), and applied to a DEAE-cellulose column (1.5 × 36 cm). The enzyme was eluted with a linear 0–0.3 M NaCl gradient in 0.02 M Tris-HCl (pH 6.7) (5-ml fractions; flow rate, 1 ml/min). Fractions containing aminopeptidase I were pooled and concentrated by $(NH_4)_2SO_4$ precipitation. After dialysis in buffer aminopeptidase I was concentrated by ultra-filtration and applied to a Sephadex G-200 column (1.8 × 117 cm); 5-ml fractions were collected (flow rate, 0.2 ml/min) and the active fractions were applied to a DEAE-cellulose column (1.8 × 8.5 cm). A linear 0–0.3 M NaCl gradient in 0.02 M Tris-HCl (pH 6.7) was used to elute active fractions.

Purification of aminopeptidase II. Purification of aminopeptidase II was achieved as above but omitting the second DEAE-cellulose column.

Assays. Protein concentrations were determined by the method of Lowry et al. [4].

Endopeptidase and aminopeptidase activities were measured according to the procedures described in the preceding publication [1].

Homogeneity. Analytical polyacrylamide gel electrophoresis in 7.5% gels was used to verify homogeneity of the purified enzyme preparations [5].

Molecular weight and subunit determination. The molecular weights of aminopeptidase I and II were determined by the method of Andrews [6]. SDS-5% polyacrylamide gel electrophoresis was used to determine the molecular weight of each subunit [7].

Effect of pH on enzyme activity. Pure aminopeptidases I and II were assayed at 2.5 and 0.16 μg protein/ml, respectively, with 1 mM L-leucyl-*p*-nitroanilide (Leu-*p*-nitroanilide) at a range of pH values. Storage stability of each enzyme was tested in the above buffers by incubating the enzyme solutions at 4°C for 12 h and then assaying for aminopeptidase at each of the temperatures shown in the Results section.

Temperature effects. Temperature profiles at an aminopeptidase I concentration of 4.0 μg/ml and aminopeptidase II concentration of 0.57 μg/ml were constructed. Heat stabilities of aminopeptidase I and II were tested by incubating 3.3 μg(I)/ml and 0.39 μg(II)/ml enzyme solutions in 0.02 M Tris-HCl buffer, pH 8.0, without substrate at 40, 50, 60 and 70°C. Samples were withdrawn at fixed intervals and surviving aminopeptidase was immediately assayed at 37°C.

Inhibitor studies. 0.5 ml of 6.7 μg/ml aminopeptidase I or 0.49 μg/ml aminopeptidase II was added to 0.5 ml of each of the potential inhibitor solutions and assayed for aminopeptidase.

Specificity studies. The procedure of Goldbarg and Rutenberg [8] was used to determine specificity for a variety (see Results) of amino acid-β-naphthylamides. Aminopeptidase I and II were reacted with 1 mM β-naphthylamides in 0.02 M Tris-HCl (pH 8.0) at final enzyme concentrations of 0.8 μg/ml and 0.29 μg/ml, respectively.

Peptide specificity was also tested with four synthetic peptides and D-leucyl *p*-nitroanilide at final enzyme concentrations of 5.7 μg/ml aminopeptidase I and 3.3 μg/ml aminopeptidase II.

Kinetic constants. 1 ml of 4.3 μg/ml aminopeptidase I or 0.92 μg/ml aminopeptidase II was mixed at 37°C with 1 ml amounts of 0.05 M Tris-HCl (pH 8.0), containing various concentrations of Leu-*p*-nitroanilide in a cuvette with a 1-cm light

path and absorbance changes were followed on a Perkin-Elmer Hitachi, Model 200, recording spectrophotometer. Lineweaver-Burk plots were used to determine K_m and V values.

Results

Enzyme isolation and purification. Aminopeptidase I and II were selectively isolated from *Alteromonas* B-207 peptidoglycan fractions and shock fluid, respectively. Typical purification procedures are summarized in Tables I and II. A 15-fold purification of aminopeptidase I and a 160-fold purification of aminopeptidase II was achieved. The two preparations were proved to be homogeneous by analytical gel electrophoresis (Fig. 1).

Molecular weight and subunit structure. The molecular weights of the two enzymes were estimated by gel filtration and SDS-polyacrylamide gel electrophoresis. A molecular weight of 450 000 was calculated from the gel filtration data of aminopeptidase I, and 113 000 by SDS-polyacrylamide gel electrophoresis. These results indicate that it is probably a tetrameric protein. The molecular weight of aminopeptidase II was estimated to be 88 000 by gel filtration and 81 000 by SDS-polyacrylamide gel electrophoresis.

TABLE I

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

Isolation steps	Volume (ml)	Total protein (mg)	Total activity (units)
1. Peptidoglycan [a] fraction	915	1 200	153
2. DEAE-batch extraction	1 175	780	111
3. $(NH_4)_2SO_4$ precipitation; dialysis	180	365	30
4. DEAE-cellulose chromatography	225	182	21
5. $(NH_4)_2SO_4$ [b] precipitation; dialysis	8.5	170	19
6. Sephadex	52	43	12
7. DEAE-cellulose chromatography	140	5.7	11.1

[a] Peptidoglycan fraction is the supernatant fluid of lysozyme-treated mureinoplasts.

[b] Steps No. 3 and 5 are composite steps.

TABLE II

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

Isolation steps	Volume (ml)	Total protein (mg)	Total activity (units)
1. Shock fluid	570	1 830	160
2. DEAE-batch extraction	1 100	1 050	126
3. $(NH_4)_2SO_4$ precipitation; dialysis [a]	57	228	76
4. DEAE-cellulose chromatography	82	11	52
5. $(NH_4)_2SO_4$ precipitation; dialysis [a]	5	8	44
6. Sephadex G-200	65	2.1	30

[a] Steps No. 3 and 5 are composite steps.

Temperature effects. Aminopeptidase I was stable at 35°C for 60 min and 40°C for a period of 20 min. After incubating the enzyme at 50°C for 10 min, approx. 10% of the activity remained. Its optimum activity, as measured by following the hydrolysis rate in a recording spectrophotometer occurred at 45°C (Fig. 2).

Aminopeptidase II was stable at 40°C and slightly activated at 50°C for 60 min. At 60°C the enzyme was activated during the first 30 min and then its activity gradually declined to 80% of the starting activity. However, at 70°C its activity decreased rapidly with incubation time. The temperature optimum of aminopeptidase II occurred at 50°C (Fig. 2).

pH optimum and stability. Aminopeptidase I exhibited optimum activity at pH 7.5 (Fig. 3). The enzyme was most stable at pH 6.2. The pH optimum of aminopeptidase II occurred at pH 8.2 (Fig. 3) and it was most stable at pH 7.5.

Enzyme inhibitors. Both EDTA and 1,10-phenanthroline inhibited aminopeptidase I (Table III). When dialyzed against EDTA it was irreversibly denatured. Approx. 20 and 45% of aminopeptidase II was reversibly (by readdition of Zn^{2+}) inhibited by EDTA and 1,10-phenanthroline, respectively. Diisopropyl-fluorophosphate failed to inhibit either aminopep-

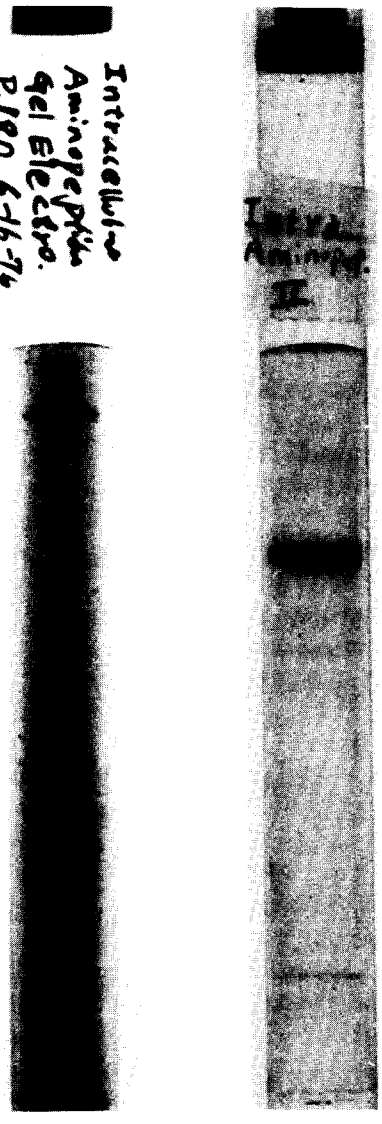

Fig. 1. Comparison of the electrophoretic mobilities of aminopeptidase I and II in analytical, native polyacrylamide gels (7.5%). The gel on the left (a) received 50 μl purified aminopeptidase I (Step 7, Table I) at a protein concentration of approx. 85 μg/ml. The gel on the right (b) received 50 μl purified aminopeptidase II (Step 6, Table II) at a protein concentration of approx. 177 μg/ml. Electrophoresis conditions were basically the same for each protein.

tidase I or II, ruling out the possibility of their being serine proteases, and apparently neither enzyme possesses a sensitive thiol group as evidenced by the failure of *p*-chloromercuribenzoate and acrylonitrile to inhibit. The results (Table III) from the addition of

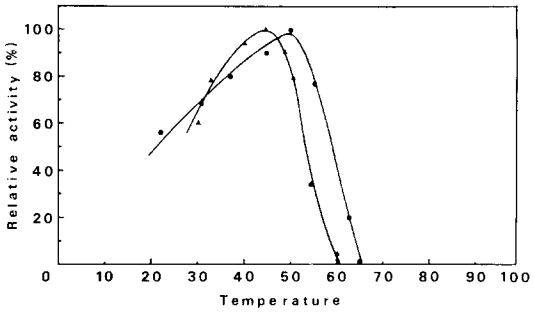

Fig. 2. Effect of temperature on the activities of aminopeptidase I and II. ▲——▲, aminopeptidase I; ●——●, aminopeptidase II. Maximum activity under the assay conditions was arbitrarily taken as 100%. See Materials and Methods for experimental details.

potential histidyl-specific reagents (diethylpyrocarbonate and iodoacetate) are equivocal.

Peptide specificity. Table IV lists the relative activities of aminopeptidase I and II against a series of L-amino acid-β-naphthylamides. Aminopeptidase I had high specificity for N-terminal L-α-Asp and L-Ala, and slightly less specificity for L-α-Glu, L-Leu and L-Orn. Aminopeptidase II had relatively high specificity for L-Leu and slightly less for L-Phe in the series of β-naphthylamides tested (Table IV).

The specificity of aminopeptidase I for various peptides is demonstrated in Table V. It had high specificity for L-α-Asp, removed Gly, L-Phe and L-Leu slowly, and did not hydrolyze peptides of L-Pro or L-Lys. Aminopeptidase II had broader

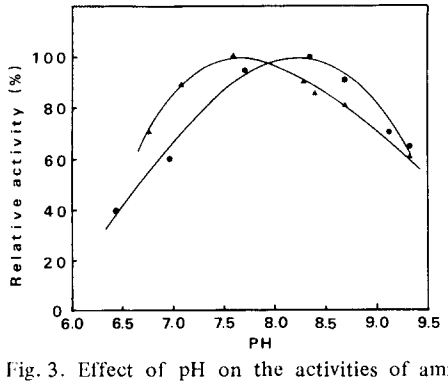

Fig. 3. Effect of pH on the activities of aminopeptidase I and II. ▲——▲, aminopeptidase I; ●——●, aminopeptidase II. Maximum activity under the assay conditions was arbitrarily taken as 100%. See Materials and Methods for experimental details.

TABLE III

INHIBITION STUDIES OF *ALTEROMONAS* B-207 AMINOPEPTIDASE I AND II

Compounds tested	Final concentrations of compounds (mM)	% Relative aminopeptidase activity [a]	
		I	II
None	0	100	100
EDTA	0.5	0	80
8-Hydroxyquinoline	0.5	98	83
Diethylpyrocarbonate	0.5	73	74
Acrylonitrile	0.5	100	91
1,10-Phenanthroline	1.0	11	55
1-Acetylimidazole	1.0	78	68
p-Chloromercuribenzoate	1.0	95	100
Iodoacetate	1.0	61	95
Diisopropylfuorophosphate	5.0	100	97

[a] Average results from two experiments are listed.

specificity toward these same peptides as shown in Table V. This enzyme removed N-terminal Gly, L-Leu, L-Phe, L-Pro and L-Val. L-Lys was hydrolyzed from the dipeptide L-Lys-L-Leu at a slow rate. Neither enzyme attacked D-leucyl-*p*-nitroanilide.

Michaelis-Menten kinetics. K_m and V values of aminopeptidase I obtained from a Lineweaver-Burk plot are 0.20 mM Leu-*p*-nitroanilide and $2.9 \cdot 10^{-2}$ units $\cdot$ min^{-1}. The calculated k_{cat} value is 2 130 min^{-1}. K_m and V values of aminopeptidase II are 0.38 mM Leu-*p*-nitroanilide and $1.6 \cdot 10^{-2}$ units $\cdot$ min^{-1}. Its k_{cat} is 3 230 min^{-1}.

TABLE IV

SPECIFICITIES OF *ALTEROMONAS* B-207 AMINOPEPTIDASE I AND II FOR VARIOUS L-AMINO ACID-β-NAPHTHYLAMIDES

Substrates (aminoacyl-β-naphthylamide)	% Relative activity [a]	
	I	II
L-Ala-	76	35
L-α-Asp-	100	2
L-α-Glu-	34	1
L-Leu-	25	100
L-Orn-	18	24
L-Phe-	10	15
L-Ser-	10	1
L-Tyr-	10	10
L-Val-	2	6

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

TABLE V

SPECIFICITIES OF *ALTEROMONAS* B-207 AMINOPEPTIDASE I AND II FOR VARIOUS PEPTIDES

↓ Bonds are readily cleaved; ↓ bonds cleaved at a slow rate.

Sites of cleavages	
Aminopeptidase I [a]	Aminopeptidase II
L-α-Asp-L-Phe-L-Ala-OCH_3	L-α-Asp-L-Phe-L-Ala-OCH_3
Gly-L-Leu-Gly [b]-Gly	Gly-L-Leu-Gly-Gly
L-Lys [b]-L-Leu	L-Lys-L-Leu
L-Pro [b]-L-Val [b]-L-α-Asp	L-Pro-L-Val-L-α-Asp
D-Leu [b]-*p*-nitroanilide	D-Leu [b]-*p*-nitroanilide

[a] Enzyme to substrate ratio = 1 : 24 600.
[b] Bonds not hydrolyzed by the enzyme.

Discussion

Initially two aminopeptidases were isolated from the soluble portion of freeze-thawed cells but the yields were extremely low because of the presence of large amounts of cytoplasmic material. Apparently, the cytoplasmic proteolytic enzymes hydrolyzed them rapidly, and the viscous intracellular material complicated and slowed the purification procedure. However, by the use of an osmotic shock procedure followed by lysozyme treatment of the resulting mureinoplasts aminopeptidase I and II were selectively released from *Alteromonas* B-207 cells without disrupting a significant amount of the cytoplasmic membranes of the cells. This was evidenced by electron microscope studies (data not shown), by the observation that no viscous material appeared in the soluble osmotic shock fluid nor in the peptidoglycan fraction, and by monitoring the absorbance of shock fluids and lyzates at 260 nm. Aminopeptidase II is found in the periplasmic spaces of the bacteria, while aminopeptidase I appears to be loosely attached to

the peptidoglycan layer in the cells.

Differences in molecular weights, pH and temperature optima, peptide specificities and inhibition by metal chelators indicate that aminopeptidase I and II are different enzymes. The relationship of these two enzymes to aminopeptidase III, the extracellular enzyme described in the preceding publication [1] remains to be determined. In terms of heat resistance, metal requirement and peptide specificity, aminopeptidase II bears some resemblance to the extracellular aminopeptidase III. Future comparative peptide mappings [9] of the three aminopeptidases may reveal homologies in this group of enzymes.

The functional roles of periplasmic aminopeptidases are probably important to the physiology of gram-negative bacteria because various aminopeptidases have been detected in them [10–12]. Lazdunski et al. [13–14] isolated an aminopeptidase specific for L-Ala-*p*-nitroanilide from the periplasmic region of *Escherichia coli*. Recently Cerny [12] demonstrated a correlation between Lazdunski's aminopeptidase and an interpeptide bridge component in the peptidoglycan of many gram-negative strains. However, the function of aminopeptidases in cell wall biosynthesis is still to be elucidated.

Although its molecular weight differs from that of the *E. coli* enzyme, aminopeptidase I may belong to the above group since its optimum pH (pH 7.5) and high specificity for L-Ala-*p*-nitroanilide are similar to Lazdunski's enzyme [14]. The other possible roles of aminopeptidase I and functions of aminopeptidase II are more speculative. They might function in the utilization of peptides as sources of amino acids [15], or they might play a role in the removal of the hydrophobic (leader or signal) peptides at N-terminal ends of nascent proteins [16].

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