原著

Aminopeptidases from *Bordetella pertussis* and rat brain: Comparison between bacterial and mammalian proteins

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Abstract

Through screening of proteases derived from *Bordetella pertussis*, we identified an alanine aminopeptidase (AAP) activity capable of specifically degrading alanyl β-naphthylamide (Ala-βNA). We subsequently conducted enzymatic studies to determine the differences between the AAP from *B. pertussis* and a rat brain-derived protease that has the same substrate specificity. After a series of purification processes, the pertussis AAP activity was purified 90-fold, and the rat brain AAP was purified 18-fold. The optimum pH for the pertussis AAP was approximately 8.0, and activity was maintained up to pH 10. For the rat brain AAP, the optimum pH was approximately 6.5 to 7.0, and 50% of the activity was retained even at pH 5.0 or pH 9.0. Although strong inhibition by *p*-chloromercuribenzoic acid (PCMB) was observed, both enzymes are most likely to be metallo-enzymes, as evidenced by inhibition by bestatin and o-phenanthroline. This report is, to our knowledge, the first description of the enzymatic properties of AAP in *B. pertussis*, including classification of the activity as a cytosolic alanine aminopeptidase (EC 3.4.11.14). The enzyme is stable between pH 7 and 10, with pH 8 being the optimum. It retained some activity at lower temperatures, exhibiting 20% activity at 10°C, but had less activity at temperatures exceeding 40°C. We have now completed cloning of the *B. pertussis* AAP-encoding gene and are investigating expression of recombinant AAP protein in *Escherichia coli* and its enzymatic activity.

Introduction

Bordetella pertussis is an aerobic, highly infectious, Gram-negative coccobacillus that causes whooping cough (pertussis), an acute respiratory tract infection in humans

characterized by paroxysmal coughs and inspiratory stridor (1,2,3).

B. pertussis utilizes amino acids, not sugars, as its energy and nitrogen sources. Based on its characteristics, we postulated that there may be specific proteases that are necessary for the release of amino acids from substrate components in media. We therefore conducted screening for proteases derived from *B. pertussis* and detected an alanine aminopeptidase (AAP) activity that corresponds to an enzyme that specifically breaks down alanyl β-naphthylamide (Ala-βNA).

We therefore purified AAP from *B. pertussis* to determine the protein's enzymatic properties. For comparison, we also purified a rat brain AAP that is known to have the same substrate specificity (4) and examined the differences between these two enzymes.

Materials and Methods

1. Materials

Rat brain was collected from SPF male Wistar rats averaging 12 weeks of age obtained from Japan SLC (Hamamatsu, Japan). Fast garnet GBC salt, diisopropyl-phosphorofluoridate (DFP), Bestatin, and Ala-βNA were purchased from Sigma (St. Louis, MO, USA). Phenylmethylsulfonyl fluoride (PMSF), *p*-chloromercuribenzoic acid (PCMB), and *o*-phenanthroline were purchased from Wako (Osaka, Japan).

2. Assay for alanine aminopeptidase activity

The enzyme activity was assayed by the method of Yoshimoto et al., with slight modifications for the use of Ala-βNA as the substrate (5,6). Briefly, 100 µL of enzyme suspension was combined with 850 µL of 20 mM Tris-HCl buffer, pH 7.6, and the mixture was preincubated at 37°C for 3 min. The reaction was initiated by adding 50 µL

of 3 mM Ala-βNA. After a 30-min incubation at 37°C, the reaction was stopped and color was developed by adding 0.5 mL of Fast garnet GBC (1mg/mL in 1 M acetate buffer, pH 4.0, containing 10% Triton X-100). The absorbance was measured at 550 nm at room temperature. One unit of the enzyme activity was defined as the amount of the enzyme that released 1 μmol of ß-naphthylamine per min at 37°C. The concentration of the enzyme protein was estimated spectrophotometrically by assuming an extinction coefficient of 10 (for a 1% solution, 1-cm path length, at 280 nm), with enzyme activity expressed in units per mg protein.

3. Cultivation of *B. pertussis* and growth conditions

The *B. pertussis* strain Tohama (7) was grown for 72 h at 37°C on Bordet-Gengou (BG) agar (Difco, Detroit, MI) supplemented with 1% glycerol, 20% defibrinated sheep blood. Liquid cultures of *B. pertussis* were grown as described previously in Stainer-Scholte medium (8). Exponentially growing *B. pertussis* was inoculated into 2.5 mL of Stainer-Scholte medium to an optical density of 0.15 at 600 nm and grown for 24 h at 37°C. Following cultivation, cells were collected by centrifugation and washed with Dulbecco's modified phosphate-buffered saline (DPBS, pH7.4) and disrupted by sonication (Bioruputor UCD-300, 4 mins, on ice). The disrupted cell suspension was used for further purification of the bacterial enzyme.

4. Purification of enzyme

All purification procedures were carried out at 4°C. The disrupted *B. pertussis* cell suspension was diluted 10-fold with 20 mM Tris-HCl buffer, pH 7.6, and fractionated by ammonium sulfate precipitation at 30 to 80% saturation. The purification method

was as previously described (5,9). Briefly, after Toyopearl HW65F gel filtration and DEAE-Toyopearl affinity chromatography, the active fraction was concentrated using ammonium sulfate precipitation. The precipitate was dissolved in 20 mM sodium phosphate buffer, pH 7.6, and subjected to chromatography on a hydroxyapatite column (3 × 10 cm). The active fractions were pooled, and then concentrated using a Vivapore concentrator (Vivascience).

For purification of the rat brain enzyme, rat brain (15 g) was homogenized in 150 mL of 20 mM Tris-HCl buffer, pH 7.6, using a glass Teflon homogenizer. As with purification of *B. pertussis*, after Toyopearl HW65F gel filtration, the active fraction was concentrated using ammonium sulfate precipitation. The precipitate was dissolved in 20 mM phosphate buffer, pH 7.6, and subjected to chromatography on a hydroxyapatite column (3 × 10 cm). The active fractions were pooled, and then concentrated using a Vivapore concentrator (Vivascience).

5. pH and temperature stability

pH stability was assessed using 20 mM sodium acetate buffer for the interval spanning pH 4.0 to 6.0; 10 mM sodium phosphate buffer for the interval spanning pH 6.0 to 8.0; and 20 mM Tris-HCl buffer for the interval spanning pH 8.0 to 10.0. Activity at each of the various pHs was tested using the same method as described above for determination of enzyme activity. Temperature stability was performed at temperatures of 10°C, 20°C, 30°C, 37°C, 40°C, 50°C, and 60°C. Activity at each of the various temperatures was tested using the same method as described above for determination of enzyme activity. Relative activities were calculated by comparison to that of the respective enzyme at pH 7.6 (optimal pH of *B. pertussis*) and 37°C, as shown in the

assay for alanine aminopeptidase activity of *B. pertussis*.

Curve fitting was performed by approximating curve, using Microsoft Excel.

Results

1. Purification of alanine aminopeptidase activities from *B. pertussis* and rat brain

The purifications of the enzymes are summarized in Table 1. The enzymes from *B. pertussis* and rat brain were partially purified by 90-fold and 18-fold, respectively, with an activity recovery of approximately 20% and 8%, respectively. As shown in the chromatograms provided in Fig. 1, under the same chromatographic condition, the enzyme from *B. pertussis* did not adsorb onto the hydroxyapatite to flow through the column, while that from rat brain was eluted at a phosphate concentration of about 50 mM.

Table 1. Partial purification of alanine aminopeptidase from *Bordetella pertussis* and rat brain.

step	*B.pertussis*				rat brain			
	Enzyme activity			Purification ratio	Enzyme activity			Purification ratio
	Unit	Recovery	S.A.		Unit	Recovery	S.A.	
Sonication	10.6	100	0.027	1	50.2	100	0.193	1
DEAE-Toyopearl	8.7	82.2	0.825	30.6	16.1	32.1	1.687	8.7
Toyopearl HW65F	8.2	77.0	1.391	51.6	10.7	21.3	2.353	12.2
hydroxyapatite	2.4	22.4	2.439	90.4	4.0	8.0	3.380	17.5

Recovery; %, S.A. ; specific activity （U / mg protein）

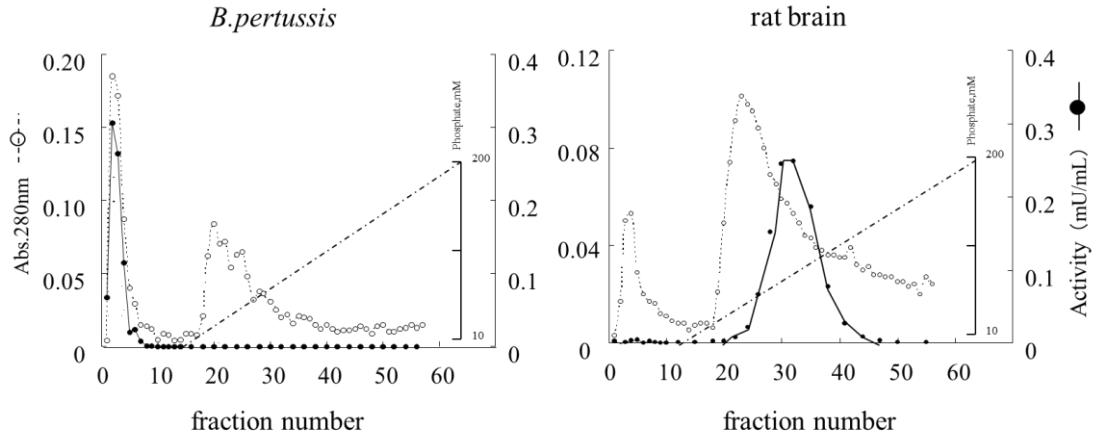

Fig. 1. Chromatography of alanine aminopeptidase on hydroxyapatite.
Step 3 fraction was applied to the column (1.8 ×15 cm) equilibrated with 10 mM phosphate buffer, pH 7.0. Elution was performed with a linear gradient of phosphate buffer (10 to 200 mM).

2. pH and thermal stability

As shown in Fig. 2, the enzyme from *B. pertussis* was stable between pH 7 and 10, with pH 8 being the optimum. However, the enzyme from rat brain showed a narrower stability range between pH 6 and 8, with pH 6.5 to 7.0 being the optimum; the activity at pH 8 was less than 80% of the maximum. The mammalian enzyme was more stable under acidic conditions than under basic conditions. Little activity was detected for the bacterial enzyme at acidic pH 5.

Temperature stability is shown in Fig. 3. The enzyme from *B. pertussis* retained some activity at lower temperatures, exhibiting 20% activity at 10°C, but had less activity at temperatures exceeding 40°C. In contrast, the rat brain enzyme retained some activity at higher temperatures of 40 to 50°C, but was inactive at temperatures of less than 20°C.

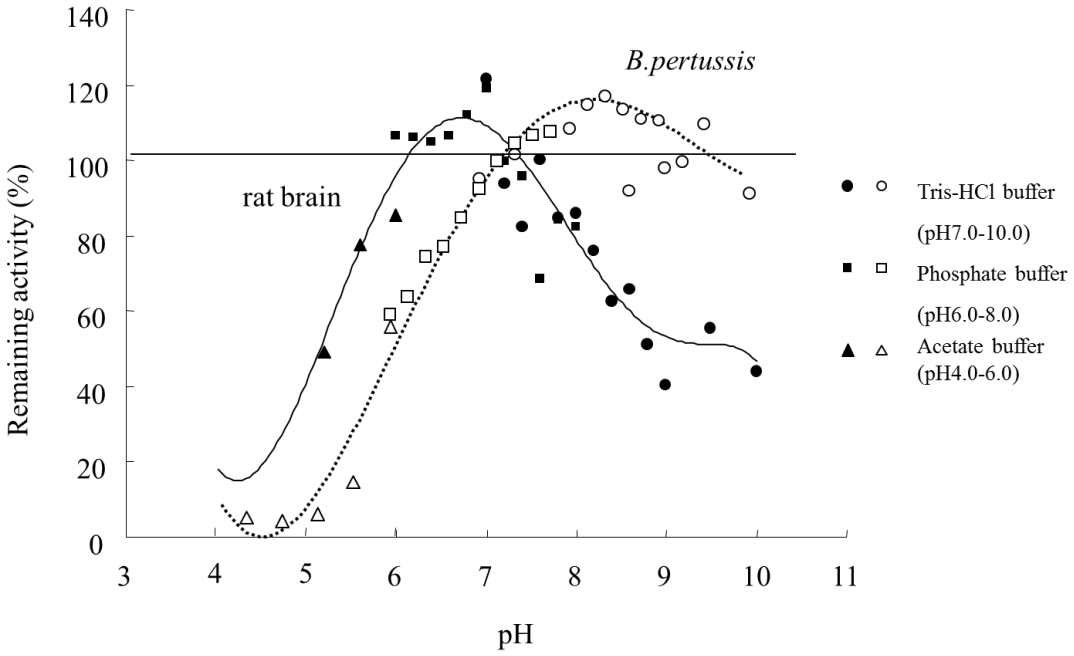

Fig. 2. pH stability of alanine aminopeptidase from *Bordetella pertussis* and rat brain.
pH stability was estimated by using 20 mM acetate buffer at 4.0–6.0, 10 mM phosphate buffer at 6.0–8.0 and 20 mM Tris-HCl buffer at 8.0–10.0. These buffers were used with the same method of enzyme activity.

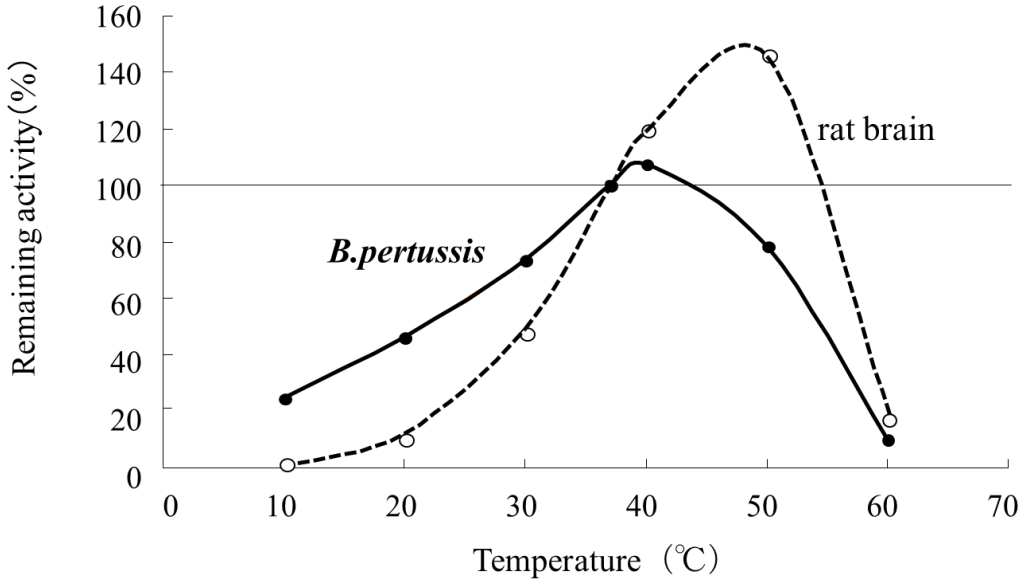

Fig.3 Temperature stability of alanine aminopeptidase from *Bordetella pertussis* and rat brain.
Temperature stability was estimated at 10°C, 20°C, 30°C, 37°C, 40°C, 50°C and 60°C respectively. These temperatures were used with the same method of enzyme activity. Relative activity against pH 7.6 and 37°C was calculated for each reaction.

3. Effects of various peptidase inhibitors

The effects of various inhibitors on the purified enzymes from *B. pertussis* and rat brain are shown in Table 2. Both enzymes were almost completely inhibited by bestatin and o-phenanthroline, indicating that those are metallo-enzymes. Although only a partial inhibition by EDTA was observed, this is usual for many metallo-peptidases including alanyl aminopeptidase (aminopeptidase N). PMSF partially inhibited the enzymes but DFP had no effect at all. PCMB showed relatively strong inhibition. Its bulky structure often affects the enzyme activity that is not dependent on thiol residue. In addition, divalent metal ions, $ZnCl_2$ and $MnCl_2$, strongly inhibited the activity.

Table 2. Effects of metal and various inhibitors on the purified alanine aminopeptidase from *Bordetella pertussis* and rat brain. The enzyme was incubated with each additive at 37°C and for 10 min, and the residual activities of the reaction mixture were assayed under the standard conditions.

reagent	final conc. (mM)	*B.pertussis* (%)	rat brain (%)
None	-	100	100
PCMB	0.11	7	16
PMSF	1	69	19
DFP	0.1	102	105
Bestatin	0.5	0	0
EDTA	1	76	33
o-phenanthroline	1	0	1
$ZnCl_2$	1	1	6
$MnCl_2$	1	9	25
$CoCl_2$	1	85	38
$MgCl_2$	1	94	81

Discussion

In the present study, we purified an AAP from *B. pertussis* and examined its enzymatic properties, including comparison to an AAP from rat brain.

B. pertussis proliferates by selectively utilizing amino acids as energy and nitrogen sources. We therefore postulated that this organism produces one or more specific proteases to digest proteins and peptides to amino acids. By conducting a screen for proteases derived from *B. pertussis*, we detected an activity that specifically breaks down Ala-βNA (data not shown).

In a comparison of pH stability, the pertussis AAP exhibited an optimal pH of approximately 8.0, and activity was retained at pHs up to 10, indicating that this enzyme prefers weakly basic environments to acidic environments. Optimal pH in growth of *B. pertussis* is 7.6, and the bacterium alkalifies the culture medium from pH 7.6 to about 9.0 as it grows (11,12). The pH stability result of the enzyme is consistent with the preferred pH of *B. pertussis*.

The rat brain AAP showed an optimal pH of approximately 6.5 to 7.0. The mammalian enzyme retained 50% activity at pH 5.0; but activity was 50% or less at approximately pH 9.0.

In an examination of thermal stability, the pertussis AAP retained approximately 20% activity at temperatures as low as 10°C, but rapidly lost activity at temperatures exceeding 40°C. This result is consistent with the optimal temperature of *B. pertussis*, which is 35–36.5°C, and multiplication stops at more than 37°C (11,12). In fact, growth of *B. pertussis* is inhibited at higher than 37°C.

By contrast, rat brain AAP had an optimal temperature of approximately 50°C, exhibiting a rapid loss of activity at temperatures of 30°C or less. We can consent to the

cases that a rat has a high temperature more than 38°C also being expected and being a result higher temperature than *B. pertussis* optimum. The rectum temperature of the rat is considered to be about 38°C, and it may be higher in the brain. Therefore, a rat brain seems to have an optimum temperature range higher than *B. pertussis.*

Unfortunately, both enzymes were still in partially purified form, so the molecular mass of these enzymes was not exclusively shown. However, characterization of aminopeptidase N (EC3.4.11.2) from *E. coli* has already been reported (10). The *E. coli* enzyme has an Mr of 99,000 and belongs to clan MA, family M1; the *E. coli* enzyme incorporates zinc in its active center (4). In humans, aminopeptidase N is also known to possess significant physiological functions as a receptor, irrespective of its enzymatic function. For example, human aminopeptidase N, which has been identified as a CD13 antigen, is known as a receptor for coronavirus transmissible gastroenteritis virus and human coronavirus 229E (13,14).

Based on differences in pH and thermal stability, it was demonstrated that the enzymatic properties differ between these two AAPs, even though the two enzymes are both proteases that degrade the same substrate. Examination of inhibition profiles revealed that both enzymes were completely inhibited by both bestatin and *o*-phenanthroline, suggesting that both activities depend on the presence of metals at the active sites. Moreover, $ZnCl_2$ and $MnCl_2$ also inhibited both enzymes. *E. coli* pepN is also inhibited by these metal ions and an inhibition of metallo-peptidase by divalent metal ions is often observed. As shown in Fig.1, the chromatogram of each AAP on hydroxyapatite differs between enzymes. While an enzyme of *B. pertussis* is eluted through 10mM phosphate buffer, AAP of rat brain is eluted through a high concentration of phosphate buffer (about 100 mM). These results indicate that

conformation, amino acid sequences or electrification of APP of *B. pertussis* is different from rat brain.

Although the pertussis enzyme exhibits the same substrate specificity as *E. coli* PepN, further investigations, including structural determinations, are planned in the future.

At the present time, we have completed cloning of the *B. pertussis* gene that encodes this AAP activity and are confirming protein expression and enzyme activity. We are seeking to further elucidate the role of this enzyme in *B. pertussis*, and hypothesize that this protein may be of use as an antigen of a future pertussis vaccine.

Acknowledgments

We are grateful to Dr. T. Yoshimoto for his useful advice throughout these experiments.

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