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# Substrate Specificity and Thermostability of the Dehairing Alkaline Protease from *Bacillus pumilus*

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**Abstract** An alkaline protease (DHAP) from *Bacillus pumilus* has shown great potential in hide dehairing. To get better insights on its catalytic properties for application, the substrate specificity and thermostability were investigated using five natural proteins and nine synthetic peptides. The results showed that DHAP could hydrolyze five proteins tested here in different specificity. Collagen, a component of animal skin, was more resistant to hydrolysis than casein, fibrin, and gelatin. Among the synthetic peptides, the enzyme showed activity mainly with tetrapeptide substrates with the catalytic efficiency in order of Phe>Leu>Ala at P1 site, although $k_m$ value for AAVA-pN is much lower than that for AAPL-pN and AAPF-pN. With tripeptide substrates, smaller side-chain group (Gly) at P1 site was not hydrolyzed by DHAP. The enzyme showed good thermostability below 60 °C, and lost activity so quickly above 70 °C. The thermostability was largely dependent on metal ion, especially $Ca^{2+}$, although other ions, like $Mg^{2+}$, $Mn^{2+}$, and $Co^{2+}$, could sustain stability at certain extent within limited time. $Cu^{2+}$, $Fe^{2+}$, as well as $Al^{3+}$, did not support the enzyme to retain activity at 60 °C even in 5 min. In addition, the selected metal ions could coordinate calcium in improvement or destruction of thermostability for DHAP.

**Keywords** *Bacillus pumilus* · Alkaline protease · Substrate specificity · Thermostability · Metal ion · Dehairing

## Introduction

Alkaline proteases are ubiquitous in occurrence in microbial world and have been widely used in various industries, especially in the food processing, detergent additives, and leather tanning, etc [1]. In addition, the alkaline proteases from bacteria have been intensively studied in protein folding, molecular structure, and catalytic kinetics and mechanism of function [2].

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In tanning industry, alkaline proteases have been shown to be prospective in application to various aspects due to the environmental problems [3]. Therefore, alkaline proteases from different resources have been characterized and evaluated as potential application [4, 5]. For instance, one of considerations for this purpose in leather tanning is the restricted substrate specificity of the protease [5].

Previously, a strain of *Bacillus pumilus* was isolated from the biological waste and has shown efficient dehairing function toward pig and buffalo hides, which contributed to the single activity of alkaline protease in the fermentation supernatant [6]. This protease (DHAP) has been purified and primarily characterized with a molecular mass of 32 kDa and isoelectric point of 9.8 [7]. And, the purified enzyme was illustrated to be optimally active at 50 °C and in pH 10.5 [7]. The gene encoding this protease has also been cloned and expressed in *Bacillus subtilis* [8]. The amino acid sequence shows that this protease belongs to the subtilisin family S. Besides, several alkaline proteases have been also purified and characterized from different strains of *B. pumilus* obtained from various resources. The results showed that these proteases have different catalytic properties [9–12], indicating potential application in various industries [13, 14], although their amino acid sequences share great similarity [15].

With our previous observations derived from the dehairing test, the purified DHAP did not damage the buffalo and pig hides [6], indicating that this protease may perform limited hydrolysis toward the collagen. In addition, thermostability is another consideration in industrial application [16]. Therefore, understanding the properties of DHAP, such as substrate specificity and thermostability, is necessary for industrial application.

In this paper, the thermostability and substrate specificity of the DHAP with several natural proteins and the synthetic peptides were investigated. The results show that this protease can hydrolyze five natural proteins with different specificity. And, synthetic tetrapeptide is the better substrate than the shorter peptides. The thermostability is largely dependent on metal ions, especially calcium; some other ions may coordinately cooperate with calcium ion in improvement or destruction of the thermostability.

## Materials and Methods

### Purification of DHAP

*B. pumilus* UN-31-C-42 was maintained and fermented in 10-l fermentor as described before [6]. After growing at 35 °C for 42 h, the fermentation broth was drained out from the fermentor. Solid $CaCl_2$ was immediately added at final concentration of 1% (*w*/*v*), while pH was adjusted to 9.0 with solid NaOH. The supernatant was harvested by centrifugation at 4 °C at 10,000 rpm in rotor JA-10 (Avanti J-E, Beckman Coulter) for 10 min. The proteins were precipitated by adding solid $(NH_4)_2SO_4$ to 60% saturation at 4 °C while stirring. The protein pellet was stored at −20 °C if needed.

For purification of DHAP, a series of chromatography was performed at 4 °C. The ammonium sulfate pellet was dissolved in buffer A (20 mM Tris–HCl, pH 8.0) plus 1 M $(NH_4)_2SO_4$ and 5 mM $CaCl_2$. After then, the resulting sample was loaded onto the hydrophobic interaction column (2.5×30 cm, Phenyl Sepharose). The protein was eluted from 1 to 0 M ammonium sulfate on Äkta Primer System (Amersham Pharmacia). Fractions with proteolytic activity were pooled and dialyzed in buffer B (buffer A plus 5 mM $CaCl_2$).

Afterward, the dialyzed sample was loaded onto the SP-Sephadex column (2.5×30 cm). However, the DHAP activity was not bound to the column and appeared in the flow

through. Therefore, the resulting sample was directly loaded onto a Q-Sephadex column (2.5×30 cm). The protein was eluted in increasing gradient from 0 to 1 M of NaCl in the buffer B. The pooled fractions containing proteolytic activity were dialyzed against the buffer B overnight and concentrated by PEG2000. The protein sample was stored in the store buffer (50% glycerol, 1 mM $CaCl_2$ and 20 mM Tris–HCl, pH 8.0) at −20 °C. The protein concentration was determined by Bradford method and on SDS-PAGE with bovine serum albumin (BSA) as standard. The concentration was calculated as 0.95 mg/ml or about 30 μM. Finally, the proteolytic activity (250,000 U) was recovered from 5 l of start fermentation broth following the above purification steps. The recovery efficiency is calculated as 12.5%.

## Proteolytic Assay with Protein Substrates

The standard protease activity assay was performed with the substrate of casein described as before [7]. The hydrolytic reaction in the boric acid–NaOH buffer (pH 9.6) was carried at 50 °C for an indicated time. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μg tyrosine per minute under this condition. As a result, the enzyme sample has 20,000 U/ml.

## Hydrolysis of Natural Proteins

As an initial assay to determine the hydrolysis of selected natural proteins, 2 μl of gelatin, fibrin, collagen, BSA, or casein (10 mg/ml) was mixed with 20 U of the purified enzyme (1.5 μM at final concentration) in 20 μl of boric acid–NaOH buffer (pH 9.6). After incubation for 15 min at 50 °C, the proteolytic reaction was terminated at 95 °C for 10 min. Then, the samples were directly loaded onto 10% SDS-PAGE.

In further comparison of the hydrolytic kinetics among the natural proteins, 50 mM substrates of gelatin, fibrin, collagen, and BSA in boric acid–NaOH buffer (pH 9.6) were prepared by taking the average molecular mass of amino acid residue in these proteins as 110 Da. For casein, molecular mass of 163.2 Da was used.

For substrate of casein, the reaction was preformed as above. But, a master reaction mixture (1 ml) was set up, which contained 20 U or 30 nM of the enzyme and 0.5 ml of 50 mM casein. After incubation at 50 °C at indicated time points, 0.2 ml of the reaction mixture was withdrawn. And, 1 ml of trichloroacetic acid (TCA) was immediately added to terminate the reaction. Finally, the hydrolytic product of tyrosine was measured as above.

To measure the hydrolysis of substrates of BSA, fibrin, collagen, and gelatin, the total free amino acid released from each protein was determined by the ninhydrin colorimetric method. Each substrate and the enzyme diluted in the boric acid–NaOH buffer (pH 9.6) were separately pre-heated for 2 min at 50 °C. Then, the reaction was started by mixing 0.5 ml of enzyme solution (20 U or 30 nM at final concentration) and 0.5 ml of substrate (25 mM at final concentration). After incubation at 50 °C for indicated time, 1 ml of TCA was added to terminate the reaction. After filtration, 0.5 ml of filtrate was mixed with 0.5 ml of indicator buffer (10.0 g $Na_2HPO_4{\cdot}12H_2O$, 6.0 g $K_2HPO_4$, 0.5 g ninhydrin, 0.3 g fructose in 100 ml) and incubated in boiling bath for 16 min. After cooling down to 20 °C in tap water for 20 min, 2.5 ml solution (2.0 g $KIO_3$, 200 ml 95% ethanol in 1,000 ml) was added. Finally, the absorbance was measured at 570 nm. The concentration of free amino acid released from the protein substrate by the DHAP was accounted with lysine as standard.

### Assay with the Synthetic Peptide Substrates

Nine synthetic peptide substrates purchased from Sigma-Aldrich (St. Louis, MO, USA) were used here to probe the substrate specificity. They are L-Leu-*p*-nitroanilide (L-pN), *N*-succinyl-L-Phe-*p*-nitroanilide (F-pN), *N*-benzoyl-L-Tyr-*p*-nitroanilide (Y-pN), Boc-*O*-benzyl-Ser-Gly-Arg-*p*-nitroanilide (SGR-pN), *N*-succinyl-Gly-Gly-Gly-*p*-nitroanilide (GGG-pN), *N*-succinyl-Ala-Ala-Val-*p*-nitroanilide (AAV-pN), *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (AAPL-pN), *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF-pN), and *N*-succinyl-Ala-Ala-Val-Ala-*p*-nitroanilide (AAVA-pN). These synthetic peptides were dissolved in dimethylsulfoxide as a stock of 0.1 M and stored at −20 °C.

The hydrolytic mixture (100 μl) was set up in 50 mM Tris–HCl buffer (pH 8.5) plus 2 mM $CaCl_2$ with proper amounts of each substrate and enzyme. The mixture was initially incubated at 50 °C for 2 min, and then 0.4 U enzyme (6 nM at final concentration) was added to start the hydrolytic reaction. The substrate hydrolysis was carried out by monitoring the release of *p*-nitroaniline on line at 410 nm at 50 °C in the spectrophotometer UV2450 (Shimadzu, Japan). The concentration of *p*-nitroaniline was calculated by using the molar extinction coefficient of 8,800 $M^{-1}$ $cm^{-1}$ at 410 nm.

For calculation of catalytic constants, the initial rate was measured for each substrate in a range of various concentrations.

### Effects of Metal Ions on Thermostability

As an initial assay, the enzyme in the presence of 3 mM $CaCl_2$ was first incubated at various temperatures for 0, 5, 15, and 30 min. After the reaction mixture was set back to ice water for 5 min, the survival activity was immediately determined as above using 0.1 mM AAPL-pN as substrate and 0.4 U or 6 nM of DHAP. The secondary concentration of calcium ion was titrated to determine the effects on thermostability at 60 °C.

On the other hand, eight metal ions, including $Mg^{2+}$, $Al^{3+}$, $Ca^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Cu^{2+}$, and $Zn^{2+}$, were selected to study the effects on thermostability at 60 °C as above. The concentration of each metal ion tested here was 5 mM.

The coordinate effects of $Ca^{2+}$ and the selected metal ions ($Mg^{2+}$, $Al^{3+}$, and $Zn^{2+}$) on the thermostability were analyzed as above.

## Results

### Hydrolysis of Natural Proteins by DHAP

The initial results showed that this protease can hydrolyze all of the five natural proteins: casein, BSA, fibrin, gelatin, and collagen revealed by SDS-PAGE analysis (Fig. 1a). Further kinetic analysis showed that DHAP had different hydrolytic activities toward the five proteins (Fig. 1b). The apparent rate constant ($k_{obs}$) were used in this work to compare the hydrolytic difference toward various substrates because the reaction was followed by the first-order reaction. Among the five protein tested here, casein is the best substrate for this DHAP with a $k_{obs}$ value of 0.0980 $min^{-1}$. Collagen and BSA are the substrates more difficult to be hydrolyzed with $k_{obs}$ values of 0.0336 and 0.0178 $min^{-1}$, respectively. In other words, the hydrolytic rate for collagen in this condition is about three times lower than that for casein.

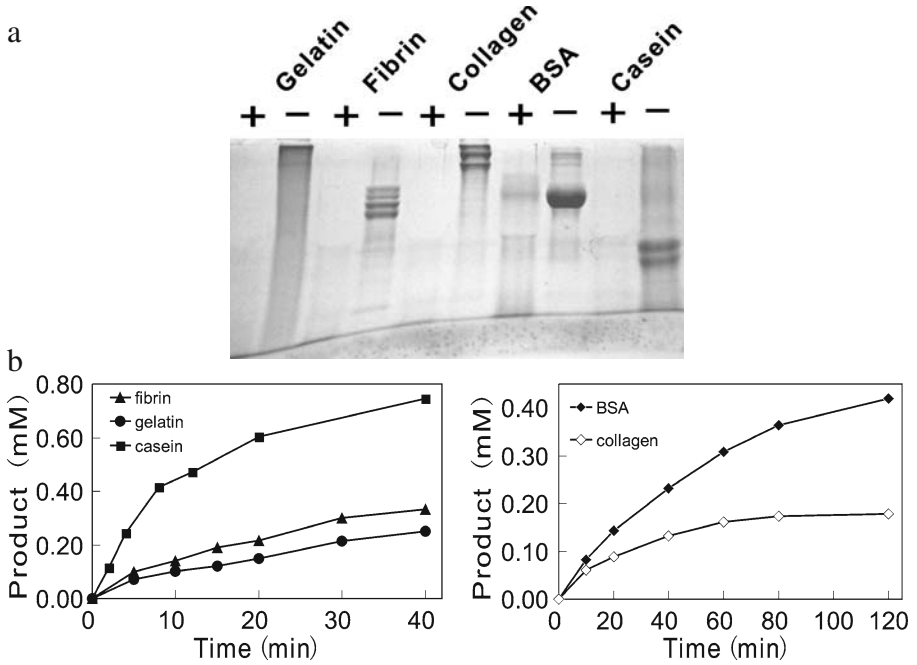


**Fig. 1** Hydrolysis of five natural proteins by DHAP. **a** SDS-PAGE analysis of the hydrolysis of five natural proteins (20 μg) indicated at the *top* by 20 U or 1.5 μM of enzyme; *plus or minus signs* mean the addition of the enzyme or not in the catalytic reaction. **b** Kinetic analysis of the hydrolytic reactions. The reaction was set up with 20 U or 30 nM of enzyme and 50 mM of each natural protein at 50 °C. The apparent rate constant ($k_{obs}$, per $min^{-1}$) was calculated by fitting the first-order equation: 0.0366±0.001 for collagen, 0.0178± 0.0007 for BSA, 0.0981±0.0086 for casein, 0.0448±0.0024 for fibrin, 0.0380±0.006 for gelatin, respectively

## Hydrolytic Kinetics of Synthetic Peptides by DHAP

Nine synthetic peptides were selected to evaluate the substrate specificity of this protease. The results showed that the enzyme has remarkable hydrolytic activity only toward AAPL-pN, AAPF-pN, and AAVA-pN, respectively. Measurement of the kinetic constants of the hydrolytic reactions revealed the $k_m$ values for AAPF-pN and AAPL-pN are in the same magnitude (Table 1). But, the $k_m$ value for AAVA-pN is unexpected to be the lowest, $2.82\times10^{-5}$ M, about ten and 12 times lower than that of AAPF-pN and AAPL-pN, respectively (Table 1). However, $k_{cat}$ value for AAVA-pN is 64 $S^{-1}$, only equal to about 1/35 and 1/17 of

**Table 1** Kinetic constants of DHAP against synthetic substrates.

| Substrate | $k_m$ (M) | Vmax (mmol/mg min) | $k_{cat}$ ($S^{-1}$) | $k_{cat}/k_m$ ($M^{-1}$ $S^{-1}$) |
|---|---|---|---|---|
| AAPF-pN | $3.52\times10^{-4}$ | 4.3077 | 2,230 | $6.34\times10^{6}$ |
| AAPL-pN | $2.70\times10^{-4}$ | 2.1524 | 1,112 | $4.12\times10^{6}$ |
| AAVA-pN | $2.82\times10^{-5}$ | 0.1246 | 64 | $2.28\times10^{6}$ |

Experiments were carried out in duplicate with 6 nM of enzyme at 50 °C in the presence of 2 mM $Ca^{2+}$. The $k_m$ values were calculated by linear regression analysis, and the deviation between the two experiments was less than 10%

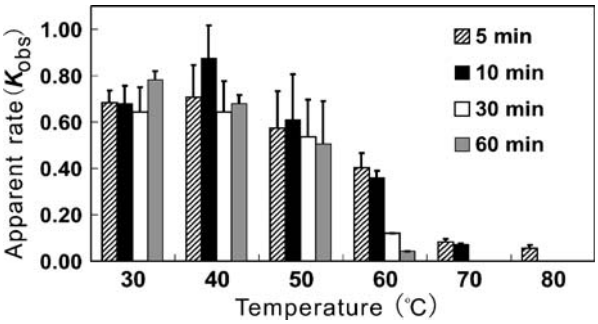


**Fig. 2** Thermostability of DHAP under various temperatures. The enzyme (0.4 U or 6 nM) prior to incubation at the indicated temperature for various time; then, the residual activity was analyzed using AAPL-pN (0.1 mM) as substrate for catalysis at 50 °C. The apparent rate constant ($k_{obs}$) is used to represent the residual catalytic activity by fitting the first-order equation

that for AAPF-pN and AAPL-pN, respectively, indicating that the catalytic efficiency for AAVA-pN is greatly lower than that for AAPF-pN and AAPL-pN.

With the shorter peptide substrates, the DHAP showed detectable proteolytic activity only with SGR-pN and AAV-pN, while the catalytic efficiency is too low to calculate the rate constants (data not shown). However, the enzyme exhibited no detectable activity with GGG-pN, F-pN, L-pN, and Y-pN.

## Effect of Calcium Ion on the Thermostability of DHAP

Previously, the DHAP has been shown to be limited in thermostability [7]. Here, we further investigated effects of calcium ion on the thermostability. As an initial analysis, the thermostability at different temperature (30 to 80 °C) was studied in the presence of 3 mM $Ca^{2+}$. The results indicate that this protease holds relative high stability from 30 to 50 °C, retaining more than 1/3 catalytic rate within 60 min. At 60 °C, this protease retained less activity for 60 min. Once the temperature was increased up to 70 °C, the stability decreased sharply and the $k_{obs}$ value reduced greatly even within 5 min (Fig. 2); almost no activity retained when incubation was up to 30 min.

So, the temperature of 60 °C was chosen to test the effectiveness of $Ca^{2+}$ concentration. The results were shown in Fig. 3. No calcium ion, in this protease, lost activity so quickly even in 5 min. And, the stability was improved with the increasing concentration of $Ca^{2+}$, ranging from 0.5 to 50 mM. Optimal activity survived in almost full for 30 min at 10 mM of $Ca^{2+}$, indicating that $Ca^{2+}$ is necessary for this protease to maintain the stability.

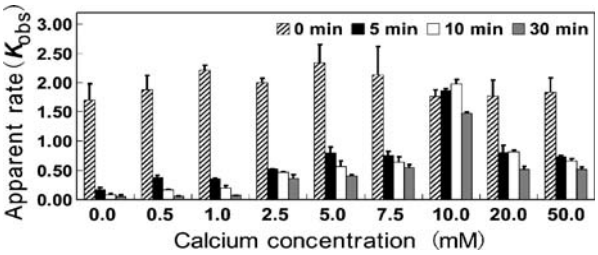


**Fig. 3** Effects of $Ca^{2+}$ concentration on thermostability of DHAP at 60 °C. The enzyme (0.4 U or 6 nM) prior to incubation at 60 °C for various times; then, the residual activity was analyzed using AAPL-pN (0.1 mM) as substrate in the presence of indicated $Ca^{2+}$ concentration. The apparent rate constant ($k_{obs}$) is used to represent the residual catalytic activity by fitting the first-order equation

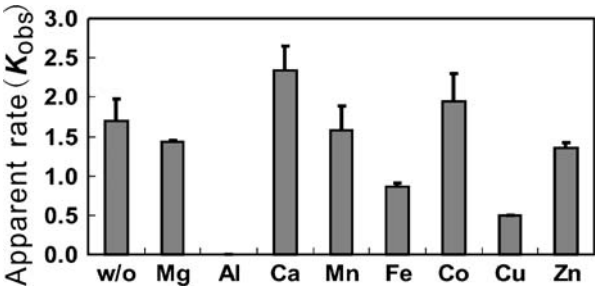


**Fig. 4** Impacts of metal ions on the catalytic rate of DHAP. The reactions were carried out using 0.1 mM AAPL-pN and 0.4 U DHAP in the presence of 5 mM of each selected metal ion

## Thermostability of DHAP with Other Metal Ions

Several common metal ions were selected to test their effects on the thermostability at 60 °C. At the beginning, we tested the impact of various metal ions on the hydrolytic reaction. $Ca^{2+}$ stimulated the activity in comparison to the control (without metal). The metal ion of $Mg^{2+}$, $Co^{2+}$, $Mn^{2+}$, and $Zn^{2+}$ at 5 mM had no or just little effect on the activity under this condition. Just $Cu^{2+}$ and $Fe^{2+}$ inhibited the activity significantly. $Al^{3+}$ completely suppressed the activity (Fig. 4).

Afterward, the impact of various metals on the thermostability was investigated. The results showed that $Co^{2+}$, $Mn^{2+}$, $Mg^{2+}$, or $Zn^{2+}$ could alternately maintain thermostability at certain extent within 10 min, but less than that of calcium (Fig. 5). For the 30-min incubation, less activity was retained only for $Mn^{2+}$ and $Mg^{2+}$, and no activity was retained for $Co^{2+}$ and $Zn^{2+}$. For $Cu^{2+}$, $Fe^{2+}$, and $Al^{3+}$, no activity was retained, even for a short time (5 min). Because $Cu^{2+}$ and $Fe^{2+}$ inhibited the activity, EDTA was to be added in the hydrolytic reaction mixture in order to deplete the retained metal ion. However, no activity was observed even for incubation at 60 °C for 5 min (data not shown). Consequently, $Cu^{2+}$, $Fe^{2+}$, and $Al^{3+}$ seem to have no effect in maintaining the stability at 60 °C.

## Coordination of Metal Ions on Thermostability

The coordinate effects of various metal ions on the activity were investigated in the presence of 1 mM $Ca^{2+}$ and selected other metal ions. As shown in Fig. 6a, $Mg^{2+}$ did not affect the hydrolytic rate; $Zn^{2+}$ reduced activity slightly with the increasing concentration (1

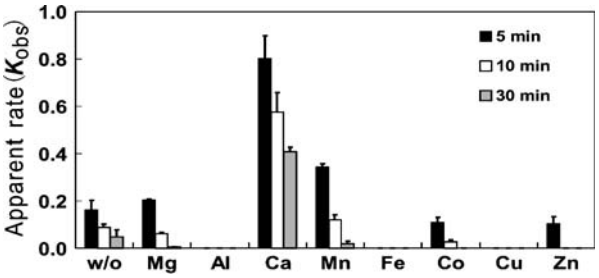


**Fig. 5** Effects of metal ions on the thermostability of DHAP at 60 °C. The enzyme (0.4 U or 6 nM) prior to incubation at 60 °C for various times; then, the residual activity was analyzed using AAPL-pN (0.1 mM) as substrate in the presence of indicated metal ions at 5 mM. The apparent rate constant ($k_{obs}$) is used to represent the residual catalytic activity by fitting the first-order equation

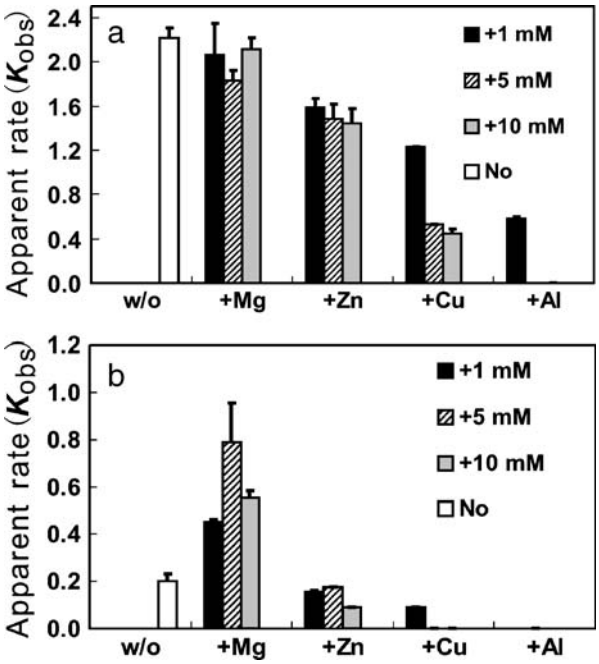


**Fig. 6** Coordinate effectiveness of selected metal ions with calcium on the catalytic activity at 50 °C (**a**) and thermostability of DHAP at 60 °C for 10 min (**b**). The hydrolytic reactions were carried out at 50 °C using 0.1 mM AAPL-pN and 0.4 U enzyme in the presence of 1 mM of $Ca^{2+}$ and each selected metal ion at an indicated concentration

to 10 mM). $Cu^{2+}$ leads to significantly decrease the catalytic rate. $Al^{3+}$ (≥5 mM) gives rise to total suppression of the hydrolytic reaction.

On the thermostability at 60 °C, $Mg^{2+}$ coordinates with $Ca^{2+}$ in maintaining the thermostability by improving the stability at concentration of 1 to 10 mM at both 10- (Fig. 6b) and 30-min incubation (data not shown). $Zn^{2+}$ leads to a slight decrease of the thermostability. $Cu^{2+}$ and $Al^{3+}$ at these concentrations lead to complete loss of the stability even at 10-min incubation (Fig. 6b).

## Discussion

Like other serine alkaline protease [17], DHAP possesses wide substrate specificity and can hydrolyze various natural proteins, but with different hydrolytic rate (Fig. 1). Among the five natural proteins tested here, collagen is somehow difficult to be hydrolyzed. Collagen is thought to be a key component of animal hides, which should not be hydrolyzed in bioprocess so as to maintain quantity of the leather product [5]. It is desired that the protease used for this purpose should be with less or without activity toward collagen. Our previous studies demonstrated that DHAP did not damage the hides in dehairing tests [6], although this protease has restricted hydrolytic activity to collagen (Fig. 1).

With the synthetic peptides tested here, DHAP could not hydrolyze synthetic mono-peptide substrates under this condition, quite different from another alkaline protease (BPP) from the same bacterium, which showed weak activity toward mono- and di-peptide substrates [9]. However, DHAP shows limited activity toward the tripeptide substrates, seemly relying upon the amino acid residue at P1 position in which larger side-chain group seems easier to be hydrolyzed. Gly at P1 position is resistant to hydrolysis by DHAP, and Val and Arg at the same position are easier to be hydrolyzed. Like other serine protease,

DHAP could efficiently catalyze the tetrapeptides. The catalytic efficiency ($k_m/k_{cat}$) toward these substrates tested here decreased in the order of Leu>Phe>Ala at P1 position. This trend is somehow different from that of other reported serine proteases. For example, SAPB from the same bacterium showed almost the same catalytic efficiency for the same three tetrapeptide substrates, although only ten amino acid residues are different between SAPB and DHAP [15]. Even if the $k_m$ for AAVA-pN is less than that for AAPF-pN and AAPL-pN (Table 1), the higher affinity to substrate (AAVA-pN) does not mean a higher hydrolytic activity, perhaps due to slower release of product from the enzyme/product complex since the $k_{cat}$ for AAVA-pN is much less than that for AAPL-pN and AAPF-pN. In addition, substrate binding sites and catalytic residues in subtilisin occur separately and independent in space [18], and site-directed mutants made in these two regions seem to not disturb each other in catalytic function [19].

This work illustrated that thermostability of DHAP is largely dependent upon metal ion, especially calcium, most like the other serine alkaline protease [20, 21]. In view of the crystal structure, there are two calcium-binding sites in subtilisin, a strong binding site A and a weak binding site B. Site A, occupied by calcium, is thought to be important to maintain the thermostability [22]. Site B, occupied by cations, has a dramatic effect on the stability [23]. However, addition of other metal ions, especially $Cu^{2+}$ and $Fe^{2+}$, leads to thermostability decrease (Fig. 6b). This effectiveness can be explained perhaps either due to the replacement of $Ca^{2+}$ in site A by other ions or due to entry into site B since $Ca^{2+}$ removal from site A or B leads to structural destruction in subtilisin Carsberg [24].

In addition, hydrolytic activity of DHAP was slightly enhanced by $Ca^{2+}$, $Mg^{2+}$, $Co^{2+}$, as well as $Mn^{2+}$ and significantly inhibited by $Cu^{2+}$ and $Fe^{2+}$. In human furin, a subtilisin-like protease, the activity was also inhibited by solvated copper, perhaps due to coordination of $Cu^{2+}$ with the catalytic histidine suggested by Podsiadlo et al. [25]. However, $Al^{3+}$ completely inhibited the activity of DHAP without any known mechanism.

In conclusion, DHAP shows unique catalytic properties in comparison with other alkaline proteases from the same bacterium, although they are highly similar in their amino acid sequences. These obvious differences not only make them to be potent in various industrial applications but also reflect the adoption of various strains to versatile niches in nature. In order to achieve these applicable purposes, it is necessary to get into deeper insights on the structure–function relationship and to improve their performance more practically through site-directed mutation or in vitro molecular evolution.

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