



## Characterization of a metalloprotease from an isolate *Bacillus thuringiensis* 29-126 in animal feces collected from a zoological garden in Japan

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**Abstract** An extracellular metalloprotease, Btmp, was partially purified from the culture supernatant of *Bacillus thuringiensis* 29-126, isolated from animal feces collected in a zoological garden in Japan, by ultrafiltration, ammonium sulfate precipitation, and a set of chromatography on Sephadex G-75 and High-Q. The molecular mass of the protease was estimated to be 60 kDa by SDS-PAGE. The enzyme showed optimum activity at 50 °C and pH 6.0, and had a half-life of 14 min at 50 °C. The enzyme activity was not influenced by Na<sup>+</sup>, K<sup>+</sup>, As<sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>, Ba<sup>2+</sup>, and phenylmethylsulfonyl fluoride, but it was moderately inhibited by Zn<sup>2+</sup> at a concentration of 1.0 mM, while the activity was significantly inhibited to less than 50 % by Cu<sup>2+</sup>, Co<sup>2+</sup>, Cd<sup>2+</sup>, and ethylenediaminetetraacetic acid. Interestingly, the enzyme was activated to 178 % by 1.0 mM of Mn<sup>2+</sup>. From these results, it may be suggested that the protease is a novel extracellular manganese-activated metalloprotease.

**Keywords** Animal feces · *Bacillus thuringiensis* · Extracellular metalloprotease · Manganese-activated enzyme

### Introduction

*Bacillus thuringiensis* is a spore-forming, Gram-positive bacterium found in soil environments and is well known for its insecticidal activity by crystalline endotoxins (Schnepf et al. 1998). It is a specific pathogen of insects, and has been intensively used as a biopesticide for over 60 years. It is genetically very close to *Bacillus cereus* and *Bacillus anthracis*, although each organism occupies a different ecological niche (Helgason et al. 2000; Gohar et al. 2005). During sporulation, it synthesizes intracellular proteolytic enzymes, and the enzymes are thought to possess hydrolytic activity and to activate the crystal proteins (Reddy et al. 2000). It has been reported that the genes *aprA* (encoding alkaline protease) and *nprA* (encoding neutral protease), which are the extracellular proteases of *B. thuringiensis*, enhanced the yield of crystal proteins by deletion experiments (Donovan et al. 1997; Tan and Donovan 2000).

Proteases were classified into four types: serine, cysteine, aspartic, and metalloprotease (Rawlings and Barrett 1993). Recently, they were classified into seven broad groups: aspartic-, cysteine-, serine-, metallo-, threonine-, glutamic-, and asparagine-protease (Oda 2012). Among the various proteases from diverse sources, microbial proteases are important industrial enzymes. They have been used in various industries: food, pharmaceuticals, baking, brewing, leather processing, and filming (Wu and Chen 2011). Metalloproteases contain one or two metal ions, usually Zn<sup>2+</sup>, Mg<sup>2+</sup>, Ni<sup>2+</sup>, or Cu<sup>2+</sup> in their active centers (Wu and Chen 2011).

The possibility of several proteases in *B. thuringiensis* has been stated, including the genes *aprA* and *nprA* (Donovan et al. 1997). Several proteases and/or genes from *B. thuringiensis* have been reported: a metalloprotease (Li and Yousten 1975), an extracellular serine protease (Epreman et al. 1981), a metalloprotease Bmp1 (Luo et al. 2013), an alkaline protease (Agasthya et al. 2013), and an alkaline thermolysin-like protease BtsTLP1 (Zhang et al. 2015). In this paper, an extracellular metalloprotease from *B.*

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*thuringiensis* 29-126, which was isolated from animal feces collected in a zoological garden in Japan, was partially purified and its biochemical characteristics were described.

## Materials and Methods

### Media

Luria broth (LB) (1.0 % tryptone, 0.5 % yeast extract, and 1.0 % NaCl) and LBS (LB supplemented with 0.7 % skim milk) media were used for the experiments.

### Selection of protease-producing strains

In the previous study, 2,953 colonies were examined from 71 fecal samples of 56 animal species collected in a zoological garden in Japan, and 531 colonies were assigned to *B. thuringiensis* on the basis of the formation of parasporal inclusions (Lee et al. 2002). They were identified as *B. thuringiensis* by further analyses of their morphologies and serotypes, and polymerase chain reaction tests (Shisa et al. 2002; Lee et al. 2003). In this study, a total of 26 *B. thuringiensis* strains, isolated from reptilian feces, were streaked onto the LBS agar plates and grown for 24 h at 37 °C for selection of protease-producing strains. Then, halos around the colonies were observed, and a strain showing the largest halo was selected. The selected strain *B. thuringiensis* 29-126 has been deposited in the Korean Collection for Type Cultures (KCTC) as KCTC 33828. The 16S rRNA was sequenced by SolGent Co. Ltd. (Daejeon, Korea) and deposited in GenBank under the accession number KX485316.

### Production of protease

The strain was cultured in 200 mL of LB in a 1 L flask for 30 h at 37 °C with 200 rpm shaking, and was sampled every 3 h to check the cell growth and the extracellular enzyme activity that was produced.

### Enzyme purification

After cultivation in 10 L broth (200 mL × 50 flasks) for 6 h, cells were harvested and supernatants were collected by centrifugation for 10 min at 12,000 × g. The resultant supernatant was concentrated to 100 mL by ultrafiltration using YM10 (Amicon, Beverly, MA, USA) and the proteins were precipitated with 80 % ammonium sulfate. After dialysis with 50 mM Tris-HCl, (pH 7.5) containing 100 mM KCl, the concentrate was loaded into a Sephadex G-75 column (72.5 × 2.8 cm) and eluted with a flow rate of 19.1 mL/h. Active fractions were pooled, concentrated with ultrafiltration, dialyzed with a low salt buffer (25 mM Tris-HCl, pH 8.0), and separated by a High-Q column (Bio-Rad, Hercules, CA, USA), which was repeated three times using both a low salt buffer and a high salt buffer (25 mM Tris-HCl, pH 8.0, 1 M NaCl) at 75 mL/h. Protein concentration was analyzed by the Lowry et al. (1951) method or monitored at A<sub>280 nm</sub> during the purification.

### SDS-PAGE and protein staining

Protein was analyzed by SDS-PAGE using a 10 % gel by the method of Laemmli (1970). Proteins in the gel were visualized according to the manufacturer's manual by a silver staining kit (GE Healthcare, Uppsala, Sweden).

### Protease assay

Enzyme activity was assayed using azocasein as the substrate by a method with a slight modification (Sarath et al. 1989; Kim et al. 2000). Briefly, the reaction mixture (200 µL) containing 0.5 % azocasein and an appropriate enzyme in 50 mM Tris-HCl (pH 7.5) were reacted for 30 min at 50 °C, and 600 µL of 10 % trichloroacetic acid was added. After centrifugation for 5 min at 12,000 × g, the supernatant was delivered to a new tube. Then, 700 µL of 1 N NaOH was added to develop the color, and the absorbance was measured at 440 nm. One unit of the enzyme was defined as the amount that produced an absorbance change of 1.0 in 30 min at 50 °C.

### Characterization of the enzyme

For optimum temperature analysis, the enzyme activity was measured at different temperatures, 30, 37, 45, 50, 60, and 70 °C. For thermostability analysis, the activity was measured by preincubating the enzyme in the absence of the substrate for 5, 10, 15, 30, and 60 min at designated temperatures prior to taking the measurement. For optimum pH analysis, the enzyme was assayed between pH 4.0 and 10.5 using 50 mM of three buffers: sodium-acetate (pH 4.0–6.0), sodium-phosphate (pH 6.0–9.0), and glycine-NaOH (pH 9.0–10.5). The influences of various cations and agents on enzyme activity were analyzed at a concentration of 1.0 mM Na<sup>+</sup>, K<sup>+</sup>, As<sup>+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ca<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, Ba<sup>2+</sup>, Fe<sup>2+</sup>, Zn<sup>2+</sup>, ethylenediaminetetraacetic acid (EDTA), and phenylmethylsulfonyl fluoride (PMSF).

## Results and Discussion

### Selection of strain and protease production

Among the 26 *B. thuringiensis* strains, a strain showing the largest halo, *B. thuringiensis* 29-126, was selected on the LBS plate (data not shown). When the strain was cultured in 200 mL of LB in a 1 L flask, they grew up to 21 h; however, it was observed that protease activity was observed earlier and reached the maximum at 6 h of culture and then decreased thereafter (Fig. 1). Similar to the result, in the nutrient broth, a metalloprotease protease activity of *B. thuringiensis* was observed starting at 5 h of growth, increased up to 9 h, then decreased to the end of culture (Li and Yousten 1975). It was also reported that a metalloprotease from *B. thuringiensis* subsp. *israelensis* was produced after 7 h of growth in nutrient-rich medium such as LB medium and reached a maximum after 12 h, and suggested that the protein-rich medium afforded the maximum amount of the protease in the shortest time

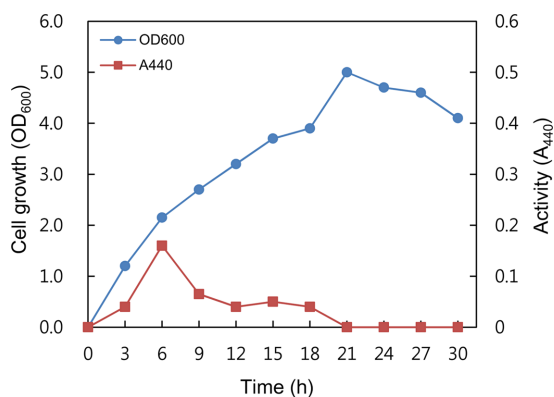


Fig. 1 Growth curve and enzyme activity of isolate 29-126

(Nisnevitch et al. 2010).

**Purification of an extracellular protease**

Protease was purified by ultrafiltration, precipitation at 80 % ammonium sulfate, and a set of chromatography on Sephadex G-75 and High-Q. In High-Q chromatography, the highest enzyme activity was measured at fraction 16 (Fig. 2A). The protease was purified 18.2 times by this procedure. However, the yield of enzyme activity was 3.1 % (Table 1). After High-Q chromatography step, specific activity was decreased compared to the previous step. It was reported the specific activity of the metalloprotease from *Bacillus polymyxa* was not increased after an ion-exchange chromatography using CM-cellulose (Fogarty and Griffin 1973). It might be suggested that some factors such as assisting ions or molecules, responsible for the activity or stability, were depleted during or after the process. In SDS-PAGE analysis, a major band was observed in fractions 15–17 of the most active fractions along with four minor bands (Fig. 2B). It was could be concluded that the major protein was a target protease based on the observations that the major band showed more than half of the total intensity of the proteins and the best correlation between the band intensity and the enzyme activity. The corresponding band could be detected as a very faint band in other series of chromatography, High-Q, High-S, and Sephadex G-75 in a small scale experiments (2 L broth) (data not shown). The molecular mass of the protease, Btmp, was estimated to be 60 kDa. Compared to other proteases from *B. thuringiensis*, the mass was similar to that of NprA (60,982 Da deduced from the DNA

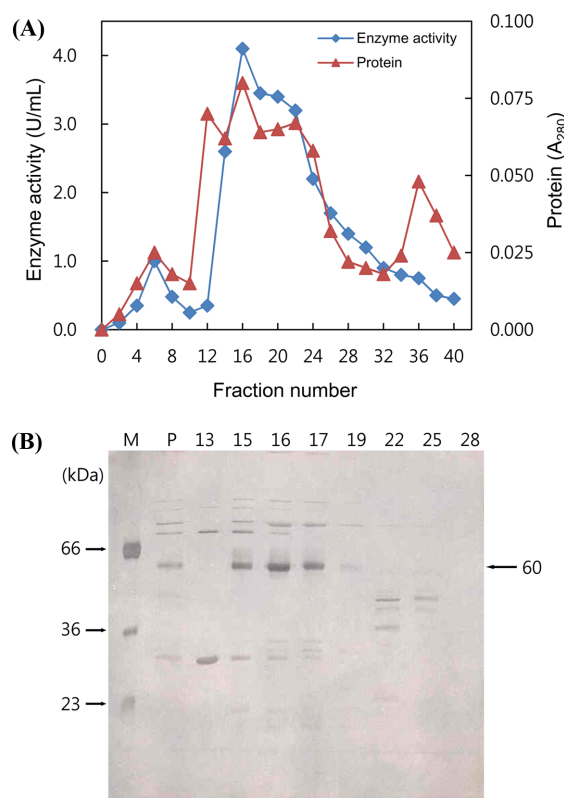
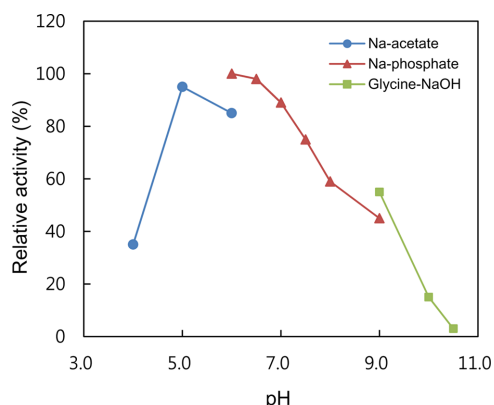


Fig. 2 High-Q chromatography (repeat III) of the concentrated enzyme (A) and silver stained gel after SDS-PAGE of the active fractions (B). M, size marker; P, pool of active fractions from repeats I and II. The numbers on the top of the panel B represent the fraction numbers of the repeat III chromatography

sequence) from *B. thuringiensis* var. *kurstaki* (Donovan et al. 1997). However, the value was not determined at the level of protein and it was mentioned that it might be processed to a 37.5-kDa metalloprotease, which was partially purified and reported by Li and Yousten (1975). The molecular mass of Btmp was different from other *B. thuringiensis* proteases: an extracellular serine protease from *B. thuringiensis* 69-6R (29 kDa) (Epreman et al. 1981), AprA from *B. thuringiensis* var. *kurstaki* (42,344 Da, deduced from DNA sequence) (Tan and Donovan 2000), Bmp1 from *B. thuringiensis* TBT-1518 (comprising several motifs with 894 amino acid residues, but without mention of the molecular mass) (Luo et al. 2013), and BtsTLP1 from *B. thuringiensis* subsp. *sichuansis* MC28 (35.6 kDa, determined by mass spectrometry)

Table 1 Purification of an extracellular metalloprotease from *B. thuringiensis* 29-126

Procedure	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Supernatant	9,460	53,460	54,800	1.0	1.0	-
Ultrafiltration	100	900	2,380	2.6	2.6	4.3
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	8.0	69.1	640	9.3	9.0	1.2
Sephadex G-75	90.0	39.6	1,998	50.5	49.0	3.6
High-Q (×3)	63.0	9.2	172.6	18.8	18.2	3.1



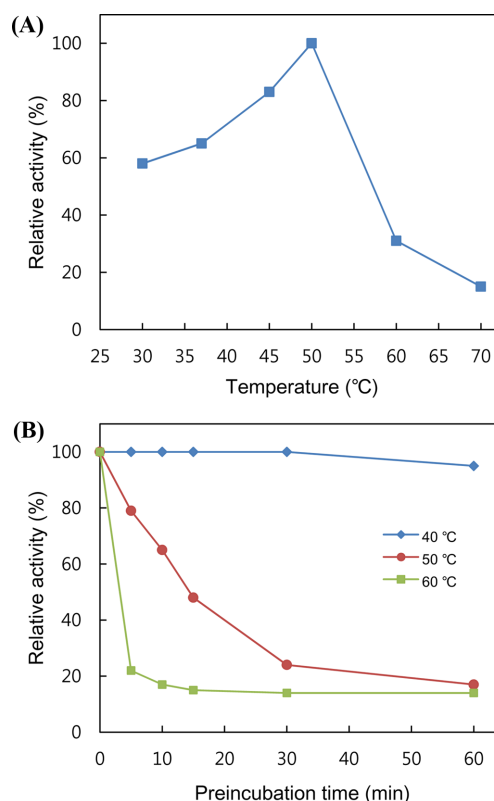
**Fig. 3** Effect of pH on the protease activity

(Zhang et al. 2015).

### Properties of the enzyme

The protease Btmp showed high activity between pH 5.0 and 7.0, and maximum activity at 6.0 in a sodium phosphate buffer or 5.0 in a sodium acetate buffer (Fig. 3). It was observed that the optimum temperature of the enzyme activity was 50 °C (Fig. 4A). The enzyme activity decreased to 80 and 63 % of maximum activity at 45 and 40 °C, respectively. After preincubation at 40 °C for up to 60 min, loss of enzyme activity was negligible (Fig. 4B). However, the enzyme was inactivated drastically after 5 min preincubation at 60 °C. In the thermostability experiments, the enzyme showed a half-life of 14 min at 50 °C. The optimum pH of Btmp was slightly lower than that of the 37.5-kDa metalloprotease and Bmp1, pH 6.5–7.5, and pH 7.4, respectively (Li and Yousten 1975; Luo et al. 2013), and was different from those of the alkaline proteases, a serine protease (Epreman et al. 1981), AprA (Tan and Donovan 2000), an alkaline protease (Agasthya et al. 2013), and BtsTLP1 (Zhang et al. 2015). The optimum temperature of Btmp was higher than that (40 °C) of Bmp1 (Luo et al. 2013). With these results, it may be concluded that Btmp is an extracellular neutral protease.

The effect of mono- and divalent cations on enzyme activity was analyzed at 1.0 mM concentration. The enzyme activity was moderately inhibited to 74 % of the activity by  $Zn^{2+}$  at a concentration of 1.0 mM, while the activity was significantly inhibited to less than 50 % by  $Cu^{2+}$ ,  $Co^{2+}$ , and  $Cd^{2+}$ , and inhibited further to 10 % by EDTA (Table 2). The enzyme activity was not significantly influenced by  $Na^+$ ,  $K^+$ ,  $As^+$ ,  $Mg^{+2}$ ,  $Ca^{2+}$ ,  $Ba^{2+}$ , and PMSF. It has been reported that the 37.5-kDa metalloprotease was largely inhibited by chelating agents but not by PMSF (Li and Yousten 1975). The metalloprotease Bmp1 was inhibited at higher concentrations of  $Zn^{2+}$  (Luo et al. 2013). Based on the results of chelation by EDTA and insignificance inhibition by PMSF, it may be concluded that the enzyme is a metalloprotease and is not a serine protease. Interestingly, the enzyme was activated to 178 % by 1.0 mM of  $Mn^{2+}$ . Several studies have reported on manganese-



**Fig. 4** Effect of temperature on the protease activity (A) and thermal stability of the enzyme (B). For thermostability experiments, the enzyme was preincubated at the temperatures for the indicated time in the absence of the substrate, and then the residual activity was measured in the presence of 0.5% azocasein as the standard assay

**Table 2** Effect of cations and effectors on the protease activity

Cation/effector	Relative activity (%)
Control	100
MgCl <sub>2</sub>	105
BaCl <sub>2</sub>	99
ZnCl <sub>2</sub>	74
CuSO <sub>4</sub>	25
CoCl <sub>2</sub>	16
MnCl <sub>2</sub>	178
CdCl <sub>2</sub>	36
CaCl <sub>2</sub>	101
Na <sub>2</sub> HAsO <sub>4</sub>	95
NaCl	95
KCl	94
EDTA	10
PMSF	94

\*The concentration of cations and effectors was 1.0 mM  
EDTA=ethylenediaminetetraacetic acid; PMSF=phenylmethylsulfonyl fluoride

activated proteases from other than *B. thuringiensis*: a metalloprotease from *B. polymyxa* (Griffin and Fogarty 1973), an extracellular protease from thermophilic *Bacillus* strain EA.1 (Coolbear et al. 1992), an alkaline protease from *B. stearo-*

*thermophilus* (33.5 kDa, Rahman et al. 1994), an alkaline serine protease from *B. pumilus* (35 kDa, Ibrahim et al. 2011), a metal ion-dependent alkaline protease from *B. aquimaris* VITP4 (34 kDa, Shivanand and Jayaraman 2011), a neutral protease from *Bacillus* strain HUTBS62 (48 kDa, Aqel et al. 2012), and a metallo-neutral protease from *B. amyloliquefaciens* (36.8 kDa, Wang et al. 2013). A 37-kDa extracellular neutral protease from *B. subtilis* produced by a gene (*nprB*) encoding a 60 kDa protease has been reported (Tran et al. 1991). However, there was no mention of the influence of  $Mn^{2+}$ . On the other hand, the inhibition by  $Mn^{2+}$  was reported in Bmp1 from *B. thuringiensis* (Luo et al. 2013). From the results and comparisons, it may be suggested that the protease in this study is a novel extracellular manganese-activated metalloprotease.

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## References

- Agasthya AS, Sharma N, Mohan A, Mahal P (2013) Isolation and molecular characterisation of alkaline protease producing *Bacillus thuringiensis*. Cell Biochem Biophys 66: 45–51
- Aqel H, Al-Quadan F, Yousef TK (2012) A novel neutral protease from thermophilic *Bacillus* strain HUTBS62. J BioSci Biotech 1: 117–123
- Coolbear T, Whittaker JM, Daniel RM (1992) The effect of metal ions on the activity and thermostability of the extracellular proteinase from a thermophilic *Bacillus*, strain EA.1. Biochem J 287: 367–374
- Donovan WP, Tan Y, Slaney AC (1997) Cloning of the *nprA* gene for neutral protease A of *Bacillus thuringiensis* and effect of *in vivo* deletion of *nprA* on insecticidal crystal protein. Appl Environ Microbiol 63: 2311–2317
- Epreman AS, Chestukhina GG, Azizbekian RR, Netyksa EM, Rudenskaia GN (1981) Extracellular serine proteinase of *Bacillus thuringiensis*. Biokhimiia 4: 920–929
- Fogarty WM, Griffin PJ (1973) Production and purification of the metalloprotease of *Bacillus polymyxa*. Appl Microbiol 26: 185–190
- Gohar M, Gilois N, Graveline R, Garreau C, Sanchis V, Lereclus D (2005) A comparative study of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* extracellular proteomes. Proteomics 5: 3696–3711
- Griffin PJ, Fogarty WM (1973) Physicochemical properties of the native, zinc- and manganese-prepared metalloprotease of *Bacillus polymyxa*. Appl Microbiol 26: 191–195
- Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I, Kolstø AB (2000) *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl Environ Microbiol 66: 2627–2630
- Ibrahim KS, Muniyandi J, Karutha Pandian S (2011) Purification and characterization of manganese-dependent alkaline serine protease from *Bacillus pumilus* TMS55. J Microbiol Biotechnol 21: 20–27
- Kim H, Yang MJ, Jung KH, Kim J (2000) Cloning and characterization of the major extracellular neutral protease (NprM) from *Bacillus megaterium* ATCC 14945. Agric Chem Biotechnol 43: 147–151
- Laemmler UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
- Lee DH, Machii J, Ohba M (2002) High frequency of *Bacillus thuringiensis* in feces of herbivorous animals maintained in a zoological garden of Japan. Appl Entomol Zool 37: 509–516
- Lee DH, Shisa N, Wasano N, Ohgushi A, Ohba M (2003) Characterization of flagellar antigens and insecticidal activities of *Bacillus thuringiensis* populations occurring in animal feces. Curr Microbiol 46: 287–290
- Li E, Yousten AA (1975) Metalloprotease from *Bacillus thuringiensis*. Appl Microbiol 30: 354–361
- Lowry OH, Rosenbrough NJ, Farr AL, Randell RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275
- Luo X, Chen L, Huang Q, Zheng J, Zhou W, Peng D, Ruan L, Sun M (2013) *Bacillus thuringiensis* metalloproteinase Bmp1 functions as a nematocidal virulence factor. Appl Environ Microbiol 79: 460–468
- Nisnevitch M, Sigawi S, Cahan R, Nitzan Y (2010) Isolation, characterization and biological role of camelysin from *Bacillus thuringiensis* subsp. *israelensis*. Curr Microbiol 61: 176–183
- Oda K (2012) New families of carboxyl peptidases: serine-carboxyl peptidases and glutamic peptidases. J Biochem 151: 13–25
- Rahman RNZA, Razak CN, Ampon K, Basri M, Zin WM, Yunus W, Salleh AB (1994) Purification and characterization of a heat-stable alkaline protease from *Bacillus stearothermophilus* F1. Appl Microbiol Biotechnol 40: 822–827
- Rawlings ND, Barrett AJ (1993) Evolutionary families of peptidases. Biochem J 290: 205–218
- Reddy ST, Kumar NS, Venkateswerlu G (2000) Identification and purification of the 69-kDa intracellular protease involved in the proteolytic processing of the crystal delta-endotoxin of *Bacillus thuringiensis* subsp. *tenebrionis*. FEMS Microbiol Lett 183: 63–66
- Sarath G, Motte RS, Wanger F (1989) Protease assay methods. In: Beynon RJ, Bond JS (eds) Proteolytic enzyme: A practical approach, IRL Press, Oxford, pp 25–55
- Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62: 775–806
- Shisa N, Wasano A, Ohgushi D, Lee H, Ohba M (2002) Extremely high frequency of common flagellar antigens between *Bacillus thuringiensis* and *Bacillus cereus*. FEMS Microbiol Lett 213: 93–96
- Shivanand P, Jayaraman G (2011) Isolation and characterization of a metal ion-dependent alkaline protease from a halotolerant *Bacillus aquimaris* VITP4. Indian J Biochem Biophys 48: 95–100
- Tan Y, Donovan WP (2000) Deletion of *aprA* and *nprA* genes for alkaline protease A and neutral protease A from *Bacillus thuringiensis*: effect on insecticidal crystal proteins. J Biotechnol 84: 67–72
- Tran L, Wu XC, Wong SL (1991) Cloning and expression of a novel protease gene encoding an extracellular neutral protease from *Bacillus subtilis*. J Bacteriol 173: 6364–6372
- Wang J, Xu A, Wan Y, Li Q (2013) Purification and characterization of a new metallo-neutral protease for beer brewing from *Bacillus amyloliquefaciens* SYB-001. Appl Biochem Biotechnol 170: 2021–2033
- Wu JW, Chen XL (2011) Extracellular metalloproteases from bacteria. Appl Microbiol Biotechnol 92: 253–262
- Zhang Z, Hao H, Tang Z, Zou Z, Zhang K, Xie Z, Babe L, Goedegebuur F, Gu X (2015) Identification and characterization of a new alkaline thermolysin-like protease, BtsTLP1, from *Bacillus thuringiensis* serovar *sichuansis* strain MC28. J Microbiol Biotechnol 25: 1281–1290