InhA-Like Protease Secreted by *Bacillus* sp. S17110 Inhabited in Turban Shell

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A strain producing a potent protease was isolated from turban shell. The strain was identified as *Bacillus* sp. S17110 based on phylogenetic analysis. The enzyme was purified from culture supernatant of *Bacillus* sp. S17110 to homogeneity by ammonium sulfate precipitation, SP-Sepharose, and DEAE-Sepharose anion exchange chromatography. Protease activity of the purified protein against casein was found to be stable at pH 7 to pH 10 and around 50°C. Approximately 70% of proteolytic activity of the enzyme was detected either in the presence of 100 mM SDS or Tween 20. The enzyme activity was enhanced in the presence of Ca^{2+}, Zn^{2+}, Mg^{2+}, but was inhibited by EDTA, indicating that it requires metal for its activity. The purified enzyme was found to be a monomeric protein with a molecular mass of 75 kDa, as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration chromatography. The purified enzyme was analyzed through peptide fingerprint mass spectra generated from matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) and a BLAST search, and identified as immune inhibitor A (inhA) deduced from nucleotide sequence of *B. cereus* G9241. Since InhA was identified as protease that cleave antibacterial proteins found in insect, inhA-like protease purified from *Bacillus* sp. S17110 might be pathogenic to sea invertebrates.

Keywords: protease, turban shell, immune inhibitor, *Bacillus*

Protease is one of the most important industrial enzymes, accounting for nearly 60% of total world wide enzyme markets (Ward, 1985; Outrup and Boyce, 1990; Cowan, 1996). The enzyme is produced from diverse organisms such as animal, plant and microbe. The enzyme could be classified as serine protease, thiol protease and metal protease as their action mode (Leighton *et al.*, 1973), and classified as acid protease, neutral protease and alkaline protease as their effect on pH.

Besides application of protease in industry, protease was found as a pathogenic factor from *Bacillus*. The insect pathogenic Gram-positive *Bacillus thuringiensis* secretes immune inhibitor A (InhA), a metallopeptidase, which specifically cleaves host antibacterial proteins. A homologue of immune inhibitor A, PrtV, has been also identified in the Gram-negative human pathogen *Vibrio cholerae* (Ogierman *et al.*, 1997). *B. thuringiensis* is highly resistant to the insect immune system due to its production of two factors, inhibitor A (InhA or InA) and inhibitor B (InhB or InB), which selectively block the humoral defense system developed by insects against *Escherichia coli* and *Bacillus cereus* (Edlund *et al.*, 1976). *B. thuringiensis* is especially resistant to cecropins and attacins, which are the main classes of inducible antibacterial peptides in various lepidopterans and dipterans (Hultmark *et al.*, 1982; Boman and Hultmark, 1987). InhA has been shown to specifically hydrolyze cecropins and attacins in the immune he-

molymph of *Hyalophora cecropia in vitro* (Dalhammar and Steiner, 1984).

In this report, we describe a bacterial strain isolated from turban shell and identified as *Bacillus* sp. S17110. We purified protease with a molecular mass of 75 kDa from the culture supernatant of the strain. The protease was identified with MALDI-TOF-MS analysis as InhA found in genome sequence of pathogenic pneumonia strain *Bacillus cereus* G9241. Since this protease has been purified in *Bacillus* isolated from turban shell, it might serve as a possible pathogenic factor to marine invertebrate. Our data for properties of the InhA-like protease indicate that the protein is alkalophilic, which probably is suitable in marine environment, and quite stable in the presence of detergent.

Materials and Methods

Microbial strain
Several isolates were obtained from turban shell collected in Gwangyang Bay, Jeonnam, Korea and checked protease activities in their culture supernatant. Three strains showed potent degradation activities against casein. The organisms were maintained on OSYM (oatmeal 1.5%, soybean meal 2%, dried yeast 1%) agar plates and the stocks were maintained in 20% glycerol at -70°C.

Phenotypic characterization
Cells grown on TSA (pancreatic digest of casein 1.5%, soybean meal 0.5%, sodium chloride 0.5%) at 30°C for 24 h were used for physiological and biochemical tests. Catalase

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Vol. 45, No. 5

Identification of immune inhibitor A from *Bacillus* sp. S17110   403

and oxidase activities were determined using 3% (v/v) hydrogen peroxide and Kovacs's reagent (Kovacs, 1956), respectively. Other physiological and biochemical tests were performed using API 20E, API 20NE and API 50CHB (bioMérieux) test panels. Enzyme activities were tested using the API ZYM kit (bioMérieux) following the manufacturer's instructions.

Phylogenetic analysis

Chromosomal DNA of the strain was isolated from the test strains using a procedure, which is a slight modification of that described in Pitcher *et al.* (1989). PCR amplification of 16S rDNA was performed using a PCR machine, and the previously described 27f and 1525r primers (Chun and Goodfellow, 1995). The 16S rDNA sequences were aligned manually with Bacillus nucleotide sequences derived from the Ribosomal Database Project (Maidak, 1996) and EMBL/GenBank database using the AL 16S program (Chun and Goodfellow, 1995). The evolutionary tree for the datasets was inferred using the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distance matrix for the neighbor-joining method was generated as described by Jukes and Cantor (Jukes and Cantor, 1969). The PHYLIP package (Felsenstein, 1993) was used for constructing the tree.

Assay of protease activity

Protease activity was determined by measuring the amount of acid-soluble material released from azocasein (Leighton, 1973). One hundred microliter of enzyme sample was added to 300 μl of 1% (w/v) azocaesin dissolved in 50 mM Tris-HCl; pH 7.0, incubated at 37°C for 60 min, added with 600 μl of ice-cold 10% (w/v) trichloroacetic acid, and then mixed immediately. The reaction solution was placed on ice for 10 min and obtained supernatant by centrifugation at 12,000×g for 15 min. The quantity of acid-soluble material in the supernatant was determined by measuring absorbance at 440 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 440 nm of 1.0 O.D. under the above mentioned assay condition.

Enzyme purification

Purification of the protease was monitored by an increase in protease specific activity. *Bacillus* sp. S17110 grown in 10 liter of OSYM medium supplemented with dextrose 4% and CaCO₃ 0.2% was harvested by centrifugation at 6,000×g for 20 min and the supernatant was used as a crude enzyme solution. Supernatant was concentrated to 500 ml by using Tangential Flow Filtration (Pellicon XL 50 cm² Device with a Biomax-10 filter; Millipore Corp.). Sodium ammonium sulfate was added to 70% saturation. The precipitate was removed by centrifugation at 20,000×g for 20 min followed by dissolution in 50 mM Tris-HCl buffer; pH 7.0 and dialyzed for 24 h at 4°C against the same buffer. The sample was loaded to SP-Sepharose column(2.0×20 cm) and washed the column with the same buffer, and eluted protein with a stepwise salt gradient of 0-1.0 M NaCl in the same buffer. The fractions that had protease activities were pooled and dialyzed against the same buffer. The enzyme sample was loaded onto a DEAE-Sepharose column (2.0×20 cm) which was

equilibrated with 50 mM Tris-HCl buffer; pH 7.0. Unbound proteins were removed by washing with the same buffer. This was followed by elution with a stepwise salt gradient of 0-1.0 M NaCl in the same buffer with a flow rate of 60 ml/h. Fractions containing the main peak of protease were pooled and concentrated to 2 ml and it was dialyzed for 24 h at 4°C against 500 ml of 50 mM Tris-HCl buffer; pH 7.0.

SDS-polyacrylamide gel electrophoresis and determination of molecular mass

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed in a 10% (w/v) acrylamide slab gel with method described by Laemmli (1970). Protein was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol and 10% (v/v) glacial acetic acid solution for 30 min, and destained with 40% (v/v) methanol and 10% (v/v) glacial acetic acid solution for overnight.

The molecular mass of the purified proteases was determined by the method described by Andrews (Andrews, 1964). Superdex 200 column (1.5×80 cm) was equilibrated in 20 mM Tris-HCl; pH 7.0 and run at a flow rate of 0.8 ml/h using 50 mM Tris-HCl; pH 7.0. Bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen A (25.0 kDa), and ribonuclease A (13.7 kDa) were used as standard of protein molecular weight markers (Amersham Biosciences). The protein concentration was determined by Bradford method (Bradford, 1976). A dye reagent was purchased from Bio-Red and bovine serum albumin (Sigma) was used as protein standard.

Enzymatic digestion of protein

Protein band was enzymatically digested in-gel in a manner similar to that previously described by Shevchenko and colleagues (Shevchenko, 1996) and using modified porcine trypsin (Promega). Gel pieces were washed with 50% acetonitrile to remove SDS, salt and stain, dried to remove solvent and then rehydrated with trypsin (8-10 ng/μl) and incubated 8-10 h at 37°C. The derivatization reaction was performed as previously described (Wang, 2004). The reagent solution was prepared by dissolving 4-sulfophenyl isothiocyanate (SPITC) in 20 mM NaHCO₃ (pH 9.5) or other salt solution to a concentration of 10 mg/ml. The sulfonation reaction was carried out in a 0.6 ml eppendorf tube by mixing 8.5 μl of reagent solution with 0.5 μl of tryptic digest. After incubation for 5-30 min at 55°C, the reaction was terminated by adding 1 μl of 5% trifluoroacetic acid (TFA). The sample was then loaded onto a micropipette tip (C18 ZipTip; Millipore, USA), washed with 3×10 μl of 0.1% TFA and followed by eluting with 1 μl of 50% acetonitrile/0.1% TFA.

Protein identification using Mass spectrometry and CAF-MALDI data

All MS and PSD (post source decay) spectra were acquired in the positive ion mode using a Ettan MALDI-TOF (Amersham Biosciences) mass spectrometer equipped with a pulsed extraction source, a 337 nm pulsed nitrogen laser and a curved-field reflectron. The acceleration voltage was 20 kV. The matrix a-cyano-4-hydroxycinnamicacid (CHCA) was prepared in 1:1 acetonitrile/water. A thin layer of the matrix was ap-

plied onto the sample plate first. This was followed by the addition of 0.5 µl of sample and 0.5 µl of matrix and was allowed to dry at room temperature.

For protein identification, the fragment masses obtained from CAF-MALDI were submitted to Sonar in the Ettan MALDI-TOF software or similar protein identification search engines (PepFrag). The mass of the non-derivatized peptide and five fragment masses or more are needed for protein identification. The amino acid sequence of the peptide was obtained from the distances between consecutive peaks (y-ions) in the PSD spectrum. By submitting the amino acid sequence, the protein was identified by a homology search using for example ProteinInfo™ (www.proteometrics.com), or in a BLAST search using the ExPASy Molecular Biology Server (www.expasy.ch).

Effect of pH and temperature on protease activity

To determine the optimum pH of the enzyme, activities of the protease were measured at various pHs between 5.0 and 10.5. To measure pH stability of the enzyme, the protease was pre-incubated in the different buffers for 1 h at 37°C at various pHs between 3.0 and 10.5 and then residual protease activities were assayed. The buffers used were 50 mM citrate buffer in the pH 3.0 to 5.6, 50 mM sodium phosphate buffer in the pH 6.0 to 6.5, 50 mM Tris-HCl buffer in the pH 6.8 to 8.0, and 50 mM glycine-NaOH buffer in the pH 8.0 to 10.5.

Effect of temperature on the protease activity was tested at 0-60°C under the standard assay condition. To examine the thermal stability of the enzyme, the residual protease activities were assayed after incubating the enzyme solutions in 50 mM Tris-HCl, pH 7.0 for 60 min at various temperatures.
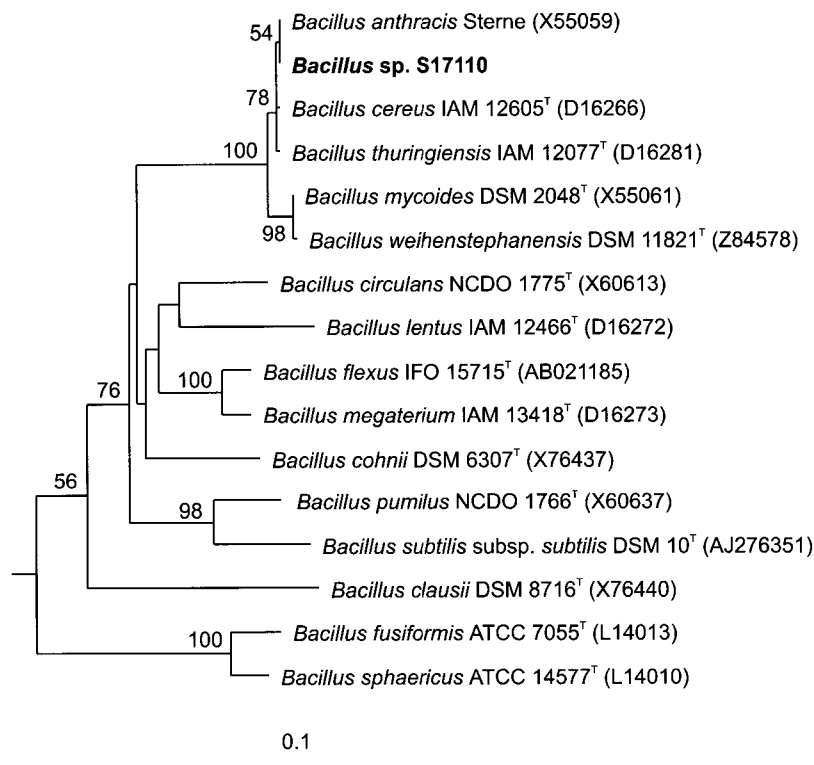

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationship between strain *Bacillus* sp. S17110 and related taxa within the genus *Bacillus*. Numbers at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1,000 resampled dataset : only values more than 50% are given. *Haloanaerobium lacuroseus* DSM 10165[T] (L39787) was used as an outgroup (data not shown). Bar, 0.1 nucleotide substitution per nucleotide position.

Table 1. Summary of the purification of protease

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	91.45	85.24	0.93	100.00	1.0
Concentrated supernatant	69.98	69.42	0.99	81.44	1.1
(NH₄)₂SO₄ fractionation	15.65	25.05	1.60	29.39	1.7
SP-Sepharose	4.50	12.68	2.82	14.88	3.0
DEAE-Sepharose	1.32	6.90	5.23	8.09	5.6

Activities were measured with azocasein as the substrate, and the purification was performed with about 10 L of 2 days cultures.

Vol. 45, No. 5

Identification of immune inhibitor A from *Bacillus* sp. S17110   405

Effect of metal ion and inhibitor on protease activity

Effect of metal ions was determined by adding each metal ion of 1 mM or 10 mM to a 1% (w/v) casein solution dissolved in 50 mM Tris-HCl; pH 7.0, and by measuring the relative proteolytic activity under the standard assay conditions. Effect of enzyme inhibitors or detergents on the protease activity was determined by measuring the residual activity under the standard assay conditions after incubating both the enzyme and inhibitor at room temperature for 1 h. Phenylmethane sulfonyl fluoride (PMSF) solution was prepared by dissolving in ethanol.

Results and Discussion

Identification of strain S17110 by biochemical and phylogenetic analysis

To obtain new potent or distinct protease we isolated microbe inhabiting in marine invertebrates and examined whether the microbes secrete protease. Three isolates were obtained from marine turban shell and characterized for their physiological and biochemical characteristics. Two isolates turned out to be same strain identified as *Staphylococcus* sp. S17111. The other strain S17110 was very close to *Bacillus cereus* (data not shown). Strain S17110 showed the highest 16S rRNA gene sequence similarity to *B. anthracis* Sterne (100%),
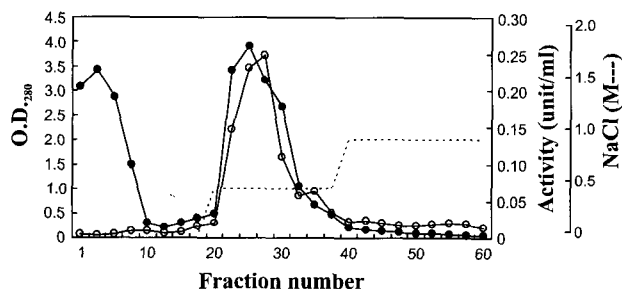

Fig. 2. SP-Sepharose column chromatography of protease. The column was equilibrated with 50 mM Tris-HCl buffer (pH 7.0). It was first washed with the equilibrating buffer and then eluted with a stepwise gradient of NaCl (0.5 M to 1.0 M) in the starting buffer. Flow rate was 2 ml/min, and fraction size was 5 ml. Symbols: ●, A280; ○, protease activity; ---, NaCl elution.
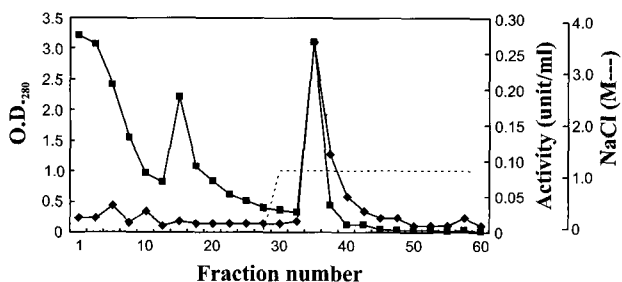

Fig. 3. DEAE-Sepharose column chromatography of protease. The column was equilibrated with 50 mM Tris-HCl buffer (pH 7.0). It was first washed with the equilibrating buffer and then eluted with a starting buffer containing 1.0 M NaCl. Flow rate was 1 ml/min, and fraction size was 5 ml. Symbols: ■, A280; ◆, protease activity; ---, NaCl elution.

B. cereus IAM 12605[T] (99.9%), *B. thuringiensis* IAM 12077[T] (99.7%), *B. mycoides* DSM 2048[T] (99.4%) and *B. wihenstephanensis* DSM 11821[T] (99.3%). The phylogenic tree produced by neighbor-joining method demonstrated that strain S17110 was most closely related to strains of *B. cereus* group (Fig. 1). The *B. cereus* group comprises the five valid species *B. cereus*, *B. mycoides*, *B. thuringiensis*, *B. anthracis* and *B. weihenstephanensis* (Pitcher *et al.*, 1989; Lechner *et al.*, 1998).

Purification of protease

The protease production was maximal at middle exponential growth phase when *Bacillus* sp. S17110 grew at 30°C in OSYM media. A summary of the purification of the protease from the culture supernatant is given in Table 1. The enzyme was purified about 5.6-fold with a yield of about 8%. Culture supernatant was concentrated by ultrafiltration and 70% ammonium sulfate precipitation. During SP Sepharose column chromatography, major part of protease eluted as a wide peak at 0.5 M NaCl (Fig. 2). The next step was performed by using DEAE-Sepharose column chromatography (Fig. 3). One sharp peak of protease activity containing 50% of the activity applied to the column was eluted at 1.0 M NaCl. This step yielded an enzyme found to be homogenous by SDS-PAGE (Fig. 4) The molecular weight of the purified protease was found to be 75,000 as determined by SDS-PAGE and gel filtration chromatography on Superdex 200 column. These results indicated that the protease is monomeric. The culture supernatant was subjected to SDS-PAGE (Fig. 4, lane 2), but any band couldn't be detected. Some bands were seen in sample concentrated with ultrafiltration (Fig. 4, lane 3). Biggest band was major protein. Most of minor bands reduced significantly in the fractions with protease activity after SP-Sepharose column chromatography (Fig. 4, lane 4). Major protease bound to DEAE-Sepharose column was detached by elution with buffer containing 1.0 M salt and resulted in one band being a mass of 75,000 Da on SDS-PAGE (Fig. 4, lane 5).
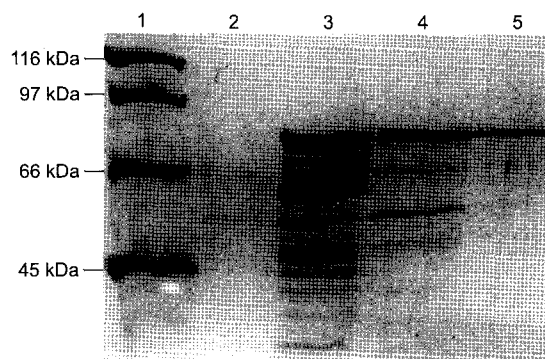

Fig. 4. SDS-PAGE of the protease purified by chromatographic methods. Lanes 1, molecular masses of protein standards, i.e., carbonic anhydrase (31.0 kDa), ovalbumin (45.0 kDa), serum albumin (66.2 kDa), and phosphorylase b (97.4 kDa), β-galactosidase (116.2 kDa); 2, supernatant; 3, supernatant concentrated; 4, fraction after SP-Sepharose column chromatography; 5, fraction after DEAE-Sepharose column chromatography.

Table 2. Matching of extraceluler protease mass fingerprint data with ZP00238828 in database

m/z Submitted	MH+ matched	Modifications	Start	End	Missed cleavage	Database sequence
883.453	883.498		469	476	0	(K) SNRPGVVR (V)
896.412	896.414		494	500	0	(K) HAYYSTR (G)
168.570	1168.562		327	336	0	(K) LGDDAIWSHR (S)
233.631	1233.660		483	493	0	(K) SVETIKPEFGK (H)
299.600	1299.603		643	651	0	(K) AHYYYLEWR (N)
423.767	1423.796		166	177	0	(K) VLVLLVEFSDYK (H)
427.674	1427.698		642	651	1	(K) KAHYYYLEWR (N)
439.643	1439.667		281	292	0	(K) GLDLSQFDQFDR (Y)
475.691	1475.725		417	430	0	(K) IAGTEPTSFSPQNK (D)
672.758	1672.834		588	601	0	(K) LQFDYITDPAVTYK (G)
717.734	1717.801		201	215	0	(K) MLFGNEPYTLFDGSK (V)
733.721	1733.796	1 Met-ox	201	215	0	(K) MLFGNEPYTLFDGSK (V)
966.790	1966.897		501	517	0	(R) GDDMHTTLETPFFDLTK (G)
982.849	1982.892	1 Met-ox	501	517	0	(R) GDDMHTTLETPFFDLTK (G)
157.826	2157.917		178	195	0	(K) HNNIDQTPGYMYSNDFSR (E)
173.749	2173.912	1 Met-ox	178	195	0	(K) HNNIDQTPGYMYSNDFSR (E)
273.939	2274.025	1 Met-ox	395	416	0	(K) YTGTGSPVEAWSLMSGGSWTGK (I)
685.025	2685.221		602	627	0	(K)GFAMDNVNVTVDGQVVFADDAEGTSK(M)

18/27 matches (28%). Acc. no.: ZP00238828, species: Bacillus, name: proteinase VCA 0223
Mass/p*I*: 77500 (Da)/5.4
The matched peptide sequences are shown in bold.
1   MRRKAPLKVL SSLAIAAIIG CTSVMSAPLA YAETPAKEKE NVSTTPIDYN LIQEDRLAEA LKERGTINPA SSKEETKKAV EKYIEKKQGD QANKEILPAD TAKEASDFVK KVKEKKMEEK EKVKKPEKNV
141 SPEQKPEPNK KQLNGQVPTS KAKQAPYKGS VRTDKVLVLL **VEFSDYKHNN IDQTPGYMYS NDFSREHYQK MLFGNEPYTL FDGSKVKTFK** QYYEEQSGGS YTTDGYVTEW LTVPGKASDY
271 GADGSSGHDN KGPKGARDLV KEALHAAAEK **GLDLSQFDQF DRY**DTNSDGN QNEPDGVIDH LMVIHAGVGQ EAGGGKLGDD **AIWSHRSKLA** IDPVAIEGTK SKVDYFGGKV AAHDYTIEPE
391 DGAVGVFAHE FGHDLGLPDE YDTKYTGTGS **PVEAWSLMSG GSWTGKIAGT EPTSFSPQNK** DFLQKNMGGN WAKILEVDYD KIKRGVGVPT YIDQSVTKSN RPGVVRVNLP GKSVETIKPE
511 **FGKHAYYSTR GDDMHTTLET PFFDLTKGTN** AKFDYKANYE LEAECDFVEV HAVTEDGTKT LIDRLGEKVV QGDKDTTDGK WIDKSYDLSQ FKGKKVKLQF **DYITDPAVTY KGFAMDHVNV**
631 **TVDGQVVFSD DAEGQSKMNL** NGFVVSDGTE KK**AHYYYLEW** RNYAGSDNGL KAGKGPVYNT

Analysis of protease by MALDI-TOF mass spectrometry
A purified protease was analyzed by MALDI-TOF-MS method. The molecular mass of the protease was determined to 77.5 kDa with MALDI-TOF-MS analysis. The protein was digested by trypsin and resulting 18 peptides were identified as the protease VCA0223 of *Bacillus cereus* G9241 (Hoffmaster *et al.*, 2004) (Table 2). The *B. cereus* G9241 was isolated from the sputum and blood of patient with pneumonia. The proteinase VCA0223 was deduced from a gene encoded by *Bacillus cereus* G9241 of which genome sequencing has been completed. Homology search of the proteinase VCA0223 revealed that it matched with immune inhibitor A (inhA).

The insect pathogenic Gram-positive *Bacillus thuringiensis* secretes immune inhibitor A, a metallopeptidase, which specifically cleaves host antibacterial proteins. The protein contains putative conserved domains of immune inhibitor A, peptidase M6. The protein has been previously identified in DNA database of many similar strains including *B. anthracis* str. Ames Ancester, *B. anthracis* str. Sterne, *B. thringiensis* serovar, and *B. weihenstephanensis*.

The protease VCA0223 of *Bacillus* sp. S17110 is composed of 701 amino acid residues whereas InhA from various strains listed above were of 799 amino acid residues. The entire region of protease VCA0223 shows 98% identity with that of *B. anthracis* str. Ames Ancester, *B. anthracis* str. Sterne, *B. thringiensis* serovar. We didn't find an InhA in *Bacillus cereus* G9241 genome sequence which is composed of appr. 799 amino acids residue and is also highly homologous with InhA (ZP00239282) of *Bacillus* sp. S17110 (Table 2). Therefore it appears that the protein purified is not degradation product of InhA that might be big, but a distinct protein.

Effect of pH and temperature on protease activity
The enzyme retained activity in the broad pH range of pH 5.0 to pH 10.5 (Fig. 5). About 70% of maximal protease activity was obtained from pH 6.0 to pH 10.5. The purified protease showed the highest activity on casein at pH 7.5 (Fig. 5A). When the pH stability of the protease was examined by incubating for 1 h or 3 h in the pH range of 3.0-10.5, their enzyme activities were very similar with samples
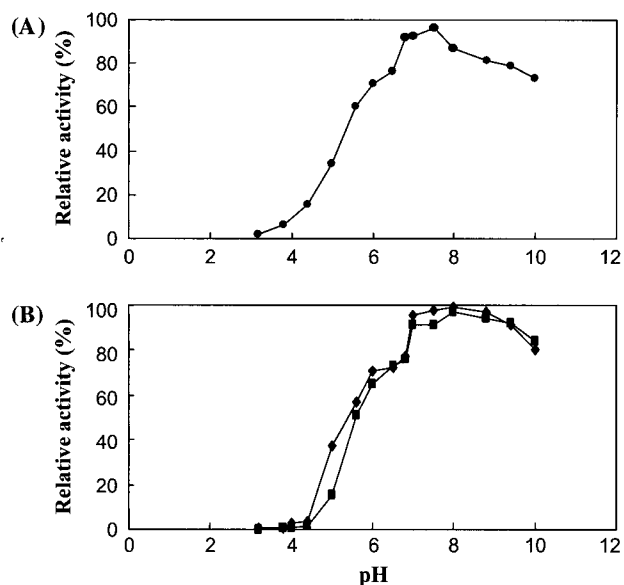
(A)

(B)

pH

Fig. 5. (A) Effect of pH on the activity of protease purified from *Bacillus* sp. 17110. The residual activity (●) was measured at 37°C after the enzyme assay carried out in buffers following: 50 mM citric acid-NaOH in the pH range of 3.0-5.6; 50 mM phosphate in the pH range of 6.0-6.5; 50 mM Tris-HCl in the pH range of 6.8-8.0; 50 mM Glycin-NaOH in the pH range of 8.6-10.5. (B) Effect of pH on the stability of protease from *Bacillus* sp. S17110. The protease was pre-incubated enzyme in the different buffers for 1 h (♦) or 3 h (■) at 37°C, and then residual protease activities were measured.
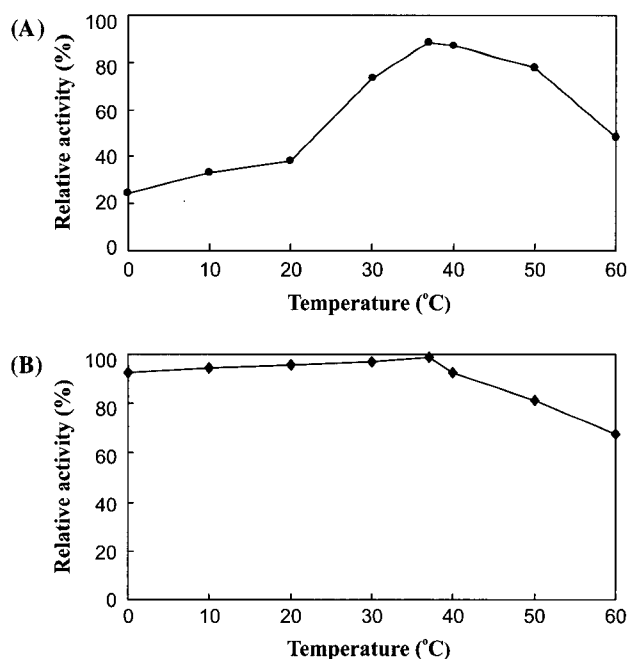
(A)

Temperature (°C)

(B)

Temperature (°C)

Fig. 6. (A) Effect of temperature on the activity of the protease purified from *Bacillus* sp. 17110. Protease activity (●) was measured at temperatures ranges of 0-60°C under the standard assay condition. (B) Effect of temperature on the stability of protease from *Bacillus* sp. S17110. The thermal stability of the enzyme was determined by measuring residual protease activities (♦) after pre-incubating for 60 min at various temperatures.

Table 3. Effects of various metal ions and reagents on activity of the protease purified from *Bacillus* sp. S17110

Metal ion	Relative activity (%)	
	1 mM	10 mM
None	100	100
CdCl₂	72	50
BaCl₂	74	80
KCl	76	83
ZnSO₄	111	109
FeSO₄	99	87
MgSO₄	104	108
CuCl₂	94	82
HgCl₂	58	45
NiCl₂	86	52
Pb(NO₃)₂	99	106
CaCl₂	105	111
MnCl₂	113	114
Li₂SO₄	81	84

Table 4. Effects of inhibitors on the activity of the protease

Inhibitor	Relative activity (%)		
	1 mM	10 mM	100 mM
None	100	100	100
PMSF	90	77	-
EDTA	88	55	-
Dithiothreitol	100	94	-
β-Mercaptoethanol	98	98	-
L-Cysteine	102	100	-
Iodoacetic acid	90	104	-
SDS	94	88	82
Triton X-100	113	112	-
Tween 20	95	94	73

without incubating at specific pHs (Fig. 5B). Almost all the protease activity was retained between pH 7.0 and pH 9.5 regardless of incubation time between 1 h and 3 h, indicating that the protease is alkalophilic.

Optimum temperature and stability for temperature of the purified protease was examined from 0°C to 60°C. Optimum temperature was 37°C (Fig. 6A). When the enzyme was pre-incubated for 60 min at 60°C, seventy percent of the activity remained (Fig. 6B).

Effect of metal ion and inhibitor on the protease activity
Table 3 showed the effects of various divalent cation on the activity of the protease. Zn^{2+}, Mg^{2+}, Ca^{2+}, and Mn^{2+} stimulated slightly enzyme activities whereas Ni^{2+}, Cd^{2+}, and Hg^{2+} inhibited the activities. 45% of the activity was lost in the presence of 10 mM divalent cation chelator EDTA (Table 4), indicating that this enzyme is metalloprotease. The enzyme remained mostly unaffected by some reducing reagents and

iodoacetic acid, which indicates lack of sulfur-containing amino acids in active site. The protease was resistant to various denaturants such as SDS, Tween 20 and Triton X-100 (Table 4). Recently, neutral metalloproteases had been isolated from *Bacillus anthracis*. Those proteases purified from culture supernatant were identified as a thermolysin like enzyme (NPR599) highly homologous to bacillolysin and InhA homologue of the *B. thuringiensis*. Interestingly both protein were inhibited by EDTA, and SDS activated Npr599 like the protease we purified, suggesting that the pathogenic factor be virulent in a denaturating condition (Chung *et al.*, 2006).

In conclusion, peptide fingerprint mass spectra analysis of the protease purified from *Bacillus* sp. S17110 revealed that it was completely identical with InhA from *Bacillus cereus* G9241 (Hoffmaster *et al.*, 2004). Overall data described in this paper suggest that the protease produced from *Bacillus* sp. S17110 be pathogenic and might be involved in infection to marine invertebrate including turban shell. Therefore it will be of significance to investigate whether the protease is prevalent in shellfish, and whether the protein plays a role as a pathogenic factor in insect or not.

Acknowledgments

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