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Identification of Three Extracellular Proteases from *Bacillus subtilis* KCTC 3014

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Abstract Three extracellular proteases (Vpr, peptidase T, and subtilisin) were identified from the culture supernatant of *Bacillus subtilis* KCTC 3014. All the proteins were partially purified as a mature form by using a DEAE-cellulose ion-exchange column chromatography. Their activities were determined by using zymography and densitometry. The relative molecular masses of Vpr and peptidase T (PepT) were determined to be 68 and 48 kDa by SDS-PAGE and zymography, respectively. However, subtilisin formed a "binding mode" at the top of the separating gel. After denaturation by boiling at 100°C for 5 min, its molecular mass was determined to be 29 kDa, whereas its activity was lost. The optimal pH of Vpr, PepT, and subtilisin were 9.0, 6.0–7.0, and 7.0–8.0, respectively. The optimal temperature of Vpr, PepT, and subtilisin was 40, 50, and 40°C, respectively. Inhibitor test revealed that Vpr and subtilisin were serine proteases and that PepT was a metalloprotease. Interestingly, we found that Vpr showed no enzyme activity on a 2DE zymogram gel. Three genes, *vpr*, *pepT*, and *apr* (encoding subtilisin protein), were cloned and their nucleotide and deduced amino acid sequences were determined.

Key words: *Bacillus subtilis* KCTC 3014, mass spectrometry, PepT, subtilisin, Vpr, zymography

Proteases are one of the major classes of extracellular proteins that the Gram-positive spore-forming bacterium *Bacillus subtilis* secretes into the culture medium at the end of the exponential phase of growth [21, 27]. The European-Japanese *B. subtilis* genome project revealed 11 different extracellular proteolytic enzymes, and the genes for these proteins have been identified [17]. The major extracellular proteases among them are subtilisin and neutral proteases encoded by *apr* and *npr* genes, respectively [12, 26, 28, 29]. These two proteins account for more than 90% of the total extracellular proteolytic activity [12]. In addition, the genes for three minor extracellular proteases, *epr* (encoding Epr protein) [24], *bpr* (encoding bacillopeptidase F) [22], and *mpr* (encoding Mpr), have also been identified [23]. Epr and bacillopeptidase F are serine protease, whereas Mpr is a minor metalloprotease [23].

Park *et al.* [20] reported extracellular fibrinolytic enzymes from *B. subtilis* 168 using a combined method of two-dimensional gel electrophoresis and zymography, and identified two extracellular proteases, WprA and Vpr [20]. To confirm the activity of Vpr, they cloned the *vpr* gene, expressed it in *Escherichia coli*, and demonstrated that the expressed Vpr protein exists in multiple forms with enzyme activity [13].

We found that the extracellular protease pattern of *B. subtilis* strains was various (Fig. 1). Among them, *B. subtilis* KCTC 3014, which shows the highest enzyme activity, was

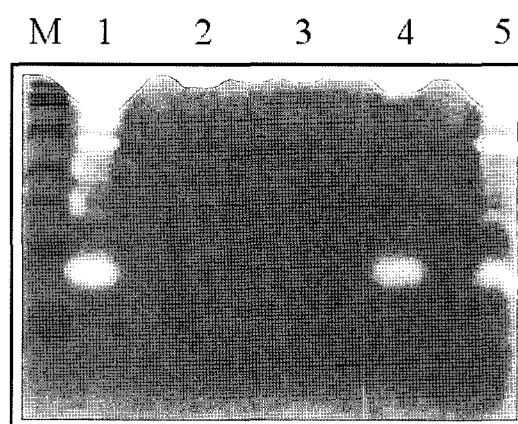

Fig. 1. Fibrin zymography of extracellular proteins of five *B. subtilis* strains.
The first dimension was 15% gel, where proteins were separated by their molecular mass. The symbols 1–5 represent *B. subtilis* KCTC 3014, *B. subtilis* KCTC 1722, *B. subtilis* KCTC 1763, *B. subtilis* DJ-2, and *B. subtilis* 168, respectively.

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used in this study. Our aim of the present study was to establish an extracellular protease map of *B. subtilis* KCTC 3014. Although the exact number of extracellular proteins of *B. subtilis* 168 has been determined by the European-Japanese *B. subtilis* genome project [17], there is as yet no well-established protease map of *B. subtilis*. In this study, we identified three extracellular proteases, Vpr, PepT, and subtilisin, in the culture supernatant of *B. subtilis* KCTC 3014 using 2DE, fibrin zymography, N-terminal amino acid sequencing, and MALDI-TOF. Consequently, we established a partial extracellular protease map of *B. subtilis* KCTC 3014, and carried out a preliminary characterization of three extracellular proteases, Vpr, PepT, and subtilisin.

MATERIALS AND METHODS

Bacterial Strain and Culture Condition

B. subtilis KCTC 3014, obtained from Korean Collection for Type Cultures (KCTC), was grown in tryptic soy broth (TSB, Difco, U.S.A.) at 37°C for 2 days. The culture supernatant was collected by centrifugation at 5,000 rpm for 20 min at 4°C.

Partial Purification of Extracellular Proteases from *B. subtilis* KCTC 3014

Two liters of the culture supernatant was concentrated by ultrafiltration with PM-10 membrane (Amicon, Inc., U.S.A.). The concentrated solution was dialyzed overnight against 20 volumes of 25 mM sodium phosphate buffer (pH 6.3), and was then loaded onto a DEAE-cellulose column (2.0×10 cm) (Pharmacia Biotech, Sweden). Proteins were eluted with 200 ml of linear gradient formed from 0 to 1 M NaCl. Active fractions were pooled and desalted by filtration with Centricon YM-10 (Millipore, U.S.A.).

Zymography

Fibrin zymogram gel was carried out as described previously [7, 14, 15]. Separating gel solution (12%, w/v) was prepared in the presence of fibrinogen (0.12%, w/v; Sigma F-8630) and 100 µl of thrombin (10 NIH units/ml; Sigma T-7513). Samples were diluted 5 times with the SDS sample buffer. After electrophoresis in a cold room (at 10 mA constantly), the gel was incubated for 30 min at room temperature on a rotary shaker in 50 mM Tris (pH 7.4), which contained 2.5% Triton X-100, washed with distilled water for 30 min to remove Triton X-100, and then incubated in the zymogram reaction buffer (30 mM Tris, pH 7.4, and NaN_3) at 37°C for 12 h. The gel was stained with Coomassie blue for 1 h and then destained. The active bands were visualized as nonstained regions of the zymogram gel.

Protease Inhibitor Assay on Fibrin Zymography

Classification of the enzymes noted on zymography [14] was performed by incubating Triton X-100-washed gels in the zymogram reaction buffer containing protease inhibitors: 10 mM PMSF (a known inhibitor of serine protease) or EDTA (a known inhibitor of metalloprotease).

Densitometric Analysis of Active Bands

For quantification, densities of the digested bands on the zymogram gel were analyzed by video densitometry using Bio 1D ver. 97.04 (Vilber Lourmat, France) [7, 8, 16].

Fibrin Plate

Enzyme activity was determined by using a fibrin plate [1]. Fibrinogen solution [5 ml of 0.6% (w/v); Sigma] in 50 mM sodium phosphate buffer (pH 7.4) was mixed with the same volume of 2% (w/v) agarose solution and 0.1 ml of thrombin solution (10 NIH unit/ml; Sigma) in a Petri dish. The Petri dish was left for 1 h at room temperature to form a fibrin clot layer. Twenty µl of the sample solution was then dropped into holes that had been made by a capillary glass tube (5-mm diameter), and the plate was incubated at 37°C for 12 h.

Enzymatic Digestion of Protein In-Gel and MALDI-TOF Mass Spectrometric Analysis and Database Search

Enzymatic digestion was performed basically as previously described [9]. The spots on the gel were excised with a razor, placed in an Eppendorf tube, and washed three times with water to remove methanol and acetic acid. Next, the gel was mashed with a glass spatula and then dried in a speed Vac vacuum centrifuge apparatus for 30 min at room temperature. The dried samples were reconstituted in 20 µl of 25 mM ammonium bicarbonate (pH 8.5) containing 0.0125 µg/µl of trypsin and incubated overnight at 37°C. After enzymatic digestion, the supernatant was removed, and the resultant peptides were extracted by adding 20 ml of 0.1% trifluoroacetic acid (TFA)-50% acetonitrile (ACN) (Merck, Darmstadt, Germany). The extraction was repeated twice with the same solution. In the final extraction, 30 µl of 100% ACN was added to completely dehydrate the gel (which turned white). Subsequently, the extracts were pooled and dried in a Speed Vac vacuum. Finally, the dried samples were dissolved in 20 µl of 0.1% TFA-ACN solution (2:1, v/v). The trypsin-digested sample was then analyzed by mass-spectrometric analysis with a PerSeptive Biosystems (Framingham, MA, U.S.A.) matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) Voyager DE-RP mass spectrometer, which was operated in delayed extraction and reflector mode. Peptide mixtures were analyzed by using a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile-0.1% trifluoroacetic acid [10]. The PEPTIDENT program of ExPASY was used for database searching.

Two-Dimensional Gel Electrophoresis

IEF was carried out using 7-cm linear immobiline IPG gels with pH range of 3–10 (Bio-Rad, Hercules, CA, U.S.A.). The

Table 1. Primer sequences for three extracellular proteases of *B. subtilis* KCTC 3014.

Primer	Nucleotide sequence	Restrict. enzyme	Accession No.
Vpr-N'	ccg gaa ttc cgA TGG ATG ACA GTG CGC CTT A	EcoRI	M76590
Vpr-C'	ccg ctc gag TTA TTC AAC AGT GAA AAG TTC T	XhoI	
PepT-N'	cgg gat ccA TGA AAG AAG AAA TCA TTG AAC	BamHI	X99339
PepT-C'	ggc tcg agT GCT TGC GCT TCA AAC TGC T	XhoI	
Subtilisin-N'	ggg ttt cat atg GCG CAG TCC GTG CCT TAC	NdeI	K02496
Subtilisin-C'	cgc gga tcc TTA CTG AGC TGC CGC CTG T	BamHI	

protein was loaded by in-gel rehydration with a reswelling solution containing 8 M urea, 0.3% DTT (w/v), and 2.0% (v/v) pH 3–10 IPG buffer. IEF was carried out for 4,000 Vh at 20°C in a IPGphor Isoelectric Focusing System (Bio-Rad), wherein the voltage was linearly increased from 250 V to 4,000 V over the first 3 h and then was maintained at 4,000 V for the final 10 h. After the IEF, the strips were equilibrated with the SDS equilibration buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 30% glycerol, and 0.05% bromophenol blue) for 15 min. The equilibrated strips were loaded into the stacking gel of the SDS gel and fibrin gel, and the 2D gel electrophoresis was performed.

N-Terminal Amino Acid Sequencing

To determine the N-terminal amino acid sequence, the protein was concentrated by acetone precipitation. After SDS-PAGE was done, the protein on the gel was transferred to a polyvinylidene difluoride (PVDF; Bio-Rad) membrane by electroblotting [19], and the membrane was stained with Coomassie blue. The stained portion was excised and directly used for the N-terminal sequencing by the automated Edman degradation method, using a Gas-phase protein sequencer (model Procise 491, ABI, U.S.A.).

Cloning of *vpr, pepT,* and *apr*

Using the amino acid sequences of three proteins (Table 2), we searched the nucleotide sequence database of National Center for Biotechnology Information and found Vpr (Accession No. M76590), PepT (Accession No. X99339), and subtilisin (Accession No. K02496) (Table 1). Three genes, *vpr*, *pepT*, and *apr*, were amplified from *B. subtilis* KCTC 3014 genomic DNA by a polymerase chain reaction (PCR) with two pairs each of primers (Table 1). PCR amplification was performed under the following conditions: 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min. The PCR-amplified DNA fragments were extracted from agarose gel and then ligated into pGEM-T Easy vector (Promega) to generate pT-*vpr*, pT-*pepT*, and pT-*apr* plasmids.

RESULTS AND DISCUSSION

Identification of Extracellular Proteases from *B. subtilis* KCTC 3014

According to the zymogram gel analysis of *B. subtilis* KCTC 3014, five active bands (EP2-6) and one active "binding mode" (EP1), which showed activity only at the top part of the separating gel, were detected (Fig. 2).

Partial Purification of Extracellular Proteases

In order to resolve the protein mixture, DEAE-cellulose ion-exchange column chromatography was performed. After fractionation by a NaCl gradient (0–1 M), the active fractions on the fibrin plate were pooled (data not shown), and salts in the fractions were removed with Centricon YM-10. The proteins were resolved by SDS-PAGE and zymogram gels (Fig. 3). Three active bands (EP1-3) were detected, whereas other bands located on low molecular range (EP4-6) were removed. The SDS gel and zymogram gel were compared, and protein bands located on clear bands of the zymogram gel were cut. Three proteins (EP1-3) were extracted and digested with trypsin. Trypsin-digested samples were then analyzed by MALDI-TOF mass spectrometry.

Identification of Vpr and PepT

The peptide fingerprint of EP2 protein revealed that it contained pure Vpr, as described previously (data not shown) [11, 20]. Park *et al.* [20] found that four different protein spots (named as S1-S4, 60–70 kDa) on a 2D gel were identified as the same Vpr protein. Of these, S2 was matched with the mature protein of Vpr [20]. On the other hand, Hirose *et al.* [11] detected Vpr protein as a single spot in

Table 2. Identification of three extracellular proteases of *B. subtilis* KCTC 3014.

Protein identity	Gene	Molecular mass (kDa)	pI	Function	Optimal T_m (°C)	Optimal pH	N-Terminal sequence	Accession no.
Minor extracellular protease	*vpr*	68	4.2–4.3	Serine protease	40	9.0	MDDSAPYIGA	AAX84518
Peptidase T	*pepT*	48	4.6–4.8	Metalloprotease	50	6.0–7.0	MKEEIIERFT	AAX58685
Subtilisin	*apr*	29	>9.0	Serine protease	40	7.0–8.0	AQSVPYGVSQ	AAY23643

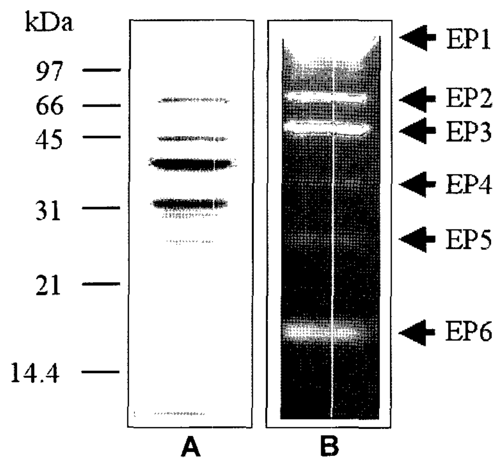

Fig. 2. One-dimensional SDS-PAGE (**A**) and fibrin zymography (**B**) of extracellular proteins of *B. subtilis* KCTC 3014.

The first dimension was 15% SDS gel or zymogram gel, where proteins were separated by their molecular mass. Proteins were visualized by silver staining in SDS gel and Coomassie blue was used for the activity staining of proteases in the zymogram.

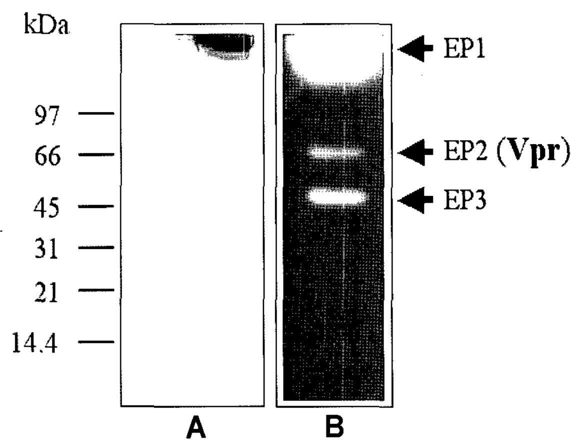

Fig. 3. One-dimensional SDS-PAGE (**A**) and fibrin zymography (**B**) of the active fraction from DEAE-cellulose ion-exchange column chromatography.

After fractionation by NaCl gradient (0–1 M) as described in Materials and Methods, the active fraction was resolved by SDS-PAGE and zymogram gels.

the conventional 2D gel. In order to obtain better resolution of proteins, we tried 2DE. As shown in Fig. 4, we also identified the mature form of Vpr (68 kDa) as a single spot on the 2D gel, whereas no activity was detected on the 2D zymogram gel. Its N-terminal sequence was determined (Table 2). The 2D zymogram showed only one active spot with an apparent molecular mass of 48 kDa, which matched with that of EP3 protein on the 1D gel (Figs. 2 and 3). Its N-terminal sequence was identified as a mature form of peptidase T (PepT). The peptide fingerprint of EP1 (binding mode) contained two or three different proteins, but any known protease was not identified.

Identification of Subtilisin

Recently, we found that recombinant subtilisins (BPN', Carlsberg, and DJ-4) formed binding mode under non-denatured condition, because of their high pI values (≥8.8) under a Laemmili buffer system (pH 8.8) [4, 8]. On the other hand, denatured subtilisin by boiling or trichloroacetic acid (TCA) precipitation could migrate into the separating gel part, and its molecular mass could be determined (29 kDa) (data not shown). In order to separate the binding mode, we tried two-step SDS gels. Protein samples (non-denatured condition) were resolved on a 12% gel. After electrophoresis, the binding mode part of the gel was cut and boiled at 95°C for 10 min (Fig. 5A). Following a brief wash with an electrophoresis running buffer, the gel slices were then applied to another gel. As shown in Fig. 5, three protein bands were separated (marked as asterisk), and one band was identified as subtilisin (Table 2) and its molecular mass was determined to be 29 kDa, but its activity was lost.

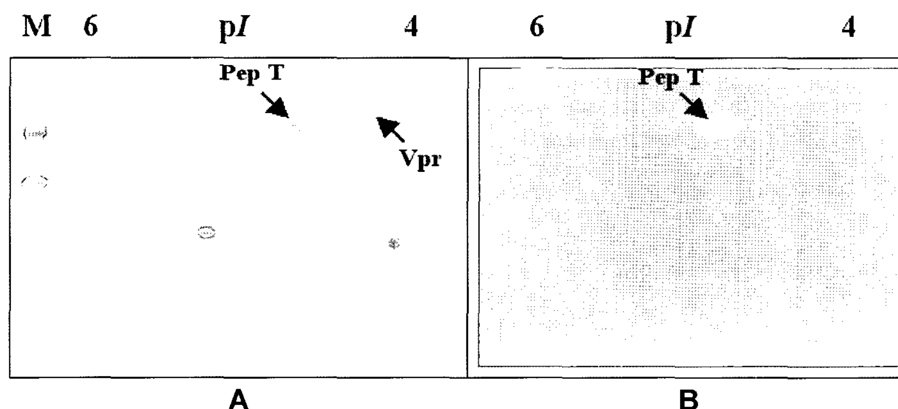

Fig. 4. Two-dimensional protein gel (**A**) and fibrin zymography (**B**) of active fraction from DEAE-cellulose ion-exchange column chromatography.

The first dimension was an isoelectric focusing (immobiline gel ranging from 4 to 6) and the second dimension included 15% SDS gel or fibrin zymogram gel.

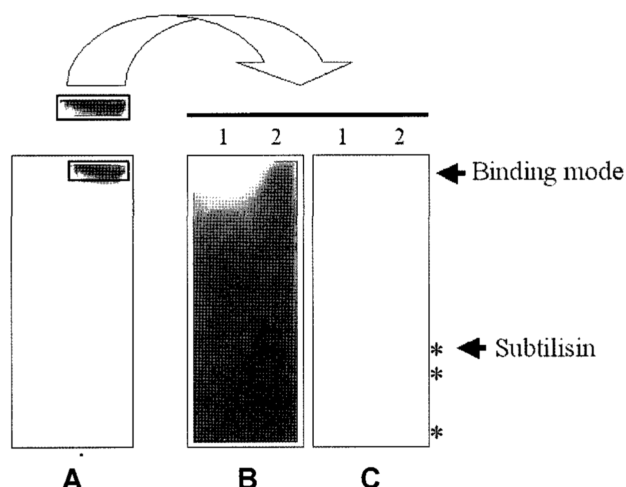

Fig. 5. One-dimensional SDS-PAGE and fibrin zymography.
After SDS gel electrophoresis with active fraction (**A**) (described in Fig. 2 and 3), the binding mode part was cut and boiled at 95°C for 10 min. Following a brief wash with an electrophoresis running buffer, the gel slices were then applied to a zymogram gel (**B**) or SDS gel (**C**).

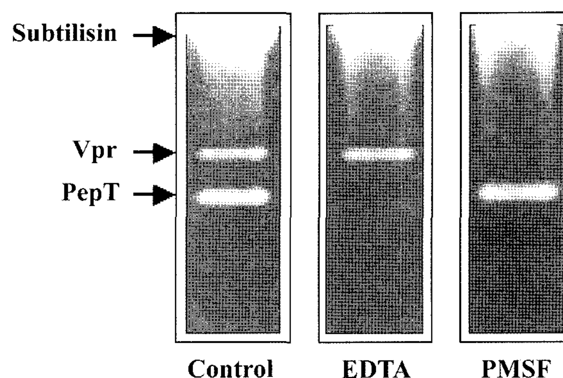

Fig. 7. Inhibitor assay in the fibrin zymography of active fraction from DEAE-cellulose ion-exchange column chromatography.
After zymogram gel electrophoresis, the gels were incubated in the zymogram reaction buffer containing protease inhibitors, 10 mM PMSF, or EDTA.

Effects of pH and Temperature on the Activity of Vpr, PepT, and Subtilisin

The effects of pH on the activity of Vpr, PepT, and subtilisin were examined using fibrin zymography and densitometry [6, 14–16]. Three proteins were incubated either in 0.1 M citrate-phosphate buffer (pH 2.0 to 5.0), sodium phosphate buffer (pH 6.0 to 7.0), tris-HCl buffer (pH 8.0 to 9.0), or glycine-NaOH buffer (pH 10.0 to 11.0). Vpr and subtilisin were found to exhibit their optimal activities at pH 9.0 and 7.0–8.0, respectively, indicating that they are alkaline proteases. The optimum pH of PepT was 6.0–7.0, suggesting that it is a neutral protease (Fig. 6 and Table 2). The optimal temperature of Vpr, PepT, and subtilisin were at 40, 50, and 40°C, respectively. As seen in Fig. 7 and Table 2, Vpr and subtilisin were inhibited by PMSF (a serine protease inhibitor),

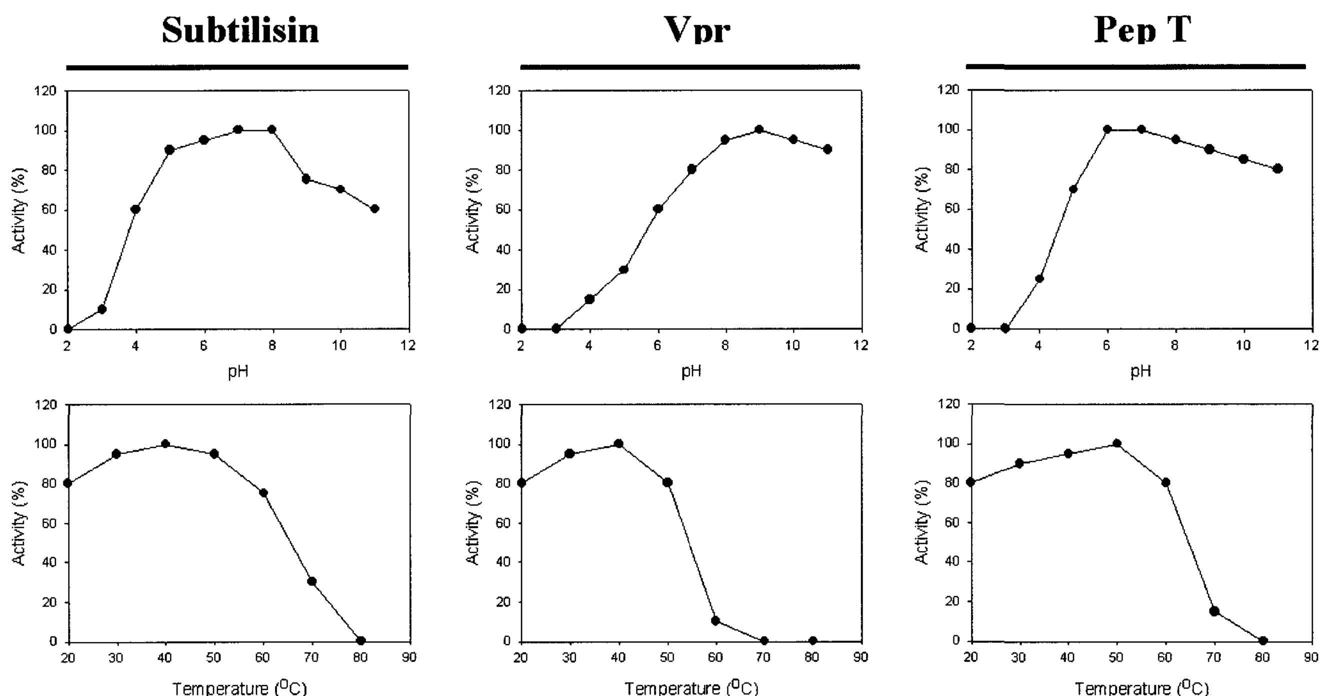

Fig. 6. Effects of pH and temperature on the activity of subtilisin, Vpr, and PepT.
The enzyme suspensions (3.0 μg) were incubated with buffers of various pHs, at 4°C for 24 h. For the effect of temperature, the enzyme suspensions (3.0 μg) were incubated at the indicated temperatures for 30 min. The samples were then applied onto zymography, and residual activity was measured by using a densitometer.

and PepT was inhibited by EDTA (a metalloprotease inhibitor). Therefore, PepT and subtilisin were classified as an alkaline serine protease, whereas PepT was a neutral metalloprotease.

Cloning of *vpr*, *pepT*, and *apr*

Three genes (*vpr*, *pepT*, and *apr*) from *B. subtilis* KCTC 3014 were cloned, and their nucleotide and deduced amino acid sequences were determined [deposited in GenBank under Accession No. AY973268 (nucleotide) and AAX84518 (amino acid) for Vpr, AY960131 (nucleotide) and AAX58685 (amino acid) for PepT, and DQ007296 (nucleotide) and AAY23643 (amino acid) for subtilisin]. The amino acid sequences of three proteins were compared with the published sequences of the other proteins. Vpr was found to be identical to minor extracellular protease (Vpr) from *B. subtilis* 168, except for three amino acid substitutions (G69E, S110T, and T401A) (Fig. 8). PepT showed 99.5% identity with peptidase T (PepT) from *B. subtilis* 168, except for two amino acid differences (K109Q and Y279H) (Fig. 9). Subtilisin was found to be identical to subtilisin DJ-4 from *Bacillus* sp. DJ-4 [5], except for three amino acid substitutions (S104Y, S189F, and C221S) (Fig. 10), showing 84% identity with subtilisin E from *B. subtilis* 168. Based on the sequence similarity search, three genes from *B. subtilis* KCTC 3014 were most closely related to the genus of *B. subtilis* 168. Therefore, we confirmed that the extracellular pattern of *B. subtilis* 168 is the same as that of *B. subtilis* KCTC 3014 (Fig. 1).

Using 2DE, Park *et al.* [20] identified four different proteins as the same Vpr protein showing enzyme activity from *B. subtilis* 168. However, we found Vpr protein as a single spot on 2D gel, as described by Hirose *et al.* [11]. Interestingly, no activity was detected on the 2D zymogram gel, which differs from the data of Park *et al.* [20].

Two types of extracellular protease are known to exist in *B. subtilis* 168; four (Bpf, Epr, Vpr, and WprA) are serine proteases and two (NprB and PepT) are neutral metalloproteases [17, 27]. All of these proteases are synthesized in the form of "prepro" enzyme [27]. In particular, four serine proteases undergo N- or C-terminal processing or proteolysis, and various active proteins are formed. Bpf is synthesized as a 92 kDa protein, and it is gradually converted through processing at both the N- and C-termini into various forms with molecular masses ranging from 80 to 40 kDa [3, 22]. Epr is also processed and the molecular mass of the active form is about 40–34 kDa [2, 24]. In the case of Vpr with molecular mass of 68 kDa, it undergoes C-terminal processing

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KCTC3014   MDDSAPYIGA NDAWDLGYTG KGIKVAIIDT GVEYNHPDLK KNFGQYKGYD FVDNDYDPKE TPTGDPRGGA TDHGTHVAGT
BS168      MDDSAPYIGA NDAWDLGYTG KGIKVAIIDT GVEYNHPDLK KNFGQYKGYD FVDNDYDPKE TPTGDPRGEA TDHGTHVAGT
BL14580    MDKSAPFIGA DQAWKSGYTG KGIKVAVIDT GVDYTHPDLK NNFGPYKGYD FVDNDYDPQE TPTGDPRGGA TDHGTHVAGT

KCTC3014   VAANGTIKGV APDATLLAYR VLGPGGSGTS ENVIAGVERA VQDGADVMNL SLGNSLNNPD WATSTALDWA MSEGVVAVTS
BS168      VAANGTIKGV APDATLLAYR VLGPGGSGTT ENVIAGVERA VQDGADVMNL SLGNSLNNPD WATSTALDWA MSEGVVAVTS
BL14580    IAANGQIKGV APEATLLAYR VLGPGGSGTT ENVIAGIEKA VADGAKVMNL SLGNSLNSPD YATSIALDWA MAEGVVAVTS

KCTC3014   NGNSGPNGWT VGSPGTSREA ISVGATQLPL NEYAVTFGSY SSAKVMGYNK EDDVKALNNK EVELVEAGIG EAKDFEGKDL
BS168      NGNSGPNGWT VGSPGTSREA ISVGATQLPL NEYAVTFGSY SSAKVMGYNK EDDVKALNNK EVELVEAGIG EAKDFEGKDL
BL14580    NGNSGPENWT VGSPGTSRVA ISVGASQLPY NEYSVTLPSY SSAKVMGYQE EKDLEALNGQ EVELVEAGLG QADDFSGKDV

KCTC3014   TGKVAVVKRG SIAFVDKADN AKKAGAIGMV VYNNLSGEIE ANVPGMSVPT IKLSLEDGEK LVSALKAGET KTTFKLTVSK
BS168      TGKVAVVKRG SIAFVDKADN AKKAGAIGMV VYNNLSGEIE ANVPGMSVPT IKLSLEDGEK LVSALKAGET KTTFKLTVSK
BL14580    KGKVAVIQRG VIPFVDKAEN AKNAGAIGAV IYNNATGEIE ANVMGNAVPT VKLSKEEGEK LVQQIKEGKH SVVFSFKLDK

KCTC3014   ALGEQVADFS SRGPVMDTWM IKPDISAPGV NIVSTIPTHD PDHPYGYGSK QGTSMASPHI AGAVAVIKQA KPKWSVEQIK
BS168      ALGEQVADFS SRGPVMDTWM IKPDISAPGV NIVSTIPTHD PDHPYGYGSK QGTSMASPHI AGAVAVIKQA KPKWSVEQIK
BL14580    KLGETIASFS SRGPVMDTWM IKPDVSAPGV NIVSTIPTHD PKNPYGYGSK QGTSMASPHV AGTAAILKQA KPDWTPEQIK

KCTC3014   TAIMNTAVTL KDSDGEVYPH NAQGAGSARI MNAIKADSLV SPGSYSYGTF LKENGNETKN ETFTIENQSS IRKSYTLEYS
BS168      AAIMNTAVTL KDSDGEVYPH NAQGAGSARI MNAIKADSLV SPGSYSYGTF LKENGNETKN ETFTIENQSS IRKSYTLEYS
BL14580    GVLMNTAEKL TDENGKPLPH NTQGAGSIRI MEALKASSIV TPGSHSYGTF LKDKGKQTKK QAFTIENLSS HRKAYQLEYS

KCTC3014   FNGSGISTSG TSRVVIPAHQ TGKATAKVKV NTKKTKAGTY EGTVIVREGG KTVAKVPTLL IVKEPDYPRV TSVSVSEGSV
BS168      FNGSGISTSG TSRVVIPAHQ TGKATAKVKV NTKKTKAGTY EGTVIVREGG KTVAKVPTLL IVKEPDYPRV TSVSVSEGSV
BL14580    FKGTGITVSG TERVVVPANQ TGKAAAKVTV NSAKTKAGTY EGTVYIREDG RKVAEIPLLL IVKEPDYPRV TSVTVEPGAK

KCTC3014   QGTVQIETYL PAGAEELAFL VYDSNLDFAG QAGIYKNQDK GYQYFDWDGT INGGTKLPAG EVYLLAYAAN KGKSSQVLTE
BS168      QGTVQIETYL PAGAEELAFL VYDSNLDFAG QAGIYKNQDK GYQYFDWDGT INGGTKLPAG EVYLLAYAAN KGKSSQVLTE
BL14580    QGAVTIEAYL PGGAEELAFL VYDENLNLLG QAGVYKNQGK GYQSYQWNGK INDAASLKSG KVYMLAYASA KGKSSYVLTE

KCTC3014   EPFTVE
BS168      EPFTVE
BL14580    DPFIVE
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Fig. 8. Comparison of the amino acid sequence of Vpr of *B. subtilis* KCTC 3014 with those of other proteases.
KCTC3014 from *B. subtilis* KCTC 3014 [Accession No. AAX84518] (this study); BS168 from *B. subtilis* 168 [Accession No. NP_391688]; BL14580 from *B. licheniformis* ATCC 14580 [Accession No. AAU25457]. Identical amino acids are asterisked.

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              **   ***   * * ****  *   * * ** **      **   *** **       * ***** **** ** *  ** ******
KCTC3014      MKEEIIERFT TYVKVDTQSD ESVDTCPSTP GQLTLGNMLV DELKSIGMQD AAIDENGYVM ATLPSNTEKD VPTIGFLAHV
BS168         MKEEIIERFT TYVKVDTQSD ESVDTCPSTP GQLTLGNMLV DELKSIGMQD AAIDENGYVM ATLPSNTEKD VPTIGFLAHV
BL14580       MKNKLIERLI SYAKVDTQSN ENSQTTPSTP GQLALANMLV EELKEIGMKD VTIDENGYVM ATLPSNTEKE VPTIGFLAHV
BC10987       MKQELIERFT RYVKIDTQSN EESHTVPTTP GQIEFGKLLV EELKEIGLTE VTMDDNGYVM ATLPANTDKD VPVIGFLAHL

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KCTC3014      DTATDFTGKN VNPQIIESYD GKDIVLNEKL QVTLSPDQFP ELSGYKGHTL ITTDGTTLLG ADNKAGIAEI MTAMDYLIKH
BS168         DTATDFTGKN VNPQIIESYD GKDIVLNEQL QVTLSPDQFP ELSGYKGHTL ITTDGTTLLG ADNKAGIAEI MTAMDYLIKH
BL14580       DTATDFTGKN VNPQVIEQYD GKDIVLNESL NVVLSPKEFP ELADYAGHTL ITTDGTTLLG ADNKAGISEI MTAMEYLIAH
BC10987       DTATDFTGKN VKPQIHENFD GNAITLNEEL NVVLTPEQFP ELPSYKGHTI ITTDGTTLLG ADDKAGLTEI MVAMNYLVHN

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KCTC3014      PEIKHGTIRV AFTPDEEIGR GPHKFDVKRF NASFAYTVDG GPLGELEYES FNAAAAKITI KGNNVHPGTA KGKMINSAKI
BS168         PEIKHGTIRV AFTPDEEIGR GPHKFDVKRF NASFAYTVDG GPLGELEYES FNAAAAKITI KGNNVHPGTA KGKMINSAKI
BL14580       PEIKHGKIRV AFTPDEEIGR GPHKFDVEAF NAKFAYTVDG GPLGELQYES FNAAAAKITC KGTNVHPGTA KGKMVNAAKI
BC10987       PQIKHGKIRV AFTPDEEIGR GPAHFDVEAF GASFAYTMDG GPLGGLEYES FNAAGAKLTF NGTNTHPGTA KNKMRNATKL

              **     **   **** **** ********    ***      * ******   *   **          **        ****
KCTC3014      AMKLNSLLPA DEAPEYTEGY EGFYHLLSIQ GDVEETKLYY IIRDFDKENF QNRKETMKRA VEELQNEYGQ DRILLDMNDQ
BS168         AMKLNSLLPA DEAPEYTEGY EGFYHLLSIQ GDVEETKLHY IIRDFDKENF QNRKETMKRA VEELQNEYGQ DRILLDMNDQ
BL14580       AMQFHAALPE NEAPEFTEGY EGFYHLLSIK GDVSETSLSY IIRDFDRDRF NERKDTVQKI ANNLKAKYGE NSVTVDMNDQ
BC10987       AMEFNGYLPV EEAPEYTEGY EGFYHLLSLN GDVEQSKAYY IIRDFDRENF EARKHNVENI VKQMQEKYGQ DAVVLEMNDQ

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KCTC3014      YYNMREKIEP VIEIVNIAKQ AMENLGIEPK ISPIRGGTDG SQLSYMGLPT PNIFTGGENF HGKFEYISVD NMVKAVNVIV
BS168         YYNMREKIEP VIEIVNIAKQ AMENLGIEPK ISPIRGGTDG SQLSYMGLPT PNIFTGGENF HGKFEYISVD NMVKAVNVIV
BL14580       YYNMREKIEP VKEIVDIAYK AMKNLDIEPV VKPIRGGTDG SQLSYMGLPC PNIFTGGENF HGKYEYISAD NMVKAANVIV
BC10987       YYNMLEKIEP VREIVDIAYE AMKSLNIEPN IHPIRGGTDG SQLSYMGLPT PNIFTGGENY HGKFEYVSVD VMEKAVQVIV

              **   ** *
KCTC3014      EIAKQFEAQA
BS168         EIAKQFEAQA
BL14580       EIVKLFEERA
BC10987       EIARRFEEQA
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Fig. 9. Comparison of the amino acid sequence of PepT of *B. subtilis* KCTC 3014 with those of other proteases.
KCTC3014 from *B. subtilis* KCTC 3014 [Accession No. AAX84518] (this study); BS168 from *B. subtilis* 168 [Accession No. NP_391688]; BL14580 from *B. licheniformis* ATCC 14580 [Accession No. AAU25457]. Identical amino acids are asterisked.

or proteolysis [25], and Park *et al.* [20] identified four different Vpr proteins with apparent molecular masses in the range of 60–70 kDa on a 2D gel. Finally, WprA is a 96 kDa polypeptide endowed with a signal peptide and a propeptide. Upon processing and export, it is also converted into two cell-wall-bound proteins, CWBP23 and 52 [18]. The reason of why we failed to establish the protease map of *B. subtilis* is that the proteases might be degraded by other proteases or themselves during the cell growth.

In conclusion, we found that *B. subtilis* KCTC 3014 secreted six extracellular proteases into the culture medium at the end of the exponential phase. Three proteases, Vpr,

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              ******* ** **********  ********* ********** ** * ****  ******* **    ******** * ****** *
KCTC3014      AQSVPYGVSQ IKAPALHSQG FTGSNVKVAV IDSGIDSSHP DLKVAGGASM VPSETNPFQD NNSHGTHVAG TVAALNNSVG
DJ-4          AQSVPYGVSQ IKAPALHSQG FTGSNVKVAV IDSGIDSSHP DLKVAGGASM VPSETNPFQD NNSHGTHVAG TVAALNNSVG
BPN'          AQSVPYGVSQ IKAPALHSQG YTGSNVKVAV IDSGIDSSHP DLKVAGGASM VPSETNPFQD NNSHGTHVAG TVAALNNSIG
BS168         AQSVPYGISQ IKAPALHSQG YTGSNVKVAV IDSGIDSSHP DLNVRGGASF VPSETNPYQD GSSHGTHVAG TIAALNNSIG
NAT           AQSVPYGISQ IKAPALHSQG YTGSNVKVAV IDSGIDSSHP DLNVRGGASF VPSETNPYQD GSSHGTHVAG TIAALNNSIG

              **** ***** ******     * *** ****** ***** **** *********  ** ***  ** *** ** **   ******* **
KCTC3014      VLGVAPSASL YAVKVLGADG SGQSSWIING IEWAIANNMD VINMSLGGPS GSAALKAAVD KAVASGVVVV AAAGNEGTSG
DJ-4          VLGVAPSASL YAVKVLGADG SGQYSWIING IEWAIANNMD VINMSLGGPS GSAALKAAVD KAVASGVVVV AAAGNEGTSG
BPN'          VLGVAPSASL YAVKVLGADG SGQYSWIING IEWAIANNMD VINMSLGGPS GSAALKAAVD KAVASGVVVV AAAGNEGTSG
BS168         VLGVSPSASL YAVKVLDSTG SGQYSWIING IEWAISNNMD VINMSLGGPT GSTALKTVVD KAVSSGIVVA AAAGNEGSSG
NAT           VLGVAPSASL YAVKVLDSTG SGQYSWIING IEWAISNNMD VINMSLGGPT GSTALKTVVD KAVSSGIVVA AAAGNEGSSG

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KCTC3014      GSSTVGYPGK YPSVIAVGAV NSSNQRASSS SVGSELDVMA PGVSIQSTLP GNKYGAYNGT CMASPHVAGA AALILSKHPN
DJ-4          GSSTVGYPGK YPSVIAVGAV NSSNQRASFS SVGSELDVMA PGVSIQSTLP GNKYGAYNGT SMASPHVAGA AALILSKHPN
BPN'          SSSTVGYPGK YPSVIAVGAV DSSNQRASFS SVGPELDVMA PGVSIQSTLP GNKYGAYNGT SMASPHVAGA AALILSKHPN
BS168         STSTVGYPAK YPSTIAVGAV NSSNQRASFS SAGSELDVMA PGVSIQSTLP GGTYGAYNGT SMATPHVAGA AALILSKHPT
NAT           STSTVGYPAK YPSTIAVGAV NSSNQRASFS SVGSELDVMA PGVSIQSTLP GGTYGAYNGT SMATPHVAGA AALILSKHPT

              *** ***  * * * * **   ********** *****
KCTC3014      WTNTQVRSSL ENTTTKLGDA FYYGKGLINV QAAAQ
DJ-4          WTNTQVRSSL ENTTTKLGDA FYYGKGLINV QAAAQ
BPN'          WTNTQVRSSL ENTTTKLGDS FYYGKGLINV QAAAQ
BS168         WTNAQVRDRL ESTATYLGNS FYYGKGLINV QAAAQ
NAT           WTNAQVRDRL ESTATYLGNS FYYGKGLINV QAAAQ
```

Fig. 10. Comparison of the amino acid sequence of subtilisin of *B. subtilis* KCTC3014 with those of other proteases.
KCTC 3014 from *B. subtilis* KCTC 3014 [Accession No. AAY23643] (this study); DJ-4 from *Bacillus* sp. DJ-4 [Accession No. AAT45900]; BPN' from *B. amyloliquefaciens* [Accession No. P00782]; BS168 from *B. subtilis* 168 [Accession No. CAB12870]; NAT from *B. subtilis* natto [Accession No. P35835]. Identical amino acids are asterisked.

Pep T, and subtilisin, were identified in active mature forms. We established a partial extracellular protease map of *B. subtilis* KCTC 3014, and characterized three extracellular proteases. Further studies are expected to identify the N- or C-terminal processed active proteins and establish their 1- or 2D protease maps. We are in the process to obtain extracellular protease profiles under various culture conditions.

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