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ARTICLE

Purification and Characterization of a Salt-Activated and Organic Solvent-Stable Heterotrimer Proteinase from *Virgibacillus* sp. SK33 Isolated from Thai Fish Sauce

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A NaCl-activated proteinase produced by *Virgibacillus* sp. SK33 was purified to homogeneity using phenyl-Sepharose and Sephadex G-75 with a yield of 12% and purification of 2.6-fold. A single protein was detected at ~32 kDa by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. However, three subunits with molecular weights of 27,858, 33,918, and 35,368 Da were obtained from MALDI-TOF mass spectra, implying that the enzyme was a heterotrimer. The isoelectric point of the proteinase was 5.4. Optimum catalytic activity was at 55 °C and pH 7.5. The enzyme showed serine characteristics as it was completely inhibited by phenylmethanesulfonyl fluoride. The purified proteinase showed broad specificity toward oxidized insulin B including Gln^4, Cys^7, Glu^{13}, Ala^{14}, $Leu^{15,17}$, $Tyr^{16,26}$, Arg^{22}, $Phe^{24,25}$, and Lys^{29}. Dominant cleavage sites of the enzyme were Tyr^{16}−Leu^{17} and Phe^{25}−Tyr^{26}, indicating that it preferably hydrolyzed aromatic amino acids located on the P_1 site. Among various substrates studied, the enzyme hydrolyzed anchovy protein to the greatest extent at 4 M NaCl. Activity increased with either $CaCl_2$ or NaCl concentration with the maximum 2-fold increase at either 50 mM $CaCl_2$ or 4 M NaCl. The enzyme was also highly stable up to 500 mM $CaCl_2$ or 4 M NaCl. The proteinase showed high stability in various organic solvents (25%, v/v) including dimethylsulfoxide, methanol, acetonitrile, and ethanol. Results of peptide mass fingerprint and de novo peptide sequencing showed that the purified proteinase is a novel proteinase. The proteinase from *Virgibacillus* sp. SK33 could have a potential application in high ionic strength environments and aqueous−organic solvent systems.

KEYWORDS: *Virgibacillus* sp.; subtilisin-like proteinase; organic solvent-stable proteinase; NaCl-activated proteinase

INTRODUCTION

Bacterial proteinases are mainly derived from the genus *Bacillus* and are widely used in various food and biotechnology industries as well as in peptide synthesis (*1*, *2*). Over 170 subtilases belonging to a superfamily of subtilisin-like serine proteinases have been reported with their complete amino acid sequences (*3*). Normally, proteinases from *Bacillus* sp. show reduced activity at increased NaCl concentrations (*4*). This is because exclusion of water molecules around the surface enzyme in high NaCl content destabilizes enzyme structure, leading to protein aggregation (*5*). Thus, most bacterial proteinases have limited application at high ionic strength environments.

A catalytic reaction of a proteinase in a nonaqueous solution is a reverse hydrolytic reaction that is commonly used in peptide synthesis. The use of proteinase for peptide synthesis offers stereospecificity and does not need side-chain protection, which although expensive and hazardous is normally required by the chemical synthesis approach (*1*). Proteinases from archaea show a greater potential for peptide synthesis because they exhibit activity and stability under high salt content with limited water activity, a condition similar to that in organic solvent systems (*6*). However, the disadvantages of using archaea's proteinases are that the solvent concentration used in the enzymatic reaction is limited by the solubility of salt and the proteinases are easily inactivated (*7*). The use of proteinases from nonhalophilic bacteria in a nonaqueous environment also encounters the instability problem of the enzyme.

Halotolerant bacteria are a good source of proteinase, showing inherent activity and stability at high ionic strength, since they thrive in 0−15% NaCl. Proteinases showing maximum activity and stability at 20−25% NaCl from halotolerant bacteria have been characterized (*8*−*11*). *Virgibacillus* is a new genus comprising a mesophilic and moderately halotolerant bacterium and is amended from *Bacillus pantothenticus* (*12*). Several species of genus *Virgibacillus* grow optimally at 5−10% NaCl (*13*, *14*). Some species of the genus are capable of growing in a wide range of NaCl, 2−23% (*15*). It has been reported that the activity of a proteinase from *Virgibacillus* sp. is strongly enhanced in the presence of NaCl (*16*). This feature is similar to that of extremely halophilic bacterial proteinases. Therefore, *Virgibacillus* might be another important source of proteinases used for catalytic

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reactions in organic environments. Thus far, there has been limited investigation of the catalytic activity of proteinases produced from halotolerant bacteria, particularly as related to high NaCl and organic environments.

Recently, *Virgibacillus* sp. SK33 (DQ910838) was isolated from fermented Thai fish sauce, and the extracellular proteinases showed activity and stability at high NaCl concentration (25%). Several molecular weights (MWs) of extracellular proteinases were observed at 56, 42, 32, 25, and 19 kDa, based on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) activity staining. There were two dominant proteinases (32 and 19 kDa) exhibiting activity at high NaCl concentration (*17*). The 19-kDa proteinase has already been purified and showed the highest activity at 20−25% NaCl (3.4−4.3 M) against both a fluorogenic substrate and anchovy protein (*18*). Another major proteinase with MW of 32 kDa from *Virgibacillus* sp. SK33 has not been previously investigated. The objectives of this study were, therefore, to elucidate the biochemical characteristics of the 32-kDa proteinase of *Virgibacillus* sp. SK33 and to demonstrate the stability of the purified enzyme in various organic solvents.

MATERIALS AND METHODS

Chemicals. *tert*-Butyloxycarbonyl(Boc)-Asp(oBzl)-Pro-Arg-4-methyl-7-coumarylamide (AMC), succinyl(Suc)-Ala-Ala-Pro-Phe-AMC, and carbobenzoxy(Z)-Phe-Arg-AMC were purchased from Bachem A.G. (Bubendorf, Switzerland). Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Z-Arg-Arg-AMC, leupeptin, trypsin inhibitor I (soybean), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), bestatin, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidine)-butane (E-64), *N*-ethylmaleimide (NEM), iodoacetic acid (IAA), dithiothreitol (DTT), 2-mercaptoethanol (*β*-ME), bovine serum albumin, L-tyrosine, casein, blue dextran, and Sephadex G-75 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), L-cysteine, imidazole, and L-histidine were purchased from Fluka (Buchs, Switzerland). Phenyl-Sepharose and Superose 6 were purchased from GE Healthcare (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). All other chemicals used were of analytical grade.

Purification. *Virgibacillus* sp. SK33 was cultivated by transferring a loopful of freshly prepared culture from halobacterium agar plate (1% yeast extract, 0.5% peptone, 1% casamino acid, 0.3% trisodium citrate, 0.2% potassium chloride, 2.5% magnesium sulfate, and 1.8% agar) containing 5% NaCl into 250-mL flasks containing 60 mL of a Ym broth composed of 1% yeast extract, 0.3% trisodium citrate, 0.2% potassium chloride, 2.5% magnesium sulfate, and 5% sodium chloride (*17*). The cultures were incubated at 40 °C at 100 rpm for 3 days. Supernatant was collected by centrifugation at 10,000 × *g*, 4 °C, for 30 min and is referred to as crude proteinase.

All purification steps were performed at 4 °C. Ammonium sulfate was slowly added to the crude proteinase to a final concentration of 0.5 M with gentle stirring. The sample was centrifuged at 10,000 × *g*, for 30 min and the supernatant was subsequently filtered through a 0.45-*μ*m cellulose acetate membrane. The filtrate (65 mL) was applied to a phenyl-Sepharose column (0.8 cm × 15 cm) equilibrated with 0.5 M $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 8. The column was washed with 3 bed volumes of the equilibrating buffer, using Biologic LP system (Bio-Rad Laboratories, Hercules, CA, USA). The bound protein was eluted with a linear gradient of 0.5 to 0 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl, pH 8 for 1 bed volume, and 5-mL fractions were collected at a flow rate of 1 mL/min. All fractions were tested for proteinase activity using Suc-Ala-Ala-Pro-Phe-AMC as a substrate. Active fractions were pooled and concentrated using ultrafiltation with 10 kDa molecular weight cutoff (MWCO) filter (Amicon, Millipore Ireland BV, County Cork, Ireland).

The second step of purification was carried out using a Purifier 10 (ÄKTA, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The concentrated sample was applied onto a Sephadex G-75 column (1.6 cm × 95 cm) equilibrated with 0.15 M NaCl, 50 mM Tris-HCl, pH 8. Three-milliliter fractions were collected at a flow rate of 0.5 mL/min. Fractions containing proteinase activity were pooled and used as purified proteinase. The protein content of the purified proteinase was determined by the modified micro Bradford assay (*19*) using bovine serum albumin as a standard.

Activity Staining. Activity staining was carried out using the method of García-Carreño et al. (*20*) with slight modifications. Purified proteinase (0.15 *μ*g/well) containing 2% SDS, 5% *β*-ME, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), and 0.05% bromophenol blue was loaded into 12.5% T SDS−PAGE (*21*). Native-PAGE was carried out in the same manner without denaturing agents. Acrylamide gel was immersed in 2% casein, 100 mM Tris-HCl, pH 7.5 at 4 °C, with shaking for 30 min and was washed twice with cold 100 mM Tris-HCl (pH 7.5). Proteolytic activity was initiated in 100 mM Tris-HCl (pH 7.5) at 55 °C for 30 min. The gel was stained in 0.1% Coomassie brilliant blue R-250, 40% methanol, and 10% acetic acid for 1 h and destained in 25% ethanol and 10% acetic acid. The clear zone indicated the presence of the proteinase.

Proteinase Activity Assay. Proteinase activity was determined by the modified method of Barrett and Kirschke (*22*) using Suc-Ala-Ala-Pro-Phe-AMC as a substrate. The reaction mixture (1 mL) containing 0.20 *μ*g of the purified proteinase, 1 *μ*M synthetic substrate, and 100 mM Tris-HCl (pH 7.5) was incubated at 55 °C for 5 min. The reaction was terminated by adding 1.5 mL of stopping solution (30% butanol, 35% methanol, and 35% deionized water, v/v). Fluorescence intensity was measured at an excitation wavelength of 380 nm and emission wavelength of 460 nm (RF-1501, Shimadzu Co., Kyoto, Japan). The unit of enzymatic activity was expressed as katal.

Biochemical Characteristics. *Determination of Molecular Weight by Gel Filtration.* The native MW of the purified proteinase was determined using Superose 6 column (1.6 cm × 87 cm) equilibrated and eluted with 0.15 M NaCl, 50 mM Tris-HCl, pH 8. Fractions of 0.75 mL were collected at a flow rate of 0.5 mL/min. Void volume (V_o) was determined using blue dextran. The ratio of elution volume (V_e) to V_o was calculated. The column was calibrated using a protein standard consisting of apoferritin (443 kDa), *β*-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrom *c* (13 kDa) (Sigma Chemical Co., St. Louis, MO, USA).

Determination of Absolute Molecular Mass by MALDI-TOF. The purified proteinase was dialyzed twice against 100 volumes of deionized water using a dialysis membrane with MWCO 10 kDa (Pierce Chemical Company, Rockford, IL, USA). The dialyzed sample was concentrated using ultrafiltation with MWCO 10 kDa (Amicon, Millipore Ireland BV, County Cork, Ireland). The sample (10 *μ*g protein/mL) was mixed with an equal volume of matrix solution (10 mg/mL α-cyano-4-hydroxy-cinnamic acid (4-HCCA) in acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) (1:2)), and subsequently the mixture was spotted onto the MALDI holder. Mass measurement was carried out using MALDI-TOF (MS model reflex V, Bruker Daltonik GmbH, Bremen, Germany) with a 2 GHz LeCroy digitizer and 337 nm nitrogen laser.

Protein Identification by Peptide Mass Fingerprint (PMF) and ESI-MS/MS. The purified proteinase was loaded onto SDS−PAGE (*21*), and the gel was stained with Coomassie brilliant blue R-250. Subsequently, tryptic digestion of the protein band was carried out on an Ettan Spot Handing Workstation robot (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) following the method of the manufacturer. The gel piece was washed twice in 50 mM ammonium bicarbonate and 50% methanol for 20 min, dehydrated in 75% ACN, and dried. The dried gel was rehydrated in 20 mM ammonium bicarbonate containing 20 ng/*μ*L sequencing grade modified trypsin (Promega Co., Madison, WI, USA) and incubated at 37 °C for 3 h. Peptides were extracted twice in 0.1% TFA and 50% ACN for 20 min. The extracted peptide was dried and dissolved in a mixture of 0.1% ACN and 0.1% TFA (1:2). The peptide sample was mixed with an equal volume of matrix solution (10 mg/mL 4-HCCA in ACN and 0.1% TFA (1:2)), applied onto a sample holder, and dried at room temperature. The PMF was determined by MALDI-TOF (MS model reflex V, Bruker Daltonik GmbH, Bremen, Germany) equipped with a 2 GHz LeCroy digitizer and 337 nm nitrogen laser. The MALDI-TOF spectrum was obtained by the positive ion mode at acceleration voltage of 20 kV and 400 ns extraction delay. The spectrum was searched using the MASCOT search engine (Matrix Science, London, U.K.) with the nonredundant protein sequence database of the National Center for Biotechnology Information (NCBI).

Protein identification and internal protein sequencing were performed using liquid chromatography−electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with the Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Co., Waltham, MA, USA). Tryptic digestion was carried out as described above. The digested peptides were mixed with 0.1% TFA and 30% ACN. Subsequently, the sample was loaded into a Zorbax 300SB-C18 column (5 μm, 5 mm × 0.3 mm) (Agilent Technologies, Inc., Palo Alto, CA, USA). The peptides were eluted with a flow rate of 100 μL/min using a linear gradient of 0−60% solvent B with 0.1% formic acid in ACN, and solvent A with 0.1% formic acid in deionized water. The tryptic-digested peptide ions were detected in a survey scan from 400 to 1600 amu. LC-MS/MS data were examined using a SEQUEST search algorithm with the nonredundant protein sequence database of NCBI. Amino acid sequences of some peptides from de novo peptide sequencing were searched using the Basic Local Alignment Search Tool (BLAST).

Isoelectric Point Determination. The isoelectric point (pI) of the purified proteinase was determined using PhastGel isoelectric focusing (IEF) with a PhastGel IEF 3−9 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The operating condition was as follows: (1) prefocusing step, 2000 V, 2 mA, 3.5 W, 15 °C, 75 Vh; (2) sample application, 100 V, 2 mA, 3.5 W, 15 °C, 15 Vh; (3) focusing step, 2000 V, 5 mA, 3.5 W, 15 °C, 75 Vh. The broad range pI standard (3−10) was used (GE Healthcare, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Silver staining was performed (*23*).

Temperature and pH Optima. The effect of temperature on the purified proteinase activity was investigated in 100 mM Tris-HCl (pH 7.5), incubated at 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80 °C, respectively, for 5 min. The optimum pH for proteinase activity was investigated at 55 °C with incubation for 5 min at various pH levels: pH 3, 4, 5, 5.5, 6, 6.5 using 100 mM sodium acetate; pH 6.5, 7, 7.5 using 100 mM Tris-maleate; pH 7.5, 8, 8.5, 9, 9.5 using 100 mM Tris-HCl; and pH 9.5, 10, 11, 12 using 100 mM phosphate buffer.

pH Stability. The effect of pH on the stability of the purified proteinase was investigated by preincubating the purified proteinase at various pH levels including sodium acetate (pH 3−6), Tris-maleate (pH 7), Tris-HCl (pH 8, 9), and phosphate buffer (pH 10−12) at a final concentration of 100 mM. The mixture was preincubated at 55 °C for 30 min. When preincubation time was reached, samples were rapidly mixed with cold 2 M Tris-HCl (pH 7.5) at a ratio of 1:1 to bring the pH of the reaction mixture to 7.5 and cooled in ice−water. The reference samples with respective pH values were run in the same manner except that their activities were determined immediately without preincubation. Relative activity was calculated using the activity of the respective references as 100%.

Thermal Stability. Thermal stability of the purified proteinase was determined by preincubating the enzyme in 200 mM Tris-HCl (pH 7.5) and either 10 mM EDTA or 10 mM $CaCl_2$, at various temperatures (35−80 °C) for 30 min. When preincubation time was attained, samples were rapidly cooled in ice−water, and proteinase activity was determined as described above. Activities of the reference samples that were not preincubated were determined at pH 7.5 in the presence of either 10 mM EDTA or 10 mM $CaCl_2$. Relative activity was calculated using the activity of the respective references as 100%.

Substrate Specificity. The substrate specificity of the purified proteinase was determined using various synthetic substrates. The reaction was carried out in 100 mM Tris-HCl (pH 7.5) and 1 μM synthetic substrates of either Boc-Asp(oBzl)-Pro-Arg-AMC, Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC, or Z-Phe-Arg-AMC.

Two dominant proteinases from *Virgibacillus* sp. SK33, namely, the 19-kDa proteinase that was purified as described by Sinsuwan et al. (*18*) and the purified enzyme from this study, were determined for specific cleavage sites using the oxidized bovine insulin chain B as a substrate. The reaction was carried out in 0.5 μg of purified proteinase, 500 μg of oxidized bovine insulin chain B, and 10 mM Tris-HCl (pH 7.5) incubated at 55 °C, for 12 h. The reaction was terminated by adding TFA to a final concentration of 0.7%. MALDI-TOF was carried out as described above. The cleavage sites of the purified proteinase on the oxidized bovine insulin chain B were determined using the ExPASy findpept tool (http://www.expasy.ch/tools/findpept.html) with tolerance mass of ± 0.5 Da.

Kinetic Parameters. The reaction was carried out using Suc-Ala-Ala-Pro-Phe-AMC as a substrate at various concentrations ranging from 10 to 300 μM at 55 °C and 100 mM Tris-HCl (pH 7.5). The K_m and V_{max} values were determined according to Michaelis−Menten kinetics using a Hanes−Woolf plot (*24*). The k_{cat} value was calculated from the equation $V_{max} = k_{cat} \times [E]_t$, where $[E]_t$ is the total proteinase concentration (μM) in reaction mixture. The molecular weight used to calculate $[E]_t$ was 97148 Da determined from MALDI-TOF.

Effect of Inhibitors and Ions on Activity. The effect of various inhibitors on proteinase activity was determined using various substances, namely, leupeptin, trypsine inhibitor I (soybean), TLCK, TPCK, PMSF, EDTA, L-histidine, imidazole, bestatin, pepstatin A, E-64, IAA, NEM, DTT, and β-ME. The effect of 1 mM metal ions ($CuCl_2$, $CdCl_2$, $CoCl_2$, $FeCl_3$, $MnCl_2$, $HgCl_2$, and $ZnSO_4$) and 10 mM of mono- and divalent cations ($LiCl_2$, NaCl, KCl, $MgCl_2$, $CaCl_2$, $SrCi_2$, and $BaCl_2$) was also investigated.

Effect of $CaCl_2$ on Activity and Stability. The effect of $CaCl_2$ on the activity was determined by measuring the proteinase activity in 200 mM Tris-HCl, pH 7.5, and in the presence of various $CaCl_2$ concentrations (0−500 mM) using the activity assay described above. The $CaCl_2$ stability of the purified proteinase was also investigated by preincubating the enzyme in 200 mM Tris-HCl (pH 7.5) at various $CaCl_2$ concentrations (0−500 mM) at 55 °C. Samples were rapidly cooled in ice−water when the preincubation time of 30 min was attained, and residual activity was determined. Relative activity was calculated using the activity of the sample without preincubation at respective $CaCl_2$ concentrations as 100%.

Effect of NaCl on Activity and Stability. The effect of NaCl on the activity was determined by measuring the proteinase activity in 200 mM Tris-HCl (pH 7.5) containing various NaCl concentrations (0−4 M). Because of phase separation caused by high NaCl content, the reaction was terminated by adding 1.5 mL of deionized water and heating at 90 °C for 5 min. NaCl stability of the purified proteinase was also determined by preincubating the enzyme in 200 mM Tris-HCl (pH 7.5) and various NaCl concentrations (0−4 M) at 55 °C for 60 min. Subsequently, the samples were rapidly cooled in ice−water, and residual activity was determined. The activity of the sample without preincubation at the respective NaCl concentration was taken as 100%.

Effect of Organic Solvents on Stability. The stability of the purified proteinase in the presence of organic solvent was determined by preincubating the purified proteinase in various organic solvents at a ratio of 3:1 at 30 °C and at a shaking speed of 200 rpm for 10 days. The purified proteinase was also tested for stability in the aqueous solution by preincubating in 200 mM Tris-HCl (pH 7.5) at 30 °C for 10 days. The reference samples were run in the same manner without preincubation. Relative activity was calculated using activity of the respective solvents as 100%.

Hydrolysis of Various Proteins. Determination of proteolytic activity toward various protein substrates, namely, acid hemoglobin (acid HB), anchovy, bovine serum albumin (BSA), and casein, was carried out. Anchovy (*Stolephorus indicus*) was homogenized in cold 50 mM Tris-maleate (pH 7) at a ratio of 1:5. Subsequently, the homogenate was gently stirred at 4 °C for 30 min. Soluble protein was collected by centrifugation at 8,000 × *g*, 4 °C for 30 min and used as anchovy substrate. Hemoglobin (2%, w/v) was dissolved in 0.1 M HCl and dialyzed twice against 100 volumes of deionized water using dialysis membrane with MWCO 10 kDa (Pierce Chemical Company, Rockford, IL, USA). The dialyzed hemoglobin was centrifuged at 8,000 × *g* for 20 min to remove insolubilized protein and used as a substrate. The protein content of all substrates was determined by the dye binding method (*19*) using bovine serum albumin as a standard.

The reaction mixture contained the purified proteinase (3.2 pkat), 2 mg/mL substrate, 200 mM Tris-HCl (pH 7.5), and 4 M NaCl and was incubated at 55 °C for 60 min. The reaction mixture containing anchovy protein included 100 μM TLCK as an inhibitor for anchovy endogeneous proteinase. The reaction was terminated by adding trichloroacetic acid (TCA) to a final concentration of 10%. The samples were cooled at 4 °C for 1 h to allow complete protein precipitation and then centrifuged at 10,000 × *g* for 10 min. TCA-soluble oligopeptide contents were determined by the Lowry method (*25*) using tyrosine as a standard. Blanks were prepared in the same manner except that the proteinase was inactivated by TCA before adding protein substrate.

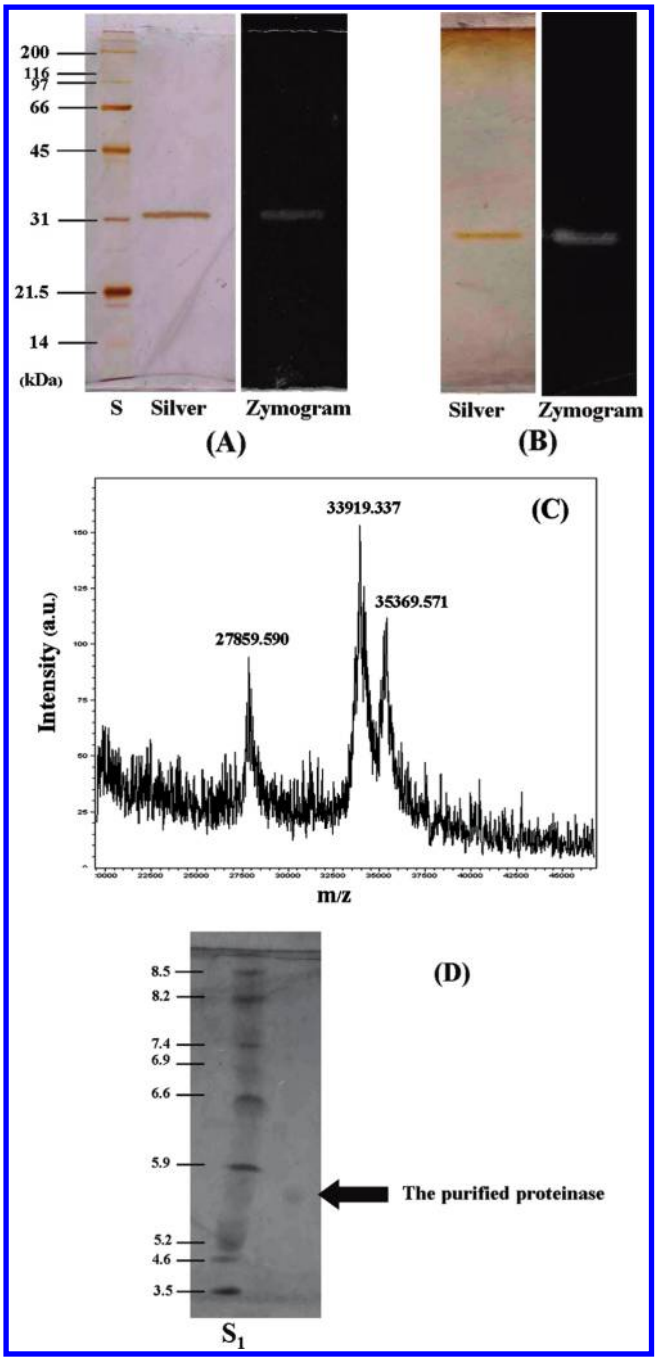

Figure 1. SDS−PAGE patterns (**A**), native-PAGE patterns (**B**), MALDI-TOF spectra (**C**), and isoelectric focusing (**D**) of the purified proteinase. S = molecular weight standard, S_1 = isoelectric focusing standard.

RESULTS AND DISCUSSION

Purification and Molecular Weight. Proteinase purification was achieved to electrophoretic homogeneity (**Figure 1A**,**B**) using two simple purification steps, phenyl-Sepharose and Sephadex G-75, with 2.6-fold increase and 12% yield (**Table 1**). It should be noted that relatively low purification fold was obtained from this study. Our previous study demonstrated that *Virgibacillus* sp. SK33 secreted at least 6 major proteinases in the Ym broth as evident on the casein zymogram and Suc-Ala-Ala-Pro-Phe-AMC was a preferred substrate of the crude enzyme (*17*). In addition, Ym medium used in this study contained amino acids and peptides from yeast extract with a low amount of proteins. Hence, the main source of contaminated proteins was other proteinases presented in the crude enzyme. Based on our purification scheme,

Table 1. Purification Table of the Purified Proteinase from *Virgibacillus* sp. SK33

step	total unit activity (pkat)	total protein (mg)	specific activity (pkat/mg protein)	fold	yield (%)
crude proteinase	2557	1.63	1573	1.0	100
crude in 0.5 M $(NH_4)_2SO_4$	2548	1.69	1507	1.0	100
phenyl-Sepharose	364	0.19	1889	1.2	14
Sephadex G-75	312	0.08	4028	2.6	12

phenyl-Sepharose and Sephadex G-75 effectively removed other contaminated proteinases that shared a common synthetic substrate, resulting in a drastically reduced total activity (**Table 1**). This was likely why only 2.6-fold purification was obtained. When a culture medium contained a proteinase inducer, such as skim milk or gelatin, purification fold was reported to increase significantly after purification as contaminated proteins were largely removed. Son and Kim (*26*) reported a 48-fold increase in the purification of proteinase from *Bacillus amyloliquefaciens* S94, which was induced by 2% skim milk.

The MW of the enzyme was estimated to be 32 kDa under denaturing conditions (**Figure 1A**), but the native form showed a molecular mass of 92 kDa based on gel filtration chromatography. This result suggested that the purified proteinase might contain 3 subunits. MALDI mass spectra also showed three peaks with MW of 27.859, 33.919, and 35.369 kDa (**Figure 1C**). These results confirmed that the purified proteinase of *Virgibacillus* sp. SK33 existed as a heterotrimer. Activity of the purified proteinase was observed on a zymogram performed under both denaturing and nondenaturing condition (**Figure 1A**,**B**). The enzyme molecule might be refolded during casein immobilization, resulting in the casein hydrolytic activity. The activity of alkaline proteinases from *Bacillus licheniformis* NH1 was also recovered on a casein zymogram after the removal of SDS by a glycine-NaOH buffer containing 2.5% Triton X-100 (*27*).

Thus far, only few endoproteinases have been reported to be oligomers. Serine endoproteinase from archaeabacterium *Pyrococcus abyssi* 549 was an oligomer with predominant MW of 150, 105, and 60 kDa (*28*), while intracellular proteinase of *P. furiosus* was a hexamer with MW of 124 kDa and a trimer with MW of 59 kDa (*29*). MW of subtilisin, a well-characterized proteinase from *Bacillus*, was 26.9−27.5 kDa (*30*), which is distinctively different from the *Virgibacillus* proteinase in this study. Despite its heterotrimer characteristic, only one band on SDS−PAGE was visualized. This could be because the MW of each subunit was too close to be completely separated on SDS−PAGE.

Isoelectric Point. The isolectric point (pI) of the purified proteinase was 5.4 (**Figure 1D**). Another dominant proteinase from *Virgibacillus* sp. SK33 with MW of 19 kDa on SDS−PAGE showed a pI value of 4.3 (*18*). Halophile proteinases usually exhibited acidic pI. The archaean serine proteinases from *Pyrococcus furiosus*, *Thermococcus kodakaraensis*, and *Halobacterium mediterranei* R4 showed pI values of 4.3, 4.2, and 4.1, respectively (*31*), and that of the purified proteinase from *Bacillus subtilis* (*natto*) was 3.9 (*32*).

Optimum Temperature and pH. Optimum temperature of the purified proteinase was at 55 °C (**Figure 2A**). It showed high activity (86−98%) over a wide temperature range of 35−60 °C. This was quite different from proteinases produced by moderate halophiles, which typically showed activity only at the relatively high temperature of 55−65 °C (*8*, *9*, *11*). The enzyme was active at pH values of pH 5−10 with the optimum catalytic activity at pH 7.5 (**Figure 2B**). The activity of the reaction containing phosphate

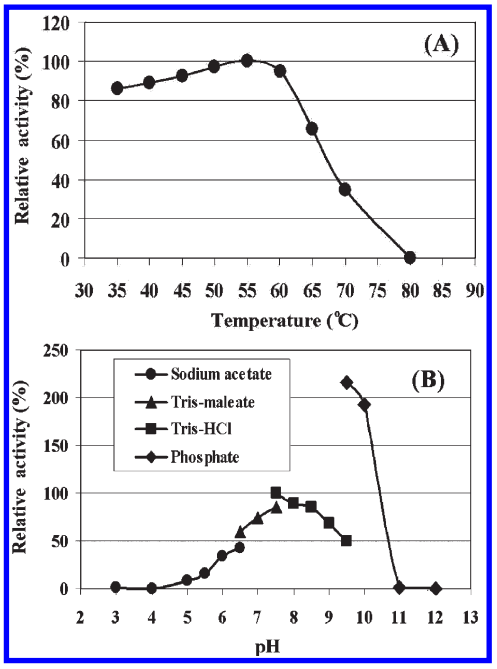

Figure 2. Temperature profile (**A**) and pH profile (**B**) of the purified proteinase.

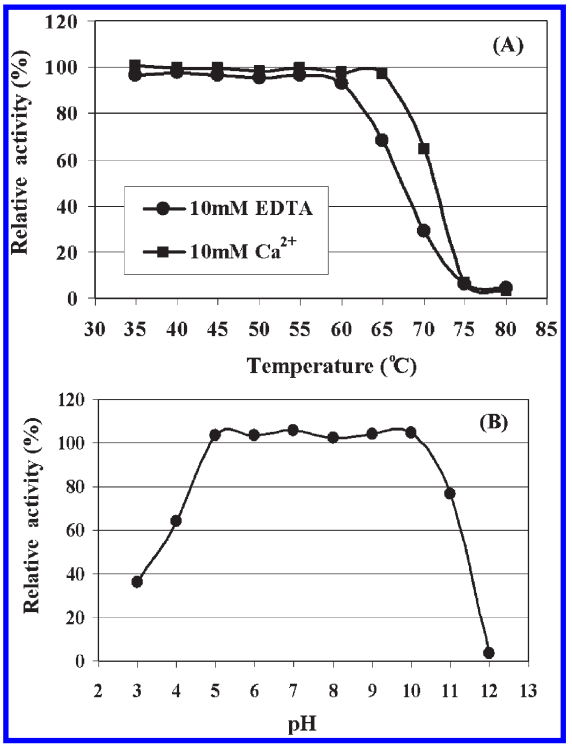

Figure 3. Thermal stability (**A**) and pH stability (**B**) of the purified *Virgibacillus* sp. SK33 proteinase. Thermal stability was carried out by preincubating the enzyme at various temperatures at pH 7.5 for 30 min, and pH stability was determined by preincubating the enzyme at varying pH values at 55 °C for 30 min.

buffer was about 4 times as high as that containing Tris-HCl at the same pH. Phosphate buffer was found to have no effect on fluorescence of the hydrolyzed product, AMC. Therefore, our result demonstrated the activation effect of phosphate ion on the proteinase activity. The purified proteinase did not exhibit activity at extreme acidic (3−4) and alkaline (11−12) pH. This characteristic was different from many bacterial serine proteinases, which are typically categorized as extremely alkaline proteinases (*2*, *4*).

Thermal and pH Stability. In the absence of Ca^{2+}, the purified proteinase showed high stability at 30−60 °C, and its stability rapidly decreased at temperature ≥65 °C (**Figure 3A**). The purified proteinase showed high thermal stability at its optimum condition (55 °C, pH 7.5), which was different from other microbial proteinases. The activity of proteinases from moderate halophiles, *Salinivibrio* sp. AF-2004 and *Pseudoalteromonas* sp. CP76, decreased to ∼20% and 40% of the original activity, respectively, after being subjected to the optimum temperature for 30 min (*8*, *11*). Our results indicated that the purified proteinase could be applied at its optimum temperature without significant reduction in stability.

The thermal stability of the purified proteinase in the presence of 10 mM Ca^{2+} increased up to 65 °C (**Figure 3A**), suggesting that Ca^{2+} exerted a positive effect on the thermal stability of the proteinase. Ca^{2+} plays an important role in enzyme stabilization at high temperatures. The thermal stability of proteinase from *Halobacillus* sp. SR5-3 increased by 10 °C with the addition of 2 mM Ca^{2+} (*10*). The thermal stability of proteinase from γ-Proteobacterium DGII in the presence of 5 mM Ca^{2+} was also higher than that without Ca^{2+} (*33*).

The purified proteinase was highly stable at a wide pH range of 5−10 (**Figure 3B**). Stability decreased at extremely acidic and alkaline pH levels (**Figure 3B**). The proteinases from moderate halophiles, *Salinivibrio* sp. AF-2004 and *Halobacillus* sp. SR5-3, were stable over broad pH ranges of 5−10 and 5−8, respectively (*10*, *11*), while the pH stability of a proteinase from an extreme halophile, *Halogeometricum borinquense* TSS101, was at pH 6−10 (*34*).

Inhibitors and Metal Ions. The proteinase was completely inactivated by PMSF, indicating a serine-like characteristic (**Table 2**). Other inhibitors did not completely inhibit the activity. Serine and metallo proteinases have mostly been found in moderate halophiles. A proteinase from *Halobacillus* sp. SR5-3, *Virgibacillus* sp. SK37, and *B. subtilis* FP-133 also showed a serine-like characteristic (*10*, *16*, *35*), while metalloproteinases were found in moderate halophiles, *Pseudoalteromonas* sp. CP76 and *Salinivibrio* sp. AF-2004 (*8*, *11*).

The activity of the purified proteinase was slightly increased by Cd^{2+}, Fe^{3+}, and Zn^{2+} but decreased in the presence of Hg^{2+} and Co^{2+} (**Table 2**). Proteinase activity from γ-Proteobacterium DGII was strongly inhibited by Mn^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Hg^{2+}, and Co^{2+} (*33*), but activity of the proteinase from *Salinivibrio* sp. AF-2004 was activated by Mn^{2+} and Co^{2+} (*11*). Monovalent cations (Li^{+}, Na^{+}, and K^{+}) showed a slight activation effect, but divalent cations (Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}) increased proteolytic activity about 1.4-fold (**Table 2**). The effect of the ions on proteinase activity varied with strains. Activity of the proteinase from *Bacillus mojavensis* was not affected by Ba^{2+}, Ca^{2+}, and Mg^{2+} (*2*), while activity of *Bacillus* sp. PS719 proteinase was activated by Ca^{2+} and Mg^{2+} (*36*). Activity of proteinase from a moderate halophile, *Pseudoalteromonas* sp. CP76, decreased in the presence of Mg^{2+}, Ca^{2+}, and Ba^{2+} (*8*).

Kinetic Constants and Substrate Specificity. The K_m and k_{cat} of the purified proteinase were 63 μM and 26 s^{-1}, respectively, with V_{max} of 0.1 μM s^{-1}. The K_m, k_{cat}, and V_{max} values of the 19-kDa purified proteinase were 27 μM, 12 s^{-1}, and 0.1 μM s^{-1}, respectively (*18*). The purified proteinase showed lower activity and substrate binding affinity than did the 19-kDa proteinase. The purified proteinase strongly hydrolyzed Suc-Ala-Ala-Pro-Phe-AMC with the rate of 26 pkat/mL, whereas other synthetic substrates for trypsin-, plasmin-, cathepsin L-, and cathepsin

B-like were not hydrolyzed. Typically, chymotrypsin- and subtilisin-like can hydrolyze aromatic and non-β-branched hydrophobic amino acid residues at P_1 (*30*). On the basis of the results with the synthetic substrate and inhibitors (**Table 2**), the purified proteinase showed subtilisin-like characteristics. *Bacillus* sp. has been known to produce various types of proteinases. *Bacillus subtilis* JM-3 produced trypsin-like proteinase (*37*), whereas *B. stearothermophilus* TLS33 produced Zn^{2+}-metalloproteinase (*38*). Aspartic proteinase was also produced by *Bacillus* sp. Wai 21a (*39*).

Table 2. Effect of Various Substances on Activity of the Purified Proteinase

substances	targeted proteinase	final concentration	relative activity (%)
leupeptin	trypsin-like and some cysteine proteinases	100 μM	72
trypsin inhibitor I (soybean)	trypsin-like proteinase	0.02 mg/mL	92
TLCK	trypsin-like proteinase	100 μM	98
TPCK	chymotrypsin-like proteinase	100 μM	83
PMSF	serine proteinase	1 mM	0
EDTA	metallo proteinase	10 mM	87
L-histidine	metallo proteinase	10 mM	76
imidazole	metallo proteinase	10 mM	114
bestatin	aminopeptidase	10 μM	90
pepstatin A	acid proteinase	10 μM	103
E-64	cysteine proteinase	10 μM	103
N-ethylmaleimide	cysteine proteinase	1 mM	105
iodoacetic acid	cysteine proteinase	1 mM	106
dithiothreitol		10 mM	106
2-mercaptoethanol		10 mM	110
metal ions			
Cu^{2+}		1 mM	96
Cd^{2+}		1 mM	122
Co^{2+}		1 mM	72
Fe^{3+}		1 mM	126
Mn^{2+}		1 mM	100
Hg^{2+}		1 mM	67
Zn^{2+}		1 mM	133
mono- and divalent cations			
Li^{+}		10 mM	113
Na^{+}		10 mM	112
K^{+}		10 mM	103
Mg^{2+}		10 mM	159
Ca^{2+}		10 mM	148
Sr^{2+}		10 mM	163
Ba^{2+}		10 mM	134

The purified proteinase showed broad specificity toward oxidized insulin B. It hydrolyzed hydrophobic ($Leu^{15,17}$), aromatic ($Tyr^{16,26}$ and $Phe^{24,25}$), and other amino acids including Gln^4, Cys^7, Glu^{13}, Ala^{14}, Arg^{22}, and Lys^{29} (**Figure 4**). The 19-kDa proteinase of *Virgibacillus* sp. SK33 showed broader specificity than did the purified proteinase. However, both proteinases from *Virgibacillus* sp. SK33 showed broader specificity than subtilisin. The common cleavage sites of subtilisins and the purified proteinase were Gln^4−His^5, Leu^{15}−Tyr^{16}, and Tyr^{16}−Leu^{17} (**Figure 4**). Major cleavage sites of two purified proteinases, namely, Tyr^{16}−Leu^{17} and Phe^{25}−Tyr^{26}, were similar to those of subtilisin BPN′. Our results indicated that the purified proteinase highly preferred the peptide bond with aromatic amino acids located in the P_1 sites. In addition, the purified proteinase exhibited an exopeptidase characteristic as it cleaved alanine at C-terminus (**Figure 4**), which is similar to *Bacillus* sp. KSM-K16 proteinses (*40*).

Effect of Ca^{2+} on Proteinase Activity and Stability. Activity of the purified proteinase increased about 2-fold at Ca^{2+} concentrations ranging from 30 to 500 mM (**Figure 5A**). Some proteinases from halophiles required Ca^{2+} for maximum activity. Activity of proteinase from the extreme halophile, *Halogeometricum borinquense* TSS101, increased ∼20% at 100−150 mM Ca^{2+} (*34*), whereas *Natrialba asiatica* 172 P1 proteinase activity was not affected by 1 mM Ca^{2+} (*42*). Activity of *Pyrococcus abyssi* proteinase decreased with 100 mM Ca^{2+} (*28*). These results suggested that the effect of Ca^{2+} on proteinase activity varied with strains.

The purified proteinase was extremely stable in the presence of Ca^{2+} up to 500 mM (**Figure 5A**). The thermal stability of proteinase from *B. stearothermophilus* F1 gradually increased with increasing Ca^{2+} concentration up to 20 mM (*43*). However, the stability of crude proteinase from *Virgibacillus* sp. SK37 gradually decreased with increasing Ca^{2+} concentration up to 100 mM (*16*). This result indicated that Ca^{2+} increased the

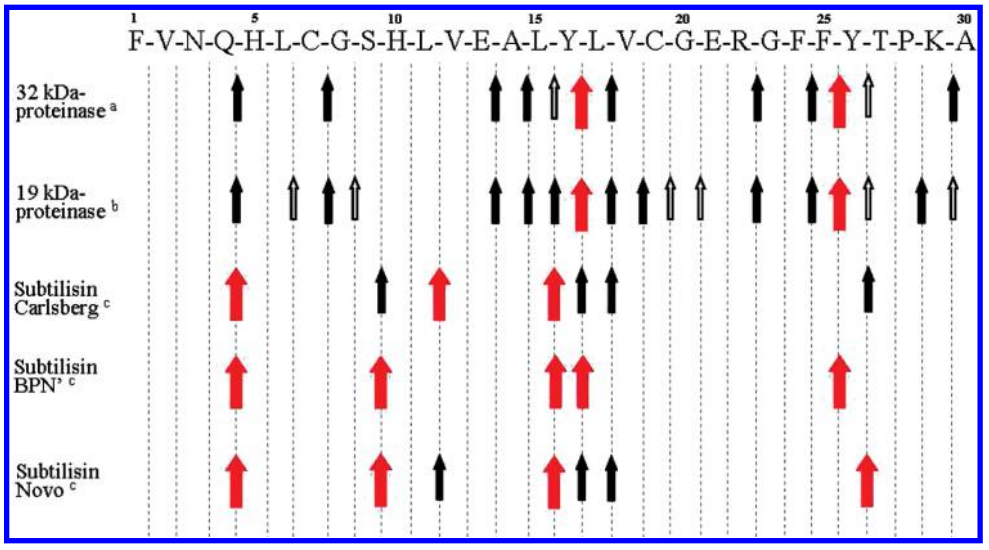

Figure 4. Cleavage sites of the purified proteinase toward oxidized bovine insulin B. Large filled arrows indicate a dominant cleavage site, small filled arrows indicate a cleavage site, small open arrows indicate an unpredictable cleavage site, the observed mass of which corresponded to ≥2 peptide sequences. Notes: (a) the purified heterotrimer proteinase; (b) the proteinase derived from *Virgibacillus* sp. SK33 was purified by the method of Sinsuwan et al. (*18*); (c) from Shimogaki et al. (*41*).

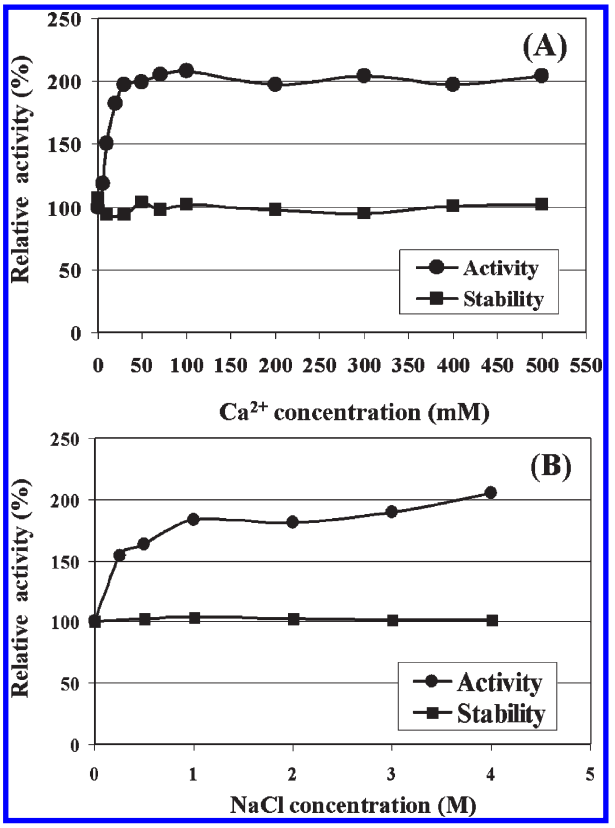

Figure 5. Effect of Ca^{2+} (**A**) and NaCl (**B**) on activity and stability of the purified *Virgibacillus* sp. SK33 proteinase. Ca^{2+} stability was carried out by preincubating the enzyme at various $CaCl_2$ concentrations (0−500 mM), pH 7.5 at 55 °C for 30 min. NaCl stability was determined by preincubating the enzyme at various NaCl concentrations (0−4 M), pH 7.5 at 55 °C for 60 min.

activity of the purified proteinase without destabilizing the structure. In general, destabilizing salts (e.g., Ca^{2+}) decrease the intramolecular hydrophobic interaction and surface tension of water, leading to protein destabilization (*44*). However, Ca^{2+} had no effect on the structural stability of the purified proteinase. It could be postulated that Ca^{2+} might strongly interact with the negatively charged surface of the enzyme, rendering more interactions with water molecules around the protein structure and increasing stability.

Effect of NaCl on Proteinase Activity and Stability. Activity of the purified proteinase increased with NaCl concentration up to 4 M (**Figure 5B**). The activity increased ∼2 times at ≥1 M NaCl, like 19 kDa-proteinase from *Virgibacillus* sp. SK33 (*18*). Proteinases from extremely halophilic bacteria normally show activity in 4 M NaCl (23.4% NaCl) and are irreversibly inactivated under low salt concentrations. Archaebacterium proteinase from *Halobacterium halobium* was completely and irreversibly inactivated at <2 M NaCl (11.7% NaCl) (*45*). On the other hand, activity of eubacterial proteinase was normally inactivated or unstable at high salt content. Activity of *B. subtilis* JM-3 proteinase decreased to ∼10% of the original at 30% NaCl (5.1 M) (*37*). Expro-I proteinase from *B. subtilis* FP-133 was stable in 0−20% NaCl (0−3.4 M), but its activity decreased with NaCl concentration (*35*). A proteinase from a moderate halophile, *Pseudomonas* sp. CP76, was unstable and inactivated at high NaCl concentration (4 M) (*8*). It should be pointed out that the purified proteinase of *Virgibacillus* sp. SK33 showed high stability and activity at 0−4 M NaCl (**Figure 5B**). This characteristic was similar to that of halophilic proteinases. Low pI value is a unique characteristic of halophilic proteinases, which implies that their surface molecules are rich in acidic amino acids (*46*). Therefore, these enzymes exhibited more effective competition with NaCl for binding water. This would ultimately lead to protection of the halophilic proteinases against salting-out.

Table 3. Proteolytic Activity in the Presence of 4 M NaCl toward Protein Substrate of Purified Proteinase from *Virgibacillus* sp. SK33

protein substrate	TCA-soluble oligopeptide content (μmol tyrosine produced/h)[a]
anchovy	0.295 ± 0.062 a
acid hemoglobin	0.063 ± 0.003 b
bovine serum albumin	0.055 ± 0.004 b
casein	0.084 ± 0.016 b

[a] a and b indicate significant difference ($p < 0.05$).

Table 4. Effect of Organic Solvents on Stability of Purified Proteinase

solvent (25% v/v)	log $P_{o/w}$[a]	relative activity (%)[b]
none		100
dimethylsulfoxide (DMSO)	−1.35	120
methanol	−0.77	102
acetonitrile	−0.34	134
ethanol	−0.31	122
isopropyl alcohol (2-propanol)	0.05	93
propyl alcohol (1-propanol)	0.25	103
ethyl acetate	0.73	81
n-butyl alcohol (1-butanol)	0.88	107
n-butyl acetate	1.78	98
chloroform	1.97	59
toluene	2.73	51
n-hexane	3.90	55
n-heptane	4.66	58

[a] Available information on http://www.srcinc.com/what-we-do/free-demos.aspx. $P_{o/w}$ is defined as the partition coefficient of the solvent in the octanol-water two-phase system. [b] Activity of each sample without incubation was taken as 100%.

The purified proteinase hydrolyzed various proteins at 4 M NaCl. It showed the highest hydrolytic activity toward anchovy protein ($p < 0.05$), and hydrolysis of acid HB, BSA, and casein by the purified proteinase was comparable ($p > 0.05$) (**Table 3**). Crude proteinase from *Virgibacillus* sp. SK33 also showed the highest proteolytic activity toward anchovy protein at 20−25% NaCl (3.42−4.28 M) (*17*). The specificity of the proteinase on protein substrates varied according to source. A proteinase from *B. subtilis* TKU007 showed the highest activity toward casein but did not hydrolyze albumin, gelatin, fibrin, and myoglobin (*47*). A proteinase from *Pseudomonas aeruginosa* PD100 hydrolyzed several proteins including collagen, fibrin, azocasein, casein, hemoglobin, BSA, ovalbumin, and elastin (*48*).

Effect of Organic Solvents on Proteinase Stability. In the presence of 0.625% organic solvent in the reaction mixture, proteinase activity decreased approximately 0−30%, depending on the type of solvent. From the viewpoint of stability, the purified proteinase was quite stable in relatively more polar solvents (DMSO, methanol, acetonitrile, ethanol, isopropyl alcohol, propyl alcohol, ethyl acetate, *n*-butyl alcohol, and *n*-butyl acetate) and unstable in nonpolar organic solvents (chloroform, toluene, *n*-hexane, and *n*-heptane, **Table 4**). A proteinase produced by the haloalkaliphilic archaeon *Natrialba magadii* was active and stable in DMSO, a more polar organic solvent (*7*). The effect of organic solvent on proteinase activity and stability is not universal and varies with strains. A proteinase from *P. aeruginosa* PST-01 was unstable in nonpolar solvents, namely, toluene, *n*-hexane, *n*-heptane, benzene, *p*-xylene, *n*-decane, and cyclohexane,

but more stable the presence of polar solvents, such as ethyleneglycol, DMSO, methanol, ethanol, isopropyl alcohol, and 1-butanol (*49*). In contrast, a proteinase from *P. aeruginosa* PseA was stable in nonpolar solvents but unstable in polar solvents (*50*). These results demonstrate that *Virgibacillus* sp. SK 33 proteinase is a solvent-tolerant enzyme and could have potential applications in aqueous−organic solvent biocatalysis.

Protein Identification. Peptide mass fingerprint (PMF) and SEQUEST search algorithm did not match the purified proteinase with any proteins in the database. The amino acid sequences of the dominant peptide mass from de novo peptide sequencing indicated that four peptides, VSVNESGR, HPGPAVSAVVALMK, EADTGVQWDHPALK, and EADTGVKWDHPALK, matched subtilisin and bacillopeptidase F from various strains, whereas three sequences, NVEWNVER, NYGYNLLHAAGDFWR, and FWYDLLHAAGDFTLK, showed significant alignment with SC protease (from *Bacillus* sp. KSM-LD1). Five peptide sequences, namely, VDVLNWR, DVVLNAFR, LLASGYEHK, MPRASLSTQHDCGANPR, and RMNTNEVQPDLSAPLNRR, showed a significant alignment with a signal peptidase I (from *Deinococcus geothermalis* DSM 11300), neutral proteinase (from *Clostridium difficile* QCD-76w55), ATP-dependent metalloprotease FtsH (from *Hydrogenobaculum* sp. Y04AAS1), acetyl-CoA acetyltransferase (from *Mycobacterium avium* 104), and protease II (from *Shewanella benthica* KT99). On the basis of its biochemical characteristics and protein identification, it can be concluded that the purified proteinase from *Virgibacillus* sp. SK33 is a novel enzyme.

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