

*Purification and characterization of
microbial protease produced
extracellularly from Bacillus subtilis FBL-1*

Article

Accepted Version

Si, J.-B., Jang, E.-J., Charalampopoulos, D. ORCID:
<https://orcid.org/0000-0003-1269-8402> and Wee, Y.-J. (2018)
Purification and characterization of microbial protease
produced extracellularly from *Bacillus subtilis* FBL-1.
Biotechnology and Bioprocess Engineering, 23 (2). pp. 176-
182. ISSN 1226-8372 doi: [https://doi.org/10.1007/s12257-017-
0495-3](https://doi.org/10.1007/s12257-017-0495-3) Available at <https://centaur.reading.ac.uk/78941/>

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Published version at: <http://dx.doi.org/10.1007/s12257-017-0495-3>

To link to this article DOI: <http://dx.doi.org/10.1007/s12257-017-0495-3>

Publisher: Springer

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2 1 **Purification and characterization of microbial protease produced**
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5 2 **extracellularly from *Bacillus subtilis* FBL-1**
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12 4 **Jin-Beom Si, Eun-Ju Jang, Dimitris Charalampopoulos, Young-Jung Wee**
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54 16 **Abstract** An ammonium sulfate precipitation of fermentation broth produced by *Bacillus*

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57 17 *subtilis* FBL-1 resulted in 2.9-fold increase of specific protease activity. An eluted protein
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2 18 fraction from the column chromatographies using DEAE-Cellulose and Sephadex G-75 had
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5 19 94.2- and 94.9-fold higher specific protease activity, respectively. An SDS-PAGE revealed a
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9 20 band of purified protease at approximately 37.6 kDa. Although purified protease showed the
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12 21 highest activity at 45°C and pH 9.0, the activity remained stable in temperature range from
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16 22 30°C to 50°C and pH range from 7.0 to 9.0. Protease activity was activated by metal ions
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19 23 such as Ca^{2+} , Mg^{2+} , Mn^{2+} , Fe^{2+} , Ca^{2+} and K^+ , but 10 mM Fe^{3+} significantly inhibited enzyme
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22 24 activity (53%). Protease activity was inhibited by 2 mM EDTA as a metalloprotease inhibitor,
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26 25 but it showed good stability against surfactants and organic solvents. The preferred substrates
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29 26 for protease activity were found to be casein (100%) and soybean flour (71.6%).
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36 28 **Keywords:** protease; *Bacillus*; metalloprotease; organic solvent; purification
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43 30 **1. Introduction**

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50 32 Proteases (E.C.3.4.21-24) catalyze the cleavage of peptide bond in protein molecules
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53 33 resulting in smaller fragments such as peptides and/or amino acids. They are distributed
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57 34 broadly in nature and a wide variety of microorganisms. Proteases are usually divided into
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2 35 two groups, exopeptidases or endopeptidases, depending on their site of hydrolysis.
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5 36 Exopeptidases break the peptide bonds formed between the end amino acid and the rest of
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9 37 peptide chain, but endopeptidases hydrolyze the peptide bonds found within the polypeptide
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12 38 or protein. There was an attempt to classify proteases based on structural features of enzyme
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16 39 active center, rather than origin, specificity, or physiological action [1]. Proteases are then
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19 40 divided into four classes based on the type of functional group present at the active site and
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22 41 their mechanism of action: 1) serine protease, 2) aspartic protease, 3) cysteine/thiol protease,
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26 42 and 4) metalloprotease [1, 2].
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30 43 Proteases are extensively used in a variety of industries, including detergent, leather,
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33 44 pharmaceuticals, food, textile, bakery, soy-processing, peptide synthesis, and X-ray film. The
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36 45 estimated value of worldwide sales of enzymes has been over 3 billion U.S. dollars, and the
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40 46 market for proteases accounts for approximately 60% of the total worldwide sale of enzymes
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43 47 [3-6]. Proteases have been isolated, purified, and identified in living organisms and bacteria.
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47 48 Microorganisms are good source of proteases due to a number of advantages; 1) the broad
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50 49 biochemical diversity, 2) the rapid growth, 3) the limited space required for cell cultivation,
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54 50 and 4) the ease at which the enzymes can be genetically manipulated to generate new
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57 51 enzymes for various applications [3]. Some bacteria, yeasts, and fungi are able to produce
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52 proteases, but only those microorganisms that produce enough amounts of extracellular
53 proteases are of industrial importance [7]. Microbial proteases are widely different not only in
54 their functions but also in their properties.

55 Recently, most of the industrial processes are carried out at harsh conditions, where the
56 enzymes are unstable under extremely high temperature, high or low pH, high concentration
57 of organic solvents and detergents, but only a limited class of proteases is recognized as
58 commercial resource. Alkaline serine proteases such as subtilisin Carlsberg, subtilisin BPN',
59 and Savinase are the major application as detergent industrial source, and some
60 metalloproteases are usually used in brewing and therapeutic industry [6].

61 We have successfully isolated and examined *B. subtilis* FBL-1 to produce potential
62 protease [7, 8]. One of the possible objectives of purifying and characterizing a bacterial
63 protease has been the production of enzymes for commercial purposes. Therefore, in the
64 present study, a bacterial protease was produced by *B. subtilis* FBL-1, which was then
65 purified and characterized by ammonium sulfate precipitation, column chromatographies, and
66 SDS-PAGE. In addition, enzymatic properties of the purified protease were further
67 investigated to characterize the effects of enzyme activity and stability on organic solvents,
68 detergents, temperature, pH, oxidizing agents, and reducing agents.

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2. Materials and Methods

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2.1. Bacterial strain

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74 *Bacillus subtilis* FBL-1 KCCM 43196 isolated from soil was procured by Food
75 Bioengineering Laboratory in Yeungnam University, Daegu, South Korea [7, 8]. Stock
76 cultures were preserved in 1.5 mL sample tubes containing 50% (v/v) glycerol at -70°C until
77 use. In order to activate cultures, strains were inoculated into tryptic soy broth (TSB; BD,
78 Sparks, MD, USA) and then grown at 37°C and 200 rpm for 15 h.

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2.2. Production of protease

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82 The cells from stock cultures were inoculated to sterile 100 mL growth medium (TSB) and
83 dispensed into 250 mL Erlenmeyer flasks, followed by incubation at 37°C for 15 h. This was
84 then inoculated aseptically at 1.5% (v/v) into 250 mL Erlenmeyer flask containing 100 mL
85 production medium, which were incubated on a shaking incubator (VS-8480SF; Vision

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2 86 Scientific Co., Daejeon, Korea) at 37°C and 200 rpm. The production medium was composed
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6 87 of 32.4 g/L, yeast extract 15.0 g/L, KH₂PO₄ 1.0 g/L, and MgSO₄·7H₂O 0.6 g/L. After 36 h of
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9 88 cultivation, cells were centrifuged at 13,000 × g and 4°C for 15 min with a high-speed
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13 89 refrigerated centrifuge (Supra 21K; Hanil Scientific Inc., Gimpo, Korea). The supernatant
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16 90 was collected and used as a crude enzyme preparation.
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23 92 **2.3. Protease activity assay**

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37 96 Protease activity was measured using casein as a substrate by the modified Folin &
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40 97 Ciocalteu's method [9]. A 20 μL of the enzyme was mixed with 500 μL of 0.5 M glycine-
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44 98 NaOH buffer (pH 9.0) containing 1% (w/v) casein and incubated at 40°C for 10 min with
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47 99 control. The enzyme reaction was stopped by addition of 2 mL of 10% (w/v) trichloroacetic
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51 100 acid. The mixture was incubated at room temperature for 15 min, followed by centrifuged at
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54 101 16,000 × g for 15 min. The supernatant was mixed with 2.5 mL of 0.5 M Na₂CO₃ and then
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57 102 500 μL of 20% (v/v) 2 N Folin & Ciocalteu's reagent was added. The mixture was incubated
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2 103 defined as the amount of enzyme required to liberate 1 μ g of tyrosine per minute under the
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6 104 standard assay conditions.
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10 11 12 106 **2.4. Measurement of protein concentration**

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19 108 Protein concentration was determined by the BCA (bicinchonic acid) method using bovine
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23 109 serum albumin as a standard [10]. During chromatographic purification steps, the protein
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26 110 content of each fraction was monitored by measuring the absorbance at 280 nm.
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31 32 33 112 **2.5. Enzyme purification**

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40 114 Culture supernatant was subjected to ammonium sulfate precipitation for purification of
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44 115 protease. Ammonium sulfate fractions of 30-80% were collected by centrifugation at 13,000
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47 116 $\times g$ and 4°C for 60 min, and the pellet was dissolved in a minimum amount of 0.1 M Tris-
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51 117 HCl buffer (pH 7.0). The protein was dialyzed against the same buffer to remove the residual
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54 118 salt at 4°C overnight with changing buffer solution. The dialysate was loaded onto a DEAE-
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57 119 Cellulose column (15 \times 300 mm), which was equilibrated with 0.1 M Tris-HCl buffer (pH
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2 120 7.0). Proteins were eluted with a linear gradient of NaCl (0-0.5 M) dissolved in the same
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5 121 buffer, and each fraction of 2.0 mL was collected. The column was washed with the same
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9 122 buffer until the absorbance of effluent at 280 nm reached zero. Enzyme activity and protein
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12 123 concentration of each fraction were measured. After then, the resultant fractions showing
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16 124 protease activity were loaded onto a Sephadex G-75 column (15 × 300 mm), which was
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19 125 equilibrated with 100 mM Tris-HCl buffer (pH 7.0). The fractions of 1 mL each were
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23 126 collected, and enzyme activity and protein concentration were measured. All purification
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26 127 procedures were carried out at 4°C.

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30 128 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 5% stacking
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33 129 gel and a 12% resolving gel was used to determine the purity and molecular weight of the
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37 130 enzyme by the method of Laemmli [11]. Protein bands were visualized by silver staining
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40 131 method. Molecular weight of the purified enzyme was estimated by comparing the relative
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43 132 mobility of standard molecular weight marker protein (Bio-rad, Hercules, CA, USA).

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50 134 **2.6. Effect of pH on protease activity and stability**

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57 136 The effect of pH on protease activity was measured at different pH values. The pH of the
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2 137 reaction mixture was adjusted to the desired values by using 0.1 M of buffers containing 1%
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5 138 (w/v) casein as a substrate as follows; citric acid buffer (pH 3.0 to 5.0), phosphate-citrate
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9 139 buffer (pH 5.0 to 7.0), Tris-HCl buffer (pH 7.0 to 9.0), and glycine-NaOH buffer (pH 9.0 to
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12 140 12.0). The pH stability of protease was determined by pre-incubation in the above mentioned
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16 141 buffers at room temperature for 30 min and 60 min. The relative activity of enzyme was
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19 142 quantified under the standard assay conditions.
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26 144 **2.7. Effect of temperature on protease activity and stability**

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33 146 The effect of temperature on protease activity was carried out by incubation of reaction
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36 147 mixture at different temperatures ranged between 30°C and 60°C in 0.5 M glycine-NaOH
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40 148 buffer (pH 9.0) containing 1% (w/v) casein as a substrate. Thermal stability of the protease
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44 149 was determined by pre-incubation of protease at 30-70°C for 30 min and 60 min. The relative
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47 150 activities were quantified under the standard assay conditions.
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54 152 **2.8. Substrate specificity**

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2 154 Substrate specificity of the protease was determined using different substrates. The reaction
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6 155 mixtures were prepared by adding 1% (w/v) of casein, bovine serum albumin (BSA), soybean
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9 156 flour, and gelatin in 0.5 M glycine-NaOH buffer (pH 9.0). The enzyme activity was
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13 157 determined as described above.

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19 159 **2.9. Effect of metal ions on protease activity**
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26 161 The effect of metal ions on enzyme activity was investigated by incubating the reaction
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30 162 mixture with NH_4Cl , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$,
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33 163 KCl , CaCO_3 , and ZnCl_2 at concentrations of 1 mM and 5 mM for 30 min and 60 min at room
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37 164 temperature, respectively. The enzyme activity measured under the absence of metal ions was
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40 165 considered as 100%.

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47 167 **2.10. Effect of detergents, oxidants, and reductants on protease activity**
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54 169 The enzyme solution was incubated at room temperature for 30 min in 0.5 M glycine-NaOH
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57 170 buffer (pH 9.0), containing 1% (v/v) Tween 20, 1% (v/v) Tween 80, 1% (v/v) Triton X-100, 1
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2 171 mM and 10 mM sodium dodecyl sulfate, 2% (v/v) H₂O₂, and 2% (v/v) 2-mercaptoethanol.

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5 172 Esterase activity was assayed by the spectrophotometric method and compared with the

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9 173 activity of the enzyme in the absence of detergent. Protease activity was measured and

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12 174 compared with the proteolytic activity of the enzyme in the absence of surfactants, oxidants,

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16 175 and reductants.

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23 177 **2.11. Effect of organic solvents on protease activity**

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29 179 The enzyme solution was incubated at room temperature for 30 min in in 0.5 M glycine-

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33 180 NaOH buffer (pH 9.0), containing 25 or 50% (v/v) of 1-butanol, benzene, *n*-hexane, 2-

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36 181 propanol, dimethyl sulfoxide (DMSO), and ethyl alcohol. Tween 80 was used as an

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40 182 emulsifier for water-immiscible solvents. Protease activity was measured and compared with

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43 183 the proteolytic activity of the enzyme in the absence of organic solvents.

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50 185 **2.12. Effect of inhibitors on protease activity**

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57 187 The effect of several inhibitors on protease activity was investigated by incubating the

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2 188 enzyme solution with ethylenediaminetetraacetic acid (EDTA, 2 mM and 10 mM),
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5 189 phenylmethylsulfonyl fluoride (PMSF, 1 mM), and diisopropyl fluorophosphates (DIFP, 0.1
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9 190 mM). The purified enzyme was pre-incubated with each inhibitor at room temperature for 30
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12 191 min and then the residual activity was measured under the standard assay condition.
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19 193 **3. Results and Discussion**

26 195 **3.1. Purification of protease**

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33 197 *B. subtilis* FBL-2 was cultivated in optimized medium for 36 h. The crude enzyme
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36 198 preparation was subjected to 30-80% ammonium sulfate precipitation, followed by dialyzed
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40 199 against 0.1 M Tris-HCl buffer (pH 7.0) at 4°C overnight by changing the fresh buffer every 4
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43 200 h. The dialyzate was loaded onto DEAE-Cellulose column at a flow rate of 2 mL/min. As
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47 201 shown in Fig. 1A, the fractions showing high protease activity (Fractions 5 to13) were pooled,
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50 202 desalted, concentrated, and loaded again onto Sephadex G-75 column. Fractions 15 to 38
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54 203 showed high protease activity (Fig. 1B). Purification factors and recoveries at each step are
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57 204 shown in Table 1. The enzyme was purified 94.89-fold with a yield of 2.3% from the crude
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2 205 extract, and the specific activity was increased to 3378.1 U/mg-protein.
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5 206 Molecular weight of the purified protease was verified by SDS-PAGE and silver staining
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9 207 method. As shown in Table 1, after ammonium sulfate precipitation step, the recovery ratio of
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12 208 protein and enzyme were as low as 2.5% and 7.1%, respectively. In addition, ammonium
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16 209 sulfate precipitation resulted in more concentrated proteins other than protease. This low
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19 210 amount of recovery for a target enzyme and low selectivity of ammonium sulfate
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23 211 precipitation might result in denser protease band of crude extract than that of ammonium
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26 212 sulfate fraction (Fig. 2). However, the protease was dramatically purified in the next
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30 213 chromatographic separation steps, which was visualized in Fig. 2. By comparing the relative
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33 214 mobility of standard marker proteins, molecular weight of the purified protease was estimated
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37 215 to be approximately 37.6 kDa, which was similar to molecular weight of protease derived
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40 216 from *B. subtilis* RKY3 (38 kDa) [12].
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47 218 **3.2. Effect of pH on protease activity and stability**

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54 220 The effect of pH on protease activity and stability was examined over a pH range from pH
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57 221 3.0 to 12.0. As shown in Fig. 3A, enzyme showed the highest activity at pH 9.0 (glycine-
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2 222 NaOH buffer), but it was declined rapidly beyond pH 9.0. As shown in Fig. 3B, the purified
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6 223 enzyme was stable between pH 7.0 and 9.0 for 30 and 60 min, respectively. In addition, the
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9 224 enzyme activity could be retained approximately 80% of its initial activity at pH 6.0 to 9.0
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12 225 after incubation for 30 min. Similar results of pH effect on protease activity have been
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16 226 reported, and the proteases produced by *B. subtilis* Y-108 [13], *B. tequilensis* P15 [14], *B.*
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19 227 *cereus* SV1 [15], and *B. cereus* AK1871 [16] showed their optimum pH at 7.5 to 8.0 and high
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23 228 pH stability at 7.0 and 9.0.

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30 230 **3.3. Effect of temperature on protease activity and stability**

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37 232 The effect of temperature on protease activity was investigated. As shown in Fig. 4A, the
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40 233 protease activity was highest at 45°C, and the enzyme activity at 50°C was sustained with
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43 234 98.5% of maximum activity. However, the protease activity was rapidly declined beyond
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47 235 50°C. As shown in Fig. 4B, the purified protease could retain 100% relative activity at 30 to
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51 236 50°C for 30 min and 60 min, respectively. Similar effects of temperature on protease activity
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54 237 have been reported. For example, the protease produced by *B. subtilis* Y-108 showed its
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57 238 optimum temperature at 50°C and thermal stability at 25 to 50°C [13]. The protease from *B.*

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2 239 *subtilis* RKY3 had its optimum temperature at 60°C, and its thermal stability was rapidly
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6 240 declined above 40°C [12].
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10 11 12 242 **3.4. Substrate specificity** 13

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19 244 The purified protease was reacted with different substrates such as casein, BSA, soybean
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23 245 flour, or gelatin. As shown in Table 2, casein was found to show the highest substrate
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26 246 specificity (100%) to the purified enzyme, followed by soybean flour (71.6%) and BSA
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30 247 (22.3%). However, the enzyme could not assimilate gelatin as a substrate. McConn *et al.* [17]
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33 248 previously reported that a neutral protease derived from *B. subtilis* was active in hydrolyzing
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37 249 casein but its ability to hydrolyze gelatin and egg albumin was only limited. This result
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40 250 suggests that the purified protease form *B. subtilis* FBL-1 show similar aspect of *B. subtilis*
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44 251 reported by McConn *et al.*.
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48 49 50 253 **3.5. Effect of metal ions on protease activity** 51

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57 255 The effect of metal ions on protease activity was shown in Table 3. The relative activity of
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2 256 protease in the presence of 10 mM Mg²⁺, Ca²⁺, and Mn²⁺ were 41%, 30%, and 23%,
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5 257 respectively. Though the enzyme was significantly inhibited by 10 mM Fe³⁺, Fe²⁺ led to
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9 258 activation of protease activity. Similar effect of metal ions on protease activity has been
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12 259 reported. Proteases produced by *B. tequilensis* P15 [14], *B. cereus* SV1 [15], *B. cereus*
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16 260 AK1871 [16], *Bacillus* sp. B001 [4], *B. mojavensis* A21 [18] and *Bacillus* sp. AK.1 [19] were
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19 261 activated by presence of Ca²⁺. Some earlier reports have also showed that thermal stability of
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23 262 protease was improved in the presence of Ca²⁺ [20]. It may be explained by strengthening the
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26 263 interactions inside protein molecules and by combining Ca²⁺ to autolysis site to prevent
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30 264 autolysis and thermal unfolding [19, 21].
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34 35 36 266 **3.6. Effect of surfactants, oxidants, and reductants on protease activity**

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43 268 The effect of various chemicals such as surfactants, oxidants, and reductants on protease
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47 269 activity was investigated. As shown in Table 4, the enzyme was stable in the presence of 1%
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50 270 (v/v) nonionic surfactants like Tween 20, Tween 80, and Triton X-100. However, the enzyme
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54 271 was inhibited by the presence of 1 mM SDS as an anionic surfactant and 69% of the enzyme
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57 272 activity was inhibited by addition of 10 mM SDS. In addition, hydrogen peroxide (H₂O₂) and
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2 273 2-mercaptoethanol inhibited the protease activity by 12% and 36%, respectively. Similar
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6 274 effects of surfactants, oxidants, and reductants on protease activity have been reported.
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9 275 Protease from *B. tequilensis* P15 was stable in the presence of nonionic surfactants such as
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12 276 Tween 20, Tween 80, and Triton X-100, but it was inhibited in the presence of anionic
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16 277 surfactant such as SDS by 47.4% [14]. However, the protease produced by *B. mojavensis* was
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19 278 stable in the presence of high concentration SDS up to 1% (w/v) [21].
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26 280 **3.7. Effect of organic solvents on protease stability**

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33 282 The relative activity of protease after exposure to organic solvents is shown in Table 5.
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36 283 Enzymes are generally inactivated in the presence of organic solvents such as 1-butanol,
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40 284 benzene, *n*-hexane, 2-propanol, dimethyl sulfoxide, or ethyl alcohol. However, the protease
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43 285 from *B. subtilis* FBL-1 was rarely inhibited by water-immiscible solvent such as *n*-hexane
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47 286 even at 25% and 50%. In addition, the enzyme was quite stable in the presence of 1-butanol
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50 287 and DMSO at 25% and 50%. Benzene and 2-propanol at 50% significantly lowered the
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54 288 enzyme activity to 69.4% and 42.8%, respectively. According to the previous studies, the
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57 289 protease from *Aeromonas veronii* PG01 [22] was inhibited in the presence of DMSO at 50%,
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2 290 the enzyme from *B. pumilus* 115B [23] was stable in the presence of hexane and benzene, the
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6 291 protease from *B. tequilensis* P15 [14] was unstable in the presence of hexane, and the enzyme
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9 292 from *B. cereus* AK187 [16] was significantly unstable in the presence of butanol.
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16 294 **3.8. Effect of inhibitors on protease activity**

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23 296 The purified protease derived from *B. subtilis* FBL-1 was completely inhibited by the EDTA
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26 297 as a metalloprotease inhibitor (Table 6). The enzyme activity was almost reduced to 11% and
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30 298 3.67% in the presence of 2 mM and 10 mM EDTA, respectively, but it was nearly not
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33 299 inhibited in the presence of PMSF and DIFP. Serine residue in the active site of the
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37 300 proteinases is irreversibly acylated by PMSF or DIFP, which results in inactivation of the
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40 301 enzymes. Therefore, the reagents such as PMSF or DIFP are serine protease inhibitors [24].
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43 302 Cysteine proteases are generally inactivated with oxidative agents, metal ions, or alkylating
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47 303 agents. However, inhibition of metalloproteases is achieved with chelating agents (EDTA) or
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50 304 sodium dodecyl sulfate [25]. Therefore, the results obtained here suggest that the purified
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54 305 protease derived from *B. subtilis* FBL-1 should be considered to be a metalloprotease.
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2 307 **4. Conclusion**
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9 309 The protease from *B. subtilis* FBL-1 was purified and characterization for industrial
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12 310 application. The enzyme showed pH stability from 7.0 to 9.0 and thermostability from 30°C
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16 311 to 50°C. The protease activity was strongly activated by divalent metal ions. EDTA as a metal
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19 312 chelator almost inhibited protease activity, but no inhibition was observed when DIFP was
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23 313 added, suggesting that the protease from *B. subtilis* FBL-1 might be classified into a neutral
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26 314 metalloprotease. In addition, the enzyme activity could be highly stable even in the presence
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30 315 of nonionic surfactants, reducing agents, or organic solvents. It's stability against various
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33 316 chemicals makes this enzyme a potential biocatalyst for industrial applications. These study
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37 317 efforts need to get more knowledge on metalloproteases in *B. subtilis* for potential industrial
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40 318 applications such as brewing and grain starch isolation industries.
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46 320 **References**
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390 serine, cysteine, and threonine proteases. *Chem. Rev.* 102: 4639-4750.

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2 391 **Figure Legends**
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9 393 Fig. 1. Chromatograms obtained by (A) DEAE-Cellulose ion-exchange chromatography and
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12 394 (B) Sephadex G-75 gel filtration chromatography. Both columns were equilibrated with 100
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16 395 mM Tris-HCl buffer (pH 7.0).
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23 397 Fig. 2. SDS-PAGE of the purified protease produced by *B. subtilis* FBL-1. Lane 1, molecular
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26 398 weight marker proteins; lane 2, crude extract; lane 3, ammonium sulfate fraction; lane 4,
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30 399 DEAE-Cellulose fraction; lane 5, Sephadex G-75 fraction.
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37 401 Fig. 3. Effect of pH on (A) protease activity and (B) stability. The buffer systems used were
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40 402 as follows: 0.1 M citric acid buffer for pH 3.0-5.0, 0.1 M phosphate-citrate buffer for pH 5.0-
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43 403 7.0, 0.1 M Tris-HCl buffer pH 7.0-9.0, and 0.1 M glycine-NaOH buffer for pH 9.0-12.0. The
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47 404 highest enzyme activity was considered as 100%, and error bars showed standard deviations
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51 405 of triplicate samples.
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57 407 Fig. 4. Effect of temperature on (A) protease activity and (B) stability. The highest enzyme
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411 Table 1. Summary of the purification step for the proteolytic enzyme from *Bacillus subtilis*

412 FBL-1

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	7,073.0	251,794.5	35.6	100	1
(NH ₄) ₂ SO ₄ (30-80%)	175.4	17,795.3	101.5	7.1	2.85
DEAE-Cellulose	3.9	12,937.1	3,352.4	5.1	94.17
Sephadex G-75	1.7	5,669.9	3,378.1	2.3	94.89

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415 Table 2. Substrate specificity of the purified protease from *Bacillus subtilis* FBL-1

Substrates (1%, w/v)	Relative activity (%)
Casein	100 ± 7.4
Bovine serum albumin	22.3 ± 1.3
Soybean flour	71.6 ± 4.3
Gelatin	0

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2 418 Table 3. Effect of metal ions on protease activity
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Metal ions	Relative activity (%)	
	1 mM	10 mM
None	100 ± 1.7	100 ± 1.6
Mg ²⁺ (MgSO ₄)	118 ± 12.9	141 ± 4.4
Fe ²⁺ (FeSO ₄)	119 ± 8.1	110 ± 3.6
Fe ³⁺ (FeCl ₃)	125 ± 4.7	47 ± 3.5
Mn ²⁺ (MnCl ₂)	123 ± 3.3	123 ± 6.5
NH ⁴⁺ (NH ₄ Cl)	105 ± 2.9	108 ± 4.6
Ca ²⁺ (CaCl ₂)	116 ± 0.8	130 ± 3.3
Ca ²⁺ (CaCO ₃)	106 ± 5.8	112 ± 2.1
K ⁺ (KCl)	107 ± 3.2	116 ± 3.0
Zn ⁺ (ZnCl)	104 ± 1.7	93 ± 4.2

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420 Table 4. Effect of surfactants, oxidants, and reductants on protease activity

Surfactants, oxidants, or reductants	Concentration	Relative activity (%)
None	-	100 ± 4.6
Tween 20	1% (v/v)	77 ± 1.2
Tween 80	1% (v/v)	88 ± 1.0
Triton X-100	1% (v/v)	77 ± 2.9
Sodium dodecyl sulfate (SDS)	1 mM	86 ± 2.1
	10 mM	31 ± 0.7
H ₂ O ₂	2% (v/v)	88 ± 2.9
2-Mercaptoethanol	2% (v/v)	64 ± 3.7

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1
2 422 Table 5. Effect of organic solvents on protease activity
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Solvents	Relative activity (%)	
	25% (v/v)	50% (v/v)
None	100 ± 2.4	100 ± 2.4
1-Butanol	77.6 ± 3.1	77.4 ± 5.8
<i>n</i> -Hexane	102.3 ± 3.5	100 ± 8.1
Benzene	66.1 ± 3.5	30.6 ± 1.0
2-Propanol	75.8 ± 4.1	57.2 ± 2.4
Dimethyl sulfoxide (DMSO)	79.3 ± 3.2	73.1 ± 1.6
Ethyl alcohol	87.6 ± 3.8	61.2 ± 3.6

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2 424 Table 6. Effect of inhibitors on protease activity
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Inhibitors	Concentration (mM)	Relative activity (%)
None	-	100 ± 2.94
Ethylene-diaminetetraacetic acid (EDTA)	2	11 ± 0.47
	10	3.67 ± 0.47
Diisoprophyl fluorophosphaete (DIFP)	0.1	86.33 ± 3.68
Phenylmethly sulfonyl fluoride (PMSF)	1	84.67 ± 6.13

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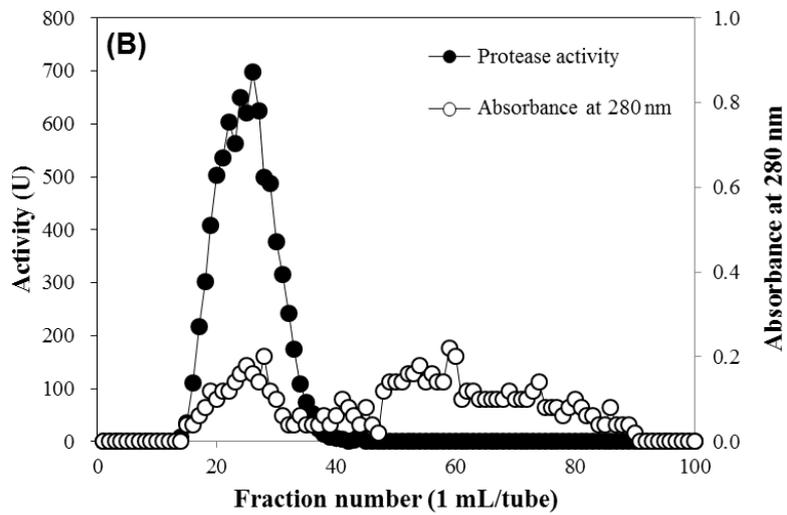
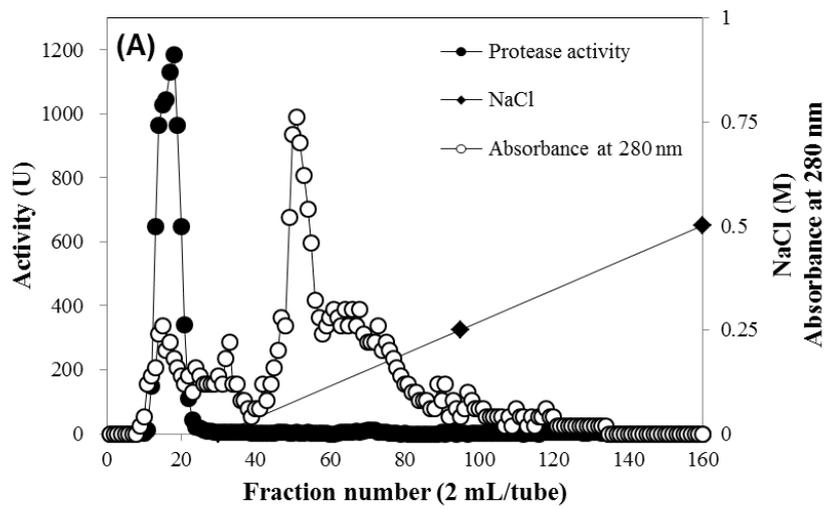


Fig. 1

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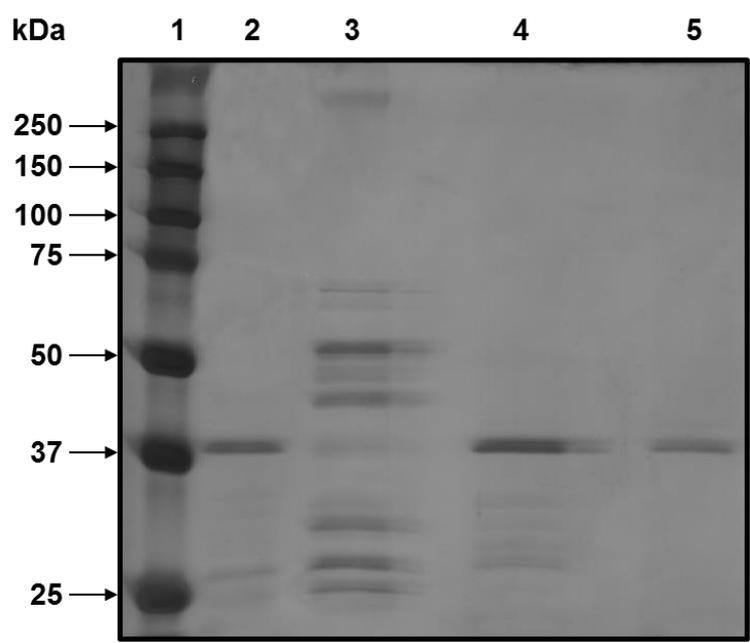


Fig. 2

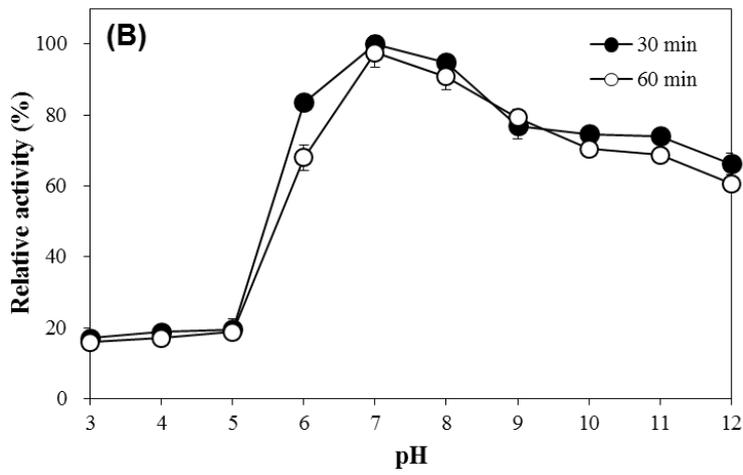
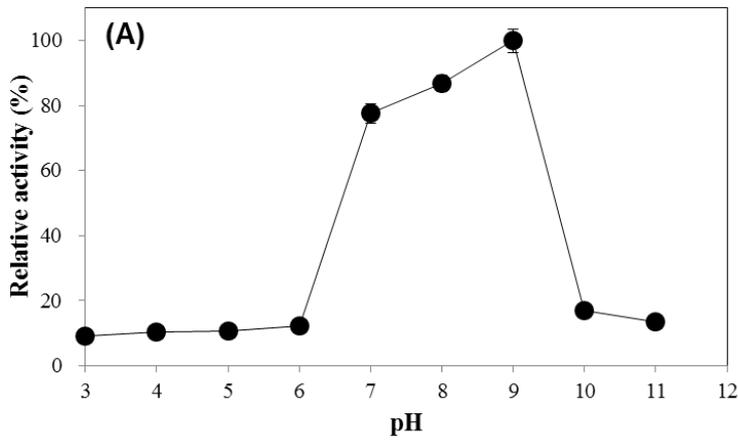
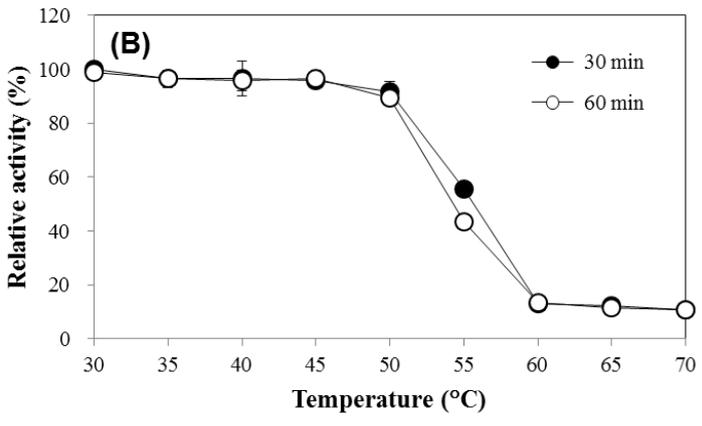
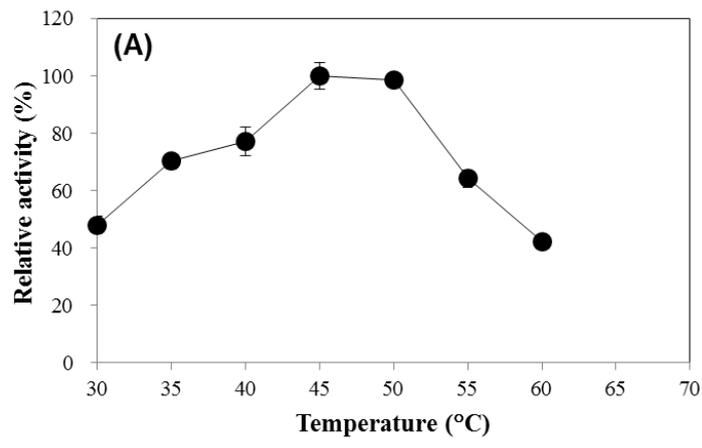


Fig. 3



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439 Fig. 4