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# Purification and Characterization of a Novel Alkaline Protease from *Bacillus horikoshii*

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**An investigation was conducted on the enhancement of production and purification of an oxidant and SDS-stable alkaline protease (BHAP) secreted by an alkalophilic *Bacillus horikoshii*, which was screened from the body fluid of a unique Korean polychaeta (*Periserrula leucophryna*) living in the tidal mud flats of Kwangwha Island in the Korean West Sea. A prominent effect on BHAP production was obtained by adding 2% maltose, 1% sodium citrate, 0.8% NaCl, and 0.6% sodium carbonate to the culturing medium. The optimal medium for BHAP production contained (g/l) SBM, 15; casein, 10; $K_2HPO_4$, 2; $KH_2PO_4$, 2; maltose, 20; sodium citrate, 10; $MgSO_4$, 0.06; NaCl, 8; and $Na_2CO_3$, 6. A protease yield of approximately 56,000 U/ml was achieved using the optimized medium, which is an increase of approximately 5.5-fold compared with the previous optimization (10,050 U/ml). The BHAP was homogenously purified 34-fold with an overall recovery of 34% and a specific activity of 223,090 U/mg protein using adsorption with Diaion HPA75, hydrophobic interaction chromatography (HIC) on Phenyl-Sepharose, and ion-exchange chromatography on a DEAE- and CM-Sepharose column. The purified BHAP was determined a homogeneous by SDS–PAGE, with an apparent molecular mass of 28 kDa, and it showed extreme stability towards organic solvents, SDS, and oxidizing agents. The $K_m$ and $k_{cat}$ values were 78.7 μM and 217.4 $s^{-1}$ for *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA at 37°C and pH 9, respectively. The inhibition profile exhibited by PMSF suggested that the protease from *B. horikoshii* belongs to the family of serine proteases. The BHAP, which showed high stability against SDS and $H_2O_2$, has significance for industrial application, such as additives in detergent and feed industries.**

**Keywords:** Alkaline protease, *B. horikoshii*, polychaeta, purification, SDS-stable

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A number of alkaline enzymes, such as proteases, amylases, and lipases, are widely used in industrial applications, including the detergent, food, animal feed, leather processing, tannery, and silk industries [16, 17]. Although a wide range of microorganisms are known to produce the enzymes, a number of *Bacillus* species from many different exotic environments have been explored and exploited for the enzyme production, because the *Bacillus* species are considered prolific producers of enzymes exhibiting significant activity and stability at considerably high pH and temperatures. Therefore, these bacteria, such as *B. licheniformis* and *B. subtilis,* are thought to be attractive organisms for industrial uses, such as food, because of their high growth rates, resulting in short culturing times; their high ability to secrete proteins into the medium; and their safety. Furthermore, there is extensive information about the biochemistry, physiology, and genetic background of *B. subtilis* and other *Bacillus* species, which facilitates development of these organisms for industrial applications [33, 49].

It is well known that proteases constitute one of the most important industrial enzymes, and the use of alkaline proteases in a variety of industrial products like detergents, food, leather, and silk has increased remarkably. Therefore, the alkaline proteases have gained renewed interest in view of their commercial significance because they can rapidly digest proteinaceous stains, such as blood and milk, on clothes even under high alkaline conditions [11, 16, 17, 30, 31, 39, 43]. The first bacterial protease used in detergent was subtilisin Carlsberg produced from *B. licheniformis* in the 1960s. Since that time, use of bacterial proteases exhibited a tendency to increase because it could reduce environmental pollution and energy waste. The first alkaline protease that differed from the subtilisin group was reported for an alkaliphilic *Bacillus* strain 221 isolated from soil, which produced large amounts at the optimum pH of 11.5 [15]. Since the highly active alkaline protease was discovered in the 1970s, a number of variants showing better washing

performance at low temperatures and high pH, such as Savinase, Maxacal (PB92), NKS-21, and M-protease, were isolated and developed for use as detergent additives [43]. Although several fungal sources are being increasingly identified [38, 44, 50], currently, a large proportion of the commercially available alkaline proteases are derived from *Bacillus* strains, such as *B. amyloliquefaciens*, *B. licheniformis*, and *B. clausii* [2, 17, 18, 21–24, 35, 36, 53]. For example, Novozymes (Denmark) manufactures industrial alkaline proteases named Alcalase and Savinae, which are produced from *B. licheniformis* and *B. clausii*, respectively; Genencor (USA) manufactures Purafect and Properase from *B. lentus* and *B. alkalophilus* PB92, respectively [36]. However, many alkaline proteases in industrial uses face limitations: (1) compatibility and stability against various detergent components, and (2) production cost. Often, enzymes could not be used in industrial processes because of the high temperatures, extreme pH conditions, and the presence of oxidizing agents, detergents, heavy metals, and organic solvents. To increase the possibility for industrial applications of the enzymes, it is necessary to reduce the production cost and to constantly improve the enzyme's working performance through screening *via* natural enzyme diversity, recombinant DNA, and fermentation technologies. Often, it has been reported that many *Bacillus*-derived alkaline proteases are active at elevated temperatures and pH, but the majority exhibited low activity and stability towards anionic surfactants and oxidants, such as peroxides and perborates [13, 43]. To overcome these shortcomings, several attempts have been made to protein engineer the commercially available enzymes to further enhance the enzyme stability and activity under harsh conditions [5]. For example, oxidatively stable mutant enzymes were obtained by replacing the Met residue with a non-oxidizable amino acid; however, it greatly reduced the enzymatic activity [7]. In addition, the thermal stability of detergent-stable serine alkaline protease produced from *B. pumilus* CBS by replacing Asn99 with Tyr99 increased approximately 3.7-fold at 60°C when compared with the wild-type enzyme [20]. Besides protein engineering, the vast marine microbial diversity has always been a useful resource for exploring newer isolates, producing novel enzymes with better functional properties. Secondly, around 30~40% of the production cost of industrial enzymes is estimated to be related to the cost of growth medium, and this is important because proteases account for over 30% of worldwide enzyme consumption [54]. With respect to nitrogen sources, soybean meal (SBM) is a potentially useful, cost-effective medium substrate owing to its easy availability and low-cost because it is produced as a by-product during oil extraction [9]. In these respects, alkaline proteases exhibiting superior performance and stability under extreme conditions suggest large commercial applicability, particularly in laundry detergent, and have become of considerable interest. Hence, further exploration of microorganisms producing alkaline proteases with low production costs is warranted.

We previously reported the optimization of alkaline protease production in several *Bacillus* species including *B. horikoshii, B. clausii* I-52, and *Bacillus* sp. I-312 [21, 22, 24]. In this paper, we report the further optimization of protease production, purification, and some biochemical properties of the purified alkaline protease (BHAP) from an alkalophilic *B. horikoshii* isolated from the hemolymph of a Korean marine polychaeta, *Periserrula leucophryna*, a unique inhabitant of the mud flats of the Yellow Sea, Korea, which has potential use in detergent formulations and other industrial applications.

## Materials and Methods

### Microorganism and Further Optimization of Protease Production

The alkaline protease-producing *B. horikoshii* used in this study was previously isolated from the hemolymph of the polychaeta, *P. leucophryna*, and the isolate was maintained on a TSB (tryptic soy broth) agar plate and stored at 4°C [21]. We examined the further optimization of BHAP production using basal medium supplemented with various carbon sources and other medium ingredients. The basal medium for the BHAP production was composed of (g/l) SBM, 15; casein, 10; $K_2HPO_4$, 2; $KH_2PO_4$, 2; $MgSO_4$, 0.06; and $Na_2CO_3$, 4. A stock solution of $Na_2CO_3$ [30% (w/v)] was sterilized separately and added to the medium before inoculation with the seed culture. The medium (100 ml) in 500 ml baffled flasks was inoculated with 1 ml of a 24 h-old seed culture and was incubated at 37°C with shaking at 250 rpm for 48 h. After adding 50 µl of 10% trichloroacetic acid (TCA) to 50 µl of the cell-free supernatant, the mixture was placed on ice for 30 min and centrifuged at 10,000 ×*g* for 10 min. The resulting precipitate was dissolved in 20 µl of SDS–PAGE sample buffer (0.05 mol/l Tris-HCl, pH 6.8, 0.1 mol/l DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue) and boiled for 5 min at 100°C. The samples were analyzed *via* SDS–PAGE (15%), as described by Laemmli [34], using a mini-gel system (Bio-Rad, USA).

### Enzyme Purification

For enzyme purification, all procedures were carried out at 4°C, unless otherwise specified. Purification of BHAP was achieved by adsorption with Diaion HPA75 (Mitsubishi Chemical, Tokyo, Japan), HIC on Phenyl-Sepharose (Amersham Biosciences, Uppsala, Sweden) and by ion-exchange chromatography on DEAE- and CM-Sepharose (Amersham Biosciences, Uppsala, Sweden). In brief, the cell-free supernatant was treated with 5% (w/v) Diaion HPA75 (Mitsubishi Chemical, Japan) with mechanical stirring at 150 rpm for 5 h. The resin was removed by suction filtration, and the filtrate was recovered. After adding $(NH_4)_2SO_4$ at a final concentration of 1 mol/l, the supernatant was recovered by centrifugation (10,000 ×*g*, 4°C, 20 min) and applied on a Phenyl-Sepharose column (2.5×10 cm), which had been previously equilibrated with buffer A (20 mmol/l sodium phosphate buffer, pH 7.0) containing 1 mol/l $(NH_4)_2SO_4$. The column was washed with the same buffer until the optical density

of the effluent at 280 nm almost reached zero. Bound proteins were eluted with buffer A at a flow rate of 100 ml/h, and 6 ml fractions were collected. The fractions exhibiting high protease activity were pooled and subsequently applied on a DEAE-Sepharose column (2.5×10 cm), which had been previously equilibrated with buffer A. The column was washed with the same buffer at a flow rate of 50 ml/h, and fractions of 5 ml were collected. The active flow-through fractions were combined and applied on a CM-Sepharose column (2.5×10 cm), which had been previously equilibrated with buffer A. After washing the column with the same buffer, the enzyme was eluted with buffer A containing 50 mmol/l NaCl. The flow rate was 50 ml/h, and fractions of 5 ml were collected. The active fractions were concentrated using Centriprep PM10, divided into aliquots, and stored at −70°C for further use.

**Protease Assay and Protein Concentration**

BHAP activity was determined using casein as a substrate at a concentration of 0.5% in 0.1 mol/l Tris-HCl buffer, pH 9 [22]. One unit of enzyme activity is defined as the amount of enzyme resulting in the release of 1 μg of tyrosine per minute at 50°C under standard assay conditions. Protein concentrations were determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard [4].

**Molecular Mass Determination**

SDS−PAGE (15%) was performed according to the method of Laemmli under reducing conditions [34]. The molecular mass was determined by interpolation from a linear semilogarithmic plot of the molecular mass *versus* $R_f$ value (relative mobility) using low molecular mass proteins standards (Pharmacia, USA). The standard proteins used were phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

**Effects of Denaturing Agents on the Enzyme Activity**

To examine the effect of denaturing agents on enzyme activity, denaturing agents including surfactants and oxidizing agents were added to the enzyme solution at the indicated concentrations and allowed to stand for 24 to 72 h at room temperature. Then, the remaining activities were measured according to the standard assay condition.

**Organic Solvent Stability of Protease**

After mixing 1 ml of the purified BHAP with distilled water or 1 ml of each organic solvent, the mixture was allowed to stand for the indicated times at room temperature. Then, the remaining activities were measured according to the standard assay conditions.

**Effects of Protease Inhibitors**

The purified BHAP was pre-incubated with several inhibitors including leupeptin and PMSF (phenylmethylsulfonyl fluoride) for 30 min at room temperature, and the remaining activity was determined under standard assay conditions. Residual activities in the presence of the inhibitors were compared with that of the controls without inhibitor.

**Substrate Specificity**

Amidolytic activity toward a variety of chromogenic peptide substrates was measured. The reaction was carried out in a total volume of 200 μl of assay buffer with the purified enzyme. Then, 10 μl of 4 mmol/l stock of each substrate was added to the reaction mixture, and the reaction was allowed to proceed for 5 min at 50°C. The proteolytic activity toward *N*-Suc-Ala-Ala-Pro-Phe-*p*NA was taken as 100%.

**N-Terminal Amino Acid Sequence Analysis**

The N-terminal amino acid sequence of the purified BHAP was determined on an ABI Procise CLC 492 protein sequencing system (Applied Biosystems Inc., USA).

## RESULTS

### Further Optimization of BHAP Production

We examined the further optimization of BHAP production by supplying various carbons sources in the basal medium (g/l): SBM, 15; casein, 10; $K_2HPO_4$, 2; $KH_2PO_4$, 2; $MgSO_4$, 0.06; and $Na_2CO_3$, 4. Production of BHAP from *B. horikoshii* was enhanced by addition of maltose and fructose (Table 1). When compared with the control (10,050 U/ml), the protease yield increased approximately 33%, 74%, and 12% by supplying 0.5%, 1%, and 1.5% fructose, respectively. Among the mono- and disaccharides tested, the maximum activity (23,520 U/ml) was obtained with a supplement of 2% maltose, which increased 2.3-fold that of the control. However, the addition of glucose (8,550 U/ml), lactose (5,330 U/ml), and galactose (3,160 U/ml) reduced protease production of 15%, 47%, and 69%, respectively. Therefore, 2% maltose was added to the culture medium for the following study. We also examined the effect of small molecular weight carbon sources, such as sodium acetate and sodium citrate, on enzyme production, using the basal medium supplemented with 2% maltose. A substantial improvement in enzyme production was noted upon addition of sodium glutamate and sodium citrate (Table 2). Protease

**Table 1.** Effects of the carbon sources on production of an alkaline protease from *B. horikoshii.*

| | Concentration (%) | Enzyme activity (U/ml, $\times 10^{-2}$) |
|---|---|---|
| Control | - | 100.5 |
| Fructose | 0.5 | 133.6 |
| | 1.0 | 174.5 |
| | 1.5 | 112.5 |
| Galactose | 0.5 | 31.6 |
| Glucose | 0.5 | 85.5 |
| Lactose | 0.5 | 53.3 |
| Sucrose | 0.5 | 91.4 |
| Maltose | 0.5 | 114.3 |
| | 1.0 | 131.6 |
| | 1.5 | 182.0 |
| | 2.0 | 235.2 |
| | 2.5 | 156.9 |

Cells were grown in basal medium supplemented with each carbon source at 37°C for 48 h. All the experiments were performed in triplicate, and the standard error in all the experimental results was within 5%.

**Table 2.** Effects of small carbon sources on production of an alkaline protease from *B. horikoshii.*

| | Concentration (%) | Enzyme activity (U/ml, $\times 10^{-2}$) |
|---|---|---|
| Control | - | 223.3 |
| Sodium acetate | 0.5 | 113.0 |
| Sodium succinate | 0.5 | 204.5 |
| Sodium glutamate | 0.1 | 204.2 |
| | 0.25 | 276.3 |
| | 0.5 | 240.7 |
| | 0.75 | 211.0 |
| | 1.0 | 181.4 |
| Sodium citrate | 0.25 | 269.7 |
| | 0.5 | 301.6 |
| | 0.75 | 342.8 |
| | 1.0 | 369.4 |
| | 1.25 | 276.4 |
| | 1.5 | 241.4 |
| Sodium propionate | 0.5 | 12.1 |
| Glycerol | 0.5 | 239.6 |
| | 1.0 | 271.8 |
| | 1.5 | 237.2 |

Cells were grown in basal medium containing 2% maltose supplemented with each small molecular weight carbon source at 37°C for 48 h. All the experiments were performed in triplicate, and the standard error in all the experimental results was within 5%.

**Table 3.** Effects of NaCl and KCl on production of an alkaline protease from *B. horikoshii.*

| Salt (%) | | Enzyme activity (U/ml, $\times 10^{-2}$) |
|---|---|---|
| NaCl | 0.0 | 373.6 |
| | 0.2 | 387.5 |
| | 0.4 | 405.2 |
| | 0.6 | 433.5 |
| | 0.8 | 459.4 |
| | 1.0 | 438.1 |
| | 2.5 | 362.2 |
| | 5.0 | 272.0 |
| | 10 | 73.4 |
| KCl | 0.0 | 365.4 |
| | 0.2 | 396.6 |
| | 0.4 | 415.1 |
| | 0.6 | 435.0 |
| | 0.8 | 389.7 |
| | 1.0 | 381.0 |
| | 2.5 | 267.9 |
| | 5.0 | 195.3 |
| | 10 | 78.7 |

Cells were grown in basal medium containing 2% maltose and 1% sodium citrate supplemented with each concentration of NaCl or KCl at 37°C for 48 h. All the experiments were performed in triplicate, and the standard error in all the experimental results was within 5%.

yield increased approximately 23% by supplying 0.25% (w/v) sodium glutamate to the basal medium containing 2% maltose when compared with that of the control (22,330 U/ml). Sodium citrate had a prominent effect on the production of BHAP among the small molecular weight carbon sources examined, and it increased approximately 35%, 53%, 65%, and 23% by supplying 0.5%, 0.75%, 1.0%, and 1.25% (w/v) sodium citrate, respectively. A maximum activity of 36,940 U/ml was obtained by supplementation with 1% sodium citrate to the basal medium containing 2% maltose (Table 2). However, supplementation with sodium propionate greatly decreased protease production by approximately 95%. It was reported that the addition of starch to the culture medium could induce protease yield and that production was dependent on starch type [8]. However, when the effects of different starches such as wheat flour and potato starch were examined, they did not significantly affect BHAP production (data not shown). When the salt effect was examined on BHAP production, the addition of NaCl or KCl at amounts less than 1% caused a slight increase in the protease production in *B. horikoshii* and showed an increase of approx. 23% and 19% by supplying 0.8% (w/v) NaCl and 0.6% KCl to the medium, respectively (Table 3). The addition of 0.8% NaCl was the effective salt concentration for BHAP production of 45,940 U/ml. To investigate the effect of medium pH on the protease production, sodium carbonate solution was added in increments ranging from 0 to 1% (w/v) to a basal medium containing 2% maltose, 1% sodium citrate, and 0.8% NaCl. An optimal protease production (56,020 U/ml) was observed at a concentration of 0.6% sodium carbonate (initial medium pH 10), indicating a typical alkalophile; however, BHAP production decreased when sodium carbonate was supplemented at more than 0.8% (Table 4). An optimum pH between 9 and 11 for growth and protease production is common among alkaliphilic and haloalkaliphilic organisms [6, 10, 22, 24, 49]. According to the final optimization experiments, the optimal medium for protease production contained (g/1) SBM, 15; casein, 10; $K_2HPO_4$, 2; $KH_2PO_4$, 2; maltose, 20; sodium citrate, 10; $MgSO_4$, 0.06; NaCl, 8; and $Na_2CO_3$, 6. A protease yield of approx. 56,000 U/ml was achieved based on the optimization (Table 4).

**Table 4.** Effect of sodium carbonate on production of an alkaline protease from *B. horikoshii.*

| Sodium carbonate (%) | Enzyme activity (U/ml, $\times 10^{-2}$) | Medium pH |
|---|---|---|
| 0.0 | 198.1 | 6.96 |
| 0.2 | 326.1 | 8.47 |
| 0.4 | 463.2 | 9.61 |
| 0.6 | 560.2 | 9.99 |
| 0.8 | 404.0 | 10.35 |
| 1.0 | 314.6 | 10.85 |

Cells were grown in basal medium supplemented with 2% maltose and 1% sodium citrate at 37°C for 48 h. The sodium carbonate solution was sterilized separately and added to the medium in increments ranging from 0 to 1.0%. All the experiments were performed in triplicate, and the standard error in all the experimental results was within 5%.

**Table 5.** Summary of the purification for an alkaline protease from *B. horikoshii*.

| Step | Unit (U, $\times 10^{-3}$) | Protein (mg) | Specific activity (U/mg protein) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Culture supernatant | 15,692.2 | 207.4 | 7,566.2 | 1.0 | 100.0 |
| Diaion HPA75 | 16,791.7 | 158.5 | 10594.1 | 1.4 | 107.0 |
| Phenyl-Sepharose | 12,565.7 | 64.9 | 19,361.6 | 2.6 | 80.1 |
| DEAE-Sepharose | 9,966.2 | 20.0 | 49,831.0 | 6.6 | 63.5 |
| CM-Sepharose | 5,345.2 | 3.2 | 223,091.7 | 29.5 | 34.1 |

**Enzyme Purification**

It is well established that downstream processing costs account for nearly 50–80% of the total production cost of enzymes/proteins [41]. The alkaline protease produced from *B. horikoshii* was purified from the 48 h cultivated supernatant using Diaion HPA75, Phenyl-Sepharose, and DEAE- and CM-Sepharose chromatographies. The final data on the purification of the protease from *B. horikoshii* are summarized in Table 5. The first step, adsorption on Diaion HPA75, was used to reduce the color intensity of the culture supernatant. SBM was used as the main medium ingredient to produce alkaline protease from many *Bacillus* species such as *Bacillus* sp. I-312 and *B. clausii* I-52 [22, 24], and this ingredient imparts a brown to dark-brown color to the fermentation medium, and the color intensity further increases because of charring of the medium components by sterilization. This caused a large increase in the color concentration during the recovery steps in the downstream processing of enzymes (*e.g.*, ultrafiltration). In addition, it created problems during the purification steps in column chromatography, concentrating in the top layer of the gel and decreasing the purification efficiency and reusability of the resins [32]. Although dialysis and diafiltration are used to reduce the color intensity, these steps are not always practical from an industrial viewpoint because these steps are time-consuming, practically expensive, and usually insufficient. Diaion HPA75, a strong anion exchanger with a functional group of dimethylethanolammonium and a mean pore size of 0.3~1.2 mm, is often used in a broad range of applications, including amino acid purification and debittering in food processing and the removal of heavy metals in waste water processing. We recently reported that HPA75 is very efficient for reducing the color intensity of bacterial culture with good recovery of the alkaline protease [29]. This step resulted in a 1.4-fold purification with a protease yield of 107% (Table 5). BHAP was purified further by HIC using Phenyl-Sepharose and ion-exchange chromatographies on a DEAE- and CM-Sepharose column. HIC on Phenyl-Sepharose was based on the hydrophobicity of the enzyme. In this step, the protease was not detected in the flow-through, and the protease was almost completely eluted by buffer A. This step resulted in a 2.6-fold purification with a specific activity of 19,360 U/mg protein. The active fractions were pooled and applied directly on a DEAE-Sepharose column. BHAP did not bind to DEAE-Sepharose, suggesting it is a basic protein. After final purification using CM-Sepharose column chromatography, the enzyme was purified to approximately 30-fold purification with a specific activity of 223,090 U/mg and an overall recovery of 34% (Table 5). The purified protease migrated as a single band in SDS–PAGE under reducing conditions, indicating that the purified protein was homogeneous. The apparent molecular mass was determined to be approximately 28 kDa, as judged by SDS–PAGE, although the size appears larger than 28 kDa by mobility difference after purification (Fig. 1).

**Effects of Metal Ions and Urea on the Enzyme Activity**

The enzyme activity increased slightly in the presence of divalent cations, such as $Ca^{2+}$ and $Mn^{2+}$, which improved by 39%, 19%, 12%, 25%, and 36% by treating with 1 mM $Ca^{2+}$, $Co^{2+}$, $Cr^{3+}$, $Cu^{2+}$, and $Mn^{2+}$, respectively. However, the enzyme was inhibited 31% in the presence of 1 mM $Hg^{2+}$ (Table 6). The purified BHAP was not affected by 1 mM EDTA, suggesting that metal ions are not essential for the enzyme activity. The enzyme activity was enhanced in the presence of high concentrations of a chemical denaturant,

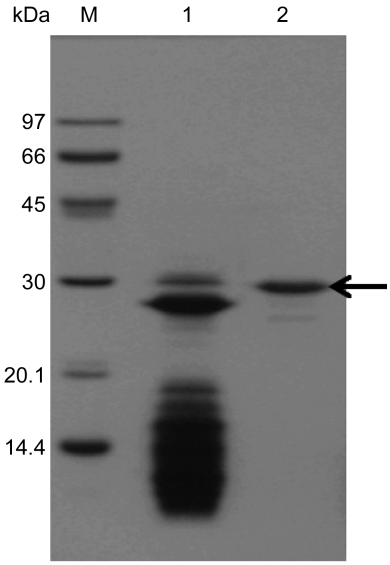


**Fig. 1.** SDS–PAGE of an alkaline protease purified from *B. horikoshii*.
Lane M, molecular mass marker; lane 1, culture supernatant; lane 2, purified protease. The arrow indicates the purified protease.

**Table 6.** Effects of metal ions and chaotropic agents on protease activity.

| Metal ions (1 mM), chaotropic agents | | Residual activity (%) |
|---|---|---|
| Control | | 100.0 |
| $Ca^{2+}$ | | 138.7 |
| $Cd^{2+}$ | | 92.6 |
| $Co^{2+}$ | | 118.8 |
| $Cr^{3+}$ | | 112.2 |
| $Cu^{2+}$ | | 125.0 |
| $Hg^{2+}$ | | 68.5 |
| $Mg^{2+}$ | | 104.2 |
| $Mn^{2+}$ | | 136.4 |
| EDTA (1 mM) | | 106.4 |
| Urea | 2 M | 110.3 |
| | 6 M | 114.5 |
| Guanidine-HCl | 2 M | 98.8 |
| | 4 M | 76.9 |

An alkaline protease from *B. horikoshii* was pre-incubated with the agents at the indicated concentrations for 24 h at room temperature, and the remaining activity was measured under optimal assay conditions.

urea, which increased activity by 10% and 15% at 2 and 6 M urea, respectively. However, its activity was slightly reduced by 23% in the presence of 6 M guanidine-HCl (Table 6).

**Effect of NaCl on the Protease Activity**

When measured, the protease activity at concentrations between 0 and 20% (w/v) NaCl increased slightly at concentrations of NaCl ranging from 0.1% to 5%, and the maximal activity was detected near 0.5–1% NaCl. The protease activity remained at high salinities (above 5%) and even retained its activity of 105%, 92%, and 74% in the presence of 5%, 10%, and 20% NaCl, respectively (Fig. 2). This result suggested that BHAP is a halotolerant alkaline protease.

**Effects of Organic Solvents on the Enzyme Activity**

Various organic solvents, such as acetone, acetonitrile, and dimethylformamide at a concentration of 50% (v/v), were

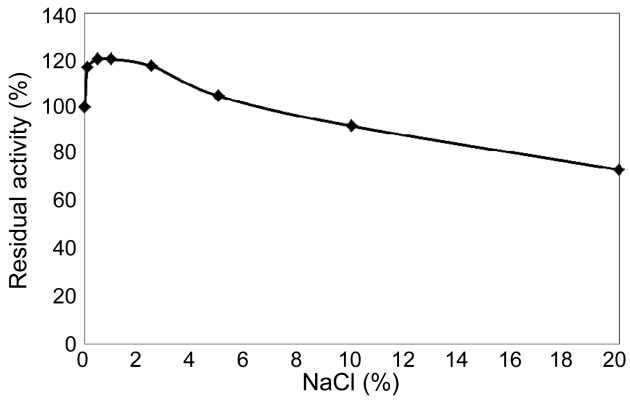


**Fig. 2.** Effect of NaCl on the protease activity.

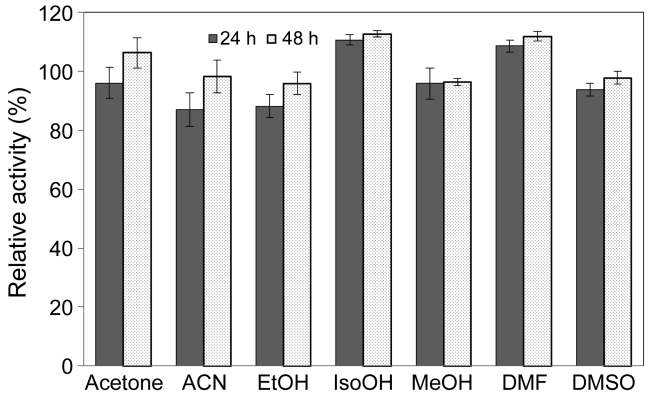


**Fig. 3.** Effects of organic solvents on the protease produced from *B. horikoshii*.

One ml aliquot of enzyme solution was incubated with an equal volume of organic solvent at room temperature for the indicated times, and their remaining protease activity was measured under standard assay conditions. The protease activity of the non-solvent-containing control was taken as 100%.

tested for their effects on enzyme stability. Fig. 3 shows the remaining activity of the protease BHAP in the presence of organic solvents. The protease was found to be stable in the solvents and retained more than 90% of activity even after treatment for 48 h, indicating that this enzyme may be useful for peptide synthesis in organic environments.

**Effects of Surfactants and Oxidizing Agents on the Enzyme Activity**

The enzyme activity of the purified BHAP was slightly increased by the non-ionic surfactants, where activity was enhanced by 9%, 21%, 18%, and 17% after treatment with 1% Brij-35, Triton X-100, Tween 20, and Tween 80 for 24 h, respectively. Moreover, the activity was enhanced by 35%, 59%, 45%, and 40% by treating with 5% Brij-35, Triton X-100, Tween 20, and Tween 80, respectively (Table 7). Furthermore, the purified BHAP showed extreme stability towards an anionic surfactant, SDS, and retained more than 95% activity upon exposure for 24 h at 2.5%

**Table 7.** Effects of non-ionic surfactants on the protease activity.

| Non-ionic surfactants (%) | | Residual activity (%) |
|---|---|---|
| Control | - | 100.0 |
| Brij-35 | 1.0 | 109.3 |
| | 5.0 | 134.8 |
| Triton X-100 | 1.0 | 121.4 |
| | 5.0 | 159.2 |
| Tween 20 | 1.0 | 117.5 |
| | 5.0 | 145.2 |
| Tween 80 | 1.0 | 116.7 |
| | 5.0 | 139.7 |

An alkaline protease from *B. horikoshii* was pre-incubated with the various agents at the indicated concentrations for 24 h at room temperature, and the remaining activity was measured under optimal assay conditions.

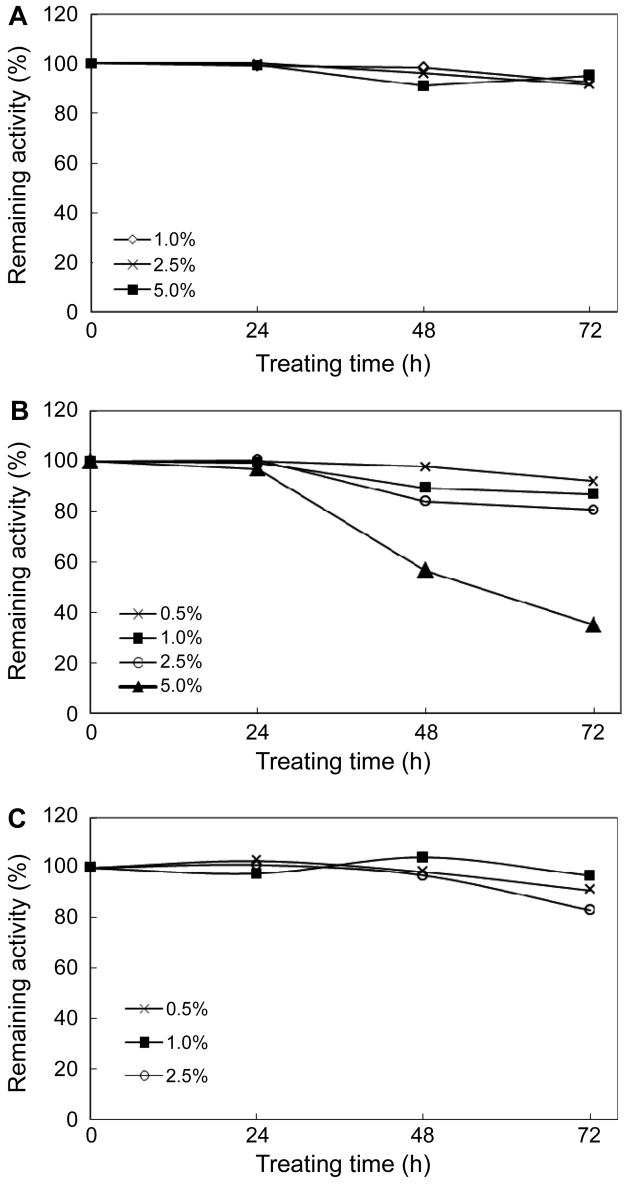


**Fig. 4.** Stability of an alkaline protease produced from *B. horikoshii* in the presence of (**A**) SDS, (**B**) hydrogen peroxide, and (**C**) sodium perborate.
Enzymes were pre-incubated with the reagents at the indicated concentrations and allowed to stand at room temperature, and their remaining protease activity was measured under standard assay conditions.

and 5% SDS. In addition, it retained more than 90% activity even after treatment with 5% SDS for 72 h (Fig. 4A). Additionally, the enzyme was barely influenced by oxidants like hydrogen peroxide, where it retained 100%, 84%, and 81% of activity upon exposure to 2.5% hydrogen peroxide for 24, 48, and 72 h, respectively. However, it decreased 3%, 42%, and 65% after treatment with 5% hydrogen peroxide for 24, 48, and 72 h, respectively (Fig. 4B). The enzyme activity also exhibited extreme stability against sodium perborate, with 101%, 97%, and 83% activity after treatment with 2.5% sodium perborate for 24, 48, and 72 h, respectively (Fig. 4C). Although few reports have been published on SDS- and $H_2O_2$-stable alkaline proteases, many of the available alkaline proteases exhibited low activity and stability toward anionic surfactants like SDS, and oxidants like hydrogen peroxide. On the contrary, we showed that BHAP produced from *B. horikoshii* was extremely stable against heavy metals, EDTA, non-ionic and anionic detergents, and oxidizing agents. Considering these results together, the *B. horikoshii* might be a potential source of alkaline protease for use as an additive in industrial applications, especially in detergent formulation.

**Table 8.** Effects of various inhibitors on the protease activity of an alkaline protease from *B. horikoshii.*

| Inhibitor | Concentration | Remaining activity (%) |
|---|---|---|
| Control | - | 100.0 |
| Aprotinin | 0.5 TIU/ml | 92.3 |
| Benzamidine | 1 mM | 109.5 |
| Chymostatin | 50 μg/ml | 94.8 |
| LBTI | 50 μg/ml | 98.4 |
| SBTI | 50 μg/ml | 94.0 |
| Leupeptin | 50 μg/ml | 90.9 |
| PMSF | 0.1 mM | 34.1 |
| | 1 mM | 13.2 |
| TLCK | 1 mM | 93.8 |
| TPCK | 1 mM | 94.5 |
| Bestatin | 50 μg/ml | 96.4 |
| Cystatin | 5 μg/ml | 105.6 |
| EDTA | 1 mM | 108.7 |

## Effects of Inhibitors

Inhibition studies primarily give an insight into the nature of the enzyme, its cofactor requirements, and the nature of the active center [46]. To know the nature of BHAP, enzyme activity in the presence of various protease inhibitors was analyzed. The results revealed that EDTA (metalloprotease inhibitor), bestatin (aminopeptidase inhibitor), and cystatin (cystein protease inhibitor) showed no effect on the protease (Table 8). Even though little or no inhibitory effect on the protease activity was observed with some serine protease inhibitors including aprotinin, benzamidine, chymostatin, and leupeptin, the enzymatic activity of BHAP was very sensitive to PMSF, one of the typical serine protease inhibitors, which inhibited 87% of its activity at a concentration of 1 mM, suggesting that BHAP belongs to the serine proteases group.

## Substrate Specificity Studies

Among the synthetic chromogenic substrates tested, BHAP efficiently hydrolyzed only *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA (AAPF), which is a specific substrate for chymotrypsin-like proteases. The $K_m$ and $k_{cat}$ values for AAPF at 37°C and pH 9 were estimated to be 78.7 μM and 217.4 $s^{-1}$,

**Table 9.** Substrate specificity of an alkaline protease purified from *B. horikoshii*.

| Chromogenic substrate | Relative activity (%) |
|---|---|
| *N*-Suc-Ala-Ala-Pro-Phe-*p*NA | 100.0 |
| *N*-Suc-Ala-Ala-Ala-*p*NA | 0.2 |
| *N*-Suc-Ala-Ala-Val-*p*NA | 0.2 |
| *N*-CBZ-Gly-Gly-Leu-*p*NA | 1.3 |
| Pyroglu-Gly-Arg-*p*NA (S-2444) | 1.5 |
| Benz-Arg-Gly-Arg-*p*NA (S-2765) | 1.3 |
| Val-Leu-Lys-*p*NA (S-2251) | 0.9 |
| Pyroglu-Phe-Lys-*p*NA (S-2403) | 1.1 |

The proteolytic activity toward *N*-Suc-Ala-Ala-Pro-Phe-*p*NA was taken as 100%.

respectively. However, it could not efficiently hydrolyze the substrates with basic amino acids at the cleavage site, such as pyroGlu-Gly-Arg-*p*NA and Val-Leu-Lys-*p*NA (Table 9).

### N-Terminal Amino Acid Sequence Analysis

The N-terminal sequence of the first 15 amino acid residues of BHAP purified from *B. horikoshii* was AQSVPYGVSQIKAPA, and it showed high homology with subtilisins and alkaline proteases from other *Bacillus* strains (Table 10). Maximal similarity of BHAP was shown with subtilisin BPN' from *B. amyloliquefaciens*; in particular, this *Bacillus* protease showed complete homology within the N-terminal region of the first 15 amino acid residues of subtilisin BPN' [52].

## Discussion

This report has described the enhanced production, purification, and some enzymatic properties of an extracellular alkaline protease from an alkalophilic *B. horikoshii* isolated from the hemolymph of a unique Korean marine polychaeta, *P. leucophryna*. It is well known that the alkaline proteases must show high activity and stability in the presence of various detergent components to use in the detergent industry; therefore, obtaining proteases with high activity and stability at highly alkaline pH and temperature may increase the use of the enzymes in the detergent industry [11, 16, 17, 30, 31, 39, 43].

We previously described the partial optimization for the production of an alkaline protease with optimal pH of 9 and temperature of approximately 50°C in *B. horikoshii*, in which SBM and casein, among the organic nitrogen sources tested, were effective nitrogen substrates for protease production [21]. In this paper, we reported the further optimization of the protease production, purification, and some biochemical properties of the purified BHAP. Following the further optimization experiments, the protease yield of approx. 56,000 U/ml was achieved, and this was an increase of 5.5-fold compared with that before optimization (10,050 U/ml). The enzymatic properties of purified BHAP were assessed by the following criteria: the apparent molecular mass of 28 kDa (Fig. 1); high stability against heavy metals except $Hg^{2+}$; no loss of activity by EDTA (Table 6); highly active in the presence of high concentration

**Table 10.** Comparison of the N-terminal amino acid sequence of an alkaline protease purified from *B. horikoshii* with other proteases from *Bacillus* species.

| Protease and microbial source | N-Terminal amino acid sequence | References |
|---|---|---|
| AP (*B. horikoshii*) | A Q S V P Y G V S Q I K A P A | This work |
| Subtilisin BPN' (*B. amyloliquefaciens*) | A Q S V P Y G V S Q I K A P A | [3] |
| Subtilisin NAT (*B. subtilis* var. natto) | A Q S V P Y G I S Q I K A P A | [3] |
| Subtilisin Calsberg (*B. licheniformis*) | A Q T V P Y G I P L I K A D K | [3] |
| Subtilisin E (*B. subtilis*) | A Q S V P Y G I S Q I K A P A | [3] |
| Subtilisin J (*B. stearothermophilus*) | A Q S V P Y G I S Q I K A P A | [3] |
| Subtilisin Sendai (*Bacillus* sp. G-825-6) | N Q V T P W G I T R V Q A P T | [3] |
| Subtilisin BSF1 (*B. subtilis* A26) | A Q S V P Y G I S Q I | [58] |
| Subtilisin NAT (*B. subtilis* natto) | A Q S V P Y G I S Q I K A P A L H S Q G Y T | [59] |
| Subtilisin FS33 (*B. subtilis* DC33) | A Q S V P Y G I P Q I K A P A | [60] |
| Subtilisin LD-8547 (*B. subtilis* LD-8547) | A Q S V P Y G I P Q I K A P A | [61] |
| Subtilisin CK (*Bacillus* sp. CK 11-4) | A Q T V P Y G I P L I K A D D | [62] |
| Esperase (*B. lentus*) | Q W V P W G I S F I N T Q Q A | [3] |
| Savinase (*B. lentus*) | A Q S V P W G I S R V Q A P A | [3] |
| AP (*B. mojavensis*) | A Q T V P H G I P L I K A D K | [3] |
| AP (*B. licheniformis* NH1) | A Q T V P Y G I P L I K A D K | [56] |
| AP (*B. pumilus* CBS) | A Q T V P Y G I P Q I K A P A | [55] |
| AP No. 221 (*Bacillus* sp.) | A Q S V P W G I S R V Q A P A | [3] |
| AP AH-101 (*Bacillus* sp.) | Q T V P W G I S F I S T Q Q A | [3] |
| DHAP (*Bacillus pumilus*) | A Q T V P Y G I P Q I K A P A V H A Q G Y | [57] |
| Elastase Ya-B (*Bacillus* sp.) | Q T V P W G I N R V Q A P I A | [3] |

of NaCl (Fig. 2); high stability towards non-ionic surfactants; high stability towards SDS and oxidants (Fig. 4); strong inhibition by PMSF, confirming it as a serine protease (Table 8); high sequence homology with the subtilisin family in the N-terminal region (Table 10).

It was reported that high concentrations of salt (*e.g.*, NaCl) was required for growth and protease synthesis in some halophilic microorganisms including *Bacillus* species and archaea [25, 51]. For example, supplementation with 5–10% NaCl to the culture medium was required to produce the maximal protease yield in haloalkaliphilic *Bacillus* sp., halotolerant *Bacillus aquimaris* VITP4, and moderately halophilic *Salinivibrio* sp. AF-2004 [1, 37, 45]. Much higher salt requirement (20~25%) for protease secretion was reported in the archaeon *Halobacterium mediterranei* and the obligatory and alkaliphilic *Bacillus* sp. P-2 [26, 47]. The purified BHAP was not affected by 1 mM EDTA, suggesting that metal ions are not essential for the enzyme activity. This property of BHAP was very useful for applications as detergent additives because chelating agents are used as detergent components by acting as water softeners and by involvement in the removal of stains from fabrics [3]. The enzyme activity of BHAP was partially stimulated in the presence of the non-ionic surfactants. It was reported that alkaline proteases from many *Bacillus* species, such as *B. mojavensis*, *B. clausii* I-52, *B. circulans*, and *B. licheniformis*, showed stimulation in their activities after treatment with non-ionic surfactants, such as Tween 20 and Triton X-100 [3, 6, 12, 14, 22, 24, 40]. The prominent property of BHAP was high stability against SDS and oxidants. Some alkaline proteases exhibiting stability against SDS were reported. For example, alkaline protease from *Bacillus* sp. KSM-K16 retained approximately 75% activity upon treatment with 5% SDS [28]. Besides *Bacillus* species, microorganisms producing detergent-stable proteases were reported. Alkaline protease from *Aspergillus parasiticus* retained 97% with addition of 2% SDS [27]. It has been reported that some alkaline proteases exhibit stability against hydrogen peroxide. We previously described the $H_2O_2$-stable alkaline proteases from some *Bacillus* species. Alkaline protease from *Bacillus* sp. I-312 retained 88% after treatment with 5% $H_2O_2$ for 72 h [24]. Alkaline protease from *B. clausii* I-52 increased slightly by approx. 11% and 17% after treatment with 1% and 5% $H_2O_2$, respectively [22]. A subtilisin-like protease from *Bacillus* sp. KSM-KP43 lost little or no enzyme activity after treatment with 10% $H_2O_2$ for 30 min [42]. In some alkaline proteases, the enzyme activity was increased in the presence of $H_2O_2$, which increased roughly 54% after treatment with 1% $H_2O_2$ for *B. mojavensis* [3] and 28% with 5% $H_2O_2$ for *B. pumilus* CBS [19]. Some alkaline proteases showing high stability against both anionic detergent and oxidants were also reported. For example, the enzyme activity of the alkaline protease was 100% and 73% retained upon treatment with 5% $H_2O_2$ and 5% SDS, respectively, from *Bacillus* sp. APP1 [6]. Alkaline protease from *B. clausii* I-52 also retained its activity of 73% and 116% upon treatment with 5% SDS and 5% $H_2O_2$ for 48 h, respectively [22]. The enzyme from *B. horikoshii* in this study also maintained its activity of 95% and 60% upon treatment with 5% SDS and 5% $H_2O_2$ for 48 h, respectively.

In conclusion, we found that *B. horikoshii* was halotolerant and alkalophilic, and BHAP produced from this isolate also was halotolerant and an alkaline protease exhibiting high stability against urea, heavy metals, and organic solvents. In particular, BHAP exhibited significant compatibility and stability toward both anionic surfactants and oxidizing agents (Fig. 4). Considering these results together, the *B. horikoshii* might be a potential source of alkaline protease for use as an additive in industrial applications, especially in detergent formulation, because proteases must be resistant to the effects of EDTA, oxidizing agents, heavy metal ions, and anionic detergents for use in the detergent industry [43].

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## Abbreviations

AAPF, *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA; BHAP, *Bacillus horikoshii* alkaline protease; EDTA, ethylenediaminetetraacetic acid; HIC, hydrophobic interaction chromatography; PMSF, phenylmethylsulfonyl fluoride; SBM, soybean meal; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TLCK, *N*α-tosyl-L-lysine chloromethyl ketone; TPCK, *N*α-tosyl-L-phenylalanine chloromethyl ketone; TSB, tryptic soy broth.

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