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Production of an extracellular protease by an Antarctic bacterial isolate (*Bacillus sp.* JSP1) as a potential feed additive[□]

*Proteasa extracelular de un aislado bacteriano Antártico (*Bacillus sp.* JSP1) con uso potencial como aditivo alimenticio para animales*

*Produção de proteasa extracelular por bactérias antárticas isoladas, *Bacillus sp.* JSP1 como um aditivo potencial em concentrado*

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Summary

Extracellular proteolytic activity was found in JSP1, an Antarctic bacterial isolate. The strain was related to *Bacillus sp.* based on 16S rRNA gene sequence analysis. The JSP1 protease was partially purified by ammonium sulfate precipitation. Optimal enzyme activity occurred at 40 °C and pH 7.4. Enzyme activity was significantly enhanced in the presence of Mg²⁺ and Ca²⁺ and was completely inactivated in presence of Cu²⁺, Zn²⁺, Hg²⁺, EDTA and SDS. The enzyme hydrolyzed casein the most effectively among the protein substrates tested. The enzyme also exhibited relatively high activity on keratin and gluten, and was active against peptidyl conjugates such as L-Leu-p-Nitroanilide and N-Succinyl-L-Phe-p-Nitroanilide. This study suggests that JSP1 protease could be utilized as a potential environmentally-friendly feed additive in animal production.

Key words: antarctic, *Bacillus sp.*, feed additive, keratin, livestock production, protease.

Resumen

Se reporta haber encontrado actividad proteolítica extracelular en una bacteria antártica denominada JSP1. Con base en el análisis de secuencia genética 16S ARNr, la cepa fué relacionada con *Bacillus*

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sp. La proteasa JSP1 fué parcialmente purificada por precipitación con sulfato de amonio. La actividad óptima de la enzima se produjo a 40 °C y pH 7,4. La actividad enzimática fue significativamente mayor en presencia de Mg²⁺ y Ca²⁺ y se inactivó completamente en presencia de Cu²⁺, Zn²⁺, Hg²⁺, EDTA y SDS. Entre todos los sustratos ensayados, el más eficientemente hidrolizado por la enzima fue la caseína. La enzima tuvo actividad relativamente alta sobre la queratina y el gluten, y participó activamente contra conjugados peptídicos como la L-Leu-p-nitroanilida y la N-succinil-L-fenilalanina-p-nitroanilida. La enzima podría ofrecer potencial para su uso como aditivo alimenticio ecológico en producción animal.

Palabras clave: antártida, aditivo alimenticio, *Bacillus sp.*, producción animal, proteasa queratina.

Resumo

A actividade proteolítica extracelular foi encontrada a partir de uma bactéria antártica denominada JSP1, mediante uma análises de sequencia do gene 16S rRNA, a cepa foi relacionada para Bacillus sp. A proteasa JSP1 foi parcialmente purificada por precipitação com sulfato de amônia. Uma óptima actividade enzimática ocorreu em 40 oC e pH 7,4. A actividade da enzima foi significativamente maior na presença de Mg²⁺ e Ca²⁺, e foi completamente inactivada em presença de Cu²⁺ + Zn²⁺, Hg²⁺, EDTA e SDS. A enzima hidrolisada de caseína foi mais eficaz entre os substratos de proteína testados, apresentando maior actividade na queratina e no glúten e foi activo contra os peptidil conjugados como L-Leu-p Nitroanilida e N-succinil-L-Phe-p-Nitroanilida. A enzima pode ser utilizado como aditivo ambiental na alimentação animal.

Palavras chave: antarctic, *Bacillus sp.*, feed additive, keratin, livestock production, protease.

Introduction

Proteases have attracted a great deal of attention due to their broad range of applications in the detergent, food, pharmaceutical, chemical, leather, paper, and pulp, and silk industries (Rai and Mukherjee, 2009). Microbial proteases comprise approximately 40% of the worldwide production of enzymes (Jaouadi *et al.*, 2008). In the field of animal nutrition, exogenous proteases have been used as feed additives. For instance, proteases help degrade soybean proteins such as glycinin and β -conglycinin, along with some protein anti-nutritional factors (lectins and trypsin-inhibitors) in inadequately processed soybean meal (Thorpe and Beal, 2001). Moreover, the combined use of proteases together with carbohydrate-degrading enzymes such as xylanase and amylase was found to enhance the nutritional value of a corn and soybean meal-based diet for poultry (Hong *et al.*, 2002; Marsman *et al.*, 1997).

In particular, *Bacillus* species, a type of exogenous spore-forming bacteria, are prolific producers of extracellular proteases (Rao *et al.*, 1998). Currently, *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus* are well-known

species employed industrially for alkaline protease production (Gupta *et al.*, 2002). Although they do not commensally colonize the gastrointestinal tract, *Bacillus* species have been shown to be effective in maintaining a favorable balance of microflora in the gastrointestinal tract and in improving the production performance of farm animals (Alexopoulos *et al.*, 2004; Kritas and Morrison, 2005).

Some useful and unusual enzymes have been reported from so-called extremophiles inhabiting Antarctica (Demirjian *et al.*, 2001). Considering that the number of microbes cultured to date remains only a tiny fraction of all microbial species on earth, the number of novel enzymes is expected to increase continuously (Park *et al.*, 2007). In this report, data are presented concerning general properties of extracellular proteolytic activity derived from an Antarctic bacterial isolate, *Bacillus sp.* JSP1.

Materials and methods

Bacterial strain and culture conditions

A bacterial isolate, JSP1, derived from Antarctic soil samples was supplied from Korea Polar Research Institute, operating the King Sejong

Station (South Korea) in Antarctica. Screening for protease activity was performed on selective agar plates [1.0% skim milk (Sigma), 0.45% $(\text{NH}_4)_2\text{SO}_4$, 0.05% yeast extract (Difco), 0.07% KH_2PO_4 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% NaCl, 0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.001% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.5% bacto agar (Difco), pH 7.4] at 28 °C by observing a clear zone of hydrolyzed casein around the colonies, as previously described (Hutadilok-Towatana *et al.*, 1999).

Growth and protease production were investigated in 100 mL of protease production medium [0.5% skim milk, 0.5% yeast extract, 0.07% KH_2PO_4 , 0.01% NaCl, 0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.4)] in a 500 mL Erlenmeyer flask, aerobically incubated with vigorous shaking (220 rpm), by monitoring the absorbance (O.D._{600nm}) for the cell growth and protease activity of the culture supernatant at 28 °C at various time points.

Taxonomic identification of strain JSP1

Genomic DNA was extracted from strain JSP1 using a FastDNA kit (Qbiogene) according to the manufacturer's protocol. The 16S rRNA gene was amplified from genomic DNA by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (William *et al.*, 1991). The amplified 1,427 bp sequences were determined by an automated ABI PRISM 3730 XL DNA analyzer (Applied Biosystems). The resulting sequences were compared with the GenBank database (NCBI) using BLAST (Altschul *et al.*, 1990). Sequences showing a relevant degree of similarity were imported into the CLUSTAL W program (Thompson *et al.*, 1994) and aligned. The evolutionary distances to other *Bacillus* strains were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004), and the phylogenetic relationships were determined using the software MEGA, version 4.0 (Tamura *et al.*, 2007).

Nucleotide sequence accession numbers

The nucleotide sequence of the 16S rRNA gene has been deposited in the GenBank database under Accession No. GU014529.

Partial purification of the enzyme

One liter of protease production medium [0.5% skim milk, 0.5% yeast extract, 0.07% KH_2PO_4 , 0.01% NaCl, 0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.4)] in two 2 L Erlenmeyer flasks was aseptically inoculated with a single colony of strain JSP1 and aerobically cultivated with vigorous shaking (220 rpm) for 96 h at 28 °C. The culture medium containing secreted protease was centrifuged at 9000 g for 30 min at 4 °C to remove the cells, and proteins in the supernatant were then precipitated with ammonium sulfate (75% saturation). The pellet was dissolved in 50 mM Tris-HCl (pH 8.0) and dialyzed overnight against 50 mM Tris-HCl (pH 7.4) at 4 °C. The dialyzed solution was used as the protease source throughout this work to examine its catalytic properties.

Zymography

Native Polyacrylamide Gel Electrophoresis (PAGE) was carried out with a Modular Mini-Protein II Electrophoresis System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Zymograms (0.1% casein in 10% polyacrylamide) were run for 6 h at 4 °C and 60 V in buffer containing 25 mM Tris-HCl (pH 8.0) and 125 mM glycine. After electrophoresis, the gel was incubated overnight at room temperature in calcium proteolysis buffer (20 mM Tris-HCl, 20 mM CaCl_2 ; pH 7.4) under gentle shaking. The gel was stained with SimplyBlueSafeStain (Invitrogen) for 30 min and destained overnight. The bands of caseinolytic activity appear white on a blue-stained background.

Preparation of keratin substrate

Keratin substrate was prepared from chicken feathers by the modification method of Nam *et al.* (2002). Briefly, ground chicken feathers (1 g) in 50 mL of dimethyl sulfoxide, were solubilized by heat treatment on a hot plate at 90 °C for 2 h. Soluble keratin was then precipitated by addition of cold acetone (200 mL) at -70 °C for 2 h followed by centrifugation at 10000 g for 20 min. The precipitate was washed twice with distilled water and then dissolved in 10 mM Tris-HCl buffer (pH 9.0).

Enzyme assay and general catalytic properties

Unless otherwise stated, the assay was performed at 40 °C for 1 h in a reaction mixture containing 460 µL of 50 mM Tris-HCl (pH 7.4), 140 µL of 3% azocasein as a nonspecific substrate, and 100 µL of enzyme. The reaction was terminated by addition of 700 µL of 10% trichloroacetic acid. One unit of the azocaseinolytic activity was defined as the amount of enzyme required to produce an increase in absorbance at 366 nm of 0.01 per minute under the given assay conditions. Protease substrate specificity was examined with casein (Sigma, St. Louis, MO, USA), gelatin (Sigma, St. Louis, MO, USA), collagen (Sigma, St. Louis, MO, USA), bovine serum albumin (Sigma, St. Louis, MO, USA), gluten (Sigma, St. Louis, MO, USA), and chicken feather keratin by a modified method of Wang *et al.* (2005). Briefly, 200 µL of enzyme was added to a reaction mixture containing 3% of each substrate in 360 µL of 50 mM Tris-HCl (pH 7.4) and incubated at 40 °C. The reaction was stopped by adding 700 µL of 10% trichloroacetic acid and centrifuged at 10000 g for 10 min.

The protein remaining in the supernatant was determined by Folin-phenol reagent (Folin and Ciocalteu, 1927). One unit of protease activity was defined as the amount of enzyme that liberated 1 µg of tyrosine per minute under the defined assay conditions. Peptidolytic activity was also assayed at 40 °C using 2 mM each of L-Leu-*p*-Nitroanilide, N-Succinyl-L-Ala-Ala-Ala-*p*-Nitroanilide, and N-Succinyl-L-Phe-*p*-Nitroanilide as substrates in 50 mM Tris-HCl (pH 7.4). The amount of *p*-Nitroaniline liberated was determined from the samples' absorbance at 405 nm.

Determination of pH and temperature optima on protease activity

To study the temperature optimum and enzyme activity, the enzyme reaction mixture was incubated at different temperatures from 0 to 80 °C in 50 mM Tris-HCl (pH 7.4) buffer using azocasein as a substrate. The pH optimum for protease activity

with azocasein substrate was determined at 40 °C in 50 mM glycine-HCl (pH 3), 50 mM sodium acetate (pH 4-5), 50 mM Bis-Tris-HCl (pH 6-7), and 50 mM Tris-HCl (pH 7.4-9.0) buffers.

Effect of reagents on enzyme activity

The effects of metal ions and inhibitors on protease activity were examined with azocasein as a substrate. Each additive (5 mM) was pre-incubated with the enzyme for 30 min at 40 °C before the standard assay was performed, and the residual activity was measured.

Results

Identification of isolated strain JSP1, protease production and partial purification of the enzyme

To identify the isolated strain JSP1 that shows protease activity (Figure 1), we cloned its 16S rRNA gene and compared the sequence with those available in the database. A phylogenetic tree based on the 16S rRNA gene sequences from 10 bacterial *Bacillus* strains showed that the JSP1 strain shared 99.7% sequence identity with the type strain, *Bacillus megaterium* IAM 13418 (Figure 2). Therefore, it was named *Bacillus* sp. JSP1.

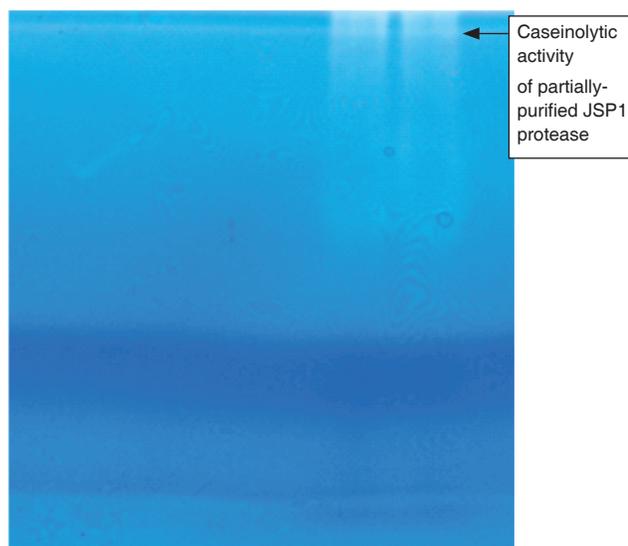


Figure 1. Caseinolytic zymogram analysis of the JSP1 protease.

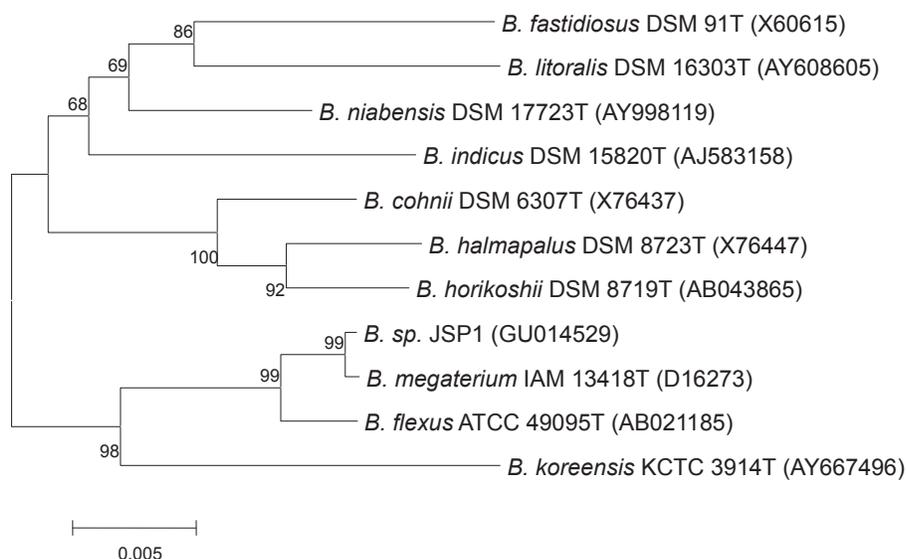


Figure 2. Phylogenetic relationships of the 16S rRNA sequences of *Bacillus* sp. JSP1 with other *Bacillus* type strains. Bootstrap values (based on 1,000 trials and only values >50%) are shown at the nodes. The GenBank accession numbers are indicated in parentheses. Bar, 0.005 substitutions per nucleotide position. B. represents the abbreviation of *Bacillus*.

Time courses of cell growth and extracellular protease activity were shown in figure 3. Protease activity was nearly proportional to cell growth during cultivation. The enzyme was steeply produced after 16 h of incubation, showing a maximum activity (21.5 0.1 U/mL) at 60 h of incubation.

The partial purification profile of the extracellular protease produced by *Bacillus* sp. JSP1 was summarized in table 1.

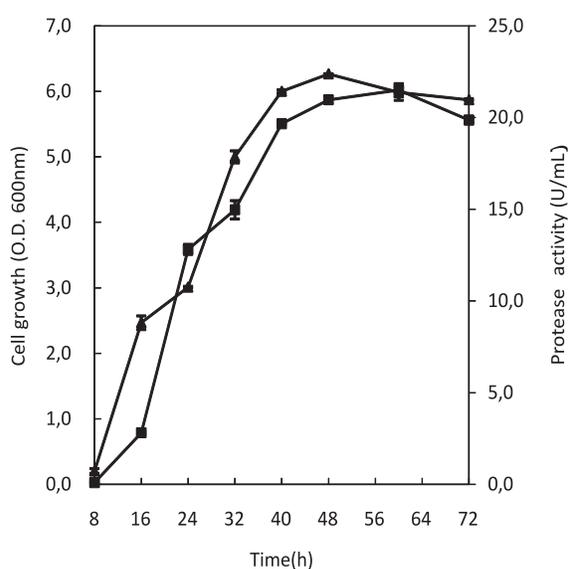


Figure 3. Growth and extracellular protease production by *Bacillus* sp. JSP1. Symbols represent protease activity (filled squares) and cell growth (filled triangles). Data represent mean standard errors from three experiments.

Table 1. Summary of partial purification profile for extracellular protease produced by *Bacillus* sp. JSP1.

Purification step	Total activity (U) ^a	Total proteins (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture supernatant	19169 ± 565	81.5 ± 2.6	235.5 ± 7.7	1.0 ± 0.0	100 ± 0.0
Ammonium sulfate precipitation	15272 ± 952	56.3 ± 0.1	271.1 ± 16.4	1.2 ± 0.1	80 ± 6.0

^a One unit (U) of the azocaseinolytic activity was defined as the amount of enzyme required to produce an increase in absorbance at 366 nm of 0.01 per minute at 40 °C and pH 7.4. Data represent mean ± standard errors from three experiments.

Effect of pH and temperature on enzyme activity

Optimal protease activity occurred at pH 7.4, while over 60% of the peak activity was achieved between pH 6.0 and 8.0. However, activity was nearly completely inactivated at acidic pH (pH 3.0-5.0) and above pH 8.5 (Figure 4A). As shown in figure 4B, JSP1 protease showed optimal activity at 20 – 40 °C and retained more than 35% of the activity at 5 – 50 °C.

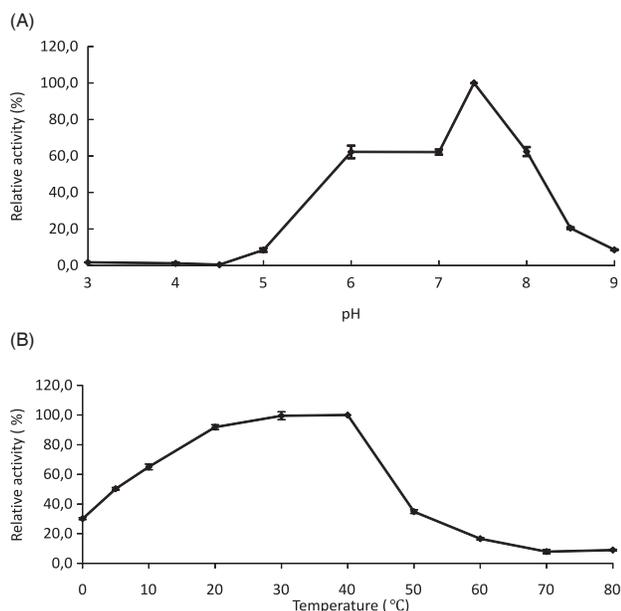


Figure 4. pH and temperature activity profiles.

(A) Effect of pH on JSP1 protease activity. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity equates to 11.9 U/mL). Data represent mean standard errors from three experiments.

(B) Effect of temperature on JSP1 protease activity. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity equates to 14.5 U/mL). Data represent mean standard errors from three experiments.

Substrate specificity

JSP1 protease hydrolyzed casein the most effectively among the protein substrates tested (Table 2). The enzyme also showed relatively high activity on keratin, which is the most abundant structural protein in skin, hair, wool, and feathers (Tatineni *et al.*, 2008) and gluten, which is a useful protein source in cattle feed (Firkins *et al.*, 1985; Ohajuruka and Palmquist, 1989). Low levels of hydrolysis were observed with gelatin, collagen, and bovine serum albumin (Table 2). Among the peptidyl-*p*-Nitroanilide substrates tested, the enzyme was active against L-Leu-*p*-Nitroanilide and N-Succinyl-L-Phe-*p*-Nitroanilide which is cleaved by subtilisin-like and chymotrypsin-like enzymes (Hutadilok-Towatana *et al.*, 1999), and exhibited no activity on N-Succinyl-L-Ala-Ala-Ala-*p*-Nitroanilide.

Table 2. Substrate specificity of the JSP1 protease.

Substrates	Enzyme activity (U ^{a,b} /mL)
Casein	7.70 ± 0.03 ^a
Gelatin	1.10 ± 0.03 ^a
Collagen	0.90 ± 0.00 ^a
Bovine serum albumin	0.50 ± 0.08 ^a
Chicken feather keratin	4.10 ± 0.13 ^a
Gluten	3.90 ± 0.21 ^a
L-Leu- <i>p</i> -Nitroanilide	0.017 ± 0.0002 ^b
N-Succinyl-L-Ala-Ala-Ala- <i>p</i> -Nitroanilide	0.000 ± 0.0000 ^b
N-Succinyl-L-Phe- <i>p</i> -Nitroanilide	0.011 ± 0.0003 ^b

^a One unit (U) of protease activity is defined as the amount of enzyme that liberates 1 µg of tyrosine per min at pH 7.4 and 40 °C. ^b One unit (U) of peptidolytic activity is defined as the amount of enzyme that liberates 1 µmol of *p*-Nitroaniline per min at pH 7.4 and 40 °C. Data represent mean standard errors from three experiments.

Effect of various reagents on enzyme activity

The protease activity in the presence of various metal ions or chemicals was shown in figure 5. Amongst the metal ions, Mg²⁺ and Ca²⁺ were highly effective at stimulating JSP1 protease, increasing activity by 40% and 55%, respectively. In contrast, enzyme activity was significantly reduced (50-78% of the control activity) in the presence of Fe²⁺, Co²⁺, and Ni²⁺. Although Cu²⁺, Zn²⁺, and Hg²⁺ almost completely inactivated the enzyme, no important effect on the activity was observed with Mn²⁺ and Ba²⁺. The enzyme was also completely inactivated by EDTA and Sodium Dodecyl Sulfate (SDS), but moderately inhibited by Phenyl Metyl Sulfonyl Fluoride (PMSF).

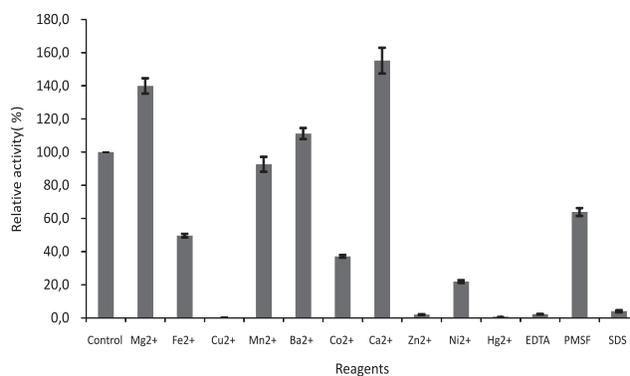


Figure 5. Effects of metal ions and chemicals on the azocasein-hydrolytic activity of the JSP1 protease. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity equates to 15.2 U/mL) and the value below 0.8% is unseen. Data represent mean standard errors from three experiments.

Discussion

In the present study, the extracellular protease secreted by an Antarctic strain, *Bacillus* sp. JSP1, was partially characterized, and most of its properties were found to be distinct from those of other proteases from *Bacillus* strains.

The JSP1 protease is nearly a neutral protease, with an optimal pH of 7.4, while most known *Bacillus* species produce commercial proteases that are highly active at pH 7.0 and 11.0, with an optimum around pH 8.0-10.0 (Davail et al., 1994; Hutadilok-Towatana et al., 1999; Jaouadi et al., 2008).

The optimum temperature of 40 °C for the protease is similar to the temperature (35 °C) for the serine protease of the psychrophilic bacterium, *Colwellia* sp. NJ341 (Wang et al., 2005). Moreover, the JSP1 protease maintains 30% of its highest activity at 0 °C, which is one of the typical characteristics found in cold-active enzymes (Wang et al., 2005; Zhang and Zeng, 2008).

The JSP1 protease showed the highest activity on casein, much like the alkaline serine proteases from *Bacillus pumilus* CBS (Jaouadi et al., 2008), *Bacillus stearothermophilus* F1 (Rahman et al., 1994) and *Bacillus* sp. KSM-K16 (Kobayashi et al., 1995). Unexpectedly, the enzyme could hydrolyze keratin which, like other insoluble proteins, is an unacceptable substrate for common proteases such as trypsin and pepsin (Letourneau et al., 1998; Papadopoulos et al., 1986). In the animal feed industry, feather waste can be a potential alternative to more costly dietary ingredients for animal feedstuffs (Shih, 1993). Worldwide, commercial poultry processing produces millions of tons of feathers per year, which are currently converted to feather meal through steam pressure and chemical treatment (Shih, 1993). Although chemical treatment renders keratin waste more digestible, it is high-priced and destroys certain amino acids (Papadopoulos et al., 1986).

The nutritional enhancement of feather meal by the enzymatic treatment might significantly improve amino acid availability of feather keratin (Odetallah et al., 2003). Until now, known keratinolytic enzymes have been mainly produced by mesophilic fungi (Santos et al., 1996), actinomycetes (Böckle et al., 1995), some thermophilic *Bacillus* sp. (Kim et al., 2001), and some thermophilic anaerobes (Nam et al., 2002). To our knowledge, little has been known about keratinolytic activity detected in Antarctic *Bacillus* strains.

Calcium ions are generally known to be involved in maintaining the activity of *Bacillus* serine proteases (Hutadilok-Towatana et al., 1999; Jaouadi et al., 2008). However, the JSP1 protease seems to belong to the metalloprotease rather than to the serine protease family, because the activity of this enzyme was strongly inhibited by a chelating agent, EDTA, but only partially inhibited by PMSF, a well-known inhibitor of serine proteases (Hutadilok-Towatana et al., 1999). This reasoning may be also supported by the observed Zn²⁺-dependent inhibition, because excess zinc inhibits some metalloproteases (Auld, 1995). It is interesting that the enzyme exhibited keratinolytic activity, despite the fact that metalloproteases are not frequently associated with keratinolytic activity (Tatineni et al., 2008). The enzyme was sensitive to anionic SDS addition, indicating that hydrogen bonds may play a pivotal role in maintaining enzyme activity (Wang et al., 2005).

The JSP1 protease may offer potential for use as an environmentally-friendly feed additive to improve the production performance of farm animals, due to its broad substrate specificity and relatively desirable activity levels at physiologically relevant pH and temperature. Additionally, the keratinolytic activity of the enzyme will help to conduct biotechnological processes of the keratinous biomaterials from poultry and leather industries. A more detailed characterization of the enzyme such as gene cloning, protein engineering, and fermentation technology is warranted to maximize the catalytic efficiency and productive yield of the enzyme.

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