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Purification and Characterization of a Novel Salt-tolerant Protease Produced by *Saccharomyces* sp. B101 Isolated from Baker's Dough Yeast

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Abstract The proteolytic enzyme from *Saccharomyces* sp. B101 was purified to homogeneity by ammonium sulfate fractionation, ultrafiltration, diethyl aminoethyl (DEAE)-Sephadex A-50 ion-exchange chromatography, and Sephadex G-100 gel filtration chromatography from the culture supernatant of *Saccharomyces* sp. B101. The specific activity and the purification fold of the purified enzyme were 4,688.9 unit/mg and 18, respectively. The molecular weight of the purified enzyme was estimated to be 33 kDa by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The optimum pH and temperature for the enzyme activity were pH 8.5 and 30°C, respectively. The enzyme activity was relatively stable in the pH range of 6.5-8.5 at below 35°C. The salt-tolerance and stability for the enzyme activity were relatively stable even at NaCl concentrations of 10 and 15%. The activity of enzyme was inhibited by Ag^{2+} and Fe^{2+}, and activated by Mn^{2+}. In addition, the enzyme activity was potently inhibited by ethylenediaminetetraacetic acid (EDTA) and phenylmethyl sulfonylfluoride (PMSF). Based on these findings we concluded that the purified enzyme was a serine protease. *Km* and *Vmax* values for hammastein milk casein were 1.02 mg/mL and 278.38 unit/mL, respectively.

Keywords: *Saccharomyces* sp. B101, salt-tolerant protease, purification, kinetics

Introduction

The use of microbes in industry is extending rapidly and will require a wider range of organisms than has been used so far. Readily accessible and full systematic information about microorganisms is critical for food bio-processes, and this information makes it possible for us to select suitable organisms for specific research and industrial uses from a greater variety of strains. In soy sauce production, salt-tolerant yeasts and enzymes are very important to flavor formation. Most yeast grow actively at higher salt conditions than those optimal for bacteria; hence it is usually easy to keep large-scale cultures of yeast free from fast-growing, contaminating microorganisms. In addition, a new process using immobilized salt-tolerant yeasts has been developed and shown to be very effective for accelerating flavor formation. Recently, many yeast strains have been developed to suit a specific need (1,2).

There are two strategies for adapting to the presence of high salt in different organisms. One is to exclude sodium from the cytoplasm and to accumulate high concentrations of compatible solutes to avoid water loss (excluder organisms) and the other is to use and accumulate high Na^+ concentrations without becoming intoxicated (includer organisms) (3,4). Halotolerant microorganisms, including *Halobacterium salinarium*, *Bacillius* sp., *Halobacterium halobium*, *Halomonas* sp., *Halobacillus* sp., *Virgibacillus* sp., *Oceanobacillus* sp., etc (5-7) have been a well-known source for obtaining salt-tolerant proteases, and the characteristics for the protease were investigated. In addition, even though *Bacillus subtilis* JM3 protease (8) isolated from anchovy sauce with 26% NaCl concentration was relatively salt-tolerant, this was still not sufficient to be used as a starter enzyme in fish sauce industry. In case of yeast, there were reports on producing of extracellular proteases from *Saccharomycopsis lipolytica* (9,10) and several strains isolated from traditional *meju* (11). However, with the exception of *Zygosaccharomyces rouxii* (12), very little is known about the production of salt-tolerant extracellular enzymes (13). *Saccharomyces cerevisiae* is also known to secret the extracellular protease with salt-tolerant activity (14).

With increasing demand of salt-tolerant proteases in processing of protein foods such as fish sauce, high protease activity is required to reduce processing expenditure and to improve product quality. There have been no published reports of a strong salt-tolerant protease from microorganisms surviving at high salt concentrations, i.e., greater than 25%. In this study we isolated a microorganism producing protease showing high activity in high salt conditions. Here we describe the purification and characteristics of the salt-tolerant protease from *Saccharomyces* sp. B101 as well as discuss the potential roles and uses of protease in food bio-processing.

Materials and Methods

Microorganism and cultivation *Saccharomyces* sp. B101 producing a salt-tolerant protease was isolated in the laboratory from bakers' dough yeast purchased from

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Choheung Chemical Co., Ltd. (Seoul, Korea) (15). *Saccharomyces* sp. B101 was cultivated in 500-mL Erlenmeyer flasks containing 200 mL of culture broth consisted of 1% dextrose, 0.5% peptone, 0.3% yeast extract, 0.3% malt, and 2% defatted soybean flour (pH 6.8) at 30°C for 2 days in a shaking incubator at 150 rpm.

Protease assay Enzyme activity was determined according to the modified method of Anson (16). One mL of enzyme solution was added to 5 mL of 0.6% casein solution with 1/15 M phosphate buffer (pH 7.0) at 37°C for 10 min. The reaction was stopped by adding 5 mL of 0.44 M trichloroacetic acid (TCA), and the solution was allowed to stand for 30 min. The resulting solution was then filtered with Whatman No. 2. Two mL of filtrate was mixed with 5 mL of 0.55 M Na_2CO_3 solution and 1 mL of 1 N Folin reagent, and then staying for 30 min at room temperature (25°C). The absorbance was measured at 660 nm with spectrophotometer (HP 8452A series; Hewlett-Packard Co., Palo Alto, CA, USA), which was converted to the amount of tyrosine equivalent by applying a standard curve. One unit (U) of protease activity was defined as the amount of enzyme releasing 1 mmol of tyrosine equivalent per 10 min.

Enzyme purification Fungal cells were harvested from the 500 mL culture broth by centrifuging at 3,500×g for 15 min after culturing at 30°C for 2 days on a rotary shaker of 150 rpm. The remaining culture supernatant was collected as a crude enzyme solution, and ammonium sulfate was added to precipitate any enzyme. The resultant precipitate of crude enzyme was dissolved in 20 mM NaOAc buffer (pH 6.5). The crude enzyme solution was dialyzed against the same buffer for 48 hr at 4°C, and then concentrated by ultrafiltration (cut-off membrane 10,000 Da). The concentrated enzyme solution was applied to dimethyl aminoethyl (DEAE)-Sephadex ion exchange column (3.5×50.0 cm), preequilibrated with the same buffer, and eluted with NaCl gradient of 0-0.5 M at a flow rate of 0.4 mL/min at 4°C. Active fractions containing greater than 50% of maximal peak activity were pooled and then concentrated by ultrafiltration. For further purification, gel filtration with Sephadex G-100 column chromatography (1.6×70.0 cm) was performed as needed with the same buffer at 4°C. The concentration of protein was determined according to the Lowry method (17) with bovine serum albumin as a standard protein.

Molecular weight determination The molecular weight of the enzyme was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with standard proteins (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) consisted of phosphorylase B (Mw 110,000), bovine serum albumin (Mw 90,000), ovalbumin (Mw 52,200), carbonic anhydrase (Mw 36,200), soybean trypsin inhibitor (Mw 29,000), and lysozyme (Mw 21,400). SDS-PAGE was carried out according to the method of Laemmli (18).

Effect of pH and temperature on the enzyme activity The protease activity of the enzyme was assayed at various temperatures of 20-60°C and pH 7.0. The enzyme activity was also determined at 37°C and a pH range of 3.5-10.5, respectively, in citrate buffer for pH 3.5-4.5, phosphate buffer for pH 5.5-7.5, and in glycine-NaOH buffer for pH 8.5-10.5.

Effect of pH and temperature on the enzyme stability The enzyme solution was first held for 30 min at the pH range of 3.5-10.5 and room temperature, and concurrently incubated for 30 min at a temperature range of 20-60°C in water bath. The protease activity was assayed at pH 7.0 and 37°C.

Effect of NaCl concentration on enzyme activity The salt-tolerant protease activity of the enzyme was assayed at 5-20% NaCl concentrations in 1/15 M phosphate buffer (pH 7.0). The effect of NaCl concentration on enzyme activity was determined by adding 500 μL of enzyme to 500 μL of 0.6% casein at 5-20% NaCl concentrations.

Effect of metal ions and inhibitors The effects of metal ions were investigated by using $AgNO_3$, $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, KH_2PO_4, $MgSO_4$, $MnSO_4$, and $ZnSO_4$. The effects of protease inhibitors were also studied by using ethylenediamine tetraacetic acid (EDTA), iodoacetic acid, phenylmethyl sulfonylfluoride (PMSF), 2,4-dinitrophenol, 2-mercaptoethanol, and *O*-phenanthroline. The purified protease was preincubated in the absence and the presence of bivalent cations such as Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Ba^{2+}, and Fe^{2+} and other inhibitors with a final concentration of 1 mM in 1/15 M phosphate buffer (pH 7.0) at 25°C for 30 min. The protease activity was measured at pH 7.0 and 37°C. The remaining activity was expressed as a percentage of the activity compared with control.

Measurement of kinetic constants The kinetic constants, K_m and V_{max} for the hydrolysis by the purified enzyme were determined by Lineweaver-Burk plot (19). Casein dissolved in 1/15 M phosphate buffer, pH 7.0 was used as a substrate at various concentrations of 1-10 mg/mL.

Results and Discussion

Purification of the enzyme The protease from the culture fluid of *Saccharomyces* sp. B101 after ammonium sulfate precipitation and ultrafiltration was separated into 3 peaks using a DEAE-Sephadex A-50 column. Only the largest peak showed strong activity (Fig. 1), and then this fraction was then applied to the Sephadex G-100 gel column for further analysis. Only one large protein peak with protease activity was obtained after this step (Fig. 2). Purification results are summarized in Table 1. After the final purification step, the purified enzyme had a specific activity of 4,688.9 units/mg protein, which indicated that the enzyme was purified 18-fold with approximately 3% of its yield.

Molecular weight The molecular weight of the purified protease was estimated to about 33 kDa by 12% SDS-PAGE, as shown in Fig. 3. It was larger than that of the alkaline protease (27-30 kDa) from *S. lipolytica* CX161-1B (20) and extracellular protease (30 kDa) from *Candida lipolytica* (21), respectively, but lower than that of antarctic yeast *Candida humicolas* extracellular protease (36 kDa) (22), and neutral protease (42 kDa) (23) from *S. lipolytica.*

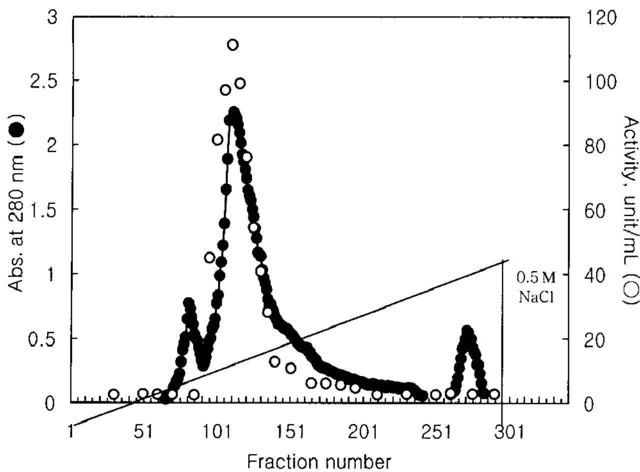

Fig. 1. Ion-exchange chromatogram of protease from *Saccharomyces* sp. B101 separated using DEAE-Sephadex. The protein was applied to the column (3×50 cm) equibrated with 20 mM NaOAc buffer (pH 6.5). The protein was eluted with a 0-0.5 M NaCl linear gradient at the flow rate of 0.4 mL/min at 4°C. The fraction volume was 2 mL.

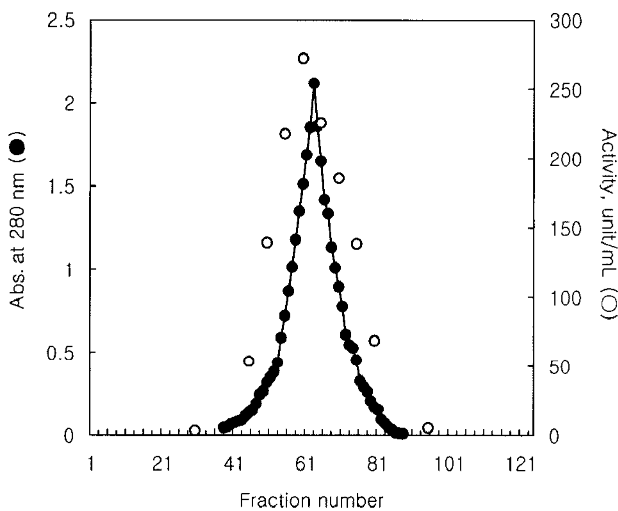

Fig. 2. Gel permeation chromatogram of protease from *Saccharomyces* sp. B101 separated using Sephadex G-100. The protein was eluted through the column (1.6×70 cm) equibrated with 20 mM NaOAc buffer (pH 6.5) at the flow rate of 0.3 mL/min at 4°C. The fraction volume was 3 mL.

However, it was similar to the molecular weight (32 kDa) of alkaline proteinases from *Yarrowia lipolytica* 504D and *Fomitella fraxinea* (24,25).

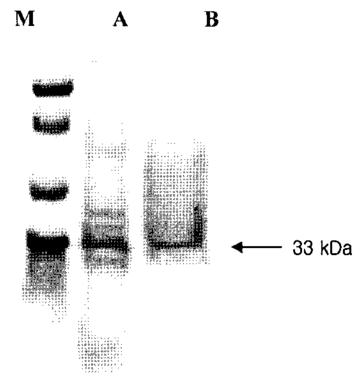

Fig. 3. SDS-polyacrylamide gel electrophoresis of the protease from *Saccharomyces* sp. B101. M, reference protein; A, crude enzyme; B, purified enzyme.

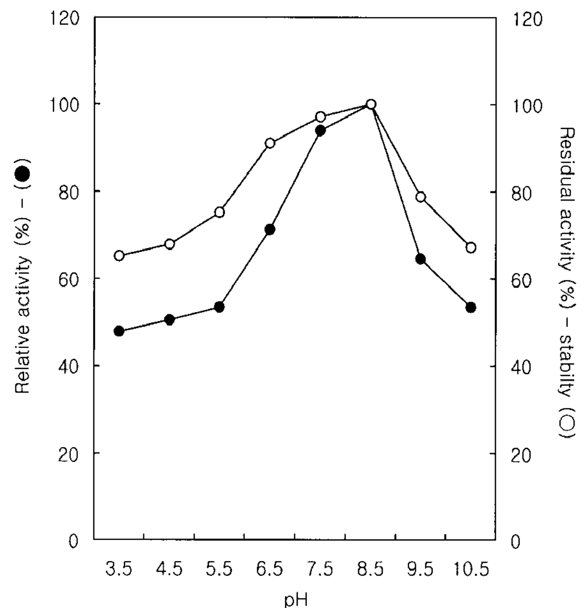

Fig. 4. Optimum pH and pH stability of the protease from *Saccharomyces* sp. B101.

Effect of pH on the activity and stability The optimum pH for protease activity was determined over the pH range from 3.5 to 10.5. As shown in Fig. 4, the optimal pH for the hydrolysis of casein was 8.5. Protease from *Saccharomyces* sp. B101 had higher proteolytic activity at weak alkaline conditions (pH 7.5-8.5) than at the acidic region. Therefore, *Saccharomyces* sp. B101 protease was an alkaline protease.

Table 1. Purification of a novel salt-tolerant protease from *Saccharomyces* sp. B101[1)]

Purification steps	Total activity (Unit)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	248,411.7	956.5	259.7	100.0	1.0
Ammonium sulfate	83,311.7	49.0	1,699.8	33.5	6.5
Ultrafiltration	28,989.4	14.0	2,069.4	11.6	7.9
Sephadex A-50	14,968.7	5.3	2,800.6	6.1	10.7
Sephadex G-100	7,698.8	1.6	4,688.8	3.1	18.1

[1)]The enzyme assay for proteolysis was carried out with 0.6% casein in 1/15M phosphate buffer (pH 7.0) at 37°C for 10 min. The enzyme unit (U) was defined as the amount of enzyme releasing 1 mmol of tyrosine equivalent per 10 min.

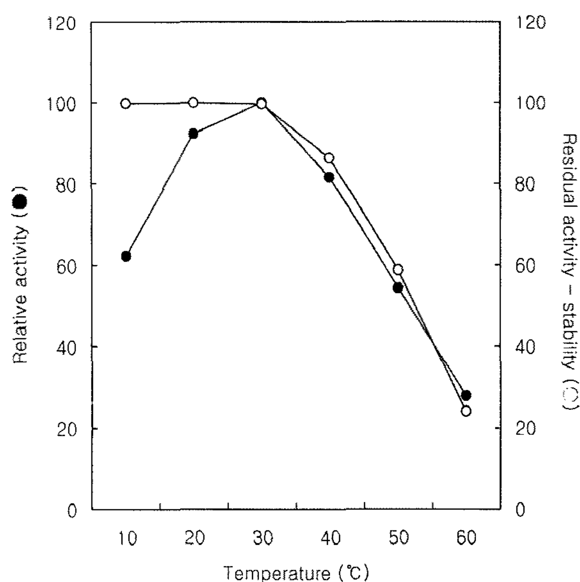

Fig. 5. Optimum temperature and thermal stability of the protease from *Saccharomyces* sp. B101.

Optimal pH for *Saccharomyces* sp. B101 protease activity was lower than those of alkaline extracellular protease produced by *S. lipolytica* CX161-1B, pH 9-10 (20), and alkaline protease from *Y. lipolytica* 504D, pH 9.5 (24). When investigating the enzyme stability at various pHs, *Saccharomyces* sp. B101 protease exhibited high stability within a range from neutral to weak alkaline pH (6.5-8.5), but lost its activity above pH 8.5 (Fig. 4). Similar results have been reported for the alkaline serine protease from *Aspergillus terreus* (26) and a novel protease from *B. cereus* KCTC 3674 (27). While these enzymes from *A. terreus* and *B. cereus* were inactivated at pH 7.5, the enzyme from *Saccharomyces* sp. B101 is more stable within a neutral pH range.

Effect of temperature on the activity and stability The effect of temperature on the activity and stability of the protease also examined in the range of 10-60°C at pH 7.0. As shown in Fig. 5, the optimum temperature for the enzyme activity was approximately 30°C. This optimum temperature belongs to a range of 30-35°C for for *Saccharomyces carlsbergensis* proteolytic enzyme reported by Maddox and Hough (28) and 37°C of *A. terreus* (IJIRA 6.2) alkaline serine protease (26) and *C. humicola* extracellular protease (22), which were a little higher than that of *Saccharomyces* sp. B101 protease in this study. The purified enzyme was stable up to 40°C and its stability sharply decreased at higher temperatures (Fig. 5), which showed its non-thermostability. The temperature of optimal stability for *S. cerevisiae* B101 protease was higher than its optimal activity temperature. Eighty-five and 60% of residual activity were shown at 40 and 50°C, respectively. Thermal inactivation was observed to be more rapid at 60°C. The temperature for optimal stability of *Saccharomyces* sp. B101 was similar to that of bakers' yeast proteinases A observed by Meussdoerffer *et al.* (29). But, optimal stability temperature of this protease was lower than those of proteases produced by *Bacillus* sp. YG95 and *Bacillus* sp. DJ-2 (30,31).

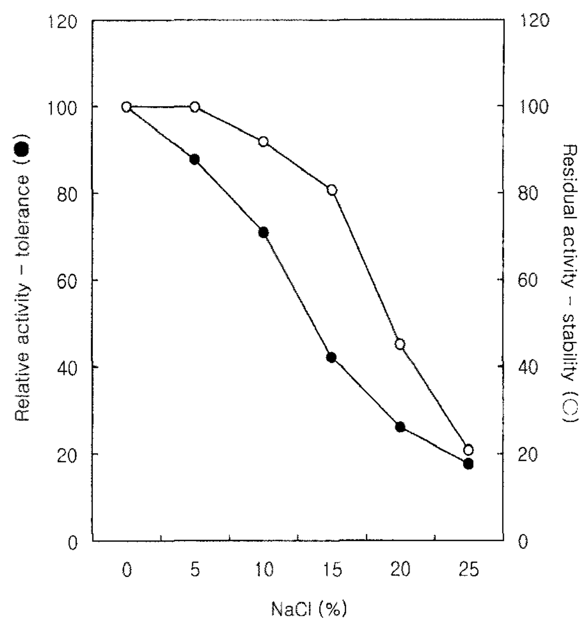

Fig. 6. Effect of salt concentrations on the activity and stability of the protease from *Saccharomyces* sp. B101. The salt-tolerant protease activity (●) was assayed at various NaCl concentrations of 5-25% and pH 7 in 1/15 M phosphate buffer. The enzyme stability (○) was determined by adding 500 μL of protease to 500 μL of 0.6% casein at 5-25% NaCl.

Effect of salt on the activity and stability The effect of NaCl concentration on protease activity and stability were shown in Fig. 6. Relative activity of *Saccharomyces* sp. B101 protease decreased with increasing NaCl concentration. Protease activity for salt-tolerance was investigated at salt concentrations of 0-25% at pH 7.0, and the result showed that 90 and 70% of residual activity were remaining at NaCl concentrations of 5 and 10%. This finding was similar to those reported by Yaichi *et al.* (32) using protease from salt tolerant *A. oryzae*. These results suggest that the protease from *Saccharomyces* sp. B101 can hydrolyze anchovy protein in anchovy sauce where the NaCl concentration is greater than 15%. The salt stability of the enzyme was also investigated in the concentration range of 5-25% NaCl by measuring the residual enzyme activity. As shown in Fig. 6, the purified enzyme was stable in solutions containing up to 15% NaCl but its stability sharply decreased at NaCl concentrations above 15%. The relative activity of this purified protease decreased with increasing NaCl concentration. But, the salt concentration of this protease's optimal stability in this study was lower than that of the extracellular serine protease of the haloalkaliphilic *Natrialba magadii* (formerly *Natronobacterium magadii*) living in hypersaline environments (>15% NaCl) (33). The salt-tolerant enzyme activity of this yeast protease was higher than that of *B. subtilis* JM-3 protease (8) isolated and purified from anchovy sauce with a 26% NaCl concentration.

Effect of metal ions on the enzyme activity The effects of metal ions on the protease activity are shown in Table 2. Under the reaction conditions used, Mn^{2+} with 1 mM increased the enzyme activity by 5% when compared to the control of absence for metal ion. Ag^{2+}, Cu^{+}, Mg^{2+}, and Fe^{2+}

Table 2. Effect of metal ions on the protease activity from *Saccharomyces* sp. B101

Ion	Metal (1 mM)	Relative activity (%)
None		100
K^+	KH_2PO_4	93
Ba^{2+}	$BaCl_2$	101
Ca^{2+}	$CaCl_2$	96
Co^{2+}	$CoCl_2$	101
Cu^{2+}	$CuSO_4$	83
Fe^{2+}	$FeSO_4$	54
Ag^{2+}	$AgNO_3$	73
Mg^{2+}	$MgSO_4$	92
Mn^{2+}	$MnSO_4$	105
Zn^{2+}	$ZnSO_4$	94

Table 3. Effect of various inhibitors on the protease activity

Reagent[1] (1 mM)	Relatve activity (%)
None	100
EDTA	66
Iodoacetic acid	99
PMSF	75
2,4-Dinitrophenol	99
2-Mercaptoethanol	98
O-Phenanthroline	50

[1]EDTA, ethylenediamine tetraacetic acid; PMSF, phenylmethy sulfonyl fluoride.

ions showed a somewhat inhibitory effect. Ca^{2+}, Zn^{2+}, Co^{2+}, and Ba^{+} had no inhibitory effect on the enzyme activity. This result was similar to those reported by Choi *et al.* (34). The protease from *Saccharomyces* sp. B101 was not greatly influenced by the metal ions used in this study.

Effect of inhibitors on the enzyme activity The effects of inhibitors were also investigated in 1/15 M phosphate buffer at pH 7.0 (Table 3). The enzyme activity was inhibited by protease inhibitors such as PMSF with 1 mM, which were specific for the inhibition of serine protease. Therefore, the enzyme from *Saccharomyces* sp. B101 can be classified as a serine protease based on its sensitivity to PMSF. EDTA and *O*-phenanthroline showed an inhibitory effect on the enzyme activity. The inhibitory effect of EDTA was shown to be the strongest among the various inhibitors used. In general, EDTA and *O*-phenanthroline has been known to remove the essential metal ion from the enzyme molecule causing the inactivation of the enzyme. These results indicate that the protease from *Saccharomyces* sp. B101 is a serine protease which requires metal ion group for the enzyme activity. The dual sensitivity to EDTA and PMSF has been reported for certain other bacterial and fungal proteases and is similar to earlier published values (35).

Enzyme kinetics To evaluate the kinetic constant for the purified enzyme, the initial velocities of the enzyme reactions were determined depending on various concentrations of the casein substrate. As shown in Fig. 7, the kinetic constant, K_m for the proteolysis was determined by using a

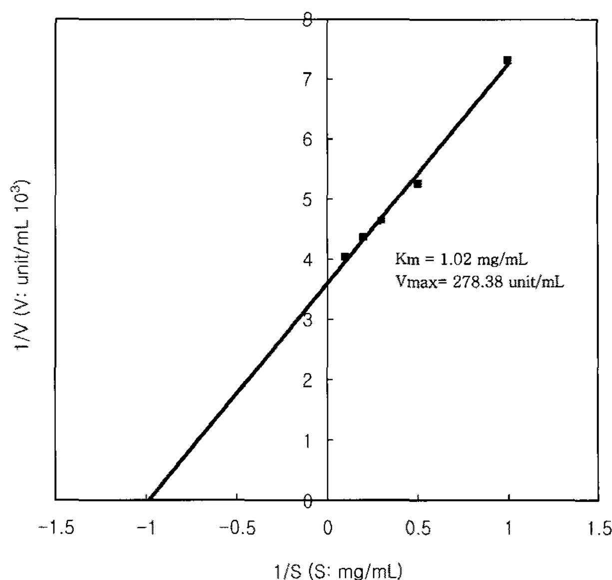

Fig. 7. Lineweaver-burk plot for casein by protease from *Saccharomyces* sp. B101.

Lineweaver-Burk plot. The reaction appears to follow Michaelis-Menten kinetics. The K_m was evaluated to be 1.02 mg/mL and the V_{max} was 278.38 unit/mL. The K_m value obtained was similar to that (1.3 mg/mL) of the alkaline protease as reported by Bae and Park (36), and it was similar to the K_m value for the protease from *Rhizopus oryzae* (37).

Acknowledgments

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References

1. Bibek R. Fundamental Food Microbiology. CRC Press Ltd., New York, NY, USA. pp. 109-113 (1996)
2. Catrinus van der S, Johnnes T, Rene HW. Enhancing and accelerating flavor formation by salt-tolerant yeasts in Japanese soy-sauce processes. Trends Food Sci. Tech. 43: 6-9 (2001)
3. Almagro A, Prista C, Benito B, Loureiro-Dias MC, Ramos J. Cloning and expression of two genes coding for sodium pumps in the salt-tolerant yeast *Debaryomyces hansenii.* J. Bacteriol. 183: 3251-3255 (2001)
4. Ramos J. Contrasting salt tolerance mechanisms in *Saccharomyces cerevisiae* and *Debaryomyces hansenii.* pp. 377-390. In: Recent Research Developments in Microbiology. Pandalai SG (ed). Research Signpost Publishers, Trivandrum, India (1999)
5. Barbosa DC, Weid IVD, Vaisman N, Seldin L. Halotolerant spore-forming Gram-positive bacterial diversity associated with *Blutaparon portulacoides* (St. Hill) mears, a pioneer species in Brazillian coastal dunes. J. Microbiol. Biotechn. 16: 193-199 (2006)
6. Jeong SC, Hyun KW, Lee JS. Isolation of a halotolerant yeast and the production of extracellular protease. Korean J. Biotechnol. Bioeng. 16: 158-162 (2001)
7. Kim DC, Oh NS, In MJ. Effect of carbon and nitrogen sources on cell growth and halotolerant alkaline protease production in *Halomonas marisflava* isolated from salt-fermented food. Food Sci. Biotechnol. 13: 837-840 (2004)
8. Kim WJ, Kim SM. Purification and characterization of *Bacillus subtilis* JM-3 protease from anchovy sauce. J. Food Biochem. 29: 591-609 (2005)

9. Bae IH, Kang KH. Caseinolytic action of extracellular protease of *Saccharomycopsis lipolytica*. Korean J. Dairy Sci. 11: 194-293 (1989)
10. Lance SD, O'donnel MM, Kaczmarek FS, Pereira DA, Dezeeuw JR, Franke AE. Cloning and sequencing of the alkaline extracellular protease gene *of Yarrowia lipolytica*. J. Bacteriol. 169: 4621-4629 (1987)
11. Lee JS, Yi SH, Kwon SJ, Ahn C, Yoo JY. Enzyme activities and physiological functionality of yeasts from traditional *meju*. Korean J. Appl. Microbiol. Biotech. 25: 448-453 (1997)
12. Yoo JY, Kim HG, Kwon DJ. Improved process for preparation of traditional *ganjang*. J. Korean Soc. Food Sci. Nutr. 27: 268-274 (1989)
13. Ichiro Y, Hirata D, Machida M, Fukui S. Cloning and expression in *Saccharomyces cerevisiae* of the secretable acid protease gene from *Sacchromycopsis fibuligera*. Agr. Biol. Chem. 50: 109-113 (1986)
14. Watanabe Y, Oshima N, Tamai Y. Co-expression of the Na^+/H^+-antiporter and H^+-ATPase genes of the salt-tolerant yeast *Zygosaccharomyces rouxii* in *Saccharomyces cerevisiae*. FEMS Yeast Res. 5: 411-417 (2005)
15. Hwang JY. Purification and characterization of a novel salt-tolerant protease from *Saccharomyces* sp. B101. MS thesis, Konkuk University, Seoul, Korea (2003)
16. Anson ML. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. J. Gen. Physiol. 22: 79-91 (1939)
17. Lowry OH, Rosebrough NJ, Farr AL, Randull RJ. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275 (1951)
18. Laemmli UK. Cleavage of structural protein during the assembly of the heat of bacteriophage T4. Nature 227: 680-685 (1970)
19. Lineweaver H, Burke D. Determination of enzyme dissociation constants. J. Am. Chem. Soc. 56: 658-666 (1934)
20. Ogrydziak DM, Scharf SJ. Alkaline extracellular protease produced by *Saccharomycopsis lipolytica* CX161-1B. J. General Microbiol. 128: 1225-1234 (1982)
21. Bae IH, Kang KH. Studies on extracellular protease from *Saccharomycopsis lipolytica* (*Candida lipolytica*): Purification and properties of enzyme. Korean J. Appl. Microbiol. Bioeng. 15: 286-293 (1987)
22. Ray MK, Devi KU, Kumar GS. Extracellular protease from the antarctic yeast *Candida humicola*. Appl. Environ. Microb. 58: 1918-1923 (1992)
23. Ahmed THA, Kennedy EH, Ahearn DG. Purification and characterization of a neutral protease from *Saccharomycopsis lipolytica*. J. Bacteriol. 130: 1125-1129 (1997)
24. Kim CH, Yoo CB. Charaterization of alkaline proteinase from *Yarrowia lipolytica* 504D. Korean J. Microbiol. 34: 82-86 (1998)
25. Lee JS, Baik HS, Park SS. Purification and characterization of two novel fibrinolytic proteases from mushroom, *Fomitella fraxinea*. J. Microbiol. Biotechn. 16: 264-271 (2006)
26. Syamal KC, Matsumura N, Ranu RS. Purification and characterization of an extracellular alkaline serine protease from *Aspergillus terreus* (IJIRA 6.2). Curr. Microbiol. 40: 239-244 (2000)
27. Kim SS, Kim YJ. Purification and characterization of a novel extracellular protease from *Bacillus cereus* KCTC 3674. Arch. Microbiol. 175: 458-461 (2001)
28. Maddox IS, Hough JS. Proteolytic enzymes of *Saccharomyces carlsbergensis*. Biochem. J. 117: 843-852 (1970)
29. Meussdoerffer F, Tortora P, Holzer H. Purification and properties of proteinase A from yeast. J. Biol. Chem. 255: 12087-12093 (1980)
30. Byun YG, Joo HK, Lim MH, Kim SH, Lee KS. Purification and characterization of proteolytic enzyme from *Bacillus* sp. YG95 by traditional *meju*. Agr. Chem. Biotechnol. 41: 349-354 (1998)
31. Choi NS, Yoo KH, Hahm JH, Yoon KS, Chang KT, Hyun BH, Maeng PJ, Kim SH. Purification and characterization a new peptidase, bacillopeptidase DJ-2, having fibrinolytic activity: Produced by *Bacillus* sp. DJ-2 from *doenjang*. J. Microbiol. Biotechn. 15: 72-79 (2005)
32. Yaichi F, Itoh H, Fukase T, Motai H. Continuous protease production in a carbon-limited chemostat culture by salt tolerant *Aspergillus oryzae*. Appl. Microbiol. Biot. 30: 604-608 (1989)
33. Maria IG, Studdert CA, Sanchez JJ, DeCastro RE. Extracellular protease of *Natrialba magadii*: Purification and biochemical characterization. Extremophiles 4: 181-188 (2000)
34. Choi C, Choi KS, Cho YJ, Lim SI, Lee SH, Son JH, Choi HJ, Lee HD. Characteristics and action pattern of protease from *Bacillus subtilis* CCKS-118 in Korean traditional soy sauce. J. Korean Soc. Appl. Biol. Chem. 39: 460-465 (1996)
35. Bae IH, Kang KH. Purification and properties of extracellular protease of *Saccharomycopsis lipolytica*. Korean J. Dairy Sci. 15: 286-293 (1987)
36. Bae M, Park PY. Purification and characterization of thermotolerable alkaline protease by alkalophilic *Bacillus* sp. No. 8-16. Korean J. Microbiol. Bioeng. 17: 545-551 (1989)
37. Banerjee R, Bhattacharya BC. Kinetic properties of extracellular alkaline protease of *Rhizopus oryzae*. J. Ferment. Bioeng. 75: 380-382 (1993)