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Article

Production and Characterization of an Alkaline Protease from *Bacillus licheniformis* MH31

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A alkalophilic strain, *Bacillus licheniformis* MH31 producing an alkaline protease was isolated from mine soil of Boryeong in Korea. Production of a high level of alkaline protease was achieved 42 h after incubation when the bacterium was grown at pH 9.0 and 35°C in Horikoshi medium supplemented with 0.5% (w/v) starch and 1% (w/v) skim milk as carbon and nitrogen source, respectively. The molecular weight of partially purified enzyme was estimated to be 30 kDa by SDS-PAGE and its optimum pH was pH 10. The enzyme showed optimum temperature at 50°C, and was stable up to 60°C after 1 h incubation. The protease was strongly inhibited by 1 mM of PMSF which was known well as strong inhibitor of serine proteases, but almost not inhibited by 5 mM of EDTA and 1,10-phenanthroline. When the protein hydrolysis products of 1% skim milk by partially purified protease was compared with available commercial proteases using HPLC analysis, most of hydrolysis products were detected below molecular weight of 10,000 and the hydrolysis ratio of purified enzyme was 24.8% lower than those (above 32%) of commercial proteases.

Key words: *alkaline protease, Bacillus licheniformis MH31, production, characterization*

Proteolytic enzymes constitute one of the most important group of industrial enzymes and especially, alkaline proteases represent one of the largest enzyme useful in the detergent industry which contribute to more than 30% of the total global enzyme market. The industrial applications of alkaline protease in detergent as well as leather tanning, pharmaceutical, and food industries have increased remarkably.[1,2,3,4]

Bacteria of the genus *Bacillus* are known to produce a group of commercially important enzymes including proteolytic enzymes. Currently approximately 25% of the world-wide available alkaline proteases are derived from *Bacillus* strains.[5,6] The representative speciés of the *Bacillus* group, which is *B. licheniformis*, *B. pumilus*, and *B. subtilis*, were known to the major source of commercial alkaline protease production and studies on characterization and industrial application of their enzymes have been reported by investigators.[7,8,9]

In this paper, we report the medium condition for the optimal production of extracellular alkaline protease from *Bacillus licheniformis* MH31 and the enzymatic properties of partially purified alkaline protease.

Materials and Methods

Microorganism and medium. A genus *Bacillus* strain producing an alkaline protease was isolated from korea mine soil of Boryeong in Korea by selective screening on skim milk agar plate. The isolate was identified by as *Bacillus licheniformis* MH-31 on the basis of 16S rRNA gene alignment with GeneBank database. Horikoshi[10] medium (yeast extact 5 g · l^{-1}; peptone 5 g · l^{-1}; glucose 10 g · l^{-1}; K_2HPO_4 1 g · l^{-1}; $MgSO_4 \cdot 7H_2O$ 0.2 g · l^{-1}; Na_2CO_3 10 g · l^{-1}, pH 9) was used to the basal media for production of alkaline protease. To determine the optimal medium components for enzyme production, *Bacillus licheniformis* MH-31 was cultured with basal media containing skim milk, tryptone, casaamino acid, soytone and bacto peptone as nitrogen source and fructose, lactose, maltose, soluble starch, sucrose and xylose as carbon source, respectively.

Protease assay. Extracellular protease activity was determined by Fugiwara method[11] using 1.0% casein in 0.1 M Tris-HCl buffer (pH 8.0) as a substrate. The assay mixture consisted of 2 m*l* of substrate and 0.5 m*l* of enzyme solution suitably diluted with 0.1 M Tris-HCl buffer (pH 8.0). The reaction mixture was incubated at 40°C for 30 min and the reaction was terminated by the addition of 2.5 m*l* of 10% trichloroacetic acid (TCA), and then centrifuged at 5,000 × g for 10 min to remove the resulting precipitate. Protease activity was determined by estimating the amount of tyrosine in the supernatant according to Lowry[12] method. One unit of protease activity was defined as the amont of enzyme required to release 1 μg of tyrosine · ml^{-1} · min^{-1} under the reaction conditions.

Partial purification of the enzyme. The culture broth containing extracellular protease was precipitated with ammonium sulphate to a final concentration of 70%. The saturated solution was lefted overnight at 4°C, centrifuged and

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the precipitate was then dissolved in the a minimal volume of 100 mM sodium phosphate buffer (pH 7.5) and dialyzed against the same buffer for 24 h. The dialyzed protein was loaded on to the anion-exchanger, Resource Q column (1.5 × 12 cm, Pharmacia Biotech, Sweden), which had been previously equilibrated with 50 mM sodium phosphate buffer (pH 7.5), and then eluted with the same buffer by the flow rate of 20 m*l* · h^{-1}. The bound fractions were separated by increaing ionic strength with the same buffer containing 0.5 M NaCl and monitored at A_{280} (GradiFrac™ System, Pharmacia Ltd). Active fractions with high protease activity were pooled, and concentrated using a Centriprep-10 (Amicon), divided into small aliquots, and stored at −70°C for further use.

Effect of Temperature and pH. The optimal pH of partially purified protease was determined with 1% (w/v) skim milk as substrate dissolved in different buffers (citrate phosphate, pH 5~6; sodium phosphate, pH 7.0; Tris-HCl, pH 8.0; and glycine-NaOH, pH 9~12). The pH stability of alkaline protease was determined by measuring the remaining activity after incubating enzyme in different buffers pH (6~12) at 4°C for 2 h. The optimum temperature of protease activity was determined with reaction mixture incubated at different temperatures ranging from 30 to 70°C for 30 min. For determination of thermostability, 0.1 m*l* of enzyme was preincubated with 0.9 m*l* of glycine-NaOH buffer, pH 9, at different temperatures ranging from 30 to 70°C for 60 min. After incubation, the residual activity was measured under standard assay condition.

Effect of Inhibitiors. The partially purified protease was preincubated with different inhibitors such as 1 mM PMSF (Phenyl Methyl Sulfonyl Fluoride), 1 mM TLCK (N-alpha-*p*-tosyl-L-lysine chloromethyl ketone), 5 mM 1,10-phenanthroline, 5 mM EDTA, and 5 mM of various metal ions for 1 h under optimum reaction conditions. Residual activities in the presence of the inhibitors were compared with the controls without inhibitor.

Analysis of protein hydrolytes. To compare the protein hydrolysis products by MH 30 protease and other commercial proteases which were originated from *Bacillus subtilis* (Neutrase), *Aspergillus oryzae* (Amano M), *Bacillus stearothermophilus* (Amano S), HPLC analysis was performed with the reaction mixtures of each enzyme and 1% skim milk as substrate. The reaction mixtures containing 2 m*l* of 1% substrate and 0.5 m*l* of each enzyme solution suitably diluted with 50 mM Tris-HCl buffer (pH 7.6) were incubated at 40°C. The aliquots of 1.0 m*l* were withdrawn at specified time intervals (0, 0.5, 1, 2, 6, 12, 18 and 24 h) and boiled immediately for 10 min to terminate the reaction. The hydrolysis product of aliquots were separated using HPLC column (TSK-G200SW, Japan) with 50 mM Tris-HCl buffer (pH 7.6) and were detected at A_{280}.

Results and Discussion

The organism. A genus *Bacillus* producing an alkaline protease was isolated from mine soil of Boryeong in the Korea. Bacterial cell which showed the chemotaxanomic characteristics of Gram-positive, nonmotile aerobic, regular rods, nonpigment and spore forming was identified as *Bacillus ligheniformis* MH31 by aligning a 16S rRNA sequence with the Genebank database (Fig. 1).

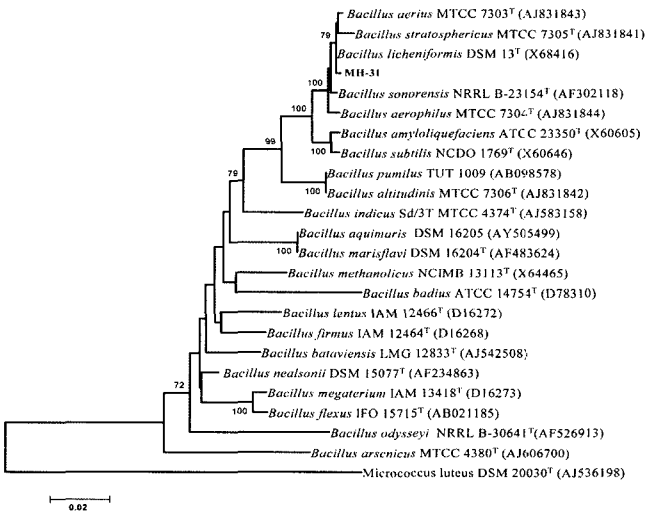

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of MH-31 and other related taxa based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1,000 replications) greater than 70% are shown at branch points. Bar, 0.02 substitutions per nucelotide position. This tree was constructed using the neighbour-joining method (Saitou & Neil, 1987) with a Kimura (1983) two parameter distance matrix and pairwise deletion.[13,14]

Effect of carbon and nitrogen sources. The optimal medium for protease production was investigated by comparing the effect of carbon and nitrogen components supplemented in Horikoshi medium. The maximum protease activity was obtained in presence of 1% (w/v) starch as the carbon source and the optimum concentration of starch for protease production was in the range of 0.5~1% (Table 1). However The protease production was markedly reduced by addition of 1% (w/v) glucose, lactose, and xylose. These results are

Table 1. Effects of carbon source on cell growth and protease production by *B. licheniformis* MH-31

Carbon source	Dry cell weight (g/L)	Protease activity (U/mL)
Fructose	1.24	0.36
Glucose	1.18	0.42
Lactose	1.36	0.84
Maltose	1.42	2.26
Starch	1.65	6.80
Sucrose	1.12	0.94
Xylose	1.26	0.24

The strain was grown on the alkaline medium with initial pH 9.0 supplemented 0.5% starch and each 0.2% carbon sources at 40°C for 42 h. The results are expressed as the means of three separate experiments.

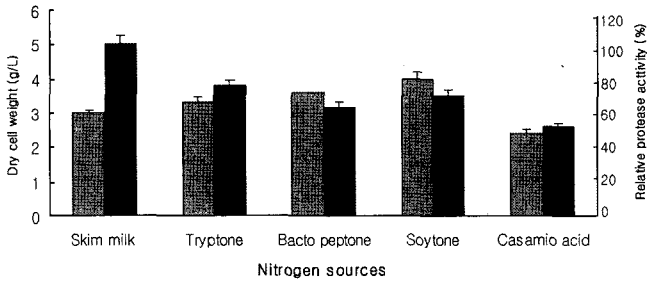

Fig. 2. Effects of nitrogen sources on alkaline protease production by *B. licheniformis* MH-31. The strain was grown in alkaline medium with initial pH 9 supplimented each 1.0% nitrogen sources at 40°C for 42 h. The results are expressed as the means S.E. from three separate experiments. $p < 0.01$. Dry cell weight (□), relative protease activity (■).

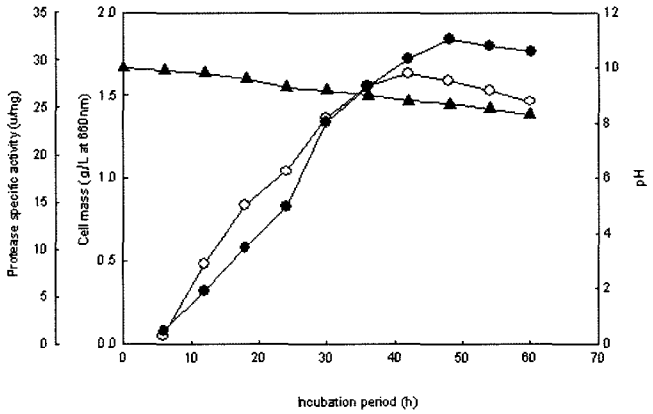

Fig. 3. The profile on cell growth and protease production of *B. licheniformis* MH-31. Cell mass (●), protease activity (○), and pH (▲).

somewhat different from those obtained using other *Bacillus licheniformis* strain[15,16], in which glucose and lactose were reported as effective carbon sources for protease production. The maximum production of protease was observed in the medium supplemented with 1% (w/v) skim milk as nitrogen source (Fig. 2) and the proteolytic activity of 12 U ml^{-1} was higher than approximately 20~40% as compared with those of other nitrogen components.

Enzyme production. The growth profile of *B. licheniformis* MH31 is presented in Fig. 3. The extracellular protease secreted by the isolate showed the time dependent increase along with specific growth of cell mass. Maximum protease production (28.2 U mg^{-1}) occurred at the end of the exponential phase after fermentation 42 h, and thereafter, the proteolytic activity remained constant until the end of the stationary phase when cell mass starts to decrease. During cultivation, the pH of the culture broth decreased from 9.0 to 8.0 and then remained constant during prolonged cultivation. The time at which enzyme production reached its maximum value during cultivation almost coincided with the time at which maximum cell density, as being similar result with that reported by *Bacillus pumilus*.[8] From the obtained results, maximal

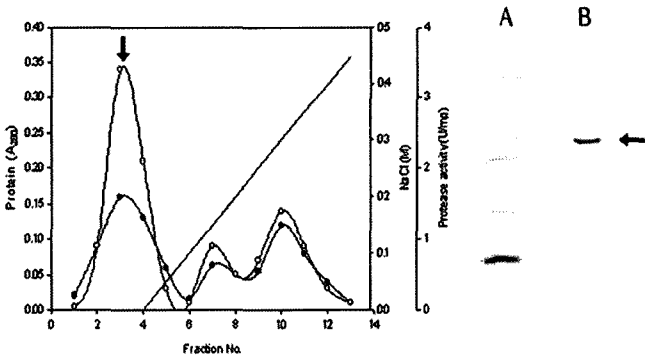

Fig. 4. Elution profile of alkaline protease from *B. licheniformis* MH-31 on Resource Q anion exchange column. The enzyme eluted with a linear gradient of NaCl (0-0.5 M) concentration in 50 mM Tris-HCl buffer (pH 7.8). Protein (●), protease activity (○) A: Maker protein; BSA (66,000), Ovalbumin (45.000), Pepsin (34,700), Trypsinogen (24,000), Lactoglobulin (18,400), Lysozyme (14,300). B: purified protease.

production of alkalin protease was achieved when MH 31 strain was incubated for 42 h in the Horikoshi medium containing 0.5% starch and 1% skim milk as carbon and nitrogen sources, respectively, at pH 9.0 and 34°C.

Characeristics of alkalin protease

Partial purification of alkalin protease. The *B. lichenifirmis* MH31 alkaline protease was partially purified by using single step ion-exchange chromatography on a Resource Q column with 50 mM Tris-HCl buffer (pH 7.8). The alkaline protease eluted out as unbound fractions without the interaction with anion Q-sepharose column (Fig. 4), presuming isoelectric point (pI) of the enzyme might be above pH 7.8. The protease activity in active fraction represented 243 U mg^{-1} of specific activity with 5.7-fold purification and the molecular weight of partially purified enzyme was estimated to be approximately 30 kDa by SDS-PAGE (Fig. 4).

Effect of pH and thermperature. The partially purified protease exhibited optimum pH at pH 10. Although the enzyme activities decreased to approximately 60% of its maximal value at pH 12 after incubation for 6 h, the enzyme was stable in a broad pH range from 6.0 to 11 (Fig. 5). The enzyme showed optimum temperature at around 50°C (Fig. 6), and was stable up to 55°C after 1 h incubation. However, the activity showed a marked decrease at over 60°C within 30 min after incubation. These results prsented the protease secreted from *B. lichenifomis* MH31 is a typical of highly pH stable alkaline protease with moderate heat stability.

Protease inhibitors. The effect of metal ions and synthetic inhibitors on the partially purified protease was investigated. Enzyme activity was strongly inhibited above 95% by 1 mM of PMSF which was known as the serine protease inhibitors[17,18], while it was not inhibited by 1mM of the trypsin selective reagent TLCK. In addition, the protease activity was resistant to inhibition by 5 mM of EDTA and 1,10-phenanthroline, indicating that the enzyme is not a metalloprotease (Table 2). Among metal ions, Mn^{2+} and Ca^{2+} marginally stimulated the

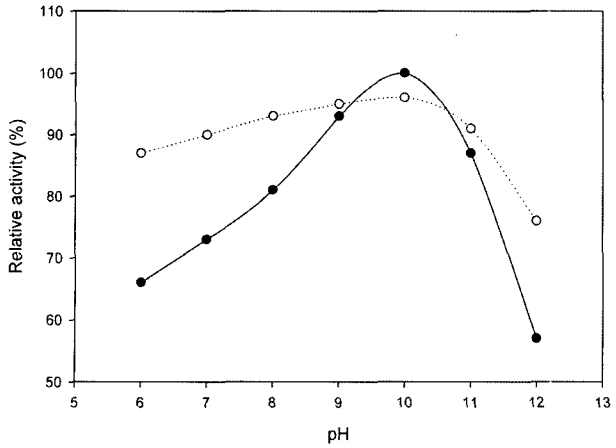

Fig. 5. Effect of pH on activity and stability of alkaline protease from *B. licheniformis* MH-31. The protease was assayed in the pH range of 6.0-12 using different buffers. The pH stability was determinded by incubating the enzyme with equal amount of buffers of different pHs for 48 h at room temperature and thereafter the relative activity was determined un der standard assay conditions. Optimal pH (●), pH stability (○).

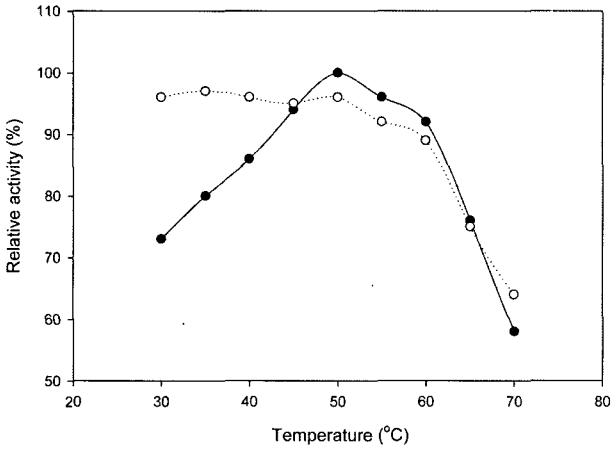

Fig. 6. Effect of temperature on activity and stability of alkaline protease from *B. licheniformis* MH-31. The optimal temperature was determined by assaying protease activity at temperatures between 30 and 70°C. The temperature stability was determined by incubating enzyme at various temperature in range on 30-70°C for 2 h and the residual enzyme activity was estimated at regular intervals. Optimal temperature (●), Temperature stability (○).

protease activity up to 20% of maximum activity.

Analysis of protein hydrolysis products. Hydrolysis rates of MH 31 protease and commercially available proteases (Neutrase, Amano M, and Amano S) originated from other microorganisms were estimated by comparing the reaction products between enzyme and substrate (1% skim milk) using HPLC column. Proteolytic activity of all protease tested was not increased more than after 60 min reaction. Seven hydrolysis products were detected in the reaction mixture of MH 31 protease and 1% skim milk after 60 min and its hydrolysis ratio was 24.8% (Table 3). These results was somewhat lower

Table 2. Effect of specific inhibitors and metal ions on alkaline protease from *B. licheniformis* MH-31

Metal ions/Inhibitor	Concentration (mM)	Residual acitivity (%)
Control	0.0	100
[1]PMSF	1	5.0
[2]TLCK	1	92
1,10-phenanthroline	5	90
[3]EDTA	5	86
$MnSO^4$	5	108
$CaCl_2$	5	119
$ZnSO_4$	5	96
$CuSO_4$	5	93

Various inhibitors were mixed with enzyme and then incubated at 40°C for 1 h, and relative activity was estimated with the means of triplicate experiments under standard assay conditions.
[1]Phenylmethyl sulfonyl fluoride,
[2]N-alpha-p-tosyl-L-lysine chloromethyl ketone
[3]Ethylene diamine tetra acetic acid

Table 3. Comparison of hydrolysis products on *B. licheniformis* MH-31 protease and commercial proteases using HPLC column

RT	Sample Casein*	1% skim milk			
		MH-31	Neutrase	Amano S	Amano M
6.1	37.2	19.6	21.1	16.8	18.0
7.1					
8.3	23.3				1.0
9.0			3.6	1.8	4.0
9.8	8.6				
10.4	9.0	3.2	6.8	3.4	11.0
11.3	6.0		9.0		7.5
12.2	9.3	8.0	4.5	9.7	
13.3		33.4	45.8	29.0	38.9
13.8	3.0				18.2
14.2				11.9	
14.5		8.7			
15.3	2.0		0.6	11.9	0.9
15.8	1.6	5.3			5.3
16.9				5.3	
18.2		3.5	8.5	10.3	2.4
18.8					
Hydrolysis ratio (%)	20.04	24.8	39.8	38.2	32.94
Enzyme content	-	0.015%	0.001%	0.001%	0.001%
Fragment Number	9	7	8	9	10

The reaction aliquots which were incubated at 40°C for 60 min were separated using HPLC column (TSK-G200SW, Japan) with 50 mM Tris-HCl buffer (pH 7.6).
*commercial hydrolyzed αs-casein

than proteases from other microorganism tested which showed 32%~39% of hydrolysis ratios and 8~10 fragments, respectively. Most of products among the hydrolysis proteins were low proteins below molecular weight of 10,000 identical

of those from other commercial proteases.

Conclusion

An alkalophilic bacteria which showed wide clear zone on Horikoshi agar plate containing 1% skim milk was isolated from mine soil of Boryeong in korea. The isolate was identified as *Bacillus lichenifirmis* MH31 on the basis of analysis of chemotaxanomic and 16S rRNA sequence. MH31 strain prefers 0.5~1% (w/v) starch and 1% (w/v) skim milk as carbon and nitrogen source, respectively and the growth of cell and production of alkaline protease exhibited the time dependent increase during cultivation. Maximal enzyme production was achieved at 42 h around the end of exponential growth when the bacterium was incubated in Horikoshi medium supplemented with 0.5% starch and 1% skim milk at pH 9.0 and 34°C for 46 h. A alkaline protease of approximately 30 kDa which was partially purified by using single step of anion-exchange chromatography, exhibited optimum pH at pH 10 and was stable in a broad pH range from 6.0 to 11, indicating a typical characteristic of alkaline proteases. The protease showed optimum temperature at 50°C, and was stable up to 60°C after 1 h incubation. The protease was strongly inhibited by 1 mM PMSF, but resistant to inhibition by 5mM of EDTA and 1,10-phenanthroline, suggesting that it is a member of the serine protease family as well as non-metalloprotease. Hydrolysis ratio of MH 31 protease on 1% skim milk as substrate was 24.8% and its hydrolysis products were seven fragments with molecular weight of below 10,000, although it was somewhat lower than proteases (Neutrase, Amano M, and Amano S) originated from other microorganism tested, which showed 32%~39% of hydrolysis ratios and 8~10 fragments, respectively. Conculusively, the protease secreted from *B. lichenifirmis* MH31 is a typical of alkaline serine protease, and might be a useful source of protease for industrial applications such as laundry detergent, dairy production and leather industry.

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