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Purification and Characterization of *Streptococcus mutans* Cell Wall Hydrolase from *Bacillus subtilis* YL-1004

OHK, SEUNG-HO[1], YUN-JUNG YOO[2], AND DONG-HOON BAI*

Department of Food Engineering, Dankook University, Chunan 330-714, Korea
[1]Brain Korea 21 Project of Medical Sciences, and [2]Department of Oral Biology, Yonsei University, Seoul 120-749, Korea

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Abstract *Bacillus subtilis* YL-1004 was isolated from soil for the development of agents to control dental caries. This strain produced an extracellular lytic enzyme that hydrolyzed the *Streptococcus mutans* cell wall. The lytic enzyme was purified to homogeneity by affinity chromatography and gel permeation chromatography to give a single band on SDS-PAGE and non-denaturing polyacrylamide gel electrophoresis. The molecular weight of the enzyme was deduced from SDS-PAGE and gel chromatography to be 38 kDa and the pI to be 4.3 from isoelectric focusing. Sirty % of its lytic activity remained after incubation at 50°C for 30 min, and its optimal temperature was 37°C. The enzyme showed its highest activity at pH 8.0 and was stable at pHs ranging from 4.0 to 9.0. Treatment with several modifiers showed that a cysteine residue was involved in the active site of the enzyme. This lytic enzyme from *Bacillus subtilis* YL-1004 exhibited specificity towards Streptococci and also showed autolytic activity on *Bacillus subtilis* YL-1004.

Key words: *Streptococcus mutans*, *Bacillus subtilis*, lytic enzyme, autolysis

Streptococcus mutans is known to be very potent in creating dental caries. In order to control dental caries, a number of therapies have been developed that inhibit the adherence or growth of oral Streptococci. Until now, fluoride has been most widely used to control dental caries. Among the several species of Streptococci, *Streptococcus mutans* and *Streptococcus sobrinus* are the most predominant strains in human dental caries [10, 13, 22, 33]. *Streptococcus mutans* was first isolated by Clarke [2] and has been reported to have the capacity of inducing dental caries [11, 12, 26]. *Streptococcus mutans* can adhere to the tooth surface and produce water insoluble glucans from sucrose [8], which enable Streptococci to colonize the tooth surface. Several types of antiplaque agents, including the use of enzymes [4], such as glucanases or dextranases [14, 15, 25, 34], have been proposed to prevent dental caries, to inhibit adherence to the tooth surface, or antimicrobial agents [31] to eradicate the growth of cariogenic organisms.

For several decades, various types of enzymes and bacteriocins have been isolated from soil bacteria [20, 24, 28] to develop microbial agents that have medical and industrial usage. The current authors have isolated and purified several types of lytic enzymes from soil [27, 36], and, with respect to antiplaque agents, several strains of bacteria producing a lytic enzyme against *Streptococcus mutans* have been isolated. In this study, strain YL-1004, which produces a bacteriolytic enzyme, active on *Streptococcus mutans*, was isolated and the lytic enzyme was purified and characterized.

Materials and Methods

Screening of *Streptococcus mutans* Cell Wall Lytic Enzyme

The *Streptococcus mutans* ATCC25175 was cultured in 10 l of BHI broth (Difco Co., U.S.A.) and harvested by centrifugation. The collected cell pellet of *Streptococcus mutans* was resuspended in 50 ml of 0.9% saline and autoclaved. These autoclaved cells were then added into a sterile SA (glucose 1%, yeast extract 0.5%, polypeptone 0.5%, 1.5% agar) medium in a ratio of 1:99 (v/v) to make an SASM agar plate. In order to screen the lytic enzyme producing bacteria, soil samples from several areas of Korea were collected. The soil samples were suspended in 0.9% sterile saline and plated onto the SA medium and incubated at 37°C for 3 days. The strains isolated from the soil samples were transferred onto a SASM agar plate by toothpick and incubated at 37°C for three days. Following incubation, the lytic enzyme-producing strains produced clear zones around the colonies.

*Corresponding author
Phone: 82-41-550-3562; Fax: 82-41-550-3566;
E-mail: baidh@anseo.dankook.ac.kr

Production and Affinity Adsorption Chromatography of Lytic Enzyme

Strain YL-1004, which produces a lytic enzyme, was cultured in 1 l of a SB (1% glucose, 0.5% yeast extract, 0.5% polypeptone) medium at 37°C for 2 days. The pH of the culture supernatant was adjusted to 5.0 with 1 N HCl, and the supernatant was subjected to affinity adsorption chromatography using a *Streptococcus mutans* cell pellet. The *Streptococcus mutans* was cultured in BHI broth (Difco Co., U.S.A.) at 37°C and harvested. The cell pellet was washed with a 50 mM Tris/HCl (pH 8.0) buffer and subsequently washed three times with 50 mM Na-acetate (pH 5.0) buffer and mixed with the culture supernatant of strain YL-1004. The mixture was incubated for 2 h at 4°C with mild stirring. Then, the *Streptococcus mutans* cells were collected by centrifugation and a desorption procedure was carried out. The collected *Streptococcus mutans* cell pellet was suspended in a 50 mM Tris/HCl (pH 8.0) buffer, incubated at 37°C for 10 min, and then centrifuged. This procedure was repeated three times and the supernatants were combined. The resulting supernatant was concentrated by ultrafiltration (Amicon Co., U.S.A.). The concentrated lytic enzyme was subjected to gel permeation chromatography using a Sephadex G-75 (Sigma Co., U.S.A.), which was pre-packed and washed with a 50 mM Tris/HCl (pH 8.0) buffer at 4°C. The standard markers used for the molecular weight determination in gel permeation chromatography were lysozyme (14.4 kDa), bovine trypsinogen (24 kDa), and bovine serum albumin (66 kDa) (Sigma Co., U.S.A.).

Assay for Bacteriolytic Enzyme Activity

The bacteriolytic enzyme activity was determined using the modified method of Hayashi and Kasumi [16]. The lyophilized cells of *Streptococcus mutans* were suspended in a 50 mM Tris/HCl buffer (pH 8.0) to give an initial absorbance of 1.0 at 660 nm. Two-tenth ml of the enzyme solution was added to 2 ml of this cell suspension and the reaction mixture incubated at 37°C for 10 min. The reduction in absorbance at 660 nm was measured. One unit of bacteriolytic enzyme activity was defined as the amount of the enzyme that caused a decrease in the absorbance of 0.001 per minute.

RESULTS

Screening and Identification of Microorganism

About three thousand organisms were tested in this study and several strains that exhibited lytic activity on *Streptococcus mutans* were isolated. Among these strains, YL-1004 showed the highest lytic activity (Fig. 1). Strain YL-1004 was observed to be about 2 μm long (Figs. 2 and 3), Gram-positive, an endospore forming rod, and strict aerobe. It produced catalase, and hydrolyzed lactose, maltose, sucrose, starch, gelatin, and mannitol. It also formed indole, grew in a medium containing 10% NaCl (Table 1), and was identified as *Bacillus subtilis*.

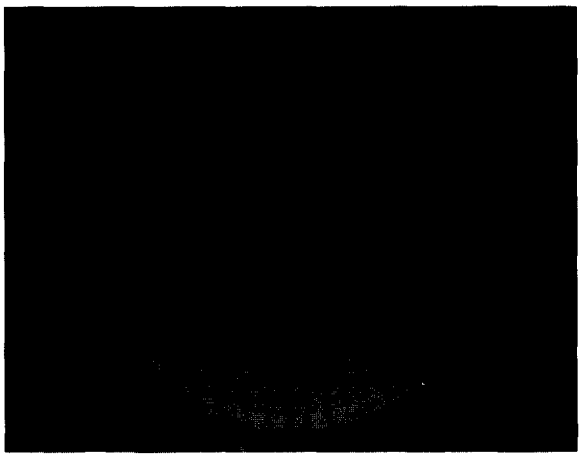

Fig. 1. Lysis of *Streptococcus mutans* on agar plate.
Bacillus subtilis YL-1004 was inoculated on a BHI agar plate containing a cell suspension of *Streptococcus mutans*. The diameter of the clear zone around the paper disc was measured.

Enzyme Production and Purification

The growth of *Bacillus subtilis* YL-1004 continued for 36 h until it reached the stationary phase, yet the lytic enzyme production rapidly increased after 6 h. The highest activity was monitored between 18 and 30 h, and after 36 h when the cell growth reached the stationary phase, the lytic

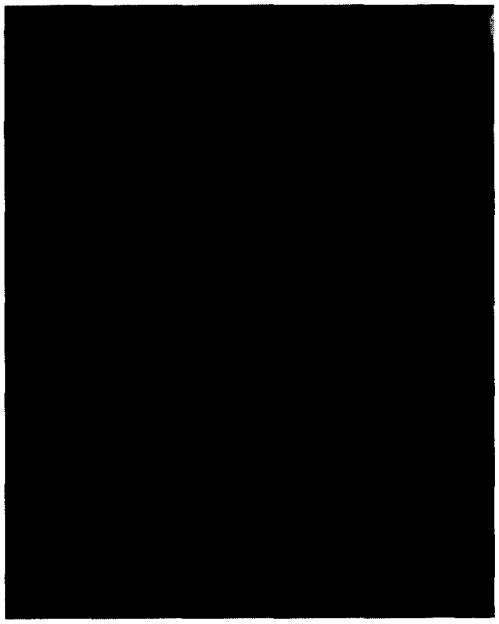

Fig. 2. Scanning electron microscopy of strain YL-1004.

Fig. 3. Transmission electron microscopy of strain YL-1004.

activity gradually decreased (data not shown). This was the similar phenomenon as observed for *N*-acetylmuramyl-L-alanine amidase from *Bacillus subtilis* 168 [37], *N*-acetylmuramidase from *Lactobacillus acidophilus* [3], and *N*-acetylmuramidase from *Streptococcus faecium* [17]. Therefore, it would appear that the lytic enzyme from *Bacillus subtilis* YL-1004 is related to the growth and enlargement of the cell wall peptidoglycan and cell division and separation. Pooley and Shockman [29, 30], Archibald and Coapes [1], and Koch [21] all previously reported an "inside-to-outside" growth mechanism. For rod-shaped Gram-positive bacteria, it has been shown that a newly synthesized peptidoglycan is first attached underneath the pre-existing peptidoglycan layer. This is followed by the specific cleavage of the covalent bonds in the stress-bearing layer by a peptidoglycan hydrolase or lytic enzyme. As a result, the new material is automatically pulled into the layer under stress.

The culture broth grown at 37°C for 24 h was centrifuged at 6,000 ×g for 5 min and the supernatant was used for enzyme purification. For the simple purification of the lytic enzyme, affinity adsorption chromatography was applied to cell pellet using *Streptococcus mutans*, as described in Materials and Methods. The lytic enzyme exhibited an affinity for the cell wall, interacted with the cell wall or cell pellet of the microorganism, and hydrolyzed the cell wall, thereby finally lyzing the cell. Purification fold through affinity adsorption chromatography was 146-fold. For further purification, the lytic enzyme was subjected to Sephadex G-75 gel permeation chromatography. Finally, 25% of the total activity was recovered (Table 2).

Table 1. Physico-chemical characteristics of strain YL-1004.

Characteristics	YL-1004
Shape	rod
Gram staining	positive
Spore	+
Catalase	+
Anaerobic growth	-
Acid from	
D-glucose	+
L-arabinose	+
D-xylose	+
D-mannitol	+
Hydrolysis of	
casein	+
gelatin	+
starch	+
Degradation of tyrosine	-
Determination of phenylalanine	-
Growth at pH	
6.8 nutrient broth	+
5.7	+
Growth at	
5°C	+
10°C	+
30°C	+
40°C	+
50°C	+
55°C	-
65°C	-

Through affinity adsorption chromatography using a *Streptococcus mutans* cell pellet and gel permeation chromatography, the lytic enzyme was purified to homogeneity. The resulting lytic enzyme migrated as a single band in SDS-PAGE and nondenaturing PAGE at a region of molecular weight of 38 kDa (Figs. 4A and 4B). Gel permeation chromatography with a Sephadex G-75 also exhibited the same size (data not shown). Other lytic enzymes or autolysins have also been reported from *Bacillus subtilis* strain 168. Two types of *N*-acetylmuramyl-L-alanine amidase with molecular weights of 30 kDa and 50 kDa, respectively, have been purified [37]. Yet, the current lytic enzyme from *Bacillus subtilis* YL-1004 exhibited a molecular weight of 38 kDa and possessed no amidase activity (data not shown). Isoelectric focusing of the lytic

Table 2. Purification table of lytic enzyme from *Bacillus subtilis* YL-1004.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Culture broth	4411.8	15000	3.4	100.0	1.0
Affinity chromatography	16.7	8320	496.4	55.5	146.0
Sephadex G-75	6.0	3750	625.0	25.0	183.8

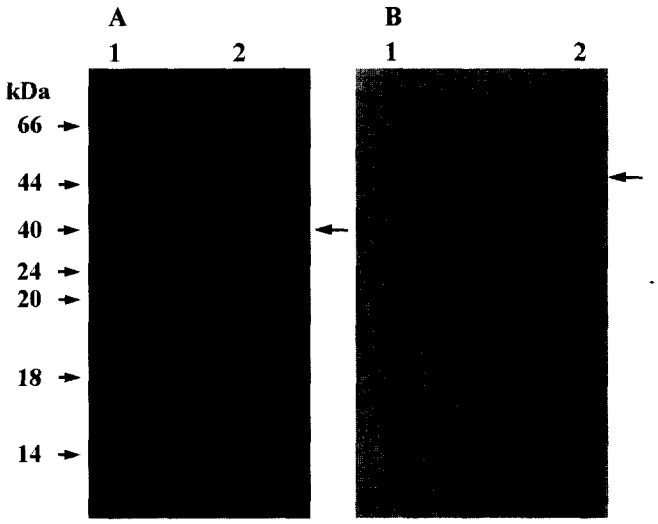

Fig. 4. SDS-PAGE (A) and nondenaturing PAGE (B) of lytic enzyme.
Panel A, lanes: 1, standard molecular weight markers (lysozyme, 14.4 kDa; trypsin inhibitor, 20 kDa; bovine trypsinogen, 24 kDa; bovine carbonic anhydrase, 29 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; egg ovalbumin, 45 kDa; bovine serum albumin, 66 kDa); 2, lytic enzyme. Panel B, lanes: 1, culture supernatant; 2, lytic enzyme from *Bacillus subtilis* YL-1004.

enzyme in native acrylamide gel electrophoresis showed that the pI of the lytic enzyme was 4.3 (Fig. 5). The lytic enzyme migrated as a single band slightly below the

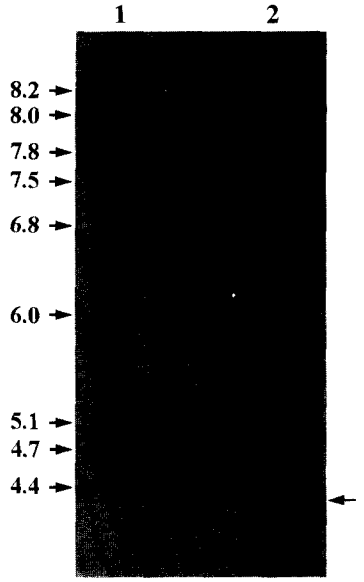

Fig. 5. Isoelectric focusing in nondenaturing acrylamide electrophoresis of lytic enzyme using wide range ampholytes (pH 3–10).
Lanes: 1, standard pI markers (phycocyanin, pI 4.45, 4.65, 4.75; β-lactoglobulin, pI 5.1; bovine carbonic anhydrase, pI 6.0; human carbonic anhydrase, pI 6.5; equine myoglobin, pI 6.8, 7.0; human hemoglobin A, pI 7.1; human hemoglobin C, pI 7.5; lentil lectin, pI 7.80, 8.00, 8.20; cytochrome c, pI 9.6); 2, lytic enzyme.

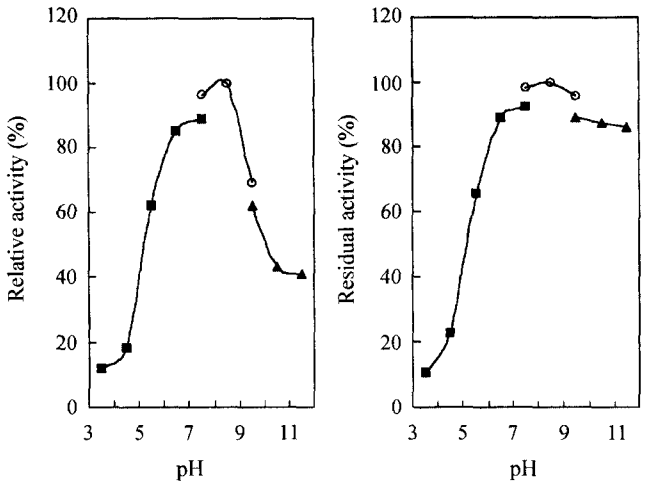

Fig. 6. Effect of pH on enzyme activity (A) and stability (B) of lytic enzyme.
A 50 mM Na-phosphate buffer, 50 mM Tris/HCl buffer, and 50 mM Glycine/NaOH buffer were used for pH 3–7, pH 7–9, and pH 9–11, respectively. For the enzyme stability test, the concentrated enzyme was dissolved in each buffer for 30 min and diluted with a 50 mM Tris/HCl buffer (pH 8.0). The highest point (pH 8.0) was considered as 100% when calculating the relative and residual activities.

standard pI marker, phycocyanin. In addition, it was found that the lytic enzyme from *Bacillus subtilis* exhibited its highest lytic activity at pH 8.0, at which point the enzyme was negatively charged.

Properties of Lytic Enzyme

The lytic activity was measured at various pHs and temperatures. The effect of pH on the enzyme activity and stability is shown in Fig. 6. When the enzyme was incubated at pH 6–9 for 30 min, more than 90% of the lytic activity remained, whereas only 12% of the residual activity was found at pH 4.0. The lytic enzyme exhibited its highest activity at pH 8.0, whereas only about 60% activity was observed at pH 5.0, and almost all activity was lost at pH 4.0. The effect of temperature on the enzyme activity and stability is shown in Fig. 7. The enzyme was found to be stable up to 40°C for 30 min, while only 64% of the lytic activity remained at 50°C. The optimal temperature for the lytic activity was 37°C.

Effect of Inhibitors on Lytic Activity

The effects of several inhibitors on the activity of the enzyme were examined by measuring the residual activity after incubation at 37°C for 10 min. The lytic activity of the enzyme was inhibited by 1 mM *p*CMB, iodoacetate, and ZPCK, yet not by PMSF, TPCK, NBS (*N*-Bromosuccinimide), and 2,3-butanedione. The *p*CMB and iodoacetate inhibited the enzyme activity down to 9.2% and 12.0% of the original activity (Table 3). The incubation of the enzyme for 10 min with 1 mM ZPCK, which irreversibly binds to the cysteine residue of proteins [5],

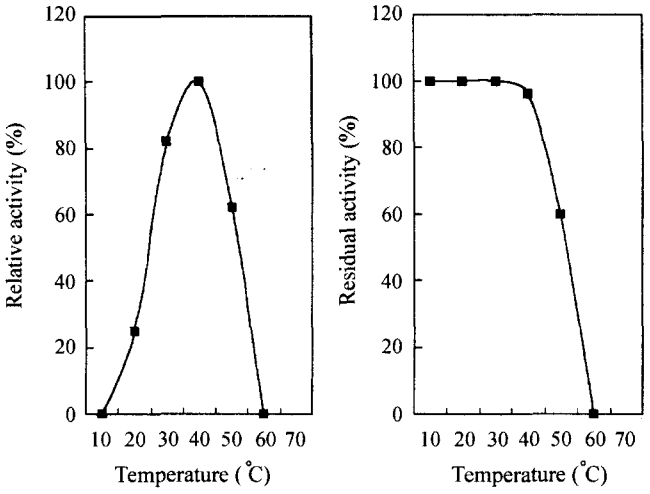

Fig. 7. Effect of temperature on enzyme activity (A) and stability (B) of lytic enzyme.
For the enzyme stability test, an enzyme solution was incubated at each temperature for 30 minutes and then applied to the enzyme activity assay at 37°C. The highest point was considered as 100% when calculating the relative and residual activities.

completely inhibited the lytic activity. NBS and 2,3-butanedione are known as inhibitors of the tryptophan and tyrosine residues of proteins, respectively [5], thereby indicating that none of these amino acids appeared to be involved in the active site of the lytic enzyme. PMSF and TPCK are known as inhibitors of the serine residue of proteins [5]. Therefore, serine also was not apparently involved in the active site of the lytic enzyme. 2,3-Butanedione degrades tryptophan and tyrosine residues very fast [5] under conditions including oxygen and light. When 2,3-butanedione was added to the enzyme solution at a concentration of 1 mM and irradiated with 366 nm of UV light, the lytic activity of the enzyme remained unchanged (data not shown), therefore implying that the

Table 3. Effect of chemical modifiers on lytic activity.

Chemical modifiers* (1 mM)	Activity (U/ml)	Residual activity (%)**
None	82.5	100.0
NBS	76.5	92.8
2,3-Butanodiene	82.5	100.0
*p*CMB	7.8	9.2
I_2	2.7	3.3
Iodoacetate	9.9	12.0
PMSF	78.6	95.9
TLCK	82.5	100.0
TPCK	68.4	82.9
ZPCK	0.0	0.0

*NBS, *N*-Bromosuccinimide; *p*CMB, *p*-chloromercuribenzoate; PMSF, phenyl methanesulfonyl fluoride; TLCK, L-1-chloro-3-(4-tosyl-amido)-7-amino-2-heptanone; TPCK, L-1-chloro-3-(4-tosyl-amido)-7-phenyl-2-butanone; ZPCK, N-CBZ-L-phenylalanine chloromethyl ketone.
**Residual activity was tested after incubation at 37°C for 10 min using chemicals.

Table 4. Lytic action spectra of lytic enzyme.

Strains		Activity (U/ml)	Relative activity (%)*
Streptococcus mutans	ATCC 25175	78.6	100.0
Streptococcus sobrinus	ATCC 27607	73.3	93.3
Streptococcus sanguis	ATCC 10556	75.0	95.4
Streptococcus salivarius	ATCC 9758	70.9	90.2
Lactobacillus helveticus	IFO 3809	9.8	12.5
Bifidobacterium infantis	ATCC 15697	25.2	32.1
Bacillus subtilis	KFCC 11316	69.2	88.0
Bacillus megaterium	KFCC 11776	75.6	86.2

*The lytic activity on *Streptococcus mutans* ATCC 25175 was considered as 100% when calculating the relative activity.

cysteine residue of the lytic enzyme seems to play an important role in the enzyme activity.

Lytic Action of Lytic Enzyme

The lytic enzyme was also tested against some oral and other bacteria (Table 4). Four species of Streptococci and several other bacteria were tested. All types of *Streptococcus mutans*, *Streptococcus sanguis*, and *Streptococcus salivarius* were found to be highly susceptible to the lytic enzyme, while *Lactobacillus helveticus* and *Bifidobacterium infantis* were relatively resistant. This result infers that the lytic enzyme has a specificity to cell wall types. The lytic enzyme from *Bacillus subtilis* YL-1004 showed a high lytic activity on *Bacillus* sp. This may have been due to the similarity in the cell wall structure between *Bacillus subtilis* YL-1004 and other *Bacillus* species. Therefore, it can be speculated from this result that the lytic enzyme may exhibit autolytic activity on the donor strain itself. Ghuysen and Hakenbeck [9] reported that several lytic enzymes are produced during the growth phase to produce or substitute new cell wall peptidoglycan. Several reports have suggested a correlation between a deficiency in autolytic activity and the failure of cells to separate several coccal and rod-shaped species [4, 7, 32, 35]. To confirm this, a cell pellet was suspended in 50 mM Tris/HCl (pH 8.0) to give an optical density of 1.0 and then tested using the lytic enzyme. The optical density of the reaction mixture decreased to 0.68 within 1 h (data not shown). Further studies on the properties of this lytic enzyme are in need to understand its role and action. The lytic mechanism and specificity of this lytic enzyme on several different cell wall types are currently under investigation.

DISCUSSION

Lytic enzymes have been reported from many kinds of organisms and these enzymes can be categorized into four groups, including *N*-acetylmuramidases, *N*-acetylglucosaminidases,

N-acetylmuramyl-L-alanine amidases, and endopeptidases, according to the mechanism of the lytic action [9]. Because of the diversity in the components and compositions of cell wall peptidoglycans, the lytic activities and lytic spectra are different from each other. Among these lytic enzymes, egg white lysozyme is widely used, yet *Streptococcus mutans* is less susceptible to lysozyme. Therefore, a lytic enzyme that hydrolyzes the *Streptococcus mutans* cell wall was screened from soil bacteria in this study and purified to homogeneity through affinity chromatography. The enzyme was found to have a molecular weight of about 38 kDa and exhibited autolytic activity on *Bacillus subtilis* itself. The lytic enzyme was produced extracellularly in the late logarithmic and stationary phases, indicating that the lytic enzyme may be associated with sporulation of the bacterium. Several kinds of lytic enzymes produced by *Bacillus* sp. have been reported to have autolytic activity and be related to spore formation [9]. The current authors also previously reported several lytic enzymes isolated from soil bacteria [18, 19, 23, 27]. Kim *et al.* [19] isolated two enzymes from *Bacillus licheniformis* with molecular weights of 27 kDa and 48 kDa. They also reported that neither of these enzymes showed any autolytic activity on synergistic effect with the other. Lee *et al.* [23] reported a lytic enzyme with a molecular weight of 24 kDa, which exhibited lytic activity on *Streptococcus mutans*. However, the lytic enzyme isolated in this study showed a different molecular weight from other such enzymes. *N*-Terminal amino acid sequence of the secreted lytic enzyme was determined to be Ser-Ala-. Foster [6] reported three major autolysins isolated from *Bacillus subtilis* 168, which had molecular weights of 30 kDa, 50 kDa and 90 kDa. One was identified as glucosaminidase, while the others were amidases. Therefore, the protease activity and glucanase activity of the current lytic enzyme were tested. The lytic enzyme showed protease activity on BSA, however, it has not yet shown any glucanase activity (data not shown).

The lytic enzyme isolated and characterized in this study has potential to be used in many ways, such as the treatment of dental caries and dental hygiene. Therefore, other studies, such as the long-term stability of this enzyme and its safety towards humans, should be performed. On the other hand, the external use of this enzyme to control dental caries can be acceptable without further safety data. Additional studies on the lytic mechanism and genetic information of this lytic enzyme are currently underway.

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