

## Purification and Characterization of Cell Wall Hydrolase from Alkalophilic *Bacillus mutanolyticus* YU5215

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**Abstract** *Streptococcus mutans* has the capacity of inducing dental caries. Thus, to develop a novel way of preventing dental caries, a cell wall hydrolase-producing strain was isolated and its characteristics were investigated. Among 200 alkalophilic strains isolated from soil, 8 strains exhibited lytic activities against *Streptococcus mutans*. However, strain YU5215 with the highest cell wall hydrolase activity was selected for further study. Strain YU5215 was identified as a novel strain of *Bacillus* based on analyzing its 16S rDNA sequence and *Bergey's Manual of Systematic Bacteriology*, and thus designated as *Bacillus mutanolyticus* YU5215. The optimal conditions for the production of the cell wall hydrolase from *Bacillus mutanolyticus* YU5215 consisted of glucose (0.8%), yeast extract (1.2%), polypeptone (0.5%), K<sub>2</sub>HPO<sub>4</sub> (0.1%), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.02%), and Na<sub>2</sub>CO<sub>3</sub> (1.0%) at pH 10.0. *Bacillus mutanolyticus* YU5215 was cultured at 30°C for 72 h to produce the cell wall hydrolase, which was then purified by acetone precipitation and CM-agarose column chromatography. The molecular weight of the lytic enzyme was determined as 22,700 Da by SDS-PAGE. When the cell wall peptidoglycan of *Streptococcus mutans* was digested with the lytic enzyme, no increase in the reducing sugars was observed, while the free amino acids increased, indicating that the lytic enzyme had an endopeptidase-like property. The amino terminus of the cell wall peptidoglycan digested by the lytic enzyme was determined as a glutamic acid, while the lytic site of the lytic enzyme in the *Streptococcus mutans* peptidoglycan was identified as the peptide linkage of L-Ala and D-Glu.

**Key words:** Cell wall hydrolase, *Streptococcus mutans*, *Bacillus mutanolyticus* YU5215, endopeptidase, D-glutamic acid

Dental caries is a progressive infectious disease initiated by complex interactions between oral bacteria, food debris, and oral saliva. As such, acidic glycoproteins in the saliva attach to the tooth surface, thereby providing oral bacteria a place to inhabit. *Streptococcus mutans*, one of the major oral bacteria, degrades sucrose to make insoluble glucan through the action of glycosyl transferase [8, 13, 16]. In turn, this insoluble glucan also attaches to the tooth surface, thereby providing other oral bacteria a place to inhabit. The resulting complex of glucan and various oral bacteria then creates an oral biofilm, a mature stage of dental plaque. At this stage, filamentous bacteria, such as Treponeme and Actinomycetes, form a major part of the oral biofilm. As dental plaque accumulates, acidic compounds, such as fructose and other fatty acids, degrade the enamel surface of the teeth, which is called dental caries [20, 30, 31, 33]. Since the first step of dental caries is the formation of insoluble glucan, the growth of *Streptococcus mutans* is a major cause of this disease [34, 36].

The basic methods used to prevent dental caries include brushing, antibiotic therapy, and the enzymatic degradation of dental plaque [2, 8, 32]. The mechanical eradication of dental plaque, for example with a toothbrush, is the most widely used method, yet the removal of oral bacteria is still limited. Meanwhile, antibiotic therapies, such as spiramycin, vancomycin HCl, and chlorohexidine, also have problems in relation to various side effects and resistance, and although the enzymatic degradation of dental plaque would seem to be an effective way of controlling dental caries, it is not a preventive method. Therefore, a new approach to preventing dental caries is needed that has specificity to oral pathogenic bacteria.

Accordingly, we previously isolated and purified several types of lytic enzymes from soil [26, 27, 38], and in the

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context of identifying anti-plaque agents, isolated several strains of bacteria that produce lytic enzymes against *Streptococcus mutans*. As such, in our present study, we isolated *Bacillus mutanoliticus* YU5215, which produces a cell wall hydrolase that is active against *Streptococcus mutans*. The cell wall hydrolase was then purified and its mechanism was characterized.

## MATERIALS AND METHODS

### Screening of *Streptococcus mutans* Cell Wall Hydrolase

The *Streptococcus mutans* ATCC25175 was cultured in 10 l of a BHI broth (Difco Co.) for 3 days at 37°C and harvested by centrifugation. The collected cell pellet of *Streptococcus mutans* was resuspended in 50 ml of 0.9% saline and autoclaved, then the autoclaved cells were added to a sterile Horikosh-1 (1% glucose, 0.5% polypeptone, 0.5% yeast extract, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.02% MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0% NaHCO<sub>3</sub>, 1.5% agar at pH 10.2) medium at a ratio of 1:99 (v/v) to make a Horikosh/SM agar plate. To screen for cell wall hydrolase-producing bacteria, soil samples were collected from several areas in Korea, suspended in 0.9% sterile saline, then plated onto the Horikosh-1 medium and incubated at 30°C for 3 days. Next, the strains isolated from the soil samples were transferred onto a Horikosh/SM agar plate using a toothpick and incubated at 30°C for three days. Following incubation, the resulting cell wall hydrolase-producing strains created zones around the colonies.

### Isolation of Cell Wall Peptidoglycan from *Streptococcus mutans*

The cell wall peptidoglycan was isolated using the method of Potvin *et al.* [28] with a slight modification. A *Streptococcus mutans* cell culture broth was collected by centrifugation and washed with a 25 mM Tris-HCl (pH 9.0) buffer. The collected cell pellet was then freeze-dried and 1 g of the dried cells suspended in 80 ml of a 4% (w/v) SDS solution. Next, the cell suspension was incubated for 90 min at room temperature with shaking, followed by sonication at 0°C for 20 min. To inactivate any autolysins still incorporated in the cell extract, the suspension was boiled at 100°C for 15 min, then the undisturbed cells removed by centrifugation at 6,000 ×g for 10 min. The cell extract was collected again by centrifugation at 15,000 ×g for 15 min, then the pellet was resuspended in 80 ml of a 1% Triton X-100 solution, incubated at room temperature for 30 min with shaking, and re-collected by centrifugation. Thereafter, the pellet was washed with 80 ml of sterile distilled water three times and suspended in a minimal amount of a 0.02% (w/v) sodium azide solution. The resulting cell wall peptidoglycan suspension was then stored at 4°C and used for further studies.

### Assay for Cell Wall Hydrolytic Activity

The bacteriolytic enzyme activity was determined using a modified version of the method of Hayashi and Kasumi [14]. Lyophilized *Streptococcus mutans* cells were suspended in a 50 mM Tris/HCl buffer (pH 9.0) to give an initial absorbance of 1.0 at 660 nm. A 0.1 ml of the enzyme solution was then added to 2.9 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, and one unit of bacteriolytic enzyme activity was defined as the amount of enzyme that caused a decrease in the absorbance of 0.001 per min.

### 16S rDNA Sequencing

The 16S rDNA was amplified through a polymerase chain reaction (PCR) using chromosomal DNA as a template. The primers used for this reaction were: forward (Eubacterial 27F: 5'-AGA GTT TGA TCM TGG CTC AG-3'); reverse (Universal 1492R: 5'-GGY TAC CTT GTT ACG ACT T-3'). A thermocycler (Perkin-Elmer, USA) was used for the amplification. After a denaturing step at 94°C for 4 min, 30 cycles of reactions were executed under the reaction conditions of 2 min at 94°C, 1 min at 55°C, and 2 min at 72°C. After being developed on a 0.8% agarose gel, the PCR product was excised and purified using a Bio101 gene cleaning kit (Bio-Rad, U.S.A.). The purified PCR product was then cloned into a pGEM-T vector (Promega Co.) for maintenance, and a PCR fragment of about 1.5 kbp used for sequencing through an ALF Red automated DNA sequencer (Pharmacia, Uppsala, Sweden).

### Quantitative Analysis of Free Amino Acids and Reducing Sugar

The cell wall peptidoglycan was suspended in a 10 mM Tris-HCl (pH 9.0) buffer to create an optical density of 1.0 at 660 nm. One ml of the enzyme solution was then added to 29 ml of the suspension and incubated at 60°C. One ml of this reaction mixture was collected at each time point, centrifuged, 500 µl of the supernatant freeze-dried, then resuspended in 120 µl of distilled water for the following assays.

A quantitative analysis of the free amino acids in the sample suspensions was carried out using the method of Ghuyssen and Hackenbeck [9] with slight modifications. A sodium borate reagent (1% Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O, w/v, 80 ml) and 0.1 M FDNB (1-fluoro 2,4-dinitrobenzene in 99% ethanol, Sigma Co., 10 µl) were added to 20 µl of the above sample suspensions and incubated at 60°C for 30 min. After incubation, 300 µl of 4 N HCl was added and the samples were incubated at 100°C for 2 h for hydrolysis. The optical density at 420 nm was measured and the standard curve was constructed with glycine (0–2.0 µmol/ml).

The amount of reducing sugar was measured using the DNS method [1], and the standard curve was constructed with glucose (0–15.0 µmol/ml).

### N-Terminal Amino Acid Analysis of Cell Wall Hydrolase

Edmann degradation and a peptide sequencer (Milligen 6600B) were used for the *N*-terminal sequencing. After SDS-PAGE, the polyacrylamide gel and a PVDF membrane were soaked in a blotting buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3) for 30 min. The purified cell wall hydrolase in the polyacrylamide gel was then transferred to the PVDF membrane using a Mini Trans Blot Assembly (Bio-Rad) at 100 V for 1 h. The membrane on which the protein was blotted was stained with 0.2% (w/v) coomassie brilliant blue R-250, 50% methanol, and 10% acetic acid for 1 min and destained with 5% methanol. The stained protein band was then excised and used for the *N*-terminal amino acid sequencing.

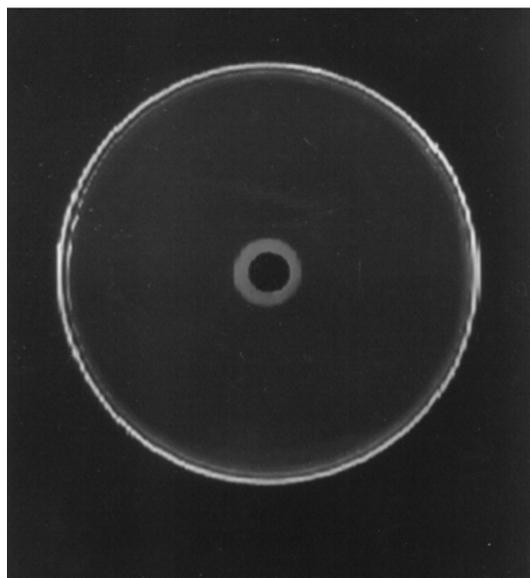
### Analysis of N-Terminal Amino Acid of Digested Peptidoglycan

The *N*-terminal amino acid in the peptidoglycan digested by the cell wall hydrolase was determined using the method of Ghuysen and Hackenbeck [9]. As such, the digested peptidoglycan was freeze-dried and resuspended in 300 ml of 1% Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, then 60 µl of 0.1 M FDNB (1-fluoro-2,4-dinitrobenzene) was added and the mixture incubated at 60°C for 30 min. Any remaining FDNB was broken down by adding 180 µl of conc. HCl. The free DNP (dinitrophenyl) derivatives were removed by extracting with ethyl ether three times and evaporating in a 37°C water bath. The DNP-peptide in the water phase was purged with N<sub>2</sub> gas and hydrolyzed at 110°C for 16 h, then extracted with ethyl ether and freeze-dried. Next, the hydrolyzed DNP-peptide was dissolved in 20 µl of 0.1 N NH<sub>3</sub> and thin layer chromatography applied using a Silica gel-60 F<sub>254</sub> plate (Merck Co.). The developing solution was prepared by mixing chloroform, methanol, and acetic acid at 85:14:1, respectively. DNP-derivatives of D-alanine, L-alanine, D-glutamic acid, and L-lysine were all used as standards.

## RESULTS AND DISCUSSION

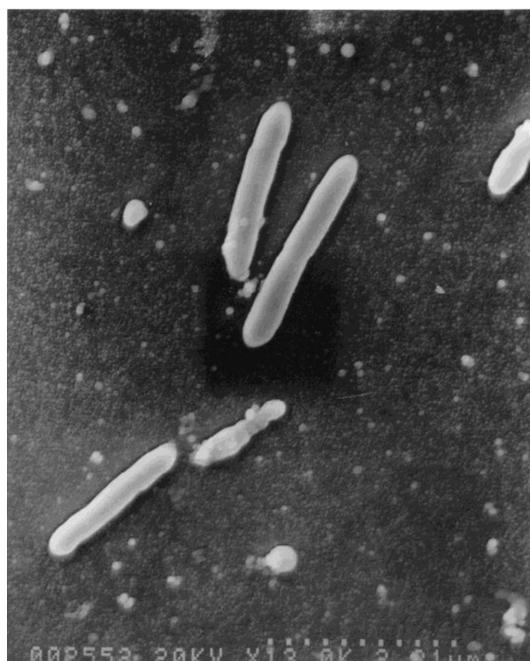
### Screening and Identification of Microorganism

Several strains were isolated that exhibited cell wall hydrolytic activity towards *Streptococcus mutans*. First, 16 candidate strains were selected that showed a clear zone on an agar plate containing *S. mutans*. Among these 16 strains, 8 strains that showed a relatively larger clear zone were cultured in 50 ml of a Horikosh-1 broth at 30°C for 24, 36, 48, 60, and 72 h to confirm the cell wall hydrolytic activity. After centrifuging the culture broths, a cell wall hydrolytic activity assay was applied to each culture supernatant according to the method described in Materials and Methods. Strain YU5215 exhibited the highest lytic activity among them and its activity was also confirmed on a Horikosh/SM agar plate (Fig. 1).



**Fig. 1.** Lysis of *Streptococcus mutans* on Horikosh/SM agar plate by cell wall hydrolase produced by strain YU5215. A paper disc (5 mm in diameter) pretreated with 100 µl of the culture supernatant was applied to a Horikosh/SM agar plate containing a cell suspension of *Streptococcus mutans*.

For the identification of strain YU5215, Gram staining and scanning electron microscopy (Hitachi S-800) were carried out. Strain YU5215 was observed to be motile, about



**Fig. 2.** Scanning electron microphotograph of strain YU5215. The cells were cultured in a Horikosh-1 broth for 3 days at 30°C and dehydrated. A Hitachi S-800 scanning electron microscope was used. Magnitude: ×13,000.

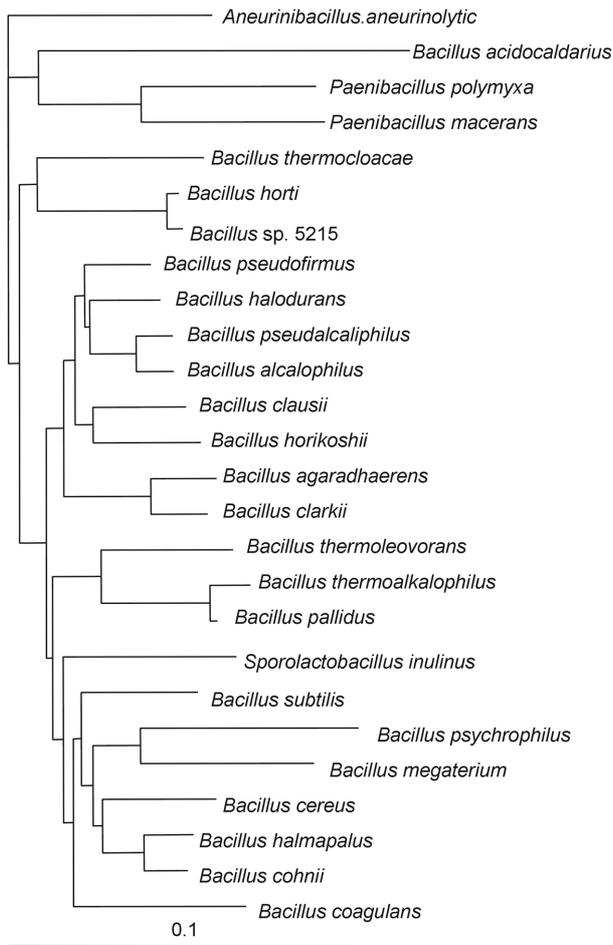
2 µm long (Fig. 2), Gram-positive, an endospore forming rod, and strict aerobe. As such, it was identified as a *Bacillus* sp. Based on a 16S rDNA sequence analysis, it was revealed that the 16S DNA of strain YU5215 was composed of 1,522 bp nucleotides and showed a 98% homology with the alkalophilic *Bacillus horti*. The sequence for strain YU5215 was registered in the GenBank under accession number

BankIt210541. A phylogenetic tree using the CLUSTAL W program also showed a high homology to *Bacillus horti* (Fig. 3). Although the 16S rDNA sequence of strain YU5215 was highly homologous, it was still considered to be a new strain and named *Bacillus mutanolyticus*.

**Production and Purification of Cell Wall Hydrolase from *Bacillus mutanolyticus* YU5215**

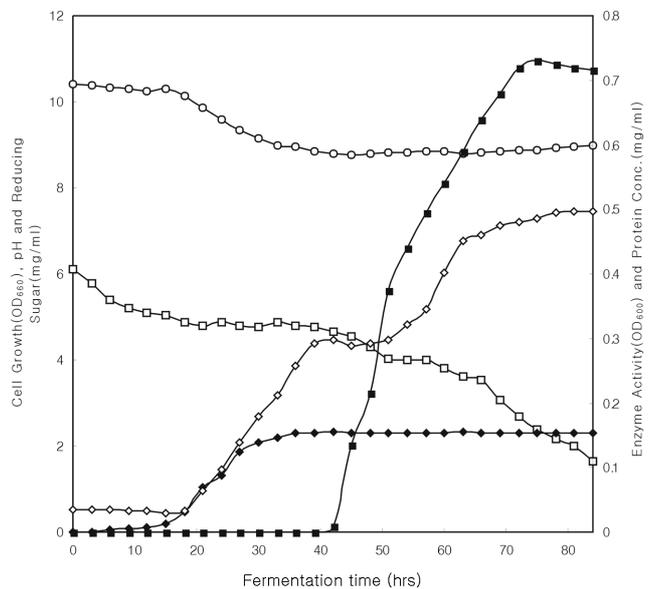
*Bacillus mutanolyticus* YU5215 was cultured under various conditions to optimize its growth and enzyme production. As such, the optimal conditions for the production of the cell wall hydrolytic enzyme were 0.8% glucose, 0.5% polypeptone, 1.2% yeast extract, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.02% MgSO<sub>4</sub>·7H<sub>2</sub>O, and 1.0% Na<sub>2</sub>CO<sub>3</sub> at pH 10.0. *Bacillus mutanolyticus* YU5215 was inoculated into 5 ml of this medium and cultured at 30°C overnight as a seed culture, then transferred into 50 ml of the medium and incubated overnight as a mid-culture. Next, the mid-cultured broth was inoculated into a 5-l fermenter containing 4 l of the enzyme production medium at 350 rpm, 3.5 vvm, and 30°C. The bacterial growth increased for 36 h, then reached a steady state. Meanwhile, the cell wall hydrolytic enzyme was produced from 42 h and reached maximum production after 74 h. The pH of the culture broth gradually decreased to 9.0 until 36 h, then remained at 8.9 (Fig. 4).

To purify the cell wall hydrolase, the culture supernatant was collected after centrifugation, followed by sequential

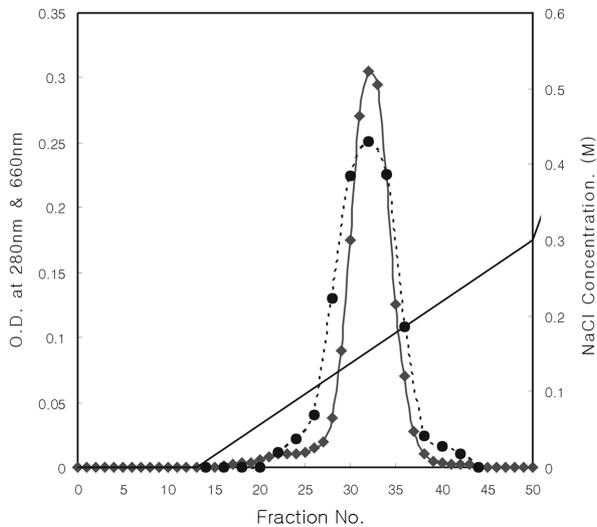


**Fig. 3.** Phylogenetic tree showing relationship between isolated alkalophilic *Bacillus* sp. 5215 and other species belonging to the genus *Bacillus*.

Scale bar, 0.1 estimated substitution per nucleotide position. *Aneurinibacillus aneurinolyticus* ATCC 12856, D78455; *Bacillus horti* K13, D87035; *Bacillus subtilis* NCDO 1769, X60646; *Bacillus thermoalkalophilus* DSM 6866, Z26931; *Paenibacillus polymyxa* NCDO 1774, X60632; *Bacillus thermocloacae* DSM5250, Z26939; *Sporolactobacillus inulinus* NRIC 1134, D16284; *Bacillus coagulans* NCDO 1761, X60614; *Bacillus cereus* NCDO 1771, X55060; *Bacillus thermoleovorans* JCM 7361, Z26923; *Bacillus psychrophilus* ATCC 23304, X60634; *Bacillus megaterium* DSM 32, X60629; *Paenibacillus macerans* NCDO 1764, X60624; *Bacillus pseudocaliphilus* DSM8725, X76449; *Bacillus halmapalus* DSM8723, X76447; *Bacillus agaradhaerens* DSM8721, X76445; *Bacillus alcalophilus* DSM485, X76436; *Bacillus clarkii* DSM8720, X76444; *Bacillus halodurans* DSM8718, X76442; *Bacillus clausii* DSM8716, X76440; *Bacillus pseudofirmus* DSM8715, X76439; *Bacillus cohnii* DSM6307, X76437; *Bacillus acidocaldarius* DSM 446, X60742; *Bacillus horikoshii* DSM8719, X76443; *Bacillus pallidus* DSM3670, Z26930.



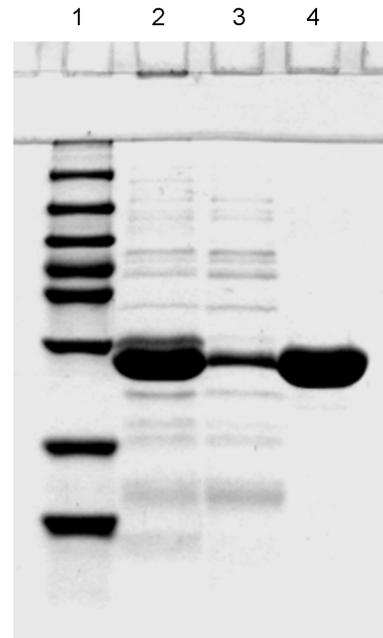
**Fig. 4.** Time course of cell wall hydrolase production in a 4-l jar fermenter. *Bacillus mutanolyticus* YU5215 was inoculated into 5 ml of the medium and cultured at 30°C overnight as a seed culture. The seed culture was then transferred to 50 ml of the medium and incubated overnight as the mid-culture. Finally, the mid-cultured broth was inoculated into a 5-l fermenter containing 4 l of the enzyme production medium at 350 rpm, 3.5 vvm, and 30°C. ◆, cell growth; ◇, protein concentration; □, reducing sugar; ■, enzyme activity; ○, pH.



**Fig. 5.** CM-agarose column chromatogram of cell wall hydrolase produced by *Bacillus mutanolyticus* YU5215.

A dialyzed acetone precipitate was applied to CM-agarose column chromatography. The crude enzyme solution was loaded and passed through the CM-agarose column pre-equilibrated with a 10 mM sodium phosphate (pH 6.0) buffer. The proteins absorbed to the column were eluted with 0.0–1.0 N NaCl as a continuous gradient. ◆, O.D. at 280 nm; ●, activity ( $\Delta$ O.D. at 600 nm); —, NaCl gradient.

purification steps, acetone precipitation, CM-agarose chromatography, and PM-10 membrane ultrafiltration. As such, ethyl alcohol, methyl alcohol, or acetone were added to the culture supernatant, the mixture agitated slowly at 4°C, then centrifuged at 8,000  $\times g$  for 20 min, and the pellets suspended using a 50 mM Tris/HCl (pH 9.0) buffer. Among several alcohol precipitations, 73% of the total enzyme activity was recovered when 75% (v/v) acetone was used. Therefore, this crude enzyme solution, acetone precipitant, was freeze-dried, resuspended in a 10 mM sodium phosphate (pH 6.0) buffer, and dialyzed. Successive CM-agarose column chromatography was then applied to the dialyzed crude enzyme solution. When the column was eluted with 0–1.0 N NaCl, most of the enzyme activity was eluted at 0.15 N NaCl (fraction nos. 27–37) (Fig. 5). The active fractions were then collected and concentrated using an ultrafiltration kit (Model KMC-86S, KMC Co.; PM-10 membrane, Amicon). The purified enzyme exhibited a single band in SDS-PAGE with a molecular weight of 22.7 kDa (Fig. 6). From 4 l of culture broth (850 mg of total protein), 130 mg of the purified enzyme was recovered



**Fig. 6.** SDS-PAGE of cell wall hydrolase produced by *Bacillus mutanolyticus* YU5215.

Lanes 1, protein markers; 2, culture broth; 3, acetone precipitation; 4, CM-agarose chromatography.

and about 41% of enzyme activity was recovered. The procedures used to purify the cell wall hydrolase are also summarized in Table 1.

#### ***N*-Terminal Amino Acid Analysis of Cell Wall Hydrolase**

SDS-PAGE was applied to the purified cell wall hydrolase, which was also transferred to a PVDF membrane. After Edmann degradation, a peptide sequencer (Milligen 6600B) was used for *N*-terminal sequencing. The amino acid sequence of the active cell wall hydrolase from *Bacillus mutanolyticus* YU5215 was Gln-Ser-Ile-Pro-Trp-Gly-Ile-Thr-Arg-Val (Table 2), which showed homology to an alkaline protease [11], serine protease [24], and subtilisin [10], yet not an exact match.

#### **Hydrolysis of Cell Wall Peptidoglycan**

To elucidate the mechanism of the cell wall hydrolase produced by *Bacillus mutanolyticus* YU5215, the cell wall peptidoglycan was digested and the free amino acids and reducing sugar were analyzed. As shown in Fig. 7,

**Table 1.** Purification process of lytic enzyme from *Bacillus mutanolyticus* YU5215.

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Fold
Culture broth	850	1,546,000	1,819	100	1
Acetone precipitation	440	1,135,600	2,581	73	1.42
CM-agarose chromatography	140	684,500	4,889	44	2.69
Ultrafiltration	130	635,000	4,185	41	2.69

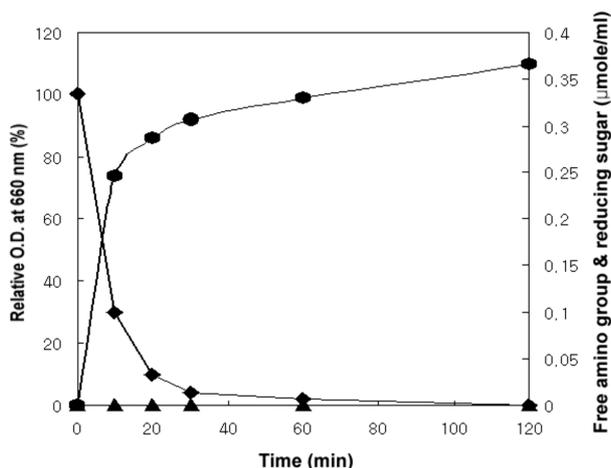
**Table 2.** Amino terminus of lytic enzyme produced by *Bacillus mutanolyticus* YU5215.

Proteins	Amino terminus	GenBank accession no.
<i>Bacillus</i> sp. 5215	QSIPWGITRV	BankIt210541
Alkaline protease	AQSVPWGISRV	1094073
Serine protease	AQSVPWGISRV	3114348
Subtilisin	QSVPWGISRV	267046

the optical density of a reaction mixture containing the cell wall peptidoglycan and enzyme solution at 660 nm was reduced when incubated at 60°C, indicating that the peptidoglycan was degraded by the enzyme. In addition, the amount of free amino acid in the reaction mixture rapidly increased as soon as the reaction started, while the amount of reducing sugar remained constant.

#### N-Terminal Amino Acid of Digested Peptidoglycan

Since the cell wall hydrolase exhibited endopeptidase activity, to determine the amino-terminal amino acid of the peptide subunit released from the peptidoglycan digested by the cell wall hydrolase, the *N*-terminal amino acid of the peptide subunit was dinitrophenylated with 0.1 M FDNB and developed on silica gel thin layer chromatography. As a result, the *N*-terminal amino acid released by the enzyme digestion was determined to be a D-glutamic acid (Fig. 8), indicating that the cell wall hydrolase produced by *Bacillus mutanolyticus* YU5215 was an endopeptidase that specifically

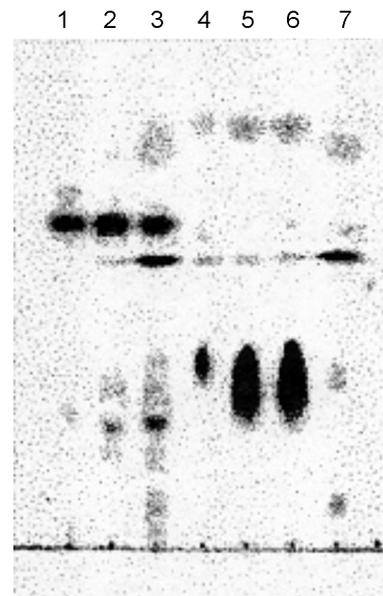
**Fig. 7.** Release of free amino acids and reducing sugar from peptidoglycan digested by cell wall hydrolase of *Bacillus mutanolyticus* YU5215.

The *Streptococcus mutans* cell wall peptidoglycan suspended in a 10 mM Tris-HCl (pH 9.0) buffer was used as the substrate. The free amino acids was dinitrophenylated with 0.1 M FDNB, then the optical density at 420 nm was measured and a standard curve made with glycine (0–2.0 μmol/ml). The amount of reducing sugar was measured using the DNS method [1]. The standard curve was made with glucose (0–15.0 μmol/ml). ◆, Relative O.D. at 660 nm; ●, free amino group; ▲, reducing sugar.

hydrolyzed the peptide bond related to the amino group in glutamic acid (Fig. 9).

*Streptococcus mutans* was first isolated by Clarke [3] and has been reported to induce dental caries [12, 13, 25]. *Streptococcus mutans* can adhere to the tooth surface and produce water insoluble glucan from sucrose [7], which enables Streptococci to colonize the tooth surface. Since several bacteriolytic proteins have been reported in many microorganisms [21, 22], we attempted to identify novel cell wall hydrolytic enzymes with a specificity for *Streptococcus mutans*. Plus, as a novel strategy for controlling dental caries, cell wall hydrolase-producing strains were isolated and their characteristics were investigated. Among 200 alkaliphilic strains isolated from soil, strain YU5215 showed the highest cell wall hydrolytic activity. Through identification processes according to *Bergey's Manual of Systematic Bacteriology*, strain YU5215 was identified as a *Bacillus* sp., then 16S rDNA sequencing was carried out for further identification. Although the 16S rDNA sequence of strain YU5215 was highly homologous to *Bacillus horti* [39], *Bacillus horti* has been reported as a Gram-negative bacillus. Therefore, strain YU5215 was concluded to be a new strain and named *Bacillus mutanolyticus*.

Bacterial cell wall peptidoglycans consist of glycan strands and a peptide subunit, therefore, the peptidoglycan

**Fig. 8.** Silica gel thin layer chromatogram of *Streptococcus mutans* cell wall peptidoglycan digested by cell wall hydrolase of *Bacillus mutanolyticus* YU5215.

The hydrolyzed DNP-peptide was dissolved in 20 μl of 0.1 N NH<sub>3</sub> and applied to thin layer chromatography with Silica gel-60 F<sub>254</sub> plate. The developing solution was prepared by mixing chloroform, methanol, and acetic acid in a ratio of 85:14:1, respectively. Lanes 1, lytic enzyme; 2, intact cell wall peptidoglycan; 3, cell wall peptidoglycan digested by lytic enzyme; 4, DNP-L-lysine; 5, DNP-L-alanine; 6, DNP-D-alanine; 7, DNP-D-glutamic acid.



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