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Purification and Biochemical Characterization of Bacteriolytic Enzyme from *Bacillus subtilis* YU-1432 Active against *Porphyromonas gingivalis*

Han-Seung Lee[1] and Hyungjae Lee[2]*

[1]*Department of Bio-Food Materials, College of Medical and Life Sciences, Silla University, Busan 617-736, Republic of Korea*

[2]*Institute of Life Sciences & Resources and Graduate School of Biotechnology, Kyung Hee University, Yongin 446-701, Republic of Korea*

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YU205, a bacteriolytic enzyme produced by *Bacillus subtilis* YU-1432 exhibited lytic activity against *Porphyromonas gingivalis* causing periodontal disease. Specific activity and purification yield of YU205 were increased by 522.0 times and 21.9%, respectively by 80% acetone precipitation, followed by DEAE-Sepharose and CM-Sepharose column chromatography. The molecular mass of YU205 was estimated to be 29.0 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and N-terminal sequencing identified fifteen amino acid residues, AQSVPYGISQIKAPA. YU205 was stabilized against thermal inactivation at 1 mM of $CaCl_2$. The essential amino acids for the lytic activity were deduced to be tyrosine, methionine, and serine. YU205 showed at least 23.4 times higher substrate specificity to *P. gingivalis* than other proteolytic enzymes tested. These results suggest that the purified enzyme may have potential for the application to food or dental care products to prevent periodontal diseases.

Key words: *Bacillus subtilis*, lytic enzyme, periodontal disease, *Porphyromonas gingivalis*, substrate specificity

Periodontal diseases including gingivitis and periodontitis are inflammatory responses on subgingival tissues surrounding teeth, which are caused by several Gram-negative, anaerobic, pathogenic microorganisms in dental plaque. *Porphyromonas gingivalis* is a Gram-negative, rod-shaped, anaerobic bacterium and has been found to cause severe chronic periodontal disease [Loesche and Grossman, 2001]. It has been known that formation of dental plaque on teeth aggravates periodontal infection of *P. gingivalis* by providing favorable, anaerobic growth condition. Periodontitis can progress to irreversible destruction of alveolar bone around teeth, and consequentially causing teeth loss without subjective symptoms. Moreover, periodontal diseases have been assumed to be implicated in cardiovascular diseases including atherosclerosis, stroke, and myocardial infarction [Seymour *et al.*, 2007].

Several control methods have been developed to prevent periodontal diseases. Antibiotics such as tetracyclines, metronidazole, and chlorhexidine have been mainly used to treat periodontal diseases [Mombelli and Samaranayake, 2004; Horz and Conrads, 2007], However, the antibiotics can inhibit the growth of beneficial microorganisms in oral microbiota. In addition, overuse of the antibiotics may result in a high incidence of antibiotic-resistant periodontal microorganisms. Scaling, root planning or surgery can physically remove dental plaques and infected tissues. However, the causative microorganisms can recur in the treated tissues [Bollen and Quirynen, 1996]; therefore, because these current control methods cannot effectively prevent the diseases, development of alternative or complementary control methods are necessary.

The genus *Bacillus* has been known to produce several lytic enzymes active against pathogenic bacteria [Yu, 1997]. In previous studies, several alkaliphilic *Bacillus* spp. were reported to produce lytic enzymes degrading the target bacterial cell walls [Jung *et al.*, 1991; Yu *et al.*, 1992; Jung *et al.*, 1993]. Moreover, two lytic enzymes active against *Streptococcus mutans* causing dental caries were purified and characterized from two different

*Corresponding author
Phone: +82-31-201-2123; Fax: +82-31-204-8116
E-mail: hl252@khu.ac.kr

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Bacillus spp. [Lee *et al.*, 1997; Kim *et al.*, 1999].

In the present study, bacterial strains were isolated from soil to screen for the production of lytic enzyme specifically active against *P. gingivalis*. A lytic enzyme was purified from one of the bacterial isolate showing the highest level of the lytic activity and its amino acids on the N-terminus were determined. In addition, the enzyme was characterized to determine the essential amino acid residues for the activity, and the substrate specificity of the enzyme to *P. gingivalis* was investigated.

Materials and Methods

Preparation of *P. gingivalis*. *P. gingivalis* W50 was grown in *P. gingivalis* broth (PGB) medium: 1.85% (w/v) brain heart infusion (BHI; BD, Sparks, MD) supplemented with 0.5% (w/v) yeast extract (BD), 1 μg/mL hemin (Sigma, St. Louis, MO), 5 μg/mL menadione (Sigma) and 0.05% (w/v) cysteine (Sigma). After the medium was equilibrated under anaerobic condition (85% N_2, 5% H_2, and 10% CO_2) for 24 h, *P. gingivalis* W50 was cultured in the pretreated medium under the same anaerobic condition for 48 h. To prepare the substrate cells for lytic activity assay, the culture broth of *P. gingivalis* W50 was centrifuged at 6,000 *g* for 10 min. The harvested cells were rinsed twice with 0.85% (w/v) NaCl and stored at −20°C prior to use. To isolate *P. gingivalis* colonies, 5% (v/v) sheep blood and 1.5% (w/v) agar was added to the PGB medium. *P. gingivalis* culture plates were incubated at 37°C in anaerobic jars with GasPak™ EZ (BD).

Screening and identification of lytic enzyme producer. Soil samples collected in Korea were suspended with 5 mL of sterile 0.85% (w/v) NaCl and left at room temperature for 1 h. The supernatant of the suspension was spread on an isolating medium [1.0% (w/v) glucose, 0.5% (w/v) yeast extract, and 1.5% (w/v) agar]. The plates were incubated at 30°C for 72 h or until bacterial colonies were observed. To screen bacterial strains exhibiting bacteriolytic activity against *P. gingivalis*, the bacterial isolates were spotted onto the isolating medium containing *P. gingivalis* W50 cells as a substrate to 10% (v/v) final concentration. After incubation at 30°C for 3 days, bacterial isolates exhibiting clear zone formed by lysis of the substrate cells were selected.

A bacterial strain (YU-1432) showing the largest inhibition zone was selected as a lytic enzyme producer. The isolate was identified by three different methods: API 50 CHB, a Gram-positive bacteria identification kit (bioMérieux Korea, Seoul, Korea); analysis of the fatty acid composition of the cell wall using gas chromatography; and 16S rDNA sequencing. Chromosomal DNA of the producer was extracted as described by Sambrook and Russell [2001] and used as a template for amplification of 16S rDNA by polymerase chain reaction (PCR). The PCR was performed using a set of primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') complementary to a conserved sequence in 16S rRNA genes of bacteria [Lane, 1991]. The PCR conditions were as follows: 1 cycle at 94°C for 3 min, 30 cycles composed of 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min, followed by 1 cycle of 72°C for 8 min. The PCR product was sequenced and analyzed by NCBI BLAST homology search (BLASTN) to identify the producer strain.

Lytic activity assay. Lytic activity was assayed using a modified method of Hayashi *et al*. [1981]. The harvested *P. gingivalis* W50 cells were resuspended with 50 mM Tris-HCl (pH 7.6) to 1.0 of absorbance at 660 nm. Two milliliters of the suspension was mixed with 200 μL of enzyme solution, and incubated at 37°C for 10 min. After incubation, the absorbance of the mixture was measured at 660 nm. One unit of lytic activity is defined as the amount of enzyme required to decrease absorbance at 660 nm by 0.001 for 1 min.

Purification and characterization of lytic enzyme. The producer strain was cultured in 3 L of a production medium [1% (w/v) glucose, 0.5% (w/v) yeast extract, and 0.5% (w/v) polypepton (pH 7.0)] in a 5-L jar fermenter (KFC SY-500, KFC, Inchon, Korea) at 30°C for 16 h with stirring (60 rpm). After removing cells by centrifugation at 6,220 *g* for 15 min, 4 volumes of pre-chilled 100% acetone was added to 1 volume of the supernatant. The mixture was then gently stirred at 4°C for 6 h. The resulting precipitate was separated by centrifugation at 17,000 *g* for 15 min and resuspended with 50 mL of sterile distilled water. The precipitate was loaded onto DEAE-Sepharose Fast Flow (Sigma), equilibrated with buffer A [10 mM Tris-HCl (pH 8.6)]. The column was sequentially washed with buffer A and 0.1 M NaCl in buffer A. The active fractions from the column were pooled, adjusted to pH 6, and then loaded onto CM-Sepharose Fast Flow (Sigma), equilibrated with buffer B [10 mM sodium phosphate-citric acid (pH 6.0)]. The column was washed with buffer B first, and then eluted with 0.1 and 0.3 M NaCl in buffer B. The active fractions from CM-Sepharose were pooled and stored at −20°C. The protein elution from chromatography was monitored at 280 nm. Protein concentration was determined by the method of Bradford [1976] using bovine serum albumin (BSA) as a standard (Sigma).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and N-terminal sequencing. SDS-PAGE (12% running gel) was performed at 20 mA for 2 h. The purified lytic enzyme was loaded on the gel

and electrophoresized. After electrophoresis, one of the duplicate SDS-PAGE gels was stained with 0.05% Commassie brilliant blue R-250 (Sigma) for 2 h, followed by destaining with 10% acetic acid. The other gel was transferred onto polyvinylidene difluoride (PVDF) membrane by electroblotting using a Mini Trans-Blot® Cell (Bio-Rad, Hercules, CA). The band corresponding to the enzyme on the membrane was sequenced to determine the N-terminal sequences of the enzyme by Edman degradation.

Biochemical characterization of lytic enzyme. pH and temperature stability of the lytic enzyme was determined after incubation for 2 h between pH 3 to 11 and between 4 and 80°C, respectively. The effect of metal ions (1 mM) on the lytic enzyme was determined after 10 min incubation of the enzyme at room temperature in fifteen metal ions (Table 2). After the incubation, the residual activity of the enzyme was measured. The effects of the eight chemical modifiers (5 mM) as inhibitors of the lytic enzyme were determined by measuring the residual activity of the enzyme after 10 min incubation at room temperature (Table 3).

Protease activity assay. Protease activity was assayed with a modified protocol of Dosoretz *et al.* [1990]. Azocasein (Sigma) was suspended in 50 mM Tris-HCl (pH 7.6) to 2% final concentration. The prepared azocasein solution (250 μL) was mixed with 150 μL of enzyme solution and incubated at 37°C for 30 min. The reaction was terminated by addition of 1.2 mL of 10% trichloroacetic acid (TCA; Sigma) and left at room temperature for 15 min. As a negative control, the same enzyme reaction mixture was mixed with 1.2 mL of 10% TCA without incubation. After centrifugation of the mixture at 8,000 *g* for 5 min, 1.2 mL of the supernatant was added to 1.4 mL of 1.0 N NaOH, and the absorbance of the final mixture was measured at 440 nm. One unit of protease activity was defined as the amount of enzyme required to increase absorbance by 0.001 at 440 nm.

Substrate specificity and lytic activity spectrum of lytic enzyme. The purified lytic enzyme and six commercially available proteases (Sigma) were assayed for their lytic and proteolytic activity (Table 4). The ratio of lytic to protease activity of each enzyme was compared to determine substrate specificity.

Microorganisms were cultured in nutrient broth (BD) for bacteria for 12 h and potato dextrose broth (PDB; BD) for fungi for 3 days at their respective optimal temperatures (Table 5). Each substrate cell of the microorganisms was prepared as described above, and lytic activity against the prepared cells was assayed as described in the previous section.

Results and Discussion

Isolation and identification of producer strain. Total 1,234 bacterial strains were isolated from the soil samples and screened for production of bacteriolytic activity against *P. gingivalis* W50. A bacterial strain (YU-1432) resulting in the largest zone of lysis was selected as the producer strain of lytic enzyme. The strain was a Gram-positive, rod-shape, spore-forming bacterium and could grow under aerobic and anaerobic (85% N_2, 5% H_2, and 10% CO_2) conditions. Biochemical characterization determined by carbohydrate fermentation and cell wall fatty acid composition analysis of YU-1432 revealed that YU-1432 is a *Bacillus* spp. (data not shown). In addition, YU-1432 showed the highest identity (99%) to many *Bacillus subtilis* strains including *B. subtilis* strains ZWQ-1, BS11, PAB1C8, and JM1C6 by 16S rDNA sequencing. Based on these results, the producer was designated as *B. subtilis* YU-1432.

Purification and N-terminal sequencing of lytic enzyme. The optimal pH and temperature for the lytic enzyme production were determined to be 7.0 and 37°C, respectively (data not shown). The highest amount of the enzyme was achieved by 16 h incubation in the production medium (data not shown). Table 1 shows the purification results of the lytic enzyme produced by *B. subtilis* YU-1432. Acetone precipitation yielded 97.6% recovery of total activity from the culture supernatant. The unbound fractions on DEAE-Sepharose chromatography retained the lytic activity (Fig. 1a); in this step, the specific activity was increased by 243.1-fold and overall recovered activity was 88,500 units (Table 1). The pooled active fractions from the column were loaded onto CM-Sepharose chromatography. The fractions showing lytic activity were eluted with 0.1 M NaCl in buffer B from the chromatography (Fig. 1b). After the serial column chromatography, purification fold was increased by 522.0 times, and 21.9% of total activity was recovered (Table 1). The molecular mass of the enzyme was estimated to be approximately 29.0 kDa by SDS-PAGE (Fig. 1c), and the purified lytic enzyme was designated as YU205. The molecular mass of YU205 was found to be different from those of two other lytic enzymes from *Bacillus* spp., 45.0 and 27.0 kDa [Jung *et al.*, 1991; Kim *et al.*, 1999]. Fifty microliters of YU205, the purified enzyme, was spotted on a 2% (w/v) agar plate containing the prepared *P. gingivalis* W50 cells to 10% (v/v) final concentration. The purified YU205 was found to degrade the substrate cells (Fig. 1d), confirming that the purified enzyme is responsible for the lytic activity against *P. gingivalis*.

The N-terminal sequence of YU205 was determined to

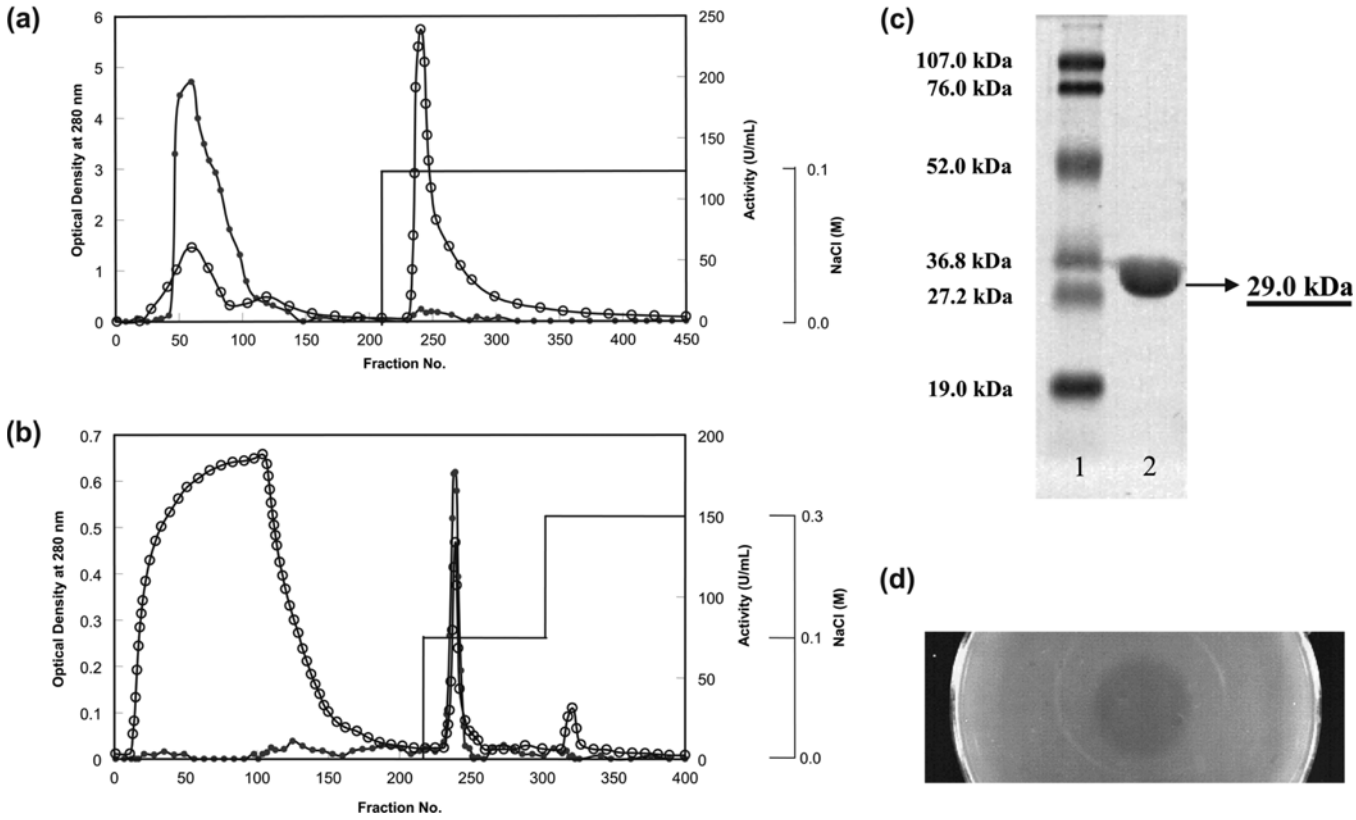

Fig. 1. Chromatogram of lytic enzyme YU205 on DEAE-Sepharose Fast Flow (a) and CM-Sepharose Fast Flow (b). ○, absorbance at 280 nm; ●, activity (U/mL). SDS-PAGE of active fraction from CM-Sepharose Fast Flow (c). Lane 1: SDS-PAGE standard marker (Sigma); lane 2: the purified lytic enzyme YU205. In (d), lysis zone of *P. gingivalis* W50 by the purified lytic enzyme, YU205 from CM-Sepharose chromatography is shown.

be AQSVPYGISQIKAPA. Using NCBI BLAST homology search, the N-terminal sequence was found to be identical to those of several enzymes produced by *Bacillus* spp., such as subtilisin E [Wong *et al.*, 1984], nattokinase [Fujita *et al.*, 1993], several fibrinolytic enzymes including subtilisin FS33 [Wang *et al.*, 2006], and subtilisin D5 [Choi *et al.*, 2009]. This result indicates that YU205 is a subtilisin-like serine-type enzyme showing lytic activity; therefore, YU205 could act on certain peptide bond of peptidoglycan existing in the *P. gingivalis* cell wall to hydrolyze the bond, resulting in cell lysis.

Biochemical characterization of YU205. YU205 was stable within the range of pH 6.0 to pH 11.0 and up to 40°C. YU205 lost the activity at 50°C or higher temperature. Furthermore, the presence of 1-5 mM $CaCl_2$ increased the thermal stability of YU205, showing 80% of residual activity even after 2 h incubation at 50°C; therefore, Ca^{2+} ion was able to stabilize YU205 against thermal inactivation.

The lytic activity of YU205 was increased by up to 20% in the presence of Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, and Ni^{2+} ions, compared with that of YU205 without metal ions (Table 2). On the contrary, the enzyme was completely inhibited in the presence of Fe^{3+} ion (2% residual activity). The inactivated YU205 with Fe^{3+} could not recover the lytic activity by the addition of

Table 1. Purification of YU205 from *B. subtilis* YU-1432

Steps	Total protein (mg)[a]	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	2,860	260,260	91	1.0	100
Acetone precipitation	180	254,000	1,411	15.5	97.6
DEAE-Sepharose chromatography	4.0	88,500	22,125	243.1	34.0
CM-Sepharose chromatography	1.2	57,000	47,500	522.0	21.9

[a]The protein concentration was determined by Bradford assay using BSA (Sigma) as a standard protein.

Table 2. Effects of metal ions on the lytic activity of YU205

Metal ions (1 mM)	Relative activity (%)[a]
None	100
$AgNO_3$	94
$CaCl_2$	120
$CoCl_2$	96
$CuCl_2$	85
$FeCl_3$	2
$FeSO_4$	76
$HgCl_2$	84
KCl	116
$MgCl_2$	112
$MgSO_4$	108
$MnCl_2$	120
NaCl	108
$NiCl_2$	104
$PbCl_2$	96
$ZnSO_4$	78

[a]The relative activity represents the ratio (%) of YU205 activity treated with each metal ion to non-treated YU205 activity.

Table 3. Effects of chemical modifiers on the lytic activity of YU205

Chemical modifier (5 mM)	Relative activity (%)[a]
None	100
N-bromosuccinimide (NBS)	0
Iodine (pH 6.0)	91
(pH 8.0)	28
Diethyl pyrocarbonate (DECP)	113
p-chloromercurybenzene (pCMB)	105
Iodoacetate	0
Hydroxylamine	118
Phenylmethylsulfonyl fluoride (PMSF)	13

[a]The relative activity represents the ratio (%) of YU205 activity treated with each chemical modifier to non-treated YU205 activity.

ethylenediaminetetraacetic acid (EDTA), a chelating agent of metal ions, indicating that YU205 is irreversibly inactivated by Fe^{3+} ion (data not shown).

When treated with *N*-bromosuccinimide, YU205 completely lost its activity (Table 3), implying that either tryptophan or tyrosine may be involved in the enzymatic reaction of YU205 [Lundblad and Noyes, 1984]. Moreover, iodine is known to inhibit tryptophan and tyrosine under acidic and alkali conditions, respectively. Although iodine (pH 6.0) slightly lowered the activity of YU205 (9% reduction in the activity), 72% of the lytic activity was lost by the addition of iodine (pH 8.0), implying that tyrosine may exist on the active site of YU205. Iodoacetate that modifies methionine completely inhibited the lytic activity of YU205. When treated with PMSF that modifies serine residue, the residual activity of the treated YU205 was only 13% of the non-treated YU205 activity, indicating that serine may be involved in the lytic activity. According to the overall results, tyrosine, methionine, and serine were deduced to be essential amino acid residues for the lytic activity of YU205.

Table 4. Comparison of substrate specificity of YU205 and proteolytic enzymes

	Lytic activity (U/mL)	Protease activity (U/mL)	[Lytic activity/ protease activity][a]
YU205	240.0	1.8	135.6
Subtilisin BPN'	73.5	27.5	2.8
Subtilisin carlsberg	32.5	10.6	1.2
Protease XIV	35.5	10.6	3.4
Protease XXIII	28.0	25.8	1.1
Protease XXV	36.5	11.8	3.1
Trypsin	14.1	2.4	5.8

[a]The ratio of lytic activity to protease activity of each enzyme was calculated to represent the substrate specificity of each enzyme to *P. gingivalis* W50.

Substrate specificity and lytic activity spectrum of YU205. The substrate specificity of YU205 was compared with those of selected commercial proteases produced by several *Bacillus* spp. and other microorganisms (Table 4). The substrate specificity of each enzyme was determined by comparing the ratio of lytic to protease activity of the enzymes; higher ratio indicates higher substrate specificity to the target cells. The ratio of YU205 (135.6) was at least 23.4 times higher than those of the other proteolytic enzymes tested, indicating that YU205 exhibited the highest substrate specificity to *P. gingivalis*. This substrate specificity may be attributed to existence of high number of peptide bonds more vulnerable to YU205 in the peptidoglycan of *P. gingivalis* cell wall than the other enzymes tested. Consequently, YU205 is expected to more effectively control periodontal disease caused by *P. gingivalis* than the other enzymes tested.

YU205 exhibited the lytic activity against Gram-positive and -negative bacteria except *Bifidobacterium longum* and *Escherichia coli* (Table 5). *P. gingivalis* W50 showed the highest sensitivity to YU205, and the relative activity of YU205 against *B. subtilis* KCCM 11315 was as high as 88%. However, among the fungi tested, the enzyme exhibited low level of lytic activity only against *Candida albicans* (4% relative activity).

Table 5. Lytic activity spectrum of YU205

Strain	KCCM[a] No.	Relative activity (%)[b]
Gram-postive bacteria		
Lactobacillus plantarum	11322	50
Bifidobacterium longum	11953	0
Bacillus subtilis	11315	88
Staphylococcus aureus	11335	25
Streptococcus mutans	40105	40
Gram-negative bacteria		
Escherichia coli	11234	0
Pseudomonas aeruginosa	11266	40
P. gingivalis W50		100
Yeast		
Candida albicans	11474	4
Saccharomyces cerevisiae	32016	0
Mold		
Penicillium chrysogenum	60010	0
Aspergillus niger	11239	0

[a]KCCM: Korea Culture Center of Microorganisms
[b]The relative activity was expressed as the ratio (%) of YU205 activity against each microorganism to YU205 activity against *P. gingivalis* W50.

In conclusion, the lytic enzyme, YU205 showed the highest level of lytic activity against *P. gingivalis* and higher level of substrate specificity to *P. gingivalis* resulting in higher number of periodontal diseases than the other enzymes tested. These results suggest that YU205 may be a promising candidate for application to various types of dental care or food products to control periodontal diseases.

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