- Research Letter -

Cloning and Expression of the Mutanase Gene of *Paenibacillus humicus* from Fermented Food

Hideaki Tsumori,^a Takiti Kawauti,^b Atsunari Shimamura,^a Nobuhiro Hanada,^b Yutaka Sakurai,^c and Kazuo Yamakami^{*,c}

^aDepartment of Chemistry, National Defense Medical College, 3–2 Namiki, Tokorozawa 359–8513, Japan, ^bDepartment of Translational Research, School of Dental Medicine, Tsurumi University, 2–1–3 Tsurumi, Tsurumi-ku, Yokohama 230–8501, Japan and ^cDepartment of Preventive Medicine and Public Health, National Defense Medical College, 3–2 Namiki, Tokorozawa 359–8513, Japan

(Received February 3, 2010; Accepted April 20, 2010; Published online April 23, 2010)

There has been much interest in mutanases, α -1,3-glucanases, as they have the potential for preventive agents in the oral cavity. Mutanases have been reported in some bacteria and fungi but remain a relatively uncharacterized family of enzymes. We screened bacterial mutanase in fermented food for the application of the enzyme to preventive medicine. Immersed solutions of fermented soybeans, natto, on mutan-containing agar plates exhibited mutanhydrolyzing activity after incubation. We isolated a microorganism that hydrolyzed mutan from the fermented soybeans and named it Paenibacillus humicus strain NA1123. The gene for the mutanase was cloned, and the nucleotide sequence of the gene consisted of 3441-bp open reading frame that encoded a predicted 1146-amino acid polypeptide including a 33-amino acid signaling peptide. The predicted molecular mass of the matured enzyme was 115399. The protein is composed of an N-terminal domain and a C-terminal domain, that are connected by a sequence composed of proline and threonine repeats. The deduced amino acid sequence of the present enzyme showed similarity to that of the mutanase MuC1 of strain KSM-M126 and the mutanase MuE of strain KSM-M318 of Paenibacillus sp. with 77.2% and 73.5% identity, respectively. We confirmed that the recombinant mutanase exhibited mutan hydrolyzing activity.

Key words — mutanase, fermented soybean, *Paeni-bacillus humicus*, glucanase

INTRODUCTION

Extracellular polysaccharides, which form part of the biofilm on tooth surfaces, are produced by microbial flora in the human oral cavity and play a role in the adherence and proliferation of bacterial aggregates on tooth surfaces in humans.^{1,2)} These polysaccharides play a significant role in the development of tartar, plaque and dental caries.¹⁾ Mutan is a major component of exo-polysaccharides produced by tooth-colonizing streptococci such as Streptococcus mutans.³⁾ It is important to develop agents that can degrade dental plaques and thus prevent caries. Mutan is water-insoluble glucan composed of an α -1,3-glucosidic linkage. Previously, dextranases, α -1,6-glucanase, were investigated for the prevention of dental caries.¹⁾ The enzymes hydrolyze soluble but not insoluble glucans. The degradation of water-insoluble glucans could prevent dental caries. Mutanase, α -1,3-glucanase (EC3.2.1.59) shows substrate specificity for the hydrolysis of α -1,3-glucan. The enzyme is capable of removing biofilms synthesized by oral bacteria in *vitro* and reducing plaque formation *in vivo*.⁴⁾

Mutanases could be useful in preventive oral hygiene, but there is limited information on its molecular characteristics. Previously, mutanases have been observed in some fungi and bacteria such as *Penicillium*, *Bacillus* and *Paenibacillus*.^{4–6)} Mutanases would have the advantage of inhibiting and controlling plaque formation, although mutanase activity in food has not been investigated to date. Therefore, it is worth investigating new efficient sources of mutanases. In the present study, we isolated and identified the mutan-hydrolyzing bacterial strain *Paenibacillus humicus* (*P. humicus*) NA1123 from the fermented soybeans, natto, which expresses mutanase activity *in vitro*.

MATERIALS AND METHODS

Materials — Escherichia coli (E. coli) JM109

^{*}To whom correspondence should be addressed: Department of Preventive Medicine and Public Health, National Defense Medical College, 3–2 Namiki, Tokorozawa 359–8513, Japan. Tel.: +81-4-2995-1563; Fax: +81-4-2996-5195; E-mail: yamakami@ndmc.ac.jp

and *E. coli* BL21 (DE3) were obtained from Takara (Otsu, Japan) and Promega (Madison, WI, U.S.A.), respectively. The vectors pGEM-T Easy and pCold I were obtained from Promega and Takara, respectively. Mutan was synthesized by recombinant glucosyltransferase with sucrose following the previously reported methods.⁶

Isolation and Identification of Microorganisms — To screen bacteria capable of degrading mutan, we focused on fermented soybeans, natto. Twenty-five packages of commercially available fermented soybeans (Takano Foods Co., Omitama; Tokiwa Co., Fuchu; Fura Co., Shizuoka; Kajinoya Co., Kawasaki; Azuma Co., Utsunomiya; Mizkan Co., Handa; and Asahimatsu Co., Iida, Japan) were purchased from the grocery stores in Saitama, Japan. A single bean (approximately 0.2 g wet weight) from each sample package was immersed in 1 ml of phosphate buffered saline, pH 7.2 at 27°C for 1 hr. One hundred microliters of these solutions were spread on agar plates containing 1% mutan and incubated at 37°C for 48 hr. Positive colonies growing on the plates and forming clear halos of degraded mutan were isolated by successive streaking onto the plates. The colony that formed the largest halo was selected for the present study.

Bacterial genomic DNA was prepared using a DNA isolation kit (Quiagen, Hilden, Germany). The 16S rDNA was amplified in the polymerase chain reaction (PCR) with the following primers: forward 5'-GTTTGATCCTGGCTCA-3' and reverse 5'-TACCAGGGTATCTAATCC-3'. PCR was performed in a reaction mixture (20 µl) containing 5 ng of chromosomal DNA, 10 pmol of each primer, 4 nmol of each deoxynucleoside triphosphates, 30 nmol MgCl₂, and 0.5 units of LA Taq polymerase (Takara). Thermocycling consisted of one cycle at 94°C for 1 min, followed by 30 cycles at 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. The amplified DNA was sequenced using an ABI 310 DNA sequencer with the ABI Prism BigDye Terminator Cycle Sequence kit (Applied Biosystems, Foster City, CA, U.S.A.). The sequences determined were analyzed with GENETYX analysis software (Genetyx Co., Tokyo, Japan). Database searches were performed with the BLAST and FASTA programs from the National Center for Biotechnology Information (Bethesda, MD, U.S.A.). Characteristics of microorganisms were assayed with the API 50 CH kit (BioMerieux, Tokyo, Japan).

Cloning of the Mutanase Gene ---- Genomic DNA of P. humicus strain NA1123 was prepared as described above, and part of the mutanase gene was amplified from the DNA by PCR. The forward primer 5'-GCGTTCCCGCAATGGATTCAAG-3' and reverse primer 5'-GGAATTGTCACCGTATTGCC-3' were designed based on the conserved region of mutanase genes.^{4, 6, 7} The compositions of PCR were the same as described above, except for the primers. Thermocycling consisted of one cycle at 94°C for 10 min, followed by 30 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min. DNA fragments located on the upstream and downstream sides of the partial mutanase gene were obtained using an LA-PCR in vitro cloning kit (Takara) and nucleotide sequences were determined. The complete mutanase gene was cloned by PCR with appropriately designed primers based on the sequences determined above.

Preparation of Recombinant Mutanase —— The complete open reading flame (ORF) of mutanase without a signal peptide was amplified from the DNA of strain NA1123 using a sense Nde I-linker primer 5'-ATCGAAGGTAGGCATATGGCAGGA-GGCGCGAATCTG-3' and an antisense Xba Ilinker primer 5'-GATTACCTATCTAGACTATTT-GATCGTCAGGTTGAAC-3'. The compositions of PCR were the same as described above, except for the primers. Thermocycling consisted of one cycle at 94°C for 10 min, followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 4 min. A 3.3-kb DNA fragment was cloned into the Nde I and Xba I sites of pCold I, which was indicated as pPHM17, and was transformed into E. coli BL21 (DE3). The E. coli BL21 (DE3) harboring pPHM17 was cultured in Luria-Bertani broth containing ampicillin (100µg/ml) at 37°C at 200 rpm until the absorbance at 600 nm reached about 0.5, and then isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the culture for up to 0.1 mM which was further cultured at 15°C for 24 hr. After centrifugation at $8200 \times q$ for 10 min at 4°C, the pellet was treated with xTractor buffer (Takara). The recombinant enzyme was purified with TALON metal affinity resin (Takara). Zymographic analysis of recombinant mutanase was performed by isoelectrofocusing on polyacrylamide gel in the presence of Ampholine (pH 3.5 to 10; GE Healthcare, Piscataway, NJ, U.S.A.). The gel was overlayed with 1% agar containing 1% mutan in 50 mM sodium phosphate buffer, pH 6.5, and incubated at 37°C for 3 hr. The position of activity was observed as the clear

band on the agar.

N-Terminal Amino Acid Sequence of Intrinsic Mutanase — The intrinsic enzyme was obtained in the supernatant of 2 l of culture broth. The enzyme was precipitated with 60% saturation of ammonium sulfate, and the fraction was dialyzed against 10 mM sodium phosphate buffer, pH 6.5, for 24 hr. The dialyzed solution was used as the intrinsic mutanase. Mutanase was blotted onto PVDF (Millipore, Billerica, MA, U.S.A.) following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein band was analyzed using a PPSQ-21A protein sequencer (Shimadzu, Kyoto, Japan).

RESULTS

Isolation and Identification of Mutanase-Producing Bacteria in Fermented Soybeans

The screening of 25 packages of fermented soybean samples resulted in the detection of bacterial strains capable of hydrolyzing mutans in their immersion solutions. All samples demonstrated the activity, which produced halos on mutan-containing agar plates at up to 48 hr. The most active isolate that produced largest halo (Okame natto, Takano Foods Co.) among these samples was selected for the present study (Fig. 1). The bacteria isolated were rod shaped, Gram positive, spore producing, and aerobic. The nucleotide sequence of 16S rDNA was assigned the GenBank accession number AB540163. In addition, the nucleotide sequence of the 16S rDNA of our sample had the 99.6% identity to that of P. humicus strain PC-147 (GenBank accession number AM411528). On the basis of these findings, the bacterial strain was named NA1123 of P. humicus.

Nucleotide and Deduced Amino Acid Sequences of the Mutanase Gene

The mutanase gene was cloned in PCR using the primers designed based on the *N*-terminal amino acid sequence of the intrinsic enzyme and the sequence of the previously reported the *Paenibacillus* sp. strain RM1 (GenBank accession number E16590). The gene cloned and sequenced had a long single ORF of 3441-bp starting from the ATG codon and ending with the TAG stop codon (Fig. 2). The putative ribosome-binding sequence was located upstream of the ORF, separated by 5-bp from the start codon.



Fig. 1. Mutan-Hydrolyzing Activities of Fermented Soybeans Mutan hydrolyzing activities were assayed for the immersion solution of single fermented soybeans on agar plates containing 1% mutan after incubation for 48 hr at 37°C. The formation of halos indicated mutan hydrolyzing activity.

The ORF for strain NA1123 mutanase encoded 1146-amino acid residues. The predicted signal sequence contained some basic amino acid residues (Arg², Arg⁴ and Lys⁶), and the helix-breaking residues Pro^{30} . Thus the mature mutanase contained 1113-amino acid residues with a deduced molecular mass of 115399, a value similar to that of the intrinsic enzyme in SDS-PAGE (Fig. 3A). The nucleotide sequence of the mutanase of the present *P. humicus* strain NA1123 was deposited in the GenBank database under accession number AB498092. The *N*-terminal amino acid sequence of the intrinsic enzyme was found to be N-AGGANLTLGKT-C.

The deduced amino acid sequence of the putative mutanase showed close similarity to that of the putative mutanase MuC1 of *Paenibacillus* sp. strain KSM-M126 (GenBank accession number AB257603) with 77.2% identity. The putative mutanase MuE of *Paenibacillus* sp. strain KSM-M318 (GenBank accession number AB292235) was also similar with 73.5% identity.

Mutanase Activity of the Recombinant Enzyme

To investigate the mutanase activity, His-tagged fusion mutanase was constructed (Fig. 3A). The recombinant enzyme exhibited mutan-hydrolyzing activity on the zymographic analysis (Fig. 3B).

DISCUSSION

The present study was initiated to screen fermented soybeans for insoluble glucan hydrolyzing activity for possible application as a preventive oral hygiene. *P. humicus* strain NA1123 was isolated as the mutan-degrading bacteria from the fermented soybeans. Mutanases derived from microorganisms, such as *Paenibacillus* sp. strain RM1

20 30 40 50 100 110 120 AAAGGAGGATCGCCAACCAATCATCCCCAGCAAAGAAGGTGATGGCAGCCCAAGAATTGAAAGCGCTTTGAATTTGGAATATACGGATTTGGCCGACCTGCTGATTCAAGC 180 120 140 150 160 170 190 200 210 220 GATTATGCGCGGAACCAATCGAACCCGAGGAGGAGTATATGCGTATCCGCACTAAATATATGAACTGGATGTTGGTGGCCGTCCTGATCGCCGCCGGCTTCTTCCAGGCTGCCGGCCCC M R I R T K Y M N W M L V L V L I A A G F F Q A A G P 80 290 300 310 320 330 340 350 3 RBS 270 280 360 250 260 ATCGCTCCCGCCACCGCTGCAGGAGGCGCGAATCTGACGCTCGGCAAAAACCGTCACCGCCAGCGGCCAGTCGCAGACGTACAGCCCCGACAATGTCAAGGACAGCAATCAGGGAACTTAC I A P A T A∱A G G A N L T L G K T V T A S G Q S Q T Y S P D N V K D S N Q G T Y 370 880 - 390 - 400 - 400 420 430 440 450 460 470 44 W E S T N N A F P Q W I Q V D L G A S T S I D Q I V L K L P S G W E T R T Q T L 490 500 510 520 530 540 550 560 570 580 590 6 600 SIQGSANGSTFTNIVGSAGYTFNPSVAGNSVTINFSA 610 620 630 640 650 660 670 680 690 700 71 ASA 710 720 RYVRLNFTANTGWPAGQLSELEIYGATA<u>PTPTPTPTPT</u> 730 740 750 760 770 780 790 800 810 820 830 840 <u>P T P T P T P T P T V T P A P S A T P T P T P P A G S N I A V G K S I T A S S S 850 860 870 880 890 900 910 920 930 940 950 96</u> Αθαυκ. Τ Q Τ Υ 970 Y V A A N A N D N N T S T Y W E G G S N P S T L T L D 0 980 990 1000 1010 1020 1030 1040 105 FGSNQSITS\ 1050 1060 1070 1080 KLNPASEWGTRTQTIQVLGADQNAGSFSNLVSAQSYTF 1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 12 1200 AATCCCGCGAACCGGCAATACGGTGACGATTCCGGTCTCCCGCGACGGTCAAGCGCCTCCAGCTGAACATTACGGCGAACTCCGGCGCCCCGGCCAGATTGCCGAGTTCCCAAGTGTTC A T G N T V T I P V S A T V K R L Q L N I T A N S G A P A G Q I A E F Q V F 1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 13 ΝP 1320 GGCACGCCAGCGCCTAATCCGGACTTACCGGCATGTCCTGGACTCCGGCTGTCTTCTCCGGCGGCGGCGACATTACGCTGAACGCCGTCGTCAAGAACATCGGAACTGCAGC IT G M S W T P S S P V E S G D I T L N A V V K N I G T A A 50 1360 1370 1380 1390 1400 1410 1420 1430 14 APNPDLT GTP 1330 1340 1350 1440 GCAGGCGCCACGACGGTCAATTTCTACCTGAACAACGAACTCGCCGGCACCGCTCCGGTAGGCGCGCTTGCGGCAGGAGCTTCTGCAAATGTATCGATCAATGCAGGCGCCAAAGCAGCC T V N F Y L N N E L A G T A P V G A L A 1460 1470 1480 1490 1500 1510 A A G A S A N V S I N A G A K A A 1450 1520 1530 1540 1550 GCAACGTATGCGGTAAGCGCCAAAGTCGACGAGAGGAACGCCGTCATCGAGGAGAAGGCAACAACAGCTACTCGAACCCGACTACTCGTCGTAGCGCCGGTGTCCAGCTCCGAC A T Y A V S A K V D E S N A V I E Q N E G N N S Y S N P T N L V V A P V S S S D 1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 168 L V A V T S W S P G T P S Q G A A V A F T V A L K N Q G T L A S A G G A H P V T 1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 180 GTCGTTCTGAAAAACCGTGCGGAGCGACGCTGCAAACCTTCACGGGCACCTACACAGGTTCCCTGGCAGCAGGCGCATCGCGCAATATCAGCGTGGGCAGCTGGACGGCAGCGACCGGC KNAAGATLQTFTGTYTGSLAAGASANISVGSWTA 10 1820 1830 1840 1850 1860 1870 1880 1890 1900 191 A S V V L 1020 1810 1010 TYTVSTVAADGNEIPAKQSNNTSSASLTVYSARGASMPY 1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 204 AGCCGTTACGACACGGAGGATGCGGTGCTCGGCGGCGGAGCTGTCCTGAGAACGGCGCCGACGTTCGATCAGTCGCTCATCGCTTCCGAAGCATCGGGACAGAAATACGCCGCACTTCCG
 R
 Y
 D
 F
 D
 Q
 S
 R
 Y
 D
 E
 A
 L
 G
 G
 A
 L
 R
 T
 A
 Q
 S
 R
 J
 T
 F
 D
 Q
 S
 A
 L
 G
 G
 A
 L
 R
 T
 D
 T
 F
 D
 Q
 S
 L
 I
 A
 S
 G
 K
 A
 L
 P
 Z050
 2060
 2070
 2080
 2090
 2110
 2120
 2130
 2140
 2150
 21
S N G S S L Q W T V R Q G Q G G A G V T M R F T M P D T S D G M G Q N G S L D V 2170 2180 2190 2200 2210 2220 2230 2240 2250 2250 2260 2270 22 2280 Y V N G T K A K T V S L T S Y Y S W Q Y F S G D M P A D A P G G G R P L 2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 FRFD 2390 2400 E V H F K L D T A L K P G D T I R V Q K G G D S L E Y G V D F I E I E P I P A A 2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2550 G A V A N D G K D D L A A F K A A 60 2570 2580 2590 2600 26 PANSVSV VTAAVAAGKS VAR ТЕҮ 2530 2540 2550 2560 2610 2620 2630 2640 CTCTACATCCCGGAAGGCACCTTCCACCTGGAGCAGCATGTGGGAGATCGGCTCGGCCACCAGCATGATCGACAACTTCACGGTCACCGGGTGCCGGCATCTGGTATACGAACATCCAGTTC L Y I P E G T F H L S S M W E I G S A T S M I D N F T V T G A G I W Y T N I Q F 2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 27 2760 ACGAATCCCAATGCATCGGGCGGCGCGCATCTCCCCTGAGAATCAAAGGAAAGCTTGATTTCAGCAACATCTACATGAACTCCCACCTGCGTTCCCGTTACGGGCAGAACGCCGTCTACAAA T N P N A S G G G I S L R I K G K L D F S N I Y M N S N L R S R Y G Q N A V Y K 2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2860 2870 287 2880 G F M D N F G T N S I I H D V W V E H F E C G M W V G D Y A H T P A I Y A S G L 2880 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 30 2000 GTCGTGGAAAAACAGCCGCATCCGCAACAATCTTGCCGACGGCATCAACTTCTCCGCAGGGAACGAGCAACTCGACCGTCCGCAACAAGCAGCATCCGCAACAACGGCGATGACGGCCTCGCC VVENSRIRNNLADGINFSQGTSNSTVRNSSIRNNGDDGLA 3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 31 GTCTGGACGAGCAACACGAACGGCGCCTCGGCCGGGGGGGAACAACACCCTTCTCCTACAACACGGTCGAGAACAACTGGCGCGCGGCGGCGCCATCGCCTTCTTCGGCGGCGGCGCCACAAG VWTSNTNGAPAGVNNTFSYNTIENNWRAAAIAFFGGSGHK 3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230 324 GCTGACCACAACTACATCATCGACTGTGTGGGGGGCTCCGGCATCCGGATGAATACGGTGTTCCCAGGCTACCACTTCCAGAACAACACCGGGCATCACCTTCTCGGATACGACGATCATC A D H N Y I I D C V G G S G I R M N T V F P G Y H F Q N N T G I T F S D T T I I 3260 3270 3280 3290 3300 3310 3320 3330 3340 3350 3360 F T N I D I I N 3450 3460 T S Q D L Y N G E R G A I D L E A S N D A I K N V T F 70 3380 3390 3400 3410 3420 3430 3440 34 A Q R N S G 3370 3470 3480 GACGGCGTTCAGATCGGCTATGGCGGCGGCGTTCGAGAACATCGTGTTCAACAACATCACGATCGACGGCACCGGCCGCGACGGGATATCGACATCCGCGTTCTCGGGACCTCATCTTGGC D G V Q I G Y G G G F E N I V F N N I T I D G T G R D G I S T S R F S G P H L G 3490 3500 3510 3520 3530 3540 3550 3560 3570 3580 3580 3600 GCAGCCATCTATACGTACACGGGCAACGGCTCGGCGACGTTCAACAACCTGGTGACCCGGAACATCGCCTATGCAGGCGGCAACTACATCCAGAGCGGGTTCAACCTGACGATCAAATAG A A I Y T Y T G N G S A T F N N L V T R N I A Y A G G N Y I Q S G F N L T I K * 3610 3620 3630 3640 GCTGCAAAAAAAGGAAGCTCCTCGGAGCTTCCTTTTTT

Fig. 2. Nucleotide Sequence and Deduced Amino Acid Sequence of Mutanase Gene from Paenibacillus humicus NA1123

The deduced amino acid sequence of mutanase is shown bellow the corresponding nucleotide sequence, indicated by a single letter. Numbering of the nucleotide sequence starts from the initial codon of the gene. The putative ribosome-binding site is indicated by an underlining with RBS. The cleavage site of the putative signal peptide is shown by an arrow. The stop codon is shown by an asterisk. The *N*-terminal amino acid sequence of the intrinsic mutanase is indicated by broken underlining. Proline-Threonine repeats are indicated by dotted underlining.



Fig. 3. Polyacrylamide Gel Electrophoresis of Recombinant Mutanase Expressed in *E. coli*

(A) SDS-PAGE of recombinant mutanase with protein staining. The *E. coli* was cultured at 15°C for 24 hr after adding 0.1 mM IPTG. Lane M, molecular weight standard proteins; lane 1, crude enzyme preparation obtained from *P. humicus* NA1123 (20 μ g protein). An arrowhead indicates the band of intrinsic mutanase; lane 2, *E. coli* cell lysate harboring mutanase (22 μ g protein); lane 3, recombinant mutanase purified with His-tag affinity resin (4.4 μ g protein). (B) Isoelectrofocusing of recombinant mutanase on polyacrylamide gel; lane 1, protein staining of *E. coli* cell lysate harboring mutanase (10 μ g protein); lane 2, protein staining of recombinant mutanase purified with His-tag affinity resin (2.0 μ g protein); lane 3, zymogram of *E. coli* cell lysate (2.0 μ g protein); lane 4, zymogram of purified recombinant mutanase (0.5 μ g protein). An arrow indicates the band of mutanase.

and Bacillus circulans strain KA-304, were isolated previously.^{7,8)} Other mutanases were characterized, and some examined for dental caries preventive efficacy.⁹⁾ Those samples, however, were obtained from the soil. Therefore, it is necessary to search for mutanase-producing microorganisms in food which can degrade the biofilm in the oral cavity as preventive agents. In the present study, we found mutan-hydrolyzing activities in the 25 samples of fermented soybeans. These samples therefore contain microorganisms that can produce mutanase. To the best of our knowledge, this is the first demonstration of the presence of microbial mutanase in a food product. However, we could not determine quantitative data on the activity of the individual samples in the plate assay used.

The deduced amino acid sequence of the present mutanase identified has 77.2% identity to that of mutanase MuClof *Paenibacillus* sp. KSM-M126.⁹⁾ In addition, these mutanases has similar molecular masses. The deduced molecular mass of the mutanase of NA1123 and MuCl of KSM-M126 were 115399 and 119514, respectively. The feature of signal sequence of the present enzyme (Arg², Arg⁴,

 Lys^6 and Pro^{30}) is resemble to that of the mutanase from Paenibacillus strain KSM-M35 (Lys⁶, Lys⁸, Lys¹¹ and Pro³⁰).⁹⁾ The N-terminal and C-terminal regions of the mutanase MuC1 are divided by proline-threonine repeat linker. The proposed function of proline-threonine sequence is considered as the linker or hinge between these domains.⁹⁾ The N-terminal region has been described as a carbohydrate-binding domain, and the C-terminal region corresponds to a catalytic domain.⁹⁾ However, the position of amino acid residues for active center of the domain is not vet clarified. In this study, we confirmed that the recombinant enzyme showed mutan-hydrolyzing activity. It was reported that the sequence-based families of glycosidases show a divergence of substrate specificity.¹⁰⁾ The sequence similarities between the present enzyme and mutanase MuC1 of Paenibacillus sp. indicate that these enzymes should demonstrate the same catalytic mechanisms.^{9,11,12} However, the sequence of the present mutanase has a low 43.7% identity to that of mutanase MuA of Paenibacillus sp. (GenBank accession number AB257601).¹³⁾ The precise catalytic property of the present mutanase should be clarified in future studies.

We screened the mutan hydrolyzing activity in the foods and found the mutanase in fermented soybeans. The present study was the first to demonstrate that mutanase in the food should be used as an agent for the prevention of oral plaque and resulting dental caries, since the ingestion of the mutanase isolated is expected to degrade the cariogenic biofilm. We will characterize the present mutanase for the application *in vivo*.

REFERENCES

- 1) Rosan, B. and Lamont, R. J. (2000) Dental plaque formation. *Microbes Infect.*, **2**, 1599–1607.
- Tsumori, H. and Kuramitsu, H. (1997) The role of the *Streptococcus mutans* glucosyltransferases in the sucrose-dependent attachment to smooth surfaces: essential role of the GtfC enzyme. *Oral Microbiol. Immunol.*, **12**, 274–280.
- Davey, M. E. and Costerton, J. W. (2006) Molecular genetics analyses of biofilm formation in oral isolates. *Periodontol.*, 2000, 42, 13–26.
- Sumitomo, N., Saeki, K., Ozaki, K., Ito, S. and Kobayashi, T. (2007) Mutanase from a *Paenibacillus* isolate: Nucleotide sequence of the gene and properties of recombinant enzymes. *Biochim. Bio-*

phys. Acta, 1770, 716-724.

- 5) Shalom, G., Pratten, J., Wilson, M. and Nair, S. P. (2008) Cloning, heterologous gene expression and biochemical characterization of the α -1,3-glucanase from filamentous fungus *Penicillium purpurogenum*. *Protein Expr. Purif.*, **60**, 170–175.
- Yano, S., Wakayama, M. and Tachiki, T. (2006) Cloning and expression of an α-1,3-glucanase gene from *Bacillus circulans* KA-304: The enzyme participates in protoplast formation of *Schizophyllum commune. Biosci. Biotechnol. Biochem.*, **70**, 1754– 1763.
- Shimotsuruma, I., Kigawa, H., Ohdera, M., Kuramitsu, H. K. and Nakashima, S. (2008) Biochemical and molecular characterization of a novel type of mutanase from *Paenibacillus* sp. strain RM1: Identification of its mutan-binding domain, essential for degradation of *Streptococcus mutans* biofilms. *Appl. Environ. Microbiol.*, **74**, 2759–2765.
- Fuglsang, C. C., Berka, R. M., Wahleithner, J. A., Kauppinen, S., Shuster, J. R., Rasmussen, G., Halkier, T., Dalboge, H. and Henrissat, B. (2000) Biochemical analysis of recombinant fungal mutanases. A new family of α-1,3-glucanases with novel carbohydrate-binding domains. J. Biol.

Chem., 275, 2009-2018.

- 9) Hakamada, Y., Sumitomo, N., Ogawa, A., Kawano, T., Saeki, K., Ozaki, K., Ito, S. and Kobayashi, T. (2008) Nucleotide and deduced amino acid sequences of mutanase-like genes from *Paenibacillus* isolates: Proposal of a new family of glycoside hydrolases. *Biochimie*, **90**, 525–533.
- Davies, G. J., Wilson, K. S. and Henrissat, B. (1997) Nomenclature for sugar-binding subsites in glycosyl hydrolases. *Biochem. J.*, **321**, 557–559.
- Kimura, T., Nakagawa, K., Saito, Y., Yamagishi, K., Suzuki, M., Yamaki, K., Shinmoto, H. and Miyazawa, T. (2004) Determination of 1deoxynojirimycin in mulberry leaves using hydrophilic interaction chromatography with evaporative light scattering detection. *J. Agric. Food Chem.*, 52, 1415–1418.
- Pleszczyjska, M., Marek-Kozaczuk, M., Wiater, A. and Szczodrak, J. (2007) *Paenibacillus* strain MP-1: A new source of mutanase. *Biotechnol. Lett.*, 29, 755–759.
- Henrissat, B., Sulzenbacher, G. and Bourne, Y. (2008) Glycosyltransferases, glucoside hydrolases. *Curr. Opin. Struct. Biol.*, 18, 527–533.