— Research Letter —

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

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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 mutan-hydrolyzing 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, *Paenibacillus 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

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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 $MgCl_2$, 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′-ATCGAAGGTAGGCATATGGCAGGAGGCGCGAATCTG-3′ and an antisense *Xba* I-linker primer 5′-GATTACCTATCTAGACTATTTGATCGTCAGGTTGAAC-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 (100 μg/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 × g 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.

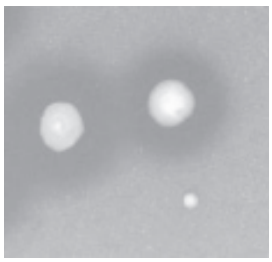

Fig. 1. Mutan-Hydrolyzing Activities of Fermented Soybeans

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 (Arg^2, Arg^4 and Lys^6), 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

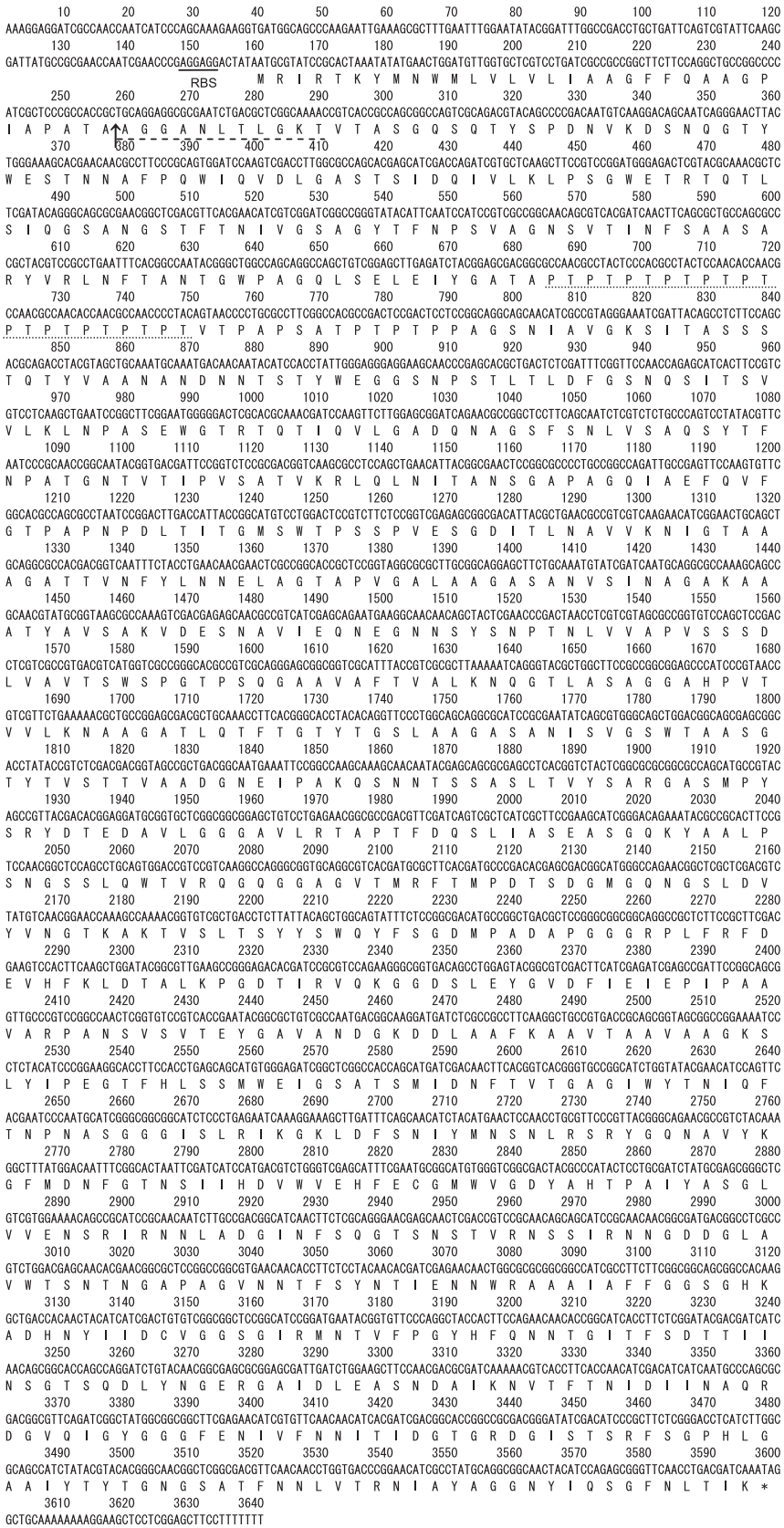

Fig. 2. Nucleotide Sequence and Deduced Amino Acid Sequence of Mutanase Gene from *Paenibacillus humicus* NA1123

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.

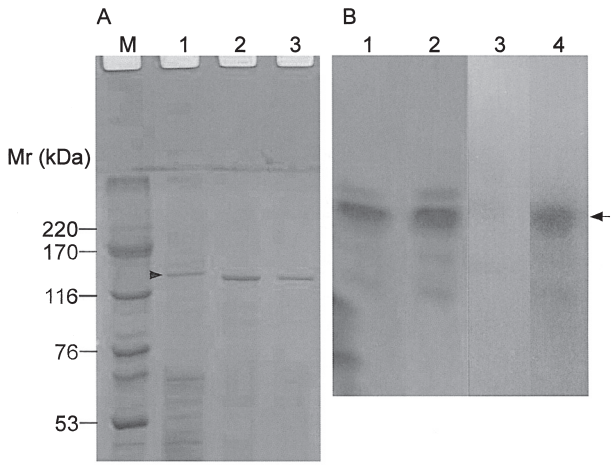

Fig. 3. Polyacrylamide Gel Electrophoresis of Recombinant Mutanase Expressed in *E. coli*

(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 μg protein). An arrowhead indicates the band of intrinsic mutanase; lane 2, *E. coli* cell lysate harboring mutanase (22 μg protein); lane 3, recombinant mutanase purified with His-tag affinity resin (4.4 μg protein). (B) Isoelectrofocusing of recombinant mutanase on polyacrylamide gel; lane 1, protein staining of *E. coli* cell lysate harboring mutanase (10 μg protein); lane 2, protein staining of recombinant mutanase purified with His-tag affinity resin (2.0 μg protein); lane 3, zymogram of *E. coli* cell lysate (2.0 μg protein); lane 4, zymogram of purified recombinant mutanase (0.5 μg 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 MuC1of *Paenibacillus* sp. KSM-M126.[9] In addition, these mutanases has similar molecular masses. The deduced molecular mass of the mutanase of NA1123 and MuC1 of KSM-M126 were 115399 and 119514, respectively. The feature of signal sequence of the present enzyme (Arg^2, Arg^4, Lys^6 and Pro^{30}) is resemble to that of the mutanase from *Paenibacillus* strain KSM-M35 (Lys^6, Lys^8, Lys^{11} and Pro^{30}).[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 yet 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*.

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