

Nucleotide Sequence and Expression of the Gene Encoding α -Glucosidase Found in Downstream of the Mycodextranase Gene from *Streptomyces* sp. J-13-3

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Abstract

Mycodextranase from *Streptomyces* sp. (strain J-13-3) is an α -glucanase that cleaves α -1,4-bonds of alternating α -1,3- and α -1,4-linked D-glucan (nigeran) to produce nigerose and nigeran tetrasaccharide. The gene encoding α -glucosidase found in downstream of the mycodextranase gene was analyzed. The deduced α -glucosidase amino acid sequence was 537 amino acids long with a calculated molecular mass of 60,263 Da. Comparison of the encoded amino acid sequence with those of other α -glucosidases showed that the α -glucosidase was a member of the glycoside hydrolase family 13 and had highly conserved four regions for catalysis and substrate binding in α -amylase family. The gene product expressed *in vitro* hydrolyzed *p*-nitropheny α -glucose and maltose, but not isomaltose and nigerose. These results indicated that the gene product must be an α -1,4-glucosidase (EC 3.2.1.20).

Key words: *Streptomyces* sp.; α -glucosidase gene; sequencing; expression.

Introduction

Nigeran (mycodextran) found in the hyphal wall of fungi such as *Aspergillus niger* is a water-insoluble and unbranched D-glucan consisting of alternating α -1,3- and α -1,4-linkages. Mycodextranase (EC 3.2.1.61) attacks α -1,4-bonds in nigeran to give nigerose and nigeran tetrasaccharide as sole reaction products. Our previous report describes the cloning and nucleotide sequencing of the mycodextranase gene from *Streptomyces* sp. (strain J-13-3).⁽¹⁾ The mycodextranase was newly classified as glycoside hydrolase (GH) family 87.⁽²⁾ Thereafter, two proteins from *Streptomyces* genome sequencing^(3,4) and α -1,3-glucanases (EC 3.2.1.59) from *Bacillus circulans*⁽⁵⁾ also belonged to the family 87 based on amino acid sequence similarities to the mycodextranase. Furthermore, another open reading frame (ORF) with the ATG initiation codon preceded by a potential ribosome-binding site (RBS) was also found in downstream from the mycodextranase gene and its deduced 57 amino acid sequence had similarity to amino terminal region of the α -glucosidase from *Streptomyces lividans*⁽⁶⁾ (pMS13-10 in Fig.1). In this report, nucleotide sequencing of the gene encoding possible α -glucosidase showed that the enzyme belongs to the GH family 13 based on the

amino acid sequence similarities. We also examined expression of the α -glucosidase gene *in vitro* system and the gene product hydrolyzed *p*-nitropheny α -glucose and maltose, but not isomaltose and nigerose.

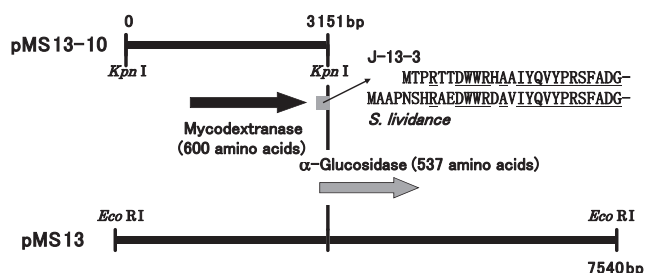


Fig. 1. Mycodextranase and α -glucosidase gene map in insert DNA of pMS13 cloned from *Streptomyces* sp. J-13-3.

The *Kpn* I fragment of the insert DNA of pMS13 was subcloned into the *Kpn* I site of pUC19, which was used to transform *E. coli* JM109. The construction plasmid was designated pMS13-10, as described previously.⁽¹⁾ Alignment of amino-terminal amino acid sequences of α -glucosidases from *Streptomyces* sp. J-13-3 and *Streptomyces lividans* was also showed.

Materials and Methods

Materials, bacterial strains, and plasmids

The materials used in this study included Quantam Prep Plasmid Miniprep kit from Bio-Rad (Hercules, CA, U.S.A.), GFX Gel Band Purification kit from Amersham Biosciences (Uppsala, Sweden), restriction endonucleases, LaTaq DNA polymerase with GC buffer I, and DNA ligation kit from Takara Shuzo (Kyoto, Japan), ampicillin sodium salt, IPTG (isopropyl-thio- β -D-galactoside), X-gal (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside), and all other chemicals from Wako Pure Chemical Co. (Osaka, Japan). Two strains (JM109 and DH5 α) of *Escherichia coli* and three plasmids (pUC19, pT7Blue(R), and pURE1) were used as hosts and vectors for cloning and expression of the gene, respectively. The plasmid pMS13 carrying about an about 7.5-kb insert of *Streptomyces* sp. (strain J-13-3) gene was used as a template DNA (Fig.1).⁽¹⁾ Transformants of *E. coli* were grown at 37°C in Luria-Bertani (LB) medium containing 100 μ g/ml of ampicillin.

Nucleotide sequencing and homology search

The nucleotide sequences of recombinant plasmid (pMS13) were analyzed for both strands by the primer walking method with a BigDye terminator cycle sequencing ready reaction kit using the ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA), as described previously.^(1,7) The nucleotide sequence data were analyzed using DNASIS-Mac (Hitachi Software Eng. Co., Tokyo). A computer assisted homology search and alignment were done using programs (FASTA and CLUSTAL W) in DNA Data Bank of Japan (DDBJ) online site.

Expression of the α -glucosidase gene *in vitro* system

The α -glucosidase gene was amplified from the pMS13 DNA by polymerase chain reaction (PCR) using forward *Nde* I (underlined) -linker primer (5'-TTCA TATG-ACCCCGC-GCACCACCGACTGGTG-3') and reverse *Xho* I (underlined) - linker primers (3'-CGCAGACCGACGCATGCCGG-ACT-C-ATC-G-ATT-GAGCTC CC-5'). PCR was done in a reaction mixture (50 μ l in GC buffer I) containing 100 ng of pMS13 DNA as template, 30 pmol of each primer, 200 μ M of each dNTP, and 2.5 units of LaTaq DNA polymerase.^(7,8) After initial denaturation (1 min at 94 °C), amplification protocol (30 sec at 94 °C, 30 sec at 55 °C, and 2 min at 72 °C) was repeated for 30 cycles, and followed by the final 72°C exten-

sion step for 5 min. The amplified DNA fragment was purified and cloned into the TA site of pT7Blue(R) (Novagen, Madison, WI), which was then used to transform *E. coli* DH5 α (Jet Competent cells, BioDynamics Laboratory, Inc. Tokyo, Japan). Transformant of *E. coli* harboring the plasmid was selected from white colonies on LB agar plate containing 100 μ g/ml of ampicillin, IPTG (0.1mM), and X-gal (40 μ g/ml). The plasmid DNA was digested with *Nde* I and *Xho* I, extracted from agarose gel, and cloned into the *Nde* I and *Xho* I sites of pURE1 (Post Genome Institute, Tokyo, Japan), which was then used to transform *E. coli* JM109 (Takara Shuzo). A recombinant plasmid with about 1.5 kb insert (pURE1-11) was selected from the transformants by colony PCR using forward (T7 promoter, 5'-TAATACGACTCACTATAGG-3') and reverse (5'-AAAAATAGG CGTATCACG-3') primers and its nucleotide sequence of the inserted α -glucosidase was confirmed using the same primers. The purified pURE1-11 DNA (2 pmol) was added to reaction mixture (50 μ l) of PURESYSYSTEM (Post Genome Institute, Tokyo, Japan) for transcription, reacted at 37°C for 1 hr, and then water (50 μ l) was added to the mixture. Ribosomal proteins (more than molecular weight of 100,000) in the enzyme solution were removed by ultrafiltration (YM-10, Millipore Corp., Bedford, MA).

Protein analysis and enzyme assays

The proteins in the enzyme solution were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with a 10% polyacrylamide slab gel.⁽⁹⁾ *p*-Nitropheny α -glucose was used as a substrate for α -glucosidase activity. In a assay, 10 μ l of the enzyme solution after ultrafiltration was incubated with 250 μ l of 0.6% substrate solution, 115 μ l of water, and 125 μ l of 0.2 M phosphate buffer (pH 6.8) at 37°C for 24 hr. After the reaction was stopped by addition of 500 μ l of 0.25 M Na₂SO₄ and the absorbance at 400 nm was measured. In substrates of disaccharides (nigerose, maltose and isomaltose), 25 μ l of the enzyme solution after ultrafiltration was incubated with 50 μ l of 0.4% substrate solution, and 25 μ l of 0.2 M phosphate buffer (pH 6.8) at 37°C for 24 hr. After the reaction, liberated glucose was measured by glucose oxidase method.

Results

Sequencing and analysis of α -glucosidase gene

pMS13 was sequenced by primer walking, and the putative

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ACCGGAACCGCC GAGGCACCCTC AACATGACCGCC GGCTTACCATC CAGCGCGGCAGC GGCAATCCGGC TGGTAACCCGA ACCGACCGCGGG CCCGGCTTGCTC CCTGACCGGCC 120
T G T A E A P L N M T G G F T I Q R G S G N S G W *
GGCCCCGGGTTCC GCCCTACCCCGA AGACCCGTGGCC CCGGCCAAGTC CCCGCCGCTGG GCCCGCGCACTG TTCGCACCACAC ACGAAGCCAGGC ACCATGACCCCG CGCACCACCGAC 240
M T P R T T D 7
TGTGGCGCTCAC GCAGCCATCTAC CAGGTCTACCGC CGCAGCTTCGCC GACGGCAACGGC GACGGCATCGGC GACCTGGCGGGG GTGGCTCCAGA CTGGACTACCTC CAGCACCTCGGC 360
W W R H A A I Y Q V Y P R S F A D G N G D G I G D L A G V R S R L D Y L Q H L G 47
ATCGAAGCCATC TGGTCTCTCCC TGGTACCTGAGC CCCATGGCGGAC GCCGGTACGAC GTCACCGACTTC CGCCGGATCGAC CCCCGTTCGGC ACCCTCGCCGAA GCCGAGAAACTC 480
I E A I W F S P W Y L T P M A D A G Y D V T D F R R I D P R F G T L A E A E K L 87
ATCAGCGAAGCC CACGAACCGCAG ATCCGCATCATC GTCGACGCTCGT CCCAACCCAGCTC TCCAACGAACAC GCCTGGTTTACC GCCGCCCTCAAC GCCGAGCCCGGC TCACCCGAAGCC 600
I S E A H E R D I R I I V D V V P N H V S N E H A W F T A A L N A E P G S P E R 127
GAACTGTTCTGG TTCCGCGAAGGC CGCGGCAGCAGC GCGCAACTACCC CCCAACGCATGG CAGTCCATCTTC GGCCTCCCGCC TGGACCCGGGTG CCCGACGGTCCAG TGGTACCTGCAC 720
E L F W F R E G R G D D G E L P P N A W Q S I F G G S A W T R V P D G Q W Y L H 167
CTGTTCCGCCCC GAACAGCCCGAC CTCACCTGGCCG AACCCCTCGTC CGGGCCGAGTAC GAAGACATCTTC CGTCTTGGTTC GACCGGGGAGTG GACGGCATCCGC GTCGACTCCGCC 840
L F A P E Q P D L N W R N P L V R A E Y E D I L R F W F D R G V D G I R V D S A 207
GCCATGTGCAGC AAGGACCCGGAC CTGCCCGACTAC GACCACAGCGTC TTCCCGACCCC CACCCCTATGTC GATCTCGACGAG AACACGACATC TACCGCGGCTGG CGCGCCGTCGCC 960
A M C S K D P D L P D Y D H S V F P D P H P Y V D L D E N H D I Y R G W R A V A 247
GACTCTACTCC GAGCCCCGGCGC CTGATCGGGGAG GTATGGCTCGCC GACCCCGACCGC TTCGCCAGTAC CTGGCGCCGAC GAACTCCACTCG GCCTTCAACTTC GACTTCTCCGGT 1080
D S Y S E P R A L I G E V W L A D P D R F A Q Y L R A D E L H S A F N F D F L G 287
TGCAGTTGGGAC CCCACTGCTCTG CGTACCCTCATC GACCAGACCCTG GCCGCCACGTC GGCCTGGGGGCC CCCAGCACCTGG GTGCTCTCAAC CACGACGTCACC CGCATGTCACC 1200
C S W D P T A L R T V I D Q T L A A H V G V G A P S T W V L S N H D V T R H V T 327
CGTACGCGCCGC CCCGACACCAGC ACCGCCCGCGGT ATCCGCCAGTAC AACCCCGTCGTC GACGAAGACCTC GGCCTCCCGCC GCCCGTCCCGCA GCCCTGCTACC ATGGCCCTGCC 1320
R Y G R P D T S T A A G I R Q Y N P V V D E D L G R R R A R A A A L L T M A L P 367
GGTGCCTCTAC ATCTACCAGGGC GAGGAACTGGGC CTGCCCGAGGTC GAGGACATCCC GCCCACCGCCGC GACGACCCATC TACCACCTAGC AACGGAGAGGAT CCCGGCCCGGC 1440
G A V Y I Y Q G E E L G L P E V E D I P A H R R D D P I Y H R T N G E D P G R D 407
GGCTGCCCGCTC CCCTGCGCCGG AACCGCACCCAGC GAGCCCTACGGC TTCAGCCCGGAC GGCCTCCCGATC CCCTGGCTCCCG CAGCCCGCGACC TGGCGCGGCTG ACCCGCGCGCT 1560
G C R V P L P W N G T T E P Y G F S P D G A A S P W L P Q P A T W A R L T A A A 447
CAGGAGCCGAT CCCACTCCATG CTCACCTCTAC CGCCAGGCACTT GCCCGTCGCGGC GCCGAACCCGCC CTGGGGGACGGG CCCATGAGTGG CTCGACTACGCA CCCGACGTTCTC 1680
Q D A D P H S M L T L Y R Q A L A R R A A E P A L G D G P M Q W L D Y A P D V L 487
GCCTTACCAGGC GGTGAGGCTTC ACCTGCATCGTC AACCTCTCCGCC ACCCCCGTACAA CTCCCGGACGAC GCCCAGGTCCTC GTCACTTCCAG CCGCTGAGCCAG GCGTGTGCTGCC 1800
A F T R G E S F T C I V N L S A T P V Q L P D D A Q V L V T S Q P L S Q G V L P 527
GTCGACACCAGC GTCTGGCTGCTG ACGGCCGAGGCC CGCGCCGCGGCG TGTGGTGTGTC CACTGTCGACCC ACAGCCCGGTCG CGTCTACGGTGC GCGCGGAGGGC AGTTTCCGGCC 1920
V D T S V W L R T A * 537

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Fig. 2. Nucleotide and deduced amino acid sequences of α -glucosidase gene in downstream from the mycodextranase gene.

The deduced amino acid sequences of the two genes are shown below the nucleotide sequence. The C-terminal amino acid sequence (T to W) of the maycodextranase is shaded. The putative ribosome-binding sequence (SD, Shine-Dalgarno) is underlined and shaded. The presumptive start and stop codons are shaded, and the stop codon is indicated by an asterisk. The palindromes sequences are shaded with dotted underline.

α -glucosidase gene ORF was localized to a 1,920-bp region in downstream from the mycodextranase gene (Fig. 2). The ORF was 1,611 nucleotide long with a ATG initiation codon at position 220 and a TGA stop codon at position 1,831. The initiation codon was preceded by a potential RBS (AAGG), which was complementary sequence at the 3' end of 16S rRNA of *Streptomyces lividans*.⁽¹⁰⁾ A palindrome sequence, a possible transcriptional termination, was found in downstream from the stop codon. The deduced putative α -glucosidase was 537 amino acids long with a calculated molecular mass of 60,263 Da. The coding sequence had a high overall G+C content (70mol%), which is typical of *Streptomyces* genes, and a strong tendency (90mol%) to have G or C in the third position of the triplet.

Homology search of α -glucosidase gene product

As shown in Fig. 3, a homology search using the FASTA program found that the deduced amino acid sequence of strain J-13-3 had high similarity to α -glucosidases from *Streptomyces lividans* (63 % identity)⁽⁶⁾ and *Streptomyces coelicolor*

(52% identity),⁽³⁾ α -1,4-glucosidase (EC 3.2.1.20) from *Thermomonospora curvata* (55% identity),⁽¹¹⁾ and oligo-1,6-glucosidase (EC 3.2.1.10) from *Bacillus flavocaldarius* (40 % identity).⁽¹²⁾ These known four α -glucosidases were classified as GH family 13 containing α -amylase. Our putative and four known α -glucosidases had three acidic residues (two Asp and one Glu) for catalysis and two His residues for substrate-binding in four highly conserved regions (CR I-VI in Fig. 3). In addition, two regions also had been conserved in all α -glucosidases (CR 1-2 in Fig. 3).

Expression of α -glucosidase gene

The homology of the putative gene product with different bacterial α -glucosidases predicted that the gene product would have a similar enzyme activity. For further biochemical characterization, the gene was amplified by PCR and placed under the control of the T7 promoter in the plasmid pURE1. After transfection into *E. coli* JM109, the gene product was expressed *in vitro* transcription with the constructed plasmid (pURE1-11) DNA. The expressed proteins were analyzed

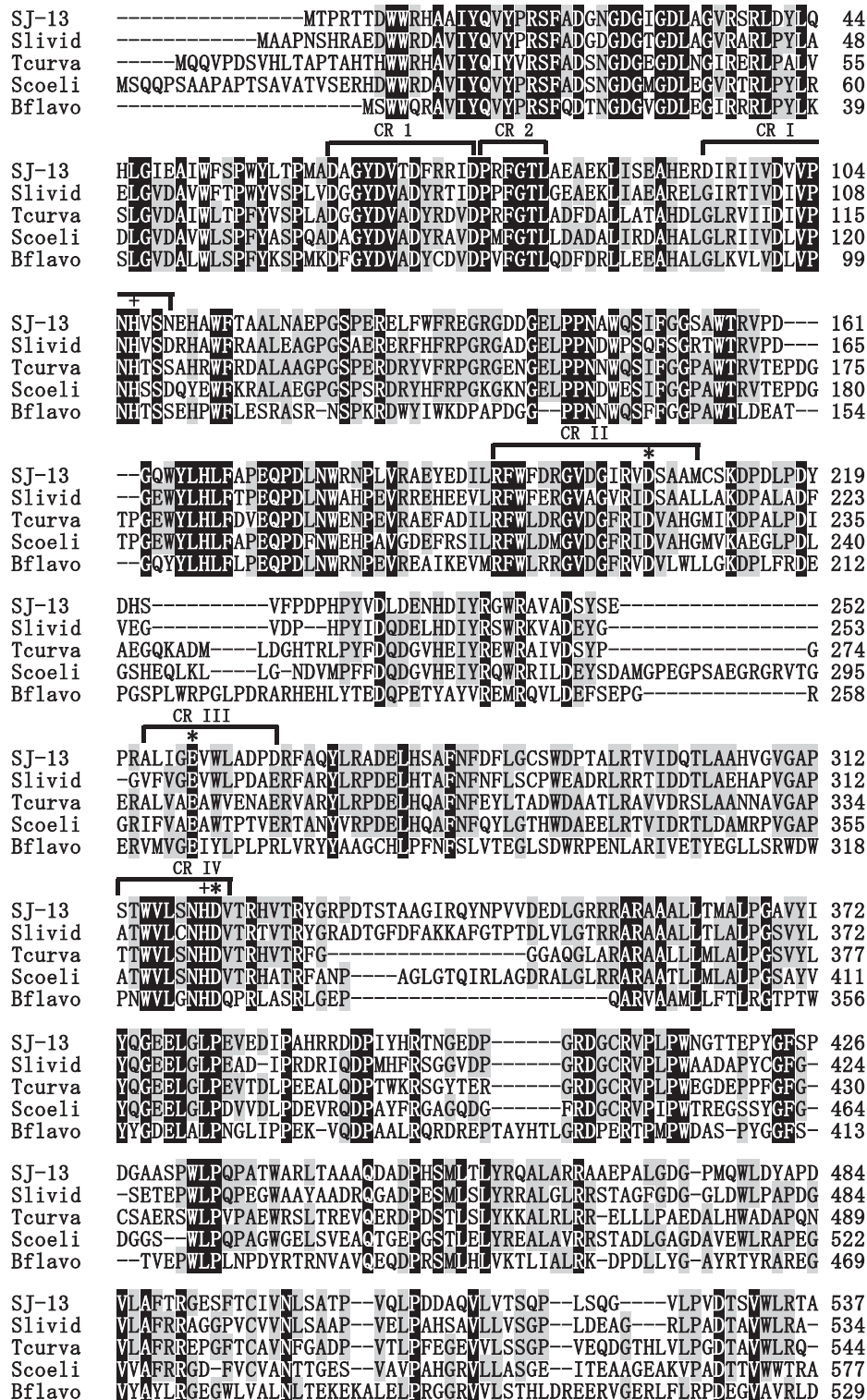


Fig. 3. Alignment of the amino acid sequences of α -glucosidase from the strain J-13-3 and four α -glucosidases belonging to GH family 13.

Amino acid residue numbers in each line are shown at the right of the figure. Residues conserved in all five α -glucosidases are indicated by white type on a black background. Identical residues that occur more than three times are shaded. The α -glucosidases were encoded in *Streptomyces sp.* J-13-3 (SJ-13, UniProt accession no. BA143475.1), *Streptomyces lividans* (Slivid, AAC46450.1), *Thermomonospora curvata* (Tcurva, AAA57313.1), *Streptomyces coelicolor* (Scoeli, CAB90874.1), and *Bacillus flavocaldarius* (Bflavo, BAB18518.1). Three acidic residues (two Asp and one Glu) for catalysis and two His residues for substrate-binding in four highly conserved regions (CR I-VI) are indicated by an asterisk and plus mark, respectively.

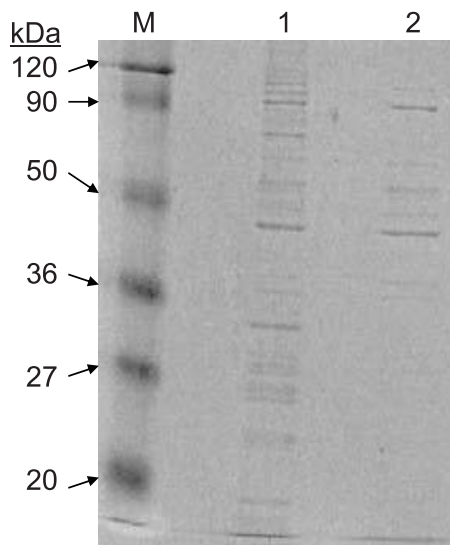


Fig. 4. SDS-PAGE of the proteins expressed *in vitro* transcription system using pURE1-11. Lane 1, expressed proteins; lane 2, proteins after removal of ribosomal proteins.

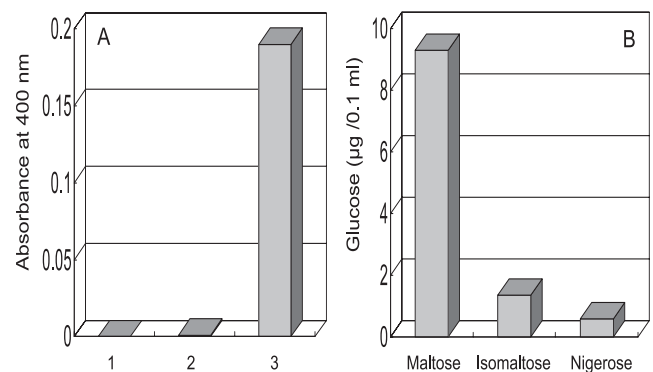


Fig. 5. Substrate specificity of α -glucosidase expressed *in vitro* transcription system. (A) Activity of α -glucosidase was assayed using *p*-nitropheny α -glucose as a substrate. Lane 1, no proteins; lane 2, proteins expressed using negative control plasmid; lane 3, α -glucosidase expressed using pURE1-11. (B) Activity of α -glucosidase expressed using pURE1-11 was assayed in substrates of α -1,4 (maltose), α -1,6 (isomaltose), and α -1,3 (nigerose)-linked disaccharides.

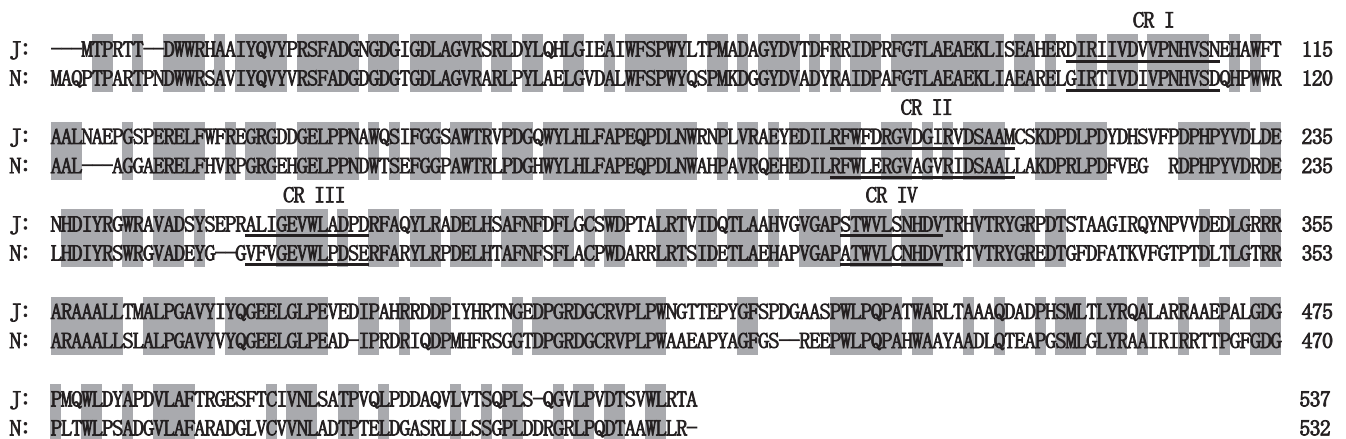


Fig. 6. Alignment of the amino acid sequences of α -glucosidases from *Streptomyces* sp. J-13-3 and NRRL 30748. Amino acid residue numbers in *Streptomyces* sp. J-13-3 (J, GenBank/UniProt accession no. AB523790/BAI43475.1) and NRRL 30748 (N, DQ351275.1/ABC87502.1) are shown at the right of the figure. Amino acids conserved in the two sequences are shaded. Four conserved regions (CR I-VI) in α -glucosidases are underlined.

by SDS-PAGE (Fig. 4). Several protein bands were detected in 50 kDa region around before and after ultrafiltration of expressed reaction mixture for removal of ribosomal proteins (lane 1 and 2 in Fig. 4), indicating that the α -glucosidase could not be purified. The α -glucosidase activity was measured by monitoring the release of *p*-nitrophenol from *p*-nitropheny α -glucose. The enzyme activity was significantly detected in the presence of pURE1-11 DNA (column 3 in Fig. 5A), but not in the presence of negative control plasmid (column 2 in Fig. 5A). Furthermore, measurement by monitoring the release of glucose from disaccharides showed that maltose with α -1,4 bond was hydrolyzed, but α -1,6 (isomaltose) and

α -1,3 (nigerose)-linked disaccharides were scarcely hydrolyzed by the α -glucosidase (Fig. 5B). These results indicated that the gene product must be an α -1,4-glucosidase (EC 3.2.1.20).

Discussion

Degradation of starch to maltose by many bacteria is catalyzed by α -amylase and is followed by hydrolysis to glucose by the action of either intra- or extracellular α -glucosidases. The amino-terminal signal peptide found on most extracellular proteins serves to initiate export across the cytoplasmic mem-

brane in bacteria.⁽¹³⁾ The signal sequence generally consists of a positively charged segment with Arg residues, a central hydrophobic segment, and a cleavage segment started with the helix-breaker Pro residue.⁽¹³⁾ Mostly Ala, Gly, Ser, and Thr have been found in -1 site, and Ala, Gly, Leu, Ser, Thr, and Val in -3 site from the cleavage position (-3,-1 role of von Heijne)⁽¹⁴⁾ No these general patterns found in the amino-terminal peptides of J-13-3 α -glucosidase (1-44 amino acids in Fig. 3). The α -1,4-glucosidase from *T. curvata* and oligo-1,6-glucosidase from *B. flavocaldarius* have been reported to be intracellular enzymes. These results indicated that J-13-3 α -glucosidase also must be intracellular enzyme.

The gene encoding α -glucosidase found in downstream of the mycodextranase gene of the strain J-13-3. The similar positional gene has been reported in *Streptomyces* sp. (strain NRRL 30748)⁽¹⁵⁾ and therefore amino acid sequences of the two α -glucosidases were aligned (Fig. 6). The deduced amino acid sequence of J-13-3 strain had high similarity to that of NRRL 30748 strain (62 % identity). Four highly conserved regions (CR I-VI in Fig. 3) were also detected in the α -glucosidase of strain NRRL 30748 (Fig. 6). The presence of the putative genes in downstream of the mycodextranase gene predicted that the gene products would have enzyme activity hydrolyzing nigerose produced in nigeran degrada-

tion by the mycodextranase. However, the α -glucosidase expressed *in vitro* scarcely hydrolyzed nigerose and isomaltose. The oligo-1,6-glucosidase from *B. flavocaldarius* and the recombinant α -glucosidase (587 amino acids) from *Bacillus* sp. (strain SAM1606)⁽¹⁶⁾ with a broad substrate specificity also hydrolyzed nigerose, and Tyr or Gly residue in conserved region (EXY or EXG in CR III) was most important for the enzyme activity with the broad substrate specificity. But there was Trp residue in the positions of α -glucosidases from four *Streptomyces* strains and *T. curvata* (EXW in CR III of Fig. 3 and 6). These results suggested that J-13-3 α -glucosidase probably did not contribute utilization of nigeran.

Our preliminary experiments demonstrated that the recombinant α -glucosidase was produced in insoluble forms (*i.e.* in inclusion body) by *E. coli* using pUC19 and pET23a(+).^(7,17) Therefore, in this study, we used *in vitro* expression system for the α -glucosidase gene product and active α -glucosidase could be synthesized. However, the amount of active α -glucosidase felled to be very small. Yeast (*Pichia pastoris*) expression systems have been used successfully for the production of mammalian, bacterial, and fungal proteins.⁽¹⁸⁾ The J-13-3 α -glucosidase gene expression for large scale protein production is thus prime candidate for investigation in the yeast system.

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(Received October 31, 2009)

放線菌J-13-3株のマイコデキストラナーゼ遺伝子の下流に見出された α -グルコシダーゼ遺伝子の塩基配列と発現

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放線菌J-13-3株のマイコデキストラナーゼは、 α -1,3と α -1,4が交互に連結したD-グルカンであるニゲラン中の α -1,4結合を分解してニゲロースとニゲラン四糖を生産する α -グルカナーゼである。マイコデキストラナーゼ遺伝子下流に見出された α -グルコシダーゼをコードする遺伝子を解析した。 α -グルコシダーゼは537残基のアミノ酸からなり、分子量は60,263と推定された。推定アミノ酸配列を他の α -グルコシダーゼと比較した結果、 α -アミラーゼが属する糖質分解酵素ファミリー13に属し、触媒や基質との結合に関与する4つの領域が高度に保存されていた。試験管内で発現させた本遺伝子の発現産物はp-ニトロフェニル α -グルコースとマルトースを分解したが、イソマルトースとニゲロースは分解しなかったことから、 α -1,4-グルコシダーゼ (EC 3.2.1.20) であると考えられた。