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Identification and Characterization of Dextran α-1,2-Debranching Enzyme from *Microbacterium dextranolyticum*

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Abstract: Dextran α-1,2-debranching enzyme (DDE) releases glucose with hydrolyzing α-(1→2)-glucosidic linkages in α-glucans, which are made up of dextran with α-(1→2)-branches and are generated by *Leuconostoc* bacteria. DDE was isolated from *Microbacterium dextranolyticum* (formerly known as *Flavobacterium* sp. M-73) 40 years ago, although the amino acid sequence of the enzyme has not been determined. Herein, we found a gene for this enzyme based on the partial amino acid sequences from native DDE and characterized the recombinant enzyme. DDE had a signal peptide, a glycoside hydrolase family 65 domain, a carbohydrate-binding module family 35 domain, a domain (D-domain) similar to the C-terminal domain of *Arthrobacter globiformis* glucodextranase, and a transmembrane region at the C-terminus. Recombinant DDE released glucose from α-(1→2)-branched α-glucans produced by *Leuconostoc citreum* strains B-1299, S-32, and S-64 and showed weak hydrolytic activity with kojibiose and kojitriose. No activity was detected for commercial dextran and *Leuconostoc citreum* B-1355 α-glucan, which do not contain α-(1→2)-linkages. The removal of the D-domain decreased the affinity for α-(1→2)-branched α-glucans but not for kojioligosaccharides, suggesting that D-domain plays a role in α-glucan binding. Genes for putative dextranases, oligo-1,6-glucosidase, sugar-binding protein, and permease were present in the vicinity of the DDE gene, and as a result these gene products may be necessary for the use of α-(1→2)-branched glucans. Our findings shed new light on how actinobacteria utilize polysaccharides produced by lactic acid bacteria.

Key words: actinobacteria, dextran, exopolysaccharide, GH65, kojibiose, lactic acid bacteria

INTRODUCTION

Dextran is a polymer of D-glucose that has an α-(1→6)-linkage, although lower numbers of branch linkages are frequently seen. This polysaccharide is produced by several lactic acid bacteria (Lactobacillales), including *Leuconostoc*, *Lactobacillus*, and *Streptococcus* species, and the structure depends on the bacterial species and strains with different ratios of α-(1→2), α-(1→3), and α-(1→4) linkages to α-(1→6)-linkages being produced.[1)2)] This variety is mostly caused by the product specificity of glucansucrases, some of which also create additional linkages in addition to α-(1→6)-linkages.[3)] α-Glucan produced by *Leuconostoc mesenteroides* NRRL B-512F is composed of 95 % of α-(1→6)-linked glucose and 5 % of α-(1→3)-linked glucose.[4)] *Leuconostoc citreum* (formerly *L. mesenteroides*) NRRL B-1299 is known to produce α-glucan with a high concentration of α-(1→2) and a low concentration of α-(1→3)-linkages in addition to α-(1→6)-linkage.[1)5)6)7)] The percentage of α-(1→2)-linkages in α-glucan is reported to be relatively high, with 27 and 35 % of the total being present in fractions of lower and higher solubility, respectively, after ethanol precipitation.[5)6)7)] B-1299 α-glucans were divided into five fractions by solubility and molecular weight size, which all comprised 23−36% α-(1→2)-linked glucose.[8)9)] *L. citreum* B-1299 possesses bifunctional α-1,2-branching dextransucrase DSR-E that synthesizes dextran with α-(1→2) branch linkages, and α-1,2-branching

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Abbreviations: CBB, Coomassie Brilliant Blue; CBM, carbohydrate-binding module; DDE, dextran α-1,2-debranching enzyme; MdDDE, *Microbacterium dextranolyticum* dextran α-1,2-debranching enzyme; GH, glycoside hydrolase; GP, glycoside phosphorylase; LC-MS/MS, liquid chromatography-tandem mass spectrometry; TLC, thin-layer chromatography.

sucrase BRS-A, which introduces α-(1→2) branches to dextran.[10)11)12)] Contrarily, *L. citreum* NRRL B-1355 produces alternansucrase that synthesizes alternan, which has alternate α-(1→6) and α-(1→3)-linkages but no α-(1→2)-linkage.[13)] The fraction of these linkages has been calculated by methylation studies and nuclear magnetic resonance, and pinpointing more precise location and frequency of branching is difficult.

Dextranase is an *endo*-acting glycoside hydrolase (GH) that breaks down α-(1→6)-linkages in dextran and is found in the glycoside hydrolase family 31 (GH31),[14)15)] GH49,[16)17)18)] and GH66[19)20)] protein families according to the CAZy classification.[21)] GH15 glucodextranase and GH13 subfamily 31 (GH13_31) oligo-1,6-glucosidase are *exo*-acting GHs that hydrolyze α-(1→6)-linkages in dextran and isomaltooligosaccharides and generate β-glucose and α-glucose, respectively.[22)23)] GH27 isomalto-dextranase and GH49 dextran 1,6-α-isomaltotriosidase break down dextran into isomaltose and isomaltotriose, respectively.[24)25)] Nevertheless, there are a small number of enzymes that are known to degrade the aforementioned branching in dextran.

Dextran α-1,2-debranching enzyme (DDE, EC 3.2.1.115, branched-dextran exo-1,2-α-glucosidase) was initially discovered in 1978 and can break down α-(1→2) branches of α-glucan from *L. citreum* B-1299.[26)] Two α-glucan-degrading enzymes were identified in the culture supernatant of actinobacteria *Microbacterium dextranolyticum* (formerly *Flavobacterium* sp. M-73)[26)] and were named dextranase I (DDE) and dextranase II (dextran 1,6-α-isomal totriosidase).[27)28)29)] DDE is a 125-kDa protein, and the ratio of enzyme secreted into the culture medium to that found on the membrane surface was reported to be 8:1.[29)] DDE-based hydrolysis has been used to analyze the structure of oligosaccharides generated from the B-1299 α-glucan.[30)31)32)] DDE is effective for revealing the chemical structure of α-glucans from lactic acid bacteria with different structures, although the amino acid sequence cannot yet be determined.

In this study, we purified DDE from *M. dextranolyticum* culture medium and determined the amino acid sequence. Based on the bacterial genome, a gene for DDE was found, and recombinant enzymes were characterized. This study suggests a novel mechanism involving DDE for polysaccharide utilization by actinomycetes.

MATERIALS AND METHODS

Materials and strains. Reagents used were of analytical grade and were bought from FUJIFILM Wako Pure Chemicals Co. (Osaka, Japan) or Nacalai Tesque, Inc. (Kyoto, Japan) unless otherwise specified. Kojibiose, kojitriose, and nigerose were purchased from Biosynth Carbosynth (Newbury, UK). Isomaltose and isomaltotriose were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Isomaltooligosaccharides were kindly provided by Hayashibara Co., Ltd. (Okayama, Japan). α-Glucans from *L. citreum* S-32 and S-64 strains were prepared according to prior instructions.[33)] α-Glucans from *L. citreum* NRRL B-1299 and *L. citreum* NRRL B-1355 were kindly provided by Dr. Mikihiko Kobayashi. *M. dextranolyticum* NBRC 14592 was provided by NITE Biological Resource Center (Chiba, Japan). *Escherichia coli* DH5α and BL21-CodonPlus(DE3)-RIPL (Agilent Technologies, Inc., Santa Clara, CA, USA) were utilized for DNA manipulation and protein expression, respectively.

Purification of native DDE. A single colony of *M. dextranolyticum* was inoculated into 5 mL of medium (pH 7.0) containing 1 % peptone, 2 % yeast extract, and 1 % $MgSO_4 \cdot 7H_2O$ and then precultured with shaking at 30 ˚C for 24 h. In order to produce enzymes, the bacterium was further cultured in 2 L of medium (pH 7.0) containing 0.3 % yeast extract, 0.2 % $(NH_4)_2HPO_4$, 0.1 % KH_2PO_4, 0.05 % KCl, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.001 % $FeSO_4 \cdot 7H_2O$, and 0.5 % dextran 40,000 at 30 ºC for 24 h. After centrifugation at 8,000 × G for 10 min, ammonium sulfate was added to the supernatant to a final concentration of 70 % saturation in order to precipitate the protein. The resultant pellet was dissolved in 20 mM Tris-HCl buffer (pH 8.0) and then dialyzed against the same buffer. The protein was further purified using anion-exchange chromatography using a HiTrap DEAE FF column (Cytiva, Tokyo, Japan) pre-equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The material was eluted using a linear gradient of 0−1 M NaCl in 20 mM Tris-HCl buffer (pH 8.0) at a flow rate of 3.0 mL/min. Active fractions were collected, and their purity was determined by SDS-PAGE using either silver-staining or Coomassie Brilliant Blue (CBB) staining. Protein concentrations were determined with the bicinchoninic acid method using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The 122-kDa band was extracted from the CBB-stained SDS-PAGE gel and destained using 50 mM NH_4HCO_3/50 % acetonitrile. Proteins in the sliced gel fragments were reduced and alkylated by treatment with 10 mM dithiothreitol/50 mM NH_4HCO_3 for 45 min at 56 ˚C and 55 mM iodoacetamide/50 mM NH_4HCO_3 for 30 min at room temperature. After being washed with acetonitrile, the proteins were trypsinized (Promega Co., Madison, WI, USA) at 37 ˚C overnight. Tryptic peptides were extracted from the gel fragments with 50 % acetonitrile that included 3 % formic acid, and the extracts were then concentrated in a vacuum centrifuge. Tryptic peptides were analyzed with LC-MS/MS using a linear ion trap time-of-flight mass spectrometer, NanoFrontier eLD (Hitachi High-Technologies Corporation, Tokyo, Japan), coupled with a nanoflow HPLC, NanoFrontier nLC (Hitachi High-Technologies). Tryptic peptides were separated on Capillary EX-Nano Column (0.05 × 150 mm, GL Sciences, Japan) and eluted with a linear gradient of 5 to 40 % of solvent A (2 % acetonitrile and 0.1 % formic acid) and solvent B (98 % acetonitrile and 0.1 % formic acid) for 60 min at a flow rate of 200 nL/min. The eluent was ionized using a nanoelectrospray ionization source equipped with SilicaTip (New Objective, Inc., Woburn, MA, USA), and MS and MS/MS spectra were obtained in a positive ion mode at a scan mass range of m/z 200−2,000. To identify proteins, MS and MS/MS data were examined using a *de novo* sequencing and protein identification software PEAKS v. 7.0.[34)]

Expression and purification of recombinant DDE. Genomic DNA of *M. dextranolyticum* was extracted using DNeasy Blood & Tissue Kit (QIAGEN Gmbh, Hilden,

Germany). The gene encoding *M. dextranolyticum* DDE (MdDDE) was PCR amplified from the genomic DNA using ExTaq DNA polymerase (Takara Bio Inc., Kusatsu, Japan) and a pair of primers, 5′-ACCACACCACATGCACCAG ACGAAGGAGAA-3′ and 5′-CCGCGCTCCTCCATACTG ACATGTCGGCAT-3′, before it was ligated into T-vector pMD19 (Takara Bio). These primers were designed based on the *M. dextranolyticum* whole genome shotgun sequence (GenBank JAFBBR010000001.1). DNA segments encoding residues 45−1175 (named $MdDDE_{45–1175}$) and 45−913 ($MdDDE_{45–913}$) were PCR amplified using KOD One polymerase (Toyobo Co., Ltd., Osaka, Japan) and primers: 5′-GCGGCAGCCATATGGCTAGCGCCGAGAGCGG TTCGGCGCCGAC-3′ (forward) for both; 5′-TCGAGTGC GGCCGCAAGCTTCTAGGCTTTCAGCGCGACG AAG-3′ (reverse) for $MdDDE_{45–1175}$; and 5′-TCGAGTGCGG CCGCAAGCTTCTACGCCGTGCGGGTCGGAATGC-3′ (reverse) for $MdDDE_{45–913}$. Resultant fragments were cloned into pET-28a, predigested with NheI and HindIII, using In-Fusion HD Cloning Kit (Takara Bio). Constructed plasmids were verified using DNA sequencing. The nucleotide sequence of *MdDDE* was submitted to GenBank/DDBJ database under the accession number LC738866.

E. coli BL21-CodonPlus(DE3)-RIPL cells carrying the required plasmid were grown in Luria-Bertani medium (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl) supplemented with 50 μg/mL kanamycin, 25 μg/mL streptomycin, and 25 μg/mL chloramphenicol at 37 ˚C until the optical density reached 0.6. Protein expression was initiated by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 0.1 mM and by further culturing at 20 ˚C overnight. Cells were collected using centrifugation and then kept at −30 ˚C until needed. Cells were resuspended in 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 20 mM imidazole, and cOmplete™ protease inhibitor cocktail (F. Hoffmann-La Roche AG, Basel, Switzerland) and then sonicated. The supernatant after centrifugation (20,640 × G, 4 ˚C, 20 min) was added to a Ni-Sepharose excel (Cytiva) column that had been pre-equilibrated with the same buffer. After being washed with the buffer, the recombinant proteins were eluted with 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 250 mM imidazole. Fractions containing the recombinant enzymes were concentrated, and buffer was altered to 50 mM sodium acetate buffer (pH 5.6) using an Amicon Ultra 30K ultrafiltration device (Merck, Darmstadt, Germany). Protein concentrations were estimated using absorbance at 280 nm and the molecular absorption coefficients of 201,110 and 167,190 M^{-1} cm^{-1} for $MdDDE_{45–1175}$ and $MdDDE_{45–913}$, respectively.

Activity measurement of DDE. For each purification stage of native DDE, 4 μL of enzyme fraction, 1 μL of 50 mg/mL S-64 α-glucan, and 5 μL of 100 mM sodium acetate buffer (pH 5.6) were combined and incubated at 30 ˚C for 15 min.

To explore pH effects for recombinant MdDDE activity, enzymes (20 nM) were incubated with 5 mg/mL S-64 α-glucan in McIlvaine buffer (pH 2.2−8.0) at 30 ˚C for 5 min. For temperature dependence, reaction mixtures with 20 nM enzyme, 5 mg/mL S-64 α-glucan, and 50 mM sodium acetate buffer (pH 5.6) were created and evaluated between 20 to 70 ˚C. To test pH stability, enzyme concentration was adjusted to 200 nM using Britton−Robinson buffer (phosphate−acetate−borate buffer, pH 2.0−11) and incubated at 4 ˚C for 12 h. Incubated enzymes were twofold dilute with 50 mM sodium acetate buffer (pH 5.6) before evaluation. To assess thermostability, enzymes (200 nM) in 50 mM sodium acetate buffer (pH 5.6) were incubated at 20 to 80 ˚C for 10 min and then stored on ice until use. Each incubated enzyme (final concentration of 20 nM) was reacted with 5 mg/mL S-64 α-glucan in 50 mM sodium acetate buffer (pH 5.6) at 30 ˚C for 5 min. All reactions were quenched by heating at 100 ˚C for 5 min. The amount of released glucose was measured using the glucose oxidase−peroxidase method with a LabAssay Glucose Kit (FUJIFILM Wako Chemicals Co., Osaka, Japan).

For investigation of substrate specificity of recombinant DDE, reaction mixtures containing 20 nM $MdDDE_{45–1175}$, 0.5 % (w/v) α-glucan or 10 mM oligosaccharide as a substrate, and 50 mM sodium acetate buffer (pH 5.6) were incubated at 30 ˚C and then analyzed by thin-layer chromatography (TLC) using silica gel 60 F_{254} aluminum sheet (Merck). Oligosaccharides used were trehalose (α-D-Glc-(1↔1)-α-D-Glc), kojibiose (α-D-Glc-(1→2)-D-Glc), nigerose (α-D-Glc-(1→3)-D-Glc), maltose (α-D-Glc-(1→4)-D-Glc), and isomaltose (α-D-Glc-(1→6)-D-Glc). Samples spotted on TLC sheets were developed using 1-butanol:ethanol:water at 5:5:2 (v/v) twice, followed by spraying with 5 % (v/v) sulfuric acid/methanol solution and charring at 110 ˚C. S-64 α-glucan and dextran 40,000 were incubated along with 20 nM MdDDE and 8 U/mL of *Chaetomium* sp. dextranase (Megazyme, Wicklow, Ireland) and analyzed by TLC in the same way.

Initial velocities of the hydrolytic reactions for B-1299, S-32, and S-64 α-glucans were determined using reaction mixtures containing 20 nM enzyme, 50 mM sodium acetate buffer (pH 5.6), and 0.2−5 mg/mL α-glucan incubated for 5 min at 30 ˚C. For kojibiose and kojitriose, reaction mixtures containing 200 nM enzyme, 50 mM sodium acetate buffer (pH 5.6), and 5−30 mM substrate were incubated at 30 ˚C for 10 min. All reactions were quenched by heating at 100 ˚C for 5 min. The amount of released glucose was measured using the glucose oxidase−peroxidase method with a LabAssay Glucose Kit (Fujifilm Wako Chemicals). Kinetic tests were carried out three times for each reaction. The kinetics parameters were determined via nonlinear regression analysis using KaleidaGraph (Synergy Software, Reading, PA, USA).

Servers and software. Signal peptides of MdDDE and related enzymes were predicted using SignalP.[35] Domain organization was projected based on the findings of InterPro[36] and Conserved Domain Database of NCBI.[37] Structure models of MdDDE were created using the whole amino acid sequence and ColabFold (AlphaFold2 with MMseqs2 sequence clustering module).[38][39] Diagrams of protein structures were created using PyMOL software (Schrödinger, Inc., New York, NY, USA). Amino acid sequence alignment was carried out using ClustalOmega.[40] Alignment data were produced with ESPript 3.0.[41]

RESULTS

Purification and identification of enzyme.

M. dextranolyticum was cultured in media containing B-1299 α-glucan or dextran 40,000 (no α-(1→2)-linkage),

and DDE activity was found in both culture supernatants as previously described.[28] In order to purify DDE, the bacterium was grown in 2 L of culture containing dextran 40,000 as the only carbon source, and the supernatant was harvested. Based on the DDE activity toward *L. citreum* S-64 α-glucan, which was reported to contain 5 % α-(1→2)-linked and 19 % α-(1→2)/α-(1→6)-linked glucose,[33] the enzyme was purified using ammonium sulfate precipitation and anion-exchange chromatography and was seen as a practically single band on SDS-PAGE (Fig. 1). The molecular mass of the purified protein was determined to be approximately 122 kDa by SDS-PAGE, which is comparable to the previously reported size.[28] The purified enzyme displayed a specific activity of 81.2 U/mg, and 15.4-fold purification was achieved with a yield of 2.48 % (Table 1).

To determine the proteins in the 122-kDa band of SDS-PAGE, peptides that had been trypsinized in gels were examined by LC-MS/MS. MS and MS/MS search using a database of proteins predicted from the shotgun genome sequence of *M. dextranolyticum* DSM 8607 (GenBank JAFBBR010000001.1) found four proteins from the known GH families (Table S1 and Fig. S1). The most prominently detected protein was a putative endoglucanase (GenBank MBM7461785.1) identified as belonging to GH9, where only GHs working on β-linkages are discovered.[21][42] The second most significant protein was a putative trehalose/maltose hydrolase-like predicted phosphorylase (MBM7463898.1), which was homologous to enzymes belonging to GH65, a family that includes GHs and glycoside phosphorylases (GPs) that act on α-glucosidic linkages in oligosaccharides such as maltose phosphorylase, kojibiose phosphorylase, and trehalase.[43][44][45] Kojibiose hydrolase, a member of GH65 that hydrolyzes α-(1→2)-glucosidic linkage in kojioligosaccharides, has recently been discovered in bacteria.[46][47] A third protein was revealed to belong to GH97, where anomer-inverting α-glucoside hydrolases, anomer-retaining α-galactosidases, and anomer-retaining β-L-arabinopyranosidase have been found.[48][49][50] However, the GH97 protein lacks a putative signal peptide in contrast to the other two proteins found and shares a substantially larger degree of sequence identity with retaining α-galactosidases than with inverting α-glucoside hydrolases. The estimated molecular masses of these detected proteins were about 120 kDa, which is comparable to that determined by SDS-PAGE. As a result, the GH65 protein (hereafter MdDDE) was a candidate for DDE; alternative proteins with comparable molecular masses may have been discovered due to insufficient purification.

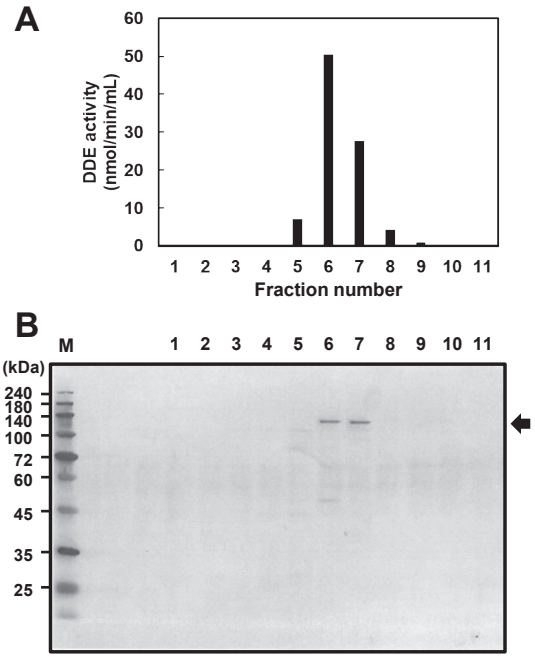

Fig. 1. Purification of native MdDDE.

(A) Hydrolytic activity of HiTrap DEAE FF-separated fractions (Nos. 1−11) was measured using S-64 α-glucan as a substrate. (B) SDS-PAGE analysis of HiTrap DEAE FF-separated fractions. Protein bands were visualized using silver-staining. The black arrow indicates the size of the 122-kDa band used for the LC-MS/MS analysis. Lane M, molecular weight marker PM1700 (SMOBIO); lanes 1−11, Nos. 1−11 fractions of anion-exchange chromatography.

Structure prediction.

MdDDE shares 33 and 29 % total sequence identity with *Flavobacterium johnsoniae* GH65 kojibiose hydrolase (FjGH65A)[46] and human GH65 protein glucosylgalactosylhydrolxylysine α-glucosidase (HsPGGHG),[51] respectively. On the other hand, MdDDE exhibits a lower level of sequence identity (24 %) with *Caldicellulosiruptor saccharolyticus* GH65 kojibiose phosphorylase (CsKP, ABP66077.1).[44] MdDDE was anticipated to be a multi-domain protein made up of an N-terminal signal peptide, carbohydrate-binding module family 35 (CBM35) domain, GH65-conserved domains (N-domain, linker, A-domain, and C-domain), DOMON_like superfamily domain (D-domain), and C-terminal transmembrane region (Fig. 2A). The CBM35 domain shares 26 % identity with a CBM35 domain of *Paenibacillus* sp. 598K GH31 α-glucosyltransferase Ps6GT31A[52] and was discovered to be inserted in the N-domain based on the AlphaFold2 prediction (Fig. 2B). The D-domain is joined by a brief linker after the C-domain and shares 44 % identity with the C-terminal domain of *Arthrobacter globiformis* GH15 glucodextranase.[22]

Table 1. Purification of DDE from *M. dextranolyticum* NBRC 14592.

Fraction	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Culture supernatant	1900	2400	13000	5.3	100	1.0
$(NH_4)_2SO_4$ precipitation and dialysis	55	27	1300	50	10	9.5
HiTrap DEAE FF	10	3.9	320	81	2.5	15

One unit (U) of the enzyme activity was defined as the amount of enzyme liberating 1 μmol glucose per min under the stated conditions.

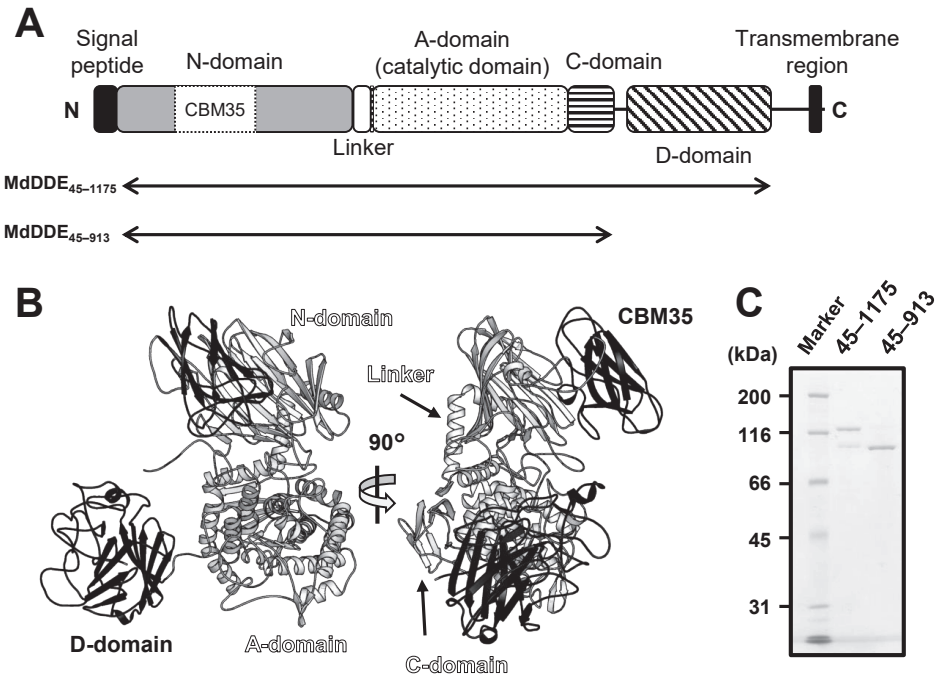

Fig. 2. Domains of native and recombinant MdDDE.

(A) Predicted domain organization of MdDDE: the signal peptide (residues 1−44, black), N-domain (45−145 and 283−453, gray), inserted CBM35 domain (146−282, dotted box), linker (454−489, white), A-domain (495−837, dot pattern), C-domain (490−494 and 838−913, horizontal stripes), D-domain (923−1175, diagonal pattern), and C-terminal transmembrane region (1244−1263, black). The regions of recombinant MdDDE45–1175 and MdDDE45–913 are indicated as two direction arrows. (B) AlphaFold2 model of MdDDE. The disordered regions at the N- and C-termini (residues 1−44 and 1176−1273, respectively) are not shown for clarity. The GH65-conserved domains (N-domain, linker, A-domain, and C-domain) are indicated as *white* and the CBM35 domain and D-domain as *black*. (C) SDS-PAGE analysis of purified recombinant MdDDE45–1175 and MdDDE45–913 stained with Coomassie Brilliant Blue.

Activity of recombinant MdDDE.

In order to test if MdDDE demonstrates DDE activity and D-domain functionality, two truncated versions (designated MdDDE45–1175 and MdDDE45–913) were created and produced in *E. coli*. Both recombinant proteins lacked the N-terminal signal peptide and the C-terminal transmembrane region, and the latter variant also lacked the D-domain (Fig. 2A). The recombinant proteins were purified to homogeneity using immobilized metal affinity chromatography (Fig. 2C) to produce yields of roughly 400−500 μg/L of culture.

First, we evaluated the hydrolytic activity of MdDDE45–1175 toward α-glucans containing various α-glucosidic linkages. When B-1299 and S-64 α-glucans, which primarily consist of α-(1→6)-linkages with a relatively high amount of α-(1→2) branches, were used as a substrate, only a spot corresponding to glucose was discovered by TLC (Fig. 3A). In contrast, no new spot was observed in the reaction mixtures containing B-1355 α-glucan, dextran 40,000, or soluble starch, which hardly ever contain α-(1→2)-glucosidic linkages. Overnight incubation with 10 mM α-glucobioses (trehalose, kojibiose, nigerose, maltose, and isomaltose) (data not shown) did not produce TLC-detectable hydrolysis, although a small quantity of hydrolytic activity toward kojibiose was found by the glucose oxidase−peroxidase method (described below). These findings show that MdDDE does have DDE activity. MdDDE also released glucose from *L. citreum* S-32 α-glucan, in which α-(1→2) linkages were not analyzed in the previous study, suggesting that S-32 α-glucan contained α-(1→2)-linked glucose (Fig. 3A). Additionally, S-64 α-glucan and dextran 40,000 were incubated with MdDDE45–1175 and *Chaetomium* sp. endodextranase (Fig. 3B). The endodextranase alone hardly degraded S-64 α-glucan and produced low amounts of glucose and isomaltooligosaccharides compared with that produced from dextran 40,000. The co-incubation with MdDDE45–1175 did not affect the degradation pattern of dextran but did increase the amount of isomaltose produced when S-64 α-glucan was used as a substrate. These results support the hypothesis that MdDDE45–1175 removed α-(1→2) glucose branches in S-64 α-glucan, facilitating the degradation of the α-(1→6)-linkages in the main chain by the endodextranase.

MdDDE45–1175 and MdDDE45–913 showed the highest activity toward S-64 α-glucan between pH 5.0 and 5.6 (Fig. 4A), which is consistent with native DDE activity.[30] The optimum temperatures of MdDDE45–1175 and MdDDE45–913 were 40 and 30 °C, respectively (Fig. 4B), which are lower than that of native DDE (50 °C).[30] MdDDE45–1175 possessed > 80 % activity after incubation at pH 7.0−9.0, whereas MdDDE45–913 retained > 80 % activity at pH 5.0−9.0 (Fig. 4C). A similar trend was observed for native DDE, which was stable on the alkaline side of the pH optimum.[30] MdDDE45–1175 and MdDDE45–913 exhibited > 80 % residual activity after incubation at ≤ 40 and ≤ 45 °C, respectively (Fig. 4D). The optimum temperature of MdDDE45–1175 was higher than that of MdDDE45–913, but MdDDE45–1175 was less stable after 10 min of heat treatment. This may be because MdDDE45–913 refolded more easily than MdDDE45–1175 while the enzymes were stored on ice after heat treatment.

Kinetic analysis. The K_m value of MdDDE45–1175 for B-1299 α-glucan was 0.332 mg/mL, which is comparable with a previously reported K_m value (0.27 mg/mL) for native DDE[30] (Table 2). The K_m values for S-32 α-glucan and S-64

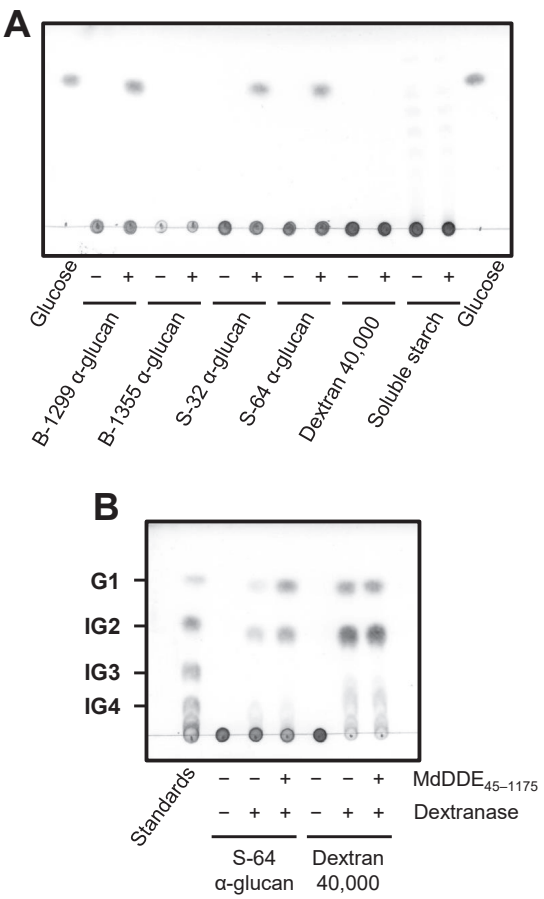

Fig. 3. TLC analysis of hydrolytic activity of recombinant MdDDE$_{45–1175}$.

(A) Hydrolytic activity toward various α-glucans. Reaction mixtures containing 2 nM enzyme and 5 mg/mL α-glucan were incubated at 30 ºC for 12 h. Glucose was used as a standard. *Plus* and *minus* indicate whether MdDDE$_{45–1175}$ was added or not. (B) Co-incubation of MdDDE$_{45–1175}$ (2 nM) and endodextranase from *Chaetomium* sp. (8 U/mL) with S-64 α-glucan and dextran 40,000 at 30 ˚C for 13 h. Glucose and isomaltooligosaccharides (isomaltose to isomaltooctaose) were used as standards. *Plus* and *minus* indicate whether each enzyme was added or not. Abbreviations: G1, glucose; IG2, isomaltose; IG3, isomaltotriose; and IG4, isomaltotetraose.

α-glucan were approximately 2.6- and 3.0-fold higher than that for B-1299 α-glucan, respectively, although there was not much difference among the k_{cat} values for these substrates. MdDDE$_{45-1175}$ exhibited hydrolytic activity toward kojibiose and kojitriose with K_m values of 12.1 and 35.3 mM and k_{cat} values of 3.13 and 6.66 s^{-1}, respectively. These K_m values are much higher than those of FjGH65A for kojibiose and kojitriose, whereas the k_{cat} values are much lower than those for FjGH65A; the k_{cat} values for S-64, S-32, and B-1299 α-glucans were comparable with those of FjGH65A. These results indicate that MdDDE prefers α-(1→2)-glucosidic linkages in *Leuconostoc* α-glucans to linkages present in kojioligosaccharides.

In comparison with MdDDE$_{45-1175}$, MdDDE$_{45-913}$, which lacked the D-domain, had a reduced k_{cat} value of approximately 50 %, whereas the K_m values were increased threefold for the S-64, S-32, and B-1299 α-glucans (Table 2). Moreover, there were no significant differences observed in either the k_{cat} or K_m values for kojibiose and kojitriose between MdDDE$_{45-913}$ and MdDDE$_{45-1175}$. These results suggest that the D-domain affects α-glucan recognition and/or release of product α-glucan.

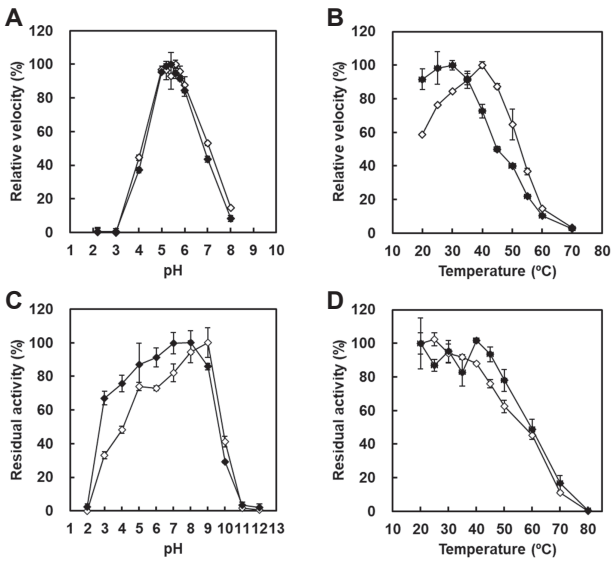

Fig. 4. Effect of pH and temperature on hydrolytic activity of recombinant MdDDE.

pH dependence (A), temperature dependence (B), pH stability (C), and thermostability (D) of the recombinant enzymes. pH dependence was measured at 30 ˚C using McIlvaine buffer (pH 2.2−8.0), and temperature dependence was measured at 20−70 ˚C using 50 mM sodium acetate buffer (pH 5.6). pH stability was measured at 30 ˚C using enzymes after incubation in Britton−Robinson buffer with varying pH for 12 h at 4 ˚C. Thermostability was measured at 30 ˚C using enzymes preincubated at varying temperatures for 10 min. S-64 α-glucan was used as a substrate for all the measurements. Plots and error bars represent the average and the standard error of triplicate measurements, respectively, for each enzyme: *open diamond*, MdDDE$_{45–1175}$; *filled diamond*, MdDDE$_{45–913}$.

DISCUSSION

In this study, we purified native DDE, whose sequence had long been unknown, from *M. dextranolyticum* and enzymatically characterized the recombinant proteins based on this. The recombinant enzyme exhibited the same activity and substrate specificity and had similar pH and temperature profiles as the native enzyme, indicating that the MdDDE protein was indeed DDE. According to the definition of DDE (EC 3.2.1.115), the International Union of Biochemistry and Molecular Biology (IUBMB) comments that DDE does not hydrolyze disaccharides or oligosaccharides containing linear 1,2-α-glucosidic linkages. This is because native DDE was not found to hydrolyze kojibiose in the previous study.[30] In contrast to this statement, the present study demonstrated that MdDDE had very low activity against kojibiose and kojitriose. Judging from the sequence, this enzyme is a member of the GH65 family and was expected to be a membrane enzyme localized to the bacterial cell surface. The bacterium is reported to produce DDE in both cell-bound and extracellular forms in a ratio of approximately 8:1,[29] suggesting that the purified native DDE was a free enzyme that was released from the cell surface by proteolysis.

GH65 consists mainly of GPs from bacteria and GHs from eukaryotes that act on α-glucosidic linkages,[43][44][45] and recently the first bacterial GH, kojibiose hydrolase (EC 3.2.1.216), was discovered in *Flavobacterium johnsoniae* (FjGH65A) and *Mucilaginibacter mallensis*.[46][47] Moreover, the biochemical and crystallographic studies of FjGH65A

Table 2. Hydrolytic activity of DDE for α-glucans and kojioligosaccharides.

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (mg/mL)	k_{cat}/K_m ($s^{-1}\,mg^{-1}\,mL$)
$MdDDE_{45-1175}$	S-64 α-glucan	111 ± 2	1.01 ± 0.05	110
	S-32 α-glucan	99.0 ± 0.9	0.872 ± 0.027	113
	B-1299 α-glucan	96.3 ± 0.9	0.332 ± 0.014	289
	B-1355 α-glucan	ND [d]	ND	ND
	Dextran 40,000	ND	ND	ND
	Soluble starch	ND	ND	ND
	Kojibiose	3.13 ± 0.10	12.1 ± 0.9 [e]	0.260 [f]
	Kojitriose	6.66 ± 0.54	35.3 ± 4.6 [e]	0.188 [f]
$MdDDE_{45-913}$	S-64 α-glucan	53.3 ± 6.4	3.41 ± 0.80	15.6
	S-32 α-glucan	48.5 ± 5.3	3.16 ± 0.70	15.3
	B-1299 α-glucan	50.1 ± 1.7	0.977 ± 0.107	51.3
	Kojibiose	2.32 ± 0.06	11.0 ± 0.8 [e]	0.210 [f]
	Kojitriose	4.11 ± 0.23	24.7 ± 2.5 [e]	0.166 [f]
Native DDE [a]	B-1298 α-glucan [c]	NA [g]	0.21	NA
	B-1299 α-glucan	NA	0.27	NA
	B-1397 α-glucan [c]	NA	0.91	NA
FjGH65A [b]	Kojibiose	108	0.28 [e]	399 [f]
	Kojitriose	46.5	0.13 [e]	273 [f]
	B-1299 α-glucan	weak activity	–	–

[a]Values were referred from Mitsuishi *et al.*[30] [b]Values were referred from Nakamura *et al.*[46] [c]α-Glucans produced by *L. mesenteroides* strains B-1298 and B-1397 contain 19 and 14 % α-(1→2) linkages at branch point, respectively.[30] [d]ND, not detected. [e]Values are represented in mM unit. [f]Values are represented in $s^{-1}mM^{-1}$ unit. [g]NA, not available because the molar concentration of native DDE was unknown.

revealed catalytic residues and the anomer-inverting mechanism of GH65 GHs.[46] MdDDE was identified as a GH that released glucose from α-(1→2)-branched α-glucan in the present study and is therefore the second bacterial GH65 GH following kojibiose hydrolase. The sequence alignment with other GH65 enzymes revealed that both general acid (Glu666) and general base (Glu811) catalysts, which are essential for hydrolytic activity, are conserved (Fig. 5A). In addition, MdDDE lacks the double serine motif that is conserved among GH65 GPs for binding phosphate. These findings support the assertion that MdDDE is indeed a GH member of GH65.

The AlphaFold2 model of MdDDE was compared with the crystal structure of FjGH65A complexed with glucose (Fig. 5B). The two catalytic glutamate residues mentioned above are spatially conserved. The residues recognizing glucose (named Glc − 1) at subsite − 1 (subsite nomenclature according to Davies *et al.*[53]) are completely conserved (not shown), and most of the residues recognizing glucose (Glc+1) at subsite +1 were shared with FjGH65A and CsKP, both of whose substrate is kojibiose.[44][46] Since the subsite +1 residues vary among GH65 GPs that act on α-glucobioses other than kojibiose, e.g., maltose phosphorylase,[43][44][46] the combination of these residues is important for the specificity toward kojibiose. Thr407 in FjGH65A is replaced by Phe601 in MdDDE, which may influence the difference in oligosaccharide/polysaccharide specificity. This region containing Arg74, which forms a hydrogen bond with O6 of Glc+1 in FjGH65A, is not conserved in MdDDE, and the space corresponding to this was wide open. FjGH65A has very low activity against B-1299 α-glucan and high activity against kojibiose, but MdDDE showed the opposite specificity (Table 2). FjGH65A was predicted to be unable to recognize the kojibiose structure in the middle of the glucan chain because of Arg74, but MdDDE was expected to be able to recognize and degrade the α-(1→2)-branch of the glucan chain because of the space corresponding to the subsite +2NR (nomenclature according to Cartmell *et al.*[54]) (Fig. 5C). It is necessary to experimentally analyze the structure of MdDDE to investigate how the enzyme

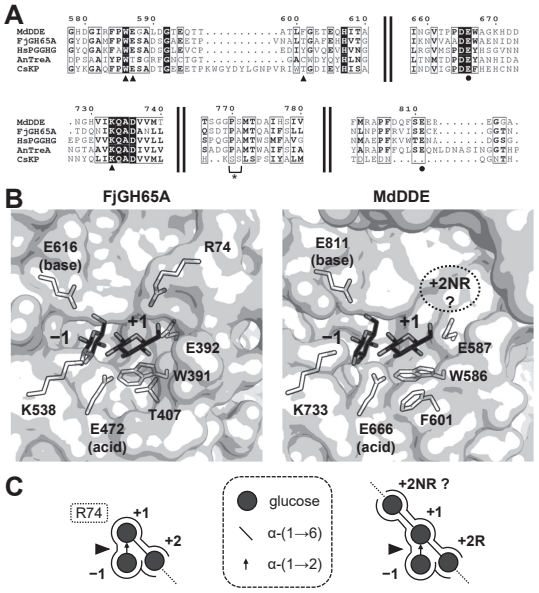

Fig. 5. Catalytic site of MdDDE.

(A) Multiple sequence alignment of MdDDE and GH65 enzymes. Amino acid sequences were obtained from GenBank: *Flavobacterium johnsoniae* kojibiose hydrolase (FjGH65A, WP_044048041.1); *Homo sapiens* protein glucosylgalactosylhydroxylysine α-glucosidase (HsPGGHG, NP_079368.1); *Aspergillus nidulans* trehalase (AnTreA, EAA66407.1); and *Caldicellulosiruptor saccharolyticus* kojibiose phosphorylase (CsKP, ABP66077.1). Symbols: *filled circle*, catalytic acid and base; *filled triangle*, residues interacting with glucose at subsite +1 (Glc+1); *asterisk*, double serine motif essential for phosphate-binding in GH65 GPs. (B) Catalytic sites of the crystal structure of FjGH65A complexed with glucose (PDB 7FE4, *left*) and MdDDE AlphaFold2 model (*right*). The catalytic residues and the residues interacting with Glc+1 are shown as a *white* stick model and glucose molecules at subsites − 1 and +1 as a *black* stick model. Two glucose molecules in 7FE4 are superimposed into the MdDDE model (*right*). The hypothetical subsite +2NR is indicated as a dotted circle. (C) Schematic drawings of subsites in FjGH65A (*left*) and MdDDE (*right*). Sugar and linkage symbols are shown in the *middle*. Black triangles indicate cleavage points by each enzyme.

recognizes the α-(1→2)-branch in the α-glucan.

The kinetic study suggests that the D-domain is involved in the recognition of α-glucan chains. The D-domain is classified in the Glucodextran_C family of the DOMON_like superfamily. Proteins of this family adopt a β-sandwich fold, which is similar to CBM9 proteins,[55] and the Glucodextran_C domain has been found in not only *A. globiformis* glucodextranase but also in GH57 amylopullulanase.[56][57] CBMs are noncatalytic modules with sugar-binding ability found in carbohydrate-active enzymes and are involved in assisting enzyme functions, such as proximity effect and targeting function.[58] However, Glucodextran_C domains have not been revealed to bind sugars and have not been identified as a CBM. A BLASTP search using the D-domain as a query found GH65 and GH15 proteins as well as bacterial proteins belonging to the GH49 family, where dextranases are classified (data not shown). To establish whether the D-domain is a CBM, further experiments are needed to prove direct interaction between this domain and α-glucans.

The shotgun genome data of *M. dextranolyticum* DSM 8607 (= NBRC 14592) identified genes for putative GHs, sugar-binding proteins, permease, and ROK transcriptional regulators that were located (JOE64_002368 to JOE64_002374) in the vicinity of the *MdDDE* gene (JOE64_002367) (Fig. 6A). There are two genes for GH49 proteins (GH49-1 and GH49-2, 63 % identity with each other), both of which have a signal sequence, a GH49 catalytic domain, and a domain homologous to the D-domain. GH49-2 is presumed to have a transmembrane region at the C-terminus. GH49-1 and GH49-2 are most homologous to *Arthrobacter* sp. KQ11 dextranase[59] with sequence identities of 55 and 61 %, respectively. JOE64_002370 has no signal sequence and belongs to the GH13 subfamily 31 (GH13_31), which contains *exo*-acting enzymes such as oligo-1,6-glucosidase that hydrolyzes isomaltooligosaccharides.[23] Dextran 1,6-α-isomaltotriosidase (dextranase II) was previously found in *M. dextranolyticum* culture supernatant along with DDE.[27][28][29] Therefore, the MdDDE and proteins encoded in the vicinity of MdDDE gene are presumed to be involved in the metabolic pathway that degrades α-(1→2)-branched α-glucan extracellularly to oligosaccharides and intracellularly to glucose (Fig. 6B). DDE homologs are found in *Rarobacter faecitabidus* (TQL64194.1) and *Luteimicrobium xylanilyticum* (QFU99754.1) and are classified into the GH65:10.1 group based on CUPP analysis.[60] In the GH65:10.1, most proteins are derived from actinobacteria, including *Streptomyces* spp. These findings suggested that actinobacteria possessing MdDDE homologs may utilize α-(1→2)-branched α-glucan produced by *Leuconostoc* spp.

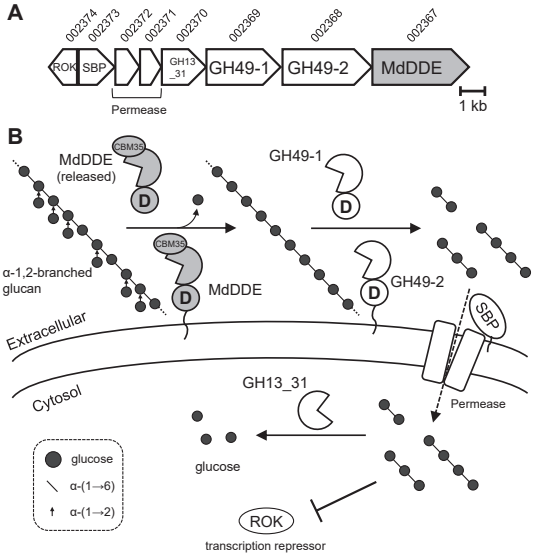

Fig. 6. Proposed function of MdDDE.

(A) Putative gene locus around *MdDDE* gene based on the whole genome shotgun sequence of *M. dextranolyticum* DSM 8607 (GenBank, JAFBBR010000001.1). Numbers above the genes represent locus tag numbers (JOE64_002367 to JOE64_002374). (B) Proposed mechanism of degradation of α-1,2-branched α-glucan by MdDDE and enzymes encoded in the locus in (A). Putative dextranases, GH49-1 and GH49-2, have a domain (*circle* labeled with D) that is homologous to D-domain of MdDDE, while GH49-2 possesses a transmembrane region at the C-terminus. Abbreviations: ROK, repressor, open reading frame, kinase; SBP, sugar-binding protein.

In summary, we identified DDE from *M. dextranolyticum* as a multi-domain enzyme with a catalytic domain classified as GH65. The enzymatic analysis of the recombinant MdDDE suggested that this specifically hydrolyzes α-(1→2)-linked branches in B-1299 and S-64 α-glucans and that the Glucodextran_C family D-domain may recognize α-glucan chains. DDE may be involved in the utilization of α-glucans from lactic acid bacteria by actinobacteria. Therefore, this enzyme may be useful in further detailed structural analysis of α-glucans produced by lactic acid bacteria.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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