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Expression of Cyclomaltodextrinase Gene from *Bacillus halodurans* C-125 and Characterization of Its Multisubstrate Specificity

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Abstract A putative cyclomaltodextrinase (BHCD) gene was found from the genome of *Bacillus halodurans* C-125, which encodes 578 amino acids with a predicted molecular mass of 67,279 Da. It shares 42-59% of amino acid sequence identity with common cyclomaltodextrinase (CDase)-family enzymes. The corresponding gene was cloned by polymerase chain reaction (PCR) and the dimeric enzyme with C-terminal 6-histidines was successfully overproduced and purified from recombinant *Escherichia coli*. BHCD showed the highest activity against β-CD at pH 7.0 and 50°C. Due to its versatile hydrolysis and transglycosylation activities, BHCD has been confirmed as a member of CDases. However, BHCD can be distinguished from other typical CDases on the basis of its novel multisubstrate specificity. While typical CDases have over 10 times higher activity on β-CD than starch or pullulan, the CD-hydrolyzing activity of BHCD is only 2.3 times higher than pullulan. In particular, it showed significantly higher activity ratio of maltotriose to acarbose than other common CDase-family enzymes.

Keywords: *Bacillus halodurans* C-125, cyclomaltodextrinase (CDase), gene cloning, enzymatic characterization, multisubstrate specificity

Introduction

Three groups of enzymes, cyclomaltodextrinases (CDases; EC 3.2.1.54), maltogenic amylases (MAases; EC 3.2.1.133), and neopullulanases (NPases; EC 3.2.1.135), have been separately reported. Even though these enzyme groups have their own EC numbers, they are known to share very similar enzymatic properties, substrate specificities, and primary structures. Lee *et al.* (1) reported that these 3 types of enzymes are nearly indistinguishable from one another and can be classified into the same group of enzyme. Especially, their primary and the tertiary structures revealed that they are very closely related with each other. Herein, the term of 'CDase-family enzymes' was suggested on the basis of their much higher CD-degrading activity than the other substrates and it enables these enzymes to be distinguished from other typical amylases (2,3). For the past decades, a number of CDase-family enzymes have been reported from a variety of microbial origins such as *Bacillus* (4-8), *Paenibacillus* (9), *Clostridium* (10), *Thermoactinomyces* (11), *Klebsiella* (12), *Thermus* (13), and *Lactobacillus* (14) species. In particular, Oh *et al.* (2) showed that the CDase-family genes are widely distributed in various *Bacillus* genomes.

Three-dimensional structures of CDase-family enzymes from *Thermoactinomyces* (15), *Thermus* (16), and *Bacillus* (17) showed that these enzymes possesses the extra N-terminal domain (about 130 amino acid residues) absent in other typical amylases. Kim *et al.* (18) suggested that the corresponding N-domain of CDase-family enzymes plays important roles in both the formation of homo-dimeric structure and the modulation of multisubstrate specificity.

Typical CDase-family enzymes not only hydrolyze carbohydrate substrates, but also transfer the hydrolyzed sugar moiety simultaneously to various acceptor molecules by forming α-(1,3)-, α-(1,4)-, or α-(1,6)-glycosidic linkages. Moreover, MAases are known to hydrolyze acarbose, a potent inhibitor of amylolytic enzymes, to glucose and acarviosine-glucose (13,19). Their versatile hydrolysis and transglycosylation activities can be applicable to the enzymatic modification of natural compounds, such as functional food and pharmaceuticals (20-22). Accordingly, the multisubstrate specificities of CDase-family enzymes have been investigated against a variety of donor and acceptor molecules, which can be closely related to their primary, tertiary, and quaternary structures. However, there have been some problems to compare their multisubstrate specificities due to the high complexity in activity assay.

The complete genome of an alkaliphilic bacterium, *Bacillus halodurans* C-125 (JCM 9153), was recently sequenced and its special mechanism of adaptation to an alkaline environment has been highly focused (23). In this study, a putative CDase gene has been found from the genome of *B. halodurans*. Molecular cloning and expression of the gene was tried for the enzymatic characterization, especially for its multisubstrate specificity.

Materials and Methods

Bacterial strains and plasmids *Escherichia coli* MC1061 was used as a host for the cloning and expression of the target gene. The resulting transformant was grown in

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Luria-Bertani (LB) medium containing ampicillin (100 μg/mL) at 37°C. Purified genomic DNA (BAA-125^{TM}) of *Bacillus halodurans* C-125 (JCM 9153) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The commercial T-cloning vector of pMD18-T (Takara Biomedical Inc., Otsu, Japan) was used for the direct cloning of polymerase chain reaction (PCR) products. The expression vector pHCXHD (24), modified from pHCEII-*Nde*I (BioLeaders Co., Daejeon, Korea), was applied to the constitutive expression of BHCD gene.

Chemicals and enzymes *Taq* DNA polymerase and restriction endonucleases were purchased from Roche Applied Science (Mannheim, Germany) and Takara Biomedical Inc., respectively. *AccuPrep* Plasmid extraction kit, PCR purification kit, and oligonucleotide primers were provided by Bioneer Co. (Daejeon, Korea).

PCR amplification and gene cloning Based on the known coding sequence for BHCD, a set of oligonucleotide primers, BHCD-N (5'-TTCATATGAAAGCTACAATCTA CCAT-3') and BHCD-C (5'-TCTCGAGTTTAAGAACCA TCGCGG-3'), were designed and used for the PCR amplification of the BHCD gene. PCR amplification was performed using the Px2 thermal cycler (Thermo-Hybaid, Middlesex, UK) as follows: an initial denaturation step for 1 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min 30 sec at 72°C, and a final cycle of 10 min at 72°C. Amplified DNA fragment was cloned into the pMD-18T cloning vector, which was designated as pMD-BHCD. The entire nucleotide sequence of BHCD gene was determined by SolGent Co. (Daejeon, Korea) using a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Gene expression and purification of BHCD After the treatment of pMD-BHCD with *Nde*I and *Xho*I, the fragment of 1.7 kb was ligated with an expression vector pHCXHD. The resulting plasmid was designated as pHCXBHCD, which was introduced into *E. coli* MC1061 cells by $CaCl_2$ method. Recombinant *E. coli* harboring pHCXBHCD was cultivated in LB broth containing ampicillin (100 μg/mL) at 37°C for 12 hr and the grown cells were disrupted by sonication (VCX750; Sonics & Materials, Inc., Newtown, CT, USA). Finally, the recombinant BHCD with a C-terminal 6 histidine-tag was simply purified to apparent homogeneity using the AKTA Prime^{TM} system with HisTrap-FF column (GE Healthcare, Uppsala, Sweden).

Gel permeation chromatography (GPC) Superdex-200 column (10×300 mm; GE Healthcare) was used to estimate the apparent molecular weight and oligomeric state of the recombinant BHCD at room temperature. Protein samples were applied to the column equilibrated with 50 mM of sodium phosphate buffer (pH 7.0) and eluted with the same buffer at a flow rate of 0.5 mL/min. Protein molecular weight markers (Sigma-Aldrich, St. Louis, MO, USA), including thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa), were used to determine the apparent molecular weight of the enzyme.

Determination of enzyme activity Purified BHCD was reacted with 1% of each substrate in 50 mM sodium phosphate buffer (pH 7.0) at 50°C for an appropriate time to determine its hydrolyzing activity. The 3,5-dinitrosalicylic acid (DNS) reducing sugar method (25) was used for the hydrolyzing activity assay against β-CD, pullulan (Wako Pure Chemical Industries Ltd., Hyogo, Japan), or soluble starch (Showa Chemical Co., Tokyo, Japan). The activity on acarbose (Carbosynth Ltd., Berkshire, UK) or maltotriose (Fluka Biochemika, Buchs, Switzerland) was determined by using AceChem Glucose kit (YD Diagnostics Co., Yongin, Korea) based on the glucose oxidase-peroxidase method (26). One unit of the hydrolyzing activity towards β-CD (CU), soluble starch (SU), or pullulan (PU) is defined as the amount of enzyme producing 1 μmol equivalent of maltose for 1 min, whereas the activity against acarbose (AU) or maltotriose (MU) is determined on the basis of glucose equivalent instead of maltose. The protein concentration was measured using the BCA^{TM} protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA) with bovine serum albumin as a standard.

Analysis of enzyme reaction products Thin layer chromatography (TLC) and high performance anion exchange chromatography (HPAEC) analyses were performed to determine the hydrolysis or transglycosylation products generated from various substrates. For the hydrolysis, the appropriate amount of BHCD was reacted with 1% each substrate at its optimal reaction conditions for 1 hr, while the transglycosylation was performed with 5% acarbose (donor) and 10% α-methyl glucopyranoside (acceptor) for 24 hr. For TLC analysis, the resulting products were separated by the K5F silica gel TLC plate (Whatman International Ltd., Maidstone, UK) with the solvents of isopropanol/ethylacetate/water (3:1:1). The plate was visualized by dipping it into a solution containing 0.3% *N*-(1-naphthyl)-ethylenediamine and 5% H_2SO_4 in methanol, and then heating it for 10 min at 110°C. In order to separate the acarbose derivatives from maltooligosaccharides, however, the only hydrolysates from maltotriose and acarbose were separated by using the $60F_{254}$ silica gel plate (Merck, Darmstadt, Germany). A CarboPac PA1 column (0.4×25 cm, Dionex Co., Sunnyvale, CA, USA) was used for HPAEC analysis (Bio-LC ICS-3000; Dionex) with an electrochemical detector (ED40; Dionex). Samples were eluted with a linear gradient from 100% buffer A (150 mM NaOH in water, Fisher Scientific, Rockford, IL, USA) to 30% buffer B (600 mM of sodium acetate in buffer A, Sigma-Aldrich) over 30 min. The flow rate of the mobile phase was maintained at 1.0 mL/min.

Results and Discussion

Primary structure analysis of BHCD gene To date, most of CDase-family enzymes have been mainly found from bacterial origins and Oh *et al.* (2) reported that these types of genes can be widely distributed in a variety of microbial genomes. Park *et al.* (3,27) proposed that the intracellular CDase can open the ring structure of CDs and hydrolyze further to maltose, the best substrate for the intracellular α-glucosidases. Nevertheless, the precise roles of these enzymes in bacterial cells have not been elucidated

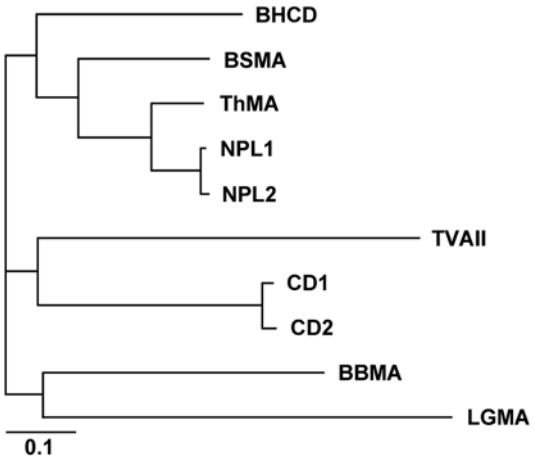

Fig. 1. Phylogenetic relationships between BHCD and other CDase-family enzymes. Based on amino acid sequences, the neighbor joining method of CLUSTAL W was used to build the tree. BHCD, CDase from *Bacillus halodurans* C-125 (in this study); BSMA, MAase from *B. stearothermophilus* ET1 (6); ThMA, MAase from *Thermus* sp. IM6501 (13); NPL1, NPase from *B. stearothermophilus* TRS40 (4); NPL2, NPase from *B. stearothermophilus* IMA6503 (8); TVAII, α-amylase II from *Thermoactinomyces vulgaris* R-47 (11); CD1, CDase from alkalophilic *Bacillus* sp. I-5 (5); CD2, CDase from alkalophilic *Paenibacillus* sp. A11 (9); BBMA, MAase from *B. subtilis* SUH4-2 (7); LGMA, MAase from *Lactobacillus gasseri* ATCC 33323 (14).

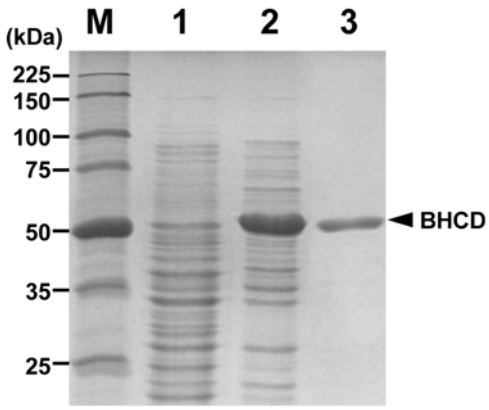

Fig. 2. Gene expression and purification of BHCD. SDS-PAGE analysis showed the expression level and the purity of BHCD: lane M, protein molecular weight marker; lane 1, crude extract from *E. coli* harboring pHCXHD (negative control); lane 2, crude extract from *E. coli* harboring pHCXBHCD; lane 3, BHCD purified by HisTrap-FF column chromatography.

yet. In this work, a putative CDase gene (BH2927; GenBank Accession No. BA000004) was found from the genome of *B. halodurans* C-125 and the corresponding ORF was designated as BHCD gene. The structural gene for BHCD consists of 1,734 nucleotides encoding the protein with 578 amino acid residues and its primary structure was comparatively analyzed with other CDases, MAases, and NPases.

In order to verify the relationship with other known CDase-family enzymes, a phylogenetic tree was constructed and shown in Fig. 1. The predicted amino acid sequence of BHCD shares 58.6% identity with *B. stearothermophilus* MAase (BSMA; 6), 56.8% identity with *B. stearothermophilus* NPase (4), 56.0% with *Thermus* MAase (ThMA; 13), 49.1% with alkalophilic *Bacillus* CDase (5), 47.8% with *Bacillus subtilis* MAase (BBMA; 7), 43.1% with *Thermoactinomyces vulgaris* amylase II (TVAII; 11), and 41.8% with *Lactobacillus gasseri* MAase (LGMA; 14). Moreover, its primary structure showed that BHCD possesses the common N-terminal amino acid residues and the well-conserved motif sequence I (Asp-Ala-Val-Phe-Asn-His), II (Gly-Trp-Arg-Leu-Asp-Val-Ala-Asn-Glu), III (Glu-Ile-Trp-His), and IV (Leu-Leu-Gly-Ser-His-Asp) commonly found in CDase-family enzymes.

Gene cloning and expression of BHCD Approximately 1.7 kb of BHCD gene was amplified by PCR with a set of primers, BHCD-N and BHCD-C. The resulting fragment was cloned into pMD18-T vector and its entire nucleotide sequence was confirmed by DNA sequencing analysis. After *Nde*I and *Xho*I treatment, the fragment was subcloned into an expression vector pHCXHD and the resulting plasmid was designated as pHCXBHCD. Recombinant BHCD with C-terminal 6-histidines was constitutively overexpressed in *E. coli* harboring pHCXBHCD and easily purified to apparent homogeneity via Ni-NTA column chromatography (Fig. 2). For the precise comparison between BHCD and other CDase-family enzymes, ThMA was also expressed and purified from recombinant *E. coli* MC1061 harboring p6xHTMX (13) by the same manner.

Enzymatic characterization of BHCD The molecular mass of the recombinant BHCD with a histidine tag was calculated to be about 68.3 kDa on the basis of its deduced amino acid sequence. GPC analysis showed that BHCD is present at a molecular mass of approximately 131 kDa, implying that it may exist in a functional homo-dimer in solution (Fig. 3). Even though a tetrameric MAase (14) and a dodecameric CDase (1) were reported to date, the homo-dimeric structure has been known as the most common feature of CDase-family enzymes including ThMA (16), NPase (17), and TVAII (15). In addition, Kim *et al*. (18) revealed that the oligomeric structure of ThMA affects its multisubstrate specificity and its extra N-domain plays an important role in the dimer formation. Accordingly, the dimeric BHCD containing N-domain could be categorized to the CD-degrading enzyme family on the basis of its primary, tertiary, and quaternary structures.

To determine the optimal reaction conditions, the effects of temperature and pH on enzyme activity and stability were examined. As shown in Fig. 4, BHCD showed the highest activity at 50°C in 50 mM sodium phosphate buffer (pH 7.0). Although the optimal temperature is 50°C, its activity decreased rapidly at temperature above 55°C. On the other side, BHCD was very stable at the broad pH range from 6.0 to 8.5 (data not shown). Recently, a MAase (TpMA) with high thermostability has been found from an archaeal hyperthomophile, *Thermoplasma volcanium* GSS1, which showed the highest activity at 75-80°C (28). However, most known mesophilic or thermolabile CDase-family enzymes have been reported to show the highest activity at 40-60°C and pH 5.5-8.0, respectively (27).

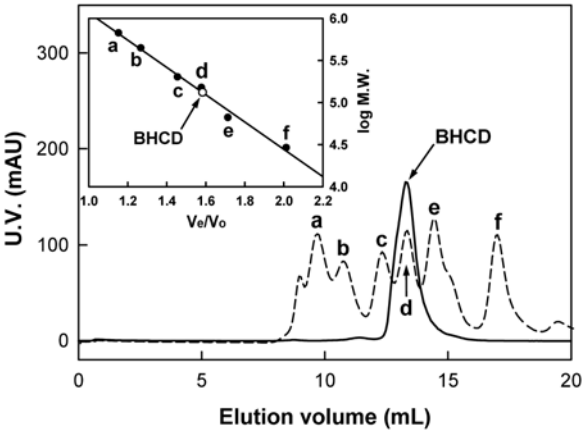

Fig. 3. Gel permeation chromatography of recombinant BHCD. Molecular weight of BHCD was estimated by comparing the ratio of V_e/V_0 (V_e, the elution volume; V_0, the void volume) using Superdex-200 GPC column. The purified BHCD was drawn as a solid line and the molecular weight markers (a dashed line) were used as the mixture of 6 proteins: a, thyroglobulin (669 kDa); b, apoferritin (443 kDa); c, β-amylase (200 kDa); d, alcohol dehydrogenase (150 kDa); e, bovine serum albumin (66 kDa); f, carbonic anhydrase (29 kDa).

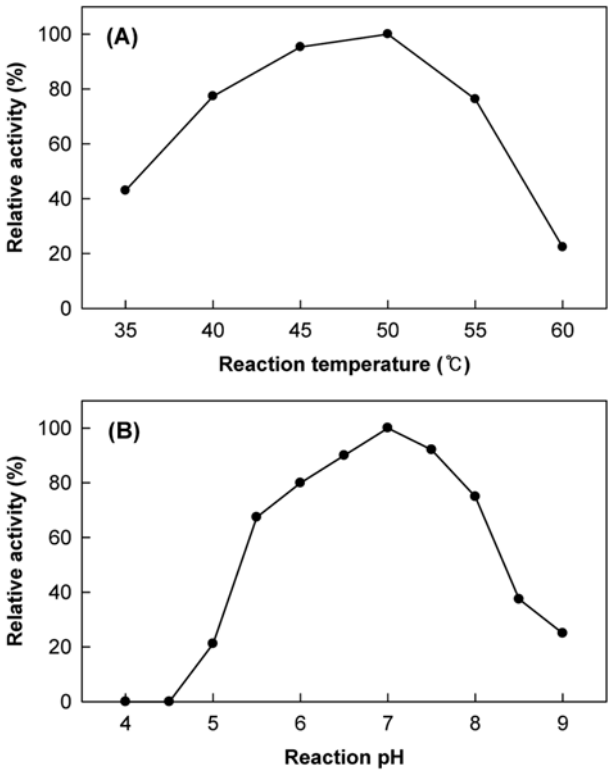

Fig. 4. Effects of reaction temperature (A) and pH (B) on BHCD activity. Optimal reaction temperature and pH of BHCD were determined on the basis of its hydrolyzing activity on β-CD. A variety of reaction buffers were tested as follows: sodium acetate (pH 4.0-6.0); sodium phosphate (pH 6.0-7.5); Tris-HCl (pH 7.5-8.0); borate-NaOH (pH 8.0-9.0).

Multisubstrate specificity of BHCD CDase-family enzymes have been known to possess the versatile hydrolysis and transglycosylation activities against various substrates, including β-CD, pullulan, acarbose, as well as starch and maltooligosaccharides. Due to its complexity in activity assay on various substrates, it is difficult to compare the differences in multisubstrate specificity among CDase-family enzymes. In this work, therefore, ThMA was selected as a model enzyme standing for the typical MAases, which was simultaneously tested and directly compared with the results from BHCD.

As shown in Table 1, ThMA possesses much higher CD-degrading activity over starch or pullulan. It showed the activity ratio of 14.8:1.0:1.1 against β-CD, soluble starch, and pullulan, respectively. Although BHCD showed the highest substrate preference towards β-CD, it exhibited the different hydrolyzing activity ratio of 4.7:1.0:2.0 towards β-CD, soluble starch, and pullulan, respectively. The activity of BHCD on starch or pullulan is not much lower than on β-CD, which can discriminate it from other typical CDase-family enzymes, such as ThMA. Meanwhile, BHCD showed 2.5 and 4.6 times higher activities against starch and pullulan, respectively, than those of ThMA. On the contrary, ThMA exhibited 2.7 times higher maltotriose-hydrolyzing activity than BHCD. Accordingly, BHCD has the enhanced activity on polymeric substrates, such as pullulan and starch, while ThMA favors the oligomeric substrate such as maltotriose.

Typical α-amylases can not hydrolyze acarbose, but can make a complex with acarbose. Although the acarbose-hydrolyzing activity was not reported from all CDases and NPases, almost MAases have been known to hydrolyze acarbose to glucose and acarviosine-glucose. It can be the unique feature clearly distinguishing the CDase-family enzymes from the other α-amylases. Compared with ThMA, however, BHCD showed significantly low specific activity on acarbose and high activity ratio of maltotriose to acarbose. Oh *et al.* (14) reported that *Lactobacillus gasseri* MAase (LGMA) showed extremely low acarbose-degrading activity and novel action pattern on pullulan to produce maltotriose and panose. In particular, it was recently found

Table 1. Comparison of the multisubstrate specificity between ThMA and BHCD

Enzyme	Specific activity (U/mg)[1)]					Activity ratio[2)]			
	β-CD	Pullulan	Starch	Maltotriose	Acarbose	C/S	P/S	M/S	M/A
ThMA	65.2±0.9	5.0±0.1	4.4±0.1	48.9±0.3	27.3±0.3	14.8	1.1	11.1	1.8
BHCD	52.9±0.3	22.8±0.5	11.2±0.1	17.9±0.1	3.0±0.1	4.7	2.0	1.6	6.0

[1)]Each hydrolyzing activity on β-CD, pullulan, or starch was determined by DNS reducing sugar assay, whereas the activity against maltotriose or acarbose was assayed by GOD-POD method.
[2)]C, β-CD; P, pullulan; S, soluble starch; M, maltotriose; A, acarbose.

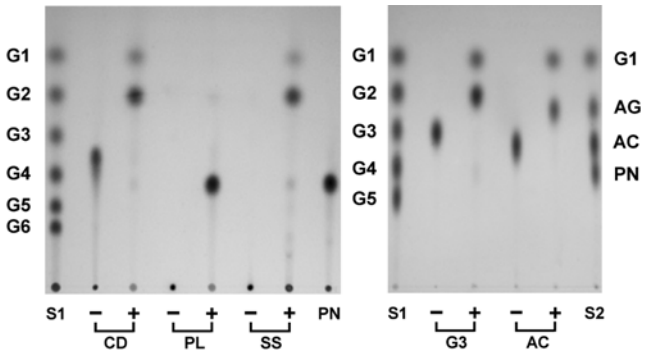

Fig. 5. TLC analysis of hydrolysis patterns of BHCD on various substrates. BHCD was reacted with 1% of each substrate; CD, β-CD; PL, pullulan; SS, soluble starch; G3, maltotriose; AC, acarbose; S1, standards for maltooligosaccharides; S2, the standard mixture of glucose (G1), AG (acarviosine-glucose), AC (acarbose), and PN (panose); reaction products with (+) or without (-) enzyme.

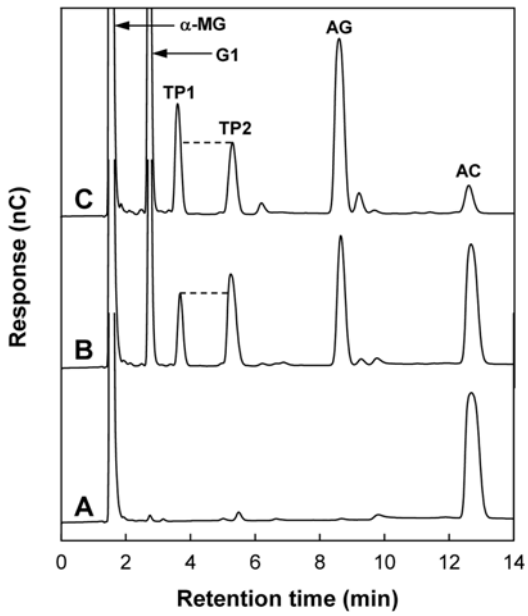

Fig. 6. Time-course HPAEC analysis of acarbose transglycosylation products. BHCD was reacted with 5% acarbose (donor) and 10% α-methyl glucopyranoside (acceptor) at 50°C for 24 hr. At each time interval of (A) 0, (B) 2, and (C) 60 min, the reaction mixture was taken and analyzed by HPAEC. α-MG, α-methyl glucopyranoside; G1, glucose; AG, acarviosine-glucose, AC, acarbose; TP1 and TP2, transfer product 1 and 2.

that multisubstrate specificity or acarbose hydrolysis pattern of ThMA could be modulated via the intensive combinatorial mutagenesis of the residues near the extra-sugar binding space (29). Even though the corresponding amino acid residues of BHCD are completely identical to those in ThMA wild-type, the accumulation of the knowledge about structure-function relationship will enable the scientists to expect and evaluate the changes of enzymatic properties in advance.

Hydrolysis and transglycosylation of BHCD In order to understand the action patterns of BHCD, its hydrolysis and transglycosylation products were analyzed and compared with other known CDase-family enzymes. As expected, BHCD could hydrolyze β-CD and soluble starch mainly to maltose, whereas it hydrolyzed pullulan to panose (Fig. 5). BHCD also produced glucose and maltose by cleaving α-(1,4)-glycosidic linkage of maltotriose, while acarbose was hydrolyzed to glucose and acarviosine-glucose (Fig. 5). As a result, the hydrolysis patterns of BHCD are very similar to those of ThMA, a typical MAase.

Meanwhile, the acarbose transglycosylation products with α-methyl glucopyranoside acceptor were examined by HPAEC analysis. According to the transglycosylation with ThMA, α-(1,6)- and α-(1,3)-transfer products were gradually produced, but α-(1,4)-transfer product was readily hydrolyzed again (13). At the early stage of transglycosylation, BHCD also transfer an acarviosine-glucose moiety to an α-methyl glucopyranoside acceptor by forming mainly α-(1,4)-glycosidic linkages. As time goes and the amount of acarbose (donor) are consumed, however, α-(1,4)-transfer product (peak of TP2) was rapidly re-hydrolyzed by BHCD and α-(1,6)-transfer product (peak of TP1) gradually increased (Fig. 6). As a result, α-(1,6)-TP1 remained as a major transfer product, due to its resistance to hydrolyzing activity of BHCD. These transglycosylation patterns of BHCD can be similar to those of ThMA.

In conclusion, BHCD is an amylolytic enzyme sharing the common enzymatic properties with known CDase-family enzymes. Compared with other CDases, however, it has significantly enhanced activity on the polymeric substrates and relatively weak activity on oligomeric substrates. In addition to hydrolysis, BHCD possesses the considerable level of transglycosylation activity similar to known MAases, which can be applicable to the production of any functional carbohydrate materials. On the basis of the primary and the tertiary structure comparison among various CDase-family enzymes, the protein engineering approaches in future will promote the development of novel carbohydrate-active enzymes with more versatile hydrolysis or transglycosylation activity.

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