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RESEARCH NOTE

Novel Quantitative Method for the Degree of Branching in Dextran

Young-Min Kim, Atsuo Kimura, and Doman Kim

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Abstract A novel quantitative method for the determination of degree of branching in *Leuconostoc mesenteroides* B-512F dextran was developed by using the combination of 3 dextran-degrading enzymes. First, *Paenibacillus* sp. endo-dextranase was randomly degraded B-512F dextran into linear or branched isomalto-oligosaccharides with various degree of polymerization (2-8). Second, *Streptococcus mutans* dextran glucosidase hydrolyzed linear or branched isomalto-oligosaccharides into glucose and branched isomalto-penta-saccharides. Third, the branched isomalto-penta-saccharide was degraded into glucose by using *Bacteroides thetaimicron* α-glucosidase. The number of branching points in B-512F dextran (5.42%) was determined by the difference in the amount of glucose in the reaction digest between BTGase-PDex and DGase-PDex treatments.

Keywords: dextran, endo-dextranase, exo-dextranase, degree of branching

Introduction

Dextrans comprise a class of bacterial D-glucans of various structures with contiguous α-(1→6) glucosidic linkage in the main chain and α-(1→2), α-(1→3), or α-(1→4) branch glucosidic linkages, depending on the specificity of the particular dextransucrase (1). The percent and the manner in which the branches are arranged give rise to differences in the structures. Methylation and NMR analyses have shown that a commercially produced *Leuconostoc mesenteroides* B-512F dextran consists of glucose moiety linked mainly (95%) α-(1→6) linkages with the remainder (5%) being α-(1→3) branch glucosidic linkages (1). Limitations of NMR spectroscopy (2) prevent the quantitative determination of the number of branching points.

Enzymatic hydrolysis can provide both qualitative and quantitative determinations by their region-selectivity and product analysis. Dextran-degrading enzymes have been isolated from various sources (1) and are divided into several families on the basis of the similarities in the amino acid sequences (http://afmb.cnrs-mrs.fr/CAZY/). According to the sequence similarities (3), dextran-degrading enzymes have been divided in glycoside hydrolase (GH) family 13 (dextran-glucosidase, 4), 15 (gluco-dextranase, 5), 27 (isomalto-dextranase, 6), and 49 (isomaltotrio-dextranase, 7). Endo-dextranases (EC 3.2.1.11) have been classified into 2 GH families, 49 and 66, with no sequence similarities between the 2 families (8). Enzymes originating from *Penicillium* (9) and *Streptococcus mitis* (10) hydrolyze α-(1→6)-D-glucosidic linkages from the non-reducing at random. However, hydrolysis of the main chain of α-(1→6)-D-glucans always stops one D-glucose residue away from a branching point. These properties produce a branched hydrolysis product from B-512F dextran (9). Recently, a novel type α-(1→3) branched dextran hydrolyzing enzyme was classified in the GH family 97 (11). *Bacteroides thetaimicron* α-glucosidase (BTGase) belonging to GH family 97 displays broad substrate specificity for disaccharides (12). This makes it attractive for the determination of dextran fine structure.

In this study, a novel method was developed to measure the number of branching points in B-512F dextran by the use of a combination of a recombinant endo-dextranase from *Paenibacillus* sp. (PDex) with each recombinant exo-

Doman Kim (✉)
School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Korea
Tel: +82-62-530-1844; Fax: +82-62-530-0874
E-mail: dmkim@jnu.ac.kr

Young-Min Kim
Eco-Friendly Biomaterial Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup, Jeonbuk 580-185, Korea

Atsuo Kimura
Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

enzyme of *Streptococcus mutans* dextran-glucosidase (DGase) or of *B. thetaimicron* α-glucosidase (BTGase). This method can be used for the determination of branching from various dextrans that have various commercial applications.

Materials and Methods

Materials Dextran from *L. mesenteroides* B-512F was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dextrans from *L. mesenteroides* B-742CB and B-1299C were prepared by previous methods (13,14) using dextransucrases. Isomalto-oligosaccharides (degree of polymerization from 2 to 9) were kindly provided by Dr. John F. Robyt, Iowa State University. Whatman TLC plates were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were of reagent grade and commercially available.

Enzyme preparation Recombinant PDex, DGase, or BTGase with a His tag was produced in *E. coli* BL21 (DE3) CodonPlus RIL (Stratagene, San Diego, CA, USA) containing the expression plasmids derived from pET23d or pET28a (Novagen, Darmstadt, Germany) as described previously (4,12,15). Isopropyl β-D-thiogalactopyranoside was added (0.2 mM) to the cultures, and cells were cultured for another 24 h at 18ºC. Recombinant DGase, BTGase, or PDex was purified from cells harvested from 1 L culture broth by Ni-chelating Sepharose column chromatography as described previously (4,12,15) to homogeneity. The bound enzyme was eluted with 20 mM sodium phosphate buffer (pH 7.2) containing 300 mM imidazole, and dialyzed against 20 mM sodium acetate buffer (pH 5.5 for PDex, pH 6.0 for DGase, and pH 6.5 for BTGase). The protein was concentrated using an Amicon Ultra 10,000 molecular weight cut-off (Millipore, Billerica, MA, USA). Protein concentration of cell-free extract was determined by the method of Bradford (16) with bovine serum albumin as a standard. The purified protein concentration was calculated by quantification of each amino acid by the ninhydrin colorimetric method using JLC-500/V (Jeol, Tokyo, Japan) after hydrolysis of 30 μg of the purified enzymes in 6 N HCl at 110ºC for 24 h.

Enzyme assay The hydrolytic activity of DGase or BTGase was measured in the standard reaction condition containing 20 mM sodium acetate buffer (pH 6.0 for DGase or pH 6.5 for BTGase), and 2 mM *p*-nitrophenyl α-D-glucopyranoside (*p*NPG, Nacalai Tesque, Tokyo, Japan), and enzyme was diluted in 50 mM designated buffer containing 0.05 mg/mL bovine serum albumin at 37ºC. After incubation, the enzyme reaction was stopped by adding 2 volumes of 1 M sodium carbonate. The amount of *p*-nitrophenol released was measured by determining the absorption at 400 nm in a 1-cm cuvette, considering a molar extinction coefficient of 5,560/M·cm. The amount of reducing sugars liberated was measured by the copper-bicinchoninate method (17) using glucose as a standard. Dextranase activity was assayed by incubation with B-512F dextran in 20 mM sodium acetate (pH 5.5) at 35ºC. The liberated glucose was quantified by the glucose oxidase-peroxidase method (18) using Glucose AR-II (Wako Pure Chemical, Osaka, Japan). One unit (U) of activity was defined as the amount of enzyme that caused the release of 1 μmol of reducing sugar/min, under the conditions of assay.

Carbohydrate analysis To investigate hydrolysis by each enzyme, 1 mL of PDex (10 nM), DGase (6 nM), or BTGase (10 nM) was incubated at 35ºC for 24 h in 20 mM sodium acetate buffer (pH 6.0) containing 1%(w/v) B-512F dextran. Aliquots (50 μL) were withdrawn at the different time intervals and carbohydrate released was analyzed by TLC or glucose oxidase-peroxidase method (18). TLC analysis was done on silica gel coated glass plate (Fisher Scientific) with nitroethane:nitromethane: ethanol:water:1-propanol (1:2:3:4:5, v/v/v/v/v) as a solvent system. After irrigation, each TLC plate was dried and visualized by dipping in a solution containing 0.3%(w/v) *N*-(1-naphthyl)-ethylenediamine (Sigma-Aldrich) and 5%(v/v) sulfuric acid in methanol, and heating at 120ºC for 5 min (19).

Isolation of hydrolysis product The reaction mixture (50 mL) contained 150 nM of PDex, 90 nM of DGase, and 1%(w/v) B-512F dextran in 20 mM sodium acetate buffer (pH 6.0) at 35ºC for 48 h. The enzyme reaction was stopped by boiling for 10 min. Reaction aliquots were passed through Amberlite MB-3 (Sigma-Aldrich) and concentrated to 1 mL by vacuum evaporation. Then, samples were applied to HPLC system consisting of a RS Pak DC-613 column (6 mm i.d.×150 mm, Showa Denko, Tokyo, Japan). Elution conditions were: injection 50 μL; detection, a RI detector model D-2000 (Hitachi Co., Tokyo, Japan); solvent, acetonitrile:water (61:39, v/v); flow rate, 0.9 mL/min; column temperature, 60ºC. Isolated compounds were analyzed for the molecular mass by LC/MS (Aglient, Bremen, Germany). Standard sugars were glucose (Glc), isomaltose (IM2), isomalto-triose (IM3), isomalto-tetraose (IM4), isomalto-pentaose (IM5), isomalto-hexaose (IM6), and isomalto-heptaose (IM7). To determine the oligosaccharide branching, the isolated products were methylated using Hakomori reagent, followed by acid hydrolysis with 2 M trifluoroacetic acid, and analyzed for the methylated products using TLC (20). The methylated and acidic

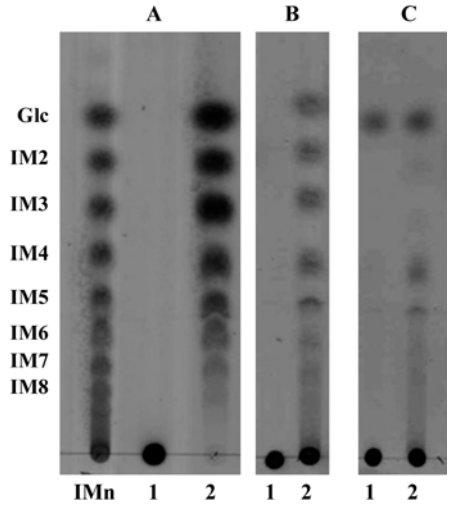

Fig. 1. TLC of PDex hydrolysis products from B-512F, B-742CB, and B-1299C dextran. A, B-512F dextran; B, B-742CB dextran; C, B-1299C dextran. IMn, isomalto-oligosaccharides standards; Glc, glucose; IM2, isomaltose; IM3, isomalto-triose; IM4, isomalto-tetraose; IM5, isomalto-pentaose; IM6, isomalto-hexaose; IM7, isomalto-heptaose; IM8, isomalto-octaose. 1, unhydrolysed dextran; 2, reaction digest by PDex

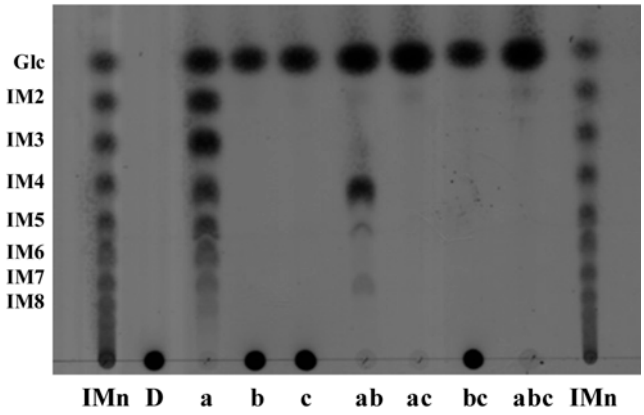

Fig. 2. TLC of reaction digest of B-512F dextran by PDex, DGase and/or BTGase. IMn, isomalto-oligosaccharides standards; Glc, glucose; IM2, isomaltose; IM3, isomalto-triose; IM4, isomalto-tetraose; IM5, isomalto-pentaose; IM6, isomalto-hexaose; IM7, isomalto-heptaose; IM8, isomalto-octaose. D, B-512F dextran standard; a, PDex treatment; b, DGase treatment; c, BTGase treatment; ab, PDex and DGase treatment; ac, PDex and BTGase treatment; bc, DGase and BTGase treatment; abc, PDex, DGase, and BTGase treatment

hydrolysis products were separated by 2 ascents on Whatman K6 plate with acetonitrile:chloroform:methanol (3:9:1, v/v/v), followed by development of the plate as previously described (20).

Results and Discussion

Action of PDex on 3 dextrans Each dextran (B-512F, B-742CB, and B-1299C) was incubated at 35°C with PDex (10 nM) in a digest (1 mL) that contained 20 mM sodium acetate buffer (pH 5.5). Portions were withdrawn after 24 h for the determination of hydrolysis. As shown in Fig. 1, the B-742CB or B-1299C dextran was resistant by PDex described for other endo-dextranases (9,10); the highly branched α-(1→3) and α-(1→2) linkagees were not hydrolyzed. B-512F dextran was completely degraded to isomalto-oligosaccharide with degree of polymerization (DP) of 2 to 8. Compared with *Lipomyces starkeyi* KSM 22 DXAMase (76.8%, 21) or *P. funiculosum* dextranase (66.7%, 21), PDex showed efficient hydrolytic activity toward B-512F dextran. For determining the amount of branching point, B-512F dextran was further investigated by mixed enzyme system.

Dextran hydrolysis with a trio of enzymes The hydrolysis products of 1%(w/v) B-512F dextran by DGase, BTGase, or PDex, respectively, are shown in Fig. 2. Several isomalto-oligosaccharides were produced by the reaction with PDex. BTGase and DGase digests showed glucose release of 23 and 32%, respectively, of control dextran. PDex and DGase treatment (reaction ab in Fig. 2) with B-512F dextran produced 94.5% glucose and unidentified products having R_f between isomalto-tetraose and isomalto-pentaose on TLC. On the other hand, the combination of BTGase and PDex (reaction ac in Fig. 2) produced only glucose (100%) indicative of complete hydrolysis of dextran to D-glucose by 2 enzymes (reaction ac). Although the treatment with DGase+PDex produced unidentified isomalto-oligosaccharides and glucose, BTGase in reaction ac degraded the unidentified products in reaction ab (Fig. 2). BTGase did not stop at the branching points composed of α-(1→6) glucosidic main chain with α-(1→3) in B-512F dextran. These results seemed to arise from broad substrate specificities of BTGase such as α-(1→2), α-(1→3), α-(1→4), or α-(1→6) glucosidic disaccharides, maltooligo-saccharides, and soluble starch (12). This complete hydrolysis of dextran was applicable for sugar processing and structural determination of various D-glucans. Lee *et al*. (22) reported that DXAMase could be used to remove soluble polysaccharide containing dextran, starch, and pectin in sugar processing. The glucanhydrolase treatment of sugar cane juice resulted in a good quality of sugar crystals. Similar to DXAMase, the combination of PDex and 2 exo-dextranases (Fig. 2) also would be applicable to produce good quality of sugar and avoid monetary problems associated with excessive levels of dextran in raw sugar.

Isolation and structural analysis of the unidentified products The unidentified products (19 mg) were isolated

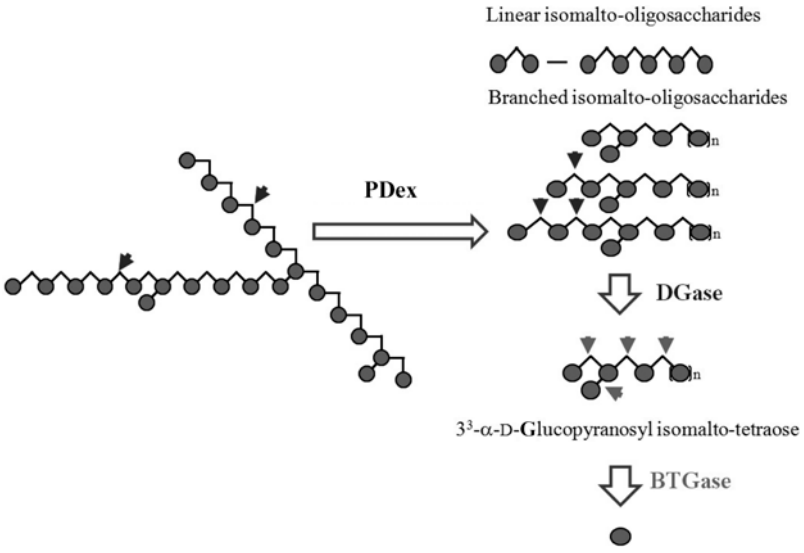

Fig. 3. Schematic description of B-512F dextran hydrolysis using the 3 enzymes. PDex, *Paenibacillus* sp. endo-dextranase; DGase, *Streptococcus mutans* dextran glucosidase; BTGase, *Bacteroides thetaimicron* α-glucosidase

by HPLC using Shodex RS pack DC-16 column eluted with 61%(v/v) acetonitrile. LC/MS analysis suggested that the molecular mass was 851 $[M+Na]^+$ for unidentified products, in agreement with that of the expected compounds binding with monosodium ion (data not shown). NMR analysis did not clearly analyze the structure of unidentified products due to high overlapping peaks (data not shown). From the methylation analysis, the isolated products were identified as 2,4-di-methyl D-glucose and 2,3,4-tri-*O*-methyl-D-glucose, indicating that the major branch glucosidic linkages in this dextran is α-(1→3) linkage. Endo-dextranase stops activity one D-glucose away from a branching point (9). Because DGase hydrolyzes only α-(1→6) glucosidic linkage at the non-reducing end of substrate (4), the hydrolysis could be stopped at the branching points of dextran. Also, these products were not hydrolyzed by DGase. From the data, the isolated products were determined to be composed of 5 residues of glucose containing α-(1→3) branch glucosidic linkage. These results suggest that the unidentified product is 3^3-α-D-glucopyranosyl isomalto-tetraose.

Determination of degree of branching in B-512F dextran The different amount of glucose in the reaction between reactions ab and ac (Fig. 2) indicated the amount of unidentified products. After oxidation of D-glucose by excess sodium borohydroxide, the unidentified products further hydrolyzed by BTGase. Then, the amount of the released glucose was same as the difference between reactions ab and ac (Fig. 2), indicating the unidentified product was branch oligosaccharides from B-512F dextran. The amount of branching was 5.42% of original dextran. This result was in good agreement with previous reported 5% branching point in B-512F dextran (1,2). This enzymatic method would be very useful for the determination of the amount of branching in various structural dextrans (Fig. 3).

In this study, a new method for analyzing the amount of branching point was developed by using mixture of enzymes. The degree of branching of highly branched dextrans, such as B-742CB or B-1299C dextran could not be determined due to limited PDex hydrolysis activity. The development of stronger hydrolytic enzymes to hydrolyze B-742CB or B-1299C dextran is in progress.

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