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A practical approach to producing isomaltomegalosaccharide using dextran dextrinase from *Gluconobacter oxydans* ATCC 11894

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Abstract

Dextran dextrinase (DDase) catalyzes formation of the polysaccharide dextran from maltodextrin. During the synthesis of dextran, DDase also generates the beneficial material isomaltomegalosaccharide (IMS). The term megalosaccharide is used for a saccharide having DP = 10–100 or 10–200 (DP, degree of polymerization). IMS is a chimeric glucosaccharide comprising α-(1→6)- and α-(1→4)-linked portions at the nonreducing and reducing ends, respectively, in which the α-(1→4)-glucosyl portion originates from maltodextrin of the substrate. In this study, IMS was produced by a practical approach using extracellular DDase (DD_{ext}) or cell surface DDase (DD_{sur}) of *Gluconobacter oxydans* ATCC 11894. DD_{sur} was the original form, so we prepared DD_{ext} via secretion from intact cells by incubating with 0.5% G6/G7 (maltohexaose/maltoheptaose); this was followed by generation of IMS from various concentrations of G6/G7 substrate at different temperatures for 96 h. However, IMS synthesis by DD_{ext} was limited by insufficient formation of α-(1→6)-glucosidic linkages, suggesting that DDase also catalyzes elongation of α-(1→4)-glucosyl chain. For production of IMS using DD_{sur}, intact cells bearing DD_{sur} were directly incubated with 20% G6/G7 at 45 °C by optimizing conditions such as cell concentration and agitation efficiency, which resulted in generation of IMS (average DP = 14.7) with 61% α-(1→6)-glucosyl content in 51% yield. Increases in substrate concentration and agitation efficiency were found to decrease dextran formation and increase IMS production, which improved the reaction conditions for DD_{ext}. Under modified conditions (20% G6/G7, agitation speed of 100 rpm at 45 °C), DD_{ext} produced IMS (average DP = 14.5) with 65% α-(1→6)-glucosyl content in a good yield of 87%.

Key points

- *Beneficial IMS was produced using thermostabilized DDase.*
- *Optimum conditions for reduced dextran formation were successfully determined.*
- *A practical approach was established to provide IMS with a great yield of 87%.*

Keywords *Gluconobacter oxydans* · dextran dextrinase · dextran · isomaltomegalosaccharide · chimeric glucosaccharide

Introduction

Dextran dextrinase [DDase; (1→4)-α-D-glucan:(1→6)-α-D-glucan 6-α-D-glucosyltransferase; EC 2.4.1.2; a member of glycoside hydrolase family 15] synthesizes a water-soluble dextran from maltodextrin. The enzyme was discovered by Hehre (1951) from the extracellular viscous material of ropy beer produced by *Acetobacter capsulatum* ATCC 11894 (later reassigned to *Gluconobacter oxydans* ATCC 11894) or *Acetobacter viscosum* ATCC 11895 during brewing. DDase substrates are a series of maltooligosaccharides other than maltose (G2), which is a less efficient substrate, on which DDase catalyzes two reactions (Hehre 1951; Yamamoto et al., 1993a): i) α-(1→4)-transglucosylation, which transfers one glucose residue from the nonreducing end of the donor substrate to the nonreducing end of the acceptor substrate to form an α-(1→4)-glucosidic linkage; ii) dextran-forming α-(1→6)-transglucosylation, which is similar to the former reaction except for the consecutive formation of α-(1→6)-glucosidic linkages to produce polysaccharide dextran. Additionally, DDase can catalyze a third reaction to form an α-(1→6)-glucosidic linkage from an α-(1→6)-glucosyl-linked substrate (e.g., isomaltooligosaccharide) (Yamamoto et al., 1993b). These three reactions are thought to be catalyzed by a single active site, since DDase possesses the same catalytic residues as other glycoside hydrolase family 15 enzymes (Sadahiro et al., 2015). DDase-synthesizing dextran has both α-(1→6)- and α-(1→4)-glucosidic linkages (Yamamoto et al., 1993a), suggesting that α-(1→4)-transglucosylation occurs during dextran formation. Many researchers have characterized the structure of dextran and proposed a mechanism for DDase action (Mountzouris et al., 1999; Suzuki et al., 1999; Wang et al., 2011; Wichienchot et al., 2009). However, there is no report on the structural aspects of a product with a far lower molecular weight (MW) synthesized by DDase, so it is of interest to determine the conformation of the shorter intermediate saccharide formed before dextran, e.g., a megalosaccharide.

In 1959, French and coworkers (Thoma et al., 1959) defined megalosaccharides as sugars with sizes between oligosaccharides (DP = 2–9; DP, degree of polymerization) and polysaccharides. The properties of polysaccharides appear with DPs greater than 100 or 200, so the DPs of megalosaccharides are considered to be 10–100 (or 10–200). Linear-type α-(1→6)-glucosyl megalosaccharide (IMS) of approximately 1.8 kDa (average DP = 11) was produced from maltodextrin using heterologously expressed recombinant DDase (Lang et al., 2014; Sadahiro et al., 2015). As mentioned above, DDase catalyzes α-(1→6)-transglucosylation from a donor to an acceptor substrate. Therefore, the reducing end segment of the formed IMS possesses an α-(1→4)-glucosyl portion originating from the acceptor substrate of maltodextrin, indicating that IMS is a "chimeric" saccharide with α-(1→6)-glucosyl and α-(1→4)-glucosyl regions.

It was found that 1.8-kDa IMS enhanced the water solubility of lipophilic compounds such as quercetin (flavonoid) and ethyl red (an environmental pollutant) (Shinoki et al., 2013; Lang et al., 2014), whereas this property was not found in dextran (dextran-4) having an MW of 4.0–6.0 kDa (average DP = 30) (Sundari et al., 1991). Solubilization by IMS constituted a valuable function increasing flavonoid uptake in the rat intestine (Shinoki et al., 2013) and assisting the azoreductase-associated degradation of ethyl red (Lang et al., 2013; Lang et al., 2014). In the latter case, the enzymatic degradation was more efficient than that of cyclodextrin [cyclic α-(1→4)-glucooligosaccharide], which increased the water solubilities of nonpolar compounds (e.g., ethyl red) by forming an inclusion complex with its hydrophobic cavity. The interaction between cyclodextrin and ethyl red was so strong that most ethyl red could not dissociate from the complex, causing inhibition of enzymatic degradation due to possible steric hindrance from cyclodextrin.

However, IMS interacted weakly with ethyl red by involving C-H groups at the surface of the linear sugar chain, and no inhibition occurred (Lang et al., 2014). This weak interaction allowed IMS to facilitate dissociation of water-insoluble compounds from complexes, so IMS satisfies various demands from industrial fields. Recently, it was reported that IMS directly enhanced the caveolae-associated barrier function of tight junctions in the rat intestinal epithelium (Hara et al., 2017), a further contribution to food science. Considering its application to the food industry, IMS produced by native enzymes might be preferred to IMS produced by genetically engineered approaches. Another matter to be considered is dextran formation, which simultaneously occurs during generation of IMS, indicating that dextran is a byproduct. This highly viscous polysaccharide requires great care during further processing (e.g., isolation of IMS). It is of great interest to find conditions that enhance IMS production and reduce dextran formation.

It has long been believed that *G. oxydans* ATCC 11894 has two functionally different DDases that exist extracellularly and intracellularly. However, our data (Sadahiro et al., 2015) suggested that both enzymes were identical and shared the same protein structure and properties. Intracellular DDase is present on the cell surface in the outer membrane/periplasmic space (Sadahiro et al., 2015), and maltodextrin assists its secretion (Suzuki et al., 1999). In this study, IMS production was investigated using a practical approach, in which we reacted G6/G7 (G6 and G7, maltohexaose and maltoheptaose, respectively) with extracellularly liberated DDase (DD_{ext}) or cell surface-bound DDase (DD_{sur}) native enzyme. DD_{ext} was prepared from cells by treatment with maltodextrin. For DD_{sur}, intact cells were used directly for production. Synthetic conditions using DD_{sur} or DD_{ext} were investigated, and efficient generation of IMS was accomplished.

Materials and methods

Localization of DDase in cells of *G. oxydans* ATCC 11894

Seed culture was performed by growing *G. oxydans* ATCC 11894 in 20 mL of FY (5% fructose and 0.5% yeast extract, pH 6.0) at 30 °C for 1 d with a rotary shaking velocity of 200 rpm (rev/min). Then, 2.5 mL of seed culture was transferred to 250 mL of FY and cultivated for 2 d under identical conditions using a 1 L baffled flask (AGC Techno Glass, Yoshida, Japan) (designated FY-culture). Cell growth was monitored with turbidity of the medium (absorbance at 600 nm, OD_{600}), enabling us to estimate the cell dry weight from the relation that 1.24 mg/mL of cells gave an OD_{600} of 1. To study the influence of maltodextrin on the cell localization of DDase, we added 1% G6/G7 [Fujioligo G6 and G7 product; donated by Nihon Shokuhin Kako (Tokyo, Japan); the contents (w/w) of G6 and G7 were nearly identical] to the 1 d-cultivated FY-culture and incubated for another 1 d under the same conditions as the FY-culture (designated as G6/G7-FY-culture).

Reportedly (Sadahiro et al., 2015), DDase was located in the medium (i.e., DD_{ext}) and at the cell surface (i.e., DD_{sur}) of *G. oxydans* and negligibly at its cytosol (DD_{cyt}; cytosolic DDase). By following our method with modification, DD_{ext}, DD_{sur}, and DD_{cyt} were prepared from FY- or G6/G7-FY-culture medium. The culture broth (5 mL) at the early stationary phase of cell growth was centrifuged at 8,000× *g* for 20 min at 4 °C and separated into supernatant (DD_{ext}) and cells (DD_{sur} and DD_{cyt}). The supernatant of FY-culture was used to measure the activity of DD_{ext}. In G6/G7-FY-culture, DD_{ext} was strongly bound to the dextran formed (Suzuki et al., 2000; Suzuki et al., 2001); therefore, after separation of cells, DD_{ext}-binding dextran was obtained from the supernatant as a precipitate by further centrifugation at 18,000× *g* for 1 h at 4 °C, then

suspended in 1 mL of 25 mM sodium acetate buffer (pH 4.2) for the activity assay. To measure the activity of DD_{sur}, cells from FY- or G6/G7-FY-culture were washed twice with 1 mL of 20 mM sodium acetate buffer (pH 5.3) and suspended in the same buffer (1 mL), and then an assay of activity was performed using cell suspension. For DD_{cyt}, washed cells from 5 mL of culture broth were lysed with 1 mL of reagent (CelLytic B Bacterial Cell Lysis/Extraction; Sigma–Aldrich, St. Louis, MO, USA), stirred for 10 min at room temperature, and then centrifuged at 13,000× *g* for 20 min at 4 °C to remove cell debris. The supernatant obtained was used to assay DD_{cyt} activity.

DDase activity was determined by incubating a reaction mixture (80 μL) containing 15 mM maltotetraose (G4; Hayashibara, Okayama, Japan), 25 mM sodium acetate buffer (pH 4.2), and DDase at 35 °C. Portions of the reaction mixture (25 μL) were withdrawn after 3, 10, and 20 min and heated at 100 °C for 10 min to terminate the enzyme reaction. Maltopentaose (G5) produced from G4 was quantitated by high-performance anion exchange chromatography equipped with a pulsed amperometric detector (Dionex, Sunnyvale, CA, USA) using a CarboPac PA1 anion exchange column (4 × 250 mm; Dionex) eluted by 600 mM sodium hydroxide at 0.8 mL/min. Sorbitol (Nacalai Tesque, Kyoto, Japan) served as an internal standard. One unit of DDase activity was defined as the amount of enzyme needed to form 1 μmol of G5 under the above conditions. All assays were performed in triplicate.

SDS–PAGE was performed with a 10% polyacrylamide gel to analyze the cell distribution of DDase by following our previously reported method (Sadahiro et al., 2015) with a molecular mass standard (Mark 12; Invitrogen, Carlsbad, CA, USA). We treated cells from FY-culture with various saccharides [0.5% glucose, G2 (Nacalai Tesque), G4, G5 (Wako Pure Chemical, Osaka, Japan), maltodextrin with average DP = 18 (G18; amylose EX-I; Hayashibara)] for 30 min and prepared the fractions of DD_{ext} and DD_{sur}. 1-Butanol extraction was employed for preparation of a combined fraction of DD_{cyt} and DD_{sur} from G5-treated cells (Yamamoto et al., 1992).

IMS production by DD_{ext} or intact cells harboring DD_{sur}

For the preparation of DD_{ext}, cells (equivalent to 496 mg dry cells) were obtained from FY-culture by centrifuging broth at 8,000× *g* at 4 °C for 20 min. A suitable amount of cells was suspended with 10 mL of 0.5% G6/G7 in 50 mM sodium acetate buffer (pH 4.2) for 3 h at 30 °C with agitation at 200 rpm, then cells were removed by centrifugation as mentioned above and supernatant was dialyzed to 25 mM sodium acetate buffer (pH 4.2). Supernatant containing 0.1 unit/mL DD_{ext} (prepared from cells with an OD_{600} of 20; 24.8 mg dry cells) was incubated with 5, 10, 15, 20, and 25% G6/G7 at various temperatures (30, 35, 40, and 45 °C) in 1 mL of reaction mixture containing 25 mM sodium acetate buffer (pH 4.2) without agitation. At the indicated time, the reaction was terminated by heating at 100 °C for 10 min.

The aforementioned cells with OD_{600} value of 5, 10, or 20 (6.20, 12.4, or 24.8 mg dry cells, respectively) were used directly for IMS production by incubating with 20 mL of 20% G6/G7 in 25 mM sodium acetate buffer (pH 4.2) at 30 °C for 1 h while shaking with 100 rpm agitation (the first process to secrete DD_{sur} and thermally stabilize enzyme), followed by further incubation at 45 °C for 6 or 7 d with shaking at 50 or 100 rpm (the second process to produce IMS). The effect of agitation in the second process was also investigated using 200 mL flasks with or without a baffled device (AGC Techno Glass). At the indicated time, an appropriate amount of recovered suspension was centrifuged at 8,000× *g* for 20 min at

4 °C to discard cells. The supernatant was heated at 100 °C for 10 min and used for further analysis of IMS and dextran formation.

DD_{ext}-catalyzed IMS production was reinvestigated under suitable conditions. DD_{ext} was prepared from cells with an OD_{600} of 20 (24.8 mg dry cells) by incubating with 1% G3 at 30 °C for 3 h with rotary shaking at 200 rpm. After removal of cells, DD_{ext} (0.1 unit/mL) was incubated in 50 mL of reaction mixture containing 20% G6/G7 and 25 mM sodium acetate buffer (pH 4.2) at 45 °C for 7 d with agitation at 100 rpm in a 200 mL baffled flask.

Fractionation of dextran and IMS by methanol precipitation

The resultant reaction mixture was subjected to alcohol fractionation to separate dextran and IMS. We added methanol (Nacalai Tesque) to the reaction mixture at a final concentration of 40% (v/v) and then left it to stand at 4 °C for 30 min. The resultant dextran precipitate was collected by centrifugation at 13,000× *g* and 4 °C for 10 min, and the pellet was washed with cold 40% methanol. The concentration of methanol in the supernatant was elevated to 90% (v/v) to precipitate IMS, followed by the same centrifugation used to precipitate dextran. The pellet of IMS obtained was washed with 90% methanol to remove residual oligosaccharides and free reducing sugars. Both dextran and IMS were dried *in vacuo*. The yield was expressed as the ratio (%) of the amount of IMS (or dextran) obtained divided by the amount of substrate G6/G7 used.

Structural analysis of IMS

^{1}H-NMR analysis of IMS was used to determine the average DP and glucosidic linkage according to the method described previously (Lang et al., 2014) with a Bruker ASX-300 (300 MHz) spectrometer (Rheinstetten, Germany). Amounts of IMS and dextran were estimated using the phenol–sulfuric acid method (Dubois et al., 1959), with glucose as the standard. The MW of IMS was determined using gel filtration HPLC equipped with a column (Shodex OHpak SB-803 HQ, 8.0 × 300 mm; Showa Denko, Tokyo, Japan) eluted with ultrapure water (generated by Direct-Q 3UV; Millipore, Molsheim, France) at 0.20 mL/min. Separated saccharides were detected by a Corona Charge Aerosol Detector (ESA Biosciences, Chelmsford, MA, USA). The molecular size markers used were Shodex Standard P-82 Pullulans (P-5 to P-100; 5.9–107 kDa; Showa Denko) and G7. IMS was subjected to overnight enzyme digestion with α-amylase (5 μg/mL) from porcine pancreas (Type I-A; Sigma–Aldrich, Steinheim, Germany) at pH 6.9 and 37 °C in the presence of 0.01% $CaCl_2$ (Lang et al., 2014) or dextran glucosidase (5 μg/mL) from *Streptococcus mutans* at pH 6.0 and 37 °C for 24 h (Saburi et al., 2006). The resulting hydrolysate was analyzed by following our previous method (Lang et al., 2014).

Results

Localization of *G. oxydans* ATCC 11894 DDase

The localization of DDase in cells cultivated with FY-culture was investigated by measuring the enzyme activities of DD_{ext}, DD_{sur}, and DD_{cyt} (Fig. 1a). DD_{sur} displayed the highest activity of 0.017 unit/(mg dry cells), while the activities of DD_{ext} and DD_{cyt} were quite small, indicating that most DDase was located at the cell surface. When *G. oxydans* was cultured with glucose, enzyme activity appeared for both DD_{ext} and DD_{sur}

and was absent for DD_{cyt} (Sadahiro et al., 2015), demonstrating the different localizations for glucose- (DD_{ext} and DD_{sur}) and fructose-culture (DD_{sur}). Thus, the carbon source for monosaccharides might influence the extracellular localization of DDase. In an attempt to secrete DDase from the cell surface into the culture medium, G6/G7-FY-culture was employed, followed by estimation of DD_{ext}, DD_{sur}, and DD_{cyt} activities (Fig. 1b). The DD_{sur} almost disappeared, and the main enzyme activity, DD_{ext} with 0.021 unit/(mg dry cells), was observed extracellularly, meaning that G6/G7 in the culture medium promoted the secretion of DDase from the cell surface. Furthermore, the effects of various saccharides on the secretion of DD_{sur} were analyzed using 0.5% glucose, G2, G4, G5, and G18. The results are shown in Table 1 and Fig. 1c for G5. When cells were treated with G5, the 180 kDa DDase protein (Sadahiro et al., 2015) appeared in the extracellular fraction (lane i, Fig. 1c). It was missing in cells (lane ii), indicating that G5 promoted the secretion of DDase. In Table 1, maltodextrins with DP ≥ 3 completely secreted DDase from the cell surface, while partial secretion appeared for glucose and G2.

IMS production by DD_{ext} secreted using G6/G7

DD_{ext} was utilized in our first attempt to produce IMS. For this, DD_{ext} was obtained from cells grown in FY-culture by treatment with 0.5% G6/G7. To determine the favorable treatment time, we examined time-dependent changes in DDase secretion (Fig. 1d). DD_{ext} was liberated immediately after 30 min, and dextran was also formed. Its activity displayed a maximum value at 1.5 h and then gradually decreased. G6/G7 disappeared at approximately 2.5 h, so we recovered DD_{ext} at 3 h and subjected it to further experiments. DD_{ext} tightly bound to dextran was formed, and neither dissociated in response to the usual dialysis approach (Suzuki et al., 1999). Dextran-bound DDase might be thermostable (≤ 45 °C; Yamamoto et al., 1992), since DDase without dextran exhibits less stability (≤ 35 °C; unpublished data of Saburi W).

The resulting DD_{ext} was utilized for IMS production over 96 h by varying the substrate concentration of G6/G7 (5–25%) and reaction temperatures (30–45 °C). The results are summarized in Table 2. IMS formation increased with increases in substrate concentration, and the highest production was obtained from 25% G6/G7 at 45 ºC. Interestingly, dextran production decreased with increasing substrate concentration. As a result, dextran was synthesized with a small yield of 2.7% from 25% G6/G7 at 45 ºC. However, the percentage of α-(1→6)-linkage content in the formed IMS was very small, probably due to formation of α-(1→4)-linked glucomegalosaccharides (i.e., maltomegalosaccharides). Therefore, we studied the generation of IMS with intact cells harboring DD_{sur} to compare the production efficiency with that of DD_{ext}.

IMS production by intact cells bearing DD_{sur}

Using intact cells, we reconfirmed the interesting results showing that a high substrate concentration increased IMS production and decreased dextran formation. DD_{sur}-bearing cells were incubated with 5 or 20% G6/G7 at 45 °C for 96 h, and the IMS and dextran formed were analyzed by gel filtration HPLC (Fig. 2a). The reaction with 20% G6/G7 exhibited a noticeable reduction in dextran (> 100 kDa) and an increase in IMS (approximately 2.3 kDa) compared to the levels for 5% G6/G7, resulting in the same phenomena observed for DD_{ext}. Furthermore, the ratio of IMS to dextran, estimated from their peak areas in the chromatogram for a reaction with 20% G6/G7, was higher than that seen with 5% G6/G7, which also

suggests greater productivity for IMS formation with 20% G6/G7. Thus, a substrate concentration of 20% G6/G7 was used for further studies.

When using DD_{ext}, incubation at 45 ºC provided the highest production of IMS (Table 2); therefore, a reaction temperature of 45 ºC was recommended. However, DD_{sur} was thermolabile at 45 ºC, since it is stable at temperatures of 35 °C and under. Temperature stability could be improved by binding dextran, so the synthetic procedure was separated into two processes. First, G6/G7 was incubated with cells at 30 °C to generate dextran together with dextran-binding DDase, and then the resultant thermostable enzyme was used for IMS production at 45 ºC (the second process). During the first process, DDase secretion from cells also occurred in the presence of G6/G7 (Table 1).

Cells with an OD_{600} = 5 (6.20 mg of dry cells) were subjected to IMS production with agitation at 50 rpm in a nonbaffled flask. Nevertheless, the percentage content of α-(1→6)-linkage remained at a low level of 7.5% (Table 2), as with the previous DD_{ext} results. Therefore, we monitored the time-dependent changes in the percentage of α-(1→6)-linkage content together with the production of IMS and dextran by varying the amounts of cells, agitation speed, and flask type (with or without the baffles) (Fig. 3). As shown in Fig. 3a–3c, IMS production increased immediately after starting the second process, while dextran was not synthesized rapidly. Agitation efficiency, controlled by agitation speed and the use of nonbaffled or baffled flasks, largely influenced the production of both IMS and dextran. Strong agitation enhanced the former and suppressed the latter (Fig. 3a and 3b). It appeared that formation of the α-(1→6)-linkage of IMS increased in a sigmoidal manner and reached its maximum value of approximately 60% (Fig. 3d–3f), for which ^{1}H-NMR data indicating the progression of α-(1→6)-linkage formation (Fig. 3d) are shown in Fig. 2b. Larger amounts of cells (OD_{600} = 20; 24.8 mg of dry cells) accelerated the generation of α-(1→6)-linkages, which reached a plateau at 96 h (Fig. 3f), indicating that the most favorable production of IMS was obtained at 96 h (Fig. 3c and 3f). Table 2 summarizes the production of cell-associated IMS over 96 h.

Reinvestigation of IMS production using DD_{ext}

As mentioned above, the formation of α-(1→6)-linkages in IMS was improved with high DDase activity (amounts of cells) and agitation efficiency, allowing us to reinvestigate IMS production using DD_{ext}. G3 enabled the secretion of DD_{sur} into the medium (Table 1), so DD_{ext} was prepared from the FY-medium-cultivated cells (OD_{600} = 20; 24.8 mg dry cells) treated with G3. As seen in Fig. 3h, surprisingly, the α-(1→6)-linkage content increased rapidly, reached a maximum of 65% at 24 h, and then maintained this level. IMS production gradually increased and reached a maximum level at 4–5 d (Fig. 3g). The improved reaction at 4 d provided the best yield (87%) and production (174 mg/mL) of IMS (Table 2), which had an α-(1→6)-linkage content of 65%. A low yield of dextran, 6.0%, was found. The obtained IMS was abbreviated as IMS-A and used for structural analysis.

Structural analysis of IMS-A

^{1}H-NMR analysis indicated that IMS-A possessed an average DP = 14.5. Considering its α-(1→6)-linkage content of 65%, IMS-A contains 9.4 and 5.1 α-(1→6)- and α-(1→4)-linked glucose units, respectively. IMS-A was digested by porcine pancreatic α-amylase (Lang et al., 2014) to form a saccharide (IMS-B) with DP = 12.0 and α-(1→6)-linkage content of 78%, meaning that the conformation of IMS-B comprised 9, 2, and 1

unit(s) of α-(1→6)-glucoside, α-(1→4)-glucoside and reducing terminal moieties, respectively. The structure of IMS-B was confirmed by treatment with dextran glucosidase. This enzyme is an α-glucosidase that specifically hydrolyzes the substrate at its nonreducing terminal α-(1→6)-linkage (Saburi et al., 2006), resulting in the production of G3 from IMS-B. Scheme 1 shows the conformation of IMS-A determined from the data obtained.

Discussion

Our first attempt to establish practical production of IMS involved the use of DD_{ext}. When *G. oxydans* was cultivated with fructose, most DDase was located on its cell surface (Fig. 1a). The secretion of DD_{sur} is important for IMS production, so various saccharides were tested to determine their secretory abilities. Maltodextrins with DP = 3–18 were efficient candidates, since their treatment of cells liberated DD_{sur} completely (Table 1); this led us to use G3 and G6/G7. Both glucose and G2 partially secreted DD_{sur}, but the mechanism cannot be clearly explained. These saccharides might interact with unknown component(s) at the cell surface to promote DD_{sur} liberation.

Although the DD_{ext} prepared was not completely purified, it is considered adequate for practical IMS production. Furthermore, DD_{ext} binds strongly to dextran and forms a macromolecule of dextran-bound DD_{ext}, which could be recovered by centrifugation at high speed. This simple procedure is thought to constitute affinity purification (Suzuki et al., 1999) and provide DD_{ext} with high purity. A further advantage of dextran enhances thermostabilization of DD_{ext} with an increase of 10 °C (dextran-bound DD_{ext}, ≤ 45 °C; free DD_{ext}, ≤ 35 °C), enabling production of IMS at the higher temperature of 45 °C (Table 2). This is a well-known phenomenon in which the coexistence of products, substrates, or reversible inhibitors (e.g., competitive inhibitors) protects the enzyme from heat inactivation by forming a complex. Both favorable effects of dextran assisted DDase-mediated IMS generation, while dextran itself was a byproduct. Another factor is substrate concentration, and higher levels increased and decreased the production of IMS and dextran, respectively (Table 2). This phenomenon is probably explainable with the reaction mechanism of DDase. The enzyme catalyzes the two-substrate reaction by using the same donor and acceptor substrates of G6/G7, meaning that one glucose unit of donor G6/G7 is transferred to another G6/G7 of an acceptor. This transfer reaction is sequentially repeated to elongate the α-(1→6)-glucosyl chain of dextran. At high substrate concentrations (15–25% G6/G7; Table 2), acceptor species are more than adequate for receiving the glucose unit from donor G6/G7, thereby impeding the sequential reaction generating dextran. Conversely, many acceptor substrates assist in the accumulation of IMS, an intermediate species in dextran formation. On the other hand, at a low substrate concentration (5% G6/G7), only the polysaccharide was observed, probably due to the presence of proper levels of donor and acceptor, which enabled sequential elongation to form dextran. However, IMS formed by DD_{ext} was found to possess a small percentage of α-(1→6)-linkage content, the possible reason for which is discussed below.

Our second attempt utilized intact cells, and IMS was generated using a two-step procedure. Formation of thermostable dextran-bound DD_{ext} was performed under mild conditions at 30 °C (the first process) prior to IMS production (the second process). During the first process, cell-bound DD_{sur} was also secreted extracellularly (DD_{ext}) by G6/G7 to form a macromolecule of dextran-binding DD_{ext}. Interestingly, formation of α-(1→6)-linkages progressed in a sigmoidal fashion (Fig. 3d–3f), indicating the presence of another

reaction prior to generation of the α-(1→6)-linkage. DDase catalyzes the formation of α-(1→4)-linkages by transglucosylation, so this transfer reaction occurs more quickly in the early stage than α-(1→6)-linkage formation. The resulting α-(1→4)-linked saccharides (maltodextrins) are further attacked by DDase and converted to IMS. Agitation of the reaction mixture and an increase in the amounts of cells enhanced the α-(1→6)-linkage content of IMS (Fig. 3f). The former effect was possibly due to mixing of dextran-bound DD_{ext} and G6/G7. Without agitation, this macromolecule cannot react efficiently with the substrate due to sedimentation. This is the reason why our first trial using DD_{ext} without agitation produced IMS with a small percentage of α-(1→6)-linkage content. Furthermore, the high agitation speed, 100 rpm, inhibited the formation of dextran (Fig. 3b) compared with slower agitation at 50 rpm (Fig. 3a). We cannot explain this phenomenon, but it is possible that agitation at high speed generates small dextran particles, resulting in a reduction in its yield (Kim D, personal information).

From our second attempt using intact cells, we learned the importance of agitation efficiency and high substrate concentration, so we reinvestigated IMS production using DD_{ext} (Fig. 3g and 3h); this resulted in a great yield of IMS with high α-(1→6)-linkage content (Table 2). The results obtained indicated that the preparation of 6 L of cell culture (OD_{600} = 20; 24.8 mg dry cells/mL) enabled practical production of IMS on a 100 g scale. The resulting IMS-A (Scheme 1) was digested with α-amylase to produce IMS-B. IMS-B was nearly identical to the IMS we previously synthesized with recombinant DDase (plus α-amylase treatment) and used for our studies of flavonoid uptake in rat intestine (Shinoki et al., 2013), solubilization of insoluble azobenzene dye (Lang et al., 2014), and enhancement of the barrier function of intestinal tight junctions (Hara et al., 2017). Isomaltooligosaccharides (average DP = 3.3) functioned in the large intestine (Iwaya et al., 2012), indicating that the long α-(1→6)-portion might be less susceptible to digestion in the small intestine. The detailed characterization of IMS will be reported in a separate paper.

Author contributions WL and AK conceived and coordinated the study. WL, YK, JS, WS, and RS conducted experiments. WL, YK, JS, WS, RS, TT, MO, HM, NS, DK, and AK analyzed the data. WL and AK wrote the manuscript. All authors read and approved the final manuscript.

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Data availability The data that support the findings of this study are available from the corresponding authors on reasonable request.

Declarations

Ethics approval This article does not involve any human subjects. The authors did not perform any animal-based experiments in this study.

Conflict of interest The authors declare no competing interests.

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Figure captions

Fig. 1 Localization of DDase in *G. oxydans* cells cultivated with FY- or G6/G7-FY-culture and secretion of DDase by incubation of cells with G5 or G6/G7.

(**a**) and (**b**) DDase activity measured using intracellular, extracellular, and cell surface fractions of *G. oxydans*. (**a**) FY-culture; (**b**) G6/G7-FY-culture; black bar, DDase activity. (**c**) SDS–PAGE of DDase from G5-treated cells. Lane M, molecular weight markers; lane i, DD_{ext}; lane ii, DD_{cyt} and DD_{sur} (1-butanol-extracted cells); lane iii, DD_{cyt} and DD_{sur} (1-butanol-extracted cells without G5 treatment; control for lane ii); arrow, DDase. (**d**) Secretion of DDase by treatment of 0.5% G6/G7. The cells grown in FY-culture were suspended in 0.5% G6/G7 at pH 4.2 and 30 °C with agitation at 200 rpm. Open circles, DD_{ext} activity; closed circles, G6/G7; triangles, dextran formed.

Fig. 2 Gel filtration and ^{1}H-NMR analyses of saccharides produced from 5% or 20% G6/G7 by intact cells.

(**a**) Gel filtration HPLC analysis of IMS and dextran. Intact cells bearing DD_{sur} (0.1 unit/mL) were incubated with 5% or 20% G6/G7 for 96 h at pH 4.2 and 45 °C without agitation. Upper panel, 5% G6/G7; lower panel, 20% G6/G7; i, dextran with more than 100 kDa; ii, IMS with 2.3 kDa; iii, oligosaccharide and glucose with 0.21–0.46 kDa; iv, buffer. (**b**) Partial ^{1}H-NMR spectra of IMS. IMS analyzed was the reaction product in Fig. 3a and 3d. The lower, middle, or upper panel corresponds to IMS, formed at 2, 4, or 6 d and having 8.0, 41.3, or 62.1% α-(1→6)-linkage content, respectively. H1(→4), anomeric proton signal of a glucose unit with an α-(1→4)-linkage; H1(→6), that with an α-(1→6)-linkage; H1α, anomeric proton signal for the α-glucose unit at the reducing terminal; H1β, that of the β-glucose unit.

Fig. 3 Production of IMS by intact cells or DD_{ext}.

IMS production was performed under various conditions by incubating 20% G6/G7 with cells (**a–f**) or DD_{ext} (**g and h**). **a–c and g**, production of IMS (closed triangles) and dextran (closed circles); **d–f and h**, percentage of α-(1→6)-linkage content in IMS (open circles); **a and d**, cells (OD_{600} = 10, 12.4 mg dry cells) agitated at 50 rpm in a nonbaffled flask; **b and e**, cells (OD_{600} = 10, 12.4 mg dry cells) agitated at 100 rpm in a baffled flask; **c and f**, cells (OD_{600} = 20, 24.8 mg dry cells) agitated at 100 rpm in a baffled flask; **g and h**, DD_{ext} (0.1 unit/mL) agitated at 100 rpm in a baffled flask.

Scheme 1 Proposed structure of IMS-A.

Open arrow, site cleaved by porcine pancreatic α-amylase.

Table 1. Secretion of DDase from cells treated with various 0.5% saccharides

0.5% Saccharide	Cell surface (DD_{sur})	Extracellular fraction (DD_{ext})
None[a]	+++	–
Glucose	++	+
G2	++	+
G3[a]	–	+++
G4	–	+++
G5	–	+++
G6/G7[a]	–	+++
G18	–	+++

+, Detected; –, not detected.

[a]Identified by activity; other saccharides were determined by SDS–PAGE.

Table 2. IMS production by DD_{ext} or intact cells for 96 h under various conditions

Origin of DDase (unit/mL) (mg dry cells)	G6/G7 (%)	Temperature (°C)	Agitation (rpm)	IMS Formation (mg/mL)	IMS %Y_{IMS}[a]	IMS %α-(1→6)[a]	Dextran Formation (mg/mL)	Dextran %Y_{Dex}[a]
DD_{ext}[*]								
0.1[**]	5	30	-[b]	0.2 ± 0.2	0.4	28.3	9.8 ± 1.6	19.6
	5	35	-	0.3 ± 0.1	0.6	44.7	10.6 ± 2.2	21.1
	5	40	-	0.2 ± 0.4	0.3	38.3	9.3 ± 0.3	18.6
	5	45	-	2.0 ± 0.4	4.0	28.6	10.2 ± 0.2	20.4
	10	30	-	12.9 ± 2.1	12.9	6.7	9.8 ± 2.5	9.8
	10	35	-	11.0 ± 2.2	11.0	7.4	7.4 ± 0.3	7.4
	10	40	-	13.6 ± 0.4	13.6	12.2	8.9 ± 0.6	8.9
	10	45	-	21.1 ± 2.0	21.1	10.0	5.5 ± 0.1	5.5
	15	45	-	36.0 ± 4.6	24.0	5.3	3.3 ± 0.1	2.2
	20	45	-	65.6 ± 2.0	32.8	2.4	8.8 ± 0.1	4.4
	25	45	-	88.4 ± 4.2	35.4	3.5	6.8 ± 1.9	2.7
Cells[***]								
6.20	20	45	50 (N)[c]	83.2 ± 2.1	41.6	7.5	13.5 ± 2.5	6.8
12.4	20	45	50 (N)	72.7 ± 2.0	36.4	39.2	27.4 ± 5.4	13.7
12.4	20	45	100 (B)[d]	130 ± 9.0	65.0	32.8	1.5 ± 0.1	0.75
24.8	20	45	100 (B)	103 ± 7.8	51.4	61.2	25.4 ± 1.9	12.7
DD_{ext}[*]								
0.1[**]	20	45	100 (B)	174 ± 4.2	87.0	65.0	12.0 ± 2.0	6.0

[*]DD_{ext}, unit/mL. [**]DD_{ext} with 0.1 unit/mL was obtained from 24.8 mg of dry cells; [***]cells, mg dry cells; [a]Percentage conversion of G6/G7 into IMS (%Y_{IMS}) or dextran (%Y_{Dex}). %α-(1→6), percentage content of α-(1→6)-linkage; [b]-, no agitation; [c]N, nonbaffled flask was used with an agitation speed of 50 rpm; [d]B, baffled flask was used with an agitation speed of 100 rpm.

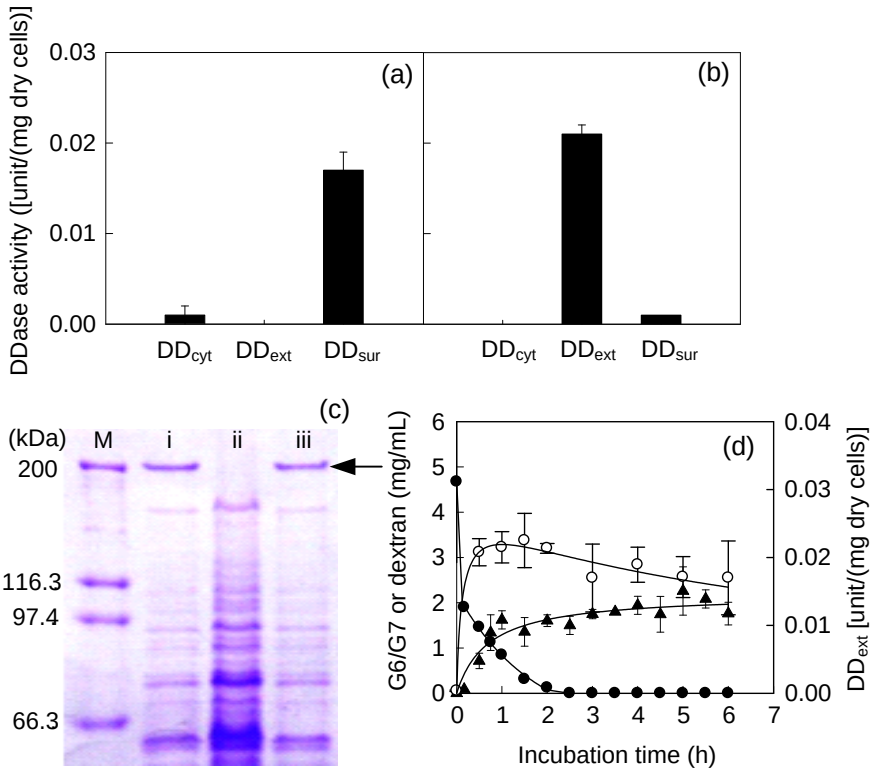


Fig. 1. Localization and secretion of DDase.

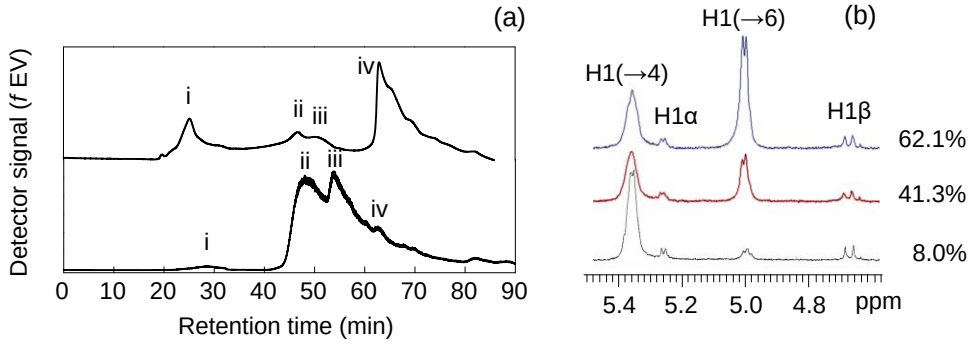


Fig. 2. Gel filtration and ^{1}H-NMR analyses.

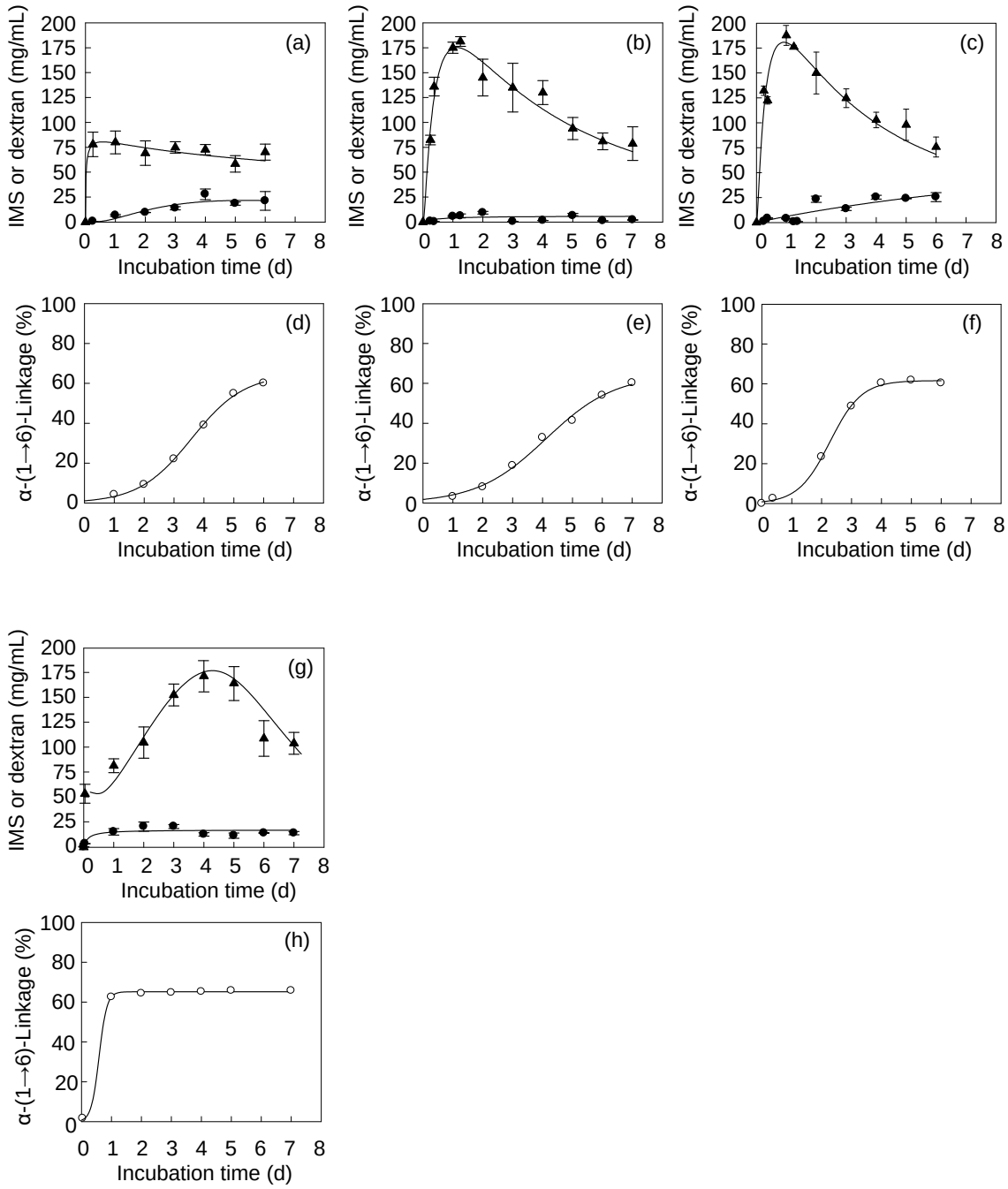
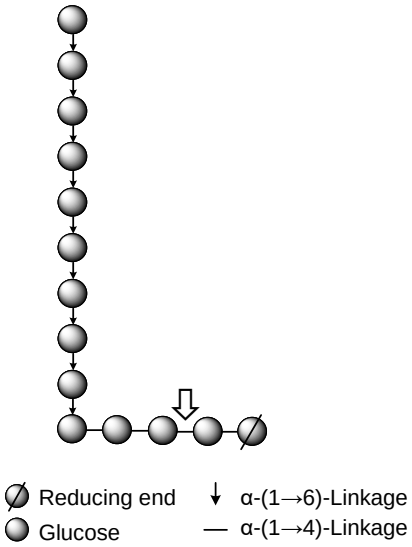


Fig. 3. Production of IMS.



Scheme 1. Proposed structure of IMS-A.