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A Novel Dextran Dextrinase from *Gluconobacter oxydans* DSM-2003: Purification and Properties

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Abstract Dextran has already been widely applied in food, pharmaceutical, and chemical industries. In this study, a novel intracellular dextran dextrinase (DDase, EC 2.4.1.2) from *Gluconobacter oxydans* DSM-2003 exhibiting catalytic activity to synthesize dextran from maltodextrin was purified to homogeneity by ultrasonic cell disruption, ion exchange chromatography, and gel filtration. This procedure showed 187.5-fold purification from the cell-free extract with 41.9 % yield. And the apparent molecular weight was estimated to be 62 kDa by SDS-PAGE. It was different from the reported literatures, which found that the molecular weight of intracellular and extracellular DDase of *G. oxydans* ATCC-11894 was 300 and 152 kDa, respectively. Otherwise, it showed different physicochemical characteristics (optimal temperature and pH, thermal, pH stability, effect of metal ions) from the DDase of *G. oxydans* ATCC-11894. This indicated that DDase of *G. oxydans* DSM-2003 was a novel one compared to the reported literatures.

Keywords *Gluconobacter oxydans* · Dextran dextrinase · Enzyme purification · Catalytic property

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Introduction

Dextran, synthesized from sucrose by the transglucosylation of dextransucrase (DSase, EC 2.4.1.5) of *Leuconostoc mesenteroides*, is an extracellular bacterial polymer of D-glucopyranose. It has a substantial number of α(1,6) linkage in main chain and a variable amount of α(1,2), α(1,3), α(1,4) branched linkages [1, 2]. To date, it has already been widely applied in pharmaceutical, food, and chemical industries [3, 4].

Dextran dextrinase (DDase, EC 2.4.1.2), produced by certain strains of *Gluconobacter oxydans*, can convert maltodextrins to dextran [5, 6]. The enzyme catalyzes the transfer of an α(1,4)-linked glucosyl unit from a donor to an acceptor molecule, forming an α(1,6) linkage: consecutive glucosyl transfers result in the formation of high molecular weight dextran from maltodextrin. Up to now, it was found that only *G. oxydans* ATCC-11894 could produce DDase. Yamamoto et al. reported that *G. oxydans* ATCC-11894 produced DDase mainly intracellularly, and the molecular weight was estimated to be 300 kDa by SDS-PAGE [7]. *G. oxydans* ATCC-11894 could also secrete an extracellular DDase in culture medium with glucose and a low level (0.05 %) of maltodextrins as carbon source. Suzuki et al. purified this enzyme by a simple one-step centrifugation at 20,000×*g* for 20 min at 4 °C. The enzyme was tightly bound to the dextran formed. The molecular mass of extracellular DDase was estimated to be 152 kDa by SDS-PAGE, just one half of the intracellular variant [8]. These two kinds of DDase both had the ability to convert short-chain amylose to dextran. The yields of dextran synthesized by intracellular and extracellular DDase were 57.6 and 74 %, respectively [9].

The differences in structure between *Gluconobacter* and *Leuconostoc* dextran have been further studied. Dextran synthesized by DSase from *L. mesenteroides* NRRL B-512F is the only one produced commercially. It is a homopolysaccharide containing 95 % α(1,6) glycosidic linkages in main linear chain and 5 % α(1,3) in branched linkages [10]. The structure analysis of *Gluconobacter* dextran synthesized by DDase from *G. oxydans* ATCC-11894 showed the existence of α(1,4) branches and α(1,4) linkages in α(1,6) glucosyl linear chains. It was constructed with 6.23 % branching points and 6.53 % α(1,4)-linked glucosyl residues [11].

Gluconobacter dextran solutions displayed shear-thinning flow behavior. According to the previous research, the novel polysaccharide showed lower viscosity than *Leuconostoc* dextran of similar molecular weight as a consequence of its higher degree of branching. It might be suitable for certain food applications not associated with thickening functionality, such as a source of dietary fiber or a low-calorie bulking agent for sweeteners [9]. Thus, Naessens et al. reported that both the intracellular and extracellular forms of *G. oxydans* DDase could be promising alternatives to DSase as a biocatalyst for the synthesis of dextran and oligodextrans. Hence, DDase needs to be studied not only for understanding the enzyme characteristics and structure, but also for finding out new DDases suitable for the above-mentioned specific applications.

In our previous report, we found that the cell-free extract of *G. oxydans* DSM-2003 could use in nature widely occurring maltodextrin to produce dextran by transglucosylation [12]. This homogeneous dextran contained a small proportion of α(1,4) branches in α(1,6) glucosyl main linear chain. This result indicated that dextran synthesized by *G. oxydans* DSM-2003 was a novel one compared with the dextran structure in the former reports [2, 11]. In this study, we described the corresponding polysaccharide synthase, a novel dextran dextrinase produced from *G. oxydans* DSM-2003. Purification and characterization of the enzyme has been elucidated.

Materials and Methods

Bacterial Strain and Culture Medium

G. oxydans DSM-2003 was isolated from ropy beer by the authors and preserved in DSMZ (German Collection of Microorganisms and Cell Cultures). *G. oxydans* grew first in a preculture containing 8 % (*w*/*v*) D-sorbitol, 2 % (*w*/*v*) yeast extract, 0.2 % (*w*/*v*) KH_2PO_4, and 0.05 % (*w*/*v*) $MgSO_4 \cdot 7H_2O$ at 30 °C and 200 rpm. After 24 h cultivation, 5-ml precultures were diluted into 50 ml production medium [13], and grew under the same conditions like the preculture.

DDase Assay

The DDase activity was determined according to the method described by Naessens et al. with modification [14]. It was based on the release of *p*-nitrophenol from *p*-nitrophenyl-α-D-glucopyranoside (NPG). A 4-ml reaction mixture, containing 0.125 mM NPG in 50 mM phosphate buffer (pH 6.5) and DDase solution was incubated at 30 °C for 30 min. The reaction was stopped by adding 0.5 ml of 2 M carbonate–bicarbonate buffer (pH10.7), and then nitrophenol was determined spectrophotometrically at 410 nm (ε=17.8 $mM^{-1}cm^{-1}$) [15]. One unit of DDase activity was defined as the amount of enzyme which releases 1 μmol of *p*-nitrophenol from NPG per minute under the standard assay condition.

Measurement of Protein

Protein concentration was measured by the Bradford method, using bovine serum albumin as a standard. In the purification procedure, protein in the column effluents was monitored by measuring the absorbance at 280 nm.

Purification of Dextran Dextrinase

All procedures were performed at 4 °C, and 50 mM Tris–HCl buffer (pH7.4) was used unless otherwise stated. All chromatographic separations were performed using AKTA-fast protein liquid chromatography system with a Frac-950 fraction collector (Amersham Biosciences, USA). Every fraction containing protein was assayed by the standard procedure, and the eluant fractions with higher specific activity (in units per milligram) than that of the last step were selected for combining.

Step 1: preparation of crude enzyme

Cells from the culture broth were harvested by centrifugation at 5,000×*g* for 10 min, washed with 50 mM Tris–HCl buffer (pH7.4), resuspended in the same buffer and disrupted by ultrasonication (VCX 750, Sonics, USA). The mixture was centrifuged at 10,000×*g* for 20 min and then the supernatant was filtrated using 0.22 μm membrane to remove small cell debris.

Step 2: strong anion-exchange chromatography

The crude enzyme solution was loaded on a HiTrap Q Fast Flow column (ϕ 1.6×2.5 cm, Amersham Biosciences) equilibrated with 50 mM Tris–HCl buffer (pH7.4), and the enzyme adsorbed was eluted from the column with 0.1 M NaCl in the Tris–HCl buffer at a flow rate of 1 $ml\,min^{-1}$.

Step 3: desalting

The enzyme solution was loaded on a HiPrep 26/10 Desalting column (2.6×10 cm, Amersham Biosciences) equilibrated with 50 mM Tris–HCl buffer (pH7.4), and proteins were eluted with the same buffer at a flow rate of 5 mlmin^{-1}.

Step 4: weak anion-exchange chromatography

The enzyme solution was loaded on a HiPrep ANX Fast Flow column (ϕ 1.6×2.5 cm, Amersham Biosciences) equilibrated with 50 mM Tris–HCl buffer (pH7.4), and the enzyme adsorbed was eluted from the column with 0.1 M NaCl in the Tris–HCl buffer at a flow rate of 1 mlmin^{-1}.

Step 5: gel filtration chromatography

The concentrated enzyme solution was loaded on a HiLoad 16/60 Superdex 75PG column (ϕ 1.6×60 cm, Amersham Biosciences) equilibrated with 50 mM Tris–HCl buffer containing 0.1 M NaCl at pH7.4, and proteins were eluted with the same buffer at a flow rate of 0.5 mlmin^{-1}.

Gel Electrophoresis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine protein purity and the molecular mass of the purified enzyme. Protein bands were visualized with Coomassie brilliant blue R-250.

Biochemical Characterization of the Purified DDase

The effect of pH on enzyme activity was determined by varying the pH between 3 and 11 at 30 °C. The pH was maintained by 50 mM citrate buffer (pH3–5), 50 mM phosphate buffer (5–9) and 50 mM carbonate buffer (pH9–11). The effect of temperature on the enzyme activity of DDase was determined in the range of 10–60 °C at pH 6.5.

The thermal stability of DDase was measured under the conditions: DDase was preincubated for 3 h in the absence of substrates at 10–60 °C, respectively. The residual activity was measured at 30 °C. To examine the pH stability of DDase, the enzyme solution was preincubated in various buffer between pH3 and 11 for 3 h, the remaining activity was assayed in pH6.5 phosphate buffer.

The effects of various metal ions ($BaCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_3$, $FeSO_4$, $MgCl_2$, $NiCl_2$, $MnSO_4$ and $ZnCl_2$) at 0.5 mM on DDase activity were assayed by preincubating the enzyme with the individual metal ions in phosphate buffer (pH6.5) at 30 °C for 3 h. The enzyme activities of DDase were then measured by the standard procedure. The extent of inhibition or activation of enzyme activity was described as percentage of the ratio of residual activity to complete enzyme activity in the control sample without addition of metal ions.

Preparation of Dextran from Maltodextrin

For preparation of dextran, 50 ml of 0.25 % maltodextrin (Sinopharm Group, China) in 50 mM phosphate buffer (pH6.5) was mixed with 10 ml purified DDase (0.1 U ml^{-1}), and incubated for 20 h at 30 °C. The reaction was stopped by heating at 100 °C for 20 min. The dextran content in the reaction mixture was monitored by gel permeation chromatography.

Measurement of Molecular Weight and Content of Dextran

The molecular weight and content of dextran was determined by gel permeation chromatography (GPC). A DAWN HELLOS system (Wyatt) with a Shodex Ohpak SB-803 HQ column (8.0×300 mm) was applied. The column was eluted with 0.1 M $NaNO_3$ at 0.5 ml min^{-1} at 40 °C.

Results and Discussion

Purification of DDase from *Gluconobacter oxydans* Culture

DDase was purified from wet cells as described above by ultrasonication, ion exchange chromatography, and gel filtration. The purification results are summarized in Table 1. These chromatography methods resulted in a 187.5-fold over the crude enzyme extract with an overall recovery of 41.9 % and had a specific activity of 112.5 Umg^{-1} of protein. The purified DDase showed a single protein band by SDS-PAGE when stained with Coomassie Brilliant Blue. And its molecular mass was estimated to be 62 kDa by SDS-PAGE (Fig. 1), which coincided with the result estimated by Sephacryl S-100 gel filtration chromatography (data not shown). This indicated that the enzyme migrated as a monomer in gel electrophoresis and thus may also be present as a monomer in solution. The purified enzyme appeared as a single band with a molecular weight of approximately 62 kDa, which was different from the reported literatures [7, 16]. They found that the molecular weight of intracellular and extracellular DDase of *G. oxydans* ATCC-11894 was 300 and 152 kDa, respectively. Up to now, researchers were not able to explain the structural difference between the extracellular and intracellular enzymes from the same strain.

Properties of DDase

The biochemical properties of DDase were characterized. The maximal activity occurred at 35 °C (Fig. 2), then it displayed a sharp decrease at temperatures higher than 35 °C. Correspondingly, it was stable below 30 °C for 3 h (Fig. 2), 3 h of incubation at 35 °C would make DDase lose half value of its maximal activity. DDase exhibited the maximum activity at pH6.3 (Fig. 2). pH stability, expressed as residual activity after 3 h, showed that DDase barely lost activity between pH5.0 and 9.5. While in acid conditions of pH4.0 and

Table 1 Purification of DDase from *Gluconobacter oxydans* DSM 2003

Procedure	Total activity (U)	Specific activity (U mg^{-1} protein)	Total protein (mg)	Yield (%)	Purification (fold)
Cell extract	101.8	0.6	169.7	100	1
Membrane filtration	97.6	1.2	81.3	95.9	2
HiTrap Q Fast Flow	96.7	19.4	5.0	95	32.3
Desalting	89.5	27.9	3.2	87.9	46.5
HiTrap ANX Fast Flow	61.7	63.2	1.0	60.6	105.3
Gel filtration	42.7	112.5	0.4	41.9	187.5

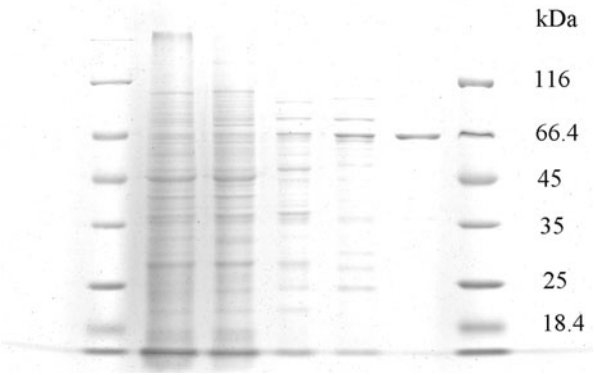

Fig. 1 SDS-PAGE of DDase purified from *Gluconobacter oxydans* DSM-2003. *Lane 1* cell extract, *lane 2* membrane filtration fraction, *lane 3* Q FF ion exchange fraction, *lane 4* ANX FF ion exchange fraction, *lane 5* gel filtration fraction, *lane 6* molecular marker

alkaline conditions of pH10.5, it was partially denatured with 50 % of maximal activity vanished.

Effects of metal ions on DDase's activity were studied. At 30 °C, the enzyme was activated slightly by Mn^{2+}, Ca^{2+}, Mg^{2+}, and Ba^{2+} ions, yet strongly inhibited by Fe^{2+}, Zn^{2+}, and Cu^{2+} ions, especially by Cu^{2+} up to 85 % (Fig. 3).

Otherwise, it showed different physicochemical characteristics (optimal temperature and pH, thermal, pH stability, effect of metal ions) from the DDase of *G. oxydans* ATCC-11894. The optimal temperature and pH of the purified DDase were 35 °C and pH6.3, respectively. These properties were different from the reported literature that the optimal temperature and pH of intracellular DDase of *G. oxydans* ATCC-11894 were 37–45 °C and pH4–4.5, respectively [7]. It was also found that the purified DDase was stable below 30 °C and between pH5 and 9 for 3 h. This difference indicated that DDase of *G. oxydans* DSM-2003 was a novel one compared to the reported literatures [7, 16].

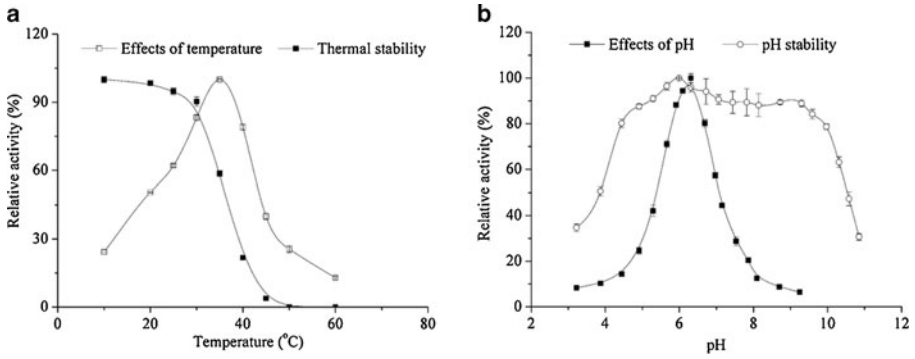

Fig. 2 **a** Effect of temperature on purified DDase activity. Experiments were performed at pH6.5 and relative enzyme activity was calculated by compared with enzyme activity at 35 °C. **b** Effect of pH on purified DDase activity. Experiments were performed at temperature of 30 °C and relative enzyme activity was calculated by compared with enzyme activity at pH6.3

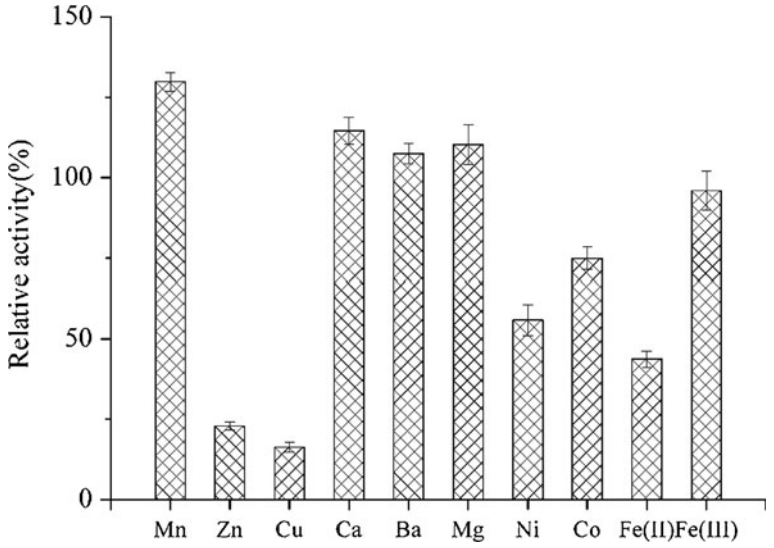

Fig. 3 Effect of various metal ions on the activity of DDase. Experiments were performed at 30 °C and pH6.5 and relative enzyme activity was calculated by compared with enzyme activity of control

Preparation of Dextran from Maltodextrin

In our previous research, we have found that the cell-free extract of *G. oxydans* DSM-2003 had the ability of producing a novel dextran from maltodextrin [12]. The structure analysis showed that the first peak (11.5 min) was a kind of dextran and the second was maltodextrin.

There are also two peaks in the GPC spectrum of reaction mixture by purified DDase (Fig. 4) and it was almost the same with what we found in previous research. After a few hours' reaction, a portion of substrate with small molecular weight was used by DDase, so the second peak in Fig. 4a eluted a little earlier than normal.

The GPC analysis (Fig. 4) showed that the purified DDase possessed the ability to produce dextran from maltodextrin.

Compared to the substrate used by dextransucrase (EC 2.4.1.5), DDase (EC 2.4.1.2) can use widely occurring in nature maltodextrin to produce dextran by transglucosylation. Furthermore, there are also some differences in the yield and structure of dextran. The theoretical yield of dextran from sucrose using dextransucrase is 50 % because a fructose molecule is liberated from sucrose. However, DDase can more efficient produce dextran, a maximum yield with 74 % was reported by using short-chain amylase as substrate [8]. *Gluconobacter* dextran synthesized by DDase from *G. oxydans* ATCC-11894 and DSM-2003 was composed of α(1,6)-linked chains containing α(1,4) branching points [11, 12]. According to Naessens' research, *Gluconobacter* dextran solution displayed lower viscosity with a similar molecular weight because of its higher degree of branching [9]. Yamamoto et al. found that *Gluconobacter* dextran was digested slightly by rat intestinal acetone powder; the hydrolysate was a mixture of high molecular weight polymers. The low digestibility suggested the possibility that it might be used as a dietary fiber [11]. In conclusion, *Gluconobacter* dextran might be more suitable for certain food use applications, such

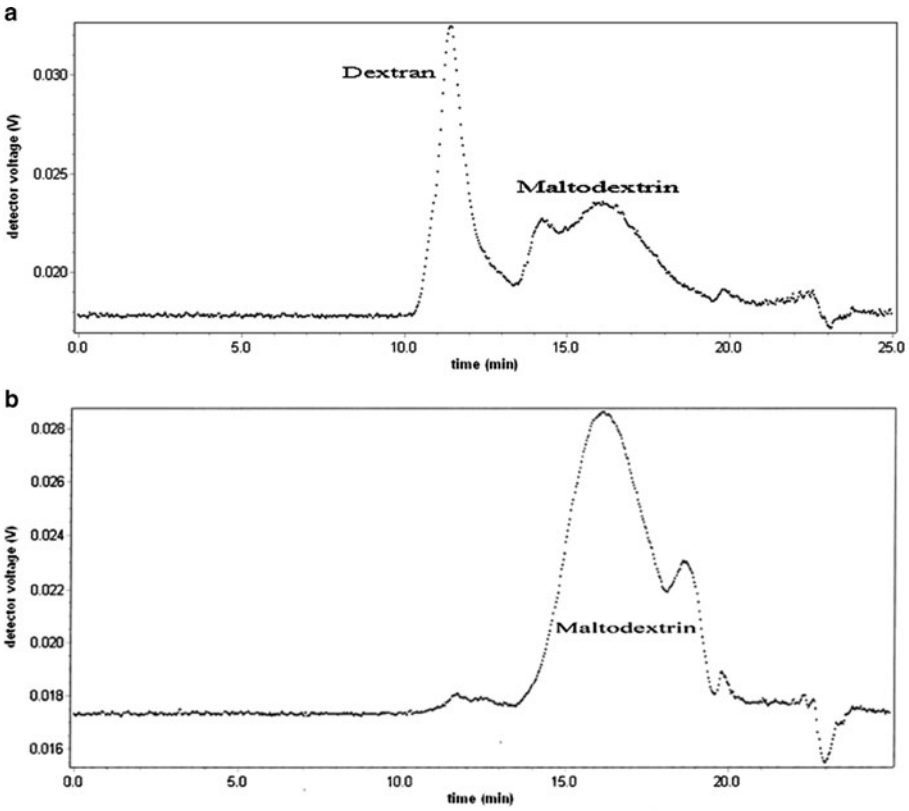

Fig. 4 The gel permeation chromatogram of reaction products (**a**) and substrate maltodextrin (**b**)

as a source of dietary fiber, cryostabilizer, fat substitute, or low-calorie bulking agent for sweeteners [9]. Thus, Naessens et al. reported that both the intracellular and extracellular forms of *G. oxydans* DDase could be promising alternatives to DSase from *L. mesenteroides* as a biocatalyst for the synthesis of dextran and oligodextran.

Conclusion

In the present study, we described the purification and characterization of DDase from *G. oxydans* DSM-2003. The purified enzyme appeared as a single band with a molecular weight of approximately 62 kDa, and it showed different physicochemical characteristics from the DDase of *G. oxydans* ATCC-11894. This indicated that DDase of *G. oxydans* DSM-2003 was a novel one compared to the reported literatures and it would have potential application in the food industries.

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