Acetobacter capsulatum ATCC11894株起源の菌体外デキストリンデキストラナーゼの簡易精製法と基本性質

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Note

Simple Purification and Characterization of an Extracellular Dextrin Dextranase from *Acetobacter capsulatum* ATCC 11894

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Dextrin dextranase (DDase, EC2.4.1.2) was extracellularly secreted in a culture medium of *Acetobacter capsulatum* ATCC 11894 containing both glucose (5.45%) and an extremely small amount of dextrin (0.05%) as the essential carbon sources. The enzyme was simply purified by only one-step centrifugation at 4℃ and 20,000×*g* for 20 min, and immediately dialyzed against 50 mM acetate buffer (pH 4.5) at 4℃ for 2-3 days. The purified DDase gave a single protein band on both Native- and SDS-PAGE. The molecular mass of the enzyme was estimated to be about 152 kDa by SDS-PAGE. The optimum pH and temperature of the enzyme were 5.2 and 38℃, respectively. The enzyme retained its original activity up to 45℃, and was stable in the range of pH 4.1-5.4 at 4℃ for 24 h. The enzyme was completely inactivated by 1 mM of Hg^{2+}, Pb^{2+} or $KMnO_4$. The purified enzyme effectively synthesized α-1,6-glucan from maltooligosaccharides. The average molecular mass of the product dextran was estimated to be about 1270 kDa.

Only a few reports concerning dextrin dextranase (DDase, EC2.4.1.2) have been published so far.[1-8] In 1947, Shimwell found an acetic acid bacteria grown in a ropy beer and isolated it carefully.[1] In 1951, Hehre and Hamilton reported that (1) a viscous material was produced from dextrin as glucosyl donor by DDase from *Acetobacter capsulatum* ATCC 11894 or *A. viscosum* ATCC 11895, and (2) DDase mainly reacted with a series of maltooligosaccharides except maltose and did not react with amylose, amylopectin, glycogen, cyclodextrin, sucrose, glucose and others.[2-4] But, at that time, the enzyme itself was only a cell-free extract and a high level of enzyme purification was not done. In 1992-1994, Yamamoto *et al.* reported the purification procedures of intracellular DDase from *A. capsulatus* ATCC 11894[5] and some of its physicochemical and enzymatic properties.[6,7] They also referred (1) that the molecular structure of dextran produced by DDase was quite different from that of *Leuconostoc* dextran,[5] and (2) the effective production of glucosyl-steviosides by α-1,6 transglucosylation of DDase.[8]

Recently, we found that *A. capsulatum* ATCC 11894 effectively produced extracellular DDase. In this paper, we report the simple purification procedures of extracellular DDase, some of its physicochemical and enzymatic properties and a comparison of our DDase with the reported intracellular type.[5-8]

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Abbreviations : HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PCMB, *p*-chloromercuribenzoate.

EXPERIMENTAL

Acetobacter capsulatum ATCC 11894 was a hearty gift by Dr. E. J. Hehre of Albert Einstein

College of Medicine in N.Y. The standard culture medium for enzyme production was a 3.6-liter liquid medium containing 0.5% yeast extract (Difco Laboratories), 5.45% glucose·H_2O (Nihon Syokuhin Kako Co., Ltd.), 0.05% Pine-dex #3 (Matsutani Kagaku Kogyo, Ltd.). The preculture broth (200 mL) of *A. capsulatum* ATCC 11894, cultivated at 25℃ for 24 h, was inoculated into a standard culture medium (3.6 L), and then cultivation was done by using a Jar-fermenter (Tokyo Rikakikai Co., Ltd., Model MBF-250M) at 25℃ for 36 h, 120 rev/min agitation and 2.5-liter air/min.

The cell-free culture broth was obtained using a refrigerated centrifuge (Tomy Co., Ltd., Model CX-250) at 4℃ and 2000×*g* for 20 min, and used as the starting material for enzyme purification. Purified maltopentaose (Nihon Syokuhin Kako Co., Ltd.) was used as the substrate throughout this work. The reaction mixture for DDase assay contained 40 μL of 40 mM maltopentaose, 40 μL of 50 mM acetate buffer (pH 4.5), and 40 μL of DDase solution. The reaction mixture was incubated at 37℃ for an appropriate period and a 15-μL aliquot of the mixture was withdrawn to assay the enzyme activity. One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the production of 1.0 μg dextran/min under the experimental conditions. The amount of product dextran was analyzed by using a HPLC equipment with LC-10AD pump, RID-6A refractive index detector, and C-R7A integrator (all Shimadzu Co.) under the following conditions: column, Aminex HPX-42A (7.8×300 mm, Bio-Rad Laboratories); column temperature, 80℃; mobile phase, distilled water at the flow rate of 0.7 mL/min.

Total sugar content was determined by the phenol-sulfuric acid method[9] using D-glucose as a standard. The total number of reducing ends in the product dextran was determined by the Park-Johnson method modified by Hizukuri *et al.*[10] Protein content was measured by the method of Lowry *et al.*[11] Native- or SDS-PAGE was done in a 5-20% gradient of acrylamide (Pagel NPG-520L, Atto Co.) with Tris-glycine buffer (pH 6.8) according to the method of Davis[12] or Laemmli,[13] respectively. In both cases, the gel plates were stained with a Silver Stain II Kit (Wako Pure Chemical Industries, Ltd.),[14,15] and the molecular mass was estimated by comparison with the molecular markers (Bio-Rad Laboratories). Carbohydrate was stained by periodic acid-Schiff staining (PAS).[16]

The effects of pH and temperature on the activity of the purified DDase were studied under the standard assay conditions using 50 mM acetate buffer (pH 3.5-6.0) and at different reaction temperatures. The effects of pH and temperature on the stability of the purified enzyme were also studied. Each DDase solution was individually adjusted to pH values from 3.5 to 6.0 by dialysis at 4℃ overnight, and then diluted with 50 mM acetate buffer (pH 4.5). Each sample was examined for remaining DDase activity by the standard assay. The enzyme was incubated at 30℃ for 10 min with each 1 mM compound (except PCMB, 0.2 mM), and the remaining enzyme activity was examined by the standard assay.

^{1}H-NMR data were obtained at 30℃ in 3 mm sample tubes by a Fourier transform NMR spectrometer (JEOL, Model JNM-EX 500). Chemical shifts are expressed in δ downfield shift from 3-(trimethylsilyl)-sodium propionate as an external standard in D_2O.

RESULTS AND DISCUSSION

The DDase activity, turbidity, pH, and total sugar content of each culture broth were measured. The courses of DDase production by *Acetobacter capsulatum* ATCC 11894 are summarized in Fig. 1. The enzyme activity and total sugar contents in the supernatant of the dialyzed cell-free culture broth were separately measured under the given conditions. It was found that both DDase and dextran presented in the culture broth did not precipitate after centrifugation up to 6000×*g* for 20 min, but almost all of the dextran-DDase complexes in culture broth were able to precipitate at 20,000 ×*g* for 20 min. As shown in Fig. 2, the DDase activity curve coincided well with the total sugar content curve. This result indicates that the enzyme is tightly bound to formed polysac-

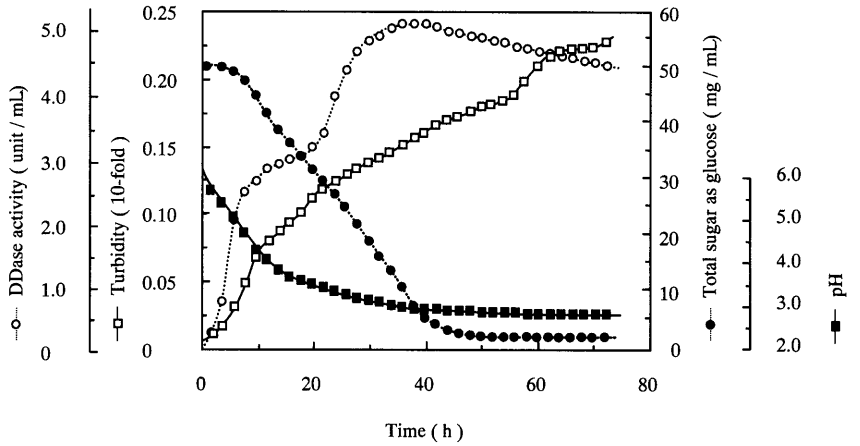

Fig. 1. Courses of cell growth of *Acetobacter capsulatum* ATCC 11894 and dextrin dextranase production.

○, DDase activity (U/mL); □, turbidity (10-fold); ●, total sugar as glucose (mg/mL); ■, pH. The experimental details are given in the text.

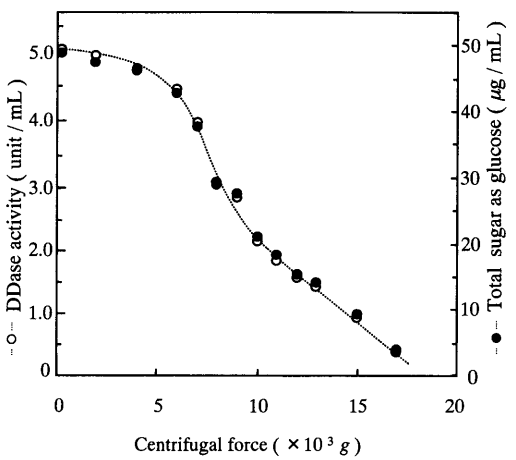

Fig. 2. Profiles of DDase activity and the product dextran during refrigerated centrifugation.

○, DDase activity (U/mL); ●, total sugar as glucose (μg/mL).

charides (dextran). Therefore, it was concluded that the cell-free culture broth should be centrifuged at least at 20,000×*g* for 20 min to recover the enzyme activity effectively. The active pellet was dissolved in 50 mM acetate buffer (pH 4.5), and the resulting suspension was dialyzed against the same buffer for 3 days to obtain the purified enzyme. In this stage, the enzyme was found to be an absolutely homogeneous protein judging from both Native- and SDS-PAGE. The purified enzyme solution gave an intense opaque color due to the dextran in the culture medium (8.4 mg/mg protein, calculated from data in Table 1). The amount of dextran bound to enzyme protein gave no influence on enzyme assay.

To determine the accurate protein contents in the purified enzyme solution, the accompanying dextran should be removed as much as possible. Therefore, the purified enzyme preparation was kept and autolyzed at 15℃ for 3 days.

Table 1. Recoveries and activities of the enzyme during purification.

Procedure	Volume (mL)	Total sugar (g)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
(1) 1st Centrifugation (2000×*g*, supernatant)	3600	30.8	18357	43.1	425.9	1.00	100
(2) 2nd Centrifugation (20000×*g*, pellet)	117	0.468	14409	20.5	702.9	1.65	78.5
(3) Dialysis	125	0.173	14409	20.5	702.9	1.65	78.5

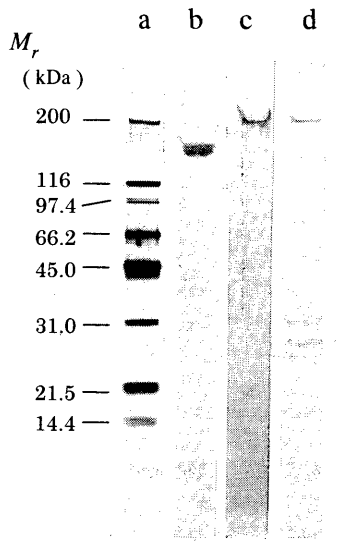

Fig. 3. Native- and SDS-PAGE of the purified DDase.

Lane a, marker proteins; lane b, purified enzyme on SDS-PAGE; lane c, purified enzyme on Native-PAGE; lane d, purified enzyme on Native-PAGE and PAS staining. The molecular markers used were as follows: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa).

Table 1 summarizes the simple purification steps of DDase. This purification procedure gave a high level of enzyme activity yield. In this method, we did not use any $(NH_4)_2SO_4$ or ethanol to precipitate the enzyme. Therefore, we can reuse the culture medium as a recycler, if necessary. The molecular mass of DDase was estimated to be about 152 kDa by SDS-PAGE, based on the mobilities of the standard proteins. The molecular mass of our extracellular enzyme is just the half size compared to that of the intracellular enzyme.[5] As can be seen in Fig. 3, the mobility of both protein (lane c) and carbohydrate (lane d) coincided well with each other.

The optimum pH and temperature of the purified DDase were 5.2 and 38℃, respectively. The enzyme was retained its original activity up to 45℃, and was stable in the range of pH 4.1-5.4 at 4℃ for 24 h. The purified DDase was completely inactivated by heating at 60℃ for 10 min. These properties of the extracellular

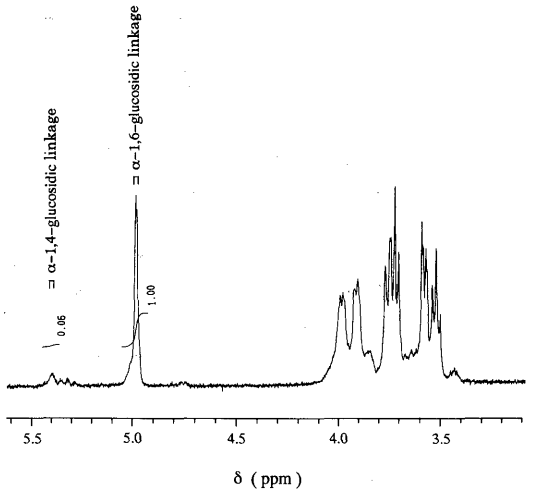

Fig. 4. ^{1}H-NMR spectrum of the product dextran (D_2O, 30℃, 500 MHz).

DDase for pH and temperature were identical with the reported intracellular DDase.[5] The enzyme was inactivated completely with 1 mM of Hg^{2+}, Pb^{2+} or $KMnO_4$, however 1 mM of Zn^{2+}, Cd^{2+} or Cu^{2+} partly inhibited enzyme activity. Other metal ions tested had no inhibitory effect on the activity of the enzyme.

Figure 4 shows the ^{1}H-NMR spectrum of the product dextran. In this case, the glycosidic protons signals in the region of δ 4.98-5.39 were more clearly differentiated. The prominent peak at δ 4.98 (doublet, $J_{1,2}$ 3.05 Hz) was attributed to glycosidic protons involved in the α-1,6 linkage and the minor ones at 5.39-5.30 to that in the α-1,4 linkage, based on earlier data. These relative intensities should reflect the ratio of α-1,4 to α-1,6 linkages. The ratio of α-1,4 to α-1,6 was therefore calculated to be about 1 : 20. The average molecular mass of the product dextran was estimated to be about 1270 kDa by determining the total number of reducing terminals in the polysaccharides (10 mg/mL).

The general properties of our purified extracellular DDase resembled well those of the intracellular enzyme from *A. capsulatus* ATCC 11894 reported previously,[5,6] but the molecular masses of the enzymes were clearly different from each other. We also estimated the molecular mass of the product dextran to be about

1270 kDa. We will present the chemical structures of oligosaccharides produced from maltooligosaccharides by transglucosylation and also its action mechanism in our next paper.

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Acetobacter capsulatum ATCC 11894 株起源の菌体外デキストリンデキストラナーゼの簡易精製法と基本性質

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酢酸菌 *Acetobacter capsulatum* ATCC 11894 株は，グルコースと微量のデキストリンを炭素源として液体培養すると，菌体外にグルコシル基転移酵素デキストリンデキストラナーゼ (EC 2.4.1.2) を産生することが知られている．当該酵素は，各種クロマトグラフィーの操作を経ることなく，高速冷却遠心分離 (4℃, 20,000×*g*, 20 分間) のみにて，簡易かつ高純度に精製された．精製酵素の分子量は SDS-PAGE 法から，約 152 kDa と推定された．また，当該酵素は，pH 5.2 および 38℃において最大活性を示した．精製酵素を各種 pH で 4℃, 24 時間処理すると，pH 4.1-5.4 の範囲で 100%の残存活性が認められた．本酵素活性は，1 mM の Hg^{2+}, Pb^{2+} あるいは $KMnO_4$ により完全に阻害された．当該酵素により，マルトース以外の一連のマルトオリゴ糖から生成される α-1,6-グルカンは，^{1}H-NMR 分析の結果から，わずかに α-1,4-グルコシド結合の分枝を有することが示唆された．生成 α-1,6-グルカンの分子量は，およそ 1270 kDa と推定された．