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Extracellular and cell-associated forms of *Gluconobacter oxydans* dextran dextrinase change their localization depending on the cell growth

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ABSTRACT

Gluconobacter oxydans ATCC 11894 produces dextran dextrinase (DDase, EC 2.4.1.2), which synthesizes dextran from the starch hydrolysate, dextrin and is known to cause ropy beer. *G. oxydans* ATCC 11894 was believed to possess both a secreted DDase (DD_{ext}) and an intracellular DDase (DD_{int}), expressed upon cultivation with dextrin and glucose, respectively. However, genomic Southern blot, peptide mass fingerprinting and reaction product-pattern analyses revealed that both DD_{ext} and DD_{int} were identical. The activity in the cell suspension and its liberation from the spheroplast cells indicated that DD_{int} was localized on the cell surface. The localization of DDase was altered during the culture depending on the growth phase. During the early growth stage, DDase was exclusively liberated into the medium (DD_{ext}), and the cell-associated form (DD_{int}) appeared after depletion of glucose from the medium.

1. Introduction

Dextran is a bacterial homopolysaccharide of glucose composed of an α-(1 → 6)-linked main chain with branches [1–3]. Dextransucrase (EC 2.4.1.5) from *Leuconostoc mesenteroides* is the most well-known dextran-synthesizing enzyme. It produces dextran for use in the pharmaceutical and biochemical fields as a plasma volume expander and a carrier for column chromatography [3]. While dextransucrase synthesizes dextran from sucrose [4], dextran dextrinase (DDase) uses dextrin (maltooligosaccharides) as a substrate and catalyzes a successive α-(1 → 6)-glucosyl transfer reaction to produce dextran [5,6]. Particular strains of *Gluconobacter oxydans* are known to produce DDase [7–9]. *G. oxydans* ATCC 11894, isolated as a bacterium causing ropy beer [10], secreted extracellular DDase (DD_{ext}) by cultivation in the presence of dextrin [5,11]. This strain also produced intracellular DDase (DD_{int}) in the presence of glucose. DD_{int} was extracted from the cells with *n*-butanol [8]. Both DD_{ext} and DD_{int} synthesized dextran from maltooligosaccharides. They catalyzed α-(1 → 6)-glucosyl transfer from maltooligosaccharides [5,8,12] and isomaltooligosaccharides [6,13]. In addition, DD_{int} catalyzed an α-(1 → 4)-transfer from maltooligosaccharides [6], whereas DD_{ext} did not [13]. For the glucosyl transfer from maltooligosaccharides, the acceptor for DD_{ext} was limited to saccharides having α-(1 → 4/6)-linkages at the non-reducing termini [13], but DD_{int} transferred glucosyl residues to various saccharides such as glucose and oligosaccharides containing a glucosyl group [14]. The relationship between DD_{ext} and DD_{int} has been examined to some extent [15], but it remains unclear.

In this paper, we clarify that DD_{int} and DD_{ext} are identical with respect to the reactions they catalyze and their protein sequences. Furthermore, we also describe that DD_{int} is present on the cell surface and that its unique alteration of localization occurs during fermentation.

2. Materials and methods

2.1. Fermentation of G. oxydans and fractionation

G. oxydans ATCC 11894 was cultured according to the method of DD_{int} production [8] in a 500-mL baffled flask with 100 mL PYG medium at 30 °C for 21 h until the optical density at 600 nm (OD) reached 0.63 (i.e., 63-OD units). During cultivation, the concentration of glucose was monitored with the Glucose C-II-Test Wako (Wako Pure Chemical Industries, Osaka, Japan). Culture supernatant containing DD_{ext} was collected by centrifugation at 2000×*g* at 4 °C for 20 min and concentrated to 1.7 mL using a Vivaspin 20 (MWCO 10,000; Sartorius Stedim Biotech, Goettingen,

Abbreviations: B4, 6^{III}-*O*-α-D-glucosyl-maltotriose; B5, 6^{IV}-*O*-α-D-glucosyl-maltotetraose; DDase, dextran dextrinase; DD_{ext}, extracellular DDase; DD_{int}, intracellular DDase; rDD_{ext}, recombinant DD_{ext}; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; OD_{600}, optical density at 600 nm.

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Germany). The precipitated cells were washed with 10 mM sodium acetate buffer (pH 4.8) twice to remove DD_{ext} and were divided in half (31-OD units of cells for each). One part was suspended in 350 μL buffer and subjected to *n*-butanol extraction as previously described [8,16]. The aqueous layer was obtained as the *n*-butanol extract (containing cell-surface DDase). The other half was suspended in 350 μL of 50 mM HEPES buffer (pH 7.0), and disrupted by sonication. The supernatant prepared by centrifugation was regarded as the cell-free extract (containing cellular DDase). The cell debris was sonicated again to obtain the second sonicated extraction in a similar fashion as for the treatment of the first extract.

2.2. Preparation of recombinant DD_{ext}

The expression plasmid for recombinant DD_{ext} (rDD_{ext}) was constructed as described elsewhere [17] using pCold I DNA (Takara Bio, Otsu, Japan) and the gene coding DD_{ext} (GenBank: LC008541, location in 4725-8579). *Escherichia coli* BL21(DE3)-CodonPlus-RIL (Agilent Technologies, Santa Clara, CA, USA) was used as a host. The production was induced with 0.5 mM isopropyl β-D-thiogalactoside (Wako) for 22 h at 15 °C. The cells were sonicated in 50 mM HEPES buffer (pH 7.0). The N-terminal His-tagged rDD_{ext} was purified by chromatography using a Ni-chelating Sepharose column (GE Healthcare, Uppsala, Sweden). rDD_{ext} was dialyzed against 20 mM sodium acetate buffer (pH 5.3), and concentrated by ultrafiltration using a Vivaspin 20 (MWCO 10,000). Protein concentrations were determined from amino acid analysis of the hydrolysates. Southern blotting analysis was done using the DNA fragment corresponding to the N-terminal region of DDase [18].

2.3. Electrophoresis analyses

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) [19] and native PAGE [20,21] were performed using a 6% separation gel. Protein bands were visualized by Rapid CBB KANTO (Kanto Chemical, Tokyo, Japan). For activity staining, the gel after native PAGE was equilibrated with 0.1 M sodium acetate buffer (pH 4.8), and kept on the 1.5% agar plate containing a 50 mM maltohexaose/maltoheptaose mixture (Nihon Shokuhin Kako, Tokyo, Japan) in the same buffer (pH 4.8) at 37 °C for 30 min. The gel was rinsed, and the formed polysaccharides were visualized by periodic acid-Schiff staining (Schiff's Reagent Solution; Nacalai Tesque, Kyoto, Japan) [22].

2.4. Confirmation of dextran formation from maltooligosaccharides

Enzymes were reacted with 50 mM maltotetraose (G4; Nihon Shokuhin Kako) in 250 mM sodium acetate buffer (pH 4.8) at 37 °C for 13 h, and inactivated by heating at 100 °C for 5 min. Maltooligosaccharides were digested by β-amylase from soy bean (1.7 mg/mL; Nagase ChemteX Corporation, Osaka, Japan) in 250 mM sodium acetate buffer (pH 5.4) at 37 °C for 1 h. After heating at 100 °C for 3 min, carbohydrates were precipitated by incubation in 40% methanol at 4 °C for 40 min. The precipitate was collected by centrifugation at 13,000×*g* at 4 °C for 10 min, dried, and dissolved in water. The carbohydrates were incubated with dextranase L (Amano Enzyme) in 250 mM sodium acetate buffer (pH 4.5) at 37 °C for 30 min. The products were analyzed by thin-layer chromatography (TLC) as described elsewhere [23].

2.5. Analysis of the initial reaction products from G4

The culture supernatant and the *n*-butanol extract at 22- and 37-h cultivation, respectively, were prepared as described in Section 2.11. The crude enzyme samples (derived from culture containing cells at 0.054-OD units; 40 μL) were reacted in 80 μL with 15 mM G4 in 250 mM sodium acetate buffer (pH 4.8) at 35 °C. Aliquots (15 μL) were taken at the indicated times, and heated at 100 °C for 3 min. The samples were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a CarboPac PA1 column (4 × 250 mm; Dionex, Sunnyvale, CA, USA). An isocratic mobile phase with 640 mM sodium hydroxide and a flow rate of 0.8 mL/min were applied.

2.6. Preparation of 6^{III}-O-α-D-glucosyl-maltotriose (B4) and 6^{IV}-O-α-D-glucosyl-maltotetraose (B5)

B4 and B5 were produced by the α-(1 → 6)-transglucosylation of α-glucosidase from *Aspergillus niger* (Transglucosidase L; Amano Enzyme) as previously reported [24]. G4 (100 mM) was reacted with the α-glucosidase in 25 mM sodium acetate buffer (pH 4.2) at 37 °C for 30 min, followed by digestion with β-amylase as described in Section 2.4.

2.7. Analysis of acceptor specificity

The culture supernatant (equivalent to a culture containing 0.038-OD units of cells; 1 μL) and the *n*-butanol extract (from 0.090-OD units; 1 μL), prepared in Section 2.1, were incubated in a volume of 4 μL with 2.5 mg/mL amylose EX-I (Hayashibara, Okayama, Japan; donor substrate) and 50 mM acceptor candidates, trehalose (Nacalai Tesque), kojibiose (Wako), nigerose (Wako), maltose (Nihon Shokuhin Kako), isomaltose (Tokyo Chemical Industry, Tokyo, Japan), sucrose (Nacalai Tesque), and cellobiose (Nacalai Tesque), in 25 mM sodium acetate buffer (pH 4.8) at 37 °C for 18 h. Reaction products were analyzed by TLC as described in Section 2.4.

2.8. Peptide mass fingerprinting (PMF) analysis

From the culture supernatant and *n*-butanol extract (Section 2.1), DD_{ext} and DD_{int} were separated by SDS–PAGE, respectively, and subjected to in-gel digestion with trypsin [25]. Peptides obtained were purified using a C18 Zip-Tip (Millipore, Billerica, MA, USA), and analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) using a Voyager DE-STR (Applied Biosystems, Framingham, MA, USA) in the peptide sensitivity reflector mode. α-Cyano-4-hydroxycinnamic acid (Sigma–Aldrich, St. Louis, MO, USA) was used as the matrix. The masses collected from the samples were assigned to the known mass of peptides deduced from the DD_{ext} sequence (PeptideMass tool, http://web.expasy.org/peptide_mass).

2.9. Activity in n-butanol extract and cell suspension

The cells (69-OD units) after 61-h cultivation were washed twice with 10 mM sodium acetate buffer (pH 4.8), and divided in half to prepare the *n*-butanol extract and cell suspension (each made up to 300 μL containing 10 mM sodium acetate buffer, pH 4.8). A portion of each sample (from 0.058-OD units of cells; 5 μL) was incubated in 20 μL with 15 mM G4 and 250 mM sodium acetate buffer (pH 4.8) at 35 °C for 2 h. Aliquots were taken at 0, 10, 30, 60, and 120 min, and carbohydrates were analyzed by TLC as described in Section 2.4.

2.10. Spheroplasting

Spheroplasts were prepared according to a previous report [26]. Briefly, cells (35-OD units) harvested after 26-h of cultivation were incubated with 0.4 mg/mL lysozyme (Nacalai Tesque) in 4 mL of

0.1 M Tris–HCl (pH 8.0) containing 0.5 M sodium chloride at 30 °C for 1 h with gentle shaking. The spheroplast formation was verified by the cell lysis under hypotonic conditions shown as a decrease in OD_{600}. The supernatant (the outer membrane/periplasmic fraction) and the precipitate (spheroplast fraction) were separated by centrifugation.

2.11. Change of DDase localization during cultivation

Cultivation (250 mL) of *G. oxydans* cells was done as described in Section 2.1. Aliquots of 40 mL were taken to prepare the culture supernatant and the *n*-butanol extract. For the SDS–PAGE analysis, samples from 5-OD unit equivalents were used. The protein samples containing DD_{ext} from the culture supernatant were prepared by the precipitation method with centrifugation (17,800×*g*, 4 °C, 40 min) after concentration using an ultrafiltration apparatus (Vivaspin 20) [11]. No DD_{ext} was remaining in the supernatant as verified by SDS–PAGE analysis of precipitates with acetone. For the activity measurement, the samples were incubated with 15 mM G4 for 10, 20, and 30 min, and the produced maltopentaose (G5) was quantified by HPAEC-PAD (as described in Section 2.5). G5 (5–100 μM) was used as the standard for quantification. Sorbitol (100 μM) was used as an internal standard. One unit was defined as the amount of enzyme that catalyzes the formation of 1 μmol G5 per min.

3. Results

3.1. Two DDases produced by G. oxydans

G. oxydans ATCC 11894 cells were cultured for 21 h at 30 °C under the conditions for the DD_{int} production [8], using 5 g/L glucose as the sole carbon source. The OD reached 0.63, and the pH and glucose concentration of the culture medium decreased from pH 7.0 to pH 3.7 and 6.8 mg/L, respectively. The culture supernatant and the cells were separated and then both cell-free and *n*-butanol extracts were prepared. By SDS–PAGE (Fig. 1A), a protein band migrating at 180 kDa was found in all three samples and identified as rDD_{ext}. The intensity of this protein was high in the *n*-butanol extract and low in the cell-free extract. Native-PAGE followed by protein staining and DDase-activity staining was done (Fig. 1B). A single band with the same migration as rDD_{ext} was observed by activity staining for all three samples. The amount of DDase in the *n*-butanol extract was comparable with that in the total extract judging from their protein band intensities (Fig. 1A and B; DDase-activity seemed to partly decrease upon *n*-butanol-treatment). The lower recovery of DD_{int} from the second sonic extraction compared with other proteins suggests that DD_{int} is easily extractable, particularly with *n*-butanol.

Polysaccharides produced from 50 mM G4 by DDases contained in the culture supernatant and the *n*-butanol extract were confirmed. The polysaccharides were resistant to β-amylase, but susceptible to dextranase, generating isomaltose (Fig. 1C). Hence, both polysaccharides were dextran, and the enzymes in the two fractions possessed DDase activities. DDase in the *n*-butanol extract is DD_{int} because it was prepared reported [8], and the other released from cells is DD_{ext}.

3.2. Reactions catalyzed by DD_{ext} and DD_{int}

Initial reaction products of DD_{ext} and DD_{int} from G4 were analyzed by HPAEC-PAD (Fig. 2A). G3 and G5, judging from their retention times, were increased as the reactions proceeded. The α-(1 → 6)-glucosyl transfer product, B5, was not detected in either sample. Therefore, DD_{ext} and DD_{int} catalyzed an α-(1 → 4)-glucosyl transfer on G4 during the initial stage of reaction, with no evidence of the ability to catalyze an α-(1 → 6)-glucosyl transfer reaction.

The acceptor specificity of two DDases was analyzed by TLC (Fig. 2B). Seven disaccharides served as acceptor substrates in the absence or presence of amylose (average degree of polymerization was 18; donor substrate). Both DDases catalyzed the same product pattern. In the presence of amylose, glucosyl transfer products were detected when kojibiose, nigerose, isomaltose, and cellobiose were employed as acceptor substrates. Maltose acted as both donor and acceptor in the glucosyl transfer reaction, and therefore DDase generated glucose, G3, and panose from maltose. Both enzymes catalyzed glucosyl-transfer reactions with broad acceptor specificity toward disaccharides. The products catalyzed by DD_{ext} and DD_{int} were indistinguishable from each other.

3.3. DD_{ext} and DD_{int} are structurally identical

PMF was employed to investigate the protein identity. The two DDases were extracted from the SDS–PAGE gel, and the tryptic peptides prepared were analyzed by MALDI-TOF. The detected masses from both samples were almost identical (Fig. 3). Besides 6 peptide masses derived from trypsin, 5 peptide masses expected from the theoretical protein sequence of DD_{ext} were detected in

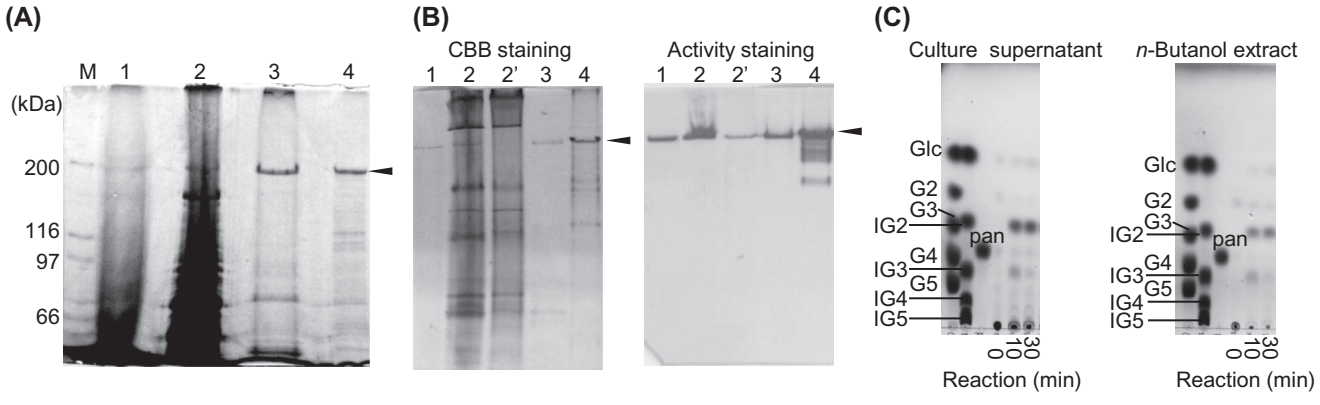

Fig. 1. Electrophoresis analyses of *G. oxydans* DDases and TLC analyses of the products of DDase catalysis. (A) SDS–PAGE. Lane 1, culture supernatant; lane 2, cell-free extract; lane 3, *n*-butanol extract (each extract from 18-OD unit cells, and culture supernatant from culture medium containing 7.6-OD unit cells); lane 4, rDD_{ext} (5 μg); M, size makers. The gel was stained with CBB. The rDD_{ext} is indicated by an arrow. (B) Native-PAGE. The samples are the same as in (A), but the extracts from 0.90-OD unit cells, and the supernatant from 0.76-OD unit cells-containing culture medium. Lane 2′, the second sonication extract. The gels were stained with CBB (left), and using DDase activity (right) (see Section 2.3). (C) TLC analysis. Products of DDase in culture supernatant (left) and in *n*-butanol extract (right) were digested by dextranase (Section 2.4). Glc, glucose; G2–G5, maltose to maltopentaose; IG2–IG5, isomaltose to isomaltopentaose; pan, panose.

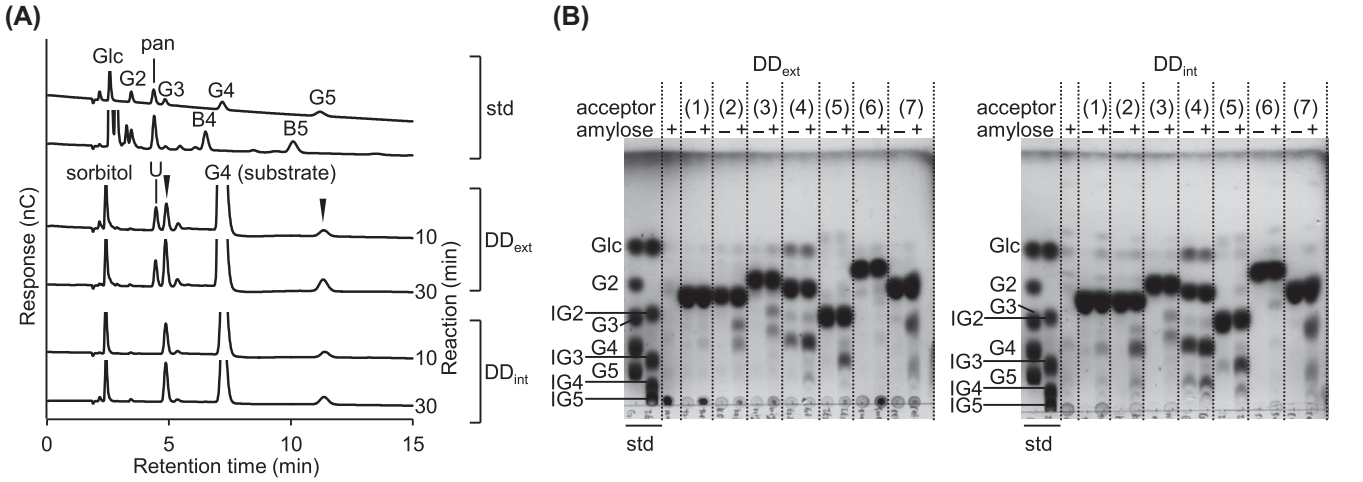

Fig. 2. Product analyses of DD_{ext} and DD_{int}. (A) HPAEC-PAD analysis of the reaction products from 15 mM G4 (Section 2.5). Products (G3 and G5) are indicated by triangles. U, unknown compounds from medium. (B) TLC analysis of the transfer products of DD_{ext} (left) and DD_{int} (right) from 50 mM acceptor candidate (1, trehalose; 2, kojibiose; 3, nigerose; 4, maltose; 5, isomaltose; 6, sucrose; 7, cellobiose) with 0 and 2.5 mg/mL amylose (− and +, respectively; donor substrate) (Section 2.7).

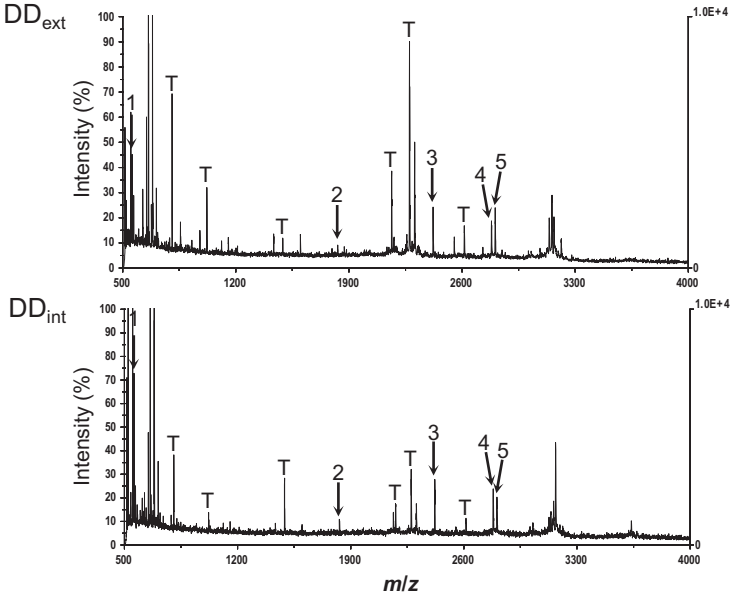

Fig. 3. PMF of DD_{ext} and DD_{int}. Tryptic peptide masses were measured by MALDI-TOF. Arrow and the letter "T" indicate the peptide fragments derived from DDase and trypsin, respectively.

Table 1
Tryptic peptide masses derived from DD_{ext} and DD_{int}.

Calculated mass[a] (Da)	Position[a]	DD_{ext} (Da)	DD_{int} (Da)	Peptide No.[b]
559.3351	539–542	559.4808	559.5446	1
1829.8704	214–229	1829.9489	1830.0437	2
2419.1888	543–565	2419.2071	2419.3752	3
2780.2944	620–643	2780.3132	2780.4611	4
2803.4009	326–352	2803.3502	2803.6153	5

[a] The deduced amino-acid sequence of the DD_{ext} gene.
[b] The peptide fragment number is indicated by arrows in Fig. 3.

both samples (Fig. 3 and Table 1). These results indicate that DD_{ext} and DD_{int} share the same protein sequence, and our Southern hybridization analysis demonstrates that DDase is coded by a single gene.

3.4. Localization of DD_{int}

The subcellular localization of DD_{int} was investigated. First the *n*-butanol extract and cell suspension, prepared from the same amount of cells, were separately incubated with G4 and the products were analyzed by TLC (Fig. 4A). In both reactions, disproportionation of maltooligosaccharides proceeded to almost the same degree for 2 h, with similar product patterns, suggesting that almost all *n*-butanol-extractable DD_{int} was located on the surface of the cells. We also generated spheroplasting *G. oxydans* cells by lysozyme treatment to obtain protein fractions from the outer membrane/periplasmic space and the spheroplast. SDS–PAGE analysis clearly indicated that DDase was predominantly present in the outer membrane/periplasmic fraction (Fig. 4B). This supported the idea that DD_{int} is localized on the surface in the outer-membrane/periplasmic space, and a negligible amount of DD_{int} occurs in the cytoplasm.

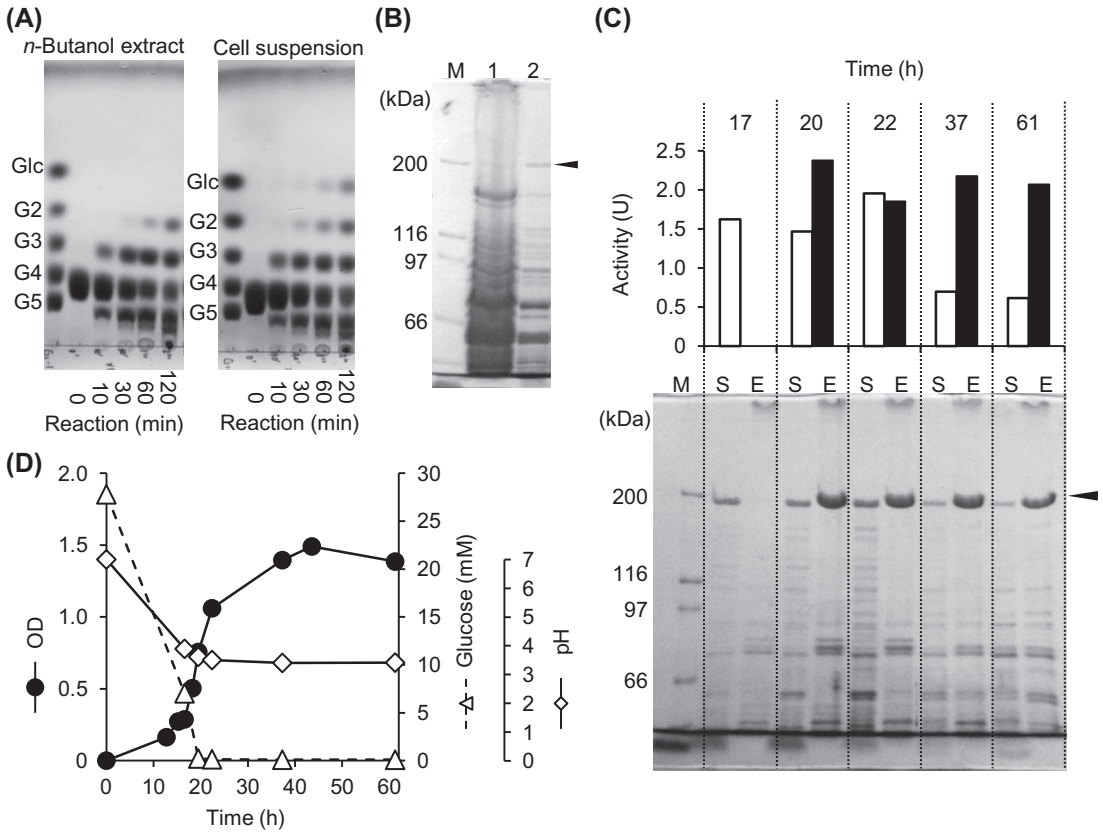

Fig. 4. Localization of DDase and its occurrence during cultivation. (A) TLC-comparison of product formation from 15 mM G4 of *n*-butanol extract (left) and cell suspension (right) (Section 2.9). (B) SDS–PAGE of proteins from spheroplasted cells (lane 1) and outer membrane/periplasmic fractions (lane 2) (Section 2.10). (C) Change of activity and protein amount of DDase in the culture supernatant (S) and *n*-butanol extract (E). The activities from culture containing 5-OD unit cells are shown. The protein was analyzed by SDS–PAGE. (D) Growth curve of *G. oxydans* in the glucose-containing medium. OD (●), pH (◇), and glucose (△) were monitored.

3.5. Change of DD_{int} and DD_{ext} expression during the cultivation

G. oxydans was cultured in the presence of glucose, and the presence of DD_{int} and DD_{ext} was investigated using activity measurements and SDS–PAGE (Fig. 4C). A total of 19 h after the start of the culture, glucose was depleted and the pH values decreased to 3.4 (Fig. 4D). During the early logarithmic growth phase (17 h), when 7 mM glucose still remained in the medium, only DD_{ext} was detected (Fig. 4C). At 20 h when glucose was depleted and the pH had dropped to 3.6, DD_{ext} still remained in the supernatant accompanied by the appearance of DD_{int}. At longer cultivation times, the amount of DD_{int} was not significantly changed, while DD_{ext} gradually decreased. Consequently, the expression of DDase was drastically influenced by cell-growth with only DD_{ext} evident at the early stage and predominantly DD_{int} present at the later stage.

4. Discussion

G. oxydans ATCC 11894 has been reported to produce DDase both extracellularly and intracellularly depending on whether dextrin or glucose is supplied in the culture medium, and therefore DD_{ext} and DD_{int} have been regarded as different enzymes [8,11]. However, our PAGE and PMF results (Figs. 1 and 3) clearly demonstrate that the two enzymes are identical. Previously, the reactions catalyzed by the two DDases were reported to be different: only DD_{int} assisted the α-(1 → 4)-glucosyl transfer on maltooligosaccharides with broad acceptor specificity [6,13,14]. However, we found that DD_{ext} also predominantly the catalyzed α-(1 → 4)-transfer on G4 during the initial stage of the reaction (Fig. 2A), and acted on various disaccharides with the same broad acceptor specificity as DD_{int} (Fig. 2B). Consequently, with respect to enzymatic properties, the two enzymes appear to be identical. Although DDase synthesizes dextran with α-(1 → 6)-glucan linkages, evidence for α-(1 → 4)-transfer is unclear. Analysis of this phenomenon is currently in progress.

Although DD_{int} has been regarded as an intracellular enzyme, our study provides evidence for its cell surface localization. Gram-negative bacteria including *G. oxydans* have three layers in their envelope: the outer membrane, the peptidoglycan cell wall, and the cytoplasmic or inner membrane [27]. The removal of the peptidoglycan cell wall by spheroplasting revealed that DD_{int} occurred in the outer membrane/periplasmic spaces, but not in the cytoplasmic space (Fig. 4B). The outer membrane harbors lipoproteins and β-barrel proteins [27], and is covered with lipopolysaccharides [28]. The biomaterials associated with the outer membrane are extracted with *n*-butanol [29–31]. Therefore, in addition to the results from the spheroplasting fractionation, the efficient extraction of DD_{int} with *n*-butanol also supported that DD_{int} was localized to the cell surface.

The mechanism of association of DDase with the outer membrane remains to be determined, but we observed fluctuation of the localization of the two states (cell surface-bound and free forms) of DDase during cell growth (Fig. 4C and D). Only DD_{ext} was found in the early phase, whereas DD_{int} appeared during the middle stage and later. The reasons behind the changes in alteration of the protein localization are unknown, but the extent of cell growth, the glucose concentration, and the medium pH may be possible contributing factors (Fig. 4D). Previously, DD_{ext} was produced by cultivation in a dextrin-containing medium [5,11], whereas DD_{int} was only generated in a glucose medium [8].

Therefore, dextrin may be responsible for liberation of DDase from cells. Naessens et al. provided evidence that addition of maltodextrin to the medium decreased DD_{int} and increased DD_{ext} compared with the maltodextrin-free fermentation [15], suggesting that maltodextrin and its related carbohydrates, for example products of DDase catalysis, are the DDase-releasing factors. In addition, maltodextrin may function as an inducer of the DDase expression in *G. oxydans*. DDase production in culture containing 63-OD unit cells was 180 μg for DD_{ext} [11], and 40 μg for DD_{int} [8]. In the work reported herein, approximately 40 μg of DDase was obtained (estimated from the band intensity from SDS–PAGE) from the equivalent amount of cells (Fig. 1A). The previous work [15] also clearly demonstrated that the production yield of total DDase increased several times in the presence of maltodextrin. Therefore, maltodextrin or its related compounds are possible inducers of DDase expression in addition to the releasing factors from the cell surface. Glucose is also a releasing factor candidate because DD_{int} is not observed in the early stage (where glucose is still present), meaning that the entire amount of DD_{int} is released from cells by glucose (Fig. 4C and D). After the depletion of glucose in the medium, rapid accumulation of DD_{int} was observed. Therefore, the change of the localization and amount of DDase during the fermentation in the present study may be explained as follows: (1) glucose is a possible releasing factor of DDase from cells; (2) after depletion of glucose, the liberation was terminated, and the produced DDase was accumulated on the cell surface; (3) the DDase in the medium was gradually decreased by possible protease catalyzed degradation. Further investigation into the mechanism of the DDase liberation is currently underway in our laboratory.

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References

[1] J.F. Robyt, Mechanisms in the glucansucrase synthesis of polysaccharides and oligosaccharides from sucrose, Adv. Carbohydr. Chem. Biochem. 51 (1995) 133–168.
[2] K. Yamamoto, K. Yoshikawa, S. Okada, Structure of dextran synthesized by dextrin dextranase from *Acetobacter capsulatus* ATCC 11894, Biosci. Biotechnol. Biochem. 57 (1993) 1450–1453.
[3] M. Naessens, A. Cerdobbel, W. Soetaert, et al., *Leuconostoc* dextransucrase and dextran: production, properties and applications, J. Chem. Technol. Biotechnol. 80 (2005) 845–860.
[4] E.J. Hehre, Production from sucrose of a serologically reactive polysaccharide by a sterile bacterial extract, Science 93 (1941) 237–238.
[5] E.J. Hehre, The biological synthesis of dextran from dextrins, J. Biol. Chem. 192 (1951) 161–174.
[6] K. Yamamoto, K. Yoshikawa, S. Okada, Detailed action mechanism of dextrin dextranase from *Acetobacter capsulatus* ATCC 11894, Biosci. Biotechnol. Biochem. 57 (1993) 47–50.
[7] E.J. Hehre, D.M. Hamilton, Bacterial conversion of dextrin into a polysaccharide with the serological properties of dextran, Proc. Soc. Exp. Biol. Med. 71 (1949) 336–339.
[8] K. Yamamoto, K. Yoshikawa, S. Kitahata, et al., Purification and some properties of dextrin dextranase from *Acetobacter capsulatus* ATCC 11894, Biosci. Biotechnol. Biochem. 56 (1992) 169–173.
[9] X. Mao, S. Wang, F. Kan, et al., A novel dextran dextrinase from *Gluconobacter oxydans* DSM-2003: purification and properties, Appl. Biochem. Biotechnol. 168 (2012) 1256–1264.
[10] J.L. Shimwell, A study of ropiness in beer. The predisposition of beer to ropiness, J. Inst. Brew. 53 (1947) 280–294.
[11] M. Suzuki, T. Unno, G. Okada, Simple purification and characterization of an extracellular dextrin dextranase from *Acetobacter capsulatum* ATCC 11894, J. Appl. Glycosci. 46 (1999) 469–473.
[12] M. Suzuki, T. Unno, G. Okada, A kinetic study of an extracellular dextrin dextranase from *Acetobacter capsulatum* ATCC 11894, J. Appl. Glycosci. 47 (2000) 27–33.
[13] M. Suzuki, T. Unno, G. Okada, Functional characteristics of a bacterial dextrin dextranase from *Acetobacter capsulatum* ATCC 11894, J. Appl. Glycosci. 48 (2001) 143–151.
[14] K. Yamamoto, K. Yoshikawa, S. Okada, Substrate specificity of dextrin dextranase from *Acetobacter capsulatus* ATCC 11894, Biosci. Biotechnol. Biochem. 58 (1994) 330–333.
[15] M. Naessens, A. Cerdobbel, W. Soetaert, et al., Dextran dextrinase and dextran of *Gluconobacter oxydans*, J. Ind. Microbiol. Biotechnol. 32 (2005) 323–334.
[16] R.K. Morton, Separation and purification of enzymes associated with insoluble particles, Nature 166 (1950) 1092–1095.
[17] M. Okuyama, M. Kitamura, H. Hondoh, et al., Catalytic mechanism of retaining alpha-galactosidase belonging to glycoside hydrolase family 97, J. Mol. Biol. 392 (2009) 1232–1241.
[18] J. Sambrook, D.W. Russell, Molecular Cloning: A Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press, New York, 2001.
[19] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[20] L. Ornstein, Disc electrophoresis – I. Background and theory, Ann. N. Y. Acad. Sci. 121 (1964) 321–403.
[21] B.J. Davis, Disc electrophoresis – II. Method and application to human serum proteins, Ann. N. Y. Acad. Sci. 121 (1964) 404–427.
[22] R.M. Zacharius, T.E. Zell, J.H. Morrison, et al., Glycoprotein staining following electrophoresis on acrylamide gels, Anal. Biochem. 30 (1969) 148–152.
[23] L. Ngiwsara, G. Iwai, T. Tagami, et al., Amino acids in conserved region II are crucial to substrate specificity, reaction velocity, and regioselectivity in the transglucosylation of honeybee GH-13 α-glucosidases, Biosci. Biotechnol. Biochem. 76 (2012) 1967–1974.
[24] J.H. Pazur, A. Cepure, S. Okada, et al., Comparison of the action of glucoamylase and glucosyltransferase on D-glucose, maltose, and malto-oligosaccharides, Carbohydr. Res. 58 (1977) 193–202.
[25] A. Shevchenko, H. Tomas, J. Havlis, et al., In-gel digestion for mass spectrometric characterization of proteins and proteomes, Nat. Protoc. 1 (2006) 2856–2860.
[26] K. Matsushita, E. Shinagawa, O. Adachi, et al., Spheroplast of acetic acid bacteria, Agric. Biol. Chem. 45 (1981) 1515–1518.
[27] T.J. Silhavy, D. Kahne, S. Walker, The bacterial cell envelope, Cold Spring Harb. Perspect. Biol. (2010) a000414.
[28] Y. Kamio, H. Nikaido, Outer membrane of *Salmonella typhimurium*: accessibility of phospholipid head groups to phospholipase c and cyanogen bromide activated dextran in the external medium, Biochemistry 15 (1976) 2561–2570.
[29] R.K. Morton, Methods of extraction of enzymes from animal tissues, Methods Enzymol. 1 (1955) 25–51.
[30] K.B. Gondolf, S.R. Batsford, A. Vogt, Isolation of an outer membrane protein complex from *Borrelia burgdorferi* by *n*-butanol extraction and high-performance ion-exchange chromatography, J. Chromatogr. A 521 (1990) 325–334.
[31] D.C. Morrison, L. Leive, Fractions of lipopolysaccharide from *Escherichia coli* O111:B4 prepared by two extraction procedures, J. Biol. Chem. 250 (1975) 2911–2919.