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Characterization of *Leuconostoc mesenteroides* B-742CB Dextransucrase Expressed in *Escherichia coli*

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Abstract Recombinant *E. coli* DH5α harboring a dextransucrase gene (*dsrB742*) produced an extracellular dextransucrase in a 2% sucrose medium. The enzyme was purified by DEAE-Sepharose and Phenyl-Sepharose column chromatographies upto a 142.97-fold purification with a 11.11% recovery to near homogeneity. The enzyme had a calculated molecular mass of 168.6 kDa, which was in good agreement with the activity band of 170 kDa on a nondenaturing SDS-PAGE. An expression plasmid was constructed by inserting the *dsrB742* into a pRSET expression vector. The activity after expression in *E. coli* BL21(DE3)pLysS increased about 6.7-fold compared to the extracellular dextransucrase from *L. mesenteroides* B-742CB. The expressed and purified enzyme from the clone showed similar biochemical properties (acceptor reaction, size of active dextransucrase, optimum pH, and temperature) to B-742CB dextransucrase, however, the ability to synthesize α-(1→3) branching decreased in comparison to that of *L. mesenteroides* B-742CB dextransucrase.

Key words: Dextransucrase, *Leuconostoc mesenteroides*, expression, clone, dextran, acceptor reaction

Dextransucrase (EC 2.4.1.5) synthesizes glucans from sucrose. Glucans are mainly composed of chains of D-glucose units linked by α-(1→6) linkages with varying amounts and arrangements of branch linkages, such as α-(1→2), α-(1→3), and α-(1→4), to the main chain depending on the kind of dextransucrase [33]. Dextransucrases are extracellular glucansucrases that are produced by various species of *Leuconostoc* and *Streptococcus*. Different kinds of glucansucrases are produced by different strains of species of *Leuconostoc* or *Streptococcus* that synthesize glucans with different structures and properties [13]. The *Leuconostoc* species require sucrose in the culture medium as an inducer for the elaboration of glucansucrases, whereas the glucansucrases of the *Streptococcus* species are constitutive [20]. One exception are the constitutive mutants produced from *L. mesenteroides* NRRL B-742 [22, 32], B-1355 [20], B-1299 [23], and B-512FM [15, 16, 20, 21] by Kim and Robyt. These mutants produce dextransucrases in a glucose or fructose medium as the carbon source instead of sucrose.

It has been previously reported that *L. mesenteroides* B-742 produces two kinds of dextransucrases with sucrose. One enzyme synthesizes a dextran containing α-(1→4) branch linkages and the other synthesizes a dextran with a very high degree of single α-(1→3) branched glucose residues [22, 33]. From *L. mesenteroides* B-742, Kim and Robyt isolated a dextransucrase constitutive mutant, *L. mesenteroides* B-742C (B-742C). After further mutation of B-742C, two different constitutive mutants, *L. mesenteroides* B-742CA (B-742CA) and *L. mesenteroides* B-742CB (B-742CB), were isolated [22]. B-742CB produces extracellular dextransucrases on 2% glucose with higher activities than those produced by B-742 produced on a 2% sucrose medium. Kim *et al.* [19] cloned and sequenced a dextransucrase gene (*dsrB*742; GenBank Acession No. AF294469) that expresses a dextransucrase synthesizing mostly α-(1→6) linked dextran with a low amount of α-(1→3) branching from *L. mesenteroides* B-742CB. The nucleotide sequence of *dsrB*742 shows one open reading frame (ORF) composed of 4,524 bp encoding the dextransucrase, while crude intracellular dextransucrase shows an activity band of 184 kDa on a nondenaturing SDS-PAGE. The amino acid sequence of *L. mesenteroides* B-742CB dextransucrase (DSRB742) shows 50% similarity with DSRA from *L. mesenteroides* B-1299 [28], 70% similarity with FMCMDS from *L. mesenteroides* B-512 [35]

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and 45–56% similarity with *Streptococcal* glucosyltransferases (GTFs) [3, 6, 7, 29].

B-742CB, grown on sucrose, produces a dextransucrase that synthesizes both soluble and insoluble dextrans. It would thus appear that the two glucans (soluble and insoluble) synthesized by B-742CB dextransucrase have only a slight susceptibility toward endodextranase and are identical in their structures [22]. This enzyme can modify cellulose or synthesize glucooligosaccharides by successive transfers of glucosyl units from sucrose to the nonreducing end of acceptors such as maltose, isomaltose, oligosaccharides, and cellulose [17, 18].

This paper describes the purification of the dextransucrase expressed from the *L. mesenteroides* B-742CB gene cloned in *E. coli*, and its characterization was compared with that of the parental enzyme.

MATERIALS AND METHODS

Bacterial Strains, Vectors, and Culture Conditions

L. mesenteroides B-742CB was the source of the *dsrB742* gene. *Escherichia coli* DH5α and a pGEM-3Zf(-) (Promega, U.S.A.) vector were used for all the cloning steps [19]. *E. coli* BL21(DE3)pLysS (Invitrogen, The Netherlands) and a pRSET vector were used for the protein expression. The *L. mesenteroides* B-742CB was grown on an LM medium containing 2% (w/v) glucose or sucrose [14]. For the plasmid isolation and recombinant DNA experiments, *E. coli* was grown in a Luria-Bertani (LB) medium with ampicillin (50 g/ml) at 37°C with vigorous shaking.

DNA Manipulation

Routine DNA manipulation, including plasmid purification and *E. coli* transformation, was performed as described by Maniatis *et al.* [19, 25]. The plasmid DNA was isolated from an overnight culture of *E. coli* using the alkaline lysis method [25]. The genomic DNA of *L. mesenteroides* B-742CB was extracted as previously described [19]. A partial *Pst*I digest of *L. mesenteroides* genomic DNA was ligated to a linearized (with *Pst*I) and dephosphorylated pGEM3Zf(-). The ligated mixture was then transformed into *E. coli* DH5α using the method of Maniatis *et al.* [25].

Expression of Dextransucrase Gene

To express and prepare a dextran-free dextransucrase (DSRB742), the DNA insert (*dsrB742*) of pDSRB742 was ligated into a pRSET vector which was predigested by *Pst*I. The expression plasmid was then transformed into BL21(DE3)pLysS. A fresh single colony grown on LB plates containing 50 μg/ml ampicillin was used to inoculate 5 ml of LB broth containing 50 μg/ml ampicillin. The broth was cultured at 28°C overnight with shaking. These cultures were used to inoculate 5 ml of the same broth at a ratio of 1:100 (v/v), then the broth was incubated upto the early exponential phase ($OD_{600\,nm}$ = 0.2) at 28°C. Next, 0.4 mM IPTG was added and the cells were cultivated for 3 h at 28°C. The cells were then pelleted by centrifugation and suspended in 200 μl of a 20 mM sodium acetate buffer (pH 5.4). The cells were disrupted by sonication. The supernatant was then obtained by centrifugation at 26,000 ×g for 10 min, and used in an SDS-PAGE analysis and activity staining.

Purification of Dextransucrase

The recombinant *E. coli* DH5α was cultivated in an LB medium containing 2% sucrose and 50 μg/ml ampicillin for 27 h at 28°C. The culture supernatant was collected by centrifugation (11,000 ×g, for 15 min) and concentrated with polyethylene glycol (PEG, Mw=1,500, a final concentration of 20%). The extracellular activity was recovered in the dextran-rich phase [36]. To remove the dextran bound to the enzyme, *Penicillium* dextranase (1 unit/ml) was added to the enzyme solution and stirred for 1 h at 24°C. The dextranase-treated enzyme was then dialyzed against 20 mM sodium acetate buffer (pH 5.4) containing 1 mM $CaCl_2$. The dialyzate was loaded onto a DEAE-Sepharose column (2.5×10 cm) equilibrated with the same buffer. The column was washed with 250 ml of the buffer, and the adsorbed protein was eluted with a linear gradient of NaCl (0–0.5 M) in the buffer. The fractions exhibiting dextransucrase activity were pooled and dialyzed against a 20 mM sodium acetate buffer (pH 5.4). The resulting dialyzate was applied to a Phenyl-Sepharose column (2×13 cm) equilibrated with the same buffer. The dextransucrase fraction was eluted with a linear gradient of decreasing ionic strength of sodium acetate buffer (starting from 20 mM to 0 mM). The amount of protein was determined by the Bradford method [5] using bovine serum albumin as the standard.

Electrophoresis

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli [24]. The protein was stained with Coomassie Brilliant Blue R-250. The dextransucrase activities were detected by incubating the gels in 100 mM sucrose overnight followed by staining for polysaccharide using a periodic acid-Schiff procedure [27].

Acceptor Reaction

The oligosaccharide synthesis was carried out at 28°C with 50 mM sucrose, 50 mM maltose, and dextransucrase (0.2 U/ml) from recombinant *E. coli* in a 20 mM sodium acetate buffer (pH 5.2) containing 1 mM $CaCl_2$ and 1 mg/ml Tween 80. The reaction was allowed to continue for 16 h, and then the reaction products were analyzed by TLC with two ascents of 2:5:2 (v/v/v) nitromethane: 1-propanol: water. The carbohydrates were visualized by dipping the plate into 0.3% N-(1-naphthyl)-ethylenediamine and 5% sulfuric acid in methanol and by heating at 120°C for 10 min [37].

Table 1. Activity of dextransucrase produced from working strains.

Strains	Enzyme activity (unit/ml broth)	Ratio of relative activity production
L. mesenteroides B-742CB[a]	0.15 (extracellular)	1
E. coli DH5α[b]	0.0343 (extracellular)	0.23
E. coli BL21(DE3)pLysS[c]	0.75 (soluble fraction of cell lysate) 0.28 (insoluble fraction of cell lysate)	6.7

[a]LM medium containing 2% sucrose was used.
[b]*E. coli* DH5α harboring pDSRB742 was cultivated in LB medium containing 2% sucrose and expressed extracellular dextransucrase.
[c]*E. coli* BL21(DE3)pLysS harboring pDSRB742 produced a dextransucrase by IPTG induction in two fractions of the cell lysate.

Analysis of Dextranase Hydrolyzates of Dextran

The dextran was prepared by the addition of an equal volume of dextransucrase to 100 mM sucrose (pH 5.2). The reaction was allowed to proceed at 28°C until the sucrose had been completely consumed. The digested dextran was precipitated with ethanol (up to 67%, v/v). The precipitates were dried and dissolved in a 20 mM sodium acetate buffer (pH 5.2). The dextrans (1%, w/v) were then incubated with *Penicillium* dextranase (60 U/ml) at 37°C overnight. The reaction digests were analyzed by TLC.

Structure Analysis of Dextran

To analyze the structure of the dextran synthesized by the dextransucrase expressed in *E. coli*, the dextran was methylated using a Hakomori reagent followed by acid hydrolysis with 2M trifluoroacetic acid, and analyzed for methylated dextran using TLC [30]. The methylation products were separated on Whatman K6 plates using 3:9:1 (v/v/v) acetonitrile:chloroform:methanol, as described above [37].

Effects of Temperature and pH

The effects of temperature on the enzyme activity and stability were studied between 15°C and 70°C. The thermostability of the enzyme activity after treating for 10, 20, 30, 40, and 60 min at the designated temperature was determined. After incubation, the enzyme activity was assayed as described above using 100 mM sucrose. The effect of pH on the enzyme activity was determined by varying the buffer pHs between 3.0 and 8.0. Sodium citrate buffer (20 mM) was used for pHs 3.0 to 4.0, sodium acetate buffer for pHs 4.5 to 5.5, and sodium phosphate buffer for pHs 6.0 to 8.0. To determine the pH stability of the dextransucrase, the enzyme was incubated at the indicated pH for 72 h and the residual activity was assayed.

RESULTS AND DISCUSSION

Expression of *dsrB742*

An extracellular dextransucrase from recombinant *E. coli* was induced by the addition of sucrose into the culture medium. To determine the optimum sucrose concentration for enzyme production, recombinant *E. coli* was cultured in LB broth containing various concentrations of sucrose (0%, 1%, 2%, 3%, 4%, and 5%). To prepare a high dextran-free dextransucrase activity from the culture supernatant, the insert DNA (*dsrB742*) was ligated into a pRSET vector, and the expression plasmid was transformed into *E. coli* BL21(DE3)pLysS. *dsrB742* was induced by IPTG (final concentration of 0.4 mM) and the highest activity was obtained 3 h post-induction; thereafter, the activity gradually decreased.

The expression level of the recombinant dextransucrase by the host cell [DH5α, BL21(DE3)pLysS] was compared with the activity from the *L. mesenteroides* B-742CB culture (Table 1). The recombinant *E. coli* DH5α secreted an extracellular dextransucrase in LB broth containing sucrose, however, the activity decreased to 23%. The clone did not produce an extracellular dextransucrase in a culture medium without sucrose. Thus, it would appear that sucrose required to induce dextransucrase. A 6.7-fold increase in activity was obtained from the culture of *E. coli* BL21(DE3)pLysS transformed with a pDSRB742 expression plasmid after IPTG induction.

Purification of Enzyme

The culture supernatant from recombinant *E. coli* DH5α harboring pDSRB742 was concentrated with PEG and treated with *Penicillium* dextranase to remove any dextran bound to the enzyme. The crude enzyme was purified by DEAE-Sepharose and Phenyl-Sepharose chromatographies. As shown in Fig. 1(A), the active fraction was eluted with 0.1 M NaCl on DEAE-Sepharose, and 42.58% of the total dextransucrase activity with a 7.31-fold purification was recovered. After Phenyl-Sepharose [Fig. 1(B)], the enzyme was purified 143-fold to near homogeneity with a specific activity of 12.61 U per mg of protein with a 11.11% yield. The enzyme was completely separated from other proteins, and on SDS-PAGE gel with silver staining, a 170-kDa polypeptide was seen (Fig. 2). The purification procedures and results are summarized in Table 2.

Acceptor Reaction of Cloned DSRB742

In the presence of sucrose and maltose, the enzyme catalyzed the synthesis of oligosaccharide, as shown in Fig. 3. The recombinant enzyme, like the native B-742CB dextransucrase,

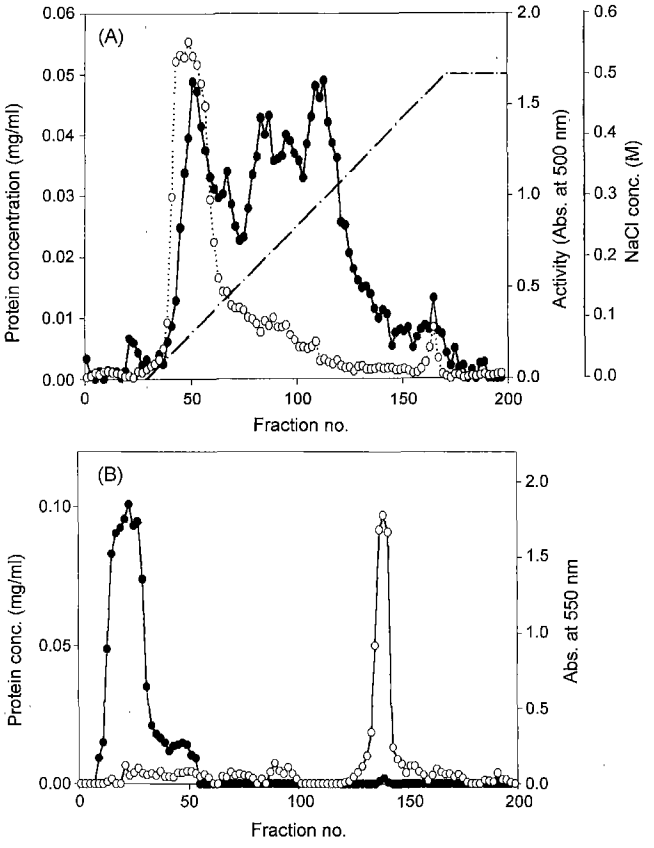

Fig. 1. Chromatographs of dextransucrase from recombinant *E. coli* DH5α harboring pDSRB742.
(A): DEAE-Sepharose column chromatography, (B): Phenyl-Sepharose column chromatography. Each fraction was collected and the protein was measured by the Bradford method (-●-) and tested for dextransucrase activity by under standard assay conditions (-○-).

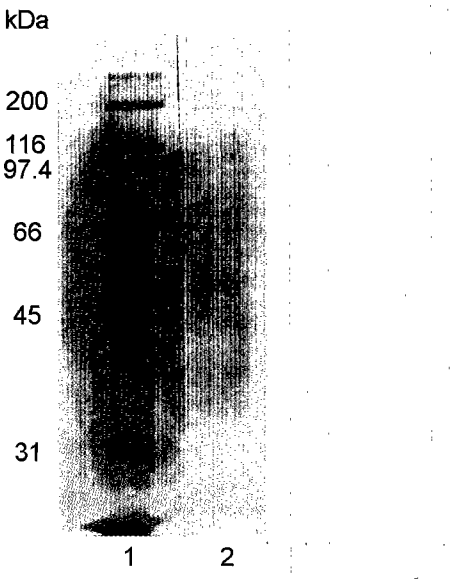

Fig. 2. SDS-PAGE of purified dextransucrase from culture supernatant of recombinant *E. coli* DH5α harboring pDSRB742.
Lane 1, size marker (kDa). Lane 2, purified dextransucrase.

synthesized acceptor products such as panose (6^2-α-D-glucopyranosylmaltose), 6^2-α-isomaltosylmaltose, 6^2-α-isomaltotriosylmaltose, 6^2-α-isomaltotetraosylmaltose, and branched oligosaccharides.

Dextranase Hydrolyzates of Dextran by Cloned DSRB742

The dextran prepared from recombinant *E. coli* harboring pDSRB742 was hydrolyzed with *Penicillium* dextranase and the resulting hydrolyzate was compared with that of B-742CB dextran (Fig. 4). The hydrolyzate was composed of glucose, isomaltose, and branched saccharides, identical to that of B-742CB dextran. B-742CB dextran showed a relatively high resistance to endodextranase hydrolysis. However, DSRB742 dextran was more susceptible to *Penicillium* dextranase than B-742CB dextran. Although *L. mesenteroides* B-742CB and B-1355 formed highly branched dextrans and alternan that were resistant to dextranase hydrolysis, the dextransucrases [expressed from a clone of B-1299; 28] and alternansucrase [expressed from a clone of B-1355; 2] formed mostly α-(1→6) linked dextrans.

There are several enzymes and proteins involved in sucrose metabolism, such as glucosyltransferases (GTFs), which include WSG (water-soluble glucan) synthesase (GTF-S) [10, 11] and WIG (water-insoluble glucan) synthase (GTF-I) [1, 13], glucan-binding proteins (GBP) [26, 34], and dextranase (Dex), responsible for the cleavage of α-(1→6) linkages in glucan molecules [4, 9]. Another protein (Dextran inhibitor, Dei) in oral *Streptococci*, which can inhibit dextranase activity, has been reported [12, 31], and it is also involved in the glucan metabolism. It inhibits endogenous dextranase, which can make more branches in glucan with the cooperation of the glucosyltransferase GTF-I. This inhibition causes the accumulation of water-soluble glucans [36].

Table 2. Summary of purification of dextransucrase from *E. coli* DH5α harboring pDSRB742.

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Recovery (%)	Purification fold
Culture supernatant	373.248	32.928	0.0882	100	-
Concentration with PEG	156.7	27.14	0.1732	82.4	1.96
Treatment with dextranase	111.6014	25.185	0.2256	76.48	2.56
DEAE-Sepharose chromatography	21.244	14.02	0.645	42.58	7.31
Phenyl-Sepharose chromatography	0.29	3.6579	12.61	11.11	142.97

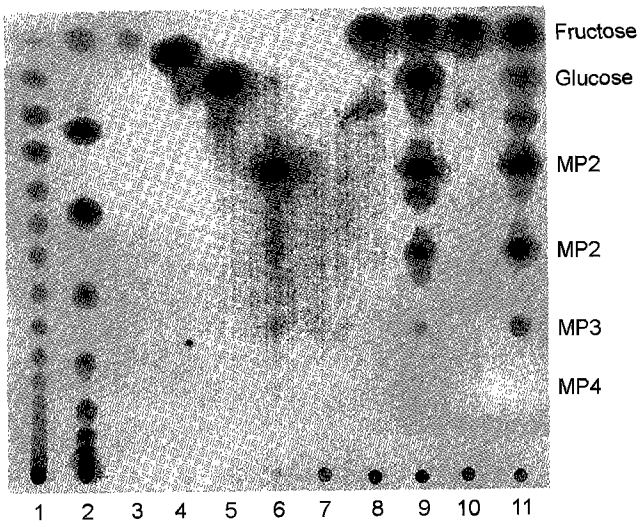

Fig. 3. Thin-layer chromatogram of acceptor reaction products of dextransucrase from recombinant *E. coli* DH5α harboring pDSRB742.

Lane 1: maltodextrin series; Lane 2: isomaltodextrin series; Lane 3: fructose; Lane 4: sucrose; Lane 5: maltose; Lane 6: panose; Lane 7: B-742CB dextransucrase; Lane 8: reaction product of B-742CB dextransucrase with sucrose; Lane 9: maltose acceptor product of B-742CB dextransucrase; Lane 10: reaction product of dextransucrase expressed in *E. coli* with sucrose; Lane 11: maltose acceptor product of dextransucrase expressed in *E. coli*. MP1, Panose; MP2, 6^2-α-isomaltosylmaltose; MP3, 6^2-α-isomaltotriosylmaltose; MP4, 6^2-α-isomaltotetraosylmaltose.

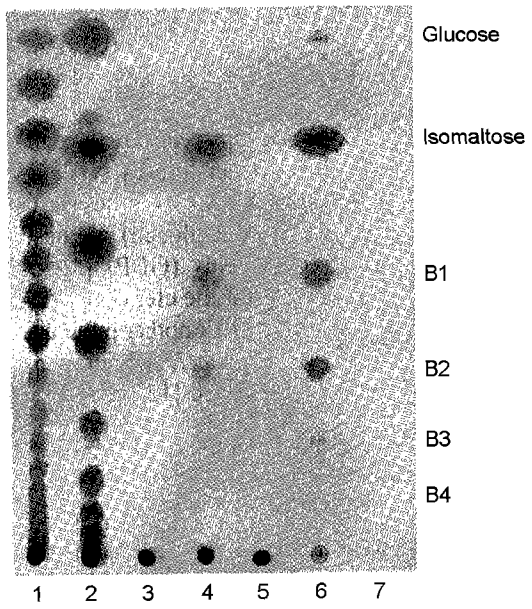

Fig. 4. Thin-layer chromatogram of the dextranase hydrolysis product of dextransucrase from recombinant *E. coli* DH5α harboring pDSRB742.

Lane 1: maltodextrin series; Lane 2: isomaltodextrin series; Lane 3: B-742CB dextran; Lane 4: dextranase hydrolysis product of B-742CB dextran; Lane 5: dextran of dextransucrase expressed in *E. coli*; Lane 6: dextranase hydrolysis product of dextran prepared by dextransucrase expressed in *E. coli*; Lane 7: dextranase. B1-B4, branched hydrolysis products.

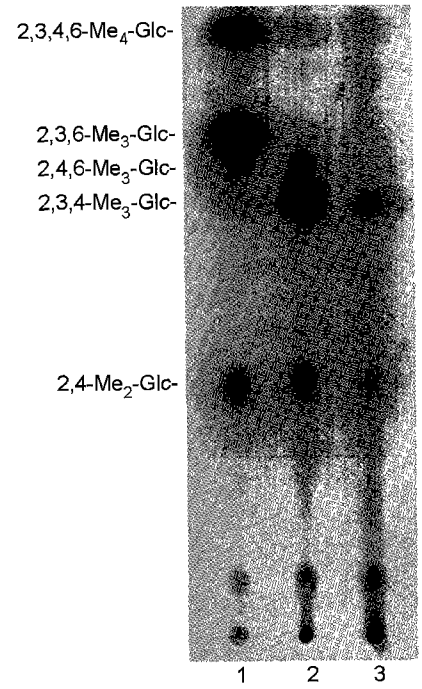

Fig. 5. Methylation of dextran synthesized by dextransucrase from recombinant *E. coli* DH5α harboring pDSRB742.

Lane 1: maltotriose, Lane 2: B-742CB dextran, Lane 3: dextran of dextransucrase expressed in *E. coli*.

Accordingly, there must be other factors which can affect the formation of branched dextrans in addition to the dextransucrase gene [8, 16]. Further studies are currently in progress for the isolation of other genes for the related proteins and to investigate the conditions of dextran synthesis.

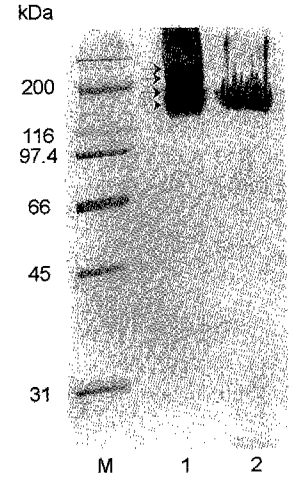

Fig. 6. Activity staining of dextransucrase from recombinant *E. coli* DH5α harboring pDSRB742.

The active band was stained by the Periodic acid-Schiff (PAS) staining procedure. The arrows indicate the four active bands of B-742CB dextransucrase. M: size marker (kDa). Lane 1: PAS staining of B-742CB dextransucrases. Lane 2: PAS staining of dextransucrase from cloned *E. coli*.

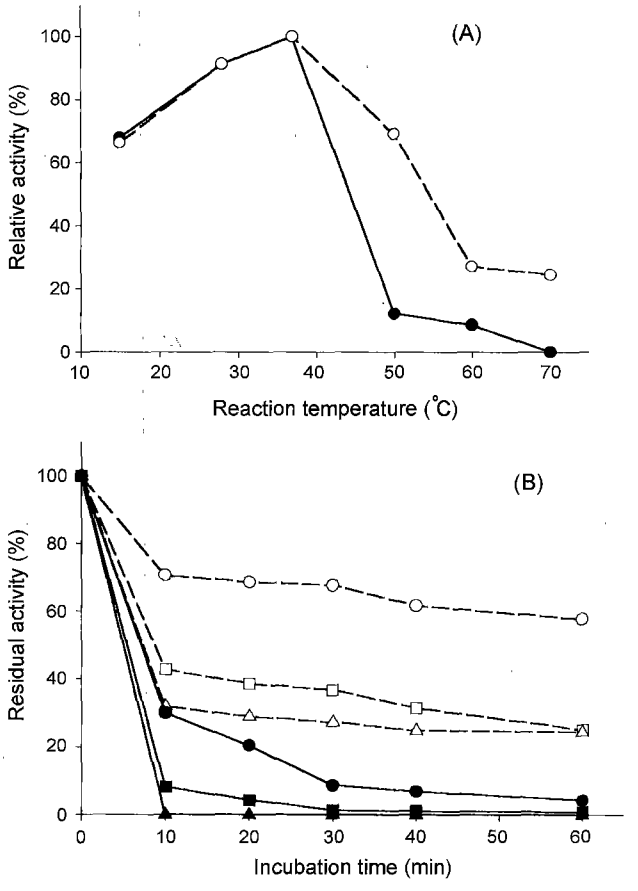

Fig. 7. Optimum temperature (A) and thermostability (B) of dextransucrase from recombinant *E. coli* DH5α harboring pDSRB742.
(A): The closed and open circles indicate the dextransucrase activities of DSRB742 and B-742CB, respectively. (B): Residual activity of dextransucrase from DSRB742: -●- 50°C, -■- 60°C, -▲- 70°C, Residual activity of dextransucrase from B-742CB: -○- 50°C, -□- 60°C, -△- 70°C.

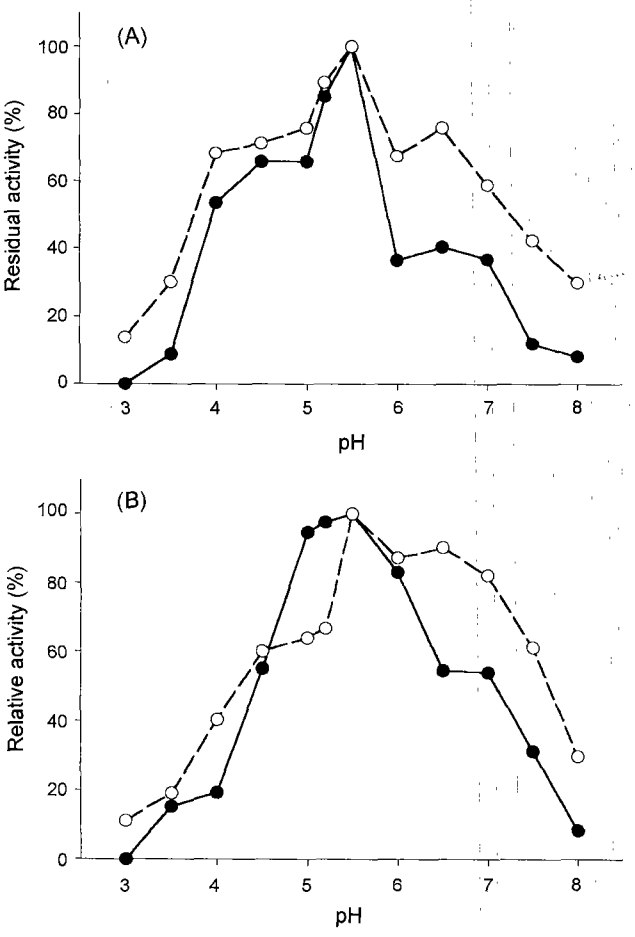

Fig. 8. Optimum pH (A) and pH stability (B) of dextransucrase from recombinant *E. coli* DH5α harboring pDSRB742.
The following buffers were used: sodium citrate (pH 3.0–4.0), sodium acetate (pH 4.5–5.5), sodium phosphate (pH 6.0–8.0). The closed and open circles indicate the dextransucrase activities from DSRB742 and B-742CB, respectively.

Structural Analysis of Dextran

To identify the structure of dextran, methylation and subsequent acid hydrolysis analyses were performed (Fig. 5). DSRB742 dextran showed α-(1→6) and α-(1→3) linkages as evidenced by 2,3,4-tri-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-glucose. The purified dextransucrase from *dsrB742* synthesized more α-(1→3) branch linkages (10%) than crude DSRB742 (3%).

Activity Staining of Dextransucrase

To confirm the active band of the dextransucrase from recombinant *E. coli* harboring pDSRB742, the enzyme was subjected to nondenaturing SDS-PAGE followed by incubation in sucrose solution and PAS staining. As shown in Fig. 6, dextransucrase activity was detected at 170 kDa. This molecular mass corresponded to a 168.6 kDa protein as calculated from the amino acid sequence. There were four active bands detected in the *L. mesenteroides* B-742CB dextransucrase (Fig. 6, lane 1), however, recombinant *E. coli* harboring pDSRB742 only expressed the smallest active band (Fig. 6, lane 2). It has been previously reported that the dextransucrases from the culture supernatants of B-512F, B-1299, B-1355, and B-742 using sucrose consist of more than one activity band [20-23]. Interestingly, there was no difference in the structure of the dextrans by each band of dextransucrase. Also, the dextransucrase prepared using glucose only showed one active band. Since dextransucrase-bound dextran may affect the formation of mutiple dextransucrase forms, further detailed studies of other factors need to be investigated.

Effects of Temperature and pH

The recombinant *E. coli* harboring pDSRB742 showed optimal dextransucrase activity at pH 5.5 and 37°C [Fig. 7(A), Fig. 8(A)], which was identical as for *L. mesenteroides* B-742CB. The enzyme was most active at pH 5.5 and

relatively stable within a range of pHs 5.0–6.0. Over 80% enzyme activity remained at pHs 5.2 and 5.5, whereas the activity decreased rapidly at other pHs [Fig. 7(B)]. The pH stability of the dextransucrase from DSRB742 showed a similar pattern for B-742CB dextransucrase, although B-742CB dextransucrase maintained a higher activity and stability at pH 6.0 and higher [Fig. 8(B)]. The temperature stability of the enzyme from the cloned *E. coli*, when incubated for 60 min at 50°C, 60°C, and 70°C, was 4, 0.8, and 0%, respectively [Fig. 7(B)]. The stability of the dextransucrase from the cloned *E. coli* at 50°C was 40% lower than that of B-742CB. This change in the thermostability may have occurred, since the dextran has been bound the dextransucrase. The dextransucrase produced by B-742CB grown on glucose or sucrose showed the same optimum pH for the activity and stability. However, the optimum temperature for dextransucrase reaction and stability showed a higher temperature stability for the dextransucrase prepared from a sucrose-grown culture than for the dextransucrase from a glucose-grown culture. The IPTG-induced dextransucrase showed lower thermostability than B-742CB grown on sucrose.

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