

## Cloning of Dextranucrase Gene from *Leuconostoc citreum* HJ-P4 and Its High-Level Expression in *E. coli* by Low Temperature Induction

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**A dextranucrase (LcDS) gene from *Leuconostoc citreum* HJ-P4 has been amplified and cloned in *E. coli*. The LcDS gene consists of 4,431 nucleotides encoding 1,477 amino acid residues sharing 63–98% of amino acid sequence identities with other known dextranucrases from *Leuc. mesenteroides*. Interestingly, 0.1 mM of IPTG induction at 15°C remarkably increased the LcDS productivity to 19,187 U/l culture broth, which was over 330-fold higher than that induced at 37°C. Optimal reaction temperature and pH of LcDS were determined as 35°C and pH 5.5 in 20 mM sodium acetate buffer, respectively. Meanwhile, 0.1 mM CaCl<sub>2</sub> increased its activity to the maximum of 686 U/mg, which was 2.1-fold higher than that in the absence of calcium ion. Similar to the native *Leuconostoc* dextranucrase, recombinant LcDS could successfully produce a series of isomaltooligosaccharides from sucrose and maltose, on the basis of its transglycosylation activity.**

**Keywords:** *Leuconostoc citreum* HJ-P4, dextranucrase (DSase), gene cloning, overexpression, low-temperature induction

*Leuconostoc* is one of the major lactic acid bacteria easily found in a variety of traditional fermented foods in the world. Recently, full genome sequences of *Leuc. mesenteroides* subsp. *mesenteroides* ATCC 8293 [11] and *Leuc. citreum* KM20 [5] were determined, and the resulting genetic information has been intensively studied with respect to their physiological functions in common fermented foods [25]. Specifically, *Leuc. citreum* was highly focused as the most prevalent lactic acid bacterium of a Korean traditional fermented vegetable, well-known as *kimchi*. *Leuconostoc* is commonly known to produce extracellular dextranucrases (DSases; E.C. 2.4.1.5), which can transfer the glucose moiety of sucrose to a variety of acceptor molecules by

forming  $\alpha$ -(1,6)-glycosidic linkages in series [30]. Because of its powerful transglycosylation activity, DSase can be applicable to the development of new functional carbohydrate materials, such as dextran polymers, for high value-added food additives or pharmaceuticals [1, 7, 20, 23]. For the past decades, many DSases have been reported from several strains of *Streptococcus* [29] and *Lactobacillus* [8], but mainly from *Leuc. mesenteroides* [3, 15, 16, 31, 34].

DSase expression is commonly induced when *Leuconostoc* strain is grown on sucrose-rich media. However, dextran polymers coproduced during the fermentation process can be associated with the extracellular DSase in the medium, which significantly inhibits the successive enzyme purification procedures and decreases the recovery yield of DSase. To date, a variety of techniques have been tried to solve this problem in DSase production and purification [10]. Kim and Robyt [4] developed the constitutive mutant of *Leuc. mesenteroides* B-1299CB4, producing DSase on glucose medium without sucrose induction. In addition, heterologous expression approaches, as alternative ways, have been tried to produce dextran-free DSases in *Escherichia coli* [3, 15, 18, 31, 34], *Bacillus megaterium* [12], and *Lactococcus lactis* [21]. Nevertheless, the expression level in heterologous systems has not been sufficient yet, due to the high molecular mass of DSases of over 160,000 Da. In order to stimulate the industrial application of DSase, its productivity can be much improved by the advanced genetic engineering or fermentation technology.

In this study, therefore, the DSase (LcDS) gene has been cloned and characterized from *Leuc. citreum* HJ-P4. This high dextran-forming lactic acid bacterium was already isolated from a pickled cucumber fermented at low temperature below 10°C [2] and used for the production of long-chain isomaltooligosaccharides [9] via its high DSase activity. Specifically, the effects of cultivation temperature on both constitutive and inducible expressions in *E. coli* were comparatively examined, which will be applied to the heterologous gene expression of DSase for industrial purposes.

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## MATERIALS AND METHODS

### Bacterial Strains and Culture Media

*Leuc. citreum* HJ-P4 (Korean Agricultural Culture Collection; KACC 91035) was cultivated at 30°C on lactobacilli MRS media (proteose peptone 10 g, beef extract 10 g, yeast extract 5 g, dextrose 20 g, polysorbate 80 1 g, ammonium citrate 2 g, sodium acetate 5 g, magnesium sulfate 0.15 g, dipotassium phosphate 2 g) and MRS media containing 2% sucrose for genomic DNA preparation and LcDS production, respectively. *Escherichia coli* MC1061 was used for DNA manipulation and constitutive expression of the LcDS gene, whereas *E. coli* BL21 Star (DE3) (Novagen, Darmstadt, Germany) was used as a host for inducible expression. The resulting *E. coli* transformants were grown in Luria–Bertani medium (LB; 1% bactotryptone, 0.5% yeast extract, 1% NaCl) containing ampicillin (100 µg/ml) at 37°C. Culture media used here were purchased from Difco Laboratories (Detroit, MI, U.S.A.)

### Gene Cloning and DNA Sequencing

A set of primers, LcDSX2-N (5'-TTTTTCTAGAATGCTGTCTATGACCGCTAC-3') and LcDSX-C (5'-TTTTCTCGAGAGCGACTGAGACAAAGTAAAC-3'), was synthesized by Bioneer Co. (Daejeon, Korea) and used for the PCR amplification of the LcDS gene by using *i*MAX polymerase (Intron Inc., Seongnam, Korea) and a Px2 thermal cycler (Thermo-Hybaidd, Middlesex, U.K.). Amplified DNA fragment was cloned into the pRBC-TA vector (Real Biotech Co., Taipei County, Taiwan), which was designated as pTA-LcDS. The entire nucleotide sequence of the LcDS gene was fully determined by SolGent Co. (Daejeon, Korea) using a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The resulting sequence of LcDS was deposited as GenBank Accession No. AB362781.

### Construction of Expression Vectors

After the treatment of pTA-LcDS with XbaI and XhoI, the fragment of 4.4 kb was ligated with the expression vector pHCXHD [22] modified from pHCEII-NdeI (BioLeaders Co., Daejeon, Korea), and the resulting plasmid was designated as pHCLcDS for the constitutive expression. For the inducible expression, the plasmid pHCLcDS was digested with NdeI and XhoI, and subcloned into pET-21a (Novagen), which was designated as pETLcDS. As a result, the LcDS with C-terminal 6 histidine residues was expressed from *E. coli* harboring each recombinant plasmid.

### Temperature-Dependent Expression of LcDS

Recombinant *E. coli* MC1061 harboring pHCLcDS was cultivated in LB broth containing ampicillin (100 µg/ml) at 37°C for an appropriate time. In order to induce LcDS expression, *E. coli* BL21 Star (DE3) harboring pETLcDS was cultivated at 37°C for 2 h and then final 0.1 mM of IPTG was added to the culture broth. After IPTG addition, the cultivation temperature was changed to 15, 20, 30, and 37°C, respectively. The grown cells were disrupted by sonication (VCX750, Sonics & Materials, Inc., Newtown, CT, U.S.A.) and the LcDS activity was determined at each time point. Finally, the LcDS with C-terminal histidine-tag was simply purified by using a nickel–nitrilotriacetic acid (Ni–NTA; Qiagen, Hilden, Germany) column chromatography.

### Reducing Sugar Assay for LcDS Activity

Purified LcDS was incubated with 150 mM of sucrose in 20 mM sodium-acetate buffer (pH 5.5) in the presence of 0.1 mM CaCl<sub>2</sub> at

35°C for an appropriate time. The 3,5-dinitrosalicylic acid (DNS) reducing sugar assay method [14] was used to determine the quantity of fructose released from sucrose. One unit of LcDS activity is defined as the amount of enzyme producing 1 µmol equivalent of fructose for 1 min.

### Determination of Hydrolysis and Transglycosylation Activities of LcDS

The total reaction rate of LcDS was obtained by determining the increase of fructose, and the rate of sucrose hydrolysis was estimated as the increase of free glucose molecules. Accordingly, the transglycosylation (or polymerization) activity of LcDS was obtained from the difference between total activity and hydrolyzing activity. In this study, the amount of fructose or glucose in the reaction mixtures was simultaneously determined by using the Glucose-Fructose Kit (Roche, Mannheim, Germany) according to the supplier's instruction.

### Analysis of Enzyme Reaction Products

For the transglycosylation reactions, 9.31 units of LcDS was reacted with 300 mM of sucrose (donor) or 60 mM maltose (acceptor) in 20 mM sodium acetate buffer (pH 5.5) with 0.1 mM CaCl<sub>2</sub>. After the reaction at 35°C for 12 h, the reaction products were analyzed by thin layer chromatography (TLC) with the solvents of acetonitrile/water (85:15). Isomaltooligosaccharides with high degree of polymerization were further analyzed with nitromethane/water/1-propanol (2:3:5). The silica gel K5F TLC plate (Whatman, Maidstone, U.K.) was visualized by dipping it into a solution containing 0.3% *N*-(1-naphthyl)-ethylenediamine and 5% H<sub>2</sub>SO<sub>4</sub> in methanol and then heating it for 10 min at 110°C.

## RESULTS AND DISCUSSION

### Molecular Cloning of LcDS Gene

Based on the unique conserved sequences among DSase genes, Koh *et al.* [6] previously developed two PCR primers, mesF (5'-GTAGATGCTGTTGATAACGTT-3') and mesR (5'-TTGCCATGTATTGACCATCA-3'), for the quantitative population analysis of *Leuc. mesenteroides*. The genomic library of *Leuc. citreum* HJ-P4 was prepared by Sau3AI treatment and ligation with pUC18 plasmid vector. Using the combination of mesR with M13 sequencing primer, the N-terminal region of LcDS was successfully amplified by PCR against the genomic library as a template. In the same way, the partial nucleotide sequence for the C-terminal region of LcDS was determined using PCR with mesF and M13 primers. According to the sequence information of both N- and C-terminal ends, the entire LcDS gene was amplified by using genomic DNA template with two specific PCR primers, LcDSX2-N and LcDSX-C. The resulting 4.4 kb of PCR fragment was ligated with a TA-cloning vector (Real Biotech Co.), which was designated as pTA-LcDS.

DNA sequencing analysis revealed that the LcDS gene consists of 4,431 nucleotides encoding 1,477 amino acid residues. The primary structure of LcDS was compared

with the other known DSases. LcDS shares the highest amino acid sequence identity of 97.5% with DSases from *Leuc. mesenteroides* NRRL B-1299 [15]. However, the other known *Leuc. mesenteroides* DSases, such as DsrX [31], DexYG [34], DsrS [16], showed about 63% of identity with LcDS. Meanwhile, less than 46% of sequence identities were found in the comparison with *Lactobacillus* [8] or *Streptococcus* [29] DSases. In addition, LcDS also possesses the well-known primary structures, including conserved regions I–IV, glucan binding domain, and YG-repeat [17, 30], commonly found in various DSases.

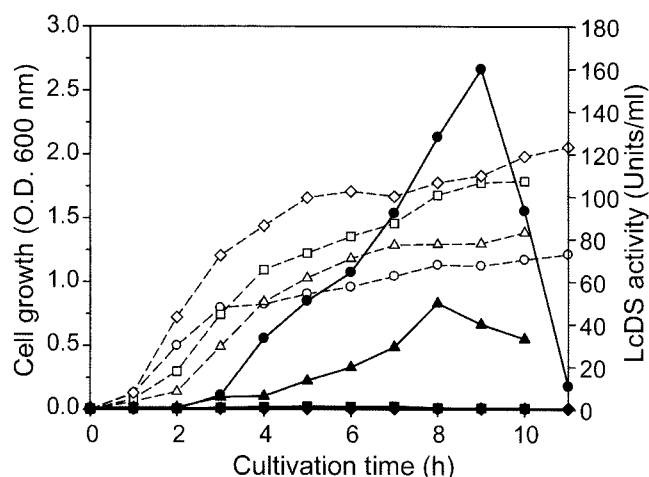
### Heterologous Expression of LcDS in *E. coli*

Two different *E. coli* vectors, pHCXHD and pET-21a, were used for the constitutive and the inducible expressions of LcDS, respectively. The LcDS gene of pTA-LcDS was subcloned into the position between XbaI and XhoI of pHCXHD, which was designated as pHCLcDS. For the construction of inducible pETLcDS, pHCLcDS was cleaved with NdeI and XhoI and the resulting fragment was inserted into pET-21a. *E. coli* harboring pHCLcDS or pETLcDS was expected to produce the recombinant LcDS fused with N-terminal 12 extra amino acids (Met-Glu-Phe-Glu-Leu-Gly-Thr-Arg-Gly-Ser-Ser-Arg) and C-terminal 6 histidines.

After transformation, each recombinant *E. coli* was cultivated at 37°C until its activity reached to the maximum. The LcDS activity increased slowly to the maximal value and then decreased rapidly to the bottom level (data not shown). Based on the activity assay, however, it was verified that the expression level of LcDS from both systems at 37°C was too low to continue the further purification and characterization experiments.

### Effect of Low Induction Temperature on LcDS Expression

Owing to its strong T7 RNA polymerase promoter, pET expression vectors are known to cause insoluble inclusion bodies in *E. coli* cells. Theoretically, the very rapid or high expression of a specific foreign gene can be toxic for the cells, and the alteration of culture conditions may alleviate the codon-usage-based expression problems [28]. Most DSases are very large proteins, which can often create critical problems in their heterologous expression. Yang *et al.* [33] reported that the expression at 25°C gave twice the amount of recombinant gloschedobin expressed at 37°C. Meanwhile, a rice lipoxigenase [26] was successfully expressed by lowering the cultivation temperature from 37 to 15°C. Moulis *et al.* [18] suggested that a lower temperature could slow the DSase expression and facilitate protein folding. They used an arabinose-inducible vector for the expression of *Leuc. mesenteroides* Dsr-S in *E. coli*. As a result, the maximal DSase productivity of 5,850 U/l culture broth was obtained at 23°C by 0.002% of L-arabinose induction.



**Fig. 1.** Effects of induction temperature on the cell growth and LcDS productivity in the inducible *E. coli* expression system.

In order to induce the LcDS expression, final 0.1 mM IPTG was added to the culture broth after 2 h cultivation. At each induction temperature of 15 (circle), 20 (triangle), 30 (square), and 37°C (diamond), the cell growth (open symbol and dashed line) and the LcDS activity (closed symbol and solid line) were monitored for 11 h.

Accordingly, the optimization of induction conditions, such as IPTG concentration and cultivation temperature, was experimentally tried to improve the LcDS productivity. Although there was little effect of inducer concentration on the enzyme productivity, the addition of 0.1 mM IPTG resulted in the highest expression of LcDS. Specifically, lowering the induction temperature to 15°C caused the dramatic increase of LcDS expression level, whereas the cell growth at various temperatures showed no significant differences among them (Fig. 1). The LcDS activity was gradually increased to the peak after 7 h induction at 15°C and decreased rapidly to the bottom. It means that LcDS might be inactivated at the late stage of fermentation. Yalin *et al.* [31] and Zhang *et al.* [34] reported that DsrX and DexYG showed almost the same up and down expression patterns as LcDS.

In the present study, low temperature induction at 15°C showed over 330-fold higher LcDS productivity of 19,187 U/l culture broth than that induced at 37°C (Table 1). When the induction temperature was lowered below 20°C, the LcDS productivity began to increase significantly. At the temperature below 15°C, however, the growth of *E. coli* was too slow to reach the desirable expression level. Even though LcDS was partly expressed as an insoluble form, a considerable portion of recombinant enzyme was successfully produced as a soluble protein, which could be simply purified to homogeneity by using a Ni-NTA column chromatography (Fig. 2). Compared with the previous reports on low temperature induction or expression [26, 27, 32, 33], this remarkable productivity enhancement of DSase can be highly significant and also applicable to the heterologous production of any foreign proteins with high molecular mass.

**Table 1.** Effects of induction temperature on LcDS productivity in constitutive or inducible expression systems.

Expression Type (Vector)	Induction temp. (°C)		LcDS productivity (U/l broth)	Ratio (%)
	Before	After		
Constitutive <sup>a</sup> (pHCLcDS)	37	15	183.5	1.0
	37	37	3.7	0.02
Inducible <sup>b</sup> (pETLcDS)	37	15	19,186.8	100.0
	37	20	7,187.3	37.5
	37	30	176.1	0.9
	37	37	56.2	0.3

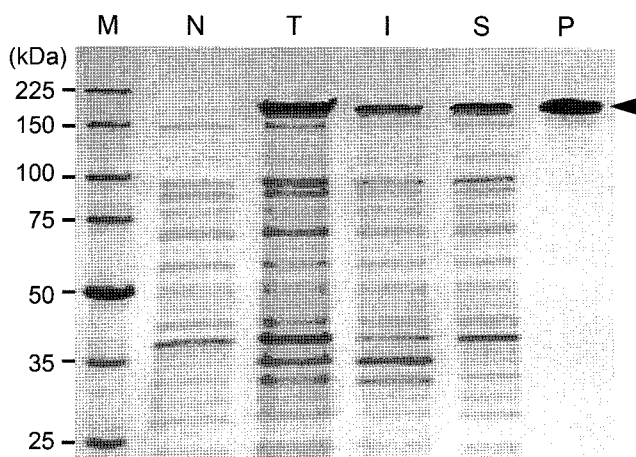
<sup>a</sup>No inducer (IPTG) was added to the culture broth for the constitutive expression.

<sup>b</sup>The cultivation temperature was changed after the induction by adding 0.1 mM IPTG.

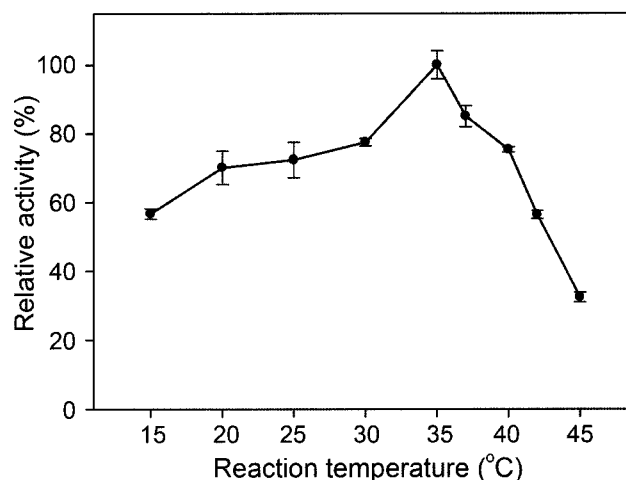
### Enzymatic Characterization of Recombinant LcDS

To determine the optimal reaction conditions of LcDS, the effects of temperature and pH on enzyme activity and stability were examined. *Leuc. mesenteroides* DSases are known to have their optimal reaction temperature and pH ranging 30–35°C and 5.0–6.0, respectively [17, 30]. As shown in Figs. 3 and 4, LcDS showed the highest activity at 35°C in 20 mM sodium acetate buffer (pH 5.5) in the presence of 0.1 mM CaCl<sub>2</sub>. Although the optimal temperature of LcDS is 35°C, its activity decreases rapidly at temperature above 37°C. On the other side, LcDS is very stable at the broad pH range from 4.0 to 8.5 (data not shown).

Recently, Kang *et al.* [3] reported that 1 mM CaCl<sub>2</sub> enhanced the activity of *Leuc. mesenteroides* DSase (DSRBCB4) up to 189%. Interestingly, the activity of DsrX

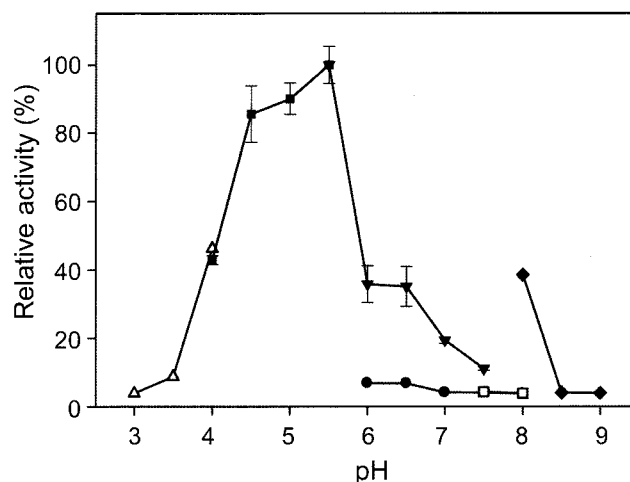
**Fig. 2.** High-level expression and purification of the recombinant LcDS.

Recombinant *E. coli* harboring pETLcDS was cultivated at 15°C after 0.1 mM IPTG induction. The expression level of LcDS was confirmed by SDS-PAGE analysis. Lane M, protein molecular mass markers; lane N, total extract without induction (negative control); lane T, total extract with induction; lane I, insoluble fraction with induction; lane S, soluble fraction with induction; lane P, LcDS purified by a Ni-NTA chromatography.

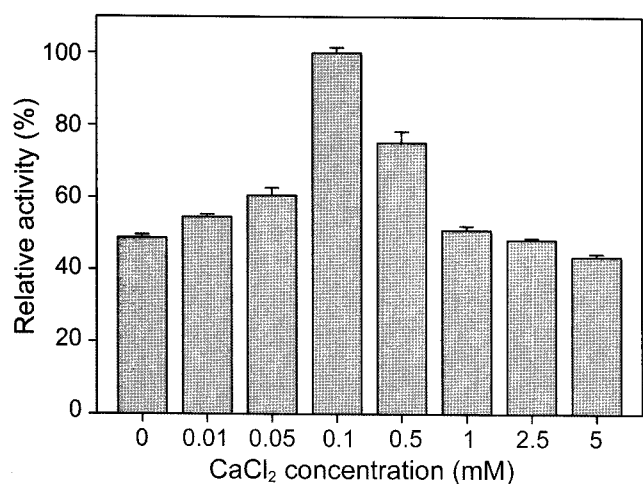
**Fig. 3.** Effects of reaction temperature on LcDS activity.

At various temperatures from 15 to 45°C, the LcDS was reacted with 150 mM sucrose in 20 mM sodium acetate buffer (pH 5.5) for 10 min and the amount of fructose released was determined by DNS reducing sugar assay.

from *Leuc. mesenteroides* was not affected by adding 1 mM CaCl<sub>2</sub> [31]. It was supposed that the effects of calcium ion on the enzyme activity depend on the origin of DSase. In this work, therefore, calcium dependency of LcDS was investigated. LcDS activity was significantly enhanced by adding 0.1 mM CaCl<sub>2</sub>, which was 2.1 times higher than that in the absence of calcium ion (Fig. 5). On the contrary, 1 mM of CaCl<sub>2</sub>, commonly used in the previous reports, could not increase the LcDS activity. According to Miller and Robyt [13], calcium ion was an activator for DSase at low concentration, whereas a weak competitive

**Fig. 4.** Effects of reaction pH on LcDS activity.

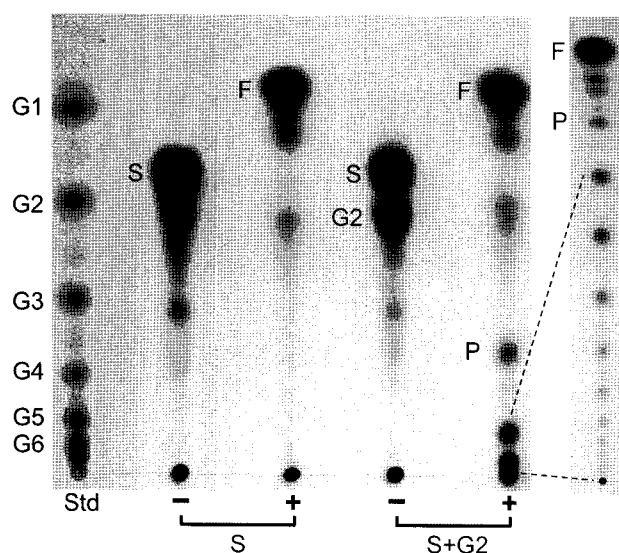
The LcDS was reacted with 150 mM sucrose in various buffers (pH 3 to 9) at 35°C for 10 min and the amount of fructose released was determined by DNS reducing sugar assay. Sodium citrate pH 3.0–4.0 (△); sodium acetate pH 4.0–5.5 (■); sodium phosphate pH 5.5–7.5 (▼); malate pH 6.0–7.5 (●); Tris-HCl pH 7.5–8.0 (□); borate pH 8.0–9.0 (◆).



**Fig. 5.** Effects of CaCl<sub>2</sub> concentration on LcDS activity. LcDS was pre-incubated for 1 h in a solution containing various concentrations of CaCl<sub>2</sub> and then it was reacted with 150 mM sucrose. The amount of fructose released was determined by DNS reducing sugar assay.

inhibitor above 1 mM. Therefore, the calcium effects on DSase activity should be carefully examined and compared against various different concentrations.

Native DSase of *Leuc. citreum* HJ-P4 was known to produce dextran polymers from sucrose. To verify its transglycosylation activity, recombinant LcDS was reacted with sucrose (donor) and maltose (acceptor). As shown in Fig. 6, LcDS produced mainly dextran polymers from only sucrose, whereas synthesized a series of isomaltooligosaccharides



**Fig. 6.** TLC analysis of transglycosylation products catalyzed by LcDS.

For the formation of isomaltooligosaccharides, LcDS was reacted with 300 mM sucrose (S), or 300 mM sucrose and 60 mM maltose (S+G2). The resulting products were separated by the solvents of acetonitrile/water (85:15) for the left panel and nitromethane/water/1-propanol (2:3:5) for the right panel. Std, maltooligosaccharide standard from glucose (G1) to maltohexaose (G6); F, fructose; S, sucrose; P, panose; G2, maltose; (-), without LcDS; (+), with LcDS.

**Table 2.** Effects of calcium chloride concentration on transglycosylation and hydrolysis activities of LcDS.

CaCl <sub>2</sub> (mM)	Activity (U/ml) <sup>a</sup>		Ratio (%) <sup>b</sup>	
	T	H	T	H
0.0	4.83	1.69	74.1	25.9
0.05	5.00	1.62	75.5	24.5
0.1	6.32	3.04	67.5	32.5
0.5	5.57	2.44	69.5	30.5
1.0	5.38	1.49	78.3	21.7

<sup>a</sup>The abbreviations of T and H correspond to the transglycosylation and the hydrolysis activities of LcDS, respectively.

<sup>b</sup>Based on the total activity of LcDS as 100%, T and H correspond to the relative transglycosylation and hydrolysis activities, respectively.

including mainly panose from sucrose with maltose acceptors. Accordingly, recombinant LcDS is likely to be a typical DSase and its enzymatic properties correspond to the native enzyme from *Leuc. citreum* HJ-P4.

Using the Glucose-Fructose Kit (Roche), the ratio of polymerization to hydrolysis of LcDS was compared against various concentrations of CaCl<sub>2</sub> (Table 2). In the absence of calcium ion, recombinant LcDS catalyzed much higher transglycosylation (74%) than hydrolysis reaction (26%). Although LcDS showed the highest total enzyme activity at 0.1 mM CaCl<sub>2</sub>, its ratio of transglycosylation to hydrolysis was changed to 67.5:32.5.

For the past decades, a variety of intensive researches of *Leuconostoc* DSases have been mainly focused on their applications. However, Moulis *et al.* [19] and Robyt *et al.* [24] have recently focused on the understanding of the mechanism in dextran formation by DSase family enzymes.

In this study, the first *Leuc. citreum* DSase (LcDS) gene has been cloned and expressed in *E. coli*, which showed relatively high specific activity of 686 U/mg compared with the others. Moreover, the expression level of LcDS was remarkably enhanced *via* low temperature induction at 15°C, which can be applicable to the heterologous expression of useful foreign genes with high molecular size. Although no three-dimensional structure of any DSase has been available yet, further investigations should be tried to elucidate the mechanism of calcium-dependent DSase activity for hydrolysis and transglycosylation.

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