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Characterization of Dextransucrases from *Leuconostoc mesenteroides* NRRL B-1299

MARGUERITE DOLS,[1] MAGALI REMAUD-SIMEON,[1] RENÉ-MARC WILLEMOT,[1] MICHEL VIGNON,[2] AND PIERRE F. MONSAN*,[1]

[1]*Centre de Bioingénierie Gilbert Durand, UMR CNRS 5504 & Lab. Ass. INRA, I.N.S.A., Complexe Scientifique de Rangueil, 31 077 Toulouse cedex, France;* [2]*CERMAV CNRS, BP 53X, Grenoble, France*

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ABSTRACT

Leuconostoc mesenteroides NRRL B-1299 dextransucrase was fractionated into soluble (SGT) and insoluble (IGT) enzyme preparations differing by their dextran content. In spite of this, they displayed the same Km for sucrose (10 g/L) and the same activation energy (35 kJ/mol). But the presence of cells and insoluble dextran led to the IGT behaving like an immobilized enzyme: stabilization against thermal denaturation and diffusional limitations at low substrate concentrations were observed. On the other hand, the behavior of SGT was influenced by the presence, in the preparation, of soluble dextran that reduced enzyme inhibition by excess substrate. SGT and IGT present very different pH profiles. In the presence of 4 g/L of soluble dextran, IGT was activated and displayed the same susceptibility to pH as SGT. The activation of IGT was highly dependent on the nature of the acceptor added but also on the pH of the reaction medium. IGT and SGT synthesize both soluble and insoluble polymer containing $\alpha(1 \rightarrow 2)$, $\alpha(1 \rightarrow 3)$, and $\alpha(1 \rightarrow 6)$ linkages. A larger amount of insoluble dextran is elaborated by SGT. The polymer structures, examined by ^{13}C NMR spectrometry, revealed that they differ mainly by their $\alpha(1 \rightarrow 3)$ linkage content (from 0 to 11%). This linkage seems to be partly responsible for the dextran insolubility and can be

*Author to whom all correspondence and reprint requests should be addressed. E-mail: monsan@insatlse.insa-tlse.fr

completely eliminated by carrying out the synthesis of soluble polymer at pH 7.4 with SGT.

Index Entries: Dextransucrase; glucosyltransferase; dextran; pH; *Leuconostoc mesenteroides*.

INTRODUCTION

Dextransucrase is a glucosyltransferase (E.C. 2.4.1.5) that catalyzes the synthesis of dextran from sucrose *(1)*. Dextran polymer is composed of α-D-glucopyranosyl residues linked through α(1 → 6) linkages in the linear chain and α(1 → 2), α(1 → 3), and/or α(1 → 4) linkages at branching points *(2)*. The structure of the dextran produced is highly dependent on the glucosyltransferase producing strain of bacteria considered *(3)* and, to a lesser extent, on the degree of purification of the enzyme *(4)*.

Dextransucrase catalyzes the transfer of glucopyranosyl residues in two different ways. The glucosyl moiety coming from sucrose is first transferred to a covalent glucosyl-enzyme intermediate *(2,5–7)*. In the absence of added acceptor, polymer synthesis occurs by a single-chain reaction *(2,6)* at the reducing end of the molecule *(5,8)*. Dextransucrases do not need any dextran primer *(9–11)*. In the presence of an acceptor, the enzyme transfers the glucosyl residue at the nonreducing end of the added molecule *(12,13)*. The product of the acceptor reaction with fructose can be either sucrose-isotope exchange-or leucrose *(14,15)*. Leucrose is not an acceptor for dextransucrase and the reaction stops. But with some other sugars, like maltose or isomaltose, the acceptor reaction product can serve as an acceptor substrate for subsequent steps *(16,17)*. Chain growth occurs by a series of single glucosyl transfers (multichain reaction). To a lesser extent, the enzyme also transfers glucosyl residues onto water molecules and the result is sucrose hydrolysis *(6)*.

All these reactions compete for the glucosyl enzyme intermediate. The addition of a good acceptor like maltose accelerates the reaction *(16)*. Dextran can also act as an acceptor *(13)*; the result is the addition of a glucosyl residue at the nonreducing end of the dextran molecule *(18)* or the formation of a branch linkage *(19)*. In most cases, added dextran accelerates the overall reaction rate. This has been attributed to acceptor reactions, but also to an effect of dextran on the enzyme conformation *(9,10)*. The effectiveness of each type of dextran as an acceptor is highly dependent on the nature of the dextran, on the degree of purification of the enzyme, and on the enzyme/dextran concentration ratio *(20,21)*. *Leuconostoc mesenteroides* dextransucrases are induced by sucrose *(22)* and dextran is always produced together with the enzyme during culture. Therefore, dextransucrase preparations always contain dextran, which may affect the enzyme properties. Carbohydrate-free dextransucrase can be obtained only after numerous purification steps. The recent isolation of constitutive mutants

of *L. mesenteroides (23,24)* may help in the study of dextran-free enzymes. However, the low production levels limit their use.

L. mesenteroides NRRL B-1299 dextransucrase produces a highly α(1 → 2) branched dextran that contains from 27 to 35% α(1 → 2) branch linkages as well as a limited amount of α(1 → 3) branch linkages *(25–28)*. The major part of the dextransucrase activity produced by *L. mesenteroides* NRRL B-1299 is mainly insoluble *(29,30)*, but both soluble and insoluble dextransucrases produce gluco-oligosaccharides that contain an α(1 → 2) linkage *(31)*. This linkage makes these oligosaccharides resistant to hydrolysis by digestive enzymes and opens a wide field of applications in health and nutrition *(32,33)*. These molecules are now produced on the industrial scale *(34)*. Optimization of the enzymatic reaction is thus of interest. Here, is presented the characterization of the two enzyme preparations obtained after a very simple fractionation scheme with efficient recovery of enzyme activity.

MATERIALS AND METHODS

Dextransucrase Production

L. mesenteroides NRRL B-1299 was supplied by the NRRL (Peoria USA). The cells were stored in 18% w/v glycerol at –18°C. The standard medium had the following composition: 40 g/L sucrose, 20 g/L yeast extract (Biomérieux), 20 g/L K_2HPO_4, 0.2 g/L $MgSO_4$ 7 H_2O, 0.01 g/L $MnSO_4$ H_2O, 0.01 g/L NaCl, 0.02 g/L $CaCl_2$, and 0.01 g/L $FeSO_4$ 7 H_2O. The phosphate buffer pH was adjusted to 6.9 with orthophosphoric acid. Sucrose, yeast extract, phosphate, and salts were sterilized separately. Cultures were carried out in a 2-L reactor (Sétric Génie Industriel, Toulouse, France) with a 1.5 L working volume, at a temperature of 27°C for 7 h. Aeration was 0.75 L/min and stirring at 200 RPM. The pH was maintained at 6.7 with 4N NaOH. The cultures were inoculated with 2% of the total volume with a 9-h overnight culture.

Biomass Measurements

Optical density measurements were not convenient for biomass measurement during cultures on sucrose medium because of the presence of dextran that invalidated both optical density and gravimetry. Therefore, cell numeration was carried out using a graduated Thoma's cell and phase contrast microscopy; the data were converted to cell dry weight (g/L) by means of a calibration graph elaborated during culture on glucose medium. A change of 10^9 cells/mL was equivalent to 0.57 g of dry matter/L.

Dextran Analysis

A measurement 4.5 mL of culture medium were centrifuged (7000*g*, +4°C, 20 min) and the pellet was washed twice with ultrapure water. It was dried and the insoluble dextran content was calculated by subtracting the

cells' dry weight (obtained from cell counting) from the total dry weight measured. The soluble dextran content of the supernatant was measured by HPLC.

Dextransucrase Fractionation

The two forms of *L. mesenteroides* NRRL B-1299 dextransucrase were separated by centrifugation of 1 L of culture broth at 7000*g*, +4°C for 30 min (Table 1). The insoluble fraction (IGT) associated with biomass and insoluble dextran was then suspended with 400 mL of sodium acetate buffer (20 m*M* pH 5.4) and stored at –18°C. IGT preparation contained both 7.9 g/L cells and 11.8 g/L insoluble dextran. The soluble form (SGT) was concentrated from the supernatant by means of aqueous two-phase partition between dextran and PEG *(35)*. The dextran phase was harvested by centrifugation at 7000*g*, +4°C for 30 min, and dissolved in 125 mL of sodium acetate buffer (20 m*M* pH 5.4) and stored at –18°C. The final SGT preparation contained 41.3 g/L soluble dextran. The specific activities of the two fraction are reported in Table 1. No significant loss of activity occurred during the enzyme fractionation. No significant interfering activity (levansucrase, invertase, or sucrose phosphorylase) was detected (see specific measurements).

Dextransucrase Assay

Standard Assay

One unit of dextransucrase activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol of fructose per min at 30°C in 20 m*M* sodium acetate buffer, pH 5.4 with 100 g/L sucrose, 0.05 g/L $CaCl_2$ and 1 g/L NaN_3 that inhibits bacterial growth. Fructose assay was carried out by dinitrosalicylic acid assay *(36)*. Sucrose concentration, pH, and temperature were modified during the experiment as specified in the text. For pH higher than 6.0, acetate buffer was replaced by phosphate buffer (20 m*M*). It was checked that the nature of the buffer did not influence the enzyme activity.

Specific Measurements

The absence of levansucrase or invertase activity from the final dextransucrase preparations was checked by specifically measuring fructose and glucose concentrations using an Aminex HPX87K Bio-Rad column (*see* HPLC analysis). The only monosaccharide detected was fructose.

The sucrose-phosphorylase activity of the final dextransucrase preparations was also determined in the following conditions: 70 m*M* K_2HPO_4, pH 6.8; 100 m*M* sucrose; 0.5 m*M* NADP; 5 m*M* $MgCl_2$; 0.1 m*M* glucose-1,6-diphosphate; 30 U/mL phosphoglucomutase; 20 U/mL glucose-6-P-dehydrogenase. The activity was evaluated spectrophotometrically by measuring the appearance of NADPH at 340 nm.

Table 1
Characteristics of the Soluble and Insoluble Dextransucrase Preparations Obtained after Fractionation

	Insoluble fraction, IGT	Soluble fraction, SGT
activity, U/mL	12.0	4.0
soluble dextran, g/L	0	41.3
cells + insoluble dextran, g/L	19.7	0
U/mg cells	1.5	—
U/mg dextran	1.0	0.1

The two preparations were obtained from 1.0 L of culture broth, containing 5400 dextransucrase units (90% insoluble), 3.0 g/L cells, and 10.0 g/L dextran (about 50% insoluble). The activity recovery was 97.8%.

Dextran Purification

Soluble and insoluble dextran were separated by centrifugation (7000*g*, +4°C, 20 min). The insoluble material was recovered from the pellet after sucrose exhaustion, washed twice and freeze-dried. It was composed of less than 2% (w/w) of cells, introduced in the initial medium with IGT, and more than 98% of newly synthesized insoluble dextran. Soluble dextran was purified from the supernatant by ethanol precipitation, washed twice and freeze-dried.

The soluble and insoluble exogenous dextrans added to the enzyme digests were synthesized using the soluble dextransucrase preparation in the conditions described for standard assay.

HPLC Analysis

Fructose, glucose, and mannitol concentrations were measured using an Aminex HPX87K Bio-Rad column, at 65°C, with 10 m*M* K_2HPO_4 as eluent, and a constant flowrate of 0.5 mL/min. The analyses were made using a Hewlett Packard 1050 series system consisting of a pump, an injector and a HP 1047A refractometer.

Soluble dextran concentration in the supernatant was measured by gel permeation chromatography (Si100 Merck column), at room temperature. The eluant was ultrapure water, at a constant flow rate of 0.5 mL/min.

Leucrose was separated from sucrose and fructose with a C18 column (Bischoff chromatography) with a constant flow rate of 0.5 mL/min of ultrapure water, at room temperature.

^{13}C NMR Spectrometry

^{13}C experiments were obtained with AC 300 Bruker spectrometer, at operating frequency of 75.468 MHz. Samples were examined as solutions in D_2O (10–15 mg in 0.35 mL of solvent) at 70°C in 5 mm diameter spinning

tubes (internal acetone ^{13}C (CH_3): 31.5 p.p.m. relative to Me_4Si). Quantitative ^{13}C spectra were recorded using the INVGATE Bruker sequence, with 90° pulse length (6.5 ms), 15000 Hz spectral width, 8K data points, 0.54 s acquisition time, a relaxation delay of 1.5 s, and 100,000 scans were accumulated.

RESULTS AND DISCUSSION

Effect of Sucrose Concentration on Dextransucrase Activity

Up to 100 g/L sucrose, soluble and insoluble dextransucrases had a Michaelis-Menten behavior (Fig. 1). Both enzymes displayed the same Km for sucrose (10 g/L). Between 100 and 150 g/L the two forms reached maximum reaction velocity, but IGT had a maximum rate at higher sucrose concentrations than SGT. Above 150 g/L, IGT, and SGT were both inhibited by sucrose. However, SGT was less sensitive to high sucrose concentrations. It kept a residual activity of more than 50% at 600 g/L sucrose, when the insoluble form kept less than 30% residual activity.

Up to 150 g/L sucrose, IGT activity could be restricted by diffusional limitations. The insoluble enzyme is linked to the cells and trapped in the insoluble dextran matrix. As a result, it behaves like a gel-entrapped enzyme at low sucrose concentrations. Higher substrate concentrations should attenuate the difference between SGT and IGT; this was not what was observed. The lower inhibition by excess substrate observed with SGT than with IGT is in opposition with the expected effect of diffusional limitations on IGT activity. An important difference between the two reaction media comes from the presence of 4 g/L of soluble dextran introduced with SGT preparation (0.4 U/mL). Initially there was no endogenous soluble dextran in the insoluble preparation (Table 1). As shown in Fig. 2, addition of 4 g/L soluble dextran in the reaction medium activates IGT, for sucrose concentrations ranging from 200 and 600 g/L, whereas addition of 4 g/L insoluble dextran had no effect on IGT activity. The presence of soluble dextran in the SGT preparation seems to be the reason for the lower inhibition observed at high sucrose concentrations. Soluble dextran probably limits dextransucrase inhibition by acting as an acceptor. In fact, it has been shown that maltose also limits *L. mesenteroides* NRRL B-1299 insoluble dextransucrase inhibition at high sucrose concentrations *(33)*, with concomitant oligosaccharide synthesis.

The inhibition of dextransucrase activity at high sucrose concentrations can also be attributed to sucrose binding to the acceptor binding site *(2,37)* or to a separate binding site *(38)* of low affinity for sucrose. It is possible that soluble dextran partially protects the inhibitor site, preventing sucrose from binding and inhibiting the enzyme. Insoluble dextran, which is less available to the enzyme because of the resulting heterogenous medium, may be less effective in binding it.

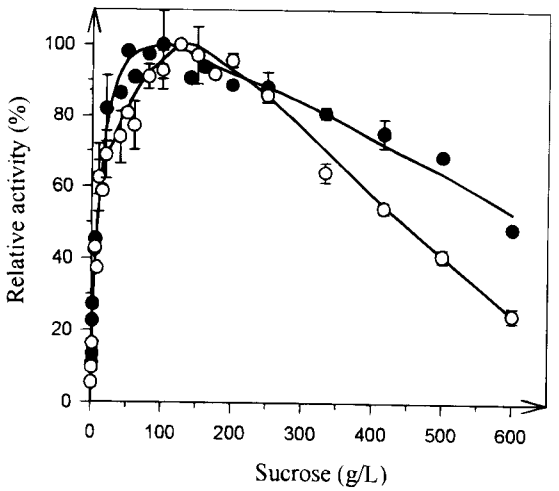

Fig. 1. Influence of sucrose concentration on initial dextransucrase activity. ● SGT, 0.4 U/mL. ○ IGT, 0.6 U/mL. The reactions were run at 30°C, pH 5.4. The references used for calculation of the relative activities were the activities measured with the corresponding enzyme at 125 g/L sucrose.

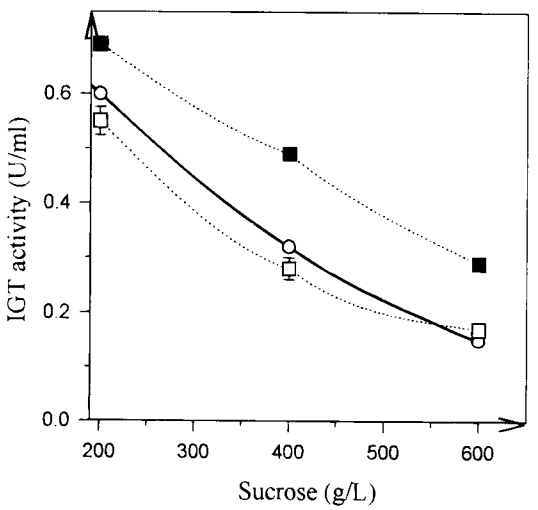

Fig. 2. Influence of high sucrose concentration on initial IGT activity in the presence of added dextran. ○ IGT, 0.6 U/mL; □ IGT, 0.6 U/mL + insoluble dextran 4g/L; ■ IGT, 0.6 U/mL + soluble dextran 4g/L. The reactions were run at 30°C, pH 5.4.

Effect of Temperature on Dextransucrase Activity

The two enzymatic preparations showed the same profile of activation by temperature (Fig. 3). IGT had an optimum temperature of 40°C and SGT of 36°C. This difference might be a result of IGT entrapment in insoluble dextran that protects it against partial denaturation.

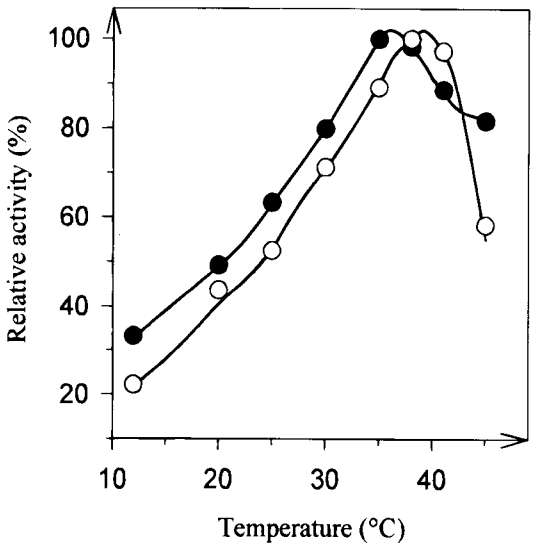

Fig. 3. Effect of temperature on initial dextransucrase activity. ● SGT, 0.4 U/mL. ○ IGT, 0.6 U/mL. The reactions were run at pH 5.4, 100 g/L sucrose. The references used for calculation of the relative activities were the activities measured at 36°C (SGT) and 40°C (IGT).

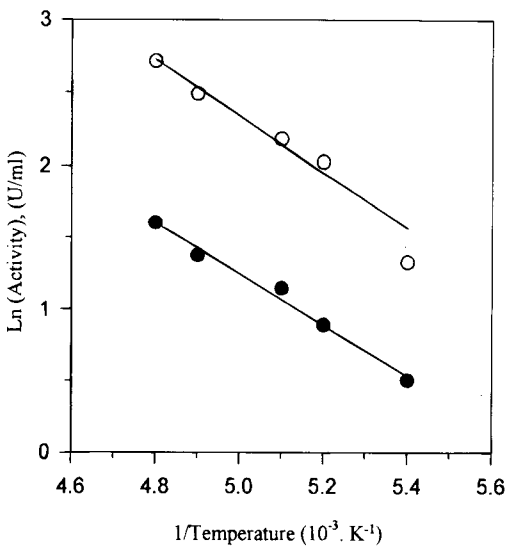

Fig. 4. Activation energy of SGT and IGT ● SGT, 0.4 U/mL. ○ IGT, 0.6 U/mL. The reactions were run at pH 5.4, 100 g/L sucrose.

The two forms had an activation energy (Ea) of the same order of magnitude: Ea = 35.0 kJ/mol for SGT and 35.4 kJ/mol for IGT (Fig. 4). Moreover, the Ea of *L. mesenteroides* NRRL B-1299 dextransucrase is lower than that of *L. mesenteroides* NRRL B-512F: 47 kJ/mol-44 kJ/mol (2,37)

showing that k_{cat} B-1299 is higher than k_{cat} B-512F. *L. mesenteroides* NRRL B-512F dextransucrase produces a polymer containing 95% α(1 → 6) linkages and 5% α(1 → 3) linkages *(3)*, i.e., much more linear than that of strain NRRL B-1299. The difference in the energy of activation of dextran synthesis between strain B-512F and strain B-1299 can be a result of greater diffusional limitations *(39)* in the case of strain B-1299.

Effect of pH

As shown in Fig. 5, IGT and SGT had rather different pH profiles. The pH range for maximal activity was 5–5.5 for SGT and 5.4–6.5 for IGT. At first sight, SGT was much more sensitive to pH inhibition than IGT.

Four g/L soluble B-1299 dextran were added to the IGT reaction mixture containing 100 g/L sucrose (Fig. 6). At extreme pH, the activity was little changed, but at pH values ranging from 5.0 to 5.5 the enzyme was highly activated. The pH profile of IGT in the presence of soluble dextran was similar to the profile observed for SGT. The fact that 3 g/L maltose had the same effect as dextran suggests that this activation was because of an acceptor reaction. But the degree of activation of dextransucrase was highly dependent on the nature of the acceptor added. Four g/L insoluble dextran also exerted pH-dependent activation on IGT, but to a lesser extend. This dextran can not be a good acceptor because of its insolubility. On the other hand, T70 dextran had no effect on IGT activity. Maybe this dextran is too linear to be a good acceptor for B-1299 dextransucrase.

Effect of pH on the Nature of the Products Synthesized by SGT and IGT

Whatever the pH of the reaction, SGT and IGT both synthesized leucrose and soluble and insoluble dextran. Less leucrose was synthesized when the pH was increased (Fig. 7). The acceptor reaction with fructose was less efficient at high pH. IGT always synthesized higher proportions of soluble dextran than SGT. Moreover, soluble dextran synthesis increased with pH for both SGT and IGT preparation. The soluble products needed more ethanol to precipitate, when produced at high pH; this indicates that these soluble dextran molecules had probably a lower molecular weight.

The structure of the various dextrans was analyzed by ^{13}C NMR spectrometry (Table 2). The α(1 → 6) and α(1 → 2) linkage content of the dextran was little affected by the reaction conditions: enzyme preparation or reaction pH. On the contrary, the dextrans differed by their α(1 → 3) linkage content. First, insoluble dextran always contained a higher proportion of α(1 → 3) linkages than soluble dextran. This was particularly obvious for the IGT dextrans whose structure was little influenced by pH. With SGT, the dextran structure was much more sensitive to pH. In fact, by carrying out the synthesis at pH 7.4, it was possible to completely eliminate

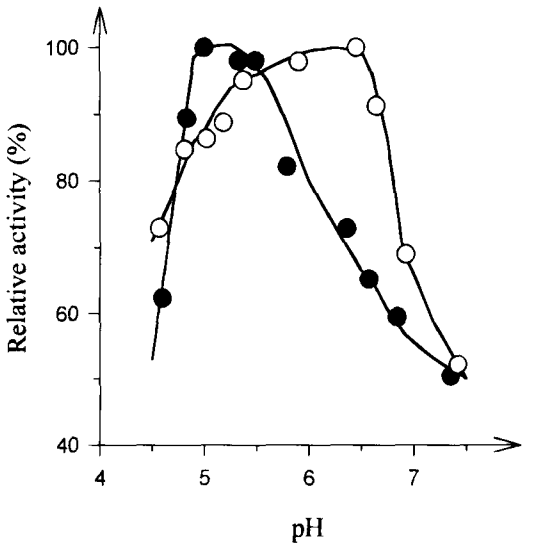

Fig. 5. Effect of pH on initial dextransucrase activity. ● SGT, 0.4 U/mL. ○ IGT, 0.6 U/mL. The reactions were run at 30°C, 100 g/L sucrose. The references used for calculation of the relative activities were the activities measured at pH 5.2 (SGT) and pH 6.5 (IGT).

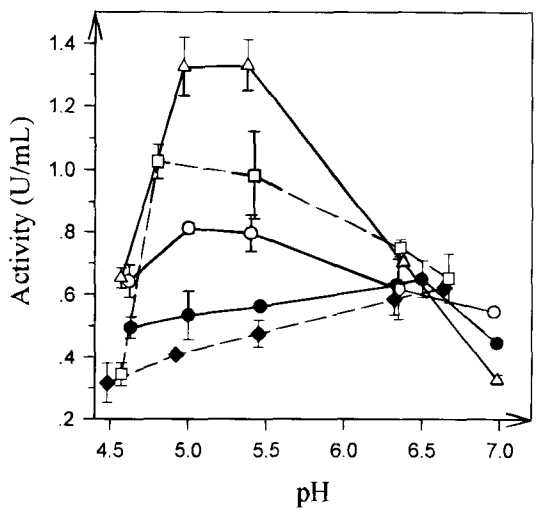

Fig. 6. Effect of pH on initial IGT activity in the presence of various products. ● IGT alone; ○ with 4 g/L insoluble dextran; △ with 4g/L soluble dextran; □ with 3 g/L maltose; ◆ with 4 g/L T70 dextran. The reactions were run with 0.6 U/mL IGT, at 30°C, 100 g/L sucrose.

the presence of α(1 → 3) linkages in the soluble dextran, while the α(1 → 3) linkage content of insoluble dextran was unchanged.

More globaly, when the synthesis pH was increased, the proportion of soluble polymer increased, the size of the soluble polymer formed was

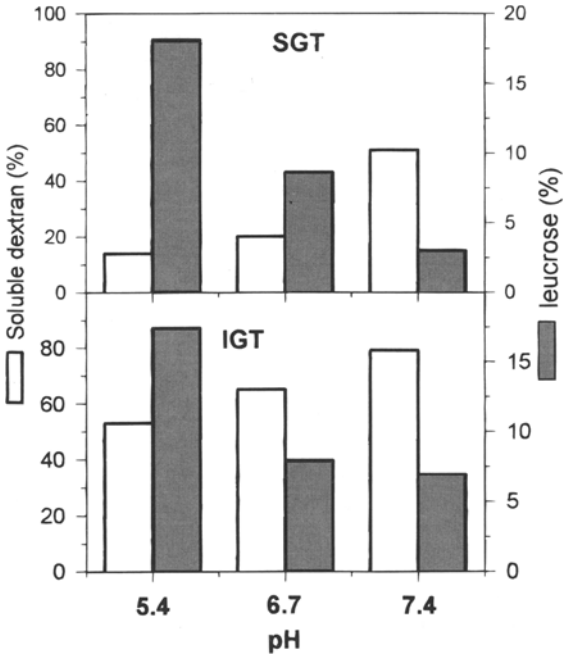

Fig. 7. Influence of pH on the nature of the products (dextran and leucrose) synthesized by SGT and IGT. SGT 0.4 U/mL, IGT, 0.6 U/mL. Reactions were run at 30°C, 100 g/L sucrose, pH 5.4, 6.7, and 7.4. The bars represent the percentage of soluble dextran versus total dextran produced and leucrose yield (g of leucrose produced/g of sucrose consumed).

Table 2
Dextran Analysis by ^{13}C NMR Spectrometry

	SGT				IGT			
pH of synthesis	5.4		7.4		5.4		7.4	
Dextran solubility	S	I	S	I	S	I	S	I
$\alpha(1 \rightarrow 6)$ (%)	63.8	60.8	62	58	68	63	62.6	63
$\alpha(1 \rightarrow 2)$ (%)	28	27.6	28	31	29	27	34	26
$\alpha(1 \rightarrow 3)$ (%)	8.2	11.6	0	10	3	8	3.4	11

[a]S: soluble; I: insoluble.
The dextran was synthesized by complete reaction at the indicated pH, with 100 g/L sucrose and 0.4 U/mL SGT or 0.6 U/mL IGT.

decreased as well as the $\alpha(1 \rightarrow 3)$ linkage content of the total dextran. These results suggest that the solubility may be influenced by the size of the polymer and the $\alpha(1 \rightarrow 3)$ linkages content. Moreover, high pH also negatively influenced the acceptor reaction (Figs. 5 and 6). So, $\alpha(1 \rightarrow 3)$

branch linkage synthesis, which decreases with increasing reaction pH, could occur by a dextran-acceptor reaction, as proposed by Robyt and Tanigushi *(26)* for strain B-512F dextransucrase. The α(1 → 2) linkages do not seem to be synthesized by the same mechanism.

CONCLUSION

Soluble and insoluble dextransucrases from *L. mesenteroides* NRRL B-1299 are known to synthesize α(1 → 2) oligosaccharides of industrial interest in the presence of maltose *(34)*. In this context, it was of importance to characterize the two enzyme preparations more precisely. Soluble (SGT) and insoluble (IGT) dextransucrase preparations were obtained by fractionation of *L. mesenteroides* NRRL B-1299 culture medium. The presence of cells and insoluble dextran gave the IGT preparation (devoid of soluble dextran) a behavior close to that of an immobilized enzyme. This affects its behavior to thermal denaturation and brings about diffusional limitations at low substrate concentrations. On the other hand, SGT is very rich in soluble dextran that influences its behavior by binding the protein and stimulating acceptor reactions. It strongly influences SGT sensitivity to excess substrate inhibition. Furthermore, soluble dextran exerts a pH-dependent activation on dextransucrase activity. This activation also appears, but to a lesser extent, with insoluble dextran and maltose, and is probably linked to acceptor reactions. Nevertheless, the differences between the two enzyme preparations have little influence on their Km for sucrose or their activation energies. Finally, the chemical characterization of the products suggests that the α(1 → 3) linkage is synthesized by a mechanism different from that of the α(1 → 2) and α(1 → 6) linkages. The α(1 → 3) linkage should then be synthesized by acceptor reaction, mainly at low pH, and thus lead to the insolubility of the dextran.

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