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ACCEPTOR REACTION OF A HIGHLY PURIFIED DEXTRANSUCRASE WITH MALTOSE AND OLIGOSACCHARIDES. APPLICATION TO THE SYNTHESIS OF CONTROLLED-MOLECULAR-WEIGHT DEXTRANS*

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ABSTRACT

Kinetic studies and product characterization of the acceptor reaction of a highly purified dextransucrase isolated from cultures of *Leuconostoc mesenteroides* suggested that the introduction of increasing amounts of maltose in the medium of reaction produced an increase of the kinetic constants, K'_m and V_{max}, of the enzyme for sucrose. A Box–Hunter mathematical optimization concerning the products of the reaction with maltose showed that the sucrose-to-maltose ratio is of prime influence upon the yield of the oligosaccharides produced and that their weight-average molecular weight (M_w) is a linear function of that ratio. In view of directly synthesizing controlled-molecular-weight dextrans, various populations of oligosaccharides were produced and used as glucosyl acceptors in a second-step reaction with sucrose. The molecular weight and polydispersity of the second-step products were related to the sucrose-to-acceptor ratio and to the characteristics of the oligosaccharide acceptor.

INTRODUCTION

Dextransucrase (EC 2.4.1.5) is a glucosyltransferase that catalyzes the transfer of an α-D-glucopyranosyl group from sucrose to form dextran, an α-D-(1→6)-linked polysaccharide having a molecular weight $>10^6$.

In the early fifties, Koepsell *et al.*[1] and Tsuchiya *et al.*[2] observed that the introduction of some sugars in the reaction mixture of dextransucrase with sucrose leads to the synthesis of oligosaccharides at the expense of high-molecular-weight dextrans. The glucosyl residue from sucrose was transferred by dextransucrase to a free hydroxyl group of those sugars that were called acceptors. Many different

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sugars act as acceptors and various authors have classified them according to their capacity to divert the glucosyl residues from dextran to form oligosaccharides and to their effect on the rate of reaction[1,3–6]. Among all acceptors tested, maltose and isomaltose have been demonstrated to be the most effective[1,4,5]. In their presence, a series of homologs is synthesized: with isomaltose, isomalto-oligosaccharides are formed (oligodextrans)[4,7], and with maltose, oligosaccharides terminating in panose (6-*O*-α-D-glucopyranosylmaltose) at the reducing end[4]. Those acceptor-reaction products may, in turn, be used as acceptors to produce directly relatively high-molecular-weight dextrans (M_r of 40 000 and 70 000 used as plasma substitute)[1,2,8,9]. Because of its high acceptor efficiency and its disponibility, maltose has been the most widely used acceptor for the synthesis of oligosaccharides by dextransucrase[1,2,7,8,10]. Processes using the maltose-reaction products as glucosyl acceptors to synthesize controlled-molecular-weight dextrans have never been developed industrially, because of the difficulties in the analysis and purification of the products and of the high cost of the purified enzyme.

The application of gel permeation chromatography for the determination of the molecular-weight distribution of dextrans[11] eliminated most of the analytical problems. A new process involving the production of dextransucrase by fed-bath culture[9] of *Leuconostoc mesenteroides* and its purification by phase partition[12], makes available large quantities of highly purified enzyme which may be efficiently used for the synthesis of controlled-molecular-weight dextrans.

EXPERIMENTAL

Dextransucrase production. — Dextransucrase was produced by fed-batch cultures of *Leuconostoc mesenteroides* NRRL B-512F in a mixture containing sucrose (40 g/L), yeast extract (20 g/L), K_2HPO_4 (20 g/L), iron-dextran (0.01 g/L), and oligoelements, as already described[13]. The pH was maintained at 6.7 by adding an alkaline sucrose solution (2M sodium hydroxide, sucrose 600 g/L) and the temperature was 27°. Final culture dextransucrase activity was 8.8 U/mL.

Dextransucrase purification. — After cell removal by centrifugation (20 min, 4°, 10 000 *g*), exocellular dextransucrase was purified by aqueous two-phase partition between the dextran present in the supernatant and poly(ethyleneglycol) (M_r 1500) added to the culture supernatant. Dextransucrase was thus obtained in the dextran-rich phase in a concentrated, highly purified form. Successive phase-partition steps resulted in an enzyme preparation having a specific activity of 175 U/mg of protein and 1.35 U/mg of dextran in a final yield of 95%. Such a dextransucrase preparation was free of levansucrase activity[12].

Dextransucrase kinetics and oligosaccharide synthesis. — Reactions took place at 20° in 20mM sodium acetate buffer (pH 5.2), $CaCl_2$ (0.05 g/L), dextransucrase (1 U/mL), and various sucrose and maltose concentrations. Dextransucrase activity was determined by measuring the initial reaction rate of fructose released from sucrose. Fructose was determined with u.v. test 139 106 from Boehringer–

Mannheim. A standard unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 μmol of D-fructose per min at 30°, in the presence of 100 g of sucrose/L.

Determination of molecular-weight distribution of dextrans. — The molecular-weight distribution of dextrans was determined by gel permeation chromatography (g.p.c.) using a l.c. system consisting of an M 6000 A pump, a U6K injector, and an R 401 differential refractometer, all purchased from Waters–Millipore. Columns (250 mm length × 10.7 mm diameter) were packed with silica gels Lichrosorb Si 60 and Si 100 from Merck. The method employed was universal calibration described by Sun and Wong[14], and Alsop *et al.*[11] where M_w is the weight-average molecular weight, M_n the number-average molecular weight, and where M_w/M_n is the polydispersity of the polymer population. The dextrans used as g.p.c. standards (T10, T40, T70) were purchased from Pharmacia (Sweden).

Sugar analysis. — Oligosaccharides were analyzed by reverse-phase chromatography using the same l.c. system. The column was a μ-Bondapack C18 purchased from Waters–Millipore. Separation conditions were the same as previously described[15,16].

RESULTS

Kinetic study of dextransucrase in the presence of maltose as glucosyl acceptor. — Initial reaction rates were measured for various substrate (sucrose) and acceptor (maltose) concentrations allowing the effect of maltose on kinetic constants to be determined by use of Lineweaver–Burk plots. Fig. 1 shows the significant effect of increasing maltose concentration on the apparent Michaelis constant, K'_m and on the maximal reaction rate, V_{max}. With increasing concentration from 0 to 200 g/L,

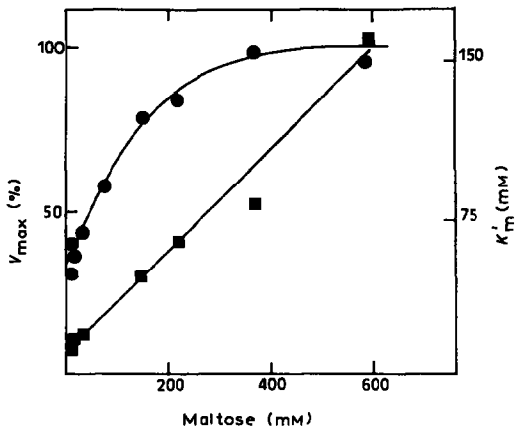

Fig. 1. Effect of maltose as glucosyl acceptor on kinetic constants of dextransucrase: (■) K'_m and (●) V_{max}.

the K'_m value for sucrose increased linearly from 12 to 163mM and the V_{max} value from 5.8 to the maximal value of 19.1 U/mL.

Factors affecting the oligosaccharide synthesis in the acceptor reaction with maltose. — In order to quantify the effect of various parameters, such as temperature, sucrose, maltose, and enzyme concentration, on the oligosaccharide synthesis,

TABLE I

FACTORS WITH THEIR CORRESPONDING LEVELS IN THE BOX–HUNTER METHOD

Factors	*Units*	*Level* (−)	*Level* (+)
Temperature	°(C)	4.0	29.5
Enzyme activity	U/mL	0.2	5.0
Maltose	g/L	2.0	100.0
Sucrose	g/L	20.0	200.0

TABLE II

AVERAGE RESPONSES FOR ALL SUCROSE–MALTOSE COMBINATIONS IN THE BOX–HUNTER METHOD

Sucrose (g/L)	*Maltose (g/L)*	*Sucrose/ maltose*	*Yield of oligosaccharides*[a]	*Yield of h.mol.wt. dextrans*[b]	M_w *of oligosaccharides*
200	2	100.0	49	54	4400
20	2	10.0	35	25	900
200	100	2.0	85	0	670
20	100	0.2	49	0	500

[a]Calculated as the ratio of oligosaccharides (g/L)/[maltose (g/L) + 0.474 sucrose (g/L)]. [b]Calculated as the ratio of high-molecular-weight (h.mol.wt.) dextrans (corresponding to the peak eluted at void volume in g.p.c. experiments)/0.474 sucrose (g/L).

TABLE III

CHARACTERIZATION AND YIELD OF THE PRODUCTS SYNTHESIZED IN OPTIMIZED SUCROSE-TO-MALTOSE RATIOS

Sucrose (g/L)	*Maltose (g/L)*	*Sucrose/ maltose*	*Yield of products (%)*		
			Oligosaccharides[a]	*H.mol.wt. dextrans*[b]	*Leucrose*[c]
120	57	2.1	88	0.8	2.4
120	50	2.4	94	0.9	2.4
120	39	3.1	92	1.4	2.5
120	29	4.1	93	3.9	3.2
200	40	5.0	89	3.7	5.2
200	31	6.5	88	4.1	5.5

[a]Calculated as the ratio of oligosaccharides (g/L)/[maltose (g/L) + 0.474 sucrose (g/L)]. [b]Calculated as the ratio of high-molecular-weight (h.mol.wt.) dextrans (corresponding to the peak eluted at the volume in g.p.c. experiments)/0.474 sucrose (g/L). [c]Calculated as the ratio of leucrose/sucrose.

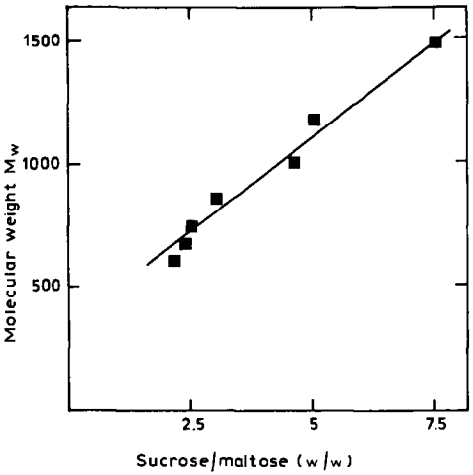

Fig. 2. Weight-average molecular weight (M_w) of the oligosaccharides produced in the presence of maltose as glucosyl acceptor in function of the ratio sucrose/maltose (S/M).

various experiments according to the method of Box and Hunter of mathematical optimization[17] were performed. Table I shows the four parameters with their corresponding levels. The three responses measured were: (a) The yield of oligosaccharide calculated as oligosaccharide (w/v)/[maltose (w/v) + 0.474 sucrose (w/v)] where 0.474 sucrose corresponds to glucose incorporated into oligosaccharide), (b) The weight-average molecular weight (M_w), (c) The yield of high-molecular-weight (h.mol.wt.) dextrans calculated as the ratio of h.mol.wt. dextran (corresponding to the peak eluted at void volume in g.p.c. experiments) to 0.474 sucrose (w/v). The ratio of maltose to sucrose was shown to drastically affect the three responses measured. Table II shows the average of the results obtained for all combinations of maltose and sucrose. Temperature and enzyme concentration show no significant effects between both levels used.

Characterization of oligosaccharides produced in the presence of maltose. — The products of various syntheses using maltose as glucosyl acceptor were analyzed by g.p.c. and reverse-phase chromatography. In addition to the dextran and oligosaccharide yields, the leucrose yield has been reported in Table III. Leucrose (5-*O*-α-D-glucopyranosyl D-fructopyranose) is always present as a product of dextransucrase action on sucrose, as it results from the transfer of a glucosyl unit onto fructose[4,18]. Leucrose is not an acceptor, and its synthesis reduces the oligosaccharide yield[19]. As may be seen in Fig. 2, the weight-average molecular weight of the oligosaccharides may be represented as a linear function of the sucrose-to-maltose ratio. We derived a simple relation (*1*) between the molecular weight of the oligosaccharides and the sucrose-to-maltose (S/M) ratio:

$$M_w = 340\ (1 + 0.48\ \text{S/M}) \qquad (1)$$

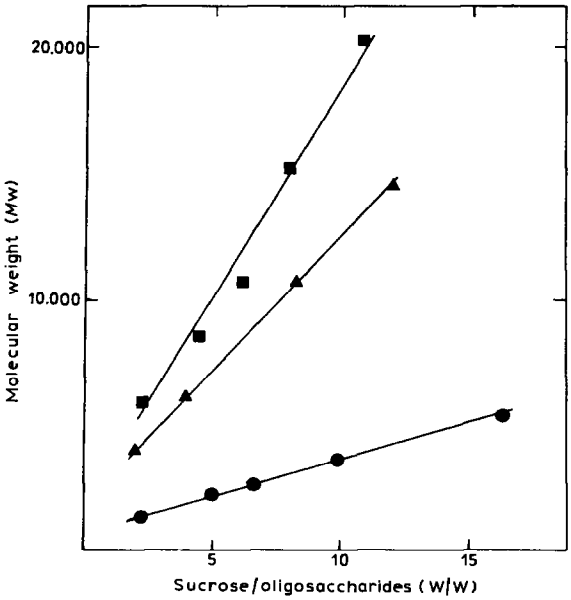

Fig. 3. Weight-average molecular weight (M_w) of the dextrans produced in the presence of oligosaccharides as glucosyl acceptors in function of the ratio sucrose/oligosaccharides (S/A), weight by weight: (●) oligosaccharides A (M_w 830); (▲) oligosaccharides B (M_w 1150); and (■) oligosaccharides C (M_w 1970). The experimental conditions for oligosaccharide synthesis were: for oligosaccharides A, sucrose (120 g/L) and maltose (40 g/L); for oligosaccharides B, sucrose (200 g/L) and maltose (40 g/L); and for oligosaccharides C, sucrose (200 g/L) and maltose (20 g/L). The experimental conditions for dextran synthesis were: 20mM sodium acetate buffer (pH 5.2), $CaCl_2$ (0.05 g/L), and dextransucrase (1 U/mL) at 20°; the sucrose concentration was 12% (w/v) for oligosaccharides A and 15% (w/v) for oligosaccharides B and C.

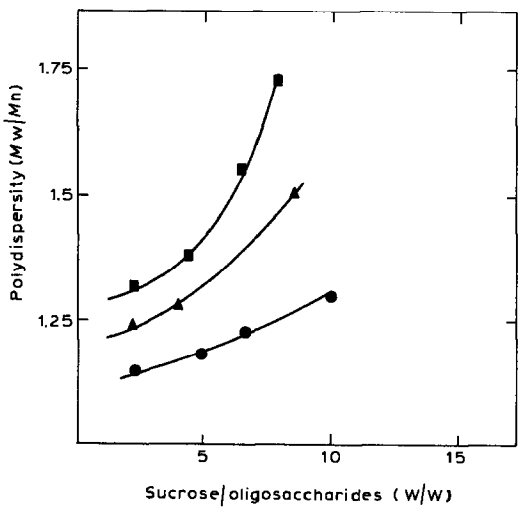

Fig. 4. Polydispersity (M_w/M_n) of the dextrans produced in the presence of oligosaccharides as glucosyl acceptors in function of the ratio sucrose/oligosaccharides (S/A), weight by weight: (●) oligosaccharides A (M_w 830); (▲) oligosaccharides B (M_w 1150); and (■) oligosaccharides C (M_w 1970). Experimental conditions as in legend to Fig. 3.

Use of oligosaccharides as glucosyl acceptors. Characterization of the second-step reaction products. — We achieved different acceptor reactions with maltose, at different S/M ratios, and obtained three populations of oligosaccharides of increasing mol. wt. and polydispersity: oligosaccharides A (S/M 3, M_w 830), oligosaccharides B (S/M 5, M_w 1150), and oligosaccharides C (S/M 10, M_w 1970). The solutions of these reaction products were concentrated and used as glucosyl acceptors in the presence of the reaction coproduct, fructose. They were chosen in order to quantify the effect of the acceptor characteristics (M_w and polydispersity) on the second-step reaction products. Figs. 3 and 4 show the change of M_w and polydispersity of the dextrans produced as a function of the sucrose(w/v)-to-oligosaccharide acceptor (w/v) (S/A) ratio. As in Fig. 2, the value of the M_w of the product is a linear function of the S/A ratio, and the slope increased with the M_w of the oligosaccharide acceptor. Moreover, the polydispersity of the product increased with the S/A ratio and the mol. wt. of the acceptor (Fig. 4).

DISCUSSION

In agreement with the model of dextransucrase mechanism presented by Ebert and Schenk[3], the presence of maltose allows D-glucopyranosyl residues to be trapped before their addition to the growing dextran-chain and then eliminates this step. Because of the steric hindrance of the h.mol.wt. dextran-chain, this transfer step is the limiting one if synthesis is achieved without acceptor addition. The release of the oligosaccharides from the active site of the enzyme and the change in destination of the D-glucosyl residues considerably accelerates the reaction rate and requires high sucrose concentrations to achieve enzyme saturation. As seen from Fig. 1, the apparent Michaelis constant, K'_m, of dextransucrase for sucrose increased linearly from 12, in the absence of maltose, to 163mM, in the presence of a 586mM maltose concentration. Moreover, maltose addition resulted in a 3-fold increase in the maximal velocity, V_{max}, of the reaction. In fact, in the presence of a very efficient acceptor such as maltose, the formation of the D-glucosyl–enzyme complex, the first obligatory step before sugar transfer to the acceptor, becomes the limiting step of the reaction.

Not only does maltose affect the kinetics of dextransucrase reaction but also the characteristics of the oligosaccharides synthesized. As may be seen in Table II, a high S/M ratio enhances the production of h.mol.wt. dextrans, and decreases the yield of oligosaccharides. Similarly, the M_w of the oligosaccharides produced is higher. These results confirm with more accuracy those obtained by Koepsell *et al.*[1] and Tsuchiya *et al.*[2], and also demonstrate that, in order to obtain a high yield of oligosaccharides, the first synthesis step must be achieved at a relatively low S/M ratio. The yields of oligosaccharides, h.mol.wt. dextrans, and leucrose obtained with S/M ratios in the range of 2.1–6.5 are shown in Table III. At such ratios, the oligosaccharide yield is high and the h.mol.wt. dextran yield is very low. A high sucrose concentration leads to a high leucrose yield, resulting from an increased

release of fructose in the reaction mixture. As fructose shows a much lower acceptor efficiency than maltose[2–5], leucrose synthesis will be dependent on the initial sucrose-to-acceptor ratio. This is confirmed by an increase in leucrose yield with S/M ratio when the sucrose concentration remains constant. Moreover, the M_w of the oligosaccharides synthesized is a linear function of the S/M ratio (Fig. 2). Equation *1* obtained from the experimental curve is identical with Eq. *2* previously suggested by Behrens *et al.*[10], where $M_{w,A}$ is the mol. wt. of the acceptor and 0.474 sucrose corresponds to the glucose residue from sucrose incorporated into oligosaccharide:

$$M_w = M_{w,A}\,(1 + 0.474\ \text{sucrose/acceptor}) \qquad (2)$$

Equation *1* has been derived from the data given in Fig. 2, corresponding to synthesis conditions resulting in high oligosaccharide yields. This equation is, thus, only valid when the synthesis reaction gives oligosaccharide yields >85% (Table III). This explains, for example, the lack of fitness of the data given in Table II (which correspond to low oligosaccharide yields) with Eq. *1*, but the value corresponding to a S/M ratio 2.0 (oligosaccharide yield, 85%).

These results show that it is possible, by varying the conditions of reaction, to control very accurately the M_w of the first-step synthesized oligosaccharides. When used as glucosyl acceptors in a second-step reaction with sucrose and dextransucrase, these oligosaccharides show the same behavior as maltose. The M_w of the product is a linear function of the S/A ratio (Fig. 3) and the slope increased with the acceptor M_w. When purified monodisperse preparations of oligosaccharides (d.p. 3–5) were used as acceptors, the M_w of the products thus obtained was consistent with a Behrens-type equation[6]. Thus, a similar relationship can be expected when a broader range of oligosaccharides are used as acceptors.

This result may be explained by considering Fig. 4 which shows that the polydispersity of the products presents the same evolution as their M_w values. This phenomenon is greatly increased in the case of the high-molecular-weight acceptor. The S/A ratio and the polydispersity of the acceptor have an effect of prime importance upon the quality of the second-step products. As fructose is not removed from the oligosaccharide acceptor mixtures from the first-step synthesis and is also produced during the second-step reaction, it drastically affects the yield of the second-step products by allowing the formation of leucrose. Thus, in a multistep process for the production of controlled-molecular-weight dextrans (for example, plasma substitutes), it would be necessary to maintain a low S/A ratio and to eliminate fructose, a reaction coproduct, after each reaction step.

In conclusion, we have determined the effect of reaction conditions on the yield, the polydispersity, and the molecular weight of the products obtained in the acceptor reaction using highly purified dextransucrase as catalyst. Our results show that, in a two-step synthesis process and with a precise control of the reaction conditions, it is possible to obtain a controlled-molecular-weight preparation at

least up to 20 000 presenting the same polydispersity as commercially available dextrans prepared by acidic hydrolysis of high-molecular-weight polymers.

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