Acceptor reactions and mechanism of Leuconostoc mesenteroides B-512FM dextranucrase

Aziz Tanriseven
Iowa State University

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Acceptor reactions and mechanism of

*Leuconostoc mesenteroides* B-512FM dextranucrase

by

Aziz Tanriseven

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1993
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<td>dp</td>
<td>degree of polymerization</td>
</tr>
<tr>
<td>IU</td>
<td>International Units of enzyme activity</td>
</tr>
<tr>
<td>M</td>
<td>molar (moles per liter)</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<td>K_i</td>
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GENERAL INTRODUCTION

*Leuconostoc mesenteroides* B-512F dextranucrase, an industrially important enzyme, synthesizes dextran using the glucosyl part of sucrose as shown below:

Dextranucrase

\[ n \text{ Sucrose} \rightarrow \text{Dextran} + n \text{ Fructose} \]

B-512F dextran has 95% α(1-6) linkages and 5% α(1-3) branch linkages\(^1\) (see Fig. 1). Dextrans have been used to make Sephadex and blood plasma substitute\(^2\). Recently a solution of dextran with a molecular weight of 40,000 has been used for the preservation of livers for transplantation\(^3\). The molecular weight of dextran largely depends on the concentration of sucrose used, and low concentration of sucrose produces high molecular weight dextran\(^4\).

In addition to sucrose, it was found that α-D-glucopyranosyl fluoride\(^5\) and p-nitrophenyl-α-D-glucopyranoside\(^6\) are also substrates for dextranucrase. The enzyme has a molecular weight of 177-195 kDa\(^7,8\). It is composed of three monomers\(^2\) and the monomer is 65 kDa. The pH optimum of the purified enzyme is 5.0-5.5\(^9\) and the isoelectric point of dextran-free enzyme is 4.0\(^9\). The enzyme is stabilized by its product, dextran\(^8,10\).

Normally dextranucrase catalyzes the formation of dextran by polymerization of the glucosyl part of sucrose, but acceptor
reactions take place when carbohydrates other than sucrose are added to sucrose-dextran-sucrase digests. In acceptor reactions, some of the glucosyl groups of sucrose molecules are transferred to the carbohydrate, which is called an acceptor. Acceptor reactions lead to formation of oligosaccharides and decreased synthesis of dextran. There are many carbohydrates which act as acceptors. The acceptor reactions of dextran-sucrase have been used industrially for the production of controlled molecular weight dextran and leucrose, α-D-glucopyranosyl-(1-5)-D-fructopyranose.

The mechanism of biosynthesis of dextran has been studied by several investigators. The mechanism proposed by Robyt et al. is generally accepted in the field (see Fig. 2). This mechanism proposes that dextran synthesis proceeds by a two-site insertion mechanism in which covalent glucosyl and dextran-syl enzyme intermediates are formed, and the glucosyl unit is added to the reducing-end of the growing dextran chain.

Explanation of dissertation format

This dissertation consists of three papers, the first two of which have been published. The process described in the third paper will be patented. References for each paper are given at the end of the paper. References for the general introduction and literature review are listed under the additional references after the general summary.
LITERATURE REVIEW

Mechanism of dextran biosynthesis

There have been many arguments concerning the mechanism of the biosynthesis of dextran by dextransucrase since there are two possible mechanisms for the biosynthesis of linear polysaccharides. In the first mechanism, the repeating units are added sequentially to the nonreducing end of a growing chain, but in the second mechanism, the repeating units are added sequentially to the reducing end by insertion between a carrier and the growing chain.

A mechanism for the biosynthesis of dextran should consist of the following: how the biosynthesis starts, how a dextran chain grows (i.e., from reducing end or nonreducing end), how the branching occurs, and how dextran is released from the enzyme.

Hehre suggested that the growth of dextran chains is a stepwise propagative reaction where glucosyl group of a sucrose molecule is transferred to nonreducing end of a growing dextran chain. A dextran primer is required in this mechanism. As of 1993, all of the dextransucrase preparations contained some dextran due to the fact that dextransucrase is induced by sucrose, and sucrose must be present in the growth medium, and therefore sucrose acts as a substrate for the enzyme in the biosynthesis of dextran. Recently in our laboratory, a constitutive mutant of Leuconostoc mesenteroides
B-512FM that produced dextranucrase from a glucose medium was obtained. The dextranucrase secreted by this mutant bacterium didn't have any dextran contamination, but it still synthesized dextran using sucrose, indicating the mechanism suggested by Hehre is not correct, that is, dextran primer is not required.

Tsuchiya and Neely proposed that the dextran chain grows from its nonreducing end. They also proposed that sucrose serves as glucosyl donor and acceptor in the beginning of the dextran synthesis, thus a glucose unit is transferred to glucosyl part of another sucrose molecule forming isomaltosyl fructoside. They proposed that subsequent transfer of glucose units to isomaltosyl fructoside eventually leads to the formation of dextran chains. Isomaltosyl fructoside, the first compound formed in the mechanisms proposed by Tsuchiya and Neely, was synthesized and reacted with dextranucrase in the presence of sucrose. This compound inhibited dextran synthesis and caused the formation of oligosaccharides showing that the mechanisms proposed by Tsuchiya and Neely were not correct.

Ebert and Schenk proposed an insertion mechanism for dextran biosynthesis, where glucose groups of sucrose molecules are added to the reducing end of the growing dextran chain. They did not present any direct experimental evidence, and their mechanism was based primarily on the logical
Finally long awaited evidence about dextran sucrase action was presented by Robyt et al., showing that dextran is synthesized by dextran sucrase by the transfer of glucose from sucrose to the reducing end of a growing dextran chain. In the mechanism proposed (see Fig. 2), dextran sucrase has two nucleophilic catalytic groups which attack sucrose to give glucosyl complexes through C1 carbon of glucose. Then, C4-OH of one of these glucose units makes a nucleophilic attack onto the C4 of the other glucose unit forming an α-(1-6) glucosidic linkage and releasing the nucleophile, which forms a new enzyme-glucosyl complex by attacking another sucrose molecule. The C4-OH of this newly formed glucosyl unit then attacks C1 of the isomaltosyl group forming isomaltotriosyl-enzyme complex. As long as sucrose is available, the process continues with the catalytic groups alternatively forming covalent complexes with glucose and the growing dextran chain. Dextran chain growth will be stopped when an acceptor reaction takes place between an acceptor, e.g. fructose or glucose, and the enzyme-dextranosyl complex leading to release of the dextran from the enzyme. If dextran is the acceptor, then, this acceptor reaction forms branch linkages in dextran10.

B-512FM dextran has 5% α-(1-3) branch linkages and some of the branches are one glucose unit long, others are longer (see Fig. 1). To explain the branching in dextran, different
mechanisms were proposed: It was suggested that a branching enzyme is responsible for the formation of branches. Bovey proposed that branches are formed in dextran by glucosyl transfer from sucrose molecule to the C₁-OH group of an α-(1-6) linked chain. Successive glucosyl transfers to the glucose at the branching point could then lead to longer branches. He also suggested that the enzyme necessary for branching could be either the dextransucrase itself, or a special branching enzyme. Dextran branching enzymes have never been observed.

Ebert and Brosche proposed a different mechanism for branch formation where a free dextran molecule acts as an acceptor releasing dextran from a dextran-dextransucrase complex and forming a branch linkage. In their experiment, a low molecular weight [³H] dextran was added to sucrose-dextransucrase digest and found that ³H label incorporated into a higher molecular weight dextran. They didn't show that the newly formed dextran was synthesized by acceptor reactions of the added [³H] dextran. It could have been formed by the transfer of glucose units to the nonreducing end of the [³H] dextran. They also didn't show that the branch linkage was on the acceptor dextran.

Robyt and Taniguchi proposed a mechanism for the formation of branch linkages by dextransucrase, giving conclusive experimental evidence: Immobilized dextransucrase
using Bio-Gel was labeled by incubating it with [\(^{14}\)C] sucrose. Extensively washed immobilized dextran was treated with a low molecular weight nonlabeled dextran. This process released the label from the immobilized enzyme as [\(^{14}\)C] dextran, but no [\(^{14}\)C] dextran was released in buffer alone. Structural analysis of the released [\(^{14}\)C] dextran showed that it was slightly branched and the acceptor dextran formed an \(\alpha\)-(1\(-\)3) branch linkage between a C\(_{\text{i}}\)-OH on the acceptor dextran and the C\(_{\text{i}}\) of the reducing end of a dextranosyl-dextranase complex (see Fig. 3).

In 1978, a general mechanism for the acceptor reactions of dextranase was proposed by Robyt and Walseth\(^{19}\). The acceptor reactions take place by nucleophilic displacement of glucosyl and dextranosyl groups from covalent enzyme-complex by a specific hydroxyl group of an acceptor molecule. The acceptor reactions stop the dextran chain growth in dextran biosynthesis and do not start dextran polymerization by acting as primers (see Fig. 3).

**Acceptor reactions**

Dextranase synthesizes dextran from sucrose but, in the presence of acceptor sugars, some of the glucosyl moieties of sucrose are transferred to acceptors forming oligosaccharides at the expense of formation of dextran, the natural product\(^{31,32}\).
Dextranucrase

Sucrose + Acceptor \rightarrow Acceptor + Dextran + Fructose

product(s)

There are more than thirty different acceptors which include monosaccharides, oligosaccharides, and polysaccharides\(^\text{12}\). Some acceptor products, e.g. the acceptor products of maltose and isomaltose, themselves act as acceptors and a homologous series of acceptor products are formed, while other acceptors, e.g. D-fructose and lactose, give only a single acceptor product\(^\text{10,31}\).

Robyt and Walseth\(^\text{10}\) studied the mechanism of the acceptor reactions using sucrose and \(^{14}\)C-labeled acceptors and they showed that both glucosyl and dextranosyl groups were transferred to the nonreducing end of the acceptors. They proposed that the acceptor reactions take place by displacing glucosyl and dextranosyl groups from the covalent enzyme-complex and that the acceptor reactions terminates dextran chain growth by displacing it from the active-site.

Ebert and Schenk\(^\text{14}\) showed that some acceptors increase the rate of reaction with B-512F dextranucrerase and some decreased the rate. Maltose and isomaltose are the most efficient acceptors\(^\text{33}\).

The acceptor reactions of dextranucrerase with several acceptors have been used industrially to produce leucrose\(^\text{13}\), a
noncariogenic disaccharide, and controlled molecular weight dextrans\textsuperscript{7} for medical purposes, such as blood plasma substitute.

\textbf{Substrate inhibition}

Several researchers observed that dextran synthesis follows the Michaelis-Menten equation up to 200 mM sucrose, and thereafter the rate of dextran synthesis decreases\textsuperscript{14,16,27,35}.

Ebert and Schenk\textsuperscript{14} assumed that the decrease of dextran synthesis was due to the binding of sucrose to an acceptor binding site, where it acts as an acceptor, a weak one. They assumed that some enzyme forms an enzyme-acceptor complex with sucrose, the rate of dextran synthesis would decrease.

Stringer and Tsuchiya\textsuperscript{16} and Neely\textsuperscript{26,37} also interpreted sucrose inhibition as due to binding of sucrose molecules to the acceptor sites. Neely\textsuperscript{26} further suggested that sucrose binds to an acceptor binding site in such a way that no reaction occurs.

Sucrose inhibition was explained by Bovey\textsuperscript{27} as the binding of a second sucrose molecule to dextransucrase to form an inactive dextransucrase-glucosyl-sucrose complex which is incapable of synthesizing dextran because of the blocked active-site.

We have recently observed that dextransucrase was almost totally inhibited in 2.4M sucrose solution and the enzyme was active when sucrose solution is diluted by addition of buffer.
Figure 1. Structure of B-512F dextran
Figure 2. Reducing end polymerization mechanism of dextranucrase.

X = nucleophiles at the active site

○ ▽ = sucrose, ○ = glucose, ▽ = fructose
Figure 3. (A): Mechanism of acceptor reactions, □□ = isomaltose, □ = reducing glucose unit; (B): mechanism for forming branch linkages in dextran. An exogenous dextran acts as an acceptor and displaces the glucosyl or dextranosyl unit forming an α-(1-3) linkage.
PAPER I

INHIBITION OF DEXTRAN SYNTHESIS BY ACCEPTOR REACTIONS OF DEXTRANSUCRASE, AND THE DEMONSTRATION OF A SEPARATE ACCEPTOR BINDING-SITE

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ABSTRACT

Hanes-Woolf plots of dextranucrase-sucrose digests with methyl α-D-glucopyranoside and with methyl 6-deoxy-α-D-glucopyranoside showed that they were weak, apparent competitive inhibitors with $K_i$ values of 97 and 267 mM, respectively; a methyl 6-deoxy-6-fluoro-α-D-glucopyranoside plot showed that it was a very weak, noncompetitive inhibitor with a $K_i$ of 400 mM. A Michaelis plot with 6-deoxysucrose, a known competitive inhibitor with a $K_i$ of 1.6 mM, converged with a sucrose plot at 500 mM sucrose. A Michaelis plot with methyl α-D-glucopyranoside, a relatively good acceptor but poor competitive inhibitor, did not converge with the sucrose plot at 600 mM sucrose. Extrapolation of the sucrose and the methyl α-D-glucopyranoside plots showed that convergence would occur at = 2 M sucrose. Based on its concentration and its $K_i$ value, the methyl α-D-glucopyranoside plot should have converged with the sucrose plot at 200 mM sucrose if it were competing with sucrose for the sucrose binding-sites. From these observations, it is concluded that methyl α-D-glucopyranoside does not bind at the sucrose binding-sites when it acts as an acceptor, but it binds at a separate acceptor binding-site. This is further corroborated by the observation that methyl 6-deoxy- and 6-deoxy-6-fluoro-α-D-glucopyranoside analogues are bound very poorly, whereas the corresponding sucrose analogues, 6-deoxysucrose and 6-deoxy-6-
fluorosucrose are bound approximately ten times more tightly than sucrose. The very high concentration of sucrose (= 2M) required for the convergence of the methyl α-D-glucopyranoside plot with the sucrose plot indicates that sucrose binds only very weakly to the acceptor binding-site.
Leuconostoc mesenteroides B-512FM dextranucrase (EC 2.4.1.5) catalyzes the polymerization of the glucosyl moiety of sucrose to form dextran, which has 95% \( \alpha-(1\rightarrow 6) \) linkages and 5% \( \alpha-(1\rightarrow 3) \) branch linkages:

\[
\text{Dextranucrase} \\
n \text{sucrose} \quad \rightarrow \quad \text{Dextran} + n \text{Fructose}
\]

The reaction is essentially nonreversible. \( \alpha\)-D-Glucopyranosyl fluoride\(^1\) and p-nitrophenyl \( \alpha\)-D-glucopyranoside\(^2\), are also glucosyl donors for the synthesis of dextran by this enzyme.

The reaction mechanism of dextran formation by dextranucrase has been studied by several groups\(^3\)\(^-\)\(^9\). Robyt et al.\(^4\) studied the mechanism by pulse and chase techniques with \( [^{14}\text{C}] \text{sucrose} \) and Bio-Gel P-2 immobilized dextranucrase. They found that glucosyl and dextranosyl covalent enzyme complexes were formed and that the glucosyl unit was added to the reducing end of the growing dextran chain.

When various carbohydrates are added to a sucrose-dextranucrase digest, some of the glucosyl groups are transferred to the added carbohydrates\(^8\)\(^-\)\(^11\), which are called acceptors. Many different carbohydrates act as acceptors, including monosaccharides, oligosaccharides, and polysaccharides. In some of these reactions, the acceptor
products themselves act as acceptors, and a homologous series of acceptor products are formed, whereas other acceptors give only a single acceptor-product\(^3\). Maltose, isomaltose, and methyl α-D-glucopyranoside are examples of acceptors that give a homologous series; D-fructose, lactose, and raffinose are examples of acceptors that give single products\(^3\).

Using radioactively labeled acceptors such as \(\text{[^3H]}\)methyl α-D-glucopyranoside, D-\(\text{[^3H]}\)glucose, D-\(\text{[^14C]}\)glucose, and D-\(\text{[^3H]}\)fructose, Ebert and Schenk\(^3\) showed that a single acceptor molecule was incorporated into a dextran molecule.

Robyt and Walseth\(^10\) studied the mechanism of the acceptor reactions by charging the enzyme with sucrose followed by reactions with \(\text{[^14C]}\)-labeled D-glucose, D-fructose, and maltose in the presence and absence of nonlabeled sucrose. Each acceptor gave a high-molecular-weight dextran and a low-molecular-weight saccharide, with the acceptor attached to the products at the reducing end. It was proposed that the acceptor reaction proceeds by displacing glucosyl and dextranosyl groups from the covalent enzyme-complex and that the acceptor reaction terminates dextran polymerization by displacing it from the active site.

Robyt and Walseth\(^10\) also showed that, for the acceptors giving a homologous series of products, the first acceptor product was an acceptor to give the second acceptor product, which was an acceptor to give the third, etc., with each
succeeding acceptor product being produced in a smaller amount than was the preceding one.

Robyt and Taniguchi\textsuperscript{11} showed that a similar reaction takes place when dextran chains are the acceptor. These investigators showed that exogenous dextran displaces dextran and glucose from the active site to give $\alpha-(1\rightarrow3)$ branch linkages between the acceptor dextran and the displaced dextran chain and the displaced glucose residue.

To study the mechanism of the acceptor reaction further, we have carried out kinetic studies with methyl $\alpha$-D-glucopyranoside, methyl 6-deoxy-$\alpha$-D-glucopyranoside, and methyl 6-deoxy-6-fluoro-$\alpha$-D-glucopyranoside as acceptors and 6-deoxysucrose as an inhibitor.
MATERIALS AND METHODS

Carbohydrates and reagents

[U-14C]Sucrose was obtained from Sigma Chemical Co. (St. Louis, MO). 6-Deoxysucrose (β-D-fructofuranosyl 6-deoxy-α-D-glucopyranoside) was synthesized as previously described12. Methyl 6-deoxy-α-D-glucopyranoside was prepared by the reduction of methyl 6-chloro-6-deoxy-α-D-glucopyranoside13 with Bu3SnH. Methyl 6-deoxy-6-fluoro-α-D-glucopyranoside was synthesized by first tritylating and then benzoylating methyl α-D-glucopyranoside, followed by the removal of the trityl group with acid. The free C-6 hydroxyl group was replaced by fluorine by reaction with diethylaminosulfur trifluoride (DAST), followed by the removal of the benzoyl groups with NaOMe in MeOH.

Enzyme

L. mesenteroides B-512FM dextranucrase (EC 2.4.1.5) was prepared as previously described14. A radiochemical assay15 using [U-14C]sucrose was used to determine the activity of the enzyme. Assays were conducted at 25° and pH 5.3 with 25 mM acetate buffer. The amount of labeled glucose incorporated in methanol-insoluble dextran was measured. Activity is given in International Units (IU), which is the amount of enzyme incorporating one μmol of D-glucose into dextran per min. The specific activity of the enzyme was 63 IU/mg of protein.
Enzyme digest conditions

All digests were carried out at 25° in 25 mM acetate buffer (pH 5.3) containing 1 mM calcium chloride, 0.1 mg/mL sodium azide, and 0.1 mg/mL Tween 80 (refs. 16, 17).

Digest conditions to obtain Hanes-Woolf plots

All digests had a total volume of 150 μL. Various sucrose concentrations (5, 10, 15, 20, and 25 mM) were used. The concentrations of methyl α-D-glucopyranoside and methyl 6-deoxy-α-D-glucopyranoside were 50 and 100 mM, and the concentration of methyl 6-deoxy-6-fluoro-α-D-glucopyranose was 100 mM. The amounts of enzyme used with methyl α-D-glucopyranoside, methyl 6-deoxy-α-D-glucopyranoside, and methyl 6-deoxy-6-fluoro-α-D-glucopyranoside were 34, 17, and 34 mIU, respectively. The initial velocities were determined by taking aliquots at various times (8, 16, 24, and 32 min) and the amount of dextran synthesized was measured as a function of time. The slope of the resulting line was determined by a linear least-squares analysis.

Digest conditions to obtain Michaelis plots

All digests had a total volume of 160 μL. Sucrose concentrations of 25, 50, 100, 200, 300, 400, 500, and 600 mM were employed. The concentrations of methyl α-D-glucopyranoside and 6-deoxysucrose were 50 and 2 mM,
respectively. The amount of enzyme in both digests was 58 mIU. The initial velocities were measured by taking aliquots at various times (10, 20, 30, and 40 min) and determined as already described.

**Determination of the amount of dextran synthesized**

Aliquots (25 μL) were taken from each digest and spotted onto Whatman 3MM filter paper squares (1.5 x 1.5 cm). These papers were immediately put into MeOH and stirred for 10 min. Washing in MeOH was repeated five times to remove any labeled D-fructose, D-glucose, unreacted sucrose, and low-molecular-weight oligosaccharide acceptor-products. A control assay using only sucrose was also carried out. The filter papers were dried, and a liquid scintillation spectrometer was used to count the radioactivity on the papers. For digests with sucrose concentrations 200 mM and above, the 25 μL aliquots were added to 0.3 M sodium hydroxide, which was neutralized after 60 min to give a 1:5 dilution. This solution (25 μL) was spotted onto paper for counting. The dilution diminishes the relatively high concentrations of unreacted sucrose, increasing the efficiency of the sucrose removal from the paper. The dilution also keeps the amount of dextran precipitated on the paper in the proper range for a quantitative determination.
RESULTS AND DISCUSSION

The action of dextransucrase can be measured in two general ways: (I) by measuring the amount of dextran synthesized, as has been done in this study, and (2) by measuring the amount of D-fructose formed. The latter is attained by either measuring the reducing value, as has been used by Koepsell et al.\(^ \text{18} \), Tsuchiya et al.\(^ \text{19} \), Ebert and Schenk\(^ \text{20} \), and Bovey\(^ \text{21} \), or by measuring the D-fructose enzymically\(^ \text{22,23} \). The reducing value method has its limitations in that it not only measures the release of D-fructose, it also measures any other reducing compounds that are produced, such as D-glucose and reducing acceptor-products. The enzymic method also has limitations as it measures both D-fructose and D-glucose, and gives low D-fructose values because of the action of D-fructose as an acceptor with dextransucrase to give leucrose. Within these limitations, the measurement of D-fructose gives the approximate determination of the overall reaction of sucrose, measuring both the amount of dextran synthesized and the amount of acceptor products formed.

Because the primary reaction of dextransucrase is the synthesis of dextran from sucrose, we have measured the rate of reaction of dextransucrase by measuring the rate of incorporation of D-glucose into dextran\(^ \text{15-17} \). We consider that this is the best and most specific method for measuring the reaction of dextransucrase, and it is especially the best
method for determining the effects of acceptors on the synthesis of dextran.

By using the determination of the rate of formation of D-fructose, several investigators\textsuperscript{4,18,21} have found that some acceptors (such as methyl $\alpha$-D-glucopyranoside, maltose, and isomaltose) produce an increase in the overall rate of reaction of sucrose with dextranse. In the present study, we have found by measuring the rate of dextran synthesis and using Hanes Woolf plots (Fig. 1-3) that methyl $\alpha$-D-glucopyranoside and methyl 6-deoxy-$\alpha$-D-glucopyranoside were very weak competitive inhibitors, with $K_i$ values of 97 and 267 mM, respectively, and that methyl 6-deoxy-6-fluoro-$\alpha$-D-glucopyranoside was a very weak noncompetitive inhibitor, with a $K_i$ value of 400 mM. It was expected that because of its structural similarity to the glucopyranosyl moiety of sucrose, methyl $\alpha$-D-glucopyranoside would bind at the sucrose binding sites. This also was suggested by the binding of $\alpha$-D-glucopyranosyl fluoride at the sucrose site where it acts as a substrate (namely, a glucosyl donor) for dextranse\textsuperscript{1}.

If an inhibitor is indeed competing with the substrate for the substrate binding site, higher concentrations of the substrate should eliminate the inhibition. Michaelis plots of the initial velocities of the incorporation of D-glucose into dextran versus sucrose concentration in the presence and absence of two inhibitors, 6-deoxysucrose and methyl $\alpha$-D-
glucopyranoside are given in Fig. 4. This plot shows that the rate of dextran synthesis by dextranse follows the Michaelis-Menten equation up to 200 mM sucrose. Above this concentration, the rate of dextran synthesis decreases. This was interpreted by Stringer and Tsuchiya\textsuperscript{5} and Bovey\textsuperscript{21} to be due to substrate (sucrose) inhibition, and by Ebert and Schenk\textsuperscript{1} to be due to sucrose acting as an acceptor. It is known that as the concentration of an acceptor is increased, the rate and amount of dextran synthesized is decreased\textsuperscript{9}. Alsop\textsuperscript{14} has shown that as the concentration of sucrose is increased, the concentration of free D-fructose and D-glucose is also increased due to their formation by the reaction of dextranse with sucrose. This increase in the concentration of D-fructose and D-glucose produces a concomitant decrease in the rate of dextran synthesized because of the diversion of the D-glucose moiety of sucrose away from its incorporation into dextran, and its incorporation into acceptor products by reaction with free D-fructose and D-glucose acceptors. Thus, it is not only sucrose itself that gives the sucrose-dependent inhibition but it is the formation of acceptor products as well. It has been suggested that sucrose acts as an acceptor and in doing so inhibits the synthesis of dextran\textsuperscript{3}. Low-molecular-weight sucrose acceptor products, for example, tri-, tetra-, and penta-saccharides, however, have never been observed.
The Michaelis plot in the presence of 2 mM 6-deoxysucrose, a known potent competitive inhibitor with a $K_i$ value of 1.6 mM (ref. 25), shows (Fig. 4) that the inhibition is completely eliminated at 500 mM sucrose. The Michaelis plot, in the presence of 50 mM methyl $\alpha$-D-glucopyranoside, a relatively good acceptor but a poor competitive inhibitor, shows that the inhibition is not eliminated at 600 mM sucrose (Fig. 4). Extrapolation of the methyl $\alpha$-D-glucopyranoside and the sucrose lines show that they do converge at an extremely high sucrose concentration of about 2 M.

A comparison of the inhibition curves of Fig. 4 for the two inhibitors is valid even though the concentrations of the two inhibitors were different by a factor of 25; i.e., 50 mM for methyl $\alpha$-D-glucopyranoside and 2 mM for 6-deoxysucrose. The $K_i$ values are vastly different - 97 mM for methyl $\alpha$-D-glucopyranoside and 1.6 mM for 6-deoxy-sucrose. The ability of sucrose to relieve the inhibition depends upon the ratio of the inhibitor concentration to the $K_i$, or $[I]/K_i$. The lower the ratio, the easier it is for sucrose to compete with the inhibitor and to eliminate the inhibition. In the inhibition studies, the ratio for methyl $\alpha$-D-glucopyranoside was (50:97 = 0.52) and the ratio for 6-deoxysucrose was (2:1.6 = 1.25). Thus, the ratio for methyl $\alpha$-D-glucopyranoside was 2.5 times lower than the ratio for 6-deoxysucrose. If methyl $\alpha$-D-glucopyranoside was competing with sucrose for the same
binding site, the increase in the sucrose concentration to 200 mM (500 mM : 2.5) should have eliminated the inhibition as it did for 6-deoxysucrose.

Because an increase in the sucrose concentration effectively eliminated the inhibition by 6-deoxysucrose but had little effect on the inhibition by methyl α-D-glucopyranoside, it is concluded that the sucrose analogue, 6-deoxysucrose, is binding at the sucrose binding site, but the acceptor-inhibitor, methyl α-D-glucopyranoside, is not binding at the sucrose binding site and, therefore, is binding at a separate acceptor binding site. The very high concentrations (2 M) of sucrose required to have the methyl α-D-glucopyranoside and sucrose lines merge further indicates that sucrose is weakly binding at the acceptor binding site as was previously postulated by Ebert and Schenk

The presence of a separate acceptor binding site is corroborated by the very weak competitive inhibition of methyl 6-deoxy-α-D-glucopyranoside (Kᵢ = 267 mM) and the strong competitive inhibition of 6-deoxysucrose (Kᵢ = 1.6 mM), and the very weak noncompetitive inhibition of methyl 6-deoxy-6-fluoro-α-D-glucopyranoside (Kᵢ = 400 mM) and the strong competitive inhibition of 6-deoxy-6-fluoro-sucrose (Kᵢ = 0.8 mM). If methyl 6-deoxy-α-D-glucopyranoside and methyl 6-deoxy-6-fluoro-α-D-glucopyranoside were binding at the same site as sucrose, it would be expected that they would be as potent
inhibitors as are the 6-deoxy- and 6-deoxy-6-fluoro-sucrose analogues. Because the methyl α-D-glucopyranoside analogues are not strong inhibitors, it can be concluded that they are not binding to the sucrose binding site.

We, therefore, propose that the active-site of L. mesenteroides B-512FM dextranulcrose has separate sucrose binding sites and acceptor binding site(s). A similar conclusion was reached by Bovey\(^{21}\), when he observed the activation effects of methyl α-D-glucopyranoside on the reaction of dextranulcrose with sucrose, by using a reducing value method to measure the rates. He further concluded that the acceptor does not compete with sucrose but it does compete for some intermediate formed later in the process, a conclusion that we also make from this study. It is reasonable to expect that all of the acceptors should bind at the same acceptor binding site, even though they might bind with different affinities. Ebert and Schenk\(^{3}\) found that some acceptors increased the rate of reaction of sucrose with B-512F dextranulcrose and some decreased the rate. These effects were additive when a mixture of acceptors were used, indicating that all of the acceptors were binding at the same site. Mayer et al.\(^{23}\) found that methyl α-D-glucopyranoside and maltose compete for the same binding site on Streptococcus sanguis dextranulcrose and suggested that all acceptors are binding at this same site.
A proposed active-site model for dextransucrase is shown in Fig. 5A. In this model there are two sucrose binding sites that are required for the synthesis of dextran and one acceptor binding site. The number of acceptor sites is not known, other than there is at least one, but there may be more than one. The incorporation of a separate acceptor binding site into the model is a refinement of the original acceptor-mechanism proposed by Robyt and Walseth. The acceptor binding site is placed between the two X-glucosyl (or dextransyl) groups (see Fig. 5B). The binding of the acceptor to the acceptor binding site, would thus, block the insertion of the glucosyl residue into the growing dextran chain, giving inhibition of the synthesis of dextran and the diversion of the glucosyl residue into acceptor products. Similarly, the acceptor can also react with the dextransyl chain (Fig. 5C) to release it from the active site and terminate its polymerization. It is further proposed that the acceptor binding site has a minimum of two glucosyl subsites as maltose, which has two glucosyl moieties, is the most effective acceptor.

Thus, although such acceptors as methyl α-D-glucopyranoside and maltose are binding at an acceptor binding site rather than at the sucrose binding sites, their kinetic effects on the initial velocity of dextran synthesis, at relatively low sucrose concentrations, appears to be
competitive with sucrose in a Hanes-Woolf plot or in a Lineweaver-Burk plot because they are competing with dextran for the glucosyl intermediate that arises from sucrose, instead of competing with sucrose for the same binding site.
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Figure 1. Hanes-Woolf plots of dextran sucrase digests with α-methyl-D-glucopyranoside. v on the Y-axis is reported as μmol of D-glucose incorporated into dextran per minute.
Figure 2. Hanes-Woolf plots of dextranase digests with 6-deoxy-α-methyl-D-glucopyranoside. v on the Y-axis is reported as μmol of D-glucose incorporated into dextran per minute.
Figure 3. Hanes-Woolf plots of dextran sucrase digests with 6-deoxy-6-fluoro-α-methyl-D-glucopyranoside. 

$v$ on the Y-axis is reported as $\mu$mol of D-glucose incorporated into dextran per minute.
Figure 4. Michaelis-Menten plots of dextran-sucrase reaction with 2 mM 6-deoxysucrose and 50 mM methyl-α-D-glucopyranoside. V on the Y-axis is reported as μmol of D-glucose incorporated into dextran per minute.
Figure 5. Model of the active site of Leuconostoc mesenteroides B-512FM dextranulose.

Symbols: X', catalytic nucleophiles; 0, glucopyranosyl residues; 0, reducing-end glucopyranoside residues; , α-1-6 glycosidic bond; — , α-1-4 glycosidic bond.

A: active site with two catalytic nucleophiles, two sucrose binding sites, and the acceptor binding site.

B: active site showing the glucosyl and dextranosyl enzyme complex, an acceptor (maltose) in the acceptor binding site, and its reaction with the glucosyl complex to give a trisaccharide acceptor product.

C: active site showing the acceptor reaction with the dextranosyl complex to give reducing-end terminated acceptor dextran product.

D: active site, without acceptor, showing the reaction of the glucosyl residue with the dextranosyl unit to give the addition of glucose to the reducing-end of the growing dextran chain. The sucrose binding sites have been omitted for clarity in C and D.
PAPER II
INTERPRETATION OF DEXTRANSEUCRASE INHIBITION
AT HIGH SUCROSE CONCENTRATIONS

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ABSTRACT

When acceptor reactions were carried out at high sucrose concentrations (≥ 200 mM), dextran synthesis was inhibited and the acceptor reactions were increased. A model, based on the known mechanisms of dextran synthesis and acceptor reactions, is proposed to explain the inhibition of dextran synthesis and the increase in the acceptor products at high sucrose concentrations. According to the model, sucrose binds to a third, low affinity binding site, allosterically changing the conformation of the active site so that dextran cannot be formed but acceptor products can be formed.
INTRODUCTION

*Leuconostoc mesenteroides* B-512FM dextran sucrase catalyzes the formation of dextran using the glucosyl part of sucrose. B-512F Dextran is a polysaccharide composed of D-glucose units linked by 95% α-(1-6) linkages and 5% α-(1-3) branch linkages.

It has been shown that dextran synthesis proceeds by a two-site insertion mechanism in which glucosyl and dextranosyl covalent enzyme intermediates are formed with dextran polymerization taking place by the addition of the glucosyl unit to the reducing end of the growing dextranosyl chain\(^1\).

When carbohydrates in addition to sucrose are present in dextran sucrase digests, some of the glucosyl groups of sucrose are transferred to the carbohydrates and are diverted from entering dextran\(^2-5\). These carbohydrates have been called acceptors and include several monosaccharides, oligosaccharides, and polysaccharides\(^3,6\). Some acceptor products, e.g., the acceptor products of maltose, isomaltose, and methyl-α-D-glucopyranoside, themselves can act as acceptors and a homologous series of acceptor products are formed\(^3\). Some acceptors, such as, D-fructose, lactose, and raffinose, give only single acceptor products\(^1,4\).

The formation of acceptor products takes place by the acceptor displacing the glucosyl and/or the dextranosyl groups from the covalent enzyme-complex\(^4\). Using B-512FM
dextran sucrase, we have shown that the acceptors do not compete with sucrose for the sucrose binding-site but bind at a separate acceptor binding-site. It has also been shown in our laboratory that the enzyme has two sucrose binding-sites and one acceptor binding-site.

By measuring either the formation of dextran or the release of fructose, it was shown that dextran synthesis follows the Michaelis-Menten equation up to 200 mM sucrose, and thereafter the rate of dextran synthesis decreases. This decrease was interpreted by Ebert and Schenk to be due to the binding of sucrose to an acceptor binding site, where it acts as an acceptor. Stringer and Tsuchiya interpreted sucrose inhibition as a possible competition between sucrose and the acceptor, methyl-\(\alpha\)-D-glucopyranoside, for a putative acceptor-site since methyl-\(\alpha\)-D-glucopyranoside relieved the inhibition when the amount of fructose released was measured. Neely also explained the inhibition as due to the binding of a second sucrose molecule to dextran sucrase-sucrose complex, in which the enzyme had a greater affinity for the first sucrose molecule than for the second sucrose molecule, with the second sucrose molecule binding to the acceptor-site, thus, inhibiting the reaction.

Bovey explained the sucrose inhibition as the binding of a second molecule of sucrose to dextran sucrase to form an inactive dextran sucrase-glucosyl-sucrose complex although no
evidence was presented.

The present work was undertaken to explain sucrose inhibition of dextranucrase because of the various interpretations that have been proposed with essentially the absence of supporting evidence.
EXPERIMENTAL

Chromatography

Thin layer chromatography (TLC) was conducted on Whatman K5 plates using three ascents of solvent 1, acetonitrile/water 85:15 (v/v) and/or three ascents of solvent 2, nitromethane/water/1-propanol 2:3:5 (v/v/v) and two ascents of solvent 3, acetone/chloroform/water 85:10:5 (v/v/v). The carbohydrates were visualized by dipping the plates into 5% (v/v) sulfuric acid in ethanol containing 0.5% thymol and 0.5% α-naphtol or 20% (v/v) sulfuric acid in methanol, followed by heating for 10 minutes at 110 °C. The sulfuric acid in methanol system was used only for the determination of dextran.

Enzyme

*L. mesenteroides* B-512FM dextranucrase was prepared as previously described\(^\text{16}\). The activity of the enzyme was determined by a radiochemical assay\(^\text{17}\) using [U-\(^{14}\)C] sucrose. Assays were conducted at 25°C and pH 5.4 with 25 mM acetate buffer containing 1 mM calcium chloride, 0.1 mg/ml sodium azide, and 0.1 mg/ml Tween 80. The amount of radioactive glucose incorporated into methanol insoluble dextran was determined by liquid scintillation spectrometry. The activity is given in international units (IU), i.e., the amount of enzyme necessary to incorporate one μ mol of D-glucose into
dextran in one minute. The activity of the enzyme used was 120 IU/ml and the specific activity was 90 IU/mg of protein.

**Enzyme-digest conditions**

The following digests containing 2.4 IU dextransucrase in 25 mM acetate buffer (pH 5.4) were prepared:

Digest 1: 5 ml of 50 mM sucrose;

Digest 2: 5 ml of 50 mM sucrose containing 50 mM methyl-\(\alpha\)-D-glucopyranoside;

Digest 3: 0.5 ml of 500 mM sucrose;

Digest 4: 0.5 ml of 500 mM sucrose containing 500 mM methyl-\(\alpha\)-D-glucopyranoside.

The reactions were carried out at room temperature (23°C) for 4 hours. TLC, using solvent 1, showed that all the sucrose had been converted into products. At the end of the reaction, digests 3 and 4 were added to 4.5 ml of acetate buffer so that all of the digests had the same final volume (5 ml) for analysis of the products.

The amounts of dextran formed, using solvent 2, and remaining methyl-\(\alpha\)-D-glucopyranoside, using solvent 3, in the
digests were determined by TLC densitometry (Uniscan, Analtech, Inc Newark, DE). Dextran with a molecular weight of 10,500 (Sigma, St. Louis, MO) was used as standards for the determination of dextran.
RESULTS

Fig. 1 shows the TLC of the reaction digests. Using TLC densitometry, the reaction digests were analyzed as shown in Table I and the results are as follows. All the glucosyl units of sucrose were incorporated into dextran when 50 mM sucrose (Digest 1) was used. In digest 2 (50 mM sucrose and 50 mM methyl-\(\alpha\)-D-glucopyranoside), 77.6% of the glucosyl units of sucrose were incorporated into dextran and 22.4% of the glucosyl units were transferred to acceptors. When 500 mM sucrose (Digest 3) was used, 94.2% of the glucosyl units of sucrose were incorporated into dextran and the remaining (5.8%) were transferred to fructose, the by-product of the reaction, to form leucrose. In digest 4 (500 mM sucrose and 500 mM methyl-\(\alpha\)-D-glucopyranoside), almost all of the glucosyl units of sucrose (95.0%) were transferred to acceptors, and there was only a small amount (5.0%) of dextran formed.

To determine the effect of the sucrose concentration on the acceptor reactions, we have used the same amounts of enzyme, sucrose, and methyl-\(\alpha\)-D-glucopyranoside in Digests 2 and 4 and found that only 7.6% of the methyl-\(\alpha\)-D-glucopyranoside reacted in Digest 2, which consisted of 5 ml of 50 mM sucrose and 50 mM methyl-\(\alpha\)-D-glucopyranoside, but 38.6% of the methyl-\(\alpha\)-D-glucopyranoside reacted in Digest 4, which consisted of 0.5 ml of 500 mM sucrose and 500 mM methyl-\(\alpha\)-D-glucopyranoside. This result showed that even though the
amounts of sucrose, acceptor, and enzyme were the same in the two digests, there was more acceptor products formed inDigest 4 in which the concentration of sucrose was high and the sucrose was inhibiting dextran synthesis.
DISCUSSION

It has been shown by several studies that the rate of dextran formation decreases if the sucrose concentration is greater than 200 mM\(^7,9-11\). Alspop\(^8\) reported that the size and the amount of dextran synthesized by dextranucrase is dependent primarily on the initial concentration of sucrose. When 2\% w/v (58 mM) sucrose was used, almost all of the glucosyl groups of sucrose were incorporated into high molecular weight dextran, but when the concentration of sucrose was increased, the amount and molecular weight of dextran decreased and some oligosaccharides were formed. The amount of D-glucose, which is obtained when water acts as an acceptor, also increased. These changes were much more obvious at sucrose concentrations greater than 200 mM in which the rate of dextran formation decreased as the sucrose concentration increased.

Tsuchiya\(^9\) used 70\% (2 M) sucrose and found that the products were mostly oligosaccharides, with some low molecular weight dextran (≤35,000 Da), and no high molecular weight dextran.

Our results confirmed that the amount of dextran synthesized depends on the sucrose concentration in which concentrations greater than 200 mM give lower amounts of dextran. Our results also show that acceptor reactions are not affected by high sucrose concentrations.
At high sucrose concentrations in the presence of equimolar amounts of good acceptors, such as methyl-α-D-glucopyranoside or maltose, dextransucrase does not catalyze the synthesis of dextran but does catalyze the synthesis of acceptor-products. For example, 500 mM sucrose in the presence of 500 mM methyl-α-D-glucopyranoside, dextransucrase gave very small amounts of dextran and transferred most of the glucose units of sucrose to methyl-α-D-glucopyranoside and subsequent acceptor-products (Fig. 1). At an identical ratio of 1:1 acceptor to sucrose but at a concentration of 50 mM, instead of 500 mM, acceptor products were produced along with dextran. Similar results have been obtained using sucrose and maltose20.

Ebert and Schenk13, Stringer and Tsuchiya19, and Neely14,15 each interpreted the sucrose inhibition of dextransucrase by high sucrose concentration (i.e., ≥ 200 mM) as the binding of sucrose at the acceptor binding-site. Their interpretations do not explain the increase in acceptor reactions that occur at high sucrose concentrations.

Bovey11 postulated that dextran synthesis occurs by the formation of a glucosyl-enzyme complex in which the glucose is transferred to a primer. He also postulated that acceptor reactions occur by a different route in which the acceptor reacts with an enzyme-sucrose complex instead of an enzyme-glucosyl complex. Both of these proposed mechanisms have been
shown by Robyt et al.¹ and Robyt and Walseth² to be incorrect. Based on these incorrect mechanisms, Bovey¹¹ interpreted inhibition by high sucrose concentrations as the result of sucrose reversibly binding to the enzyme-glucosyl complex, which is the first step in his mechanism of dextran synthesis. This dextransucrase-sucrose-glucosyl complex was postulated to be incapable of further reaction because the sucrose was blocking the active-site. Bovey then proposed that the reason acceptor reactions were not affected by high sucrose concentrations was because they were occurring through an enzyme-sucrose complex that could not react with a second sucrose molecule.

In view of what is now known about the mechanisms of dextran synthesis¹ and acceptor reactions⁴ of dextransucrase, we propose the following model to explain the simultaneous sucrose inhibition of dextran synthesis and the increased synthesis of acceptor products at high sucrose concentrations. It is known that the active site of dextransucrase has two high affinity sucrose binding-sites¹,⁸ and one acceptor binding-site⁸. Acceptors can bind in the acceptor-site and react with either the glucosyl or the dextranosyl groups that are covalently attached to the X-nucleophilic groups at the enzyme active-site⁷. Sucrose does not bind in the acceptor binding-site, even at very high sucrose concentrations⁷. At high sucrose concentrations (i.e. above 200 mM), it is
proposed that sucrose binds at a low affinity, third sucrose binding-site, that allosterically changes the conformation of the active-site (see B in Fig. 2). This allosteric binding does not affect the binding of sucrose at the sucrose binding sites nor does it affect the formation of glucosyl-enzyme covalent complexes. The binding of sucrose in the allosteric site changes the conformation of the active-site in such a way as to prevent the interaction of the two glucosyl groups to give dextran elongation. The allosterically inhibited enzyme, however, still allows an acceptor to bind at the acceptor binding-site and interact with one of the glucosyl-enzyme complexes to give acceptor products (see Fig. 2 B and C).

Methyl-α-D-glucopyranoside and the subsequent acceptor products, which are mainly produced by allosterically inhibited dextranucrase, release glucosyl units from both the uninhibited dextranucrase and the allosterically inhibited dextranucrase. This explains the incorporation of almost all the glucosyl units of sucrose into acceptor products, rather than into dextran in the digest containing 500 mM sucrose and 500 mM methyl-α-D-glucopyranoside.

At high sucrose concentrations, in the absence of acceptors, the allosterically inhibited enzyme can still form dextranucrase-glucosyl complexes, but it does not synthesize dextran due to the change in the conformation of the active-site (Fig. 2 B). In the absence of acceptors, the glucosyl
units can be released by acceptor reactions with water. This, thus, explains the observed increase in D-glucose when the sucrose concentrations are greater than 200 mM\textsuperscript{18}. The increase in the amounts of D-glucose, along with the concomitant presence of D-fructopyranose, provide acceptors that also can release D-glucose from the active-site and form oligosaccharides that were observed by Alsop\textsuperscript{18} and Tsuchiya\textsuperscript{19}.

In summary, then, the sucrose allosteric inhibition of dextran sucrase explains all of the observed effects of high (> 200 mM) sucrose concentrations: namely, the inhibition of dextran synthesis, the increase in the formation of acceptor products, and the increase in the formation of D-glucose and oligosaccharides.
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20 D. Su and J. F. Robyt, manuscript in preparation.
Table I. Percentages of the total D-glucose from sucrose in products of *Leuconostoc mesenteroides* B-512FM Dextranucrase Digests

<table>
<thead>
<tr>
<th>Digests</th>
<th>% Dextran</th>
<th>% Acceptor product(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 5 mL 50 mM sucrose</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2. 5 mL 50 mM sucrose + 50 mM methyl-α-D-glucopyranoside</td>
<td>78</td>
<td>22</td>
</tr>
<tr>
<td>3. 0.5 mL 500 mM sucrose</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>4. 0.5 mL 500 mM sucrose + 500 mM methyl-α-D-glucopyranoside</td>
<td>5</td>
<td>95</td>
</tr>
</tbody>
</table>

* Each digest contained 2.4 IU of enzyme.
Figure 1. TLC analysis of dextran sucrase digests with different amounts of sucrose and methyl-α-D-glucopyranoside.

Lane 1: 50 mM sucrose digest
Lane 2: 50 mM sucrose + 50 mM methyl-α-D-glucopyranoside digest
Lane 3: 500 mM sucrose digest
Lane 4: 500 mM sucrose and 500 mM methyl-α-D-glucopyranoside digest
Lane 5: Methyl-α-D-glucopyranoside, fructose, glucose and sucrose standards
Lane 6: Isomaltodextrin series standards
IMn: Isomaltodextrins of d.p. n
APn: Acceptor product where n represents first, second, third, etc. product formed.
α-Me-Glc: Methyl-α-D-glucopyranoside
Glc: Glucose
Fru: Fructose
Suc: Sucrose
Leu: Leucrose

TLC was run on Whatman K5 plates using three ascents of solvent 2 up to the middle of the plate, and then three ascents of solvent 1.
Fig. 2 Model for inhibition of *L. mesenteroides* B-512FM dextransucrase by high sucrose concentrations.

A, fully active uninhibited dextransucrase active-site, showing two high affinity sucrose binding-sites (b and b'), a lower affinity allosteric sucrose binding-site (c), and an acceptor binding-site (a); B, allosterically inhibited dextransucrase by high sucrose concentration in which the conformation of the active-site has been changed so that dextran synthesis cannot occur; C, acceptor reaction of allosterically inhibited dextransucrase in the presence of methyl-α-D-glucopyranoside acceptor and the formation of acceptor-product, methyl-α-isomaltoside.
PAPER III
A NEW METHOD FOR THE PREPARATION OF SUGAR MIXTURES CONTAINING
ISOMALTODEXTRINS FROM SUCROSE

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We have produced a sugar mixture containing isomaltodextrins, fructose, glucose, and leucrose using a new technique. Sucrose was first treated with yeast invertase to obtain invert sugar, a mixture of fructose and glucose. After inactivating the invertase, the invert sugar was added to sucrose and reacted with Leuconostoc mesenteroides B-512FM dextranucrase to produce isomaltodextrins and leucrose. Depending upon the molar ratio of invert sugar to sucrose, isomaltodextrins with different degree of polymerization (DP) were obtained.

This sugar mixture can be produced in large quantity very cheaply and could find many uses in the food industry due to its sweetness and health benefits. Isomaltodextrins are known to enhance the Bifidobacterium, beneficial bacteria, that are naturally found in the human large intestine.
INTRODUCTION

Sucrose is the most commonly used sweetener. It, however, contributes significantly to the formation of dental plaque and caries\(^1,2\). Some of the glucose of sucrose is converted into glucan by *Streptococcus mutans* in the oral cavity. This glucan binds to teeth and aids in the adhesion of *Streptococcus mutans* to teeth surfaces. The resulting fructose is fermented to lactic acid by these bacteria and this acid is held close to teeth surfaces, where it attacks the enamel, leading to dental caries. Restricting sucrose in diet does decrease the numbers of *Streptococcus mutans* in the dental plaque\(^3,4\).

Several kinds of new sugars have been developed to prevent tooth decay. Sucrose was converted into isomaltulose\(^5\), using immobilized \(\alpha\)-glucosyltransferase from *Protaminobacter rubrum*. This sugar does not cause tooth decay and has 42\% of the sweetness of sucrose. Leucrose also has been produced using sucrose, fructose, and dextranase\(^6,8\). Leucrose, \(\alpha\)-D-glucopyranosyl (1-5)-D-fructopyranose, is a non-cariogenic sugar with a sweetness about 50 to 60 \% that of sucrose. Leucrose is not fermented by several kinds of oral microorganisms and inhibits the uptake and fermentation of sucrose by *Streptococcus mutans* NCTC 10449\(^9\).

It has been found that several different kinds of carbohydrates will enhance the growth of *Bifidobacteria* in the
human large intestine\textsuperscript{10-13}. The \textit{Bifidobacteria} occur naturally in the human intestine and contribute many healthful benefits\textsuperscript{13-15}, including synthesis of B-complex vitamins, reduction in serum cholesterol, anticarcinogenic effect, and resistance to some infections. One of the carbohydrates enhancing the \textit{Bifidobacteria} is 6'-galactosyllactose, which is present in human milk. This sugar promoted the growth of piglets and could be used in infant formula and animal feed. It has been produced synthetically using lactose and \(\beta\)-galactosidase in Japan\textsuperscript{16}. It was also shown in France that when pigs were fed with oligosaccharides which can reach large intestine without being digested in small intestine, the pigs were significantly heavier than the control pigs. The average weight increase over the controls was 9\% in three months. It is thought that these oligosaccharides enhance the beneficial bacteria, \textit{Bifidobacteria}, in the colon\textsuperscript{17}.

There are two ways to increase the number of \textit{Bifidobacteria}: (a) having bifid-amended foods and beverages, which is becoming common in Japan and Europe\textsuperscript{14}; there are also some US milk companies that are adding live \textit{Bifidobacteria} to their dairy products, and (b) eating specific carbohydrates that promote the growth of \textit{Bifidobacteria} in the large intestine.

The carbohydrates that enhance the \textit{Bifidobacteria} in the human intestine include isomaltodextrins\textsuperscript{10-12}, fructooligosaccharides\textsuperscript{10,13}, and galactooligosaccharides\textsuperscript{10}.
Isomaltodextrins selectively promote the growth of *Bifidobacteria* and thereby inhibit the growth of harmful bacteria, such as *Clostridia* that cannot use them	extsuperscript{18}. When rats were given drinking water with 3% isomaltodextrins, no adverse biochemical effects were observed, and mutagenicity tests using bacteria were negative	extsuperscript{19}. It was also shown by another study that the levels of serum triacylglycerol and free fatty acids were significantly lower when isomaltodextrins or fructooligosaccharides were ingested than it was when sucrose or maltose were ingested	extsuperscript{20}. Isomaltodextrins also reduce the harmful cariogenic effects of sucrose, when sucrose and isomaltodextrins are consumed together	extsuperscript{21,22}. Further, it has been shown that isomaltodextrins increase B-lymphocytes, thus, helping the immune system fight against infection	extsuperscript{23}. It was also found that solutions composed of isomaltodextrins can be administered intravenously to humans to prevent the dextran-antibody precipitation reaction, which is observed in about 5% when a solution of dextran is given to humans as a plasma substitute	extsuperscript{24}.

A health drink, containing isomaltodextrins as a sweetening agent, has been patented for its promotion of bifidus bacteria in the large intestines. It has been claimed that the drink is especially good for infants, the elderly, and the ill patients	extsuperscript{25}. Several food preparations, containing isomaltodextrins also have been patented in Japan	extsuperscript{26,27}. Animal
feeds containing isomaltodextrins as an additive have also been prepared in Japan.

Using sugars in food preparations that are enhancers for the *Bifidobacteria* and have no cariogenity and are not caloric will be very beneficial.

We have developed a process by which it is possible to produce healthful sugar mixtures using sucrose, yeast invertase, and *Leuconostoc mesenteroides* B-512FM dextranucrase. The components of the sugar mixture are fructose, glucose, leucrose, and isomaltodextrins. The reactions taking place during the process are shown in Fig. 1. First sucrose is hydrolyzed by yeast invertase to glucose and fructose, then sucrose and dextranucrase are added to produce leucrose and a mixture of isomaltodextrins. This mixture of sugars could have many health benefits to man as the isomaltodextrins are *Bifidobacteria* enhancers, and leucrose is a noncariogenic sweetener. Fructose and glucose are also sweet and do not contribute to the formation of dental plaque, a prequiste for tooth decay. The sugar mixture can be produced on a large scale, inexpensively by using immobilized invertase and dextranucrase. As indicated above this sugar mixture can find many applications. It could replace sucrose in some food preparations and high fructose corn syrup.
EXPERIMENTAL

Chromatography

Thin layer chromatography (TLC) was performed on Whatman K5 silica gel plates (Whatman Chemical Separatins, Inc., Clifton, NJ). TLC plates were developed in one of the following solvent systems: (A) acetonitrile/water 85:15 (v/v) or (B) nitromethane/water/1-propanol 2:3:5 (v/v/v). The carbohydrates were visualized by dipping the plates into a solvent system composed of 5% sulfuric acid, and 0.5% α-naphthol in ethanol, followed by heating for 10 minutes at 120°C.

Enzymes

Leuconostoc mesenteroides B-512FM dextranucrase was prepared as previously described. Dextranucrase activity was determined by a radiochemical assay using [U-14C] sucrose and is given in International Units, IU, which is the amount of enzyme necessary to incorporate 1 μ mole of D-glucose from sucrose into dextran in 1 minute at pH 5.4 and 25°C. The activity of dextranucrase was 42 IU/ml. Yeast invertase (grade V) was purchased from Sigma Chemical Co., St. Louis, MO. and had an activity of 30 units/mg. One unit of Invertase hydrolyzes one μ mole of sucrose to glucose and fructose in one minute at pH 4.5 at 55°C.
Preparation of sugar mixtures containing isomaltodextrins from sucrose

Sucrose solution (1.5 M), prepared using 5 g of sucrose and 25 mM acetate buffer (pH 5.4), was reacted with 50 mg of yeast (*Saccharomyces cerevisiae*) invertase at room temperature (23°C) for one hour. TLC (two ascents of solvent A) showed that the reaction was complete. Invertase was inactivated by heating at 95°C for 20 minutes. Invert sugar solution was added to sucrose, followed by dextran sucrase to obtain two different digests (6 mL total volume each). The first digest consisted of 0.5M invert sugar, 1M sucrose, and 30 IU dextran sucrase. The second digest consisted of 1M invert sugar, 1M sucrose, and 30 IU dextran sucrase. The reactions were allowed to go for 16 hours at room temperature (23°C). TLC (two ascents of solvent A) showed that all of the sucrose had been consumed. The components of the two digests were analyzed by TLC (10×20 cm Whatman K5 plate, by first using three ascents of solvent B up to two thirds of the plate followed by four ascents of solvent A to the top of the plate) (See Fig. 2). The amount of each carbohydrate in the two digests was determined using an imaging densitometer (Bio-Rad Laboratories, Inc., Hercules, CA) and standards (fructose and glucose).
RESULTS AND DISCUSSION

The results are shown in Table I. Digest I had fructose, glucose, leucrose, and isomaltodextrins up to DP of 12. Digest II had fructose, glucose, leucrose, and isomaltodextrins up to DP of 6.

Isomaltodextrins have been produced by using different methods and carbohydrates such as starch\textsuperscript{10,11}, glucose\textsuperscript{32}, maltose\textsuperscript{32}, dextran\textsuperscript{13,34}, and sucrose along with glucose\textsuperscript{35}. Starch is treated first with thermostable bacterial \(\alpha\)-amylase, then a mixture of soybean \(\beta\)-amylase and \textit{Aspergillus niger} transglucosidase, a type of \(\alpha\)-glucosidase. The sugar mixture contains glucose, maltose, and isomaltodextrins. This sugar mixture is used presently as a syrup in soft drinks, confectionery products, bakery goods, and several other food preparations\textsuperscript{30}. In a recently developed method in Japan, bacterial saccharifying \(\alpha\)-amylase from \textit{Bacillus subtilis} and neopullulanase are added to a gelatinized starch solution\textsuperscript{31}. The resulting syrup consisted of glucose, maltose, panose, and isomaltodextrins. Glucose or maltose and cellulase from \textit{Aspergillus niger} were also used for the production of isomaltodextrins\textsuperscript{32}. A novel endodextranase from \textit{Fusarium} sp\textsuperscript{33}, and an exo-type dextranase from \textit{Arthrobacter japonicus}\textsuperscript{34} were used to produce isomaltodextrins from dextran. These preparations, however, are all quite expensive. Sucrose along with glucose and dextranucrase have also been reported for
the synthesis of isomaltodextrins. In our new method, we use sucrose, yeast invertase, and *L. mesenteroides* B-512FM dextran sucrase for the production of sugar mixtures containing isomaltodextrins. The cost involved in the process is quite low. Yeast invertase, one of the enzyme used, is a thermostable enzyme and has been produced industrially on a large scale and is very inexpensive. Even whole yeast cells can be used as invertase after a short heat treatment or a brief contact with concentrated ethanol. We have shown that whole yeast cells can be used as invertase: 1 g of Fleischmann's yeast was suspended in 4 mL of distilled water and kept in a water bath at 70°C for four minutes, then cooled in an ice-water bath. These heat treated yeast cells were non-viable and incapable of fermenting glucose and fructose, but they had invertase activity. Dextran sucrase, the second enzyme used in the process, is also an industrially important enzyme and is produced on a large scale. We have successfully immobilized dextran sucrase and invertase and used them for the production of isomaltodextrins. The product distribution was similar to those obtained using the soluble enzymes.

The sugar mixture obtained from our process has isomaltodextrins, fructose, glucose, and leucrose. The sugar mixture is sweet due to the presence fructose, glucose, and leucrose, and has the many health benefits of isomaltodextrins. It can be produced cheaply on a large scale
and has potential use in soft drinks and many other food preparations that could be marketed as a health food.
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Figure 1. Reactions taking place for the production of sugars containing isomaltodextrins from sucrose using yeast invertase and dextranucrase.
Table I. Percentage of carbohydrates in Digest I\textsuperscript{a} and Digest II\textsuperscript{b}

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Digest I</th>
<th>Digest II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>52.1\textsuperscript{c}</td>
<td>50.9\textsuperscript{c}</td>
</tr>
<tr>
<td>Glucose</td>
<td>13.3</td>
<td>19.0</td>
</tr>
<tr>
<td>Leucrose</td>
<td>9.4</td>
<td>9.6</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>2.7</td>
<td>5.1</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>2.5</td>
<td>5.4</td>
</tr>
<tr>
<td>Isomaltotetraose</td>
<td>3.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Isomaltopentaose</td>
<td>4.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Isomaltohexaose</td>
<td>3.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Isomaltoheptaose</td>
<td>2.8</td>
<td>-</td>
</tr>
<tr>
<td>Isomaltooctaose</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>Isomaltonanoose</td>
<td>1.7</td>
<td>-</td>
</tr>
<tr>
<td>Isomaltodecaose and bigger</td>
<td>2.3</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Digest I consisted of 0.5M invert sugar, 1M sucrose, and Dextranucrase (30 IU).

\textsuperscript{b}Digest II consisted of 1M invert sugar, 1M sucrose, and Dextranucrase (30 IU).

\textsuperscript{c}Percentages determined by quantitative TLC, using an imaging densitometer.
Figure 2. TLC analysis of dextranucrase digests

Lane 1: Digest I, 0.5M invert sugar + 1M sucrose
Lane 2: Digest II, 1M invert sugar + 1M sucrose
Lane 3: Fructose, glucose, and sucrose standards
Lane 4: Isomaltodextrins standards

Fru: Fructose
Glc: Glucose
Suc: Sucrose
Iso: Isomaltulose
Leu: Leucrose
IMn: Isomaltodextrins of dp n

TLC was run on Whatman K5 plate using three ascents of solvent B up to two thirds of the plate, and then four ascents of solvent A to the top of the plate.
GENERAL SUMMARY

When acceptors and sucrose are present in a digest, dextransucrase carries out two different reactions, dextran synthesis and formation of oligosaccharides by transfer of glucose units to the nonreducing end of acceptors. During the acceptor reactions, it was natural to assume that the acceptors compete with sucrose for the sucrose binding-site because of the structural similarities between sucrose and the acceptors such as glucose, maltose, and methyl α-D-glucopyranoside. Although Hanes-Woolf plot showed that methyl α-D-glucopyranoside was an apparent competitive inhibitor for dextransucrase, a Michaelis-Menten plot using methyl α-D-glucopyranoside in the presence of high concentration of sucrose showed that it was not a real competitive inhibitor. We have also found that methyl 6-deoxy-α-D-glucopyranoside and methyl 6-deoxy-6-fluoro-α-D-glucopyranoside were weak inhibitors, whereas the corresponding sucrose analogues 6-deoxysucrose and 6-deoxy-6-fluorosucrose are very potent inhibitors for dextransucrase. From these findings, it is concluded that the acceptor, methyl α-D-glucopyranoside, does not compete with sucrose for the sucrose binding-sites and it binds at a separate acceptor binding-site.

Dextran synthesis follows Michaelis-Menten equation up to 200 mM sucrose concentration. At high sucrose concentrations
(≥200 mM), the rate of dextran formation decreases and acceptor reactions are increased. We have proposed a model to explain the inhibition of dextran synthesis and the increase in the acceptor products at high sucrose concentrations. According to the model, sucrose binds to a third low-affinity binding-site changing the conformation of the active site so that dextranucrase cannot synthesize dextran but can carry out acceptor reactions to form oligosaccharides.

Isomaltodextrins are known enhancers of Bifidobacteria, a beneficial bacteria naturally found in human large intestine. We have developed a new method for the preparation of sugar mixtures containing isomaltodextrins. Invert sugar, obtained by hydrolysis of sucrose by yeast invertase, was added to sucrose and dextranucrase to synthesize leucrose and isomaltodextrins.
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