Carbohydrate Research, 211 (1991) 337–342
Elsevier Science Publishers B.V., Amsterdam

Note

Acceptor activity of affinity-immobilized dextransucrase from *Streptococcus sanguis* ATCC 10558

Susan F. Kobs*
Department of Chemistry, The Ohio State University, Columbus, OH 43210 (U.S.A.)

(Received March 14th, 1990; accepted in revised form September 5th, 1990)

INTRODUCTION

Dextransucrase (sucrose: 1,6-α-D-glucan 6-α-D-glucosyltransferase, EC 2.4.1.5) is produced by various bacteria including *Leuconostoc* and *Streptococcus* species. This enzyme synthesizes dextran by polymerization of glucosyl moieties from the substrate sucrose with the concomitant production of fructose. The enzyme catalyzes four discrete reactions: (*i*) glucosyl transfer to acceptor molecules, (*ii*) autopolymerization, (*iii*) sucrose hydrolysis, and (*iv*), an isotope exchange reaction[1]. These reactions probably proceed *via* a glucosylated enzyme intermediate[2,3]. Dextransucrase may possess multiple active sites or a catalytic domain which contains areas both for catalysis (*i.e.*, formation of the glucosyl–enzyme intermediate) and acceptor substrate binding[4].

There have been numerous attempts to prepare immobilized forms of dextransucrase[5–7]. Major problems with previous preparations include large losses of activity upon immobilization[6] and alterations in the reaction kinetics of the immobilized enzyme[5]. The products of the enzymatic reaction either act as acceptor molecules (D-fructose) or clog the pores of the support matrix (dextran), thereby greatly limiting the utility of the immobilized enzyme preparation[5]. This report describes the use of fully active, affinity-immobilized[8] dextransucrase (from *Streptococcus sanguis* ATCC 10558) and the role this system could play in the study of the reaction pathways and the structural characteristics of this enzyme.

EXPERIMENTAL

Materials. — Sephadex G-200 (40–120 μm) was from Pharmacia Fine Chemicals; D-glucose, sucrose, D-fructose, and D-glucitol were obtained from Sigma Chemical Co. [U-^{14}C-Glucose]sucrose and [U-^{14}C]maltose were obtained from New England Nuclear Corp. and Amersham Corp., respectively. Bicinchoninic acid (BCA) protein assay was

* Address for correspondence: Comprehensive Cancer Center, The Ohio State University, 410 W. 12th Avenue, Columbus, OH 43210.

0008-6215/91/$ 03.50

from Pierce Chemical Co. Sodium dodecyl sulfate (SDS) was obtained from Bio-Rad Laboratories.

Methods. (*a*) *Preparation of dextransucrase.* — Crude concentrated dextransucrase (5.6 U/mL) was prepared from cultures of *Streptococcus sanguis* ATCC 10558. Purified enzyme (>95% homogeneity; specific activity >73 U/mg) was prepared as previously described[9].

(*b*) *Immobilization of dextransucrase on Sephadex G-200.* Sephadex G-200 was equilibrated in Buffer A (10mM sodium phosphate, pH 6.3, 0.02% NaN_3). Dextransucrase (50 mL; 5 U/mL) was added to 50 mL of settled resin, and the mixture was slowly stirred for 30 min at 4°. The mixture was poured into a column, and the resin was washed with Buffer A until the absorbance of the eluate was <0.01 a.u. at 280 nm. The resin was transferred to a graduated cylinder, allowed to settle, and the gel volume was matched (v/v) with Buffer A to give a 1:1 gel suspension. Much (>55%) of the activity originally in solution was immobilized on the resin (4.7–5.2 U/mL of gel suspension). This preparation was stable for at least one week when stored at 4°.

(*c*) *Acceptor activity of immobilized dextransucrase.* The 1:1 gel suspension (1 mL) was aspirated into a 3-mL plastic syringe. An aliquot of sucrose solution was pipetted into the end of the syringe, and a filter (Gelman Acro LC3A, 0.45 μm) was affixed to the tip. The reaction solution was mixed by rotating the syringe barrel. At intervals the supernatant fluid was expressed through the filter and an aliquot removed for analysis.

(*d*) *Preparation and hydrolysis of* ^{14}*C-labeled Sephadex G-200.* An aliquot of 1:1 gel suspension (1.0 mL) was aspirated into a 3-mL plastic syringe. [U-^{14}C-Glucose]sucrose (30mM, 0.01 μCi) was added, and a filter was attached to the syringe. The solution was mixed by swirling the contents in the syringe barrel. At intervals the supernatant fluid was expressed through the filter and an aliquot was removed for fructose and radioactivity determinations. When the reaction was complete, the contents of the syringe were quantitatively transferred to a column (0.7 x 7 cm), and the gel was washed extensively (50 column volumes) with Buffer A. Dextransucrase was eluted by washing the column with Buffer A containing 0.1% (w/v) SDS, and the resin was washed with water to remove the detergent and buffer salts. All column eluates were analyzed for radioactivity, protein, and sugar content. The ^{14}C-labeled gel was transferred to a 16 × 125 mm screw-cap tube, excess water was removed, and the gel was lyophilized to dryness. The dried gel was reduced with $NaBH_4$ (100 μL of 20 mg/mL $NaBH_4$, freshly prepared) for 16 h at 50°. The solution was cooled, neutralized by addition of N HCl (50 μL), and lyophilized to dryness. The reduced gel mixture was hydrolyzed in 2M HCl (1.0 mL, 5 h at 96°), lyophilized to dryness, and re-lyophilized twice from water to remove trace amounts of HCl. A control sample of [U-^{14}C]maltose was similarly reduced and hydrolyzed. Components of the hydrolyzates were separated by paper chromatography, and the chromatograms were analyzed by liquid scintillation counting.

(*e*) *Analyses.* Dextransucrase activity (in solution) and D-fructose concentration were determined using a coupled-enzyme assay system[1]. Protein was measured by absorbance at 280 nm or by the BCA protein microassay. The anthrone assay[10] was utilized to measure sugar content, and sodium dodecyl sulfate was analyzed using a dye-binding assay[11].

Paper chromatography (descending technique) was performed on Whatman 3MM paper using 9:1:1 2-butanone–acetic acid–H_3BO_3–saturated water as solvent. The chromatograms were cut into 0.5 × 2-cm strips which were immersed in ScintiVerse E cocktail (10 mL), and the radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 460 CD instrument). A test chromatogram of D-glucose, D-glucitol, sucrose, maltose, and oligosaccharide standards was stained using a modified silver nitrate dip procedure[12]. Thin-layer chromatography was run using H_3BO_3-impregnated Silica Gel G plates developed in 3:1:1 2-butanone–acetic acid–water solvent. Plates were exposed to Kodak SB film for 5–7 days at −70°, and the film was subsequently developed using Kodak film-processing reagents.

RESULTS AND DISCUSSION

Dextransucrase was affinity immobilized on Sephadex G-200 gel with at least 90% retention of enzyme activity (activity present in solution prior to immobilization). The immobilized enzyme exhibits exclusively acceptor activity, and the kinetics of this reaction are remarkably similar to the enzyme-acceptor activity in solution. The double-reciprocal plots are linear (Fig. 1) in contrast to results with other immobilized

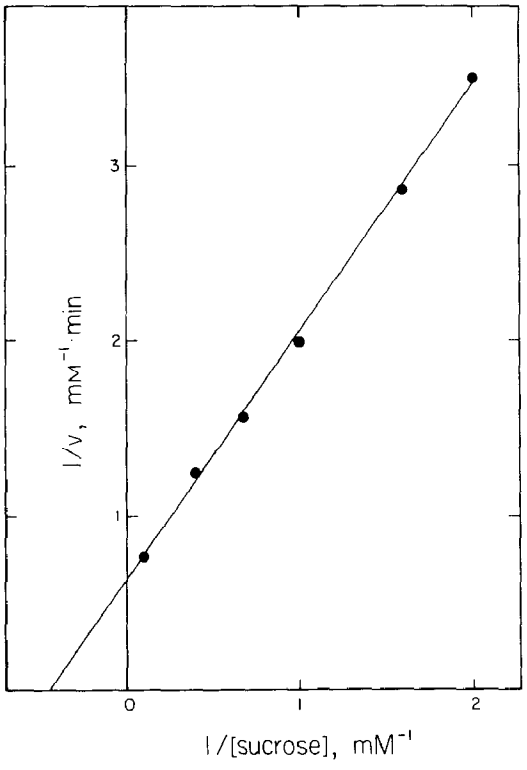

Fig. 1. Kinetics of reaction of sucrose with affinity-immobilized dextransucrase. Reactions were run at 25° using 1:1 gel suspension (1.0 mL) as described in the Experimental; sucrose concentration ranged from 0.5 to 10mM. D-Fructose concentration was measured using the coupled-enzyme assay system.

preparation of the enzyme[5] which show saturation effects. The K_m (2.3mM) and V_{max} (83.1 μmol/min/mg) are comparable with values for the reaction in homogeneous solution[1].

The immobilized enzyme hydrolyzes sucrose, adding the glucosyl portion of this substrate to the non-reducing end of the dextran acceptor (in this case, Sephadex G-200 polymer) and producing the by-product, D-fructose, in >95% of the theoretical yield (Fig. 2). This is the characteristic acceptor reaction catalyzed by dextransucrase[1]. Analysis by paper and thin-layer chromatography indicates that this reaction is nearly quantitative, as only trace amounts of sucrose remain following reaction completion (data not shown).

The ^{14}C-labeled Sephadex G-200 gel produced *via* the acceptor reaction with ^{14}C-glucose-labeled sucrose as the donor substrate was analyzed for the presence of ^{14}C-labeled D-glucitol. The presence of D-glucitol in the polymer would indicate either (*1*)

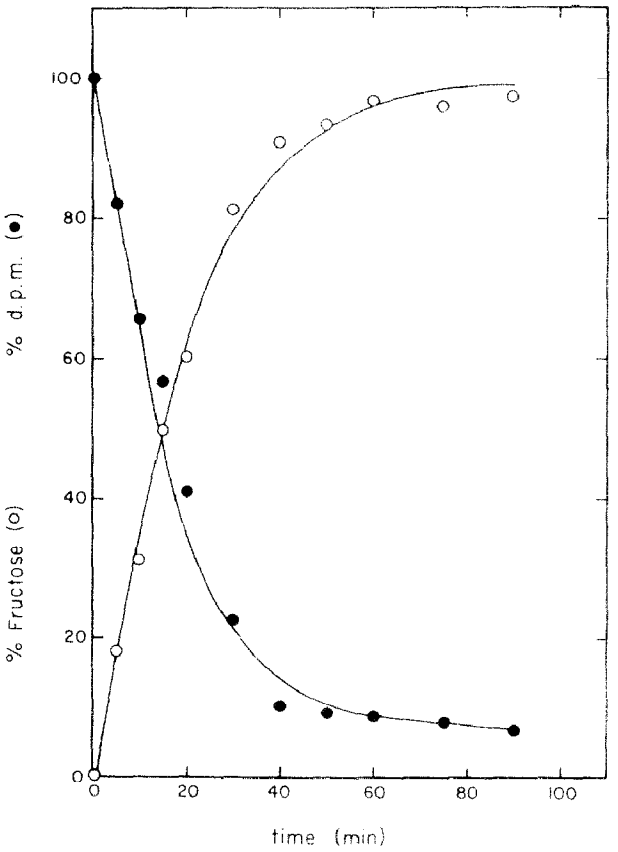

Fig. 2. Time course of the reaction of [U-^{14}C-D-glucose]sucrose with affinity-immobilized dextransucrase. The reaction was run at 25° using the 1:1 gel suspension (1.0 mL) and 30mM sucrose (0.01 μCi ^{14}C) as described in the Experimental. At intervals, aliquots of the supernatant fluid were analyzed for D-fructose content and radioactivity; ○ = percent of the theoretical yield of fructose, ● = percent of the d.p.m. of ^{14}C-labeled sucrose.

that addition of D-[^{14}C]glucose was to the reducing end of dextran chains or (*ii*) that the enzyme was not immobilized though interaction with the catalytic site which was free to synthesize oligomer(s) by the autopolymerization pathway. Following reduction and hydrolysis of ^{14}C-labeled Sephadex gel, paper chromatography showed that no ^{14}C-labeled D-glucitol was present (Fig. 3). Also, no labeled oligomer or polymer was found after extensive washing of the gel (prior to reduction/hydrolysis), and no sugar was detected in column eluates that contained protein. The initial column washes contained large amounts of D-fructose and a quantity of unreacted sucrose that was barely detectable on paper chromatograms.

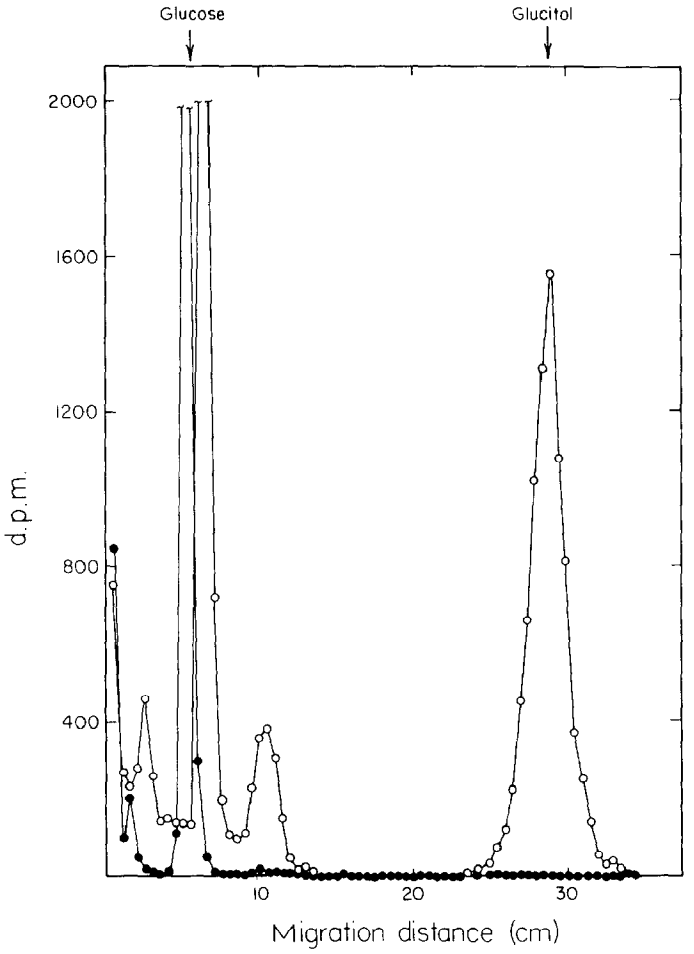

Fig. 3. Chromatographic analysis following hydrolysis of reduced ^{14}C-labeled Sephadex G-200. [The affinity-immobilized dextransucrase preparation was reacted with ^{14}C-labeled sucrose as described in the legend for Fig. 2. The ^{14}C-labeled gel was reduced with $NaBH_4$ and hydrolyzed in HCl; the products were separated by paper chromatography as described in the Experimental. The chromatogram was cut into strips which were analyzed by liquid scintillation counting; ● = ^{14}C-labeled Sephadex G-200 hydrolyzate, ○ = [^{14}C]maltose hydrolyzate. The positions of D-glucose and D-glucitol standards (stained by $AgNO_3$ dip procedure) are indicated by arrows].

These data indicate the presence of a catalytic domain in this enzyme that contains two non-equivalent sites. One site confers the ability to bind to acceptor molecules, such as dextran (Sephadex) with high affinity. This characteristic has been exploited for purification of dextransucrase[9]. The other site within the catalytic domain is responsible for formation of the glucosyl-enzyme intermediate by sucrose hydrolysis. Transfer of this D-glucose residue to the acceptor molecule would then complete a catalytic cycle for the acceptor reaction pathway. This type of mechanism has been postulated for dextransucrase[4].

The immobilized dextransucrase preparation is quite stable (retention of >85% acceptor activity) at 4° and even during the course of lengthy (>2 h) reactions at 25°. The dextranyl chains on the Sephadex gel may provide some stability against thermal denaturation of the enzyme[13].

In summary, dextransucrase was affinity immobilized to Sephadex G-200 gel yielding an enzyme preparation which retains most (>90%) of its original activity. The kinetics of the immobilized enzyme are similar to the enzyme in homogeneous solution (K_m = 2.3mM, V_{max} = 83.1 μmol/min/mg) for the acceptor reaction activity. The limitations (*e.g.*, saturation effect, low activity) found with dextransucrase immobilized by conventional chemical methods are not seen with affinity-immobilized enzyme. D-Fructose is produced in stoichiometric amounts as a by-product of the polymerization reaction and is easily removed by simply washing the gel with aqueous solution. The enzyme may be eluted and quantitatively recovered, and the modified support can be obtained free of protein, buffer salts, and monosaccharides.

Affinity-immobilized enzyme systems may be useful in the study of polymerization reactions of this type. This particular affinity-immobilized preparation has been used (this study) to examine the reactions of dextransucrase and to identify functional domains of the enzyme (manuscript in preparation).

REFERENCES

1 R. M. Mayer, *Methods Enzymol.*, 138 (1987) 649–660.
2 G. A. Luzio, V. K. Parnaik, and R. M. Mayer, *Carbohydr. Res.*, 121 (1983) 269–278.
3 G. Mooser and K. R. Iwaoka, *Biochemistry*, 28 (1989) 443–449.
4 J. F. Robyt and S. H. Eklund, *Bioorg. Chem.*, 11 (1982) 115–132.
5 P. Monsan, F. Paul, D. Auriol, and A. Lopez, *Methods Enzymol.*, 136 (1987) 239–254.
6 J. F. Robyt, B. K. Kimble, and T. F. Walseth, *Arch. Biochem. Biophys.*, 165 (1974) 634–640.
7 V. K. Parnaik, G. A. Luzio, D. A. Grahame, S. L. Ditson, and R. M. Mayer, *Carbohydr. Res.*, 121 (1983) 257–268.
8 B. Mattiasson, *Methods Enzymol.*, 137 (1988) 647–656.
9 S. F. Kobs, D. W. Husman, K. M. Cawthern, and R. M. Mayer, *Carbohydr. Res.*, 203 (1990) 156–161.
10 S. Seifter, S. Dayton, B. Novic, and E. Muntwyler, *Arch. Biochem. Biophys.*, 25 (1950) 191–200.
11 R. Amons and P. I. Schrier, *Anal. Biochem.*, 116 (1981) 429–443.
12 W. E. Trevelyan, D. P. Proctor, and J. S. Harrison, *Nature*, 166 (1950) 444–445.
13 R. M. Willemot, P. Monsan, and G. Durand, *Ann. N.Y. Acad. Sci.*, 542 (1988) 169–172.