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AN ENZYME FROM STREPTOCOCCUS MUTANS FORMS BRANCHES ON DEXTRAN IN THE ABSENCE OF SUCROSE

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An enzyme in glucosyltransferase preparations from Streptococcus mutans catalyzed the transfer of [^{14}C]glucopyranoside from purified isomaltosaccharides, each containing [^{14}C]glucopyranoside at its non-reducing terminus, to acceptor dextran, in the absence of sucrose. Half of the radioactivity present in the resulting [^{14}C]dextrans was resistant to hydrolysis by amylo-1,6-glucosidase. Treatment of the [^{14}C]dextrans with endodextranase resulted in extensive hydrolysis and produced [^{14}C]-labeled limit oligosaccharides containing branch sites. Acetolysis of the [^{14}C]-labeled limit oligosaccharides yielded [^{14}C]nigerose, thus indicating the formation of branch sites on dextran in the absence of sucrose. The enzyme catalyzing this reaction has not been identified but appears to be independent of the major extracellular glucosyltransferases of S. mutans.

The synthesis of dextran (1,6-α-D-glucan) from sucrose by dextransucrase (sucrose: 1,6-α-D-glucan 6-α-glucosyltransferase, EC 2.4.1.5) has been the subject of numerous studies, yet agreement upon the mechanisms for propagation of the linear 1,6-α-D-glucan chains and for attachment of branches to these chains has not been reached (1,2). Experimental evidence lends support to two proposed branching mechanisms: the acceptor reaction, catalyzed by dextransucrase, is suggested to account for branching during dextran synthesis by Leuconostoc mesenteroides enzyme (3), while transglucosylation from sucrose to 1,6-α-D-glucan, catalyzed either by a 3-α-D-glucosyltransferase (1,4-6) or by dextransucrase (7) has been suggested to account for branch forma-

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tion by Streptococcus mutans enzymes. An early suggestion that a branching enzyme, similar to the glycogen- and amylopectin-branching enzymes, catalyzed dextran branch formation (8) was not supported by subsequent experiments (2). This report presents evidence indicating the presence, in preparations or glucan-synthesizing enzymes from Streptococcus mutans, of an enzyme capable of branch formation in the absence of sucrose.

Materials and Methods

Streptococcus mutans strain 6715-49 was obtained from Dr. Richard Cowman, Dental Research Unit, VA Hospital, Miami, Florida, while mutant 27 of strain 6715-13 (9), which produces abnormally high levels of dextransucrase and glucosyltransferase, was obtained from Dr. Michael Freedman, University of Connecticut. These bacteria were grown as previously described (10). Dextransucrase and 3-α-D-glucosyltransferase activities were obtained from $(NH_4)_2SO_4$ precipitates of cell-free culture liquors by affinity chromatography (11) on 0.2% NaN_3, and subsequent ion exchange chromatography on either DEAE Bio Gel A (Bio Rad Laboratories, Richmond, CA) or DEAE-Trisacryl M (LKB, Gaithersburg, MD), equilibrated with 0.01 M, pH 5.5 sodium acetate buffer and eluted with a nonlinear gradient of 0-0.25M NaCl, in the same buffer. The major dextran-binding protein (11) was removed from the protein preparation, prior to ion exchange chromatography, by gel filtration on Bio Gel P10 (Bio Rad) equilibrated with the above buffer. This major protein eluted at V_o. Glucan synthesizing activity was detected as previously described (12). Protein was assayed with Coomassie Blue G250 (13).

A series of isomaltosaccharides, each labeled with a single [U-^{14}C]glucopyranosyl residue on its non-reducing terminus, was prepared by the method of Walker (14), using purified dextransucrase from S. mutans (Figure 1, peak 7; 20 IU/mg protein). [U-^{14}C]Sucrose was obtained from New England Nuclear, Boston, MA. [^{14}C]Isomaltose was prepared by digesting 0.075mg of [^{14}C]isomaltotriose, prepared as above, with 190 I.U. (0.5mg) of endodextranase (EC 2.3.1.11; Penicillium sp.; Grade I, Sigma Chemical Co., St. Louis, MO) in 0.25 M, pH 6.0 sodium phosphate buffer for 60 hrs. The products were resolved by gel filtration on Bio Gel P2-400 (1.6 X 180 cm), equilibrated with 0.02% NaN_3, and examined by paper chromatography on Whatman #1 paper in ethyl acetate:pyridine:H_2O (10:4:3). All label was recovered in isomaltotriose (2%) or isomaltose (98%). Limit dextran was prepared by exhaustive digestion of clinical dextran (MW 60,000-90,000; ICN Nutritional Biochemicals, Cleveland, OH) with Asperigillus niger amylo-1,6-glucosidase (EC 3.2.1.33), as previously described (15).

Possible branching enzyme activity was detected by assaying the transfer of radioactivity from alcohol-soluble donor [^{14}C]-isomaltosaccharides to alcohol-insoluble acceptor dextrans. [^{14}C]Isomaltosaccharides (0.1ml, 12 nmoles, 1.8 X 10^5 CPM) and limit dextran or clinical dextran (0.05ml, 6.4mg) were incubated with enzyme (0.1ml) and 0.25 M, pH 4.5 sodium acetate (0.05ml) for 18 hrs at 37°C. A 0.05ml aliquot was removed from each

reaction mixture and assayed for alcohol-insoluble radioactivity (10). Larger reaction mixtures, containing twice the proportions of components were sampled as above, then heated (100°C, 15 min) and subjected to gel filtration on columns of Bio Gel P2-400 (1.6 X 90 cm) in 0.02% NaN_3 to separate the products of the reaction. The P2-400 columns were calibrated with known isomaltosaccharides. Radioactivity present in each column fraction (2ml) was determined by liquid scintillation spectrometry of aliquots (0.2ml) dissolved in scintillation cocktail containing Scintisol (Isolab, Inc., Akron, OH). Assays to determine the effects of pH upon enzyme activity utilized sodium acetate buffer or sodium phosphate buffer at 0.25 M.

[^{14}C]-Labeled acceptor dextrans (3mg) isolated during gel filtration (Figure 2) were digested with amylo-1,6-glucosidase (25 I.U.) or Penicillium sp. dextranase (44 I.U.) in 0.05 M, pH 6.0 sodium phosphate buffer (0.5ml) for 24 hrs at 37°C. The amylo-1,6-glucosidase digests were assayed for release of glucose by the glucose oxidase method (16), and for alcohol-insoluble radioactivity (10). The dextranase digests were chromatographed on a column of Bio Gel P2-400 and radioactivity was detected in fractions, as described above. Radioactive limit oligosaccharides obtained by repeated dextranase digestion were subjected to acetolysis (17) and products were examined by chromatography, as above.

Results and Discussion

An enzyme catalyzing the transfer of radioactivity from donor [^{14}C]isomaltosaccharides to acceptor dextran was detected in the crude protein preparation from strain 6715-49 cultures and in the dextran-binding proteins eluted from Sephadex G50 affinity chromatography columns. Fractionation of the latter proteins by ion exchange chromatography consistently resulted in copurification of the transferring activity and dextransucrase activity (Figure 1, peak 7). Maximum transferring activity in this dextransucrase preparation occurred at pH 4.5 to 5.0. Analysis of a 3-α-D-glucosyltransferase preparation (Figure 1, peak 5) failed to detect transferring activity at any pH.

The products of the transfer reaction consisted of [^{14}C]-labeled acceptor dextran, [^{14}C]glucose and various unidentified [^{14}C]-labeled oligosaccharides larger than the donor isomaltosaccharides (Figure 2). The omission of acceptor dextran from reaction mixtures resulted in production of [^{14}C]glucose and [^{14}C]-labeled oligosaccharides larger than the donors. A survey

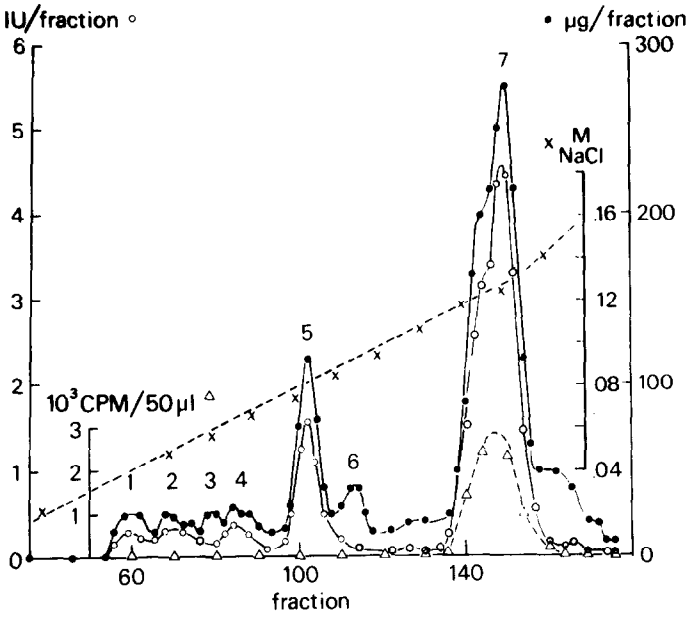
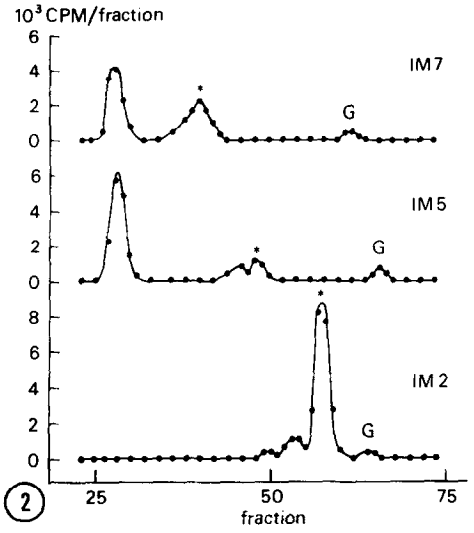


Figure 1. Ion exchange chromatography of the affinity-purified (11) glucosyltransferases of strain 6715-49 on DEAE Bio Gel A (2.6 X 10 cm bed). Numerals above profile refer to protein peaks. Fractions (5 ml) were assayed for protein (●), glucosyltransferase activity (○), transferring activity (△) and NaCl (x).

of a panel of [^{14}C]-labeled isomaltosaccharides ranging from isomaltose to isomaltoheptaose indicated that isomaltopentaose was the best donor while isomaltose was not a donor (Figures 2 and 3). Protein preparations with strong transferring activity were examined for the presence of α-1,6-glucan glucohydrolase (EC 2.3.1.70), an enzyme produced by S. mutans which could conceivably catalyze a transfer reaction under our assay conditions (18). Selected protein preparations catalyzed release of glucose from some isomaltosaccharides in the absence of acceptor, but none hydrolyzed isomaltose (Figure 3). It was considered unlikely, therefore, that α-1,6-glucan glucohydrolase accounted for the transferring activity, since its presence would have been readily detected by hydrolysis of isomaltose (19,20).

The [^{14}C]-labeled product of the transfer reaction was isolated and examined to determine the nature of the glucosidic linkages formed during the reaction. Amylo-1,6-glucosidase



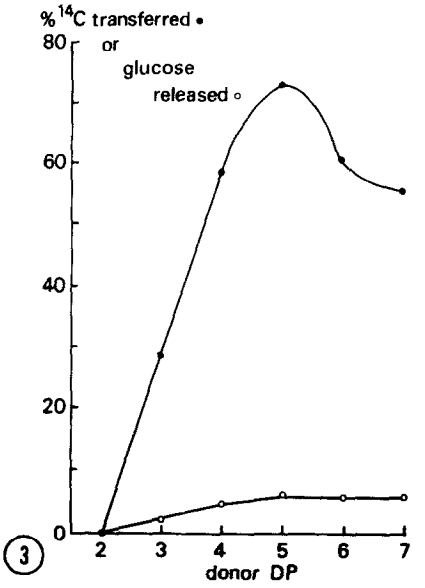


Figure 2. Gel filtration of the products of the transfer reaction on Bio Gel P2-400 columns (1.6 X 90 cm gel beds). Asterisks indicate the V_e of the [^{14}C]-labeled isomaltosaccharide donors. Fractions were 2 ml. G: glucose; IM2: isomaltose donor; IM5: isomaltopentaose donor; IM7: isomaltoheptaose donor.

Figure 3. Percent of [^{14}C]glucose transferred from a series of [^{14}C]-labeled isomaltosaccharide donors to acceptor dextran and the percent of total glucose released from donors in the absence of acceptor. Glucose release was detected by the glucose oxidase method (16). Donor DP is the degree of polymerization of [^{14}C]-labeled donor isomaltosaccharides.

catalyzed rapid release of the [^{14}C]glucose present in [^{14}C]dextran formed from clinical dextran acceptor and [^{14}C]isomaltopentaose donor (48% released after six hrs). The release of [^{14}C]-glucose essentially ceased after six hours, however, and half of the incorporated [^{14}C]glucose remained resistant to hydrolysis by this exo-enzyme, while unlabeled glucose continued to be released at the expected rate (14% after 24 hrs). Similar treatment of [^{14}C]dextran formed from limit dextran acceptor and a [^{14}C]isomaltopentaose donor revealed that no detectable unlabeled glucose was released, as expected, and that two-thirds of the incorporated [^{14}C]glucose was resistant to hydrolysis. A considerable portion of the [^{14}C]glucose incorporated into acceptor dextrans during the transfer reaction was, therefore, resistant to amylo-

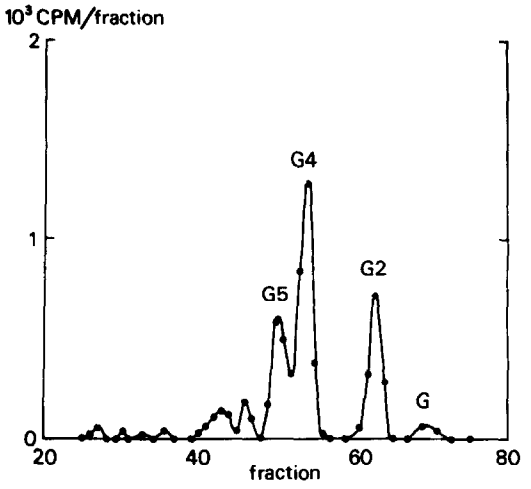


Figure 4. Bio Gel P2-400 (1.6 X 90 cm) gel filtration of endodextranase limit products from [^{14}C]dextran formed from [^{14}C]isomaltopentaose donor and limit dextran acceptor. Fractions were 2 ml. G: glucose; G2: isomaltose (identified by paper chromatography); G4: tetrasaccharide (unidentified); G5: pentasaccharide (unidentified).

1,6-glucosidase action, a result suggesting that the transfer of [^{14}C]glucose from donor to acceptor resulted in the formation of glucosidic linkages other than 1,6-α-D on the acceptor dextran.

Endodextranase treatment of the [^{14}C]dextran formed from a limit dextran acceptor and a [^{14}C]isomaltopentaose donor yielded [^{14}C]glucose, [^{14}C]isomaltose and unidentified [^{14}C]-labeled limit oligosaccharides (Figure 4). These [^{14}C]-labeled limit oligosaccharides, which resisted subsequent treatment with amylo-1,6-glucosidase, endodextranase or a mixture of amylo-1,6-glucosidase and endodextranase, were combined, dried and subjected to acetolysis. Paper chromatography revealed that [^{14}C]glucose was the major acetolysis product, but that approximately 20% of the label was present as [^{14}C]nigerose (3-α-D-glucopyranosyl-D-glucose). The recovery of [^{14}C]nigerose as an acetolysis product indicated the de novo formation of 1,3-α-D-glucosidic linkages during the transfer reaction and the presence of an enzyme capable of forming branch linkages on dextran in the absence of sucrose.

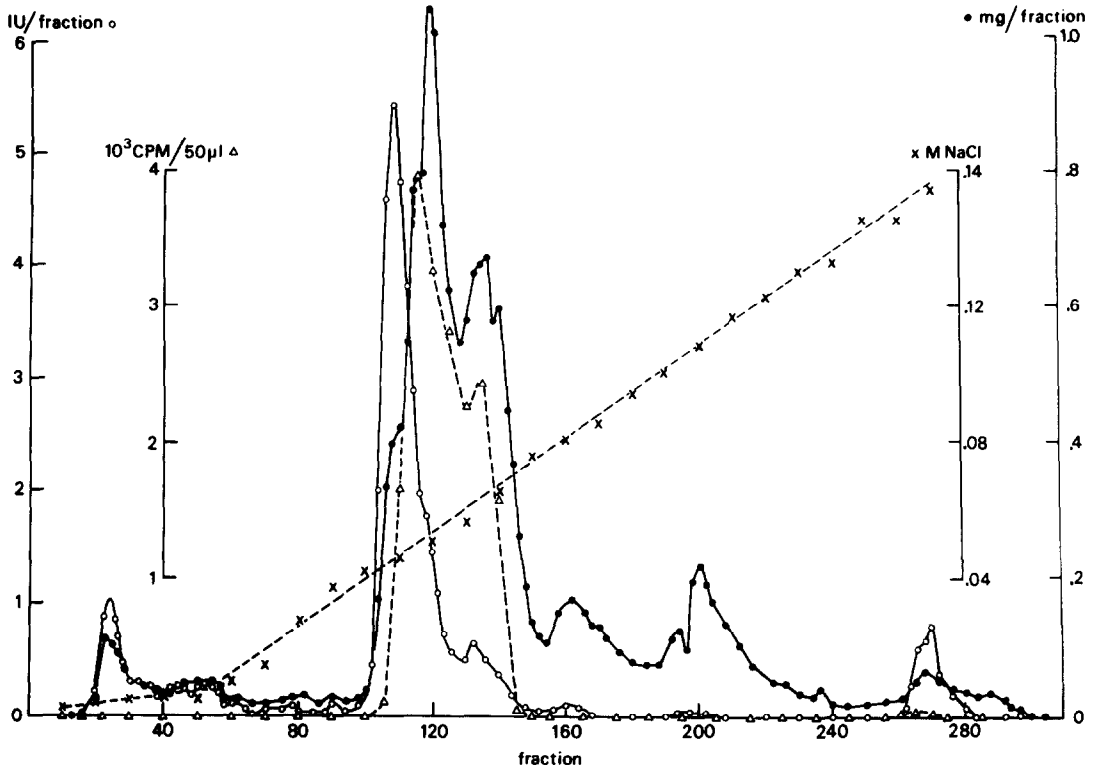


Figure 5. Ion exchange chromatography of affinity-purified (11) glucosyltranferases from mutant 27 of strain 6715-13 on DEAE-Trisacryl M (2.6 X 10 cm). Fractions (5 ml) were assayed as described in Figure 1 legend.

The association of branching activity with dextransucrase activity, observed in enzyme prepared from strain 6715-49 (Figure 1), was not observed when enzyme was prepared from mutant 27 of strain 6715-13; rather, the major portion of branching activity was eluted with 3-α-D-glucosyltransferase activity (Figure 5). Branching activity, therefore, could not be exclusively ascribed to either dextransucrase or 3-α-D-glucosyltransferase, since it did not consistently associate with either enzyme. The branching activity observed may have been catalyzed by the dextran-branching enzyme proposed by Bovey (8).

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