Properties of Glucosyltransferase and Glucan Transferase from Spinach

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ABSTRACT
A glucosyl and a glucosyl-glucan transferase activity from spinach (Spinacia oleracea L. var. Matador) leaves have been partially purified and characterized. The latter activity (fraction 1 after diethylaminoethylcellulose chromatography) is responsible for the transfer of glucosyl as well as of maltosyl, maltotriosyl, and higher homologous residues to glucose giving rise to maltose and the corresponding larger molecules. This fraction also shows β-amylase activity. The transfer takes place only to glucose; maltose, as well as other α-1,4-glucans, serve as donors. The enzyme fraction 2 is amylase-free and catalyzes only the transfer of glucosyl moieties, again with high acceptor specificity to glucose. Maltose and larger α-1,4-glucans, with the exception of maltotriose and maltotetraose, act as donors. The physiological function of these enzymes may be the formation of oligosaccharide primers for starch synthetase or phosphorylase.

Maltose was first recognized to participate in 1,4- to 1,4-glycosyl transfer reactions with extracellular enzymes of Escherichia coli (1, 7), which synthesized members of the maltodextrin series from maltose. Enzymes catalyzing analogous transfer reactions have been reported in extracts of Bacillus macerans (25), blood plasma (22), germinated green gram (24), and milled rice (6). Similarly, a recently reported cabbage leaf enzyme (17) produced several oligosaccharides from concentrated maltose solutions. In 1961 Edelman and Keys (8), described an enzyme from wheat germ which catalyzed the transfer of the nonreducing end glycosyl moiety of maltose to 14C-glucose with reformation of 14C-maltose, as in reaction 1.

\[ \text{reaction 1} \]

\[
0-\beta + \beta^* \rightarrow 0-\beta^* + 0
\]

In reactions 1 through 5, 0 represents a glucosyl unit, \( \beta \) represents a reducing glucosyl unit, \( \beta^* \) represents a 14C-labeled glucosyl unit, \( \beta \) represents an α-1,4-linkage, and \( \beta^* \) represents continuation of such bonding as in amylase.

The enzymatic activities described in this paper catalyzed reaction 1 as well as some reactions similar to, but not identical with, those of D-enzyme (EC 2.4.1 f; 1,4-glucan:α-1,4-glucan 4-glucosyltransferase) from potato (15, 26, 27, 30, 31). Other properties of the enzymatic reactions upon preliminary purification are described.

MATERIALS AND METHODS

Sources of Chemicals. Litter soluble starch and shellfish glycogen were purchased from Schuhardt, Munich. Amylopectin was prepared from fresh potato juice from which the amylase was precipitated with 1-butanol. Pure maltose was a generous gift from the laboratory of J. F. Robyt. Other maltodextrins were prepared from a fungal amylase digest of amylase precipitated three times (Sigma, St. Louis), separated on a charcoal-celite column (10), and purified by paper chromatography on formic acid and water-washed Whatman No. 1 paper. Other oligosaccharides were from commercial sources and were purified in the same way. Other materials were reagent grade.

Radioactive compounds were obtained from the Radiochemical Centre, Amersham, with the exception of the following: 2-deoxy-glycosyl and α-D-glucose-1-P were from New England Nuclear, Boston: 6-deoxy-glucose was purchased from Koch-Light, Colnbrook and tritiated at Amersham; sorbitol was prepared by sodium borohydride reduction of glucose (30); maltose was hydrolyzed from starch of Lamium leaves after having undergone 1 hr of photosynthesis in "CO2 (16); and maltotriose and maltopentaose were purified from the alcoholic extract of Eschichtum after 1 hr of photosynthesis in "CO2 (16). These oligosaccharides were purified by paper chromatography.

Enzyme Preparation and Assay. Spinach (Spinacia oleracea L. var. Matador) was collected fresh either from garden or greenhouse immediately prior to each enzyme preparation. In a typical preparation, 18 g of washed and chilled spinach leaves were homogenized for 5 sec at top speed of a Waring Blender in 150 ml of buffer at 4°C. The buffer used throughout these studies contained 0.02 M sodium glycerophosphate and 0.001 M diethiothreitol at pH 8.0. The brei was filtered through four layers of cheesecloth and fractionated between 40 and 52% saturation of ammonium sulfate. The redissolved protein was centrifuged for 40 min at 95,000g. The supernatant was dialyzed overnight against 1 liter of the buffer and frozen in small portions for use as needed. Stored in this manner, full activity was maintained over a 3-month period. Unless specifically stated, experiments were conducted with material at this stage of purity.

Chromatography of dialyzed ammonium sulfate fractions of the spinach homogenate was carried out in 1 x 10 or 1 x 20 cm columns containing Whatman DE-52 microgranular
DEAE-cellulose which had previously been washed, sized, and equilibrated with the standard buffer. Fractions of 5 ml were collected after charging the column with 200 mg of protein. When the 260 nm absorbance returned to baseline after unadsorbed protein material eluted, stepwise increases in potassium chloride concentration eluted adsorbed protein.

The reaction mixture for the standard assay of enzymatic activity contained 1.6 \times 10^{-4} \text{M maltose}, 2.6 \times 10^{-4} \text{M } ^{14}\text{C-glucose} (308 mCi/mmole), and approximately 80 \mu\text{g} of protein in a total volume of 30 \mu\text{l} of the standard buffer. After incubation at 20 \text{C} for 30 min, the enzymatic reaction was stopped by spotting 20 \mu\text{l} of the digest mixture on Whatman No. 1 paper under unheated forced air. The 20-\mu\text{l} aliquots dried in less than 1 min. Variations from this standard procedure are mentioned in the appropriate legends. After chromatography radioactive areas were located by strip-scanning or by radiography on Agfa x-ray paper. These areas were then cut out and the radioactivity was counted on paper in 15 ml of toluene scintillation solution containing 5 g/l of 2,5-diphenyloxazole. The counting efficiency was 70\%.

**Chromatography and Distribution of Radioactivity.** Unless otherwise stated, all chromatography was descending in 1-butanol-pyridine-water-acetic acid (60:40:30:3; v/v/v/v) on Whatman No. 1 paper between 25 and 27 C.

The distribution of radioactivity within reaction products was determined according to the methods Walker and Whelan (30) by sodium borohydride reduction, acid hydrolysis, and descending chromatographic separation of glucose and sorbitol in ethyl acetate-acetic acid-saturated boracic acid (9:1:1; v/v/v) on Whatman No. 1 paper at between 18 and 20 C. In some cases, the distribution of radioactivity in maltotriose was determined by incubation overnight at 40 C in 0.15 ml of a solution containing 500 IU of 3X crystallized \beta-amylase (Serva, Heidelberg) in 10 ml of 0.1 M pyridine-acetate buffer at pH 4.8. The products were separated by chromatography. Radioactive maltpentaose was similarly treated with a 100-fold diluted \beta-amylase solution.

**General Methods.** Substrate concentrations were determined by the phenol-sulfuric acid method for total carbohydrate (5). Reducing values were determined by the method of Nelson (23). Protein was determined using the Lowry method (20) against BSA standards. Reducing sugars on chromatograms were made visible by AgNO3-NaOH reagents (29).

**EXPERIMENTAL AND RESULTS**

**Characterization of Transfer Reaction.** When crude and ammonium sulfate fractionated preparations of the glucan transferase were incubated with soluble starch and \(^{14}\text{C-glucose}, the labeled products were maltose, maltotriose, and maltotetraose as estimated by comparison with chromatographic mobilities of standard compounds; small traces of higher oligosaccharides were also found. No branched (a-1-6-linked) oligosaccharides were detected by chromatographic observation. Nearly identical product distributions as with soluble starch were obtained with glycogen and amylopectin serving as donor in the reaction with \(^{14}\text{C-glucose}.

The location of the radioactivity in the maltose, maltotriose, and maltotetraose, obtained as above, was determined by sodium borohydride reduction, hydrolysis, and chromatography (30). In each case, label was recovered only in sorbitol, thereby indicating that the reducing ends of these compounds had been labeled and that the mechanism of transfer for the formation of each compound was as in equations 2, 3, and 4.

\[
\begin{align*}
\theta^* + 0 &- 0 - 0 - 0 - 0 - 0 - 0 - 0 \quad \vdash \\
0 &- 0^* + 0 - 0 - 0 - 0 - 0 - 0 \\
0 &- 0 - 0 - 0^* + 0 - 0 - 0 - 0 \quad \vdash \\
0 &- 0 - 0 - 0 + 0 &- \theta^* \quad (5)
\end{align*}
\]

When the maltotriose product was hydrolyzed with \beta-amylase, the sole labeled hydrolysis product was glucose. Because of the specificity of \beta-amylase, the labeled glucose corresponded to the reducing end of the maltotriose. This specific hydrolysis proved that the linkage formed in the transfer was a-1,4 and that maltosyl residues had been transferred, as in reaction 3. Only when maltpentaose served as donor in the reaction with \(^{14}\text{C-glucose} were significant levels of labeled maltpentaose recovered. Products of hydrolysis of this labeled product with \beta-amylase were labeled maltotriose (the sole radioactive hydrolysis product) and unlabeled maltose. In this case maltpentaosyl residues had been transferred as in reaction 5.

Figure 1 shows a radioautogram of the chromatographically separated products of six experiments in which the maltodextrins, maltose through maltpentaose, and \(^{14}\text{C-glucose were used as substrates. The largest product in each experiment, except in the case of maltpentaose, was the size of the added donor. Because the products were labeled in the reducing end, the enzyme was capable of transferring to \(^{14}\text{C-glucose, residues of one less than the length of the original donor. Just as in reactions with starch as donor, maltose, maltotriose, and maltpentaose were products in cases in which the donors were larger than maltotetraose. Hardly detectable in Figure 1 and on the original radioautogram itself were the labeled maltodextrin products larger than the donors in each experiment. Material at the origin was also slightly radioactive after 15 min incubations. Another point of interest in Figure 1 was the systematically low yield of products one glucose unit shorter than the original donor, as for example with maltpentaose as donor, only a trace of maltotriose was formed, and with maltpentaose as donor, a minor amount of maltotetraose was labeled.

**Reaction Parameters.** The \(K_m\) for glucose was determined to be approximately \(3 \times 10^{-4} \text{M}\) at saturating maltose concentrations in reaction 1. Except for the data presented in Table II, most assays were conducted at nonsaturating substrate concentrations in order to conserve substrates. In Figure 2, the kinetic parameters for the catalysis of reaction 1 are plotted. The reaction linear with protein concentration to about 60 \mu\text{g/30 ml} (Fig. 2A). The temperature dependence of the reaction is shown in Figure 2B. The reaction rates were linear with time at 20, 27, and 35 C for 33 min. At 41 C the initial reaction velocity was greater than at 35 C but decayed after 6 min. The \(pH\) activity curve (Fig. 2C) exhibited a plateau from \(pH\) 6 to 9, both in sodium glycophosphate and in sodium phosphate buffer.

The activity of the enzyme for catalysis of reaction 1 was increased in the presence of diethiothreitol. Interestingly enough, mercaptoethanol did not substitute in this role but in fact inhibited activity. In Table I are tabulated the activities of four enzyme preparations containing equal amounts of protein dissolved either in buffer alone, in buffer plus 0.01 M diethiothreitol, in buffer plus 0.01 M mercaptoethanol or in buffer plus 0.01 M CaCl\(_2\). The activities of the solutions were tested before and after being held at 4 C for 24 hr. As can be
FIG. 1. Transfer products from maltodextrin donors. The conditions were the same as in the standard assay, except that the donors were varied and their concentration was $8.0 \times 10^{-2}$ M. Glucose, 3-O-methyl-d-glucose, α-D-methylglucoside, α-D-glucosamine, 2-deoxy-D-glucose, 6-deoxy-D-glucose (20 μCi $^{3}$H).

α-C-Maltose was also tested for its acceptor ability by incubation with soluble starch and enzyme. The data in Table II, experiment 1, indicated that maltose had about 10% the acceptor ability of glucose. However, the counts in maltotriose most likely resulted from side reactions such as the transfer of maltosyl units from starch to hydrolyzed $^{3}$C-glucose; since with $^{3}$C-maltose as acceptor, about 3% of the radioactivity was present in glucose at the end of the experiment. Also, maltotriose did not seem to serve significantly as acceptor. Experiment 2, Table II, compares the effectiveness of potential donors containing other than α-1,4-linkages. Reactivity of β-1,4- and β-1,6-linkages may be eliminated; however, slight action on α-1,6-linkages was repeatedly observed with the ammonium sulfate fractionated enzyme. In experiment 3,
Table II. Products of Transfer from Reaction of Various Acceptors and Donors

| Expt. No. | Acceptor | Donor | Radioactivity in Maltooligosaccharides
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cpm</td>
</tr>
<tr>
<td>1</td>
<td>14C-G1</td>
<td>Soluble starch</td>
<td>136000</td>
</tr>
<tr>
<td></td>
<td>14C-G2</td>
<td>Soluble starch</td>
<td>1834</td>
</tr>
<tr>
<td></td>
<td>14C-G3</td>
<td>Soluble starch</td>
<td>303</td>
</tr>
<tr>
<td>2</td>
<td>14C-G1</td>
<td>Maltose</td>
<td>135956</td>
</tr>
<tr>
<td></td>
<td>14C-G1</td>
<td>Isomalto</td>
<td>130224</td>
</tr>
<tr>
<td></td>
<td>14C-G1</td>
<td>Panose</td>
<td>157884</td>
</tr>
<tr>
<td></td>
<td>14C-G1</td>
<td>Cellobiose</td>
<td>160849</td>
</tr>
<tr>
<td></td>
<td>14C-G1</td>
<td>Gentiobiose</td>
<td>160361</td>
</tr>
<tr>
<td>3</td>
<td>Water</td>
<td>14C-glucose</td>
<td>27876</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>14C-maltose</td>
<td>40614697</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>14C-maltotriose</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>14C-maltopentaose</td>
<td>275</td>
</tr>
</tbody>
</table>

1 Backgrounds of paper blanks were subtracted.

2 Glucose, maltose, maltotriose, maltotetraose, and maltopentaose are represented, respectively, by G1, G2, G3, G4, and G5.

Table II, are presented hydrolysis data for the uniformly labeled maltooligosaccharides. 14C-Glucose is not condensed in this system to yield maltose. Less than 5% of the 14C-maltose and 14C-maltotriose were hydrolyzed. The extent of hydrolysis of 14C-maltopentaose and further experiments indicated that 14C-amylose contaminated the ammonium sulfate fractionated preparations.

DEAE-cellulose Chromatography. Chromatography of dialyzed ammonium sulfate fractions of microgranular DEAE-cellulose permitted separation of glucan transferase activities into two fractions as seen in Figure 3. Both were identified with the standard assay. 13C-amylose, as judged by increase in reducing value of soluble starch digests at pH 4.8, was eluted with one of the glucan transferase peaks by buffer alone (fraction 1), whereas the second glucan transferase activity eluted free of amylose activity with 0.10 M KCl in the buffer (fraction 2). The specific glucan transferase activity of fraction 2 was only 1.2 times greater than that of the dialyzed ammonium sulfate fraction which was applied to the column. The action of the amylose-free glucan transferase (fraction 2), on soluble starch and 13C-glucose yielded only reducing-end labeled maltose. Fraction 1 enzymes yielded labeled maltose, maltotriose, and maltotetraose under the same conditions. Reaction of 14C-glucose and pure maltooligosaccharides also differed: the fraction 1 enzyme yielded the spectrum of products of crude preparations seen in Figure 1, but with the amylose free fraction 2 little reaction could be detected with maltotriose, and maltotetraose. Maltose was the sole labeled product in reactions with 14C-glucose and donors, maltose, maltopentaose, maltolhexaose, and maltopentaose. Figure 4 shows the relative rates of maltose formation from reaction of 14C-glucose and the pure maltooligosaccharides with the fraction 2 preparation. Maltose was clearly the best substrate for this enzymatic activity.

**DISCUSSION**

The amylose-free fraction 2 enzyme described above appears to be similar to that described by Edelman and Keys (8). No
Plant distinguishing properties spinach in amylase and glucose. L-arabinose, the transfer or methyl-D-glucoside, for as donors for the transfer to similar enzymes both investigated and glycogen described in Whelan facts of nonreducing end and the 10% the acceptor was incapable Whelan of maltodextrin. Although the spinach leaf glucantransferase preparations contained interfering activities, it is tempting to draw a parallel to the action of D-enzyme on the basis of the transfer patterns from higher maltodextrins to 14C-glucose (Fig. 1). Formation of products which required the transfer of the penultimate linkage from the reducing end of the maltodextrin was unfavorable.

Other dextrin-4-glucantransferases have been described in human saliva (28), liver (21, 31), rabbit muscle (3), and yeast (18). The glucan transferase from rabbit muscle showed preference for maltotriosyl transfer and maltohexaose was the smallest donor acted upon (3). The yeast enzyme reportedly transferred both maltosyl and maltotriosyl units from maltohexaose (18). It transferred maltosyl units from maltopentaose, and although slowly, from maltotetraose as well. The yeast enzyme had no activity with maltose and maltotriose.

The rat liver glucosyltransferase appears to be associated with α-glucosidase activity of lysosomes (14). The rat muscle (3) and yeast (18) enzymes are highly purified, and it appears that these homogenous proteins also carry amylo-1,6-glucosidase activity. Slight α-glucosidase activity was observed in the spinach glucosyl- and glucan transferase preparations (Table II), and so the possibility is open that these activities are likewise associated in spinach.

De Fekete and Vieweg (4) have proposed the regulation of

![Graph](image-url)

**Fig. 3.** Elution profile of a DEAE-cellulose chromatogram of spinach glucan transferase and the corresponding levels of amylase and glucantransferase activity in every fifth fraction. □: CPM in maltose; ■: β-amylase reducing value.

distinguishing properties can be found in the information at hand. For both enzymes maltose and other α-1,4-glycans serve as donors for the transfer of glucosyl units to the C-4 position of glucose. Neither enzyme could utilize d-mannose, d-galactose, l-arabinose, d-xylose, cellobiose, isomaltose, α-methyl-d-glucoside, or d-glucose 1-phosphate as acceptor in the transfer reaction.

The amylase-containing fraction 1 activity has characteristics similar to the activity of D-enzyme, which has been extensively investigated by Whelan and co-workers (15, 26, 27, 30, 31). Both enzymes transfer maltosyl and maltotriosyl residues from higher maltodextrins, amylose, and outer chains of amylopeptin and glycogen to glucose. Also maltose appeared to have about 10% the acceptor capacity of glucose. However, unlike the activities described in this paper, maltose was never produced in significant quantities from the action of D-enzyme (26, 27). Maltose was also inactive as a donor for D-enzyme. These two facts enabled Whelan (15, 31) to establish that D-enzyme was incapable of glucosyl transfer and thereby to establish which maltodextrin linkages were immune to its action. The nontransferable linkages were the single terminal linkage at the nonreducing end and the linkage penultimate to the reducing end of a maltodextrin.

Although the spinach leaf glucantransferase preparations

![Graph](image-url)

**Fig. 4.** Dependence of reaction rate of fraction 2 glucan transferase activity on maltose ( ), maltopentaose ( ), maltohexaose ( ), and maltoheptaose ( ) concentrations. Glucose: 2.3 × 10⁻⁶ M; 40 μg of protein in a total volume of 25 μl was used with each of the four indicated donor concentrations.
phosphorylase in starch synthesis by maltose. Since maltose is formed by the transfer of glucose from polysaccharides as well as from other oligosaccharides, the enzymes described in this paper may be more closely involved in phosphorylase regulation than amylase, as they proposed. Many of the glucosyl- and glucan transferases discussed above were capable of building maltodextrin products larger than the substrates. The characteristic was not a strong point of the spinach glucosyl- and glucan transferases, as only traces of higher oligosaccharides were observed on the radioautograms such as the one shown in Figure 1. The strong point of these enzymes appears to lie with production of maltose, maltotriose, and maltotetraose, which have been shown to have priming activities for starch synthetase (11) and phosphorylase (32). The existence of these oligosaccharides in photosynthetic tissue (9, 12, 16, 19) may be attributed to the glucosyl- and glucan transferase activities described here.

LITERATURE CITED