Beta-Glucosyl-Transferase from Germinated Barley [1]

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ABSTRACT

Beta-glucosyl-transferase was prepared by chromatographing the albumin portion of 0.1*N* NaCl extracts of germinated barley on DEAE-cellulose. Transferase activity was exhibited when cellobiose, cellotriose, laminaribiose, laminaritriose, gentiobiose, 3-O-β-D-cellobiosyl-D-glucose, or 3-O-β-D-cellotriosyl-D-glucose was the substrate, but not when maltose, sucrose, or glucose was the substrate. Transferase activity was indicated by the presence on chromatograms of oligosaccharides other than the substrates. Extended incubation resulted in hydrolysis of the substrates to glucose following an initial lag phase. The hydrolysis proceeded by removal of glucose units from the nonreducing ends of the substrates. The enzyme exhibited lower K_m values on trisaccharides and tetrasaccharides than on disaccharides, but higher maximum velocities on disaccharides. The highest velocity was exhibited on the beta-1,3-linked disaccharide laminaribiose.

Several beta-glucosidase activities have been reported in germinated barley (1, 2, 3). The reports of the activities are usually based on the ability of extracts or partially purified preparations to hydrolyze the substrates in question. In general, there is very little information showing whether each activity results from an individual enzyme or whether several of the activities result from a single enzyme. The general similarity of beta-linked glucose polymers presents a situation in which a single enzyme might well be able to catalyze the hydrolysis of more than one species.

Beta-glucosyl-transferase refers to an enzyme which can transfer a glucose moiety from a donor to an acceptor molecule. In this report the term is used to indicate the transfer of glucose residues from one oligosaccharide to another or to glucose without participation of phosphorylated sugars. Thus the reaction may be represented as follows:

$$\text{glucosyl-R}_1 + \text{enzyme} \rightarrow \text{glucosyl-enzyme} + \text{R}_1$$
$$\text{glucosyl-enzyme} + \text{R}_2 \rightarrow \text{glycosyl-R}_2 + \text{enzyme.}$$

If the transferase considered here acts in the usual fashion, the linkage would be cleaved between the oxygen bridge and the carbon-1 of the glucose that is transferred. Transfer of the glucosyl moiety to water rather than to the hydroxyl group of another carbohydrate would represent hydrolysis. Thus a beta-glucosyl-transferase with a low specificity for the linkage cleaved could be responsible for several oligosaccharase activities (4).

MATERIALS AND METHODS

Enzymes

Sodium chloride extracts of germinated barley were dialyzed against water (5). The albumin fraction was chromatographed on DEAE-cellulose columns.

Substrates

Purified barley beta-D-glucan was prepared as described previously (6).

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Laminaribiose and laminaritriose were prepared by separating partial acid hydrolysates of laminarin on carbon-celite columns (7). Cellotriose was prepared similarly from cellulose. 3-O-β-D-cellobiosyl-D-glucose and 3-O-β-D-cellotriosyl-D-glucose were prepared from barley beta-D-glucan (8). The remaining substrates were purchased from chemical suppliers.

Ion-Exchange Resin

DEAE-cellulose was pretreated as described by Peterson and Sober (9), except that after a final submersion in alkali the resin was washed in distilled water until the pH decreased to 8.5 to 9.

Analytical Methods

The degree of polymerization of oligosaccharides was determined by the method of Timell (10). Periodate oxidation was performed according to the Smith degradation (11). The chromatographic solvents used were n-butanol-pyridine-water (10:3:3)(I) and ethyl acetate-acetic acid-saturated boric acid in water (9:1:1)(II)(12). Glucose production was measured by a modification of the method published by Worthington (13). When glucose production was used as a measure of transferase activity the assay procedure was as follows: The transferase was allowed to act at pH 4.7 and 30°C. in 0.04*N* sodium acetate buffer. At the appropriate times 1-ml. aliquots were removed and placed in tubes containing 1 ml. of 0.04*N* NH_4OH. The higher pH (9.2) stopped the transferase action. Subsequently 8 ml. of combined glucostat reagent was added; reaction was allowed to continue for 10 min., and was stopped by adding 3 drops of 12*N* HCl. The resulting absorbance was measured at 400 mμ and converted to a glucose value by use of a standard curve. A combined reagent of chromogen dissolved in 41 ml. of water and glucostat reagent in 41 ml. of 0.1*N* sodium phosphate buffer, pH 6.75, was used in place of the solution listed by Worthington (13). This adjustment resulted in a pH of approximately 7.1 during the glucostat reaction. Measurements made over a wide range of pH values require appropriate adjustments in the normality of the NH_4OH used to stop the transferase action. A lag period in glucose production by transferase was observed (see Fig. 5). Reaction velocities were based on linear portions of the glucose production curves. One unit of activity was defined as that amount of enzyme producing 1 μ*M* of glucose per min.

RESULTS

Transferase activity was first detected in the enzyme fractions by experiments designed to learn what products are formed during hydrolysis of barley beta-D-glucan (B-G). Enzyme fractions were incubated with B-G and the products were separated by paper chromatography. Gentiobiose, which travels with an R_g of approximately 0.33 in solvent I, was formed by certain enzyme fractions. The relative levels of gentiobiose formed by fractions from a DEAE-cellulose separation of albumin are shown in Fig. 1. Since gentiobiose and laminaritriose both exhibit R_g values of approximately 0.33 in solvent I, the following tests were invoked to show that the compound was gentiobiose. It exhibited a D.P. of 2.1 and the largest fragment remaining after periodate oxidation, sodium borohydride reduction, and mild

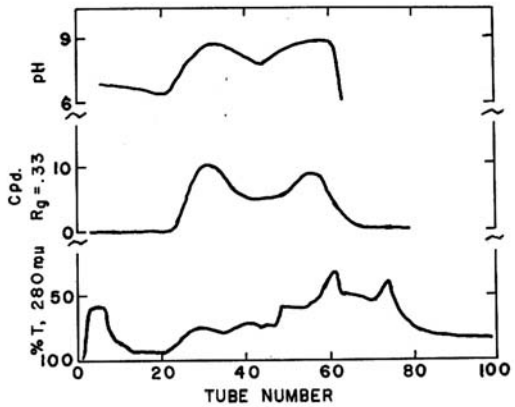
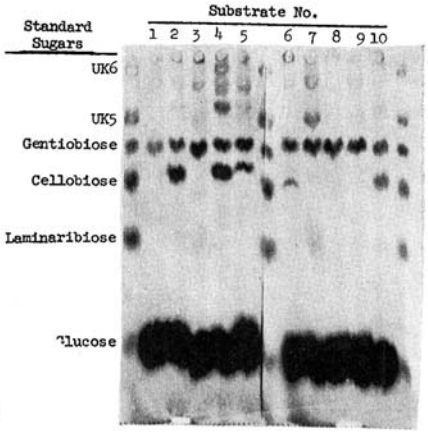


Fig. 1. Separation of albumin on DEAE-cellulose columns: 230 mg. of protein was applied to a column 1.1 cm. diam. × 30 cm. The column was eluted with successive linear gradients. Gradient I was composed of 300 ml. water and 300 ml. 0.1*N* NaCl; gradient II of 400 ml. 0.033*N* NaCl and 400 ml. 0.033*N* NaCl-0.1*M* phosphate, pH 4.3. Distilled water was initially passed through the column, gradient I was started at tube 14, and gradient II was started at tube 35. Flushing the column with 0.25*M* phosphate, pH 4.0, did not remove any additional enzyme. The relative levels of gentiobiose (cpd. $R_g = 0.33$) were estimated by comparing the size and darkness of the spots to standards.

hydrolysis was glycerol. Also the compound moved with gentiobiose in solvent II. (Gentiobiose moves at approximately twice the rate of laminaritriose in solvent II.) The presence of gentiobiose showed that the enzyme fractions contained beta-glucosyl transferase activity, since there are no $(1 \rightarrow 6)$ bonds in B-G. Endo-beta-glucanase activity as shown by a decrease in viscosity of B-G solutions is present along with the transferase activity.

The enzyme fractions contained in tubes 27–32 (Fig. 1) were combined and tested for transferase activity on a series of oligosaccharides. Typically, enzyme and substrate in buffer were incubated under toluene for 3 days at room temperature, after which the products were separated, with the use of solvent I. The results of one such series of tests are shown in Fig. 2. Companion chromatograms were irrigated with solvent II to verify the presence of gentiobiose. Gentiobiose was the major new oligosaccharide formed from all the beta-linked substrates; other new oligosaccharides were formed in lesser amounts. No action was exhibited on the alpha-linked disaccharide maltose, on sucrose, or on glucose. The rate of formation of gentiobiose varied with the substrate (Fig. 3). With laminaritriose as the substrate, the peak level of gentiobiose occurred after 1 day. With cellobiose and cellotriose as substrates it occurred after 3 days. With all three substrates the level of gentiobiose reached a peak and then decreased to much lower levels after 5 days of hydrolysis.

The second most prevalent new oligosaccharide formed depended on the substrate. With cellobiose and cellotriose as substrates it moved faster than cellotriose, so was not the reformation of cellotriose. The compound formed when laminaribiose was the substrate moved more slowly than laminaritriose



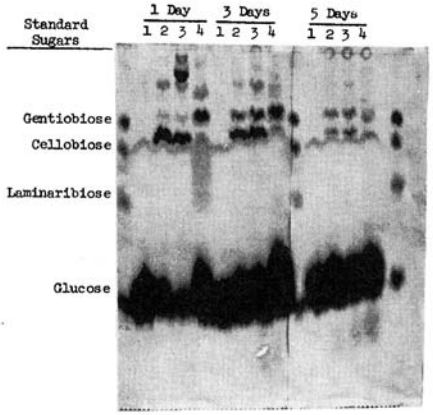


Fig. 2 (left). Formation of gentiobiose from several substrates. The substrates were incubated with transferase for 3 days (under toluene), after which the products were separated by irrigation with solvent I for 4 days. Substrates 1 through 10 respectively were laminaribiose, cellobiose, laminaritriose, cellotriose, laminaribiose plus cellotriose, gentiobiose, 3-O-β-D-cellobiosyl-D-glucose plus 3-O-β-D-cellotriosyl-D-glucose, laminaribiose plus 3-O-β-D-cellobiosyl-D-glucose, laminaribiose plus 3-O-D-cellotriosyl-D-glucose, and cellobiose plus 3-O-β-D-cellobiosyl-D-glucose. The distances traveled by reference compounds are shown on both sides and in the center of the chromatogram.

Fig. 3 (right). Effect of incubation time on the types and amounts of products formed when transferase acts on glucose (No. 1), cellobiose (No. 2), cellotriose (No. 3), and laminaribiose (No. 4). Incubation times were 1 day, 3 days, and 5 days, after which the products were separated by irrigation for 4 days with solvent I. The distances traveled by reference compounds are shown on both sides of the chromatogram and between the 3-day and 5-day samples.

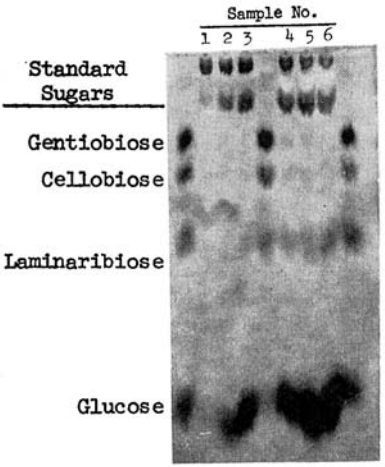
but faster than the product from cellobiose (Fig. 3). The structures of these compounds were not determined, but work by Anderson and Manners (14) with barley transferase showed formation of compounds with a glucose attached in the 6 position.

After the effect of time on the level of gentiobiose present had been observed (Fig. 3), the fractions from a DEAE-cellulose column were monitored; one-tenth the amount of enzyme, and twice the 1 ml. of enzyme which was used in Fig. 3. With 2-ml. aliquots only a very small amount of gentiobiose was present after the 3-day incubation. At the 0.1-ml. level of enzyme more gentiobiose was present, and the activity appeared as one broad peak rather than two fractions, as appears from the data in Fig. 1. These results suggest that the two peaks in Fig. 1 were artifacts of the size of the enzyme aliquots used. Transferase activity also was present in the column front. The results clearly show that quantitative assessment of transferase action by the appearance of gentiobiose will have to be based on a series of time intervals (or enzyme concentrations) to make certain that the peak concentration of gentiobiose has not occurred before the sample is taken.

For the kinetic experiments the enzyme was prepared by combining the fraction corresponding to tubes 25 through 58 (Fig. 1). The combined

enzyme was freeze-dried, dissolved in one-tenth its original volume of water, and desalted on a Sephadex G-25 column. It was necessary to use the rate of formation of glucose as an index of the transferase activity in the kinetic experiments because of the physical problem of preparing and quantitating the results from a long series of chromatograms. Preliminary experiments showed that the rate of glucose formation from laminaribiose was somewhat greater than twice that from cellobiose. Since this apparently agreed with the rate of formation of gentiobiose as estimated from the chromatograms (Fig. 3), it was assumed that glucose formation did reflect transferase activity.

The effect of pH on the rate of glucose formation when the enzyme acts on 3-O-β-D-cellobiosyl-D-glucose and 3-O-β-D-cellotriosyl-D-glucose is shown in Table I. The Michaelis constants and maximum velocities of the enzyme acting on several substrates are presented in Table II. The order of appearance of products during the action on 3-O-β-D-cellotriosyl-D-glucose is shown in Fig. 4 and the progress curve of glucose formation when the enzyme is acting on 3-O-β-D-cellobiosyl-D-glucose is shown in Fig. 5. The effects of heating the enzyme on the rates of formation of glucose from several substrates are shown in Table III.



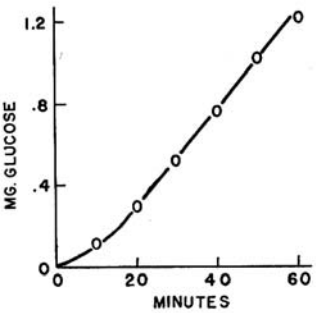


Fig. 4 (left). The order of appearance of products when transferase acts on 3-O-β-D-cellotriosyl-D-glucose. Samples 1 through 6 were incubated respectively for 30 sec., 10.5 min., 20.5 min., 30.5 min., 40.5 min., and 50.5 min. The slowest-moving component is the substrate 3-O-β-D-cellotriosyl-D-glucose; the next slowest is 3-O-β-D-cellobiosyl-D-glucose. Reference compounds were spotted on both sides and in the center of the chromatogram.

Fig. 5 (right). Progress of glucose formation during action of transferase on 3-O-β-D-cellobiosyl-D-glucose.

DISCUSSION

The results in Fig. 2 show that germinated barley extracts contain an enzyme which can transfer beta-glucosyl moieties from one carbohydrate chain to another or to glucose. The enzyme did not act on the alpha-linked maltose, on sucrose, or in systems containing only glucose. The transfer of

the glucosyl group to other carbohydrates occurred even in quite dilute solutions (2 g./liter); this suggests a relatively high preference for transfer to a second carbohydrate over a transfer to water. The appearance of relatively large amounts of trisaccharide during the period that free glucose and gentiobiose are building up (action on cellobiose, Fig. 3) further indicated the preference for carbohydrate acceptors over water. In this respect the enzyme appears to differ from the barley beta-glucosyl-transferase reported by Anderson and Manners (14); high concentrations of cellobiose (54 g./liter) were required by their enzyme to obtain products resulting from transfer to other carbohydrates. However, they did not report any experiments testing the effect of incubation time on the types and amounts of products present in the system. Such experiments should be performed, since the results in Fig. 3 show that compounds such as gentiobiose are formed and then degraded by the enzyme.

Enzyme Properties

The results in Table I show a rather broad pH optimum of 4.5 to 5 for action on 3-O-β-D-cellobiosyl-D-glucose and 3-O-β-D-cellotriosyl-D-glucose. Action on laminaribiose and cellobiose showed even broader pH curves with maxima in the same range. There was no indication of different

TABLE I

EFFECT OF pH ON THE VELOCITY OF GLUCOSE FORMATION

SUBSTRATE[a]	pH					
	3.65	3.98	4.34	4.64	5.12	5.68
	μM/hr.	*μM/hr.*	*μM/hr.*	*μM/hr.*	*μM/hr.*	*μM/hr.*
Cpd. 5	0.48	0.55	0.61	0.62	0.59	0.49
Cpd. 6	0.49	0.52	0.58	0.57	0.58	0.48

[a]Cpd. 5: 3-O-β-D-cellobiosyl-D-glucose; cpd. 6: 3-O-β-D-cellotriosyl-D-glucose.

pH responses in activity on the four compounds.

Lower K_m values were exhibited toward tri- and tetrasaccharides than toward disaccharides (Table II). Higher maximum velocities were exhibited

TABLE II

MICHAELIS CONSTANTS AND MAXIMUM VELOCITIES OF GLUCOSE PRODUCTION FOR TRANSFERASE ACTING ON SEVERAL SUBSTRATES

SUBSTRATE	Km × 10^{-5}	V	SUBSTRATE	Km × 10^{-5}	V
	M/liter	*Units*		*M/liter*	*Units*
Cellobiose	90	1.4	3-O-β-D-cellobiosyl-D-glucose	10	0.78
Gentiobiose	70	1.4			
Laminaribiose	50	2.6	3-O-β-D-cellotriosyl-D-glucose	4	0.61
Cellotriose	20	0.67			
Laminaritriose	30	1.1			

on the disaccharides. The results also show a considerably greater affinity for substrates containing (1 → 3)-linked glucose units than for those containing (1 → 4)-linked glucose units.

The order of appearance of products (Fig. 4) clearly shows that glucose

units are removed from the nonreducing end when 3-O-β-D-cellotriosyl-D-glucose is the substrate. Removal of glucose from the reducing end would have left cellotriose, which moves more slowly than the initial compound that was actually formed. Action on 3-O-β-D-cellobiosyl-D-glucose shows the same pattern. In this case laminaribiose is the earliest disaccharide formed. If it is assumed that transfers from laminaritriose and cellotriose also proceed from the nonreducing end of the molecule, the K_m values in Table II indicate that whether the bond to be cleaved is $(1 \rightarrow 3)$ or $(1 \rightarrow 4)$ has some but not a great deal of effect on the affinity of enzyme for substrate. The results show a much more definite relation between the bond broken and the rate at which glucose is formed; e.g., the results in Table II show that when oligosaccharides of the same degree of polymerization are compared, glucose is formed at much higher rates when $(1 \rightarrow 3)$ bonds are attacked than when $(1 \rightarrow 4)$ or $(1 \rightarrow 6)$ bonds are attacked. The obvious conclusion is that the velocity constant for cleavage of $(1 \rightarrow 3)$ bonds is higher than that for cleavage of $(1 \rightarrow 4)$ or $(1 \rightarrow 6)$ bonds. Unfortunately, the experimental design is such that the results can be interpreted in more than one way. The fact that glucose formation rather than disappearance of substrate is measured leads to the possibility that some rate-controlling step may intervene between the removal of glucose from the primary substrate and its transfer to water. Of course it also is possible that separate transferases might be involved.

The results in Fig. 5 show a definite lag in glucose production when the enzyme acts on 3-O-β-D-cellobiosyl-D-glucose. Similar lag periods were observed on the other substrates, but they varied in magnitude. One interpretation of curves which show initial lag periods such as that in Fig. 5 is that small amounts of a second substrate are present, for which the enzyme exhibits a much lower K_m than for the main substrate (15). Such a possibility seems rather remote in this case because of the high purity of the substrate (8). Instead, it seems more likely that the reaction pattern is such that certain levels of intermediates such as gentiobiose and other newly formed oligosaccharides must be attained before glucose production reaches its maximum value. The effect could be either an activation phenomenon or simply a matter of reaching an adequate level of an intermediate to sustain the maximum velocity.

The formation of glucose by the preparation may result as suggested (4) from transfer of glucosyl residues to water by a transferase. It might also result from the presence of other enzymes; for example, a disaccharase or trisaccharase. This question was approached by testing the effect of heat on the rates of formation of glucose from the various substrates. The results (Table III) show differences in the heat stabilities of the several activities. Action on cellobiose was most stable and action on laminaritriose was least stable, which may indicate separate enzymes. Of course similar results could occur if one enzyme hydrolyzed both substrates and changes in conformation of the enzyme caused the activity on laminaribiose to decrease more than the activity on cellobiose. The heat-inactivation did not proceed by first-order kinetics with any of the substrates, which also suggested that more

TABLE III

EFFECT OF HEATING AT 41°C. ON VELOCITY OF GLUCOSE FORMATION; ACTIVITY REMAINING

	Substrate[a]						
	C_2	C_3	L_2	L_3	Gent.	Cpd. 5	Cpd. 6
hours at 41°C.	%	%	%	%	%	%	%
0	100	100	100	100	100	100	100
3	73	44	37	14	24	27	44
6	67	38	33	14	17	.....	.....

[a] C_2, cellobiose; C_3, cellotriose; L_2, laminaribiose; L_3, laminaritriose; gent., gentiobiose; cpd. 5, 3-O-β-D-cellobiosyl-D-glucose; cpd. 6, 3-O-β-D-cellotriosyl-D-glucose.

than one enzyme is involved in formation of glucose from each substrate. The transferase activity, as reflected by the rate of formation and disappearance of gentiobiose, was also decreased by heating. There was a very large decrease in activity during the first 3 hr. and a much smaller decrease during the next 3 hr. The concentration of gentiobiose was determined by comparing the darkness and size of spots on chromatograms; hence, the quantitative precision was not high enough to relate it precisely to the rate of glucose production from any particular substrate, although it did appear to correlate more closely to those showing about a three-fourths drop in activity during the first 3 hr.

Heat-inactivation experiments did show clearly that the transferase which acts on cellobiose will not act on native beta-D-glucan (B-G). Heating the preparation at 41°C. eliminated its endo-beta-glucanase activity and its transferase activity on B-G. Contrarily, heating had only a slight effect on the transferase activity as reflected by the formation of gentiobiose from cellobiose.

It would appear that further experiments, particularly with inhibitors, should lead to a clarification of the system being studied. However, any approach suffers very serious limitations because of the analytical methods for measuring the enzyme activities. Perhaps the most troublesome problem is that glucose production rather than substrate depletion must be measured, because of the lack of a simple accurate method for measuring cellobiose concentration in the presence of gentiobiose, and so on. The experimental results show that much of the glucose is transferred to other compounds before being transferred to water. Thus, differential inhibition of the action on two different substrates might indicate two enzymes, but it also might indicate that transfer to or from one substrate was effected to a different extent than transfer to or from another substrate, even though both are catalyzed by a single enzyme. Because of the difficulties posed by the necessity to test for transferase activity by measuring glucose production, present effort is being directed toward purifying and obtaining the transferase as a homogeneous protein. The use of an enzyme shown to be homogeneous by physical methods will facilitate learning the sequence of reactions and the mechanism of the reactions catalyzed by the transferase, because the results then can be interpreted in the light of the knowledge that just one enzyme is present.

Acknowledgments

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