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Characterization of Intestinal Invertase as a Glucosido-Invertase

II. Studies on Transglycosylation by Intestinal Invertase

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Purified hog intestinal invertase produced a number of oligosaccharides in a concentrated sucrose solution. Since no oligosaccharides were produced from a mixture of glucose and fructose under the same conditions, the mechanism for oligosaccharide formation is a transglycosylation.

At the initial stage of the reaction more fructose than glucose was liberated. Intestinal invertase therefore transfers *glucose*, which also appears from the structure of the oligosaccharides produced. This is in accordance with the classification of intestinal invertase as a glucosido-invertase (α-D-glucopyranosidase).

The origin and structure of some of the oligosaccharides produced were studied. The product by transfer of glucose to sucrose was identified as α-D-glucopyranosyl (1 → 4) α-D-glucopyranosyl (1 → 2) β-D-fructofuranoside. The name *maltosucrose* is proposed for this trisaccharide.

Maltosucrose was rapidly hydrolyzed by intestinal invertase. During this hydrolysis, sucrose was formed as an intermediate product. The maltose linkage of the trisaccharide is therefore hydrolyzed before the sucrose linkage.

No resynthesis of sucrose could be demonstrated during the hydrolysis of sucrose in the presence of ^{14}C-labelled glucose or fructose. The ^{14}C-hexoses were, however, incorporated into other oligosaccharides.

It is generally assumed that two different kinds of invertases exist, namely, fructosido-invertases (β-D-fructofuranosidases) and glucosido-invertases (α-D-glucopyranosidases). It has been suggested that intestinal invertase is a glucosido-invertase. The experimental evidence for this theory is, however, incomplete [1]. For this reason, a study of the transglycosylation by intestinal invertase was performed, the results of which are reported in the present paper.

During the action of glycosidases upon a *concentrated* substrate solution, a number of oligosaccharides are usually * formed by a process called transglycosylation [3]. Since this phenomenon occurs with highly purified glycosidase preparations as well, it is assumed that hydrolysis and transglycosylation are catalyzed by the same enzyme [3].

With invertases two different kinds of transglycosylation have been observed, namely *transfructosylation* and *transglucosylation* **. It has been demonstrated that yeast invertase [3] and invertase from *Penicillium spinulosum* [3,4], which undoubtedly are *fructosido*-invertases, have trans-*fructosyl*ase activity. Honey invertase [3,5,6] on the other hand, which is a *glucosido*-invertase, has trans*glucosyl*ase activity. The hypothesis that these enzymes are fructosido- and glucosido-invertases is therefore supported by the transglycosylase activities, which seem to provide a valuable basis for the classification of invertases.

MATERIALS AND METHODS

Purified intestinal invertase (from pig) was obtained by mutual displacement chromatography on TEAE-cellulose [2]. The preparation used had a maltase/invertase activity quotient of 0.6 and an invertase activity of 250 units per mg of protein [8]. It contained no trehalase or maltase III activity [7].

Sucrose, glucose and fructose had analytical grade purity. Sucrose and glucose were obtained from J. T. Baker Co., fructose from Pfanstiehl Chemical Inc.

^{14}C-Labelled glucose and fructose (uniformly labelled) were obtained from The Radiochemical Centre (England).

Paper chromatography of sugars was performed by the descending technique on Whatman No. 1 paper. The lower end of the paper was serrated so as to allow the solvent to drip off (Fig. 1). 2—5 μl spots of the solution to be analyzed were applied to the paper, and after drying the chromatogram was equilibrated and run with ethyl acetate:acetic acid:water 9:2:2 (v/v). For separation of the oligosaccharides produced by transglycosylation, the chromatogram was run for 3 days. Under these conditions the sucrose spot travelled about 15 cm.

As *general reagent* for the detection of the sugar spots, the *periodate* reagent described by Movery [9], was used. This reagent had the great advantage over the more commonly used aniline phthalate reagent that it had the same sensitivity to non-reducing oligosaccharides as to reducing sugars.

For detection of free *reducing groups* in oligosaccharides ammoniacal silver nitrate [10] was used. This reagent reacted with glucose, fructose and maltose, but did not produce any colour even with great amounts of non-reducing oligosaccharides such as sucrose, raffinose, melezitose or trehalose.

Fructose-containing oligosaccharides were detected with the modified Seliwanoff reagent described by Hattori and Shiroya [11]. This reagent is specific for ketoses. Fructose, sucrose, raffinose and melezitose produced brilliantly red spots with this reagent, while glucose, maltose and trehalose did not produce any colour at all.

For the chromatography of ^{14}C-labelled sugars papers were used which had previously been cut into 1.5 cm broad strips, 1 cm apart, held together by intact paper [12]. The lower end of these papers, too, was serrated. One sugar spot was applied to each strip. After the run, the distribution of the radioactivity was measured for each strip with a Geiger-Müller counter. The paper strip automatically moved in front of the tube, and the time for 100 counts was recorded for each cm length of the strip. No back-ground correction was made (Figs 2 and 3). After counting, the sugar spots were localized with the periodate reagent.

* No transglycosylation could be demonstrated with intestinal trehalase [2].

** Trans*glycos*ylation is the name for the transfer of any sugar residue while trans*glucos*ylation means the transfer of *glucose*.

For the *quantitative determination* of glucose and fructose, papers were used which had been cut in the same way as in the case of ^{14}C-labelled sugars. The chromatograms were run for 20 h only. The sugar spots were localized by marker sugars run on parallel strips. 3 cm long pieces of the paper containing the sugars to be determined were eluted in small beakers with 5 ml of water at room temperature for 20 min with occasional shaking. A sample of the eluate, containing 1 – 9 μg of glucose or fructose, was used for quantitative determination with the highly sensitive method of Park and Johnson [13]. The colour produced was measured at 690 mμ with a Beckman B spectrophotometer.

Chromatography of sugars on carbon-celite columns was performed as described by Whistler and Durso [14]. 300 g each of Darco G-60 and celite were mixed and packed to a column 9 × 25 cm. The column was washed with 1 l of 0.1 N HCl, 3 – 4 l of 50 % (by vol.) ethyl alcohol in water and finally with water. After adsorption of the sugar mixture to the column, the monosaccharides were eluted with 0 – 2.5 %, the disaccharides with 5 – 7.5 % and the trisaccharides with 10 – 15 % ethyl alcohol. The amount of sugar in the effluent was measured with the anthrone method, performed as described by Scott and Melvin [15], but with heating for 7.5 min at 100°C instead of for 16 min at 90°C. The column had a capacity of 10 g of sugar (determined with maltose). 10 – 15 l of solvent was used for the elution of each class of sugar.

Raybin's diazouracil test [16]. 10 mg of the sugar to be tested was dissolved in 0.5 ml of 0.1 N NaOH. 0.5 ml of a freshly prepared 0.4 % solution of diazouracil in water was added. If the reaction was positive, a *green colour* developed in a few minutes at room temperature. After 10 min 1 drop of 0.1 M $MgCl_2$ was added. When the reaction was positive, a stable *blue precipitate* was formed. If the reaction was negative, no colour developed, and after addition of $MgCl_2$ a red precipitate was formed.

Sucrose and raffinose gave positive Raybin's test, while glucose, fructose, maltose and melezitose gave negative reaction, in accordance with earlier findings [16,17]. The reaction is specific for the sucrose structure [17].

Determination of reducing sugars produced from oligosaccharide A. The enzymatic digestion of oligosaccharide A was performed in 0.1388 M (7 %) solution with 0.05 M maleate buffer pH 6.5. The reducing sugars produced were determined with the method of Somogyi and Nelson [18,19], after precipitation of the proteins with the Zn-Ba-reagent of Somogyi [20].

RESULTS AND DISCUSSION

Demonstration of oligosaccharide formation. In a solution containing 30 %* sucrose, 0.05 M maleate buffer pH 6.5 and 38 units of purified intestinal invertase per ml, glucose and fructose were rapidly formed at 37°C. During the course of the reaction, however, a number of oligosaccharides were formed, too, all of which moved slower than sucrose on the paper chromatogram with the solvent used.

One of these, oligosaccharide A, was formed very early in the reaction (Fig. 1). It has the R_s value (*i.e.* mobility/mobility of sucrose) 0.38. Later in the reaction two oligosaccharides were formed with mobility intermediate between oligosaccharide A and sucrose, namely, oligosaccharides B_1 and B_2 with R_s values 0.83 and 0.70, respectively. In addition one oligosaccharide with low mobility, oligosaccharide C with R_s 0.17, was formed.

When a 30 % solution of invert sugar was incubated during the same conditions, only glucose and fructose were found on the paper chromatogram. Intestinal invertase therefore has no demonstrable synthetic action. The oligosaccharides formed from sucrose must therefore be formed by *transglycosylation.*

* Per cent means g of solute per 100 ml of solution.

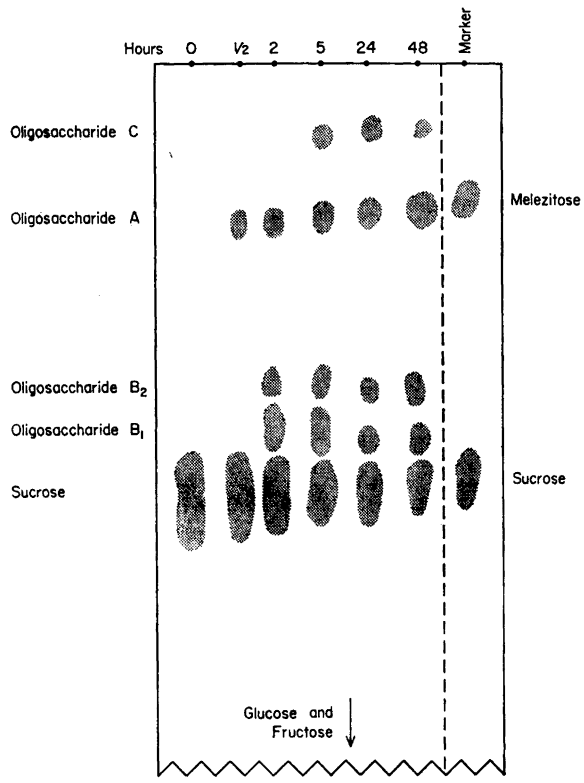

Fig. 1. Formation of oligosaccharides by transglycosylation. Purified intestinal invertase (38 units/ml) in 30 % sucrose with 0.05 M maleate buffer pH 6.5 at 37°C. Chromatogram developed during 3 days.

Oligosaccharides formed by transglycosylation are usually hydrolyzed when the reaction has been in progress for a sufficiently long time. In the present case, the reaction was followed for 14 days. Although the oligosaccharide spots had become smaller after this time, they were still clearly visible. This fact may be explained by inhibition of the enzyme brought about by the great amounts of monohexoses present. Pure oligosaccharide A was found to be rapidly hydrolyzed by intestinal invertase.

Amounts of glucose and fructose produced. The relative amounts of glucose and fructose produced by an invertase preparation during the action on a concentrated sucrose solution are dependent upon the nature of the transglycosylation. For each fructosyl group transferred one glucose molecule is liberated, and for each glucosyl group transferred, one fructose molecule is liberated.

During the action of intestinal invertase on a 30 % sucrose solution, the amount of fructose liberated at the initial stage of the reaction (5—10 %

Table 1. Properties of sugars produced by action of intestinal invertase in 30 % sucrose solution at pH 6.5 and 37°C.

	Reaction with ammoniacal $AgNO_3$ (reducing power)	Reaction with Seliwanoff reagent (fructose)
Fructose	pos.	pos.
Glucose	pos.	neg.
Sucrose	neg.	pos.
Oligosaccharide B_1	pos.	pos.
Oligosaccharide B_2	pos.	neg.
Oligosaccharide A	neg.	pos.
Oligosaccharide C	neg.	pos.

hydrolysis) was 1.5 times the amount of glucose liberated, determined by quantitative paper chromatography. The transglycosylation by intestinal invertase must therefore be a transfer of *glucose*. Since it has earlier been found that fructosido-invertases transfer fructose, while glucosido-invertases transfer glucose [3], this fact affords strong evidence that intestinal invertase is a glucosido-invertase. This conclusion implies, of course, that hydrolysis and transglycosylation are catalyzed by the same enzyme. This, however, seems probable since purified intestinal invertase (with 250 times increased specific activity) was found to produce the same oligosaccharide spots as a crude preparation of intestinal glycosidases.

Properties of the oligosaccharides produced. Some information about the properties of the different sugars formed may be obtained on the paper chromatogram by use of specific spray reagents. In the present case ammoniacal silver nitrate was used for the demonstration of reducing end groups, and a modified Seliwanoff reagent for the demonstration of fructose in oligosaccharides. The results with these reagents are listed in Table 1.

Oligosaccharide A is non-reducing and contains fructose. This may be expected if oligosaccharide A is formed by transfer of a glucosyl radical to sucrose. This is the probable way for the formation of oligosaccharide A, since it appears very early in the reaction. The isolation of oligosaccharide A and the determination of its structure are described below.

Oligosaccharide C is also non-reducing and contains fructose. Since this sugar has a low chromatographic mobility, it is probably a tetrasaccharide, possibly formed by transglycosylation to oligosaccharide A. This theory is supported by the fact that a sugar with the same mobility as oligosaccharide C is produced when intestinal invertase acts upon a solution of pure oligosaccharide A.

Oligosaccharides B_1 and B_2 are reducing sugars. Their relatively high chromatographic mobility indicates that they may be disaccharides. This theory is supported by the fact that they were eluted (together with sucrose) by 5 % ethyl alcohol from a carbon-celite solumn. The slowest moving one, oligosaccharide B_2, contains no fructose, and has the same chromatographic mobility as maltose. It may be identical with this sugar.

If oligosaccharides B_1 and B_2 are disaccharides, they may be formed either by transfer of glucose to free fructose and glucose, or by partial hydrolysis of a trisaccharide. By the use of ^{14}C-labelled fructose and glucose it was possible to identify oligosaccharides B_1 and B_2 as the products of transglucosylation to free fructose and glucose, respectively (see below).

Isolation and structure of oligosaccharide A. 60 g of sucrose were dissolved in 0.05 M maleate buffer pH 6.5 to a final volume of 200 ml and 1 ml of a purified invertase preparation, containing 600 units of invertase and 3 mg of protein, was added. As preservative, 5 ml of toluene was added. After 7 days at 37°C the solution had a reducing power corresponding to 25 % hydrolysis of the sucrose. The reaction was then interrupted by boiling for 5 min.

By paper chromatography the digest was found to contain fructose, glucose, sucrose and the series of oligosaccharides described above. The whole solution was filtrated through a 9 × 25 cm carbon-celite column. The amount of sugar was about 6 times the capacity of the column. Because greater molecules are adsorbed with greater force, however, the solution passing through the column contained only monosaccharides, sucrose and oligosaccharide B_1 and B_2, while all the oligosaccharide A was adsorbed to the column. The disaccharides adsorbed to the column were eluted with 15 l of 7.5 % ethyl alcohol. Oligosaccharide A was then eluted with 8 l of 12.5 % ethyl alcohol. This fraction was evaporated *in vacuo*, lyophilized and dried *in vacuo* at 50°C over P_2O_5. In this way 1.66 g of chromatographically pure oligosaccharide A was obtained (when 1 mg of the preparation was chromatographed it formed only one spot, which had the same mobility as oligosaccharide A).

The preparation did not reduce Somogyi's copper reagent. During total acid hydrolysis (boiling with 1 N HCl for 15 min) a mixture of glucose and fructose was obtained. With the aid of quantitative paper chromatography the mixture was found to contain two parts glucose and one part fructose. By cryoscopy (in water) the molecular weight of oligosaccharide A was determined at 470 (calculated for a trisaccharide 504.4). The sugar is therefore a non-reducing trisaccharide, composed of one fructose and two glucose molecules.

Raybin's diazouracil test was positive for oligosaccharide A, which demonstrates that fructose and one of the glucose molecules are joined by a sucrose link. This was later supported by the observation of a non-reducing, fructose-containing sugar, with the same mobility as sucrose, as intermediate product during the hydrolysis of oligosaccharide A by intestinal invertase.

Since the fructofuranoside link of sucrose is very easily split by acids, it was thought possible to split this link by mild acid hydrolysis while preserving the other link of the trisaccharide. When the trisaccharide was boiled with 0.05 N HCl for 15 min only fructose and a reducing disaccharide, which did not contain fructose, and which had the same chromatographic mobility as maltose, were found. 500 mg of oligosaccharide A were partially hydrolyzed by 0.05 N HCl and applied to a 3.5 × 20 cm carbon-celite column. The fructose was eluted with 2 l of 2.5 % ethyl alcohol and discarded. The disaccharide was eluted with 2 l of 7.5 % ethyl alcohol, evaporated *in vacuo* and lyophilized. The yield was 193 mg of chromatographically pure sugar.

This sugar was identified as maltose. It had the same chromatographic mobility as maltose, $[\alpha]_D^{20}$ + 130.0° (5 % in water) after slight downwards mutarotation (maltose $[\alpha]_D^{20}$ + 130.4° [17]). It gave a phenylosazone which (crystallized from ethyl alcohol) had the same crystal form as maltosazone. Acetylation with sodium acetate as catalyst yielded a β-octaacetate with m.p. 156—159°C (first crystallization from ethyl alcohol) maltose α-octaacetate 157—159°, mixed m.p. 156—158°).

Oligosaccharide A is therefore α-D-glucopyranosyl (1→ 4) α-D-glucopyranosyl (1→ 2) β-D-fructofuranoside. A sugar with this structure has earlier ben isolated from the oligosaccharides produced from sucrose by honey invertase [6]. The properties of our preparation agreed well with those reported for this trisaccharide. $[\alpha]_D^{20}$ + 122.1° (2.7 % in water) (reported $[\alpha]_D^{25}$ + 121.8°) [6] Acetylation with pyridine as catalyst [6,21] yielded a hendecaacetate with $[\alpha]_D^{20}$ + 83° (amorphous, 1.18 % in $CHCl_3$) (reported $[\alpha]_D^{25}$ + 86.0°) [6]. A suitable name for α-D-glucopyranosyl (1→ 4) α-D-glucopyranosyl (1→ 2) β-D-fructofuranoside should be *maltosucrose*.

Enzymatic hydrolysis of oligosaccharide A. In a 0.1388 M (7 %) solution of oligosaccharide A (maltosucrose), containing 0.05 M maleate buffer pH 6.5 and 600 units of purified intestinal invertase per ml, the oligosaccharide was rapidly hydrolyzed. After 30 min at 37°C, glucose, fructose and one intermediate disaccharide with the same chromatographic mobility as sucrose, were formed. The disaccharide was non-reducing and contained fructose, which supports the hypothesis that it is sucrose, formed by partial hydrolysis of oligosaccharide A.

A faint spot with the same chromatographic mobility as oligosaccharide C was also observed after 30 min. This supports the belief that oligosaccharide C is formed by transglycosylation to oligosaccharide A.

After 5 h at 37°C, only glucose and fructose were found on the paper chromatogram. The hydrolysis of oligosaccharide A therefore goes on until completed.

The *rate* of hydrolysis of oligosaccharide A in 0.1388 M solution was studied with more dilute enzyme solutions. The amount of reducing sugars produced from oligosaccharide A was 0.24 times the amount produced from sucrose during the same conditions. Calculated as the number of glycosyl linkages hydrolyzed per time unit, the rate for hydrolysis of oligosaccharide A is 0.48 times the rate for sucrose hydrolysis when calculated as hydrolysis to glucose and sucrose, or 0.32 times when calculated as complete hydrolysis into glucose and fructose.

Transglycosylation to free hexoses. The reaction mixture contained 30 % sucrose, ^{14}C-labelled fructose or glucose (about 1 mg/ml) which gave 1 250 000 cpm/ml with the counter used, 0.05 M maleate buffer pH 6.5 and 50 units/ml of purified intestinal invertase. After different times of reaction at 37°C, 5 μl samples were applied to a paper chromatogram. Each spot thus contained 6 250 cpm of ^{14}C-labelled sugar.

As is seen in Figs. 2—3 fructose and glucose are incorporated into oligosaccharides B_1 and B_2, respectively. This reaction is very rapid during the first hour, but then proceeds much slower since the ^{14}C-labelled monohexoses

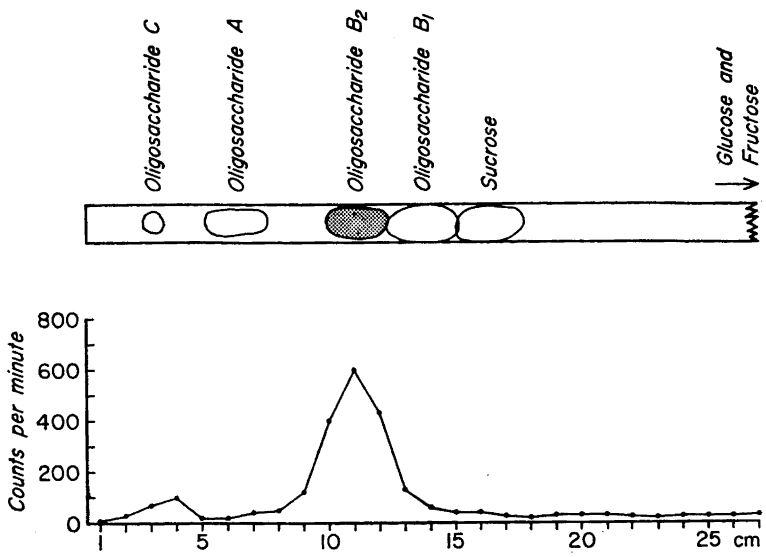

Fig. 2. Transglycosylation by intestinal invertase with ^{14}C-glucose as receptor. Incubated at 37°C for 23 h. For experimental conditions, see text. Glucose is incorporated into oligosaccharide B_2. The spot applied to the chromatogram contained 6 250 cpm of ^{14}C-labeled sugar. 25 % of the activity is localised in the oligosaccharide B_2 spot.

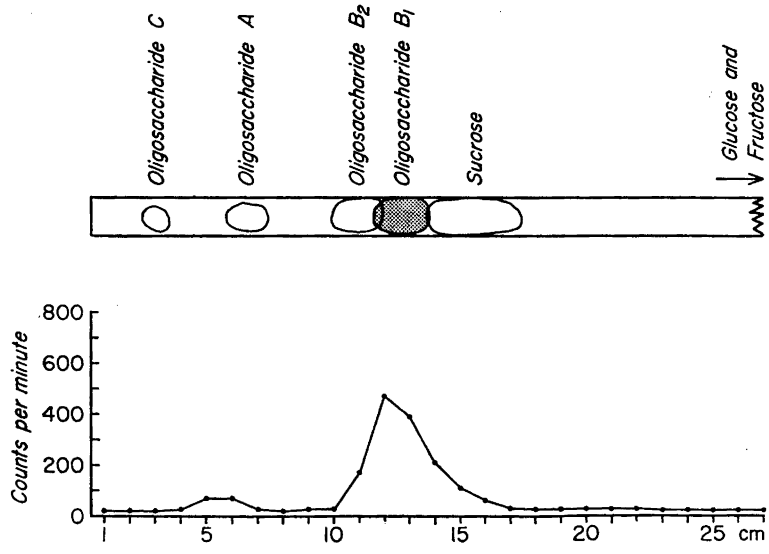

Fig. 3. Transglycosylation by intestinal invertase with ^{14}C-fructose as receptor. Incubated at 37°C for 5 h. For experimental conditions, see text. Fructose is incorporated into oligosaccharide B_1. The spot applied to the chromatogram contained 6 250 cpm of ^{14}C-labeled sugar. 20 % of the activity is localised in the oligosaccharide B_1 spot.

are diluted with inactive hexoses produced by hydrolysis. In both cases a very small peak of activity is also found, with chromatographic mobility intermediate between oligosaccharides A and C. Since no sugar spot could be detected in this place, these peaks must be caused by sugars which are formed only in very small amounts.

The incorporation of fructose and glucose into oligosaccharides B_1 and B_2, respectively, is in accordance with the fact that oligosaccharide B_1 contains fructose while oligosaccharide B_2 does not (see above), since intestinal invertase transfers *glucose*. The results show that oligosaccharides B_1 and B_2 are formed by transfer of glucose to free fructose and glucose respectively.

No resynthesis of sucrose by transglycosylation could be demonstrated. Resynthesis of sucrose (with glucose as receptor) has been observed with mold invertase, but could not be demonstrated with yeast invertase [3].

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