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Characterization of Intestinal Invertase as a Glucosido-Invertase

I. Action on Different Substrates

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The basis for classification of intestinal invertase as a glucosido-invertase is discussed.

The action of a preparation of intestinal glycosidases on some di- and trisaccharides, related to sucrose, has been studied.

No action upon raffinose [*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] or gal-sucrose (α-D-galactopyranosyl-β-D-fructofuranoside) could be detected. Intestinal invertase can therefore not hydrolyze these sugars. This supports the view that intestinal invertase has a great specificity for the glucopyranoside part of the sucrose molecule.

Melezitose [*O*-α-D-glucopyranosyl-(1→3)-*O*-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside] was hydrolyzed, but at a very slow rate. The substitution in the fructofuranoside part of the sucrose molecule must therefore have a marked influence on the action of intestinal invertase. At present, however, we do not know, if sucrose and melezitose really are hydrolyzed by the same enzyme.

An attempt to demonstrate, if the sucrose linkage in melezitose can be hydrolyzed before the turanose linkage, had no success. This failure was explained by the slow rate of melezitose hydrolysis.

The results indicate that intestinal invertase has a very high degree of specificity for the glucopyranosyl part of sucrose, but that also alterations in the fructofuranosyl part of the molecule interfere strongly with the action of the enzyme. Further specificity studies require more purified enzyme preparations.

The division of invertases into glucosido- and fructosidoinvertase was first made by Kuhn, grounded on their inhibition by glucose or fructose, respectively [1–3]. Such inhibitions, however, show many irregularities [4–6], and are therefore no practical ground for the classification of invertases.

Later Kuhn *et al.* proposed the use of raffinose [*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] and melezitose [*O*-α-D-glucopyranosyl-(1→3)-*O*-β-D-fructofuranosyl(2→1)-α-D-glucopyranoside] for the characterization of invertases [6]. These sugars are regarded as sucrose, where the glucosyl or the fructosyl parts, respectively, are substituted with a

third sugar. Glucosido-invertases should be able to hydrolyze melezitose and fructosido-invertases raffinose (into fructose and melibiose) in addition to sucrose.

That intestinal invertase cannot hydrolyze raffinose was observed by Bierry [7] already in 1912. Recent reports support this statement [8].

The behavior of intestinal invertase against melezitose has never been investigated, although the interest for such an investigation has been expressed [9].

Recently has been reported the synthesis of a new disaccharide, gal-sucrose (α-D-galactopyranosyl-β-D-fructofuranoside), which is a sucrose where the glucose has been replaced by galactose [10]. The only structural difference from an ordinary sucrose molecule is therefore that the hydrogen and the hydroxyl at the fourth carbon atom of the glucosyl part have shifted place. This substance seems very interesting for testing the specificity demands of invertases for the glucopyranosyl part of sucrose.

In the present paper are reported some experiments on the action of an intestinal glycosidase preparation upon raffinose, melezitose, and gal-sucrose. Turanose (3-*O*-α-D-glucopyranosyl-D-fructose) is also included, because it is a part of the melezitose molecule. For the substrates hydrolyzed, the optimum pH, relative rate of hydrolysis, K_s, and v_{max} have been determined.

As source of enzyme was selected swine intestinal mucosa. In this material invertase (together with maltase, lactase, and trehalase) has been found localized in the microsome fraction [11], obtained by differential centrifugation. The enzymes were extracted from the microsomes by sodium desoxycholate.

MATERIALS AND METHODS

Substrates. A sample of gal-sucrose was kindly supplied by D. Feingold, G. Avigad, and S. Hestrin, who recently reported the synthesis of this sugar [10]. The other substrates were commercial preparations of analytical grade purity, sucrose from J. T. Baker Co., raffinose and melezitose from Pfanstiehl Chemical Co., and turanose from Nutritional Biochemicals Corporation.

Glycosidase preparation. Pieces of swine intestine were taken from the slaughterhouse immediately after the slaughter, and chilled with ice during the transport to the laboratory. The pieces were cut from the proximal jejunum, because this part seems to be richest in invertase [12]. The mucosa was scraped off, homogenized in 0.25 M sucrose (previously chilled to 0° to prevent hydrolysis), and the microsomes were isolated by differential centrifugation [11]. The glycosidases were eluted from the microsomes with 0.5 % sodium desoxycholate (kindly supplied by A. Norman). The desoxycholate and the remaining sucrose were removed by dialysis against phosphate buffer pH 7.5, and then the buffer was removed by dialysis against distilled water. The suspension thus obtained contained 80—90 % of the invertase present in the original homogenate, and corresponding amounts of maltase, lactase, and trehalase [11].

Determination of enzymatic activity. Samples of enzyme solution were mixed at 37°C with buffered substrate solutions, to give a concentration of 0.1 M buffer (acetate or phosphate) and, when not otherwise noted, 0.1460 M substrate (5 % sucrose or turanose, 7.89 % melezitose . $2H_2O$, or 8.65 % raffinose . $5H_2O$). Toluene was used as preservative. The degree of hydrolysis was not allowed to exceed 5 % per hour. During these conditions, the reaction was found to be of zero order up to at least 10 % hydrolysis, and the (initial) reaction velocity was found to be proportional to the amount of enzyme present.

Gal-sucrose, of which only a total amount of 2 mg was available, was used in lower concentration as described later.

The degree of hydrolysis was determined by different methods, depending of the properties of the substrates.

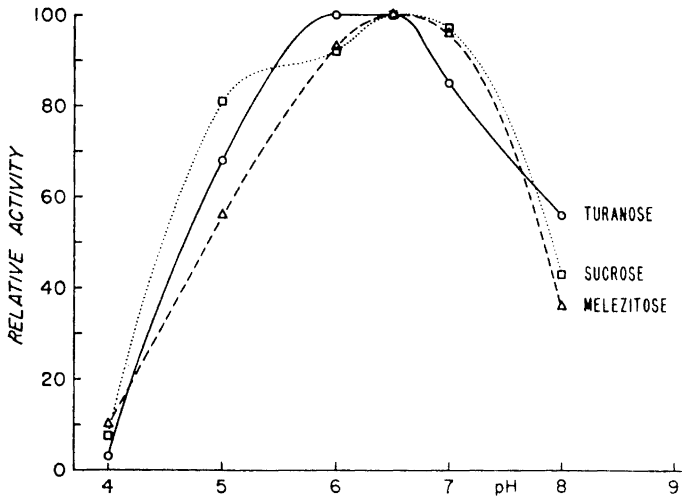

Fig. 1. Influence of pH on the hydrolysis of sucrose, melezitose and turanose. Buffers: 0.1 M acetate pH 4.0—5.0, 0.1 M phosphate pH 6.0—8.0. Temperature 37°C. Substrate concentration 0.1460 M.

1) Sucrose, raffinose, and melezitose are nonreducing. For determination of the monosaccharides produced from these sugars was used a modification of the 3,5-dinitrosalicylic acid method of Sumner [13,14], with spectrophotometric reading of the obtained colour [15]. No protein precipitation is necessary with this reagent.

2) Gal-sucrose is also nonreducing, but because this sugar was used in a very dilute solution, the highly sensitive method of Somogyi and Nelson [16,17] was used, after previous precipitation of proteins with Zn-Ba reagent [18].

3) Turanose is a reducing disaccharide. The liberated glucose was determined by the specific enzymatic method of Keilin and Hartree [19].

Samples of 0.4 ml of the reaction mixture were added 1.6 ml of 0.1 M acetate buffer pH 5.0, and the glycosidase action was interrupted by boiling in water-bath for 2 min. (The addition of acid buffer before boiling was found to be essential since turanose is a very alkali-labile sugar, and if it is boiled at a pH higher than 6.0, part of the turanose will be decomposed, leaving free glucose. This buffer will not interfere with the following enzymatic determination of glucose.) 1.0 ml of this solution was transferred to a Warburg flask (without central cylinder). 2.0 ml of a 0.1 M acetate buffer pH 5.6, containing 0.1 ml of catalase solution (Catalase, sterile solution, Worthington) and 0.02 ml of 99.5 % ethanol per 2 ml, were added. In the side arm of the flask was placed 0.5 ml of a 10 % glucose oxidase solution (Glucose oxidase, crude, Sigma). This contains no enzyme acting upon turanose (but it contains maltase) and might therefore in this case be used without further purification. After equilibration at 25°C, the tap was closed and the flask tipped. When no more oxygen was absorbed (after 30—45 min) the readings of the manometers were recorded. 124.4 mm^3 of oxygen absorbed corresponds to 1.0 mg of glucose oxidized.

Paper chromatography of sugars was performed by the ascending technique on 30 cm long pieces of paper (Whatman No. 1). A roll of equal breadth, containing about 120 cm of filter paper was placed on the top to absorb the solvent. The chromatograms were developed for 24 h with ethyl acetate:acetic acid:water 9:2:2 v/v. The sugar spots were detected with aniline hydrogen phthalate [20].

RESULTS

Sucrose was rapidly hydrolyzed by the enzyme solution, with an optimum pH between 6.3 and 6.8 (Fig. 1). The amount of invert sugar produced per

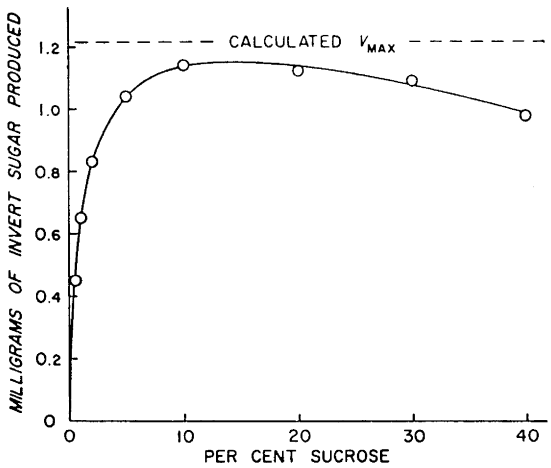

Fig. 2. Influence of substrate concentration upon the hydrolysis of sucrose. Reaction volume 2.0 ml in 0.1 M phosphate buffer pH 6.5. Incubated for 1 h at 37°C. Mg of produced invert sugar determined by the 3,5-dinitrosalicylic acid method. For calculation of v_{max} see Fig. 3.

time unit increased with increasing substrate concentration up to 10 % (0.2920 M) sucrose, but at higher substrate concentration the amount of invert sugar produced (as measured with the 3,5-dinitrosalicylic acid method) decreased somewhat (Fig. 2). This decrease may be caused by transglycosidation, which is marked at concentrations higher than 10 % sucrose, but hardly detectable at 5 % [21]. From the values obtained at substrate concentrations below 10 % the theoretical v_{max} for infinite substrate concentration and the K_s (*i. e.* the substrate concentration at which the initial rate of hydrolysis is half of the v_{max}) were calculated by the graphical method of Lineweaver and Burk [22] as modified by Dixon [23] (Fig. 3). The v_{max} was found to be 1.2 times the rate of reaction in 0.1460 M sucrose, and the K_s was calculated to 2.5×10^{-2} M sucrose.

No action upon raffinose could be detected. A solution of raffinose was mixed with enzyme solution to give a concentration of 0.1460 M raffinose in 0.1 M phosphate buffer pH 6.5. No reducing sugars could be detected after 72 h at 37°C, and only raffinose was found on a paper chromatogram. During the conditions used, a hydrolysis at a rate only 1/1000 of that of sucrose hydrolysis would have been easily detected.

Gal-sucrose (α-D-galactopyranosyl-β-D-fructofuranoside) was available only in a minute quantity (2 mg), and was therefore used in a considerably more diluted solution than the other substrates. For comparison of the results, it was therefore necessary to study the rate of hydrolysis in an equally diluted sucrose solution (Table 1). It is seen, that even in a 0.05 % solution of sucrose, this sugar is rapidly hydrolyzed. Corresponding experiments were then performed with weighed amounts of gal-sucrose (Table 2). As blanks were used

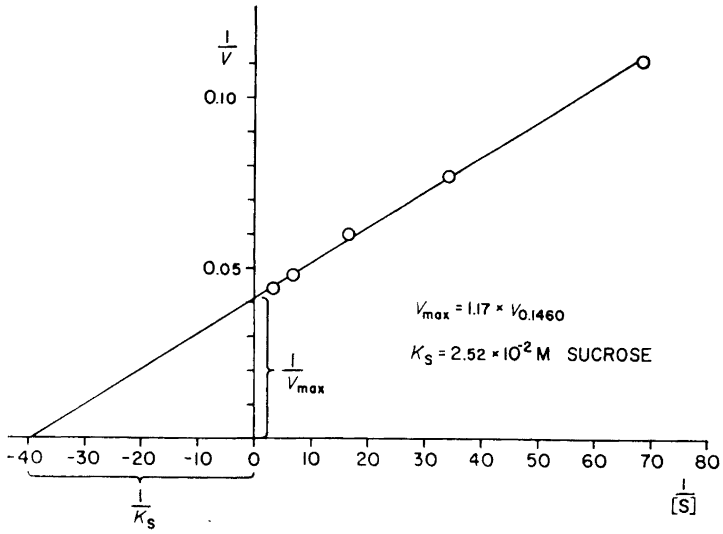

Fig. 3. Graphical determination of v_{max} and K_S for invertase. Temperature 37°C. 0.1 M phosphate buffer pH 6.5.

mixtures of enzyme and buffer, without sugar, incubated in the thermostat for the same time. It was also found necessary to correct the values obtained with gal-sucrose for a small amount of reducing sugar present as a contamination in this sugar (Table 2). No hydrolysis of gal-sucrose could be detected. During the conditions used a hydrolysis at a rate of only 1/500 of that of sucrose would have been easily demonstrated.

Table 1. Hydrolysis of a dilute sucrose solution by intestinal invertase. Sucrose solution, 1 mg/ml in 0.2 M phosphate buffer pH 6.5, is mixed with an equal volume of enzyme solution. After incubation at 37°C, the protein is precipitated with Zn-Ba-reagent, and reducing sugars are determined by the method of Somogyi and Nelson. The sucrose is rapidly hydrolyzed.

	Time of incubation	*D* 500	*D* 660	Reduction power (expressed as μg of invert sugar per 2 ml	% hydrolysis
Blank without sugar	0	0.038	0.160	(24)	—
» » »	3 h	0.050	0.190	(32)	—
1 mg of sucrose	0	0.040	0.161	24	(0)
1 » » »	10 min	0.360	1.225	212	20.3
1 » » »	1 h	1.200	(>2.5)	740	70.5
1 » » »	3 h	1.530	(>2.5)	950	87.5

Table 2. Incubation of gal-sucrose with intestinal invertase. Weighed amounts of gal-sucrose were dissolved in 1.0 ml of 0.2 M phosphate buffer pH 6.5. 1.0 ml of enzyme solution was added, and the sample was incubated in water-bath at 37°C. Reducing sugars were determined by the method of Somogyi and Nelson. No hydrolysis could be detected.

	D 660	% hydrolysis
Expt. 1.		
0.460 mg of gal-sucrose without enzyme (reducing contaminants in gal-sucrose)	0.063	—
Expt. 2.		
0.820 mg of gal-sucrose incubated for 4 h	0.267	0
Blank without sugar, incubated for 4 h	0.200	
Reduction by 0.820 mg of gal-sucrose *	0.057	
Blank value:	0.257	
Expt. 3.		
0.640 mg of gal-sucrose incubated for 4 h	0.242	0
Blank without sugar, incubated for 4 h	0.191	
Reduction by 0.640 mg of gal-sucrose *	0.044	
Blank value:	0.235	
* calculated from Expt. 1		

Turanose was hydrolyzed with optimum pH between 6.0 and 6.5 (Fig. 1). The initial rate of hydrolysis in 0.1460 M turanose at pH 6.5 and a temperature of 37°C was 0.15 times the rate of sucrose hydrolysis during the same conditions. The K_s was calculated to 2.8×10^{-2} M turanose, and the v_{max} to 1.2 times the rate at 0.1460 M substrate concentration. By paper chromatography turanose was shown to be completely hydrolyzed into glucose and fructose.

Melezitose was also hydrolyzed by the enzyme solution, with an optimum pH equal to that for sucrose hydrolysis (Fig. 1). The rate of melezitose hydrolysis was, however, very low. At 0.1460 M melezitose concentration, pH 6.5, and a temperature of 37°C, the amount of hexoses produced was only 0.003 times the amount produced from sucrose under the same conditions. Calculated as the number of glucosidic linkages split per time unit, the rate of melezitose hydrolysis is 0.004 times the rate of sucrose hydrolysis. The hydrolysis of melezitose proceeds to completion.

In order to find out which of the two glycosidic linkages in melezitose that is hydrolyzed first, the reaction was followed on repeated paper chromatograms, but none of the two possible intermediate disaccharides (sucrose or turanose) could be demonstrated at any stage of the reaction. This is apparently caused by the fact, that these disaccharides are hydrolyzed at a rate so great, compared with melezitose, that whichever of the linkages in the trisaccharide that is hydrolyzed first, the liberated disaccharide is almost instantaneously split. In calculating the rate of melezitose hydrolysis, it must therefore be more correct to compare the number of *completely* hydrolyzed

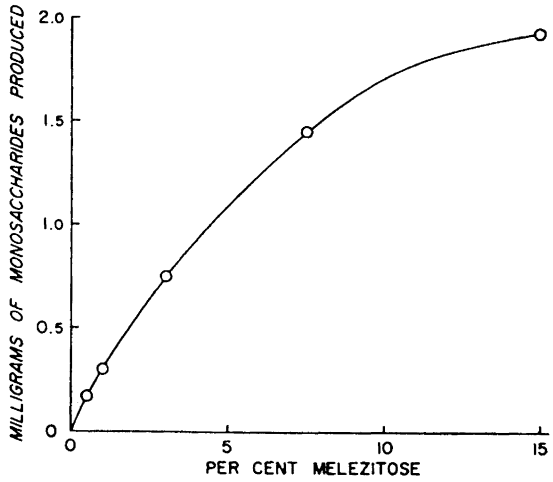

Fig. 4. Influence of the substrate concentration of the hydrolysis of melezitose. Reaction volume 2.0 ml in 0.1 M phosphate buffer pH 6.5. Incubated for 24 h at 37°C. Mg of produced monosaccharides determined by the 3,5-dinitrosalicylic acid method.

melezitose molecules with the number of hydrolyzed sucrose molecules. Calculated in this way, the rate of melezitose hydrolysis is only 0.002 times the rate of sucrose hydrolysis during the conditions used.

The rate of melezitose hydrolysis is rather much increased by increasing the substrate concentration, indicating that the enzyme has a low affinity for melezitose (Fig. 4). The K_s was calculated to 2.6×10^{-1} M melezitose and the v_{max} to 2.8 times the rate at 0.1460 M melezitose concentration.

It is not proven, if sucrose and melezitose really are hydrolyzed by the same enzyme. Both activities are inhibited by tris(hydroxymethyl)-aminomethane (buffer grade, Sigma) at alkaline pH (Fig. 5). This inhibition is caused by competition with the substrate for the enzyme. However, this inhibition seems to be common for several glycosidases, since Larner *et al.*[24] have found that both oligo-1,6-glucosidase and maltase (which they have separated from each other) are inhibited by tris(hydroxymethyl)aminomethane.

DISCUSSION

The findings of Bierry [7], that intestinal invertase has no action upon raffinose, was confirmed. Furthermore, it was found that the new disaccharide gal-sucrose was not hydrolyzed. These facts demonstrate, that intestinal invertase has a very high degree of specificity for the α-D-glucopyranosyl part of sucrose.

Melezitose, on the other hand, was hydrolyzed by the enzyme preparation used, but at a very slow rate as compared with sucrose. At 0.1460 M substrate concentration the hydrolysis of melezitose proceeded with a rate that was only

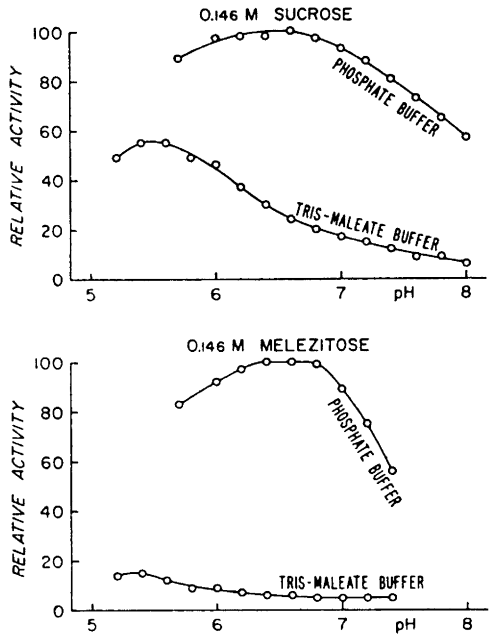

Fig. 5. Inhibition of the hydrolysis of sucrose and melezitose by tris(hydroxymethyl)-aminomethane at alkaline pH. Buffers: 0.05 M phosphate or maleate, respectively, with 0.05 M tris(hydroxymethyl)aminomethane. Maleate buffer without tris(hydroxymethyl)-aminomethane gave the same values as phosphate buffer.

0.002 times the rate for sucrose hydrolysis during the same conditions. The K_s for melezitose hydrolysis is about ten times greater than the K_s for sucrose hydrolysis, which indicates that the slow hydrolysis of melezitose is partly caused by a low affinity for this substrate. By comparing the v_{max} for the two substrates, however, one can see that the most important factor is a slow formation of the hydrolysis products from the enzyme-melezitose complex.

These calculations are valid only for the first hydrolyzed link in melezitose. Because melezitose is hydrolyzed so slowly, compared with sucrose or turanose, it was not possible to determine which of the linkages in the trisaccharide that was hydrolyzed first.

The calculations also presume that melezitose and sucrose are hydrolyzed by the same enzyme. At present no proof for this has been obtained. Conclusions must therefore be drawn with care. The action of purified preparations of intestinal invertase upon melezitose and turanose is under investigation.

It can, however, be stated, that, even if the substitution in the fructofuranosyl part of sucrose does not totally prevent the action of invertase, it interferes strongly with the reaction. It seems therefore probable that intestinal invertase has a marked specificity also for the fructofuranosyl part of the sucrose molecule.

This is in accordance with the recently observed multiple nature of intestinal glycosidases, which contests the view of Weidenhagen, who thought that mammalian intestine contains one single α-D-glucopyranosidase, hydrolyzing maltose, sucrose, and other α-D-glucopyranosides [25,26]. It has been shown that intestinal glycosidase preparations are without action upon α-methylglucoside [27–29]. The activities against *iso*maltose (6-*O*-α-D-glucopyranosyl-D-glucose) and nigerose (3-*O*-α-D-glucopyranosyl-D-glucose) have been partially separated from maltase activity [30–32]. The trehalase activity of intestinal mucosa has been totally separated from invertase and maltase activities, and the invertase and maltase activities partially separated from each other [11]. For a further study of the specificity demands of intestinal invertase, it is therefore necessary to separate the enzyme from the other glycosidases.

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