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Hog Intestinal Isomaltase Activity

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Homogenates of hog intestinal mucosa have *isomaltase* activity, caused by at least three different enzymes.

About 3/4 of the total isomaltase activity is caused by an enzyme (or enzymes) which seems to have mainly isomaltase activity. This enzyme has no invertase or maltase activity, but it possibly hydrolyzes phenyl-α-D-glucopyranoside. The name *specific isomaltase* is suggested for this enzyme.

The remainder of the isomaltase activity is caused by two hog intestinal maltases, which hydrolyze isomaltose slowly.

Separation of the specific isomaltase from the other hog intestinal α-D-glucopyranosidases was not successful. It could be stated, however, that the specific isomaltase has transglycosylase properties which clearly differ from those of the isomaltase activity accompanying the two maltases.

The influence of freezing and thawing upon the specific isomaltase activity is complex, and involves a decrease as well as an increase of this activity.

The hydrolysis of *isomaltose* (6-α(-D-glucopyranosyl)-D-glucose) by enzyme preparations from hog intestinal mucosa has recently been studied by Larner, Mc Nickle and Gillespie [1-7]. These authors postulated that isomaltose and maltose were hydrolyzed by two different enzymes in these preparations, although they were able to separate these two activities only partially. A hog intestinal maltase preparation was obtained, which had no demonstrable isomaltase activity, but the isomaltase activity* was never purified from maltase activity [3,6].

More recently, however, it has been demonstrated that hog intestinal *maltase* activity is not caused by a single enzyme, but can be separated into three different fractions (maltase I—III) with different enzymatic properties.[8-10] One of these fractions, maltase I, had no detectable isomaltase activity, while the other two, maltase II and III, hydrolyzed isomaltose slowly [10].

The present investigation was undertaken to elucidate further the relation between the *maltase* and *isomaltase* activities of hog intestinal mucosa.

* Larner *et al.* name this activity *oligo-1,6-glucosidase* (to distinguish it from *amylo*-1,6-glucosidase). It seems more logical to call it *isomaltase* activity in agreement with general nomenclature used for other glycosidase activities.

MATERIALS AND METHODS

Enzyme preparations

Crude hog intestinal glycosidase preparations. Pieces of hog small intestine were cut within a few minutes after slaughter and chilled with crushed ice during the transport to the laboratory. In some experiments the mucosa was scraped off (as done by Larner *et al.*[3]) and homogenized for 2 min in an "Ultra-Turrax" homogenizer with an equal volume of saline or buffer. In other experiments the mucosa was squeezed out manually and solubilized as described earlier [11]. Whichever procedure was used, the relation of the isomaltase activity to the invertase and maltase activity of the preparation obtained was the same.

Purified hog intestinal maltase II and purified hog intestinal maltase III were obtained by mutual displacement chromatography of solubilized hog intestinal glycosidase preparations as described earlier [9].

Substrates

Isomaltose (6-(α-D-glucopyranosyl)-D-glucose) was prepared by enzymatic hydrolysis of dextran as described by Jeanes *et al.*[12] *Dextran* (average molecular weight M_w (end group analysis) 17 600, M_n (light scattering) 40 400) was obtained as a gift from A. B. Pharmacia (Uppsala, Sweden). *Penicillium funiculosum* was obtained from Centraalbureau voor Schimmelkultures (Baarn, Nederland). The mold was cultured on a solution containing 2 % dextran and 3 % yeast extract (from Difco Lab., Detroit, Mich.) at room temp. for 9—10 days during shaking. The culture filtrate had dextranase activity of 18 units/ml (determined essentially as described by Tsuchiya *et al.*[13], but the amount of reducing sugar produced was measured with the 3,5-dinitrosalicylic acid reagent [17] instead of Somogyi's copper reagent).

For preparations of isomaltose 4 g of dextran was dissolved in 40 ml of water. To this solution was added 20 ml of 0.1 N acetate buffer pH 5.1, 20 ml of culture filtrate from *Penicillium funiculosum*, and 5 ml of toluene. After 23 h, at 37°C the reducing power of the solution corresponded to 77 % of hydrolysis to isomaltose. After removing toluene, the solution was boiled for a few minutes to interrupt the enzymatic hydrolysis. The solution was then poured onto a 9 × 25 cm carbon-celite column [14]. The glucose was eluted with 5 l of distilled water, and then the isomaltose was eluted with 5 l of 5 % (by vol.) ethanol. The isomaltose fraction was evaporated at reduced pressure to a volume of about 50 ml and then lyophilized (the sugar was markedly hygroscopic and crystallization was not successful). The powder obtained was dried *in vacuo* over P_2O_5 at 50°C. The yield was 1.75 g of chromatographically homogeneous sugar (when 1 mg of the sugar was run on a paper chromatogram it formed only one spot). The chromatographic mobility was somewhat lower than that of maltose, R_M value (mobility/mobility of maltose) 0.75 in ethyl acetate: acetic acid : water 9:2:2 (v/v). The molecular weight of the sugar was determined at 325 by cryoscopy (calcd. for $C_{12}H_{22}O_{11}$ 342.3). The optical rotation agreed well with that reported for isomaltose, measured $[\alpha]_D^{20}$ +117.5° (10 % in water), reported for isomaltose +122.0° [12], +103.2° (amorphous) [15], +120° (cryst.) [15]. Acetylation with sodium acetate as catalyst yielded the β-octaacetate (amorphous, in spite of several attempts crystallization was not achieved) which had $[\alpha]_D^{20}$ +97.5° (2 % in $CHCl_3$), reported for isomaltose-β-octaacetate +96.9° [12,16].

Phenyl-α-D-glucopyranoside was prepared as described earlier [10]. The other substrates used were commercial preparations of analytical grade purity [11].

Determination of glycosidase activity

The reaction mixture in all cases had 0.1388 M substrate concentration and contained 0.05 M maleate buffer pH 6.5 and small amount of toluene as preservative. The degree of hydrolysis was determined with the methods described earlier [9-10]. One unit of glycosidase activity is the amount which causes 1 % of hydrolysis in 2 ml of reaction mixture in 1 h at 37°C during these conditions. The degree of hydrolysis was not allowed to exceed 10 %.

The hydrolysis of isomaltose was determined with Tauber and Kleiner's acid copper reagent as described earlier for maltose [9]. Since isomaltose had stronger reducing power with this reagent than maltose, the sugar determination was made with 1.0 mg of isomaltose instead of 2.5 mg of maltose. The modified copper reagent (Caputto's reagent) which was used for K_s and optimum pH determinations with maltose could not be used for isomaltase determinations, because isomaltose, itself, reduced this reagent too strongly.

Adsorbents

Cellulose ion exchangers. DEAE-cellulose was prepared from Solkafloc cellulose powder SW 40 A by the method of Peterson and Sober [20]. The product contained 0.8 mequiv. titrable hydroxyl groups per gram dry weight. *TEAE-cellulose* was prepared from DEAE-cellulose by ethylation as described by Porath [21]. CM-cellulose was prepared from Whatman cellulose powder by the method of Peterson and Sober [20]. It contained 0.3 mequiv. titrable acid groups per gram dry weight.

Alumina Cγ-gel was prepared as described by Willstätter and Kraut [22]. The gel was stored, suspended in distilled water, for six months at room temperature before use. The amount of gel used in the experiments will be expressed as mg dry weight, determined after drying to constant weight at 100°C.

Calcium phosphate gel (for column chromatography) was prepared by the method of Tiselius et al.[23]

Paper chromatography of sugars was performed as described earlier [14].

RESULTS AND DISCUSSION

Freshley prepared homogenates of hog small intestinal mucosa hydrolyzed *isomaltose* with a rate which was 8—10 % of the rate for the hydrolysis of maltose. The relation between the isomaltase and maltase activities was the same in all parts of the small intestine. Some different α-D-glucopyranosidase activities of a representative homogenate from hog small intestine are recorded in Table 1.

The separation of several different hog intestinal α-D-glucopyranosidases has been reported previously [8-10,17]. Two of the different α-D-glucopyranosidases separated had *isomaltase* activity, namely maltase II and maltase III.

Table 1. Some α-D-glucopyranosidase activities of a freshly prepared homogenate (1:2 in saline) of hog intestinal mucosa. The protein content of the homogenate was 9.5 mg/ml.

	Units/ml
Invertase	32
Maltase (total)	62
Maltase I *	19
Maltase II	21
Maltase III **	22
Isomaltase	6.8
Phenyl-α-D-glucopyranosidase	3.6

* Calculated as 0.6 times the invertase activity [10].
** Determined after inactivation of maltase I and II by heat [9].

The rate for the hydrolysis of isomaltose by these fractions was 2 and 5 %, respectively, of the rate for the hydrolysis of maltose [10]. Since each of the maltase II and III contributes only 1/3 to the total maltase activity of crude preparations, the isomaltase activity of these purified maltase fractions can contribute only 1/4 of the total isomaltase activity of the crude preparation. The existence therein of a *specific hog intestinal isomaltase* therefore was postulated.

The question then arises, if the isomaltase activity of the purified maltase II and III preparations really is caused by these enzymes *per se*, or if is caused by contamination with the specific isomaltase.

In order to study the properties of the specific hog intestinal isomaltase, attempts were made to separate this enzyme from the other intestinal α-D-glucopyranosidases.

Solubilization. Like the other hog intestinal glycosidase activities, the isomaltase activity is localized to the microsome fraction, obtained by differential centrifugation [6,11]. Like the invertase and maltase activities, the isomaltase activity could be solubilized with trypsin (in the presence of phosphate buffer) and precipitated with ethanol in the same way as described earlier for the other activities [11], without losses of isomaltase activity.

It has been reported that hog intestinal isomaltase is inactivated by high speed centrifugation [6]. This, however, could not be verified. Even when homogenates of intestinal mucosa (in the presence of 0.01 M veronal buffer pH 7.5) were centrifuged at 100 000 × *g* for 3 h (Spinco preparative ultracentrifuge, rotor 40) no losses of isomaltase activity were recorded. All the activity was found in the sediment, when fresh homogenates were used, or in the supernatant, when solubilized preparations were used.

Ion exchange chromatography. Chromatography of the solubilized glycosidase preparations on DEAE-cellulose or TEAE-cellulose, which has earlier been used for the separation of the other hog intestinal α-D-glucopyranosidases [9,11], was associated with losses of more than 50 % of the isomaltase activity. When adsorption to TEAE-columns, however, was performed at +4°C and elution completed within 60 min, 90—100 % of the isomaltase activity was eluted with 0.07—0.15 M phosphate buffer (pH 6.0) together with the invertase and maltase activities. DEAE- and TEAE-cellulose, therefore, were not useful for the purification of hog intestinal iso-maltase.

Neither the isomaltase, invertase or maltase activities were adsorbed to CM-cellulose in 0.01 M phosphate buffer pH 6.5, although 50 % of the protein content of the glycosidase preparation was adsorbed.

Adsorption to alumina Cγ-gel. At pH 5.0 (in 0.02 M acetate buffer) the invertase, maltase and isomaltase activities were adsorbed to alumina Cγ-gel. When pH was increased, the activities were released all together, and at pH 6.0 (0.02 M phosphate buffer) no adsorption occured.

The influence of varying the amount of adsorbent in the presence of 0.02 M acetate buffer pH 5.0 was studied (Table 2). The activities investigated, however, showed no difference in their degree of adsorption during these conditions which was sufficiently great to be used for their separation.

Adsorption to calcium phosphate gel. This adsorbent was used in columns, as described by Tiselius *et al.*[23], and the experiments were performed in the

Table 2. Adsorption of intestinal α-D-glucopyranosidases on alumina Cγ-gel in 0.02 M acetate buffer, pH 5.0. The solution contained 0.8 mg/ml of protein, 10 units/ml of invertase, 21 units/ml of maltase, 2.5 units/ml of isomaltase and 0.9–7.2 mg/ml (dry weight) of alumina Cγ-gel. After shaking for 30 min at room temperatur, the solution was centrifuged and the supernatant analyzed.

Mg/ml of alumina C γ-gel	Per cent adsorbed			
	Protein	Invertase activity	Maltase activity	Isomaltase activity
0.9	35	40	23	40
1.8	53	83	65	75
3.6	66	96	90	95
7.2	70	100	96	95

presence of phosphate buffer pH 6.8. The invertase, maltase and isomaltase activities were weakly adsorbed to calcium phosphate, for the adsorption step it was necessary to use 0.001 M buffer. Whether gradient elution or step-wise elution was used, the invertase, maltase and isomaltase activities were eluted all together with 0.002—0.005 M buffer. Calcium phosphate is therefore no suitable adsorbent for the separation of these activities. (The trehalase activity, however, was not eluted until the buffer concentration exceeded 0.005 M, and was therefore separated from the other activities. The separation of hog intestinal trehalase on ion exchange columns has been reported earlier [11,17]).

Heat inactivation. The isomaltase activities of the purified maltase II and III preparations followed the same heat inactivation patterns as the maltase activities, *i.e.* that of the maltase II fraction was rapidly inactivated at 60°C, while that of the maltase III fraction was not inactivated at temperatures below 70°C (in presence of 0.01 M phosphate buffer pH 6.0) [10].

In one experiment, a crude solubilized glycosidase preparation, after dialysis against 0.01 M phosphate buffer pH 6.0, was heated at 60°C. The preparation had protein content 5.2 mg/ml and contained 38.5 units/ml of invertase, 97 units/ml of total maltase and 7.6 units/ml of isomaltase. During heating, all the invertase activity and 62 % of the total maltase activity were abolished within 20 min. The remaining 38 % of the maltase activity (*i.e.* maltase III [8]) was not affected during heating for 60 min. The isomaltase activity decreased by 74 % within 20 min. but was then not further diminished. Apparently the specific isomaltase was inactivated (together with the weak isomaltase activity of maltase II) within the first 20 min. The quotient between that isomaltase activity which survived heating and the maltase activity which was not abolished, fits well with the isomaltase/maltase activity quotient of a purified maltase III preparation [10].

Transglycosylation properties. The purified maltase II and maltase III as well as a crude glycosidase preparation, formed a number of oligosaccharide spots when acting in a concentrated (30 %*) isomaltose solution. The oligosaccharides formed by the crude preparation were, however, not identical with those formed by the purified maltase preparations.

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

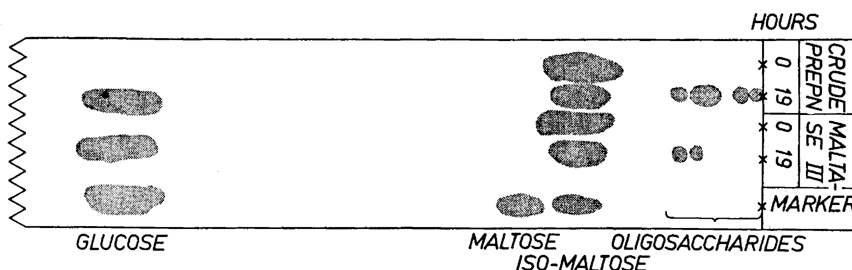

Fig. 1. Oliogosaccharides produced by transglycosylation by hog intestinal isomaltase. The *crude preparation* was a freshly prepared solubilized hog intestinal glycosidase preparation. In this case the reaction mixture contained 12 units of isomaltase, 108 units of (total) maltase and 15 mg of protein/ml. In the other case a *purified maltase III preparation* was used, and the reaction mixture contained 12 units of isomaltase, 263 units of maltase (III) and 1.5 mg of protein/ml. In both cases the reaction mixture containe 30 % isomaltose, 0.05 M maleate buffer and a few drops of toluene/ml. Before incubation and after incubation at 37°C for 19 hours 5 μl spots of the reaction mixture were applied to a chromatogram, which was developed with ethyl acetate: acetic acid: water (v/v) for 3 days.

In Fig. 1. is shown a chromatogram from one such experiment. In one case the reaction mixture contained a crude preparation, yielding 12 units/ml of isomaltase, 108 units/ml of (total) maltase and 15 mg/ml of protein. In the other case it contained a purified maltase III preparation, yielding 12 units/ml of isomaltase, 263 units/ml of maltase and 1.5 mg/ml of protein. In both cases the reaction mixture contained 30 % isomaltose, 0.05 M maleate buffer pH 6.5 and a few drops of toluene/ml. After different time of incubation at 37°C, 5 μl spots were applied to a paper chromatogram, which was developed for 3 days.

After the reaction had proceeded for 19 h, the reaction mixture with the crude glycosidase preparation contained *four* different oligosaccharides, formed by transglycosylation, which were separated on the paper chromatogram. In the reaction mixture with the purified maltase III, however, only *two* oligosaccharides could be detected (Fig. 1), although the reaction mixture in both cases contained equally many units of isomaltase activity per ml. Even when the reaction time was extended to two and three days, only two oligosaccharides could be detected in the reaction mixture which contained purified maltase III. A purified maltase II preparation showed the same behaviour as maltase III. It is therefore apparent that the specific isomaltase which is present in crude preparations, is able to form some oligosaccharides by transglycosylation which cannot be formed by the purified maltase II and III. This indicates that the isomaltase activity precent in the purified maltase II and maltase III preparations is *not* caused by contamination with specific isomaltase.

After a crude preparation had been heated at 60°C for 1 h (inactivating the specific isomaltase and maltase II but not the maltase III) it formed only two oligosaccharides in a 30 % isomaltose solution. These oligosaccharides had

the same mobility as those formed by the purified maltase II and maltase III preparations.

Effect of freezing. It has been reported by Larner *et al.*[6] that freezing and thawing of particulate intestinal glycosidase preparations *increases* the isomaltase and maltase activities by more than 200 %. In another paper[3], however, the same authors state that freezing and thawing *reduces* the isomaltase activity of crude preparations.

The present author has *not* observed any remarkable change of the maltase or invertase activities of either particulate or solubilized intestinal glycosidase preparations, although these preparations usually were stored in the frozen state in order to preserve their activity. Nor was any change of these activities observed when intestinal microsomes were frozen and thawed repeatedly as an attempt to solubilize the glycosidases[11].

For studing the effect of freezing and thawing upon hog intestinal isomaltase activity a solubilized crude glycosidase preparation was used which contained 15 mg/ml of protein in 0.01 M phosphate buffer pH 6.0. About 5 ml of the solution in a test tube was frozen by dipping the tube into a mixture of dry ice and ethanol at −80°C. After freezing, which took less than one min, the tube was held in the freezing mixture for 10 min, and then thawed in a water-bath at 37°C. As is seen in Table 3, the isomaltase activity had decreased by 1/3 after repeating this procedure three times. By further repeating of the freezing and thawing the isomaltase activity was, however, not further decreased, although the isomaltase activity still was three times that which could be caused by the isomaltase activity of maltase II and maltase III in the preparation. It was also found that the preparation after this treatment still formed all the four oligosaccharides in a 30 % isomaltose solution which were formed by the crude preparation before freezing. It is therefore not possible to *completely* inactivate the specific isomaltase by repeated freezing and thawing.

Exactly the same resultes were obtained when the freezing mixture consisted of sodium chloride and ice at −15°C. The temperature used seems therefore to be without importance for the results.

The effect of keeping the preparation in the frozen state for a longer time was also studied. The same enzyme preparation was used, but in this case the preparation was distributed in a series of test tubes, so that the content of each tube was frozen and thawed only once. The tubes were stored at −16°C

Table 3. Effect of repeated freezing and thawing upon some hog intestinal α-D-glucopyranosidase activities. For experimental conditions, see text.

	Activity (units per ml)			
	Isomaltase	Maltase	Invertase	Phenyl-α-D-glucopyranosidase
Before freezing	13.6	130	61	8.3
Frozen and thawed once	11.8	131	62	8.8
Frozen and thawed three times	9.1	115	62	8.2

Table 4. Effect of storage in the frozen state upon some hog intestinal α-D-glucopyranosidase activities. For experimental conditions, see text.

	Activity (units per ml)			
	Isomaltase	Maltase	Invertase	Phenyl-α-D-gluco-pyranosidase
Before freezing	13.6	130	61	8.3
After 4 days at −16°C	11.0	135	55	8.0
After 12 days at −16°C	8.2	148	55	7.0
After 33 days at −16°C	18.0	165	57	8.5

and each tube was thawed just before analysis. The effect of storage in the frozen state upon the different α-D-glucopyranosidase activities is shown in Table 4. The iso-maltase activity decreased by a little more than 1/3 within the first two weeks, but then *increased* to 1/3 more than its original value. In this experiment the maltase activity, too, increased somewhat, but not so much as reported by Larner *et al.*[6]

The effect of freezing during a still longer time was studied by analysis of a solubilized glycosidase preparation which had been dialyzed against distilled water and kept frozen at −16°C for eight months. This preparation contained 23.5 units/ml of invertase and 45 units/ml of maltase, *i.e.* the maltase/invertase activity quotient was 1.9, which agrees well with that of fresh preparations. The isomaltase activity of the preparation which had been frozen for eight months was 5.5 units/ml, *i.e.* the isomaltase activity was 12 % of the maltase activity, which is somewhat higher than the value usually obtained with fresh preparations (8−10 %). When acting in a 30 % solution of isomaltose, this preparation formed all the four different oligosaccharides which are formed by a fresh crude intestinal glycosidase preparation.

The isomaltase activities of *purified maltase II and maltase III preparations* were not changed by storage in the frozen state.

The procedure of freezing apparently has a complex effect upon the isomaltase activity of hog intestinal mucosa, with an initial *decrease* of the isomaltase activity followed by a more slow *increase* of this activity. A more thorough investigation of this phenomenon must wait until a method has been found for the purification of the specific isomaltase of hog intestinal mucosa. One explanation may be that the specific isomaltase is a mixture of several different enzymes which are influenced in different ways by freezing, but no other indications for such a heterogenity have appeared.

Phenyl-α-D-glucopyranosidase activity was studied in some experiments. Of the different hog intestinal α-D-glucopyranosidases separated earlier, two had phenyl-α-D-glucopyranosidase activity, namely maltase II and maltase III. The phenyl-α-D-glucopyranosidase activity of these fractions was 3 and 5 %, respectively, of their maltase activity (calculated as number of molecules hydrolyzed per time unit)[10]. Fresh mucosa homogenates had phenyl-α-D-glucopyranosidase activity about 6 % of their (total) maltase activity. This means that the phenyl-α-D-glucopyranosidase activity of the maltases II and III in the homogenates can contribute only 1/2 to the total phenyl-α-D-

glucopyranosidase activity. The remainder of the phenyl-α-D-glucopyranosidase activity may be caused by the specific isomaltase.

During heating at 60°C, the major part (about 2/3) of the phenyl-α-D-glucopyranosidase activity of crude preparations was abolished together with the invertase (= maltase I), maltase II and specific isomaltase. After heating the preparation had phenyl-α-D-glucopyranosidase/maltase (III) activity quotient which was the same as that of a purified maltase III preparation.

The effect of freezing and thawing upon the phenyl-α-D-glucopyranosidase activity of crude preparations varied somewhat. In some experiments slightly decreased activity was observed on freezing and thawing (Table 4) while in other cases no change could be stated (Table 3). It can not at present be stated whether that part of the phenyl-α-D-glucopyranosidase activity which is not caused by maltase II and maltase III is caused by the specific isomaltase or by some other enzyme.

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