

## Characterization of Hog Intestinal Invertase as a Glucosido-Invertase

### III. Specificity of Purified Invertase

ARNE DAHLQVIST

*Department of Physiological Chemistry, University of Lund, Lund, Sweden*

The specificity of purified hog intestinal invertase has been studied.

Hog intestinal invertase, purified by mutual displacement chromatography on TEAE-cellulose, hydrolyzed *sucrose* and *turanose* (3-( $\alpha$ -D-glucopyranosyl)-D-fructose). Heat inactivation and inhibition experiments indicated that a single enzyme in this preparation hydrolyzed both *sucrose* and *turanose*.

*Maltose* was also hydrolyzed by the purified hog intestinal invertase. *Maltose* and *sucrose* inhibited the hydrolysis of each other competitively, and the values of  $K_i$  for *maltose* and *sucrose* as inhibitors for each other agreed with the values of  $K_s$  for each sugar as substrate, indicating the hydrolysis of each of these sugars by the same enzyme.

The purified invertase, however, is responsible for only 1/2 of the total *turanase* activity and 1/3 of the total *maltase* activity of crude hog intestinal glycosidase preparations.

*Melezitose* ( $\alpha$ -D-glucopyranosyl (1 $\rightarrow$ 3)  $\beta$ -D-fructofuranosyl-(2 $\rightarrow$ 1) $\alpha$ -D-glucopyranoside) was *not* hydrolyzed by the purified enzyme preparation. Crude hog intestinal glycosidase preparations have *melezitase* activity caused by two other  $\alpha$ -D-glucopyranosidases, but these enzymes have no invertase activity.

The characterization of hog intestinal invertase as a *glucosido-invertase* ( $\alpha$ -D-glucopyranosidase) was recently confirmed by a study of the transglycosylation catalyzed by this enzyme<sup>1</sup>. It may therefore be expected that hog intestinal invertase should hydrolyze, in addition to *sucrose*, a number of other  $\alpha$ -D-glucopyranosides, e.g. *maltose*, *isomaltose* (6-( $\alpha$ -D-glucopyranosyl)-D-glucose), *trehalose* (1-( $\alpha$ -D-glucopyranosyl)- $\alpha$ -D-glucopyranoside), *melezitose* ( $\alpha$ -D-glucopyranosyl (1 $\rightarrow$ 3)  $\beta$ -D-fructofuranosyl (2 $\rightarrow$ 1)  $\alpha$ -D-glucopyranoside), *turanose* (3-( $\alpha$ -D-glucopyranosyl)-D-fructose), *phenyl- $\alpha$ -D-glucopyranoside*, or *methyl- $\alpha$ -D-glucopyranoside*.

*Methyl- $\alpha$ -D-glucopyranoside* is *not* hydrolyzed by crude hog intestinal preparations<sup>2,3</sup>, and is therefore *not* a substrate for hog intestinal invertase.

The hydrolysis of *melezitose* and *turanose* by crude hog intestinal preparations has been reported previously<sup>4</sup>. Since the preparation used contained several different  $\alpha$ -D-glucopyranosidases<sup>5-10</sup>, however, these findings do not prove that *melezitose* and *turanose* are hydrolyzed by intestinal *invertase*.

Hog intestinal *invertase* was recently purified by ion exchange chromatography, and was then separated from all the *trehalase*, *isomaltase* and *phenyl- $\alpha$ -D-glucopyranosidase* activity of the original preparation<sup>5,8,9</sup>. These glucosides are therefore not substrates for hog intestinal *invertase*. The *invertase* was also separated from 2/3 of the *maltase* activity of the original preparation<sup>6,7,9</sup>. However, 1/3 of the original *maltase* activity accompanied the *invertase* activity during its purification, and was not separable from the *invertase* activity by heat inactivation at varying pH-values or by ion exchange chromatography<sup>6,7</sup>. The question therefore arises, if this part of the *maltase* activity is caused by the same enzyme as the *invertase* activity.

In the present paper will be reported a study of the specificity of purified intestinal *invertase*. The aim the study was to answer the following two questions:

1) Is the hydrolysis of *melezitose* and *turanose* by crude hog intestinal preparations caused by the same enzyme as is responsible for the *invertase* activity?

2) Is that part of the *maltase* activity (= 1/3 of the total *maltase* activity), which it was not possible to separate from the *invertase* activity, caused by the same enzyme as the *invertase* activity?

## MATERIALS AND METHODS

### Enzyme preparations

*Purified hog intestinal invertase* was obtained by mutual displacement chromatography upon TEAE-cellulose<sup>7</sup>. The preparation had *maltase* activity which was 0.6 times the *invertase* activity, and was identical with the *purified hog intestinal maltase I* described in previous papers<sup>7,9</sup>. The specific *invertase* activity of the preparation was 292 units per mg of protein. One unit of glycosidase activity is the activity which causes 1 % of hydrolysis in 2 ml of reaction mixture in 1 h at 37°C under the conditions used.

*Purified hog intestinal maltase II* and *purified hog intestinal maltase III* were the same preparations as have been described in previous papers<sup>7,9</sup>. The specific *maltase* activity of these preparations was 100 and 200 units per mg of protein, respectively. These two *maltase* preparations had no *invertase* activity<sup>9</sup>.

*Crude hog intestinal glycosidase preparation* was obtained as described earlier<sup>5</sup>. This preparation contained all the hog intestinal glycosidase activities observed earlier<sup>4-10</sup>. The specific *invertase* activity of the crude preparation was 20 units per mg of protein. The *maltase* activity of the crude preparation was 2 times the *invertase* activity, and it contained *maltase I*, *II*, and *III* in about equal proportions, expressed as number of units per ml.

### Substrates

*Sucrose*, cryst., was obtained from J. T. Baker Co. *Maltose* (monohydrate), cryst., was obtained from Merck A.G. (Germany). Since the commercial *maltose* was found to contain contaminating sugars, which moved slower than *maltose* in paper chromatography (higher homologues), it was purified on carbon-celite columns<sup>1,7</sup>. The product was chromatographically pure. *Melezitose* (dihydrate) was obtained from Pfanstiehl Chemical Co., and *turanose* from Nutritional Biochemicals Corporation. All substrates used were chromatographically homogenous.

## Activity determination

*Determination of glycosidase activity* was performed at 0.1388 M substrate concentration in the presence of 0.05 M maleate buffer pH 6.5. The degree of hydrolysis of the different substrates was determined with the methods described earlier <sup>4,7</sup>. (In order to spare enzyme, the 3,5-dinitrosalicylic acid method in some cases was replaced by the more sensitive Somogyi-Nelson method <sup>4</sup> for the determination of the hydrolysis of melezitose. The values obtained by these two methods agreed very well.)

*Determination of the hydrolysis of sucrose and maltose in mixture.* For the separate determination of the hydrolysis of sucrose and maltose, when the reaction solution contained these two substrates *in mixture*, it was necessary to determine the amounts of glucose and fructose liberated by quantitative paper chromatography. One molecule of sucrose hydrolyzed liberates one molecule of fructose and one molecule of glucose. One molecule of maltose hydrolyzed liberates two molecules of glucose. The number of sucrose and maltose molecules, respectively, which are hydrolyzed in a mixture of these two substrates therefore may be calculated.

The quantitative determination of glucose and fructose on paper chromatograms was performed with 5  $\mu$ l spots of the reaction mixture as described earlier <sup>1</sup>.

## RESULTS AND DISCUSSION

## Invertase activity

The invertase activity of the purified hog intestinal invertase preparation had the same characteristics as that of a crude hog intestinal glycosidase preparation. The optimum pH was 6.5 (Fig. 3) in accordance with that for a crude preparation <sup>4</sup>. The  $K_s$  was determined at  $2.6 \times 10^{-2}$ , which may be compared with the value  $2.5 \times 10^{-2}$  obtained for a crude preparation <sup>4</sup>. It has been demonstrated earlier that the  $K_s$  value for the invertase activity was the

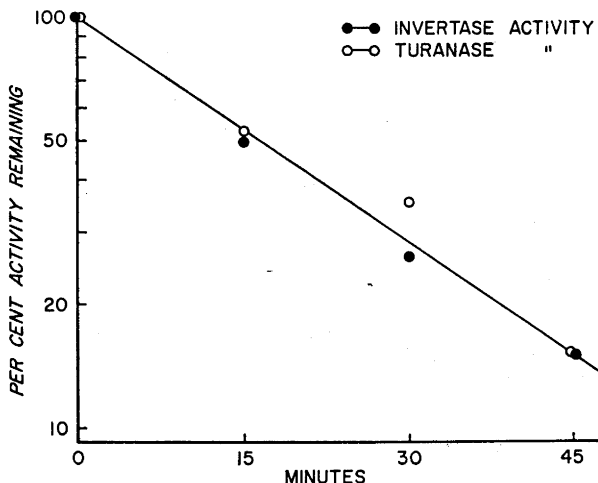


Fig. 1. Heat inactivation of purified hog intestinal invertase in 0.01 M phosphate buffer pH 6.0 at 48°C. The solution contained 305 units/ml of invertase, 23 units/ml of turanase and 1.05 mg/ml of protein. The inactivation of the invertase and turanase activities ran parallel.

**Table 1.** Action of purified hog intestinal invertase upon sucrose and turanose alone and in mixture. The reaction mixture contained 0.01 mg/ml of protein. After incubation at 37°C for 1 h, the amount of glucose liberated was determined with glucose oxidase <sup>4</sup>.

Concentration of substrate	Mg of glucose produced in 2 ml of reaction mixture
0.10 M sucrose	3.2
0.05 M sucrose	2.5
0.10 M turanose	<0.5
0.05 M turanose	<0.5
0.05 M sucrose +0.05 M turanose }	1.4

same in different fractions of the single invertase peak obtained by anion exchange chromatography of a solubilized hog intestinal glycosidase preparation <sup>7</sup>. These facts indicate that the invertase activity of hog intestinal glycosidase preparations is exerted by one single enzyme.

#### Turanase activity

The purified invertase preparation had turanase activity which was 7.5 % of the invertase activity, whereas a crude hog intestinal glycosidase preparation had turanase activity which was 15 % of the invertase activity <sup>4</sup>.

The  $K_s$  for the turanase activity of the purified invertase preparation was determined at  $1.0 \times 10^{-2}$ , which is lower than the value obtained <sup>4</sup> with a crude glycosidase preparation ( $2.8 \times 10^{-2}$ ). When the purified invertase was inactivated at 48°C in the presence of 0.01 M phosphate buffer pH 6.0, the inactivation of the turanase activity ran completely parallel with the inactivation of the invertase activity (Fig. 1), which supports the theory that the invertase and turanase activities of this fraction are caused by the same enzyme. This theory was also supported by the fact that the amount of monosaccharides produced by a certain amount of the enzyme acting upon the two substrates *in mixture* was less than the sum of the amounts of monosaccharides produced when the same amount of enzyme in separate experiments was acting upon each substrate alone (Table 1).\*

\* In a later experiment the inhibition of the hydrolysis of a series of sucrose solutions (0.025–0.100 M) by the presence of turanose (0.025 M) was determined. The amount of glucose liberated in the reaction mixtures was determined with glucose oxidase as described earlier for turanose <sup>4</sup>. Since turanose is hydrolyzed considerably slower than sucrose, the approximation was made that all the glucose liberated was produced from sucrose. (This approximation will cause the  $K_i$  value measured to be somewhat *too high*. From the relative rates of hydrolysis of turanose and sucrose and the  $K_s$  values for these activities the error to be expected was calculated at about 1/3 of the  $K_i$  value). It was found that turanose inhibited the hydrolysis of sucrose *competitively* and  $K_i$  for turanose as inhibitor was calculated at  $1.2 \times 10^{-2}$  which accords with the  $K_s$  value for the hydrolysis of turanose. This strongly supports the theory that sucrose and turanose are hydrolyzed by the same enzyme in the preparation studied. (For definitions and calculations see the experiments with sucrose and maltose described below.).

The remainder of the turanase activity of the crude glycosidase preparation appeared to be present in the *maltase II* and *maltase III* fractions<sup>3</sup>. The turanase activity of these fractions showed the same heat inactivation patterns as did the maltase activity<sup>3,9</sup>. At 72°C, the inactivation of the turanase activity of the maltase III preparation ran parallel to that of the maltase activity (Fig. 2). The turanase activity was 4 % of the maltase activity for maltase II and 6 % for maltase III. This means that the turanase activity of a crude preparation<sup>4</sup> quantitatively fits very well with the sum of the turanase activities of the invertase (= maltase I), maltase II and maltase III in the crude preparation. (In the calculation it is assumed that the activities of the maltase I, II and III in the crude preparation were equal, and that the maltase/invertase activity quotient was 2.0.)

### Melezitase activity

The purified invertase had *no measurable melezitase activity*. With the conditions used, this means that the amount of monosaccharides produced from melezitose was less than 0.05 % of that produced from sucrose. All of the melezitase activity of the crude preparation<sup>4</sup> could be shown to reside in the maltase II and maltase III fractions.

The purified *maltase III* hydrolyzed melezitose to *glucose and sucrose* as demonstrated by paper chromatography. Only the turanose link of the trisaccharide was hydrolyzed. *Maltase II*, too, had some melezitase activity (with glucose and sucrose as the hydrolysis products), but this activity was weaker in relation to the maltase activity than that of maltase III. The melezitase activities followed the same heat inactivation patterns as did the maltase activities<sup>9</sup> of these two fractions. At 72°C the inactivation of the melezitase activity of a maltase III preparation ran parallel with that of the maltase activity (Fig. 2). The melezitase activity<sup>\*</sup> was 0.1 % of the maltase activity for maltase II and 0.4 % for maltase III. With the same assumptions as made above in calculating the turanase activity, these enzymes would cause a melezitase activity of the crude preparation 0.17 % of the (total) maltase activity, which is somewhat higher than the value obtained, 0.10 % \*\*, in earlier experiments<sup>4</sup>. Maltase II and III, therefore, are the only

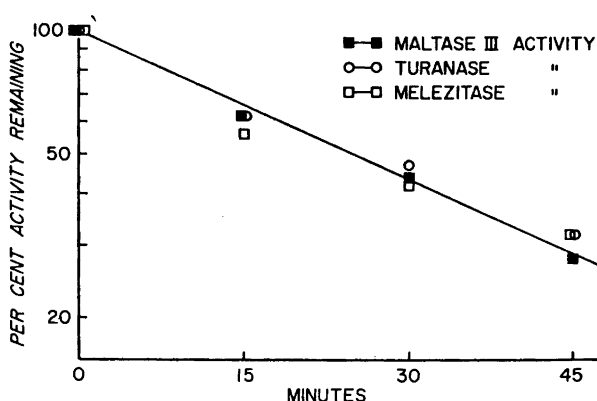


Fig. 2. Inactivation of a purified hog intestinal maltase III preparation at 72°C in the presence of 0.01 M phosphate buffer pH 6.0. The solution contained 235 units/ml of maltase III, 14 units/ml of turanase, 0.9 units/ml of melezitase and 1.18 mg/ml of protein.

The inactivation of the maltase, turanase and melezitase activities ran parallel.

\* Calculated as number of melezitose molecules hydrolyzed to glucose and sucrose, compared with the number of maltose molecules hydrolyzed by the same amount of enzyme.

\*\* Calculated as number of melezitose molecules *completely* hydrolyzed<sup>4</sup>.

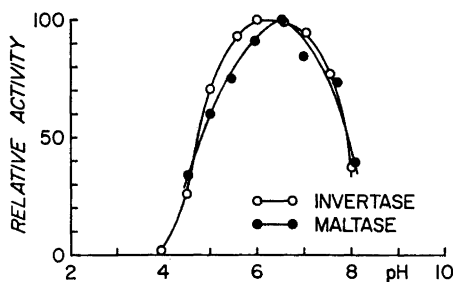


Fig. 3. pH activity curves for the invertase and maltase (I) activities of a purified hog intestinal invertase preparation. Temperature 37°C, 0.1388 M substrate concentration. Buffers: 0.05 M acetate pH 4.0–5.0, 0.05 M maleate pH 5.5–6.5, 0.025 M veronal pH 7.0–8.0. pH was measured with a glass electrode.

enzymes in crude hog intestinal glycosidase preparations which hydrolyze melezitose.

When a crude glycosidase preparation was heated at 60°C, in the presence of 0.01 M phosphate buffer pH 6.0, the amount of reducing sugars produced from melezitose per time unit decreased by 70 % within 30 min but was then not further diminished. After heating, the invertase (= maltase I) and maltase II (containing weak melezitase activity) are inactivated, but maltase III (containing the majority of melezitase activity) remains unaffected. This marked decrease in the amount of reducing sugars liberated from melezitose is chiefly caused by the inactivation of *invertase*. Although the invertase has no melezitase activity *per se*, it hydrolyzes the sucrose formed from melezitose hydrolysis to two molecules of reducing sugar. In the absence of invertase, melezitose is hydrolyzed by maltase III only to glucose and sucrose, and one molecule, rather than three, of reducing sugar is released for each melezitose molecule hydrolyzed.

### Maltase activity

The purified hog intestinal invertase preparation had maltase activity (= maltase I) which was 0.6 times the invertase activity. This activity represents 1/3 of the total maltase activity of crude preparations. Attempts to separate the maltase (I) activity of the purified invertase preparation from the invertase activity were not successful. With rechromatography on ion exchange columns the two activities formed a homogenous peak<sup>6</sup>. When the preparation was exposed to heat, the two activities were inactivated completely similarly, even when such experiments were performed in a series of buffers of varying pH<sup>6</sup>. In frontal analysis the maltase I and invertase activities appeared as one common, homogeneous front<sup>7</sup>, and they were not separable by mutual displacement chromatography<sup>7</sup>. The pH activity curve of the maltase I activity was similar to that of the invertase activity (Fig. 3) but differed from those of the maltase II and III activities<sup>9</sup>.

All these facts support the theory, that the maltase I activity is caused by same enzyme as the invertase activity. The ability to independently determine the hydrolysis of sucrose and maltose, by quantitative paper chromatography of the hydrolysis products, when these two substrates are mixed with each other, offers one additional method for proving the identity of the maltase I

and invertase activities. If one enzyme catalyzes the hydrolysis of two different substrates, these two substrates must act as competitive inhibitors for each other. The invertase activity should be competitively inhibited by maltose, and the maltase I activity competitively inhibited by sucrose. In addition the inhibitor constant ( $K_i$ ) for maltose as a competitive inhibitor for the invertase must have the same value as the substrate constant ( $K_s$ ) for maltose as substrate for maltase I, and vice-versa, if it possible to ascribe the invertase and maltase I activities to the same enzyme.

*Definitions.*  $K_s$  = the dissociation constant for the enzyme-substrate complex (Michaeli's constant).  $K_s$  is measured as the substrate concentration at which the initial velocity of reaction is half of the  $V_{\max}$  (provided no (competitive) inhibitor is present) \*.

$K_i$  = the dissociation constant for the enzyme-inhibitor complex. When the inhibition is *competitive*,  $K_i$  is calculated from the influence of the presence of the inhibitor upon the *apparent*  $K_s$  value for the substrate.

$v$  = the measured initial velocity for the hydrolysis of the substrate.

$V_{\max}$  = the initial velocity for the hydrolysis of the substrate when the enzyme is 100 % saturated with substrate.

[s] = the substrate concentration  
[i] = the inhibitor concentration.

The constants were obtained graphically by the method of Lineweaver and Burk <sup>11</sup> as modified by Dixon <sup>12,13</sup> (Fig. 4).

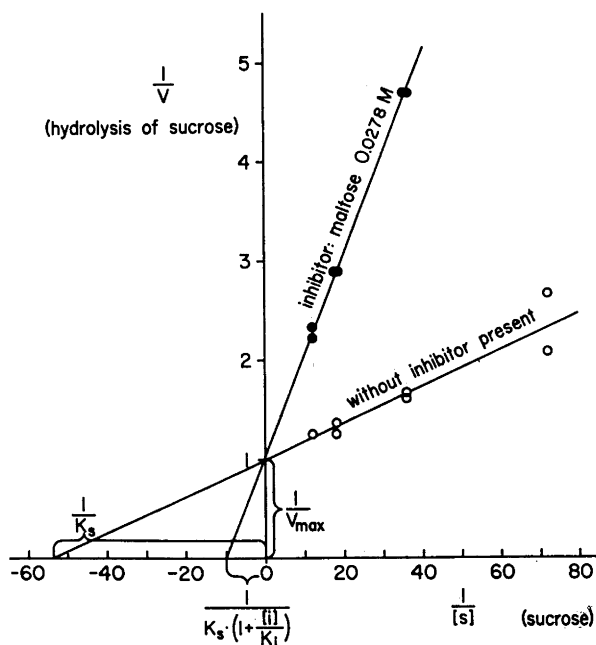
*Experimental conditions.* The reaction mixture contained 0.05 M maleate buffer pH 6.5, and the incubation of the substrate with the enzyme was performed for 60 min at 37°C. After this time the reaction was interrupted by boiling in a water-bath for 2 min.

The enzyme used was the purified invertase preparation. The substrate concentration varied within the range of 0.10–0.01 M, and the inhibitor concentration was 0.0139 or 0.0278 M in the experiments where inhibitor was present. In each experiment the hydrolysis at a series of different substrate concentrations was measured, at a constant concentration of inhibitor. The degree of hydrolysis was never allowed to exceed 20 % at the lowest substrate concentration used in the experiment. The quantitative determination of glucose and fructose, after elution from the paper chromatograms, was made with amounts of 1–8  $\mu$ g.

It is essential for the accuracy of the results that no transglycosylation <sup>1</sup> occurs. In the experiments with sucrose as substrate, without maltose present, there was no measurable difference between the amount of glucose and the amount of fructose liberated, and this demonstrates that no transglycosylation occurs at the substrate concentrations used.

*Inhibition of invertase activity.* In one series of experiments *sucrose* was used as *substrate*. One such experiment is illustrated in Fig. 4. Without inhibitor present, the  $K_s$  for the invertase activity was determined at  $2 \times 10^{-2}$

\* Measured in this way, the  $K_s$  is not the true dissociation constant for the enzyme-substrate complex, but with most enzymes it has probably approximately the same value <sup>13</sup>.



**Fig. 4.** Inhibition of the invertase activity of a purified hog intestinal invertase preparation by maltose. The  $V_{\max}$  for the hydrolysis of *sucrose* was unaltered by the addition of inhibitor (maltose) which demonstrates that the inhibition was *competitive*. The  $K_s$  (for *sucrose*) and the  $K_i$  (for maltose as inhibitor of the invertase activity) were calculated from the intercept with the abscissa as seen in the figure.

with the chromatographic method, which may be compared with the value  $2.6 \times 10^{-2}$  earlier obtained with the 3,5-dinitrosalicylic acid method<sup>7</sup>. Maltose was found to inhibit the invertase activity competitively with a  $K_i$  value at about  $1 \times 10^{-2}$ , which agrees with the value for  $K_s$  for maltase I obtained earlier ( $1.1 \times 10^{-2}$ ) with another method<sup>7</sup>.

*Inhibition of maltase I activity.* In another series of experiments *maltose* was used as *substrate*. Without the presence of *sucrose* the  $K_s$  for the maltase I activity was determined at  $1 \times 10^{-2}$ . *Sucrose* inhibited the maltase I activity competitively with  $K_i$  at  $2 \times 10^{-2}$ , which agrees with the  $K_s$  value for the invertase activity obtained earlier.

These results clearly demonstrate that the *maltase I* activity, which represents one third of the total maltase activity of crude preparations, and which closely follows the *invertase* activity during its purification, is exerted by the same enzyme as the invertase activity.

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