

## Barley Malt $\alpha$ -Glucosidase

### II. Studies on the Substrate Specificity

O. B. JØRGENSEN

*Department of Technical Biochemistry, The Technical University of Denmark\**

An enzyme with  $\alpha$ -glucosidase activity isolated from barley malt and purified to some extent shows activity toward different substrates containing  $\alpha$ -1,4- and  $\alpha$ -1,6- glucosidic linkages, *e.g.* isomaltose, maltose, and panose. Heat inactivation, inhibition, and other experiments suggest that only one enzyme is involved in the hydrolysis of both types of linkages. Michaelis constants  $K_m$  for isomaltose, maltose, panose, isomaltotriose, and maltotriose were found to be 11, 2.0, 6.1, 25, and 2.4 mM, respectively. Some inhibitor constants  $K_I$  are given, and the action of the enzyme preparation on phenyl  $\alpha$ -D-glucoside, methyl  $\alpha$ -D-glucoside, and sucrose is tested.

In a previous paper the extraction and purification of an  $\alpha$ -glucosidase with isomaltase activity have been reported.<sup>1</sup> It was found that the enzyme preparation was also able to catalyse the hydrolysis of maltose and panose. Maltase activity in barley malt has been known for a long time<sup>2</sup> and it was of interest to find out whether the maltase activity of the enzyme preparation was caused by the same enzyme that catalysed the hydrolysis of isomaltose. Further, it was of interest to examine if trisaccharides with  $\alpha$ -1,4- and  $\alpha$ -1,6- glucosidic linkages were hydrolysed by the same enzyme. For this reason the hydrolysis of panose, especially, was examined, but the hydrolysis of maltotriose and isomaltotriose was also studied.

#### MATERIALS AND METHODS

*Isomaltose* (*O*- $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  6)-D-glucose) was prepared as described previously.<sup>1</sup>

*Maltose* (Kerfoot). As it was not possible by several recrystallisations from 80 % ethanol to remove carbohydrates which moved more slowly on paper chromatograms, the maltose was purified by charcoal adsorption as described by Pazur and French.<sup>3</sup> This purification yielded maltose, H<sub>2</sub>O which contained less than 0.5 % of other sugars as shown by paper chromatography (chromatographically pure); optical rotation  $[\alpha]_D^{20} + 130.6^\circ$ .

\* Address: 14, Odensegade, Copenhagen Ø, Denmark.

*Panose* (*O*- $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  6)-*O*- $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  4)-D-glucose) was prepared from maltose using a transglucosylase from *Aspergillus niger* NRRL 337 as described by Pan *et al.*<sup>4</sup> As the isolated panose was contaminated with other sugars, 15 g was adsorbed on a 8  $\times$  37 cm charcoal (Darco G 60)-Celite (545) column (1:1, w/w; previously washed with water). The panose was eluted with stepwise-increasing concentrations of ethanol in water, and a panose fraction (chromatographically pure) was released at an ethanol concentration of 10 %, v/v.  $[\alpha]_D^{20} + 151^\circ$  (reported,<sup>4</sup>  $[\alpha]_D^{25} + 154^\circ$ ). Panitol was prepared by reduction with sodium borohydride. Acetylation of panitol with acetic anhydride-sodium acetate yielded the crystalline panitol-dodecaacetate (yield 61 % after two recrystallisations) m.p. 149–150°C (reported,<sup>5</sup> panitol-dodecaacetate m.p. 148–150°C).

*Isomaltotriose* (*O*- $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  6)-*O*- $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  6)-D-glucose) was isolated from the same enzymatic hydrolyzate of dextran as that used for the preparation of isomaltose, but was released from the charcoal-Celite column at a higher ethanol concentration (10–15 %, v/v). After refractionation on a similar column about 1 g (chromatographically pure) isomaltotriose was obtained,  $[\alpha]_D^{20} + 141^\circ$  (reported,<sup>6</sup>  $[\alpha]_D + 142^\circ$ ). Benzoylation gave the crystalline  $\beta$ -undecabenzoate, m.p. 227–228°C (reported,<sup>8</sup> isomaltotriose- $\beta$ -undecabenzoate m.p. 226–227°C).

*Maltotriose* was isolated from an acidic hydrolyzate of amylose as described by Whelan *et al.*<sup>7</sup> but the maltotriose was eluted from the charcoal-Celite column with a water-ethanol gradient (0–20 %, v/v ethanol). The chromatographically pure maltotriose showed  $[\alpha]_D^{20} + 156^\circ$  (reported,<sup>7</sup>  $[\alpha]_D^{15} + 160^\circ$ ). Acetylation with acetic anhydride-sodium acetate yielded the crystalline  $\beta$ -undecaacetate, m.p. 134°C (reported,<sup>8</sup> maltotriose- $\beta$ -undecaacetate 134–136°C).

*Phenyl  $\alpha$ -D-glucoside* was obtained by condensation of D-glucose- $\beta$ -pentaacetate and phenol in the presence of ZnCl<sub>2</sub> and subsequent deacetylation with barium methanolate.<sup>9</sup> Phenyl  $\alpha$ -D-glucoside,  $[\alpha]_D^{20} + 180.3^\circ$ , m.p. 173–174°C (reported,<sup>10</sup>  $[\alpha]_D^{20} + 180.8$ , m.p. 173–174°C).

*Sugar alcohols.* Solutions of isomaltose, maltose, panose, isomaltotriose, and maltotriose, 10  $\mu$ moles, in 3.5 ml were treated with 1.5 ml 1 % sodium borohydride at room temperature for 12 h. The sodium ions were removed by passing the solutions through Dowex 50 resin columns and the boric acid by distillation with methanol.

Other reagents were commercial preparations, analytical-grade purity.

*Sugar determination.* The contents of isomaltose, isomaltotriose, and panose in solutions were determined by acidic hydrolysis to glucose as by Turvey and Whelan<sup>6</sup> and the maltotriose contents as by Pirt and Whelan<sup>11</sup> followed by glucose determination using the Somogyi-Nelson method.<sup>12,13</sup>

*Paper chromatographic analysis.* Whatman No. 1 filter paper was used. The solvent systems were ethyl acetate-pyridine-water (10:4:3, by vol.)<sup>14</sup> developing for 20 h, and ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.)<sup>15</sup> developing for 40 h. Descending chromatography. Location reagent silver nitrate-sodium hydroxide.<sup>16</sup>

*Enzyme.* The enzyme was extracted from high-diastrase malt and fractionated as described in a previous paper.<sup>1</sup>

*Enzyme activity determinations* were performed as described previously,<sup>1</sup> using a tris-glucose-oxidase reagent. Tris-buffer in the concentration used totally inhibits the  $\alpha$ -glucosidase activity. This method can be used for determination of the enzymatic action on all the substrates except phenyl  $\alpha$ -D-glucoside, as the phenol interferes with the glucose-oxidase reagent. The action on phenyl  $\alpha$ -D-glucoside was measured by determination of the phenol released as by Arnaud<sup>17</sup> (UV-absorption at 285  $m\mu$  in 0.1 N NaOH); it is necessary in this method to have an enzyme blank and a substrate blank as both enzyme and substrate absorb at 285  $m\mu$ .

The activity determinations were performed at 37°C at pH 4.6 (acetate buffer) with substrate concentration 2 mM and reaction time 1 hour, unless otherwise stated. In none of the experiments did the hydrolysis of the substrate exceed 5 %, and it was found that below this limit the degree of hydrolysis of the different substrates used was apparently proportional with the time and the amount of enzyme used.

*Enzyme unit.* One unit (U) of enzyme activity is defined as the amount of enzyme required to hydrolyse 1  $\mu$ mole isomaltose in a substrate concentration of 2 mM at pH 4.62 in 1 min at 37°C. 1 U = 1000 mU.

## RESULTS

*pH-Optimum of isomaltase, maltase, and panase activity.* The pH-activity curves for the three activities were identical when the activity at different pH-values was measured for the same degree of saturation of enzyme with the three substrates. The pH-optimum was found to be about pH 4.6.

*Experimental.* Substrates: isomaltose 2.2 mM, maltose 0.4 mM and panose 1.2 mM. Enzyme: 0.72 mU, 0.05 mU, and 0.36 mU, respectively, per 0.5 ml reaction mixture. Buffers: 0.18 M acetate-buffers pH 3.5–5.6.

*The influence of heat inactivation on isomaltase, maltase, and panase activity.* Enzyme at pH 8.5 was heated in a water-bath at the required temperature for 10 min and then cooled in an ice-bath. The remaining enzyme activity was determined on the three substrates. Fig. 1 shows the results. In another experiment the enzyme was kept at 40°C and after different periods of heating samples of the enzyme were withdrawn and cooled in an ice-bath, and again remaining enzyme activity was determined. The results are shown in Fig. 2, the heat inactivation follows a first order reaction over a wide range.

*Acid inactivation.* By adjusting the enzyme to pH 2.7 with 0.1 N HCl at 25°C and keeping this pH for 4 min a fall is caused in the isomaltase, maltase, and panase activity of 55 %, 55 %, and 57 %, respectively.

*Inhibition by tris.* 2-Amino-2-hydroxymethylpropane-1,3-diol (tris) inhibits the  $\alpha$ -glucosidase activity. Examination of the inhibition, using the method of Dixon<sup>18</sup> (plotting the reciprocal of the initial velocities *versus* the inhibitor concentration for two different substrate concentrations) showed that the inhibition was competitive. The inhibitor constant  $K_I$  was found as the abscissa of the triple point between the two substrate curves and the straight line  $1/v = 1/V$ ,  $V$  being the maximum velocity (Fig. 3). The  $K_I$ -values for the three

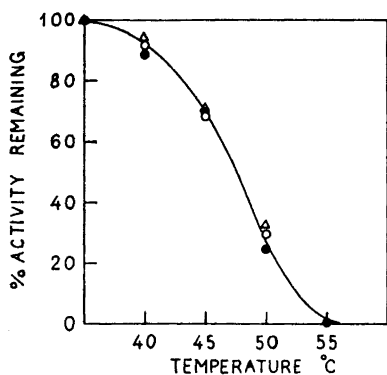


Fig. 1. Heat inactivation of isomaltase, maltase, and panase activity from barley malt at different temperatures. Heating time 10 min, pH 8.5.  $\circ$  isomaltase,  $\bullet$  maltase, and  $\triangle$  panase activity.

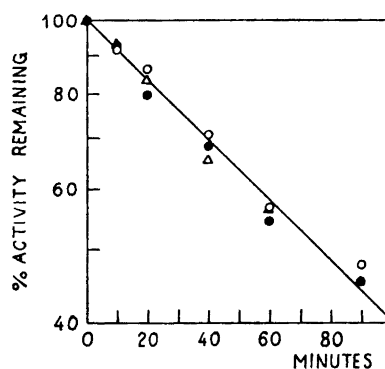


Fig. 2. Heat inactivation of isomaltase, maltase, and panase activity from barley malt using different heating times at 40°C and pH 8.5.  $\circ$  isomaltase,  $\bullet$  maltase, and  $\triangle$  panase activity.

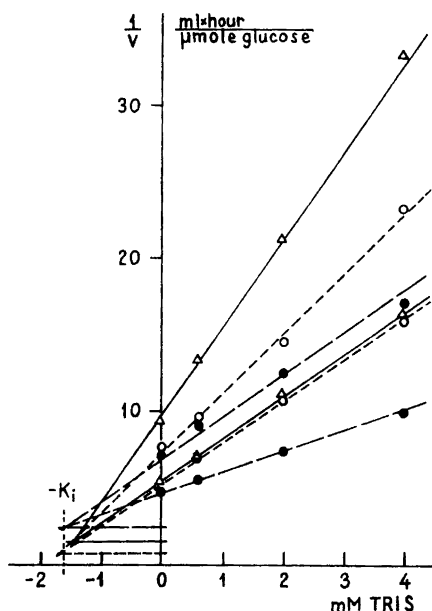


Fig. 3. Competitive inhibition of isomaltase, maltase, and panase activity of barley malt with 2-amino-2-hydroxymethylpropane-1,3-diol (tris). Reciprocal of initial velocities versus inhibitor concentrations. Substrates: ○ isomaltose, ● maltose, and △ panose.

substrates were almost the same, namely 1.7, 1.6, and 1.5 mM tris for isomaltase, maltase, and panase activity, respectively.

*Experimental.* Substrates: isomaltose 1.3 and 2.0 mM, maltose 1.0 and 2.0 mM, and panose 1.0 and 2.0 mM. Tris concentration 0, 0.6, 2.0, and 4.0 mM. Enzyme: 0.79 mU, 0.05 mU, and 0.40 mU per 0.5 ml reaction mixture for the isomaltase, maltase, and panase activity.

*Inhibition by 1,5-D-gluconolactone and D-glucose.* The isomaltase and maltase activity was competitively inhibited by 1,5-gluconolactone, and, using Dixon's method, the  $K_I$ -values were found to be 0.95 mM 1,5-gluconolactone for both activities. Glucose inhibited competitively both the isomaltase and the maltase activity, the  $K_I$ -values were found to be about 1 mM glucose for both activities.

*Experimental.* Substrates: isomaltose 1.3 and 2.0 mM, maltose 1.0 and 2.0 mM, 1,5-gluconolactone (the solution was 24 h old) 0, 0.28, 0.56, and 0.84 mM. Glucose 0, 0.1, 0.2, and 0.3 mM. Enzyme: 0.91 mU and 0.06 mU per 0.5 ml reaction mixture for the isomaltase and maltase activity, respectively.

*Inhibition of maltase activity by isomaltose.* The enzymatic hydrolysis of maltose was measured in the presence of isomaltose. As the rate of hydrolysis of isomaltose is considerably lower than the rate of hydrolysis of maltose (with the same amount of enzyme and the same substrate concentration), only a small error is introduced by correcting for the amount of isomaltose hydrolysed as if the hydrolysis of isomaltose was not affected by the maltose. Assuming this, it was found that the hydrolysis of maltose was competitively inhibited by isomaltose with a  $K_I$ -value of 9.5 mM isomaltose.

*Experimental.* Substrate: maltose 1.0 and 2.0 mM, inhibitor: isomaltose 0, 1.0, 2.0, and 4.0 mM. Enzyme: 0.06 mU per 0.5 ml reaction mixture.

*Mixed substrates.* When isomaltose and maltose in high concentrations were acted on by the same amount of enzyme, the sum of the amounts of glucose released was greater than the glucose released from a mixture of the two substrates incubated with the same amount of enzyme, see Table 1.

*Experimental.* Substrates: a) 35 mM isomaltose, b) 7 mM maltose, c) 35 mM isomaltose and 7 mM maltose. Enzyme: 0.05 mU per 0.5 ml reaction mixture.

*Ratio of maltase activity to isomaltase activity at different purification steps of the enzyme.* Table 2 shows the ratio of maltase activity to isomaltase activity at different purification steps of the enzyme.<sup>1</sup> As will be seen, the ratio of the two activities is fairly constant throughout the purification procedure.

*Determination of  $K_m$  and  $V$  for the hydrolysis of isomaltose, maltose, panose, isomaltotriose, and maltotriose.* The enzyme hydrolysed the oligosaccharides

Table 1. "Mixed substrates". Three reaction solutions with maltose, isomaltose, and a mixture of maltose and isomaltose incubated with the same amount of enzyme for the same reaction time.

Substrate	$\mu\text{g}$ glucose in 0.5 ml reaction mixture	
	found	calculated
7 mM maltose	30.3	
35 mM isomaltose	7.3	
7 mM maltose } 35 mM isomaltose }	21.4	21.8

Table 2. Ratio of maltase activity to isomaltase activity at different purification steps of the enzyme.<sup>1</sup>

	$\frac{\text{Maltase activity}}{\text{Isomaltase activity}}$
Raw extract	17
I. Clear filtrate	17
II. After pH 5.5 precipitation	16
III. After ammonium sulphate precipitation	15
IV. After pH 5.1 precipitation	15
V. After bentonite adsorption	16

Table 3.  $K_m$ - and  $V$ -values for the hydrolysis of different oligosaccharides by an  $\alpha$ -glucosidase from barley malt.

Substrate	$K_m$ mM	$V$
		$\frac{\mu\text{mole glucose}}{\text{hour} \times \text{ml} \times \text{mU}}$
Isomaltose	11	0.9
Maltose	2.0	3.9
Panose	6.1	0.9
Isomaltotriose	25	1.3
Maltotriose	2.4	2.4

mentioned at different rates. For comparison of the activities the  $K_m$ -values for the different substrates were measured. The reciprocal of the initial velocities was plotted against the reciprocal of the substrate concentrations (Lineweaver and Burk<sup>19</sup>). The results are given in Table 3 and Fig. 4.

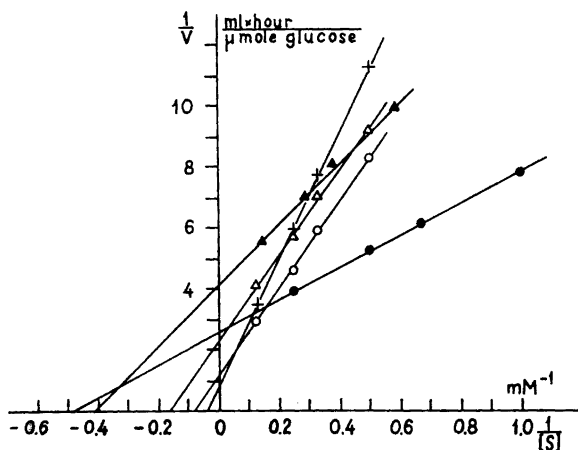


Fig. 4. Lineweaver-Burk plot for elucidation of  $K_m$  and  $V$  for the hydrolysis of: ○ isomaltose, ● maltose, △ panose, + isomaltotriose, and ▲ maltotriose, catalysed by an  $\alpha$ -glucosidase from barley malt.

*Experimental.* Substrates: isomaltose, isomaltotriose, and panose 2.0, 3.0, 4.0 and 8.0 mM; maltose 1.0, 1.5, 2.0, and 4.0 mM; maltotriose 1.7, 2.6, 3.4, and 6.8 mM. Enzyme: 0.50 mU per 0.5 ml for the isomaltose and isomaltotriose substrates, 0.05 mU per 0.5 ml for the maltose and maltotriose substrates and 0.25 mU per 0.5 ml for the panose substrates.

*Hydrolysis of other  $\alpha$ -glucosides.* The hydrolysis of the different substrates was performed with the same amount of enzyme and the substrate concentrations 2.0 mM with the exception of dextran (0.16 %). The rate of hydrolysis of the substrates is given relative to the hydrolysis of maltose: maltose 100, phenyl  $\alpha$ -D-glucoside 11, isomaltose 6, sucrose  $\sim$ 1, and methyl  $\alpha$ -D-glucoside  $\sim$ 0.5. Dextran was hydrolysed with a relative rate less than 0.2, but it was shown by paper chromatography that glucose was released from dextran. These values cannot be correlated directly with the enzyme affinity for the different substrates as the enzyme under the condition applied has not been saturated with substrate to the same extent. The hydrolysis of sucrose is apparently caused by the  $\alpha$ -glucosidase and not by  $\beta$ -fructosidase activity which may be present as a contaminant, as raffinose is not hydrolysed (shown by paper chromatography).

*Hydrolysis of panitol, isomaltotriitol, and maltotriitol.* The hydrolysis of the three substrates was examined by paper chromatography. It was found that at the beginning of the reaction panitol was hydrolysed to glucose and maltitol, isomaltotriitol gave glucose and isomaltitol, and maltotriitol was

hydrolysed to glucose and maltitol. Glucitol could not be detected until a considerable release of glucose from the substrates had taken place.

*Experimental.* Substrates: 4.0 mM. Enzyme: 0.4 mU, 0.8 mU, and 0.08 mU per 0.5 ml reaction mixture for the panitol, isomaltotriitol, and maltotriitol, respectively.

#### DISCUSSION

Maltase activity in barley malt has often been reported. In a previous paper the extraction and purification of isomaltase activity in barley malt have been reported.<sup>1</sup> As it was found that this enzyme preparation was capable of hydrolysing maltose it was of interest to find out whether the isomaltase and maltase activity was caused by the same enzyme. Further the enzyme was able to catalyse the hydrolysis of other oligosaccharides containing  $\alpha$ -1,6- and  $\alpha$ -1,4-glucosidic linkages *e.g.* panose, and it was examined whether the panase activity was caused by the same enzyme as the two activities mentioned above.

The heat inactivation and acid inactivation experiments in which the isomaltase, maltase, and panase activity is inactivated to the same extent suggest that these activities are due to the same enzyme. Amines often inhibit glycosidases and this also applies in this case. The competitive inhibition of the three activities caused by 2-amino-2-hydroxymethylpropane-1,3-diol (tris) showed the same inhibitor constant 1.6 mM tris. The isomaltase and maltase activity was competitively inhibited by 1,5-gluconolactone with the same inhibitor constant  $K_I$  0.95 mM gluconolactone. This strongly suggests that the same active site is involved in the catalysis of the hydrolysis of the three substrates. Further, the hydrolysis of maltose is competitively inhibited by isomaltose with a  $K_I$ -value of about 9.5 mM isomaltose. This value is very close to the  $K_m$ -value (11 mM isomaltose) for the hydrolysis of isomaltose by the enzyme and this makes it probable that the two activities are due to the same enzyme. (If it is assumed that the hydrolysis of isomaltose is inhibited by the maltose present with a  $K_I$ -value about 2 mM maltose (the  $K_m$ -value for maltose) the  $K_I$ -value 9.5 mM isomaltose found is about 15 % too low). The result of the "mixed substrate" experiment again supports the hypothesis of a single active site. Assuming that maltose and isomaltose compete for each other with  $K_I$ -values 2 mM maltose and 11 mM isomaltose (the  $K_m$ -values), the hydrolysis of the two substrates when mixed can be calculated using the equations  $v = V/(1 + K_m/[S])$  and  $v = V/[1 + K_m(1 + [I]/K_I)/[S]]$ . The calculation gives 21.8  $\mu$ g glucose which corresponds to the amount found (Table 1).

The ratio of isomaltase activity to maltase activity was found to be fairly constant throughout the purification procedure of the enzyme. This makes it plausible that even in the unpurified malt extract (extracted at pH 8.5) only one enzyme activity causes the hydrolysis of both isomaltose and maltose.

All the experiments confirm the theory that the hydrolysis of the  $\alpha$ -1,4- and  $\alpha$ -1,6-glucosidic linkages in the substrates examined is catalysed by the same enzyme.

The enzyme preparation also catalysed the hydrolysis of maltotriose and isomaltotriose and as these substrates contain linkages similar to those in

isomaltose, maltose, and panose, it seems very probable that maltotriose and isomaltotriose are hydrolysed by the same enzyme that hydrolyses isomaltose, maltose, and panose. As isomaltose and isomaltotriose are hydrolysed the enzyme is different from the limit dextrinase in barley malt described by Mac William and Harris.<sup>20</sup>

The hydrolysis of panitol and maltotriitol giving glucose and maltitol and the hydrolysis of isomaltotriitol giving glucose and isomaltitol at the beginning of the reaction suggest that the same enzyme is responsible for the hydrolysis of these substrates. From this experiment it is deduced that the enzyme attacks the oligosaccharides from the non-reducing end-group.

Comparing the  $K_m$ -values (Table 3) it is seen that the affinity for substrates containing  $\alpha$ -1,4-glucosidic linkages is greater than the affinity for substrates containing  $\alpha$ -1,6-glucosidic linkages (assuming that the  $K_m$ -values are equal to or approach the substrate constants  $K_s$  which has not been shown for the enzyme examined). From the  $K_m$ -values it is further seen that the affinity for the trisaccharides is lower than the affinity for the disaccharides and this is again lower than the affinity of the enzyme for glucose ( $K_I$ -value about 1 mM glucose). If the affinity of the enzyme for higher maltodextrins is decreasing it is to be expected that the enzyme will show little or no ability to hydrolyse starch. This problem has not been studied, but, as mentioned, the enzyme preparation showed a weak hydrolysis of dextran.

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