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Barley Malt α-Glucosidase with Isomaltase Activity

I. Extraction and Purification

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Optimum conditions for extraction of isomaltase activity (α-1,6-glucosidase activity) from barley malt have been studied, and the subsequent purification of the activity has been examined. A six times increase in the specific activity was obtained. pH-Optimum, temperature optimum, K_m and Q_{10} for the isomaltase activity are reported.

The presence of enzymes which hydrolyse isomaltose (*O*-α-D-glucopyranosyl-(1→6)-D-glucose) has been ascertained in enzyme preparations from microorganisms and animal tissues. This paper reports the extraction and purification of isomaltase activity from barley malt.

Enzymes with α-1,6-glucosidase activities in barley malt have special interest in relation to the degradation of unfermentable α-limit dextrins.

Mac William and Harris,[1] and Mac William [2] have shown the existence in barley malt of two enzymes capable of hydrolysing α-1,6-glucosidic links, *viz.* an R-enzyme type (starch 6-glucanohydrolase) and a limit dextrinase (dextrin 6-glucanohydrolase), but neither of these enzymes was able to catalyse the hydrolysis of isomaltose. An enzyme in barley malt capable of hydrolysing "Fishers isomaltose" has been reported by Myrbäck and Ahlborg [3], whether this activity is identical with the one reported here is uncertain.

MATERIALS AND METHODS

Isomaltose (*O*-α-D-glucopyranosyl-(1→6)-D-glucose) was prepared from an enzymatic hydrolyzate of dextran essentially by the method of Jeanes *et al.*[4] A submerge culture of *Penicillium funiculosum* Thom R. 1768 was prepared according to Dahlqvist [5]. Culture filtrate (90 ml) containing 1710 dextranase units determined as described by Tsuchiya *et al.*[6] was added to 270 ml of a 6.7 % solution of dextran (Dextran 10, Pharmacia) in 0.03 M acetate buffer pH 5.1. After 24 h at 37°C the reaction mixture was boiled and 10 g of baker's yeast was added. After shaking for 24 h at 30°C the yeast was removed by centrifugation. The solution was concentrated to 75 ml *in vacuo* and adsorbed on a 8 × 37 cm charcoal (Darco G 60)-Celite (545) column, 1:1 w/w (previously washed with water). The isomaltose was eluted with stepwise-increasing concentrations of ethanol in water. After refractionation on a similar column, about 6 g isomaltose (lyophilized) was obtained.

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The isomaltose was shown by paper chromatography to contain less than 0.5 % of other sugars, the optical rotation was $[\alpha]_D^{20}$ + 122° (reported [4] $[\alpha]_D^{20}$ + 122.0°). Acetylation with acetic anhydride-sodium acetate yielded the crystalline β-octaacetate (yield 50 % after two recrystallisations), m.p. 145–146°C (reported [4] isomaltose-β-octaacetate m.p. 145.2–146.2°C).

Barley malt. All the experiments were performed on the same lot of high-diastase malt from Herta barley dried at a maximum of 65°C for 3 h and with a total drying time of 24 h (kindly supplied by A/S KB, Copenhagen). Water content 5.9 % and protein content 10.9 %. Before the extraction the malt was ground in a Wiley mill using a 40-mesh screen.

Isomaltose determination. The isomaltose contents of the solutions were determined by acidic hydrolysis to glucose as by Turvey and Whelan [7] and subsequent determination of the glucose, using the Somogyi-Nelson method [8,9].

Glucose determination. The glucose released in the enzyme assay was determined using Dahlqvist's [10] tris-glucose-oxidase reagent. This reagent is specific for glucose and gives no reaction with isomaltose in the concentration used in the enzyme assay procedure. Reagent: 125 mg glucose-oxidase (DeeO, Miles Chemical Company) and 2 mg peroxidase (C. F. Boehringer & Soehne, gereinigt) are dissolved in 100 ml 0.5 M tris buffer pH 7.0. After filtration 0.5 ml of a 1 % solution of *o*-dianisidine in ethanol is added.

To 0.5 ml sample in a test tube 3 ml reagent is added, and the test tube is incubated for 60 min at 37°C. (Direct sunlight should be avoided). Two standards containing 5 μg and 20 μg glucose and a water blank are run simultaneously. After the incubation the extinction is measured in a 1 cm cell at 420 mμ (Beckman spectrophotometer DU). The amount of glucose in the sample is determined from a standard curve.

Protein was determined, using the biuret reaction according to Gornall *et al.*[11] To each of 1 ml dialyzed enzyme(containing less than 6 mg protein per ml)and a water blank 4 ml biuret reagent is added. To correct for the colour of the enzyme solution a blank with enzyme and "biuret reagent" without $CuSO_4$ is prepared. After 30 min at room temperature the extinction is measured at 540 mμ in a 1 cm cell (Beckman C). Denoting the difference between the extinction of the sample and the sum of the extinctions of the two blanks E, it was found that mg protein per ml = mg P/ml = 19 E. This value was found as a mean value by checking 20 enzyme preparations at different purification steps with Kjeldahl determinations (protein factor 6.25).

Enzyme unit. One unit (U) of isomaltase activity is defined as the amount of enzyme required to hydrolyse 1 μmole isomaltose in 2 mM isomaltose at pH 4.62 in 1 min at 37°C. 1 U = 1000 mU.

The K_m-value was determined as 11 mM isomaltose and the Q_{10}-value as 1.5. From these figures it is possible to calculate the E C [12] recommended unit by multiplying the unit used here with 4.

Enzyme assay procedure. The isomaltase activity was determined as follows: In a test tube containing 300 μl 3.33 mM isomaltose in 0.13 M acetate buffer, pH 4.62, 200 μl dialyzed enzyme containing no more than 5 mU/ml is added at time zero and 200 μl dialyzed enzyme is added to another test tube containing 300 μl 0.13 M acetate buffer, pH 4.62, (blank value). After incubation for 60 min at 37°C the reaction is stopped by adding 3 ml glucose-oxidase reagent to both test tubes (tris buffer inhibits the isomaltase activity). The determination proceeds as described above. From the difference between the extinction of the sample and the blank the amount of glucose is found. The activity of the enzyme is: μg glucose × 0.23 mU/ml. The activity determinations were run in duplicate. Substrate hydrolysis did not exceed 6 % in these assays and it was found that in this interval the degree of hydrolysis was apparently proportional to the amount of enzyme used. Incubation for several hours gave nearly 100 % hydrolysis of isomaltose, which shows that the enzymatic release of glucose was not due to the hydrolysis of contaminants in the isomaltose.

Before the enzyme assay and protein determinations it is necessary to dialyze the enzyme, mainly because of the sugar and salt present. The dialysis was performed in "Visking" dialysis tubing (1 cm inflated diameter). 3 ml enzyme with 30 μl toluene added was dialyzed 72–0 h (according to the purity) against running tap water (10–12°C). After the dialysis the content of the tubes was diluted to a known volume. It was found that there was no loss in activity during the dialysis, and Ca^{2+} from the tap water had no influence.

Table 1. Extraction of isomaltase activity from barley malt at different pH-values.

pH	Activity mU/ml	Specific activity mU/mg P
3.0	0.8	0.8
4.2	1.0	0.8
5.0	1.1	0.6
5.9	1.4	0.7
6.8	2.4	1.0
7.7	5.9	2.2
8.5	7.8	2.3
9.0	8.5	2.3
10.0	5.5	1.3

RESULTS AND DISCUSSION

Extraction of isomaltase activity. In the following extraction experiments 10 g portions of malt flour were extracted with 50 ml solvent by shaking for 1 h at room temperature. A clear filtrate was obtained by filtration through S & S 572 ½ folded filter.

Extraction with water gave extracts of low activity (1.6 mU/ml). Extraction with 1—7 % salt solutions (NaCl, Na_2SO_4, $(NH_4)_2SO_4$, and $CaCl_2$) gave extracts with an activity between 4.8 and 8.0 mU/ml and with a specific activity between 1.7 and 2.9 mU/mg P. $CaCl_2$ gave slightly better results than the other salts. Table 1 gives the results from experiments with extractions at different pH-values, the pH was adjusted by addition of 0.1 N NH_3 or 0.1 N HCl. Maximum of extracted isomaltase activity was found at pH 8.5—9.0.

Extraction experiments at pH 8.5 with the addition of the following reagents which may influence the extraction of enzymes: EDTA (1 mM), Na_2SO_3 (5 mM), $KBrO_3$ (5 mM), Tween 20 (1 %) or ethanol (5 %), showed little or no effect. Addition of cysteine (1 mM), trypsin (crystalline, Novo, 0.04 %) or Na_2SO_4 (3 %) gave 10—20 % higher isomaltase activity, but as the effect was so small, no addition was used during the extraction.

The results given in Table 2 show that extraction for 24 h at 4°C gives the greatest yield of activity, but for practical reasons 1 h at room temperature was preferred.

Table 2. Influence of time and temperature on the extraction of isomaltase activity from barley malt at pH 8.5.

Extraction time, hours	Temperature °C	Activity mU/ml
1	23	8.3
3	—	7.8
24	—	1.7
24*	—	7.4
1	4	8.7
3	—	9.1
24	—	9.3
24*	—	9.0

* 0.5 % toluene added.

Purification of the isomaltase activity. Attempts to purify the extract by heat denaturation (pH 8.5) failed. Precipitation of the activity with Zn^{2+} or Pb^{2+} was possible, but caused 40—60 % loss of activity. Ethanol and acetone precipitation also proved unsatisfactory. The isomaltase activity showed a low solubility at low pH as shown in Fig. 1. Portions of the extract were precipitated by adjusting to different pH-values between 4.3 and 6.3. The precipitates were brought into solution at pH 8.5 and the activity measured. Precipitation of the extract at pH 5.5 gave maximum of purification and little loss of activity. A ten times concentration was achieved by dissolving the precipitate at pH 8.5 in one tenth of the original volume. Presence of salt, *e.g.* 1—6 % Na_2SO_4, made the precipitation at low pH-values incomplete. For this reason salt extraction of the enzyme activity was abandoned.

Further purification was difficult and only a little was achieved. It was tried to purify the enzyme solution (after the pH 5.5 precipitation) with ethanol, acetone, and $(NH_4)_2SO_4$ fractionation. Ethanol, even in very high concentrations, could not precipitate the activity. The activity was precipitated by acetone (2 vol. at pH 7.25, 3 vol. at pH 8.5, precooled to —20°C and slowly added at 0°C) with a 10—15 % loss of activity. The precipitates were more or less insoluble at pH 8.5, but active in suspension, when precipitated with less than 4 vol. of acetone. In attempts to solubilize the precipitates addition of EDTA, cysteine or urea proved to be ineffective.

The results of $(NH_4)_2SO_4$ fractionation at pH 8.5 are shown in Fig. 2. It was chosen to precipitate the isomaltase activity with 1.5 ml 3.875 M $(NH_4)_2SO_4$ per 4 ml enzyme solution which gives a precipitate purified about 1.2 times. The precipitate was dissolved at pH 8.5 and the salt was removed by precipitating the activity at pH 5.1 and dissolving at pH 8.5.

Repeating the $(NH_4)_2SO_4$ fractionation at pH 8.5, 7.0, 5.0 or in enzyme solutions diluted 5 and 10 times gave no further purification. Fractionation at this step with NaCl, $MgSO_4$, mixed salts ($NaCl/(NH_4)_2SO_4$), ethanol in 0.18 M $(NH_4)_2SO_4$, acetone, Zn^{2+}, Ba^{2+}, Mn^{2+} or Ca^{2+} showed little or no purification.

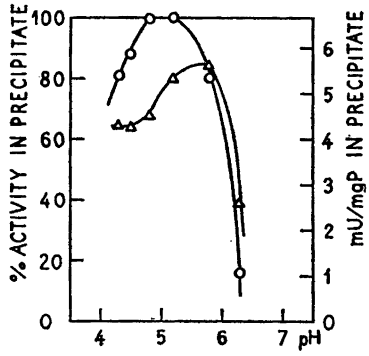

Fig. 1. Isomaltase activity precipitated at different pH-values. O—O isomaltase activity as per cent of maximal activity; △—△ specific activity (mU/mg P) in precipitates redissolved at pH 8.5.

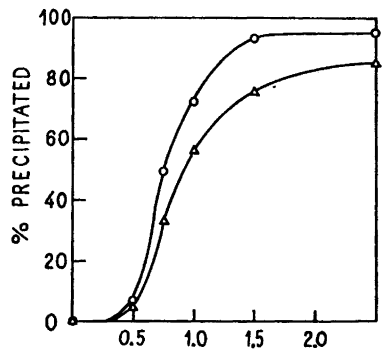

Fig. 2. Fractionation of isomaltase activity with $(NH_4)_2SO_4$ at pH 8.5. Abscissa: ml 3.875 M $(NH_4)_2SO_4$ per 4 ml enzyme (II). O—O per cent isomaltase activity precipitated; △—△ per cent protein precipitated. Precipitates redissolved at pH 8.5.

Adsorption experiments. It is possible to adsorb the isomaltase activity on potato starch in 20–25 % ethanol (v/v). Chromatography on potato starch columns, adsorbing the enzyme in 25 % ethanol (v/v) and eluting with phosphate buffer, pH 8.0, gave some purification but great loss of activity.

Adsorption on calcium phosphate gel [13] gave little purification but better purification was obtained by aluminia Cγ-gel [13] or bentonite.

Chromatography on cellulose ion exchangers. 100 mg protein was introduced on columns containing 3 g of the equilibrated ion exchange material (Whatman) at 4°C. The isomaltase activity was not adsorbed on CM-cellulose (pH 6.6, 5 mM phosphate buffer). ECTEOLA-cellulose adsorbed only 15–25 % of the activity (pH 6.6–8.5, 5 mM phosphate buffer) and these exchangers are thus unsatisfactory. On DEAE-cellulose the activity was adsorbed in a nearly irreversible manner, high salt concentrations at low pH-values release only 5–10 % of the activity from the columns. Tris buffer (0.25 M, pH 8.0) released a 4 times purified fraction, but only about 5 % of the total activity was recovered.

Further purification was possible only with great loss of activity. Adsorption of the impurities on bentonite gave a purification of about 1.2 times. A similar purification could be achieved using adsorption on aluminia Cγ-gel.

Procedure for purification of isomaltase activity from barley malt. 350 g malt flour (40-mesh) * is extracted for 1 h at room temperature by shaking with 1.75 l water containing sufficient NH_3 to produce pH 8.5 (*ca.* 380 ml 0.1 N NH_3). The extract is cooled to 0°C, and the following procedures are carried out at 0°C. The suspension is centrifuged (15 min, 2000 × *g*) and a clear extract is obtained by filtration on a large Büchner funnel mounted with a Carlson K_5 filter plate and a layer (*ca.* 2 cm) of washed cellulose floc.

The filtrate (I, *ca.* 1.1 l) is adjusted to pH 5.5 by addition of 0.1 N acetic acid (*ca.* 130 ml). The precipitate is collected by centrifugation (10 min, 2000 × *g*) and dissolved to 1/10 of the original filtrate volume at pH 8.5 (adjusted with 0.1 N NH_3) (II, 110 ml). To this solution 3.75 ml 3.875 M $(NH_4)_2SO_4$ (adjusted to pH 8.5 and measured after dilution 10 times) per 10 ml enzyme is added slowly. After a delay of 30 min the precipitate is collected by centrifugation (30 min, 2000 × *g*) and suspended in 20 ml water per 10 ml II (III, *ca.* 220 ml). pH is adjusted to 5.1 with 0.1 N acetic acid and the precipitate is collected by centrifugation (15 min, 2000 × *g*) and dissolved to the same volume as II at pH 8.5 (adjusted with 0.1 N NH_3) (IV, 110 ml). Bentonite is added slowly while stirring, the stirring is continued for 20 min and the bentonite is removed by centrifugation (10 min, 2000 × *g*) (V, 100 ml). The optimal amount of bentonite was determined and was usually found to be about 2 g of bentonite per 100 ml enzyme.

The purification procedure can be followed in Table 3.

pH-Optimum was measured as under "enzyme assay procedure", except that the acetate buffer was replaced by Sørensen citrate buffers of a different pH. The pH activity curve is shown in Fig. 3. The pH-optimum, about 4.6, remained unchanged throughout the purification procedure.

Temperature optimum was measured using the conditions given for the enzyme assay procedure but with varying temperatures. Setting the activity

Table 3. Purification of isomaltase activity from barley malt.

Step	Activity mU/ml	Total activity U	Specific activity mU/mg P
I. Clear filtrate	6.9	8.0	1.8
II. After pH 5.5 precipitation	62	7.2	6.9
III. After $(NH_4)_2SO_4$ precipitation	29	6.8	8.3
IV. After pH 5.1 precipitation	52	5.7	8.6
V. After bentonite adsorption	38	3.8	10.6

* Instead of using malt flour, the extract can be prepared using unground malt treated for 2 min in a Waring blendor before the extraction.

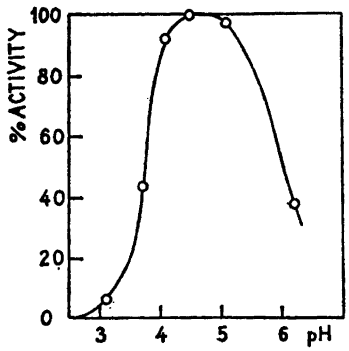

Fig. 3. Effect of pH on the hydrolysis of isomaltose by isomaltase activity from barley malt.

at 37°C arbitrarily as 100 %, the activity at 25°C, 30°C, 45°C, and 50°C was 64 %, 76 %, 96 %, and 53 %, respectively. In the temperature interval 25°C—37°C the Q_{10}-value is 1.5.

Stability. The enzyme, pH 8.0—8.5, looses *ca.* 10 % of the activity by standing for 24 h at 24°C, at 4°C no loss in activity is found. Storage for 1 month at 4°C with toluene added or at —20°C causes a 8—13 % fall in the activity.

K_m*-value.* A linear double-reciprocal plot (reciprocal of initial velocities *versus* reciprocal of substrate concentrations, Lineweaver and Burk [14]) obtained in the substrate concentration range 2—40 mM isomaltose showed $K_m = 11$ mM isomaltose.

Other enzyme activities. The purified enzyme showed a strong hydrolytic action on maltose and panose (*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose) but little or no action on methyl α-D-glucoside. When incubated with maltose (25 %, 24 h at pH 4.6) the enzyme showed transglucosylase activity. A report on these activities will be given later.

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