Purification and Substrate Specificity of Brewer's Yeast α -Glucosidase[†]

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Received April 21, 1977

Three kinds of α -glucosidase (I, II and III) were isolated from brewer's yeast. α -Glucosidases I and II were homogeneous in disc electrophoresis, but α -glucosidase III was not. Their pH-optima were found to be in the range of 6.3 to 7.1. Each of them was a typical α -glucosidase showing a preferential activity on phenyl- α -glucoside. α -Glucosidase I was so-called isomaltase which could hydrolyze isomaltose but not maltose. α -Glucosidases II and III showed no activity on isomaltose. The ratio of velocity of hydrolysis for phenyl- α -glucoside (K_m , 2.9 mM) and isomaltose (K_m , 28.6 mM) of α -glucosidase I was calculated to be 100:9 in that order, and those for phenyl- α -glucoside (K_m 's, 0.8 mM and 3.6 mM) and maltose (K_m 's, 14.3 mM and 71.4 mM) of α -glucosidases II and III were calculated to be 100:17 and 100:18, respectively.

It has been known that α -glucosidase from *Saccharomyces italicus*¹⁾ or *Saccharomyces cerevisiae*^{2,3)} is a typical α -glucosidase which shows a higher activity to aryl- α -glucoside (e.g., phenyl- and *p*-nitrophenyl- α -glucoside), sucrose and turanose than to maltose. Okada⁴⁾ and Terui *et al.*⁵⁾ pointed out that among α -glucosidases of this type, the enzyme capable of hydrolyzing isomaltose, so-called isomaltase, was present independently of maltase. The α -glucosidases obtained from yeast by Halvorson¹⁾ and Phillips²⁾ appear to be enzymes of the latter type exhibiting no activity on isomaltose.

Years ago, Khan and Eaton⁶⁾ reported that the α -glucosidase from a strain of *Saccharomyces cerevisiae* could be separated into two enzymatically active components, maltase and isomaltase, by chromatography on DEAE-Sephadex. The same results from α -glucosidases of *Saccharomyces ovoformis* were demonstrated by Lai and Axelrod.⁷⁾ However, the substrate specificities of these enzymes have not been thoroughly examined.

In the previous paper,⁸⁾ the authors reported on the properties of a partially-purified α -glucosidase from brewer's yeast. In view of the above facts, however, this enzyme preparation is likely to be a mixture of two or more α -glucosidases, whose substrate specificities differ from one another. Therefore, it seemed necessary to reexamine the substrate specificity with a highly-purified enzyme. The present paper describes the purification and the substrate specificities of brewer's yeast α -glucosidases.

MATERIALS AND METHODS

Enzyme source. Brewer's yeast was kindly supplied by Sapporo Breweries, Ltd. α -Glucosidase preparation, which was partially purified from brewer's yeast by the method described in the previous report⁸⁾ and preserved after salting-out with ammonium sulfate, was used for further purification.

Substrates. Maltose, phenyl- α -glucoside (Nakarai Chemicals Co., Ltd.), sucrose (Wako Pure Chemical Ind., Ltd.), turanose and methyl- α -glucoside (Tokyo Kasei Co., Ltd.) were obtained from commercial sources, and maltose was further purified by repeated recrystallization to remove a possible trisaccharide impurity. Nigerose and kojibiose were enzymatically synthesized through transglucosylation of buckwheat α -glucosidase.⁹⁾ Isomalto- and malto-oligosaccharides were prepared from the partial acid-hydrolyzate of

[†] This work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan on April 3, 1972, in Sendai and on April 3, 1973, in Tokyo.

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dextran (Meito Sangyo Co., Ltd.) and *Bacillus subtilis* α -amylase (Seikagaku Kogyo Co., Ltd.)-hydrolyzate of amylose, respectively, by carbon-Celite column chromatography.

Enzyme assay. α -Glucosidase activity on phenyl- α -glucoside was determined by measuring phenol liberated from phenyl- α -glucoside in accordance with a modification of the method of Robertson and Halvorson¹⁰⁾ using 2,6-dibromoquinone-chloroimide. The reaction mixture contained 0.1 ml of 1% phenyl- α -glucoside, 0.4 ml of McIlvaine buffer (pH 6.8) and 0.5 ml of enzyme solution. After a certain period of incubation at 33°C, an aliquot of the mixture was pipetted out and added to 5 ml of Clark-Lubs buffer at pH 9.8 to terminate the reaction. Then, 0.2 ml of 0.2% 2,6-dibromoquinone-chloroimide solution in ethanol was added and the mixture, after being kept for 45 min at room temperature, was diluted with water up to 10 ml for measurement of the optical density at 660 nm.

Maltase activity and isomaltase activity were determined by measuring glucose from each substrate in accordance with a modification of glucose oxidase method¹¹⁾ by the authors using "Glucostat" (Worthington Biochemicals Co.). The reaction mixture, containing 0.1 ml of 1% maltose or isomaltose, 0.4 ml of McIlvaine buffer (pH 6.8) and 0.5 ml of enzyme solution, was incubated at 33°C.

One unit of enzyme activity represents the amount of enzyme which hydrolyzed 1 μ mole of each substrate per min under the above conditions.

Protein determination. The concentration of protein was spectrophotometrically determined under the assumption that the $E_{1\text{cm}}^{1\%}$ at 280 nm was 10.0.

Homogeneity. Disc electrophoresis was conducted in diethylbarbituric acid-Tris buffer (pH 7.0) for 70 min with a current of 3 mA/tube at room temperature by the method of Reisfeld *et al.*¹²⁾ and Nagai.¹³⁾

RESULTS AND DISCUSSION

Purification of brewer's yeast α -glucosidases

An aqueous solution of the precipitate of the above partially-purified enzyme preparation was dialyzed against running tap-water and then against 0.05 M Sørensen phosphate buffer (pH 6.8) containing 1 mM β -mercaptoethanol. The solution (12 ml, about 7,600 units for phenyl- α -glucosidase) was applied to a column of Sephadex G-75 which had been equilibrated with the same buffer, and the elution was successively carried out (Fig. 1).

FIG. 1. Gel Filtration of Brewer's Yeast α -Glucosidase on Sephadex G-75 Column.

Enzyme soln., 12 ml; column size, 2.8 $\times$ 98 cm; equilibration and elution, with 0.05 M Sørensen phosphate buffer (pH 6.8) containing 1 mM β -mercaptoethanol; flow rate, 24 ml/hr.

●, OD at 280 nm; ○, phenyl- α -glucosidase activity; ⊙, maltase activity; △, isomaltase activity.

The fractions (No. 60~68) were combined and concentrated to 27.5 ml with Amicon ultra-filtrator.

The concentrated solution was subjected to chromatography on a DEAE-Sephadex A-50 column equilibrated in advance with the above buffer. After the non-adsorbed proteins were washed out from the column with the same buffer, the elution was run under a linear gradient of sodium chloride. As shown in Fig. 2, the α -glucosidases were separated into three peaks of protein, fraction numbers 201 to 210 (I), fraction numbers 214 to 229 (II) and fraction numbers 245 to 260 (III). Isomaltase activity was found to be present only in eluate I which was free from maltase activity. These isolated α -glucosidases are tentatively designated as α -glucosidases I, II and III in this paper.

Each effluent was concentrated to 4 ml and rechromatographed on a DEAE-Sephadex A-50 column (3.2 $\times$ 42 cm) under the same conditions as mentioned above. The active fractions of α -glucosidase I, II and III were collected and concentrated to 3 ml each.

The gel-filtrations of the concentrates were

FIG. 2. Chromatography of Brewer's Yeast α -Glucosidase on DEAE-Sephadex A-50 Column. Enzyme soln., 27.5 ml; Column size, 4.5×46 cm; equilibration, with 0.05 M Sørensen phosphate buffer (pH 6.8) containing 1 mM β -mercaptoethanol; elution, with the same buffer under a linear gradient of NaCl; flow rate, 37 ml/hr.
 ●, OD at 280 nm; ○, phenyl- α -glucosidase activity; ⊙, maltase activity; △, isomaltase activity; ---, concentration of NaCl.

FIG. 3. Gel Filtration of Brewer's Yeast α -Glucosidases I (a), II (b) and III (c) on Sephadex G-75 Column.

Enzyme soln., 3 ml; column size, 2.8×66 cm; equilibration and elution, the same conditions as shown in Fig. 1; flow rate, 19 ml/hr.

●, OD at 280 nm; ○, phenyl- α -glucosidase activity; △, isomaltase activity.

further conducted on a Sephadex G-75 column, as shown in Fig. 4-a, -b and -c. The eluates, α -glucosidases I (fraction No. 72~78), II (fraction No. 72~78) and III (fraction No. 73~81), were used as enzyme preparations in the following experiments.

α -Glucosidases I, II and III were purified 5.8-, 10.2- and 2.1-fold over the partially-purified enzyme preparation, and their specific activities (unit/mg protein) were 46.3, 80.8 and 17.0, respectively. The molecular weight of each enzyme was determined by thin-layer gel filtration^{14,15} with Sephadex G-150 and TLC-apparatus (Pharmacia Fine Chemicals Co.). The molecular weights of α -glucosidases I, II and III were all estimated to be approximately 5.2×10^4 .

Homogeneity of the enzymes

In order to examine the homogeneity of the enzyme preparations, disc electrophoresis with polyacrylamide-gel was carried out. As shown in Fig. 4, α -glucosidases I and II migrated as a single band. However, α -glucosidase III, which was too labile for isolation as a homogeneous protein, gave three or more bands. Neither α -glucosidase I nor II, though less labile than α -glucosidase III,

FIG. 4. Polyacrylamide Disc Gel Electrophoresis of Brewer's Yeast α -Glucosidases.

Electrophoresis was carried out in Tris-diethylbarbituric acid buffer at pH 7.0 with a current of 3 mA per tube for 70 min. The degree of polymerization of gel was 7.5%, and protein was stained with 1% Amido Black 10B in 7% acetic acid.

could be obtained as a homogeneous preparation in the absence of β -mercaptoethanol. The homogeneity was merely maintained for two to three weeks in 0.05 M Sørensen phosphate buffer (pH 6.8) containing 1 mM β -mercaptoethanol at -20°C .

Attempts to isolate α -glucosidase III as a homogeneous protein were unsuccessful, because of its instability. Therefore, this enzyme preparation was used without further purification, though the possibility was not excluded that it might contain some additional α -glucosidases whose substrate specificities were different from one another.

Effect of pH or temperature

The effect of pH on the activities of brewer's yeast α -glucosidases was investigated. As shown in Fig. 5, each of these α -glucosidases was a neutral α -glucosidase, and their pH-optima were found to be in the range of 6.3 to 7.1.

Experiments on the effect of temperature were also carried out. The temperature-optimum for the reaction of α -glucosidases I and II was about 42°C (incubation time: 10 min). However, the optimal temperature for α -glucosidase III was found at 36°C (incubation time: 5 min). α -Glucosidase III seemed to be more susceptible to thermal

FIG. 5. pH-Activity Curves of Brewer's Yeast α -Glucosidases.

Reaction mixture containing 0.5 ml of 0.2% phenyl- α -glucoside, 0.4 ml of McIlvaine buffer and 0.1 ml of enzyme solution (0.15 unit) was incubated at various pH for 10 min at 33°C .

○, α -glucosidase I; ●, α -glucosidase II; ○, α -glucosidase III.

effect than the others.

Hydrolytic activity on various substrates

Phenyl- α -glucoside, maltose, nigerose, kojibiose, isomaltose, sucrose, turanose, methyl- α -glucoside, maltotriose, isomaltotriose and phenyl- α -maltoside were used as substrates. Figure 6 shows that each of these enzymes is a typical α -glucosidase exhibiting higher hydrolytic activity on phenyl- α -glucoside.

α -Glucosidase I manifested a relatively high activity on isomaltose and methyl- α -glucoside besides phenyl- α -glucoside. Sucrose and kojibiose were degraded very slowly, but maltose, nigerose, turanose, maltotriose, isomaltotriose and phenyl- α -maltoside were not split. The facts indicate that α -glucosidase I is so-called isomaltase.^{4,7,16)}

On the other hand, α -glucosidases II and III were capable of hydrolyzing turanose, sucrose, maltose and nigerose. However, isomaltose, methyl- α -glucoside and kojibiose were not cleaved. Actions on maltotriose and phenyl- α -maltoside will be described in the succeeding section.

Either α -glucosidase I or II showed no activity on malto-oligosaccharides made up

FIG. 6. Progresses of Hydrolytic Action on Various Substrates by α -Glucosidases II (a), I (b) and III (c).

(a): Reaction mixture of 4 ml of 1% substrate, 4 ml of McIlvaine buffer (pH 6.8) and 2 ml of α -glucosidase II solution (6 units) was incubated at 33°C.

(b) and (c): Reaction mixture contained 0.5 ml of 1% substrate, 2 ml of McIlvaine buffer (pH 6.8) and 2.5 ml of α -glucosidase I solution (0.26 unit) or α -glucosidase III solution (0.23 unit).

○, phenyl- α -glucoside; ●, turanose; ⊙, sucrose; □, maltose; ■, isomaltose; ×, nigerose; △, methyl- α -glucoside; ▲, kojibiose.

of four or more glucose units or on isomalto-oligosaccharides, of more than two glucose units.

Panose and melezitose were not attacked by any of the present enzymes.

Hydrolytic activity of α -glucosidase II or III on maltose, maltotriose and phenyl- α -maltoside

As mentioned above, α -glucosidases II and III are the enzymes capable of hydrolyzing maltose, but they show no activity on isomaltose. Moreover, the actions on maltose, maltotriose and phenyl- α -maltoside were examined. The progresses of liberation of glucose from these substrates are shown in Fig. 7. The action pattern of each enzyme on maltotriose was checked by thin-layer chromatography, and glucose and maltose were recognized to accumulate as products. The fact suggests that the hydrolysis of the substrate in the initial phase of the reaction may be due to a multi-chain reaction mechanism.^{17,18} In such a mechanism, only glucosidic linkage at non-reducing end of substrate is considered to be split. Figure 7 also shows the progresses of liberation of phenol from phenyl- α -maltoside by α -glucosidases II and III. The molar ratio

FIG. 7. Progresses of Hydrolytic Action on Maltose, Maltotriose and Phenyl- α -maltoside by α -Glucosidases II and III.

(a): Reaction mixture consisting of 5 ml of 1% substrate, 6 ml of McIlvaine buffer (pH 6.8) and 1 ml of α -glucosidase II solution (2.1 units) was incubated at 33°C.

(b): Reaction mixture contained 0.5 ml of 1% substrate, 2.5 ml of McIlvaine buffer (pH 6.8) and 2 ml of α -glucosidase III solution (1.25 units).

○, phenyl- α -maltoside; ●, maltose; ⊙, maltotriose; ○---○, phenol from phenyl- α -maltoside.

for the liberation of glucose and phenol is approximately 2:1, which means that the substrate is split into glucose and phenyl- α -glucoside, and the latter immediately into

FIG. 8. Lineweaver-Burk Plots for Hydrolysis of Phenyl- α -glucoside, Maltose and Isomaltose.

Reaction mixture containing 2 ml of substrate, 1.9 ml of McIlvaine buffer (pH 6.8) and 0.1 ml of enzyme solution (0.022 unit) was incubated at 33°C. Glucose or phenol liberated from each substrate was determined.

A, B and C show α -glucosidases I, II and III on phenyl- α -glucoside, respectively; D and E, α -glucosidases II and III on maltose, respectively; F, α -glucosidase I on isomaltose.

glucose and phenol. The results of thin-layer chromatography of the action pattern indicate that the accumulation of phenyl- α -glucoside is little or even negligible. Consequently, it appears that the hydrolysis of phenyl- α -maltoside is caused by a single-chain reaction mechanism.^{19,20)}

In Fig. 8 are shown Lineweaver-Burk plots for the hydrolysis of phenyl- α -glucoside, maltose and isomaltose. From the double

reciprocal plots, Michaelis constants (K_m) for phenyl- α -glucoside were calculated to be 2.9 mM, 0.8 mM and 3.6 mM for α -glucosidases I, II and III, respectively. Only phenyl- α -glucoside is the common substrate which these enzymes can preferentially hydrolyze. The hydrolysis velocity on maltose or isomaltose was remarkably dependent on the substrate concentration. The data for various substrates tested, that is, Michaelis constants and the relative rates (V) to hydrolysis velocity of phenyl- α -glucoside, which directly expresses the velocity of cleavage of α -glucosidic bond in each substrate, were calculated from each maximum velocity (V_{max}) (Table I). Although α -glucosidase I exhibited a hydrolytic activity on sucrose and kojibiose (Fig. 6-b), the activity was too feeble for kinetic studies. As mentioned above, phenyl- α -maltoside (G-G- ϕ : G and ϕ represent a glucosyl residue or glucose and a phenyl residue or phenol, respectively) is likely to be attacked by a single-chain reaction mechanism. The processes of the reaction are as follows:

In this case, the velocity of cleavage of an α -glucosidic linkage between glucosyl residue and phenyl residue (Process II) may be much faster than that between glucosyl residues (Process I). The velocities of Processes I and II could not be separately determined by measurement in this experiment. Therefore, in

TABLE I. MICHAELIS CONSTANT (K_m) AND RELATIVE RATE (V) FOR HYDROLYSIS OF A LINKAGE OF VARIOUS SUBSTRATES

Substrate	K_m (mM)			V^a		
	I	II	III	I	II	III
Phenyl- α -glucoside	2.9	0.8	3.6	100	100	100
Phenyl- α -maltoside	—	2.8	14.3	—	34	35
Turanose	—	11.7	30.3	—	67	56
Sucrose	—	16.6	71.4	—	27	35
Maltose	—	14.3	71.4	—	17	18
Nigerose	—	20.0	100.0	—	15	14
Isomaltose	28.6	—	—	9	—	—
Maltotriose	—	6.2	14.3	—	16	30
Methyl- α -glucoside	50.0	—	—	11	—	—

^a) Relative rate for cleavage of α -glucosidic linkage, calculated from V_{max} .

Table I, the values for this substrate denote the mean value of the hydrolysis rate, calculated by assuming a single-chain reaction mechanism, in the two precesses. The V values of maltotriose were obtained from V_{max} , on the basis of the assumption that the liberation of glucose was due to a multi-chain reaction mechanism mentioned above. As shown in Fig. 7-a, the production of glucose from maltotriose by α -glucosidase II was smaller than that from maltose. However, the V values of maltose and maltotriose was nearly equal. α -Glucosidase III hydrolyzed maltotriose more rapidly than maltose, unlike α -glucosidase II.

As is obvious from the above results, it seems to be of interest that both maltase and isomaltase from brewer's yeast are typical α -glucosidases showing a preferential activity on aryl- α -glucoside such as phenyl- α -glucoside.

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