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# Two potent competitive inhibitors discriminating α-glucosidase family I from family II

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**Abstract**—The inhibition kinetics for isoacarbose (a pseudotetrasaccharide, IsoAca) and acarviosine–glucose (pseudotrisaccharide, AcvGlc), both of which are derivatives of acarbose, were investigated with various types of α-glucosidases obtained from microorganisms, plants, and insects. IsoAca and AcvGlc, competitive inhibitors, allowed classification of α-glucosidases into two groups. Enzymes of the first group were strongly inhibited by AcvGlc and weakly by IsoAca, in which the $K_i$ values of AcvGlc (0.35–3.0 μM) were 21- to 440-fold smaller than those of IsoAca. However, the second group of enzymes showed similar $K_i$ values, ranging from 1.6 to 8.0 μM for both compounds. This classification for α-glucosidases is in total agreement with that based on the similarity of their amino acid sequences (family I and family II). This indicated that the α-glucosidase families I and II could be clearly distinguished based on their inhibition kinetic data for IsoAca and AcvGlc. The two groups of α-glucosidases seemed to recognize distinctively the extra reducing-terminal glucose unit in IsoAca.


*Keywords*: Enzyme inhibitor; α-Glucosidase family; Isoacarbose; Acarviosine–glucose; Acarbose

## 1. Introduction

α-Glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) is an exo-type carbohydrase that catalyzes the liberation of α-glucose from the nonreducing end of the substrate. The enzyme is widely distributed in microorganisms, plants, and animal tissues, and the substrate specificity of α-glucosidases is known to differ greatly depending on their source.[1–3] The bacterial, yeast (*Saccharomyces cerevisiae*) and insect enzymes, named α-glucosidase I, show higher activity toward such heterogeneous substrates as sucrose and *p*-nitrophenyl α-glucoside (PNPG), and none or less toward such homogeneous substrates as maltooligosaccharides, implying that α-glucosidase I recognizes the 'glucosyl structure' in the substrate.[3] The mold, plant, and mammalian enzymes, named α-glucosidase II, hydrolyze the homogeneous substrates more rapidly than the heterogeneous substrates, indicating that this class of α-glucosidases recognizes the 'maltosyl structure.' Some of α-glucosidase II enzymes also attack such α-glucans as soluble starch and glycogen. The hydrolysis of *p*-nitrophenyl 2-deoxy-α-D-arabino-hexopyranoside was catalyzed by α-glucosidase II, but no such reaction was observed with α-glucosidase I,[4] suggesting that the 2-OH group in the glucose moiety is essential for α-glucosidase I. α-Glucosidase II catalyzed the hydration of D-glucal to produce 2-deoxy-α-D-arabino-hexose, but α-glucosidase I gave no detectable hydration product.[5] It has been reported that homology analysis of the complete

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amino acid sequences divides the α-glucosidases into two groups, family I and family II.[2,3] This approach classified α-glucosidase I into family I, and α-glucosidase II into family II. The partial amino acid sequence containing the catalytic residue (namely, the catalytic region) was also found to be different between the two families.[6] The family I α-glucosidases have four catalytic regions, which are conserved in the alpha-amylase family enzymes, such as neopullulanase, alpha amylase, and cyclomaltodextrin glucanotransferase (CGTase).[7] The family II α-glucosidases have two catalytic regions responsible for the enzyme reaction.[8,9] It has been reported that the families I and II enzymes belong to glycoside hydrolase families 13 and 31, respectively.[10,11] We are interested in the difference in reaction mechanisms of both families, as caused by their distinct active-site structures.

Amino sugar derivatives synthesized by some strains of the *Actinomycetales* inhibit the activity of carbohydrases. One of these derivatives, acarbose (Aca) is a potent inhibitor of several carbohydrases, such as α-glucosidase, glucoamylase,[12,13] alpha-amylase,[14] and CGTase.[15] In the conformation of Aca, there is a unique pseudodisaccharide moiety at the nonreducing end, which links to maltose by an α-(1→4)-bond. The pseudodisaccharide structure is composed of the sugar-like ring of (4,5,6-trihydroxy-3-hydroxymethyl-2-cyclohexene-1-yl) linking to the nitrogen atom of 4-amino-4,6-dideoxy-D-glucopyranose (4-amino-4-deoxy-D-quinovo- pyranose). Park and co-workers have modified Aca through hydrolysis and transglycosylation catalyzed by *Bacillus stearothermophilus* maltogenic amylase (BSMA).[16,17] BSMA cleaved the α-(1→4)-glucosidic bond of Aca to produce an acarviosine–glucose (AcvGlc, see Fig. 2C). BSMA also catalyzed the transglycosylation of Aca with various carbohydrate acceptors to produce transfer products. When the AcvGlc component was transferred to the 6-hydroxyl group of glucose, isoacarbose (IsoAca, see Fig. 2D) was produced. AcvGlc and IsoAca showed potent inhibitory action toward α-glucosidases obtained from baker's yeast and rat intestine, CGTase from *Bacillus macerans*, and porcine pancreatic alpha-amylase, with competitive-type, mixed-type, and mixed-type inhibitions, respectively.[18] For CGTase and alpha-amylase, the inhibitory potency of AcvGlc and IsoAca was found to be higher than that of Aca. The inhibitory abilities of Aca, AcvGlc, and IsoAca toward two α-glucosidases gave somewhat complicated data[18] (see Table 1). AcvGlc inhibited baker's yeast α-glucosidase more strongly than Aca. The inhibition of Aca showed a level similar to that of IsoAca for baker's yeast α-glucosidase and those of IsoAca and AcvGlc for rat intestinal α-glucosidases. It is known that the disturbance of carbohydrate metabolism causes diabetes, and diabetic patients need, therefore, to control their digestion of carbohydrates. In fact, Aca has

**Table 1.** Inhibition type and $K_i$ values of AcvGlc and IsoAca for α-glucosidases from various origins

| Enzyme source | α-Glucosidase family | $K_m$[a] (mM) | Substrate (pH)[c] | Inhibitor | Inhibition type[d] | $K_i$ (μM) | Ratio[b] |
|---|---|---|---|---|---|---|---|
| Brewer's yeast | Family I | 0.35 | PNPG[e] | AcvGlc | Comp | 3.0 | |
| | | | (pH 7.0) | IsoAca | Comp | 400 | 130 |
| *B. subtilis* | Family I | 5.17 | Isomaltose | AcvGlc | Comp | 0.36 | |
| | | | (pH 7.2) | IsoAca | Comp | 160 | 440 |
| Honeybee | Family I | 13.3 | Maltose | AcvGlc | Comp | 0.35 | |
| (Isozyme III) | | | (pH 5.5) | IsoAca | Comp | 7.5 | 21 |
| Baker's yeast[f] | Family I | — | Maltose | AcvGlc | Comp | 0.18 | |
| | | | (pH 7.0) | IsoAca | Comp | 405 | 2240 |
| | | | | Aca | Comp | 77.9 | 430 |
| *A. niger* | Family II | 0.75 | Maltose | AcvGlc | Comp | 7.5 | |
| | | | (pH 4.3) | IsoAca | Comp | 8.0 | 1.1 |
| *S. pombe* | Family II | 6.2 | Maltose | AcvGlc | Comp | 5.3 | |
| | | | (pH 4.5) | IsoAca | Comp | 7.8 | 1.5 |
| Buckwheat | Family II | 6.3 | Maltose | AcvGlc | Comp | 2.5 | |
| | | | (pH 5.0) | IsoAca | Comp | 2.7 | 1.1 |
| Rice | Family II | 2.0 | Maltose | AcvGlc | Comp | 1.6 | |
| | | | (pH 4.0) | IsoAca | Comp | 2.8 | 1.8 |
| Rat intestine[f] | Family II | — | Maltose | AcvGlc | Comp | 0.13 | |
| | | | (pH 7.0) | IsoAca | Comp | 0.13 | 1.0 |
| | | | | Aca | Comp | 0.059 | 0.45 |

[a] $K_m$ value for substrate used.
[b] ($K_i$ for IsoAca)/($K_i$ for AcvGlc) or ($K_i$ for Aca)/($K_i$ for AcvGlc).
[c] pH in the reaction mixture for estimating the $K_i$ value.
[d] Comp, competitive inhibition.
[e] *p*-Nitrophenyl α-glucoside.
[f] Data from Ref. 16.

been therapeutically used for this purpose.[19,20] AcvGlc and IsoAca are also used medically to slow down the digestive reactions of carbohydrases.

Park and co-workers have also reported distinct inhibitory effects of AcvGlc and IsoAca on α-glucosidases of baker's yeast and rat intestine.[18] The intestinal enzyme showed almost identical inhibitory constants ($K_i$) for AcvGlc and IsoAca. For the yeast enzyme, large and small $K_i$ values were obtained for AcvGlc and IsoAca inhibitions, respectively, their ratio being more than 2000. Since the yeast enzyme belongs to the α-glucosidase family I and the intestinal enzyme belongs to the α-glucosidase family II, there is a possibility that the two analogues are distinctively recognized by the enzymes of different families. We are interested in the difference between the reaction catalyzed by the two families, in particular, the molecular recognition of the substrate and analogues thereof. This paper describes the inhibitory effects of AcvGlc and IsoAca on several α-glucosidases belonging to families I and II.

## 2. Materials and methods

### 2.1. Materials

AcvGlc and IsoAca were prepared according to methods described previously.[18] Maltose, PNPG (Nakalai Tesque, Kyoto, Japan), and isomaltose (Wako Pure Chemical Ind., Ltd., Osaka, Japan) were commercial products. α-Glucosidases of brewer's yeast (no. 4634)[21,22] and rice (no. 9259)[23,24] were purchased from Sigma Chemical Co. (St. Louis, MO). α-Glucosidases from honeybee (isozyme III),[25,26] *Aspergillus niger*,[27–29] *Bacillus subtilis*,[30,31] *Schizosaccharomyces pombe*,[8,32] and buckwheat[24,33] were purified, and each enzyme showed a single band in SDS electrophoresis.

### 2.2. Enzyme reactions

Reaction mixtures consisting of 0.1 mL of AcvGlc or IsoAca of appropriate concentration (or $H_2O$ as a control), 0.2 mL of substrate (maltose, PNPG, or isomaltose) of various concentrations, and 0.1 mL of 0.2 M buffer were incubated at 37 °C. The substrates and buffer pHs are summarized in Table 1. Buffers of sodium acetate and sodium phosphate were used to maintain pH 4 to 5.5 and pH 6.8 to 7.2, respectively. Glucose liberated from substrates was measured by the modified glucose oxidase–peroxidase method using a Glucose AR-II kit (Wako Pure Chemical Ind., Ltd.).[34]

Quantitative analysis of the anomeric form of glucose released from maltotriose or *p*-nitrophenyl α-maltoside (Nakalai Tesque) was done by the gas–liquid chromatographic method previously reported.[35] All α-glucosidases were 'retaining' enzymes that produce α-glucose from the substrate. The transglucosylation products of rice and brewer's yeast α-glucosidases were analyzed by thin-layer chromatography (TLC).[16] The compositions of reaction mixtures for the two enzymes were the same as those already described, except for the buffers: pyridium–acetate and imidazole–HCl were used for rice and brewer's yeast enzymes, respectively.

### 2.3. Kinetic analysis of inhibition

The inhibition types for AcvGlc and IsoAca were determined by Lineweaver–Burk plots ($1/s$ vs $1/v$). Competitive inhibition was observed in each case, and therefore, we estimated the $K_i$ value using the following equation:[36]

$$K_i = i/(K_p/K_m - 1),$$

where $i$, $K_m$, and $K_p$ are the inhibitor concentration used, Michaelis constant for the substrate, and the apparent $K_m$ in the presence of inhibitor, respectively.

## 3. Results and discussion

The enzyme activities of α-glucosidases from rice and brewer's yeast were investigated in the presence of 1 mM IsoAca or 1 mM AcvGlc (Fig. 1A and D). We used the small and favored substrates, maltose and PNPG, for rice and brewer's yeast enzymes, respectively, which bound to subsites −1 and +1 of the catalytic sites when the productive enzyme–substrate intermediate was formed (see Fig. 2A and B).[34] Strong inhibition was observed when 1 mM AcvGlc was present in the reaction mixture, and neither enzyme showed any activity. The inhibitory effects of 1 mM IsoAca on the activities of two α-glucosidase were found to be distinct, with strong inhibition for the rice enzyme and weak for the brewer's yeast enzyme, which maintained about 80% of the original activity (Fig. 1D). The two enzymes fully recovered their original activities after removal of IsoAca and AcvGlc by dialysis, indicating that the inhibition by AcvGlc and IsoAca was reversible. To estimate the inhibition types and $K_i$ values, the hydrolytic rates for various concentrations of substrate and inhibitors were examined by their Lineweaver–Burk plots, as shown in Figure 1B, C, E, and F. Both IsoAca and AcvGlc were found to be competitive inhibitors. The calculated $K_i$ values are summarized in Table 1. The $K_i$ value of brewer's yeast α-glucosidase for AcvGlc ($K_{i\text{-}AG}$) was about two orders of magnitude smaller than that for IsoAca ($K_{i\text{-}IA}$), indicating that AcvGlc is more than 100 times stronger an inhibitor than IsoAca. However, the $K_{i\text{-}AG}$ and $K_{i\text{-}IA}$ values for rice α-glucosidase showed no remarkable difference: the inhibition ratio of AcvGlc and IsoAca (abbreviated as R-I/P; dividing $K_{i\text{-}IA}$ by $K_{i\text{-}AG}$) was calculated to be 1.8. The results obtained

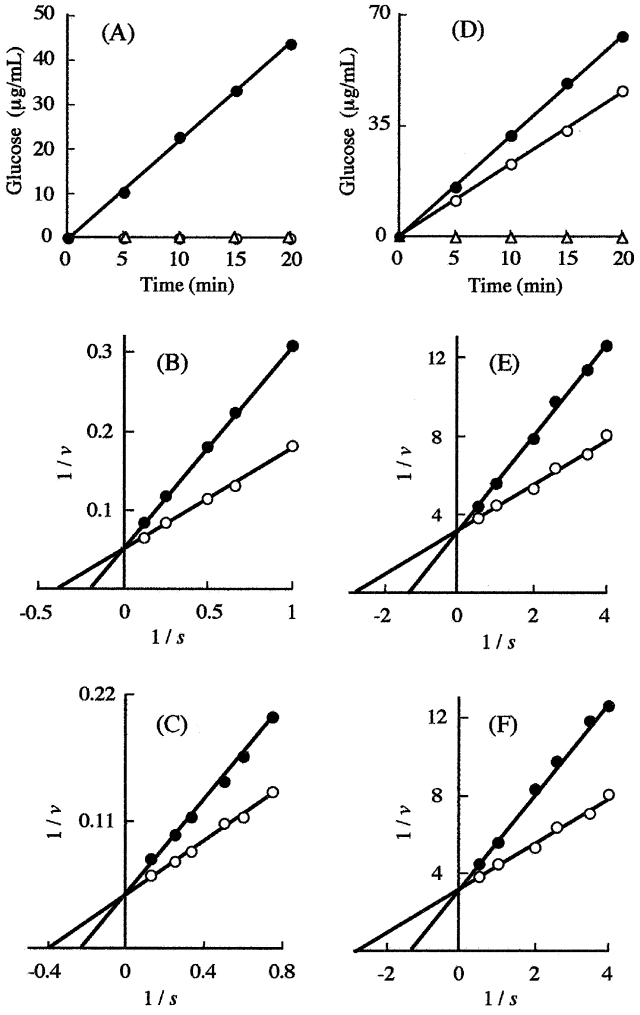


**Figure 1.** Inhibition of α-glucosidases from rice (A–C) and brewer's yeast (D–F) by AcvGlc and IsoAca. (A and D) Time course of glucose production in 1 mM IsoAca (open circles) or 1 mM AcvGlc (triangles) as compared with 0 mM inhibitors (closed circles). The vertical axis shows the amounts of glucose released in 1 mL of reaction mixture containing 2 mM maltose and 1 μg protein for A (pH 4.0 and 37 °C) or 2 mM PNPG and 1 μg protein for (D) (pH 7.0 and 37 °C). (B, C, E, and F) Lineweaver–Burk plots for the hydrolysis of maltose (B and C) and PNPG (E and F) in the presence (closed circles) and absence (open circles) of inhibitors. AcvGlc of 1.5 or 3.1 μM was used for (B) or (E), and IsoAca of 2.0 or 500 μM was used for (C) or (F), respectively. The reaction mixture (0.5 mL) containing 1 μg rice α-glucosidase or 1 μg brewer's yeast α-glucosidase was incubated at 37 °C under the conditions shown in Table 1. *s*, mM; *v*, mg glucose/min/mg protein.

agreed well with those of Park's group[16] in that the R-I/P values for the baker's yeast enzyme and the intestinal enzyme were 2240 and 0.940, respectively (see Table 1). Since the two yeast (*S. cerevisiae*) enzymes belong to the α-glucosidase family I and the rice and mammal intestine enzymes belong to the α-glucosidase family II, it is suggested that AcvGlc inhibits both families of enzymes to the same extent and IsoAca discriminates between two enzyme groups by stronger inhibition of family I and weaker of family II.

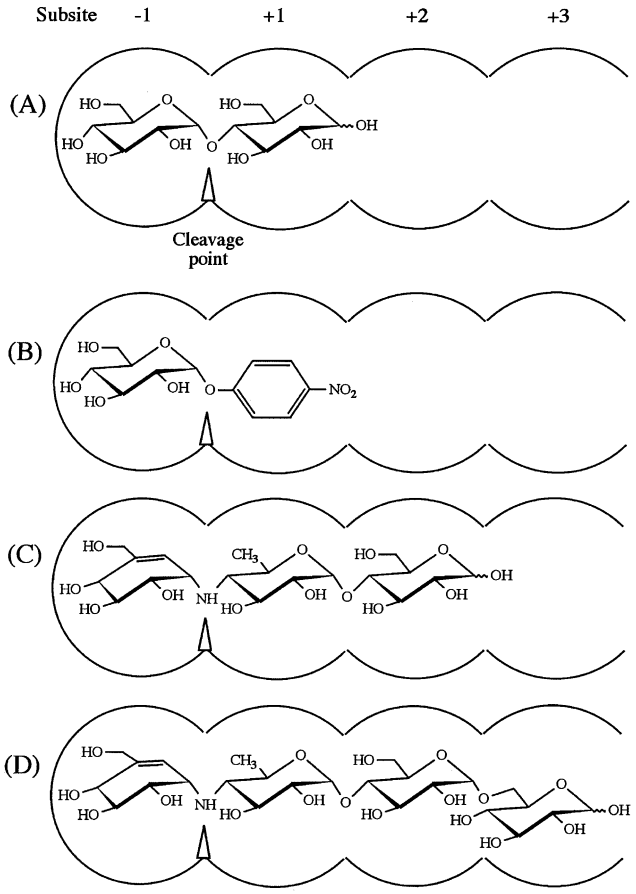


**Figure 2.** Subsite structure in catalytic site of α-glucosidase, and binding of maltose (A), PNPG (B), AcvGlc (C), and IsoAca (D). Triangle, cleavage point of substrate.

Likewise, the $K_{i\text{-}AG}$ and $K_{i\text{-}IA}$ values for various α-glucosidases belonging to families I (two enzymes) and II (three enzymes) were determined. A favored disaccharide substrate, having a large $k_{cat}$ and small $K_m$, was selected for each enzyme. Table 1 summarizes the $K_i$ values estimated for two inhibitors, along with the $K_m$ value for each substrate used. Both AcvGlc and IsoAca behaved as competitive type inhibitors for all α-glucosidases tested. Interestingly, there is an obvious difference in R-I/P between the enzymes of families I and II. The R-I/P values of the family II enzymes ranged from 1 to 1.8, showing that $K_{i\text{-}AG}$ and $K_{i\text{-}IA}$ were almost identical. The R-I/P values of the family I enzymes ranged from 21 to 440, indicating that the affinity of IsoAca was lower. IsoAca showed weaker inhibition toward family I than AcvGlc, but the binding ability of IsoAca was considered to be strong, since the $K_{i\text{-}IA}$ values of bacterial and insect enzymes were, respectively, 1/30 and 1/1800 of the levels of $K_m$ values for their favored substrates (Table 1). The $K_{i\text{-}IA}$ value of the brewer's yeast enzyme was almost identical to the $K_m$ value, which means that the affinities to IsoAca and the favored substrate were at the same levels.

The different extents of inhibition between AcvGlc and IsoAca suggested that the binding of each inhibitor to the catalytic site of the α-glucosidase families should also be distinct. The catalytic site of α-glucosidase is known to be composed of several subsites,[25,27,33,37] which independently recognize each glucosyl residue in the substrate. In Figure 2C and D, the most possible enzyme–inhibitor complexes and the subsite structure are shown. In each case, the unsaturated aminocyclitol ring residue and the 4-amino-4-deoxy-D-quinovopyranosyl residue are shown to bind to subsites −1 and +1, respectively, and an NH-group between two residues is located in the cleavage position. Eventually, AcvGlc and IsoAca occupy the subsites −1 to +2 and subsites −1 to +3, respectively. A similar binding-mode has been reported for the *Aspergillus oryzae* alpha-amylase–Aca complex, as elucidated by crystallographic analysis at 1.98 Å resolution.[38] As compared with the conformation of AcvGlc, IsoAca has an extra glucose moiety (Glc-I), which is considered to bind subsite +3 (Fig. 2). Glc-I is the only structural difference between the two compounds. The large R-I/P values for family I enzymes imply that Glc-I could obstruct the approach of IsoAca to the catalytic site by possible steric hindrance between the Glc-I moiety and the amino acid residue(s) in subsite +3. The $K_{i\text{-}IA}$ values of four family II α-glucosidases from microorganisms and plants were slightly larger than $K_{i\text{-}AG}$ (R-I/P = 1.07–1.47, Table 1), suggesting that the Glc-I structure of IsoAca could be also recognized by family II enzymes (probably by subsite +3). The finding that the α-glucosidase families I and II showed different affinities to IsoAca and AcvGlc implies that both families of enzymes might exhibit conformational variation in close vicinity to the catalytic site, that is, subsite +3.

Park and co-workers estimated the $K_i$ value of Aca ($K_{i\text{-}AB}$) for family I α-glucosidase from baker's yeast and that for family II α-glucosidase from rat intestine.[18] Aca, 4-α-(acarviosine-glucosyl)-glucose, is an isomer of IsoAca, 6-α-(acarviosine-glucosyl)-glucose. As shown in Table 1, the $K_{i\text{-}AB}$ value for the baker's yeast enzyme (77.9 μM) is 430-fold (two orders of magnitude) larger than $K_{i\text{-}AG}$ value (0.181 μM), which means that the reducing-terminal glucose unit of Aca decreases its binding ability to the active site of family I α-glucosidase. The $K_{i\text{-}AB}$ value for rat intestinal enzyme, 0.059 μM, was almost identical for AcvGlc ($K_{i\text{-}AG}$, 0.134 μM) and IsoAca ($K_{i\text{-}IA}$, 0.126 μM). The recognition of Aca and AcvGlc by families I and II enzymes was similar to that of IsoAca and AcvGlc.

It was reported that α-glucosyl-α-acarviosine-glucose (Glc-AcvGlc) is a strong inhibitor of maltogenic amylase.[39] The $IC_{50}$ value of Glc-AcvGlc was 1/5040 of that of Aca. There is, therefore, a possibility that α-glucosidase catalyzed transglucosylation in the inhibitory experiments to form potent inhibitor(s), such as glucosyl-IsoAca or Glc-AcvGlu. In this case, the Lineweaver–Burk plots are considered to lose linearity and exhibit a concave curve, since the transglucosylation activity is large when there is a high concentration of substrate. No such phenomena were observed in Figure 1B, C, E, and F. The linearity of each plot means that there is no formation of transglucosylation products in the inhibitory experiment. TLC analysis of the reaction mixtures in Figure 1 showed no detectable transglucosylation compound.

As shown already, AcvGlc and IsoAca act as highly effective and specific inhibitors with different efficacy against enzymes of both α-glucosidase families. Three potential usages are indicated for these potent inhibitors. The first is a medical application to suppress the activities of the intestinal sucrase–isomaltase complex and maltase–glucoamylase complex (family II enzymes), resulting in the decrease of glucose absorption. Consequently, diabetic patients could decrease the postprandial elevation of blood glucose level. The second application is as an insecticide through inhibition of the insect's digestive system. The strong inhibitory effects (see Table 1, the insect α-glucosidase was inhibited by AcvGlc and IsoAca with $K_i$ values of μM- and sub-nM-levels, respectively) imply that both inhibitors are insecticide candidates, especially for grain-feeding insects. A third possible usage is the selective measurement of the family I α-glucosidase activity from a mixture of families I and II enzymes by the addition of IsoAca. Figure 1A shows that 1 mM IsoAca completely inhibits the rice enzyme activity. Under the same concentration of IsoAca, the brewer's yeast enzyme maintained ~80% of the original activity (Fig. 1D). These data indicate that the activity of the family I enzyme can be measured separately in a mixture of both enzyme families by IsoAca. To achieve selective measurement of family I enzyme, two factors, described next, are of importance. The first important factor is to adjust the concentrations of IsoAca and substrate to proper levels. For determining suitable concentrations, the following equation is useful:

$$v_i/v_0 = [V \cdot s/(K_{m\text{-}M} + s + K_{m\text{-}M} \cdot i/K_{i\text{-}IA})]/[V \cdot s/(K_{m\text{-}M} + s)],$$

where $v_i$, $v_0$, $s$, $i$, $V$, and $K_{m\text{-}M}$ represent the reaction rate at an arbitrary concentration of IsoAca, the reaction rate in 0 mM IsoAca, the arbitrary concentrations of substrate and IsoAca, the maximum rate, and the Michaelis constant for substrate, respectively. To selectively measure brewer's yeast enzyme activity from the rat intestinal enzyme activity, for example, the optimum conditions are 3 mM maltose and 0.1 mM IsoAca at pH 7.0 and 37 °C. Under these conditions, the brewer's yeast enzyme showed 76% of its original activity, where the $K_m$ value for maltose was 11 mM. The activity of the rat intestinal

enzyme is estimated to be only 0.48% of the original maltase activity using the $K_m$ value for maltose of 1.05 mM. The second factor is the combination of α-glucosidases. The most appropriate case is a combination of the family I enzyme having a large $K_{i\text{-}IA}$ value and the family II enzyme having a small $K_{i\text{-}IA}$ value, such as a mixture of the yeast-type and intestine-type α-glucosidases. This approach is considered to be applicable to the fermentation process, using yeast and mold α-glucosidases. In this process, the activity of the yeast enzyme can be measured by blocking that of the mold enzyme (belonging to family II) in the presence of IsoAca.

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