S3

Preparation and Characterization of α-D-Glucopyranosyl-α-Acarviosinyl-D-Glucopyranose, a Novel Inhibitor Specific for Maltose-Producing Amylase

Myo-Jeong Kim[1] and Kwan-Hwa Park[2]

[1]Food Science Institute and School of Food and Life Science,
Inje University, Kimhae 621-749
[2]Research Center for New Bio-Materials in Agriculture and Department
of Food Science & Technology, School of Agricultural Biotechnology,
Seoul National University, Suwon, 441-744

A novel inhibitor against maltose-producing a-amylase was prepared via stepwise degradation of a high molecular weight acarbose (HMWA) using *Thermus maltogenic amylase* (ThMA). The structure of the purified inhibitor was determined to be α-D-glucopyranosyl-α-acarviosinyl-D-glucopyranose (GlcAcvGlc). Progress curves of p-nitrophenyl-α-D-maltoside (PNPG2) hydrolysis by various amylolytic enzymes, including maltogenase (MGase), ThMA, and cyclodextrinase (CDase) I-5, in the presence of acarbose or GlcAcvGlc indicated a slow-binding mode of inhibition. The inhibition potency of GlcAcvGlc for MGase, ThMA, and CDase I-5 was 3 orders of magnitude higher than that of acarbose.

INTRODUCTION

Acarbose is widely recognized as a potent inhibitor of several carbohydrases including α-glucosidase (*1*), glucoamylase (*2*), α-amylase (*1*), and cyclomaltodextrin glucanotransferase (CGTase; *3*). Acarbose is a pseudotetrasaccharide in which acarviosine, a pseudo sugar ring (4,5,6-trihydroxy-3-[hydroxymethyl]-2-cyclohexen-1-yl) at the nonreducing end, is linked to the nitrogen of 4-amino-4,6-dideoxy –D-glucopyranose (4-amino-4-deoxy-D-quinovopyranose) that is linked to maltose

by α-(1→4)-glycosidic bond (Fig. 1A). Acarbose, an inhibitor most potent against α-glucosidase, is produced by *Actinoplanes* sp. This species also produces a high molecular weight acarbose (HMWA), which is an excellent inhibitor of α-amylase (1). HMWA is a mixture of homologue series with 7-30 glucose units linked to acarviosine. Since the only difference between HMWA and acarbose is the number of glucose units attached to acarviosine, the length of glucose chain determines the specificity of a target enzyme.

Park *et al.* (4) produced various acarbose derivatives using the transglycosylation reaction of maltogenic amylases from *Bacillus stearothermophilus* (BSMA) and *Thermus* (ThMA). Among the derivatives produced, isoacarbose was found to be a potent inhibitor for porcine pancreatic a-amylase (PPA) (3). Development of acarbose derivatives as amylolytic enzyme inhibitors provides a new approach for the management of diabetes (5). And, it also provides a useful tool for
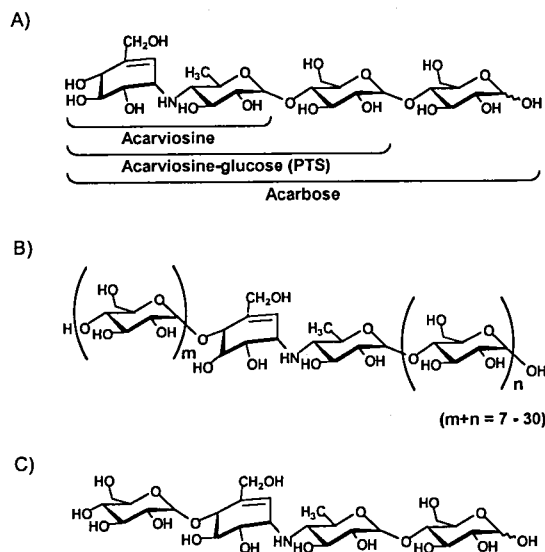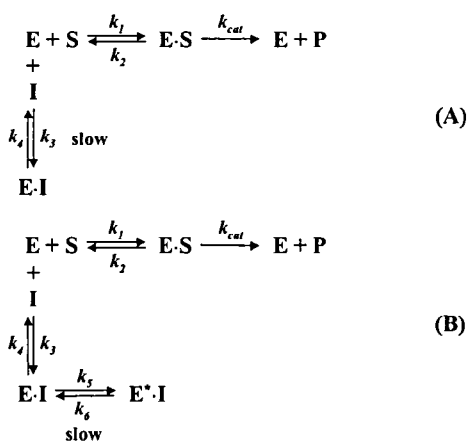

Fig. 1. Structures of the amino sugars having inhibitory effects on amylase activity. A, acarbose; B, high molecular weight acarbose (HMWA); and C, α-D-glucopyranosyl-α-acarviosinyl-D-glucopyranose.

screening a novel inhibitor against a target enzyme (3).

Slow binding, or time-dependent inhibition, is a widespread phenomenon among potent glycosidase inhibitors (6,7), where the inhibition process is relatively slow, occurring over a period of minutes or longer. Further studies on slow inhibition are required to get more detailed insight into the binding mode of

SCHEME I

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} E \cdot S \xrightarrow{k_{cat}} E + P$$
$$+$$
$$I$$
$$k_4 \Big\Updownarrow k_3 \quad \text{slow}$$
$$E \cdot I$$

(A)

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} E \cdot S \xrightarrow{k_{cat}} E + P$$
$$+$$
$$I$$
$$k_4 \Big\Updownarrow k_3$$
$$E \cdot I \underset{k_6}{\overset{k_5}{\rightleftarrows}} E^* \cdot I$$
$$\text{slow}$$

(B)

potent glycosidase inhibitors. From the kinetic point of view, most reversible time-dependent inhibition is related with one of the two mechanisms depicted below (8).

In Mechanism A, the inhibitor binds to the enzyme in a simple bimolecular reaction in a single step, but the association and dissociation rate constants (k_3 and k_4, respectively) are such that equilibrium is established slowly. In Mechanism B, binding between the enzyme and the inhibitor may have an initial fast-binding step establishing equilibrium that is defined by the on and off rate constants k_3 and k_4, just as in Mechanism A. However, binding of the inhibitor induces a reversible conformational transition or isomerization of the enzyme, leading to a tightly-bound enzyme-inhibitor complex $E^* \cdot I$, where the forward and reverse rate constants for the equilibrium between these two inhibitor-bound conformations of the enzyme are given by k_5 and k_6, respectively.

In this study, a hydrolysis product of HMWA was obtained by the action of ThMA, and its structure as well as physico-chemical properties were characterized. In addition, efficacy and specificity of the inhibitor, α-D-glucopyranosyl-α-acarviosinyl-D-glucopyranose (GlcAcvGlc), on maltose producing enzymes such as maltogenase, maltogenic amylase, and cyclomaltodextrinase were also investigated.

METHODS

Preparation of GlcAcvGlc from HMWA. For the production of HMWA, *Actinoplanes* sp. KCTC9162 was cultured in starch medium (5% soluble starch, 1% yeast extract, 0.2% K_2HPO_4). Two hundred milliliters of fresh medium containing 6 g of glass beads was prepared in a 500 mL baffled bottom flask and autoclaved at 121°C for 15 min. *Actinoplanes* sp. KCTC9162 was inoculated into the sterilized medium and cultured at 28°C for 3-5 days in a shaking incubator (300 rpm, 8480-SF, Vision Science Co., Buchon, Korea). pH of the culture broth was adjusted to 2.5 with 0.1 M of HCl and stirred at ambient temperature for 2 hr after activated charcoal (30 g/L; Sigma) was added. The mycelium and charcoal was discarded by centrifugation at 8,000 g for 15 min (VS-15, Vision Science Co.). pH of the supernatant was readjusted to 6 with 0.1 M NaOH and concentrated by ten folds using a rotary vacuum evaporator (EYELA, Tokyo, Japan). Starch and long chain dextrins in the concentrated supernatant (20 mL) were precipitated by adding 80 mL of methanol. After removal of sediment by centrifugation (4,000 g, 5 min), 800 mL of ethanol was added to 100 mL of the supernatant. Finally, HMWA was collected by centrifugation and dissolved in 200 mL of 50 mM sodium acetate buffer (pH 6.0).

To produce GlcAcvGlc, HMWA solution (200 mL) was incubated with 25,400 U of ThMA (127 U/mL) at 55°C for 24 hr. HMWA hydrolysate was filtrated

using an ultrafiltration kit (150 mL capacity, Millipore, Bedford, MA) with YM10 membrane (MW cutoff = 10000, Millipore). The filtrate was concentrated using a SpeedVac (Savant, Holbrook, NY) and the concentrate was loaded onto a BioGel P-4 column (1.5×100 cm, 45-90 mm bead size, BioRad, Hercules, CA). The fractions with inhibition activity against ThMA were concentrated and loaded onto a BioGel P-2 column (1.5×100 cm, 45-90 mm bead size, BioRad). Then, the major fractions were applied to a CM-cellulose column (3×12 cm, 25-60 mm bead size, Sigma) that was pre-equilibrated with distilled water and desorbed with 0.1M NaCl. The GlcAcvGlc fractions were collected and concentrated using a SpeedVac. Finally, the concentrated sample was desalted using a BioGel P-2 column (1.5×100 cm). The GlcAcvGlc fractions were collected and lyophilized for future uses. About 50 mg of GlcAcvGlc was obtained from a liter of culture supernatant.

Progress Curve Determinations. All reactions were carried out using PNPG2 as a substrate in 50 mM sodium phosphate buffer (pH 7.0) at 40°C. Enzyme activities were measured continuously using Ultrospec III spectrophotometer (Pharmacia, Uppsala, Sweden). Two hundreds and fifty microliters of 20 mM PNPG2 and 50 µL of inhibitor at various concentrations were transferred into a cuvette (1 cm path-length, 1mL capacity), which was preheated for 5 min in a water jacketed turret circulated with 40°C water. Enzyme solution prewarmed at 40°C for 5 min (200 µL) was added to the preheated substrate-inhibitor mixture. Total elapsed time between enzyme addition and the initiation of data collection was less than 15 sec. Formation of *p*-nitrophenol was monitored continuously at 400 nm. The data were analyzed using the nonlinear regression program of SigmaPlot (SPSS Inc., Chicago, IL) to give the individual parameters for each progress curve: v_0 (initial velocity), v_s (steady-state velocity), and k_{obs} (apparent first-order rate constant for the transition from v_0 to v_s) according to eq 1 (*8*):

$$[P] = v_s t + (v_0 - v_s)[1 - \exp(-k_{obs}t)] / k_{obs} \tag{1}$$

where P is the p-nitrophenol formed and represented by the A_{400nm} increase and t is the reaction time. K_m values of PNPG2 for each enzyme were determined from Lineweaver-Burk plot (9).

RESULTS

Production of GlcAcvGlc. *Actinoplanes* sp. KCTC9162 produces acarbose in the culture broth when maltose or glycerol was added as a carbon source (1). On the other hand, HMWA, much longer compounds with strong inhibition activity against a-amylase, were produced in the presence of starch. They have an acarviosine core structure in which 7-30 of D-glucose units are linked at both sides (Fig. 1B). To produce large amounts of HMWA, *Actinoplanes* sp. KCTC9162 was cultured for 7 days in starch medium. The culture broth turned dark to black as the growth proceeded. Strong inhibition activity against α-amylase was found in the cell-free supernatant.

HMWA was hydrolyzed with ThMA for the production of GlcAcvGlc (Fig. 2). ThMA degraded HMWA readily to maltose and smaller compounds, including GlcAcvGlc. GlcAcvGlc was not further hydrolyzed by ThMA and accumulated in a considerable amount. Since GlcAcvGlc itself is an inhibitor for maltogenic amylases (discussed below), the hydrolysis rate of HMWA decreased as the reaction proceeded.

Time-Dependent Inhibition of MGase by Acarbose and GlcAcvGlc. To investigate the inhibitory effect of acarbose and GlcAcvGlc on maltose-producing enzymes, MGase activity was assayed with the inhibitors. MGase showed time-dependent inhibition in the presence of acarbose or GlcAcvGlc (Fig. 3, panels A and B). The reaction rate of the enzyme decreased gradually as the reaction proceeded and finally reached a steady-state velocity (v_s). While the
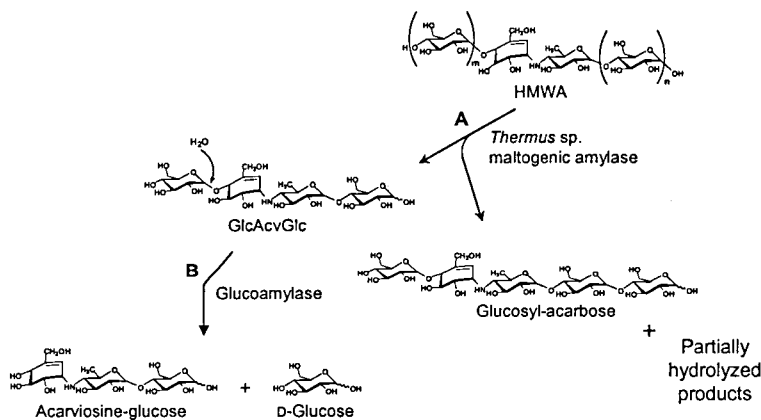
Fig. 2. A scheme for the production of GlcAcvGlc. HMWA was produced in the culture broth by *Actinoplanes* sp. KCTC9162 using starch as a carbon source. By hydrolysis of HMWA by ThMA produced GlcAcvGlc and other analogues (A). GlcAcvGlc was degraded to D-glucose and acarviosine-glucose by glucoamylase (B).

initial velocity (v_0) of the reaction was independent of the concentration of both inhibitors, v_s was reduced. As shown in Fig. 3, the progress curve was linear in the absence of the inhibitors, which indicated that there was no effect caused by substrate depletion. The progress curves obtained using various concentrations of the inhibitors were fitted to eq 1 to determine v_0, v_s, and k_{obs}. The plots for k_{obs} versus [I] are shown in panels C and D in Fig. 3. Each plot shows linear dependence on the concentration of the inhibitors, which is one of typical properties of Mechanism A involving single step of inhibition (*10*). In Mechanism A, the plot for k_{obs} versus [I] should be straight line with the following equations (*8*):

$$k_{obs} = k_4 + \frac{k_3[I]}{1+[S]/K_m} \tag{2}$$

$$K_i = \frac{k_4}{k_3} \tag{3}$$

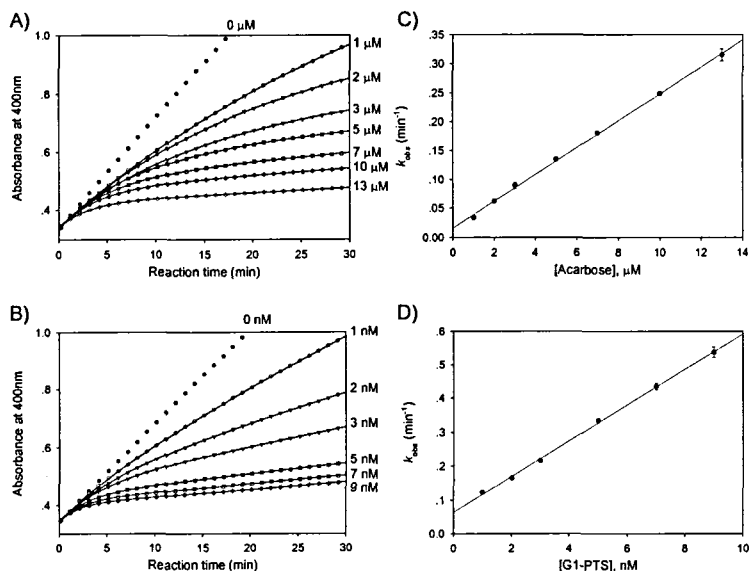where [I] and [S] are the concentration of inhibitor and substrate, respectively;

Fig. 3. Time-dependent inhibition of MGase in the presence of acarbose and GlcAcvGlc. MGase were added to the reaction mixture containing a substrate, PNPG2, and various concentrations of inhibitors. The final concentration of the enzyme was 0.5 nM. Progress curves for acarbose (panel A) and for GlcAcvGlc (panel B) were obtained continuously but only 30 data points are shown for clarity. The time-dependent loss in activity was fit to eq 1 (solid lines). The pseudo-first-order rate constants (k_{obs}) derived from the fit are plotted against the concentrations of inhibitors (panel C for acarbose and D for GlcAcvGlc). The straight lines represent the best fits of the data to eq 2, and the kinetic parameters (k_3, k_4) are derived from the fits.

k_3 and k_4, the association and dissociation rate constants of E · I complex, respectively; K_i, a dissociation constant of E · I complex; K_m, Michaelis constant. The K_m value of the substrate, PNPG2, was 7.0 mM for MGase. The results of initial velocity and plots of k_{obs} versus [I] indicated that the inhibition of MGase by acarbose or GlcAcvGlc followed Mechanism A. The kinetic parameters, k_3 and k_4, were estimated by fitting the plots of k_{obs} versus [I] to the eq 2 and then

the K_i value was calculated using the eq 3 (Table 1). The K_i value of GlcAcvGlc, 0.44 nM, was far smaller than 0.25 µM of acarbose, showing that the inhibitory activity of GlcAcvGlc towards MGase was about 560 times potent than that of acarbose. The association rate constant (k_3) of GlcAcvGlc, 147 $\mu M^{-1}min^{-1}$, was much larger than that of acarbose, 0.0647 $\mu M^{-1}min^{-1}$. The result indicated that the rate of MGase-GlcAcvGlc complex formation was faster and the binding affinity of GlcAcvGlc to MGase was better than acarbose.

Time-Dependent Inhibition of ThMA and CDase I-5 by Acarbose and GlcAcvGlc. The progress curves of ThMA and CDase I-5 were also hyperbolic representing slow-binding inhibition (Fig. 4, panels A and B; Fig. 5, panels A and B). In contrast to MGase, the initial velocity (v_0) as well as the steady-state velocity

Table 1. Kinetic parameters for the time-dependent inhibition of amylases by acarbose and GlcAcvGlc.

Enzymes	Inhibitors	Kinetic parametersa (Mechanism A)			Kinetic parametersa (Mechanism B)					Potencyb
		k_3 ($\mu M^{-1}min^{-1}$)	k_4 (min^{-1})	K_1 (nM)	k_5 (min^{-1})	k_6 (min^{-1})	k_5/k_6	K_i (mM)	K_i* (nM)	
MGase	Acarbose	0.0647± 0.0008	0.0160± 0.0014	247± 18						1
	GlcAcvGlc	147±7	0.0643± 0.0063	0.439± 0.062						563
ThMA	Acarbose				0.374± 0.010	0.0173± 0.0063	21.6	8.18± 0.60	362± 135	1
	GlcAcvGlc				3.61± 0.32	0.0333± 0.0074	108.4	0.0272± 0.0033	0.249± 0.067	1,450
CDase I-5	Acarbose				0.593± 0.038	0.0200± 0.0057	29.7	25.2± 3.5	822± 266	1
	GlcAcvGlc				3.30± 0.28	0.0688± 0.0100	48.0	0.0369± 0.0081	0.754± 0.208	1,090

a. These values were derived from k_{obs} versus [I] plots by fitting the data to eq -2 eq 5.
b. Potency was calculated by dividing K_i (or K_i*) of acarbose with K_i (or K_i*) of GlcAcvGlc.
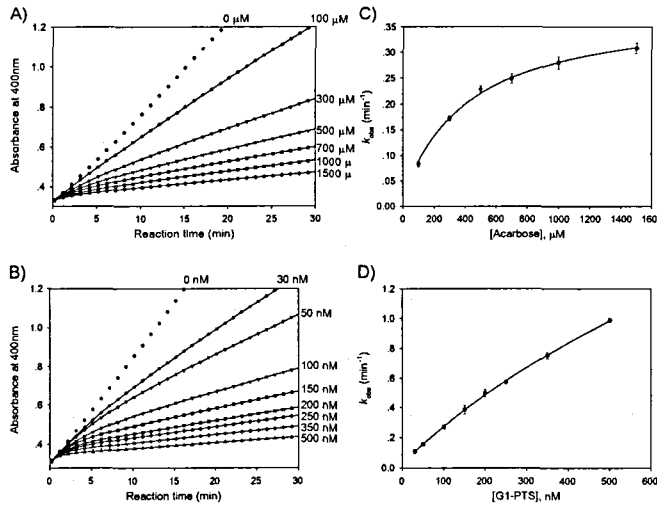
Fig. 4. Time-dependent inhibition of ThMA in the presence of acarbose and GlcAcvGlc. Each curve was obtained as described in the legend to Fig. 3. The final concentration of the enzyme was 3.4 nM. In panels C and D, the curves represent the best fits of the data to eq 4, and the kinetic parameters (K_i, k_5, k_6) are derived from the fits.
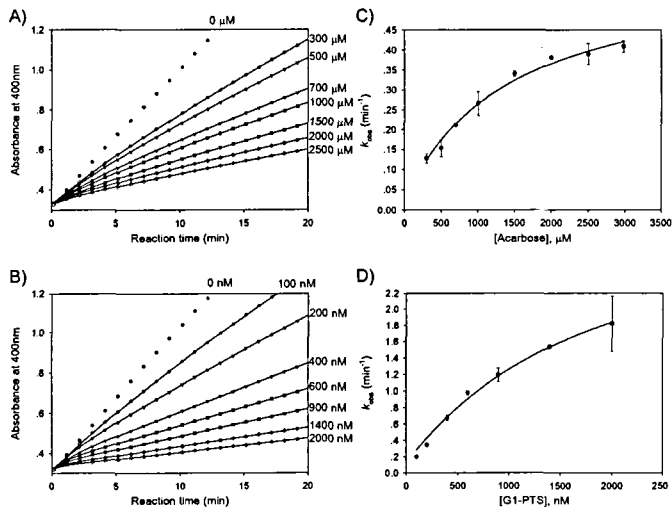

Fig. 5. Time-dependent inhibition of CDase I-5 in the presence of acarbose and GlcAcvGlc. Each curve was obtained as described in the legend to Fig. 3. The final concentration of the enzyme was 8.1 nM. In panels C and D, the curves represent the best fits of the data to eq 4, and the kinetic parameters (K_i, k_5, k_6) are derived from the fits.

(v_s) decreased as the concentration of the inhibitors increased in assays with ThMA and CDase I-5. In general, one of the different properties between Mechanism A and B is the constancy of initial velocity in the presence of slow-binding inhibitor (11). That is, $v0$ does not vary with [I] in Mechanism A, but decreases in Mechanism B. Also, the plot of $k$obs versus [I] is not linear but hyperbolic in Mechanism B and the relationship can be described as the following equations,

$$k_{obs} = k_6 + \frac{k_5[I]/K_i}{1+[S]/K_m+[I]/K_i} \tag{4}$$

$$K_i^* = K_i \frac{k_6}{k_5 + k_6} \tag{5}$$

where k_5 and k_6 are the forward and reverse isomerization rate constants, respectively; K_i^*, the overall inhibition constant. The K_m values of PNPG2 were 0.20 mM and 0.18 mM for ThMA and CDase I-5, respectively. The transition rate constants (k_{obs}) of ThMA and CDase I-5 showed a hyperbolic dependence on the concentration of the inhibitor (Fig. 4, panels C and D; Fig. 5, panels C and D), so the inhibitions of ThMA and CDase I-5 by both inhibitors followed Mechanism B. The kinetic parameters, k_5, k_6, K_i, and K_i^*, were derived from the plots by fitting the results to eqs 4 and 5, and summarized in Table 1. Both acarbose and GlcAcvGlc acted as slow-binding inhibitors for ThMA and CDase I-5. The overall inhibition constants (K_i^*) of GlcAcvGlc were far smaller than those of acarbose. K_i^* of GlcAcvGlc for ThMA and CDase I-5 was 0.25 nM and 1.3 nM, respectively, while that of acarbose was 0.36 µM and 0.82 µM, respectively. In other words, inhibitor potencies of GlcAcvGlc for ThMA and CDase I-5 were 3 orders of magnitude superior to those of acarbose. Also, forward rate constants (k_5) for the isomerization of the enzyme-GlcAcvGlc intermediate to a more stable complex is about 10 times lager than those for enzyme-acarbose complex. The result reveals that GlcAcvGlc is a more suitable

inhibitor converting the enzyme-inhibitor intermediate into the more stable form. Since the faster forward isomerization rate of the enzyme-inhibitor complex causes the shorter duration time of the complex, the steady-state concentration of E·I is decreased and the inhibition seems to follow Mechanism A (8).

Inhibition of other amylolytic enzymes by GlcAcvGlc. Table 2 shows the IC_{50} values of several amylolytic enzymes in the presence of acarbose or GlcAcvGlc. Although both maltase and sucrase from rat intestine were inhibited more efficiently by acarbose than by GlcAcvGlc, α-amylase from porcine pancreas (PPA) was more sensitive to GlcAcvGlc than acarbose. GlcAcvGlc was most effective for ThMA as expected. IC_{50} of GlcAcvGlc for ThMA, 68 nM, which was significantly less than the IC_{50} (340 nM) of acarbose, indicatied that GlcAcvGlc was a much better inhibitor of ThMA. Neither GlcAcvGlc nor acarbose was an inhibitor for sweet potato β-amylase.

Table 2. Inhibitory activities of acarbose and GlcAcvGlc against various amylolytic enzymes.

Enzymesb	IC_{50}[a] (mM)		Potency[d]
	Acarbose	GlcAcvGlc	
Maltase	0.24 ± 0.01	4.1 ± 0.2	0.058
Sucrase	0.96 ± 0.01	8.1 ± 0.2	0.12
α-Amylase	2.7 ± 0.3	1.2 ± 0.2	2.2
β-Amylase	N_c	N_c	--
ThMA	340 ± 30	0.068 ± 0.01	5,040

a. IC_{50} value represents the concentration of an inhibitor causing 50% decrease of the enzyme activity. The values are means $\pm$S.D. of three separate experiments.

b. Maltase and sucrase are from rat intestine; α-amylase, porcine pancreas; β-amylase, sweet potato; and ThMA, a maltogenic amylase from *Thermus* sp.

c. No inhibition was observed at 1 mM of the inhibitors.

d. Potency was calculated by dividing the IC_{50} of acarbose with IC_{50} of GlcAcvGlc.

DISCUSSION

In the present study, we described the production of GlcAcvGlc and its inhibition properties for maltose-producing enzymes including MGase, ThMA, CDase I-5 and some other amylolytic enzymes. Junge *et al.* (*12*) prepared acarviosine-glucose, acarviosine, and some byproducts from acarbose by acid hydrolysis, but not GlcAcvGlc by the procedure. Both GlcAcvGlc and acarbose showed time-dependent inhibitions against MGase, ThMA, and CDase I-5. The inhibition of ThMA and CDase I-5 by acarbose or GlcAcvGlc followed Mechanism B, while that of MGase followed Mechanism A in Scheme I.

GlcAcvGlc showed strong inhibitory effect especially on the maltose-producing amylases such as MGase, ThMA, and CDase I-5 (Table 1). For the three enzymes tested, K_i and K_i^* of GlcAcvGlc were in the nanomolar range, while those of acarbose were in the micromolar range, that is, the inhibition potency of GlcAcvGlc was about three orders of magnitude better than acarbose. Since ThMA readily hydrolyzes maltotetraose to two molecules of maltose rather than glucose and maltotriose (*13*), binding of maltotetraose at −2 to +2 subsite of ThMA is supposed to be thermodynamically most favorable. The binding pattern of GlcAcvGlc seems to be similar to that of maltotetraose, which is located at −2 to +2 subsite so that the acarviosine unit is positioned at −1 to +1 subsite of ThMA. CDase I-5 and MGase also produce maltose mainly from linear maltooligosaccharides (*14*), and the subsite location of the inhibitor is likely to be identical to that of ThMA. Besides acarbose inhibited ThMA with less inhibitory effect than GlcAcvGlc, it could be hydrolyzed into acarviosine-glucose and glucose by ThMA. Therefore, the acarviosine moiety of acarbose is supposed to locate at either −1 to +1 (when inhibiting) or −3 to 1 (when being hydrolyzed) subsite of ThMA (*15*). From this reason, GlcAcvGlc showed a better inhibition than acarbose for maltose-producing enzymes. GlcAcvGlc inhibited PPA more

effectively than acarbose (Table 2). This result is reasonable considering the catalytic properties of amylases. When maltotetraose specifically labeled in the reducing glucose unit was treated with PPA, 70% of maltotetraose was hydrolyzed to maltose and labeled maltose, while the first glycosidic linkage from non-reducing end was hardly cleaved (16). That is, the most favorable binding position of maltotetraose is at -2 to +2 subsite of PPA, which indicates that GlcAcvGlc can be a more potent inhibitor than acarbose. In contrast, acarbose is a better inhibitor for maltase and sucrase. This can be explained by that acarbose binds glucose-producing enzyme at -1 to +3 subsite in which the acarviosine unit of acarbose is located at -1 to +1 subsite of the enzyme. GlcAcvGlc was a poor inhibitor for β-amylase from sweet potato (Table 2). This is a somewhat unexpected result because β-amylase produces maltose from starch, implying that b-amylase might be a target enzyme of GlcAcvGlc. This result provides an indirect information that the action mechanism or substrate binding mode of β-amylase may be different from the mechanisms for other amylolytic enzymes. Actually, the primary structure of b-amylase is quite different among them (17).

REFERENCES

1. Schmidt, D. D., Frommer, W., Junge, B., Müller, L., Wingender, W., and Truscheit, E. (1981) in *First International Symposium on Acarbose* (Creutzfeldt, E. W., Ed.) pp 5-15, Excerpta Medica, Amsterdam.

2. Aleshin, A. E., Firsov, L. M., and Honzatko, R. B. (1994) *J. Biol. Chem. 269*, 15631-15639.

3. Kim, M. J., Lee, S. B., Lee, H. S., Lee, S. Y., Baek, J. S., Kim, D., Moon, T. W., Robyt, J. F., and Park, K. H. (1999) *Arch. Biochem. Biophys. 371*, 277-283.

4. Park, K. H., Kim, M. J., Lee, H. S., Han, N. S., Kim, D., and Robyt, J. F.

(1998) *Carbohydr. Res. 313*, 235-246.

5. Bischoff, H. (1995) *Clin. Invest. Med. 18*, 303-311.

6. Hanozet, G., Pircher, H.-P., Vanni, P., Oesch, B., and Semenza, G. (1981) *J. Biol. Chem. 256*, 3703-3711.

7. Lohse, A., Hardlei, T., Jensen, A., Plesner, I. W., and Bols, M. (2000) *Biochem. J. 349*, 211-215.

8. Morrison, J. F., and Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201-301.

9. Segel, I. H. (1975) in *Enzyme Kinetics*, pp. 46-49, John Wiley & Sons, New York.

10. Duggleby, R. G., Attwood, P. V., Wallace, J. C., and Keech, D. B. (1982) *Biochemistry 21*, 3364-3370.

11. Copeland, R. A. (1996) in *Enzymes*, pp 237-261, Wiley-VCH, New York.

12. Junge, B., Boshagen, H., Stoltefub, J., and Müller, L. (1980) in *Enzyme Inhibitors* (Brodbeck, U., Ed.) pp 123-137, Verlag Chemie, Weinheim.

13. Kim, T. J., Kim, M. J., Kim, B. C., Kim, J. C., Cheong, T. K., Kim, J. W., and Park, K. H. (1999) *Appl. Environ. Microbiol. 65*, 1644-1651.

14. Kim, M. J., Park, W. S., Lee, H. S., Kim, T. J., Shin, J. H., Yoo, S. H., Cheong, T. K., Ryu, S., Kim, J. C., Kim, J. W., Moon, T. H., Robyt, J. F., and Park, K. H. (1999) *Arch. Biochem. Biophys. 373*, 110-115.

15. Kim, T. J., Park, C. S., Cho, H. Y., Cha, S. S., Kim, J. S., Lee, S. B., Moon, T. W., Kim, J. W., Oh, B. H., and Park, K. H. (2000) *Biochemistry 39*, 6773-6780.

16. Robyt, J. F., and French, D. (1970) *J. Biol. Chem. 245*, 3917-3927.

17. Mikami, B., Hehre, E. J., Sachettini, J. C., and Morita, Y. (1995) in *Enzyme Chemistry and Molecular Biology of Amylases and Related Enzymes* (Yamamoto, T., Ed.) pp 145-153, CRC Press, Boca Raton.