Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 338 (2003) 1969–1980
CARBOHYDRATE RESEARCH
www.elsevier.com/locate/carres

Study of the inhibition of four alpha amylases by acarbose and its 4^{IV}-α-maltohexaosyl and 4^{IV}-α-maltododecaosyl analogues

Seung-Heon Yoon, John F. Robyt*

Laboratory of Carbohydrate Chemistry and Enzymology, Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA

Received 28 January 2003; accepted 12 June 2003

Abstract

Acarbose analogues, 4^{IV}-maltohexaosyl acarbose (G_6-Aca) and 4^{IV}-maltododecaosyl acarbose (G_{12}-Aca), were prepared by the reaction of cyclomaltodextrin glucanyltransferase with cyclomaltohexaose and acarbose. The inhibition kinetics of acarbose and the two acarbose analogues were studied for four different α-amylases: *Aspergillus oryzae*, *Bacillus amyloliquefaciens*, human salivary, and porcine pancreatic α-amylases. The three inhibitors showed mixed, noncompetitive inhibition, for all four α-amylases. The acarbose inhibition constants, K_i, for the four α-amylases were 270, 13, 1.27, and 0.80 μM, respectively; the K_i values for G_6-Aca were 33, 37, 14, and 7 nM, respectively; and the G_{12}-Aca K_i constants were 59, 81, 18, and 11 nM, respectively. The G_6-Aca and G_{12}-Aca analogues are the most potent α-amylase inhibitors observed, with K_i values one to three orders of magnitude more potent than acarbose, which itself was one to three orders of magnitude more potent than other known α-amylase inhibitors.

Keywords: Enzyme inhibitors; Acarbose; Maltohexaosyl acarbose; Maltododecaosyl acarbose; *Aspergillus oryzae* α-amylase; *Bacillus amyloliquefaciens* α-amylase; Human salivary α-amylase; Porcine pancreatic α-amylase

1. Introduction

α-amylase (EC 3.2.1.1) is an endo-acting enzyme that catalyzes the hydrolysis of the (1→4)-α-D-glycosidic linkages of starch, amylose, amylopectin, glycogen, and various maltodextrins. α-amylases are produced by a diverse variety of organisms: bacteria, fungi, plants, and animals.[1] Two kinds of α-amylases are produced by many mammals, salivary α-amylase from the parotid gland and pancreatic α-amylase from the pancreas. The digestion of food starch begins with salivary α-amylase in the mouth. This digestion is stopped by the low pH of the stomach. When the food bolus passes from the stomach into the small intestine it is neutralized and the digestion of the starch is completed by an α-amylase secreted into the small intestine from the pancreas. α-amylases from other sources, such as bacteria and fungi, are secreted into their environment where they encounter starch that is hydrolyzed to maltodextrins and transported into the cells to be further converted to D-glucose and metabolized. Plants produce α-amylases to degrade the starch that they had formed from photosynthesis for their energy needs. Because starch represents the major storage form of the energy from the sun obtained in photosynthesis, starch digestion by α-amylases is a very important process in the utilization of the sun's energy by nonphotosynthesizing organisms, hence the wide distribution of α-amylase in nature.[2]

α-amylases also have important industrial uses in the modification of starch in food materials, in the industrial production of maltodextrins, and in the modification of starch for sizing paper and textiles.[3] They are of interest in the clinical analysis of some diseases, such as pancreatitis.[4] Inhibitors of α-amylases have applications in modifying and controlling α-amylase action that have medical applications, such as the influence on blood

* Corresponding author. Tel.: +1-515-294-1964; fax: +1-515-294-0453.
E-mail address: jrobyt@iastate.edu (J.F. Robyt).

0008-6215/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0008-6215(03)00293-3

glucose, serum insulin, and starch loading tests in animals and man.[5] Various α-amylase inhibitors have previously been reported.[1]

Acarbose is a natural product produced by *Actinoplanes* sp. fermentation.[6] It is a pseudotetrasaccharide with an unsaturated cyclitol [2,3,4-trihydroxy-5-(hydroxymethyl)-5,6-cyclohexene in a D-*gluco* configuration] attached to the nitrogen of 4-amino-4,6-dideoxy-D-glucopyranose, which is linked α-(1→4) to maltose (see Fig. 5(E)). Acarbose is a strong competitive inhibitor of α-glucosidase,[7–9] α-amylase,[7–10] cyclomaltodextrin glucanyltransferase (CGTase),[9,11,12] glucoamylase,[13,14] and glucansucrases.[15,16] The mechanism of inhibition for these enzymes has been postulated to be due to the unsaturated cyclohexene ring and the glycosidic nitrogen linkage that mimics the transition state for the enzymatic cleavage of glycosidic linkages.[6,17]

Some acarbose analogues that have maltodextrin residues attached to the reducing-end and/or to the nonreducing-end of acarbose were produced by *Actinoplanes* sp. fermentation. These acarbose analogues had inhibitory activity against α-amylase and sucrase that was altered by the number of D-glucose units in the maltodextrins at the two ends.[7] Various other kinds of acarbose analogues have been obtained by the modification of the maltose unit at the reducing-end, using the transglycosylation reaction of *Bacillus stearothermophilus* maltogenic amylase (BSMA) with acarbose and several carbohydrate acceptors.[8,18,19] It was found that the removal of one D-glucose residue from the reducing-end of acarbose, to give acarviosine-glucose, inhibited yeast α-glucosidase 430-times better than acarbose. It was also found that the replacement of the maltose unit by isomaltose gave an inhibitor that inhibited porcine pancreatic α-amylase (PPA) 15.2-times better than acarbose. Lee and co-workers[19] reported that when the maltose unit of acarbose was replaced by cellobiose and lactose, the acarbose analogues were potent inhibitors for β-glucosidase and β-galactosidase, while acarbose was not an inhibitor at all.

In a recent paper, the authors[20] reported the synthesis of acarbose analogues with maltohexaose (G_6), maltododecaose (G_{12}), and maltooctadecaose (G_{18}) attached to the C-4-hydroxyl group of the nonreducing-end of acarbose by the reaction of CGTase with cyclomaltohexaose and acarbose. In the present study, we report the inhibition of four α-amylases from different origins, bacterial α-amylase from *Bacillus amyloliquefaciens*, mammalian α-amylase from porcine pancreas, human α-amylase from saliva, and fungal α-amylase from *Aspergillus oryzae,* by acarbose (Aca), 4^{IV}-α-maltohexaosyl acarbose (G_6-Aca) and 4^{IV}-α-maltododecaosyl acarbose (G_{12}-Aca).

2. Experimental

2.1. Materials

Acarbose was a gift from Dr K.-H. Park (Department of Food Science and Technology, Seoul National University, Suwon, Korea). Soluble amylose was precipitated by the addition of 1-butanol to a solution of potato starch that first had the amylose fraction removed by retrogradation.

A. oryzae α-amylase (AOA) [EC 3.2.1.1] was purchased from Sigma (St. Louis, MO). Porcine pancreatic α-amylase (PPA) and *B. amyloliquefaciens* α-amylase (BAA) were obtained from Boehringer Mannheim (Indianapolis, IN). Crystalline human salivary α-amylase (HSA) was prepared in our laboratory by the method of Fisher and Stein.[21]

B. macerans CGTase [EC 2.4.1.19] was obtained from Amano International Enzyme Co. (Troy, VA, USA). It was extensively dialyzed against 25 mM imidazole–HCl buffer (pH 6.0) to remove glucose, low-molecular weight saccharides, and other unwanted colored material. Cyclomaltohexaose was prepared in our laboratory, using *B. macerans* CGTase reaction with starch. All other chemicals were of reagent grade.

2.2. Preparation of acarbose analogues

B. macerans CGTase [EC 2.4.1.19] (15 IU) was added to 2.0 mL, containing 50 mM acarbose and 50 mM cyclomaltohexaose in 25 mM imidazolium–HCl buffer (pH 6.0). The enzyme reaction was carried out at 35 °C for 6 days with the periodic, stepwise addition of 1.0 mL of 100 mM cyclomaltohexaose solution every 1–2 days. After stopping the enzyme reaction by heating in boiling water for 5 min, insoluble matter was removed by centrifugation at 4000 rpm for 10 min, and then the supernatant was concentrated to 1.2 mL by rotary vacuum evaporation. The major reaction products, G_6-Aca, G_{12}-Aca and G_{18}-Aca, were purified by Bio-Gel P2 column (1.5 × 100 cm) chromatography (flow rate 0.06 mL/min, fraction size 1.0 mL).[20]

2.3. α-amylase reactions in the presence and absence of inhibitors

Soluble amylose (0.05–1.0% w/v) in 20 mM pyridinium acetate buffer (pH 5.5) containing 1.0 mM of $CaCl_2$ was used as substrate solution for AOA reaction, and the same concentration of soluble amylose in 25 mM imidazolium–HCl buffer (pH 6.5) containing 1.0 mM $CaCl_2$ was used as substrate solution for BAA, HSA and PPA reaction. The substrate solution (1080 μL), containing various concentrations of inhibitors (acarbose, G_6-Aca and G_{12}-Aca) was preincubated at 37 °C for 10 min. The α-amylase reactions were started by adding

120 μL of AOA (54 mU/mL), BAA (44 mU/mL), HSA (30 mU/mL) and PPA (80 mU/mL) enzyme solutions to the substrate/inhibitor solutions. One unit (U) of α-amylase was defined as the hydrolysis of 1 μmol of α-(1→4) glycosidic linkages per minute at a specified pH and temperature. A sample of 150 μL was taken every 5 min, and the reaction was stopped by adding 300 μL of 0.05 M NaOH, giving pH 12.5. The reducing value was measured by micro copper–bicinchoninate method,[22,23] using maltose as a standard.

2.4. Kinetics of enzyme inhibition

From the above α-amylase reactions, the initial velocities (v_i) were determined from the slope of the linear part of the curves of the amount of product, in terms of μg of maltose equivalents, versus time in minutes. Dixon plots[24] of $1/v_i$ versus the concentration of the inhibitor for five concentrations of soluble amylose and five concentrations of inhibitor were used to determine the type of inhibition and the inhibition constants, K_i and K_i'.

2.5. Possible reaction of the α-amylases with the inhibitors

Each of the α-amylases (1.0 IU/mL) was incubated with 1.0 mg/mL of inhibitor at 37 °C and the optimum pH of the individual α-amylase. Aliquots (0.1 mL) were taken at 0.5, 1, 2, and 3 h and the reaction stopped by heating in a boiling bath for 5 min; 5 μL of each reaction was added to a 20 × 20 cm Whatman K5 TLC plate (Fisher Scientific, Chicago, IL). The plate was irrigated 3-times with 85:20:50:70 MeCN–EtOAc–1-propanol–water, with an 18-cm irrigation path length. The carbohydrates were visualized by dipping the plate into a MeOH solution, containing 0.3% (w/v) *N*-(1-naphthyl) ethylenediamine and 5% (v/v) H_2SO_4, followed by heating at 120 °C for 10 min. The carbohydrates were quantitated on the plate, using a scanning densitometer, model GS-670 (Bio-Rad Laboratories, Hercules, CA).[25,26]

3. Results

3.1. Determination of the type of inhibition of G_6-Aca and G_{12}-Aca

The type of inhibition of acarbose, G_6-, and G_{12}-acarbose with four α-amylases was determined from the Lineweaver–Burk plots of $1/v_i$ versus 1/[S] (data not shown) and from Dixon plots of $1/v_i$ versus [I] for five substrate concentrations (Figs. 1–4). The type of inhibition was mixed noncompetitive in which both EI and ESI complexes were formed. The kinetic parameters, V_m, K_m, K_i, and K_i' were determined from the following equation, the Michaelis–Menten equation for mixed noncompetitive inhibition, using nonlinear regression analysis:[27,28]

$$v_i = \frac{V_m[S]}{K_m(1 + ([I]/K_i)) + [S](1 + ([I]/K_i'))}$$

where, V_i is the initial velocity in the absence and presence of the inhibitor; [S] and [I] are respectively, the concentrations of substrate and inhibitor; V_m is the maximum velocity; K_m is the Michaelis–Menten constant; K_i is the inhibition constant, indicating the dissociation of the enzyme–inhibitor complex (EI); and K_i' is the inhibition constant when the inhibitor binds to an enzyme–substrate complex, indicating the dissociation constant of the enzyme–substrate–inhibitor complex (ESI).[8,28] All of the data were obtained from triplicate or more determinations. The inhibition constants, K_i for the dissociation of the EI complex and K_i' for the dissociation of the ESI complex for each of the α-amylases and inhibitors are given in Table 1.

The inhibition potency, which represents the relative enzyme inhibition of an inhibitor to acarbose, was calculated from the ratio of the inhibition constant (K_i) of acarbose to the inhibition constants (K_i) of the other inhibitors for each α-amylase. The relative potency of the inhibitors for the four kinds of α-amylases is given in Table 1.

3.2. Susceptibility of α-amylases to inhibition by acarbose, G_6-Aca, and G_{12}-Aca

The susceptibility of the α-amylases to inhibition by acarbose varied with the origin of the enzyme. For example, AOA, fungal α-amylase, was inhibited the least of the four α-amylases, and PPA, mammalian α-amylase, was inhibited the most of the four α-amylases. The ratio of the inhibition constants for acarbose showed that PPA was inhibited 338-times better than AOA. G_6-Aca was the best inhibitor for all four of the α-amylases and G_{12}-Aca was a better inhibitor for all four of the α-amylases than was acarbose (Table 1). G_6-Aca had a K_i of 7 nM for PPA, indicating that it is the most potent α-amylase inhibitor known.

3.3. Inhibition of Aspergillus oryzae α-amylase

Fig. 1 shows the Dixon plots for AOA by acarbose, G_6-Aca, and G_{12}-Aca. AOA was weakly inhibited by acarbose with a K_i of 270 μM. G_6-Aca and G_{12}-Aca were much stronger inhibitors with K_i values of 33 and 59 nM, respectively. G_6-Aca inhibited AOA 8182-times better than acarbose and G_{12}-Aca inhibited AOA 4576-times better than acarbose.

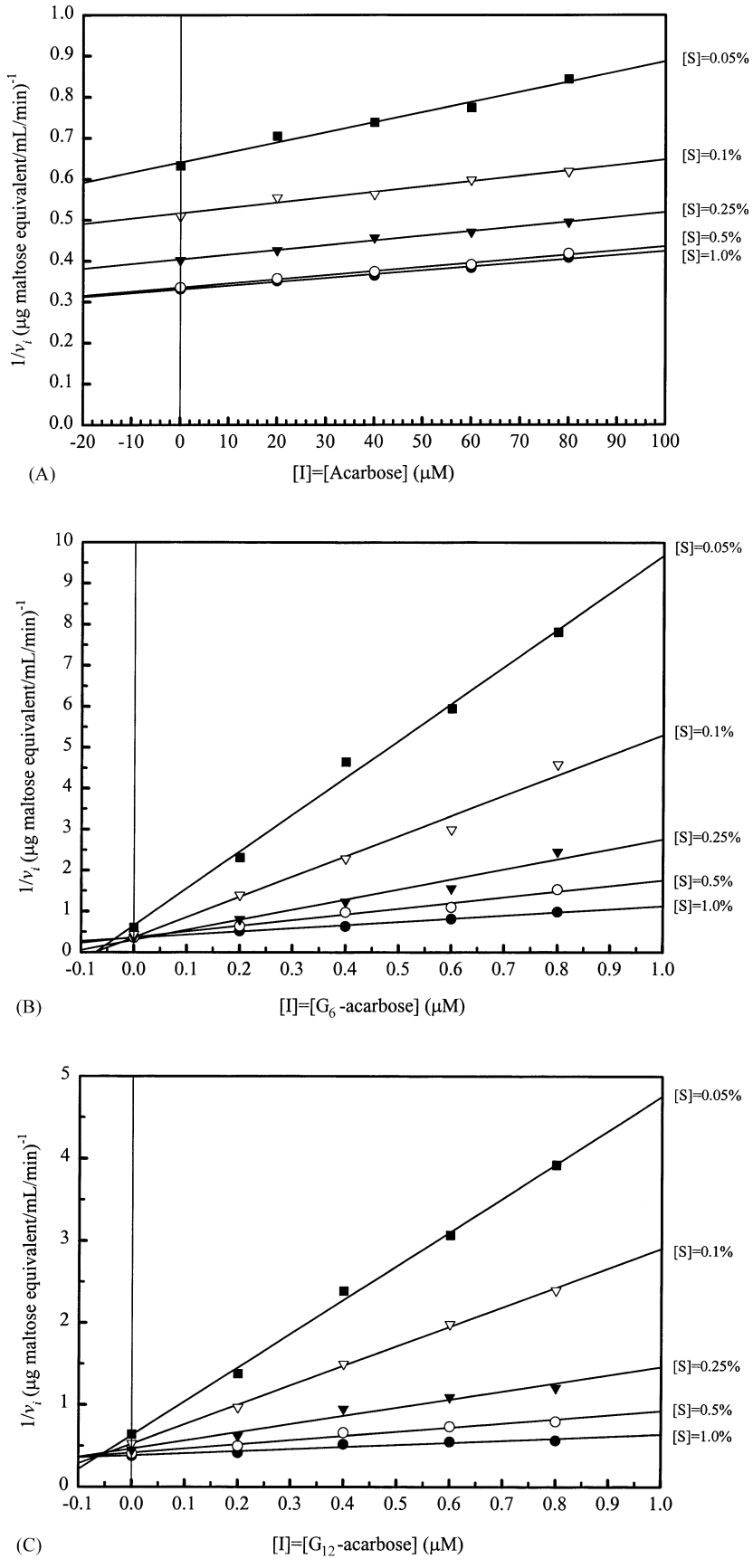


Fig. 1. Dixon plots of the reaction of AOA with different concentrations of soluble amylose [S] in the presence of (A) acarbose; (B) 4^{IV}-α-maltohexaosyl acarbose; and (C) 4^{IV}-α-maltododecaosyl acarbose.

Table 1
Inhibition constants of acarbose, maltohexaosyl acarbose (G_6-acarbose) and maltododecaosyl acarbose (G_{12}-acarbose) for AOA, BAA, HSA and PPA

α-amylase	Inhibitors	Type of inhibition	K_i (μM) [a]	K_i' (μM) [a]	Inhibition potency [b]
AOA	Acarbose	Mixed	270 ± 39	376 ± 70	1
	G_6-acarbose	Mixed	0.033 ± 0.003	0.610 ± 0.130	8182
	G_{12}-acarbose	Mixed	0.059 ± 0.020	2.94 ± 0.84	4576
BAA	Acarbose	Mixed	13.00 ± 3.66	30.69 ± 2.59	1
	G_6-acarbose	Mixed	0.037 ± 0.007	0.095 ± 0.004	351
	G_{12}-acarbose	Mixed	0.081 ± 0.026	0.188 ± 0.004	160
HSA	Acarbose	Mixed	1.265 ± 0.589	2.711 ± 0.101	1
	G_6-acarbose	Mixed	0.014 ± 0.002	0.431 ± 0.023	90
	G_{12}-acarbose	Mixed	0.018 ± 0.005	0.481 ± 0.034	70
PPA	Acarbose	Mixed	0.797 ± 0.156	0.866 ± 0.030	1
	G_6-acarbose	Mixed	0.007 ± 0.002	1.75 ± 0.49	114
	G_{12}-acarbose	Mixed	0.011 ± 0.003	1.86 ± 0.51	72

[a] K_i is the inhibition constant, defined as [E][I]/[EI]; K_i' is the inhibition constant, defined as [ES][I]/[ESI].[8,28]
[b] Inhibition potency was obtained by dividing the K_i of acarbose by the K_i of G_6-acarbose or G_{12}-acarbose.[8,19]

3.4. Inhibition of Bacillus amylolique faciens α-amylase

Fig. 2 shows the Dixon plots for BAA by acarbose, G_6-Aca, and G_{12}-Aca. The K_i inhibition constants for acarbose, G_6-Aca, and G_{12}-Aca are 13 μM, 37 nM, and 81 nM, respectively (Table 1). Acarbose was 21-times better inhibitor for BAA than it was for AOA. G_6-Aca and G_{12}-Aca inhibited BAA 351- and 160-times better than acarbose.

3.5. Inhibition of human salivary α-amylase

Fig. 3 shows the Dixon plots for HSA by acarbose, G_6-Aca, and G_{12}-Aca. The K_i inhibition constants for acarbose, G_6-Aca, and G_{12}-Aca were 1.27 μM, 14 nM, and 18 nM, respectively (Table 1). Acarbose was 213-times better inhibitor for HSA than it was for AOA and 10.2-times better inhibitor for HSA than for BAA. G_6-Aca and G_{12}-Aca inhibited HSA 90- and 70-times better than acarbose.

3.6. Inhibition of porcine pancreatic α-amylase

Fig. 4 shows the Dixon plots for PPA by acarbose, G_6-Aca, and G_{12}-Aca. The K_i inhibition constants for acarbose, G_6-Aca, and G_{12}-Aca were 0.797 μM, 7 nM, and 11 nM, respectively (Table 1). Acarbose was 338-times better inhibitor for PPA than it was for AOA, 16-times better inhibitor for PPA than it was for BAA, and 1.6-times better inhibitor for PPA than it was for HSA. G_6-Aca and G_{12}-Aca inhibited PPA 114- and 72-times, respectively, better than acarbose.

3.7. Reaction of the α-amylases with the inhibitors

Because both of the inhibitors, G_6-Aca and G_{12}-Aca, have a significant length of maltodextrins attached to acarbose, there was the possibility that the α-amylases might hydrolyze the maltodextrin chains, giving degradation of the inhibitors and the possibility of decreasing the effectiveness of the inhibition. Fig. 6 shows the TLC analysis of the four α-amylases reacting with the two acarbose analogue inhibitors from 0.5 to 3 h. Three of the amylases: PPA, HSA, and BAA (Fig. 6(A–C)) showed no degradation of either of the inhibitors over the 3-h reaction period. The reaction of AOA with G_6-Aca (Fig. 6(D)) gave 0.2% hydrolysis after 0.5 h and 12.8% hydrolysis after 3 h of reaction. The reaction of AOA with G_{12}-Aca gave 0.9% hydrolysis after 0.5 h of reaction and 51.3% after 3 h of reaction. Table 2 gives the percent of degradation of the inhibitors by AOA over the 3 h reaction period.

4. Discussion

In this study, we have found that acarbose inhibits α-amylases from different sources to different degrees. We have also found that two analogues of acarbose, α-4^{IV}-maltohexaosyl acarbose (G_6-Aca) and α-4^{IV}-maltododecaosyl acarbose (G_{12}-Aca), inhibit the α-amylases to different degrees, but with a potency that is 70–8200-times that of acarbose, depending on the α-amylase.

BAA, PPA, HSA, and AOA, are each known to have a different number of D-glucose-binding subsites at their active sites. From the study of the action pattern of BAA acting on amylose, amylopectin, glycogen and maltodextrins, Robyt and French[29] were the first to

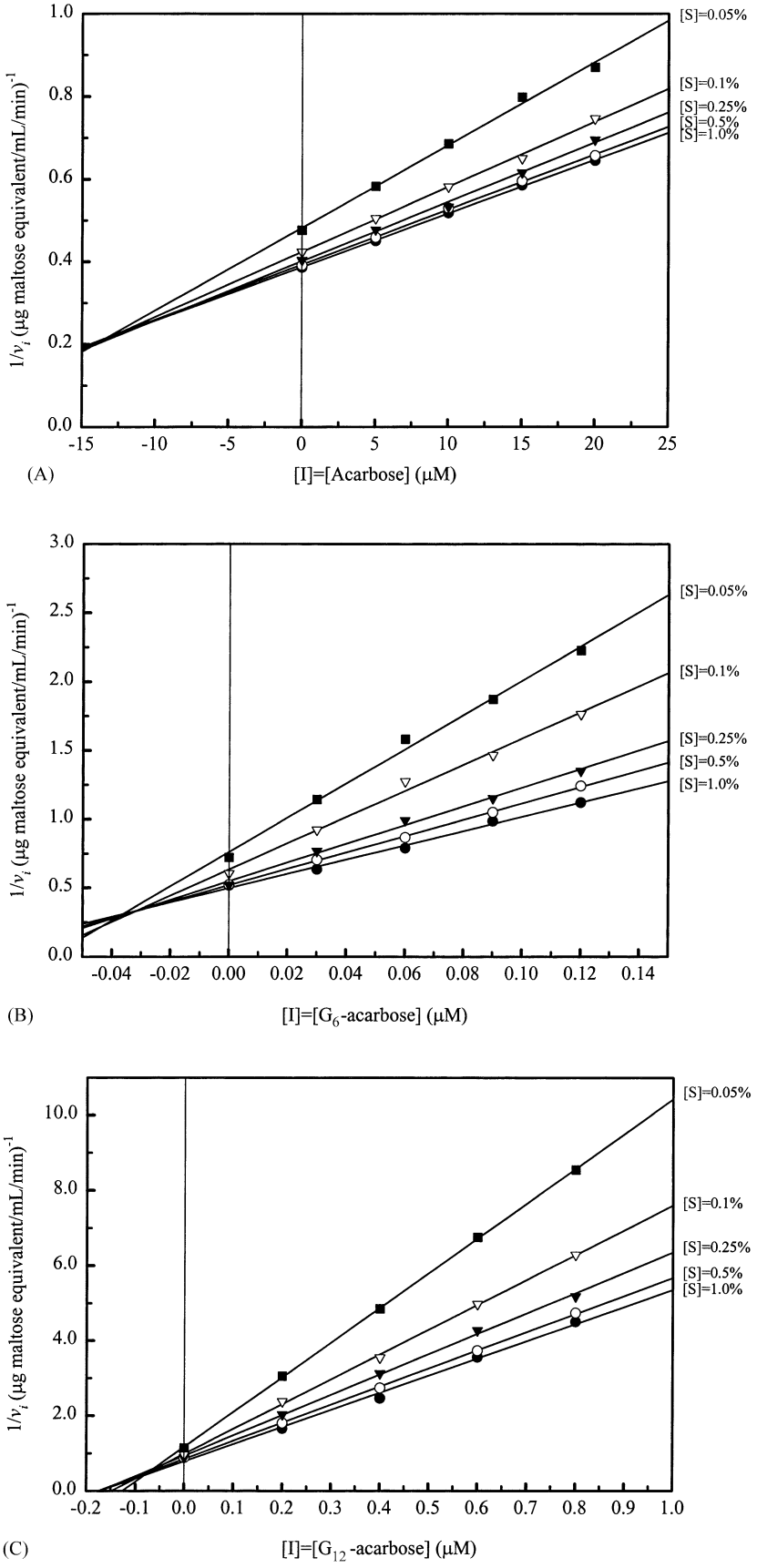


Fig. 2. Dixon plots of the reaction of BAA with different concentrations of soluble amylose [S] in the presence of (A) acarbose; (B) 4^{IV}-α-maltohexaosyl acarbose; and (C) 4^{IV}-α-maltododecaosyl acarbose.

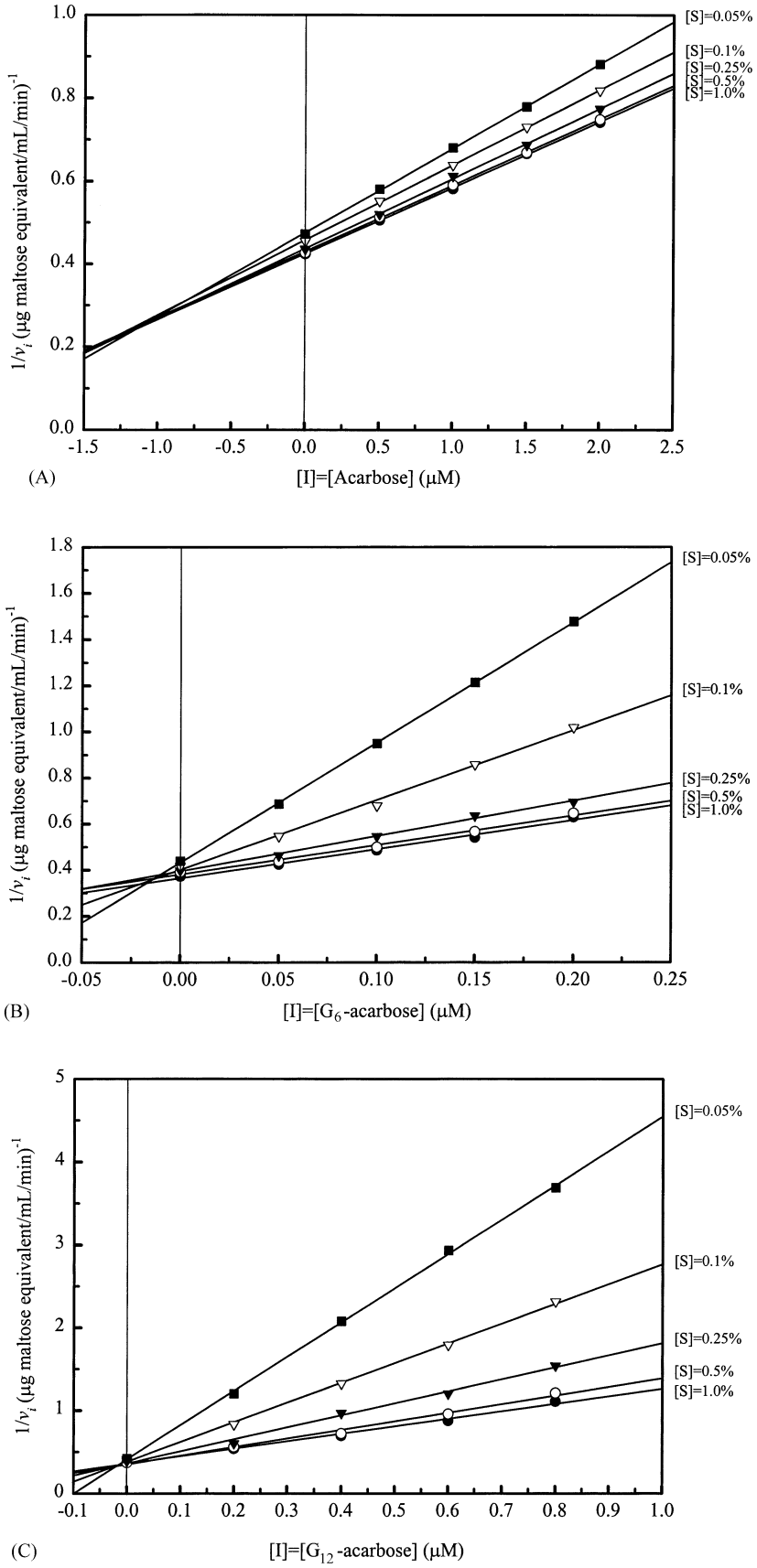
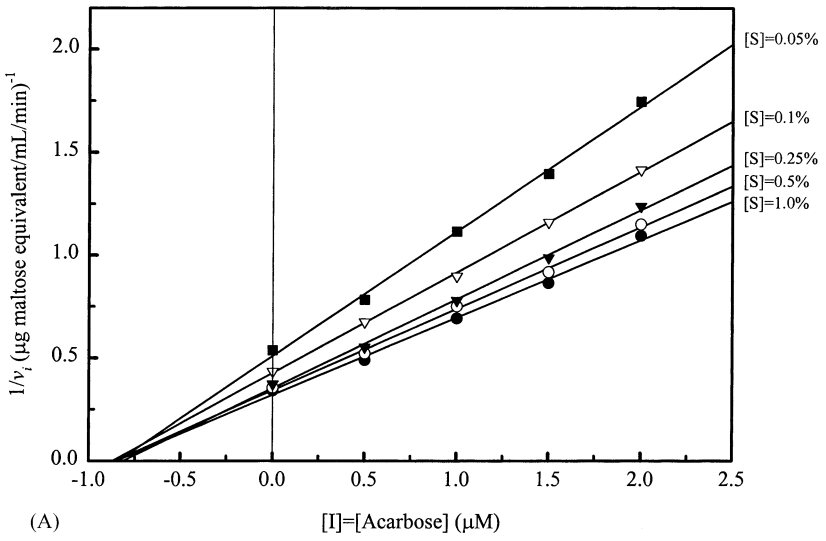
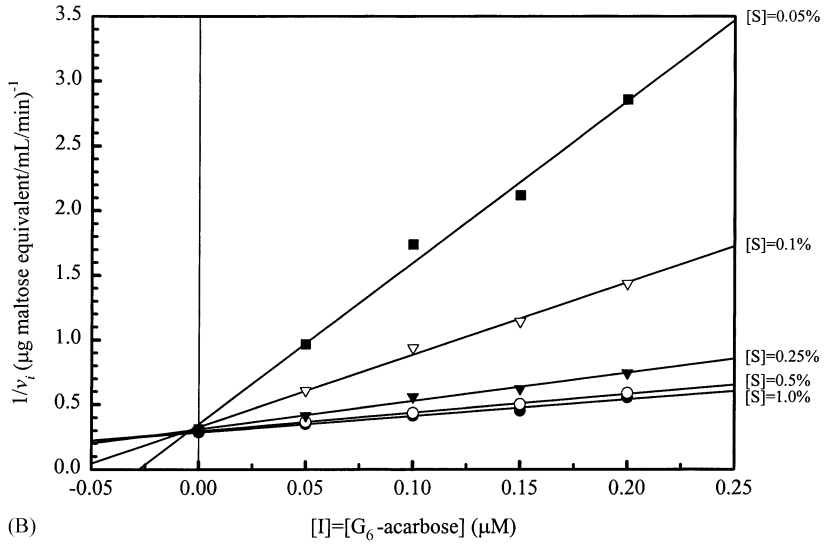


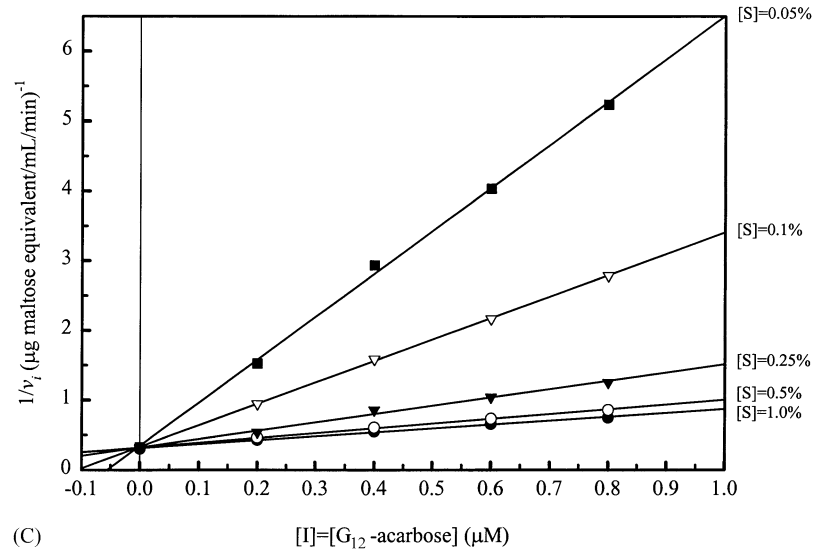
Fig. 3. Dixon plots of the reaction of HSA with different concentrations of soluble amylose [S] in the presence of (A) acarbose; (B) 4^{IV}-α-maltohexaosyl acarbose; and (C) 4^{IV}-α-maltododecaosyl acarbose.



(A)



(B)



(C)

Fig. 4. Dixon plots of the reaction of PPA with different concentrations of soluble amylose [S] in the presence of (A) acarbose; (B) 4^{IV}-α-maltohexaosyl acarbose; and (C) 4^{IV}-α-maltododecaosyl acarbose.

Table 2
Percent hydrolysis of G_6-Aca and G_{12}-Aca by *A. oryzae* α-amylase at 37 °C as a function of reaction time

Reaction time (h)	G_6-Aca + AOA		G_{12}-Aca + AOA	
	Density [a]	Percent	Density [a]	Percent
0.0	16.50	0.0	10.76	0.0
0.5	16.46	0.2	10.66	0.9
1.0	16.25	1.5	9.85	8.5
2.0	16.07	2.6	8.07	25.0
3.0	14.38	12.8	5.35	50.3

[a] Density measured by quantitative TLC, using a scanning densitometer.

postulate a D-glucose-binding subsite to explain the kinds and amounts of maltodextrin products produced. Nine subsites, with the catalytic groups located between subsites III and IV were postulated for BAA (see Fig. 5(D)). This relatively large D-glucose-binding subsite was confirmed by Thoma and co-workers[30,31] using a kinetic study with maltodextrins and the determination of the unitary free energy of D-glucose binding by each subsite. More recently, X-ray crystallographic study of a chimeric α-amylase, formed by using recombinant techniques from the genes of *B. amyloliquefaciens* and *B. licheniformis*,[32] confirmed that the chimeric α-amylase had a large, D-glucose-binding subsite of 10 D-glucose units.

From action pattern studies of PPA, Robyt and French[33] postulated that PPA has five D-glucose-binding subsites with the catalytic groups located between subsites II and III (see Fig. 5(A)). This was confirmed by Seigner and co-workers[34] by determining the energy of binding of the D-glucose residues at each subsite and by X-ray crystallographic studies, using acarbose as a substrate analogue.[35]

From action pattern studies of HSA with maltodextrins, having 2-chloro-4-nitrophenyl group at the reducing-end and 4,6-*O*-benzylidene at the nonreducing-end, Kandra and Gyémát[36] postulated that HSA had six D-glucose-binding subsites, with the catalytic groups located between subsites II and III (see Fig. 6(B)). Using phenyl α-maltodextrins modified at the nonreducing-end with iodo or 2-pyridylamino groups, Nagamine and coworkers[37] suggested that HSA has at least seven D-glucose-binding subsites. An X-ray crystallographic study of HSA indicated at least five D-glucose-binding subsites with the catalytic groups located between subsites II and III.[38]

From a kinetic study of AOA reacting with maltodextrins and the determination of the energy of binding of D-glucose to each subsite, Suganuma and coworkers[39] postulated seven D-glucose-binding subsites with the catalytic groups located between subsites III and IV. This was confirmed by X-ray crystallography.[40,41]

The X-ray crystallographic analysis of PPA in the presence of acarbose[35] and of AOA in the presence of acarbose[41] showed that the two units (acarviosine) at the nonreducing-end of acarbose, 5-hydroxymethyl-cyclohexenitol and 4-amino-4,6-dideoxy-D-glucose, are bound at subsites III and II, respectively for PPA and at subsites IV and III, respectively for AOA to produce inhibition. The two units of acarbose (acarviosine) act as a transition-state analogue for the hydrolysis of the glycosidic linkage in starch and thereby produce inhibition.[6,17] On the basis of the similarity of the reactions and the similarity in the structural features of the active sites[42–44] of various carbohydrases and related enzymes, it would be expected that the acarviosine unit of acarbose inhibits the α-amylases by binding with the two D-glucose-binding subsites that are on either side of the catalytic groups (see Fig. 5 for the binding of the acarviosine group with the active sites of the four α-amylases studied).

The binding of carbohydrate units at the active sites of α-amylases is dependent on the number of subsites and their relative affinity for binding D-glucose and related analogues, such as the 5-hydroxymethyl-cyclohexenitol and 4-amino-4,6-dideoxy-D-glucose units of acarviosine. Acarbose is an inhibitor that binds to the active sites of all four of the α-amylases studied. It inhibited PPA the best with a K_i of 0.797 ± 0.156 μM and inhibited AOA the poorest with a K_i of 270 ± 39 μM. The inhibition of HSA and BAA by acarbose was intermediate with K_i values of 1.265 ± 0.589 and 13 ± 3.7 μM, respectively.

The addition of an α-maltohexaosyl unit to the C-4 position at the nonreducing-end of acarbose, to give G_6-Aca, greatly increased the potency of the inhibition of all four of the α-amylases studied. G_6-Aca has a K_i of 7 ± 2 nM for PPA, a 72-fold increase over acarbose; G_6-Aca has a K_i of 14 ± 2 nM for HSA, a 90-fold increase over acarbose; G_6-Aca has a K_i of 33 ± 3 nM for AOA, a 8182-fold increase over acarbose; and G_6-Aca has a K_i of 37 ± 7 nM for BAA, a 351-fold increase over acarbose. Although G_6-Aca was a very potent inhibitor for PPA, the most dramatic increases in the inhibition over acarbose were for AOA and BAA. The proposed complexes of G_6-Aca with the active sites of the four α-amylases are given in Fig. 5. G_{12}-Aca was slightly less effective inhibitor for the four α-amylases than was G_6-Aca, but it was still over two orders of magnitude better than acarbose.

The study clearly shows that the attachment of maltodextrins to the nonreducing-end of acarbose greatly increased the affinity of the analogues for the active sites of the α-amylases and gave acarbose-analogue inhibitors that are much more potent than acarbose. The maltodextrin chains apparently help to

position and anchor the acarviosine unit at the subsites where the catalytic groups are located. As the number of subsites increased, however, G_6-Aca became less effective as an inhibitor, but still considerably more effective than acarbose. In fact, the inhibition of AOA increased 8200-fold with the addition of the maltohexaose unit to acarbose. Although the addition of a maltodextrin chain with twice as many D-glucose residues as maltohexaose, G_{12}-Aca, was less effective as an inhibitor than was G_6-Aca, it still was much more effective than acarbose. In contrast to acarbose, G_6-Aca and G_{12}-Aca gave the same order of magnitude (in the nM range) of inhibition, even when the α-amylases from the different sources had different numbers of D-glucose-binding subsites at their active sites.

Because G_6-Aca and G_{12}-Aca have relatively long maltodextrin chains attached to the pseudotetrasaccharide, acarbose, it might be expected that the α-amylases would hydrolyze the maltodextrin chains, decreasing their size. We, therefore, studied this possibility by

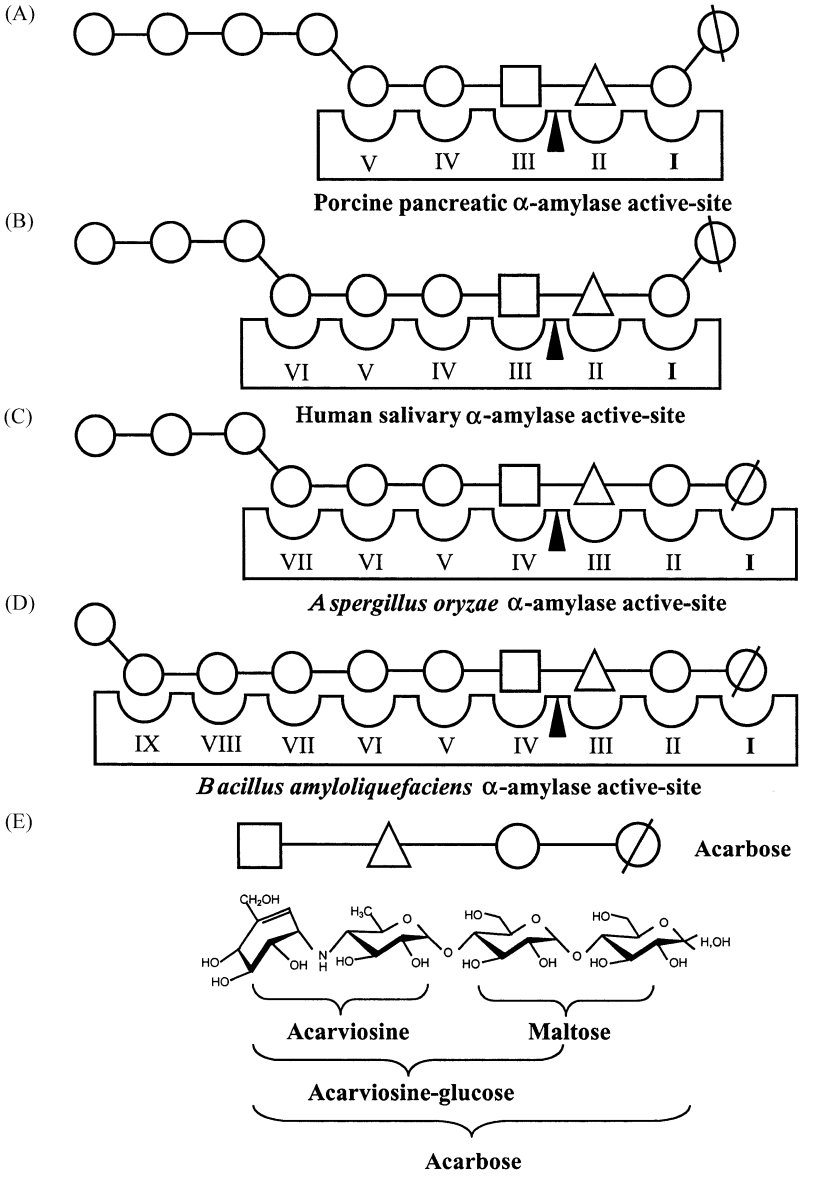


Fig. 5. D-Glucose-binding subsites at the active sites of four α-amylases and their inhibition by 4^{IV}-α-maltohexaosyl acarbose. The Roman numerals represent the D-glucose-binding subsites of the α-amylases. Subsite I begins with the subsite that would bind the reducing-end glucose residue. The catalytic-site is indicated by a black triangle. (A) PPA; (B) HSA; (C) AOA; (D) BAA; (E) molecular structure and symbolic representation of acarbose; the square is the cyclohexene; the triangle is 4-amino-4,6-dideoxy-D-glucose; the circles are D-glucose; and the reducing-end D-glucose is indicated by a circle with a slash through it.

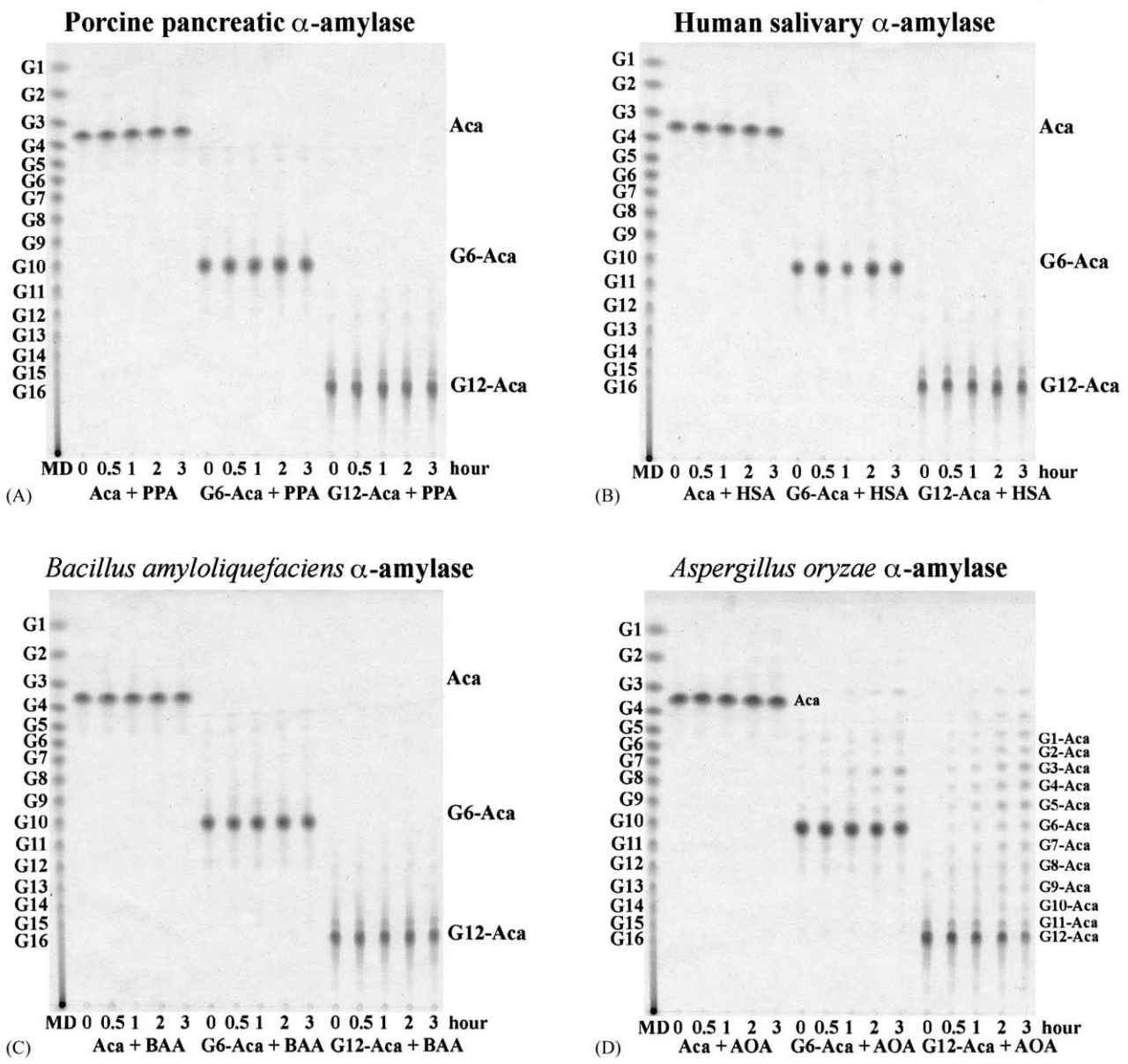


Fig. 6. TLC analysis of the reaction of the four α-amylases with acarbose, G_6-Aca, and G_{12}-Aca at 37 °C and the optimum pH of the enzymes. (A) PPA; (B) HSA; (C) BAA; (D) AOA.

incubating the maltodextrin–acarbose analogues with the four α-amylases. Samples were taken from the reactions at 0.5 h, the time period that we used to measure the initial velocities of the inhibition reactions, and every hour for 3 h. We found that PPA, HSA, and BAA did not hydrolyze the maltodextrin–acarbose analogues or acarbose at all. AOA did produce a low degree of hydrolysis ($< 1\%$) of G_6-Aca and G_{12}-Aca in the 0.5 h period; higher degrees of hydrolysis occurred in the later times of reaction. This latter hydrolysis, however, produced maltodextrin–acarbose analogues that were probably better inhibitors for AOA, especially the hydrolysis of G_{12}-Aca in which the maltodextrin chain was shortened and made comparable to G_6-Aca, which was a slightly better inhibitor than G_{12}-Aca.

Acknowledgements

The authors thank Professor K.-H. Park of Seoul National University, Korea, for the kind gift of acarbose.

References

1. Robyt, J. F. In *Starch: Chemistry and Technology*; Whistler, R. L.; BeMiller, J. N.; Paschall, E. F., Eds.; Academic Press: New York, 1984; pp 87–123.
2. Robyt, J. F. *Essentials of Carbohydrate Chemistry*, Springer-Verlag, New York, **1998**, pp. 328-333 and 337-343.
3. Crugger, W.; Crugger, A. In *Biotechnology, A Textbook of Industrial Microbiology; English Edition*; Brock, T. D., Ed.; Sinauer Assoc. Inc. and Sci. Tech. Inc.: Madison, WI, 1982; pp 163–166.
4. Nix, P. T.; Goldfarb, R. D.; Morgenstern, S.; Strong, L. J.; Trivedi, R. C. *Clin. Chem.* **1978**, *24*, 1000–1010.
5. Puls, W.; Kemp, U. *Diabetologia* **1973**, *9*, 97–101.
6. Truscheit, E.; Frommer, W.; Junge, B.; Muller, L.; Schmidt, D.; Wingender, W. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 744–761.
7. Müller, L.; Junge, B.; Frommer, W.; Schmidt, D.; Truscheit, E. In *Enzyme Inhibitors*; Brodbeck, U., Ed.; Berlag Chemie: Weinheim, 1980; pp 109–122.
8. Kim, M.-J.; Lee, S.-B.; Lee, H.-S.; Lee, S.-Y.; Beak, J.-S.; Kim, D.; Moon, T.-W.; Robyt, J. F.; Park, K.-H. *Arch. Biochem. Biophys.* **1999**, *371*, 277–283.
9. Kazaz, M. A.; Dessezux, V.; Marchis-Mouren, G.; Prodanov, E.; Santimone, M. *Eur. J. Biochem.* **1998**, *252*, 100–107.
10. Strokopytov, B.; Penninga, D.; Rozeboom, H. J.; Kalk, K. H.; Dijkhuizen, L.; Dijkstra, B. W. *Biochemistry* **1995**, *34*, 2234–2240.
11. Mosi, R.; Sham, H.; Uitdehaag, J. C. M.; Ruiterkamp, R.; Dijkstra, B. W.; Withers, S. G. *Biochemistry* **1998**, *37*, 17192–17198.
12. Aleshin, A. E.; Firsov, L. M.; Honzatko, R. B. *J. Biol. Chem.* **1994**, *269*, 15631–15639.
13. Breitmeier, D.; Gunther, S.; Heymann, H. *Arch. Biochem. Biophys.* **1997**, *346*, 7–14.
14. Kim, D.; Park, K.-H.; Robyt, J. F. *J. Microbiol. Tech.* **1998**, *8*, 287–290.
15. Devulapalle, K.; Mooser, G. *J. Carbohydr. Chem.* **2000**, *19*, 1285–1290.
16. Hillebrand, I.; Berchtold, P. In *Enzyme Inhibitors*; Brodbeck, U., Ed.; Berlag Chemie: Weinheim, 1980; pp 154–162.
17. Junge, B.; Boshagen, H.; Stoltefuss, J.; Muller, L. In *Enzyme Inhibitors*; Brodbeck, U., Ed.; Berlag Chemie: Weinheim, 1980; pp 123–137.
18. Park, K.-H.; Kim, M.-J.; Lee, H.-S.; Han, N.-S.; Kim, D.; Robyt, J. F. *Carbohydr. Res.* **1998**, *313*, 235–246.
19. Lee, S.-B.; Park, K.-H.; Robyt, J. F. *Carbohyr. Res.* **2001**, *331*, 13–18.
20. Yoon, S.-H.; Robyt, J. F. *Carbohydr. Res.* **2002**, *337*, 509–516.
21. Fisher, E. H.; Stein, E. A. *Biochem. Preps.* **1961**, *18*, 27–33.
22. Fox, J. D.; Robyt, J. F. *Anal. Biochem.* **1991**, *195*, 93–96.
23. Doner, L. W.; Irwin, P. L. *Anal. Biochem.* **1992**, *202*, 50–53.
24. Dixon, M. *Biochem. J.* **1953**, *55*, 170–171.
25. Robyt, J. F.; Mukerjea, R. *Carbohydr. Res.* **1994**, *251*, 187–202.
26. Robyt, J. F. *Encyclopedia of Separation Science*; Vol. 5; Academic Press: London, 2000; pp 2235–2244.
27. Duggleby, R.G.; Leonard, D.R. DNRPEASY, a data analysis computer program, 1992.
28. Segel, I. H. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*; John Wiley & Sons: New York, 1975; pp 100–202.
29. Robyt, J. F.; French, D. *Arch. Biochem. Biophys.* **1963**, *100*, 451–467.
30. Thoma, J. A.; Brothers, C.; Spradlin, J. *Biochemistry* **1971**, *9*, 1768–1775.
31. Thoma, J. A.; Rao, G. V. K.; Brothers, C.; Spradlin, J. *J. Biol. Chem.* **1971**, *246*, 5621–5635.
32. Brzozowski, A. M.; Lawson, D. M.; Turkenburg, J. P.; Bisgaard-Frantzen, H.; Svendsen, A.; Borchert, T. V.; Duater, Z.; Wilson, K. S.; Davies, G. J. *Biochemistry* **1996**, *39*, 9099–9107.
33. Robyt, J. F.; French, D. *J. Biol. Chem.* **1970**, *245*, 3917–3927.
34. Seigner, C.; Prodanov, E.; Machis-Moren, G. *Biochim. Biophys. Acta* **1987**, *913*, 200–209.
35. Qian, M.; Haser, R.; Buisson, G.; Duée, E.; Payan, F. *Biochemistry* **1994**, *33*, 6284–6294.
36. Kandra, L.; Gyémánt, G. *Carbohydr. Res.* **2000**, *329*, 579–585.
37. Nagamine, Y.; Omichi, K.; Ikenaka, T. *J. Biochem.* **1998**, *104*, 409–415.
38. Ramasubbu, N.; Paloth, V.; Luo, Y.; Brayer, G. D.; Levine, M. L. *Acta Crystallogr. Sect. D: Biol. Crystallogr.* **1996**, *D52*, 435–446.
39. Suganuma, T.; Matsuno, R.; Ohinishi, M.; Hiromi, K. *J. Biochem.* **1978**, *84*, 293–316.
40. Matsura, Y.; Kunusoki, M.; Harada, W.; Kakudo, M. *J. Biochem.* **1984**, *95*, 697–702.
41. Brzozowski, A. M.; Davies, G. J. *Biochemistry* **1997**, *36*, 10837–10845.
42. MacGregor, E. A.; Svensson, B. *Biochem. J.* **1989**, *259*, 145–152.
43. Svensson, B.; Søgaard, M. *J. Biotechnol.* **1993**, *29*, 1–37.
44. Svensson, B. *Plant Mol. Biol.* **1994**, *25*, 141–157.