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Enzymatic Synthesis of Novel α-Amylase Inhibitors via Transglycosylation by *Thermotoga maritima* Glucosidase

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Abstract Novel amylase inhibitors were synthesized via transglycosylation by *Thermotoga maritima* glucosidase (TMG). TMG hydrolyzes acarbose, acarviosine-glucose, and maltooligosaccharide by releasing ^{14}C-labeled glucose from the reducing end of each molecule. When TMG was incubated with acarviosine-glucose (the donor) and glucose (the acceptor), two major transfer products, compounds **1** and **2**, were formed via transglycosylation. The structures of the transfer products were determined using thin-layer chromatography (TLC), high-performance ion chromatography (HPIC), and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The results indicate that acarviosine was transferred to glucose at either C-6, to give a α-(1→6) glycosidic linkage, or at C-3, to produce an α-(1→3) glycosidic linkage. The transfer products showed a mixed-type inhibition against porcine pancreatic α-amylase; therefore, they may be useful not only as inhibitors but also as acarbose transition-state analogs to study the mechanism of amylase inhibition.

Key words: *Thermotoga maritima* glucosidase (TMG), acarbose, transfer product, inhibition, α-amylase

Introduction

Acarbose, a pseudotetrasaccharide with a pseudo-sugar ring at its nonreducing end linked to the nitrogen of 4-amino-4,6-dideoxy-D-glucopyranose, is widely recognized as a potent inhibitor of several carbohydrases, including α-glucosidase (1), glucoamylase (2), α-amylase (3), and cyclomaltodextrin glucanotransferase (CGTase) (4). Because acarbose is easy to transfer to other sugar molecules, transglycosylation is an excellent method for developing new drugs and inhibitors from acarbose, including compounds used in diabetes management (5,6). The formation of several acarbose analogs modified at their reducing ends through transglycosylation by maltogenic amylases has been reported (7-10). Li *et al.* (11) demonstrated that acarbose could be hydrolyzed by α-amylase, but that acarviosine-glucose, which is resistant to enzymatic rearrangement, could not. *Thermotoga maritima* glucosidase (TMG) hydrolyzes various maltodextrins, including cyclomaltodextrins (CDs), to glucose and maltose, and it readily degrades acarbose to acarviosine and glucose (12). In contrast to CD-/pullulan-degrading enzymes (13), TMG also shows very high hydrolytic activity toward acarviosine-glucose. Interestingly, it has strong transglycosylation activity in the presence of various acceptor molecules, forming α-(1,3)-, α-(1,4)-, or α-(1,6)-glycosidic linkages.

In this study, novel compounds were synthesized by transglycosylation using TMG with acarviosine-glucose as the donor and glucose as the acceptor. These novel compounds were purified and their molecular structures were determined by matrix-associated laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and ^{13}C nuclear magnetic resonance (NMR) analysis. In addition, we determined the effectiveness of the acarviosine-glucose transfer products as inhibitors of porcine pancreatic α-amylase.

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Materials and Methods

Analysis of the hydrolysis products of maltooligosaccharides labeled with ^{14}C Maltooligosaccharides labeled with ^{14}C at their reducing ends were produced by the modified method (14) using β-CD and D-glucose-UL-^{14}C (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The reaction mixtures were separated by descending paper chromatography on Whatman No. 3 MM chromatography paper and developed with *n*-propanol/water (65/35, v/v).

The reaction mixtures containing TMG and the ^{14}C-labeled maltooligosaccharides were separated by thin-layer chromatography (TLC). After the TLC plate was dried, it was placed in a cassette and covered with an imaging plate for more than 10 hr. The radioactivity from the ^{14}C-labeled maltooligosaccharides was measured using a BAS2500 image analyzer (Fujifilm, Tokyo, Japan).

Preparation of acarviosine-glucose To prepare acarviosine-glucose, 1%(w/v) acarbose in 50 mM sodium acetate buffer, pH 6.0, was incubated with 5 U of maltogenic amylase from *Bacillus stearothermophilus* per mg of acarbose for 12 hr. After the reaction was completed, the mixture was loaded onto an activated charcoal column (3.6×25 cm) equilibrated with distilled water. The sample

was washed with distilled water at a flow rate of 2 mL/min, and the acarviosine-glucose was eluted with 40%(w/v) ethanol. A rotary vacuum evaporator (Eyela, Tokyo, Japan) was used to remove the ethanol, and the final product was analyzed by TLC.

Transglycosylation of acarviosine-glucose by TMG The transglycosylation of acarviosine-glucose by TMG was carried out in the presence of glucose. TMG was added to a mixture of 5%(w/v) acarviosine-glucose and 10%(w/v) glucose in sodium phosphate buffer (50 mM, pH 7.0). The reaction was allowed to proceed for 18 hr. The major transfer products were purified by preparative TLC followed by gel filtration chromatography using a Bio-Gel P-2 column (1.6×90 cm; Bio-Rad, Hercules, CA, USA) to remove contaminating silica and binder compounds.

The products were then analyzed by TLC on Whatman K5F silica gel plates with isopropyl alcohol/ethyl acetate/water (3:1:0.5, v/v/v) as the solvent system. high-performance ion chromatography (HPIC) was performed using a CarboPac PA1 column (0.4×25 cm; Dionex, Sunnyvale, CA, USA) and an electrochemical detector (ED40; Dionex). Buffers A (150 mM NaOH) and B (600 mM sodium acetate in buffer A) were used for elution along with a linear gradient having different slopes depending on the samples.

Mass spectrometry The MALDI-TOF mass spectrum was collected using a Voyager TM-DE (Perceptive Biosystems, Framingham, MA, USA) system with α-cyano-4-hydroxy-cinnamic acid (α-CHCA, C-2620; Sigma-Aldrich Chemical Co.) as the matrix. One μL each of the purified sample and α-CHCA was dropped on a sample applicator and dried thoroughly. The sample plate was operated with a 24 kV acceleration voltage.

NMR spectrometry The ^{13}C nuclear magnetic resonance (NMR) spectrum was recorded using a Jeol LA-400 FT-NMR spectrometer (Jeol, Tokyo, Japan). The sample was dissolved in H_2O-d_6 at 24.9°C with tetramethyl silane (TMS) as the internal reference.

Enzyme assay for inhibition kinetics α-Amylase activity was measured by the copper-bicinchoninate reducing-value method using a microsample plate reader. A soluble starch solution was prepared in 50 mM sodium phosphate buffer containing 6 mM NaCl (pH 7.0), and 450 μL were incubated with or without inhibitors at 37°C. Each inhibitor was incubated with the enzyme solution at 37°C for 5 min. The reaction was initiated by adding 50 μL of the enzyme solution, and 200 μL aliquots of the enzyme digest were collected at various time intervals and added to 200 μL of the copper-bicinchoninate reagent. The samples were heated in a water bath at 80°C for 35 min then cooled, and the absorbances were measured using a microsample plate reader (EL340 Biokinetics Reader; Bio-Tech Instruments, Inc., Winooski, VT, USA). The kinetic parameters were calculated using Lineweaver-Burk plots, and DNRPEASY software developed by Duggleby (15) was used to analyze the inhibition of the enzyme.

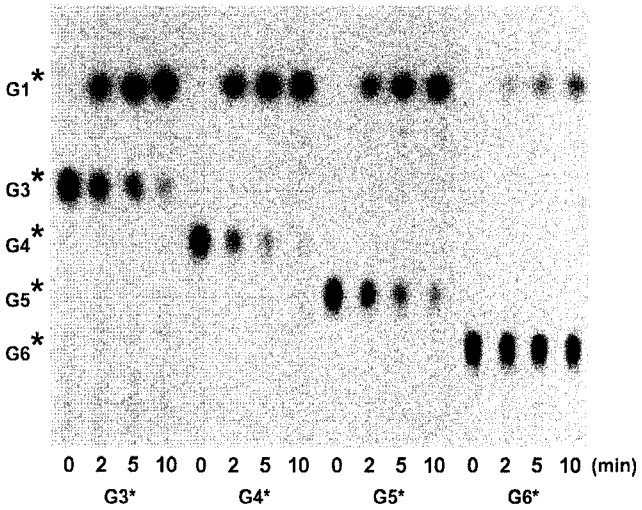

Fig. 1. TLC analysis of the maltooligosaccharide hydrolysis products produced by TMG as a function of reaction time. The substrates were labeled with [^{14}C]-D-glucose at their reducing ends.

Results and Discussion

Catalytic properties of TMG To confirm the action pattern of TMG, maltooligosaccharides of various lengths labeled with ^{14}C at their reducing ends were incubated with TMG for 2 to 10 min. The level of ^{14}C-glucose gradually increased while the levels of the labeled substrates, including maltotriose (G3), maltotetraose (G4), maltopentaose (G5), and maltohexaose (G6), decreased. This result indicates that the enzyme hydrolyzed the maltooligosaccharides by releasing the glucose unit from their reducing ends (Fig. 1). Lee *et al.* (12) found that TMG initially hydrolyzes *p*-nitrophenyl-α-D-pentaoside to give maltopentose and *p*-nitrophenol. Together with our data, this strongly suggests that TMG recognizes the glucose moiety at the reducing end of its substrates.

Transglycosylation of acarviosine-glucose with glucose by TMG The transglycosylation of acarviosine-glucose with glucose by TMG yielded 2 transfer products (Fig. 2). The major transfer products were likely a mixture of the 2 products, designated **1** and **2**. Compounds **1** and **2** were purified by preparative TLC, followed by Bio-Gel P2 gel filtration chromatography (Fig. 2; lane C and D).

Structure of the glycosidic linkage in the transfer products The purified compounds were further analyzed by MALDI-TOF mass spectrometry (Fig. 3). In both cases, one molecular ion peak appeared at 506.1 *m/z* ($[M+Na]^+$), which corresponded to the calculated molecular mass of the sodium ion adduct of acarviosine-glucose (483.4 Da). Compound **1** and **2** had identical molecular masses (483.4 Da), suggesting that the transfer products were acarviosine-glucose with different glycosidic linkages.

^{13}C-NMR analyses were subsequently carried out to determine the configuration of the glycosidic linkage between acarviosine and glucose. Chemical shifts in the ^{13}C-NMR spectrum were compared with those of acarbose and

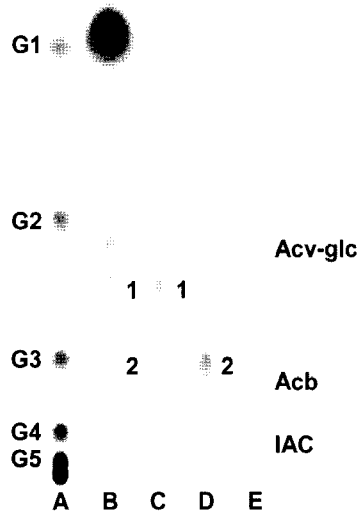

Fig. 2. TLC analysis of the transglycosylation product produced from acarviosine-glucose and glucose by TMG. Lane A, maltooligosaccharide standards; lane B, reaction mixture; lane C, acarviosyl-α-1,3-D-glucopyranoside (1); lane D, acarviosyl-α-1,6-D-glucopyranoside (2); lane E, acarbose standard (Acv-Glc, acarviosine-glucose; Acb, acarbose; IAC, Isoacarbose).

glucose (Table 1 and Fig. 4). A large chemical shift was observed in both **1** and **2**. In the case of compound **1**, a shift occurred at position C-3 of glucose, from 74.6 to 69.3 ppm, whereas compound **2** exhibited a chemical shift from 61.6 to 55.5 ppm at position C-6. These results suggest that the transfer products **1** and **2** were acarviosyl-α-1,3-D-glucopyranoside and acarviosyl-α-1,6-D-glucopyranoside, respectively.

Based on the structural determination, an action pattern for the transfer of acarviosine from acarviosine-glucose to glucose by TMG was proposed (Fig. 5). In this scheme, acarviosine-glucose is hydrolyzed to acarviosine and glucose by TMG, with water acting as an acceptor. When glucose, also an acceptor, is added, TMG transfers acarviosine to glucose, forming α-(1,3)- and α-(1,6)-linkages between acarviosine and glucose.

Inhibition of porcine pancreatic α-amylase We then determined the ability of the acarviosine-glucose transfer products to inhibit α-amylase activity. The inhibition constants, K_i and K_I, and the inhibitor potency are listed in Table 2. A Lineweaver-Burk plot showed that the inhibition of porcine pancreatic α-amylase by acarviosyl-α-1,6-D-glucose and acarviosyl-α-1,3-D-glucose followed a mixed-

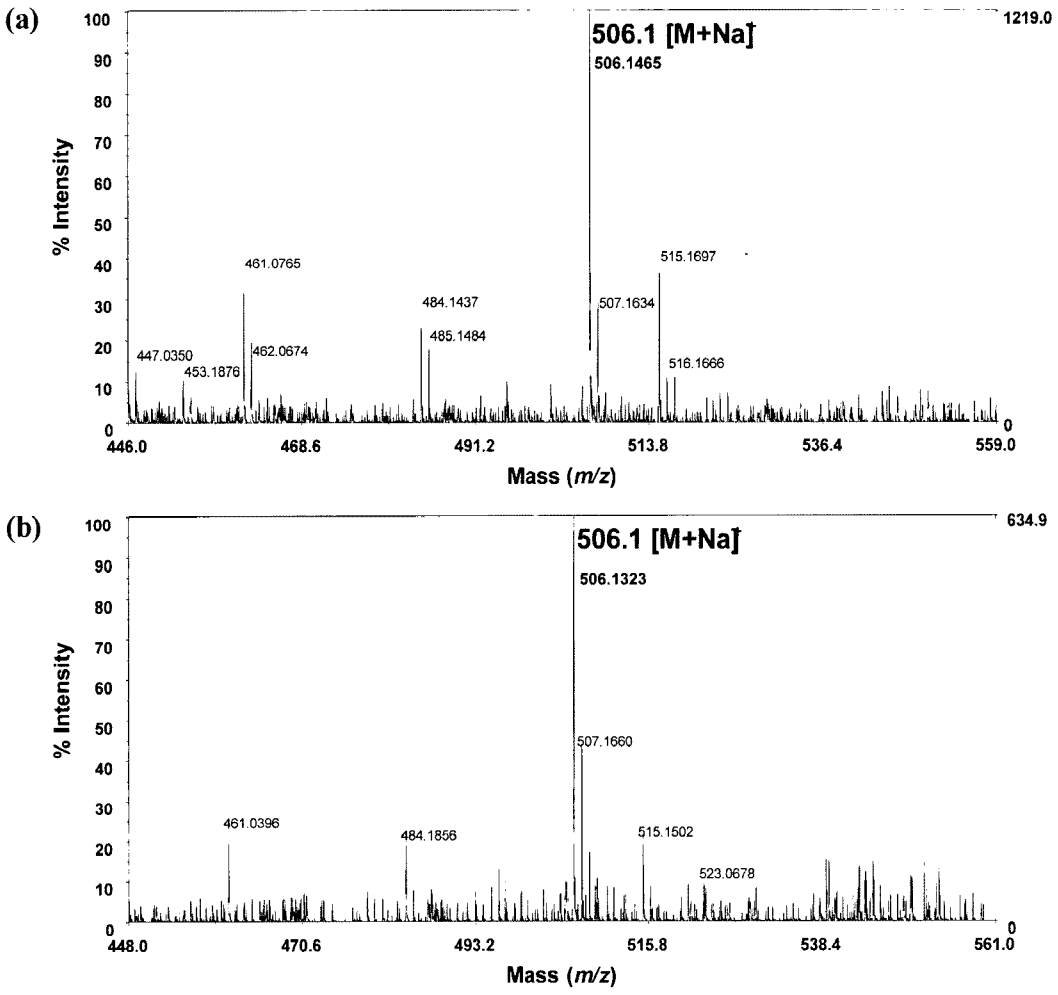

Fig. 3. MALDI TOF mass spectrometer analysis of (a) acarviosyl-α-1,3-D-glucopyranoside (1) and (b) acarviosyl-α-1,6-D-glucopyranoside (2).

Table 1. ^{13}C NMR data for acarbose and its transfer products (units: ppm)

Carbon		Acarbose (δ)	Glucose (δ)	**1** (δ1)	Differences (δ1-δ)	**2** (δ2)	Differences (δ2-δ)
Ring A	1	56.0		56.0	0	56.0	0
	2	74.0		74.6	0.6	74.0	0
	3	72.7		72.8	0.1	72.5	0.2
	4	71.2		71.2	0	71.4	0.2
	5	139.0		139.0	0	138.8	0.2
	6	123.7		123.8	0.1	121.5	2.2
	7	61.6		61.6	0	61.5	0.1
Ring B	1	99.9		100.4	0.5	97.8	2.1
	2	70.8		70.9	0.1	71.0	0.2
	3	73.0		73.0	0	73.0	0
	4	65.0		65.1	0.1	65.7	0.7
	5	69.6		69.6	0	69.6	0
	6	17.3		17.3	0	17.3	0
Ring C	1		95.6	96.0	0.4	96.0	1.4
	2		73.4	73.2	0.2	72.2	1.2
	3		74.6	**69.3**	5.3	74.2	0.4
	4		76.9	76.1	0.8	75.9	1.0
	5		71.6	71.2	0.4	71.7	0.1
	6		61.6	60.5	1.1	**55.5**	6.1

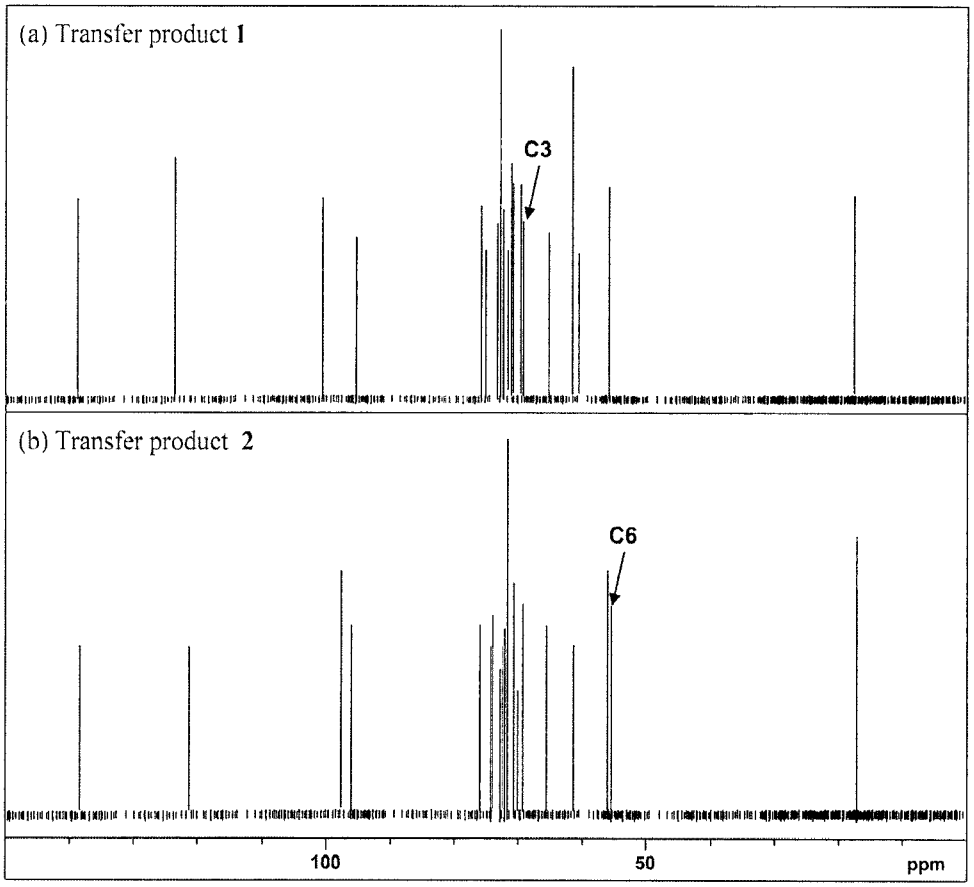

Fig. 4. ^{13}C-NMR spectrum for (a) acarviosyl-α-1,3-D-glucopyranoside 1 and (b) acarviosyl-α-1,6-D-glucopyranoside 2.

type noncompetitive pattern (Fig. 6), with K_i values of 0.527 and 0.342 μM, respectively. K_I values were also determined for 3 other acarviosine-containing inhibitors, but acarviosyl-α-1,3-D-glucose was the most potent inhibitor

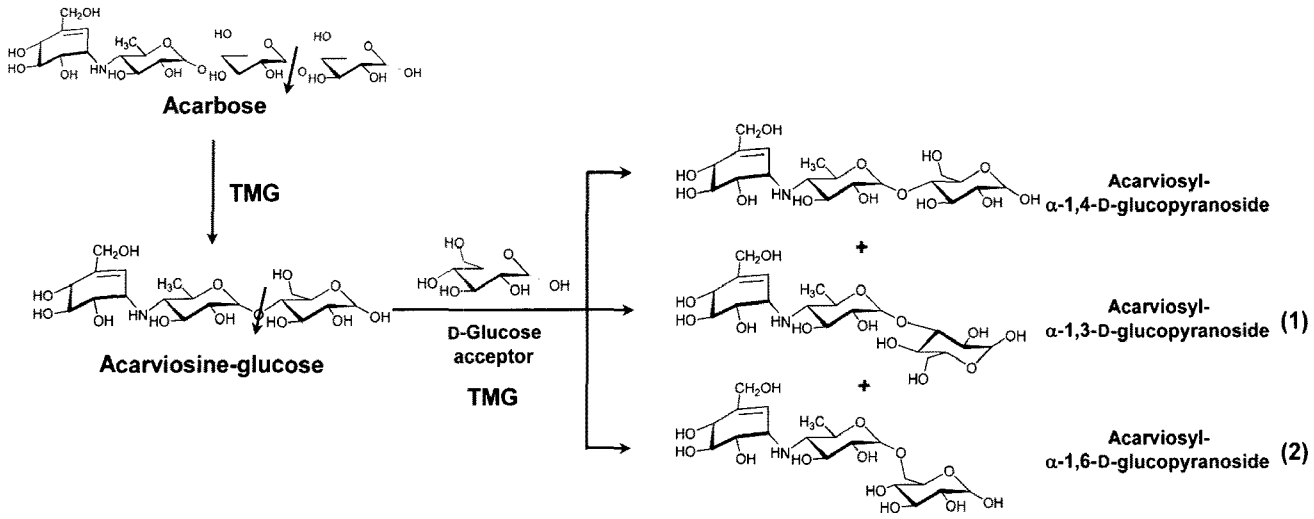

Fig. 5. Proposed transglycosylation reaction mechanism for acarviosine-glucose using TMG.

Table 2. Analysis of inhibition mediated by acarbose and its derivatives

Enzyme	Inhibitors	Type of inhibition	K_i (μM)[1)]	K_I (μM)[2)]	Inhibitor potency[3)]
α-Amylase (porcine pancreas)	Acarbose	Mixed	0.632	0.909	1.0
	Acarviosine-glucose	Mixed	0.587	0.853	1.1
	Acarviosyl-α-1,6-D-glucopyranoside	Mixed	0.527	0.886	1.2
	Acarviosyl-α-1,3-D-glucopyranoside	Mixed	0.342	0.794	1.9

[1)] K_i is the inhibition constant, defined as [E][I]/[EI].
[2)] K_I is the inhibition constant, defined as [EI][S]/[ESI].
[3)] Inhibitor potency was determined by assigning acarbose a value of 1 and dividing the Ki value of acarbose by the Ki value of the inhibitor.

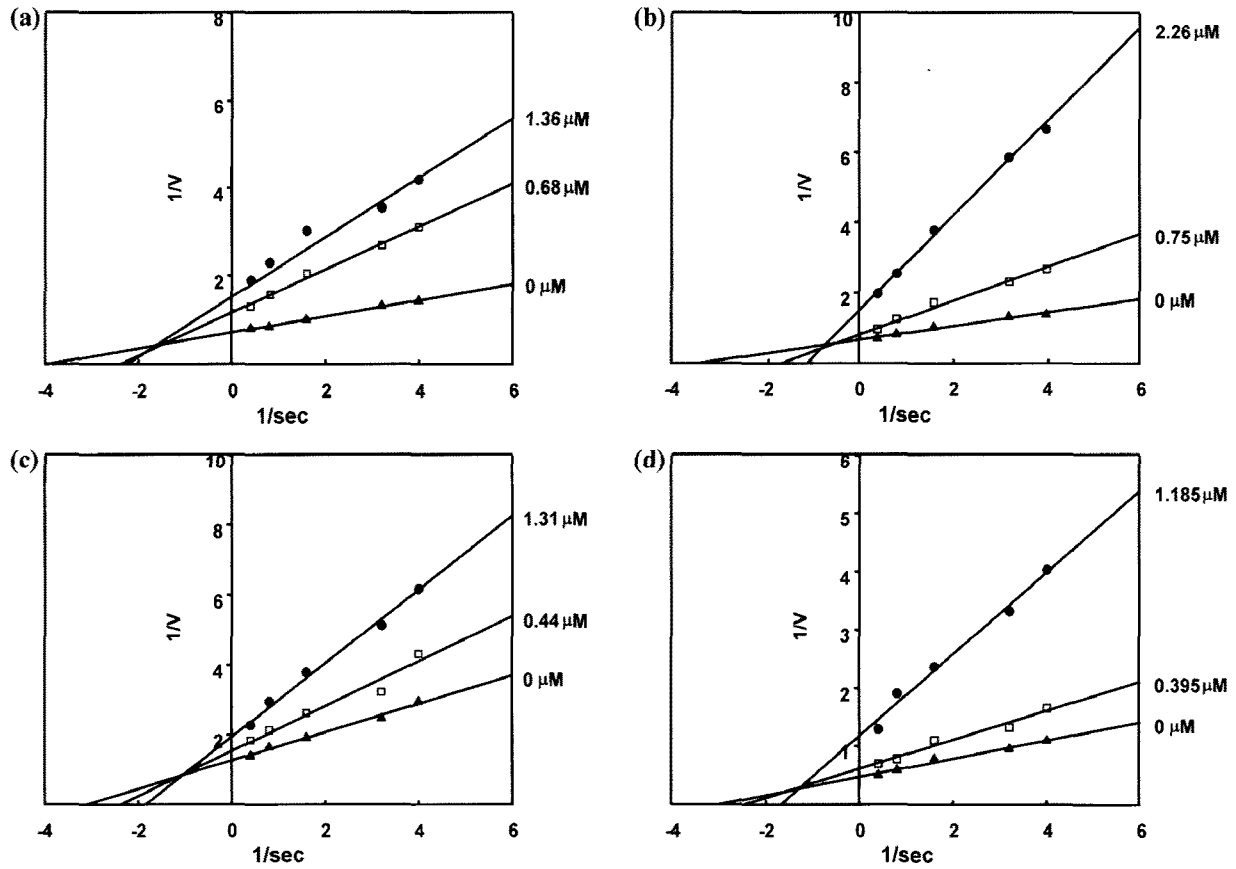

Fig. 6. Lineweaver–Burk plot for the reaction of porcine pancreatic α-amylase with the following inhibitors: (a) acarbose, (b) acarviosine-glucose, (c) acarviosyl-α-1,6-D-glucopyranoside 2, and (d) acarviosyl-α-1,3-D-glucopyranoside 1.

of α-amylase (1.9 times better than acarbose).

Several acarbose-derived α-amylase inhibitors have been enzymatically synthesized to study the mechanisms of inhibition and substrate binding in amylase and related enzymes. Considerable effort has been made to shorten the inhibitor using acarbose as the donor, since acarbose is rearranged by α-amylase. Li *et al.* (11) studied the mechanism of acarbose rearrangement in human pancreatic α-amylase using such acarbose analogs as isoacarbose and acarviosine-glucose. Similarly, the novel inhibitors acarviosyl-α-1,3-glucose and acarviosyl-α-1,6-glucose seem to be resistant to α-amylase and can therefore be used as acarbose analogs for studying inhibition.

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