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Addition of maltodextrins to the nonreducing-end of acarbose by reaction of acarbose with cyclomaltohexaose and cyclomaltodextrin glucanyltransferase

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Abstract

New kinds of acarbose analogues were synthesized by the reaction of acarbose with cyclomaltohexaose and cyclomaltodextrin glucanyltransferase (CGTase). Three major CGTase coupling products were separated and purified by Bio-Gel P2 gel-permeation chromatography. Digestion of the three products by beta-amylase and glucoamylase showed that they were composed of maltohexaose (G6), maltododecaose (G12), and maltooctadecaose (G18), respectively, attached to the nonreducing-end of acarbose. ^{13}C NMR of the glucoamylase product (D-glucopyranosyl-acarbose) showed that the D-glucose moiety was attached α-to the C-4-OH group of the nonreducing-end cyclohexene ring of acarbose, indicating that the maltodextrins were attached α-(1→4) to the nonreducing-end cyclohexene of acarbose.

Keywords: Acarbose analogues; Cyclomaltohexaose; Cyclomaltodextrin glucanyltransferase; Transglycosylation reactions; Maltohexaose; Maltododecaose; Maltooctadecaose; ^{13}C NMR spectroscopy

1. Introduction

Acarbose is a pseudotetrasaccharide with an unsaturated cyclitol [2,3,4-trihydroxy-5-(hydroxymethyl)-5,6-cyclohexene in a *gluco*-configuration] attached to the nitrogen of 4-amino-4,6-dideoxy-D-glucopyranose, which is linked α-(1→4) to maltose. Acarbose is a natural product produced by various species of *Actinoplanes*. It is a strong competitive inhibitor of α-glucosidase,[1–3] which has led to its use in the treatment of diabetes mellitus.[4] It has also been shown to be an inhibitor for α-amylase,[1–3,5] cyclomaltodextrin glucanyltransferase (CGTase),[3,6,7] glucoamylase,[8,9] and glucansucrases.[10,11]

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.[2,12]

Some naturally occurring acarbose analogues have been found that have several D-glucose residues attached to the nonreducing-end of acarbose.[1] The substitution of different saccharides for maltose at the reducing-end of acarbose has given analogues that have significantly increased inhibition and/or altered enzyme specificity.[3,13,14] Park et al.[3,13] reported the formation of several acarbose analogues modified at the reducing-end by the transglycosylation reaction between acarbose and various carbohydrate acceptors catalyzed by *Bacillus stearothermophilus* maltogenic amylase (BSMA). They 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. They also found that the replacement of the maltose unit by isomaltose gave an inhibitor that inhibited porcine pancreatic alpha-amylase 15.2-times better than acarbose. Lee et al.[14] found 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, whereas acarbose was not an inhibitor at all.

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In the present study, we report the enzymatic synthesis of new acarbose analogues in which specific maltodextrin chains, maltohexaose (G6), maltododecaose (G12), and maltooctadecaose (G18) are added to the C-4-hydroxyl group of the nonreducing-end cyclohexene ring by the reaction of acarbose with cyclomaltohexaose in a coupling reaction catalyzed by cyclomaltodextrin glucanyltransferase (CGTase).

2. Experimental

Materials.—*Bacillus 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 material. *Aspergillus niger* glucoamylase [EC 3.2.1.3] was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and was dialyzed extensively against 50 mM pyridinium–acetate buffer (pH 5.2) before using to remove glucose, other low-molecular weight saccharides, and other unwanted materials. The glucoamylase activity was analyzed by a micro glucose oxidase method.[15] Sweet potato beta-amylase [EC 3.2.1.2] was obtained from Worthington Biochemical Co. (Freehold, NJ, USA). The activity of beta-amylase was determined by measuring the amount of maltose formed from starch, using the copper–bicinchoninate micromethod.[15] Acarbose was a gift from Dr K.-H. Park (Department of Food Science and Technology, Seoul National University, Suwon, Korea). Cyclomaltohexaose (α-CD) was a gift from Ensuiko Sugar Refining Co. Ltd. (Yokohama, Japan). All other chemicals used were of reagent grade.

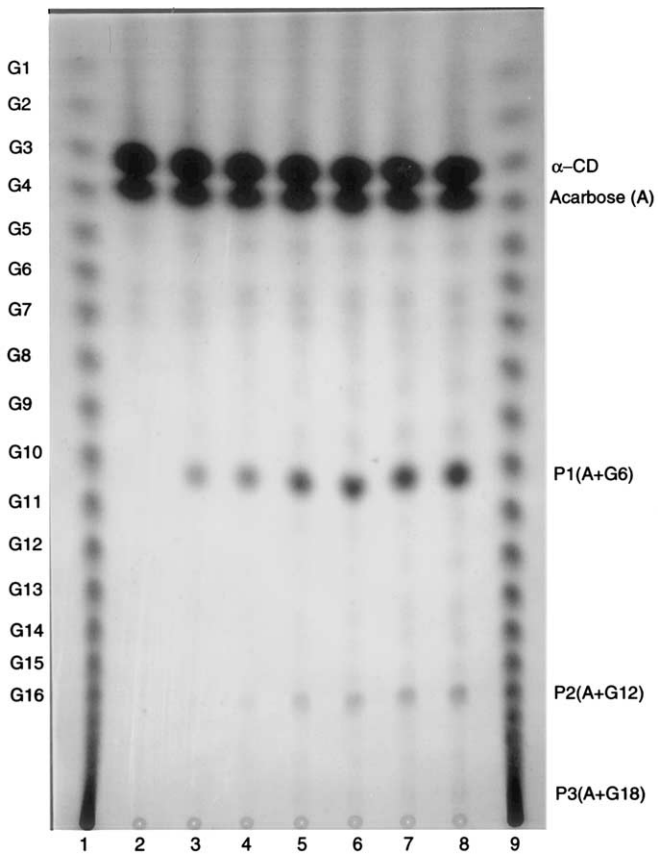

Fig. 1. Thin-layer chromatogram (10 × 20 cm Whatman K5F plate irrigated twice for 18.0 cm path-length each with 85:20:50:70 MeCN–EtOAc–1-propanol–water) of the CGTase reaction products from cyclomaltohexaose (α-CD) and acarbose. Lanes 1 and 9, maltodextrin standards; lanes 2–8, CGTase reaction products after 0, 0.5, 1, 2. 3, 4, and 5 days of reaction time, respectively.

Assay of the activity of CGTase.—CGTase activity was determined by a modification of the method of Thoma et al.[16] in which 0.1 mL of glucoamylase (10 IU/mL) in 20 mM pyridinium–acetate buffer (pH 5.0) was added to 0.3 mL of 18 mM α-CD and 18 mM methyl α-D-glucopyranoside in 20 mM pyridinium–acetate buffer (pH 5.0); 0.1 mL of CGTase was added to initiate the reaction at 37 °C; 0.1 mL samples were taken every 5 min for 25 min and the D-glucose determined by the micro glucose oxidase method.[15]

Formation of CGTase products with reaction time.—CGTase (600 mIU, 1 IU = 1 μmol of α-CD combined with 1 μmol of acceptor per min) was added to a substrate mixture composed of 100 μL of 100 mM acarbose and 100 μL of 100 mM α-CD in 25 mM imidazole–HCl buffer (pH 6.0). The enzyme reaction was carried out at 37 °C for 5 days. Aliquots (25 μL) were removed after 0.5, 1, 2, 3, 4, and 5 days, and the reaction was stopped by heating in boiling water for 5 min. The reaction products were analyzed by TLC; 4 μL of each time period of the digest was spotted onto a 10 × 20 cm Whatman K5F Silica Gel plate (Fisher Scientific, Chicago, IL). The plate was irrigated twice with 85:20:50:70 MeCN–EtOAc–1-propanol–water with an 18-cm irrigation path length. The carbohydrates on the TLC plate were visualized by dipping the plate into an 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.[17]

Preparation of CGTase coupling-reaction products.—CGTase (15 IU) was added to 1.0 mL of 100 mM acarbose and 1.0 mL of 100 mM α-CD in 25 mM imidazole–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 α-CD solution every 1 to 2 days. The reaction was stopped by heating in boiling water for 5 min. Insoluble matter in the digest was removed by centrifugation at 4000 rpm for 10 min. The reaction mixture was concentrated to 1.2 mL by rotary vacuum evaporation.

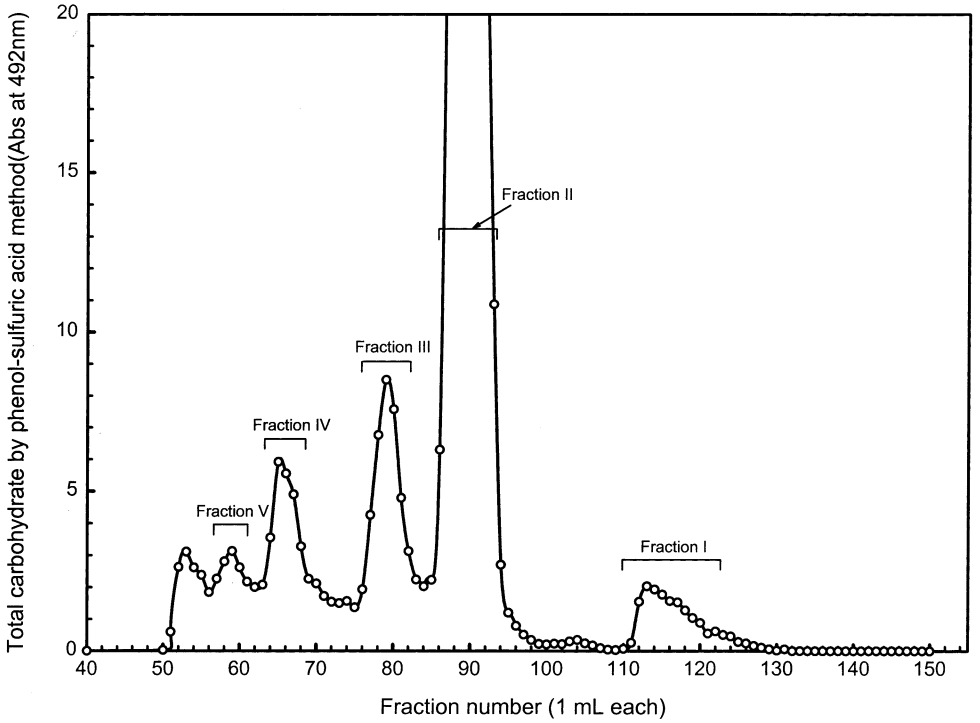

Fig. 2. Bio-Gel P2 gel-permeation column chromatogram (1.5 × 100 cm; flow rate 0.063 mL/min; fraction size, 1.0 mL) of the CGTase reaction products. Fraction I, acarbose; fraction II, α-CD; fractions III, IV, and V, products of the reaction.

Purification of the reaction products from acarbose, α-CD, and CGTase.—About 1.2 mL of the reaction digest was loaded onto Bio-Gel P2 (fine) column (1.5 × 100 cm), and eluted with deionized water at a flow rate of 0.063 mL/min, collecting 1.0 mL fractions. The total carbohydrate of each fraction was determined by the micro phenol–H_2SO_4 method,[15] and the composition of the fractions was analyzed by TLC as described above with Whatman K6F plates.

Beta-amylase digestion of the CGTase reaction products.—The purified CGTase reaction products (4 μL) were diluted to 20 μL with 20 mM sodium acetate buffer (pH 4.8) and beta-amylase (230 mIU) was added and incubated at 25 °C for 24 h. The reaction was stopped by heating in boiling water for 5 min. The beta-amylase reaction digest was analyzed by TLC as described above.

Glucoamylase digestion of the CGTase reaction products.—The purified CGTase reaction products (4 μL) were diluted to 20 μL with 20 mM pyridinium–acetate buffer (pH 5.2), to which glucoamylase (380 mIU) was added and incubated at 37 °C for 24 h. The reaction was stopped by heating in boiling water for 5 min. The glucoamylase reaction digest was analyzed by TLC as described above.

Preparation of D-glucosyl-acarbose.—For the preparation of D-glucopyranosyl-acarbose, P1 (400 μL) was diluted to 2.0 mL with 20 mM pyridinium–acetate buffer (pH 5.2) and reacted with glucoamylase (5.8 IU) at 37 °C for 24 h. D-Glucose produced from P1 by glucoamylase was removed by yeast (*Saccharomyces cerevisiae)* fermentation for 24 h at 37 °C. D-Glucopyranosyl-acarbose was purified by Bio-Gel P2 gel-permeation column (1.5 × 110 cm) chromatography similar to the conditions for the purification of CGTase reaction products.

NMR analysis.—About 100 mg of acarbose and about 30 mg of glucosyl-acarbose were exchanged three times with D_2O and were dissolved in 0.5 mL of pure D_2O, and then placed into 5 mm NMR tubes. Spectra were obtained on a Bruker DRX 500 spectrometer, operating at 500 MHz for ^1H and at 125 MHz for ^{13}C at 25 °C. Spectra of homonuclear correlation spectroscopy (COSY) and heteronuclear multiple quantum correlation spectroscopy (HMQC) were recorded and analyzed with XWINNMR (Bruker) or NMRVIEW[18] programs.

3. Results

The CGTase catalyzed coupling reaction between acarbose and α-CD gave three major products (P1, P2, and P3 in Figs. 1 and 2) and very small amounts of very minor products, even though acarbose inhibits CGTase.[3,6,7] A time-course study (Fig. 1) shows that CGTase sequentially produced P1, P2, and P3 after 0.5, 1, and 3 days, respectively, without any distinct increase of the minor products.

The three major products were separated and purified by Bio-Gel P2 gel-permeation chromatography (Fig. 2). Fractions I and II were unreacted acarbose and α-CD, respectively. Fractions III, IV, and V were the three major products, P1, P2, and P3, respectively.

P1, P2, and P3 were completely hydrolyzed by beta-amylase to give only acarbose and maltose (lane 4 in Figs. 3–5). This demonstrates that the maltooligosaccharides attached to acarbose in the three products had an even number of D-glucose residues as maltose and acarbose were the only beta-amylase products formed. The formation of acarbose by beta-amylase further suggests that the maltodextrin chains were attached to the C-4 hydroxyl group of the cyclohexene ring of acarbose as beta-amylase will only hydrolyze a terminal α-(1→4) glycosidic linkage.[19,20]

The TLC migration of P1 corresponded to a decasaccharide (Figs. 1 and 3). Glucoamylase specifically hydrolyzes D-glucose residues from the nonreducing-ends of maltodextrins and starch chains. The reaction of

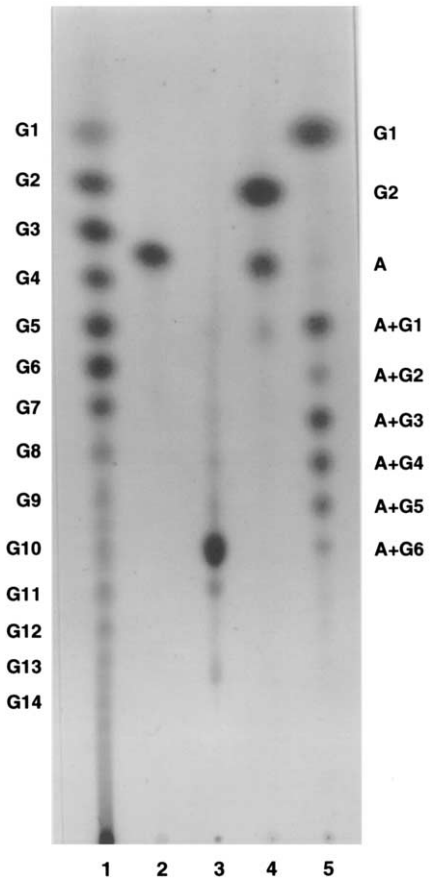

Fig. 3. Thin-layer chromatogram (10 × 20 cm Whatman K6F plate was irrigated twice for 18.0 cm path-length for each ascent with 85:20:50:70 MeCN–EtOAc–1-propanol–water) of the beta-amylase and glucoamylase reaction products of P1. Lane 1, maltodextrin standards; lane 2, acarbose standard; lane 3, P1; lane 4, beta-amylase reaction products from P1; and lane 5, glucoamylase reaction products from P1.

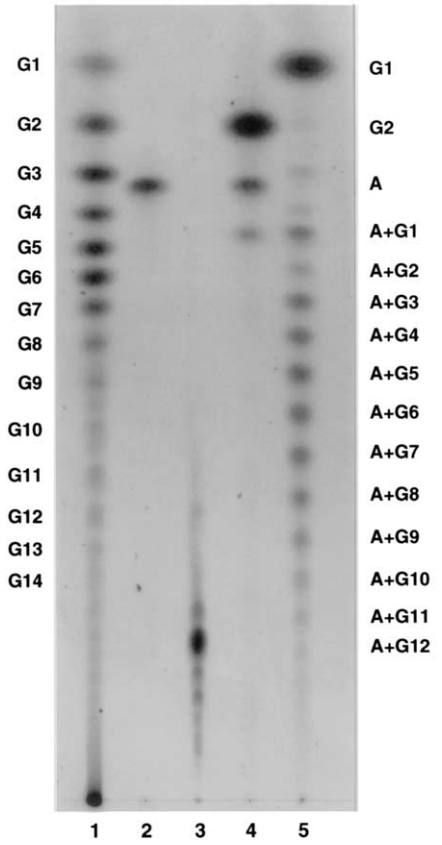

Fig. 4. Thin-layer chromatogram (10 × 20 cm Whatman K6F plate was irrigated three-times for 18.0 cm path-length for each ascent with 85:20:50:70 MeCN–EtOAc–1-propanol–water) of the beta-amylase and glucoamylase reaction products of P2. Lane 1, maltodextrin standards; lane 2, acarbose standard; lane 3, P2; lane 4, beta-amylase reaction products from P1; and lane 5, glucoamylase reaction products from P2.

glucoamylase with P1 gave partial hydrolysis that resulted in D-glucose and a series of products containing D-glucose and maltodextrin chains, having two to six D-glucose residues (lane 5, Fig. 3) attached to acarbose. The last product formed was D-glucopyranosyl-acarbose and no free acarbose was formed. These results indicate that P1 has a maltohexaose unit attached to the nonreducing-end cyclohexene ring of acarbose, giving a pseudo-decasaccharide.

P2 migrated as a hexadecasaccharide (16 monomer units) on TLC (Figs. 1 and 4). Glucoamylase hydrolyzed P2 to give D-glucose plus a series of 12 products, (A–G12)–(A–G1) (lane 5 in Fig. 4). The last product formed was D-glucopyranosyl-acarbose and no free acarbose was formed. These results show that P2 is composed of maltododecaose (G12) attached to the nonreducing-end cyclohexene ring of acarbose, giving a pseudo-hexadecasaccharide.

P3 migrated as a docosasaccharide (22 monomer units) on TLC (Figs. 1 and 5). Glucoamylase hydrolyzed P3 to give D-glucose and a series of 18 prod-

ucts. (A–G18)–(A–G1), with acarbose not being formed. Similar to the results for P1 and P2, P3 is a pseudo-docosasaccharide in which maltooctadecaose (G18) is attached to the nonreducing-end cyclohexene ring of acarbose.

The ^{13}C NMR spectrum of D-glucopyranosyl-acarbose (Fig. 6(A)) was almost identical to that of the ^{13}C NMR spectrum of acarbose (Fig. 6(B)) previously reported[21,22] with the exception of the 5-hydroxymethyl-cyclohexene ring (unit IV in Fig. 6(C)) and the D-glucopyranosyl unit (unit V in Fig. 6(C)). The ^{13}C NMR peaks of D-glucopyranosyl acarbose (Fig. 6(A)) were assigned using ^{1}H COSY and ^{1}H–^{13}C HMQC NMR (data not shown). The changes of the carbon-chemical shifts of the 5-hydroxymethyl-cyclohexene ring before and after the addition of D-glucopyranose to acarbose were, respectively, as follows; C-1, 56.5–55.4; C-2, 71.5–71.7; C-3, 73.3–71.2; C-4, 71.1–76.3; C-5, 139.4–136.8; C-6, 62.0–62.4; C-7, 124.0–126.5 ppm (Fig. 6(A and B)). This shows that there was a relatively large downfield chemical shift of 5.2 ppm for C-4 and a relatively small upfield chemical shift at C-3 of 2.1 ppm and at C-5 of 2.6 ppm of the 5-hydroxymethyl-cyclohexene ring. These chemical shifts are characteristic of the attachment of a D-glucopyranosyl unit to the C-4 position of 6-hydroxylmethyl-cyclohexene (unit IV in Fig. 6(C)) of acarbose.[23] The chemical shift of the anomeric carbon (C-1) of the D-glucopyranose (unit V in Fig. 6(C)) was 98.5 ppm characteristic of an α linkage.[23] Thus, the ^{13}C NMR results indicate that the D-glucopyranose unit was attached to the 5-hydroxymethyl-cyclohexene ring by an α-(1→4) linkage and hence the maltodextrins (G6, G12, and G18) in P1, P2, and P3 are attached to 5-hydroxymethyl-cyclohexene ring of acarbose by an α-(1→4) linkage.

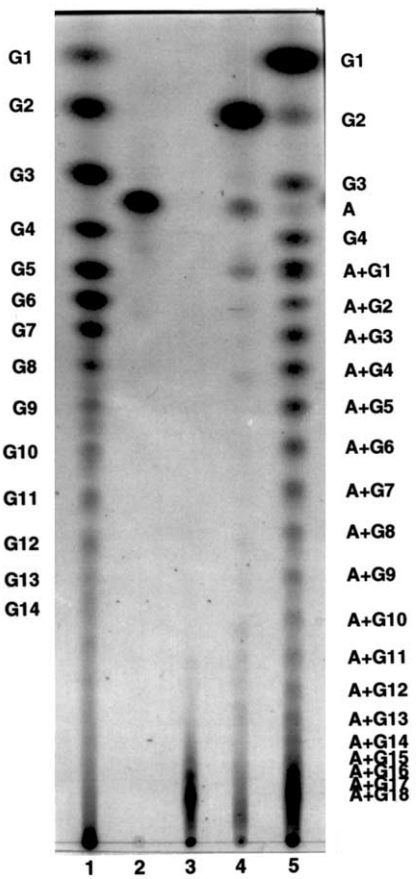

Fig. 5. Thin-layer chromatogram (10 × 20 cm Whatman K6 plate was irrigated four-times for 18.0 cm path-length for each ascent with 85:20:50:70 MeCN–EtOAc–1-propanol–water) of the beta-amylase and glucoamylase reaction products of P3. Lane 1, maltodextrin standards; lane 2, acarbose standard; lane 3, P3; lane 4, beta-amylase reaction products from P1; and lane 5, glucoamylase reaction products from P3.

4. Discussion

It is well recognized that CGTase catalyzes three reactions:[24,25] (1) the formation from starch of nonreducing, cyclic maltodextrins, containing six, seven, and eight D-glucose residues linked α-(1→4) to each other; (2) coupling reactions between cyclomaltodextrin and an added carbohydrate acceptor, such as D-glucose, maltose, panose, and so forth; and (3) disproportionation reactions between two maltodextrin chains of d.p. 6, to give (G2 + G10), (G3 + G9), (G4 + G8), and so forth.[24,25] In the present study, CGTase was used as a catalyst for the coupling of maltodextrins to acarbose by reaction of α-CD with acarbose. Three major products (P1, P2, P3) were formed sequentially and only some very minor disproportionation products were produced after the coupling reaction (Fig. 1).

Because beta-amylase is an exo-nonreducing-end acting enzyme that produces maltose as the exclusive product, the digestion of the P1, P2, P3 products with beta-amylase to produce exclusively maltose and acarbose indicated that maltodextrins, containing an even number of D-glucose residues, were attached to the nonreducing-end of acarbose.

Glucoamylase digestion of P1 gave D-glucose plus five compounds containing G5–G1 attached to acarbose. Digestion of P2 gave D-glucose plus 11 compounds, containing G11–G1 attached to acarbose. Digestion of P3 gave D-glucose plus 17 compounds, containing G17–G1 attached to acarbose. Complete digestion of the three products gave a single product that was D-glucopyranosyl acarbose. Free acarbose was never observed. D-Glucopyranosyl acarbose apparently was a potent inhibitor of glucoamylase as this enzyme is usually capable of hydrolyzing different kinds of α linkages and it would have been expected that it would have hydrolyzed the last D-glucopyranose residue from acarbose.

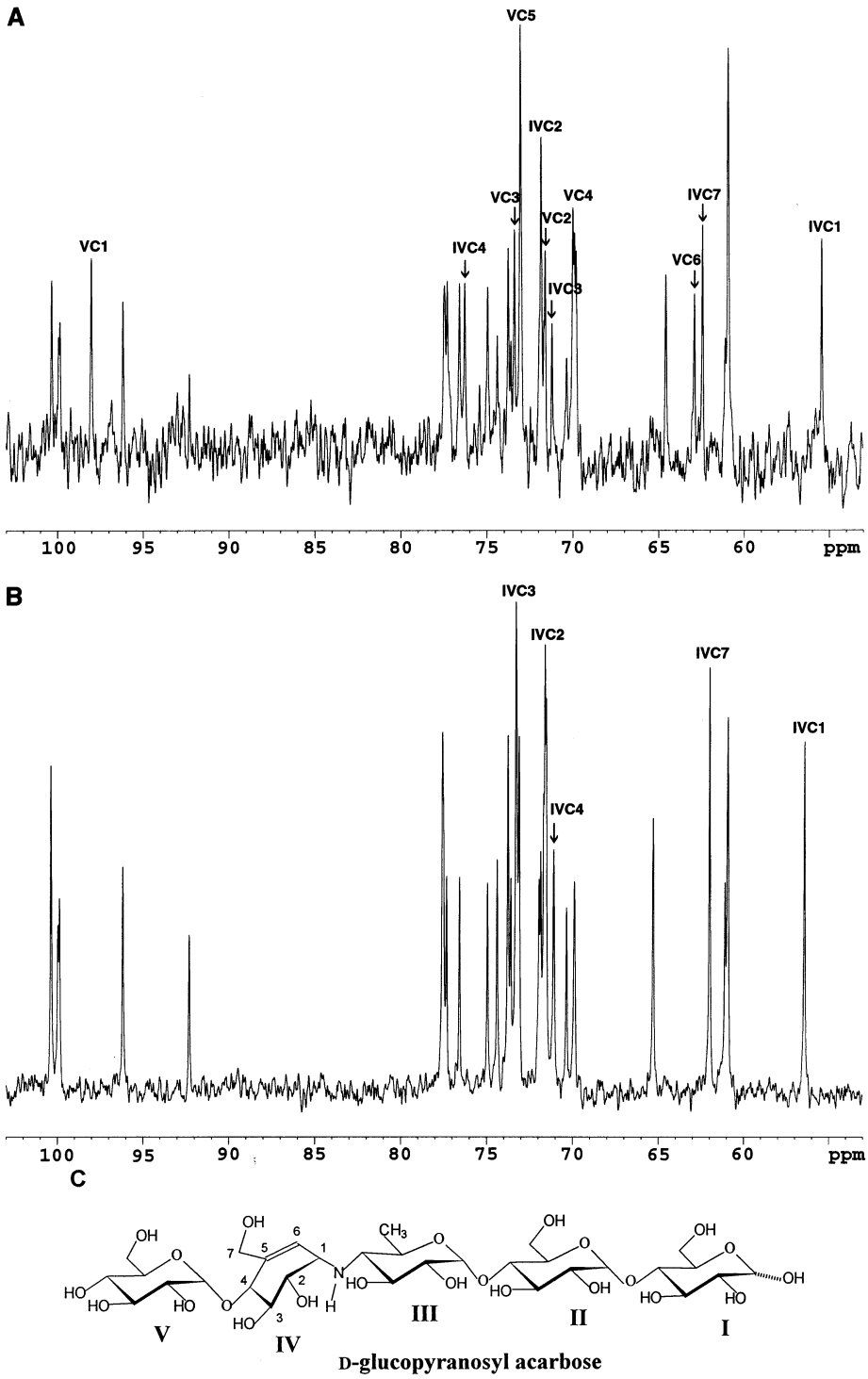

Fig. 6. 125 MHz ^{13}C NMR spectra of glucopyranosyl acarbose (A) and acarbose (B) recorded in D_2O at 25 °C and (C) proposed chemical structure of glucopyranosyl acarbose. Each of the residues of glucopyranosyl acarbose is designated by Roman numerals, starting with the reducing residue at I. The NMR peaks are designated first by the Roman numeral of the residue, followed by the particular carbon on the residue. C-4 of the cyclohexene ring would thus be IVC4, C-4 of the nonreducing D-glucopyranosyl residue would be VC4, and so forth.

The type of linkage of the maltodextrins to acarbose was determined by ^{13}C NMR of the glucoamylase product, D-glucopyranosyl acarbose, in which it was found that there was a significant downfield chemical shift of C-4 of the 5-hydroxymethyl-cyclohexene ring of 5.2 ppm, indicating that the D-glucopyranosyl unit was attached to C-4-OH of the 5-hydroxymethyl-cyclohexene ring of acarbose, and that the linkage was α be-

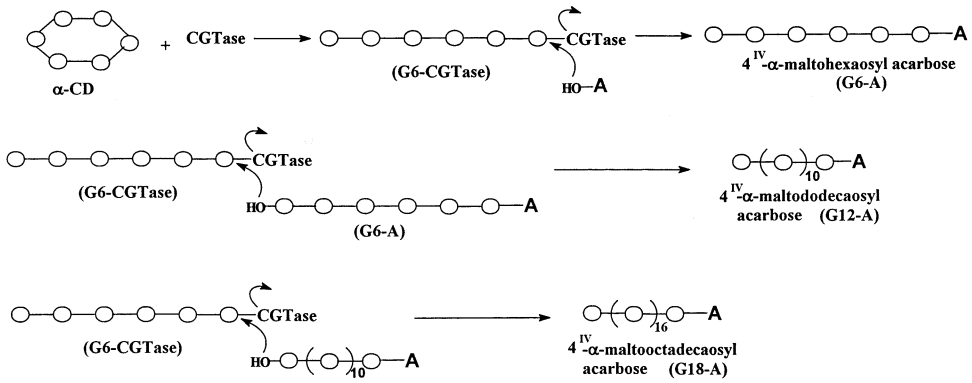

Fig. 7. Proposed mechanism for the addition of maltodextrin chains, containing multiples of six D-glucose units, to the nonreducing-end of acarbose by the reaction of acarbose with cyclomaltohexaose (α-CD) and cyclomaltodextrin glucanyltransferase (CGTase). Circles represent D-glucose residues and A represents acarbose.

cause of the formation of a peak at 98.5 ppm for the anomeric carbon of the D-glucopyranosyl unit, which is characteristic of an α linkage.[23] The maltodextrins of the three products are, thus, linked α-(1→4) to the nonreducing-end of acarbose.

The migration of the three products on TLC, their digestion by beta-amylase and glucoamylase, and the ^{13}C NMR data showed that P1 had maltohexaose (G6) attached α-(1→4) to the nonreducing-end of acarbose to give 4^{IV}-α-maltohexaosyl acarbose (G6–A); P2 had maltododecaose (G12) attached α-(1→4) to the nonreducing-end of acarbose to give 4^{IV}-α-maltododecaosyl acarbose (G12–A); and P3 had maltooctadecaose (G18) attached α-(1→4) to the nonreducing-end of acarbose to give 4^{IV}-α-maltooctadecaosyl acarbose (G18–A). P1 was formed by the reaction of acarbose with α-CD to give (G6–A); P2 was formed by reaction of P1 (G6–A) with α-CD to give (G12–A); and P3 was formed by reaction of P2 (G12–A) with α-CD to give (G18–A). There, thus, resulted a pattern of three products in which the number of D-glucose residues in the maltodextrin chains differed by multiples of six. The minor products observed in the later stages of reaction (Fig. 1) were most probably produced by disproportionation reactions between the three coupling products (P1 + P1), (P1 + P2), (P1 + P3), (P2 + P3), and (P3 + P3). The relatively low amounts of these minor products indicate that the coupling reactions were highly favored over the disproportionation reactions, even after a long reaction time of 144 h. This is interpreted by postulating that α-CD is the favored compound bound at the active-site of CGTase, which opens the cyclodextrin ring to give maltohexaose (G6) covalently linked at the active-site.[26] Acarbose then reacts with this covalent CGTase complex to give the transfer of G6 to the nonreducing-end of acarbose. The nonreducing-end of G6-acarbose can then react with the G6-enzyme complex to give G12-acarbose, which in turn can react with the G6-enzyme complex to give G18-acarbose (see Fig. 7).

In conclusion, specific, new kinds of acarbose analogues were synthesized by reaction of CGTase with α-CD and acarbose to give maltodextrins, containing multiples of six D-glucose residues added α-(1→4) to the nonreducing-end of acarbose to give G6–acarbose, G12–acarbose, and G18–acarbose.

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