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Transglycosylation reactions of *Bacillus stearothermophilus* maltogenic amylase with acarbose and various acceptors

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Abstract

It was observed that *Bacillus stearothermophilus* maltogenic amylase cleaved the first glycosidic bond of acarbose to produce glucose and a pseudotrisaccharide (PTS) that was transferred to C-6 of the glucose to give an α-(1→6) glycosidic linkage and the formation of isoacarbose. The addition of a number of different carbohydrates to the digest gave transfer products in which PTS was primarily attached α-(1→6) to D-glucose, D-mannose, D-galactose, and methyl α-D-glucopyranoside. With D-fructopyranose and D-xylopyranose, PTS was linked α-(1→5) and α-(1→4), respectively. PTS was primarily transferred to C-6 of the nonreducing residue of maltose, cellobiose, lactose, and gentiobiose. Lesser amounts of α-(1→3) and/or α-(1→4) transfer products were also observed for these carbohydrate acceptors. The major transfer product to sucrose gave PTS linked α-(1→4) to the glucose residue. α,α-Trehalose gave two major products with PTS linked α-(1→6) and α-(1→4). Maltitol gave two major products with PTS linked α-(1→6) and α-(1→4) to the glucopyranose residue. Raffinose gave two major products with PTS linked α-(1→6) and α-(1→4) to the D-galactopyranose residue. Maltotriose gave two major products with PTS linked α-(1→6) and α-(1→4) to the nonreducing end glucopyranose residue. Xylitol gave PTS linked α-(1→5) as the major product and D-glucitol gave PTS linked α-(1→6) as the only product. The structures of the transfer products were determined using thin-layer chromatography, high-performance ion chromatography, enzyme hydrolysis, methylation analysis and ^{13}C NMR spectroscopy. The best acceptor was gentiobiose, followed closely by maltose and cellobiose, and the weakest acceptor was D-glucitol.

Keywords: Maltogenic amylase; Transglycosylation; Acceptor reaction; Acarbose; Isoacarbose

1. Introduction

The enzymatic synthesis of a wide variety of oligosaccharides has been attained in vitro by transfer reactions between a segment of a donor and various kinds of acceptors. Usually the transfer takes place from a specific donor to a relatively large number of structurally different acceptors. The specificity of the transfer is dependent on the specific enzyme used, which usually determines the configuration of the glycosidic bond that is formed. The

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structure of the acceptor also often plays a role in determining the position of transfer for the formation of the glycosidic bond [1,2]. Enzymatic methods of glycosylation have many advantages over chemical methods of synthesizing oligosaccharides. For example, complicated and tedious procedures for the specific protection and deprotection of hydroxyl groups are not required and the enzymes often only transfer to one or two hydroxyl groups with the transfer to one of them greatly exceeding the other. This leads to fewer reaction steps, simpler purification procedures, and higher yields.

Many different oligosaccharides have been synthesized by enzymatic transfer reactions involving glucansucrases [1–3], cyclodextrin glucanosyltransferase [4,5], alpha-amylase [6,7], neopullulanase [8], and sialyl transferase [9], to mention only a few.

Acarbose, a pseudotetrasaccharide that has a pseudo sugar ring at the nonreducing end [4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl] linked to the nitrogen of 4-amino-4,6-dideoxy-D-glucopyranose (4-amino-4-deoxy-D-quinovopyranose), which is linked α-(1→4) to maltose (see Fig. 10 for the structure), is widely recognized as a potent inhibitor of several carbohydrases such as α-glucosidase [10], glucoamylase [11], alpha-amylase [12], and cyclodextrin glucanosyltransferase (CGTase) [13], to mention a few. Acarbose is bound in the active sites of alpha-amylase and CGTase in such a manner that the α-(1→4) glycosidic linkages are not cleaved and transglycosylation cannot occur [12]. The maltogenic amylase from *Bacillus stearothermophilus* (BSMA) [14] and *Bacillus* sp. I-5 cyclodextrinase (CDase) [15] on the other hand did bind acarbose to cleave the first glycosidic linkage and give transglycosylation of a pseudotrisaccharide (PTS) [see Fig. 10 for the structure]. Cha et al. [16] cloned the BSMA gene and showed that it hydrolyzed cyclomaltoheptaose, pullulan, starch, and acarbose and exhibited transglycosylation activity, forming both α-(1→6) and α-(1→4) glucosidic linkages [14].

In the present study, the transfer products of BSMA acting on acarbose and 17 carbohydrate acceptors are studied and their structures reported.

2. Experimental

Materials.—Acarbose was obtained from Bayer (Leverkusen, Germany) through Dr Y. Konishi at Osaka University, Japan. Acceptors were obtained from various commercial sources and were available in the laboratory.

Methods.—*Purification of recombinant BSMA enzyme*. *Escherichia coli* transformant carrying the BSMA gene [16] was cultured in a 3 L jar fermentor containing Luria–Bertani medium [17] at 37 °C for 11 h. The culture broth was centrifuged (8,000 × *g*, 4 °C, 7 min) and the cells were resuspended in 300 mL of 50 mM Tris–HCl buffer (pH 7.5). The cells were sonicated at 4 °C for 10 min (Sonicator: VC-600, Sonics & Materials Inc., Danbury, CT, USA, output 3.5, duty cycle 60, pulse ON), centrifuged (10,000 × *g*, 4 °C, 15 min), and the supernatant was used as crude BSMA. The crude BSMA was fractionated between a saturated concentration of 20% (w/v) and 50% (w/v) ammonium sulfate at 4 °C. It was dialyzed for 12 h using a cellulose dialysis membrane tube (Sigma Chemical Co., St. Louis, MO, USA, MW cut off 10,000 Da) against 50 mM Tris–HCl buffer (pH 7.5). The dialyzed enzyme solution was loaded onto a Q-Sepharose column (anion exchange, 3 × 15 cm) that was equilibrated with 50 mM Tris–HCl buffer (pH 7.5). The sample was washed with the same buffer at a flow rate of 7 mL/min, and bound proteins were eluted with a linear gradient of 0–0.5 M NaCl in 400 mL of the buffer. Active fractions from the Q-Sepharose chromatography were loaded onto a Mono-Q HR5/5 column (anion exchange; Waters, 1.0 × 10 cm), washed with the buffer at a flow rate of 1 mL/min, and eluted with a linear gradient of 0–0.5 M NaCl in 60 mL of the buffer. Active fractions from the second Mono-Q HR5/5 chromatography were concentrated and dialyzed against 20 mM Tris–HCl buffer (pH 7.5).

Assay of enzyme activity. Assay of BSMA activity was carried out according to the pro-

cedure of reducing sugar determination by the dinitrosalicylate (DNS) method [18]. The enzyme digest was composed of 250 μL of 1% (w/v) substrate solution (cyclomalto-heptaose, soluble starch, pullulan or acarbose), 160–220 μL of 50 mM sodium citrate buffer (pH 6.0), and 30–90 μL of enzyme solution. The reaction mixture was prewarmed at 55 °C for 5 min, then the diluted enzyme solution was added, and incubated for 30 min. The reaction was stopped by adding 1.5 mL of DNS solution. Absorbances were measured at 575 nm using a spectrophotometer. One unit (U) of BSMA hydrolyzing activity was defined as the amount of enzyme that forms reducing sugars to give an absorbance at 575 nm of 1.0 when the reaction was carried out at 55 °C for 30 min.

Hydrolyis and transglycosylation reactions of acarbose. The hydrolysis rate of acarbose was determined by mixing 3.9 mM acarbose in 50 mM sodium citrate buffer (pH 6.0) with BSMA. The reaction was carried out at 55 °C and samples were taken every 8 h for 24 h. The reaction was stopped by boiling for 5 min. For the acarbose transfer reaction, 10% (w/v) acarbose in 50 mM sodium citrate buffer (pH 6.0) and 20% (w/v) acceptor carbohydrate were mixed and preincubated 1 h at 55 °C. Ten units of BSMA per mg acarbose were added to the mixture and the reaction was carried out for 24 h at 55 °C. The products were analyzed by high performance ion chromatography (HPIC) and/or thin layer chromatography (TLC) after 24 h of reaction.

Preparation of isoacarbose. For the preparation of isoacarbose, the reaction condition was the same as that of the transfer reactions except that the acceptor carbohydrate was D-glucose. When the reaction was terminated, the excess amount of glucose in the reaction mixture was removed by yeast fermentation and the product was purified by gel filtration chromatography on Bio-Gel P-2 column (2 × 90 cm).

Analysis of acceptor reactions. The reaction was analyzed by TLC on Whatman K6F silica gel plates (Fischer Scientific) with 1:3:1 EtOAc–2-propanol–water. After irrigating twice, the TLC plate was dried and visualized by dipping into 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine, 5% (v/v) H_2SO_4 in MeOH, followed by heating at 120 °C for 10 min. The carbohydrates were quantitatively determined on the TLC plate by densitometry [20].

Analysis of acceptor reaction products by HPIC. The reaction mixture was mixed with an equal volume of acetonitrile, boiled for 5 min, centrifuged at $12{,}000 \times g$ for 5 min, and filtered using a membrane filter kit (0.2 μm pore diameter, Gelman Sciences); 20 μL of the sample was applied to a Dionex Carbopak PA1 column (0.4 × 25 cm, 10 μm particle diameter) and eluted with a 0 to 30% (v/v) of 600 mM NaOAc gradient and 150 mM NaOH at a flow rate of 1.0 mL/min.

Methylation analysis. The methylation was carried out according to Mukerjea et al. [19].

NMR analysis. The ^{13}C nuclear magnetic resonance spectrum was recorded with JNM LA-400 FT-NMR spectrometer (Jeol, Japan) and Heteronuclear Multiple Bond Connectivity (HMBC) mode.

3. Results

TLC and HPIC chromatograms were obtained for the acceptor reactions of BSMA with acarbose and various acceptors (Figs. 1–4). The two types of chromatography complimented each other in their ability to separate the various compounds in the reactions. The TLC mobility of compounds having α-(1→4) and α-(1→3) linkages was faster than compounds having α-(1→6) linkages while the opposite was observed for HPIC.

Analysis of the acceptor reactions with monosaccharides.—Fig. 1 shows the TLC analyses of the products in the acceptor reactions with monosaccharides. When D-glucose was added as an acceptor, one major and one minor product were formed, **3** and **4**, respectively in lane B of Fig. 1. Methylation analysis of the acceptor products (lane E in Fig. 9) gave 2,3,6-, 2,4,6-, and 2,3,4-tri-*O*-methyl-D-glucose. The 2,3,6-tri-*O*-methyl-D-glucose was formed from the first glucopyranose residue of PTS. The 2,3,4-tri-*O*-methyl-D-glucose was formed from the linkage of PTS to D-glucose

in the major acceptor product, indicating that the linkage was α-(1→6).

The formation of α-(1→6) linkages was confirmed by ^{13}C NMR analysis. The major product was isolated by column chromatography, and its ^{13}C NMR spectrum was obtained (Fig. 5). The chemical shifts for the anomeric carbons of acarbose are 100.3 and 100.4 ppm, corresponding to an α-(1→4) linkage. The chemical shifts of the anomeric carbons of the acceptor product was 98.6 and 98.7 ppm, corresponding to an α-(1→6) linkage. The presence of the α-(1→6) linkage was also confirmed by HMBC, and the structure was found to be {[4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]4-amino-4-deoxy}-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose. We have called this α-(1→6)-linked acceptor product of D-glucose, *isoacarbose*. The 2,4,6-tri-*O*-methyl-D-glucose, formed in lesser amount, was from the minor acceptor product, indicating that PTS was linked α-(1→3) to D-glucose. These results were in agreement with those obtained by HPIC (Fig. 4). The HPIC chromatogram (Fig. 4) gave a distinct separation of two

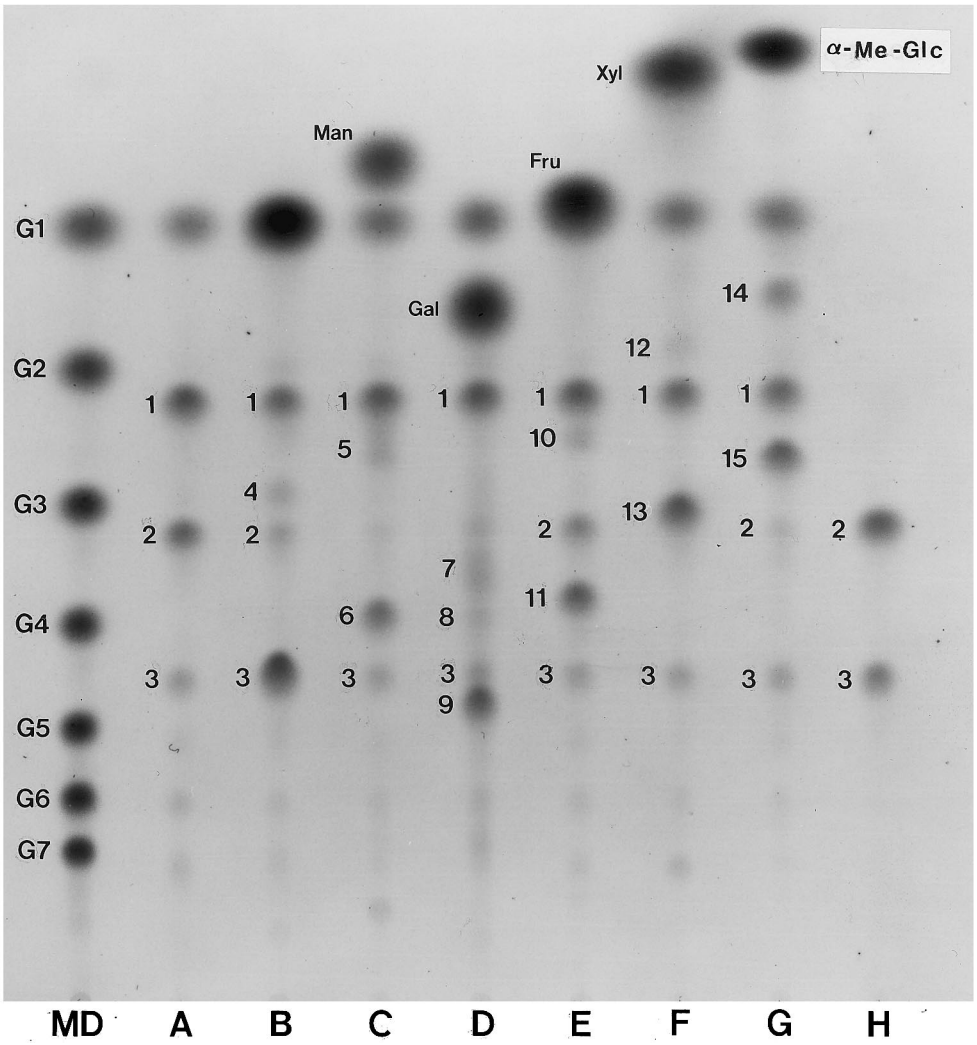

Fig. 1. TLC of the acceptor reactions of acarbose with various acceptors catalyzed by *Bacillus stearothermophilus* maltogenic amylase. MD = maltodextrin standards (G1–G7); lane A, reaction with acarbose; lane B, glucose acceptor reaction; lane C, mannose acceptor reaction; lane D, galactose acceptor reaction; lane E, fructose acceptor reaction; lane F, xylose acceptor reaction; lane G, methyl α-D-glucopyranoside; lane H, acarbose and isoacarbose standards. **1** = pseudotrisaccharide (PTS); **2** = acarbose; **3** = isoacarbose; **4** = minor glucose acceptor product; **5** = minor mannose acceptor product; **6** = major mannose acceptor product; **7** and **8** = minor galactose acceptor products; **9** = major galactose acceptor product; **10** = minor fructose acceptor product; **11** = major fructose acceptor product; **12** = minor xylose acceptor product; **13** = major xylose acceptor product; **14** and **15** = acceptor products of methyl α-D-glucopyranoside.

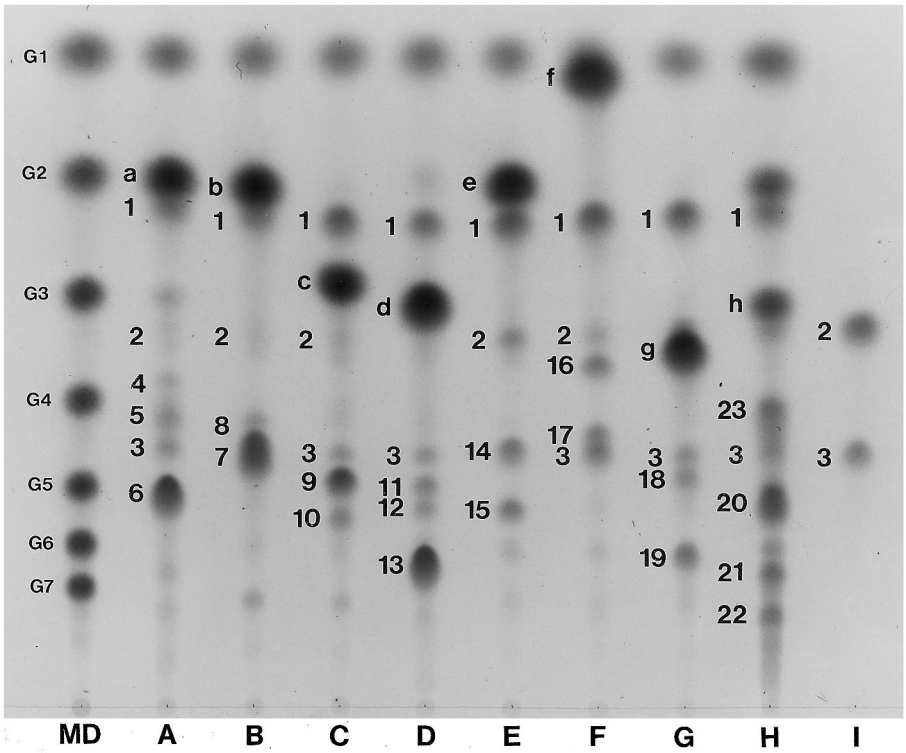

Fig. 2. TLC of the acceptor reactions of di- and tri-saccharides with acarbose. MD = maltodextrin standards (G1–G7); lane A, maltose acceptor reaction; lane B, cellobiose acceptor reaction; lane C, lactose acceptor reaction; lane D, gentiobiose acceptor reaction; lane E, α,α-trehalose acceptor reaction; lane F, sucrose acceptor reaction; lane G, raffinose acceptor reaction; lane H, maltotriose acceptor reaction; lane I, acarbose and isoacarbose standards. **1** = pseudotrisaccharide (PTS); **2** = acarbose; **3** = isoacarbose; **4** = maltose acceptor product; **5** = maltose acceptor product; **6** = maltose acceptor product; **7** and **8** = cellobiose acceptor products; **9** and **10** = lactose acceptor products; **11**, **12** and **13** = gentiobiose acceptor products; **14** and **15** = α,α-trehalose acceptor products; **16** and **17** = sucrose acceptor products; **18** and **19** = raffinose acceptor products; **20**–**22** = maltotriose acceptor products; **23** = maltotetraose (G4). a = maltose; b = cellobiose; **c** = lactose; d = gentiobiose; e = α,α-trehalose; f = sucrose; g = raffinose; h = maltotriose.

peaks that corresponded to the minor products linked α-(1→4), *acarbose*, and α-(1→3), *nigeroacarbose*. The component with the α-(1→3) linkage migrates faster than products with α-(1→4) linkages in HPIC, peaks 4 and 5 in Fig. 4 corresponding to the α-(1→3) and α-(1→4)-linked acceptors, respectively.

Reaction of acarbose in the absence of any added acceptor (lane A of Fig. 1) gave D-glucose, PTS (**1** in lane A of Fig. 1), and isoacarbose (compare **3** in lane A with **3** in lanes B and H of Fig. 1). The acceptor reaction of methyl α-D-glucopyranoside also gave two products, **14** and **15** in lane G of Fig. 1. Methylation analysis of **14** (see lane C in Fig. 9) gave 2,3,6- and 2,4,6-tri-*O*-methyl-D-glucose. The 2,3,6-tri-*O*-methyl-D-glucose comes from the first glucopyranose residue of PTS and possibly also from a minor product linked α-(1→4) and the 2,4,6-tri-*O*-methyl-D-glucose was from the methyl α-D-glucopyranoside residue of the acceptor product. Because in the methylation analysis the formation of 2,3,6-tri-*O*-methyl-D-glucose can arise from both the glucose residue of PTS and from the glucose residue from an α-(1→4)-linked product, the α-(1→4) glucose acceptor product can only be detected by measuring the relative intensities of 2,3,6- and 2,4,6-tri-*O*-methyl-D-glucose. If only an α-(1→3)-linked product is formed, the intensities of the two methylated glucoses would be the same, but if an α-(1→4)-linked product is also formed, the intensity of 2,3,6 - tri - *O* - methyl - D - glucose would be greater than the intensity of 2,4,6-tri-*O*-methyl-D-glucose. The results showed that 2,3,6-tri-*O*-methyl-D-glucose had 35% higher intensity than 2,4,6-tri-*O*-methyl-D-glucose, indicating that an α-(1→4)-linked acceptor product was formed. Methylation analysis of **15** (lane D in Fig. 9) gave 2,3,6- and

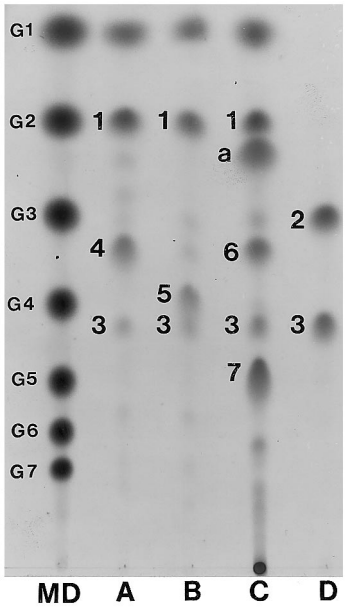

Fig. 3. TLC of the acceptor reactions of sugar alcohols with acarbose. MD = maltodextrin standards (G1–G7); lane A, xylitol acceptor reaction; lane B, D-glucitol acceptor reaction; lane C, maltitol acceptor reaction; lane D, acarbose and isoacarbose standards. **1** = pseudotrisaccharide (PTS); **2** = acarbose; **3** = isoacarbose; **4** = xylitol acceptor product; **5** = D-glucitol acceptor product; **6** and **7** = maltitol acceptor products; a = maltitol.

2,3,4-tri-*O*-methyl-D-glucose, indicating the formation of both α-(1→4)- and α-(1→6)-linked products. As with compound **14**, the 2,3,6-tri-*O*-methyl-D-glucose was from the first glucopyranosyl residue of PTS and the 2,3,4-tri-*O*-methyl-D-glucose indicates that PTS is linked α-(1→6) to methyl α-D-glucopyranoside. The acceptor reaction of methyl β-D-glucopyranoside gave acceptor products that were similar to those of methyl α-D-glucopyranoside (data not shown).

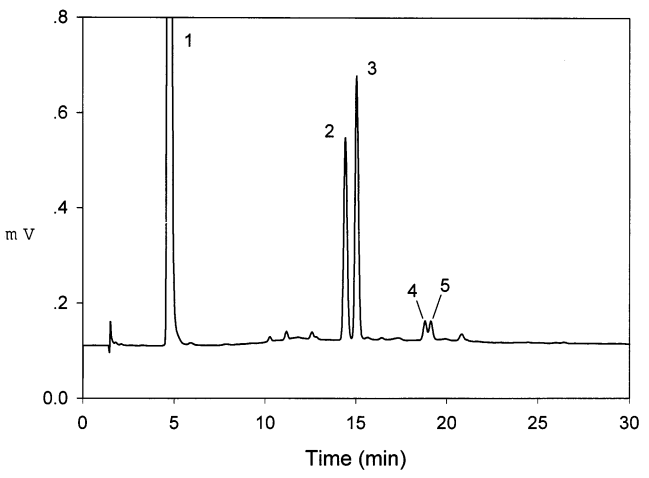

Fig. 4. High performance ion chromatogram of the acceptor reactions of glucose. **1** = glucose; **2** = pseudotrisaccharide (PTS); **3** = isoacarbose; **4** = 3^1-α-PTS-glucose; **5** = acarbose.

The acceptor reactions of D-mannose (lane C of Fig. 1) and D-galactose (lane D of Fig. 1) were similar to those of D-glucose. The major product with D-mannose is **6** in lane C and from its chromatographic mobility it is postulated to have PTS linked α-(1→6) to D-mannose and the minor product, **5** in lane C, is likewise postulated to have PTS linked α-(1→3). The major product with D-galactose is **9** in lane D and it is postulated to have PTS linked α-(1→6) to D-galactose and the minor products, **7** and **8** in lane D, are postulated to have PTS linked α-(1→3) and α-(1→4), respectively.

The major acceptor product of D-fructose (**11** in lane E of Fig. 1) is postulated to have PTS linked α-(1→5) to D-fructopyranose and the minor product, **10** in lane E, to have PTS linked α-(1→4) to D-fructopyranose. This is based on the fact that D-fructose exists primarily in the pyranose ring form in solution and that by analogy with the acceptor reactions of dextransucrase, the major product is D-glucose linked α-(1→5) to D-fructopyranose (leucrose) and the minor product is D-glucose linked α-(1→4) to D-fructofuranose (isomaltulose) [1,21]. The major product of D-xylose (**13** in lane F of Fig. 1) is postulated to have PTS linked α-(1→4) to D-xylopyranose (the pyranose ring is the major form of D-xylose in solution) and the minor product (**12** in lane F of Fig. 1) is postulated to have PTS linked α-(1→3) to D-xylopyranose.

D-Glucose, PTS, and isoacarbose were formed in all of the acceptor reactions due to the hydrolysis of acarbose to D-glucose and PTS, of which the latter is transferred to D-glucose to give isoacarbose. The principal linkage formed in the transfer reactions of PTS to the various monosaccharides was α-(1→6) with minor transfers to form α-(1→3)- and/or α-(1→4)-linkages. The exceptions being the transfer to D-fructose in which the major linkage was α-(1→5) and the minor linkage was α-(1→4), and the transfer to D-xylose in which the major linkage was α-(1→4) and the minor linkage was α-(1→3).

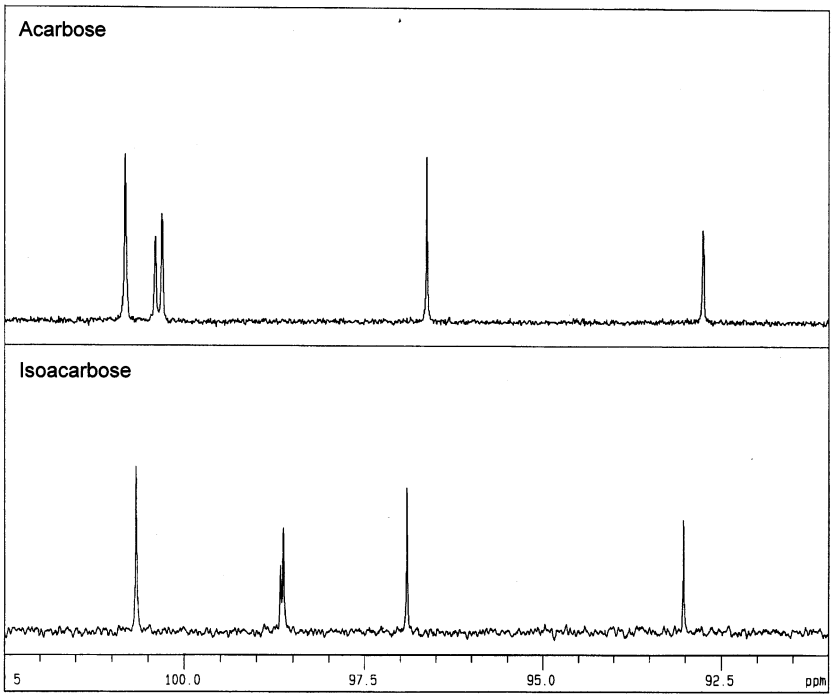

Fig. 5. ^{13}C NMR spectrum of acarbose and the carbohydrate acceptor product produced by the action of BSMA on acarbose.

Analysis of the acceptor reactions with disaccharides.—Fig. 2 shows the TLC analyses of the compounds in the acceptor reactions with various disaccharides. When maltose was the acceptor (lane A of Fig. 2), the major acceptor product was **6** with minor products **4** and **5** (Fig. 6); **3** was isocarbose formed in the transfer of PTS to D-glucose. When the maltose acceptor digest was treated with pullulanase (lane F of Fig. 7), the major acceptor product, (**6** in lane E of Fig. 7) completely disappears and PTS and maltose increase, indicating that pullulanase is hydrolyzing an α-(1→6) linkage and the major acceptor product of maltose is 6^2-α-PTS-maltose (Fig. 8(A)).

The acceptor reaction with cellobiose indicated a major acceptor product (**7** in lane B of Fig. 2) and a minor product (**8** in lane B of Fig. 2). Methylation analysis of the mixture of acceptor products from the cellobiose reaction (lane F of Fig. 9) gave 2,3,6-, 2,4,6-, and 2,3,4-tri-*O*-methyl-D-glucose with 2,3,4-tri-*O*-methyl-D-glucose in larger amounts than the 2,4,6-tri-*O*-methyl-D-glucose, indicating that the major acceptor product **7** in lane B of Fig. 2 is 6^2-α-PTS-cellobiose and the minor acceptor product **8** in lane B of Fig. 2 is 3^2-α-PTS-cellobiose. The 2,3,6-tri-*O*-methyl-D-glucose comes from both PTS and the reducing-end of the cellobiose and is the major methylated glucose produced.

The acceptor reaction with lactose gave one major product (**9** in lane C of Fig. 2) and a minor product (**10** in lane C of Fig. 2). The major product has a TLC mobility less than the major product of cellobiose, as would be expected for a compound with PTS attached α-(1→6) to the D-galactose residue of lactose. The minor product, **10**, has not been identified but could possibly be PTS linked α-(1→4) or α-(1→3) to the D-galactose residue of lactose. In the HPIC chromatographic analysis (data not shown) of the lactose acceptor products, the major product migrated faster than the minor product, supporting the conclusion above that the linkage of PTS to lactose is α-(1→6).

The acceptor product of gentiobiose gave one major product (**13** in lane D of Fig. 2). This acceptor product had a TLC mobility that was considerably slower than that of the α-(1→6)-linked acceptor product of lactose. Gentiobiose is the β-(1→6) isomer of cellobiose and would be expected to migrate slower than either cellobiose or lactose and hence its PTS acceptor product would also be

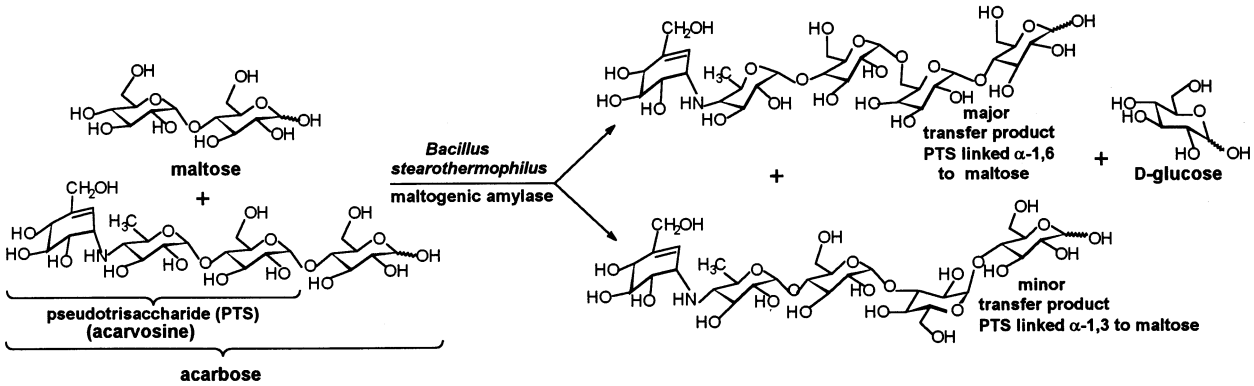

Fig. 6. Reactions of BSMA with acarbose and maltose, forming the major acceptor products of pseudotrisaccharide (PTS) linked α-(1→6) (major product) and α-(1→3) (minor product) to the nonreducing glucose residue of maltose. The formation of the α-(1→4) linkage (very minor product) is not shown here.

expected to migrate slower than the PTS acceptor products of cellobiose and lactose. The structure of the major gentiobiose acceptor product is thus postulated to be 6^2-α-PTS-gentiobiose. The minor acceptor products of gentiobiose, **11** and **12** in lane D of Fig. 2, are postulated to have PTS linked α-(1→3) and α-(1→4) to gentiobiose.

The acceptor reaction with sucrose gave two major products, **16** and **17** in lane F of Fig. 2. The structures of the two sucrose acceptor products were determined using invertase. Invertase is a β-fructofuranosidase that specifically hydrolyzes the 2-β-fructofuranosyl linkage of sucrose. Invertase reaction with the sucrose acceptor products (**4** and **5** in lane C of Fig. 7) gave acarbose and isoacarbose (**2** and **3** in lane D of Fig. 7), indicating that **4** in lane C of Fig. 7 (**16** in lane F of Fig. 2) had PTS linked α-(1→4) to sucrose and **5** in lane C of Fig. 7 (**17** in lane F of Fig. 2) had PTS linked α-(1→6) to sucrose (see Fig. 8(B,C) for a summary of the reactions of invertase with acceptor products of sucrose).

The acceptor reaction with α,α-trehalose also gave two major products, **14** and **15** in lane E of Fig. 2, similar to sucrose. From their TLC mobility and similarity to the sucrose acceptor products, the two α,α-trehalose acceptor products are postulated to have PTS linked α-(1→4) and α-(1→6), respectively.

Analysis of the acceptor reactions with trisaccharides.—The acceptor reactions with raffinose gave one major product and one minor product (**19** and **18**, respectively in lane G of Fig. 2). From the chromatographic mobility of these products and the specificity pattern that has developed for the transfer reactions of BSMA with acarbose, it is postulated that the structure of the major raffinose acceptor product is 6^{Gal}-α-PTS-raffinose and the structure of the minor acceptor product is 4^{Gal}-α-PTS-raffinose.

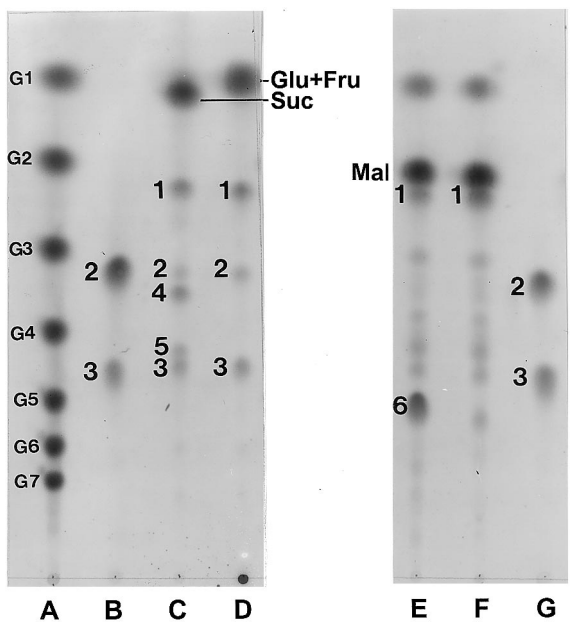

Fig. 7. TLC analysis of the reaction of the sucrose acceptor product with invertase (lane D) and the reaction of the maltose acceptor product with pullulanase (lane F). Lane A, maltodextrin standards; lane B, **2** = acarbose and **3** = isoacarbose standards; lane C, acceptor products of sucrose, **1** = pseudotrisaccharide (PTS), **2** = acarbose, **3** = isoacarbose, **4** = sucrose acceptor product I, **5** = sucrose acceptor product II; lane D = sucrose acceptor products reacted with invertase, **1** = PTS, **2** = acarbose, and **3** = isoacarbose; lane E, **1** = PTS; **6** = maltose acceptor reaction product; lane F, maltose acceptor product reacted with pullulanase, **1** = PTS; lane G, **2** = acarbose and **3** = isoacarbose standards.

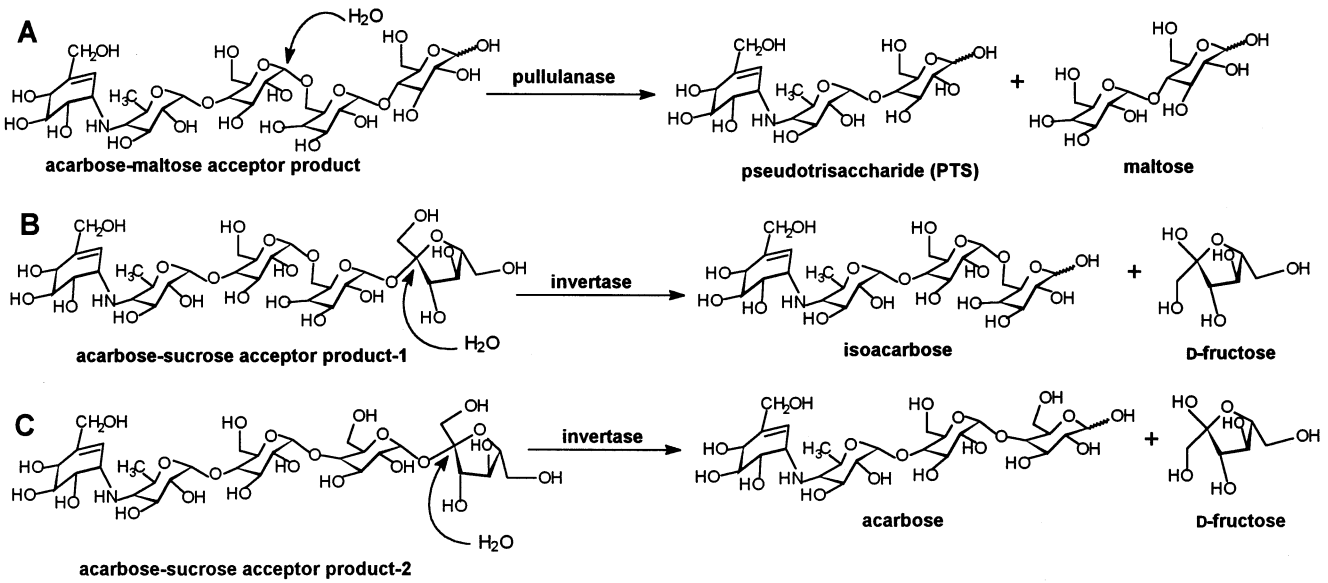

Fig. 8. Summary of the reactions of pullulanase and invertase with maltose and sucrose acceptor products. (A) Reaction of pullulanase with the PTS-maltose acceptor product. (B) Reaction of invertase with the PTS-sucrose acceptor products, linked to 6^{Glc} of sucrose and (C) reaction of invertase with the PTS-sucrose acceptor, linked to 3^{Glc} of sucrose.

The acceptor reaction with maltotriose is complex in that maltotriose can be both an acceptor for the transfer of PTS from acarbose and a hydrolytic substrate for BSMA that gives glucose and maltose. The acceptor reactions obtained with maltotriose had both maltotriose acceptor products and maltose and glucose acceptor products. This is seen in lane H of Fig. 2 in which **20** is the major maltose acceptor product (compare **20** in lane H with **6** in lane A); **21** and **22** migrate more slowly and are the acceptor products of maltotriose. The chromatographic mobilities of **21** and **22** indicate that PTS is linked α-(1→4) and α-(1→6), respectively to maltotriose, in about equal amounts.

Analysis of the acceptor reactions with sugar alcohols.—Fig. 3 shows the TLC analysis of the components in the acceptor reactions with xylitol, D-glucitol, and maltitol. Xylitol gave one major product (**4** in lane A of Fig. 3). From the chromatographic mobility, it is postulated that PTS is linked α-(1→5) to xylitol. D-Glucitol also gave one major product (**5** in lane B of Fig. 3) that is postulated to have PTS linked α-(1→6) to D-glucitol. The acceptor reaction with maltitol gave a major product and a minor product (**6** and **7**, respectively, in lane C of Fig. 3); it is postulated that the minor product, **6**, has PTS linked either α-(1→3) or α-(1→4) and the major product, **7**, has PTS linked α-(1→6) to the glucopyranose residue.

Relative amounts of the acceptor products.—The quantitative amounts of the acceptor products were determined by quantitative TLC densitometry [20]. Table 1 shows that gentiobiose was the best acceptor. The relative efficiencies of the individual acceptor reactions were determined by summing the amounts of products of each individual acceptor and dividing the sums by the sum of the products from gentiobiose. The efficiencies of maltose (99%) and cellobiose (92%) closely followed that of gentiobiose. D-Glucose, methyl α-D-glucopyranoside, D-galactose, and D-xylose were relatively strong acceptors with efficiencies between 82 and 85%. Maltitol (69%), D-fructose (66%), lactose (66%), and D-mannose (66%) were moderate acceptors. Xylitol (40%), α,α-trehalose (25%), and D-glucitol (16%) were relatively weak acceptors.

The results indicate that many different carbohydrates were acceptors for the transfer of PTS from acarbose by BSMA, and this enzyme favors an acceptor with a pyranose ring structure. Further, the transfer is primarily to a C-6, primary alcohol, to form an α-(1→6) glucosidic linkage, but not exclusively so, similar to the transfer acceptor reactions of dex-

transucrase in which the position of the transfer reaction is partially dependent on the structure of the acceptor [1].

4. Discussion

Tonozuka et al. [7] proposed that there were two stages in the transglycosylation reaction of *Thermoactinomyces vulgaris* R-47 alpha-amylase (TVA). The first is the transglycosylation products followed by the second, the hydrolysis of the accumulated products. The hydrolysis of the transglycosylation products depends on the chemical structure of the acceptor molecules. If the acceptor products contain α-(1→4)-linked glucose residues, they would be readily hydrolyzed by the alpha-amylase. However, if after hydrolysis, the resulting products do not have α-(1→4) linkages that could be hydrolyzed, the products would accumulate. For example, if the donor fragment from the transglycosylation was linked to the acceptor by an α-(1→3) or α-(1→6) glycosidic linkage, the product would not be hydrolyzed and would accumulate. In the present study, we analyzed the acceptor products after reaction had occurred for 24 h and equilibrium had been attained.

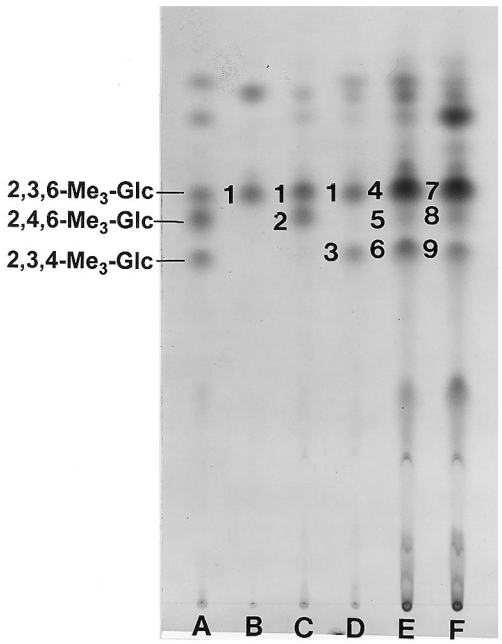

Fig. 9. TLC separation of the methylation analysis from acarbose transfer products. Lane A, *O*-methylated glucoses as standards; lane B, *O*-methylated sugars from acarbose; lane C, *O*-methylated sugars from the acceptor reaction product of methyl α-D-glucopyranoside, (**14** in lane G, Fig. 1); lane D, *O*-methylated sugars from the acceptor reaction product of methyl α-D-glucopyranoside (**15** in lane G of Fig. 1); lane E, *O*-methylated sugars from the acceptor reaction mixture of glucose in which the unreacted glucose and PTS were removed using Bio-Gel P-2 column; lane F, *O*-methylated sugars from the acceptor reaction mixture of cellobiose in which PTS and the unreacted cellobiose and acarbose were removed from the mixture, using Bio-Gel P-2 column chromatography.

TVA transferred a glucosyl residue from the donor (pullulan) to the acceptor molecules with the formation of both α-(1→4) and α-(1→6) linkages [7]. α-(1→3)-Linked acceptor products were not observed. Similar observations have been reported for neopullulanase [8] and *B. licheniformis* maltogenic amylase [6]. In the present study of the acceptor reaction of BSMA, the formation of α-(1→3) and α-(1→6) linkages were observed and the products accumulated in significant amounts.

The reactions of BSMA with acarbose, with acarbose and D-glucose, and with acarbose and maltose, are summarized in Fig. 10. Acarbose is hydrolyzed to D-glucose and PTS (reactions (A1) and (A2) in Fig. 10). When D-glucose is present in the reaction mixture, PTS is transferred from acarbose to C-4, C-3, and C-6 of D-glucose to give *acarbose*, *nigeroacarbose*, and *isoacarbose* (reactions B1, B2 and B3, respectively, in Fig. 10). When other carbohydrates, such as maltose, are present, PTS is transferred to C-6 or C-3 of the nonreducing end glucose residue (reactions (C1) and (C2), respectively, in Fig. 10). BSMA can also transfer PTS to C-4 of the nonreducing glucose residue of maltose (reaction (C3) in Fig. 10) and then hydrolyze the second α-(1→4) glucosidic linkage of acarbose, giving maltose and PTS (reaction (D) in Fig. 10).

When other acceptors are added, BSMA cleaves the first α-(1→4) glucosidic linkage of acarbose and transfers PTS to the acceptors, primarily forming α-(1→6) linkages between PTS and the acceptors, although α-(1→3) and α-(1→4) linkages are also formed. With some acceptors, such as D-fructopyranose and D-xylopyranose, α-(1→5) and α-(1→4) linkages are formed, respectively. The principal linkage was to a primary alcohol, if it was present on the acceptor.

Table 1
Products of the transfer reaction of *Bacillus stearothermophilus* maltogenic amylase with acarbose and acceptors

Acceptor	PTS[a] acceptor products[b] (mg/mL)[c]					Relative efficiency[d] (%)
	α-(1→6)	α-(1→5)	α-(1→4)	α-(1→3)	Total	
D-Glucose	7.40			1.81	9.21	82
Maltose	9.27			(1.88)	11.15	99
D-Mannose	4.20		(3.19)		7.39	66
D-Galactose	5.43		(1.45)	(2.34)	9.22	82
Lactose	5.55		(1.82)		7.37	66
Cellobiose	8.84			(1.5)	10.35	92
Gentiobiose	8.20		(1.24)	(1.80)	11.24	100
D-Glucitol	1.82				1.82	16
Xylitol		(2.66)	(0.77)	(1.08)	4.51	40
α,α-Trehalose	(1.59)		(1.17)		2.76	25
Raffinose	2.73		(1.87)		4.60	41
D-Fructose		(5.04)	(2.41)		7.45	66
Sucrose	1.97		4.05		6.02	54
Maltotriose	2.69		(3.63)		6.32	56
D-Xylose			6.54	(3.01)	9.55	85
Maltitol	3.84		(3.93)		7.77	69
Methyl α-D-glucopyranoside	4.29		2.90	2.20	9.39	84

[a] PTS is the pseudotrisaccharide produced by the cleavage of the first glucosidic linkage.
[b] The structures of the acceptor products in parenthesis were estimated by their relative mobilities on TLC and HPIC and were not definitively determined.
[c] The amounts of the acceptor products were determined by TLC densitometry [20].
[d] The relative transfer efficiencies were determined by comparing the total amounts of the acceptor products to those of gentiobiose, the best observed acceptor.

The crystallographic structures of amylolytic carbohydrases, such as alpha-amylase, CGTase, and glucoamylase complexed with acarbose have been reported [11–13]. None of these enzymes hydrolyzed the acarbose or produced transglycosylation products. The binding of the acarbose with the active sites of these enzymes are such that the potentially susceptible α-(1→4) glycosidic linkages of acarbose are not favorably positioned for the catalytic groups to produce cleavage. Brzozowski and Davies [12] have speculated that alpha-amylase might hydrolyze and produce transglycosylation of acarbose if acarbose was bound at subsites −3 to +1, placing the α-(1→4) glycosidic bonds in position for cleavage. Until the present report, the enzymatic hydrolysis of the α-(1→4) glycosidic bonds of acarbose or the transglycosylation of an acarbose fragment to acceptors has not been reported. The binding of acarbose with the subsites of BSMA must be such that the first α-(1→4) glycosidic linkage is favorably placed in conjunction with the catalytic groups to produce hydrolysis and transglycosylation. The acarbose must be so specifically bound to the active site of BSMA that only the first α-(1→4) glycosidic linkage is cleaved. The second α-(1→4) glycosidic linkage of acarbose apparently is not favorably positioned to give its cleavage and the formation of maltose and the pseudo-disaccharide, acarviosine. There is the possibility that the PTS transfer products obtained in this study might be specific inhibitors for various other carbohydrases.

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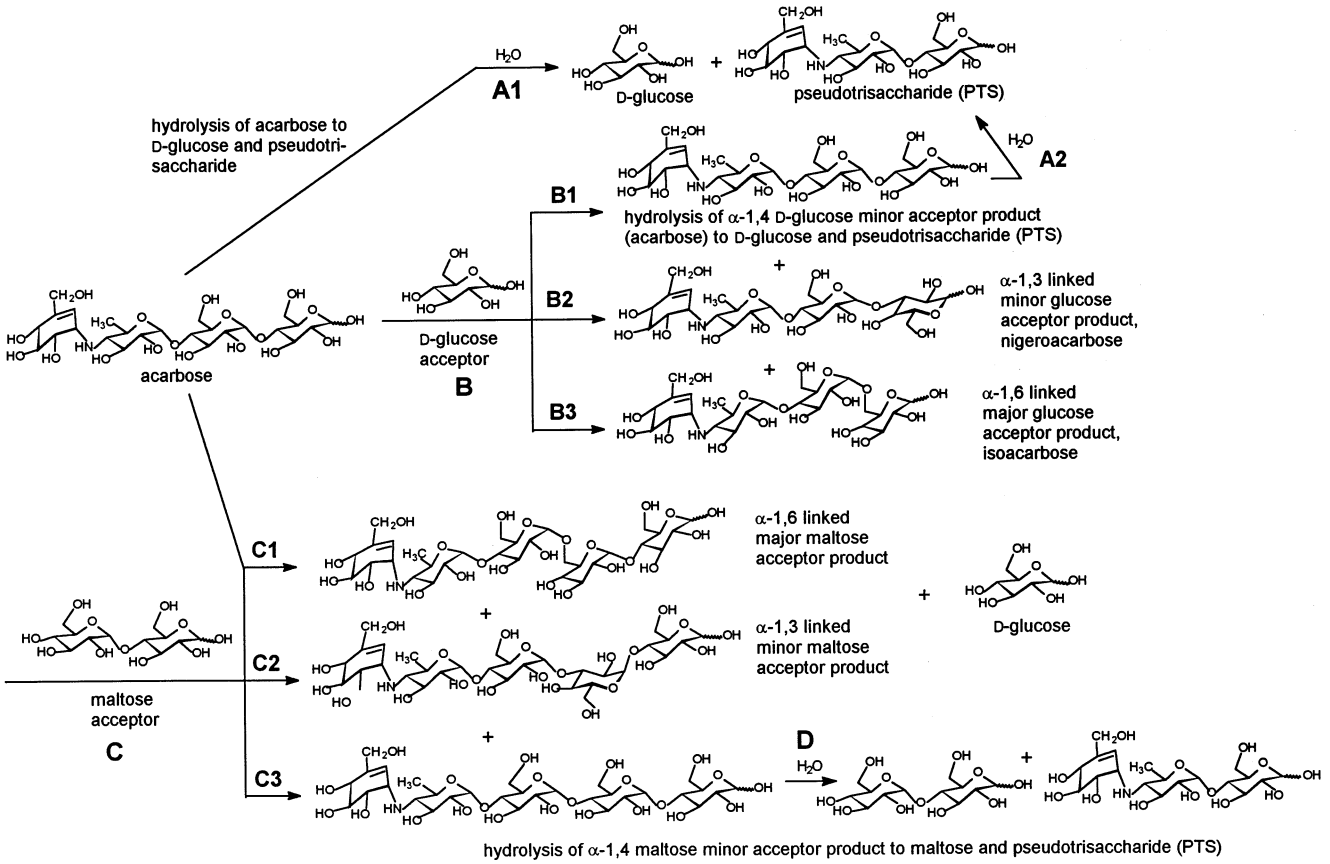

Fig. 10. Summary of the reactions of *Bacillus stearothermophilus* maltogenic amylase with acarbose to give (A) hydrolysis, (B) transfer of PTS to D-glucose, and (C) transfer of PTS to maltose.

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