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Trapping of a covalent enzyme intermediate in the reaction of *Bacillus macerans* cyclomaltodextrin glucanyltransferase with cyclomaltohexaose

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Abstract

The mechanism of catalysis of *Bacillus macerans* cyclomaltodextrin glucanyltransferase (CGTase, EC 2.4.1.19) was studied by trapping and isolating a covalent-enzyme intermediate. CGTase catalyzes an acceptor or coupling reaction between cyclomaltohexaose and a carbohydrate acceptor such as D-glucose. CGTase was incubated with ^{3}H-labeled cyclomaltohexaose in the absence of any added acceptor. After 30 s of reaction, the enzyme was rapidly denatured and precipitated by the addition of 10% trifluoroacetic acid (TFA). Extensive washing of the precipitated protein showed retention of radioactivity with the protein. The precipitate was dissolved in 0.1 M TFA, containing 6 M urea and passed over a BioGel P-10 column. The protein fraction retained 95% of its original radioactivity. The reaction with [^{3}H]cyclomaltohexaose was also stopped by the addition of TFA to give an inactive enzyme at pH 2.5. The enzyme was separated from unreacted cyclomaltohexaose on a BioGel P-10 column and was shown to be radioactive. When the radioactive protein fraction was rechromatographed on BioGel P-10, it retained 100% of the label. These results demonstrate the formation of a covalent carbohydrate–enzyme intermediate in the reactions catalyzed by CGTase.

Keywords: Cyclomaltodextrins; Cyclomaltodextrin glucanyltransferase; CGTase; Enzyme mechanism; Transglycosylation reactions

1. Introduction

Cyclomaltodextrin glucanyltransferase (CGTase, EC 2.4.1.19) is elaborated by a number of bacterial species. The first and probably best known is *Bacillus macerans* CGTase, which forms cyclomaltohexaose as the primary product from starch. The enzyme catalyzes three kinds of reactions: (1) the formation of cyclic, nonreducing, α-(1→4)-linked maltodextrins, composed of six, seven, and eight D-glucose residues, from the nonreducing-ends of starch chains;[1,2] (2) the so-called 'coupling reactions' when the cyclomaltodextrins are opened and transferred to other carbohydrates, such as D-glucose, methyl α-D-glucopyranoside, maltose, panose, and so forth;[3] and (3) disproportionation reactions between two maltodextrin molecules.[4] When cyclomaltohexaose and D-[^{14}C]glucose are the substrates in the coupling reaction, labeled maltoheptaose is first formed with the label exclusively located in the reducing-end D-glucose moiety.[5] Two maltoheptaose (G7)

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molecules then undergo the disproportionation reaction (reaction 3) to give a series of maltodextrin products, G2 + G12, G3 + G11, G4 + G10, and so forth with the reducing-end D-glucose moiety of the maltodextrins exclusively labeled. The three reactions, cyclization, coupling, and disproportionation are all transglycosylation reactions in which a glucanosyl chain is transferred to an acceptor.

In the present study, we have investigated the mechanism of action of CGTase by using cyclomaltohexaose that was labeled with ^{3}H at the six C-6 carbons in the absence of an acceptor, and rapidly quenching the reaction by addition of acid to precipitate the enzyme or by lowering the pH to 2.5 to inactivate the enzyme action.

2. Experimental

Chemicals.—Cyclomaltohexaose was a gift from Ensuiko Sugar Refining Co. Ltd. (Yokohama, Japan). DEAE-cellulose was obtained from Sigma Chemical Co. (St. Louis, MO).

Synthesis of 6-[^{3}H]cyclomaltohexaose.—6-[^{3}H]Cyclomaltohexaose was prepared by a modification of the method described by Westlake and Hill[6] for the oxidation and reduction of the primary alcohol groups of amylose. The primary alcohol groups of cyclomaltohexaose were oxidized by pyridinium dichromate, followed by reduction with sodium borotritide. Pyridinium dichromate (0.4 mmol) was dissolved in 0.15 mL of concd H_2SO_4, followed by dilution to 1 mL with deionized water. Oxidation was carried out by adding the pyridinium dichromate solution to 1 mL of 0.36 M cyclomaltohexaose and allowing the oxidation to take place at 20 °C for 18 h. The oxidation reaction was stopped by the addition of solid $BaCO_3$ until there was no color. The solution was deionized by the addition of 1 g AF11A8 ion-exchange resin (50–100 mesh, obtained from Bio-Rad) with stirring for 30–45 min. The resin was removed by centrifugation. The deionization step was repeated three times. The oxidized cyclomaltodextrin was reduced by slowly adding 5 mL of 80 mM KOH solution, containing 1 μCi (0.2 mmol) sodium borotritide, over 20 h. The reduction was terminated by neutralization with 0.6 M HCl, making the final pH 2. The acidic solution was evaporated under vacuo three-times in 5 mL of water. After the final evaporation, the solids were dissolved in 1 mL of water and added to a BioGel P-2 column (2.5 × 50 cm, 200–400 mesh), followed by elution with water, collecting 1 mL fractions; 25 μL aliquots were counted in a liquid scintillation counter. The labeled fractions were pooled and lyophilized.

Assay of the activity of CGTase.—CGTase activity was determined by a modification of the method of Thoma et al.[7] in which 0.1 mL of glucoamylase (10 IU/mL) in 20 mM pyridine/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 pyridine–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.[8]

Preparation of B. macerans *CGTase*.—The enzyme was elaborated by growing *B. macerans* ATCC 8517 on wheat bran according to the method of Kobayashi et al.[9] The enzyme was purified by a modification of the methods of Kobayashi et al.[9] Maize starch (5 g) was added to 500 mL of culture supernatant, containing 5200 units of CGTase. The starch suspension was stirred for 15 h at 3–5 °C. The starch was then removed by centrifugation and washed five-times with cold (4 °C) 33% v/v EtOH solution. The enzyme was desorbed from the starch by adding 30 mL of 10 mM acetate buffer (pH 6), containing 1 mM $CaCl_2$. The desorbed enzyme solution was filtered and concentrated to 2 mL in a dialysis bag with polyethylene glycol 20,000 at 4 °C.[10] The enzyme solution was then added to a BioGel P-10 column (2.5 × 50 cm), and eluted with 10 mM acetate buffer (pH 5). The protein peak was added to a DEAE-cellulose column (2.5 × 12.5 cm) and eluted with a 0–0.5 M gradient of NaCl at a flow rate of 0.5 mL/min and the collection of 3 mL fractions at 4 °C. Active enzyme fractions were pooled and dialyzed against 5 mM acetate buffer (pH 5), containing 0.1 mM $CaCl_2$, followed by

concentration to 1 mL in a dialysis bag, using PEG 20,000.[10] The enzyme gave a single band by SDS–PAGE. The concentration of the protein was determined by the Bradford method[11] and gave a specific activity of 220 units/mg of protein.

Trapping a CGTase complex by acid precipitation.—6-[^{3}H]Cyclomaltohexaose (50 μL, 0.1 μCi, 40 mM) was diluted to 0.5 mL and added to 0.5 mL of the concentrated CGTase (2233 units, 0.14 μmol). The reaction was allowed for 30 s at 4 °C, and then it was stopped by the addition of 10% w/v trifluoroacetic acid to give a pH below 1. The enzyme precipitated and was denatured. The precipitate was centrifuged and resuspended in 1 mL of 0.1% v/v trifluoroacetic acid, giving a pH of 2.5, and it was washed with 1 mL of water five-times by centrifugation and resuspension to remove any soluble radioactivity. The last wash gave background activity. The washed protein was dissolved in 0.2 mL of 1 M NaOH and the amount of radioactivity was determined by liquid scintillation spectrometry. The NaOH solution was washed and then diluted to 0.5 mL with 0.1% w/v TFA, containing 6 M urea. The solution was allowed to stand for 10 min at 20 °C and then it was added to a BioGel P-10 (200–400 mesh) column (1.5 × 20 cm) that had been equilibrated with the TFA–urea solution. Protein fractions (1 mL) were collected and the radioactivity determined by liquid scintillation spectrometry.

Trapping of the CGTase complex by lowering the pH to 2.5.—Another quenching method that did not precipitate the enzyme was the lowering of the pH to 2.5. After 30 s of reaction under the same conditions as above, the pH of the digest was 2.5 by the rapid addition of 0.2 mL of 0.2 M HCl. The resulting solution was then chromatographed on a BioGel P-10 (200–400 mesh) column (1.5 × 50 cm) that had been equilibrated with 10 mM HCl–50 mM KCl (pH 2.5) solution to remove unreacted labeled cyclomaltohexaose from the protein. Fractions (1 mL) were collected with a flow rate of 0.5 mL/min. The separated protein fractions were pooled and concentrated by using a Millipore Microcon 10 centrifuge filter tube. The amount of radioactivity was determined by liquid scintillation spectrometry and protein was determined by the Bradford method.[11] The enzyme was kept at pH 2.5 and 4 °C, and the cyclization activity was measured at various times (0.25–10 h) by using a modified method of Mäkelä and Korpela[12] in which 100 μL of enzyme was added to 900 μL of 50 mM imidazole–HCl buffer (pH 6.0), containing 1 mM $CaCl_2$, 0.4 mM Methyl Orange, and 0.44 mM maltodextrin with an average dp of 13; the decrease in absorbance at 528 nm was measured continuously in a Varian DMS 100 spectrophotometer with calibration by different concentrations of cyclomaltodextrin; the decrease in the absorbance, using the factor of 1.2×10^{-3} absorbance units/μmol, was determined. The inactivated protein was also rechromatographed on BioGel P-10 at various periods of time at pH 2.5, to determine if the amount of radioactivity that was initially associated with the protein was maintained on standing.

3. Results

One of the reactions catalyzed by *B. macerans* CGTase is the transfer or 'coupling reaction' in which the enzyme opens the cyclomaltodextrin ring and transfers the maltodextrin chain to a carbohydrate acceptor. The possible formation of a covalent-enzyme intermediate in the action of CGTase was studied by incubating the purified enzyme with 6-[^{3}H]cyclomaltohexaose for a limited time (30 s) in the absence of a carbohydrate acceptor. The enzyme was rapidly denatured by precipitating it with the addition of 10% w/v trifluoroacetic acid (TFA) at 4 °C. The precipitate was washed 11 times by resuspension in 0.1% w/v TFA, followed by centrifugation and the determination of the amount of radioactivity in the washings. Fig. 1 shows that radioactivity was present in seven washes, but was completely absent in washes 8–11. Through 11 washes, the precipitate remained radioactive (P in Fig. 1).

The extensively washed, radioactive protein-precipitate was dissolved in 0.5 mL of 0.1% w/v TFA, containing 6 M urea. The solution was allowed to stand 10 min at 20–21 °C and

then was added to a BioGel P-10 column that had been equilibrated with the TFA–urea solution; 1 mL fractions were collected and the radioactive fractions were pooled. The specific radioactivities of the two fractions, the precipitated and washed protein fraction (P in Fig. 1) and the protein that had been dissolved in TFA–urea and chromatographed on BioGel P-10 (BGC in Fig. 1), were nearly identical, 3.7×10^3 and 3.5×10^3 cpm/μmol, respectively. Fig. 1 shows that 95% of the radioactivity originally associated with the precipitated protein remained with the protein that was dissolved in the TFA–urea solution and chromatographed on BioGel P-10.

Besides precipitating the enzyme with high concentrations of TFA, the enzyme was inactivated by lowering the pH to 2.5 after reacting with 6-[^{3}H]cyclomaltohexaose for 30 s. At pH 2.5, the enzyme was inactive, but it was not precipitated. Fig. 2 shows the stability of CGTase at pH 2.5 and 4 °C over a 10-h period. The enzyme activity was relatively stable under these conditions and could be assayed at pH 5.2, showing retention of 90% of its activity after 30 min and 45% after 10-h at pH 2.5 and 4 °C. The inactivated enzyme was chromatographed on BioGel P-10 column after standing at pH 2.5 for 10 min. The elution profile is given in Fig. 3(A). The protein fraction (P in Fig. 3(A)) was radioactive. The amount of radioactivity in the protein indicated that 0.09 μmol of labeled cyclomaltohexaose remained with the protein after passing over BioGel P-10. Rechromatography of the labeled protein fraction on the same BioGel P-10 column (Fig. 3(B)) showed that 100% of the radioactivity from the first chromatographic fraction remained with the protein on rechromatographing.

4. Discussion

The mechanism of action of enzymes cleaving glycosidic linkages has been postulated to be of two possible types: (1) the cleavage of the glycosidic linkage to give an oxycarbonium ion; and (2) the cleavage of the glycosidic linkage by a nucleophilic displacement to give a covalent-enzyme intermediate.[13] The oxycarbonium ion mechanism is frequently postulated for enzymes that give inversion of the configuration at the anomeric carbon of

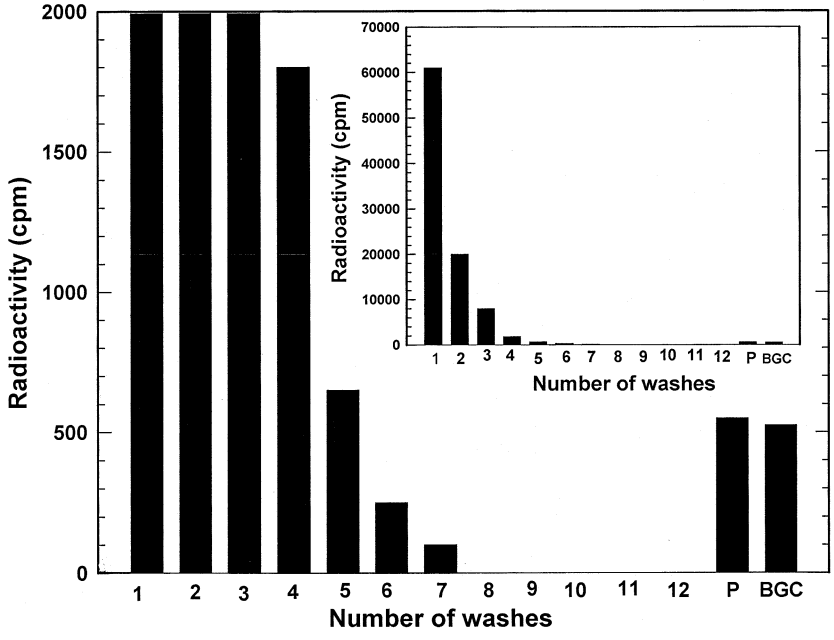

Fig. 1. Trapping a radioactive covalent enzyme-intermediate of CGTase by reaction with 6-[^{3}H]cyclomaltohexaose for 30 s in the absence of a carbohydrate acceptor and rapid precipitation of the enzyme with 10% w/v trifluoroacetic acid (TFA). The precipitated enzyme was washed 11-times by resuspension in 0.1% TFA, centrifuged, and the radioactivity measured in the supernatants. P is the amount of radioactivity remaining in the protein after 11 washes. BGC is the amount of radioactivity found in the protein after dissolving in 0.1% TFA–6 M urea, followed by chromatography on BioGel P-10.

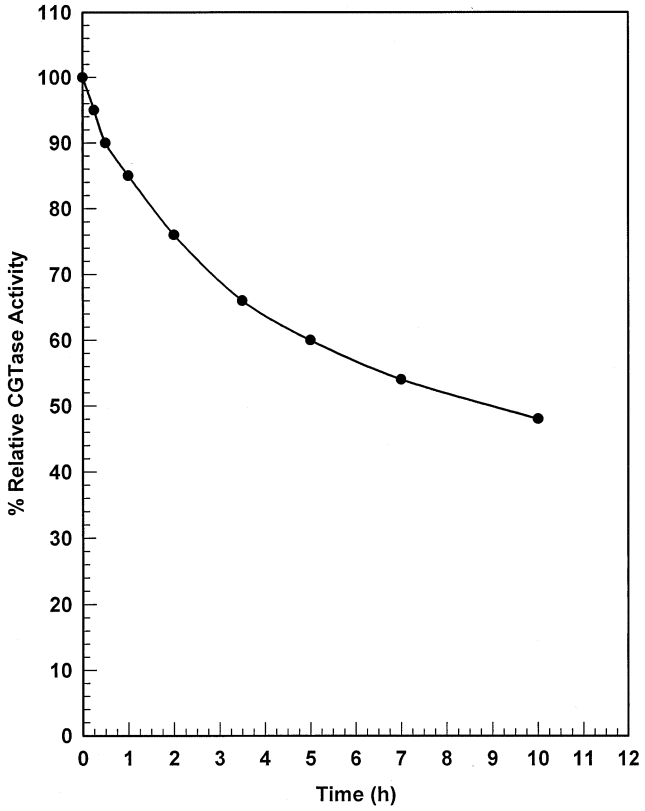

Fig. 2. Percent relative CGTase activity as a function of standing at pH 2.5. The enzyme activity, forming cyclomaltodextrins was determined using maltodextrin in 50 mM imidazole–HCl buffer (pH 6.0), containing 1 mM $CaCl_2$ at 25 °C.

the reducing residue moiety. It also has been postulated for enzymes that give retention of the configuration. The evidence for the oxycarbonium ion mechanism, especially for those enzymes that give retention, has often been derived from indirect observations and deductions, such as the inhibition of lysozyme by the 1,5-lactone of tetra-*N*-acetyl-chitotetraose $(GlcNAc)_4$, which has an SP_2 coplanar configuration at carbons 1 and 2 and the ring oxygen on the lactone moiety, identical to the configuration of an oxycarbonium ion[14] and by analogy from the mechanism of acid catalyzed hydrolysis of glycosidic linkages.[15]

The reaction catalyzed by sucrose phosphorylase, which gives α-D-glucopyranosyl phosphate from sucrose, was shown to form a covalent glucosyl–enzyme intermediate by quenching the reaction at low pH and isolating the D-glucosyl–protein complex under denaturing conditions.[16] The action of porcine pancreatic alpha-amylase was shown to form a covalent maltodextrin–enzyme intermediate by using ^{13}C-enriched maltotetraose substrate and following the reaction in 40% v/v Me_2SO by cryogenic ^{13}C NMR.[17] Spectral summation and difference techniques in this experiment revealed a broad resonance peak, whose chemical shift, relative signal intensity, and time-course formation corresponded to a β-carboxyl-acetal ester that was an enzyme maltodextrinyl-intermediate. Covalent glucosyl–enzyme intermediates have also been isolated for α-glucosidase[18] and β-glucosidase[19–21] and for *Streptococcus sobrinus* glucosyltransferase (dextransucrase).[22]

Reactions of *B. circulans* 251 CGTase, which primarily forms cyclomaltoheptaose, were shown to proceed via a double-displacement mechanism, involving a covalent glycosyl–enzyme intermediate. Attempts to trap this intermediate by using 4-deoxy-α-maltotriosyl fluoride were made as the substrate had a good leaving group (fluoride), but it could not undergo transglycosylation (disproportionation) reactions because the requisite nucelophilic hydroxyl group at carbon-4 on the nonreducing-end was absent. An inactive mutant enzyme in which Glu257 was replaced by Gln, however, had to be used to trap the intermediate because the covalent intermediate with the wild-type enzyme underwent hydrolysis with water acting as the acceptor and hence destroying the covalent intermediate.[23]

In the present study, we have been able to show the formation of a covalent-enzyme intermediate for wild-type *B. macerans* CGTase by using 6-[^3H]cyclomaltohexaose, in the absence of a carbohydrate acceptor. The covalent intermediate was trapped by rapid denaturation and by inactivation of the enzyme at pH 2.5. It was found that radioactive label was tightly associated with the precipitated and denatured enzyme by extensive washing of the precipitate, dissolution of the precipitate in a dissociating solution of 0.1% TFA and 6 M urea, followed by chromatography on BioGel P-10. The pH 2.5 inactivated enzyme was chromatographed on BioGel P-10 to remove the protein fraction from the cyclomaltodextrin fraction. It was found that after chromatography, the protein fraction still had tightly bound radioactivity. Rechromatogra-

phy of this radioactive protein fraction showed 100% retention of the radioactivity originally bound to the protein fraction.

These experiments demonstrate that *B. macerans* CGTase forms a carbohydrate-covalent intermediate when it catalyzes transglycosylation reactions. When the enzyme reacts with cyclomaltohexaose, it opens the ring and forms a covalent maltohexosyl intermediate that can be displaced by a carbohydrate acceptor to give the 'coupling reaction'. However, in the absence of an acceptor, the covalent maltohexaosyl–enzyme intermediate can be trapped by rapid denaturation or by inactivation of the enzyme at pH 2.5. When CGTase reacts with the starch chains at the nonreducing-ends, it cleaves the 5th, 6th, or 7th α-(1→4) glycosidic linkage, forming a covalent maltohexaosyl, maltoheptaosyl, or maltooctaosyl intermediate that then undergoes reaction with the C-4-hydroxyl group at the nonreducing-end of the bound chain to give cyclomaltodextrins.

The formation of covalent intermediates for CGTase indicates that the mechanism of action follows a classical S_N2-type double-displacement reaction. The first step is initiated by the nucleophilic attack by the catalytic residue (carboxylate-group) of CGTase onto the anomeric-carbon of the glycosidic linkage with the assistance of an acidic residue (carboxyl-group) donating a proton to the leaving glycosidic-oxygen atom. A covalent, β-linked carboxyl-acetal ester would, thus, be formed between the enzyme and C-1 of the cleaved maltodextrin chain. The covalently linked maltodextrin chain can then be displaced by an incoming carbohydrate acceptor or by water, giving an acceptor product or a hydrolytic product that has the retention of the α configuration.

Comparisons of the amino acid sequences of CGTase with those of *Aspergillus oryzae* alpha amylase[24] and porcine pancreatic alpha amylase[25] show that three acidic amino acid residues (Asp229, Glu257, and Asp328) are

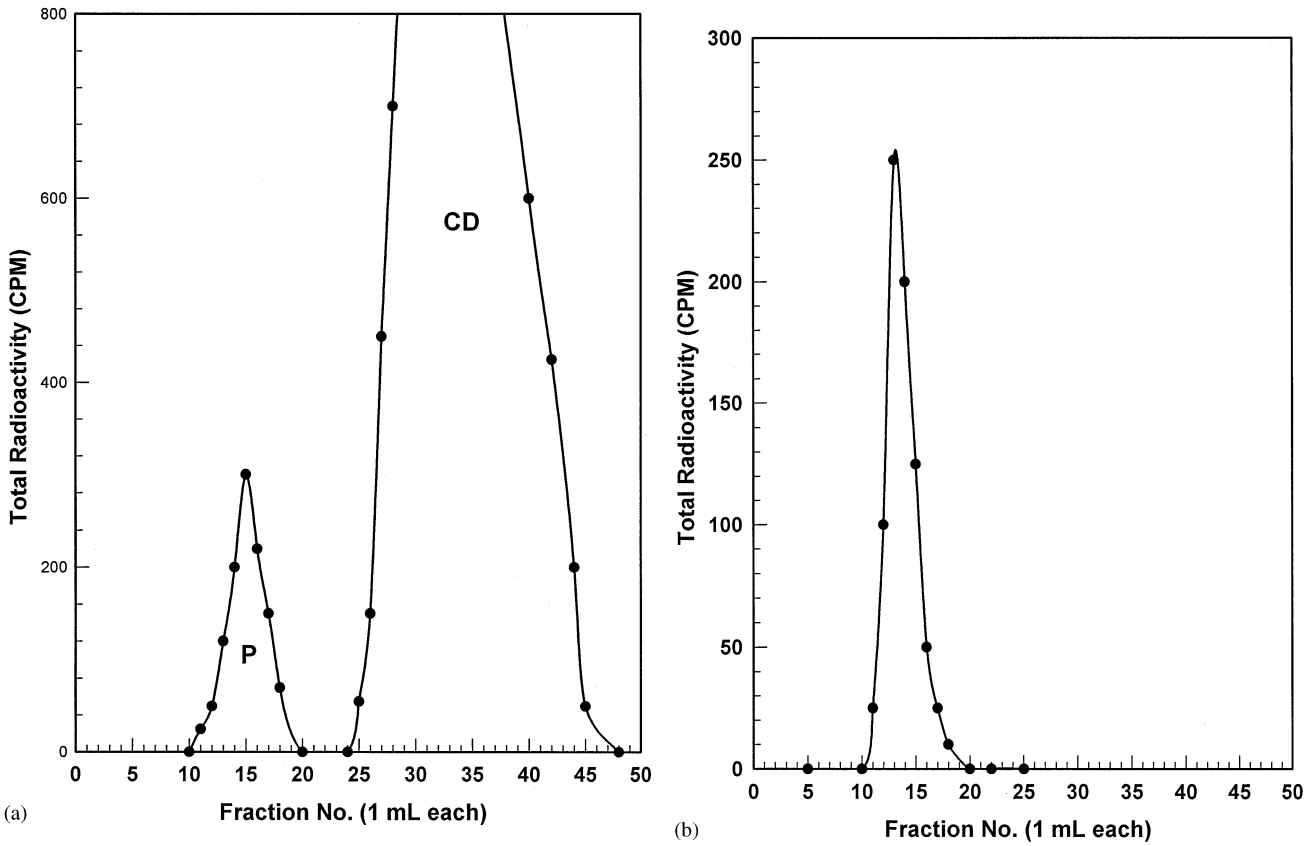

Fig. 3. Isolation of radioactively labeled enzyme-intermediate by inactivating the enzyme at pH 2.5, followed by chromatography on BioGel P-10. (A) Separation of radioactively labeled protein (P) from unreacted cyclomaltohexaose (CD). (B) Rechromatography of P on BioGel P-10.

conserved and play important roles as catalytic groups at the active-sites of both CGTases and alpha amylases.[26] The same residues are also found at the active-sites of pullulanase,[27] neopullulanase,[28] isoamylase,[29] and α-glucosidases.[30,31] This suggests that all of these enzymes form covalent intermediates and use a similar mechanism to cleave glycosidic linkages that are released from the enzyme active-sites by reaction with a hydroxyl group on a carbohydrate acceptor or by reaction with water, as has been shown for *B. macerans* CGTase (this study) and *B. circulans* 251 CGTase.[23] The results of this study, thus, reinforce the cryogenic ^{13}C NMR evidence[17] for the formation of covalent-enzyme intermediates in the reactions catalyzed by porcine pancreatic alpha amylase.

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