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Starch Branching Enzymes from Maize[1]

IMMUNOLOGICAL CHARACTERIZATION USING POLYCLONAL AND MONOCLONAL ANTIBODIES

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ABSTRACT

Spleen cells from mice immunized with starch branching enzymes were fused with cells from the mouse myeloma Sp2/0-AG14 cell line to form hybridomas. Those hybridomas producing antibodies against the branching enzyme were screened by the enzyme-linked immunosorbent assay using purified branching enzyme as the antigen. Three monoclonal cell lines (1A1D7, 1A1C3 and 4D2A9D8) were found to produce antibodies which showed positive enzyme-linked immunosorbent assay reactions with maize branching enzyme I in addition to branching enzymes IIa and IIb. Three other monoclonal cell lines (4D2D10, 4D2F9, and 2A6C12) were also selected which were found to produce antibodies showing positive enzyme-linked immunosorbent assay reactions with branching enzymes IIa and IIb only.

Amino acid composition and peptide maps obtained after trypsin or chymotrypsin digestion show that there is no difference between branching enzyme IIa and IIb but they are significantly different from branching enzyme I which, along with immunological data, suggests that only two forms of starch branching enzyme may be present in maize kernels.

Immunological cross-reaction was also found between the starch branching enzyme from maize kernels and the glycogen branching enzyme from *Escherichia coli* using polyclonal antibodies against starch branching enzyme I or IIa and IIb or *E. coli* glycogen branching enzyme, suggesting some immunological similarities between maize starch branching enzymes and *E. coli* glycogen branching enzyme.

The synthesis of the (1 → 6)-α-D-glucosidic linkages of the amylopectin fraction of starch is considered to be catalyzed by the plant branching enzyme (EC 2.4.1.18). The enzyme has been shown to modify the developing α-1,4-polyglucan by hydrolysis of an α-1,4 bond, and subsequent transfer of the excised α-1,4-glucan chain to the remaining or another α-1,4-glucan chain with formation of an α-1,6 bond. This transfer creates branch points as well as additional nonreducing ends where further synthesis of α-1,4-glucan chains can occur.

Starch branching enzyme has been reported to be present in multiple forms in spinach (9) and in normal starchy maize endosperm three different forms of branching enzyme (I, IIa, and IIb) have been reported (2). These three forms of branching enzyme were separable using DEAE-cellulose chromatography and could be distinguished by the ratio of activity using two different assay procedures. Immunological differences between these enzymes using polyclonal rabbit antiserum have also been reported (7).

In order to explore this further, the three branching enzymes from maize were purified to homogeneity and characterized on the basis of their immunological properties, using both polyclonal and monoclonal antibodies, on their amino acid composition and by comparison of the peptide maps after proteolytic digestion. It is hoped that these characterizations of the multiple forms of maize branching enzyme will help in determining their possible genetic relationships.

MATERIALS AND METHODS

Protein Samples. Starch branching enzymes I, IIa, and IIb were purified according to previously described procedures (2). It was necessary to further purify branching enzyme I in order to obtain homogeneous enzyme preparation which was achieved by further chromatography using DEAE-cellulose (DE 53) as described in "Results."

Assay of Maize Branching Enzymes. The basis of the assay is the stimulation by branching enzyme of the synthesis of α-D-glucan from α-D-glucose-1-P catalyzed by rabbit muscle phosphorylase. The mixture contained, in a volume of 0.2 ml, 0.1 M sodium citrate (pH 7.0), 1 mM AMP, 50 mM α-D-[^{14}C]glucose-1-P (5.0×10^4 cpm/μmol), 480 g of crystalline rabbit-muscle phosphorylase a, and branching enzyme. The reaction was initiated by addition of α-D-glucose-1-P and incubated at 30°C. Aliquots were taken out at 60, 90, and 120 min and incorporation of label into the glucan was assayed as previously described (9). One unit of enzyme activity is defined as 1 μmol of D-glucose incorporation into α D-glucan per min under foregoing conditions.

Protein Determination. Proteins were assayed by the method of Lowry *et al.* (12).

Electrophoresis. Slab gel electrophoresis in the presence of SDS was performed in 9% polyacrylamide gels as described by Neville (13). Various standard proteins were used in the estimation of the mol wt of the branching enzyme subunit. Protein bands were located by staining with Coomassie blue (4).

Location of Branching Enzyme Activity in Polyacrylamide Gels. Native gel electrophoresis was performed on 6% polyacrylamide gels with the Ornstein-Davis Tris-glycine buffer system (6, 14) which included 5 mM 1,4-DTT. Gels were quick-frozen in powdered dry ice and sliced in 1-mm sections. Every two consecutive sections were combined and 0.5 ml of 50 mM Tris-acetate buffer (pH 7.5) containing 10 mM EDTA and 2.5 mM DTE was added. The sections were macerated and incubated for several h at 0°C. Branching enzyme activity was measured as described above. Protein bands in the parallel lane were located by staining with Coomassie blue as described above.

Immunization of Mice. The *in vivo* immunization protocol used here is a modified method described by Vaitukaitis *et al.* (17). Separate sets of mice were immunized with either branching enzyme I (three mice) or branching enzyme IIa + IIb (four mice). Ten g of branching enzyme in Tris-acetate buffer (pH 7.5) were

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emulsified with an equal volume of Freund's complete adjuvant and were injected intradermally into the backs of 6- to 8-week-old BALB/c mice. Immunization was repeated after 1 and 3 weeks using Freund's incomplete adjuvant. Two weeks after final injection, serum was collected from these mice separately and tested for the antibody of the branching enzyme. Following this test, sera from mice within a set (*e.g.* serum from all the mice immunized with branching enzyme I) were pooled and used as a source of polyclonal serum. The animal (immunized with branching enzyme IIa + IIb) containing the highest titer was selected and was boosted intraperitoneally with 100 μg of the branching enzyme for 4 consecutive days before the day of fusion (16).

Cells. The mouse plasmacytoma Sp2/0-Ag14 (15) of BALB/c origin, an 8-azaguanine-resistant cell line mutant in hypoxanthine phosphoribosyltransferase, was kindly provided by Dr. M. E. Etzler. The cell line was propagated in Dulbecco's modification of Eagle's medium (Flow Laboratories, Inc.), streptomycin (50 g/ml), and penicillin (50 IU/ml), 4 mM L-glutamine, 1% 100 X nonessential amino acids (Flow Laboratories, Inc.), and 2% type 100 rabbit serum (Kappa Scientific Co., Escondido, CA) in a 37°C humidified incubator with a 8% CO_2 and 92% air gas phase. The hybridoma cultures were propagated in the same medium with an addition of 100 μM aminopterin and 16 μM thymidine.

Cell Fusion and Cloning. Hybridomas were produced by fusing 10^8 spleen cells (from mouse immunized with branching enzyme IIa + IIb) and 10^7 Sp2/0-Ag14 myeloma cells in 47% PEG and 7.5% DMSO. Immediately after the fusion, the cells were mixed with 2×10^7 mineral oil-induced peritoneal exudate cells and seeded into 15-mm Linbro wells. Antibody-producing cells were screened by using a solid phase ELISA[3] using purified branching enzyme-coated microtiter plates. Monoclonal antibody-producing cell lines were obtained by cloning the expanded cell culture by limiting dilution.

Production of Monoclonal Antibody towards Branching Enzyme IIa + IIb. Monoclonal cells (2×10^6 cells/injection) were injected intraperitoneally into BALB/c mice which had been pretreated with 0.5 ml of 2,6,10,14-tetramethyl pentadecane (Pristane, from Aldrich Chemical Co.) 1 week before. Most animals developed ascites within 10 to 14 d. Ascites fluid was withdrawn from the animals and the monoclonal antibodies were precipitated from the ascites fluid with 60% $(NH_4)_2SO_4$. The precipitate was dissolved in 10 mM sodium phosphate buffer (pH 7.0) and dialyzed against the same buffer containing 0.02% NaN_3.

Solid Phase Immunoenzyme Assay. Branching enzyme in varying amounts (see the details of each experiment) in 100 μl/well of coating buffer (Carbonate-bicarbonate buffer, pH 9.6) was allowed to incubate in the microtiter plate at 4° C for 3 to 4 h. The plate was then washed once with 10 mM sodium phosphate buffer (pH 7.0). The wells were filled with the same buffer containing 0.1% BSA and incubated at 4°C for 30 to 60 min in order to block the remaining electrostatic sites of the plate. The plate was then washed 3 more times with the same buffer (without BSA) and then allowed to dry by brisk inversion on tissue paper. One hundred μl of antibody solution were added to each well and allowed to incubate for 20 min at 37°C. The plates were then washed 5 times with washing buffer (0.9% NaCl, 0.05% Tween 80). One hundred μl of a 1:1,000 dilution of horseradish peroxidase conjugated to goat antimouse IgG (Cappel Laboratories, Inc., PA) were added and allowed to react for 15 min at 37°C before the plates were washed 10 times with washing buffer. A final addition of 100 μl of substrate [2 mM H_2O_2, 0.16 mM 2,2′-azino di-(3-ethyl benzthiazoline sulfonic acid) in 0.05 M citric acid (pH 4.0)] was made and the wells were read in an automatic ELISA reader (Titertek Multiskan, Flow Laboratories, Inc.).

Neutralization of Branching Enzyme Activity with Polyclonal and Monoclonal Antibody. A conventional neutralization assay involving precipitation of enzyme with the immune serum was not successful, presumably because of the presence of serum amylase which interferes with the branching enzyme assay. Monoclonal antibody from the ascites fluid, on the other hand, was unable to cause the precipitation of enzyme. It was, therefore, attempted to neutralize the branching enzyme activity using goat anti-mouse IgG conjugated to Sepharose beads (Cappel Laboratories, Inc.). Goat anti-mouse IgG covalently bound to Sepharose 4B beads (300 μl) was washed with 1 ml of PBS (pH 7.0). Washing was done twice by suspending the beads in buffer, centrifuging at 12,000*g* for 5 min, and then discarding the supernatant. After second wash, the beads were suspended in 500 μl of the same buffer. Out of this suspension, 50 μl were incubated with 15 μl of the various dilutions of preimmune and immune serum. Incubation was done at room temperature for 30 min. During incubation, the tubes were shaken frequently to keep the beads in suspension. Beads were then washed 3 times with 1 ml of PBS (pH 7.0) as described above and then finally suspended in 75 μl of the same buffer. From this suspension, 25 μl were incubated with a cocktail containing 0.25 mg of BSA, 2.5 μmol of sodium citrate buffer (pH 7.0), 63 nmol of DTT and purified branching enzyme in a total volume of 50 μl. Incubation was done at room temperature for 2 h. The mixture was then centrifuged at 12,000*g* for 5 min and then 5 μl of supernatant were taken for branching enzyme assay.

Amino Acid Analysis. The protein was reduced and carboxymethylated with iodoacetic acid by the method of Crestfield *et al.* (5) modified for use with guanidine-hydrochloride (8). Carboxymethylated protein was lyophilized and then dissolved in 70% HCOOH. Portions containing a minimum of 0.4 mg protein were placed in hydrolysis tubes and dried *in vacuo*. Constant boiling HCl was added and sealed. Samples were hydrolyzed at 110°C for 24, 48, and 72 h. Hydrolyzed protein samples were run on a Durum D-500 amino acid analyzer.

Digestion of Branching Enzymes with Trypsin. Carboxymethylated branching enzyme (300 μg) was digested with 3 μg of trypsin in a total volume of 500 μl using 100 mM ammonium bicarbonate solution (pH 7.7) at 37°C for 3 h with occasional shaking. At the end of this period, a further 3 μg of trypsin were added and incubated under the same conditions for another 3 h. At the end of incubation, samples were freeze dried. Freeze-dried material was dissolved in 50 μl of water and freeze dried again to remove ammonium bicarbonate. This process was repeated. Freeze-dried sample was then dissolved in 300 μl of 0.1% TFA in water and centrifuged for 1 min at 12,000*g* to pellet the undissolved material. From the supernatant, 100 μl were chromatographed on a reverse-phase C-4 (4 × 250 mm, VYDAC) column. The column was washed for 5 min with solvent A (0.1% TFA in water) prior to a gradient elution of 0 to 55% solvent B (0.075% TFA in acetonitrile) in 55 min. The peptides were monitored at 214 nm.

Digestion of Branching Enzymes with Chymotrypsin. Carboxymethylated branching enzyme (500 μg) was digested with 5 μg of chymotrypsin in a total volume of 500 μl using 100 mM ammonium bicarbonate solution (pH 7.7) at 25°C. Sample was constantly mixed (gently) using a magnetic stirrer. At the end of 2 h, a further 5 μg of chymotrypsin were added to the sample. Aliquots (100 μl) were taken 0, 1, 2, 4, and 6 h after the beginning of the digestion, freeze dried, and then chromatographed on a protein C-4 column in the same way as described earlier for tryptic digest samples.

Glycogen Branching Enzyme from *E. coli* and its Antiserum. Glycogen branching enzyme from *E. coli* purified according to previously described procedures (1) was used in the experiments

[3] Abbreviations: ELISA, enzyme linked immunosorbent assay; IgG, immunoglobulin G; R_m, relative migration value to the tracking dye.

reported here. Rabbit polyclonal serum against glycogen branching enzyme prepared in our laboratory (11) was used.

RESULTS

Purification of Starch Branching Enzyme I. Branching enzyme I was purified according to the previously described procedures (2) except that the enzyme obtained after Bio Gel $A_{1.5m}$ was dialyzed against 20 mM Tris-acetate buffer (pH 7.5), containing 4 mM EDTA and 2.5 mM DTE. This enzyme was applied to a column of DE 53 (at 3 mg protein/ml resin bed volume) equilibrated with above buffer. The column was washed with 2 bed volumes of buffer and the enzyme eluted with a linear gradient of 0 to 0.4 M KCl in the same buffer. Fractions containing branching enzyme activity (Fig. 1) were pooled and concentrated by ultrafiltration. The enzyme was enriched from a specific activity of 557 to 1096 after chromatography on DEAE cellulose (DE 53) with an enzyme unit recovery of about 58%.

The DE 53 fractions of branching enzyme I showed one major protein band after native PAGE which corresponded to activity measured by the procedure indicated under "Materials and Methods" and had R_m of 0.55. No activity was detected with the two very faint bands seen only if greater than 5 μg of protein were applied for electrophoresis and which migrated faster and slower than the major band.

Electrophoresis of the branching enzyme I after DE 53 in SDS in a discontinuous system showed one protein band, however, when 5 to 10 μg of protein were loaded, then 2 to 4 very faint bands were also seen. The electrophoretic mobility of the major band was compared with standard proteins subjected to electrophoresis on the same gel. A plot of R_m *versus* the logarithm of the mol wt of the standards (phosphorylase a, *E. coli* branching enzyme, BSA, ovalbumin and lactate dehydrogenase) was linear. The R_m value of 0.34 obtained for branching enzyme I corresponded to a mol wt of 82,000.

Reaction of Antibranching Enzyme I Serum with Starch Branching Enzymes I, IIa, or IIb, and *E. coli* Glycogen Branching Enzyme. Serum collected from mouse immunized with starch branching enzyme I was tested against the starch branching enzymes I, IIa, IIb or glycogen branching enzyme from *E. coli* using an ELISA assay (Fig. 2). A positive reaction was found with all three maize starch branching enzymes, the reaction of branching enzyme I being the highest. Peak absorbance for branching enzyme IIa or IIb was about 3- to 5-fold lower than that of branching enzyme I. *E. coli* glycogen branching enzyme also reacted positively and the peak absorbance was even higher than that of branching enzyme I.

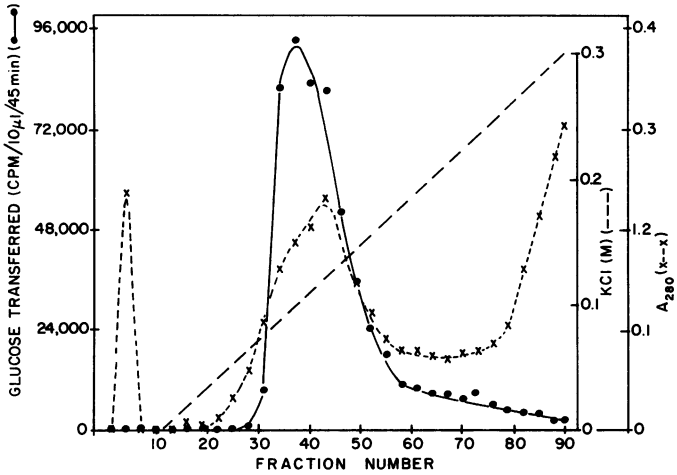

FIG. 1. Chromatography of maize branching enzyme I on DEAE-cellulose, grade DE 53; (●), branching enzyme activity; (×), A_{280}.

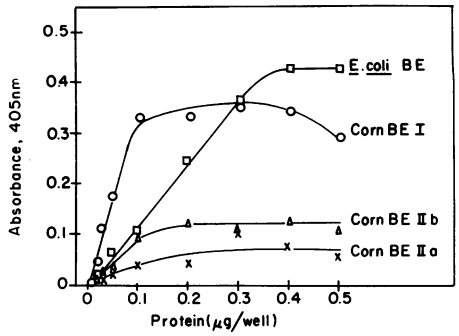

FIG. 2. ELISA of serum from mouse given an injection of maize starch branching enzyme I. Maize branching enzyme I, IIa, IIb, and *E. coli* glycogen branching enzyme were used at concentrations of 0.01 to 0.5 μg/well to coat the microtiter plates. Mouse serum and horseradish peroxidase-antimouse IgG conjugate were used at dilutions of 1:50 and 1:1,000, respectively. The plates were read using a Titertek Multiskan (Flow Laboratories, Inc.).

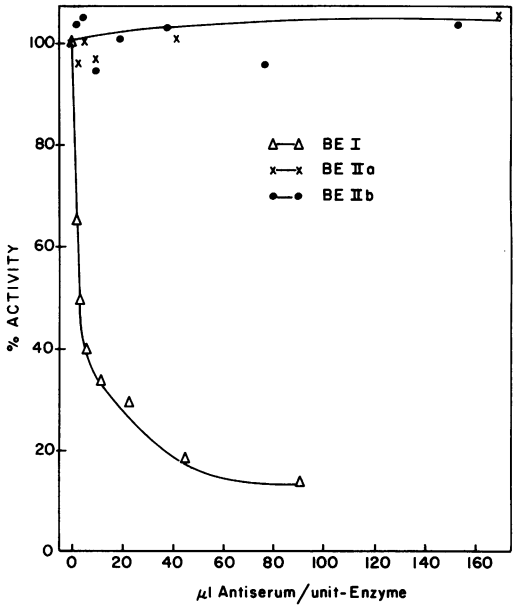

FIG. 3. Neutralization of starch branching enzyme activity by antibranching enzyme I serum. Neutralizations were performed as described in "Materials and Methods." (Δ), Branching enzyme I; (×), branching enzyme IIa; (○), branching enzyme IIb.

Neutralization of starch branching enzyme activity with branching enzyme I antiserum was also performed (Fig. 3). The activity of branching enzyme I was inhibited by more than 85% with its own antiserum. The amount of antiserum required for 50% inhibition of branching enzyme I activity was 3 μl/unit of activity for this enzyme. Branching enzyme IIa or IIb, however, showed no inhibition of activity at all.

Reaction of Antibranching Enzyme IIa + IIb Serum with Starch Branching Enzymes I, IIa, or IIb and *E. coli* Glycogen Branching Enzyme. Antiserum produced against a mixture of starch branching enzyme IIa and IIb was also tested against the starch branching enzyme from maize and glycogen branching enzyme from *E. coli* using an ELISA assay. All the enzymes reacted with the serum (Fig. 4). Interestingly, reaction of *E. coli* branching enzyme was higher than that of all three maize branching enzymes at all levels of protein tested. Among the maize enzymes, reaction of branching enzyme IIb with the serum was the highest. The reaction of branching enzyme IIa with antibranching enzyme IIa + IIb serum was about 75% of the reaction

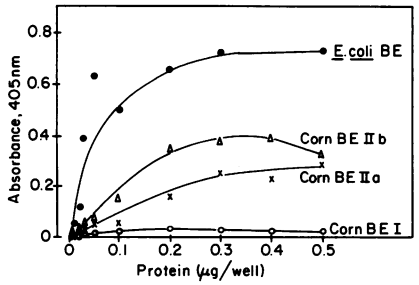

FIG. 4. ELISA of serum from mouse given an injection of maize starch branching enzymes IIa + IIb. Maize branching enzyme I, IIa, IIb, and *E. coli* glycogen branching enzyme were used at concentrations of 0.01 to 0.5 μg/well to coat the microtiter plates. Mouse serum and horseradish peroxidase-antimouse IgG conjugate were used at dilutions of 1:200 and 1:1,000, respectively.

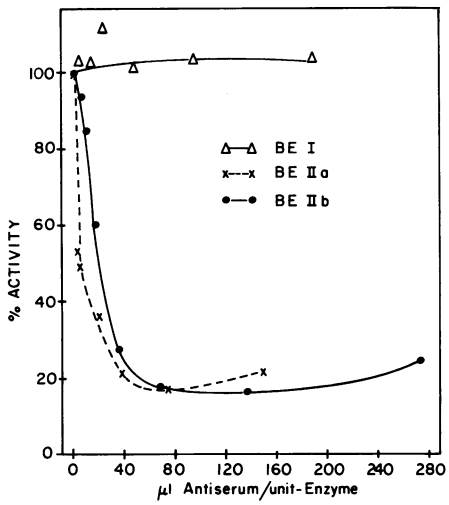

FIG. 5. Neutralization of starch branching enzyme activity by anti-branching enzyme IIa + IIb serum. (△), Branching enzyme I; (×), branching enzyme IIa; (●), branching enzyme IIb.

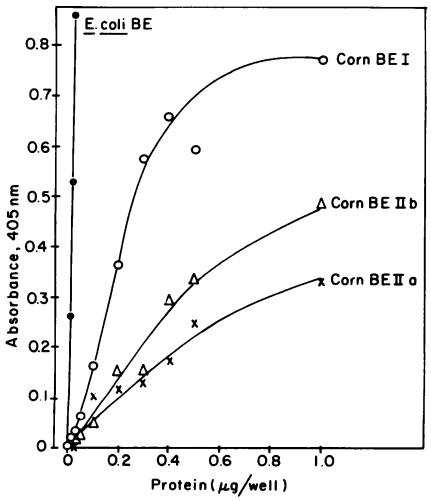

FIG. 6. ELISA of serum from rabbit given an injection of *E. coli* glycogen branching enzyme. Maize branching enzyme I, IIa, IIb, and *E. coli* glycogen branching enzyme were used at concentrations of 0.01 to 1.0 μg/well to coat the microtiter plates. Rabbit serum was used at concentrations of 1:250 and 1:10,000 for corn branching enzymes and *E. coli* glycogen branching enzyme, respectively. Horseradish peroxidase-anti-rabbit IgG conjugate was used at a dilution of 1:1,000.

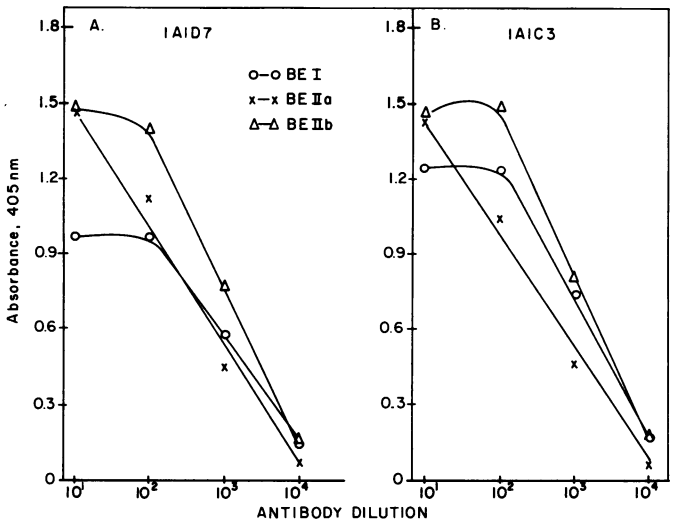

FIG. 7. ELISA using monoclonal antibodies from various clones. The microtiter plates were coated with 100 μl/well of 2.5 μg/ml of purified branching enzyme from maize as indicated. The serial dilutions of the ascites fluids were carried out with PBS containing 0.1% Tween 80. Horseradish peroxidase-antimouse IgG conjugate was used at a dilution of 1:1,000. (A), 1A1D7; (B), 1A1C3.

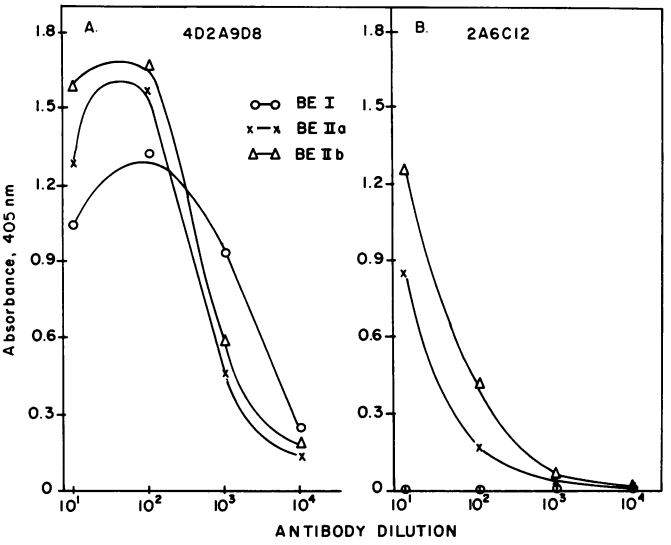

FIG. 8. ELISA using monoclonal antibodies from various clones. The details of ELISA are same as in Figure 7. (A), 4D2A9D8; (B), 2A6C12.

of branching enzyme IIb, and the reaction of branching enzyme I was 5- and 7-fold lower than that of branching enzyme IIa and IIb, respectively.

Both branching enzyme IIa and IIb activity were neutralized by antibranching enzyme IIa + IIb serum (Fig. 5). Branching enzyme IIa required 4 μl of antiserum per unit of enzyme for 50% neutralization of enzyme activity. Branching enzyme IIb required 20 μl of antiserum per enzyme unit for 50% neutralization of enzyme activity and this antiserum was ineffective in neutralizing the activity of branching enzyme I.

Reaction of Antiglycogen Branching Enzyme Serum with Starch Branching Enzymes I, IIa, or IIb and *E. coli* Glycogen Branching Enzyme. Serum collected from rabbit given an injection of glycogen branching enzyme from *E. coli* reacted positively with all three starch branching enzymes from maize (Fig. 6). Starch branching enzyme I showed the greatest reaction with anti-glycogen branching enzyme serum. A smaller reaction was

observed with branching enzyme IIb and the least reaction was observed with branching enzyme IIa. However, the reaction of glycogen branching enzyme against its own antiserum was about 230 times higher than that of maize branching enzymes. Neutralization of enzyme activity was not performed in this case.

Immunological Comparison of Starch Branching Enzymes I, IIa, or IIb, using Monoclonal Antibody from Different Cell Lines. Using an ELISA assay, the monoclonal antibodies obtained from different cell lines were tested against each of the three starch branching enzymes and the results are presented in Figures 7 through 9. Monoclonal antibodies raised from three monoclonal cell lines 1A1D7, 1A1C3, and 4D2A9D8 (Figs. 7, A and B, and 8A) reacted with all three branching enzymes whereas monoclonal antibodies obtained from cell lines 2A6C12, 4D2D10, and 4D2F9 (Figs. 8B, 9A and 9B) reacted with branching enzyme IIa and IIb only; BE I did not show any reaction at all. The magnitude of reactions with BE IIa or IIb was the same using monoclonal antibodies from cell line 4D2A9D8 (Fig 8A). Antibodies from other cell lines showed small differences.

Neutralization of branching enzyme activity using monoclonal antibodies from these cell lines were also performed. Monoclonal

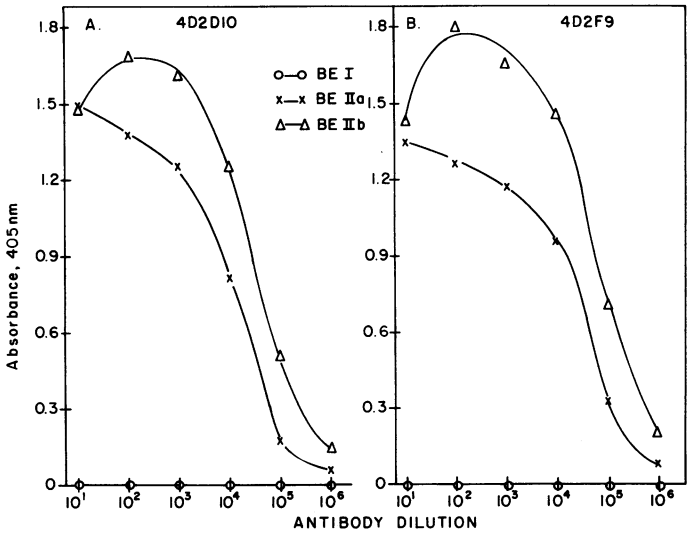

FIG. 9. ELISA using monoclonal antibodies from various clones. The details of ELISA are same as in Figure 7. (A), 4D2D10; (B), 4D2F9.

Table I. *Amino Acid Composition of Starch Branching Enzymes I, IIa, and IIb from Maize Kernels*

Amino Acid	BE I	BE IIa	BE IIb
	mol/82,000 g	*mol/80,000 g*	
Asp	79.9	87.6	93.0
Thr	38.8	34.3	32.8
Ser	52.5	47.6	46.5
Glu	74.9	65.1	57.5
Pro	39.4	38.1	34.1
Gly	68.9	63.2	62.0
Ala	67.8	42.3	36.5
CM-Cys	13.2	12.5	13.0
Val	48.7	42.0	39.5
Met	6.9	6.4	9.8
Ile	31.8	33.6	31.0
Leu	65.2	52.3	48.6
Tyr	22.6	36.2	39.6
Phe	32.4	40.6	44.4
Lys	38.6	30.6	30.7
His	18.0	30.4	35.8
Arg	39.9	42.8	43.4

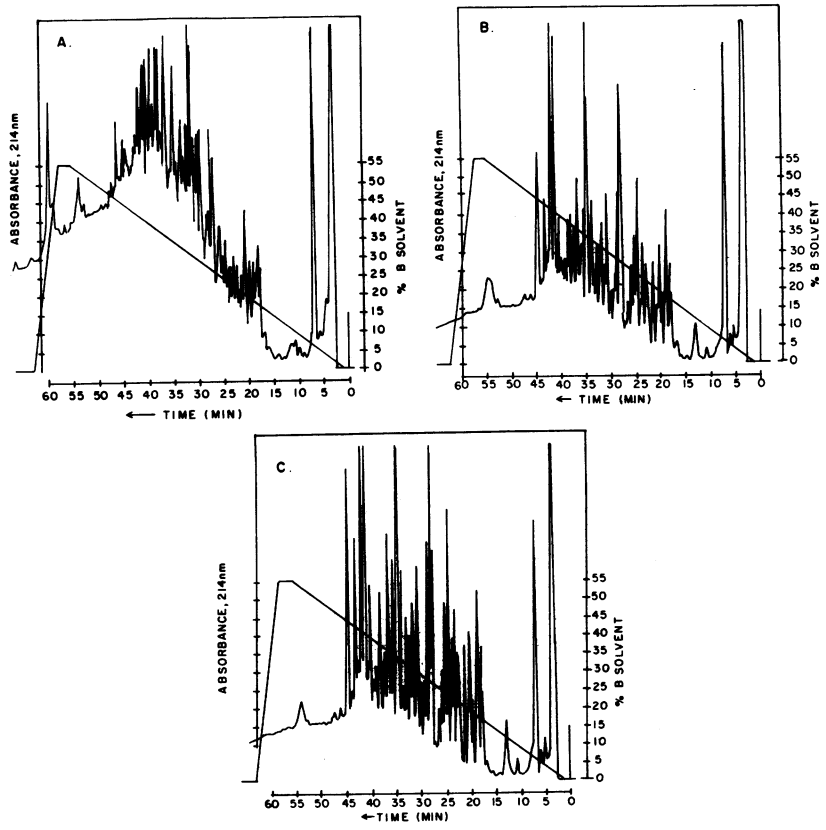

FIG. 10. Tryptic peptide maps of maize branching enzymes by reverse-phase chromatography at pH 2.0. Approximately 1.25 nmol of tryptic digest were chromatographed as described in "Materials and Methods." (A), Branching enzyme I; (B), branching enzyme IIa; (C), branching enzyme IIb.

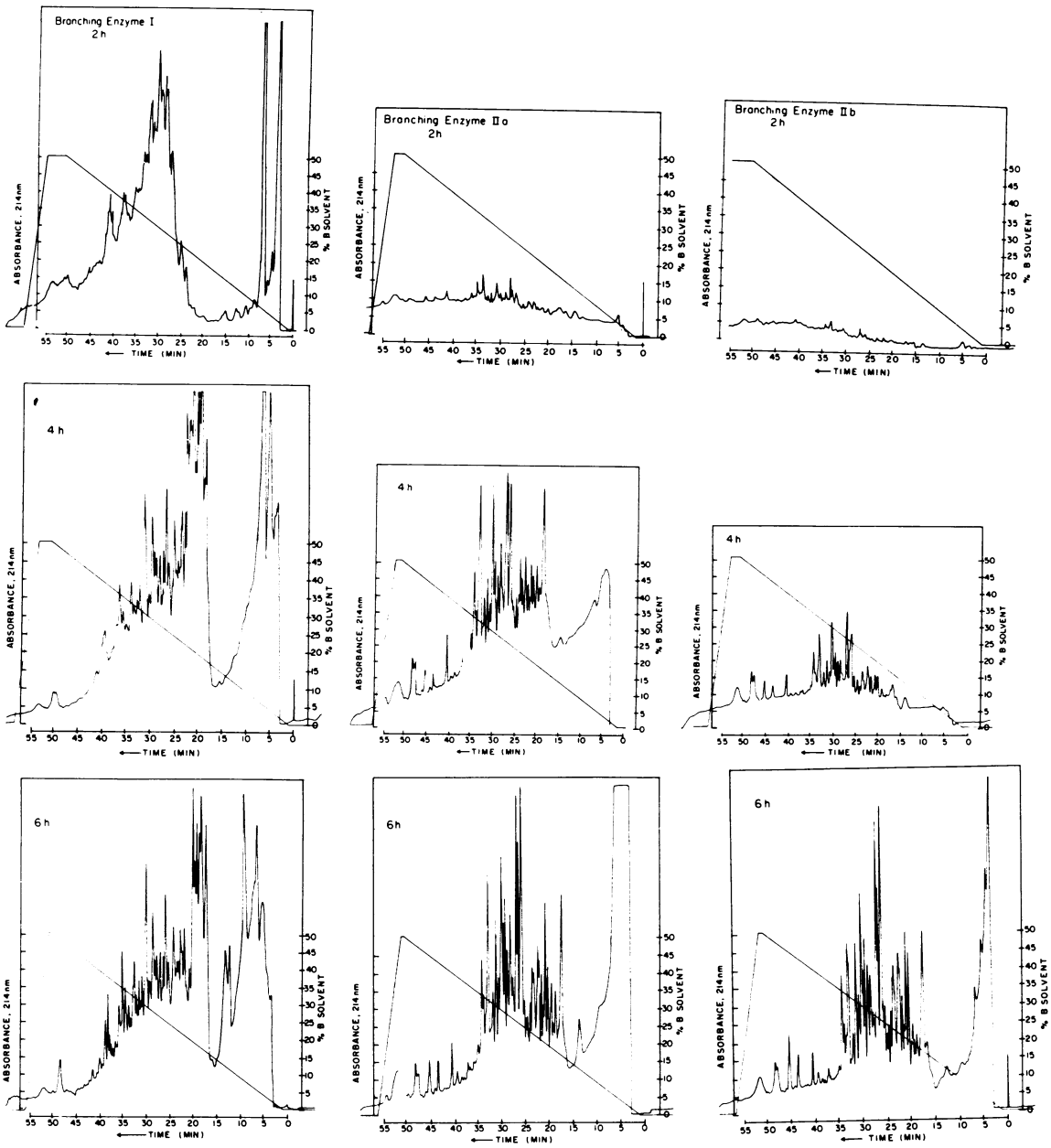

FIG. 11. Peptide maps of maize branching enzyme after digestion with chymotrypsin by reverse-phase chromatography at pH 2.0. Details of chromatography are same as in "Materials and Methods." The hours of incubation are noted in each figure.

antibodies from cell line 1A1D7 inhibited branching enzyme I, IIa, and IIb activity by 65, 50, and 50%, respectively. On the other hand, monoclonal antibodies from cell line 4D2F9 inhibited the activity of branching enzyme IIa and IIb only by 60 and 75%, respectively. Monoclonal antibodies obtained from other cell lines were unable to inhibit the activity of any branching enzyme.

Amino Acid Composition of Starch Branching Enzymes. The results presented in Table I show pronounced differences in the amino acid composition of branching enzyme I and the other two branching enzymes. Branching enzymes IIa and IIb also show small differences in their amino acid composition as seen in aspartate, glutamate, alanine, methionine, and histidine, however, these differences may not be significant.

Peptide Maps of Starch Branching Enzymes after Proteolytic Digestion. The chromatography of tryptic digests of different starch branching enzymes presented in Figure 10 show that branching enzyme I (Fig. 10A) gave a very different peptide pattern compared to branching enzymes IIa or IIb (Fig. 10, B and C) in terms of both the number of peptides and their retention times. Branching enzyme I produced over 60 peptides whereas branching enzymes IIa or IIb had about 50 peptides. There appears to be no significant difference in the number or retention times of the peptides produced by trypsin digestion of branching enzymes IIa and IIb.

In order to attempt to demonstrate differences between branching enzymes IIa and IIb, a second proteolytic enzyme, chymotrypsin, was used and generation of peptides at different time intervals was followed. No differences between the peptide maps of branching enzymes IIa and IIb were observed at the different times of incubation (Fig. 11). The profile of branching enzyme I had many peaks with similar retention times but the patterns generated at the different times of incubation are quite different compared to that of branching enzyme IIa or IIb.

DISCUSSION

The purpose of this investigation was to characterize the three branching enzymes from maize on the basis of their immunological properties, amino acid composition, and digest maps. Branching enzyme I has been purified from maize kernels to the degree required to perform these studies by chromatography on DEAE cellulose DE 53. Electrophoresis in SDS gels showed that branching enzyme I had a subunit mol wt of 82,000 which is smaller than reported earlier (2).

Immunological comparison of branching enzymes on the basis of cross-reaction with the polyclonal antisera (Figs. 2 and 4) and reaction with monoclonal antibodies (Figs. 7 to 9) suggests some homology between all three starch branching enzymes. In an earlier report, it was suggested that branching enzyme I is immunologically different from branching enzyme IIa and IIb (7). However, in the present investigation it is quite clear that in ELISA reactions branching enzyme I reacted with branching enzyme IIa + IIb antiserum (Fig. 4) and vice versa (Fig. 2). Stronger evidence is apparent from the reaction with monoclonal antibodies from cell lines 1A1D7, 1A1C3, and 4D2A9D8 (Figs. 7, A and B, and 8A) where all three branching enzymes reacted to about the same degree, suggesting the presence of those particular antigenic determinants in all three branching enzymes. One reason for no cross-reaction in the case of results reported by Fisher and Boyer (7) may be due to their lower antibody titer compared to the results presented here and in not using the ELISA technique. This suggestion is based on the comparison of amount of antiserum required for 50% inhibition of the enzyme activity. For example, the amount of antiserum required for 50% inhibition of branching enzyme I activity was 3 μl in the present study (Fig. 3) compared to 96 μl reported earlier by Fisher and Boyer (7). Additionally, the ELISA technique used in the present study is a more sensitive test for antigen-antibody reaction than Ouchterlony and enzyme neutralization studies used previously.

Results discussed above suggest some homology between all three branching enzymes from corn. Branching enzyme I, however, seems to have major differences in the structure compared to the other two branching enzymes because it does not react with antibodies produced by monoclonal cell lines 2A6C12, 4D2D10, and 4D2F9 (Figs. 8b and 9, a and b) and also on the basis of their amino acid composition (Table I) and peptide maps after trypsin or chymotrypsin digestion (Figs. 10 and 11). It is interesting to note that there are large differences in the amino acid composition of branching enzyme I compared to that of branching enzyme IIa or IIb. Similarly, the peptide maps generated after trypsin or chymotrypsin digestion also show large differences both in the number of peptides and their retention times. These results are consistent with the previously reported differences between starch branching enzyme I and the two forms of branching enzyme II on the basis of their ability to bind to DEAE-cellulose, different reactivities in the assay systems, K_m for the substrate, and mol wt (2, 3).

Maize kernels appear to contain only two types of branching enzyme, branching enzyme I and II. Both branching enzymes IIa and IIb react with the antibodies, both polyclonal and monoclonal, to about the same degree (Figs. 2 to 5, 7 to 9). There are no major differences in their amino acid composition (Table I). The peptide maps generated after digestion with trypsin were exactly the same for the two enzymes (Fig. 10). A time-course study of the generation of peptides after chymotrypsin digestion also shows identical peptide maps at each digestion time suggesting that both branching enzymes IIa and IIb are identical. Previous studies also indicate similarities between them in the mol wt, K_m for the substrate, and affinities to bind with DEAE-cellulose and aminobutyl Sepharose columns (2). The differences between these enzymes on the basis of their elution from DEAE-cellulose and aminobutyl Sepharose columns at different salt concentrations may possibly be due to the differences between these enzymes in the amounts of glucan noncovalently bound to them. Although the maize mutant, *amylose extender*, has been suggested to be the structural gene for branching enzyme IIb (3, 10), the differences observed in those cases may arise from the differences in the amounts and type of glucan associated with the branching enzyme II forms because of the alteration in the starch structure brought about by the mutation. Further studies are certainly required to establish whether distinct structural genes or duplicate structural genes are needed for expression of the branching enzymes IIa and IIb.

The immunological similarity of *E. coli* B glycogen branching enzyme and starch branching enzymes is an interesting observation. Reaction of *E. coli* glycogen branching enzyme with starch branching enzyme I antiserum and starch branching enzyme IIa + IIb antiserum was even greater than the reaction with the enzymes against which these antisera were obtained (Figs. 2 and 4) which is an interesting observation. This homology suggests that branching enzyme is a highly conserved protein and some of the antigenic determinants appear to be common in proteins from both prokaryotes and eukaryotes. However, the amino acid composition of the *E. coli* enzyme is significantly different from that observed for the maize isozymes (11).

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