Plant Physiol. (1989) 90, 75–84
0032-0889/89/90/0075/10/$01.00/0

Received for publication July 15, 1988
and in revised form December 2, 1988

Immunological Comparison of the Starch Branching Enzymes from Potato Tubers and Maize Kernels[1]

Greetje H. Vos-Scheperkeuter*, Janny G. de Wit, Anne S. Ponstein, Will J. Feenstra, and Bernard Witholt

Groningen Biotechnology Center, Department of Biochemistry, Nijenborgh 16, 9747 AG Groningen, The Netherlands (G.H.V.-S, J.G.d.W., A.S.P., B.W.); *and Department of Genetics, Kerklaan 30, 9751 NN Haren, The Netherlands* (W.J.F.)

ABSTRACT

Starch branching enzyme was purified from potato (*Solanum tuberosum* L.) tubers as a single species of 79 kilodaltons and specific antibodies were prepared against both the native enzyme and against the gel-purified, denatured enzyme. The activity of potato branching enzyme could only be neutralized by antinative potato branching enzyme, whereas both types of antibodies reacted with denatured potato branching enzyme. Starch branching enzymes were also isolated from maize (*Zea mays* L.) kernels. All of the denatured forms of the maize enzyme reacted with antidenatured potato branching enzyme, whereas recognition by antinative potato branching enzyme was limited to maize branching enzymes I and IIb. Antibodies directed against the denatured potato enzyme were unable to neutralize the activity of any of the maize branching enzymes. Antinative potato branching enzyme fully inhibited the activity of maize branching enzyme I; the neutralized maize enzyme was identified as a 82 kilodalton protein. It is concluded that potato branching enzyme (M_r = 79,000) shares a high degree of similarity with maize branching enzyme I (M_r = 82,000), in the native as well as the denatured form. Cross-reactivity between potato branching enzyme and the other forms of maize branching enzyme was observed only after denaturation, which suggests mutual sequence similarities between these species.

Starch granules contain a mixture of linear molecules (amylose) and branched molecules (amylopectin). Amylose is produced by starch synthase (ADPglucose:α-1,4-glucan 4-glucosyltransferase, EC 2.4.1.21), whereas amylopectin is produced by the combined action of both starch synthase and branching enzyme (or Q-enzyme; α-1,4-glucan:α-1,4-glucan 6-glucosyltransferase, EC 2.4.1.18) (16). The latter enzyme introduces branches into glucans by hydrolyzing an α-1,4 bond and reattaching the released α-1,4 glucan segment via an α-1,6 bond to a similar glucan chain (1). This reaction creates new nonreducing ends where further chain elongation can occur.

Purification of branching enzyme from potato tubers has been reported by several authors (2, 7, 8), some of whom state that the enzyme can be separated into two functionally different components (molecular masses of 70 and 20 kD; 8). In contrast, Borovsky *et al.* (2) have purified a homogeneous branching enzyme preparation containing a single active protein of 85 kD. Multiple forms of branching enzyme analogous to those detected in spinach leaves (10) and seeds of maize (3, 8), teosinte (5), peas (14), and sorghum (6) have not been found in potatoes. The aim of this study was to characterize the potato branching enzyme with respect to the multiple forms of maize branching enzyme.

In a previous study we have compared the granule-bound starch synthase of potato and maize (20). Identification of this starch enzyme was facilitated by the availability of the *waxy* mutant of maize (*Zea mays* L.). Recently, an amylose-free (*amf*) mutant of the potato was isolated and we have found that this mutant resembled the *waxy* mutant of maize in that it also lacked the granule-bound starch synthase (11). Thus, there are considerable similarities between at least some starch synthesizing enzymes of potato and maize.

With respect to branching enzyme, however, there appear to be major differences. In contrast to potato tubers which probably harbor only a single species of branching enzyme (2), maize kernels contain three forms of this enzyme (branching enzymes I, IIa, and IIb with molecular masses of 82, 80, and 80 kD, respectively; 3, 18). Immunological and other characteristics strongly suggest that branching enzymes IIa and IIb are very similar and perhaps even identical, whereas both are significantly different from branching enzyme I (18). All forms of branching enzyme found in teosinte, another species of the genus *Zea*, were immunologically similar to those of maize (5). In this paper we compare the branching enzymes of potato and maize, two unrelated species, using specific polyclonal antibodies directed against either the native or the denatured potato branching enzyme. Although all branching enzymes studied appeared to share some antigenic determinants, the highest degree of similarity was clearly observed between potato branching enzyme and maize branching enzyme I.

MATERIALS AND METHODS

Materials

Potato tubers (*Solanum tuberosum* L., cv. Promesse) were obtained from A. Scheperkeuter. These potatoes were harvested late in the growth season and stored at about 10°C during 6 weeks.

[1] This research was financed by the Department of Economic Affairs, the Hague, the Netherlands, through the ISP program. Some of the results presented in this paper were reported at the 2nd International Workshop on Plant Polysaccharides held in July 1987 in Grenoble, France; the abstract was published in a special issue of Food Hydrocolloids (21).

Wild-type maize seeds (*Zea mays* oh 43; No. 83496) were kindly supplied by Dr. G. B. Fletcher, Maize Genetics Corporation Stock Center (MGCSC), Department of Agronomy, University of Illinois, Urbana, IL. They were grown in a glasshouse, self-pollinated, and harvested at 32 d after pollination. The ears were frozen in liquid nitrogen and stored at −20°C until use.

DEAE-cellulose (DE-52) was obtained from Whatman Ltd., Maidstone Kent, UK. Ag-butylamine (4-aminobutyl-Sepharose), Sephacryl S-300, and protein A-Sepharose CL 4B were obtained from Pharmacia, Uppsala, Sweden. Adenosine-5′-monophosphate (AMP), rabbit muscle phosphorylase a, glucose-1-phosphate, glycogen and thimerosal (ethylmercurithiosalicylate) were purchased from Sigma Chemical Company, St. Louis and [U-^{14}C]glucose-1-phosphate (294 mCi/mmol) from Amersham Int., U.K. All materials used for immunoblotting were purchased from Bio-Rad Laboratories, Richmond, CA.

Purification of Potato Branching Enzyme

Potato tubers (5 kg) were washed, homogenized in the presence of 0.1% (w/v) $Na_2S_2O_5$, and filtered through synthetic cloth. After settling of the starch granules, concentrated Tris buffer, DTT[2] and EDTA were added to give final concentrations of 50 mM Tris-HCl (pH = 7.5), 1 mM DTT, and 10 mM EDTA. These and all further purification steps were carried out at 4°C. The homogenate was cleared by centrifugation and proteins were precipitated by making the solution to 50% saturation with respect to ammonium sulphate. The precipitate was resuspended in 10 mM Tris-HCl (pH = 7.5) containing 1 mM DTT and 10 mM EDTA, dialysed extensively, and finally cleared by centrifugation. The crude potato juice obtained in this way contained 67.7 mg protein/mL and was stored at −20°C for 11 months.

After thawing, the crude potato juice was first precipitated once more with ammonium sulphate (final saturation 40%). This second precipitation resulted in a heavy loss of protein but gain of branching enzyme activity. The precipitate was dissolved in and dialyzed against TED buffer. The dialysate was brought to 75 mM NaCl, loaded onto a DEAE-cellulose column, and eluted with a linear gradient of 75 to 375 mM NaCl in TED. Fractions were measured for protein content (A_{280}), conductivity, and BE activity (assay A).

After dilution, the BE pool was subjected to a second DEAE-cellulose chromatography, concentrated by ammonium sulphate precipitation (final saturation 50%), and dialyzed against TED. NaCl was added to a final concentration of 20 mM and the cleared preparation was chromatographed on an Ag-butylamine column. Elution was performed with a linear gradient of 20 to 200 mM NaCl. Fractions were assayed and analyzed by SDS-polyacrylamide gel electrophoresis. This chromatographic step was repeated and the BE pool was concentrated by ammonium sulphate precipitation.

[2] Abbreviations: DTT, dithiothreitol; TED, 10 mM Tris-HCl buffer (pH = 7.5) containing 1 mM EDTA and 1 mM DTT; BE, branching enzyme; anti-NPBE, antibodies raised against native potato branching enzyme; anti-DPBE, antibodies raised against denatured potato branching enzyme; PBS, phosphate buffered saline (pH = 7.5); SaCI, *Staphylococcus aureus* Cowan I.

Finally, three runs of gel filtration on Sephacryl S-300 were performed, the first one in TED buffer and the subsequent ones in a buffer with a higher ionic strength (50 mM Tris-HCl [pH = 7.5] containing 5 mM EDTA, 1 mM DTT, and 100 mM NaCl). After each step only one or two fractions with a very high specific activity of BE were selected for further purification; the final BE preparation contained about 70% pure enzyme (average M_r = 79,000, specific activity 189 units/mg protein; *cf.* Fig. 1, lane 9) and is denoted as native potato BE throughout this paper.

Some fractions of the second S-300 chromatography which contained less pure potato BE were combined (*cf.* Fig. 1, lane 11) and subjected to preparative SDS-gel electrophoresis as described previously (20). The gel-purified branching enzyme (*cf.* Fig. 1, lane 12) prepared in this way is denoted as denatured potato BE throughout this paper.

Preparation of Specific Antisera

Antisera were raised in New Zealand rabbits, both against native potato BE and against denatured potato BE. After some 7 mL preimmune serum had been collected, immunization was started by injection of the enzyme in Freund's complete adjuvant, subcutaneously. Booster injections were administered after 20, 33, and 46 d. Two antiserum batches were collected, the first (8–10 mL) after 42 d by puncturing the central ear vein, and the second (30–60 mL) after 54 d by heart puncture. Antiserum batches from one rabbit were combined and stored at −20°C in the presence of 0.01% (w/v) thimerosal.

The amounts of protein injected varied: the first injection contained 48 μg native potato BE or 62 μg denatured potato BE, whereas the booster injections contained 32 μg native potato BE or 50 μg denatured potato BE.

The antisera are referred to as anti-NPBE and anti-DPBE, respectively.

Isolation of IgG

Since total antiserum gave irreproducible results in neutralization tests, a result which has also been reported by others (18), IgG was isolated from all (anti)sera. Antiserum or control serum (4–5 mL) was applied to a 5 mL protein A-Sepharose CL-4B column, washed with PBS, and eluted with 0.1 M glycine-HCl (pH = 3.0), according to the Pharmacia protocol. After neutralization, the IgG fraction was dialyzed against PBS and stored at −20°C in the presence of 0.01% thimerosal. On the average, this procedure yielded 4.1 mg IgG per mL nonimmune (control) serum, and 9.5 mg IgG per mL antiserum.

Preparation of Crude Potato Branching Enzyme for Immunological Tests

A crude potato BE fraction was prepared from fresh potatoes cv Promesse by precipitation with ammonium sulphate (final saturation 50%) and chromatography on DEAE-cellulose as described above. After concentration, a BE preparation was obtained which contained 0.8 mg protein/mL, had a specific activity of 2.4 units/mg, and was free of amylolytic

activity; it is denoted as crude potato BE in this paper and it was used for various immunological tests.

Isolation of Maize Branching Enzymes

Branching enzymes were isolated from wild-type maize kernels according to Boyer and Preiss (3, 4). Briefly, 59.1 g kernels were homogenized in 50 mM Tris-acetate buffer (pH = 7.5) containing 10 mM EDTA and 2.5 mM DTT using a Waring blender, filtered through cheese cloth and centrifuged (45 min at 30,000 *g*). The homogenate was precipitated by making the solution to 50% saturation with respect to ammonium sulphate, and the precipitate was dissolved in and dialyzed against 50 mM Tris-acetate buffer (pH = 7.5) containing 10 mM EDTA, 2.5 mM DTT, and 10% (w/v) sucrose. After centrifugation (10 min at 30,000 *g*), the major portion of this kernel extract (about 75 mg protein) was chromatographed on a DEAE-cellulose column (1.5 × 22 cm) and eluted with a linear gradient of 0 to 0.4 M KCl (3, 4). Fractions were tested for protein content (A_{280}), conductivity and BE activity using assay A.

Four peaks of BE activity were pooled separately: pool 1 contained fractions 4 to 6, pool 2 contained fractions 16 to 18, pool 3 contained fractions 29 to 32, and pool 4 contained fractions 36 and 37. These pools were dialyzed extensively against distilled water, freeze dried, and stored at −4°C.

Assay of Branching Enzyme

Assay A

Branching enzyme activity was measured as a stimulation of the synthesis of α-D-glucan from α-D-glucose-1-P catalyzed by rabbit muscle phosphorylase *a* (3, 10). The assay mixture contained, in a total volume of 0.1 mL, 0.1 M sodium citrate (pH = 7.0), 1 mM AMP, 50 mM α-D-glucose-1-P, 0.17 μM α-D-[^{14}C]glucose-1-P (5 nCi or 11,000 dpm per sample), 20 μg of crystalline rabbit muscle phosphorylase *a*, and branching enzyme. All components of the reaction mixture were mixed shortly before use; it was checked that the glucose incorporation which occurred in the complete reaction mixture before BE was added, was negligible. The assay was started by adding the assay mix (80–90 μl) to branching enzyme (10–20 μl) and incubating at 30°C. Glucose incorporation was routinely measured during the first 90 min of the reaction and expressed as μmol glucose transferred per 90 min. In neutralization experiments, enzyme samples were sized such that glucose incorporation did not exceed 40% of the totally added label, thus ensuring that any decrease by antibody-neutralization could be observed sensitively. In some cases, the activity is expressed in enzyme units (1 unit as defined as 1 μmol of glucose incorporated into α-D-glucan per min), which was based on the measurement of glucose incorporation during only the linear phase of the reaction (10).

Assay B

Branching enzyme activity was measured as a decrease in absorbance of the amylose-iodine complex (2; assay B in refs. 3 and 4). The assay mixture contained, in a total volume of 50 μL, 0.81 mg/mL potato amylose in 0.1 M sodium citrate buffer (pH = 7) and BE. After 10 min at 24°C the reaction was stopped by adding 1 mL of a 50-fold diluted iodine stock solution (0.2% I_2/2% KI). After centrifugation (2 min in an Eppendorf centrifuge), the absorbance at 680 nm was measured. In agreement with others (2), we found that the A_{680} decreased linearly with the amount of enzyme until it reached 60% of its initial value. One enzyme unit is defined here as the amount of enzyme that decreased the A_{680} 1% per min under the foregoing conditions (2).

Protein Determination

Protein concentrations were measured by the method of Lowry *et al.* (13), using BSA as a standard.

Analytical SDS-PAGE

The protein compositions of various samples were analyzed on SDS-gels containing 8% acrylamide (19, 22). Samples were boiled in denaturation buffer, subjected to electrophoresis, and stained with Coomassie brilliant blue (19).

Immunoblotting

Proteins separated on a SDS-gel were transferred electrophoretically to a nitrocellulose filter. The blot was reacted with specific antibodies and the (crossreacting) bands were visualized with a protein A-peroxidase probe, as described by Hovenkamp-Hermelink *et al.* (11). The latter procedure was

Table I. *Purification of Potato Branching Enzyme*

Fraction	Volume	Protein	Total Activity[a]	Specific Activity[a]
	mL	*mg*	*units*	*units/mg*
1. 0–50% $(NH_4)_2SO_4$	300	20310	1488	0.1
2. 0–40% $(NH_4)_2SO_4$	175	9800	2576	0.3
3. 1st DEAE-cellulose	445	779	1558	2.0
4. 2nd DEAE-cellulose	394	473	3842	8.1
5. 1st 4-aminobutyl-Sepharose	197	79	1649	20.9
6. 2nd 4-aminobutyl-Sepharose	40	56	1691	30.2
7. 1st Sephacryl S-300	1.6	6.6	566	86.3
8. 2nd Sephacryl S-300	1.0	0.8	151	184.0
9. 3rd Sephacryl S-300	2.0	0.3	53	188.7

[a] Activity measured by phosphorylase *a* stimulation.

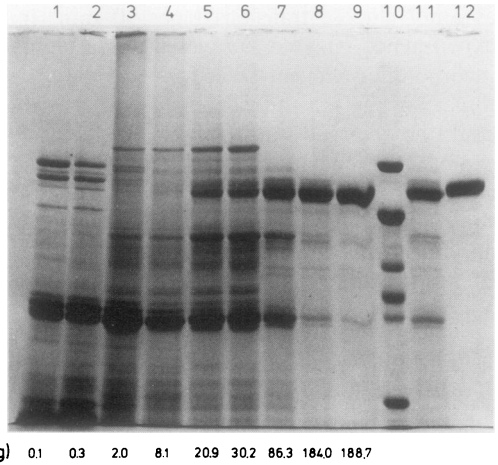

Figure 1. SDS-gel analysis of potato branching enzyme at different stages of purification. Lanes 1 to 9 illustrate the purification of native potato BE. Samples contain: potato BE after 0 to 50% ammonium sulphate precipitation (100 μg, lane 1), 0 to 40% ammonium sulphate precipitation (100 μg, lane 2), first DEAE-cellulose chromatography (80 μg, lane 3), second DEAE-cellulose chromatography (80 μg, lane 4), first Ag-butylamine chromatography (50 μg, lane 5), second Ag-butylamine chromatography (50 μg, lane 6), first S-300 gel filtration (30 μg, lane 7), second S-300 gel filtration (15 μg, lane 8), and third S-300 gel filtration (15 μg, lane 9). Lane 10 contains—from top to bottom—marker proteins of 97 kD (rabbit muscle phosphorylase *b*), 68 kD (bovine serum albumine), 55 kD (glutamate dehydrogenase), 45 kD (ovalbumine), 40 kD (aldolase), and 25 kD (chymotrypsinogen *A*). Lanes 11 and 12 illustrate the purification of denatured potato branching enzyme: partially purified BE (15 μg, lane 11) was further purified by preparative SDS-gel electrophoresis to give pure BE (15 μg, lane 12).

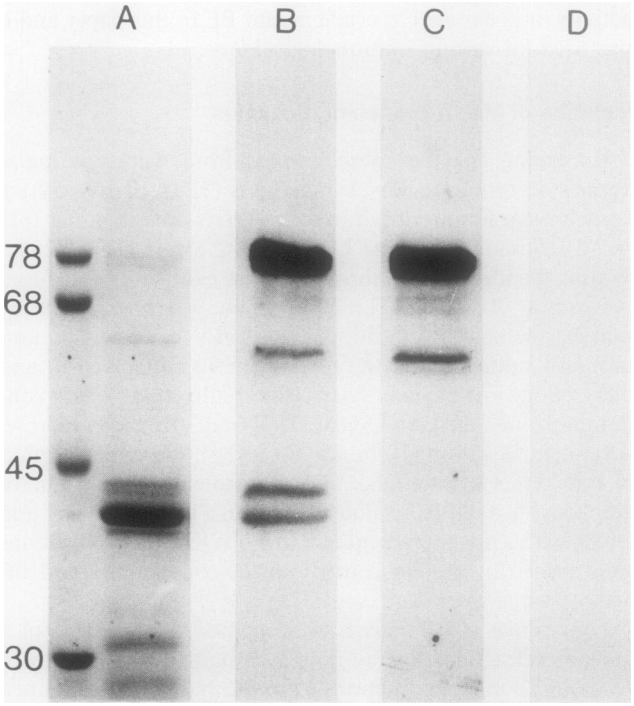

Figure 2. Immunoblotting of crude potato branching enzyme using two different anti-potato BE antibodies. Multiple samples of crude potato BE (30 μg protein) were separated on a SDS-gel. Part of the gel was stained with (A) Coomassie brilliant blue, whereas other sections were blotted to nitrocellulose and detected with anti-NPBE IgG (B, 0.6 mg IgG used), anti-DPBE IgG (C, 0.8 mg IgG used) or control antibodies (D, preimmune IgG from the same rabbit as anti-NPBE; 0.3 mg IgG used). Section A contains a different set of marker proteins than that in Figure 1: in addition to the markers of 68 and 45 kD, markers of 78 kD (ovotransferrin) and 30 kD (carbonic anhydrase) are present.

modified with respect to the one described in Vos-Scheperkeuter *et al.* (20) as follows. First, the transfer buffer contained 20% (v/v) methanol instead of SDS, and second, pretreatment of the blot with gelatin was omitted and replaced by 8 to 10 washing steps of 10 min each with incubation buffer. The latter buffer contained 20 mM Tris-HCl (pH = 7.5), 0.05% (v/v) Tween 20, and 200 mM—instead of 500 mM—NaCl. These modifications increased the sensitivity of immunodetection significantly.

Neutralization Tests

Crude potato or maize BE was incubated with increasing amounts of anti-NPBE, anti-DPBE, or nonimmune (control) IgG and incubated for 30 min at room temperature. Immediately following incubation, samples were assayed for BE activity without further manipulations, using either assay A or B. The enzyme activity found after preincubation with IgG was expressed as a percentage of that found in the absence of IgG.

Immunoadsorption Experiments

Heat-inactivated, fixed cells of *Staphylococcus aureus* Cowan I were prepared (12), washed extensively, and incubated with anti-NPBE IgG or nonimmune IgG using about 0.5 mg IgG per mL 10% (v/v) SaCI suspension. Incubation was performed in a rotation apparatus during 1 h at room temperature. The IgG-coated SaCI cells were washed by three cycles of centrifugation (7 min at 3,000 *g*) and resuspension in 10 mM Tris-HCl (pH = 7.5) containing 1 mM EDTA. Subsequently, increasing amounts of this suspension were added to the antigen preparation, *i.e.* crude potato or maize BE, and incubated for 15 min at room temperature under frequent mixing. The SaCI-bound immunocomplexes were collected by centrifugation and the residual BE activity present in the supernatant was measured. The latter activity was expressed as a percentage of the activity found when no IgG-coated SaCI cells had been added.

In some experiments the protein composition of the immunocomplexes isolated via SaCI adsorption were analyzed by SDS-gel electrophoresis as described previously (19).

RESULTS

Purification of Potato Branching Enzyme

When concentrated potato juice was chromatographed on DEAE-cellulose in TED buffer containing 75 mM NaCl, most proteins were not retarded whereas potato branching enzyme was completely adsorbed to the column (21). Elution with a 75 to 375 mM NaCl-gradient yielded a single potato BE peak

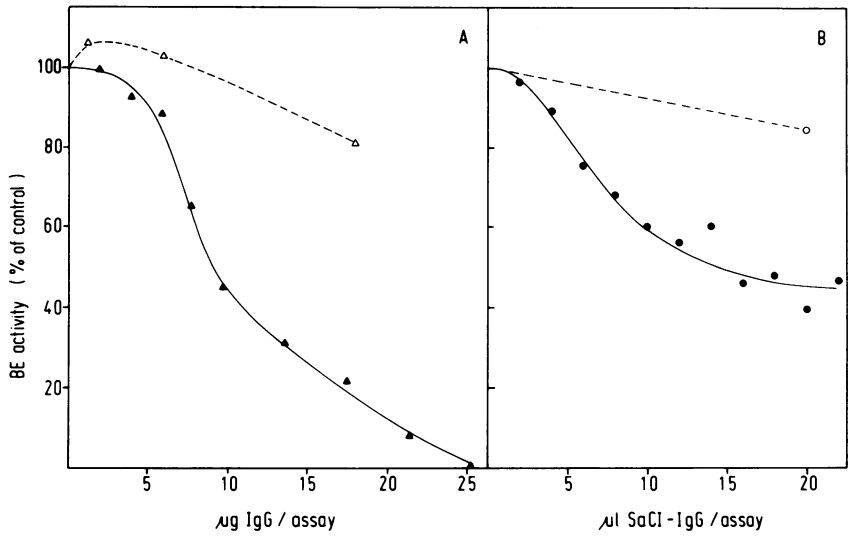

Figure 3. Neutralization (A) and immunoadsorption (B) of potato branching enzyme by antinative potato BE IgG. Crude potato BE (4 µg protein per sample) was either incubated with increasing amounts of anti-NPBE IgG and then assayed directly (A; ▲) or with increasing amounts of anti-NPBE IgG-coated SaCl-cells and assayed after removal of the immunocomplexes (B; ●). The open symbols indicate the results when nonimmune IgG was used (△, ○). The control samples (100% values) correspond to an activity of 1.12 (A) or 1.04 (B) µmol glucose transferred per 90 min.

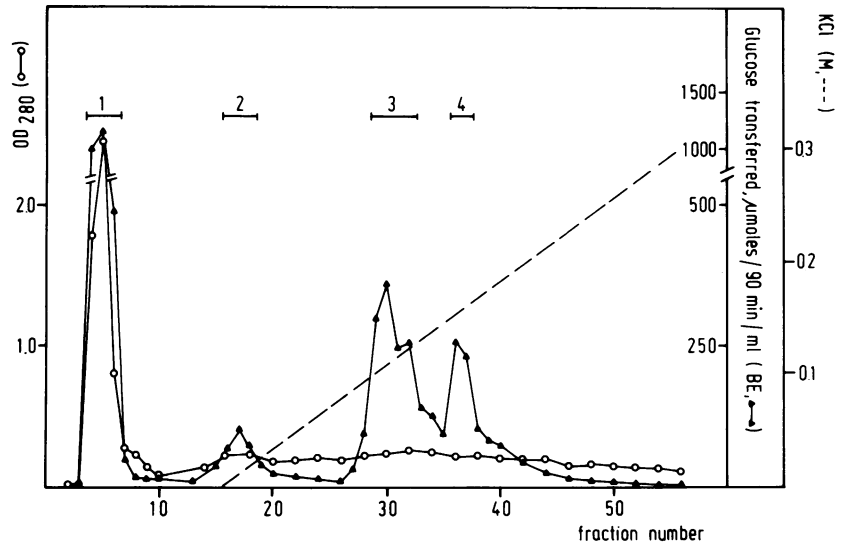

Figure 4. Chromatography of maize branching enzymes on DEAE-cellulose. A maize kernel extract (75 mg protein) was adsorbed to a DEAE-cellulose column, washed with two bed volumes starting buffer, and eluted with a 500 mL 0 to 0.4 M KCl gradient (– – –). Fractions were tested for protein content (A_{280}: ○——○) and BE activity (▲——▲). Four BE containing fractions (1–4) were pooled as indicated.

which contained only 8% of the total amount of protein applied to the column (21; Table I).

The enzyme also bound to 4-amino-butyl Sepharose and eluted as a broad peak in the middle of the salt gradient (results not shown). Chromatography on Sephacryl S-300 yielded a single, rather sharp and symmetrical peak. The elution profile on S-300 corresponded to a protein species of M_r > 70 kD, as indicated by comparison with standard proteins, and was not affected by incorporating 1% (v/v) Triton X-100 (with or without 1 M NaCl) in the buffer (results not shown).

Figure 1 and Table I give an overview of the total purification procedure. Several purification steps are required before BE can be recognized as one particular protein band in the complex protein profile of a potato tuber cell lysate. By isolating branching enzyme from the corresponding gel band a very pure—but denatured—BE fraction was obtained (Fig. 1, lane 12). BE was found to have a mol wt varying between 77,000 and 81,000, with an average of 79,000 (Fig. 1, lanes 9 and 12).

The two potato BE preparations were used to raise specific anti-NPBE and anti-DPBE antibodies. These antibodies were characterized with respect to a crude potato BE preparation in immunoblotting and neutralization experiments.

Immunoblotting of Potato BE

Anti-NPBE IgG was found to detect a major band of 79 kD, a minor band of about 63 kD and two minor bands around 40 kD in crude potato BE (Fig. 2, lane B). The 40 kD bands correspond to the major protein bands present in crude potato BE and can be identified as patatin, the major storage protein of potato tubers (17). Thus, anti-NPBE IgG is contaminated with some patatin-specific IgG which is not unexpected since the enzyme preparation used for immunization still contained trace amounts of this protein (*cf.* Fig. 1, lane 9). Anti-DPBE IgG detected the same set of bands except the patatin bands (Fig. 2, lane C). All these bands were recognized specifically since preimmune IgG detected none of them (Fig. 2, lane D). The nature of the minor 63 kD band detected by

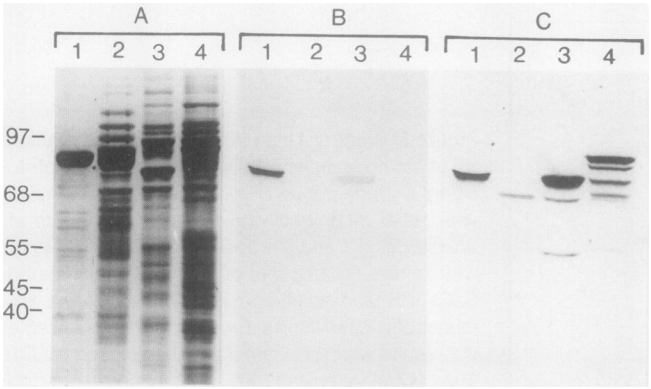

Figure 5. Immunoblotting of various forms of maize branching enzyme using potato BE-specific antibodies. Multiple samples of maize BE pools 1, 2, 3, and 4 (25–50 μg protein per sample: lanes are numbered according to pool numbers) were separated on a SDS-gel. One section of the gel was stained with Coomassie brilliant blue (panel A), whereas other sections were blotted to nitrocellulose and detected with anti-NPBE IgG (1.5 mg IgG used; panel B) or anti-DPBE IgG (2.1 mg IgG used; panel C). Marker proteins (see Fig. 1) are shown on the left.

both potato BE-specific antibodies is not clear at present; possibly, it represents a proteolytic fragment of the enzyme.

Neutralization of Potato BE

The activity of potato branching enzyme could be inhibited completely by anti-NPBE IgG (Fig. 3A, closed triangles) but not by anti-DPBE IgG (data not shown). The latter IgG caused only a partial inhibition of BE activity (maximum 20%) at concentrations higher than about 20 μg IgG/assay; since nonimmune IgG showed a similar effect (Fig. 3A, open triangles), this is considered to be an aspecific effect.

The amount of IgG required to neutralize 50% of the potato BE activity was 9.2 μg anti-NPBE IgG per 4 μg protein (Fig. 3A), which corresponds to about 10 to 20 μl antiserum per unit enzyme when the estimated loss of activity during storage and the yield of IgG isolation are taken into account. The finding that potato BE can be neutralized for 100% by anti-NPBE IgG suggests that the active center of branching enzyme is easily accessible to IgG molecules.

Despite this easy access, only 60% of the total enzyme activity could be removed from solution by immunoadsorption of the enzyme with IgGs coupled to SaCI-cells and centrifugation of the immunocomplexes (Fig. 3B, closed circles). Again, nonimmune IgG allowed some aspecific adsorption and removal of branching enzyme (Fig. 3B, open circles).

Other potato tuber enzymes involved in the metabolism of starch such as amylolytic enzymes, phosphorylase and debranching enzyme (or: R-enzyme) were also tested against our potato BE-specific antibodies in neutralization or immunoadsorption experiments; no specific inhibition was observed (data not shown).

Separation of Maize Branching Enzyme Activities

Maize branching enzyme was fractionated by DEAE-cellulose into four peaks (Fig. 4). The chromatographic profile obtained is quite similar to previously reported ones (3, 4) in that one peak does not adsorb to the column and that two closely running peaks are eluted at 0.1 to 0.2 M KCl. Based on this similarity, our peaks can be identified as BE I (pool 1), BE IIb (pool 3), and BE IIa (pool 4) (3, 4). Figure 4 shows one additional small peak (No. 2); it eluted at the very beginning of the salt gradient and may therefore represent loosely adsorbed material.

The protein composition of pools 1 to 4 were analyzed by SDS-PAGE (Fig. 5, panel A). Each of the four BE peaks appeared to have a distinct protein profile, those of peaks 2, 3, and 4 being quite complex. Pool 1 showed a relatively simple protein profile with only one abundant protein species

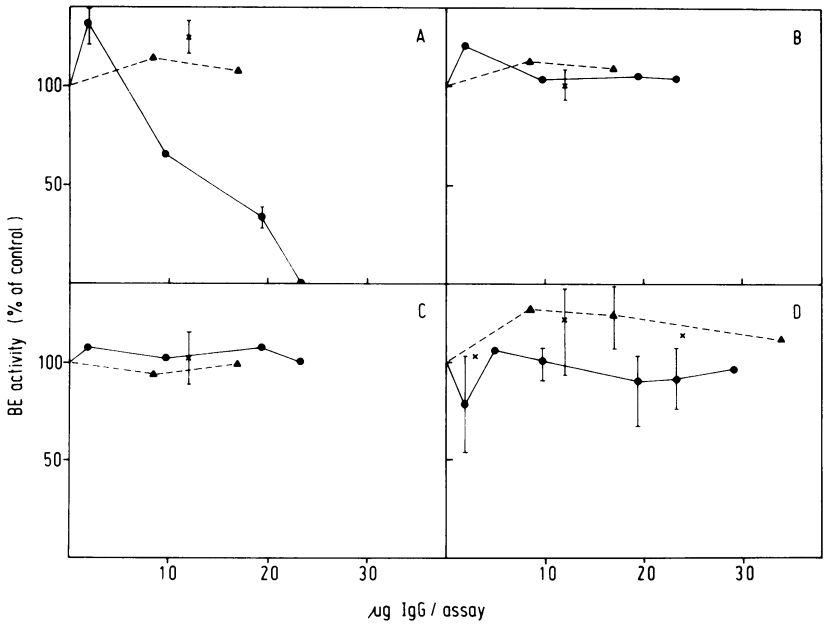

Figure 6. Neutralization of various forms of maize branching enzyme using potato BE-specific antibodies. Maize BE pools containing comparable enzyme activities were incubated with increasing amounts of anti-NPBE IgG (●——●), anti-DPBE IgG (▲- - -▲), or nonimmune IgG (×) and then assayed using assay A. Samples contained: pool 1 (1.2 μg protein transferring 1.24 μmol glucose/90 min; panel A), pool 2 (16.0 μg protein transferring 1.33 μmol glucose/90 min; panel B), pool 3 (0.5 μg protein transferring 1.71 μmol glucose/90 min; panel C), and pool 4 (0.7–1.0 μg transferring an average of 0.80 μmol glucose/90 min; panel D). When samples were measured in duplicate or triplicate, the average value is given together with the range (vertical bars). BE activity is expressed as a percent of the activity found in the absence of IgG.

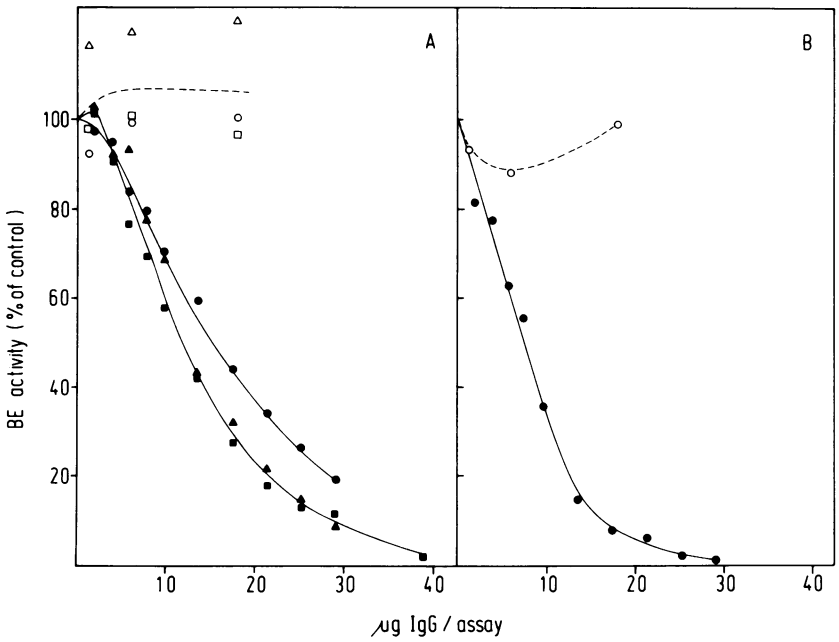

Figure 7. Neutralization of maize BE I by antinative potato BE IgG. Maize BE I (pool 1, containing a specific activity of 2.4 units/mg as measured by assay A) was incubated with increasing amounts of anti-NPBE IgG (closed symbols) or nonimmune IgG (open symbols) and then assayed using either assay A (panel A) or assay B (panel B). The different symbols represent the results of various independent experiments: the two curves of panel A represent experiments in which either 3.2 µg (▲, △; ■, □) or 8.0 µg (●, ○) protein was used per assay, whereas the curve in panel B was obtained with 8.0 µg protein per assay (●, ○). The control samples (100% values) correspond to an activity of 0.94 to 1.83 µmol glucose transferred per 90 min in assay A (panel A), or 5.51 enzyme units in assay B (panel B).

(Fig. 5A, lane 1). By comparing the electrophoretic mobility of small amounts (1–2 µg) of this major protein with those of standard proteins run on the same slab gel, the molecular mass of this protein was estimated to be 85 kD, which is slightly higher than the molecular mass of 82 kD estimated previously by us (21). Experiments described below strongly suggest that this major 85 kD protein is not identical to maize BE I.

Immunoblotting of Various Maize BE Forms

Proteins present in maize BE pools 1 to 4 were transferred from an SDS-gel to a nitrocellulose filter and immunoblotted with potato BE-specific antibodies (Fig. 5, panels B and C, respectively). Anti-NPBE reacted strongly and specifically with one particular protein band of 80 to 83 kD in pool 1, and weakly with a protein band of similar M_r in pool 3. None of the protein bands in pool 2 or pool 4 was recognized by anti-NPBE IgG (Fig. 5, panel B).

There was much more cross-reactivity when maize BE fractions were immunoblotted with antibodies directed against denatured potato BE isolated from a preparative SDS-gel (anti-DPBE; Fig. 5, panel C). The number of cross-reacting bands varied between one (in pool 1) and about six (in pool 4), while the overall patterns were distinct for each maize BE fraction. The nature of the cross-reacting bands is discussed below.

Neutralization of Various Maize BE Forms

The ability of the potato BE-specific antibodies to neutralize the activity of various maize BE fractions was tested (Fig. 6). Maize BE pools 1 to 4 (Fig. 6, panels A–D, respectively) were preincubated with either anti-NPBE IgG, anti-DPBE IgG, or control IgG and then assayed without further manipulations. Neutralization was observed only when maize BE pool I (BE I) was incubated with anti-NPBE antibodies (Fig. 6A, closed circles). Maize BE pools 2 to 4 were not inhibited by these antibodies (Fig. 6, panels B–D) in spite of the fact that pool 3 reacted with anti-NPBE in the immunoblotting experiment (Fig. 5, panel B). This apparent discrepancy may be due to the fact that, compared to pool 1, pool 3 reacted weakly in the immunoblotting experiment.

Further Characterization of the Cross-Reaction between Maize BE I and Potato BE

The neutralization experiment shown in Figure 6A was repeated in more detail (Fig. 7). Inhibition of maize BE I by anti-NPBE was specific and reproducible; half-maximal inhibition was observed with 12 to 16 µg IgG/assay, while neutralization was complete at 25 to 40 µg IgG/assay (Figs. 6A and 7A). A comparable inhibition was observed when neutralization was monitored via the alternative BE assay (*i.e.* assay B; Fig. 7, panel B).

The amount of anti-NPBE IgG required to neutralize 50% of the maize BE I activity varied between 1.9 and 3.7 µg IgG per µg maize protein in different experiments (Fig. 7A) which corresponds to about 20 to 35 µL antiserum per unit enzyme when the estimated loss of activity during storage and the yield of IgG isolation are taken into account.

Maize BE I was also reacted with SaCI-bound anti-NPBE antibodies. The results were quite similar to those obtained with immunoadsorption of potato BE (*cf.* Fig. 3, panel B): the enzyme activity of maize BE I could be decreased by immunoadsorption to a minimum of about 50% (results not shown).

Combining the results of Figures 5 and 7, it is tempting to speculate that the 80 to 83 kD maize protein in pool 1 which cross-reacts with anti-NPBE IgG in immunoblotting is identical to the enzyme which is neutralized by the same IgG. This possibility was tested by isolating the native maize enzyme from pool 1 via immunoadsorption and analyzing the obtained immunocomplex by SDS-gel electrophoresis (Fig.

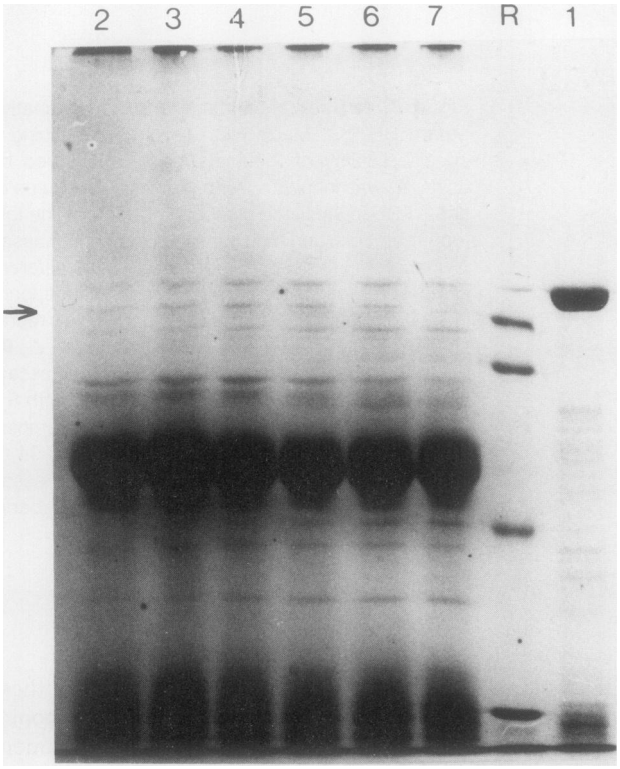

Figure 8. SDS-gel analysis of immunoprecipitates isolated from maize BE I using anti-NPBE IgG. Maize BE I (pool 1, 2.4 units/mg) was incubated with anti-NPBE IgG-coated SaCI cells (lanes 2–6) or nonimmune IgG-coated SaCI cells (lane 7) and the adsorbed immunocomplexes were analyzed on a SDS-gel. Increasing amounts of maize BE I were used, varying between 32 μg (lane 2) and 160 μg (lane 6) protein, while either 80 μL (lane 2) or 100 μL (lanes 3–7) of the IgG-coated SaCI cell suspension were added. Lane 1 contains maize BE I (pool 1; 16 μg protein), lane R shows marker proteins (same as those in Fig. 2). The arrow indicates the position of the specifically precipitated maize protein.

8). Numerous protein bands were observed, the major ones belonging to IgG molecules (H- and L-chains of about 50 and 25 kD, respectively). When the immunocomplexes obtained with anti-NPBE IgG were compared with those obtained with nonimmune IgG, only one protein band (arrow in Fig. 8) was found to be precipitated specifically. Both the molecular mass (82 kD) and the relative abundancy of this protein, suggest that the homology between native potato BE and maize BE I resides in a minor protein of slightly lower M_r than the major protein (85 kD, see above) present in this fraction.

This conclusion was strongly supported by a direct comparison between potato branching enzyme and maize branching enzyme I in an immunoblot subjected to an additional protein staining (Fig. 9). Panels B, C, and D show the immunoblots obtained with anti-NPBE, anti-DPBE, or preimmune IgG, respectively, either before (left halves) or after (right halves) the additional protein staining. The potato enzyme (M_r = 79,000) is detected as the major immunoreactive protein species which represents only a few percent of the total protein content of this crude potato BE preparation (Fig. 9, lanes p, *cf.* panels B and C *versus* A). It is evident that the only protein band in the maize BE I fraction (lanes m in all panels) which is detected specifically by both potato BE-specific antibodies is a minor protein running slightly faster than the major protein present in the same fraction.

DISCUSSION

Purification of Potato BE and Characterization of Anti-Potato BE

Potato branching enzyme has been purified to near homogeneity using several cycles of chromatography on three different types of column. Although the overall yield (3.6%) and the purification factor (1887-fold) can be calculated from Table I, these values represent no more than rough estimates since BE activity cannot be measured reliably in crude potato fractions (*cf.* Table I: note the heavy fluctuations in the total enzyme activity measured during the first few purification steps).

The procedure used by us to purify potato branching enzyme resembles that previously published by Borovsky *et al.* (2), whereas chromatography on 4-amino-butyl Sepharose was based on the successful use of this material in the purification of maize branching enzyme (3). The purification was monitored by assaying branching enzyme activity indirectly (assay A). Although it is known that multiple forms of branching enzyme may display different activities in assay A or assay B (3, 16), this did not interfere with our purification since subsequent purifications clearly showed that the potato BE peaks detected by either assay A or assay B completely coincide (data not shown).

By pooling column fractions on the basis of both enzyme measurements and SDS-gel electrophoresis, we obtained a highly purified potato BE with a M_r of 79 kD (range: 77–81 kD), which is somewhat lower than that reported by Borovsky *et al.* (85 kD; 2). Both the differences in SDS-gel types (7% tube gels *versus* 8% slab gels) and the different sets of reference proteins used probably account for this discrepancy.

The behavior of potato branching enzyme on all columns tested strongly suggests that the enzyme occurs as a single protein entity, as has been concluded previously (2). Close examination of the SDS-gel profile, however, shows that the purified protein does not run as a single sharp band, but rather as a diffuse and broad band (*cf.* Fig. 1) which, in some cases, appeared to consist of two very closely running bands (*cf.* Fig. 2). Although this opens the possibility that potatoes, like many other plants (6), contain multiple forms of branching enzyme, it is clear that additional evidence has to be obtained for the definite identification of this double band.

The two types of potato BE-specific antibodies which were used in this study, one against the native and one against the SDS-denatured enzyme, were first characterized with respect to the corresponding enzyme. Anti-NPBE IgG reacted with potato BE under both native conditions (*i.e.* in neutralization and immunoadsorption experiments) and denaturing conditions (*i.e.* in immunoblotting), whereas recognition of the enzyme by anti-DPBE IgG was only observed after SDS-denaturation (*i.e.* in immunoblotting). This result suggests that the antigenic determinants of native potato branching

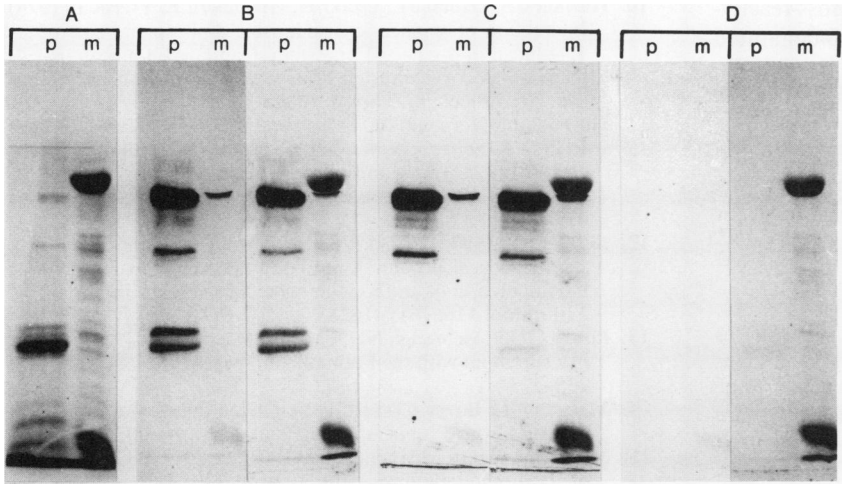

Figure 9. Immunoblotting of crude potato BE and maize BE I using two different potato BE-specific antibodies. Multiple samples of crude potato BE (30 μg, lanes p) and maize BE I (pool 1; 32 μg, lanes m) were separated on a SDS-gel. One section of the gel was stained with Coomassie brilliant blue (panel A) whereas other sections were blotted to nitrocellulose and detected with anti-NPBE IgG (1.2 mg IgG used; panel B), anti-DPBE IgG (1.7 mg IgG used; panel C), or control IgG (preimmune IgG from the same rabbit as anti-NPBE; 0.6 mg IgG used, panel D). The left halves of sections B to D show the blot directly after immunoreaction, whereas the right halves of these sections show the same blots after additional protein staining with Amido Black.

enzyme are completely distinct from those exposed after SDS-denaturation.

Relationship between Potato and Maize BE

One form of maize branching enzyme, *i.e.* BE I, could be neutralized for 100% by anti-NPBE, whereas none of the maize enzymes was inhibited by anti-DPBE. This implies that maize BE I resembles the native potato branching enzyme but not, or much less, the SDS-denatured potato enzyme, which is not surprising since the two antisera recognize quite a different set of antigenic determinants (see above).

The amounts of anti-NPBE required to neutralize 50% of the original activities were 10 to 20 μL antiserum per unit potato BE and 20 to 35 μL antiserum per unit maize BE I, respectively. From this small difference it can be concluded that at least some of the sites on maize BE I which are involved in enzymic activity are quite similar to those on potato BE.

The activity of crude potato BE or maize BE I could also be reduced significantly by adsorption to SaCI-bound anti-NPBE IgG followed by removal of the adsorbed proteins by centrifugation. In contrast to neutralization, however, immunoadsorption never resulted in complete abolishment of the enzyme activity: the activities of potato BE and maize BE I could be decreased to 40 and 50% of their originals levels, respectively. This partial elimination of BE activity by immunoadsorption to immobilized antipotato BE antibodies may be compared to the maximum inhibition of 85% observed by Singh and Preiss (18) when maize BE I activity was immunoadsorbed to immobilized antimaize BE I antibodies.

Using gel analysis of immunoprecipitates and protein staining of immunoblots, the protein species in the maize BE I fraction which reacted with anti-NPBE could be identified as a minor protein of 82 kD. Thus, our results indicate that the maize branching enzyme found in pool 1 (BE I) which is cross-reactive with potato branching enzyme is an enzyme with M_r = 82,000, as also reported by Singh and Preiss (18).

In immunoblotting, anti-DPBE reacted with 1 to 6 protein bands in all four maize BE fractions, whereas anti-NPBE reacted only with a single protein species in two BE fractions. This result is probably best explained by the previous finding that fully denatured antigens elicit a wider range of antibodies than do native antigens (15, 23). These multiple cross-reacting protein species, which were particularly observed in maize BE pools 3 and 4, suggest that maize kernels contain several proteins sharing sequence similarities with potato BE. Clearly, at least one of these bands must be identical to maize BE IIb (in pool 3) or maize BE IIa (in pool 4). From previous studies it can be estimated that the molecular mass of maize BE IIa/IIb is probably in the order of 72 to 80 kD (3, 18). Therefore, multiple cross-reacting bands in pools 3 and 4 appear to be putative candidates for these forms of maize branching enzyme (Fig. 5, panel C).

Based on both protein staining and immunoblotting, maize BE pools 1, 2, 3, and 4 appear to be clearly distinct fractions. This conclusion has some interesting implications. First, it implies that although pool 2 is a minor fraction possibly representing proteins loosely bound to the DEAE-cellulose matrix, it is clearly a BE-related fraction with unique properties as compared to the three well-known maize BE forms. The precise nature of this pool remains to be elucidated. Second, our immunoblotting experiment reveals that maize BE IIa and IIb display different reactions *versus* anti-DPBE, a result which does not support the previous suggestion that BE IIa and BE IIb may be identical (18).

Some cross-reacting protein bands appear to be common to multiple maize BE pools. In particular, a major 80 to 83 kD protein band is detected in pool 1 and pool 3 by both anti-NPBE and anti-DPBE, which indicates that these bands are immunologically related. However, their reactions *versus* the two types of antipotato BE are clearly not proportional: the band in pool 1 shows the strongest reaction toward anti-NPBE, whereas the band in pool 3 is better recognized by anti-DPBE. We therefore conclude that the 80 to 83 kD bands in pools 1 and 3 are probably not identical, a conclusion which is supported by the fact that pool 1 and pool 3 react quite differently in neutralization experiments (Fig. 6). A minor protein band of about 75 kD was detected in both pools 2 and 3 (Fig. 5, panel C); the relationship between these two bands is also not clear. Further experiments are required to elucidate the nature of these multiple cross-reacting species.

In conclusion, our results indicate that potato branching enzyme strongly resembles maize branching enzyme I in that

both enzymes are neutralized by the same IgG. Cross-reactivities have also been observed between the SDS-denatured forms of potato enzyme and those of maize BE IIa, BE IIb, and a fourth, unidentified BE fraction, indicating that the regions involved in these similarities are buried in the native enzymes. Therefore, all branching enzymes from potato and maize appear to share sequence similarities, albeit to varying extents, and may therefore derive from a common ancestor (15, 23).

ACKNOWLEDGMENTS

We would like to thank Mr. A. Scheperkeuter for supplying us with potato tubers cv Promesse, Dr. Hendri Pas for useful suggestions with respect to immunoblotting, and coworkers of the Dutch Institute for Carbohydrate Research (NIKO, Groningen) for their assistance in the large scale preparation of a potato cell lysate. We are also indebted to N. Panman for drawing and K. Gilissen for photography.

LITERATURE CITED

1. **Barker SA, Bourne EJ, Wilkinson IA, Peat S** (1950) The enzymic synthesis and degradation of starch. Part VII. The mechanism of Q-enzyme action. J Chem Soc 93–99
2. **Borovsky D, Smith EE, Whelan WJ** (1975) Purification and properties of potato, 1,4-α-D-glucan:1,4-α-D-glucan 6-α-(1,4-α-D-glucano)-transferase. Evidence against a dual catalytic function in amylose-branching enzyme. Eur J Biochem **59:** 615–625
3. **Boyer CD, Preiss J** (1978) Multiple forms of (1-4)-α-D-glucan-6-glucosyl transferase from developing *Zea mays* L. kernels. Carbohydr Res **61:** 321–334
4. **Boyer CD, Preiss J** (1981) Evidence for independent genetic control of the multiple forms of maize endosperm branching enzymes and starch synthases. Plant Physiol **67:** 1141–1145
5. **Boyer CD, Fisher MB** (1984) Comparison of soluble starch synthases and branching enzymes from developing maize and teosinte seeds. Phytochemistry **23:** 733–737
6. **Boyer CD** (1985) Soluble starch synthases and starch branching enzymes from developing seeds of sorghum. Phytochemistry **24:** 15–18
7. **Drummond GS, Smith EE, Whelan WJ** (1972) Purification and properties of potato α-1,4-glucan α-1,4-glucan 6-glycosyltransferase (Q-enzyme). Eur J Biochem **26:** 168–176
8. **Griffin HL, Wu YV** (1971) Corn and potato α-1,4-glucan:α-1,4-glucan 6-glucosyltransferase: evidence for separate hydrolytic and branching components. Biochemistry **10:** 4330–4335
9. **Hawker JS, Downton WJS** (1974) Starch synthases from *Vitis vinifera* and *Zea mays*. Phytochemistry **13:** 893–900
10. **Hawker JS, Ozbun JL, Ozaki H, Greenberg E, Preiss J** (1974) Interaction of spinach leaf adenosine diphosphate glucose α-1,4-glucan α-4-glucosyl transferase and α-1,4-glucan α-1,4-glucan-6-glucosyl transferase in synthesis of branched α-glucan. Arch Biochem Biophys **160:** 530–551
11. **Hovenkamp-Hermelink JHM, Jacobsen E, Ponstein AS, Visser RGF, Vos-Scheperkeuter GH, de Vries JN, Witholt B, Feenstra WJ** (1987) Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.). Theor Appl Genet **75:** 217–221
12. **Kessler SW** (1975) Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J Immunol **115:** 1617–1624
13. **Lowry OH, Rosebrough NH, Farr AL, Randall RJ** (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–273
14. **Matters GL, Boyer CD** (1981) Starch synthases and branching enzymes from *Pisum sativum*. Phytochemistry **20:** 1805–1809
15. **Mouhli H, Zakin MM, Richaud C, Cohen GN** (1980) Detection of the homology among the aspartokinase I-homoserine dehydrogenase I and the aspartokinase III from *E. coli* K12 by immunochemical cross-reactivity between denatured species. Biochem Int **1:** 403–409
16. **Preiss J, Levi C** (1980) Starch biosynthesis and degradation. *In* J Preiss, ed, The Biochemistry of Plants, Vol 3, Carbohydrates, Structure and Function. Academic Press, New York, pp 371–423
17. **Racusen D, Foote M** (1980) A major soluble glycoprotein of potato tubers. J Food Biochem **4:** 43–52
18. **Singh BK, Preiss J** (1985) Starch branching enzymes from maize. Immunological characterization using polyclonal and monoclonal antibodies. Plant Physiol **79:** 34–40
19. **Vos-Scheperkeuter GH, Witholt B** (1984) Assembly pathway of newly synthesized LamB protein, an outer membrane protein of *Escherichia coli* K-12. J Mol Biol **175:** 511–528
20. **Vos-Scheperkeuter GH, de Boer W, Visser RGF, Feenstra WJ, Witholt B** (1986) Identification of granule-bound starch synthase in potato tubers. Plant Physiol **82:** 411–416
21. **Vos-Scheperkeuter GH, Ponstein AS, de Wit JG, Feenstra WJ, Oostergetel GT, van Bruggen EFJ, Witholt B** (1987) Biosynthesis of starch; identification of potato starch enzymes. Food Hydrocoll **1:** 387–391
22. **Wensink J, Witholt B** (1981) Identification of different forms of the murein-bound lipoprotein found in isolated outer membranes of *Escherichia coli*. Eur J Biochem **113:** 349–357
23. **Zakin MM, Garel J-R, Dautry-Varsat A, Cohen GN, Boulot G** (1978) Detection of the homology among proteins by immunochemical cross-reactivity between denatured antigens. Application to the threonine and methionine regulated aspartokinases-homoserine dehydrogenases from *E. coli* K12. Biochemistry **17:** 4318–4323