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Purification and Partial Characterization of Potato (*Solanum tuberosum*) Invertase and Its Endogenous Proteinaceous Inhibitor

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ABSTRACT

Invertase plays an important role in the hydrolysis of sucrose in higher plants, especially in the storage organs. In potato (*Solanum tuberosum*) tubers, and in some other plant tissues, the enzyme seems to be controlled by interaction with an endogenous proteinaceous inhibitor. An acid invertase from potato tubers (variety russet) was purified 1560-fold to electrophoretic homogeneity by consecutive use of concanvalin A-Sepharose 4B affinity chromatography, DEAE-Sephadex A-50-120 chromatography, Sephadex G-150 chromatography, and DEAE-Sephadex A-50-120 chromatography. The enzyme contained 10.9% carbohydrate, had an apparent molecular weight of 60,000 by gel filtration, and was composed of two identical molecular weight subunits (M_r 30,000). The enzyme had a K_m for sucrose of 16 millimolar at pH 4.70 and was most stable and had maximum activity around pH 5. The endogenous inhibitor was purified 610-fold to homogeneity by consecutive treatment at pH 1 to 1.5 at 37°C for 1 hour, $(NH_4)_2SO_4$ fractionation, Sephadex G-100 chromatography, DEAE-Sephadex G-50-120 chromatography, and hydroxylapatite chromatography. The inhibitor appears to be a single polypeptide (M_r 17,000) without glyco groups. The purified inhibitor was stable over the pH range of 2 to 7 when incubated at 37°C for 1 hour.

Sucrose is the most abundant transportable free carbohydrate in the plant kingdom. Sucrose serves as an important reserve carbohydrate in plants, especially in such storage organs as tuber, root, and seed. During germination, sucrose is a readily degradable source of energy. In storage organs, invertase (β-fructofuranosidase; EC 3.2.1.26) functions in the hydrolytic degradation of sucrose to glucose and fructose.

Invertase was one of the first enzymes discovered; it was first isolated from yeast more than a century ago (3). The enzyme occurs widely in microbial, plant, and animal sources. The presence of invertase in potatoes was first reported over 80 years ago (10). The first evidence for an endogenous proteinaceous invertase inhibitor in potatoes was obtained from kinetic studies on invertase in crude extract (22). Pressey (18, 19) showed an inhibitor was present by its purification to homogeneity and partial characterization. Invertase inhibitors have been found also in red beet, sugar beet, and sweet potato (13, 20) and in maize endosperm (8). These higher plant invertase inhibitors are all soluble proteins and their inhibitor activity seems to be limited to acid invertases.

The present paper describes the purification of potato invertase and its endogenous inhibitor to homogeneity, with partial characterization of the two proteins.

MATERIALS AND METHODS

BSA (98–99% pure, catalog No. A-7030), sweet potato β-amylase (type 1-B, crystalline suspension, catalog No. A-7005), bovine pancreatic trypsin (type IX, crystallized, catalog No. T-0134), bovine thyroglobulin (type I, catalog No. T-1001), chicken lysozyme (grade I, 3X crystallized, catalog No. L-6876), methyl mannopyranoside (grade III, catalog No. M-6882), DEAE-Sephadex (stock A-50-120), and Con A-Sepharose 4B (catalog No. C-9017) were obtained from Sigma Chemical Co.

Human transferrin (electrophoretically pure, catalog No. 616397) and hydroxylapatite (fast flow, catalog No. 391947) were obtained from Calbiochem. Chicken ovalbumin was a gift from Dr. Robert Feeney's laboratory. A standard proteins kit for mol wt determination using SDS-PAGE was obtained from Bethesda Research Laboratories. Sephadex G-100 and Sephadex G-150, both column chromatography fine grade, were obtained from Pharmacia. All other chemicals used were of reagent grade. All water used was double deionized.

Potatoes (*Solanum tuberosum*, var russet) were purchased in local supermarkets and screened for invertase and inhibitor activities. Potatoes with high inhibitor activity were peeled, sliced, soaked in a 0.1 M $NaHSO_3$ solution, frozen rapidly, and stored at −20°C in polyethylene bags, each containing about 1 kg of sliced potatoes. Potatoes with high invertase activity were stored at 4°C in polyethylene bags for at least 3 weeks.

Screening of Potatoes for Invertase and Inhibitor

In a typical experiment, about 100 g of peeled, sliced potatoes were homogenized with 10 mL of 0.1 M $NaHSO_3$ for 2 min in a Waring blender at 4°C. The extract was filtered through eight layers of cheesecloth, and the filtrate was centrifuged at 15,000*g* for 30 min at 4°C. A portion of the supernatant was dialyzed against three changes of water (at least 50-fold dialysate/sample ratio was always used) at 4°C for 3 to 4 h each time. The remainder of the supernatant was blended for 30 min in a Waring blender at 4°C. Blending was carried out with 5 min blending periods, with 10 min cooling periods in between. The blended extract was centrifuged at 15,000*g* for 30 min at 4°C and dialyzed as above. The precipitate that formed during dialysis was removed by centrifuga-

tion, and the supernatants were assayed for invertase and inhibitor activities as described below.

Assay for Invertase Activity

Invertase activity was determined by measuring reducing sugars formed from sucrose hydrolysis by the Somogyi method as modified by Nelson (15). The incubation mixture (0.5 mL) contained a suitable amount of enzyme, 80 mM acetate buffer (pH 4.70), and 143 mM sucrose. The assay was performed at 37°C for 1 h. One-half mL of copper reagent was then added to terminate the reaction, and the tubes were immediately heated for 30 min in a boiling water bath. After cooling, 0.5 mL of arsenomolybdate reagent followed by 3.5 mL of water were added. Solutions were mixed, and 1.0 mL of each was centrifuged for 2 min using a high speed microfuge and the absorbance was read at 660 nm. Boiled enzyme blanks included as controls did not vary significantly in absorbance from reagent blanks. Centrifugation was required only with impure preparations of invertase or inhibitor. A unit of invertase is defined as the amount of enzyme that catalyzed the breakdown of 1 μmol of sucrose/min at 37°C and pH 4.70. The specific activity is expressed as units of invertase per mg of protein.

Assay for Inhibitor Activity

Inhibitor activity was determined by preincubating at 37°C a constant amount of invertase with varying amounts of inhibitor in 0.4 mL incubation mixture containing 80 mM acetate buffer, pH 4.70. At the end of 1 h of incubation, 100 μL of 720 mM sucrose (final concentration of 144 mM) was added to each tube and the remaining invertase activity left was measured as described above. Enzyme without inhibitor added was used as control. A plot of residual activity *versus* amount of inhibitor used was linear over the range of 0 to 80% inhibition. The amount of inhibitor in each sample was estimated from the corresponding plot. A unit of inhibitor was defined as that amount which inhibits one unit of invertase 50% at pH 4.70 and 37°C. The specific activity is expressed as units of inhibitor per mg of protein.

Protein Determination

Protein was determined by the method of Lowry *et al.* (12) with BSA as a standard protein. For column fractions, the protein concentrations were estimated by absorbance at 280 nm.

Purification of Invertase

Storage of potatoes at 4°C for 3 to 5 weeks usually increased the invertase content two- to threefold. However, with some batches of potatoes, the effect of storage at low temperatures was not as pronounced. Purification of invertase was always performed on low temperature-stored potatoes.

All the purification procedures were performed at 4°C and with centrifugation at 15,000*g* for 30 min, unless otherwise noted.

Potatoes (1 kg), peeled and sliced, were homogenized with 100 mL of 100 mM $NaHSO_3$ for 2 min in a Waring blender at 4°C. The extract was filtered through eight layers of cheesecloth and the filtrate was centrifuged. The supernatant, normally about 600 mL, was dialyzed against three changes of water. After dialysis, the precipitate formed was removed by centrifugation, and the supernatant was freeze-dried. The lyophilized powder was dissolved in 190 mL of 20 mM Tris-HCl/500 mM NaCl buffer (pH 7.40), containing 1 mM each $CaCl_2$, $MgCl_2$, and $MnCl_2$, and the solution was applied to a Con A-Sepharose 4B affinity column (2.5 × 10 cm) equilibrated with the same buffer. Elution (using the same buffer) proceeded until the 280 nm absorbance of the fractions was 0.040 or less. The enzyme was then eluted with 30 mM methyl α-D-mannopyranoside in the above buffer. The fractions with invertase activity were combined, concentrated 10-fold by ultrafiltration using an Amicon PM-10 membrane, and dialyzed three times against 20 mM sodium phosphate buffer (pH 6.0). After the Con A-Sepharose 4B step, the dilute solutions of invertase were very unstable, even at low temperature. Immediate and rapid concentration by ultrafiltration stopped loss of activity.

The enzyme solution from the Con A-Sepharose 4B affinity column was applied to the top of a DEAE-Sephadex A-50-120 column (2.5 × 18 cm) equilibrated with 20 mM phosphate buffer, pH 6.0. The column was washed with the same buffer until the 280 nm absorbance was zero. The enzyme then was eluted with a linear gradient produced by using 250 mL of the phosphate buffer in one chamber and 250 mL of the same buffer containing 500 mM NaCl in an identical mixing chamber. The fractions with invertase activity were combined and concentrated 10-fold by ultrafiltration as above.

The enzyme solution from the DEAE-Sephadex A-50-120 column was applied to a Sephadex G-150 column (1.5 × 90 cm) equilibrated with 50 mM sodium phosphate/100 mM NaCl buffer (pH 7.2). The column was eluted with the same buffer. The main fractions with invertase activity were combined and concentrated as above. The leading and trailing side fractions were combined, concentrated, and rechromatographed on the same Sephadex column. The main fractions were combined, concentrated, and combined with the first main pool.

The enzyme solution from the Sephadex G-50 column was dialyzed three times against 20 mM sodium phosphate buffer, pH 6.0, and rechromatographed on a DEAE-Sephadex A-50-120 column (1.5 × 18 cm) equilibrated with the same buffer. After the column was washed with the starting buffer, the enzyme was eluted with 100 mM NaCl/20 mM sodium phosphate buffer (pH 6.0). The main fractions were combined and concentrated as above. The enzyme was stored frozen in the same buffer in 25 μL aliquots at −20°C until used. Aliquots of invertase were diluted with appropriate buffers containing a final concentration of 0.1 mg/mL of BSA to stabilize the enzyme.

Purification of Inhibitor

The inhibitor content of potatoes did not vary significantly during frozen storage. All purification procedures were carried out at 4°C and with centrifugation at 15,000*g* for 30 min, unless otherwise stated.

Frozen potatoes (1 kg) were thawed at room temperature and homogenized with 100 mL of 100 mM $NaHSO_3$ for 2 min in a Waring blender at 4°C. The extract was filtered through eight layers of cheesecloth, and the filtrate was centrifuged. The supernatant (600 mL) was dialyzed against three changes of water, and the precipitate was removed by centrifugation. The supernatant was adjusted to pH 1.0 to 1.5 using concentrated HCl (acid was added very slowly and with continuous stirring) and was incubated in a water bath at 37° C for 1 h, with occasional stirring. The precipitate formed was removed by centrifugation, and the pH of the supernatant was readjusted to 4.0 with 10 M NaOH (base was added very slowly and with continuous stirring). The extract was held overnight at 4°C. Solid ammonium sulfate to give 25% saturation (at 4°C) was added to the pH 4.0 solution with continuous stirring. After standing for 1 h, the supernatant was collected by centrifugation and brought to 50% saturation with solid ammonium sulfate. After 1 h, the precipitate was collected by centrifugation and dissolved in 20 to 30 mL of 200 mM NaCl adjusted to pH 5.5.

The inhibitor solution from $(NH_4)_2SO_4$ fractionation was applied to a Sephadex G-100 column (2.1 × 110 cm) equilibrated with 200 mM NaCl. The main fractions containing inhibitor activity were combined and dialyzed against three changes of 20 mM sodium phosphate buffer (pH 6.0).

The inhibitor solution from the Sephadex G-100 column was applied to the top of a DEAE-Sephadex A-50-120 column (4 × 15 cm) equilibrated with 20 mM phosphate buffer (pH 6.0). After the column was washed with the same buffer, the same phosphate buffer, with stepwise increases of 50 mM NaCl (ranging from 50 to 500 mM NaCl), was used to elute all the protein loaded on the column. The fraction containing inhibitor (eluted with 150 mM NaCl/20 mM sodium phosphate buffer) was combined and dialyzed against three changes of 20 mM sodium phosphate buffer (pH 6.0).

The inhibitor solution from the DEAE-Sephadex A-50-120 column was then applied to a hydroxylapatite column (2.6 × 10 cm) equilibrated with 20 mM phosphate buffer (pH 6.0). After the column was washed with starting buffer, stepwise elutions with 200 mM sodium phosphate buffer (pH 6.0), and 300 mM sodium phosphate buffer (pH 6.0), were performed. The main fractions containing inhibitor (200 mM sodium phosphate buffer) were combined, dialyzed against three changes of 80 mM acetate buffer (pH 4.70), and stored frozen in the same buffer in 1.0 mL aliquots at −20°C until used.

Molecular Weight Determination

The mol wt of the enzyme and the inhibitor were determined by SDS disc gel electrophoresis according to the method of Weber and Osborn (26). A standard proteins kit from Bethesda Research Laboratories was used for calibration. The kit included myosin (H-chain, 200,000), phosphorylase *b* (97,400), BSA (68,000), chicken ovalbumin (43,000), bovine α-chymotrypsinogen (25,700), bovine β-lactoglobulin (18,400), and chicken lysozyme (14,000). The native mol wt were also determined by gel filtration on a Sephadex G-150 column (1.0 × 110 cm) according to the method of Whitaker (27). The proteins were eluted using 100 mM acetate/100 mM NaCl buffer (pH 4.70). Human thyroglobulin (669,000) was used to determine the void volume of the column. Sweet potato β-amylase (215,000), human transferrin (80,000), BSA, chicken ovalbumin, bovine pancreatic trypsin (23,300), chicken lysozyme, and horse heart cytochrome *c* (12,000) were used as standard proteins.

Electrophoresis

Disc gel electrophoresis for determining homogeneity of the proteins used the Davis (4) procedure. Protein (20–40 μg) was run on 7.5% polyacrylamide gels. Staining of some gels for glycoprotein was by the periodic acid-Schiff's base method as described by Segrest and Jackson (23).

Carbohydrate Determination

The carbohydrate content of purified invertase was estimated by the phenol-sulfuric acid method (5) using one mL of enzyme solution (about 0.15 mg/mL) in 100 mM NaCl/20 mM sodium phosphate buffer (pH 6.0). Absorbance at 490 nm was read against a blank in which sample was replaced by buffer. A standard curve was prepared using sucrose. The carbohydrate content was expressed as percent of the total organic matter.

Organic Matter Determination

The nonvolatile organic matter content of the purified invertase was determined by the chromic acid-sulfuric acid method of Johnson (9) in the same buffer described above. Reagent grade sucrose was used to prepare a standard curve.

Determination of pH-Stability and pH-Activity Profiles of Invertase

The pH-stability profile of the enzyme was determined by incubation of 2.1×10^{-9} M (0.126 μg/mL) of enzyme at various pHs, ranging from pH 2 to 10, for 1 h at 37°C, followed by assay of the remaining activity at pH 4.70 as described above. The buffer system consisted of 5 mM each of sodium borate, phosphate, and citrate. Sodium chloride was used to adjust the ionic strength of each buffer to a constant value of 0.1. The optimal pH for activity was determined by measuring the activity of 2.1×10^{-9} M enzyme in 143 mM sucrose with the same buffers at various pHs at 37°C.

For binding studies, the rate of inactivation (rate observed, k_{obsd}) of the enzyme (2.1×10^{-9} M; 0.125 μg/mL) at various pHs was also determined.

Determination of pH-Stability Profile of Invertase Inhibitor

The pH-stability profile of the inhibitor was determined by incubating 2.5×10^{-6} M (42.5 μg/mL) of inhibitor at various pHs with the same buffers used above with enzyme for 1 h at 37°C and assaying the remaining activity against 2.1×10^{-9} M (0.126 μg/mL) of the invertase. After being incubated at the different pHs, three different amounts of inhibitor from each incubation tube were preincubated with the constant amount of enzyme at pH 4.70 for 1 h at 37°C. Residual enzymatic activity was determined as described earlier.

RESULTS

Screening of Potatoes for Invertase and Invertase Inhibitor

Invertase and invertase inhibitor were present in all samples of potatoes examined. However, there was a large variation in their content. A high content of invertase activity correlated with a low content of inhibitor activity and vice versa. Most potatoes tested, however, showed intermediate levels of each protein.

Purification of invertase used potato tubers that, in addition to having high activity content in the screening test, were then incubated for at least 3 weeks at 4°C. The low temperature storage increased the invertase activity two- to threefold in most cases. If storage had to be continued much beyond 3 weeks before purification could be started, the potatoes were treated and frozen, as were the ones used in purification of the inhibitor, to prevent sprouting. Purification of the inhibitor was started with frozen-stored potatoes that had shown high inhibitor activity content in the screening test.

Assay for Invertase Activity

The Somogyi method as modified by Nelson (15) is a very sensitive and precise procedure to determine invertase activity. A molar extinction coefficient (ϵ_M) of 3.50×10^4 M^{-1}cm^{-1} at 660 nm was obtained from a plot of concentration of glucose (M) *versus* absorbance at 660 nm (data not shown). The high value of ϵ_M permitted use of very low amounts of enzyme in the experiments, with very good reproducibility. Also, potato invertase is completely inactivated upon addition of the Cu^{2+} reagent, thereby stopping further enzyme activity or binding to inhibitor.

Purification of Invertase

Purification of invertase is summarized in Table I. Attempts to fractionate the crude potato extract with ammonium sulfate were not successful, as the range of ammonium sulfate concentration in which invertase precipitated in the crude extract was very broad. About 90% of the activity precipitated between 20 and 90% ammonium sulfate saturation. Inactivation of the inhibitor, by blending of the crude extract, was used only during the screening of the potatoes for invertase and inhibitor activity. Blending was abandoned due to its irreproducibility in our hands, and because the next step in the purification, using a Con A-Sepharose 4B column, was more satisfactory in releasing the low amount of inhibitor present in the enzyme-inhibitor complex.

Affinity chromatography on Con A-Sepharose 4B (2) (Fig. 1A), in addition to producing an 11-fold increase in the specific activity of invertase, resulted in release of inhibitor from the enzyme-inhibitor complex, evidenced by recovery of more than 100% of the initial activity. It is important to note that after this step potato invertase was very unstable, losing activity rapidly even in buffer solutions stored at low temperatures. Immediate and rapid 10-fold concentration by ultrafiltration stopped the loss of activity, due to instability of the diluted enzyme. In kinetic experiments, where dilute enzyme solutions were required, addition of BSA (final concentration of 0.11 mg/mL) was enough to stabilize the enzyme. Inclusion of BSA in the reaction mixture was shown not to affect the rate of complex formation of enzyme with inhibitor (data not shown).

With some batches of potatoes not all of the invertase bound to the Con A-Sepharose 4B column. To test the possibility of column overloading, the unbound invertase activity was reloaded onto the regenerated Con A-Sepharose 4B column. No further binding occurred. This may indicate the presnce of isozymes (21) or perhaps invertase with partially degraded glyco groups (since the affinity column binds mannose). These possibilities were not investigated further. As noted in Table I, all the invertase activity was recovered in the active fraction with the batch of potatoes used here.

The first of two DEAE-Sephadex A-50-120 chromatographies (Fig. 1B) increased the purification to 193-fold with a 69% retention of activity. Most of the invertase activity lost during purification occurred during the ion-exchange chromatography steps. This loss was minimized by decreasing the time required by using small columns and appreciable flow rates with the buffers during elution. The invertase eluted as a small shoulder of the main protein peak.

Gel filtration on Sephadex G-150 resulted in a small increase in purification with good retention of the activity (Fig. 1C).

The second DEAE-Sephadex chromatography (Fig. 1D) produced an additional 7.4-fold increase in purity with 29% of activity recovery overall. The pooled DEAE-Sephadex fractions contained a single protein as indicated by SDS-PAGE (Fig. 2C) and by regular PAGE (Fig. 2A); the single band for invertase stained for both protein and carbohydrate.

Table I. *Purification of Potato Invertase*[a]

Fraction	Total Protein	Total Activity	Specific Activity	Recovery of Activity	Purification
	mg[b]	*units*[c]	*units/mg*	%	*-fold*
Crude extract	3000	125	0.042	100	1.0
Con A-Sepharose 4B	300	138	0.46	110	11.0
DEAE-Sephadex A-50-120	10.6	86	8.1	69	193
G-150 Sephadex	7.3	64	8.8	51	210
DEAE-Sephadex A-50-120	0.55	36	65.5	29	1560

[a] Based on 1 kg potatoes. [b] Protein was determined by the Lowry method (12). [c] One unit of invertase is the amount of protein that catalyzes the breakdown of 1 μmol of sucrose/min at 37°C and pH 4.70.

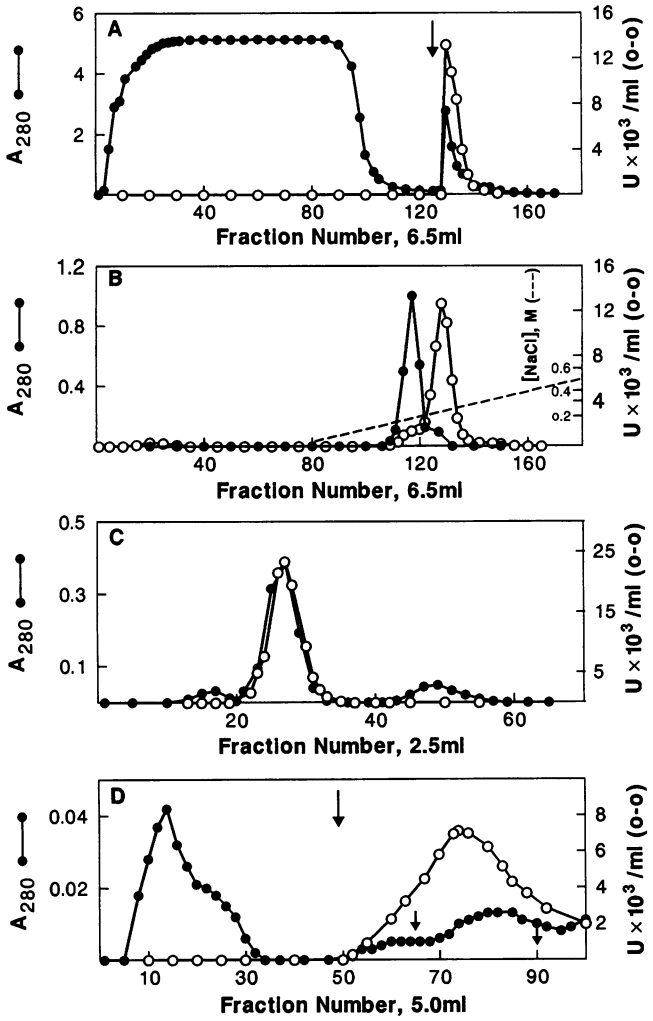

Figure 1. A, Con A-Sepharose 4B affinity chromatography of crude potato invertase. Crude lyophilized potato extract was dissolved in 20 mM Tris-HCl/500 mM NaCl buffer (pH 7.40), containing 1 mM each $CaCl_2$, $MgCl_2$, and $MnCl_2$ and was applied to the affinity column (2.5 × 10 cm) equilibrated with the same buffer. The unbound proteins were eluted with the same buffer. The bound enzyme was eluted with the same buffer containing 30 mM methyl α-D-mannopyranoside as indicated by the arrow. Protein (●——●) was monitored by absorbance at 280 nm. Invertase activity units (○——○) are defined as described in Table I. Fractions 129 to 140 were pooled, concentrated by ultrafiltration, and used in the following DEAE ion-exchange chromatography. B, First DEAE-Sephadex A-50-120 chromatography of potato invertase. The Con A-Sepharose invertase preparation was concentrated 10-fold, dialyzed three times against 20 mM sodium phosphate buffer (pH 6.0), and applied to the column (2.5 × 18 cm) equilibrated with the same buffer. Elution of the bound proteins was carried out with a linear sodium chloride gradient (.....) in the same buffer. Protein (●——●) and invertase activity units (○——○) were determined as in A. Fractions 107 to 120 were pooled, concentrated, and used in the following gel filtration chromatography. C, Sephadex G-150 chromatography of potato invertase. The first DEAE-Sephadex A-50-120 invertase preparation was concentrated 10-fold and applied to the column (1.5 × 90 cm) equilibrated with 50 mM sodium phosphate/100 mM NaCl buffer, pH 7.2. The column was eluted with the same buffer. Protein (●——●) and invertase activity units (○——○) were measured as in A. Fractions 22 to 32 were pooled, concentrated, and used in the following DEAE ion-exchange chromatography. D, Second DEAE-Sephadex A-50-120 chromatography of potato invertase. The Sephadex G-150 invertase preparation was concentrated 10-fold, dialyzed three times against 20 mM sodium phosphate (pH 6.0), and applied to the column (1.5 × 18 cm) equilibrated with the same buffer. The bound proteins were eluted with the same buffer containing 100 mM NaCl, pH 6.0, as indicated by the upper arrow. Protein (●——●) and invertase activity units (○——○) were measured as in A. Fractions 65 to 90 (lower two arrows) were pooled, concentrated, and frozen in small aliquots until used.

Purification of the Invertase Inhibitor

Purification of the inhibitor is summarized in Table II. Low pH (1.0–1.5) treatment for 1 h at 37°C completely inactivated the low content of invertase present in potatoes with high inhibitor activity. This is supported by the following evidence. In potatoes with high inhibitor activity, the invertase activity was low but always detectable; however, after the low pH treatment, it was not detectable. Furthermore, after the low pH treatment, blending did not produce an increase in invertase specific activity, in contrast with the two- to three-fold increase obtained with the same potatoes by blending before the low pH treatment. As shown later, invertase activity is lost at pH 2.3 and below.

Fractionation with solid ammonium sulfate (between 25 and 50% saturation) followed by gel filtration on Sephadex G-100 (Fig. 3A) produced a 5.6-fold increase in specific activity with 65% retention of the activity. Chromatography on DEAE-Sephadex A-50-120 increased the overall specific activity to 79-fold (Fig. 3B). Hydroxylapatite chromatography gave an additional 8-fold increase in the specific activity and constant specific activity (not shown) across the peak containing the inhibitor activity (Fig. 3C). The pooled sample from hydroxylapatite chromatography was homogeneous by SDS-PAGE (Fig. 2C) and by regular PAGE (Fig. 2B).

Molecular Weight Determination

SDS-PAGE of purified invertase and invertase inhibitor gave single bands (Fig. 2C) which had estimated M_r of 30,000 and 17,000, respectively, on the basis of their mobilities relative to those of standard calibration proteins (Fig. 4A).

The native mol wts of invertase and invertase inhibitor were estimated to be about 60,000 and 17,000, respectively, by Sephadex G-150 chromatography (Fig. 4B). These results indicate that the invertase is composed of two identical mol wt polypeptide chains and the inhibitor is composed of one. The molecular weight of the invertase inhibitor reported here agrees with the value reported by Pressey (19).

To our knowledge, this is the first report on the purification to homogeneity of potato invertase and its mol wt determination.

Carbohydrate Determination

The purified invertase was shown to be a glycoprotein. This was indicated by its binding to a Con A-Sepharose 4B column (Fig. 1A), by staining the protein with Schiff's base reagent following disc gel electrophoresis (Fig. 2A), and was further substantiated by the phenol-sulfuric acid carbohydrate determination which indicated a carbohydrate content of 10.9%

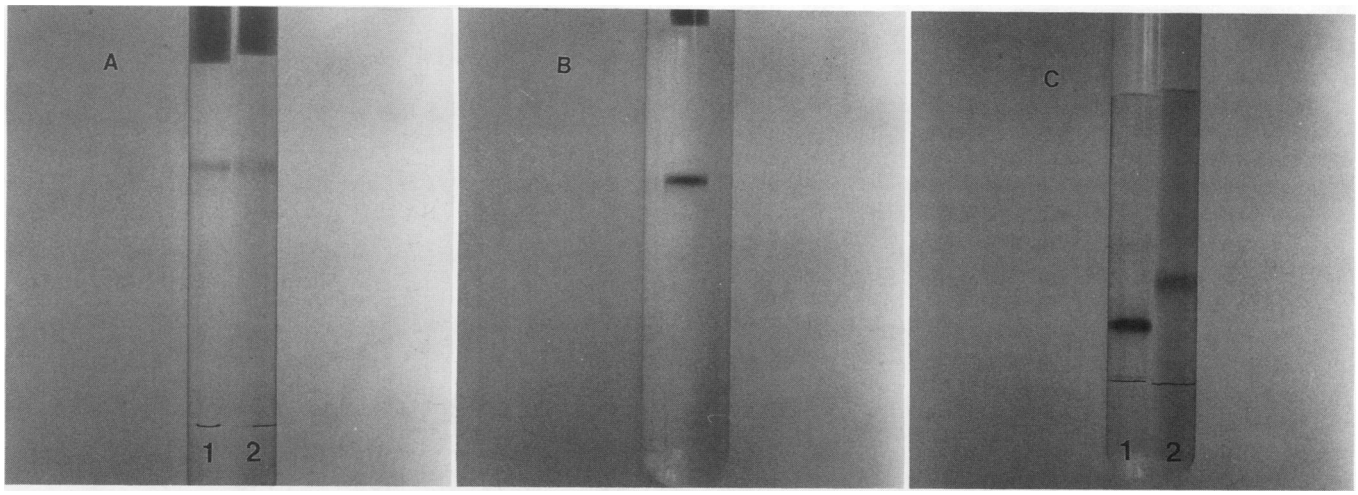

Figure 2. A, Polyacrylamide disc gel electrophoresis of native potato invertase after the second DEAE-Sephadex chromatography. Protein per gel was 20 μg. Running gel was 7.5% polyacrylamide at pH 8.9 (4). Gels were stained for protein (left) and carbohydrate (right). Tracking dye location is indicated by the fine wire at the bottom end of the gels. B, Polyacrylamide disc gel electrophoresis of native potato invertase inhibitor after hydroxylapatite chromatography. Protein per gel was 22 μg. Running gel was 7.5% polyacrylamide at pH 8.9 (4). C, SDS gel electrophoresis of purified invertase and invertase inhibitor. After the purified enzyme (25 μg, right gel) and purified inhibitor (30 μg, left gel) were treated with 1.0% SDS in 10 mM sodium phosphate buffer (pH 7.2), containing 1.0% 2-mercaptoethanol at 100°C in a water bath for 5 min, the treated protein preparations were subjected to electrophoresis in the preence of 0.1% SDS with 10% polyacrylamide gels in the phosphate buffer (26).

Table II. *Purification of Potato Invertase Inhibitor*[a]

Fraction	Total Protein	Total Activity	Specific Activity	Recovery of Activity	Purification
	mg[b]	*units*[c]	*units/mg*	%	*-fold*
Crude extract	5000	22	0.0044	100	1.0
Low pH, 37°C	3000	20	0.0067	91	1.5
$(NH_4)_2SO_4$ 25–50% cut	1500	17	0.0113	77	2.6
G-100 Sephadex	580	14.4	0.0248	65	5.6
DEAE-Sephadex A-50-120	20.5	7.1	0.346	32	79
Hydroxylapatite	2.0	5.4	2.7	25	610

[a] Based on 1 kg potatoes. [b] Protein was determined by the Lowry method (12). [c] One unit of inhibitor is the amount of protein that inhibits 1 unit of invertase 50% at 37°C and pH 4.70.

by weight, using sucrose as a standard. The monosaccharide composition of the carbohydrate portion of the enzyme was not investigated. That potato invertase is a glycoprotein is not surprising, since most known invertases are glycoproteins. Invertase inhibitor was shown not to contain carbohydrate by protein staining with Schiff's base reagent in polyacrylamide gels and by the phenol-sulfuric acid reagent.

pH Dependence of Invertase Stability and Activity

Figures 5 and 6 show the pH-stability and pH-activity profiles of invertase at 37.0°C, respectively. Stability and catalysis were optimal around pH 5.0. Rapid loss of activity was observed below pH 3.3 and above pH 6.7 with the pH-activity curve being sharper (Fig. 7). Since the pH-stability and pH-activity curves are not coincident, more than just instability determines the pH-activity curve. The substrate concentration in these experiments was 143 mM sucrose (~9 K_m). At this substrate concentration, the enzyme was 90% saturated with substrate, indicating that the difference in the pH-stability and pH-activity curves is due to the effect of pH on the catalytic breakdown of sucrose by invertase (28). Ionizable groups with pK_a values of ~3.7 and 5.9 appear to be involved in the catalyzed hydrolysis of sucrose to glucose and fructose.

The rate of inactivation of invertase did not vary substantially between pH 3.3 and 6.7, as indicated in Figure 7. However, the rate of inactivation increased dramatically below pH 3 and above pH 7.

pH-Stability Profile of Invertase Inhibitor

The inhibitor (2.5×10^{-6} M) was stable over the pH range of 2 to 7 when incubated at 37°C for 1 h. At pH 8.0, 35% of the activity was lost after 1 h at 37°C and 65% was lost at pH 9.0 and 37°C (data not shown).

DISCUSSION

The purification protocol developed here was found to be effective, producing a homogeneous protein that was 1560-fold purified with 29% recovery of activity. Selection of the

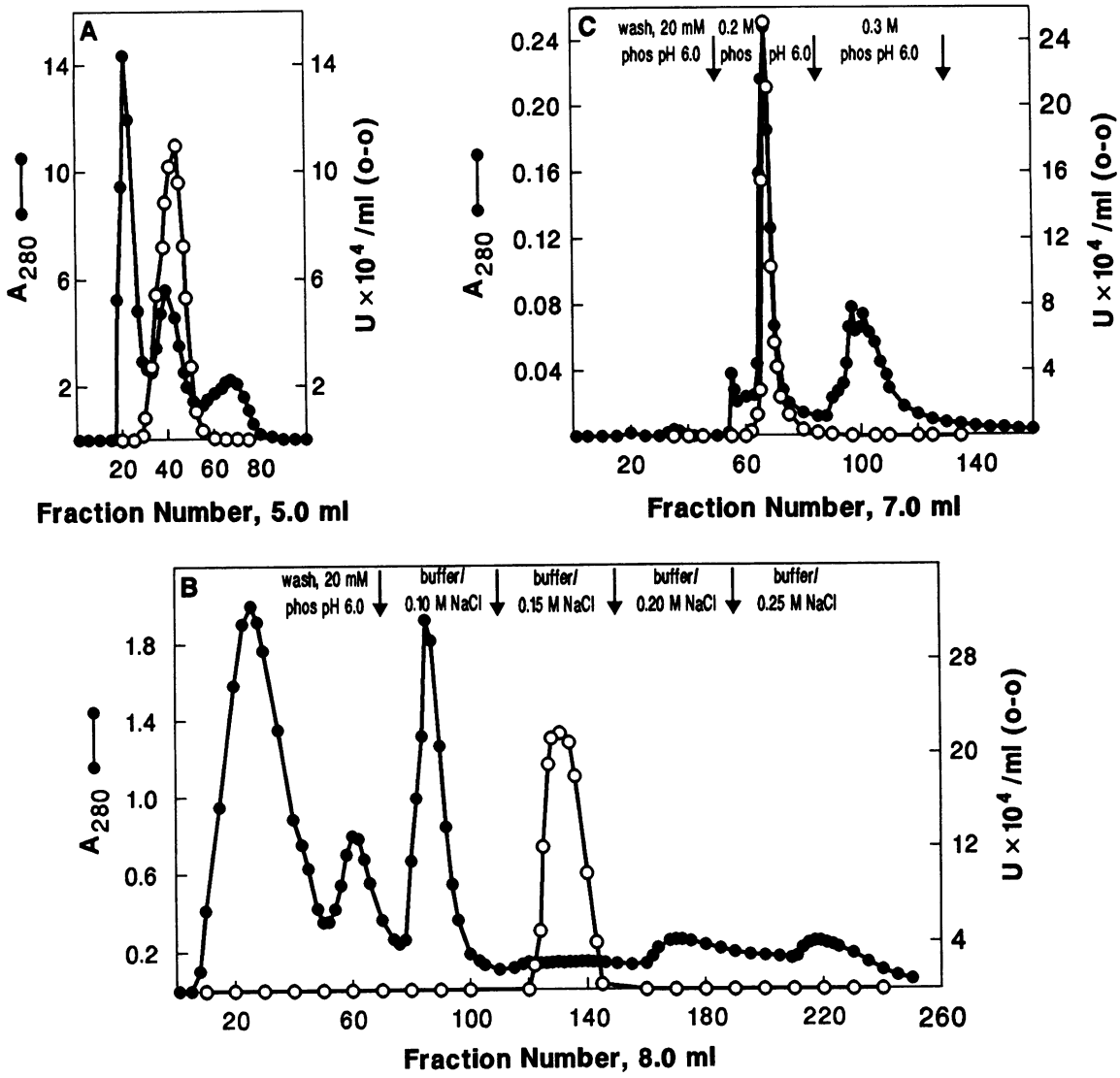

Figure 3. A, Sephadex G-100 chromatography of crude potato invertase inhibitor. Precipitate from ammonium sulfate fractionation (between 25 and 50% saturation) of the pH-treated sample was resuspended in 200 mM NaCl (pH 5.5), and applied to the column (2.1 × 100 cm) equilibrated with 200 mM NaCl. Elution was carried out with 200 mM NaCl. Protein (●——●) was monitored by absorbance at 280 nm. Invertase inhibitor units (○——○) are defined as described in Table II. Fractions 32 to 52 were pooled and used in the following DEAE ion-exchange chromatography. B, DEAE-Sephadex A-50-120 chromatography of potato invertase inhibitor. The Sephadex G-100 invertase inhibitor preparation was dialyzed three times against 20 mM sodium phosphate buffer, pH 6.0, and applied to the column (4 × 15 cm) equilibrated with the same buffer. Elution was carried out with the same buffer containing stepwise increases of sodium chloride concentration as indicated at top of the chromatogram. Protein (●——●) and invertase inhibitor units (○——○) were determined as in A. Fractions 123 to 144 were pooled and used in the following hydroxylapatite chromatography. C, Hydroxylapatite chromatography of invertase inhibitor. The DEAE-Sephadex A-50-120 invertase inhibitor preparation was dialyzed three times against 20 mM sodium phosphate buffer, pH 6.0, and applied to the column (2.6 × 10 cm) equilibrated with the same buffer. After washing the column with the same buffer, elution of the bound protein was carried out with more concentrated solutions of the same buffer (pH 6.0), as indicated. Protein (●——●) and invertase inhibitor units (○——○) were determined as in A. Fractions 65 to 71 were pooled and frozen in small aliquots until used.

starting material was a key factor in the success of the purification. The Con A-Sepharose step (2), besides producing a good increase in the specific activity, helped to dissociate the invertase-invertase inhibitor complex present in the crude extract. The DEAE-Sephadex A-50-120, used twice, was also very effective in removing impurities.

The potato invertase was shown to be a glycoprotein by periodate-Schiff base staining of the purified enzyme in polyacrylamide disc gels and by the phenol-sulfuric acid method for carbohydrate (10.9% found). This confirms the report by Anderson and Ewing (2) about the glycoprotein nature of the enzyme. This finding is not surprising, since most of the well-purified and characterized invertases are glycoproteins.

The molecular weight of the purified invertase was estimated to be 60,000 by Sephadex G-150 chromatography. This mol wt may be high due to the overestimation of the

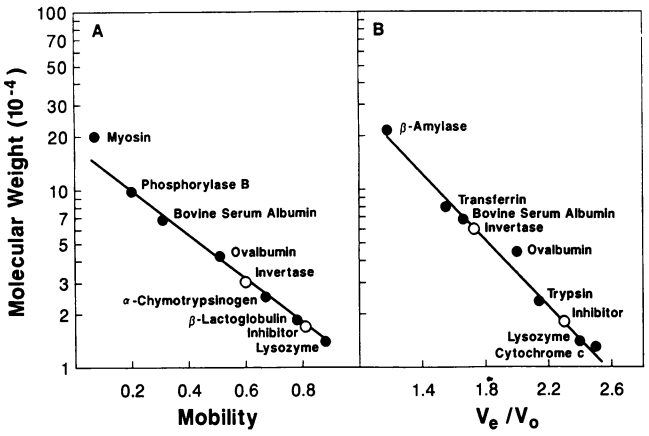

Figure 4. A, Molecular weight determinations of invertase and invertase inhibitor by SDS-PAGE. The semilogarithmic plot of molecular weight against mobility was constructed using standard proteins (●) as shown on the graph. B, Molecular weight determinations of invertase and invertase inhibitor by gel filtration on Sephadex G-150. The semilogarithmic plot of molecular weight against the ratio of the elution volume, V_e, to the void volume, V_o, was constructed using standard proteins (●) as shown on the graph. The proteins were eluted using 100 mM sodium acetate/100 mM NaCl buffer (pH 4.70).

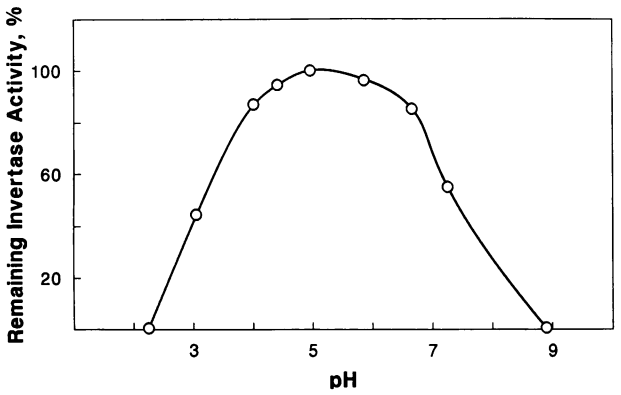

Figure 5. Stability-pH profile of potato invertase. The enzyme (2.1×10^{-9} M) was incubated in various pH buffers containing BSA (0.11 mg/mL) for 1 h at 37°C followed by assay of remaining activity with 143 mM sucrose in 80 mM sodium acetate buffer containing 0.11 mg/mL BSA (pH 4.70), for 1 h at 37°C.

mol wt of glycoproteins (27). SDS-PAGE of the purified enzyme gave a single band with an estimated M_r of 30,000, indicating that the native enzyme is composed of two identical mol wt polypeptide chains. Castor bean invertase was reported to be a heptamer (M_r of 77,800) composed of identical mol wt subunits (M_r of 11,000) (17). Soluble radish seedlings invertase was found to have a mol wt of 48,500 by gel filtration (6). Date invertase is a glycoprotein (8.2% carbohydrate) with a mol wt of about 130,000 by gel filtration; the native enzyme appears to be composed of two identical subunits with a M_r of 70,000 (1).

Potato invertase was most stable and had maximum activity around pH 5. These data place potato invertase in the group of other known acid invertases, whose stability and activity

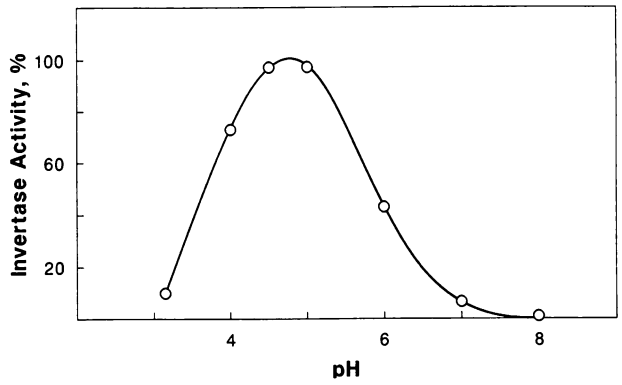

Figure 6. Activity-pH profile of potato invertase. The activity of the enzyme (2.1×10^{-9} M) was assayed using 143 mM sucrose solutions containing 0.11 mg/mL BSA at various pHs at 37.0°C for 1 h.

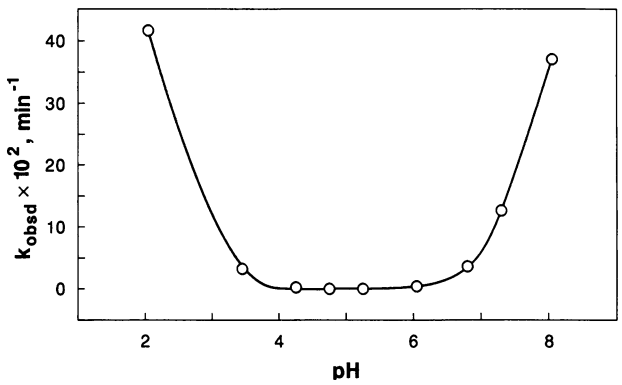

Figure 7. Rate of inactivation (k_{obsd}) of potato invertase at various pHs at 37.0°C. The enzyme (2.1×10^{-9} M) was incubated in various pH solutions containing 0.11 mg/mL BSA, and the loss of activity with time was followed using a rate assay. The rate of inactivation (k_{obsd}) was obtained from a semilogarithmic plot of remaining activity against time for each reaction.

are maximum around this pH. The pH values for half maximum activity were found to be about 3.7 and 5.9 (pH-activity curve, Fig. 6). This is consistent with the participation of carboxyl and imidazole groups in catalysis. Frost *et al.* (7), working with a partially purified potato invertase, reported evidence for the participation of carboxyl and imidazole groups in catalysis; in addition, a carboxyl group might also participate in substrate binding. All the data available from pH and chemical modification of yeast, *Neurospora*, and some higher plant invertases are consistent with participation of carboxyl and imidazole groups in catalysis (7, 14, 25).

Our preparation of potato invertase had a K_m for sucrose of 16 mM at pH 4.70 (data not shown). This value is higher than the K_m values reported for invertase from other varieties of potato. Reported K_m values for sucrose are: white rose potato invertase, 3 mM at pH 6.2 (22); Kennebec potato invertase, 5.3 mM at pH 4.5 (19); Dr. Mackintosh potato invertase, 3.3 mM at pH 4.0 and 2.7 mM at pH 4.9 (7). Our data gave a k_{cat} value of 1125 min^{-1} (moles sucrose hydrolyzed per min per mole of enzyme). Pure date invertase (1) gave a k_{cat} value of 2940 min^{-1}. The k_{cat} values for other higher plant

invertases are not available. But some comparative specific activities (units/mg) include: potato invertase, 65.5 (present report); radish seedling, 25 (6); lily pollen, 50 (24); castor bean, 166 (17); wheat, 1305 (11); *Neurospora,* 1820 (14); and yeast, 4000 (16). Wide ranges of specific activity are found for the same enzyme from different sources and even isozymes from the same organism.

The potato invertase inhibitor was purified 610-fold with an activity recovery of 25%. The protocol of purification used here was different from the one used by Pressey (18), who also obtained a homogeneous preparation of this inhibitor. Key steps in the protocol of purification developed by Pressey (18) were two treatments with alumina C_γ. Adsorption on alumina gel was tried at the beginning of our research; our results, however, were discouraging due to the irreproducibility of the alumina treatment. Here again, the selection of potatoes with high inhibitor content and the treatment at pH 1 to 1.5 were important in the success of the purification. We also modified the procedure to include hydroxylapatite chromatography which gave an eightfold purification in the final step.

The molecular weight of 17,000 for the purified invertase inhibitor is identical to the one reported earlier by Pressey (18). SDS-PAGE of the inhibitor also produced a single band with a M_r of 17,000, indicating that the native invertase inhibitor is a single polypeptide chain. Invertase inhibitors from other higher plants have similar molecular weights. Acid invertase inhibitor from sweet potato had a molecular weight of 19,500 by gel filtration (13). Pressey (20) reported mol wts of 17,800, 18,100 and 22,900 for red beet, sugar beet and sweet potato inhibitors, respectively. Pressey (20) and Matsushita and Uritani (13) did not report on the subunit composition of their inhibitors. Our purified invertase inhibitor gave a negative test for carbohydrate.

The inhibitor was stable over the pH range of 2 to 7 when incubated at 37°C for 1 h. Incubation at higher pH caused some loss of activity. At pH 8.0, 35% of the activity was lost after 1 h at 37°C and 65% was lost at pH 9.0 and 37°C. The good stability of the purified inhibitor under these relatively mild conditions is not unexpected, since proteins of low mol wt usually have a remarkable stability even under more drastic conditions.

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