Plant Physiol. (1997) 113: 235–241

Multiple Forms of ADP-Glucose Pyrophosphorylase from Tomato Fruit[1]

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ADP-glucose pyrophosphorylase (AGP) was purified from tomato (*Lycopersicon esculentum* Mill.) fruit to apparent homogeneity. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis the enzyme migrated as two close bands with molecular weights of 50,000 and 51,000. Two-dimensional polyacrylamide gel electrophoresis analysis of the purified enzyme, however, revealed at least five major protein spots that could be distinguished by their slight differences in net charge and molecular weight. Whereas all of the spots were recognized by the antiserum raised against tomato fruit AGP holoenzyme, only three of them reacted strongly with the antiserum raised against the potato tuber AGP large subunit, and the other two spots (with lower molecular weights) reacted specifically with antisera raised against spinach leaf AGP holoenzyme and the potato tuber AGP small subunit. The results suggest the existence of at least three isoforms of the AGP large subunit and two isoforms of the small subunit in tomato fruit in vivo. The native molecular mass of the enzyme determined by gel filtration was 220 ± 10 kD, indicating a tetrameric structure for AGP from tomato fruit. The purified enzyme is very sensitive to 3-phosphoglycerate/inorganic phosphate regulation.

The enzyme AGP (ATP:α-Glc-1-P adenyl transferase, EC 2.7.7.27) plays a pivotal role in starch biosynthesis in both photosynthetic and nonphotosynthetic plant tissues (Preiss, 1991; Stark et al., 1992). It catalyzes the conversion of Glc-1-P and ATP to PPi and ADP-Glc, which is the major, if not the sole, substrate for starch synthase (Preiss, 1988).

Plant AGP has been characterized as a heterotetramer (approximately 200–240 kD) composed of two small subunits (approximately 50–55 kD) and two large subunits (approximately 51–60 kD) (Kleczkowski et al., 1991). Comparison of cDNA sequences and immunological analysis revealed that small subunits from different sources are highly conserved, whereas the large subunits are divergent (Preiss et al., 1989; Okita et al., 1990; Smith-White and Preiss, 1992). Expression of both subunit cDNAs from potato tubers in *Escherichia coli* suggests that a major function of the large subunit is to modulate the regulatory properties of the native heterotetrameric enzyme, whereas that of the small subunit is catalysis (Ballicora et al., 1995). Plant AGPs from all tissues, except from wheat and barley endosperm (Duffus, 1993; Kleczkowski et al., 1993), are activated by 3-PGA and inhibited by Pi (Preiss, 1991).

In tomato (*Lycopersicon esculentum* Mill.), fruit starch is transiently accumulated during early fruit development. As the fruit ripens, the starch is degraded in concert with the accumulation of soluble sugars (hexoses) (Davies and Cocking, 1965). As the major component of the soluble solids, the soluble sugars are considered to be a primary indicator of tomato fruit quality. A comparison of tomato genotypes differing in total fruit solids content showed a positive correlation between the final levels of soluble sugars and the peak levels of starch early in fruit development (Dinar and Stevens, 1981). The latter is regulated by the starch biosynthetic capacity, as determined by levels of AGP activity rather than starch degradative capacity (Robinson et al., 1988). In view of this relationship, AGP may be an attractive biotechnological target for increasing the soluble solids content of tomato fruit. As the first step toward this goal, we report here the purification and multiple forms of AGP from tomato fruit.

MATERIALS AND METHODS

Reagents

All chemicals and coupling enzymes, unless stated otherwise, were from Sigma. Affi-Gel blue gel and nitrocellulose membranes were from Bio-Rad. Sephacryl S-300, Q Sepharose, and Mono-Q HR5/5 were from Pharmacia. BCA protein reagent was from Pierce. The Pep Tag protease assay kit was from Promega. Pharmalyte (pH 3.0–10.0) was from Sigma.

Plant Material

Tomato (*Lycopersicon esculentum* Mill. cv Laura) was grown in the greenhouse under 16 h of light/8 h of dark. Fruit harvested 2 weeks postanthesis (fresh weight about 30 g) were dissected free from the outer walls of pericarp and locule tissue and were frozen in liquid nitrogen immediately. The dissected fruit were stored at −80°C.

[1] This work was supported by National Aeronautics and Space Administration grant no. 94–0308 to H.W.J. This is paper no. D–12150–7–95 of the New Jersey Agricultural Experiment Station.

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Abbreviations: $A_{0.5}$, concentration giving 50% maximal activation; AEBSF, 4-(2-aminoethyl)benzene-sulfonylfluoride; AGP, ADP-Glc pyrophosphorylase; 3-PGA, 3-phosphoglycerate; $S_{0.5}$, concentration giving 50% maximal activity.

Assay of AGP

Assay A

The pyrophosphorylase of ADP-Glc was coupled with phosphoglucomutase and Glc-6-P dehydrogenase and assayed by measuring NADH production at 340 nm and 37°C (Plaxton and Preiss, 1987). A standard reaction mixture (1 mL) contained 80 μmol of Hepes-NaOH buffer (pH 7.4), 5 μmol of $MgCl_2$, 10 μmol of 3-phosphoglycerate, 0.2 mg of BSA, 1 μmol of ADP-Glc, 1 μmol of sodium PPi, 0.6 μmol of NAD^+, 10 μmol of Glc-1,6-bisphosphate, and 2 units each of rabbit muscle phosphoglucomutase and *Leuconostoc mesenteroides* Glc-6-P dehydrogenase. Assays were initiated by addition of PPi. The AGP activity was linear to both time and amount of enzyme extract added. One unit of activity is defined as the amount of enzyme required to produce 1 μmol Glc-1-P/min at 37°C. Specific activity is defined as units per milligram of protein.

Assay B

[^{14}C]Glc-1-P was used to measure the synthesis of [^{14}C]ADP-Glc. Standard reaction mixtures contained in 0.2 mL: 20 μmol of Hepes-NaOH (pH 8.0), 50 μg of BSA, 1 μmol of $MgCl_2$, 0.35 μmol of ATP, 0.1 μmol of [^{14}C]Glc-1-P (about 1000 cpm/nmol), and 0.12 unit of inorganic pyrophosphatase. 3-PGA (0.5 μmol) was added when the enzyme was assayed in the presence of activator. Assays were initiated by the addition of enzyme. Reaction mixtures were incubated for 10 min at 37°C and terminated by heating for 1 min in a boiling water bath. The amount of [^{14}C]ADP-Glc formed was determined as previously described (Ghosh and Preiss, 1966).

AGP Purification

Unless otherwise indicated, all purification steps were carried out at 0 to 4°C and centrifugations were conducted at 14,000*g* for 10 min. Assay A was used to monitor enzyme activity throughout the purification.

The dissected tomato fruit (1 kg) were homogenized by hand using a prechilled mortar and pestle in 650 mL of a grinding solution containing 50 mM potassium phosphate (pH 7.0), 5 mM EDTA, 1 mM dithioerythritol, 1.5 mM freshly added PMSF, 0.5 mM chymostatin, 0.5 mM leupeptin, 5 mM pepstatin A, and 0.5 mM AEBSF. The homogenate was centrifuged. The resulting supernatant was heated to 60°C in a water bath and maintained at this temperature for 4 min. Then the heated solution was cooled on ice and later centrifuged. Solid ammonium sulfate was added to the resulting supernatant. The fraction, precipitating between 35 and 55% saturation, was collected by centrifugation. The pellet was suspended in buffer A containing 50 mM Hepes-NaOH (pH 7.4), 4 mM $MgCl_2$, 1 mM EDTA, 1 mM dithioerythritol, and 10% Suc. Following desalting by ultrafiltration with an XM50 membrane (Amicon, Beverly, MA), the suspension was loaded onto an Affi-Gel blue affinity chromatography column (30-mL bed volume) equilibrated with buffer A. After the column was washed with buffer A, the enzyme was eluted with 200 mL of 10 mM ATP plus 100 mM KCl in buffer A. Following concentration and desalting by ultrafiltration the eluate was loaded onto a Q-Sepharose Fast Flow column (200-mL bed volume) equilibrated with buffer A. The enzyme was eluted with 200 mL of 0 to 0.45 M gradient of KCl in buffer A. The active fractions were pooled, desalted, and concentrated by ultrafiltration and then loaded onto a Mono-Q HR5/5 column equilibrated with buffer B, which contained 20 mM Bis-Tris-Propane (pH 7.0), 5 mM potassium phosphate, 1 mM EDTA, 10% Suc, and 1 mM dithioerythritol. The enzyme was eluted with 25 mL of linear KCl gradient (0–0.5 M) in buffer B. Active fractions were pooled, concentrated with Centricon (Amicon, Beverly, MA), and stored at −80°C.

Antibody Production and Neutralization of Enzyme Activity

A preparative SDS-PAGE with 10% separating gel was run to further separate the purified tomato fruit AGP from any contaminating proteins. The protein bands were visualized during the run by the Chromaphor Protein Visualization System (Promega). After the run the protein band at approximately 50 kD was excised and used directly as the antigen to immunize the rabbit. The antibody against tomato fruit AGP was custom-made by the BAbCO-Berkeley Antibody Co. (Richmond, CA) with their standard protocol. Neutralization of AGP activity was performed as previously described (Plaxton and Preiss, 1987).

Protease Assay

Tomato fruit crude extract (with the addition of different protease inhibitors) was tested for protease activity by the Pep Tag protease assay kit according to the manufacturer's recommendations. This qualitative assay monitors the proteolysis of small, dye-linked peptides. Digestion of the peptides alters the size and charge of the peptides and these changes are detected using agarose gel electrophoresis (White et al., 1993).

PAGE and Western Blotting

SDS-PAGE was performed using a discontinuous system with a 10% separation gel based on the procedure of Laemmli (1970). Two-dimensional PAGE was conducted as outlined by Phillips (1988). After electrophoresis proteins were either stained with a silver stain kit (Sigma) or electroblotted to nitrocellulose membranes according to the method of Towbin et al. (1979). Following electroblotting nitrocellulose membranes were treated with the following anti-AGP sera: the spinach antibody, which was raised against highly purified spinach leaf AGP holoenzyme (Morell et al., 1987); the potato antibodies, which were raised against the individually purified potato tuber AGP subunits and were specific for the individual subunit types (Nakata et al., 1994); and the tomato antibody, which was raised against tomato fruit AGP holoenzyme (this study). The antigen-antibody complex was visualized by the Proto Blot II AP System (Promega). Immunological specificity was confirmed by performing western blots in which rabbit preimmune serum was substituted for AGP immune sera.

For determination of subunit molecular weight using SDS-PAGE, a plot of relative mobility versus log molecular weight was constructed for the following standard proteins: rabbit muscle phosphorylase b (97.4 kD), BSA (66.2 kD), ovalbumin (45 kD), bovine carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD).

Native Molecular Mass Estimation

Native molecular mass estimations were made on a Sephacryl S-300 column (16 × 70 cm) using 1 mL of sample volume and 40 mM Hepes-NaOH buffer (pH 7.4) containing 150 mM KCl, 5 mM potassium phosphate, and 2 mM dithioerythritol as the equilibrium/elution buffer. The native molecular mass of AGP was determined from a plot of the partition coefficient versus log molecular mass for the following standard proteins: thyroglobulin (699 kD), ferritin (440 kD), catalase (232 kD), and aldolase (158 kD). The void volume was determined using a 1 mg/mL solution of blue dextran 2000.

Kinetic Studies

Assay B was used in all the kinetic studies. $S_{0.5}$ and $A_{0.5}$ values were determined from double-reciprocal plots using near-saturating concentrations of the nonvaried substrate (Segal, 1975). Unless otherwise indicated, the assay concentration of 3-PGA was 2.5 mM. All kinetic parameters are the means of three determinations and are reproducible to within ±10%.

Other Procedures

The protein concentration was measured by the method of Smith et al. (1985) using the prepared BCA reagent (Pierce) and BSA as the standard. Nondegraded protein extraction in the presence of TCA for western blots was performed according to the method of Wu and Wang (1984).

RESULTS AND DISCUSSION

Inhibition of Protease Activity in Tomato Fruit Crude Extract

Protease activity is a significant problem for AGP purification from plants, especially from nonphotosynthetic tissues. Proteolytic degradation of the AGP small subunit was evident during the AGP purification from maize endosperm (Plaxton and Preiss, 1987; Preiss et al., 1989). In the case of barley endosperm AGP, the small subunit was relatively resistant to proteolytic degradation but the large subunit was not, with a half-time for proteolysis at 0 to 4°C on the order of minutes, even in the presence of various protease inhibitors (Kleczkowski et al., 1993). In the purification of AGP from potato tuber, low recovery and instability of the enzyme, probably resulting from protease activity, were reported (Okita et al., 1990). Therefore, an *E. coli* mutant expressing the potato tuber AGP was used as an alternative for the enzyme purification (Iglesias et al., 1993). Low recovery and instability of the enzyme were also found in our preliminary experiments on the purification of AGP from tomato fruit.

To efficiently inhibit the protease activity in tomato fruit crude extract five protease inhibitors were included, both individually and collectively, in the grinding solution. The protease activities of the resulting extracts were determined by a highly sensitive Pep Tag protease assay. As shown in Figure 1, PMSF, chymostatin, and leupeptin were the three most effective inhibitors. With the addition of these inhibitors, the test peptide was proteolyzed to a lesser degree than the one without any inhibitors present (Fig. 1, compare lanes 3, 4, and 8 with lane 2), although no test peptide remained intact (Fig. 1, compare lanes 3, 4, and 8 with lane 1). Even with the presence of all five inhibitors, the protease activity was merely decreased but not abolished (Fig. 1, compare lanes 9 and 10 with lane 1). The remaining protease activities were much lower when the assay was done at 4°C than at room temperature. Only when the assay was done at 4°C with the addition of all five inhibitors did some of the test peptide remain intact (Fig. 1, lower part of lanes 9 and 10). These results, in agreement with previous studies by Okita et al. (1990) and Kleczkowski et al. (1993), indicate that the protease activity is also a hurdle to AGP purification from tomato fruit. The presence of protease inhibitors in the grinding solution and extraction and purification at 0 to 4°C were two effective measures in inhibiting the protease activity. Unfortunately, complete inhibition was not obtained by these measures; it seems that more effective plant protease inhibitors are needed. The Pep Tag protease assay, which can be done within 30 min with a detection limit of less than 100 pg of protease (White et al., 1993), is well suited for screening these inhibitors. It is interesting to note that PMSF and

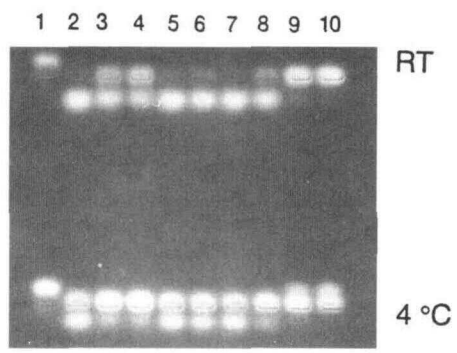

Figure 1. Effects of five protease inhibitors on the protease activity in the tomato fruit crude extract. Pep Tag protease assay was used to test the protease activity in crude extracts with or without different protease inhibitors. The top row shows the results obtained after 24 h of incubation at room temperature (RT), and the bottom row shows the same assay done at 4°C. Lane 1, Negative control, incubation with the grinding buffer; lane 2, crude extract (CE) without protease inhibitors; lane 3, crude extract plus 1.5 mM PMSF; lane 4, crude extract plus 0.5 mM chymostatin; lane 5, crude extract plus 0.5 mM AEBSF; lane 6, crude extract plus 1 mM AEBSF; lane 7, crude extract plus 5 mM pepstatin A; lane 8, crude extract plus 0.5 mM leupeptin; lane 9, crude extract plus 1× all of the five inhibitors above; and lane 10, crude extract plus 2× all of the five inhibitors above.

chymostatin, the most effective protease inhibitors in tomato fruit crude extract judged by the Pep Tag protease assay, were also the only two effective protease inhibitors among 10 protease inhibitors tested in maize endosperm (Plaxton and Preiss, 1987).

Purification of AGP from Tomato Fruit

After comparing AGP activity between different parts (the outer wall of pericarp, the locular tissue, the placental tissue, and the inner wall of pericarp) of tomato fruit during its early development, we found that the inner wall of the pericarp and the placental tissue at 2 weeks after anthesis contained the highest activities (data not shown). Thus, enzyme purification was performed using fruit dissected free from the outer wall of the pericarp and the locule tissue. A typical purification of AGP from tomato fruit is summarized in Table I. By using a procedure consisting of heat treatment, ammonium sulfate precipitation, Affi-Gel blue gel, Q-Sepharose, and Mono-Q chromatography, we were able to purify the enzyme 5018-fold, with 31% recovery, to a final specific activity of about 45 units/mg, a value comparable to that reported for the enzyme purified from potato tuber (56.9 units/mg; Okita et al., 1990). However, the purification and yield values are an overestimation because the heat treatment caused a 3.5-fold increase in total enzyme activity (Table I). Although this phenomenon was observed in other purifications (Plaxton and Preiss, 1987; Iglesias et al., 1991), the increases by the heat treatment were usually not significant (below 1.5-fold). We consistently observed a 3- to 5-fold increase in enzyme activity by the heat treatment step. As suggested by Iglesias et al. (1991), this could be attributed to the presence of an inhibitor in the crude extract. Also, it is likely that interfering reactions occurred in the crude extract that lowered the enzyme activity values in that step.

Successful enzyme purification depends on the right source of starting materials. In tomato fruit AGP activity varies among different parts of the fruit (data not shown). The outer pericarp of young tomato fruit contains photosynthetically active chloroplasts (Piechulla et al., 1987) but low enzyme activity. By removing this tissue, not only was the enzyme enriched, but many contaminating proteins, e.g. enzymes for photosynthesis, were discarded, which facilitated the subsequent purification process.

We also tried hydrophobic and affinity chromatography (using Glc-1-P-Sepharose and ATP-Sepharose) but failed to increase the enzyme specific activity because of low recovery. Dye interaction (Affi-Gel blue) and Mono-Q chromatography were found to be important and effective purification steps, but the recovery was low (Table I), possibly because of the protease activity. The dye interaction chromatography seems very promising in the purification of AGP from plants because of the relatively specific binding of the enzyme to the dye ligands and the effective elution of the enzyme with ATP.

Properties of the Antiserum against Tomato Fruit AGP

Antiserum raised in rabbits against tomato fruit AGP was tested for its reactivity with AGP by both neutralization of enzyme activity and western blot analysis. The effect of the antiserum on the activity of AGP purified from tomato fruit is shown in Figure 2. Increasing amounts of the antiserum inhibited AGP activity. Up to 80% of the AGP activity could be neutralized by the antiserum. The reactivity of the antiserum with AGP was also shown by western blot analysis (Fig. 3B). The antiserum reacted strongly with the purified tomato fruit AGP subunits (Fig. 3B, lane P). Furthermore, there was no cross-reactivity to any other polypeptides present in the crude extract (Fig. 3B, lane C), suggesting that this antiserum is also very specific.

Kinetic and Regulatory Properties of the Purified Tomato Fruit AGP

The purified tomato fruit AGP was characterized for its kinetic and regulatory properties. The enzyme is very sensitive to 3-PGA activation with an $A_{0.5}$ of 0.2 mM. Without 3-PGA in the reaction mixture, only negligible levels of enzyme activity were measured. This activating effect by 3-PGA can be neutralized by Pi (Fig. 4). The $S_{0.5}$ for ATP and Glc-1-P in the presence of 2.5 mM 3-PGA are 0.12 and 0.086 mM, respectively. A comparison of these parameters with those of potato tuber enzyme is shown in Table II.

Multiple Forms of AGP in Tomato Fruit

The purified AGP was analyzed by SDS-PAGE. Silver staining revealed only two closely migrating bands with estimated molecular weights of 50,000 and 51,000 (Fig. 3A), which are identical to those of the small and large subunits in potato tuber (Okita et al., 1990). Both bands reacted with

Table I. *AGP purification from tomato fruit*

Fraction	Volume	Protein	Activity	Specific Activity	Purification	Recovery
	mL	*mg*	*units*	*units/mg*	*-fold*	*%*
Crude	1050	15,360	135	0.009	NA[a]	NA
Heat	1032	10,384	470	0.045	5	350
Ammonium sulfate	40	312	467	1.5	167	346
Ultrafiltr.	14.5	215	425	1.98	220	315
Affi-Gel	8	25.6	135	5.27	586	100
Q-Sepharose	5	10	73	7.3	811	54
Mono-Q	1.45	0.93	42	45.16	5018	31

[a] NA, Not applicable.

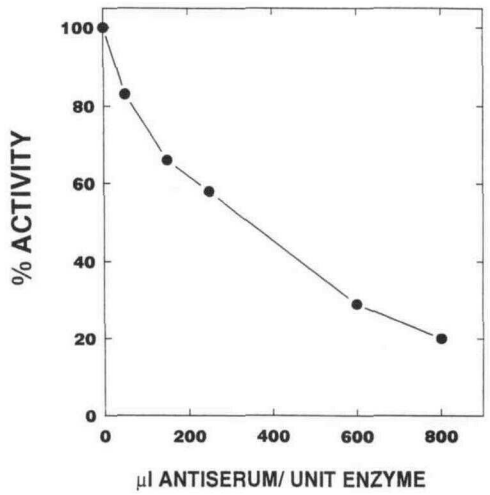

Figure 2. Neutralization of tomato fruit AGP by the antiserum raised against itself. The enzyme activity was determined after incubation with different amounts of antiserum diluted in the preimmune serum and expressed as the percentage of the control in which only the preimmune serum was incubated with the enzyme.

anti-tomato fruit AGP serum, with the lower band reacting more strongly than the upper one (Fig. 3B, lane P).

To further characterize the purified enzyme, high-resolution, two-dimensional PAGE was performed. After silver staining five major protein spots with slight differences in molecular weights and pIs were observed (Fig. 5A). All of them reacted with the anti-tomato fruit AGP serum (Fig. 5B). However, the anti-spinach leaf AGP serum detected only two spots (B_1 and B_2) with molecular weights of 50,000 (Fig. 5C), which were also recognized by the serum raised against the potato tuber small subunit (Fig.

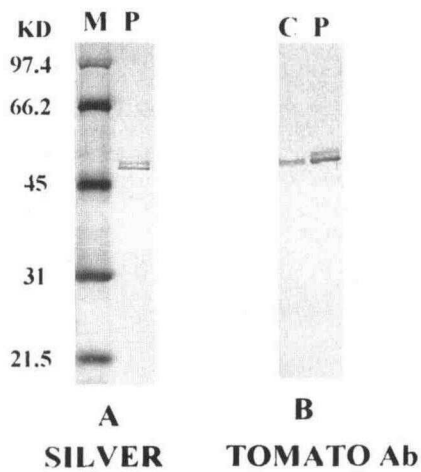

Figure 3. SDS-PAGE and immunoblot analysis of purified AGP from tomato fruit. The purified enzyme was subjected to SDS-PAGE, followed by either staining with silver (A) or transfer to a nitrocellulose filter for immunoblotting (B). A, SDS-PAGE of purified tomato fruit AGP after silver staining. B, An immunoblot of the enzyme probed with the antiserum raised against tomato fruit AGP. Lane C, Crude extract made by the TCA method (Wu and Wang, 1984) from tomato fruit; lanes P, purified tomato fruit AGP; lane M, molecular mass markers, with the molecular mass indicated at the left.

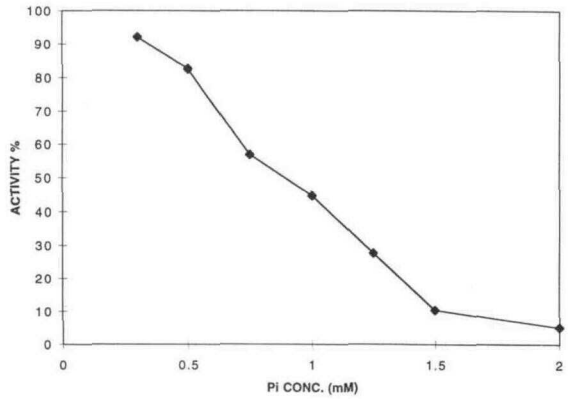

Figure 4. The effect of Pi on purified tomato fruit AGP activity. The enzyme activity was determined by assay B containing 0.5 mM 3-PGA and varying amounts of Pi and expressed as the percentage of the control in which no Pi was added.

5D). When the serum against the potato tuber, large subunit was used, the spots B_1 and B_2 reacted weakly, whereas the other spots (S_1, S_2, and S_3) showed strong reaction (Fig. 5E). The native molecular mass of AGP determined by Sephacryl S-300 gel-filtration chromatography was 220 ± 10 kD (average of three separate determinations), indicating a tetrameric structure for AGP in tomato fruit.

Based on the above immunological cross-reactivity, the five spots can be distinguished into different subunits. B_1 and B_2 are the small subunits because of their strong reaction to the antiserum raised against the potato tuber small subunit; S_1, S_2, and S_3 are the large subunits because of their specific reaction to the antiserum raised against the potato tuber large subunit. In our observation, the large subunits in tomato fruit do not necessarily have a higher molecular weight than the small subunits. For example, the molecular weight of S_1 appears identical to that of B_1 and B_2, which also explains why the 50-kD band was stronger than the 51-kD band when an SDS-PAGE gel of the purified enzyme was probed with anti-tomato fruit AGP serum (Fig. 3B). Consistent with the observation in other plants (Preiss et al., 1989; Okita et al., 1990), these results also indicate that the tomato fruit small subunits share structural homology to the small subunits in spinach leaves and potato tuber, whereas the large subunits are structurally more divergent, as revealed by the inability of the anti-spinach leaf AGP to cross-react with the tomato fruit large subunits (Fig. 5C). The conservation of the small subunits in tomato fruit and potato tuber has been corroborated by

Table II. *Kinetic parameters of purified tomato fruit AGP and comparison with the potato tuber enzyme*

Assay B (ADP-Glc synthesis direction) was used to measure the enzyme activity. Kinetic data for the potato tuber enzyme were obtained from Sowokinos and Preiss (1982).

Plant	$S_{0.5}$ of ATP (+3-PGA)	$S_{0.5}$ of Glc-1-P (+3-PGA)	$A_{0.5}$ of 3-PGA
	mM	mM	mM
Tomato fruit	0.12	0.086	0.2
Potato tuber	0.19	0.14	0.4

the analysis of their cDNA clones. The deduced amino acid sequences from these cDNA clones reveal an identity as high as 98% (Chen and Janes, 1995).

To determine whether the multiple spots for both the small and large subunits exist in tomato fruit in vivo or result from proteolytic degradation during the purification, crude extract was subjected to both SDS-PAGE and two-dimensional PAGE, followed by western blot analysis. The cross-reacting polypeptides in the crude extract made in the presence of TCA (Wu and Wang, 1984) have identical molecular weights to those in the purified enzyme when resolved by SDS-PAGE and detected by the antiserum raised against tomato fruit AGP (Fig. 3B). This result indicates that the purified enzyme is intact. The intactness of the small subunit was confirmed by the analysis of its cDNA clone. The calculated molecular mass of the mature small subunit in tomato fruit is 49.6 kD (Chen and Janes, 1995), which is exactly the size of the small subunit of the purified enzyme. In the second experiment the crude extract made in O'Farrell's lysis buffer (O'Farrell, 1975) was analyzed by two-dimensional PAGE. A pattern similar to the purified enzyme was detected by the antiserum raised against the potato tuber AGP large subunit (Fig. 6A). Moreover, two polypeptides corresponding to B_1 and B_2 reacted with the antiserum raised against spinach leaf AGP (Fig. 6B). It is therefore indicated that the multiple forms of AGP do exist in tomato fruit in vivo.

Multiple forms of AGP at the protein level were reported previously in several plants. AGP purified from pea embryos consisted of four antigenically related polypeptides, of which two seemed to be related to small subunits (Hylton and Smith, 1992). Nakamura and Kawaguchi (1992) reported six polypeptides of similar molecular weights for rice endosperm AGP, and these polypeptides could not be assigned to the small or large

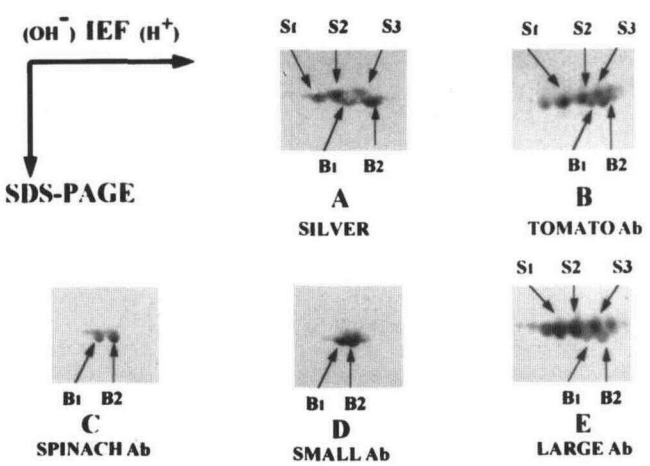

Figure 5. Two-dimensional PAGE and immunoblot analysis of purified tomato fruit AGP. The purified enzyme was subjected to two-dimensional PAGE, followed by either staining with silver (A) or transfer to a nitrocellulose filter for immunoblotting with the antisera against tomato fruit AGP (B), spinach leaf AGP (C), potato tuber small subunit (D), and potato tuber large subunit (E). The drawing at upper left illustrates the directions of the IEF and SDS-PAGE runs. Ab, Antibody.

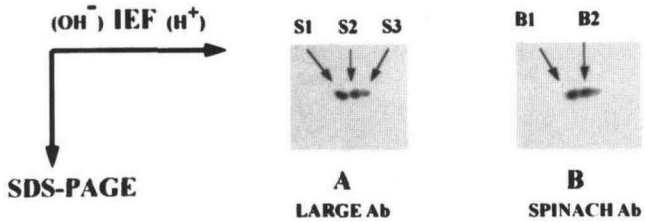

Figure 6. Two-dimensional PAGE and immunoblot analysis of tomato fruit crude extract. The crude extract made in O'Farrell's lysis buffer from tomato fruit was subjected to two-dimensional PAGE. The gel was electroblotted to a nitrocellulose filter and probed with the antiserum against the potato tuber AGP large subunit (A) and the antiserum against spinach leaf AGP (B). The drawing at left shows the directions of the IEF and SDS-PAGE runs. Ab, Antibody.

subunit. In potato tuber, AGP was composed of two types of subunits that could be distinguished by immunological cross-reactions, and several isoforms were observed for the large subunit (Okita et al., 1990). Considering the extreme sensitivity of the enzyme to proteolytic degradation (Plaxton and Preiss, 1987; Kleczkowski et al., 1993), it was argued by Kleczkowski et al. (1993) that the multiple forms in rice endosperm may be proteolytic products of AGP. The multiple large subunits in potato tuber were not consistently observed; the authors pointed out that they were dependent on the age of the protein sample and IEF conditions (Okita et al., 1990). In the present study multiple forms were consistently observed in the purified preparation and also in the crude extract made in 9.5 M urea from tomato fruit, which unequivocally demonstrated that the multiple forms exist in tomato fruit in vivo. Comparison of expression and catalytic properties among these isoforms may provide clues to biotechnological manipulation potential of this important enzyme as it relates to starch biosynthesis.

ACKNOWLEDGMENTS

We thank Dr. Jack Preiss (Michigan State University, East Lansing) for supplying the antiserum against spinach leaf AGP; Dr. Thomas Okita (Washington State University, Pullman) for providing the antisera against potato tuber AGP small and large subunits; and Mr. Binzhang Shen for valuable suggestions about the writing of this paper.

Received June 27, 1996; accepted September 23, 1996.
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