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Sink Metabolism in Tomato Fruit[1]

IV. Genetic and Biochemical Analysis of Sucrose Accumulation

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ABSTRACT

Fruit of domesticated tomato (*Lycopersicon esculentum*) accumulate primarily glucose and fructose, whereas some wild tomato species, including *Lycopersicon chmielewskii*, accumulate sucrose. Genetic analysis of progeny resulting from a cross between *L. chmielewskii* and *L. esculentum* indicated that the sucrose-accumulating trait could be stably transferred and that the trait was controlled by the action of one or two recessive genes. Biochemical analysis of progeny resulting from this cross indicated that the sucrose-accumulating trait was associated with greatly reduced levels of acid invertase, but normal levels of sucrose synthase. Invertase from hexose-accumulating fruit was purified and could be resolved into three isoforms by chromatofocusing, each with isoelectric points between 5.1 and 5.5. The invertase isoforms showed identical polypeptide profiles on sodium dodecyl sulfate polyacrylamide gel electrophoresis, consisting of a primary 52 kilodalton polypeptide and two lower molecular mass polypeptides that appear to be degradation products of the 52 kilodalton polypeptide. The three invertase isoforms were indistinguishable based on pH, temperature, and substrate concentration dependence. Immunological detection of invertase indicated that the low level of invertase in sucrose-accumulating fruit was due to low levels of invertase protein rather than the presence of an invertase inhibitor. Based on comparison of genetic and biochemical data we speculate that a gene either encoding tomato fruit acid invertase or one required for its expression, plays an important role in determining sucrose accumulation.

Soluble sugar concentration of tomato (*Lycopersicon esculentum*) fruit is a major determinant of fruit quality. A wild relative of tomato (*Lycopersicon chmielewskii*) is characterized by an extremely high level of total soluble sugars (37). This wild species accumulates sucrose rather than glucose and fructose as in *L. esculentum* and when expressed on a glucose equivalent basis, fruit of *L. chmielewskii* accumulate twice as much soluble carbohydrate as *L. esculentum* (37). The accumulation of sucrose rather than hexose may contribute to increased levels of soluble carbohydrate in several ways. First, osmotic considerations suggest that sucrose-accumulating fruit can accumulate twice as much soluble carbohydrate than a hexose-accumulating fruit while maintaining an equivalent osmotic potential. In this regard it is interesting to note that while *L. chmielewskii* fruit accumulate twice as much soluble carbohydrate on a glucose equivalent basis, the osmotic concentration of soluble carbohydrate is approximately the same in *L. chmielewskii* as *L. esculentum*. To the extent that soluble sugar uptake and accumulation is regulated by turgor (35) or by osmotic potential (3) the trait of sucrose accumulation, therefore, might favor elevated soluble sugar accumulation. In addition, sucrose is metabolically less active than hexose (24) and may be less accessible for loss through respiration, contributing to higher levels of accumulation. It is significant to note that storage organs accumulating very high levels of sugars do so by accumulating sucrose (6, 7, 13, 27). In sweet melon, sugar accumulation occurs in two stages with only low levels of hexose accumulating early in development followed by high levels of sucrose accumulating in later stages of development (30). Schaffer *et al.* (30) showed that the phase of sucrose accumulation in sweet melon was associated with the loss of soluble acid invertase activity and suggested that this loss is a general requirement for sucrose accumulation in storage organs. Other studies (20, 36) have also reported that the loss of the soluble form of acid invertase is required for the induction of sucrose accumulation in sucrose-accumulating species. Not surprisingly, fruit of *L. chmielewskii* were shown to have greatly reduced levels of invertase activity (37).

Studies have shown the presence of multiple invertases in sink tissues, including cell wall bound invertase proposed to be associated with phloem unloading and the capacity of the sink organs to accumulate assimilates (8). Soluble acid invertase is thought to be a vacuolar enzyme contributing to the hydrolysis of sucrose to hexose which may be stored (17, 18). A third form, alkaline invertase, is presumably localized in the cytoplasm and plays a role in sucrose degradation to meet the metabolic demand for glucose. Nakagawa *et al.* (21) reported two forms of acid invertase associated with the tomato fruit cell wall and Iki *et al.* (12) reported an increase in cell wall bound acid invertase during tomato fruit development. Ho and Baker (10) and Ho (11) have proposed that hydrolysis of sucrose by invertase may determine import rate and subsequent sink activity of tomato fruit by enhancing the

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source of sink sucrose gradient. However, if the high soluble sugar content of *L. chmielewskii* is attributable to high import rates, as has been suggested for high soluble sugar varieties of *L. esculentum* (9), these high rates of import are apparently not associated with high invertase levels in *L. chmielewskii*.

The primary objective of this study was to assess the feasibility of genetically transferring the trait of sucrose-accumulation from *L. chmielewskii* to *L. esculentum*. In this way we have been able to assess the number of genes that determine this trait and to establish populations in which we can assess the biochemical basis of sucrose accumulation and its effect on total soluble sugar accumulation.

MATERIALS AND METHODS

Plant Material

Two parental lines differing significantly in soluble sugars were selected for this study: *Lycopersicon esculentum*, cv UC82B (female parent) and the wild species *Lycopersicon chmielewskii* accession LA 1028 (male parent). Subsequent backcrosses utilized *L. esculentum* cv UC204C as the recurrent parent. Seed of *L. chmielewskii* LA1028 were obtained from Dr. C. M. Rick, Department of Vegetable Crops, University of California, Davis.

All plants evaluated were greenhouse grown and F_1, F_2, and BC_1F_1 plants set abundant self-pollinated fruit. However, F_3 and BC_1F_2 plants failed to set fruit and required pollination with *L. chmielewskii* LA1028 pollen to obtain fruit for sugar analyses. All crosses were made by utilizing standard methods of emasculation and pollination.

Invertase and Sucrose Synthase Assays

Five g of tomato fruit pericarp tissue was homogenized with a polytron in 5 mL of homogenization buffer containing 100 mM Hepes-KOH (pH 8.3), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EGTA, and 2 mM DTT. The extract was filtered through Miracloth, frozen in liquid N_2, and aliquots subsequently used for invertase and sucrose synthase assays. Removal of endogenous sugars before enzymatic assay was accomplished by centrifugation of samples through a 1 mL column of Sephadex G-50.

Invertase activity was assayed in 0.2 mL of 500 mM sodium acetate (pH 4.5) and 3% sucrose. The reaction mixture was incubated at 37°C for 30 min and terminated by heat denaturation. The reducing sugars formed were detected by the Nelson's reducing sugar arsenomolybdate method (23) and quantitated by measurement of absorbance at 660 nm. For pH optimum and K_m determinations, citrate buffer instead of sodium acetate buffer was used. For temperature optimum, the reaction was performed during 30 min by adding the samples to the substrate that were preincubated at each temperature. One unit of invertase was defined as the enzyme required to liberate 1 μmol of reducing sugar per min at 37°C. Sucrose synthase was assayed as previously described (28, 29), in a final volume of 0.05 mL containing 0.25 μmol UDP[^{14}C] Glc (160,000–240,000 cpm/μmol), 0.5 μmol Fru, and 5 μmol Tris-HCl (pH 8.0). After 20 min at 30°C the reaction was terminated by heat denaturation and unincorporated UDP[^{14}C]Glc was removed by mixing the reaction mixture with 0.4 g of anion exchange resin (AG1-X8, Bio-Rad). Samples were centrifuged and soluble UDP[^{14}C] sucrose counted with a liquid scintillation counter.

Sugar Determination

For sugar determination, 3 g of tissue was ground with a polytron in cold 80% ethanol and boiled twice for 20 min in a water bath. After each boiling, samples were centrifuged 10 min at 10,000 rpm, the supernatants pooled and brought to a final volume of 20 mL with 80% ethanol. Total reducing sugars were measured using Nelson's arsenomolybdate reagent (23). To determine sucrose concentration, samples were incubated with yeast invertase (Sigma) and total reducing sugar was determined as described above. Sucrose levels were inferred by the difference in reducing sugar levels assayed with and without invertase treatment. The validity of this technique was confirmed by HPLC analysis of sugar composition in some samples.

Extraction and Purification of Invertase

Six hundred grams of fresh ripe tomato pericarp tissue was homogenized in 400 mL of 10 mM Na citrate buffer (pH 4.5) containing 5 mM β-mercaptoethanol and 0.5% soluble PVP. The homogenate was centrifuged at 10,000 rpm for 30 min and the pellet washed in extraction buffer (without PVP) and recentrifuged. The pellet was resuspended in 200 mL of 50 mM Na citrate buffer (pH 5.5) containing 1.7 M NaCl and 15 mM EDTA. The homogenate was shaken for 1 h and then centrifuged at 10,000 rpm for 30 min. The supernatant, containing essentially all the invertase, was concentrated by the addition of $(NH_4)_2SO_4$ to 90% saturation and gently shaken for 1 h. The precipitate was collected by centrifugation (50 min at 10,000 rpm), dissolved in 35 mL of Con A buffer (0.1 M Na citrate [pH 6.0], 1 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$) and dialyzed overnight against Con A buffer. The enzyme solution thus obtained was applied to Con A-Sepharose 4B column (Sigma) previously equilibrated with Con A buffer. Proteins remaining in the column after extensive washing with Con A buffer were eluted with Con A buffer containing 250 mM α-methyl mannoside. The enzyme solution was subsequently dialyzed against 10 mM bis-tris propane (pH 6.5) and then concentrated by ultrafiltration using an Amicon pressure cell fitted with a PM-10 membrane. The concentrated enzyme was then applied to an anion exchange column (Mono Q, Pharmacia) equilibrated with bis-tris propane (pH 6.5). Elution of the Mono Q column was carried out using a linear gradient of 10 mM bis-tris propane (pH 6.5) containing between 0 and 1 M NaCl. The fractions with invertase activity were pooled, concentrated by ultrafiltration, and applied to a gel filtration column (Superose 6, Pharmacia) equilibrated with 10 mM bis-tris propane containing 250 mM NaCl and eluted with the same buffer. The enzyme solution obtained from the gel filtration column was concentrated by ultrafiltration and applied to a chromatofocusing column (Mono P, Pharmacia) equilibrated with 10 mM bis-tris propane (pH 6.15). The proteins retained on the column were eluted with a polybuffer at pH 4.8 (polybuffer 75, Pharmacia). Invertase was also purified from green (20 d after anthesis)

Table I. *Level of Soluble Carbohydrates in Ripe Fruits of L. esculentum, L. chmielewskii, and F_3 population*

The F_3 population is derived from the highest sucrose accumulator of the F_2 population.

Population	Hexose	Sucrose	Sucrose/Hexose Ratio	Total Sugar
	μmol GLC/g fresh wt			*μmol GLC/g fresh wt*
L. esculentum[a]	128.82	8.94	0.07	137.76
L. chmielewskii[a]	27.4	235.20	8.58	262.60
F_3 population[b]	23.09	194.36	8.42	217.45

[a] Each point represents the mean of five values. [b] Each point represents the mean of 60 values calculated from 60 different plants of the F_3 population.

and mature green (40 d after anthesis) fruit of *L. esculentum, L. chmielewskii,* and fruit of the BC_1F_2 population accumulating either hexose or sucrose. For those six purifications, 50 g of pericarp tissue was used and invertase was purified as described above.

Gel Electrophoresis and Immunoblotting

Protein was extracted from tomato fruit as described above. After $(NH_4)_2SO_4$ precipitation, the protein solution was dialyzed overnight against 10 mM bis-tris propane (pH 6.5). Proteins separated by SDS polyacrylamide gel (15) were stained with Coomassie blue or silver or electroblotted to PVDF[4] membrane (Immobilon P, Millipore) for immunodetection. Rabbit polyclonal antibodies to carrot invertase (16; obtained from Dr. M. J. Chrispeels, University of California, San Diego) were used and cross-reactive polypeptides visualized using goat anti-rabbit IgG conjugated to alkaline phosphatase.

Nondenaturing gels were carried out using a 7.5% polyacrylamide gel without SDS (15) with the pH of the running and stacking gels of 7.2 and 6.8, respectively. Invertase activity staining of nondenaturing gels was performed using the procedure for localization of enzymes producing keto sugars (5).

[4] Abbreviation: PVDF, polyvinyllidene difloride.

Protein Assay

Protein was determined by the method of Bradford (2) using a Bio-Rad protein assay kit and bovine serum albumin as standard.

RESULTS

Genetic and Biochemical Analysis

The F_1 and F_2 generations derived from a cross between *Lycopersicon esculentum* and *Lycopersicon chmielewskii* were analyzed for sucrose and hexose levels. Of the 148 plants analyzed in the F_1 generation, no sucrose-accumulating fruit were found (data not shown), suggesting that recessive gene(s) are responsible for sucrose accumulation. In the F_2 population, fruit of 6 of the 91 plants analyzed accumulated sucrose.

A single F_2 sucrose-accumulating plant was selected for further genetic analysis. This plant was self-pollinated and crossed to *L. esculentum* to produce F_3 and BC_1F_1 seed, respectively. All plants analyzed in the F_3 population accumulated sucrose rather than hexose, and the total sugar content was 57% higher than in *L. esculentum* (Table I). The average sucrose/hexose ratio of the F_3 population was 8.4, which is similar to *L. chmielewskii.* The fact that true-breeding, sucrose-accumulating lines could be obtained suggests that the trait of sucrose accumulation could be stably transferred to *L. esculentum.*

As expected from the F_1 population, all the fruit of the BC_1F_1 population accumulated hexose (data not shown). Sugar composition of fruit in the BC_1F_2 population are presented in Table II. Of the 62 BC_1F_2 plants planted, only 56 were analyzed since six of them did not set fruit. Of the plants analyzed, 46 were hexose accumulators and 10 were sucrose accumulators (Table II). Thus, the ratio of sucrose- to hexose-accumulating plants was approximately 1:5 in the BC_1F_2 population. The discrepancy between this ratio and that observed in the F_2 population (1:14) has been tentatively attributed to close linkage between the sucrose-accumulating locus and a locus conferring sterility in crosses between *L. esculentum* and *L. chmielewskii* (see "Discussion").

In young fruit of the BC_1F_2 population (20 d after anthesis), the total sugar content was similar in hexose-accumulating fruit than in sucrose-accumulating fruit, whereas in mature green fruit (40 d after anthesis) 32% more sugar was accumulated in sucrose-accumulating than hexose-accumulating

Table II. *Level of Hexose and Sucrose in Tomato Fruits of the BC_1F_2 Population*

Population	Age	Hexose	Sucrose	Sucrose/Hexose Ratio	Total Sugar
	d	*μmol GLC/g fresh wt*			*μmol GLC/g fresh wt*
Hexose accumulator[a]	20	70.9 ± 3.2	7.2 ± 1.4	0.11 ± 0.02	78.1 ± 3.9
(46/56)	40	176.3 ± 6.8	5.1 ± 1.8	0.05 ± 0.01	181.4 ± 6.5[c]
Sucrose accumulator[b]	20	36.6 ± 5.6	48.9 ± 4.51	1.34 ± 0.23	85.5 ± 7.36
(10/56)	40	71.9 ± 12.6	165.0 ± 18.9	4.22 ± 1.26	236.5 ± 18.4[c]

[a] Each point represents the mean of 46 values calculated from 46 different plants of the BC_1F_2 population. [b] Each point represents the mean of 10 values calculated from 10 different plants of the BC_1F_2 population. [c] Values for total sugar are different at the 99.9% confidence level.

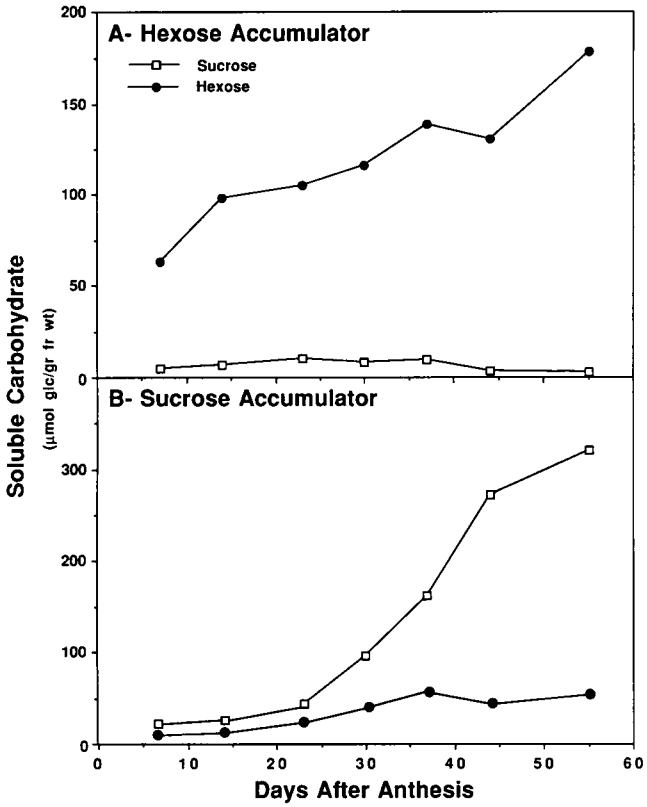

Figure 1. Level of soluble carbohydrates throughout fruit development of one hexose- (A) and one sucrose-accumulating (B) plant of the BC_1F_2 population. Each point represents the mean of three values.

fruit (Table II). In a previous study (37), we found that the accumulation of sugar in *L. chmielewskii* occurred late in development (40–60 d after anthesis). Sugar beet (6), and sweet melon (30) also accumulate much of their sucrose late in development. To evaluate the developmental pattern of sugar accumulation in fruit of the BC_1F_2 population, the level of soluble carbohydrates was measured throughout fruit development of one hexose- and one sucrose-accumulating plant (Fig. 1). In hexose-accumulating fruit little accumulation of sucrose was observed, while in sucrose-accumulating fruit, the level of sucrose rose during later stages of development, with a major increase between 30 and 40 d after anthesis. The pattern of sugar accumulation and final total soluble sugar content of the sucrose-accumulating BC_1F_2 fruit was similar to that previously described for *L. chmielewskii* fruit (37).

To assess the role of specific metabolic processes contributing to this sucrose-accumulating trait, invertase and sucrose synthase activity were assayed in fruit of the BC_1F_2 population at 20 and 40 d after anthesis (Table III). Fruit of the 46 hexose accumulators showed a high level of both invertase and sucrose synthase, while low invertase but high sucrose synthase activities were measured in fruit of the 10 sucrose accumulators.

Invertase and sucrose synthase were assayed throughout fruit development for one hexose- and one sucrose-accumulating BC_1F_2 plant (Fig. 2). The activity of invertase was very

Table III. *Activity of Enzymes Invertase and Sucrose Synthase in Tomato Fruit of the BC_1F_2 Population*

Population	Days after Anthesis	Invertase Activity	Sucrose Synthase Activity
	d	*nmol GLC/g fresh wt min*	
Hexose accumulator[a]	20	3216 ± 271	397 ± 52
(46/56)	40	12839 ± 572	ND[b]
Sucrose accumulator[c]	20	42 ± 20	346 ± 57
(10/56)	40	32 ± 29	ND

[a] Each point represents the mean of 46 values calculated from 46 different plants of the BC_1F_2 population. [b] Sucrose synthase levels not determined at 40 d after anthesis since levels are very low in both *L. esculentum* and *L. chmielewskii* at this developmental stage. [c] Each point represents the mean of 10 values calculated from 10 different plants of the BC_1F_2 population.

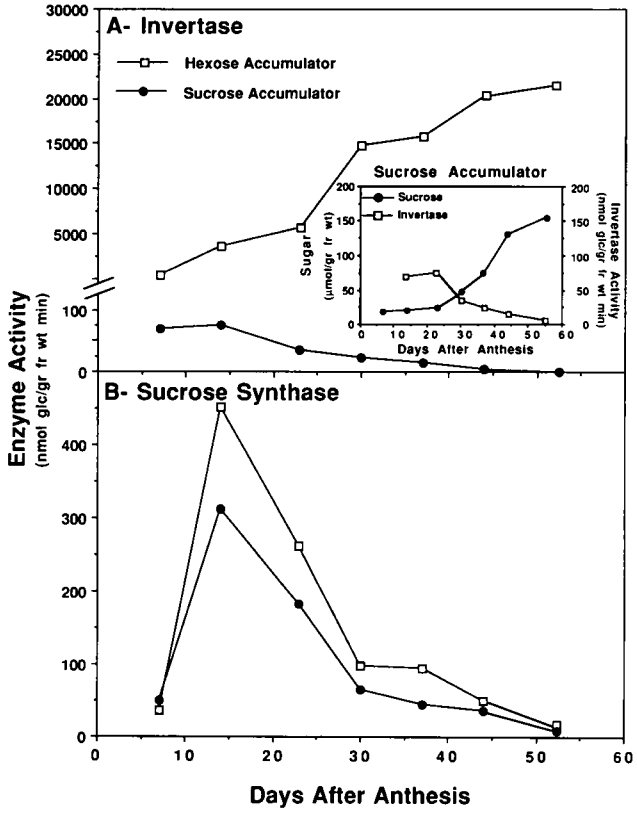

Figure 2. Level of invertase (A) and sucrose synthase (B) throughout fruit development of one hexose- and one sucrose-accumulating plant of the BC_1F_2 population. Inset (A) shows the levels of invertase and soluble carbohydrate throughout development of one sucrose-accumulating plant. Data for soluble carbohydrate are replotted from Figure 1 and the data for invertase levels are replotted from Figure 2A with an expanded scale. Each point represents the mean of three values.

Table IV. *Purification of Invertase from Ripe Fruit of L. esculentum*

Fraction	Volume	Activity	Protein	Specific Activity	Yield	Purification
	mL	*units*	*mg*	*units/mg*	%	*-fold*
Crude extract						
Soluble	1100.0	0	48.70			
Insoluble	385.0	2020	246.20	8.21	100.0	1.0
$(NH_4)_2SO_4$	25.0	2010	165.40	12.20	99.5	1.5
Con A	3.0	1307	24.90	54.49	64.7	6.4
Mono Q	13.0	992	2.55	389.02	49.1	47.4
Gel filtration	3.7	535	0.71	753.50	26.5	91.8
Mono P						
Invertase I	2.1	74	0.08	890.00	3.7	108.4
Invertase II	2.1	313	0.33	950.00	15.5	115.7
Invertase III	2.1	67	0.08	860.00	3.3	104.8

low in sucrose-accumulating fruit throughout fruit development and decreased further as fruit matured. In contrast, invertase activity was much higher in hexose-accumulating fruit, increasing as fruit matured. The accumulation of reducing sugars in hexose-accumulating fruit (Fig. 1A) was associated with the rise of invertase activity (Fig. 2A) while the accumulation of sucrose in sucrose-accumulating genotypes was associated with a decrease in invertase activity (Fig. 2A). This relationship is clearly seen in the plot of sucrose accumulation and invertase activity in the sucrose-accumulating fruit (Fig. 2A, inset). These results suggest that the accumulation of sucrose in tomato fruit requires the loss of invertase activity.

Sucrose synthase of both hexose- and sucrose-accumulating fruit reached a peak of activity 14 d after anthesis and then decreased to near zero as fruit matured (Fig. 2B). The high level of sucrose synthase in sucrose-accumulating fruit was unexpected since *L. chmielewskii* did not have any sucrose synthase activity (37). These results suggest that invertase, but not sucrose synthase, is a critical enzyme regulating sucrose accumulation in tomato fruit.

Purification of Tomato Invertase

To determine the basis for the low level of invertase activity in sucrose-accumulating tomato fruit, we purified and characterized invertase from both hexose- and sucrose-accumulating tomato fruit (Table IV). The distribution of invertase between the soluble and insoluble fraction was readily altered by varying pH of the extraction buffer (data not shown). For convenience, low pH and low salt concentration were used in the extraction buffer and under these conditions all of the invertase activity was found in the cell wall fraction. However, it is unlikely that localization in the cell wall extract reflects the subcellular localization of tomato fruit invertase *in vivo*. Invertase was subsequently solubilized by suspension of the cell wall fraction in high salt buffer and the protein solution concentrated by the addition of $(NH_4)_2SO_4$ to 90% saturation. Con A Sepharose affinity chromatography resulted in the elimination of 85% of the total protein and an increase in the specific activity of invertase by about fourfold (Table IV). Anion exchange (Mono Q) chromatography increased the specific activity another sevenfold with a recovery of more than 65% of the invertase activity. In contrast to a previous report (21) tomato fruit invertase subjected to anion exchange chromatography eluted as a single peak with a linear gradient of 1 M NaCl (Fig. 3). The resulting invertase peak was dialyzed and concentrated by ultrafiltration and applied to a gel filtration column (Superose 6) previously calibrated with protein standards of known molecular mass. The active invertase fractions eluted from the column at a position corresponding to a molecular mass of 50 to 55 kD (data not shown).

The final step of invertase purification was chromatofocusing (Mono P) and resulted in the resolution of three peaks of invertase activity (Fig. 4). The three isoforms were designated invertase I, II, III in the order of their elution from the column. Isoelectric points obtained from the pH of each fraction from the chromatofocusing column were 5.42, 5.32, and 5.12 for invertase I, II, and III, respectively (Fig. 4). Analysis of tomato fruit invertase by nondenaturing gel electrophoresis indicated the presence of two major polypeptides with invertase activity (Fig. 4, inset). The two polypeptides visualized on native gels corresponded to invertase II and III of the chromatofocusing column (Fig. 4, inset). Insufficient quantities of invertase I prevented analysis on native gels.

The overall purification of invertase I, II, and III were

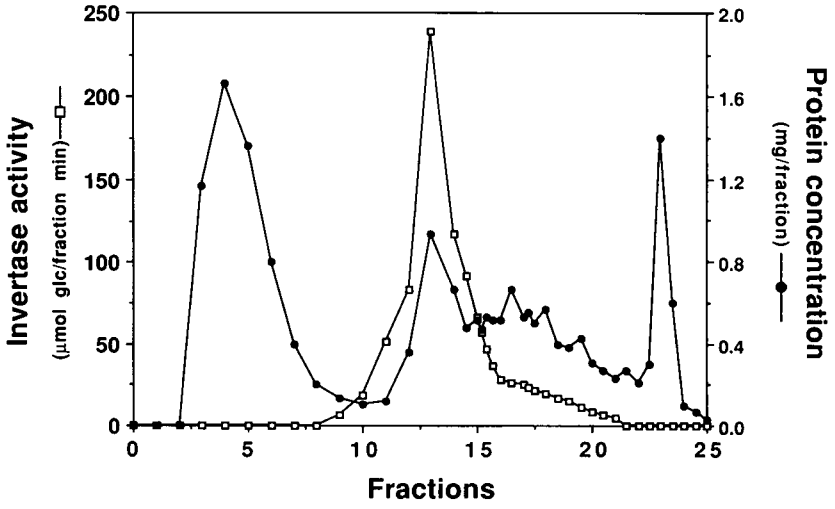

Figure 3. Anion exchange chromatography of tomato fruit invertase on Mono Q. Approximately 25 mg of protein was applied to a Mono Q column and eluted with gradient of 0 to 1 M NaCl as described in "Materials and Methods." The flow rate was 0.5 mL/min and 0.5 mL fractions were collected and assayed for invertase activity (□) and protein content (●).

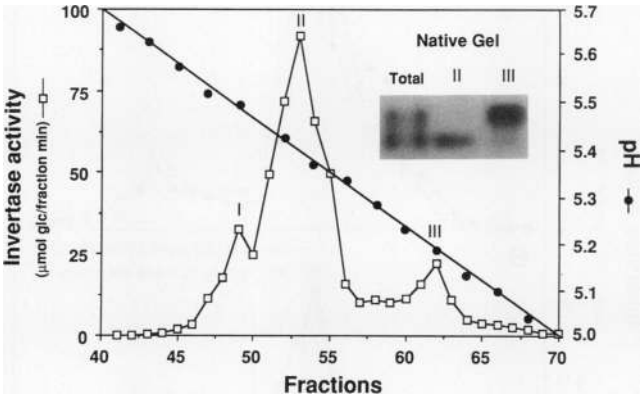

Figure 4. Chromatofocusing of tomato fruit invertase on Mono P. Approximately 2.5 mg of protein was applied to a Mono P column and eluted with polybuffer 75. The flow rate was 0.3 mL/min and 0.3 mL fractions were collected and assayed for invertase activity (□) and pH (●) determined. Peaks of invertase activity are designated isoforms I, II, and III in the order of elution from the Mono P column. Inset: A native gel of total invertase following purification on Mono Q and of peak fractions of invertase isoforms II and III following purification on Mono P. Approximately 5 μg of each fraction was separated by nondenaturing PAGE and stained for invertase activity as described in "Materials and Methods."

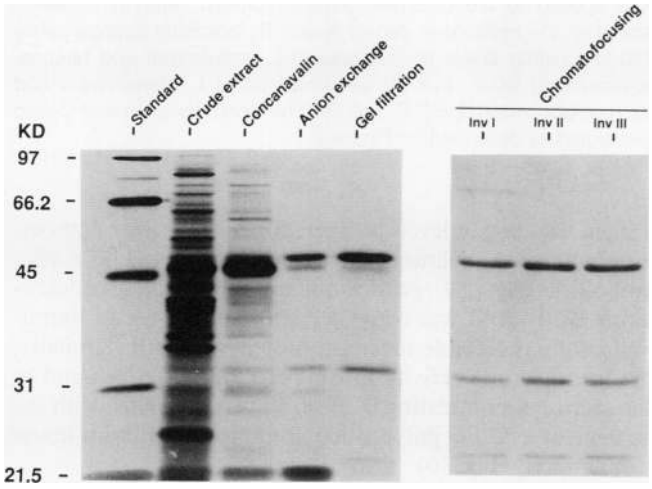

Figure 5. SDS-PAGE of tomato fruit invertase at various stages of purification. The amount of protein in each sample lane was 30 μg, crude extract; 15 μg, concanavalin; 5 μg, anion exchange; 3 μg, gel filtration; and 2 μg each, Inv I, Inv II, and Inv III. The gel was stained with Coomassie blue.

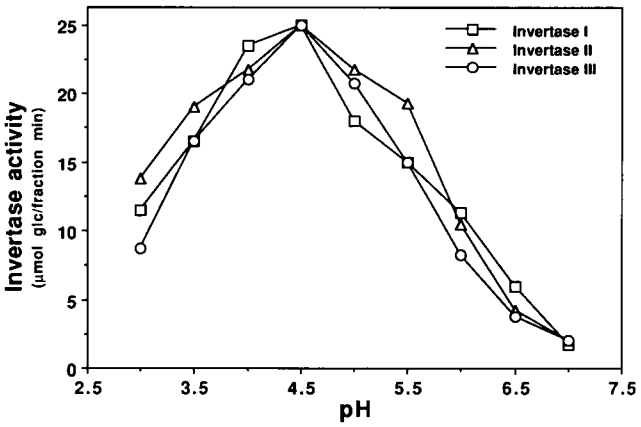

Figure 6. pH dependence of tomato fruit invertase isoforms I (□), II (△), and III (○). Approximately 30 μg of pure invertase protein was assayed.

108-, 116-, and 105-fold with a recovery of about 4, 16, and 3%, respectively (Table IV). The six-step purification procedure (Table IV) resulted in a preparation with three polypeptides detectable on an SDS polyacrylamide gel (Fig. 5). The three polypeptides have molecular masses of 52, 30, and 22 kD. Sequencing of the amino terminus of the 52 and 30 kD polypeptides yielded an identical amino terminal sequence (data not shown) suggesting that the lower molecular polypeptides (30 and 22 kD) are degradation products of the 52 kD polypeptide.

Characterization of Invertases

The effects of pH, temperature, and substrate concentration on the activity of the three invertase isoforms isolated from *L. esculentum* were examined. The three tomato fruit invertase isoforms had identical pH optima of 4.5 (Fig. 6) and the optimum temperatures for reaction of the three invertase isoforms were essentially identical (Fig. 7). The sucrose concentration dependence of the three isoforms of invertase was also determined. From the double reciprocal plot of relative activity *versus* substrate concentration, apparent K_m (Michaelis constants) for the invertase I, II, and III isoforms were calculated to be 7.9, 7.7, and 7.4 mM with V_{max} of 9.23, 9.08, and 8.42 μmol min^{-1}, respectively (Fig. 8). Based on pH, temperature, and substrate concentration dependence, the three tomato fruit invertase isoforms were indistinguishable.

Invertase of Hexose- and Sucrose-Accumulating Fruit

Invertase was also purified from fruit of *L. chmielewskii,* and from either hexose or sucrose-accumulating fruit of the BC_1F_2 population. All the purification steps were similar to those described above for *L. esculentum* invertase and the result of the final step of purification is shown in Figure 9. Chromatofocusing of invertase showed that hexose-accumulating fruit of the BC_1F_2 population have a very similar invertase isoform composition as *L. esculentum* (Fig. 9A) while invertase isoforms of sucrose-accumulating fruit of the BC_1F_2 population are similar to *L. chmielewskii* (Fig. 9B).

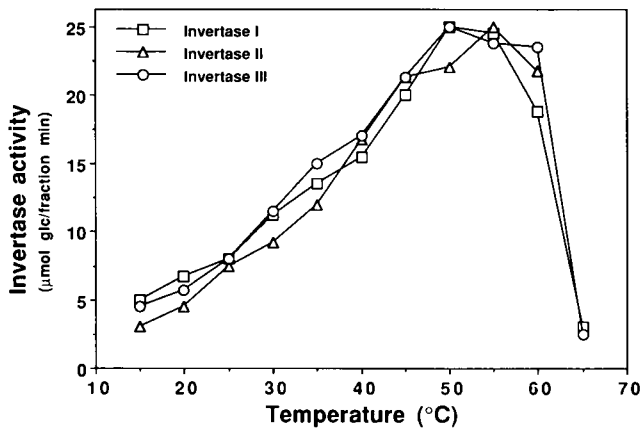

Figure 7. Temperature dependence of tomato fruit invertase isoforms I (□), II (△), and III (○). Approximately 30 μg of pure invertase was assayed.

Invertase I appears to be absent or substantially reduced in sucrose-accumulating fruit. The ratio of invertase II to invertase III is slightly different between sucrose- and hexose-accumulating fruit, being approximately two in sucrose-accumulating fruit and four to six in hexose-accumulating fruit. Despite the fact that invertase levels are greatly reduced in sucrose-accumulating fruit, the invertase that is present appears to be quite similar to the invertase in hexose-accumulating fruit, with the major form corresponding to invertase II.

Immunodetection of Tomato Fruit Invertase

Protein was extracted from tomato fruit of *L. esculentum, L. chmielewskii,* and sucrose- and hexose-accumulating fruit of the BC_1F_2 population at 20 and 40 d after anthesis. Invertase activity in fruit of *L. esculentum* and of the hexose-accumulating BC_1F_2 fruit was similar and about four times higher at 40 than at 20 d after anthesis (Table III). In contrast, the activity of invertase was very low in both *L. chmielewskii* and sucrose-accumulating fruit of the BC_1F_2 population with

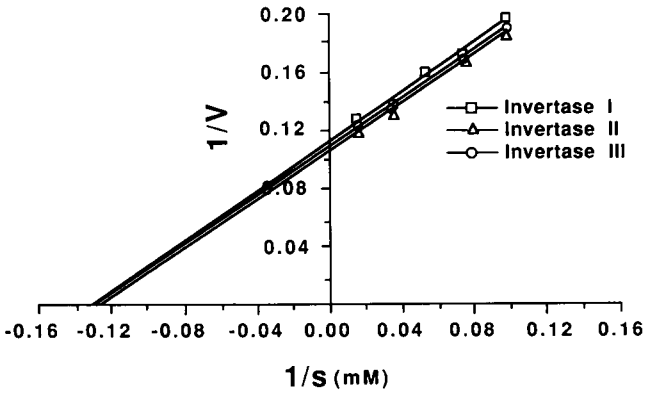

Figure 8. Double reciprocal plot of the sucrose concentration dependence of tomato fruit invertase isoforms I (□), II (△), and III (○). Approximately 10 μg of pure invertase was assayed.

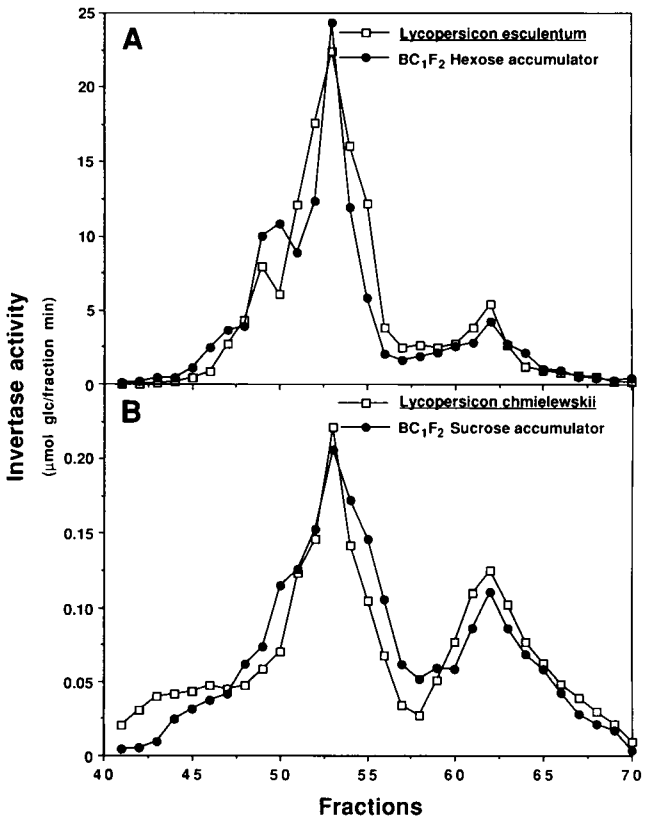

Figure 9. Chromatofocusing of invertase purified from *L. esculentum* and a hexose-accumulating fruit of a BC_1F_2 plant (A) or from *L. chmielewskii* and a sucrose-accumulating fruit of a BC_1F_2 plant (B). Total invertase purified from 50 g of pericarp tissue of each genotype was applied to the chromatofocusing column. Note the different scales of the ordinate in panels A and B, indicating approximately 100-fold higher levels of invertase in *L. esculentum* and hexose-accumulating BC_1F_2 fruit (A) as compared to *L. chmielewskii* and sucrose-accumulating BC_1F_2 fruit (B). The Mono P column was loaded and eluted as described for Figure 2.

a slight decrease in levels between 20 and 40 d after anthesis. The increase of invertase activity between 20 and 40 d after anthesis in fruit of *L. esculentum* and of the hexose-accumulating BC_1F_2 fruit was correlated with an increase in immunologically detectable invertase protein (Fig. 10). Similarly, the low invertase activity in fruit of *L. chmielewskii* and of the sucrose-accumulating BC_1F_2 fruit was associated with the absence of a 52 kD polypeptide immunoreactive with invertase antibody (Fig. 10).

DISCUSSION

The present study has demonstrated the feasibility of transferring the sucrose-accumulating trait of *L. chmielewskii* to *L. esculentum*. The accumulation of sucrose in mature fruit of the BC_1F_2 population was characterized by a sugar content (expressed on a Glc equivalent basis) twice as high as hexose-accumulating fruit. This supports the previous suggestion that transfer of the sucrose-accumulating trait to domesticated tomato may increase total fruit soluble sugar content (37).

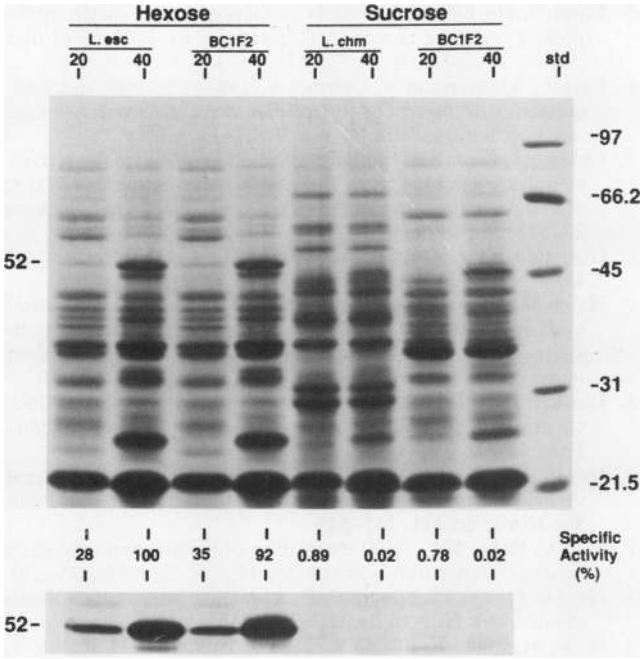

Figure 10. Immunological detection of invertase in *L. esculentum* or *L. chmielewskii* and in hexose- or sucrose-accumulating BC_1F_2 fruit. Protein from hexose-accumulating fruit (*L. esculentum* or BC_1F_2) or from sucrose-accumulating fruit (*L. chmielewskii* or BC_1F_2) at either 20 or 40 d after anthesis. Protein (100 μg) was loaded per lane and silver stained (upper panel) or blotted to PVDF membrane and invertase detected with antibodies to carrot invertase (lower panel) as described in "Materials and Methods." The relative specific activity of invertase in each extract (*L. esculentum*, 40 d; 100%) is also indicated.

Sucrose-accumulating tomato fruit were found only in the F_2, F_3, and BC_1F_2 and not in F_1 or BC_1 generations, suggesting that recessive gene(s) control the accumulation of sucrose. Of the 56 plants analyzed in the BC_1F_2 population, 10 were sucrose accumulators. Restriction fragment length polymorphism analysis of the BC_1F_2 population has shown that 16 of the 64 plants analyzed were homozygous for a *L. chmielewskii* fragment of chromosome 3 that is linked to the trait of sucrose accumulation (data not shown). Of those 16 plants, 10 set sucrose-accumulating fruit while the remaining 6 corresponded to the plants that did not set any fruit. Previous studies (A Paterson, personal communication) with *L. chmielewskii* × *L. esculentum* crosses have identified a recessive sterility allele in the region of chromosome 3 near the locus associated with sucrose accumulation. Therefore, the 6 sterile plants of the BC_1F_2 population were likely to be sucrose accumulators, suggesting an actual ratio of 1:3 sucrose accumulators in this population. The fact that the ratio of hexose- to sucrose-accumulating plants decreased from approximately 1:14 in the F_2 population to 1:5 in the BC_1F_2 population suggests that the association between sucrose accumulation and the sterility trait must be determined by linkage, not pleiotropy. The ratio of 1:5 (and potentially 1:3) sucrose accumulators in the BC_1F_2 population is close to that expected for one recessive gene controlling sucrose accumulation in tomato fruit.

Sucrose accumulation was associated with a low invertase, but a normal sucrose synthase level in fruit of the BC_1F_2 population. In *L. chmielewskii* fruit, the accumulation of sucrose was associated with a low level of acid invertase and the absence of sucrose synthase. Unexpectedly, the presence of sucrose synthase in sucrose-accumulating fruit of the BC_1F_2 population did not alter the fruit carbohydrate composition when compared to *L. chmielewskii* (37). This suggests that sucrose synthase is not directly responsible for the composition and/or concentration of sugar in tomato fruit.

Other studies have found that the loss of soluble acid invertase is required for sucrose accumulation in sugar beet (6), citrus (13), carrot root (27), and sugar cane (7). The presence of invertase has been reported to play a major role in regulating the rate of carbon import to tomato fruit (34). In the present study the high soluble sugar content of sucrose-accumulating fruit is not associated with high invertase levels and we speculate that invertase influences sugar composition in tomato fruit, with no direct effect on carbon import rates.

In order to establish the basis for low invertase levels in sucrose-accumulating tomato fruit, we purified and characterized invertase in both hexose- and sucrose-accumulating tomato fruit. A previous report of the purification of tomato invertase from ripe tomato fruit indicated the presence of two isozymes that were resolved by anion exchange chromatography (21). Although we were not able to resolve invertase isozymes by anion exchange chromatography, three isoforms were resolved by chromatofocusing. The size of all three isoforms was approximately 52 kD, as determined by both gel filtration and SDS-PAGE. Although lower molecular mass polypeptides were observed on SDS gels of purified invertase (Fig. 5), analysis of the 52 and 30 kD polypeptides indicated an identical amino terminal amino acid sequence, suggesting that the lower molecular mass polypeptides were degradation products of the 52 kD polypeptide. This conclusion was supported by the observation that antibodies raised against carrot invertase (16) cross-reacted with all three polypeptides. The agreement in molecular mass determination by both gel filtration and SDS-PAGE suggests that tomato fruit invertase is active as a monomer. The size of tomato fruit invertase is similar to other higher plant acid invertases that range in size from approximately 50 to 70 kD (3, 9). However, some acid invertases have been reported to be structurally quite distinct, most notably the acid invertase of *Ricinus communis* consisting of multiple 11 kD subunits (25).

Multiple isozymes of invertase have been reported in many species (1, 14, 19, 20, 32, 33). In tomato fruit, Nakagawa *et al.* (21) have reported two forms of invertase. Our results also indicated multiple isoforms of tomato fruit invertase but, we found no marked differences between the three isoforms with regard to pH, temperature, and substrate concentration dependence. The three invertase isoforms identified in tomato fruit are acid invertases with an optimal enzymatic activity of pH 4.5, with the relative activity for the three forms of invertase below 10% at pH 7.0. The three isoforms of invertase purified in the present study were indistinguishable by SDS-PAGE. Faye *et al.* (4) reported at least six different forms of soluble invertase in radish cotyledons, with isoelectric points ranging from 4.7 to 7.5. As in tomato fruit, the radish invertase isoforms showed an identical size when analyzed by SDS-

PAGE and because all the forms of invertase were antigenically related, Faye *et al.* (4) proposed that the difference in charge properties resulted from posttranslational processing of a single gene product. The three tomato invertase isoforms reported here are very similar and all react with an antibody to carrot invertase, suggesting that the three isoforms may similarly arise by differential processing of a single gene product.

To assess if the change in invertase activity throughout tomato fruit development was associated with a change in isoform composition, we purified invertase at three different stages of development (20, 40, and 60 d after anthesis, data not shown). Despite the large differences in activity over this developmental time, no significant differences were found in relative invertase isoform composition. Purification of invertase in both hexose- and sucrose-accumulating fruit also showed the same relative composition of invertase isoforms. Based on these results, we cannot associate the low invertase activity in sucrose-accumulating fruit relative to hexose-accumulating fruit to a difference in invertase isoform composition.

Immunological detection of invertase showed that the low activity of invertase in *L. chmielewskii* and in sucrose-accumulating fruit of the BC_1F_2 population was associated with a low level of invertase protein. The increase of invertase activity throughout fruit development in *L. esculentum* and in hexose-accumulating fruit of the BC_1F_2 population was also associated with increased levels of invertase protein. Nakagawa *et al.* (22) reported a decline in invertase activity in senescent tomato fruit which was due to catalytically inactive but immunologically detectable invertase, suggesting the presence of an invertase inhibitor. Proteinaceous invertase inhibitors have been reported in the sucrose storing red beet, sugar beet, and sweet potato (26). However, Schaffer *et al.* (30) reported that the loss of invertase activity as sucrose accumulates in sweet melon was not caused by an inhibitor of invertase activity. Based on the correlation between immunologically detectable invertase protein with levels of invertase activity, we propose that the low invertase activity in sucrose-accumulating fruit is caused by a low level of invertase protein rather than by an inhibitor of invertase activity.

The number of genes encoding invertase in any plant has not been determined. In the present study, the high degree of similarity between the different isoforms of tomato fruit acid invertase suggests that they may originate from a single gene or from closely related genes. Biochemical and genetic analysis of the BC_1F_2 population derived from a cross between *L. esculentum* and *L. chmielewskii* suggested that a single recessive gene controls sucrose accumulation. We speculate that this gene may encode tomato fruit invertase or a factor regulating its expression.

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