Plant Cell Physiol. 31(5): 655–659 (1990)

Size and Levels of mRNA for Acid Invertase in Ripe Tomato Fruit

Masakazu Endo, Hiroki Nakagawa, Nagao Ogura
and Takahide Sato

Department of Agricultural Chemistry, Faculty of Horticulture, Chiba University, 648 Matsudo, Chiba, 271 Japan

Poly(A)$^+$RNA was isolated from ripe tomato fruit and translated in a wheat germ cell-free translation system. A 74-kDa polypeptide was detected as a putative precursor of acid invertase by immunoprecipitation with antiserum raised against SDS-treated acid invertase (denatured form) from tomato fruit. The molecular mass of the mRNA for acid invertase was estimated to be about 8×10^5 Da (2.4 k nucleotides) by sucrose density gradient centrifugation. Mature, green tomato fruit contained very low levels of invertase mRNA. When mature, green tomato fruit were stored at 22°C, levels of invertase mRNA per gram fresh weight increased to a maximum after four days and then declined.

Key words: Acid invertase synthesis (in vitro) — *Lycopersicon esculentum* — Polyadenylated RNA — Tomato fruit.

Acid invertase (EC 3.2.1.26), which hydrolyses sucrose to glucose and fructose, is one of the enzymes whose activities are increased in tomato fruit during ripening (Nakagawa et al. 1970). It has been purified to homogeneity from ripe tomato fruit (Takehana and Nakagawa 1970), and antiserum has been raised against the pure protein (Iwatsubo et al. 1976). Using this antiserum, Iwatsubo et al. (1976) showed that the increase in acid invertase acitivity in ripe tomato fruit was due to the synthesis of the enzyme de novo. As a first step in the investigation of the mechanisms that controls the synthesis of acid invertase in tomato fruit, we tried to identify a precursor to acid invertase among the products of cell-free translation of poly(A)$^+$RNA prepared from ripe tomato fruit. This antiserum failed, however, to bind to any of the polypeptide bands generated by the products of translation in vitro. In the study described in this report, we prepared antiserum against SDS-treated invertase (denatured form) and, using this antiserum, we detected a 74-kDa putative precursor to acid invertase among the products of cell-free translation of the poly(A)$^+$RNA.

Abbreviations: PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride; poly(A)$^+$RNA, polyadenylated RNA; poly(U), polyuridylic acid.

Materials and Methods

Plant materials—Tomatoes (*Lycopersicon esculentum* Mill cv. Omiya) were grown at the Chiba University farm. Fruit were harvested at the mature, green stage and ripened at 22°C or 33°C for various lengths of time (Yoshida et al. 1984).

Isolation of poly(A)$^+$RNA—Nucleic acids were isolated from the tomato pericarp tissue by the SDS-phenol method (Sato et al. 1984). Nucleic acids were dissolved in distilled water and then precipitated with 3 M LiCl at 0°C for at least 2 h. The precipitate collected by centrifugation at $30,000 \times g$ for 30 min at 0°C was dissolved in distilled water and then precipitated with ethanol. Poly(A)$^+$RNA was prepared by column chromatography on poly(U)-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). The concentration of RNA was estimated from measurements of the absorbance at 260 nm (Sato et al. 1985).

Cell-free translation of poly(A)$^+$RNA and immunoprecipitation of products of translation—Poly(A)$^+$RNA was translated in a wheat germ cell-free translation system (25 μl) (Sato et al. 1980) in the presence of ^{3}H-leucine (5 μCi, 146 Ci/mmol, New England Nuclear) for 30 min at 30°C. The invertase polypeptide produced in vitro was immunoprecipitated with invertase-specific antiserum (5 μl)

then analyzed by SDS-PAGE, on a 10% gel that contained 8 M urea, and subsequent fluorography (Sato et al. 1984). The radioactivity in the gel was measured as described previously (Sato et al. 1978).

Analysis of poly(A)$^+$RNA on sucrose density gradients—A linear gradient of sucrose [5 to 20% (w/v) in 10 mM Tris-HCl buffer (pH 7.5) that contained 1 mM EDTA and 0.1% SDS] was prepared in a polyallomer tube (4.8 ml, Hitachi Co. Ltd., Japan). Poly(A)$^+$RNA (100 μg) was taken up in 100 μl of the same buffer and then heated at 60°C for 5 min, after which it was chilled on ice and loaded on the gradient. The gradient was centrifuged at 190,400 × *g* for 4 h in a Hitachi RPS 50-2 rotor at 20°C. Fractions of 0.25 ml were collected. Carrier tRNA (10 μg of wheat germ tRNA; Sigma, St. Louis, MO, U.S.A.) was added to each fraction, and then RNA was precipitated by addition of two volumes of ethanol. The precipitated RNA was washed with 70% ethanol and then taken up in sterile distilled water (Sato et al. 1985).

Extraction of invertase—Tomato pericarp tissue (20 g) was homogenized with 20 ml of chilled distilled water for 2 min in a blender. The homogenate was centrifuged at 10,000 × *g* for 15 min, and the supernatant was discarded. The resultant pellet was resuspended in 10 ml of 0.2 M Tris-HCl buffer (pH 8.5) that contained 0.5 mM PMSF and stirred for 2 h. The slurry was then centrifuged at 15,000 × *g* for 20 min and the supernatant (crude extract) was used for enzymatic assays.

Assay of invertase activity—Invertase activity was assayed by monitoring the formation of reducing groups in a reaction mixture at 30°C, which initially contained 0.25 ml of 10% sucrose, 0.25 ml of 0.4 M sodium acetate buffer (pH 4.5) and 0.5 ml of a solution of enzyme. Reducing groups formed in the reaction mixture were measured by the 3,6-dinitrophthalic acid procedure, as described previously (Nakagawa et al. 1970). One unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 1 μmol of sucrose in 1 min under the conditions of the reaction.

Purification of acid invertase from tomato fruit and preparation of antiserum—Mature, green tomato fruit were stored at 33°C for 14 days. Invertase was purified from 3 kilograms of pericarp tissue from the tomato fruit by a modified version of the method of Takehana and Nakagawa (1970). In brief, the crude extract of the tomato fruit was fractionated by column chromatography on SE-53 cellulose, Con A Sepharose and Toyopearl HW-55F. The specific activity of invertase after elution from the column of Toyopearl HW-55F was about 470 units per mg of protein, and the purity of the enzyme was about 95% as judged by SDS-PAGE. About 300 μg of purified invertase were subjected to SDS-PAGE on a 12.5% gel (90 × 100 × 1 mm^3; Laemmli 1970). After staining of the gel with Coomassie Brilliant Blue R, the portion of the gel containing the invertase polypeptide (51 kDa) was cut out and crushed by passage through a needle (#25 gauge). About 10 volumes of 50 mM ammonium carbonate containing 0.1% SDS and 0.01% 2-mercaptoethanol were added to the crushed gel and the mixture was stirred for 48 h at 37°C. After centrifugation, the supernatant was concentrated and used to raise antibody as described previously (Ozutsumi et al. 1983).

Enzyme immunoassay of invertase protein—The assay was performed using small piece of Immobilon membrane (Millipore; 1 × 1 cm^2). Four μl of diluted crude extract or of a solution of purified invertase protein were spotted on a membrane and then the membrane was washed with TBST [50 mM Tris-HCl (pH 8.0), 0.14 M NaCl, 0.05% Tween 20] supplemented with 2% milk protein for 1 h, with subsequent incubation with invertase-specific antiserum (1 : 2,000 dilution) in TBST overnight. After the membranes had been washed three times with TBST for 10 min, they were incubated with antiserum raised in goat against rabbit IgG, conjugated with alkaline phosphatase (Promega, Madison, WI, U.S.A.; 1 : 7,500 dilution) for 30 min. The membranes were washed with TBST as described above and then incubated in 1 ml of 100 mM Tris-HCl buffer (pH 9.5) that contained 100 mM NaCl, 5 mM $MgCl_2$ and 40 μg of *p*-nitrophenylphosphate for 1 h at 37°C. The alkaline phosphatase activity was determined by measuring the absorbance at 405 nm. Diluted crude extract from tomatoes at the over-ripe stage was used to generate a standard curve, which was calibrated with purified invertase protein. The alkaline phosphatase activity observed was linearly dependent on the amount of invertase protein over a range from 4 ng to 10 ng of protein per membrane.

Immunoblot analysis—Proteins were separated by SDS-PAGE on a 12.5% gel and transferred to nitrocellulose sheets in a solution of 25 mM Tris and 192 mM glycine (pH 8.3) which had been made 20% (v/v) in methanol, at 100 V for 2 h, as described by Burnette (1981). The filters were washed with TBST for 5 min and then blocked with 2% milk protein in TBST for 30 min. The filters were incubated with diluted antiserum (1 : 2,000 dilution) in TBST that contained 2% milk protein for 3 h. The filters were processed by the ProtoBlot Western Blot AP System (Promega) for detection of antibodies that reacted with the antigen.

Results and Discussion

Specificity of antiserum against acid invertase from tomato fruit—The specificity of the reaction of antiserum raised in rabbits against acid invertase with the invertase polypeptide was tested by immunoblot analysis with crude extracts of pericarp tissues from tomato fruit which had been harvested at the mature, green stage and stored at 22°C or 33°C for 10 days. As shown in Figure 1, the an-

tiserum (1 : 2,000 dilution) allowed visualization of both the purified acid invertase polypeptide (lane 4) and a single polypeptide band (invertase polypeptide) in the crude extract of tomato fruit stored at 33°C (lane 3), but it did not reveal any polypeptide bands in the crude extract of mature, green fruit (lane 1). This antiserum did, however, react with some smaller polypeptides, in addition to acid invertase, in the crude extract of tomato fruit stored at 22°C (lane 2). These smaller polypeptides may be products of the degradation of acid invertase, because prolonged storage of purified invertase gave similar bands on immunoblot analysis (data not shown).

Immunoprecipitation of a putative precursor to acid invertase of tomato fruit—Poly(A)$^+$RNA isolated from tomato fruit at the pink stage was translated in vitro. A putative precursor to acid invertase, with a molecular mass of about 74 kDa, was immunoprecipitated with invertase-specific antiserum (Fig. 2, lane 2). When preimmune serum was used, no labeled polypeptide was detected (Fig. 2, lane 1). Moreover, when invertase-specific antiserum was incubated with unlabeled purified invertase for 1 h at 30°C prior to the assay, the intensity of this band was greatly reduced (Fig. 2, lane 3). These results show that the 74-kDa polypeptide had antigenic properties in common with acid invertase. The 74-kDa polypeptide was about 23 kDa larger than the purified acid invertase (51 kDa) from tomato fruit. The acid invertase of tomato fruit has been reported to be associated with the cell wall (Nakagawa et al. 1970) and to play a role in the transport of sucrose into cells (Walker and Ho 1977). Therefore, the enzyme may

Fig. 1 Immunoblot analysis of crude extracts of the pericarp tissue of tomato fruit at various stages. Mature, green tomato fruit were stored at 22°C or 33°C for 10 days. Proteins (about 200 ng) in the crude extracts of pericarp tissue were separated by SDS-PAGE on a 12.5% gel and then transferred to a nitrocellulose filter. The filter was treated with diluted invertase-specific antiserum (1 : 2,000 dilution), then processed with ProtoBlot Western Blot AP systems (Promega) as described in Materials and Methods. The molecular mass markers were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsin (25 kDa). The mobility of the purified invertase from tomato fruit is indicated by an arrow. Lane 1, crude extract of mature, green fruit (3×10^{-4} units); lane 2, crude extract of tomato fruit stored at 22°C for 10 days (4×10^{-3} units); lane 3, crude extract of tomato fruit stored at 33°C for 10 days (4×10^{-3} units); lane 4, purified acid invertase (2×10^{-3} units).

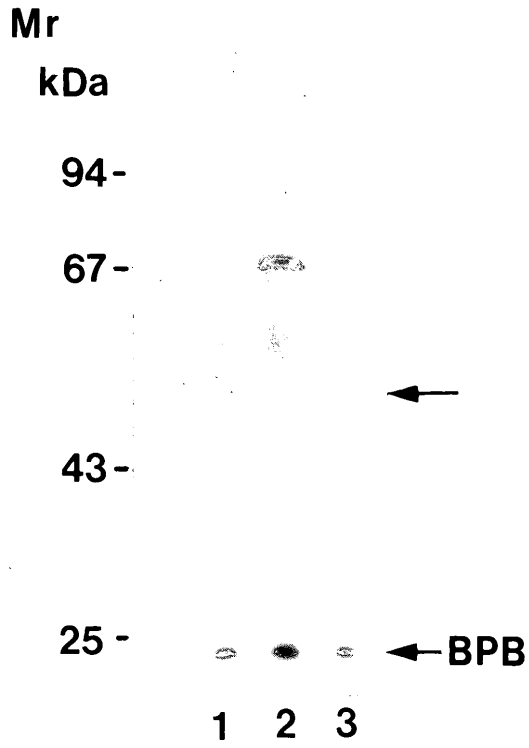

Fig. 2 Results of SDS-polyacrylamide gel electrophoresis of the immunoprecipitate of products of in vitro translation by a wheat germ cell-free system programmed with poly(A)$^+$RNA from ripe tomato fruits. The polypeptides produced in vitro were immunoprecipitated, and then they were analyzed by SDS-PAGE on a 10% gel that contained 8 M urea, with subsequent fluorography. The mobility of the purified invertase from tomato fruit is indicated by an arrow. Lane 1, polypeptides immunoprecipitated with preimmune serum (5 μl); lane 2, polypeptides immunoprecipitated with invertase-specific antiserum (5 μl); lane 3, polypeptides immunoprecipitated with invertase-specific antiserum (5 μl) which had previously been incubated with unlabeled, purified invertase (1 μg) for 1 h at 30°C. BPB, bromphenol blue.

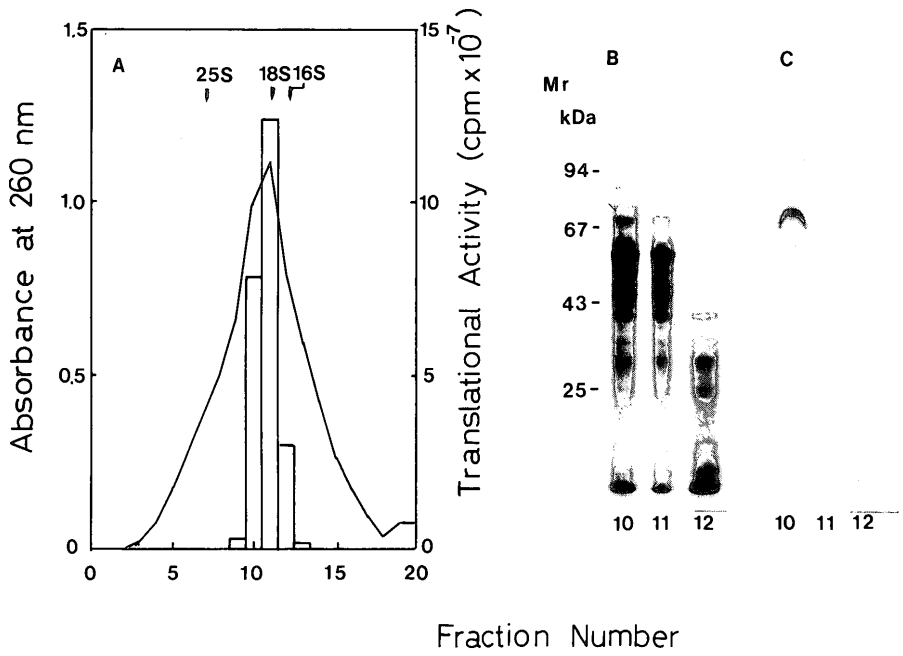

Fig. 3 Fractionation of poly(A)$^+$RNA from tomato fruit by sucrose density gradient centrifugation. Poly(A)$^+$RNA was fractionated on a gradient of 5 to 20% sucrose, as described in Materials and Method. RNA (10 μg/ml) from each fraction was translated in a wheat germ system. The polypeptides produced in vitro were treated with invertase-specific antiserum, and immunoprecipitates were analyzed by SDS-PAGE on a 12.5% gel that contained 8 M urea, with subsequent fluorography. A: Absorbance at 260 nm (—), and translational activity per fraction (bar graph). B: Total population of products of translation (about 2×10^5 cpm). C: Immunoprecipitated products of translation. Products of translation (about 1×10^6 cpm) were treated with invertase-specific antiserum.

be synthesized as a high-molecular-mass precursor polypeptide that contains signal sequences. A large precursor to acid invertase (97 kDa) was reported among the products of the cell-free translation of poly(A)$^+$RNA isolated from artichoke shoots (Goupil et al. 1988). This precursor to acid invertase was processed to the 58-kDa mature form of acid invertase. The 74-kDa precursor polypeptide of acid invertase might be processed to the 51-kDa mature form of the enzyme in the cells of tomato fruit.

Size of the mRNA for acid invertase on sucrose density gradients—The size of poly(A)$^+$RNA isolated from tomato fruit at the pink stage ranged from about 7 *S* to 25 *S* with a peak at 16–18 *S* (Fig. 3A). Translational activity was distributed in RNAs of 7 to 23 *S* with a peak at 18 *S* (Fig. 3A). The total population of polypeptides and the immunoprecipitable polypeptides produced in vitro by the fractionated RNAs were analyzed by SDS-PAGE, with subsequent fluorography (Fig. 3B, C). The RNAs in fraction 10 from the sucrose density gradient after centrifugation contained mRNA specific for the precursor to the invertase polypeptide (Fig. 3C). Our results indicate that the invertase mRNA is about 2400 nucleotides in length, large enough to contain the structural information required to code for the 74-kDa putative precursor to acid invertase.

Changes in the levels of acid invertase, translational activities of poly(A)$^+$RNA and invertase mRNA in tomato fruit during ripening—Mature, green tomato fruit contained low levels of acid invertase activity (Fig. 4A). When mature, green tomato fruit were stored at 22°C, the invertase activity increased greatly as they ripened. The level of invertase protein was also very low at the mature, green stage, but it increased in parallel with increases in invertase activity during ripening (Fig. 4A). The increase in invertase activity was due to synthesis de novo of invertase protein. Poly(A)$^+$RNA was isolated from fruit at various stages. The levels of poly(A)$^+$RNAs and their translational activities decreased rapidly during the first 4 days of storage (from the mature, green to the pink stage) by about 41 and 40% of the initial values, remained constant during the next 3 days (from the pink to the red, ripe stage), and thereafter decreased gradually by about 19 and 16% of the initial values by day 11th (over-ripe stage; Fig. 4B, C). Mature, green fruit contained very low levels of acid invertase mRNA (Fig. 4D). Levels of invertase mRNA per gram fresh weight increased, however, to a maximum on day 4 and then declined. The level on day 11 was about 37% of that on day 4. The translational activity per μg poly(A)$^+$RNA and the size distribution of products of translation in vitro of poly(A)$^+$RNAs remained unchanged during ripening (data not shown). Therefore, the decline in the levels of invertase mRNA in tomato fruit after day 4 was not due to degradation of the mRNA during the prepa-

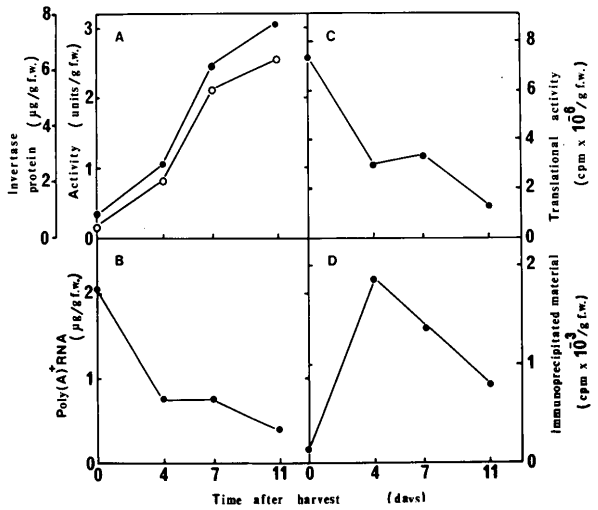

Fig. 4 Changes in the levels of invertase, translational activities of poly(A)$^+$RNA and of invertase mRNA in the pericarp tissue of tomato fruit during ripening. Tomato fruit were stored at 22°C for various lengths of time. Acid invertase and poly(A)$^+$-RNA were prepared from pericarp tissues as described in Materials and Methods. Invertase protein was measured by the enzyme immunoassay described in Materials and Methods. Poly(A)$^+$RNA was translated in a wheat germ system. Products of translation that were immunoprecipitated with invertase-specific antiserum were separated by SDS-PAGE. The radioactivity of the 74-kDa polypeptide was measured. A: Invertase activity (-○-) and protein (-●-). B: Levels of poly(A)$^+$RNA. C: Translational activity of poly(A)$^+$RNA. D: The radioactivity of the 74-kDa polypeptide.

ration of poly(A)$^+$RNA from the tomato fruit. These results suggest that the increase in acid invertase activity during ripening of tomato fruit depends on changes in the levels of its mRNA.

We thank Nami Nakano for excellent technical assistance.

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(Received May 24, 1989; Accepted May 2, 1990)