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Sucrose Synthase in Rice Plants[1]

Growth-Associated Changes in Tissue Specific Distributions

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ABSTRACT

Different parts of the rice (*Oryza sativa* L.) plant at different growth stages were analyzed for sucrose synthase (SS) by enzyme activity assay and enzyme-linked immunosorbent assay directly on the extracts or on the eluates from a gel filtration column. On a dry matter basis, the amount of soluble protein and SS activity decreased significantly, but the amount of enzyme protein changed little in growing leaves. In the grain, the SS activity was the highest at the early ripening stage and decreased later, but the amount of SS protein increased with the increase in maturity. In the root, a low activity of SS was detectable only in the tillering but not in other stages. Immunoblotting of SS protein extracted from different parts of rice showed two bands. Elution patterns of crude extracts from a gel filtration column showed the presence of several types of SS protein. Among them, two to three types with larger elution volumes had the SS activity but others with smaller elution volumes (considered as the aggregated forms) had no activity. The SS purified from different parts of the plant showed similar but distinctly different electrophoretic mobilities in a native gel. It has been concluded that different isozymes are expressed in different tissues at different growth stages.

We have demonstrated previously (1, 11) that the sugar nucleotides synthesized from sucrose and a nucleoside diphosphate such as ADP or UDP may be utilized directly in the synthesis of starch in maturing grains of rice (*Oryza sativa* L. cv NTU-1009, an *indica,* and cv Tainan 5, a *japonica* variety). For all SS[2] studied so far, UDP has been regarded as the natural nucleotide substrate, but they may also react with some other nucleoside diphosphates in place of UDP. Since sugar nucleotides are important substrates in many biosynthetic reactions, the capability of SS to synthesize various sugar nucleotides directly from sucrose, the most important form of carbohydrate transport in plants, may have important relevance in various aspects of plant anabolism.

To elucidate the biochemical roles played by the enzyme in the rice plant, we have analyzed the enzyme activity, the amount of enzyme protein, the enzyme protein pattern, and properties of the enzyme in different parts of the rice plant at different stages of growth. We also looked for diurnal changes of enzyme activity in different organs. As a result, the presence of isozymes or multienzymes as well as the growth associated changes of enzyme patterns have been revealed.

MATERIALS AND METHODS

Rice Plant

Samples of a *japonica* strain of rice, *Oryza sativa* L. cv Tainong 67, provided by the Taiwan University Experimental Farm and the Provincial Pingtung Agricultural Experiment Station were used. The rice transplanted from a nursery at the former farm on March 12, 1986, was used for the analysis of SS at different growth stages, and that transplanted in the latter on February 2, 1987, was used for studying diurnal changes of the enzyme. Before heading, the plant was divided into root and above the ground parts; after heading, it was divided into grain, leaf, stalk, and root parts. The pooled samples were kept at −20°C before use.

Enzyme Samples

The thawed sample was homogenized with four times its amount of 50 mM potassium phosphate (pH 7), containing 2-mercaptoethanol and sodium EDTA both at 1 mM in a Polytron homogenizer (Kinematica, Switzerland) at full speed intermittently four times for 30 s each, and the homogenate was centrifuged at 4°C and 7800 *g* for 20 min. The clear supernatant was divided into a number of aliquots and stored at −20°C. Before use, the frozen extract was brought to room temperature and centrifuged in an Eppendorf centrifuge to remove possible precipitates. Electrophoretically homogeneous enzymes were also prepared from these extracts according to the procedures reported previously (7).

Analytical Methods

Protein was estimated by the Lowry's method (12) using bovine serum albumin as the standard. The activity of SS was

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[2] Abbreviations: SS, sucrose synthase; EIA, enzyme immunoassay; PBST, PBS with 0.05% Tween; gelatin-NET, 50 mM Tris-HCl (pH 8), containing 0.25% gelatin/0.15 M NaCl/5 mM EDTA/0.05% Tween; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid; DAB, diaminobenzidine; Ass, protein showing positive reaction against SS specific antibody in ELISA.

estimated by a UDPG dehydrogenase coupling method as developed in our laboratory (14). The enzyme protein was analyzed by an ELISA we developed. In the assay, a rabbit IgG (10 μg/mL of 0.1 M Tris-HCl [pH 9.7]) prepared from an antiserum raised against an electrophoretically pure rice grain SS was used in coating the ELISA plate, a biotinylated IgG was prepared (5) (6 μg/mL of gelatin-NET) and used to sandwich the antigen, and a biotinylated horse radish peroxidase reagent (reagents A and B in a kit from Vector Lab., Burlingame, CA, U.S.A. were each diluted 1000-fold with gelatin-NET, mixed 1 to 1, and incubated at room temperature for 30 min before use) was fixed on the sandwich through avidin, according to the following sequence of treatments. Coat each well with 0.1 mL of IgG at 37°C for 30 min and at 4°C for 1 h; wash with PBST in an automatic washer for three cycles; add 0.2 mL of gelatin-NET and incubate at room temperature for 1 h to block nonspecific binding groups; remove gelatin-NET and wash with PBST for three cycles; add 0.1 mL of the sample, incubate, and wash as the previous step; add 0.1 mL of biotinylated IgG and incubate at 37°C for 30 min and then at 4°C for 20 min; wash with PBST for three cycles; add 0.1 mL of the avidin-biotin-horse radish peroxidase reagent; incubate at 37°C for 30 min, then at 4°C for 20 min; wash with PBST for 5 cycles; add 0.15 mL of an ABTS reagent (12 mg of ABTS and 20 μL 30% H_2O_2 in 0.1 M sodium citrate [pH 4.2]); incubate at 37°C for 20 to 30 min; read the absorbance of the developed color in an EIA reader at 405 nm.

Electrophoresis was done in a mini-gel system (either Hoefer or Bio-Rad) using a 7.5% polyacrylamide gel for the disc run (3) and a 7.5 to 15% gel for the gradient-SDS run (9). The protein bands were revealed by staining with either Coomassie brilliant blue R-250 (4) or a silver nitrate procedure which we devised to prevent negative staining of some protein bands, as follows. The glass trays and jars for the staining operation are silanized with a 5% solution of dimethyl silane in chloroform. After electrophoresis, gel slabs are kept in 50% methanol. Before staining, gel slabs are washed with three changes of water for 10 min in each washing. Gel slabs are immersed in a solution containing 0.5 mL of glutaraldehyde and 0.5 g of sodium thiosulfate in 100 mL for 1 h. After decanting off the solution, three washings with double distilled water are done. A silver solution is prepared by slowly adding 0.8 g of silver nitrate dissolved in 4 mL of water to 100 mL of an aqueous solution containing 21 mL of 0.36% NaOH and 1.4 mL of concentrated ammonium hydroxide. Treating of gel slabs with the silver solution is done in a tray for 8 to 11 min. After three washings with water, an aqueous solution containing 1 mL 0.5% citric acid, 0.1 mL formalin, and 15 mL methanol per 100 mL is used for developing the color. As soon as any background color is noticeable, the color developer is removed and an aqueous stop solution containing 10 mL of 0.5% citric acid and 0.1 mL of ethylamine per 100 mL is added. After one h, gel slabs are transferred to 50% methanol and dried according to the procedure developed by us (8). Throughout the washing, the gel is shaken gently. Western analysis of the protein bands separated on gel slabs was done on the Immobilon film (Millipore) after electrophoretic transfer in 25 mM Tris-HCl (pH 8.3), containing 0.192 M glycine and 10 to 20% methanol. The electrophoretic blotting was done in a Hoefer apparatus (TE 22) at 1 A for 1 h. In case of SDS-gel blotting, the Immobilon film was washed thoroughly with three changes of 6 M urea in PBST for removing SDS and renaturing protein before immunostaining. The immunostaining was done by a procedure essentially identical to that of ELISA except that a DAB reagent (5 mg DAB and 10 μL 30% H_2O_2 per 100 mL PBS) was used in the place of ABTS reagent.

For the molecular sieve analysis of a crude enzyme extract, the extract was saturated with ammonium sulfate and the precipitate formed was collected by centrifugation. The precipitate was dissolved in a smallest possible amount of the elution buffer and the solution was clarified by centrifugation before putting into the column. A column of Sepharose CL-6B, 2 × 90 cm, was equilibrated with 50 mM potassium phosphate (pH 7), the flow rate was adjusted to 18 mL/h, and the eluate was collected in 3 mL fractions. The column eluate was monitored simultaneously by its absorbance at 280 nm, SS activity, and the amount of SS protein by ELISA. The column was calibrated by filtering a set of standard proteins, *viz.* thyroglobulin 669 kD, ferritin 440 kD, catalase 232 kD, and aldolase 158 kD. Further purification of the enzyme was carried out on a DEAE Sephacel (Pharmacia) column (2 × 30 cm) equilibrated with 50 mM potassium phosphate (pH 7). The elution was done by a linear gradient of 0 to 0.3 M NaCl in the same buffer (150 mL each).

RESULTS AND DISCUSSION

Sampling Regime and ELISA Standardization

The samples used for the major part of work were planted in the first crop season (from late March to late July) according to the rice cropping regime prevalent in northern Taiwan. Samples were taken from the field from 3 to 4 o'clock in the afternoon. The sampling scheme is shown in Table I. The reliability of the ELISA method developed for the SS protein was checked by a checkerboard titration on a pure rice SS preparation (8). The results are presented in Figure 1.

Table I. *Sampling Scheme for Rice Plant*

Growth stages	Date	DAT[a]	DPP[b]	Plant Height
				cm
Tillering	4/17	36		26
	4/24	43		26
	5/01	50		30
Elongation	5/08	57		45
	5/15	64		50
Panicles formation	5/24	73		80
	5/31	80		80
Heading	6/18	98	−1	80
	6/28	108	9	
Milk stage	7/04	114	15	
Dough stage	7/11	121	22	
	7/19	129	30	
Ripening complete	7/23	133	34	

[a] Days after transplanting. [b] Days postpollination.

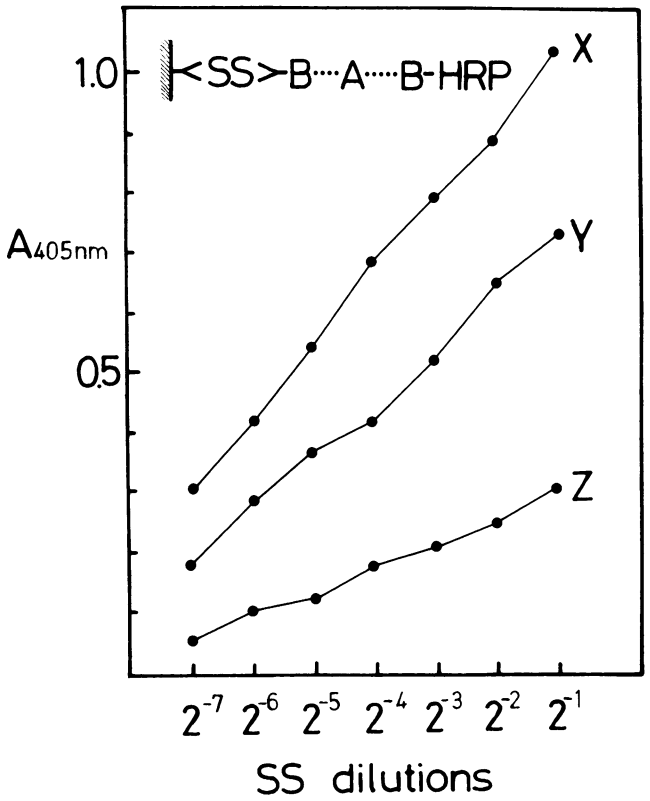

Figure 1. ELISA checkerboard titration curves of SS using the sandwich method. Assay protocol was summarized in the upper part of the figure. The avidin-biotin reagent (A···B-HRP) as supplied by Vector Lab (see "Materials and Methods") was diluted 1:500 (X), 1:1,000 (Y), or 1:6,000 (Z).

Growth Associated Changes of SS Protein and its Activity

Gathering from data for protein content (Fig. 2A), SS activity (Fig. 2B), and SS protein content (Fig. 2C) in different parts of rice plant during growth, one may conclude that: (a) In all parts of the plant, a higher SS activity per unit dry weight is present in the earlier phase of organ development. At the later phase, more SS protein but less soluble proteins are found. These results may indicate that the SS activity is needed for the early phase of organ development and the enzyme protein after inactivation is not turned over rapidly. (b) The level of SS activity in the leaf is very high before the panicles formation but declines to nil as the plant reaches the milk stage. Only a transient accumulation of the inactive SS protein is found at the milk stage. (c) In the grain, the enzyme shows the highest specific activity (either activity/dry weight or activity/soluble protein) during the early period of grain development (milk stage) when starch is accumulated at the highest rate. The change in enzyme activity and the increase in the SS protein take place at the most significant rate in the maturing grain. A dot blot hybridization test of total RNA with specific rice SS cDNA probes also showed that the amount of SS mRNA was the highest when the enzyme activity was at an exponential rise (our unpublished results). These data imply that SS is closely associated with the activity

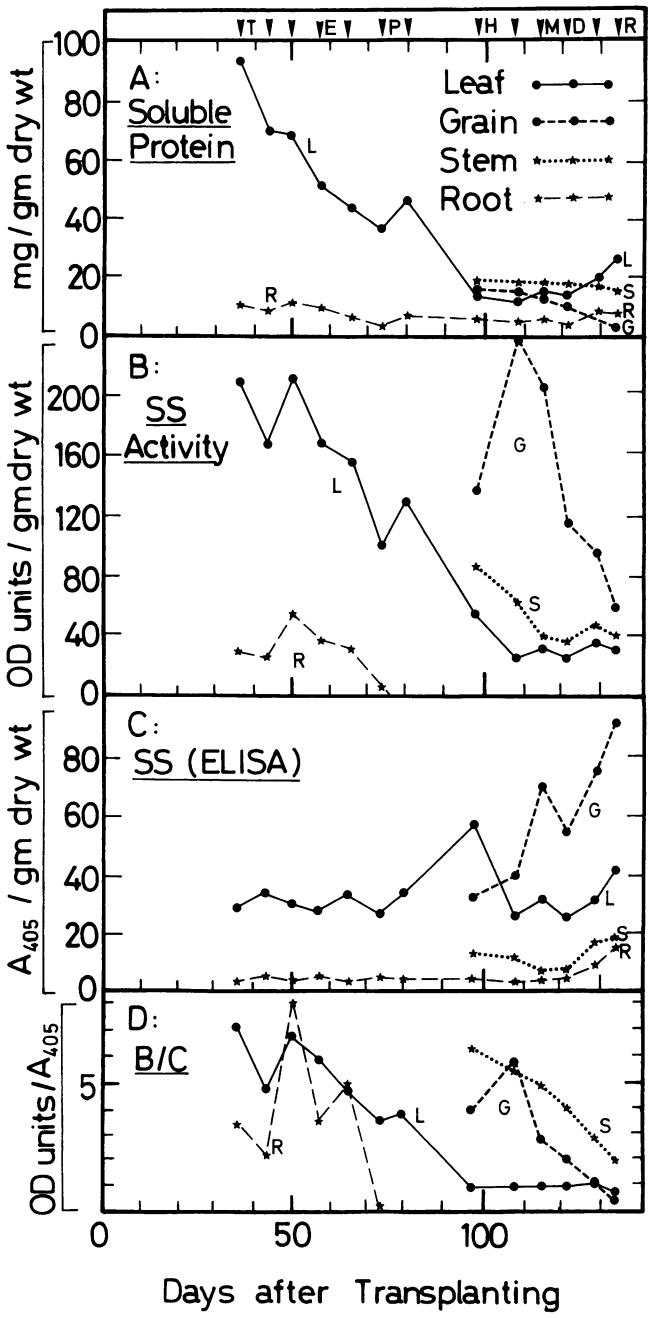

Figure 2. Changes of the amount of total protein (A), SS activity (B), the amount of SS protein (C), and specific SS activity (B/C) in different parts of the rice plant at different growth stages. Capital letters next to arrows indicate growth stages as shown in Table I, as follows: T, tillering; E, elongation; P, panicles formation, H, heading; M, milk stage; D, dough stage; R, ripening complete.

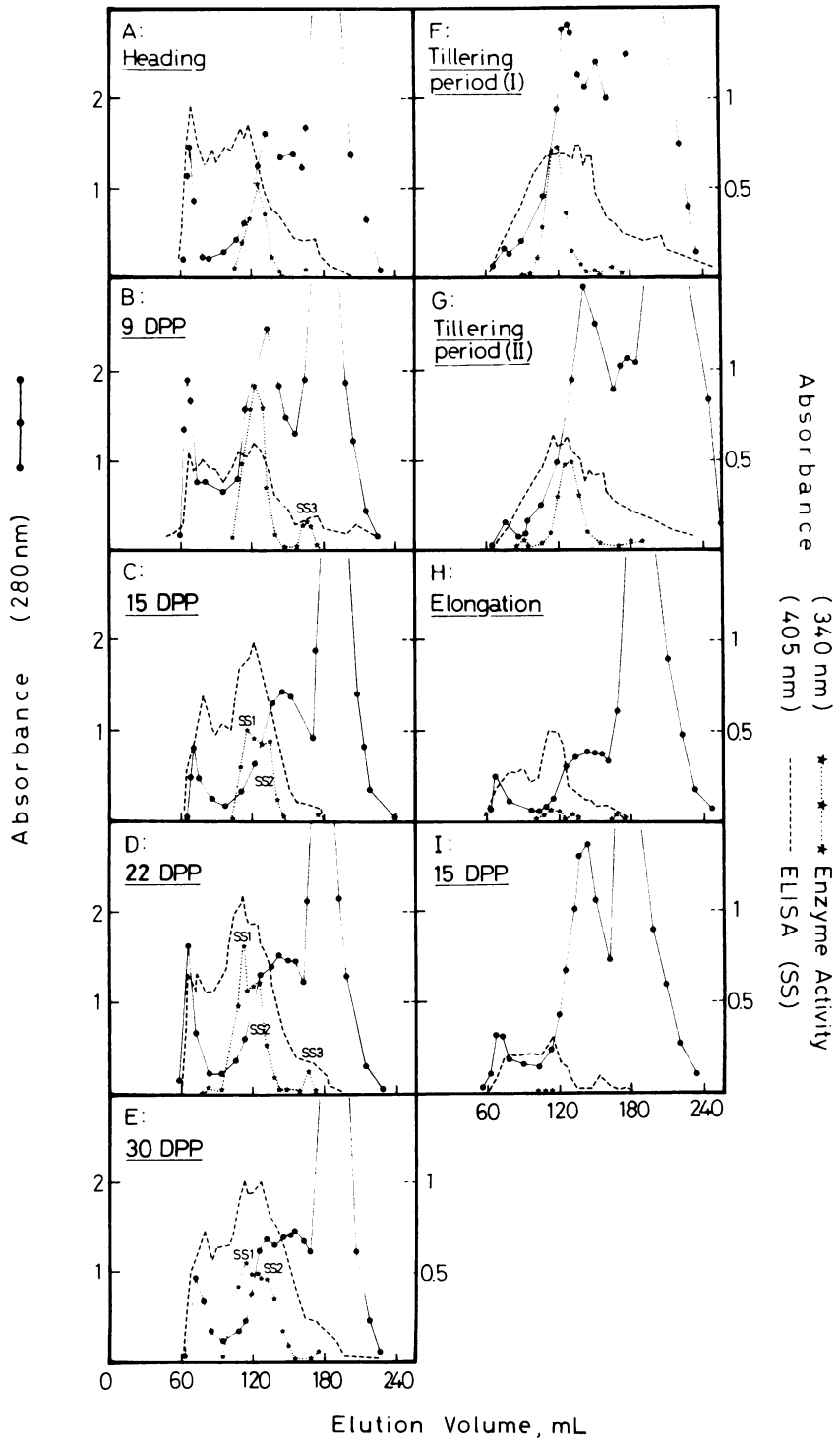

Figure 3. Gel filtration patterns of total protein, SS activity, and SS protein from rice grains (A–E) and leaves (F–I) at various growth stages. Please refer to Table I for details of growth stages.

of starch synthesis in the rice grain, in conformity with our previous finding that the reaction catalyzed by SS in rice grains can provide precursor(s) of starch synthesis (11). These results also show that, in the grain, after a rapid decline of SS activity, the SS protein still has a tendency to accumulate. It seems that the turning over of the activity does not necessarily require a severe degradation of the enzyme, and the remnant of the enzyme protein probably in an aggregated form constitutes a significant part of extractable proteins in the mature rice grain. (d) When the data points in Figure 2B are divided by the corresponding ones in Figure 2C, plots showing changes in the specific enzyme activity are obtained (Fig. 2D). One can see from this figure that the highest specific activities obtainable for enzymes from different organs are similar but not the same. This may also indicate that the rice SS is heterogeneous.

Growth Associated Changes of Enzyme Activity and Enzyme Protein Distribution Patterns studied by Gel Filtration

The crude enzyme extract after being concentrated 3 to 5-fold by vacuum evaporation in a desiccator at low temperature showed poor immunoblotting results in both gradient disc-PAGE (5–10%) and gradient SDS-PAGE (7.5–15%) runs. However, the results (not shown) were adequate in showing that SS was most prevalent in grains and that there could be more than one form of SS in the rice plant. To verify these results, a gel filtration study was done on crude enzymes from different tissues at different stages of growth.

Grains

The elution patterns obtained for the enzyme at five stages of development are given in Figure 3, A to E. These results indicated that: (a) Multiple activity peaks are present at all stages although the distribution patterns are not identical. It should be noted, however, that the patterns for before and after the milk stage show some differences; before the milk stage, only two sharp peaks located in the SS-1 and SS-3 regions are present instead of three found in later stages (SS-2 in addition). (b) There are present immunoreactive (Ass) but without enzyme activity peaks at the vicinity of void volume at all stages of plant development. Again, these aggregated forms are considered to constitute a large part of proteins in the mature grain (Fig. 2C). (c) The Ass peaks have a wider and more complex distribution pattern than the activity peaks, and in any case the enzyme activity peaks are found within the area covered by the Ass pattern. (d) We have shown previously (7) that the rice SS has a subunit with molecular mass from 80 to 90 kD, the major active form is a homotetramer, but a dimer may be present as a less active form. A similar situation has been shown to occur in the purified maize grain SS (13). The data presented here also show that the enzyme can be present in different polymeric forms, among them some with and some without activity. However, we are not sure that the complexity of elution patterns, especially those obtained for Ass, is due to the presence of either the different but analogous gene products (isozymes), or the degradation products of the enzyme in aggregated forms. (e) The major soluble rice grain proteins have a molecular mass range at ≤200 kD but they do not react with the antibody, indicating that the type of on-storage degradation of purified rice grain SS we had observed previously (7) does not take place to the SS in freeze-stored tissues.

Leaves

The distribution of SS proteins and their activities are presented in Figure 3, F to I. At the tillering stage both the activity and Ass peaks are distinct. In the early phase of tillering (Fig. 3F), one can find two activity peaks, one major and the other minor. The patterns of these two peaks are very similar to those of grains at the flowering stage. In leaves at the elongation stage as well as in grains at the milk and later stages, the major activity peak is split into two smaller peaks. However, the SS activity is lost from the leaf tissue in stages later than the milk-ripe. For the time being, we do not know whether the active enzymes found in the flowering stage grains and those in the tillering stage leaves are identical or not; they form distinct precipitin lines with the IgG raised against the pure grain SS in a double diffusion test, but the two seem not to fuse well (data not shown). However, since they are all richer in more vigorously growing tissues, they should have similar if not identical functions.

The Ass distribution patterns in leaves of early stages of development show a cluster at around the activity peak. After passing the tillering stage and as the enzyme activity greatly diminishes, Ass peaks with higher MW increase although the overall enzyme protein profile diminishes in height. This may imply that the enzyme protein tends to aggregate before being degraded beyond recognition by the antibody.

Electrophoretic Property

Two forms of milk-ripe grain SS and one form of tillering stage leaf SS could be purified to electrophoretic homogeneity by DEAE ion exchange chromatography and preparative electrophoresis as described (7). The patterns of disc PAGE of native enzymes are shown in Figure 4. Although the number of SS isozymes obtained in this type of study is limited, these results show unambiguously that there are isozymes of SS in the rice plant and the one from leaf is different from one of the grain isozymes.

Diurnal Changes

No significant diurnal changes in the protein and activity levels of SS in the grain, stem, leaf, and root could be found. The specific activity of SS in grains seemed to elevate in the day time but the change was statistically not significant (data not shown). Vassey (15) has shown that sugar beet leaves under continuous light or dark conditions for up to 15 d have little differences in the sucrose synthase activity. Thus, no changes in the enzyme activity under the diurnal cycle seems reasonable.

In summary, we may conclude that, similar to the results obtained with maize (2, 6) and wheat SS isozymes (10), different SS isozymes are present in different organs of different growth stages of rice plant as a result of development

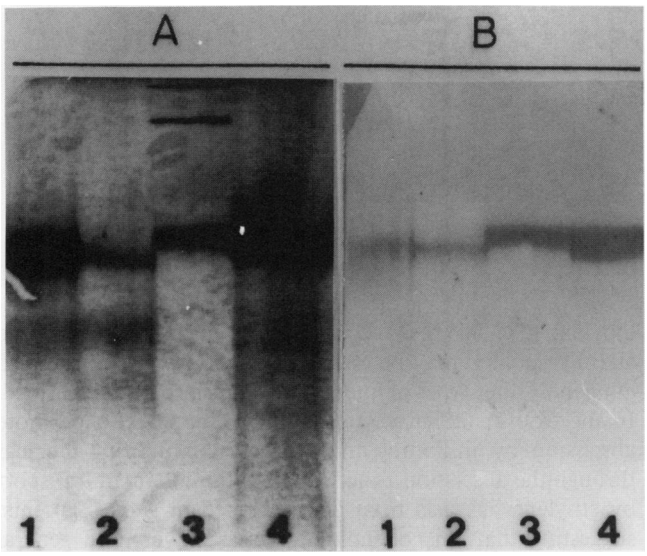

Figure 4. SS of rice grains (lanes 1, 3, and 4) and leaves (lane 2) both at the milk stage as revealed by the silver-stain of a disc-PAGE gel (A) or the immunostain of a gel blot (B). Lane 1, purified by preparative PAGE and stored for a long period o time; lane 3 and lane 4, eluted from a DEAE Sephacel column at 0.03 M and 0.07 M NaCl, respectively.

associated changes in the expression of SS genes. The complex elution patterns of SS activity from a gel filtration column was complicated further by the presence of aggregated SS proteins. The presence of higher SS activity in all organs at the earlier stage of development may indicate that the enzyme is important in supporting the plant growth by providing sugar nucleotides for the syntheses of polysaccharides, glycoproteins, glycolipids, etc. This view is supported by the results of rice SS gene cloning and gene expression studies which will be published elsewhere.

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