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Purification and Characterization of Sucrose Synthetase from the Shoot of Bamboo *Leleba oldhami*[1, 2]

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JONG-CHING SU
Department of Agricultural Chemistry, National Taiwan University, and Institute of Biological Chemistry, Academia Sinica

JEN-LEIH WU
Institute of Zoology, Academia Sinica

CHAO-LUAN YANG
Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan, Republic of China

ABSTRACT

A 108-fold purification of the sucrose synthetase from the extract of the shoot of bamboo *Lelaba oldhami* was achieved by ammonium sulfate fractionation, calcium phosphate gel adsorption, and chromatographic separations on Sephadex G-100 and diethylaminoethyl-cellulose columns. Some properties of this enzyme, namely thermal and pH stabilities, stabilization by aqueous glycerol, pH optimum, substrate specificities, effects of metallic ions, effects of sulfhydryl reagents, molecular weight, sedimentation constants, isoelectric point, and substrate saturation kinetics had been investigated.

The substrate saturation kinetics indicated that the enzyme could be an allosteric enzyme with the saccharide substrates (sucrose and fructose) serving as the homotropic allosteric effectors in regulating the biosynthesis and degradation of sucrose.

A series of studies aimed at the elucidation of the biochemical mechanism of cell wall maturation in higher plants have been conducted in our laboratory. The shoot of bamboo *Leleba oldhami* has been chosen as the main research material. Previous investigations revealed the types of glycosidic linkages present in bamboo cell wall polysaccharides (14), the distribution of sugar nucleotides in the tissue (12), and the changes of polysaccharide constituents accompanied with the growth and maturation of the plant (13). Unlike many other plants, bamboo shoot is incapable of utilizing GDP-D-glucose, which could not be detected in the plant, as the precursor for synthesizing cell wall polysaccharides. UDPG, the most abundant soluble nucleotide in bamboo shoot (12), is a good precursor for synthesizing a β-1,3-glucan (15). This synthetic ability is strongly inhibited by UDP-D-xylose (and moderately by xylose and xylobiose), which has been demonstrated to be a good precursor of bamboo shoot pentosans (16). The pentosan synthesis from the precursor UDP-D-xylose, which is rapidly isomerized to a mixture of UDP-D-xylose and UDP-L-arabinose by the pentosan synthetase preparation, is inhibited by UDP-D-galactose, the precursor of bamboo shoot galactan.

These results imply that the synthesis of different polysaccharides at the different stages of plant growth is regulated by the availability of some sugar nucleotides not only as the substrates but also as the modulators. It therefore becomes necessary to investigate into the sugar nucleotide-synthesizing systems of the plant.

UDPG is doubtless the precursor of all sugar nucleotides mentioned above. From the physiological properties of bamboo shoot and the results of a preliminary survey, we suspected that UDPG could be supplied by the reaction catalyzed by sucrose synthetase in the direction of sucrose cleavage. Sucrose synthetase from bamboo shoot may be a regulatory enzyme which is more important for synthesis of UDPG rather than for synthesis of sucrose. Here, we report the properties of sucrose synthetase purified from the shoot of bamboo *L. oldhami*, especially those relevant to the postulated physiological functions of the enzyme explained above.

EXPERIMENTAL PROCEDURES

MATERIALS

The shoots of bamboo *L. oldhami* grown in the vicinity of Taipei were sampled. Only the edible part was used as the enzyme source. All of the commercially available chemicals except UDP were used without purification. The UDP obtained from the Sigma Co. was contaminated with UDPG and UMP, the former of which interfered with the assay of sucrose synthetase by the UDPG dehydrogenase coupled method. These contaminants were removed by paper chromatography according to the method of Paladini and Leloir (10). The filter paper used for this purification procedure was prewashed in sequence with 1 M oxalic acid and distilled H_2O and dried. Calcium phosphate gel was prepared according to Keilin and Hartree (4).

METHODS

The protein content was estimated with Lowry's phenol method (6) using crystalline BSA as the standard.

In density gradient centrifugation analyses, a linear gradient of sucrose from 5 to 20% and of glycerol from 8 to 33% (specific gravities from 1.0173 to 1.0806) were employed. The density gradient columns were centrifuged in a swinging bucket rotor at 112,000g and 4 C for 10 hr. Yeast alcohol dehydrogenase ($s_{20,w}$ = 6.72) and fibrinogen ($s_{20,w}$ = 7.63, mol wt 341,000) were used as the marker proteins and the results were calculated

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[2] Part II in the series "Sucrose Synthetase." For Part I, see Yeh DB, JC Su 1972 J Chinese Biochem Soc 1: 37.

according to Martin and Ames (7). Molecular weight of the enzyme was estimated by gel filtration through a Sephadex G-200 column according to Andrews (1) and also from the sedimentation data (7). Bovine γ-globulin, bovine hemoglobin, horse heart Cyt *c*, yeast alcohol dehydrogenase, and apoferritin were used in calibrating the column.

Isoelectric focusing of the enzyme was carried out in a density gradient electrophoresis apparatus from the Instrumentation Specialties Co., Lincoln, Neb., according to the procedure described in the brochure supplied by the manufacturer (8). The carrier Ampholine with a pH range of 3.5 to 10 was purchased from LKB, Sweden. Total focusing time was 24 hr during which period the column was maintained at 10 C by circulating chilled water through the jacket of the column. The enzyme obtained from the gel filtration step was used in this experiment.

The activity of sucrose synthetase was assayed by one of the following methods according to the situation.

Method 1. Determination of UDPG by UDPG Dehydrogenase-Catalyzed Reaction. The method reported by Avigad (2) for the assay of sucrose synthetase from sugar beet was found not suitable for the assay of bamboo shoot enzyme because the bamboo shoot enzyme had a pH optimum much lower than that of bovine liver UDPG dehydrogenase. The following two-step method was developed.

The reaction mixture (0.80 ml) contained 50 μmol sucrose, 0.1 μmol UDP, 1 μmol $MgCl_2$, 0.1 μmol EDTA, 1 μmol 2-mercaptoethanol, and 25 μmol sodium phosphate (pH 6). After temperature equilibration at 37 C, the reaction was started by adding 0.1 ml of properly diluted enzyme solution. A blank without the addition of UDP was run simultaneously. After 10 min of incubation, 0.1 ml of 1 M tris-HCl (pH 8.8) was added and the mixture heated in a water bath at 68 C for 10 min to stop the reaction. Eight-tenths ml of the reaction mixture was transferred into a cuvette, to which 50 μl of 20 mM NAD^+ and 140 μl of H_2O were added. After addition of 10 μl (33 units) of UDPG dehydrogenase, the absorbance readings at 340 nm of the solution were recorded until no further increase could be noted. The net increase in the reading corrected for the blank value was taken for calculating the amount of UDPG produced. This method was the one most used in this investigation.

Method 2. Determination of Fructose by a Reducing Sugar Method. The reducing sugar formed in method 1 was estimated by Nelson's modification of Somogyi's method (9). The assay method was used when various nucleotides were tested as the substrates.

Method 3. Determination of Sucrose Formed in the Direction of Sucrose Synthesis. The method described by Leloir and Cardini (5) was followed. This method was employed for analyzing the kinetics of sucrose synthesizing reaction.

One unit of enzyme is defined as that amount of enzyme which produces 1 μmol of UDPG, of fructose, or sucrose/min under the specified assay condition, and the specific activity is the number of the enzyme unit exhibited by 1 mg of protein.

The enzyme was extracted and purified according to the following procedures. One hundred g of the edible portion of bamboo shoot, prechilled at 4 C for 2 to 3 hr and chopped into about 1-cm cubes, was homogenized with 1 ml of 1 M K-phosphate buffer (pH 7) in a Waring Blendor for 1 min. The homogenate was squeezed through two layers of cheesecloth and the milky filtrate centrifuged at 10,000*g* for 30 min. From the clear supernatant, the bulk of the enzyme was precipitated between 0.35 to 0.50 saturation of ammonium sulfate. The precipitate was dissolved in a small amount of 50 mM K-phosphate (pH 7) containing 1 mM EDTA and 5 mM 2-mercaptoethanol and dialyzed overnight against 1 mM K-phosphate (pH 7) containing 0.1 mM EDTA and 1 mM 2-mercaptoethanol.

The dialyzed enzyme solution was diluted with 5 volumes of 10 mM K-phosphate (pH 6.8), and calcium phosphate gel suspension was added to it to make a final gel to protein ratio (w/w) of 5. After stirring for 10 min, the gel was collected by centrifugation and washed stepwise with 6-ml aliquots of 16, 20, 24, and 28 mM of K-phosphate (pH 8). The last two buffer solutions contained 5% ammonium sulfate. The desorbed enzyme solutions showed nearly identical specific activities. Thus, they were combined for further purification. Solid ammonium sulfate was added to the gel eluate and the precipitate formed between 0.30 to 0.60 saturation of the salt was collected. The bulk of the precipitate was dissolved in a minimum amount of 10 mM Na-phosphate (pH 7) and the insoluble matter was removed by centrifugation. One ml of the enzyme solution from the second ammonium sulfate fractionation step was applied to a Sephadex G-100 column (2.5 × 38 cm) which was preequilibrated with 2 mM Na-phosphate (pH 7.6). Before it was introduced into the column, 50 mg of sorbitol was added/ml of the enzyme solution. The flow rate was adjusted to 0.5 ml/min and the eluate collected as 2.5-ml fractions (Fig. 1). Sucrose synthetase obtained from this purification step was free from invertase. The eluate was concentrated by ultrafiltration and could be used for the studies of kinetics and general properties.

For further purification, a DEAE-cellulose column (2 × 28 cm) preequilibrated with 2 mM Na-phosphate buffer (pH 7.6) was used. The enzyme eluate from the Sephadex G-100 column was directly introduced into the ion exchange column. After the adsorption was complete, the column was eluted by a linear gradient with 2 mM Na-phosphate (pH 7.6) as the starting buffer and 50 mM Na-phosphate (pH 6.4), which is 1 M with respect to Na-acetate, as the supplementary buffer to the mixing chamber.

Table I. Summary of purification steps.

Results are based on 1 kg of edible tissue.

Fraction	Volume	Total activity	Total protein	Specific activity	Purification	Recovery
	(ml)	(units)	(mg)	(units/mg protein)	(fold)	(%)
Crude extract	600	635	12450	0.051	1	100
First ammonium sulfate	26.6	615	3272	0.188	3.7	97
After dialysis	38.0	589	2886	0.204	4.0	92
Calcium phosphate gel	238	349	779	0.448	8.8	55
Second ammonium sulfate	5.0	313	256	1.224	24	49.5
Sephadex G-100	36.0	220	59.8	3.67	72	34.6
DEAE-cellulose	190	131	23.7	5.51	108	20.5

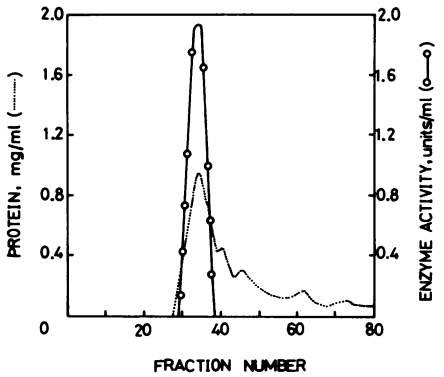

FIG. 1. Gel filtration pattern of the enzyme from the second ammonium sulfate step on a Sephadex G-100 column.

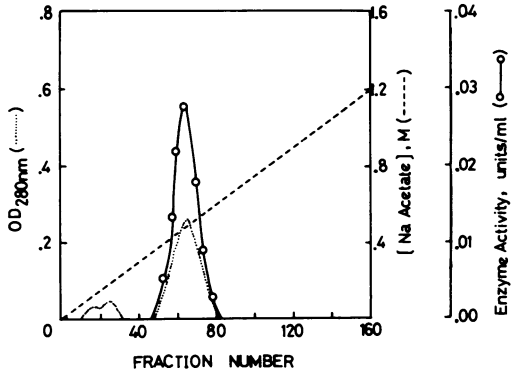

FIG. 2. Ion exchange chromatographic pattern of the enzyme from the gel filtration step on a DEAE-cellulose column.

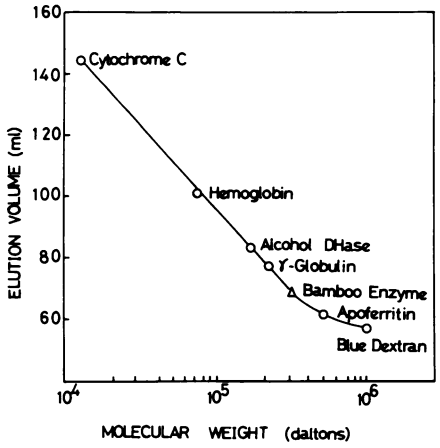

FIG. 3. Estimation of the mol wt of bamboo shoot sucrose synthetase by gel filtration chromatography on a Sephadex G-200 column. Although the true mol wt of γ-globulin is 160,000, the protein exhibits an apparent mol wt of 205,000 by gel filtration (1).

The volume of the buffer was 150 ml in each chamber. The flow rate of the buffer through the column was 1 ml/min and the eluate was cut into 2-ml fractions. The enzyme was eluted between 0.36 and 0.61 M Na-acetate (Fig. 2). The fractions containing the bulk of the enzyme activity were combined, dialyzed, and lyophilized.

RESULTS AND DISCUSSION

Through six steps of treatment, a 108-fold purification of the enzyme was achieved (Table I). The chromatographic patterns on a Sephadex G-100 column and a DEAE-cellulose column are shown in Figures 1 and 2, respectively. After the gel filtration step, the preparation was completely free from invertase activity. The final enzyme preparation showed a nearly homogeneous disc electrophoretic pattern. From these results, it has been estimated that the enzyme comprises nearly 1% of the soluble proteins in the bamboo shoot extract. The following studies were done under conditions where the initial velocities of the enzyme reaction were linear with respect to reaction time.

Heat Stability. The crude enzyme after the first ammonium sulfate step was quite stable; storage at −20 C for a month resulted in no loss of activity and 52% of the activity was lost after 6 months' storage. The enzyme after the DEAE-cellulose step became unstable and could not be stored for a prolonged time even at the freezing temperature. The enzyme showed good stability between pH 6.4 and 8.5. Heating at temperature below 50 C for 10 min resulted in no loss of the enzyme activity. Above 50 C, the enzyme activity was lost rapidly. The enzyme from the gel filtration step lost 90% of its activity after storage at 4 C for 15 days. When the enzyme was stored in 25, 20, 15, or 10% glycerol at 4 C, there remained 50, 50, 30, or 20% of the initial activity after 68 days. It is thus evident that glycerol is capable of stabilizing the enzyme in solution.

pH Optimum. The optimum pH range of sucrose synthetase assayed in the direction of sucrose cleavage was between 5.8 and 6.8 with pH 6 showing the highest reaction rate. As can be predicted, the optimum pH for the reaction in the direction of sucrose synthesis, which involves the release of 1 proton/molecule of UDPG utilized, is about 1.5 pH units higher.

Substrate Specificity. UDP, ADP, IDP, and TDP were the nucleotide substrates of sucrose synthetase. At 0.1 mM, when the activity of UDP as the substrate was taken as 100, those of ADP, IDP, and TDP were 21, 9.5, and 25, respectively. Other nucleoside diphosphates were completely inactive as the substrate for the enzyme.

Fructose is the only monosaccharide that can be the acceptor of glucosyl group from UPDG in the enzyme-catalyzed reaction. Among sucrose, maltose, lactose, cellobiose, trehalose, gentiobiose, raffinose, melibiose, and turanose, only sucrose, turanose, gentiobiose, and melibiose at 50 mM showed activities as glucosyl donors in the ratio 100:11.5:7.0:1.5.

Effectors. When the concentration was lower than 0.8 mM, Mg^{2+} was an activator; at 0.5 mM, it could enhance the activity by 18%. The rest of the divalent cations tested, namely Hg^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, and Ca^{2+} were inhibitors. Among them, Hg^{2+} was the most effective; at the concentration of 0.2 μM, it could inhibit the enzyme activity by 73%.

Cyanide, azide, and fluoride were inhibitors in decreasing order of effectiveness. At 5 μM, cyanide inhibits 80% of the enzyme activity.

Incubation with *p*-mercuribenzoate or iodoacetate at 4 C and concentration of either 0.8 or 0.4 mM resulted in the complete

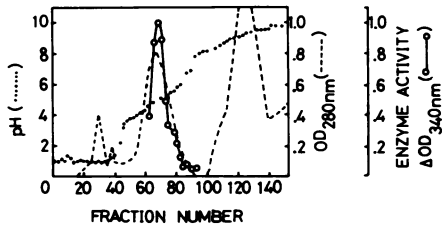

FIG. 4. Isoelectric focusing pattern of bamboo shoot sucrose synthetase in a carrier ampholyte with a pH range of 3.5 to 10.

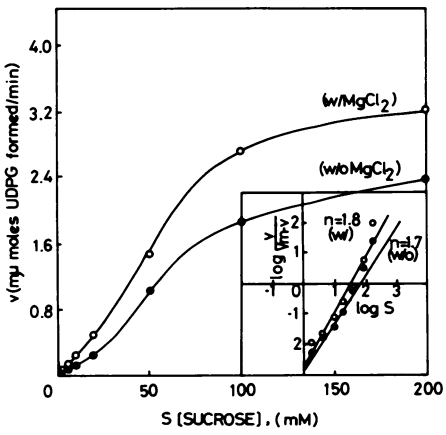

FIG. 5. Effect of sucrose concentration on the rate of enzyme-catalyzed reaction. The maximum velocity was obtained from the double reciprocal plot (not shown) and used in drawing the Hill plot. Inset shows the Hill plot from which the $S_{0.5}$ was evaluated.

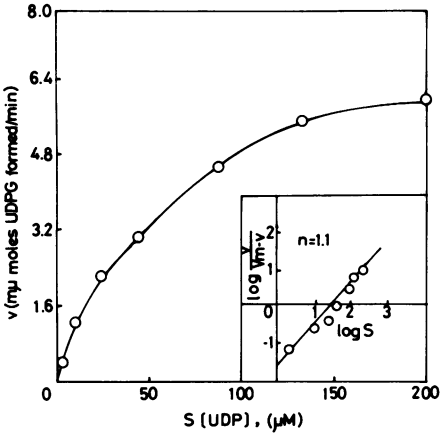

FIG. 6. Effect of UDP concentration on the rate of enzyme-catalyzed reaction. The maximum velocity and *Km* were obtained from the double reciprocal plot (not shown) and the former was employed in drawing the Hill plot as shown in the inset.

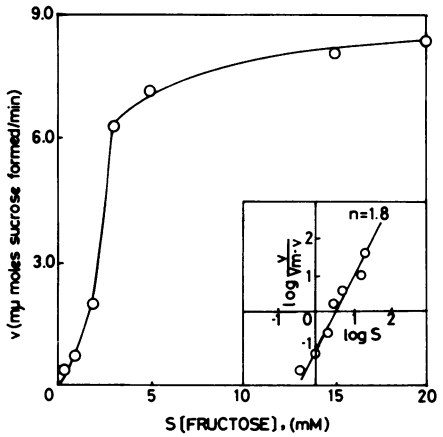

FIG. 7. Effect of fructose concentration on the rate of enzyme-catalyzed reaction. The maximum velocity was evaluated from the double reciprocal plot (not shown) and used in drawing the Hill plot (inset).

loss of the enzyme activity. When reduced glutathione was added to the *p*-mercuribenzoate-inhibited enzyme to the final concentration of 9 mM and the mixture incubated at 4 C for 20 min, the enzyme recovered 52% of its activity. From this test, it may be concluded that the enzyme is an −SH enzyme.

Molecular Weight and Sedimentation Properties. By comparing the elution volume (V_e) of the enzyme with the standard curve of log(MW) *versus* V_e obtained with standard proteins on a Sephadex G-200 column, the mol wt of bamboo shoot sucrose synthetase was estimated as 280,000 (Fig. 3).

The sedimentation constant of the enzyme was calculated to be 7 by a sucrose density gradient ultracentrifugation method. However, in a glycerol density gradient, the sedimentation constant of the enzyme was estimated to be 7.3. The decrease in the sedimentation constant of the enzyme by about 4% under the presence of its substrate sucrose was in agreement with the properties of aspartate transcarbamylase from *Escherichia coli* (3); the sedimentation coefficient of ATCase was reduced by 3.6% when succinate and carbamyl phosphate were present. This experimental result can be taken as evidence to show the binding between sucrose and the enzyme. The mol wt calculated from the sedimentation constant of the enzyme in sucrose and that of the standard protein fibrinogen was 295,000, in good agreement with that obtained by the gel filtration technique.

pI. The isoelectric point of the enzyme was found to be 5.2 by the isoelectric focusing technique (Fig. 4). Among the four protein peaks recorded automatically by a double beam UV analyzer, the largest on the far right side of the figure was due to the protein precipitate formed during the course of isoelectric focusing.

Kinetics. The saturation curves for the four substrates and their respective Hill plots are shown in Figures 5 to 8. The kinetic constants estimated from these plots are summarized in Table II.

In determining the saturation curve of one substrate, the concentration of the other substrate was fixed at a level at least two times higher than the *Km* (or $S_{0.5}$) value (fixed substrate concentrations: sucrose, 100 mM; UDP, 2 mM; fructose, 5 mM; and UDPG, 3.4 mM). For the substrate with a very low *Km* value, adequate amount was provided so as to ensure that the kinetic behavior of the reaction does not change during the course of activity measurement.

The saturation curves for the two carbohydrate substrates were both sigmoidal in shape with n values of 1.8 (sucrose with magnesium chloride and fructose) or 1.7 (sucrose without magnesium chloride), while those of the nucleotide substrates were both hyperbolic in shape with n values close to unity.

It has been revealed in our previous investigation that the bamboo shoot is very rich in UDPG and that the sugar nucleotide can also be formed by the sucrose-cleaving reaction catalyzed by a sucrose synthetase, although UDPG pyrophosphorylase, which is known to be the enzyme responsible for synthesizing UDPG from UTP and G-1-P in many living systems, is also present in the bamboo shoot (11). Our data have revealed that the sucrose-cleaving reaction catalyzed by the bamboo shoot sucrose synthetase is modulated by the availability of sucrose. Thus, sucrose is regarded as the homotropic allosteric effector of the enzyme and the enzyme is turned on only when the concentration of sucrose in the shoot tissue reaches a certain level through translocation from the mother plant, and the turning on of the enzyme makes available to the tissue UDPG, the key sugar nucleotide from which various precursors of cell wall polysaccharides are derived.

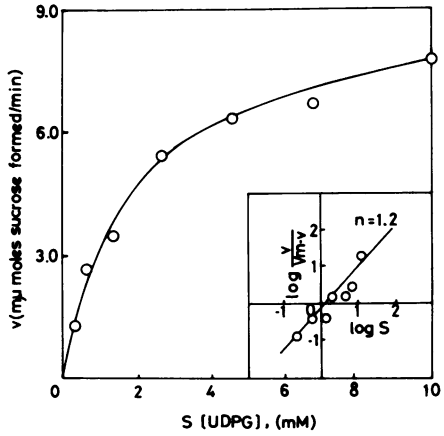

FIG. 8. Effect of UDPG concentration on the rate of enzyme-catalyzed reaction. The maximum velocity and *Km* values were estimated from the double reciprocal plot (not shown) and the former was employed in drawing the Hill plot (inset).

Table II. Kinetic constants of bamboo shoot sucrose synthetase catalyzed reaction

Substrate	Sucrose		UDP	Fructose	UDPG
	$-Mg^{2+}$	$+Mg^{2+}$			
$S_{0.5}$ (mM) or Km*	59	56	0.044	2.50	1.36
Hill coef. (n)	1.7	1.8	1.1	1.8	1.2

*$S_{0.5}$ for sucrose and fructose and Km for UDP and UDPG.

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