

Enzyme-Catalyzed Organic Synthesis of Sucrose and Trehalose with *In Situ* Regeneration of UDP-Glucose

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ABSTRACT

This paper describes cell-free enzymatic syntheses of sucrose and trehalose using partially-purified preparations of sucrose and trehalose synthetase. The coupling of the regeneration of uridine-5'-diphosphoglucose (UDP-Glc) with synthesis of the disaccharide offers a practical route to millimol quantities of these carbohydrates. The syntheses used pyruvate kinase, UDP-Glc pyrophosphorylase, and inorganic pyrophosphatase, and the regenerated UDP-Glc was cycled approximately 10 times.

Index Entries: UDP-Glc, regeneration of; sucrose; trehalose.

INTRODUCTION

Sucrose and trehalose are the major nonreducing disaccharides occurring in nature. Although these sugars are readily isolated in high purity and in large quantities from natural sources (sucrose is ubiquitous among plants (1); trehalose is abundant in fungi and insects (2)), their syntheses are still difficult chemically. Their ready availability notwithstanding, the α -glycosidic link of trehalose and sucrose still presents a significant synthetic challenge (3,4).

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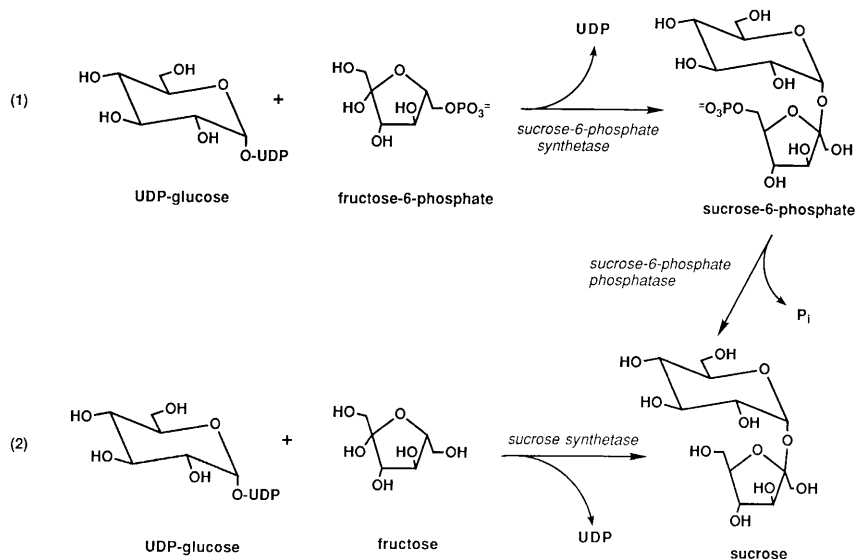


Fig. 1. Enzyme-catalyzed routes to sucrose.

We have explored the practicality of enzymatic routes to sucrose and trehalose based on partially-purified preparations of sucrose and trehalose synthetases. We selected wheat germ and brewer's yeast as the sources of sucrose and trehalose synthetases, respectively, based on their availability and low cost and on the simplicity of the enzyme isolation/purification sequence and the stability of the enzyme (5-8).

Sucrose Synthetase (EC 2.4.2.13; UDP-Glucose: D-fructose-2-glycosyltransferase)

Sucrose accumulation in plants results primarily from the sequential actions of sucrose-6-phosphate synthetase (1,8) and sucrose-6-phosphate phosphatase; this synthesis is driven by the hydrolysis of sucrose-6-phosphate (1). The direct route in which sucrose synthetase catalyzes a glycosylation of fructose by UDP-glucose (UDP-Glc), is reversible; its biological function may be to synthesize nucleoside diphosphate derivatives of glucose (NDP-glucose). This enzyme has been partially purified from numerous plant sources and purified to homogeneity from *P. aureus*, rice seed and artichoke tubers (2). In general, most preparations exhibit rather broad specificity for the nucleotide sugar and the ketoses. ADP-Glc and TDP-Glc can serve as glucosyl donors. Fructose could be replaced by ketoses, such as xylulose, sorbose, and rhamnulose, in a green pea enzyme preparation (9). Sucrose synthetase from sugar beets allows L-sorbose, levanbiose, 5-keto-D-fructose, fructose-6-phosphate, or tagatose to serve as acceptor sugars (10).

The equilibrium of reaction 2 (Fig. 1) can be readily displaced toward sucrose synthesis. High concentrations of UDP-Glc, low levels of sucrose,

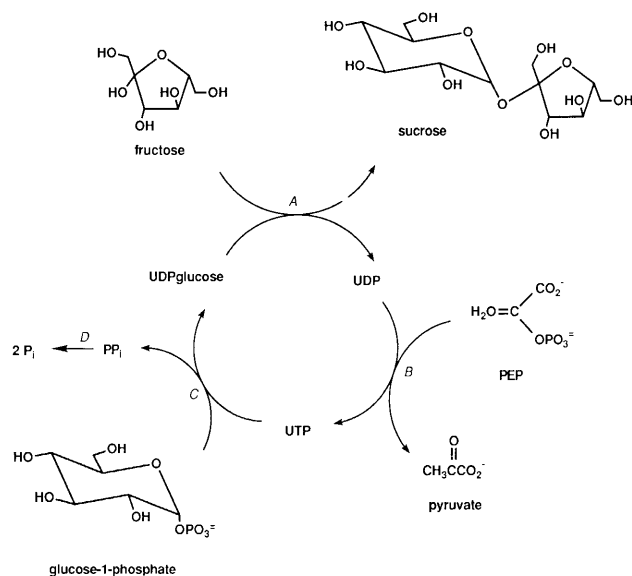


Fig. 2. General scheme for an enzyme-catalyzed synthesis of sucrose with UDP-glucose regeneration. A=sucrose synthetase; B=pyruvate kinase; C=UDP-glucose pyrophosphorylase; and D=inorganic pyrophosphatase.

and high pH favor formation of sucrose. The following effectors selectively enhance sucrose synthesis: Mg^{2+} (20 mM), glucose-1-phosphate (Glc-1-P), glucose-6-phosphate (Glc-6-P), and nucleoside triphosphates (1). The response of sucrose synthetase to these effectors makes the catalytic reaction suitable for coupling to a UDP-Glc regeneration system (Fig. 2). A coupled regeneration cycle is useful for two reasons: it provides the UDP-Glc required for synthesis in an economical way, and it drives the equilibrium toward sucrose synthesis by maintaining a high ratio of UDP-Glc to UDP.

Trehalose Synthetase

The predominant biosynthetic path to trehalose is a two-step route involving trehalose-6-phosphate (2,11,12). Trehalose-6-phosphate phosphatase activity is found as a contaminant in most impure preparations of trehalose-6-phosphate synthetase, and it is present at levels sufficient to release the unphosphorylated sugar (Fig. 3). A value of 40 for K_{eq} has been measured for this reaction at pH 6.1 (11).

In general, the trehalose synthetases accommodate only limited structural variation in either the nucleotide sugar or the sugar phosphate (2). A cell-free brewer's yeast extract produced 2-deoxy-trehalose by the transfer of a glucosyl unit to 2-deoxy-Glc-6-P (13). *Mycobacterium smegmatis* trehalose synthetase accepted GDP-glucose, ADP-glucose, CDP-glucose, and TDP-glucose as D-glucosyl donors; galactose-6-phosphate and fructose-6-phosphate were acceptor sugars in the presence of polyribonucleotides (14).

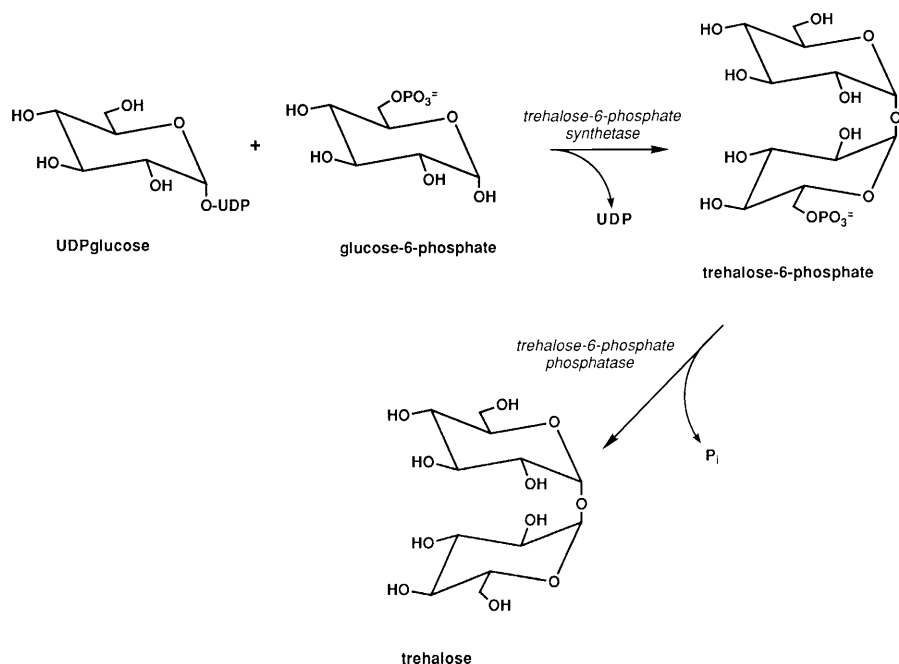


Fig. 3. Enzyme-catalyzed route to trehalose.

RESULTS

Purification and Immobilization of Sucrose Synthetase

Sucrose synthetase was prepared from untreated wheat germ by the procedure of Leloir and Cardini (5). This procedure yielded approximately 0.01 U of purified enzyme/g of wheat germ (Table 1). The purified enzyme preparation was unstable at temperatures greater than 4°C. The half-life of sucrose synthetase activity in this preparation was 30 h in buffer solution at 25°C. The activity of the enzyme was not restored by the addition of DDT. The sucrose synthetase preparations were immobilized on a cross-linked polyacrylamide (PAN) (15) gel to improve their stabilities. The yield of enzymic activity retained upon immobilization was typically 35%.

Synthesis of Sucrose

All sucrose-forming syntheses were carried out in solutions of pH 7.5 under argon at ambient temperature. Most glucosyl transfers to fructose were complete within 24 h. Sucrose was prepared using UDP-Glc, both stoichiometrically and in a coupled reaction system, in which the UDP-Glc was regenerated 10 times using pyruvate kinase (16). Pyruvate kinase and PEP were chosen for the phosphorylation step because of their stabilities in solution. Glc-6-P was also included in these reactions to balance

Table 1
Summary of the Purification Procedure
for Sucrose Synthetase from Wheat Germ^a

Fraction	Volume, mL	Units	Protein, mg	Specific activity	Yield, %
I. Crude Extract	265	–	3577	–	–
II. 1st Ammonium Sulfate	157	5.25	1994	0.0026	100
III. Manganous Chloride	155	–	– ^b	–	–
IV. 2nd Ammonium Sulfate	50	1.1	1000	0.0011 ^c	21

^aThe enzyme preparation began with 90 g of wheat germ.

^bThe dark yellow color of this fraction interfered with the colorimetric assay for protein.

^cThe large decrease in the specific activity reflects the rapid loss of activity after purification step IV.

the loss of Glc-1-P by a phosphoglucomutase present in the impure disaccharide-forming enzyme preparations. The relative concentrations of Glc-6-P and Glc-1-P that were added to the reaction mixtures were roughly proportional to their equilibrium constant ($K_{eq}=17-19$) (17). During the reaction, insoluble magnesium phosphate salts accumulated, and $MgCl_2$ was added every 3–4 d to provide sufficient levels of soluble magnesium ion for activation of all four enzymes. The reaction times were typically 7 d to maximize the turnover number of UDP-Glc and compensate for the low sucrose synthetase activity. The syntheses were terminated by removing the enzyme-containing gels from the solution.

Purification and Immobilization of Trehalose Synthetase

Trehalose synthetase was prepared from a commercial sample of dried brewer's yeast by the method of Leloir and Cabib (7). Although this procedure has been claimed to remove the phosphoglucomutase, this activity was present in our trehalose synthetase preparations. The yield of the partially-purified, trehalose-forming activity was 0.1 U/g dried yeast (Table 2). Trehalose synthetase retained all of its original activity after 12 d at 4°C (0.02% NaN_3 ; 20 mM potassium phosphate, 1 mM EDTA, pH 7.2). Our measured activity profile is consistent with Leloir's observation that 30% of the original activity was preserved after 33 mo of storage at –20°C (18). The trehalose synthetase preparation was immobilized in PAN gel (15).

Synthesis of Trehalose

Although the maximal trehalose synthetase activity is observed in 0.1M tris-maleate, pH 6.6 buffer (25), we carried out the trehalose syntheses in buffer-free aqueous solutions at pH 7.2 to facilitate product purification and optimize the stabilities and reactivities of other enzymes. In a typical synthesis, the turnover number of UDP-Glc was 10. After synthesis of

Table 2
Summary of the Purification Procedure
for Trehalose Synthetase from Yeast^a

Fraction	Volume, mL	Units	Protein, mg	Specific activity	Yield, %
I. Crude Extract	450	157	14400	0.011	100
II. 1st Ammonium Sulfate	150	44	4800	0.009	28
III. 2nd Ammonium Sulfate	125	38	3750	0.01	24
IV. Dialysis	180	34	2520	0.013	22
V. Heating at 30°C	345	42	2415	0.017	27
VI. Acetone	50	25	625	0.04	16

^aThe enzyme preparation began with 240 g of Brewer's Bottom Yeast.

1.5 mmol trehalose, the solution was separated from the insoluble enzymes by centrifugation and the solution was worked up to isolate and purify the disaccharide. The immobilized enzymes were washed and resuspended in another fresh, reactant-containing solution. Trehalose synthesis then resumed at a rate of 75% of the reaction rate in the previous cycle.

DISCUSSION

The major advantage of these enzymatic preparations of disaccharides is their avoidance of protecting groups (19). The preparations of trehalose and sucrose synthetases involved relatively rapid and simple manipulations. The impure fractions appear to be more stable than pure protein. Highly purified sucrose (10) and lactose (20) synthetase preparations were much less stable than crude preparations of each enzyme. These impure enzyme preparations have the disadvantages that they have contaminating activities (especially phosphoglucomutase) and their low activities required large volumes of gel. Wheat germ and brewer's yeast provide inexpensive sources of the enzymes, and their preparations could probably be improved. The MEEC technique (21) might be more convenient than covalent immobilization in polyacrylamide gel for these low-activity preparations; we have not, however, explored its use.

Despite the disadvantages of a partially-purified enzyme preparation, these enzyme/substrate systems are probably still simpler and less expensive than the classical chemical syntheses to the disaccharides. The most important aspect of the enzyme-catalyzed disaccharide synthesis, although not explored in this work, is the potential for synthesis of a variety of disaccharide analogs. Specific trehalose (13,14) and sucrose synthetase preparations (1,9,10) have revealed moderate flexibility in this regard. Enzyme-catalyzed syntheses of disaccharides that incorporate a nucleotide-sugar regeneration scheme should compete favorably with chemical routes to naturally-occurring and unnatural disaccharides.

EXPERIMENTAL

General

Reactions were maintained at constant pH with a pH controller coupled to a peristaltic pump. Centrifugations were carried out as part of the enzyme purification procedures in a refrigerated Sorvall RC5 Superspeed Centrifuge. Nucleotide analyses were performed on a Waters HPLC system using a μ bondapak C-18 reverse phase cartridge and UV detector. Carbohydrate analyses by HPLC used a differential refractometer detector and a 30-cm stainless steel μ bondapak-carbohydrate column.

Materials

All biochemicals, enzymes and cells (wheat germ and brewer's yeast) were obtained from Sigma Chemical Co., St. Louis, MO. PAN-1000 was prepared as previously described (15). Inorganic salts were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. Electrophoresis-grade ammonium sulfate, ion exchange resins, and gel permeation beads were obtained from Bio-Rad Labs, Richmond, CA. Water was doubly distilled from a Corning Model 3B glass still. Welding grade argon was used as an inert atmosphere in all syntheses.

Assays

Unless otherwise stated, all activities are reported in units of mol/min at 25°C. UDP-Glc was measured by reduction of NAD^+ to NADH, catalyzed by UDP-Glc dehydrogenase (EC 1.1.22) (22). Sucrose was measured using invertase (EC 3.2.1.26) (23). Trehalose was measured by trehalase-catalyzed hydrolysis to glucose, followed by analysis for glucose (24). Inorganic phosphate was determined calorimetrically (25,26). Phosphoenolpyruvate (27), pyruvate (27), and pyruvate kinase (28) were determined by standard procedures.

Measurement of Glc-6-P, Glucose, Fructose and Glc-1-P

These four reagents were sequentially measured in one assay sample. Glucose-6-phosphate was measured first with the addition of Glc-6-P (29) dehydrogenase (1 U) to a cuvet that contained the following reagents: 0.4M triethanolamine buffer, pH 7.6 (400 μL); distilled water (500 μL); 0.5M MgCl_2 (20 μL); 62.5 mM NADP^+ (15 μL); and 0–50 μL of the sample. The change in optical density was proportional to the quantity of Glc-6-P in the sample. Hexokinase (1 U) and 0.1M ATP (10 μL) were added to the above assay mixture and the change in optical density was related to the concentration of glucose (31) in the sample. Fructose (31) will also undergo phosphorylation by hexokinase to form fructose-6-phosphate. If phosphoglucosomerase (EC 5.3.19, 5 U) is added to the assay solution,

the enzyme will rapidly isomerize Frc-6-P to Glc-6-P, and the change in absorbance is proportional to the fructose concentration in the sample. Finally, Glc-1-P (32) is converted to Glc-6-P by addition of phosphoglucomutase (5 μ L) to the assay cocktail.

Enzymes

All proteins used in syntheses were attached to a water-soluble, crosslinked polyacrylamide gel by the method of condensation polymerization developed by Pollak et al. (15). Ammonium ions compete with the amine groups of triethylenetetraamine (TET) and lysine residues of the protein during the crosslinking reaction; therefore, all commercial enzymes obtained as suspensions in ammonium sulfate solution were centrifuged (15,000g, 15 min, 0°C), and the supernatants were removed as completely as possible. The pellets were dissolved in HEPES buffer (300 mM HEPES, 15 mM MgCl₂, pH 7.6) to a protein concentration of 0.5 to 2.0 mg/mL. Enzymes obtained as pure protein lyophilates were dissolved in the same HEPES buffer; lyophilates that contained buffer salts were dissolved in distilled water. Those enzymes that were isolated in the laboratory were dialyzed at 5°C against several liters of the appropriate buffer to free the dialysate of ammonium ions from prior ammonium sulfate fractionations. Protein concentrations of partially purified enzyme preparations were measured by the Lowry method (33).

Measurement of Enzyme Stability: UDP-Glc Pyrophosphorylase and Inorganic Pyrophosphatase

These two enzymes were monitored by periodic removal and assay of small aliquots (~50 μ L) of the immobilized gel. One milliliter of the gel was stored in a small, capped vial equipped with a magnetic stirring bar. No measure was taken to exclude dioxygen from these samples.

Disaccharide-Forming Enzymes

Owing to the low specific activities of these disaccharide synthetases, their stabilities as immobilized catalysts were best estimated by their performances in synthetic reactions. The reaction conditions are described under the "Synthesis of Sucrose (Trehalose)" section. One-milliliter samples of the soluble enzyme preparations were stored in capped, glass vials at ambient temperature and 4°C. Aliquots were removed at various time intervals and assayed for activity by procedures described under the "Assay of Sucrose (Trehalose) Synthetase" section.

HPLC: Analyses for Sucrose and Trehalose

The standard mobile phase for all carbohydrate analyses was acetonitrile:water, 75:25 (v/v) at a flow rate of 1.8 mL/min. Carbohydrate stan-

dards, 0.15% (w/v) solutions of each sugar (34), were used to analyze the composition of the reaction mixture. Aliquots (0.5–1.0 mL) from synthetic mixtures were routinely mixed with anion exchange resin (AG1-X8—bicarbonate form). The supernatant was passed through a 0.22-micron Millex filter before injecting the solution into the HPLC sample port. The retention times for the standards were: fructose, 4.4 min; glucose, 5.0 min; sucrose, 7.2 min; and trehalose, 8.8 min.

Preparation of Sucrose Synthetase

Sucrose synthetase was prepared from wheat germ (Sigma) by the procedure of Leloir and Cardini (6). Ninety grams of the untreated wheat germ was suspended in 300 mL of 50 mM potassium phosphatase, 1 mM EDTA buffer, pH 7.2, and homogenized in a Waring blender for 5 min, with pulsed blasts at 30-s intervals. Centrifugation (16,000g, 2°C) yielded 210 mL of crude extract, which was dialyzed against 2×4 L of distilled water for 6 h. The dialysate (265 mL) was treated with 93.4 g of solid ammonium sulfate (0.5 saturated). The ammonium sulfate suspension was stirred in the cold for 30 min before centrifugation (16,000g, 15 min, 2°C). The pellet was suspended in 100 mL of distilled water and dialyzed overnight against 2×4 L of distilled water. One-tenth volume of 1.0M manganous chloride was added to the dialysate (~157 mL) and centrifuged to remove the pellet. The supernatant (155 mL) was fractionated with 33 g of ammonium sulfate (0.3 saturation). After centrifugation, the supernatant was collected and made 0.5 saturated in ammonium sulfate. The pellet collected after centrifugation was suspended in a minimal volume of distilled water (~30 mL). This clear yellow fraction was dialyzed for 12 h against 3×4 L of distilled water at 5°C. After dialysis, the enzyme preparation was immobilized immediately.

Immobilization of Sucrose Synthetase

The sucrose synthetase preparation (45 mL, 0.2 U) was mixed with 15 mL of 0.3M HEPES, 15 mM MgCl₂, pH 7.6 buffer, and 1.0M DTT (0.375 mL) in a 250-mL beaker equipped with a stirring bar. Fifteen g of PAN-1000 was added, and the solution was vigorously stirred by hand with a thick glass rod until the mixture was homogeneous. TET (0.5M, 12.75 mL) was added to the rapidly stirred mixture. The gel set within 10 s, then sat under a moderate stream of argon for 60 min before it was crushed and washed by standard procedures (15). The gel was stored in 0.3M HEPES, 15 mM MgCl₂, pH 7.6, and 10 mM DDT buffer until use in synthetic reactions at which time the buffer solution was removed and the gel resuspended in deoxygenated distilled water before added to the reaction mixture. No enzyme substrates (i.e., sucrose, fructose, and UDP-glucose) were included in the immobilization reaction since the immobilization yields were not significantly enhanced when these reactants were included.

Assay of Sucrose Synthetase

Sucrose synthetase activity was measured by monitoring the disappearance of UDP-Glc and fructose or the appearance of sucrose. Aliquots (20 μ L) were removed from a mixture containing: 0.2M potassium phosphate pH 7.6 buffer (100 μ L); 0.1M fructose (100 μ L); 50 mM UDP-Glc (50 μ L); distilled water (650 μ L); and the enzyme solution (100 μ L). A blank reaction containing all reagents except fructose was run during each determination.

Synthesis of Sucrose

Sucrose syntheses were performed in a 1-L, three-necked, round-bottomed flask equipped with a pH controller adaptor. The pH controller maintained the reaction at pH 7.2 with 2.0N KOH. Buffer-free immobilized sucrose synthetase (170 mL) was suspended in a deoxygenated solution (180 mL) containing UDP-Glc (90 μ mol), fructose (2.1 mmol), Glc-1-P (80 μ mol), Glc-6-P (2.1 mmol), PEP (2.6 mmol; the solution also contained 0.9 mmol of pyruvate), $MgCl_2$ (10 mmol), KCl (4.5 mmol), and 5 mL of 4% NaN_3 . The reaction was charged with the regeneration enzymes: immobilized pyruvate kinase (5 U), immobilized UDP-Glc pyrophosphorylase (5 U), and immobilized inorganic pyrophosphatase (28 U). The stirred reaction mixture was kept at ambient temperature under a moderate stream of argon. The reaction course was monitored by HPLC and enzymatic analyses. When necessary, depleted PEP and fructose levels were restored to their initial concentrations. After 7 d, the reaction was terminated. The enzyme gel was removed by centrifugation, and the supernatant was worked up following the procedure used for the isolation of sucrose. The gel was resuspended in 0.3M HEPES, 15 mM $MgCl_2$, pH 7.6, and 10 mM DTT buffer briefly (ca. 10 min) to allow reactivation of any oxidized sucrose synthetase or UDP-Glc pyrophosphorylase. The gel was centrifuged, and the resulting supernatant was decanted before suspending the gel in deoxygenated water prior to use in another reaction.

Purification of Sucrose

Anionic reactants and coproducts were removed by batchwise treatment of the reaction solution with washed and filtered anion exchange resin (AG1-X4, bicarbonate form). The suspension was filtered through a Büchner funnel to remove the ion exchange resin. The filtrate was concentrated by rotary evaporation and lyophilized. The lyophilate was suspended in 50% aq. ethanol to a concn. of ~ 200 mg/mL. Precipitated inorganic salts were removed by filtration, and the supernatant liquor was again lyophilized. The lyophilate was suspended in 3 mL of distilled water and treated with activated charcoal to decolorize the solution. The concentrate was

applied to a Bio-Rad P-2 gel filtration column (1.5×46 cm) as three fractions. The samples were eluted with distilled water and collected in fractions of 6 mL. Each fraction was analyzed by HPLC for the sucrose content; those fractions containing only sucrose (fractions 9–12) were pooled, concentrated, and lyophilized. The solid was dissolved in a minimal volume of 60% (v/v) aq. ethanol, then concentrated to a syrup by rotary evaporation. The syrupy mixture was treated with hot absolute ethanol and rapidly stirred until crystalline product appeared. After concentration and lyophilization of the solution, the quantity and purity of sucrose that remained in the mother liquid were estimated by HPLC as 95 mg and 90% pure, respectively. The crystals were washed with ethanol and ethyl ether before drying *in vacuo*; mp = 184–186°C, lit. mp = 185–86°C (35). HPLC and enzymatic analyses indicated that the product of 98% purity was free of glucose and fructose. ¹³C NMR (D₂O): δ 105.2, 93.7, 82.9, 77.9, 75.5, 74.0, 73.9, 72.6, 70.7, 63.9, 62.9, and 61.7. The resonances of all twelve carbons of sucrose were resolved and corresponded to those of an authentic sample.

Preparation of Trehalose Synthetase

Brewer's bottom yeast (240 g) was suspended in 720 mL of distilled water (19). The mixture was vigorously stirred at ambient temperature for 1 h before cold storage (4°C) for 20 h with occasional stirring of the viscous cell suspension. Centrifugation (15,000g, 20 min, 2°C) yielded 450 mL of a crude extract. The murky supernatant was treated with an equal volume of cold 50% ammonium sulfate solution (for 500 mL of solution: 250 g of solid (NH₄)₂SO₄, 0.5 mmol EDTA, pH adjusted to 7.5 with NH₄OH), then was stirred for 30 min at 4°C. After centrifugation, the pellet was suspended in 1mM EDTA (100 mL). One volume of cold 50% ammonium sulfate solution was added to the enzyme suspension. The mixture was stirred for 30 min at 0°C, then was centrifuged to yield a whitish-gray pellet. The pellet was dissolved in 100 mL of 1 mM EDTA. If the solution was turbid, the suspension was centrifuged to remove the insoluble material before dialysis at 4°C against 2×4 L of 1 mM EDTA for 6 h. The dialysate was mixed with an equal volume (180 mL) of 40 mM MgSO₄ and stirred in a water bath at 50°C until the solution temperature reached 36°C. The flask was rapidly cooled in an alcohol/ice bath until the solution temperature was 2°C. After centrifugation, the supernatant was placed in a 1-L Erlenmeyer flask that was immersed in a bath at –4°C until the temperature of the cell fraction liquid reached 0°C. Over a period of 20 min, 0.35 vol of cold acetone (–4°C) was slowly dropped into the stirred solution. During this period, the temperature of the solution was allowed to drop to –3°C before centrifugation (2000g, 10 min, –5°C). The pellet was dissolved in 20 mM potassium phosphate, pH 7.0 buffer (35–40 mL). The resulting yellow solution was dialyzed overnight against 2×4 L of the same buffer.

Immobilization of Trehalose Synthetase

Trehalose synthetase dialysate (45 mL) and 15 mL of 0.3M HEPES, 15 mM MgCl₂, pH 7.6 buffer, were mixed in a 250-mL beaker equipped with a stirring bar. PAN-1000 (15g) was vigorously stirred into the enzyme-buffer solution. Immediately after homogenizing the PAN mixture, DTT (1.0M, 0.375 mL) and TET (0.5M, 12.75 mL) were added. The gel was allowed to set for 1 h under a rapid stream of argon, crushed into fine particles, and washed successively with the following: 1×1 vol of 50 mM HEPES, 50 mM (NH₄)₂SO₄, 10 mM MgCl₂, pH 7.5 buffer; 2×1 vol of 0.3M HEPES, 15 mM MgCl₂, pH 7.5, 10 mM DTT buffer. The immobilized enzyme remained suspended in the final wash buffer until use in a reaction. For use in syntheses, the immobilized enzyme was washed free of buffer and suspended in deoxygenated distilled water. Immobilization yields were not increased by the inclusion of UDP-glucose, Glc-6-P, or trehalose in the immobilization reactions.

Assay Methods for Trehalose Synthetase

Method A:

Indirect Measurement of Trehalose Synthetase Activity

The rates of change in UDP-Glc and Glc-6-P concentrations in the presence of the enzyme preparation were measured and set proportional to trehalose synthetase activity. The rate curves for UDP-Glc and Glc-6-P were similar. This procedure served as a reliable—although not a fool-proof—test for the presence of trehalose-forming activity. The reaction components were UDP-Glc (29 mM, 50 μ L), Glc-6-P (50 mM, 50 μ L), 0.125M MgSO₄, 5 mM EDTA (50 μ L) and 50–100 μ L of the enzyme preparation. At 1-h intervals, Glc-6-P and UDP-Glc concentrations were measured by the previously described spectrophotometric methods.

Method B:

Direct Measurement of Trehalose Formation Using Trehalase

Trehalase (EC 3.2.1.28) catalyzes the hydrolysis of trehalose to glucose. Glucose concentrations were measured after treatment of a reaction sample with a trehalase preparation (*see next section*). The specific activity of the trehalase DEAE-cellulose fraction was very low, and the reaction was allowed to run for long periods to ensure that all trehalose was degraded. Typically aliquots from the trehalose-forming reactions were diluted 4- to 20fold with the enzyme solution (0.0008 U trehalase/100 mmol trehalose). Under these conditions, the hydrolysis reaction was complete within 12 h. Blank control reactions containing the reaction sample diluted in 50 mM sodium phosphate, pH 5.6, 5 mM cysteine buffer were run, but these control reactions indicated that for times up to 30 h, Glc-1-P, Glc-6-P, and trehalose did not undergo any detectable nonenzymic hydrolyses to glucose.

Preparation of Trehalase from Baker's Yeast

Three 6-ounce packages (54 g) of Fleischman's compressed yeast were broken in small pieces into a 500-mL Erlenmeyer flask (36,37). The yeast cells were macerated with a mixture of toluene (20.5 mL) and 0.1M sodium acetate, pH 5.6 (21.6 mL), in a water bath for 2 h. After cooling the warm flask to 10°C, the cell suspension was diluted with 54 mL of cold distilled water and left at 5°C for 3 d with occasional stirring. The top layer of toluene and yeast stromata was siphoned off as completely as possible. The aqueous suspension was centrifuged (29,000g, 10 min, 0°C) to yield 93 mL of supernatant. Solid cysteine was added to a final concn. of 5 mM. Trehalase activity was precipitated by the addition of 53.5 g of ammonium sulfate to 93 mL of enzyme solution. The mixture was stirred for 30 min in the cold before centrifugation. The supernatant was discarded, and the pellet was suspended in 0.1M sodium acetate, pH 5.7, 5 mM cysteine buffer (12 mL), then centrifuged to remove any insoluble material. The yellow solution was applied to a Sephadex G-25 column (1.5×50 cm) and eluted with the acetate-cysteine buffer. The effluent was collected in 7.5 mL fractions and assayed for trehalase activity (*see next section*). Fractions 4 and 5 were combined, centrifuged (29,000g, 10 min, 0°C) and applied to a DEAE-cellulose column equilibrated in 50 mM sodium acetate, pH 5.6. When the enzyme solution had completely entered the cellulose matrix, the column was washed with approximately 10 mL of the equilibrating buffer before eluting the protein with 200 mL of a 0–3.0M sodium chloride gradient in the same buffer. Fractions (3.75 mL) of effluent were collected and tested for trehalase activity. The trehalase activity eluted in two peaks. All fractions containing greater than 0.0001 U of trehalase activity were combined, then dialyzed against 3×4 L of 50 mM sodium acetate, pH 5.6, 5 mM cysteine buffer for 12 h at 5°C. The colorless solution (35 mL, 0.171 U) was stored at –20°C in 4-mL fractions. A trehalase fraction used for trehalose analysis was stored at 5°C for 3 wk with no significant loss of activity.

Measurement of Trehalase Activity

Since trehalase exhibits maximal activity at pH 5.6 (23), the trehalose hydrolysis was not coupled to the indicator enzymatic assay for glucose (pH optima: hexokinase, 7.5; Glc-6-P dehydrogenase, 9.2) (29). Instead, trehalase samples (50 µL) were added to a reaction mixture containing trehalose (0.1M, 100 µL) and 0.1M sodium acetate, pH 5.6, 5 mM cysteine (350 µL) at 25°C. A sample containing no enzyme was run as a blank. Aliquots of 20 µL were periodically removed and assayed for the product glucose.

Synthesis of Trehalose

The syntheses were executed in a 1-L, three-necked, round-bottomed flask with a pH controller attachment to enable automatic adjustment to

and maintenance of pH 7.2 with 2.0N KOH. Distilled water (180 mL) containing UDP-Glc (0.1 mmol), MgCl_2 (3.6 mmol), KCl (2.3 mmol), Glc-6-P (0.55 mmol), and sodium azide (0.04%) was added to the reaction vessel and thoroughly deoxygenated with a rapid stream of argon. The rapidly-stirred mixture was charged with immobilized trehalose synthetase (50 mL, 0.124 U) at ambient temperature ($\sim 25^\circ\text{C}$) under a moderate flow of argon. After 20 h, the UDP-Glc regeneration system was coupled to the main reaction by the addition of the following: immobilized pyruvate kinase (5 mL, 1.9 U), immobilized UDP-Glc pyrophosphorylase (5 mL, 2.5 U), immobilized inorganic pyrophosphatase (3 mL, 8.7 U), PEP (0.69–0.8 mmol; containing 0.12–0.16 mmol of pyruvate); and Glc-1-P (0.23 mmol). Throughout the reaction, every component of this multiple enzymatic sequence was monitored by enzymatic assay. When trehalose synthesis came to a halt owing to the depletion of PEP and/or Glc-1-P, these reactants were added in amounts necessary to restore them to their previous levels. Trehalose was synthesized at a constant rate for 15 d. After 360 h, the reaction was terminated owing to the buildup of Glc-6-P by phosphoglucomutase activity. In a subsequent reaction, the accumulation of Glc-6-P was suppressed by periodic addition of Glc-6-P (instead of Glc-1-P) to the reactor. Separation of the catalysts from the products was achieved by centrifugation (10,000g, 5 min, 2°C). The supernatant was decanted and worked up by the purification procedure described below; the gel catalyst was resuspended in distilled water and reused in another synthesis. Typically, 75% of the initial trehalose-forming activity was recoverable.

Purification of Trehalose

The supernatant was treated with an anion exchange resin (AG1-X4, bicarbonate form) in a batchwise manner to bind the anionic reactants and coproducts. This mixture was poured through a glass-sintered filter funnel to remove the resin. Cations were removed by stirring the filtrate in cation exchange resin (AG-50W, hydrogen ion form). After the removal of the resin by filtration, the solution was concentrated by rotary evaporation. The concentrate was lyophilized to a pale yellow hygroscopic foam and dissolved in 50% aq. ethanol (~ 200 mg solid/mL of solution) to remove insoluble inorganic salts. The solution was concentrated by rotary evaporation to remove ethanol before the second lyophilization. The lyophilate (673 mg) was dissolved in a minimal volume of distilled water (~ 2 mL) and transferred to a Bio-Rad P-2 gel filtration column (1.5×46 cm) in three fractions. The samples were eluted with distilled water; fractions (6 mL) were collected and assayed for trehalose by HPLC. Trehalose was the sole carbohydrate present in fractions 8–12. For each column run, these fractions were pooled and lyophilized. The solids were suspended in boiling 80% aq. ethanol. Trehalose did not readily crystallize from these solutions. Crystallization occasionally occurred in the presence of a pure trehalose seed crystal: melting point of the crystalline solid (67 mg) was 96°C ; lit

mp for α,α -trehalose dihydrate = 96–97°C (38). In those instances where crystallization did not occur, the ethanolic solutions were concentrated by rotary evaporation, lyophilized, suspended in a measured volume of distilled water, and calibrated for trehalose concentration by HPLC and enzymatic assay methods. HPLC showed the product (413 mg) to be free of glucose and 93% pure. ^{13}C NMR (D_2O): δ 94.6 (s, C"1); 74.0 (s, C"3); 73.6 (2, C"2); 72.5 (s, C"5); 71.1 (s, C"4); 62.0 (s, C"6); and 50.2 (impurity).

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