Expression and Characterization of Sucrose Synthase from Mung Bean Seedlings in Escherichia coli

Tonomori Nakai, Naoto Tonouchi,* Takayasu Tsuchida,* Hitoshi Mori,** Fukumi Sakai, and Takahisa Hayashi†

Wood Research Institute, Kyoto University, Gokasho, Uji, Kyoto 611, Japan
*Bio-Polymer Research Co., Ltd., KSP R&D Business-park Bldg. B-1015, 2-1, Sakato 3-chome, Takatsu-ku, Kawasaki 213, Japan
**Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464-01, Japan
Received February 12, 1997

The cDNA fragment coding for mung bean (Vigna radiata Wilczek) sucrose synthase was introduced into the expression vector pET-20b resulting in the construction of plasmid pEB-01. After transformation of Escherichia coli strain BL21(DE3) cells by pEB-01 and induction with isopropyl thio-β-galactoside, high level expression of the recombinant enzyme was obtained. The enzyme had a tetrameric form that conserved the activity of sucrose synthase. Although the $K_m$ and $V_{max}$ of the recombinant enzyme acting on either UDP-glucose or fructose were very close to those of the native enzyme isolated from mung bean seedlings, the $K_m$ for sucrose was higher by a factor of 10 for the recombinant enzyme. This suggests that the recombinant sucrose synthase has a tendency to synthesize sucrose, although the native enzyme catalyzes a freely reversible reaction.

Key words: mung bean; recombinant; sucrose synthase; kinetics

Sucrose synthase (EC 2.4.1.13) catalyzes the reaction: UDP-glucose + fructose $\rightleftharpoons$ sucrose + UDP, a freely reversible reaction. The amount of the enzyme is much higher in non-photosynthetic tissues, where sucrose is the source of carbon that is translocated and cleaved by the enzyme to produce UDP-glucose for synthesis of cell walls as a major sink in plants. Therefore, the enzyme may function to produce UDP-glucose rather than to synthesize sucrose in plant tissues. UDP-glucose is not only the direct precursor of cellulose, 1,3-β-glucan, 1,3- and 1,4-mixed β-glucan, and xyloglucan, but also the starting sugar nucleotide interconverted to other UDP-sugars that are all used in the synthesis of cell wall polysaccharides. Then, UDP formed from UDP-sugars by glycosyltransferase reactions can be recycled in a short time to produce UDP-glucose by sucrose synthase. The production of UDP-glucose by the enzyme is a method of conserving energy for ATP, which only occurs in higher plants. In developing cotton fibers, the sucrose synthase, localized in arrays that parallel the helical pattern of cellulose deposition, may participate in the biosynthesis of cellulose.

In earlier studies, the mung bean sucrose synthase was isolated, its enzyme properties identified, and its cDNA cloned. The protein is a tetramer composed of identical subunits of 95 kDa, and its cDNA contains an open reading frame of 2415 bp that encodes a polypeptide of 805 amino acids with a calculated molecular mass of 92,087 daltons. The N-terminus of the enzyme is not known because its terminal amino acid is blocked.

To examine the assembly and activation of sucrose synthase subunits, we expressed recombinant sucrose synthase in Escherichia coli and characterized the polypeptide. This paper shows that the recombinant polypeptide forms a tetramer that is enzymatically active as the native mung bean enzyme, although its kinetic properties are somehow different.

Materials and Methods

Bacterial strain and plasmids. The cDNA encoding sucrose synthase from mung bean seedlings has been cloned into the plasmid pTTQ18 and the resulting plasmid pM-SS-5 sequenced. Plasmid pET-20b and its accompanying Escherichia coli strain BL21(DE3) were obtained from Novagen.

Cloning, expression, and purification of recombinant sucrose synthase. The oligonucleotides used for cloning sucrose synthase cDNA were introduced into the expression vector pET-20b (Novagen) as N-ter (nucleotide position 39–58), TGGTACGATCTGGTACCCGATGATCC, which was homologous to the coding strand and the C-ter (2494–2489, CTTGCCTGGCCACACCGGGCTTTCCCGTCCCTTTCCCTATCC) was complementary to the coding strand (reverse primers), which attached a SalI recognition site. The PCR product was done by using EX Taq polymerase (Takara) and the amplified products were purified from agarose gels. The PCR fragment was digested with SalI and cloned into pET-20b that had been digested with NdeI, filled in with the Klenow fragment and then digested with SalI. After ligation, the products were used to transform E. coli strain BL21(DE3)-competent cells and a mini-preparation was used to isolate the recombinant plasmids. The correct assembly and predicted alignment of the resulting plasmid, designated as pEB-01, was confirmed by sequencing. The whole cDNA insert according to the primer-labeled didoxygen chain-termination method. E. coli BL21(DE3) cells harboring pEB-01 were grown and the expression of the recombinant protein was induced by isopropyl thio-β-galactoside (IPTG) at a final concentration of 0.3 mM. The bacterial pellets were resuspended in 30 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM EDTA and 0.1 mM dithiothreitol (DTT). The cell suspension was frozen, thawed on ice, and then disrupted by sonication. After centrifugation, the supernatant was adjusted to 65% saturation with ammonium sulfate. The
precipitating proteins were collected and dissolved in 200 µL of 30 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 0.1 mM EDTA, and 0.1 mM DTT. The suspension was centrifuged and the resultant supernatant was put on a Superose 6 gel filtration column (1.0 × 30 cm, Pharmacia) that had been equilibrated with the same buffer. For the gel filtration, which was a part of an FPLC system (Pharmacia), the flow rate was 0.3 mL/min. The active fractions were concentrated and filtered again on the same gel. The active fractions were combined and stored at 4°C. This combined fraction was used in the experiment for a kinetic analysis.

**Electrophoresis and Western-blot analysis.** Electrophoresis was done in the discontinuous buffer system of Laemmli with 0.1% SDS at 20 mA for 70 min. Stacking and separating gels contained 4% and 5% acrylamide, respectively. After electrophoresis, proteins were stained with Coomassie brilliant blue.

For Western blotting, immunoblot reactions between enzyme and antiserum were examined on polyvinylidene difluoride (PVDF) membranes by binding peroxidase-conjugated anti-rabbit IgG (1:1000) and chemiluminescence subsequently detected by exposing to X-ray film using an ECL detection kit (Amersham). Proteins were blotted by the electroelution of proteins on the gel after SDS-PAGE onto a PVDF membrane for 2 h at 1.2 mA/cm². Dot blot analysis was done by dotting 0.5 µL of each fraction obtained from gel filtration on a nitrocellulose membrane to measure the amount of recombinant protein in the fractions. The detection procedure was the same as that for Western blotting. The intensity of the detected signal was expressed numerically by using Ibas-Kontron Image Analysis Division ver. 2.0 application software.

**Extraction of plant sucrose synthase.** Mung bean sucrose synthase was extracted from four-day-old etiolated seedlings as described by Delmer. All procedures were done at 4°C. Hypocotyls (fresh weight 123 g) of mung bean seedlings were homogenized in a mortar with 120 mL of 30 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA and 0.1 mM DTT. The homogenate was centrifuged at 22,000 g for 30 min and the clear extract was brought to 65% saturation with ammonium sulfate. The precipitate was collected by centrifugation and the precipitated pellet (0.75 µg) was stored at −85°C. A part of the precipitate was resuspended in 200 µL of 30 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA and 0.1 mM DTT, and the enzyme solution was subjected to activity and kinetic analyses. The crude extract containing sucrose synthase yielded the enzyme preparation with specific activity of 0.296 unit/mg total proteins. One unit of the activity was defined as one µmol of glucose incorporated into sucrose per hour.

**Assay for recombinant sucrose synthase.** For measurement of the incorporation of glucose from UDP-[14C]glucose into sucrose, the reaction mixture contained 3 mM UDP-[14C]glucose (420 pCi/µmol), 50 mM fructose, 50 mM Tris-HCl (pH 7.5), and the enzyme preparation (usually 20 to 30 µg of protein) in a total volume of 20 µL. For measurement of the incorporation of glucose from [U-14C]sucrose (2.5 mM/µmol), 10 mM UDP, 50 mM Tris HCl (pH 7.5), and the enzyme preparation in a total volume of 20 µL. The reaction was allowed to proceed for 5 min to form sucrose and for 30 min to form UDP-glucose, and the reaction mixture was immediately spotted on a 2-cm wide Whatman 3MM filter paper strip, followed by electrophoresis in 30 mM sodium tetraborate (pH 9.6) at 250 V for 3 h. After electrophoresis, the paper strips were dried and used for autoradiography or Fuji bio-imaging analyzer Bas2000 (Fuji Photo Film Co., Ltd.). The area corresponding to sucrose, UDP-glucose, and fructose on the paper was excised and neutralized, and its radioactivity was counted in 5 mL of a toluene scintillator.

**Results and Discussion**

**Construction of plasmid pEB-01.** A cDNA for sucrose synthase was originally obtained by immunobiochemical screening from a mung bean cDNA library constructed in the plasmid pT7TQ18. In the earlier construction of plasmid pM-SS-5, the recombinant protein derived from cDNA was fused with a part of a β-galactosidase leader peptide. To remove the sequence encoding the leader peptide, a plasmid pET vector was used because this plasmid has a high expression vector.

The full-length cDNA encoding mung bean sucrose synthase was amplified by PCR using pm-SS-5 as a template and oligonucleotide N-ter and C-ter as elongating primers. The PCR products isolated from agarose gel were digested with SalI and integrated into the expression vector pET-20b that had been digested with NdeI, filled with Klenow fragment, and then digested with SalI. After ligation, the plasmid was introduced into E. coli strain BL21(DE3)-competent cells, and the clone harboring the cDNA insert was selected. The constructed pEB-01 contained a 2415-bp fragment encoding 805 amino acids for mung bean sucrose synthase.

**Expression in E. coli of mung bean sucrose synthase.**

The expression of sucrose synthase was induced by addition of IPTG in E. coli cells harboring pEB-01. Then induced cells were sonicated and the cell lysate was used for Western blot analysis. An intense band with an apparent molecular mass of 95 kDa was produced, but not with extracts of control (Fig. 1A). Western blot analysis showed that the antiserum against mung bean sucrose synthase bound to the 95 kDa polypeptide, but extracts of the control did not cross-react with the antiserum (Fig. 1B). This is in accord with the earlier observation that the native plant enzyme is composed of identical protein subunits of approximately 95 kDa.

To identify and define the activity of sucrose synthase in the extracts, the recombinant protein from pEB-01 was incubated in the presence of UDP-[14C]glucose and fructose. The incubation mixture was analyzed by paper electrophoresis followed by autoradiography. Radioactive sucrose was observed in the reaction mixture with the extracts of native and recombinant enzymes by the autoradiogram (Fig. 2A, lanes 1 and 2), but not in the control (lane 3) and heat-treated recombinant protein (lane 4). The radioactive sucrose was also confirmed on paper chromatography after excision of the spot. When the recombinant protein was incubated with [U-14C]sucrose and UDP, radioactive UDP-glucose and fructose were observed in the auto-

---

**Fig. 1.** SDS-Polyacrylamide Gel Electrophoresis Analysis of Recombinant and Mung Bean Sucrose Synthases. A, Coomassie brilliant blue-stained polyacrylamide gel. B, protein cross-reacted with antiserum against mung bean sucrose synthase. Arrow indicates a band of recombinant sucrose synthase. Lanes 1, the enzyme preparation of sucrose synthase from mung bean seedlings; 2, extract of E. coli cells expressing recombinant sucrose synthase; 3, total extract from uninduced E. coli cells; M, molecular size marker.
between recombinant and native enzymes (Fig. 2A).

**Purification and molecular properties of recombinant sucrose synthase**

The recombinant protein that cross-reacted with polyclonal antibodies against mung bean sucrose synthase was distributed from the void volume to the position of 350 kDa (Fig. 3), although only a single peak (350 kDa) of sucrose synthase activity was obtained after gel filtration on Superose 6. This suggests that the recombinant active protein was a 350-kDa polypeptide which comprises identical polypeptide subunits (95 kDa) on SDS–PAGE (data not shown). This is in accord with the earlier observation that the native plant enzyme is a tetramer composed of identical protein subunits of approximately 95 kDa.\(^5\,^6\) There were no monomer or dimer proteins in the elution profile of gel filtration, suggesting that the recombinant protein has a strong tendency to form a structure larger than a tetramer.

The recombinant enzyme from pEB-01 could be purified to homogeneity from cell lysate by gel-filtration chromatography because a homotetramer of the protein was one of the largest proteins in molecular size produced in *E. coli*. The yield of enzyme recovered was calculated to be approximately 10.5 mg/liter bacterial culture. The specific activity of the protein was 1.08 unit/mg enzyme in the formation of UDP-glucose and fructose, and 11.3 unit/mg enzyme in the formation of sucrose and UDP. This specific activity in the formation of sucrose was almost the same as that of the enzyme purified from mung bean (5.80 unit/mg) as reported by Delmer.\(^5\)

**Kinetic properties**

As shown in the Table, the *K*<sub>m</sub> of sucrose synthase in crude extract from mung bean seedlings were almost the same as those (2 mM for fructose, 0.21 mM for UDP-glucose, and 17 mM for sucrose) of the purified enzyme as reported earlier,\(^4\,^5\) probably because the values obtained were measured under at the optimized conditions of the assay reaction for sucrose synthase.

The *K*<sub>m</sub> and *V*<sub>max</sub> of purified recombinant enzyme acting on either UDP-glucose or fructose were almost the same to those of the native enzyme isolated from mung bean seedlings. However, the *K*<sub>m</sub> for sucrose was much higher by a factor of 10 for the recombinant enzyme. The data shows that the recombinant sucrose synthase has almost the same affinity for UDP-glucose and fructose but a lower affinity for sucrose. This is in full agreement with the earlier observation (Fig. 2B, lane 2) that the amounts of UDP-glucose and fructose formed from sucrose with the recombinant enzyme were much lower than those with the native enzyme.

It seems likely that the binding site of sucrose in the recombinant enzyme is somehow difficult to bind to sucrose. One of the possibilities is that phosphorylation is required for sucrose synthase to bind to sucrose because the recombinant enzyme is not phosphorylated in *E. coli*. In fact, phosphorylation (SS1 and SS2) of maize sucrose synthase identified as Ser-15 activated the formation of UDP-glucose and fructose from sucrose plus UDP by increasing the affinity of the enzyme for sucrose.\(^19\) Based on comparison of the amino acid consensus sequence,\(^19\) the reaction mixture after paper electrophoresis (Fig. 2B). The relative amounts of UDP-glucose and fructose formed from sucrose were almost equivalent in both lanes 1 and 2, suggesting ineffectiveness of any invertase and other enzyme activities in the sucrose synthase reaction. However, the amounts of UDP-glucose and fructose formed from sucrose with the recombinant enzyme were much lower than those with the native enzyme (Fig. 2B, line 2). This suggests that the activity for the formation of UDP-glucose and fructose from sucrose was lower for the recombinant enzyme, although the formation of sucrose from UDP-glucose and fructose showed similar levels between.
Ser-11 in mung bean sucrose synthase may be the site of phosphorylation. Other possibilities are glycosylation and N-terminal amino acid processing of the protein, which do not occur in E. coli. These modifications might cause the low affinity for sucrose of the recombinant protein.

Nevertheless, the recombinant sucrose synthase is useful for the synthesis of sucrose because of its low affinity for sucrose. This will be useful for the kind of carbohydrate engineering that would make valuable contributions to oligosaccharide synthesis.

Acknowledgment. We thank Steven C. Huber (USDA/ARS Plant Science Research) for his valuable discussions during the final preparation of this paper.

References