**Study on structure and function of sucrose phosphate synthase from sugarcane**

サトウキビ由来 sucrose phosphate synthase の構造機能研究

A doctoral thesis

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**Abbreviation**

DTT; dithiothreitol

| | |
|---|---|
| EDTA; | ethylenediaminetetraacetic acid |
| F6P; | fructose-6-phosphate |
| GFP; | green flouresence protein |
| G6P; | glucose-6-phosphate |
| GTD; | glucosyl-transferase domain |
| IPTG; | isopropyl β-D-1-thiogalactopyranoside |
| LB; | luria broth |
| PHD; | phosphohydrolase domain |
| PMSF; | phenylmethylsulfonyl fluoride |
| SDS-PAGE; | sodium dodecyl sulfate poly-acrylamide gel electrophoresis |
| SPS; | sucrose phosphate synthase |
| S6P; | sucrose-6-phosphate |
| TEV; | tobacco etch virus |
| UDP-G; | uridine diphosphate glucose |

# General Introduction

## Sucrose biosynthesis

It has been discovered that sucrose is the most common sugar in plant life,

including unicellular algae and cyanobacteria. Sucrose biosynthesis occupies a crucial role in the functional biology of plant, whereas the function of sucrose in microorganism is still unanswered. The recent biochemical and molecular studies of sucrose biosynthesis in prokaryotic offer new insight into the origin of sucrose metabolism. Sucrose is the major products of plant photosynthesis transported from leaves to provide the rest of plant for growth, development, signal transduction, and storage reserves for allocation carbon resources in some plants (Lunn, J. E., et al 2003b; Salerno, G. L., et al 2003).

The enzymes are responsible for sucrose metabolism has been characterized in sucrose biosynthesis related proteins (SBRPs) including sucrose-phosphate synthase (SPS), sucrose synthase (SuS), and sucrose-phosphate phosphatase (SPP) (Cumino, A., et al 2002; Salerno, G. L., et al 2003). SPS plays a key role in the pathway of sucrose synthesis, which catalyzes the formation of sucrose-6-phosphate (S6P) from uridine diphosphate glucose (UDP-G) and fructose-6-phosphate (F6P) as substrates (Amir, J., et al 1982; Leloir, L. F., et al 1954; Huber, S. C., et al 1996). S6P is further hydrolyzed to sucrose by SPP. The hydrolysis of the intermediate by SPP leads to an essentially irreversible pathway providing an efficient production of substrate. SuS involves a reversible reaction in the sucrose synthesis and cleavage. However, plant SuS generally has been associated with cleavage of sucrose and UDP to fructose and UDP-G. Another enzyme has been linked with sucrose cleavage is invertase. Otherwise, invertase catalyzes the irreversible cleavage sucrose to glucose and fructose (Fig. 1) (Koch, K., et al 2004; Salerno, G. L., et al 2003; Winter, H., et al 2000).

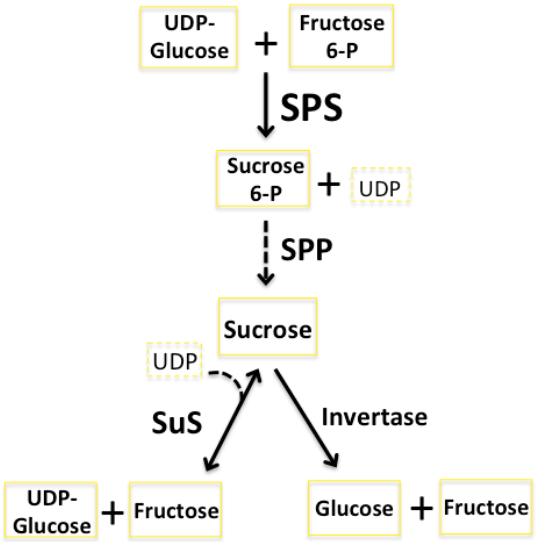



**Fig. 1-1 Scheme of the sucrose biosynthesis**

The structure of SBRPs in plant and cyanobacteria are suggested to be multiple-domain proteins and it may have originated from primordial functional domains of sucrose biosynthesis. The domainal protein of the most SBRPs is a functional glucosyl-transferase domain (GTD) and phosphohydrolase domain (PHD) (Fig. 1-2). SuS and SPS are sharing conserved residues with functional GTD and cyanobacterial SPP defined a PHD of SBRPs. The characteristic of the structural features of SBRPs is including the extension of N-terminal at GTD of SuS, N-terminal at GTD of plant SPS, and C-terminal at PHD of plant SPS. All of the extensions have been suggested to represent oligomeric proteins (Cumino, A., et al 2002; Salerno, G. L., et al 2003).

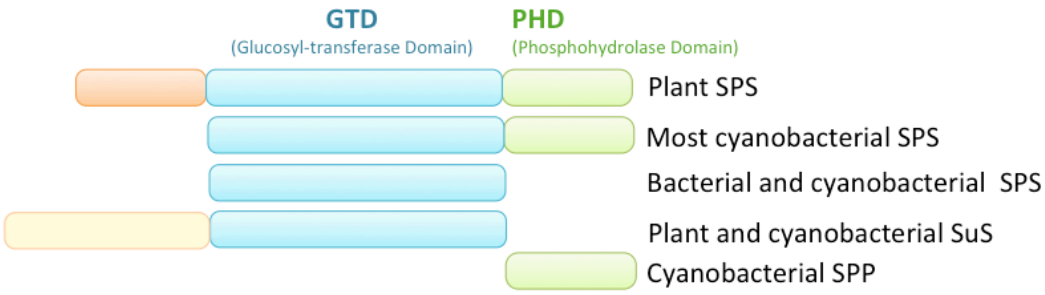



**Fig. 1-2 Structure analysis of SBRPs**

The biochemical studies in less-complex organism, such as cyanobacteria, proteobacteria, and lower plants, has recently increased our knowledge of new perspective on plant sucrose metabolism and the structural studies of SBRPs will give an important contribution to their catalytic and regulatory properties (Salerno, G. L., et al 2003). However, the crystal structure of plant SPS which has pivotal role for synthesis of sucrose is still unknown. Therefore, structural studies of plant SPS is necessary to draw more extensive story of mechanism on sucrose metabolism.

**Regulation of sucrose phosphate synthase (SPS) in higher plants**

SPS catalyzes the transfer of glycosyl group of UDP-G and F6P as substrates to S6P. It has been believed that SPS plays the physiological role of regulating photosynthetic carbon flux into sucrose. The activity of SPS is regulated by diurnal cycles of day/night (Ohsugi, R., et al 1987) and other environmental factors such as osmotic stress (Quick, P., et al 1989) and cold temperature (Hurry, V. M., et al 1994).

Plant SPS is under complex regulation involving allosteric control by metabolites, such as glucose-6-phospate (G6P) and inorganic phosphate (Pi) (Fig. 1-3). An increase in G6P and a decrease in Pi, as might occur during dark to light transition

and it would favor activation of SPS. Simultaneously, the catalytic activity would be increased as the result of allosteric regulation (Huber, S. C., et al 1996).

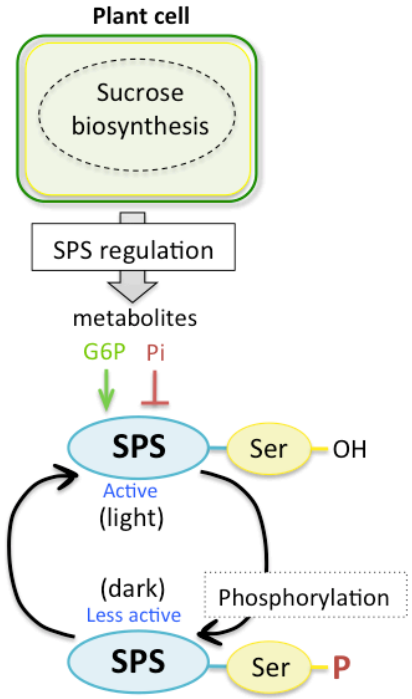



**Fig. 1-3 SPS regulation in higher plant**

Plant SPSs have a molecular mass around 120 kDa and consists of three domains. The central region contains glycosyltransferase domain responsible for the catalytic function of SPS (Castleden, C. K., et al 2004). The C-terminal region resembles sucrose phosphate phosphatase and the N-terminal region has no clear similarity with any other proteins. It has been proved by biochemical and sequential analysis that SPS has three different domains: the minimal catalytic SPS unit which defines a functional GTD; GTD with PHD; and plant SPS which consists of N-terminal regulatory region, GTD, and PHD (Fig. 1-2) (Salerno, G. L., et al 2003). The domains arrangement would be responsive to different mechanism for controlling the metabolism of sucrose. Notably, cyanobacteria SPSs (Cumino, A., et al 2002) and

bacterial SPSs (Chua, T. K., et al 2008) which do not appear to have N-terminal region are not activated by allosteric effectors.

The knowledge of plant SPS regulatory function still remains unclear. With the aim of studying biochemical and structural basis of plant SPS, it would give an important insight into the reaction mechanism of plant SPS as well as the bacterial enzyme.

**Characterization of SPS from sugarcane**

Sucrose is the main storage reserve in sugarcane (*Saccharum officinarum*) and the accumulation of sucrose has been dependent on enzymatic activity of sucrose synthesis, such as SPS (Zhu, Y. J., et al 1997; Castleden, C. K., et al 2004). In some plants, including sugarcane, SPS plays role not only as a regulator of photosynthetic genes expression but also of non-photosynthetic genes. In the previous study, it was identified two cDNAs encoding sugarcane SPS with different characterization in the photosynthetic and non-photosynthetic tissues (Sugiharto, B., et al 1997).

Two clones encoding SPS were obtained from cDNA library of sugarcane leaves. The comparison between deduced amino acid of sugarcane SPS, *So*SPS1 and *So*SPS2, and SPSs from other species showed that *So*SPS1 had the highest homology about 95% identical to maize SPS and less but significant homology to spinach SPS (56%), sugar beet SPS (56%), and potato SPS (55%). *So*SPS2 had significant homology to maize (50% identical), spinach (58%), sugar beet (57%), and potato (56%). The corresponding sequences revealed that 49% identity between *So*SPS1 and *So*SPS2

(Sugiharto, B., et al 1997).

It also has been reported that *So*SPS1 and *So*SPS2 are accumulated in different organs and functional differentiation. *So*SPS1 was differentially regulated in response to light and predominantly detected in leaves, whereas *So*SPS2 was not affected by light and slightly expressed at similar level in leaves and roots (Sugiharto, B., et al 1997).

Carbon partitioning is a critical process in distributing chemical energy converted by plant through photosynthesis. As noted above, the regulation of *So*SPS1 activity is representative for sucrose biosynthesis enzyme which is regulated in the photosynthetic tissue. In this thesis, *So*SPS1 has been used for understanding the plant SPS regulation in sugarcane.

## Study 1.
## Expression of *So*SPS1 in E. coli

## Introduction

The crystal structure of SPS from the nonphotosynthetic bacterium *Halothermothrix orenii* was reported and the structure revealed the mechanisms of substrate binding and glycosyl transfer (Chua, T. K., et al 2008). This knowledge could give an important insight into the reaction mechanism of plant SPS as well as the bacterial enzyme. However, the structural basis for the regulatory function of plant SPS still remains totally unknown. Unlike plant SPS, bacterial enzyme activity is not allosterically regulated. Approximately 180 amino acids at N-terminal region and C-terminal region of plant SPS are missing in *H. orenii* bactaria SPS (Chua, T. K., et al 2008).

It was previously cloned cDNAs from sugarcane leaves, *So*SPS1 and *So*SPS2 encoding two isoforms of SPS. *So*SPS1 is abundantly expressed in sugarcane leaves and is considered to be a representative of the isoenzyme responsible for photosynthetic carbon allocation with regulatory function (Sugiharto, B., et al 1997). In this study, cDNA of *So*SPS1 was expressed in *E. coli* and with the aim of studying structure-function relationship of the regulatory mechanism of the plant enzyme, the recombinant plant SPS with quality and amount sufficient for biochemical analysis has been prepared.

## Material and methods

*Construction of various expression plasmids of sugarcane So*SPS1 *cDNA in E. coli*

To allow the expression of full size enzyme encoded by sugarcane *So*SPS1

(gene accession No. AB001337 in GenBank) in *E. coli*, *Nco*I/*Sac*I fragment of *So*SPS1 including the coding region of the N-terminal part of SPS was ligated into pTrc99A vector (Pharmacia) and the remaining coding region of *So*SPS1 starting from *Xho*I up to *Sph*I (filled in) was inserted into the downstream of the N-terminal part. These steps yielded pTrcSPS(Full) for the expression of full-length SPS under control of *trc* promoter.

To produce SPS fused with a tag, the full coding region of *So*SPS1 was inserted into pTrcHisA with an N-terminal $His_6$ tag (Life Technologies, Invitrogen). The coding region was amplified by PCR using forward and backward primers generating *Sbf*I and *Eco*RI sites. The PCR amplified fragment was digested with *Sbf*I and *Eco*RI and inserted into *Pst*I and *Eco*RI sites of the vector.

*Expression and purification of So*SPS1 *in Escherichia coli*

The constructed plasmids were introduced into *E. coli* BL21 (DE3) competent cells (Novagen) to express pTrc99A-*So*SPS1, pTrcHisA-*So*SPS1, and *So*SPS1 mutants. The transformed bacterial cells were grown in 50 ml of Luria Broth (LB) media containing 50 μg/ml of ampicillin overnight at 30°C. The seed culture was inoculated into a fermenter containing 8 L of LB media with 50 μg/ml of ampicillin and further grown for 3-5 hr until an early log phase. Then, the culture temperature was shifted to 20°C, IPTG was added at a final concentration of 0.05 mM, and the culture was continued overnight. The cells were harvested by centrifugation at 6,000 rpm for 6 min at 4°C and stored at -20°C until use.

The frozen bacterial cells were suspended in extraction buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF], and disrupted by sonication on ice. Cell homogenate was centrifuged at 15,000 rpm for 15 min at 4$^{o}$C and the resulting supernatant was mixed gently with DE52 anion exchange cellulose (Whatman). The mixture was filtered through Miracloth (Calbiochem) and the filtrate was directly loaded onto a column of cOmplete His-Tag Purification Resin (Roche) equilibrated with 50 mM Tris-HCl (pH 7.5) /150 mM NaCl. The column was washed with the same buffer containing 30 mM imidazole and 10% glycerol (w/v) and the resin binding proteins were eluted by increasing concentration of imidazole to 120 mM. The eluted sample from the His-tag resin column was concentrated and applied to size exclusion chromatography on a Superdex 200 column (GE Healthcare Life Sciences) and eluted with 50 mM Tris-HCl (pH 7.5) /150 mM NaCl.

Purification of the full length SPS without His tag was carried out by a combination of ion-exchange, hydrophobic, and size exclusion chromatographies as described in Fig. 2-1.

*Partial purification of SPS from sugarcane and maize leaves*

Frozen leaves were pulverized with liquid $N_2$ and the proteins were extracted in 3 volumes (v/w) of an extraction buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, and 10% PVPP]. The homogenate was centrifuged at 14,000 rpm at 4$^{o}$C for 15 min and the supernatant fluid was filtered through two layers cheesecloth. Crude SPS was recovered by PEG-8000 fractionation between 6% and

12% according to the method described previously (Torres, W. -K., et al 1987; Walker, J. L., et al 1989). The SPS-containing precipitate was resuspended with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and10% glycerol (v/v).

*SDS-PAGE and Western blot analysis*

Proteins in the total bacterial extract or in fractions obtained during purification were separated by SDS polyacrylamide-gel (10%) electrophoresis (PAGE) as described by Laemmli (1970). The gels were directly stained with Coommassie Brilliant Blue or electroblotted onto Immobilon-P transfer membrane (Millipore) for immunodetection. The polyclonal antibody was prepared against C-terminal 362 amino acid residues of the coding region of *So*SPS1. Proteins reacted with the antibody was visualized with alkaline phosphatase-conjugated goat secondary antibodies (BioRad) using the NBT/BCIP for color development. The quantification of protein bands stained with Coommassie Brilliant Blue and visualized by the color development was performed by densitometry using ImageJ software (http://imagej.nih.gov/ij/).

*Assay of SPS activity*

SPS activity was assayed as described previously (Huber, S. C., et al 1985) with an appropriate modification. The assay mixture (50 μl) contained 50 mM Hepes-NaOH (pH 7.5), 20 mM $MgCl_2$, 20 mM F6P, and 20 mM UDP-G. The mixture was incubated at 25-27$^{o}$C for 10 min and reaction was stopped by an addition of 35 μl of 1 M NaOH, followed by incubation at 95$^{o}$C for 10 min to decompose unreacted F6P.

To determine sucrose formed by the enzyme reaction, 125 μl of 0.1% (w/v) resolcinol in 95% ethanol and 30% (w/v) HCl was added and heated at 85°C for 8 min. The developed color of sucrose derivative was measured at absorbance 520 nm with using a microtiter plate reader (SH-1000, Colona Electric).

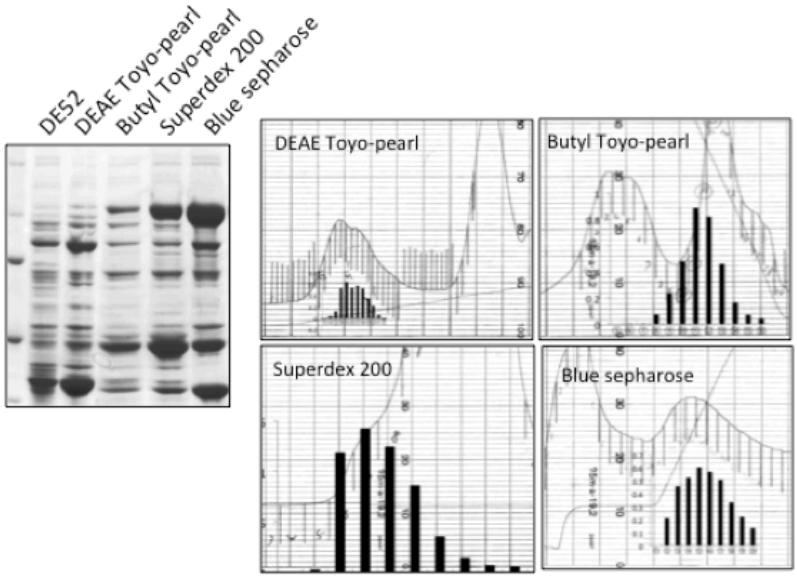



**Fig. 2-1** The pTrc99A-SPS was purified by a combination of several conventional chromatographic steps. The purification procedure consisting of ion exchange chromatography (DE52 and DEAE Toyo-pearl), hydrophobic chromatography (butyl Toyo-pearl), gel filtration chromatography (superdex 200), and affinity chromatography (blue-sepharose). Each purification step was monitored by enzymatic activity and the histogram on the chromatography chart showed the activity of SPS.

**Result**

*Expression, purification and limited-proteolysis of SPS in E. coli*

A full length cDNA of sugarcane SPS inserted into pTrc vector was introduced into *E. coli* cells and its expression was checked by Western blot analysis of the total bacterial cell extracts. As shown in Fig. 2-2A, two major bands with different mobility on the SDS-PAGE gel were detected in the SPS gene transformed cells, but not in the control cells; a larger band with around 120 kDa and a shorter one with 100 kDa were designated as Form A and Form B, respectively. Form A was firstly appeared in the bacterial cells at an early stage of growth, while Form B was increased in the cells of prolonged cultivation. Enzyme activity of SPS was measured using the total extracts of bacterial cells which accumulated the two forms with varying ratios (Fig. 2-2B). The activity level was varied during a time course of the cultivation and the activity profile was similar with the accumulation profile of Form B, suggesting that Form B might be enzymatically more active than Form A.

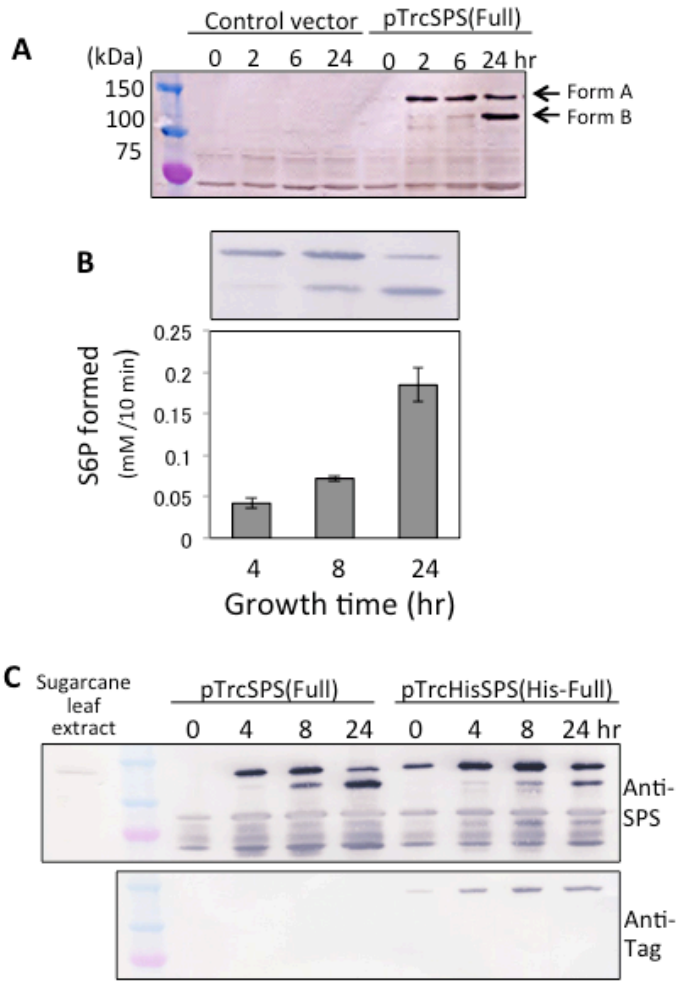
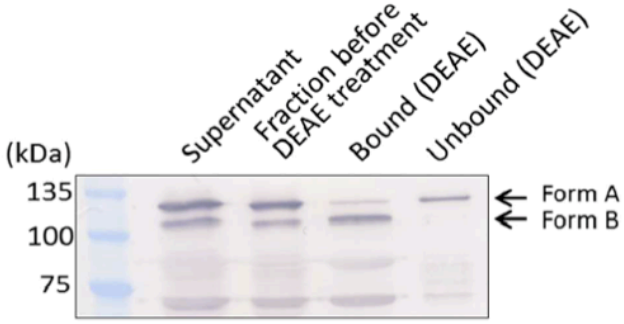



**Fig. 2-2 Expression of SPS in *E. coli* cells.** (A) Detection of SPS in *E. coli* transformed with pTrcSPS(full) or the original vector pTrc99A (control vector). The bacterial cells were grown for 0 to 24 hr and the total bacterial cell proteins were separated by SDS-PAGE, followed by immunodetection with polyclonal antibodies against SPS protein. Two major bands, Form A and B, were differentially accumulated in a culture-time dependent manner. (B) Activity and accumulation levels of SPS. The total extracts prepared from the comparable amounts of bacterial cells cultured for 4, 8, and 24 hr were subjected to activity assay and Western blotting. (C) Detection of SPS in *E. coli* transformed with pTrcSPS(full) or pTrcHisSPS(full). The total bacterial cell proteins were analyzed as in (A) by using polyclonal antibodies against SPS protein or monoclonal antibody against the tag region. Sugarcane leaf extracts were also analyzed in order to compare molecular sizes of authentic and recombinant SPSs.

The gel mobility of the two forms of recombinant SPS was compared with SPS prepared from sugarcane leaves and Form A was close in size with the authentic enzyme (Fig. 2-2C). To examine whether Form B was a proteolytic product of Form A by trimming the terminal region of SPS polypeptide, a 6-histidine-epitope-tag (His-tag) was fused to the N-terminus of SPS and its expression profile was monitored by immunoblotting using two different antibodies against SPS polypeptide and the epitope tag. As shown in Fig. 2-2C, the His-tag SPS (HisFull) was also processed to be the two forms, and Form A of HisFull retained the reactivity with the tag antibody as expected, but Form B did not. This suggested that Form B was most likely a product with the N-terminal region trimmed.

The two forms showed distinctive affinity with DEAE cellulose; Form A and Form B were recovered in the unbound and bound fractions, respectively, and the fraction containing Form B showed an activity higher than that containing Form A (Fig. 2-3). As shown in Fig. 2-1, Form B was successfully purified to a certain level of homogeneity by a combination of conventional chromatographic procedures. No clear date on the N-terminal amino acid sequence of Form B was obtained by automatic Edman degradation procedure, suggesting that its N-terminus might be blocked. Form A is hardly purified due to its non-binding nature to ion exchange resin (Fig. 2-3).




| Fraction | S6P formed (mM / 10 min) |
|---|---|
| Fraction before DEAE-resin treatment | 0.096 |
| Bound to DEAE-resin | 0.156 |
| Unbound to DEAE-resin | 0.019 |

**Fig. 2-3 Partial purification to obtain Form A SPS.** Recombinant SPS without His tag (pTrc99A-SPS) was introduced into *E. coli* cells and two forms of SPS was separated by DEAE cellulose. However, full length SPS (Form A) is hardly purified due to its non-binding to ion exchange resin. The unbound protein containing Form A fraction showed a lower activity than bound protein containing Form B fraction. The bound protein showed rising of S6P formed by ten-fold than unbound protein.

*In vitro enzymatic regulation of wild-type* and *alanine substitution So*SPS1 *mutant*

Plant SPS was clearly activated by addition of G6P and deactivated by addition of Pi. The effectors are also involved in the light modulation by reversible seryl phosphorylation. G6P is known to be an allosteric effector in many plant SPSs. SPSs from sugarcane and maize leaves are members of such allosteric enzymes. Maize SPS has a high sequence homology with sugarcane SPS (Sugiharto, B., et al 1997). By using SPS partially purified from maize leaves as a control of authentic enzyme, it was further examined how allosteric characteristics were retained in Form B of recombinant *So*SPS1 and full-length SPS from maize SPS.

The present result shows the sigmoidal curve for maize SPS kinetic properties and it indicated that maize SPS is activated on G6P activation, whereas, the Form B of recombinant *So*SPS1 has not been affected by G6P (Fig. 2-4).

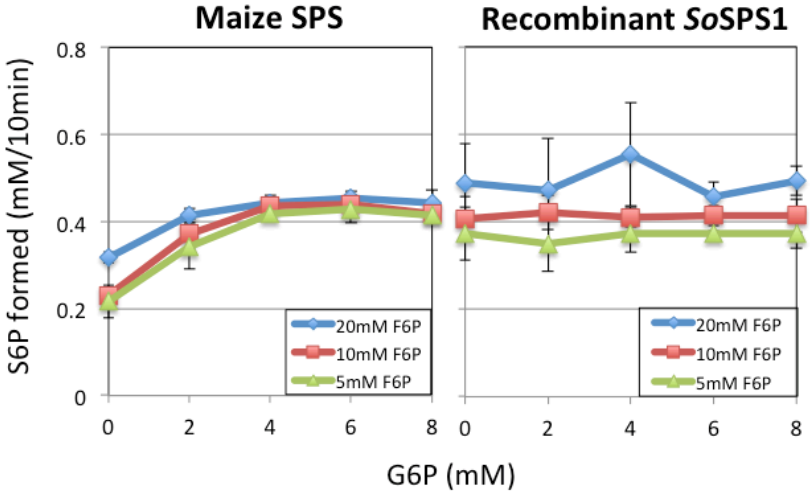



**Fig. 2-4 Kinetic properties of maize SPS and sugarcane recombinant SPS in the presence of G6P**

Using the expression vector of the His-tag SPS, the deletion of N-terminal, C-terminal, N- and C- terminal derivatives were designated as ΔN, ΔC, and ΔNC, respectively. Their expression profiles were immunologically monitored using antibody against the tag region. All genes encoding for the deletion region of SPSs were expressed in the bacterial cells after overnight cultivation (Fig. 2-5). However, the activity level showed the contrast result between deletion of N-terminal and C-terminal regions. The activity of C-terminal deletion mutant was completely lost, while N-terminal deletion mutant showed the retained SPS activity.

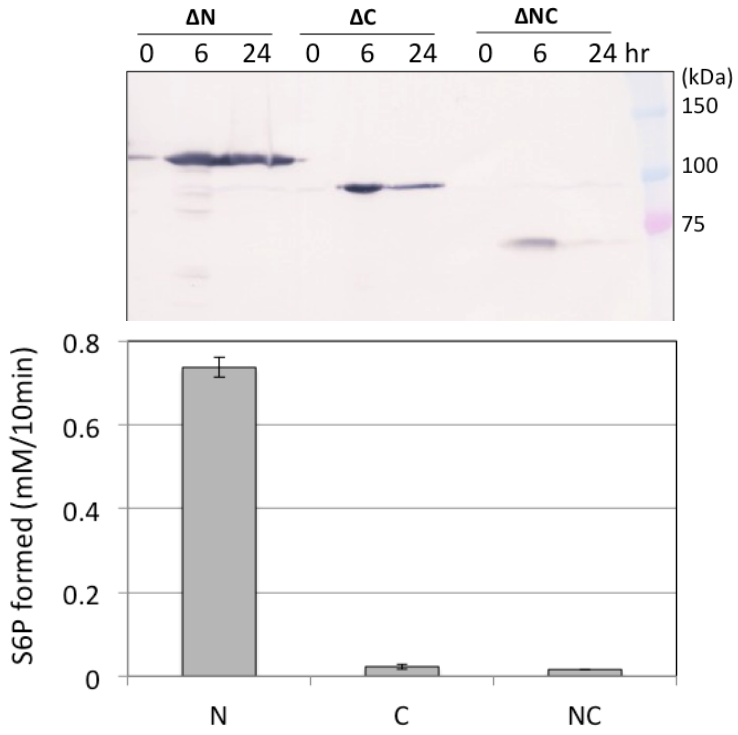



**Fig. 2-5 The expression of N-terminal and C-terminal deletion of SPS**

**Discussion**

There are several papers reporting proteolytically cleaved of SPSs from plant leaves in the course of purification to produce two fragments around 90-100 kDa and 30 kDa. Recombinant SPS expressed in the bacterial cells was also processed to a cleaved form shorter by 20-30 kDa than the original size. All recombinant enzymes were shown to be active, but it was not clearly described whether their activity was modulated by the metabolic effectors or not. This is most probably because the full length and cleaved enzymes could not be separately characterized. More extensive and detailed studies were apparently necessary for understanding of structure-function relationship of plant SPSs. In this study, it has been expressed sugarcane SPS in *E. coli* and observed that the original SPS with a size of 120 kDa was trimmed down to be a shorter form with 100 kDa during bacterial cultivation. When His-tag was fused at the N-terminus of SPS, the trimmed form had no tag-region (Fig. 2-2), indicating that the proteolytic cleavage occurs at the N-terminal side.

It was also reported the expression of SPS from plant leaves in *Escherichia coli* (Worrell, A. C., et al 1991; Sonnewald, U., et al 1993; Chen, W. L., et al 2007), but resulting recombinant enzymes were not activated by G6P and Pi. The expressed protein from *E. coli* did not show a clear property of the allosteric regulation and the reason for that is not clearly understood yet (Chen, W. L., et al 2007). It suggested that the loss of allostery properties due to the cleavage of N-terminal region. As some bacterial SPSs lacking the N- and C-terminal regions of plant SPSs show no allosteric regulation, possible allosteric properties of the truncated sugarcane enzyme are

interesting.

To clarify the functional regulatory properties of sugarcane SPS, it was expressed the mutant with N-terminal, C-terminal, both N- and C-terminal deletion. The result showed that deletion of N-terminal SPS activity is retained. Conversely, deletion of C-terminal region showed the loss of enzyme activity. It suggested that C-terminal region of SPS has a crucial role in catalytic enzyme regulation and N-terminal deletion is related to enzymatical properties and allostery regulation. Further detailed studies of N-terminal region of sugarcane SPS will be undertaken to elucidate the mechanism of SPS allostery regulation.

## Study 2.

## The importance of N-terminal *So*SPS1

## Introduction

Plant SPSs have a molecular mass around 120 kDa and consists of three domains. The central region contains glycosyltransferase domain responsible for the catalytic function of SPS (Castleden, C. K, et al 2004). The C-terminal region resembles sucrose phosphate phosphatase and the N-terminal region has no clear similarity with any other proteins. The SPSs from the photosynthetic cyanobacteria (Lunn, J. E., et al 1999) and nonphotosynthetic bacteria (Huynh, F., et al 2004) lack the N-terminal domain or both terminal domains of plant SPS, respectively, and unlike plant SPS, their enzyme activity is not allosterically regulated (Lunn, J. E., et al 2003a; Chua, T. K., et al 2008). This indicates that the domain(s) characteristic of plant SPS is(are) contributed to the regulatory properties and that, in particular, the unique N-terminal region is likely to be involved in the regulation of enzyme activity.

In this study, cDNA of *So*SPS1 was expressed in *E. coli* and insect cells and recombinant enzymes with various lengths of the N-terminal deletion were produced. According to the result of study 1, it suggested that the N-terminal region of sugarcane SPS is not essential for the catalytic reaction itself, but plays a role in the allosteric regulation.

## Material and methods

*Construction of N-terminal truncation So*SPS1 *cDNA in E. coli*

A series of mutant SPSs with the N-terminal truncation was also constructed in a similar way of the his-tagged full-length enzyme. The truncations of the N-terminal

region were generated by PCR using six different forward primers with *Sbf*I site and the backward primer containing *Eco*RI site as used for full-length SPS. The PCR product of the N-terminal truncated forms designated as HisΔN1, HisΔN2, HisΔN3, HisΔN4, HisΔN5, and HisΔN6 were subcloned into pGEM-T vector (Promega) that contains 3'-T overhangs at the insertion site and then cut with *Sbf*I and *Eco*RI. The resulting DNA fragments encoding the N-terminal truncated SPSs were inserted into *Pst*I and *Eco*RI sites of pTrcHisA. All the SPS genes thus constructed were verified by DNA sequencing.

*Expression and purification of N-terminal truncated of So*SPS1 *in Escherichia coli*

The constructed plasmids were introduced into *E. coli* BL21 (DE3) competent cells (Novagen) to express the full length SPS and various N-terminal truncated forms. The transformed bacterial cells were grown in 50 ml of Luria Broth (LB) media containing 50 μg/ml of ampicillin overnight at 30$^{o}$C. The seed culture was inoculated into a fermenter containing 8 L of LB media with 50 μg/ml of ampicillin and further grown for 3-5 hr until an early log phase. Then, the culture temperature was shifted to 20$^{o}$C, isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.05 mM, and the culture was continued overnight. The cells were harvested by centrifugation at 6,000 rpm for 6 min at 4$^{o}$C and stored at -20$^{o}$C until use.

The frozen bacterial cells were suspended in extraction buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF], and disrupted by sonication on ice. Cell homogenate was centrifuged at 15,000 rpm for 15

min at 4°C and the resulting supernatant was mixed gently with DE52 anion exchange cellulose (Whatman). The mixture was filtered through Miracloth (Calbiochem) and the filtrate was directly loaded onto a column of cOmplete His-Tag Purification Resin (Roche) equilibrated with 50 mM Tris-HCl (pH 7.5) /150 mM NaCl. The column was washed with the same buffer containing 30 mM imidazole and 10% glycerol (w/v) and the resin binding proteins were eluted by increasing concentration of imidazole to 120 mM. The eluted sample from the His-tag resin column was concentrated and applied to size exclusion chromatography on a Superdex 200 column (GE Healthcare Life Sciences) and eluted with 50 mM Tris-HCl (pH 7.5) /150 mM NaCl.

*Expression of So*SPS1 *in Sf9 insect cells and purification of the full-size SPS*

*So*SPS1 cDNA was PCR-amplified with primers shown in Fig. 3-1. A 5'-untranslated leader sequence of a lobster tropomyosin cDNA (L21) was included in the forward primer for the enhancement of protein expression **(**Sano, K. I., et al 2002). The PCR product was ligated into pGEM-T Easy vector (Promega), cut with *Not*I and *Eco*RI, and then subcloned into the pFastBac1 (Thermo Fisher Scientific) vector for baculovirus expression in Sf9 insect cells. A tobacco etch virus (TEV) protease cleavage site, $His_8$-myc-$His_8$ tag, and EGFP were introduced at the carboxyl terminus of *So*SPS1.

The bacmid and baculovirus of *So*SPS1 were generated using Bac-to-Bac system (Thermo Fisher Scientific). Sf9 cells were grown to ~$4.0 \times 10^6$ cells/ml in an Erlenmeyer flask containing PSFM-J1 medium (Wako) and infected with P2 virus at

28°C. After 24 hr post-infection, the cells were further incubated under low temperature at 20°C for 48 hr. The cells were harvested by centrifugation at 4,000*g* for 5 min at 4°C, resuspended in extraction buffer [50 mM Tris-HCl (pH7.5), 1 M NaCl, 20 mM imidazole (pH7.5), 2 mM DTT, 2 mM benzamidine], and then disrupted by sonication. The insoluble fraction was removed by centrifugation at 40,000*g* for 20 min at 4°C and the supernatant was mixed gently with Ni-NTA Agarose (Qiagen) resin for 30 min at 4°C. The resin was washed thoroughly with wash buffer A [50 mM Tris-HCl (pH7.5), 0.3 M NaCl, 20 mM imidazole (pH7.5), 2 mM DTT]. SPS-EGFP was eluted with elution buffer [50 mM Tris-HCl (pH7.5), 0.3 M NaCl, 250 mM imidazole (pH7.5), 2 mM DTT]. SPS-EGFP fraction was batch incubated with 2 ml of GFP-nanotrap resin for 30 min at 4°C with gentle rotation (Rothbauer, U., et al 2008). The resin was washed with wash buffer B [20 mM Tris-HCl (pH7.5), 0.5 M NaCl, 5 mM DTT]. SPS without the tag was eluted from GFP-nanotrap resin by cleaving the linker between SPS and $His_8$-myc-$His_8$-EGFP Tag with TEV protease for 4 hr at 4°C, leaving the tag bound to the resin. The eluted SPS was concentrated and further purified by size-exclusion chromatography on a column of Superdex 200 (GE Healthcare Life Sciences) in buffer [20 mM Tris-HCl pH7.5, 0.5 M NaCl, 5 mM DTT].

| Primers | Sequence (5' to 3') |
|---|---|
| L21-Full-SPS-F | GCGGCCGCAACTCCTAAAAAACCGCCACCATGGCCGGGAACGAG TGG |
| SPS-nonST-R | GAATTCCATGCCGCTAGAAGTCTTGGAGAC |

**Fig. 3-1** The primers that used to construct full-length SPS expressed in Sf9 insect cells

*SDS-PAGE and Western blot analysis*

Proteins in the total bacterial extract or in fractions obtained during purification were separated by SDS polyacrylamide-gel (10%) electrophoresis (PAGE) as described by Laemmli (1970). The gels were directly stained with Coommassie Brilliant Blue or electroblotted onto Immobilon-P transfer membrane (Millipore) for immunodetection. The gels were directly stained with Coommassie Brilliant Blue or electroblotted onto Immobilon-P transfer membrane (Millipore) for immunodetection with the polyclonal antibodies against SPS or monoclonal antibody against Xpress™ epitope contained in the tag (Invitrogen). Proteins reacted with the antibody was visualized with alkaline phosphatase-conjugated goat secondary antibodies (BioRad) using the NBT/BCIP for color development. The quantification of protein bands stained with Coommassie Brilliant Blue and visualized by the color development was performed by densitometry using ImageJ software (http://imagej.nih.gov/ij/).

*Assay of SPS activity*

SPS activity was assayed as described previously (Huber, S. C., et al 1985) with an appropriate modification. The assay mixture (50 μl) contained 50 mM Hepes-NaOH (pH 7.5), 20 mM $MgCl_2$, 20 mM F6P, and 20 mM UDP-G. The mixture was incubated at 25-27$^{o}$C for 10 min and reaction was stopped by an addition of 35 μl of 1 M NaOH, followed by incubation at 95$^{o}$C for 10 min to decompose unreacted F6P. To determine sucrose formed by the enzyme reaction, 125 μl of 0.1% (w/v) resolcinol in 95% ethanol and 30% (w/v) HCl was added and heated at 85$^{o}$C for 8 min. The

developed color of sucrose derivative was measured at absorbance 520 nm with using a microtiter plate reader (SH-1000, Colona Electric).

**Result**

*Production of the N-terminal truncated forms of SPS*

As the N-terminal truncation would give a significant effect on its enzymatic nature as described above, a series of the N-terminal truncated forms of SPS with different lengths deleted was considered to be useful for further investigation of an effect of the N-terminal region, if any, on the enzyme activity. Using the expression vector of the His-tag SPS as described above, the N-terminal truncated derivatives designated as HisΔN1 to HisΔN6 were constructed (Fig. 3-2A). Their expression profiles were immunologically monitored using two antibodies against SPS polypeptide and the tag region (Fig. 3-2B). All genes encoding for the N-terminal truncated forms were expressed in the bacterial cells and polypeptides with shorter sizes corresponding to the length of truncation were found as a major band in bacterial cells grown for 4 hr, which was reacted with both antibodies. After overnight cultivation, additional polypeptide with the size equivalent with Form B appeared in all the truncated SPSs except for HisΔN6. These shorter polypeptides were not reacted with the tag specific antibody, indicating that the limited proteolysis occurred irrespective of the length of the N-terminal region. HisΔN6 did not produce such shorter band and this was most probably due to the lack of the proteolytic site, indicating that the cleavage site should be within the regions between HisΔN5 and HisΔN6. It is noted that HisΔN6 lost Ser162,

which was proposed to be one of phosphorylated serine residues involved in the modulation of SPS activity (Sugiharto, B., et al 1997). Accumulation level of HisΔN1 became lower than a limit of our immunological detection during the overnight growth.

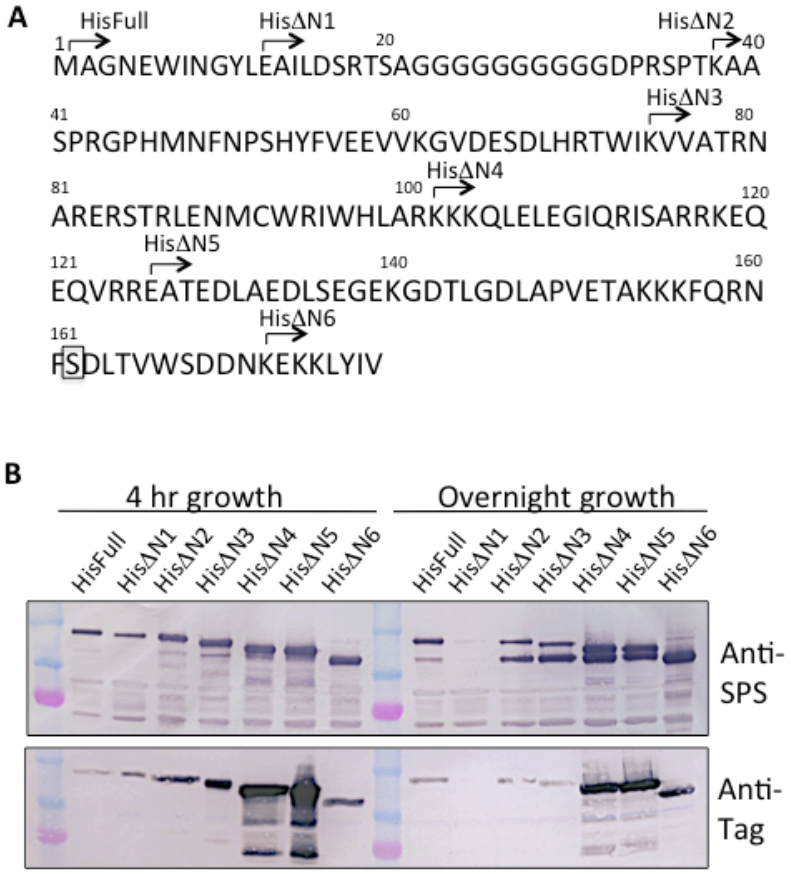



**Fig. 3-2 Expression of the N-terminal truncated forms of SPS in *E. coli*.** (A) Sites of the N-terminal truncation of SPS. *So*SPS1 genes with deletion of the increasing length of the N-terminal coding region were inserted into pTrcHis vector to construct fusion with his-tag. The resulting truncated forms of SPS were designated as HisΔN1 to HisΔN6. Ser162 marked with box is proposed to be a phosphorylation site (Sugiharto, B., et al 1997). (B) *E. coli* cells transformed with the N-terminal truncated constructs were grown for 4 hr or overnight. The total cell proteins from each transformant were analyzed by Western blotting using antibodies against SPS polypeptide and the tag region as in Fig. 1C. Major SPS bands with decreasing size corresponding to the length of truncation were detected with both antibodies.

The N-terminal truncated forms of SPS were subjected to chromatography on a Ni-resin column and representative data of HisΔN5 is shown in Fig. 3-3A. This purification step gave us an advantage to remove efficiently the limited proteolysis product. Fractions containing HisΔN5 were combined and further subjected to a size exclusion chromatography. HisΔN5 was eluted as a single major peak at a retention volume corresponding to a molecular size of 370 to 380 kDa (Fig. 3-3C), indicative of an oligomeric structure in a native state. HisΔN4 and HisΔN6 were also eluted in the same profile with HisΔN5 (data not shown). All truncated forms except for HisΔN1 were thus successfully prepared by using the Ni-resin column as shown in Fig. 4A. Purification of HisFull and HisΔN1 was not successful, since they were hardly bound to the Ni-resin column. The reason for this phenomenon was not clear, but it presumes that the His-tag region might be masked within the whole structure of SPS molecule in a manner not accessible with the resin. The presence of His-tag had been confirmed in an SDS-denatured state by immunoblotting with the tag-specific antibody (Fig. 3-2B).

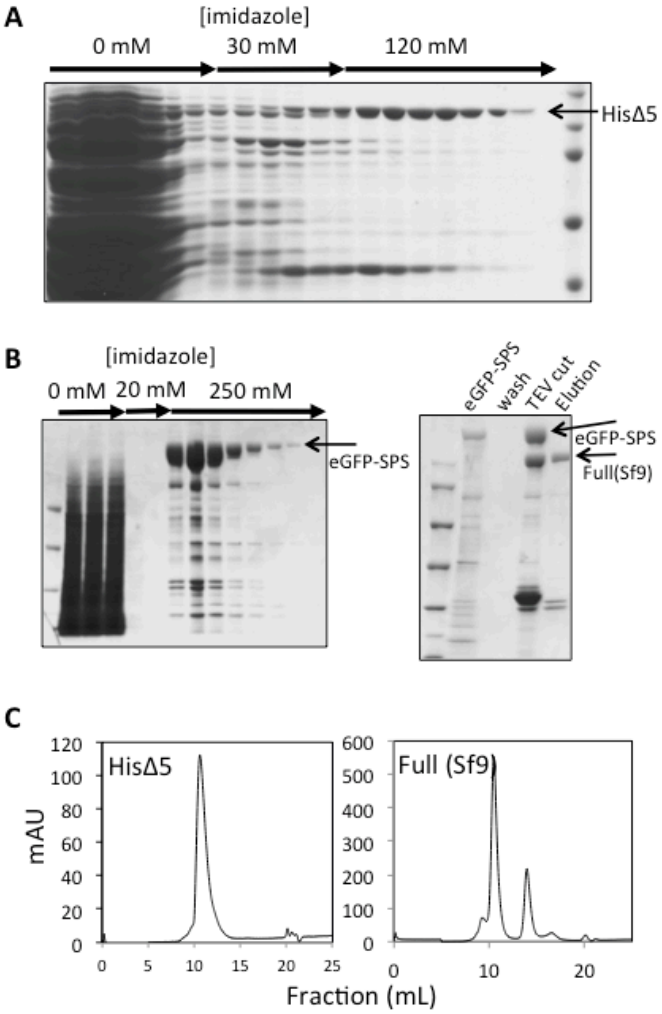



**Fig. 3-3 Purification of the N-terminal truncated and full-length forms of SPS expressed in *E. coli* and insect cells, respectively**. (A) Chromatography of HisΔN5 on a column of Ni-NTA resin. (B) Sequential chromatography of Full (Sf9) on columns of Ni-NTA resin and GFP nano-trap resin. (C) Size exclusion chromatography of HisΔN5 and Full (Sf9). HisΔN5 and Full (Sf9) obtained as described above were further purified by size exclusion chromatography on a column of Superdex S-200.

*Expression of SPS in insect cells*

Preparation of the full length SPS was not successful in an *E. coli* recombinant expression system. Then, baculovirus expression system using the Sf9 insect cell line was applied. The C-terminus of SPS fused with His tag and EGFP was expressed in Sf9

cells and resulting expressed SPS was purified by successive chromatographies on columns of Ni resin and GFP-nanotrap resin (Fig. 3-3B). The EGFP tag region was removed by digestion with TEV protease and the full length SPS thus obtained, Full(Sf9), was confirmed to have an molecular size equivalent with authentic SPS from sugarcane leaves (data not shown). Full(Sf9) was subjected to a size exclusion chromatography and eluted with an elution time comparable with that of HisΔN5 (Fig. 3-3C).

*Effect of the N-terminal truncation on enzyme activity*

Activity of Form B and the truncation forms of HisΔN2 to HisΔN6, using at least partially purified preparation (Fig. 3-4A), were measured in the presence of the saturating concentrations of F6P and UDP-G, together with that of the full length SPS. As shown in Fig. 3-4B, the truncated forms showed an increasing tendency of the specific activity and this was the most remarkable for HisΔN6 that showed an increase in the activity higher by ten-fold than that of the full-length SPS. These results were in good agreement with the above-mentioned observation that activity of SPS in the *E. coli* cells became higher when accumulation level of Form B was increased over that of Form A.

As the activity of plant SPS is known to be affected by G6P, assay were carried out under increasing concentrations of two substrates in the presence or absence of G6P (Fig. 3-5A and B). The activity of the full length SPS was significantly enhanced in the presence of G6P and affinities for the two substrates were increased. The activity of the

N-terminal truncated forms was not significantly influenced by the addition of G6P under all conditions tested in this experiment and this was a sharp contrast with the results of full-length enzyme. It is noteworthy that affinities for UDP-G and F6P of the N-terminal truncated forms were high even in the absence of G6P. These combine data suggested that the N-terminal region of SPS, which is not proximal with the catalytic domain in the primary structure, would give a significant generative influence on the catalytic domain to lower enzyme activity and that thus, the deletions of this N-terminal region was resulted in a disappearance of the suppressive effect.

It was further examined how allosteric characteristics were retained in the full length and N-terminal truncated SPSs (HisΔN3 and HisΔN4). Under three different concentrations of F6P, G6P-dependent activation of SPS activity was measured. As shown in Fig. 3-6, the full length recombinant SPS was found to be activated by G6P in a concentration dependent manner up to around 5 mM, and this tendency was comparable with that of maize leaf SPS. Such clear allosteric property was not observed in the N-terminal truncated enzymes.

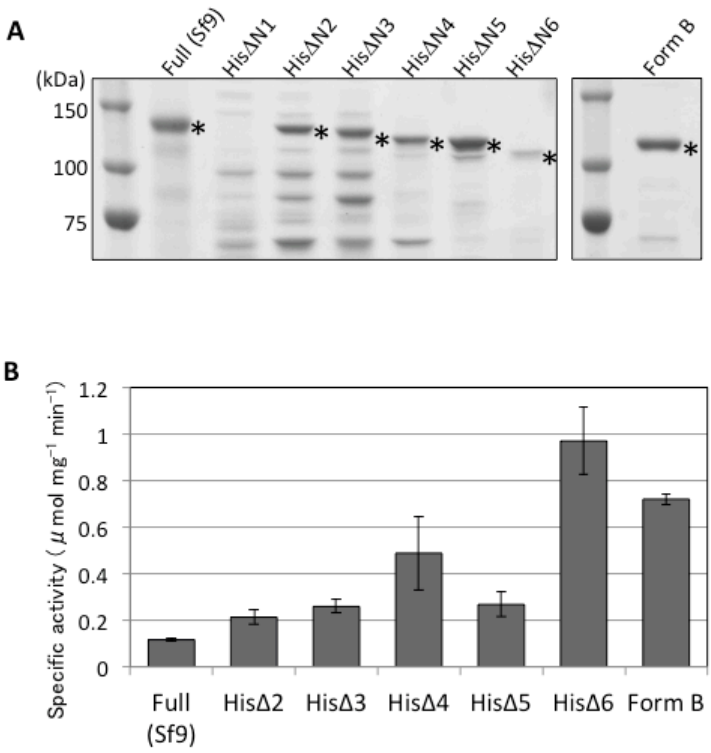



**Fig. 3-4 Comparison of activity of the N-terminal truncated and full-length forms of SPS.** (A) SDS-PAGE analysis of various forms of SPS used for activity measurement. All the N-terminal truncated forms of SPS were prepared by His-tag affinity chromatography as described in Fig. 3A. HisΔN1 was not sufficiently obtained due to a low expression in the bacterial cells (Fig. 3-2B). HisΔN4, HisΔN5, and HisΔN6 were further purified by size exclusion chromatography on a Superdex S-200 column. Full (Sf9) was prepared as described in Fig. 3B. Amounts of sample loaded on a gel were based on the activity unit to give an assay condition suitable for colorimetric quantification of enzyme reaction. Protein bands were visualized by Coomassie Brilliant Blue staining and quantification of various forms of SPS (protein bands marked by star) were carried out by densitometry using imageJ software with an appropriate standard protein. (B) Specific activity of the N-terminal truncated and full-length forms of SPS. The enzyme activity was measured in the presence of saturated levels of two substrates, 10-20 mM UDP-G and 20 mM F6P. Specific activity was calculated with the concentration of each enzyme determined as in (A).

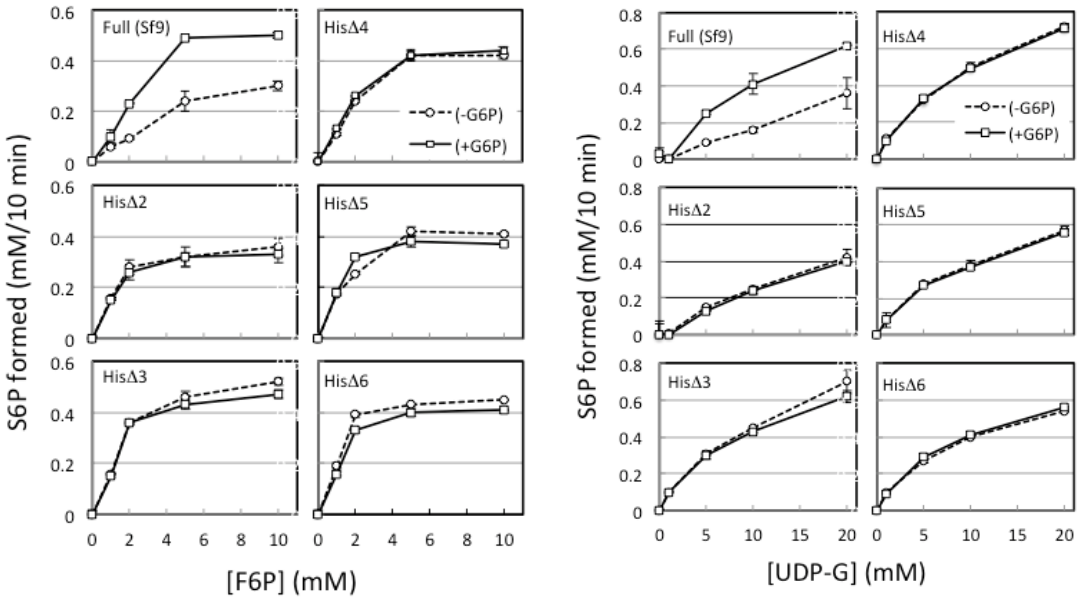



**Fig. 3-5 Kinetic properties of the N-terminal truncated and full-length forms of SPS.** (A) Activity of various forms of SPS was measured in the assay mixture containing 20 mM UDP-G and increasing concentrations of F6P from 0 to 10 mM in the presence or absence of 6 mM G6P. The amount of S6P formed for 10 min after an addition of each enzyme was determined calorimetrically as described in materials and methods. (B) Activity of various forms of SPS was measured in the assay mixture containing 10 mM F6P and increasing concentrations of UDP-G from 0 to 20 mM in the presence or absence of 6 mM G6P. Reaction rate was determined as in (A).

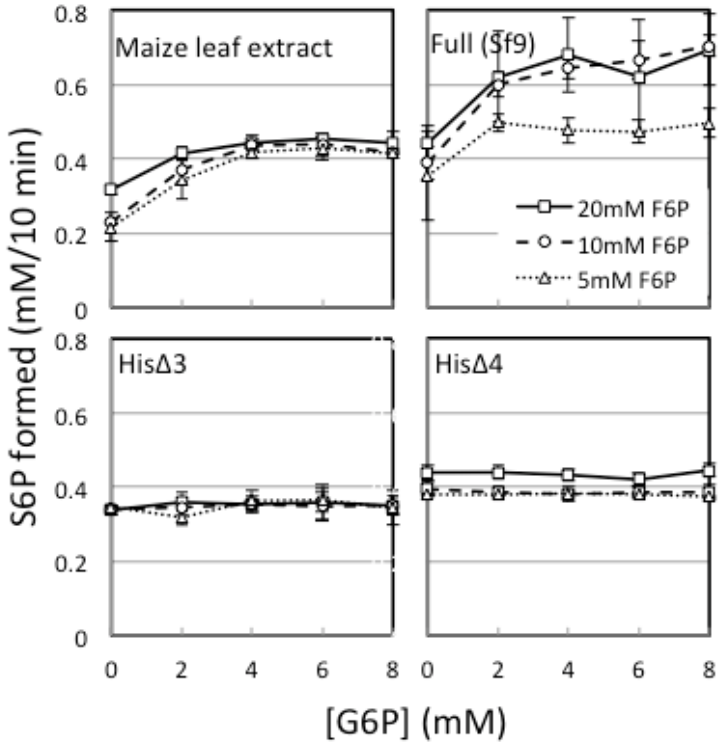



**Fig. 3-6 An allosteric effect of G6P on the activity of recombinant SPSs and authentic SPS from plant leaves.** Activity of Full (Sf9), HisΔN3, and HisΔN4 was measured in the assay mixture containing 20 mM UDP-G and 5, 10, or 20 mM F6P. Effect of allosteric activator G6P was examined by the addition of increasing concentrations from 0 to 8 mM. As a control for the allosteric activation of SPS by G6P, a crude SPS fraction prepared from maize leaves was also assayed side by side.

**Discussion**

The activity of plant SPS, a key enzyme in sucrose biosynthesis, is modulated by metabolic effectors, such as G6P and inorganic phosphate. To elucidate the regulation of SPS activity, the enzyme needs to be prepared in an amount suitable for kinetic analyses. It has obtained a series of mutants of SPS with the N-terminal truncation of increasing lengths from 37 to 171 residues (Fig. 3-2A). Purification of SPS with His-tag resin allowed us to separate the original and its trimmed forms of SPS. As the full-length SPS was not successfully prepared in *E. coli* cells, we utilized insect cells for preparation of recombinant SPS. The full-length enzyme was successfully purified as a fusion with the C-terminal His-tag and EGFP, which could be cleaved off during purification (Fig. 3-3B).

By kinetic analysis of the full length and truncated forms of sugarcane SPS thus prepared, three important results were obtained: 1) the full length enzyme showed a remarkable allosteric activation by G6P, while none of the truncated enzymes tested here had such characteristics (Fig. 3-6), 2) specific activity of the full length and truncated SPSs showed a tendency that longer truncation has higher activity (Fig. 3-4), and 3) whether G6P was added or not, the truncated forms showed similar substrate affinity with F6P and UDP-G, while the full length enzyme showed a higher substrate affinity when G6P was present (Fig. 3-5A and 3-5B).

Present biochemical studies has clarified the functional roles of the N-terminal region of sugarcane SPS. In conclusion, N-terminal region of sugarcane SPS is crucial for the allosteric regulation, but no relationship between allosteric regulation

and specific activity. It was revealed by the truncated forms showed the G6P-independent and that the increasing lengths of the deletions were resulted in an enhancement of the catalytic efficiency. These results also indicated N-terminal region may function like a suppressor domain for the enzyme activity. It would be necessary to further analysis both the full length and the N-terminal truncated forms for elucidation of the precise model of the allostery by crystal structure studies.

**Study 3.**

**The structural studies of *So*SPS1**

## Introduction

It has been reported the crystal structure of SPS from *Halothermothrix orenii* and it reveals the mechanisms of substrate binding and glycosyl transfer in nonphotosynthetic bacterium (Chua, T. K., et al 2008). However, the structural basis for the regulatory function of plant SPS still remains unknown. Plant SPSs consist of three domains, glucosyltransferase domain (GTD) (55 kDa), the C-terminal phosphohydrolase domain (30 kDa) and the N-terminal domain (20 kDa). The N-terminal domain has no apparent homology with known motifs (Cumino, A., et al 2002). Notably, cyanobacteria SPSs (Lunn, J. E., et al 1999) and *H. orenii* SPS (*Ho*SPS) (Chua, T. K., et al 2008), which lack the N-terminal domain of plant SPSs, are not allosterically regulated. As noted above, modulation in plant SPS implies an involvement of the N-terminal domain in the allosteric regulation.

According to the background of the research, it assumes that the combined studies of functional and structural information from recombinant *So*SPS1 may represent an accurate model for reaction mechanism of SPS. Therefore, the objective of research is characterization of recombinant *So*SPS1 through crystal structure determination.

## Material and methods

*Expression and purification of N-terminal truncated form of So*SPS1 *cDNA in E. coli*

Constructed plasmid of pTrcHisA-SPS was introduced into *E. coli* BL21 (DE3) competent cells (Novagen) to express HisΔN5 and HisΔN6 form of SPS. The

transformed bacterial cells were grown in 50 ml of Luria Broth (LB) media containing 50 μg/ml of ampicillin overnight at 30$^{o}$C. The seed culture was inoculated into a fermenter containing 8 L of LB media with 50 μg/ml of ampicillin and further grown for 3-5 hr until an early log phase. Then, the culture temperature was shifted to 20$^{o}$C, isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.05 mM, and the culture was continued overnight. The cells were harvested by centrifugation at 6,000 rpm for 6 min at 4$^{o}$C and stored at -20$^{o}$C until use.

The frozen bacterial cells were suspended in extraction buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF], and disrupted by sonication on ice. Cell homogenate was centrifuged at 15,000 rpm for 15 min at 4$^{o}$C and the resulting supernatant was mixed gently with DE52 anion exchange cellulose (Whatman). The mixture was filtered through Miracloth (Calbiochem) and the filtrate was directly loaded onto a column of cOmplete His-Tag Purification Resin (Roche) equilibrated with 50 mM Tris-HCl (pH 7.5) /150 mM NaCl. The column was washed with the same buffer containing 30 mM imidazole and 10% glycerol (w/v) and the resin binding proteins were eluted by increasing concentration of imidazole to 120 mM. The resulted sample from the His-tag resin column which contains SPS activity was selected and applied for additional purification step onto Source-Q (GE Healthcare Life Sciences) ion chromatography system. The eluted sample from Source-Q chromatography system was concentrated and injected to size exclusion chromatography on a Superdex 200 column (GE Healthcare Life Sciences) and eluted with 50 mM Tris-HCl (pH 7.5) /150 mM NaCl.

*Expression of So*SPS1 *in Sf9 insect cells and purification of the full-size SPS*

The bacmid and baculovirus of *So*SPS1 were generated using Bac-to-Bac system (Thermo Fisher Scientific). Sf9 cells were grown to ~4.0 × $10^6$ cells/ml in an Erlenmeyer flask containing PSFM-J1 medium (Wako) and infected with P2 virus at 28°C. After 24 hr post-infection, the cells were further incubated under low temperature at 20°C for 48 hr. The cells were harvested by centrifugation at 4,000*g* for 5 min at 4°C, resuspended in extraction buffer [50 mM Tris-HCl (pH7.5), 1 M NaCl, 20 mM imidazole (pH7.5), 2 mM DTT, 2 mM benzamidine], and then disrupted by sonication. The insoluble fraction was removed by centrifugation at 40,000*g* for 20 min at 4°C and the supernatant was mixed gently with Ni-NTA Agarose (Qiagen) resin for 30 min at 4°C. The resin was washed thoroughly with wash buffer A [50 mM Tris-HCl (pH7.5), 0.3 M NaCl, 20 mM imidazole (pH7.5), 2 mM DTT]. SPS-EGFP was eluted with elution buffer [50 mM Tris-HCl (pH7.5), 0.3 M NaCl, 250 mM imidazole (pH7.5), 2 mM DTT]. SPS-EGFP fraction was batch incubated with 2 ml of GFP-nanotrap resin for 30 min at 4°C with gentle rotation (Rothbauer, U., et al 2008). The resin was washed with wash buffer B [20 mM Tris-HCl (pH7.5), 0.5 M NaCl, 5 mM DTT]. SPS without the tag was eluted from GFP-nanotrap resin by cleaving the linker between SPS and $His_8$-myc-$His_8$-EGFP Tag with TEV protease for 4 hr at 4°C, leaving the tag bound to the resin. The eluted SPS was concentrated and further purified by size-exclusion chromatography on a column of Superdex 200 (GE Healthcare Life Sciences) in buffer [20 mM Tris-HCl pH7.5, 0.5 M NaCl, 5 mM DTT].

*Crystallization trial of N-terminal truncated form SPS*

The final purified protein from size exclusion chromatography on a Superdex 200 column was concentrated to over than 6 mg/ml and conducted the crystallization screening. The following 96-well with different temperatures and screening conditions were tested for crystallization trial, such as SaltRx (Hampton Research) which screens based on salt varying concentration and pH, PEGRx (Hampton Research) which is using polymer reagents and pH in low to medium ionic strength, and Index (Hampton Research) which is designed by specially formulated reagent zones to identify reagent class and pH are effective to producing crystals. The 100nl of the protein sample was aliquoted into the sitting drop well in 96-well plate using the Mosquito (TTP LabTech).

The remaining purified protein was further conducted Dynamic Light Scattering (Malvern Instruments) for particle size characterization of protein and Size-Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS).

**Result**

*Purification of the N-terminal truncated form expressed in E. coli and full-length SPS expressed insect cells*

The crude extracts of *E. coli* cells expressing N-terminal truncated forms, HisΔN5 and HisΔN6, and crude extracts of insect cells expressing full-length SPS were applied for various purification systems including ion exchange and the affinity columns as described above.

The eluted samples of HisΔN5 and Full (Sf9) were further purified by size

exclusion chromatography on a column of Superdex S-200. HisΔN5 and Full (Sf9) were eluted at comparable elution volumes of 10.4 ml and 10.5 ml, respectively. Calibration of molecular size was carried out with marker proteins; thyrogloburin (669 kDa) at 9.1 ml, ferritin (440 kDa) at 10.2 ml, aldolase (158 kDa) at 12.1 ml, ovalbumin (44 kDa) at 14.8 ml, carbonic anhydrase (29 kDa) at 16.2 ml, and ribonuclease A (13.7 kDa) at 17.8 ml (Fig. 3-3C). These results suggested that the molecular size of native SPS from N-terminal truncated form and full-length are similar about over than 120 kDa which indicates the formation of oligomer.

*Crystallization trial and particle size characterization of N-terminal truncated form SPS*

The crystallization of HisΔN6 was conducted with temperature up to 4$^{o}$C and 20$^{o}$C, however the result showed no crystal formed. In other hand, the molecular weight of N-terminal truncated SPS has been analyzed using light scattering. Measurement of molecular weight by SEC-MALS gives an indication of the oligomeric state (dimer).

**Discussion**

Our present data give a clear piece of evidence that the N-terminal region composed of at least 171 residues of sugarcane SPS is crucial for its allosteric sensitivity to G6P and that the N-terminal truncation as short as 37 residues leads the mutant enzyme to be constitutively active with the allosteric sensitivity lost. The N-terminal region of plant SPS was postulated to be intrinsic disordered by bioinformatics analysis (Chua, T. K., et al 2008). It has been proposed that polypeptide

regions with intrinsic disorders play a role for modulation of allostery (Ferreon, A. C., et al 2013), such as optimizing high or low affinity binding and controlling the ability to interact the protein surface (Hilser, V. J., et al 2007). Therefore, plant SPS might be one of examples for such allosteric modulation.

The modular architecture of sucrose biosynthesis enzymes has recently been suggested to represent oligomeric proteins, including SPS and sucrose synthase (SUS) (Salerno, G. L., et al 2003). All SPSs and SUSs share 20-33% homology for the sequences of GTD (Cumino, A., et al 2002) and the 3-D structures of GTDs from *Arabidopsis thaliana* SUS (*At*SUS) and bacterial SPS (*Ho*SPS) were well superimposed (Zheng, Y., et al 2011). Furthermore, *At*SUS appears as a tetramer to generate the most compact oligomer, which revealed the N-terminal half and C-terminal extensions compromise the subunit interface (Zheng, Y., et al 2011). The similarity between GTDs of SPSs and yeast glycogen synthase (Gsy2p) gave a possible mechanism for plant SPS regulation (Chua, T. K., et al 2008). In eukaryotes, glycogen synthase is oligomeric and the modulation of activity involves phosphorylation and G6P allostery (Pederson, B. A., et al 2000). The G6P binding to Gsy2p, leading a substantial conformation reorganization at the subunit interface, increases the catalytic efficiency (Baskaran, S., et al 2010). By analogy, it could be hypothesized that the N-terminal domain of plant SPS plays a direct role in allostery by inducing a structural perturbation upon binding to G6P, which might include three-model states as an unphosphorylated state with intermediate activity, phosphorylated state with reduced activity, and G6P-bound state with high activity. Our present work has not given any information on phosphorylation

status of recombinant SPS except that loss of Ser162 in HisΔN6, which is one of phosphorylated serine residues (Sugiharto, B., et al 1997), has no significant effect on SPS activity (Fig. 3-5) and further works will be necessary to draw more extensive story of the allostery of plant SPS.

Based on these results, the modulation model of sugarcane SPS has been proposed on Fig. 4-1. This model revealed that the oligomeric state has no correlation to allosteric regulation at N-terminal region. The functional intactness of N-terminal region in SPS has been noted like a suppressor domain for enzyme activity. In other hand, binding of G6P at N-terminal region has induced the enhancement of UDP-G substrate affinity binding and SPS becomes more active.

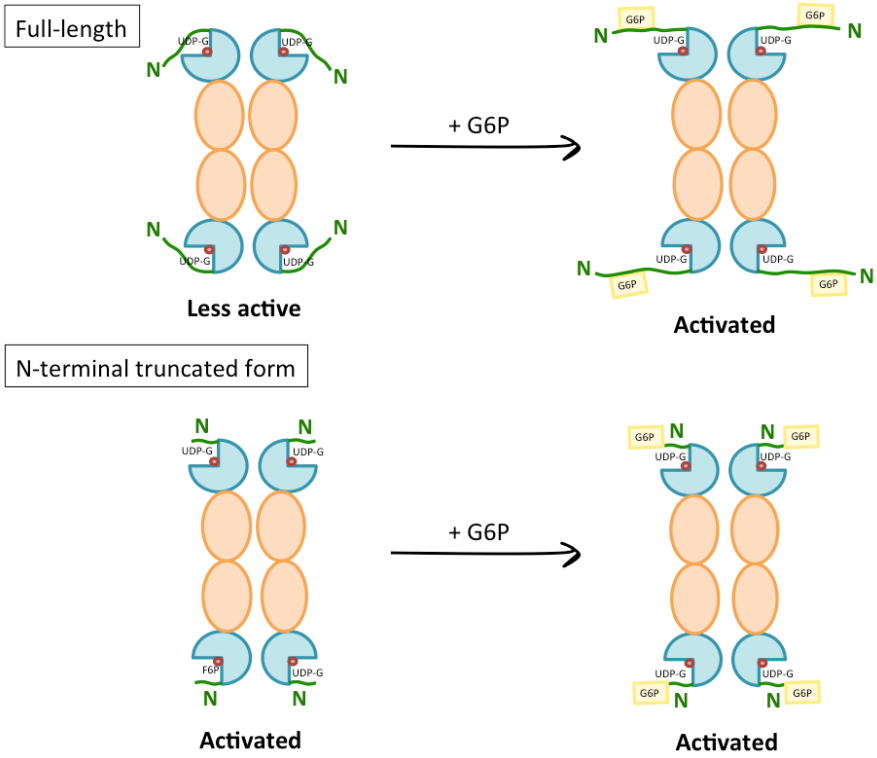



**Fig. 4-1 A proposed model of sugarcane SPS for the functional intactness of N-terminal region and allosteric regulation**

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