Structure and Physiological Function of Starch Phosphorylase from Sweet Potato Roots

- Regulation by Proteolytic Modification & Phosphorylation

莊榮輝 Rong-Huay Juang

Department of Biochemical Science & Technology, National Taiwan University

國立台灣大學 生化科技學系
Glycogen phosphorylase is regulated complicatedly to control the carbohydrate metabolism.
Comparison of alpha glucan phosphorylases

Starch phosphorylase has two isoforms (L-SP and H-SP). L-SP is the major isoform in the sweet potato roots and other plants.
SP catalyzes the reversible phosphorolysis of starch

Starch \[ \text{Phosphorylation} \]

Phosphate + \[ n \]

Starch biosynthesis

Glc-1-P

Primer-independent activity

Starch phosphorylase might involve in starch biosynthesis


Starch phosphorylase increases proportionally when the roots accumulate starch

Primary fibrous roots
Pencil roots
Storage roots

Root size in diameter

5 cm
0 cm

SP activity
- Potato
  - Mingo-Castel et al. 1976
  - Albrecht et al. 2001
- Maize
  - Liu & Shannon 1981
- Rice
  - Baun et al. 1970
- Wheat
  - Schupp & Ziegler 2004

SP expression
- Rice
  - Ohdan et al. 2005
- Potato
  - Brisson et al. 1989
  - St-Pierre & Brisson 1995
  - Duwenig et al. 1997
  - Albrecht et al. 2001
- Spinach
  - Duwenig et al. 1997
- Pea
  - van Berkel et al. 1991

Protein interaction
- Wheat
  - Tetlow et al. 2004
L-SP is phosphorylated and bound with SBE


Protein Phosphorylation in Amyloplasts Regulates Starch Branching Enzyme Activity and Protein–Protein Interactions

Ian J. Tetlow, Robin Wait, Zhenxiao Lu, Rut Akksaeng, Caroline G. Botwright, Behjat Kosar-Hashemi, Matthew K. Morell, and Michael J. Emes

Departments of Botany and Molecular Biology and Genetics, University of Guelph, b Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College, c Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, d School of Biological Sciences, University of Manchester, Manchester, M13 9PT, UK, e Dipartimento di Biologia Vegetale, University of Naples [Federico II], 80139, Naples, Italy, f Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Australia

Protein phosphorylation in amyloplasts and chloroplasts of *Triticum aestivum* (wheat) of intact plastids with γ-32P-ATP. Among the soluble phosphoproteins detected in vitro, the starch branching enzyme (SBE) were phosphorylated in amyloplasts (SBEI, SBEIIa, and SBEIIb), and SBEIIb and SBEIIla were shown to be phosphorylated after sequencing of the immunoprecipitated protein using quadrupole-orthogonal acceleration time of flight mass spectrometry. Phosphorylated SBE forms indicated that the proteins are all phosphorylated on Ser residues, and incubation of intact amyloplasts with γ-32P-ATP showed that granule-associated forms of SBEII and two granule-associated forms of starch synthase (SS) are phosphorylated. Analysis of SBE activity in amyloplasts and chloroplasts showed that phosphorylation activation of SBEII, whereas dephosphorylation using alkaline phosphatase reduced the catalytic activity of SBEII, and dephosphorylation had no effect on the measurable activity of SBEI in amyloplasts. Both granule-bound forms of SBEII in amyloplasts were unaffected by treatment with ATP. Precipitated with SBEI in a phosphorylation-dependent manner, suggesting that SBEII and SBEIIla complexes within the amyloplast in vivo. Conversely, dephosphorylation of immuno-precipitated SBEIIla by protein phosphatase and indicate a wider role for protein phosphorylation in the control of starch anabolism and catabolism.
Starch is synthesized by elongation-branching-trimming cycles.

Ball S et al. (1996) From glycogen to amylopectin - a model for the biogenesis of the plant starch granule. Cell 86: 349-352
IS THERE A ROLE FOR PHOSPHORYLASE IN STARCH SYNTHESIS?

Until the discovery of the glucosyl transferases that transfer glucose from nucleoside diphosphate glucose to the nonreducing ends of growing starch or glycogen molecules, it was assumed that the enzyme responsible for lengthening this enzyme [Nelson O, Pan D (1995) Starch synthesis in maize endosperms. Annu Rev Plant Physiol Plant Mol Biol 46: 475-496] and the amount of Pi in homogenates of starch-synthesizing storage tissues would be inimical to starch synthesis, it was necessary to postulate that much of the Pi was effectively sequestered away from the sites of starch synthesis. Since the discovery of these glucosyl transferases (34, 54), many investigators have tacitly assumed that they are responsible for all starch synthesis. The GBSS and the SSSs, which catalyze essentially irreversible reactions, clearly are better suited to fulfill the synthetic role. The mutations (bt2 and sh2) that so drastically lower the ADPGlc pyrophosphorylase activity attest to the major role of the ADPGlc to starch glucosyl transferases. Yet there is no evidence to demonstrate conclusively that an α-glucan phosphorylase does not make a contribution. Phosphorylase activity in the developing endosperm increases...
The DNA complexity analysis (PC/GENE) reveals that L78 might be derived from an intron sequence during the evolution of SP gene.
L-SP has a 78-amino acid insertion (L78) in the middle of the molecule, which blocks the glucan binding site of L-SP.
Analysis of the amino acid sequence on L78 and its C-terminal flanking residues shows several unique structural features. A “PEST sequence” is found in the middle of L78.
L-SP is proteolytic modified but still keeps its activity

**A** SDS-PAGE:

<table>
<thead>
<tr>
<th></th>
<th>not-heated</th>
<th>heated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Days</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4 Days</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>8 Days</td>
<td>2</td>
<td>4</td>
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</tbody>
</table>

**B** Disc-PAGE:

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<th>0</th>
<th>1</th>
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<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C** Activity staining:

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<thead>
<tr>
<th></th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
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<tbody>
<tr>
<td>SP</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The proteolytic modification of partially purified L-SP (A). Although L-SP molecule is nicked, it keeps its native quaternary structure (B) and catalytic activity (C)

Monoclonal antibodies (J3b and H7c) simplify the SDS-PAGE patterns of L-SP during the proteolytic procedure, and reveal two final stable fragments of L-SP (N and C).
L-SP is modified by controlled proteolytic process


**L-SP** 955 aa (108.5 kDa)

**SP110** 910 aa (103.4 kDa)

**SP108** 896 aa (101.8 kDa)

**L78** (8.7 kDa) 411 aa (46.8 kDa)

**SP110 & SP108** Minor cuttings sites on L78-C

**N480** 480 aa (54.5 kDa) Major cuttings at X & Z

**N436** 436 aa (49.7 kDa) Multiple minor cutting sites on L78-C

**E15** (EKNEGLDAA) 436 aa (49.7 kDa)

**C460** 460 aa (52.1 kDa) Synthetic peptides

**C416** 416 aa (47.3 kDa)

**N436** 436 aa (49.7 kDa) *EKDEDPVPAP*

**C416** 416 aa (47.3 kDa)
L1 and L2 peptides are completely removed from L78

Reconstituted proteolysis

Why an intron was evolved to express, and then the expressed peptide was cut away?
Proteolysis increases the affinity to starch, but not Glc-1-P

\[ \text{Starch}_n + \text{Glc-1-P} \rightarrow \text{Starch}_{n+1} + P_i \]

A. Fixed [Glc-1-P]

B. Fixed [soluble starch]

The proteolytic modified L-SP shows higher affinity toward one of its substrate (starch, A)
But the intact L-SP has higher affinity toward Glc-1-P (B)
Removing L78 exposes starch binding site on L-SP

Proteolytic modification

Starch binding site blocked

Catalytic site

Starch binding site opened

Catalytic site

The starch binding site is opened by removing peptides on L78
The affinity to starch is increasing after proteolysis.


Native-PAGE

Proteolytic modification

Days

kDa

- starch

+ starch

The modified L-SP is retarded in native electrophoresis gel containing soluble starch.
L78 as a **molecular switch** in regulating L-SP catalytic direction

Intact L-SP binds Glc-1-P preferentially and the L78 blocks the starch binding site.
Primer-independent glucan biosynthesis from single Glc-1-P

Glucan is synthesized in vitro by L-SP from single Glc-1-P in the absence of a primer.

\[ \text{Glc-1-P} + \text{Glc-1-P} \rightarrow \text{Glc-Glc-1-P} \] (the initiation step)

Amylose (straight-chain starch)
The amylose synthesized is radioactive

The radioactive Glc-1-P is covalently bound to amylose

Chen et al, (2006) submitting
Further purification by ion exchange

Chen et al, (2006) submitting

b

DEAE Sephacel

Absorbance at 650 nm

Radioactivity (cpm)

Fraction number

0.5

1.0

Amylose

0.5 M NaCl

L-SP

0.5 M NaCl
The amylose contains Glc-1-P moieties.
Glc-1-P consumption has three phases, suggesting a mechanism for glucan polymerization. The phases are:

1. **Slow initiation phase**
2. **Rapid elongation phase**
3. **Steady-state phase**

The degree of polymerization reaches several thousands, as indicated by Chen et al. (2006) submitted.
The PI activity of L-SP is lost when its L78 is removed.

Does L78 serve as the “primer” for amylose synthesis? Or an anchoring point for Glc-1-P?

Chen et al, (2006) submitting
Active site of L-SP and possible PI action mechanism.
Action mechanism for PI amylose synthesis (1)

Glc-1-P on A site loses its phosphate as interacting with the phosphate on the cofactor PLP.
The C-1 on Glc (A site) becomes a carbonium ion after releasing the phosphate. The released phosphate attracted a proton from the hydroxyl group (C-4) of the B site Glc-1-P.
The negatively charged alkoxide attacks the carbonium ion producing a new glycosidic bond.
L-SP is predicted as phosphorylated

Several phosphorylation sites are predicted in the helices on L78

L-SP is phosphorylated by a kinase in sweet potato roots.

Purified L-SP was phosphorylated in the plant.

Ammonium sulfate fractions contained a kinase activity.

L-SP is found phosphorylated in sweet potato roots, or in vitro phosphorylated by a protein fraction from the root extract.

L-SP is phosphorylated specifically on its L78 insertion.

Phosphorylase molecules lacking L78 insertion cannot be phosphorylated.

Ser on L-SP is the target for the kinase

MBP, myelin basic protein; S, phospho-Ser; T, phospho-Thr; P_i, inorganic phosphate; x, the origin spot of the sample; * indicates phosphopeptides by partial hydrolysis

L-SP is phosphorylated specifically on Ser \textsuperscript{71} of L78

Ser \textsuperscript{71} on L78 is the only phosphorylation site on L-SP by the kinase.
Although this kinase could also phosphorylate L-SP from potato, the exact phosphorylation site and mechanism are unclear.
What is the possible physiological function for the phosphorylation of L-SP?
Phosphorylated L-SP has no change in its kinetic parameters

\[
\text{Starch}^{(n)} + P_i \rightleftharpoons \text{Starch}^{(n-1)} + \text{Glc-1-P}
\]

### Synthetic direction (Chen et al. 2002)

<table>
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<tr>
<th></th>
<th>(K_m)</th>
<th>(K_{cat}) (1/s)</th>
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</thead>
<tbody>
<tr>
<td><strong>L-SP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmodified</td>
<td>0.077 ± 0.015</td>
<td>1.052 ± 0.311</td>
</tr>
<tr>
<td>Phosphorylated</td>
<td>0.070 ± 0.016</td>
<td>1.090 ± 0.320</td>
</tr>
</tbody>
</table>

\(a\) [Glc-1-P] = 4 mM; \(b\) [soluble starch] = 0.3%

### Phosphorolytic direction (Mori et al. 1993)

<table>
<thead>
<tr>
<th></th>
<th>(K_m)</th>
<th>(K_{cat}) (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L-SP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmodified</td>
<td>0.115 ± 0.023</td>
<td>1.498 ± 0.562</td>
</tr>
<tr>
<td>Phosphorylated</td>
<td>0.108 ± 0.021</td>
<td>1.443 ± 0.568</td>
</tr>
</tbody>
</table>

\(c\) \([P_i]\) = 5 mM; \(d\) [soluble starch] = 0.2%
Phosphorylated L-SP is sensitive to proteolytic modification

a
Coomassie Brilliant Blue R staining

b
mAb J3b

c
mAb anti-L78P


Is the phosphorylation of L-SP a signal for its proteolytic modification on L78?
How is the phosphorylation connected to proteolysis?

Phosphorylation of L78 might trigger the removal of the L78 insertion, and change the catalytic behavior of L-SP from starch synthesis to phosphorolysis.

A high MW complex (HX) expressing SP activity is found.
HX consists of L-SP and 20S proteasome

<table>
<thead>
<tr>
<th>no.</th>
<th>Full protein name</th>
<th>Matched peptide</th>
<th>Sequence coverage (%)</th>
<th>Match score</th>
<th>Species</th>
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<td><em>Petunia x hybrida</em></td>
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</table>

6 % native-PAGE
CBR staining

(L-SP) → Native-PAGE → Band 1 → LC/MS/MS

HX

SDS-PAGE → Band 1~7 → LC/MS/MS

Unpublished
Double diffusion reveals the components of HX

HX can not be reconstituted by just mixing L-SP and proteasome in the test tube (Control)
Both L-SP and proteasome are detected in amyloplast

Immunostaining

Control (no primary Ab)

J3b (anti L-SP)

M71 (anti proteasome)

Unpublished
Blue-native 2D PAGE and immunostaining for HX

Only H7c could stain the HX band on the native PAGE.

N-terminal half of L-SP including L78 is buried inside the proteasome.
The degradation of L-SP is protected by proteasome inhibitor

mAb H7c

<table>
<thead>
<tr>
<th></th>
<th>L-SP only</th>
<th>Extract only</th>
<th>L-SP + Extract</th>
<th>L-SP + Extract + Inhibitor</th>
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<td>SP110</td>
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<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
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<tr>
<td>F50s</td>
<td>![Image]</td>
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<table>
<thead>
<tr>
<th></th>
<th>E64</th>
<th>MG132</th>
<th>Lactacystin</th>
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<tbody>
<tr>
<td>SP110</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
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<tr>
<td>F50s</td>
<td>![Image]</td>
<td>![Image]</td>
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</table>

10% SDS-PAGE

Unpublished
The degradation of L-SP is protected by proteasome inhibitor

mAb H7c

L-SP only

Extract only

L-SP + Extract → L-SP activity

![Image of 7.5% native PAGE gel with bands for L-SP only, Extract only, L-SP + Extract, and L-SP + Extract + Inhibitor for E64, MG132, and Lactacystin.](Unpublished)

L-SP

E64

MG132

Lactacystin

0 1/2 1 2 3 0 1/2 1 2 3 0 1/2 1 2 3 (day)
Phosphorylation might control the proteolysis of L78 via UPS

Primer-independent activity is contributed by L78. L78 was removed by proteolytic modification induced by the PEST signal or the phosphorylation-UPS pathway.
and many others…

We ❤️ sweet potato