Structure and Physiological Function of Starch Phosphorylase from Sweet Potato Roots

- Regulation by Proteolytic Modification & Phosphorylation

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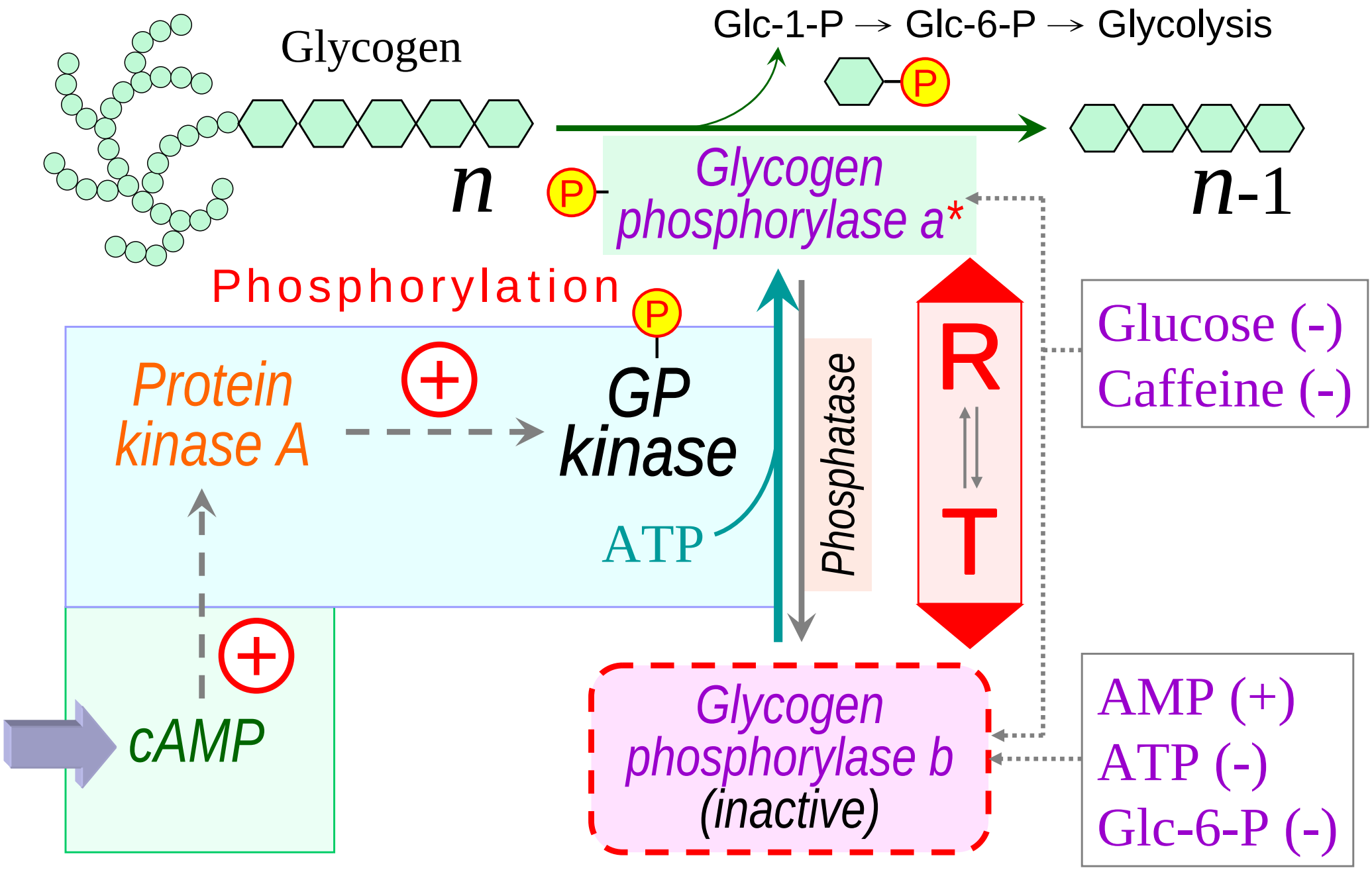
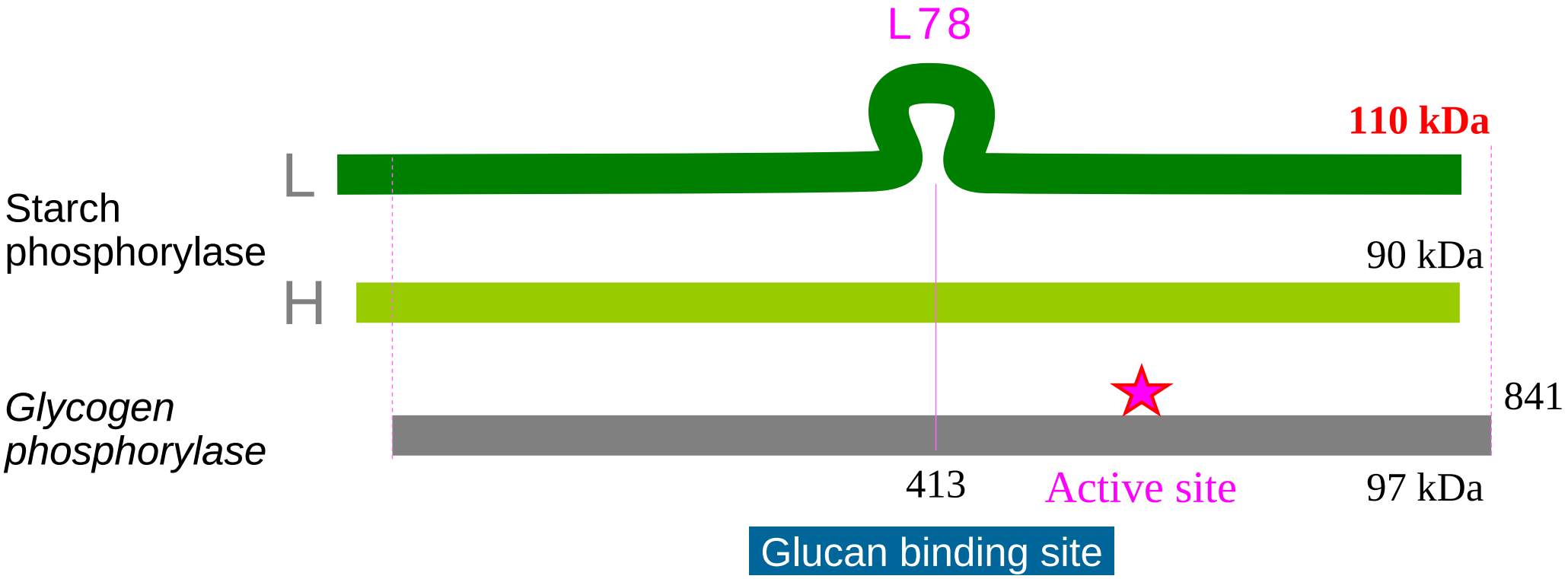
國立台灣大學 生化科技學系

Glycogen phosphorylase 肝糖磷解酶



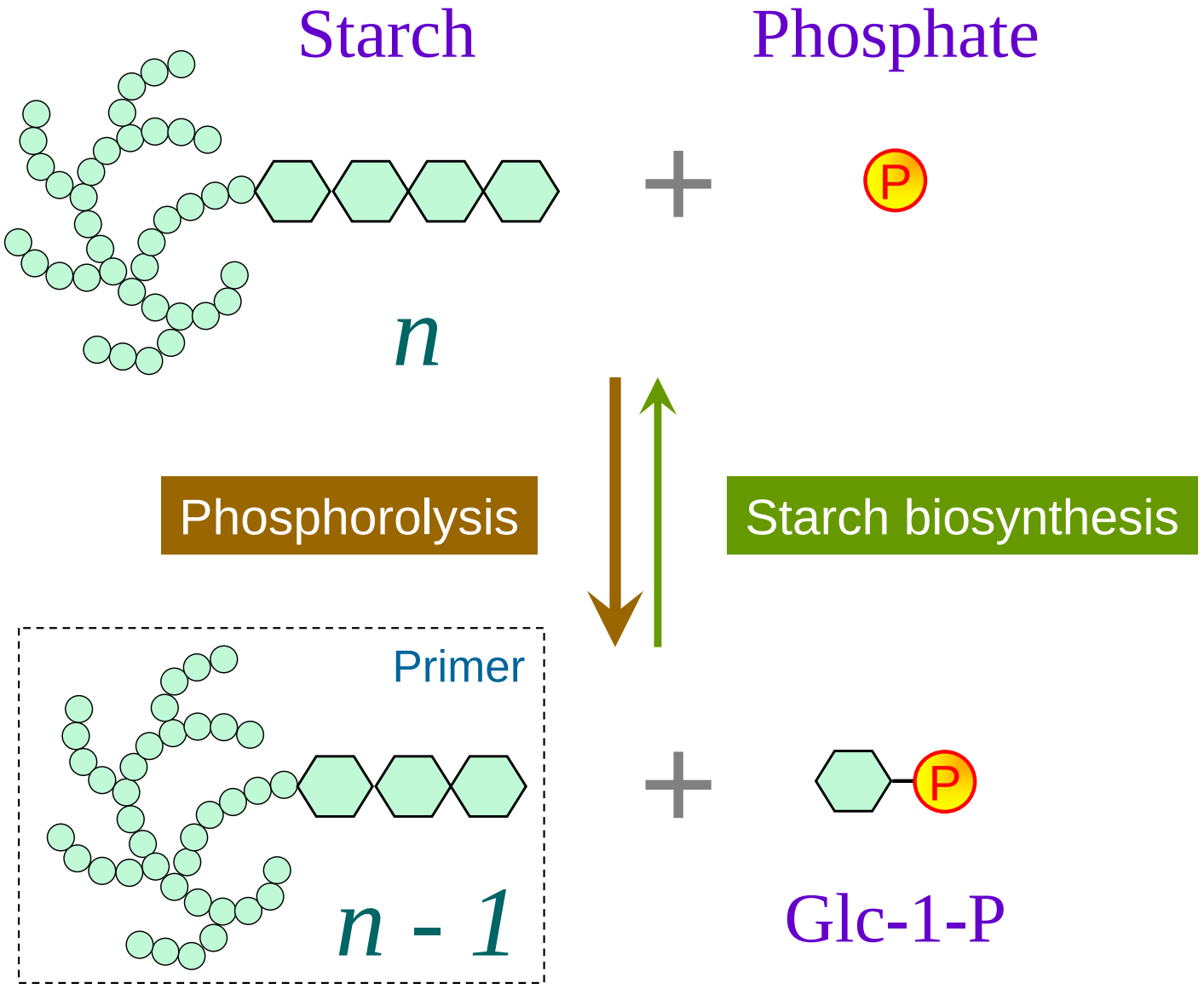
Glycogen phosphorylase is regulated complicatedly to control the carbohydrate metabolism

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

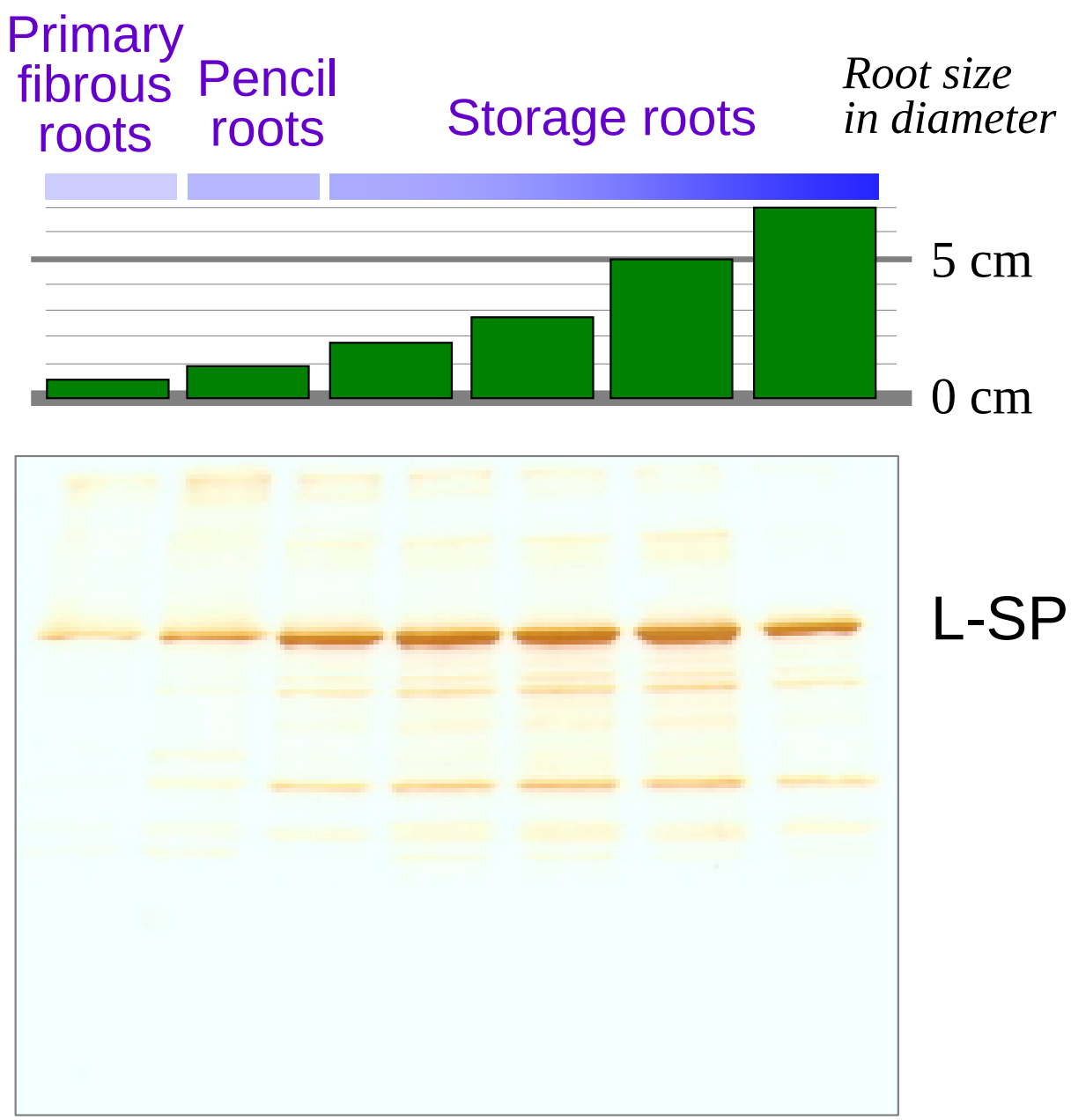
SP catalyzes the reversible phosphorolysis of starch



Hanes CS (1940) Proc Roy Soc (London) B128: 421-450
Hanes CS (1940) Proc Roy Soc (London) B129: 174-208

Starch phosphorylase might involve in starch biosynthesis

Chang et al, (2000) Bot Bull Acad Sin **41**:105-111



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

Starch phosphorylase increases proportionally when the roots accumulate starch

L-SP is phosphorylated and bound with SBE

The Plant Cell, Vol. 16, 694–708, March 2004, www.plantcell.org © 2004 American Society of Plant Biologists

Tetlow et al. ***J. Exp. Bot.*** (2004) 55: 2131-2145

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

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Behjat Kosar-Hashemi,[f] Matthew K. Morell,[f] and Michael J. Emes[a]**

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[f] Division of Plant Industry, Commonwealth Scientific and Industrial Research Organi

Protein phosphorylation in amyloplasts and chloroplasts of *Triticum aestivum* (wh of intact plastids with γ-^{32}P-ATP. Among the soluble phosphoproteins detected in enzyme (SBE) were phosphorylated in amyloplasts (SBEI, SBEIIa, and SBEIIb), an and SBEIIa) were shown to be phosphorylated after sequencing of the immuno using quadrupole-orthogonal acceleration time of flight mass spectrometry. P phorylated SBE forms indicated that the proteins are all phosphorylated on S associated phosphoproteins after incubation of intact amyloplasts with γ-^{32}P-A forms of SBEII and two granule-associated forms of starch synthase (SS) are phos of SBE activity in amyloplasts and chloroplasts showed that phosphorylation acti whereas dephosphorylation using alkaline phosphatase reduced the catalytic a and dephosphorylation had no effect on the measurable activity of SBEI in amylo of both granule-bound forms of SBEII in amyloplasts were unaffected by experiments using peptide-specific anti-SBE antibodies showed that SBEIIb an precipitated with SBEI in a phosphorylation-dependent manner, suggesting tha plexes within the amyloplast in vivo. Conversely, dephosphorylation of immun disassembly. This article reports direct evidence that enzymes of starch metabol by protein phosphorylation and indicate a wider role for protein phosphorylatio control of starch anabolism and catabolism.

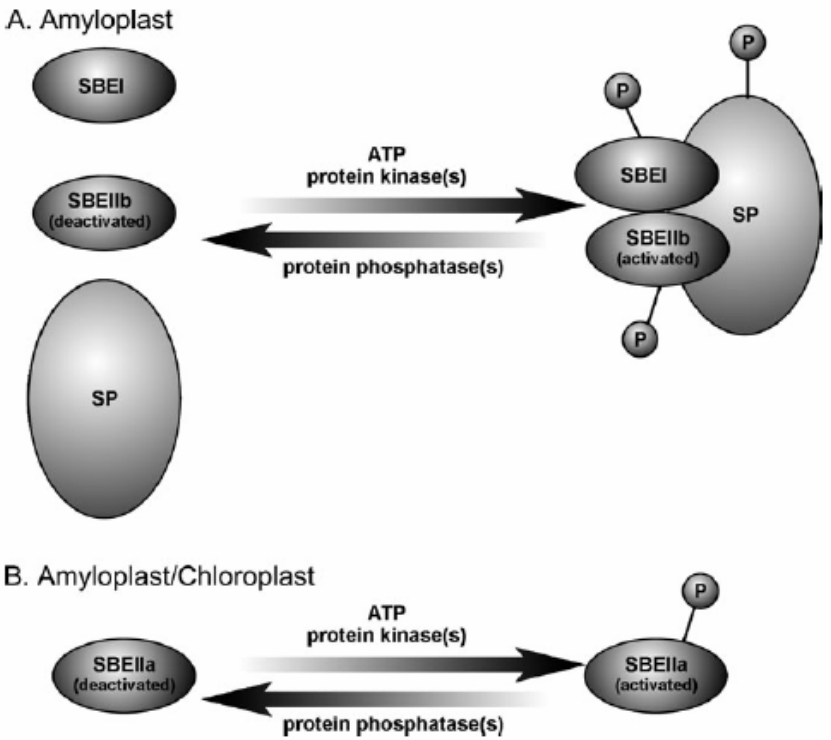
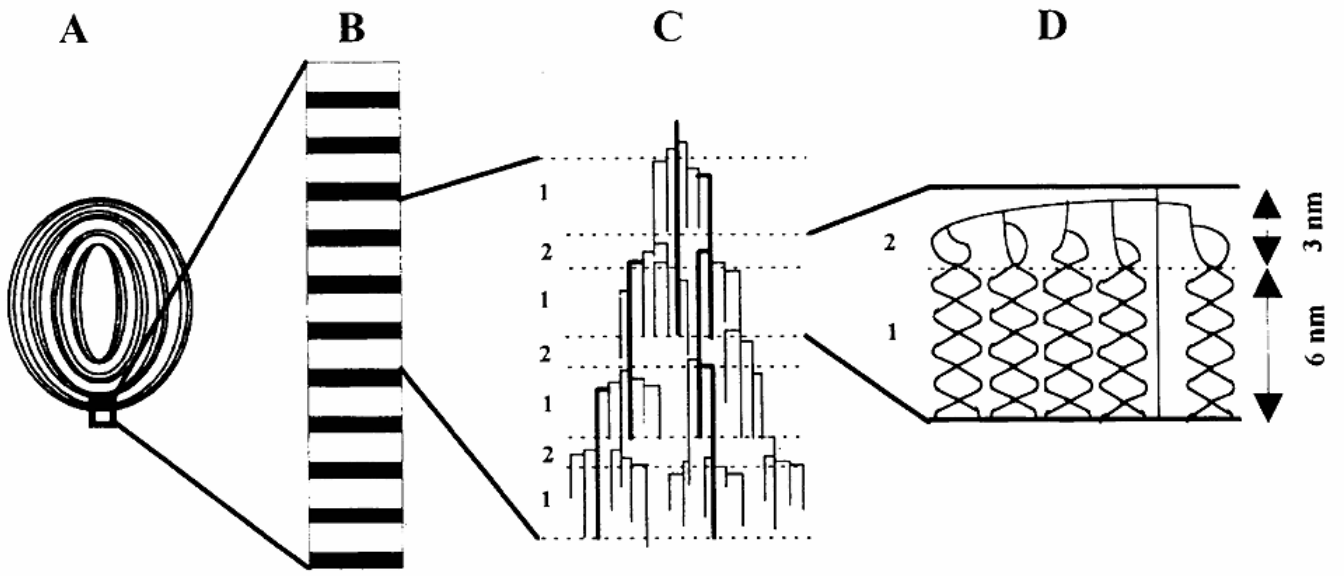
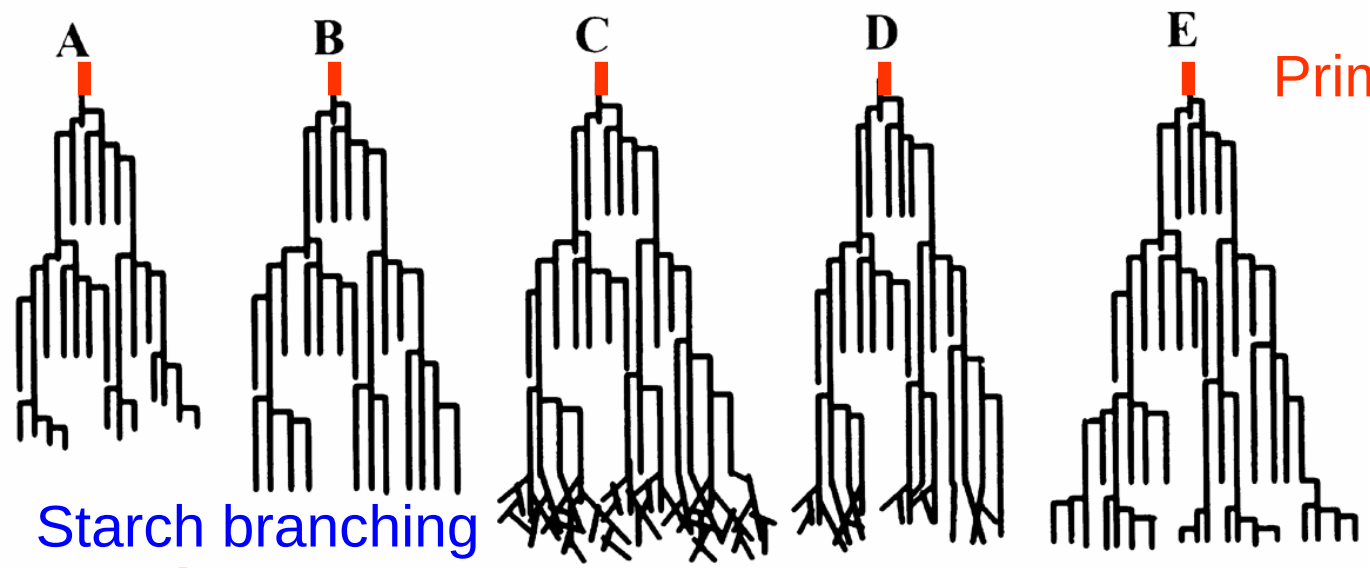


Fig. 2. Model of phosphorylation-dependent protein complex formation involved in storage starch biosynthesis. Activation of SBEIIa (in chloroplasts and amyloplasts, A and B), and activation and complex formation involving SBEI, SBEIIb, and SP by protein phosphorylation in the amyloplast stroma (A) stimulates amylopectin biosynthesis. The functional relationships between the different components of the putative protein complex are unclear. It is notable that in mutants lacking SSIIa, that starch granules are also observed to be devoid of SSI, SBEIIa, and SBEIIb, suggesting that these components may also be capable of forming a complex under *in vivo* conditions.

Starch is synthesized by elongation-branching-trimming cycles

Starch granule





Primer

Starch elongation

Starch synthase

Starch branching

Branching enzyme

Branch trimming

Debranching enzyme

Starch elongation

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?"

IS THERE A ROLE FOR PHOSPHORYLASE IN STARCH SYNTHESIS?

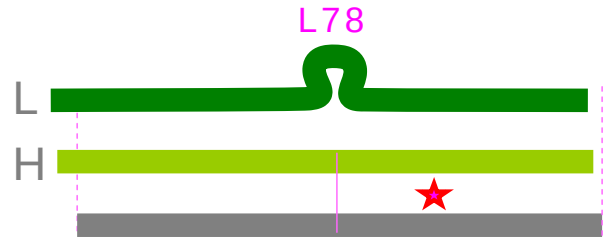
Until the discovery of the glucosyl transferases that transfer glucose from nucleoside diphosphate glucoses to the nonreducing ends of growing starch or glycogen molecules, it was assumed that the enzyme responsible for lengthening

Nelson O, Pan D (1995) Starch synthesis in maize endosperms.
Annu Rev Plant Physiol Plant Mol Biol **46**: 475-496

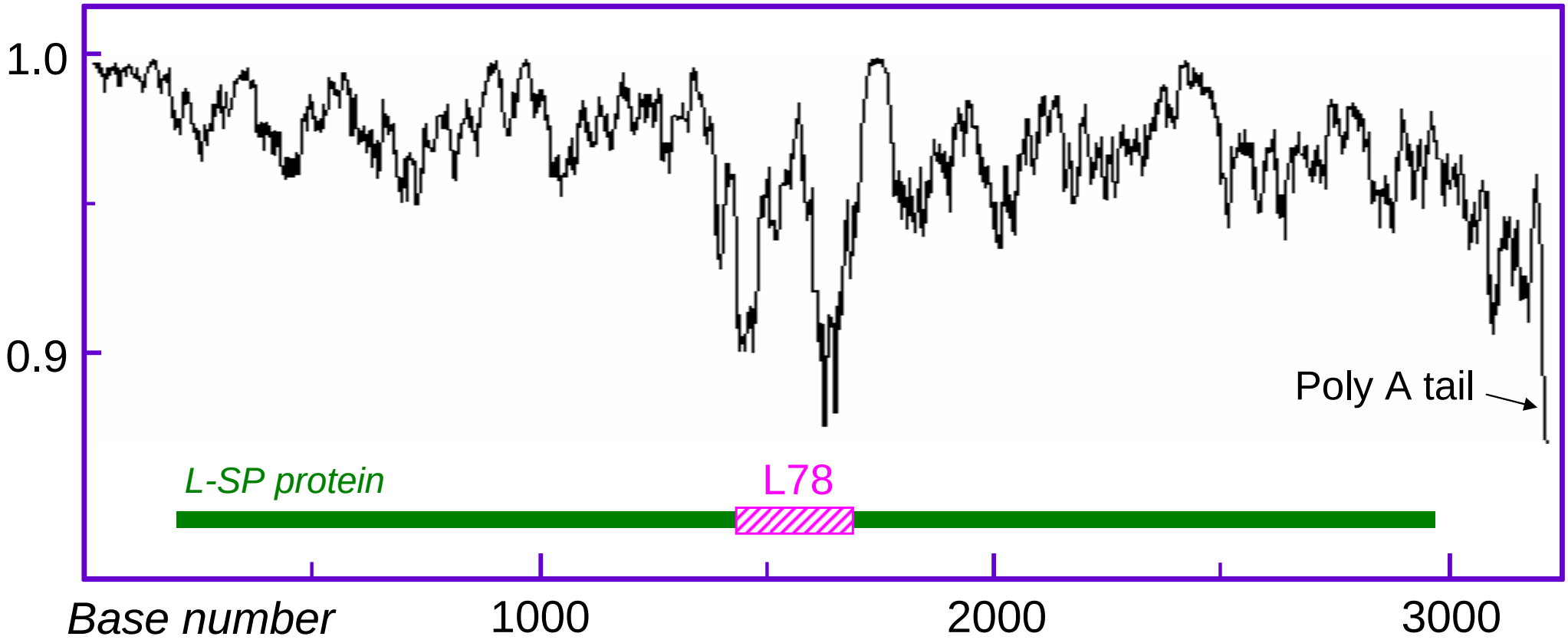
this enzyme and the amount of Pi in homogenates of starch-synthesizing storage tissues would be inimical to starch synthesis, it was necessary to postulate that much of the Pi was effectively sequestered away from the sites of starch synthesis. Since the discovery of these glucosyl transferases (34, 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

L78 might be evolved from an intron

Chen et al, (2002) Physiologia Plantarum **114**:506-515



DNA complexity of L-SP cDNA

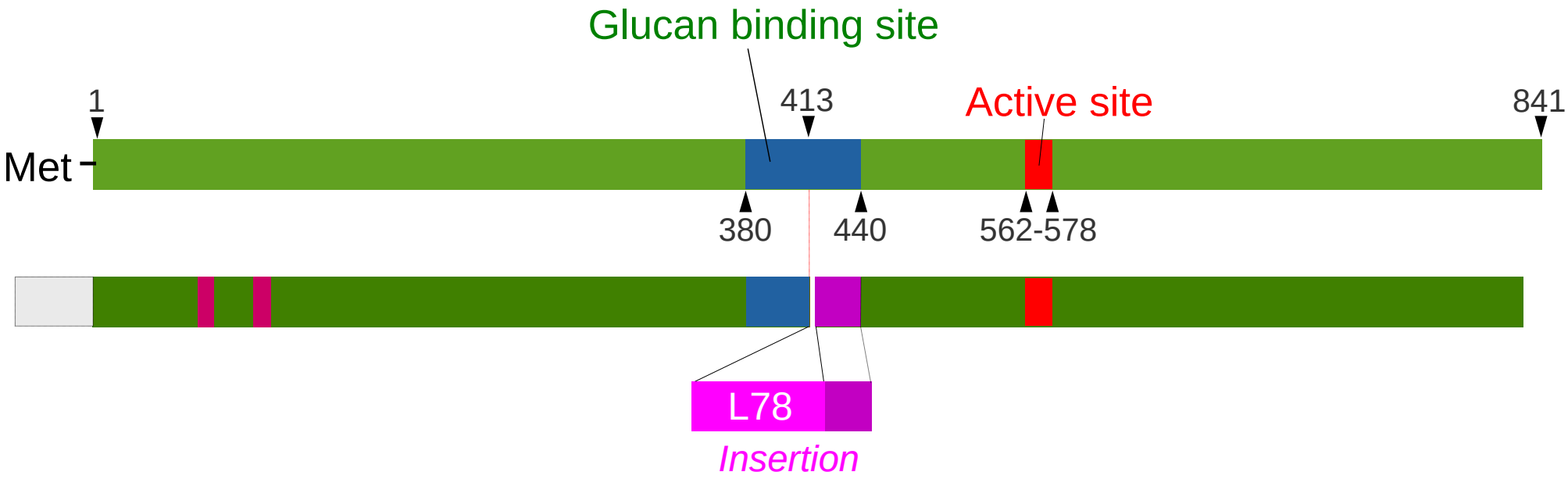


The *DNA complexity* analysis (PC/GENE) reveals that L78 might be derived from an intron sequence during the evolution of SP gene

L78 is inserted in the glucan binding site of L-SP

Chen et al, (2002) Physiologia Plantarum **114**:506-515

Glycogen phosphorylase

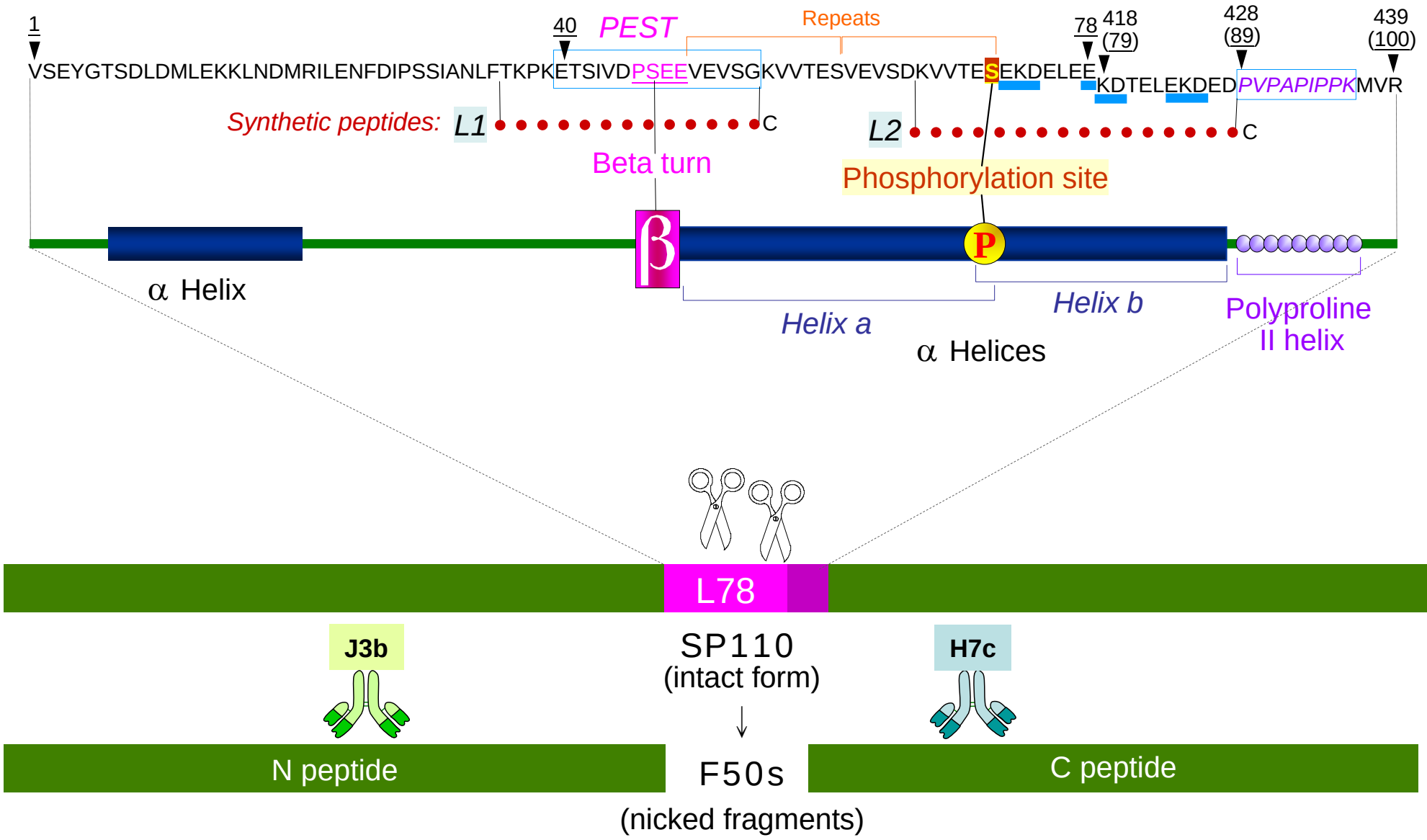


L-form starch phosphorylase (L-SP)
(low affinity type)

L-SP has a 78-amino acid insertion (L78) in the middle of the molecule, which blocks the glucan binding site of L-SP

L78 sequence reveals unique structural features

Chen et al, (2002) Physiologia Plantarum **114**:506-515

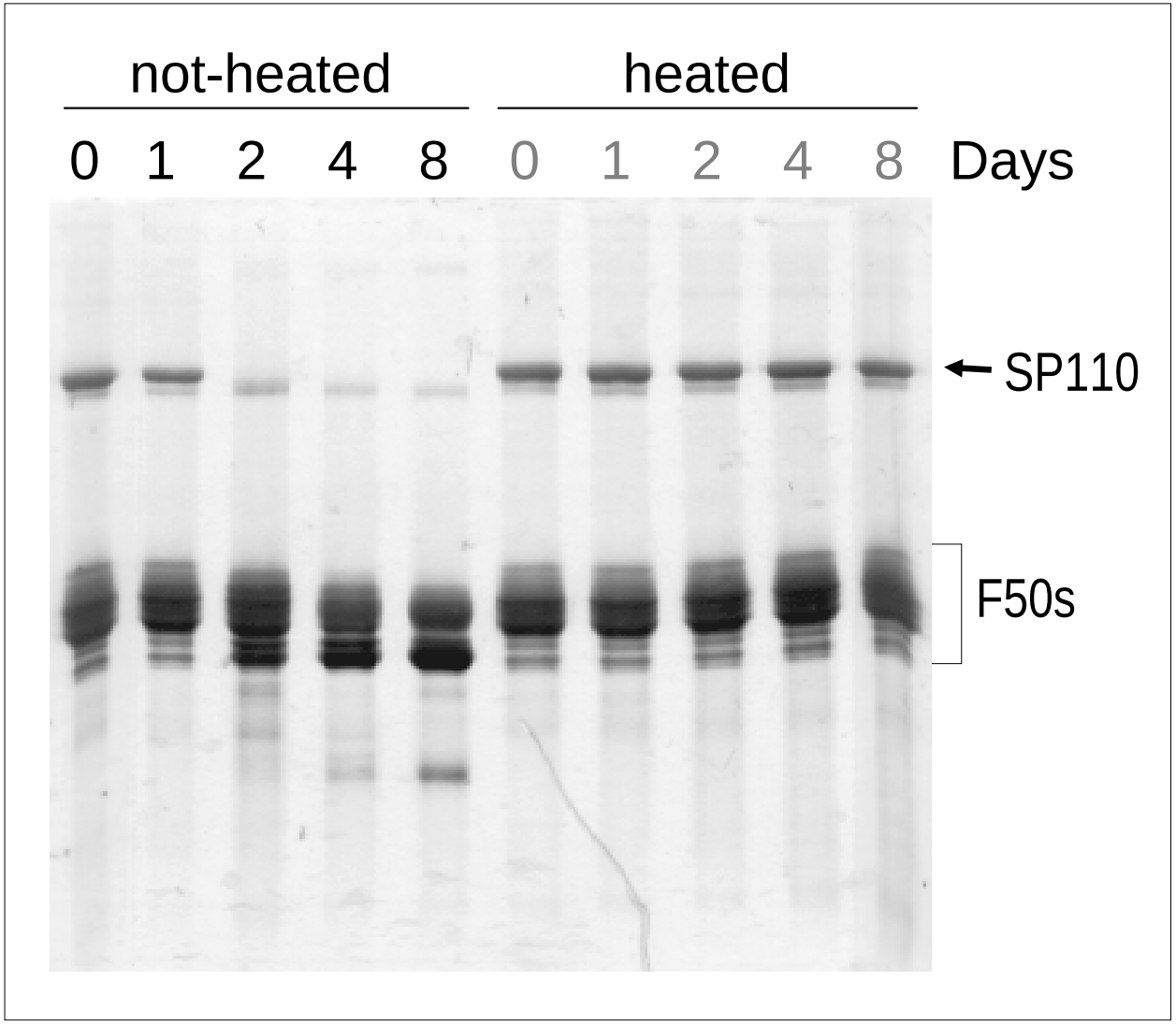


Analysis of the amino acid sequence on L78 and its C-terminal flanking residues shows several unique structural features. A "*PEST sequence*" is found in the middle of L78

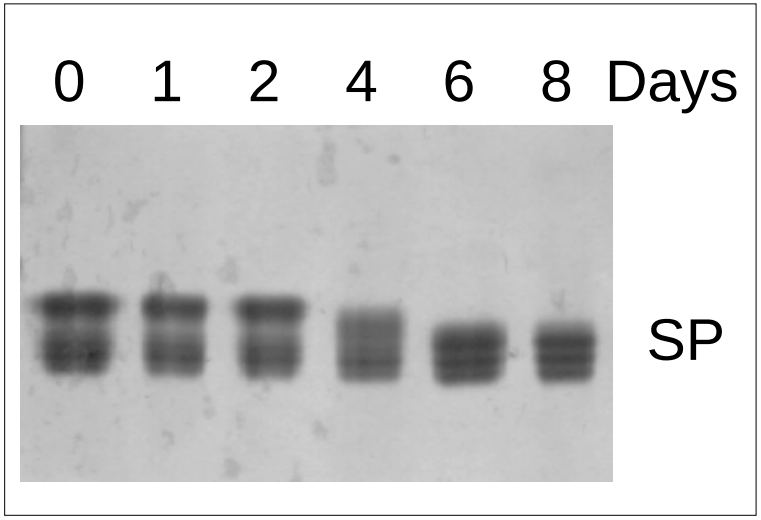
L-SP is proteolytic modified but still keeps its activity

Chen et al, (2002) Physiologia Plantarum **114**:506-515

A SDS-PAGE:



B Disc-PAGE:

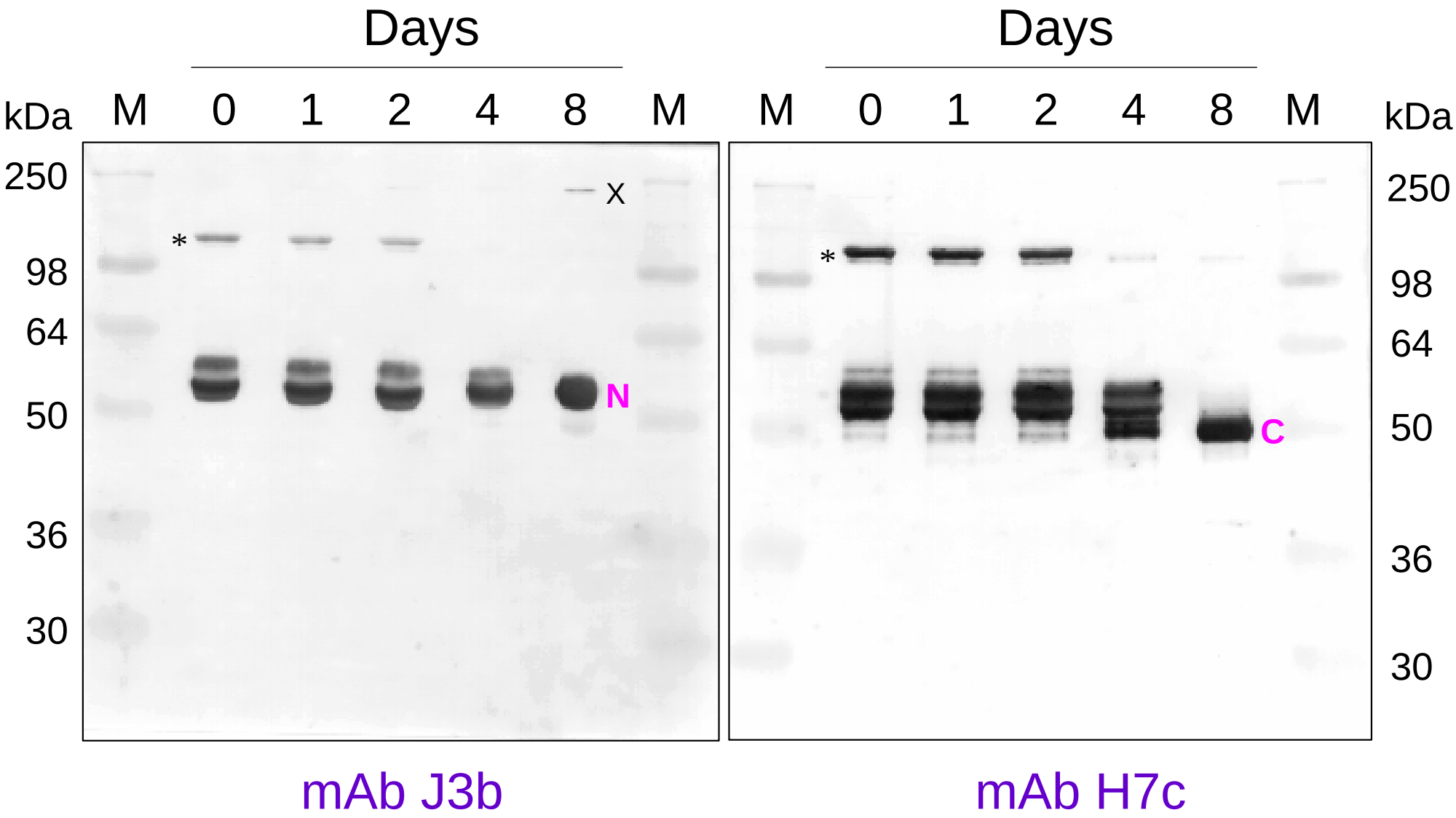


C Activity staining:

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)

L-SP is modified into two final stable fragments

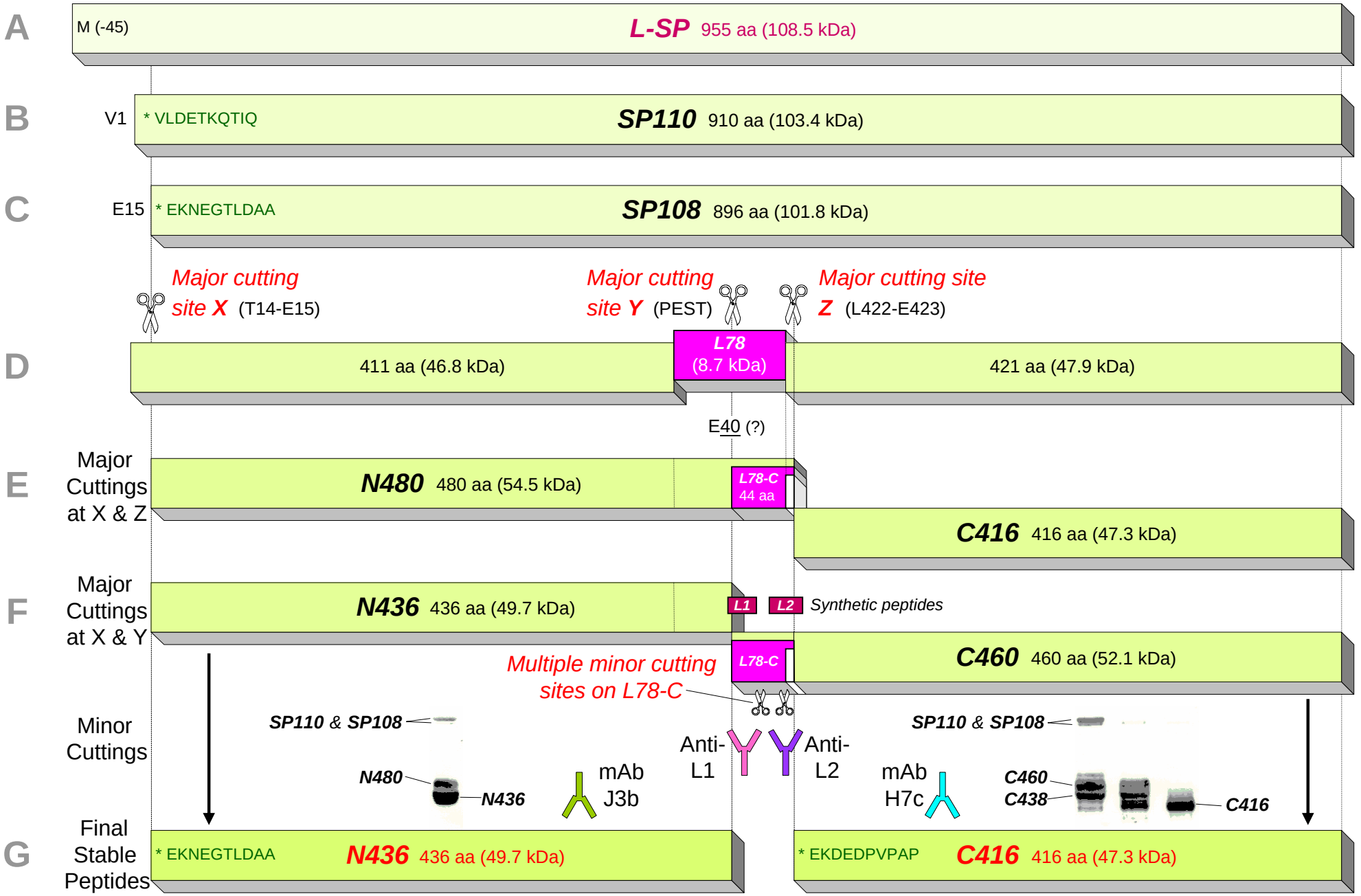
Chen et al, (2002) Physiologia Plantarum **114**:506-515



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)

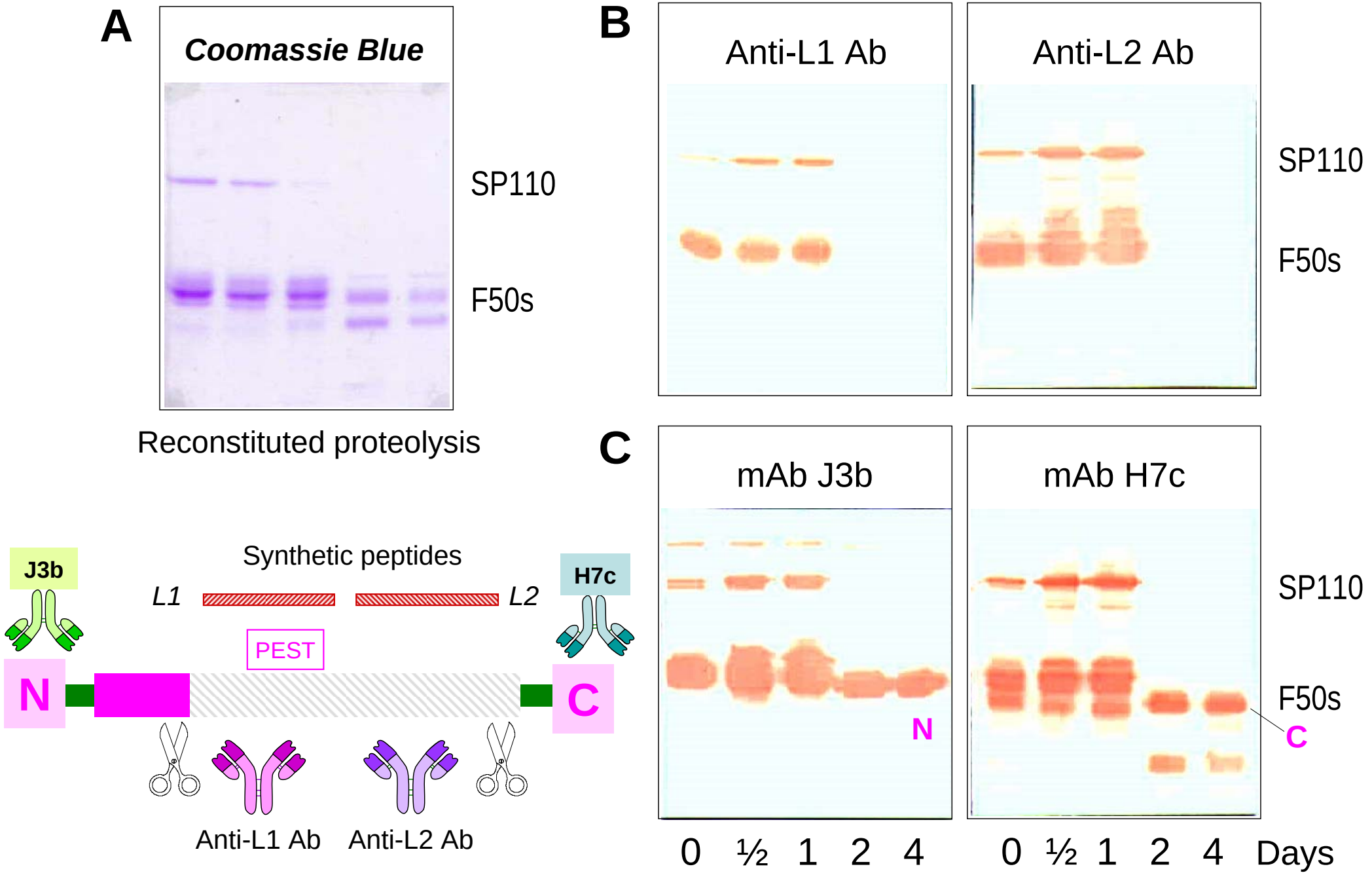
L-SP is modified by controlled proteolytic process

Chen et al, (2002) Physiologia Plantarum **114**:506-515



L1 and L2 peptides are completely removed from L78

Chen et al, (2002) Physiologia Plantarum **114**:506-515



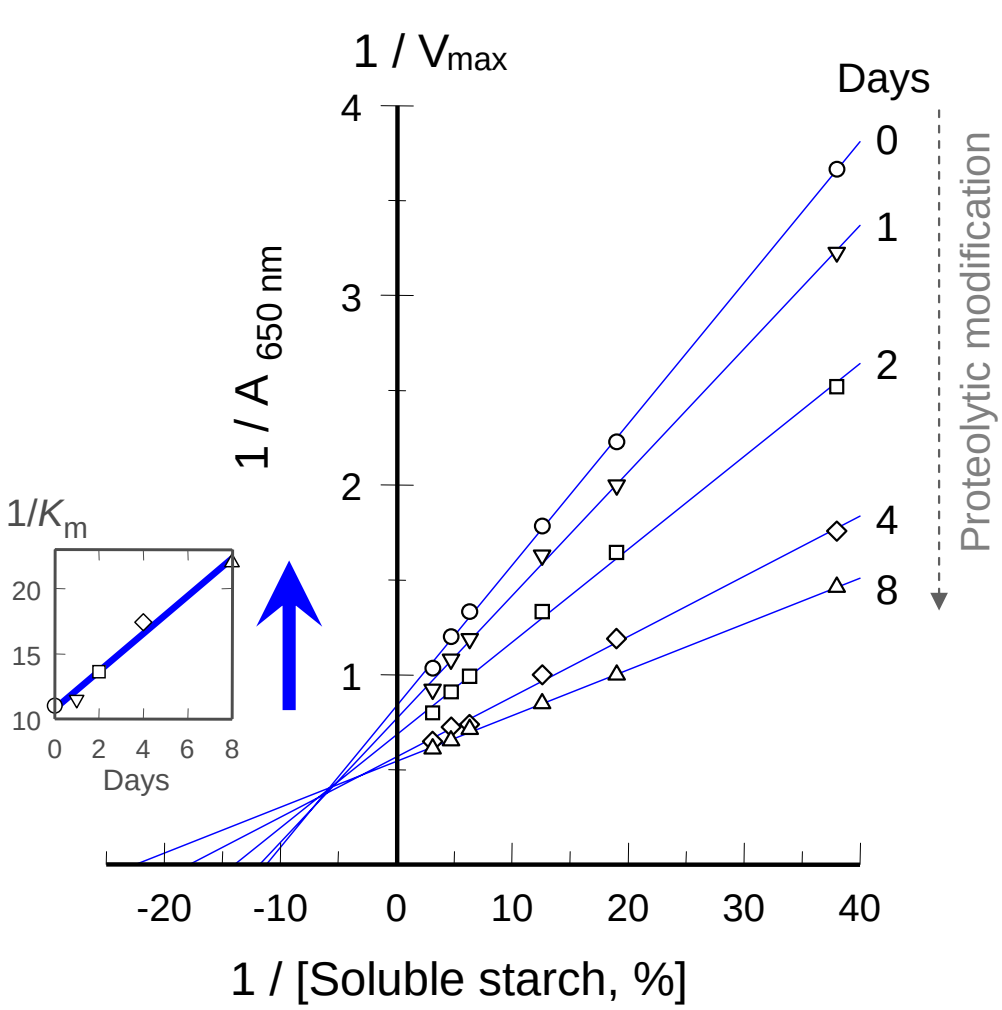
Why an intron was evolved to express, and then the expressed peptide was cut away?

Proteolysis increases the affinity to starch, but not Glc-1-P

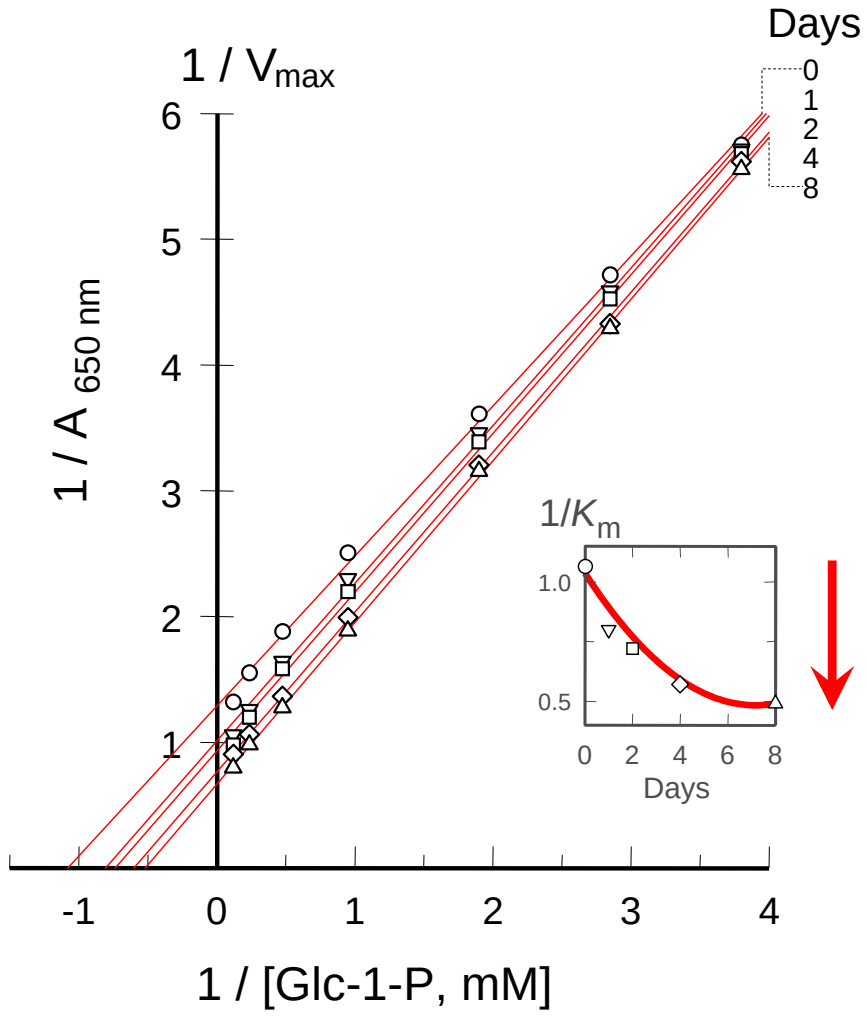
Chen et al, (2002) Physiologia Plantarum **114**:506-515

$$\text{Starch}_n + \text{Glc-1-P} \longrightarrow \text{Starch}_{n+1} + P_i$$

A Fixed [Glc-1-P]

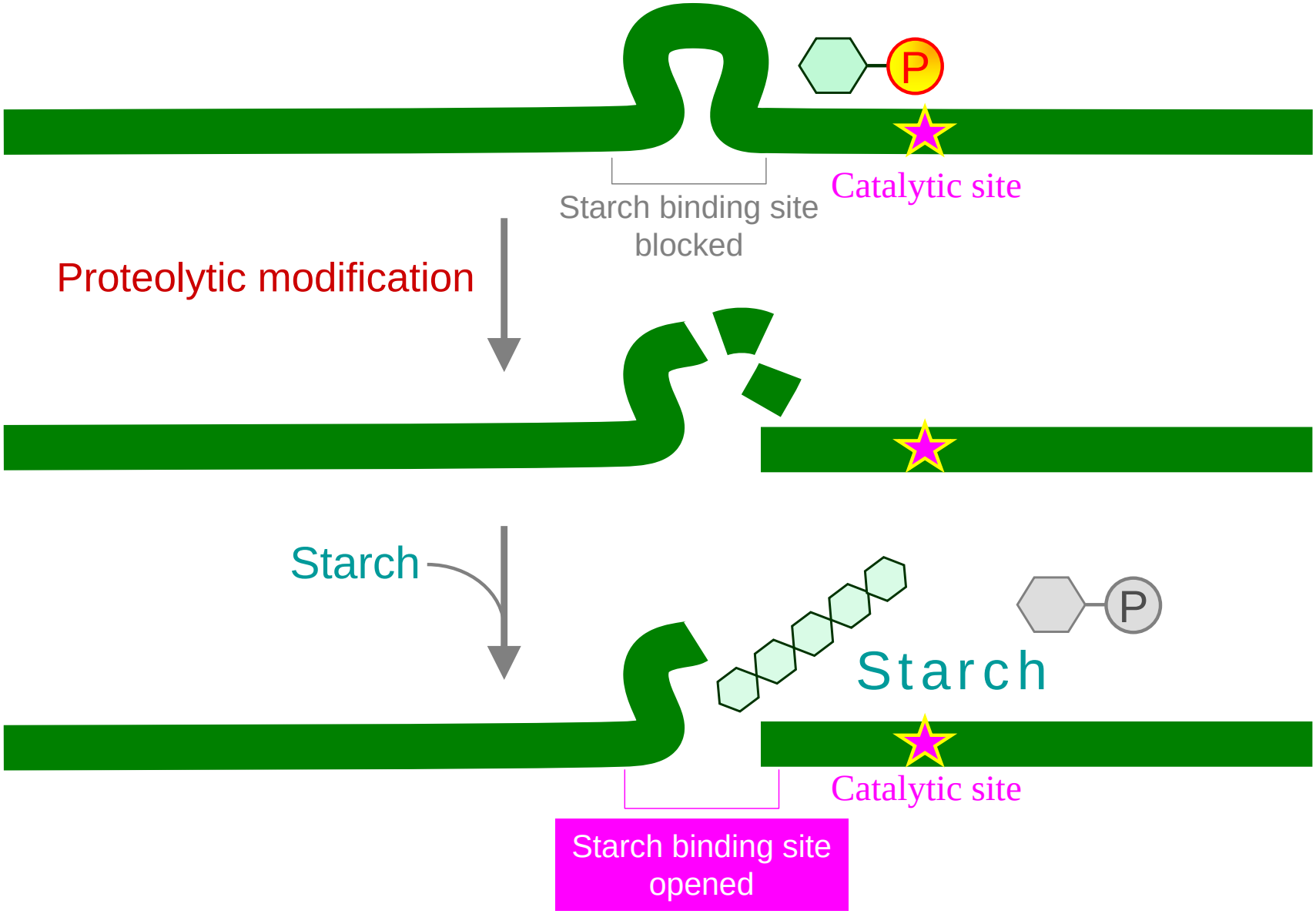


B Fixed [soluble starch]



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

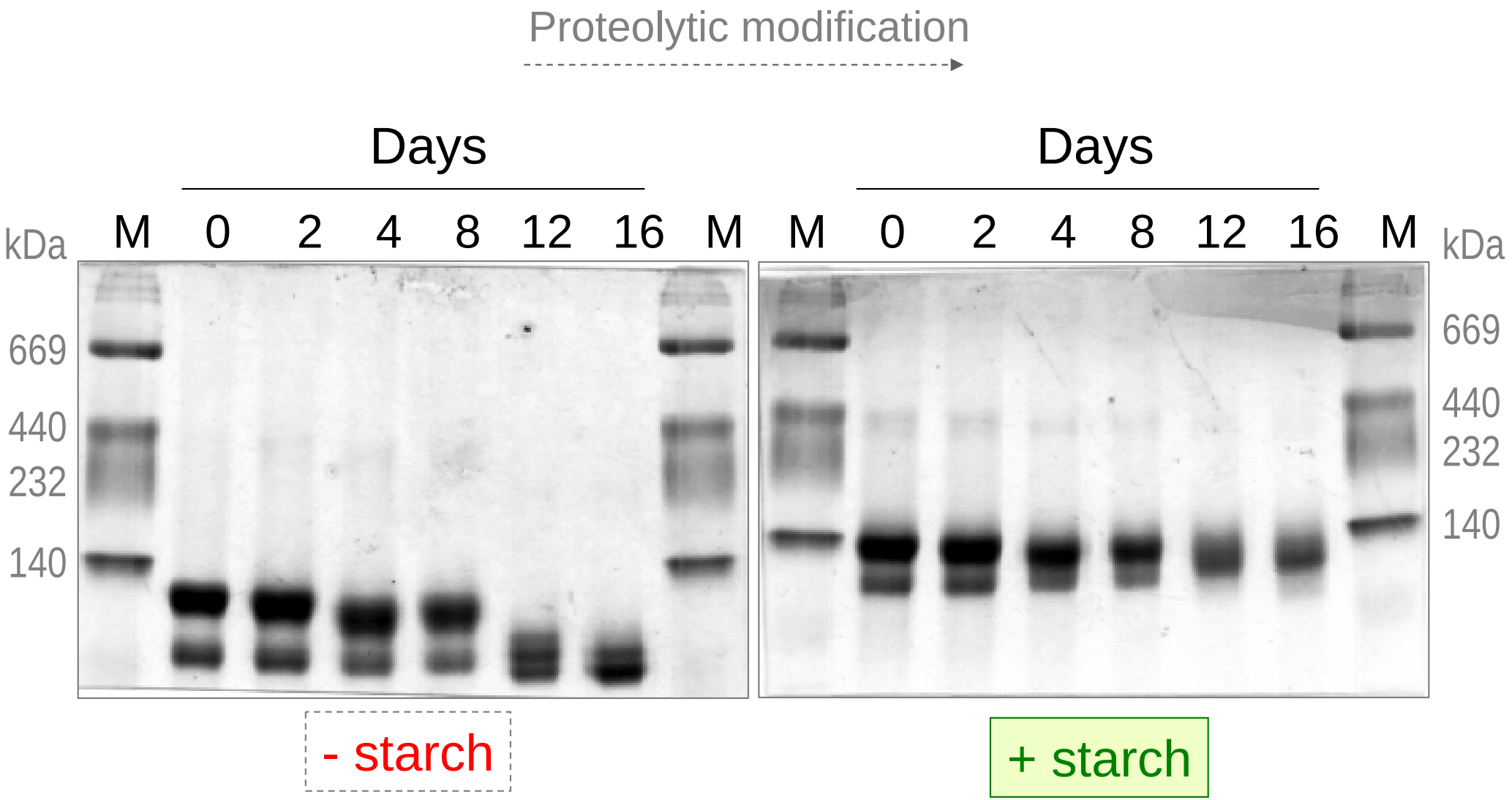


The starch binding site is opened by removing peptides on L78

The affinity to starch is increasing after proteolysis

Native-PAGE

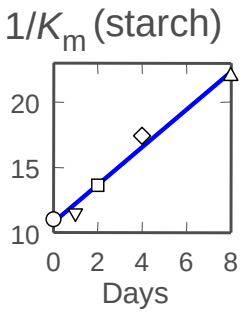
Chen et al, (2002) Physiologia Plantarum **114**:506-515



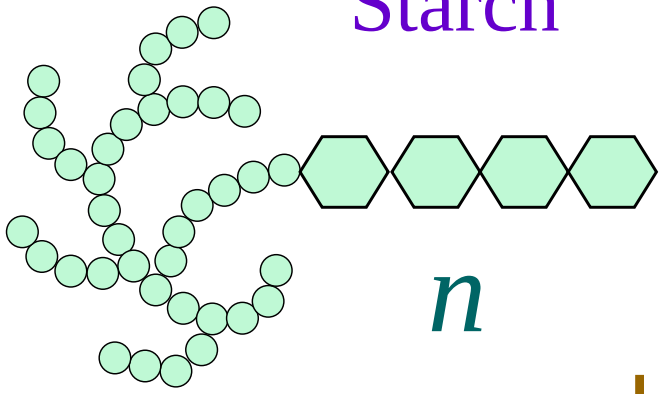
The modified L-SP is retarded in native electrophoresis gel containing soluble starch

L78 as a ***molecular switch*** in regulating L-SP catalytic direction

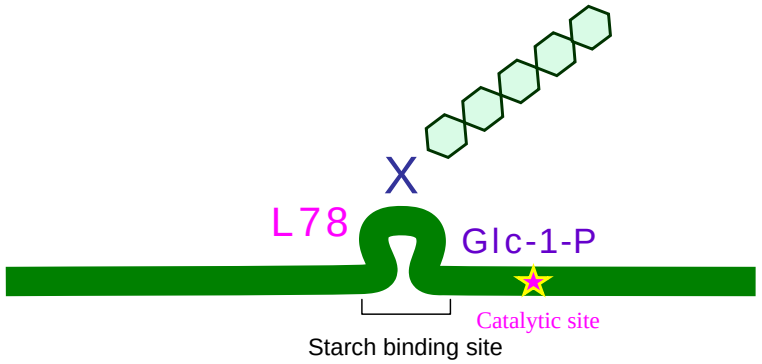
L78 removed



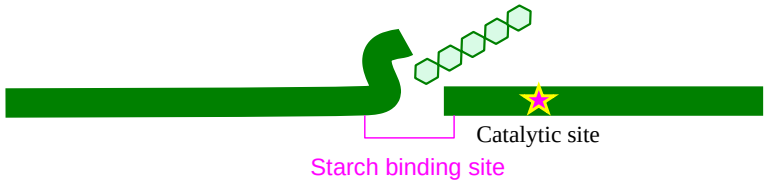
Starch



Phosphate

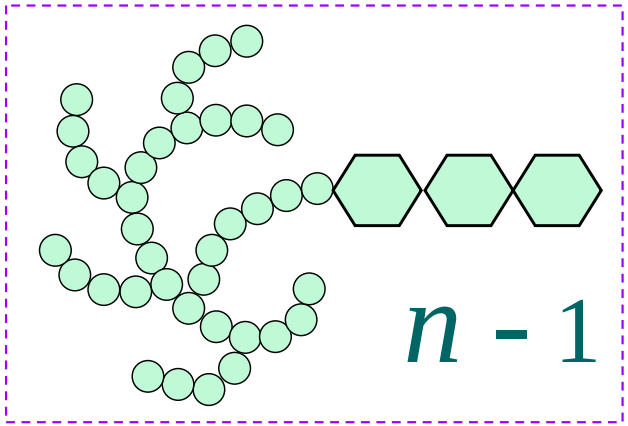


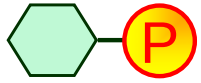
Phosphorolysis



Starch biosynthesis

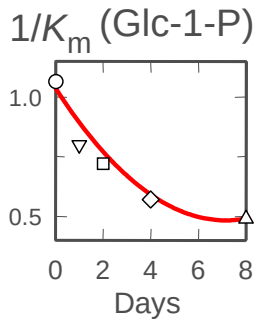
No primer ?
(primer-independent)





Glc-1-P

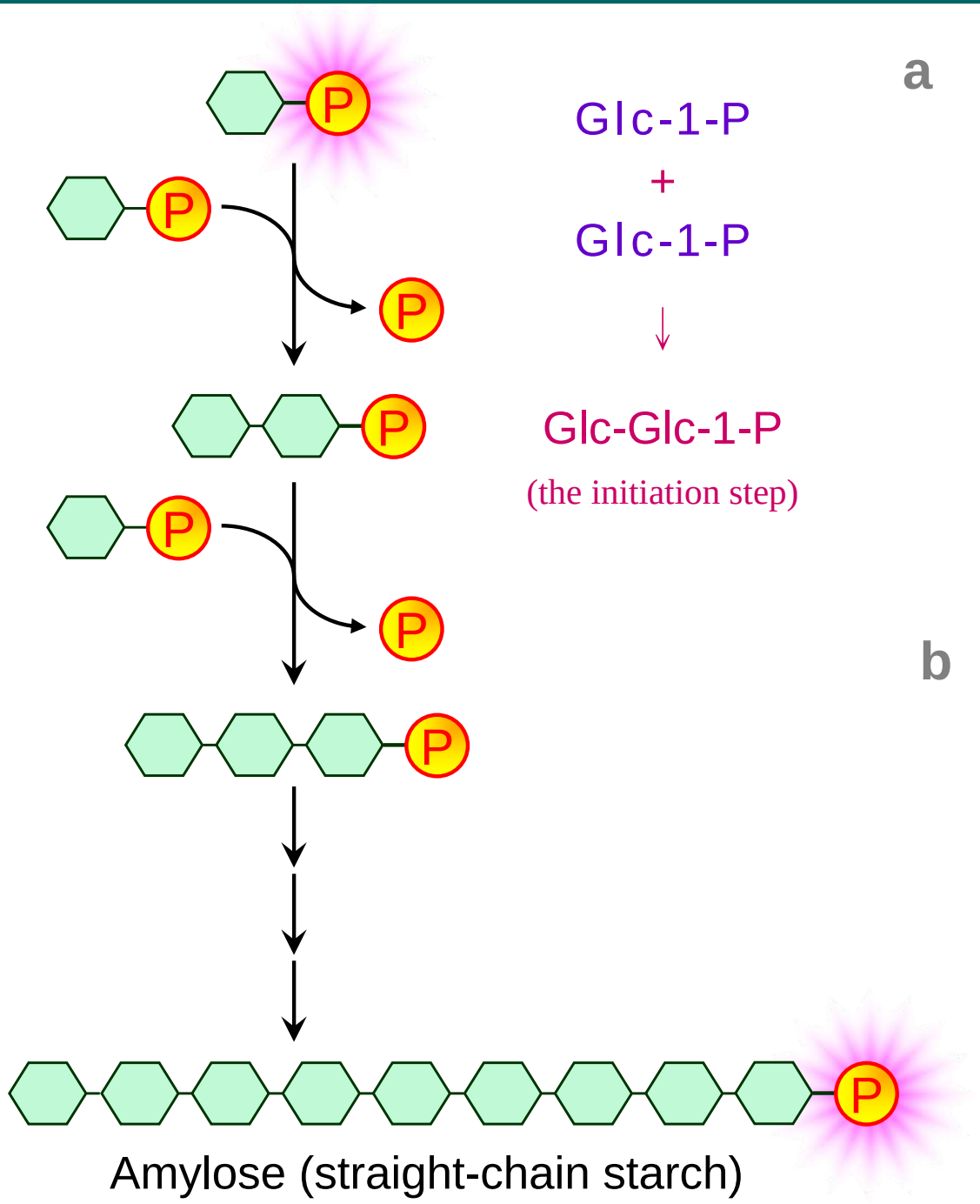
L78 intact



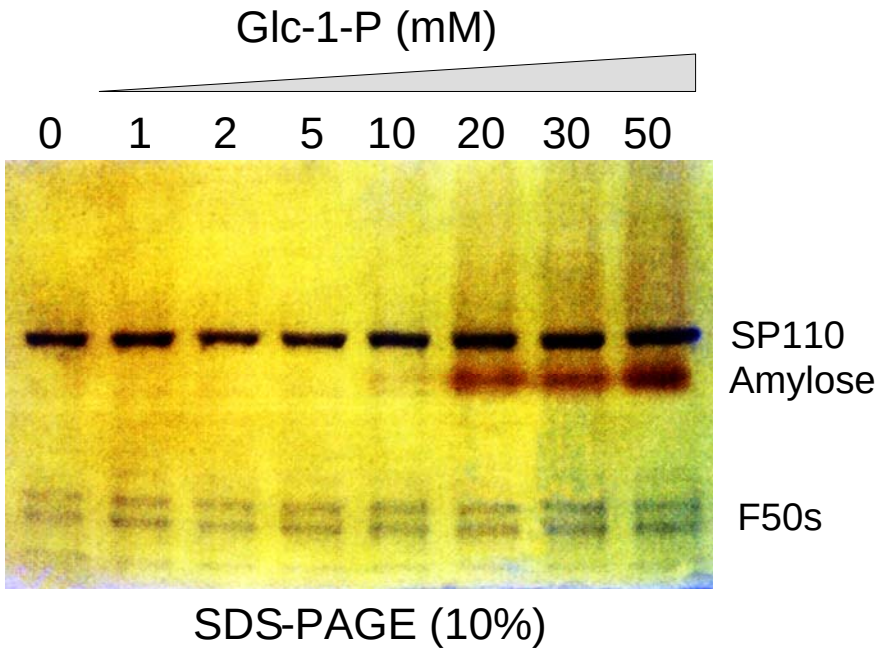
Intact L-SP binds Glc-1-P preferentially and the L78 blocks the starch binding site

Primer-independent glucan biosynthesis from single Glc-1-P

Chen et al, (2006) *submitting*



a



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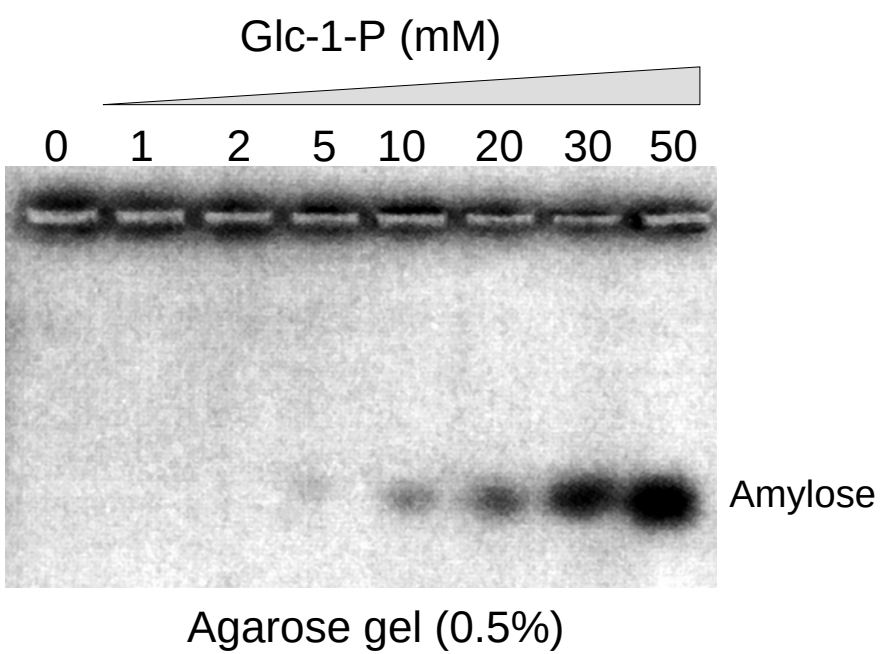
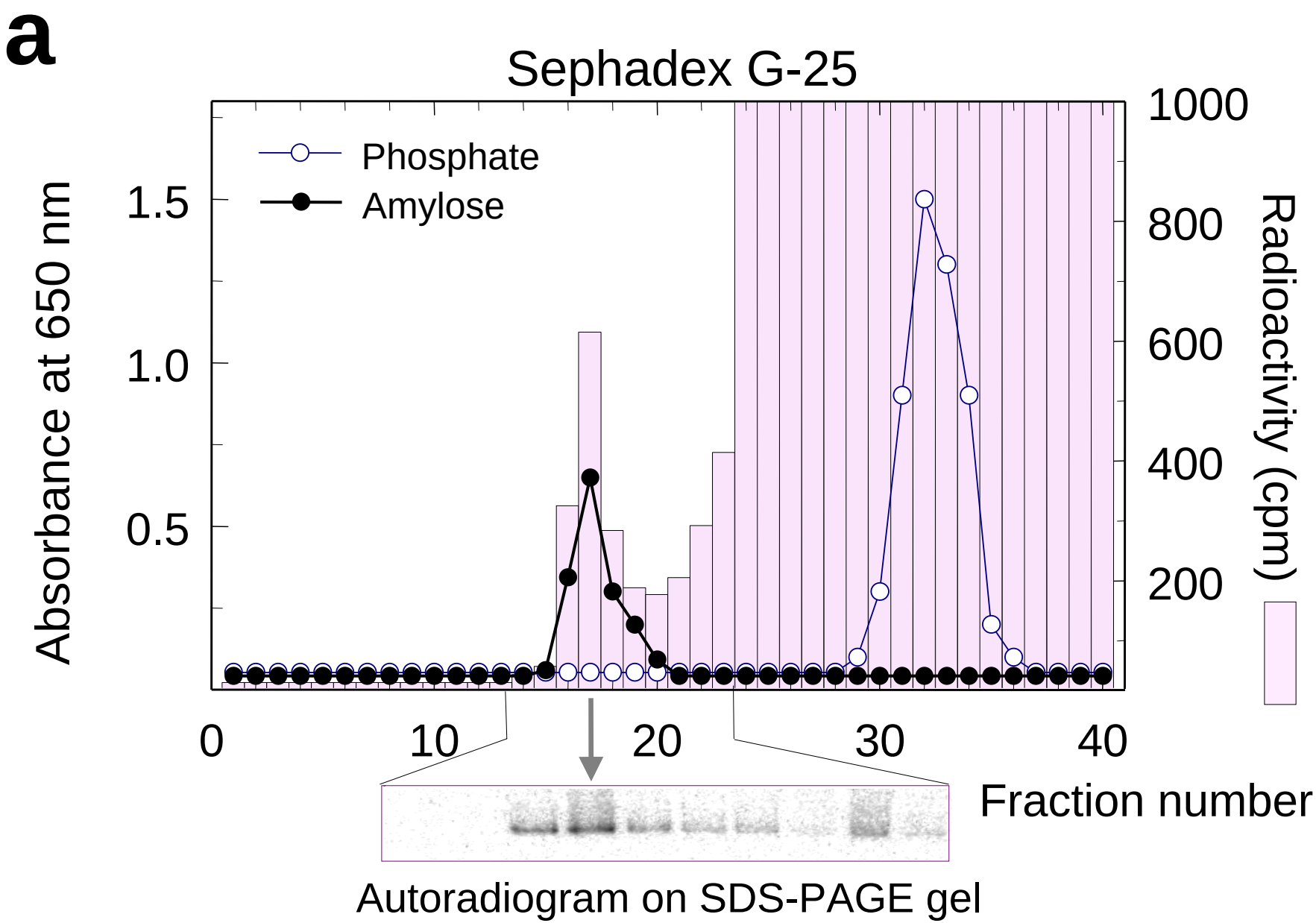


Glucan is synthesized *in vitro* by L-SP from single Glc-1-P in the absence of a primer

The amylose synthesized is radioactive

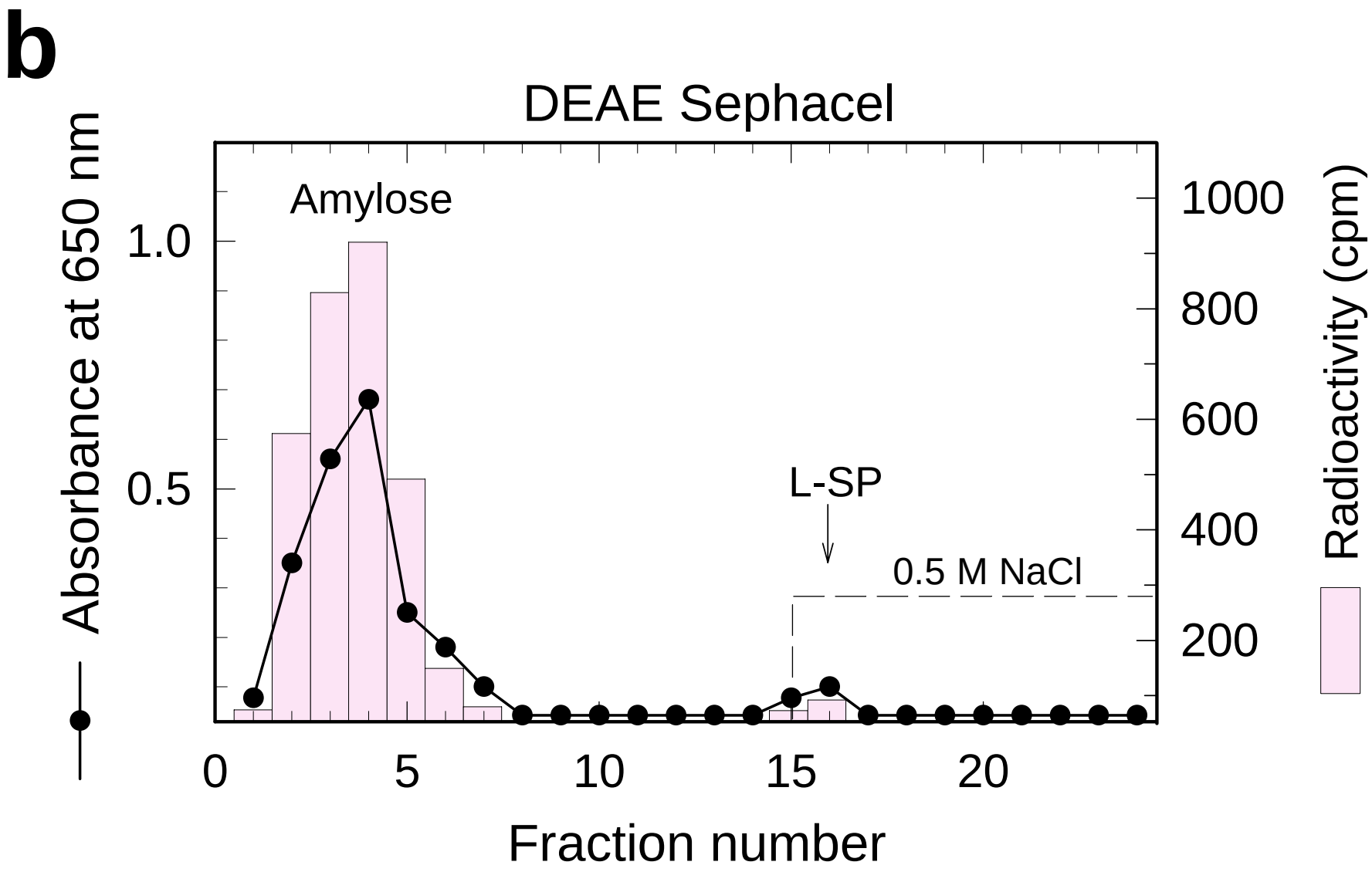
Chen et al, (2006) *submitting*



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

Further purification by ion exchange

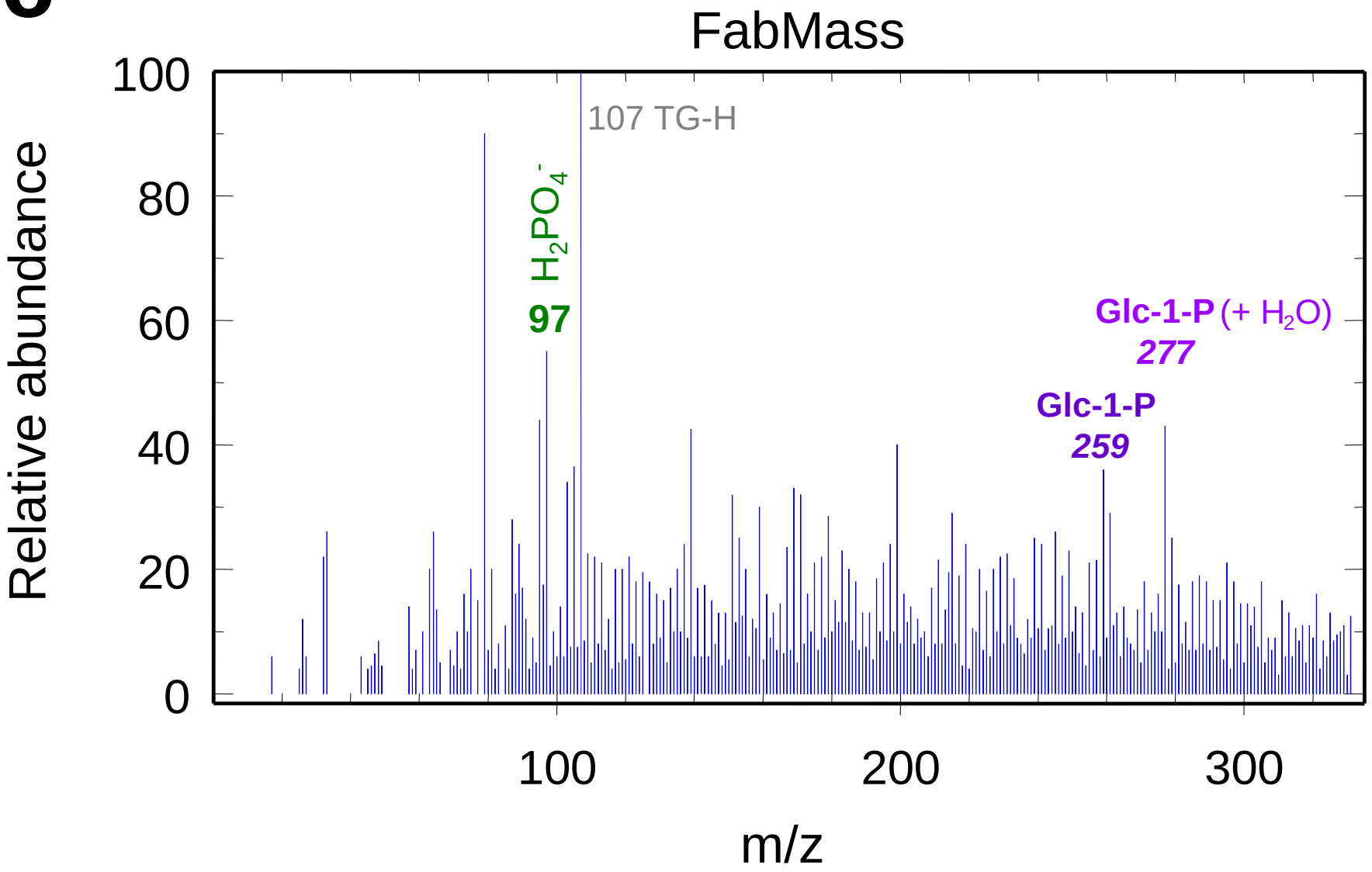
Chen et al, (2006) *submitting*



The amylose contains Glc-1-P moieties

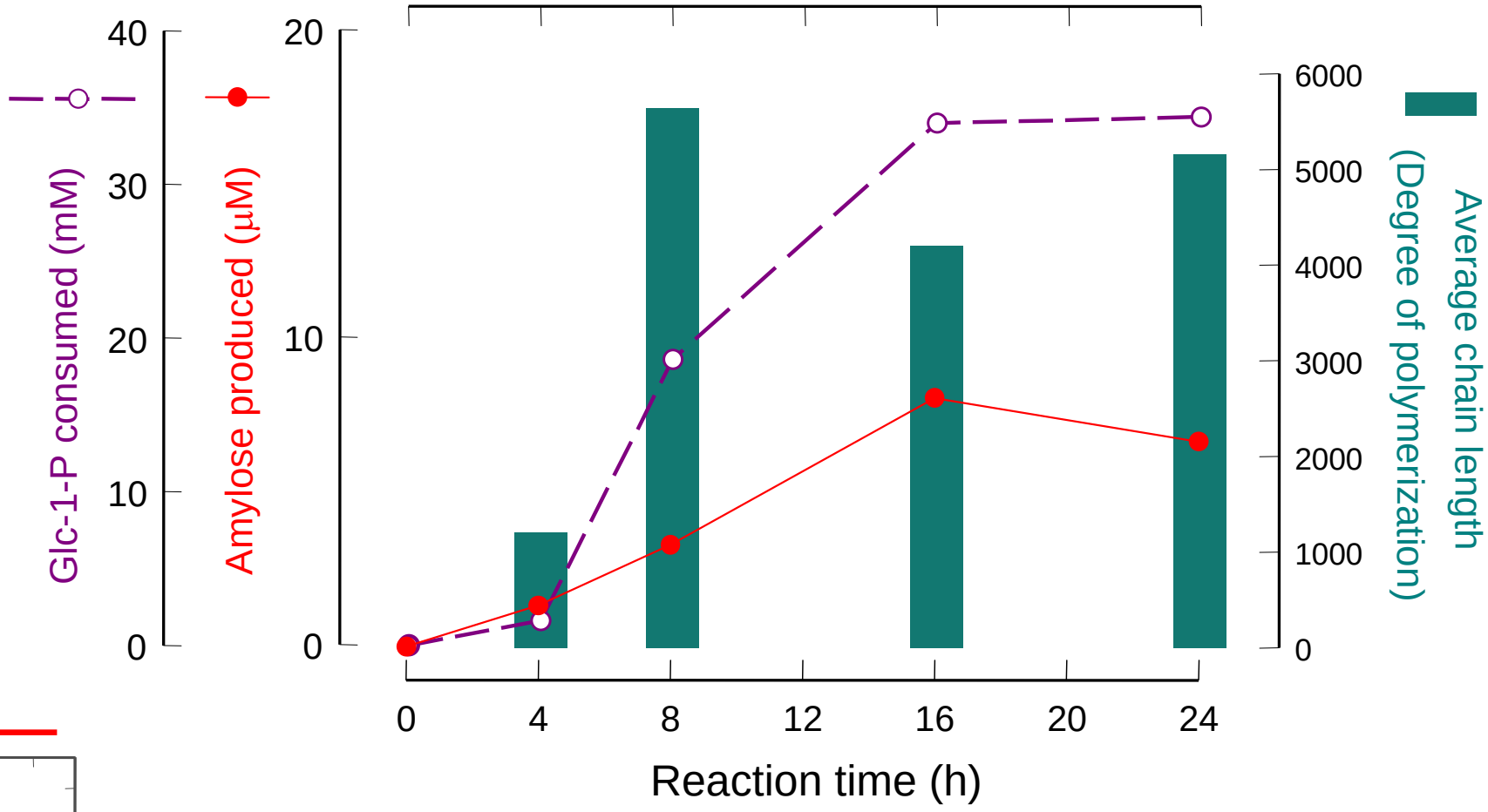
Chen et al, (2006) *submitting*

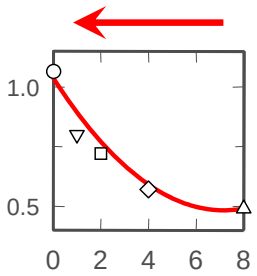
c

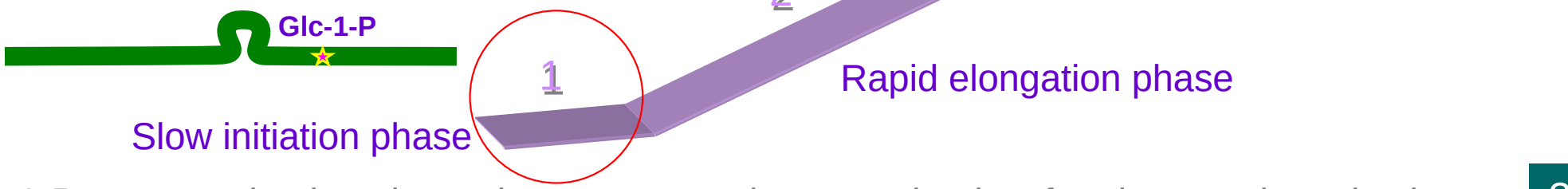


Degree of polymerization reaches several thousands

Chen et al, (2006) *submitting*



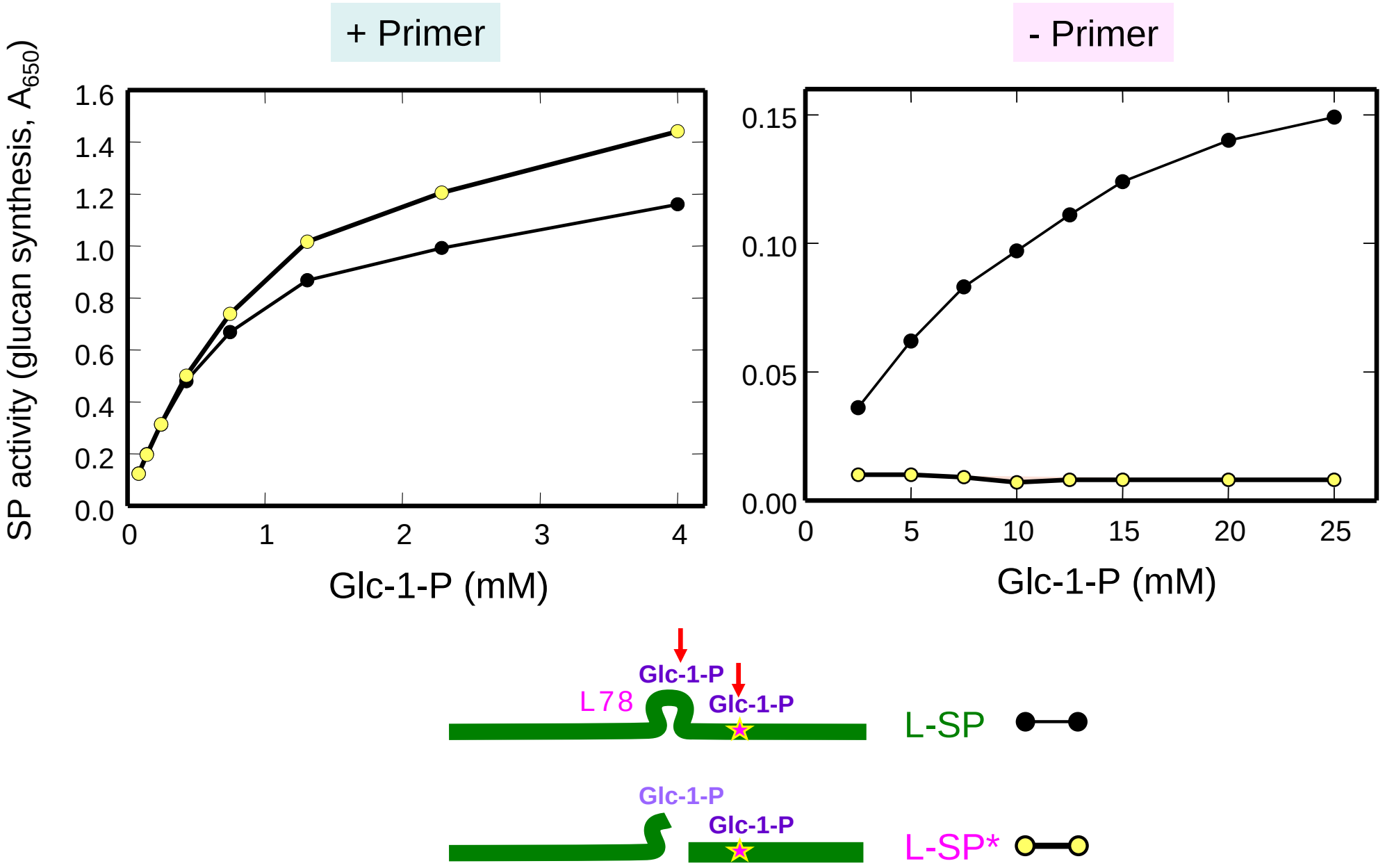




Glc-1-P consumption has three phases, suggesting a mechanism for glucan polymerization

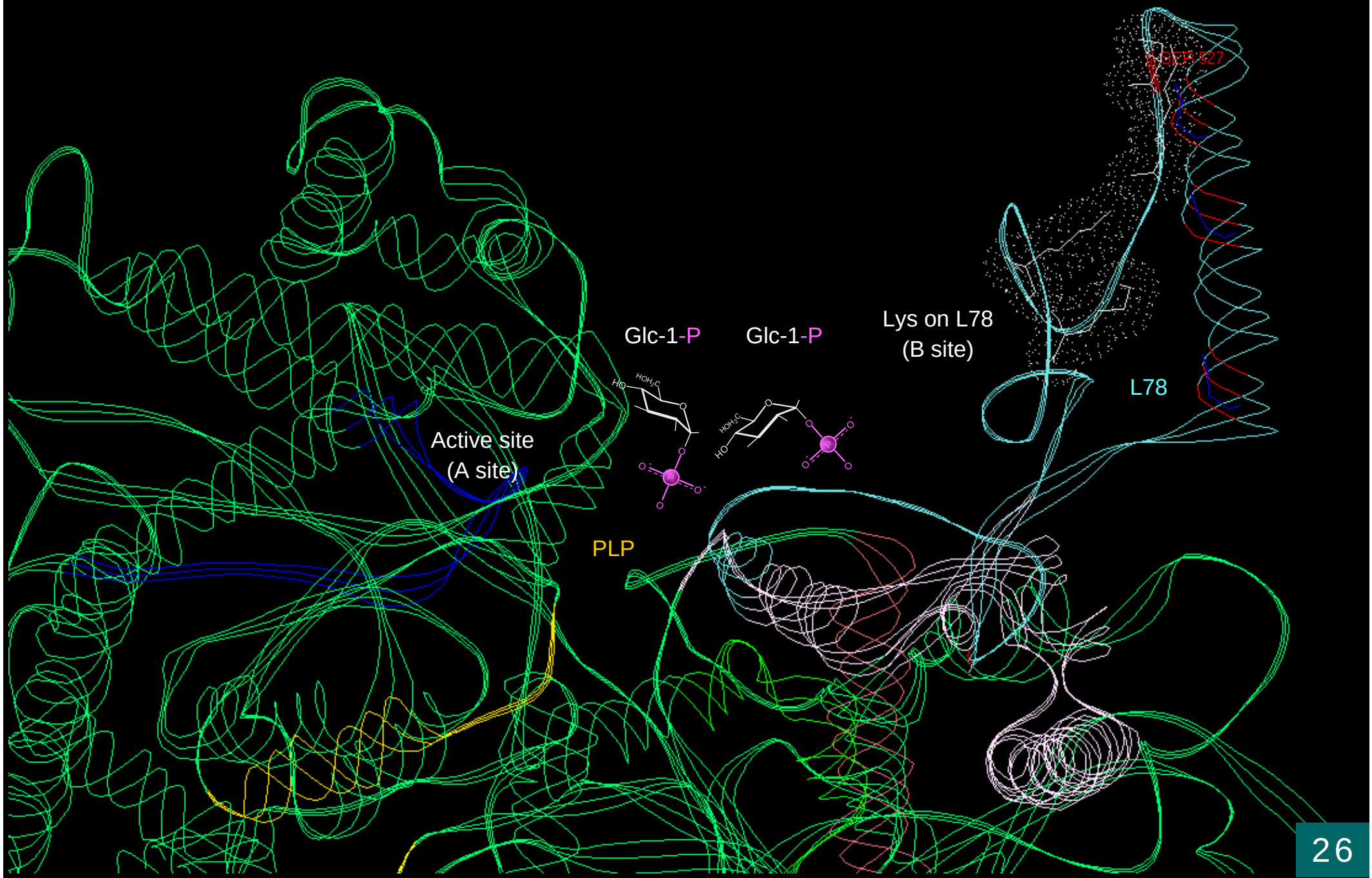
The PI activity of L-SP is lost when its L78 is removed

Chen et al, (2006) *submitting*



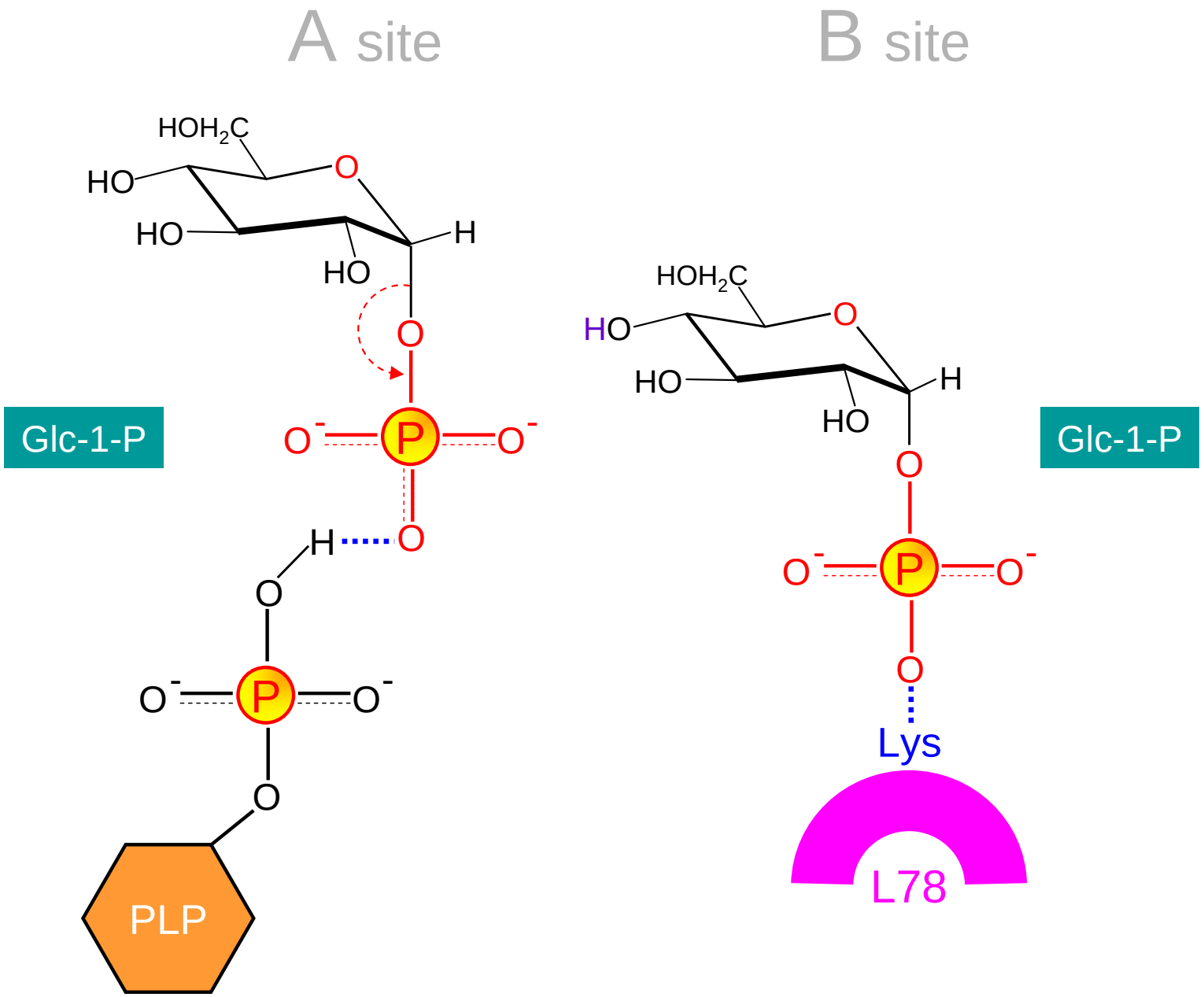
Does L78 serve as the “primer” for amylose synthesis ? Or an anchoring point for Glc-1-P?

Active site of L-SP and possible PI action mechanism



Action mechanism for PI amylose synthesis (1)

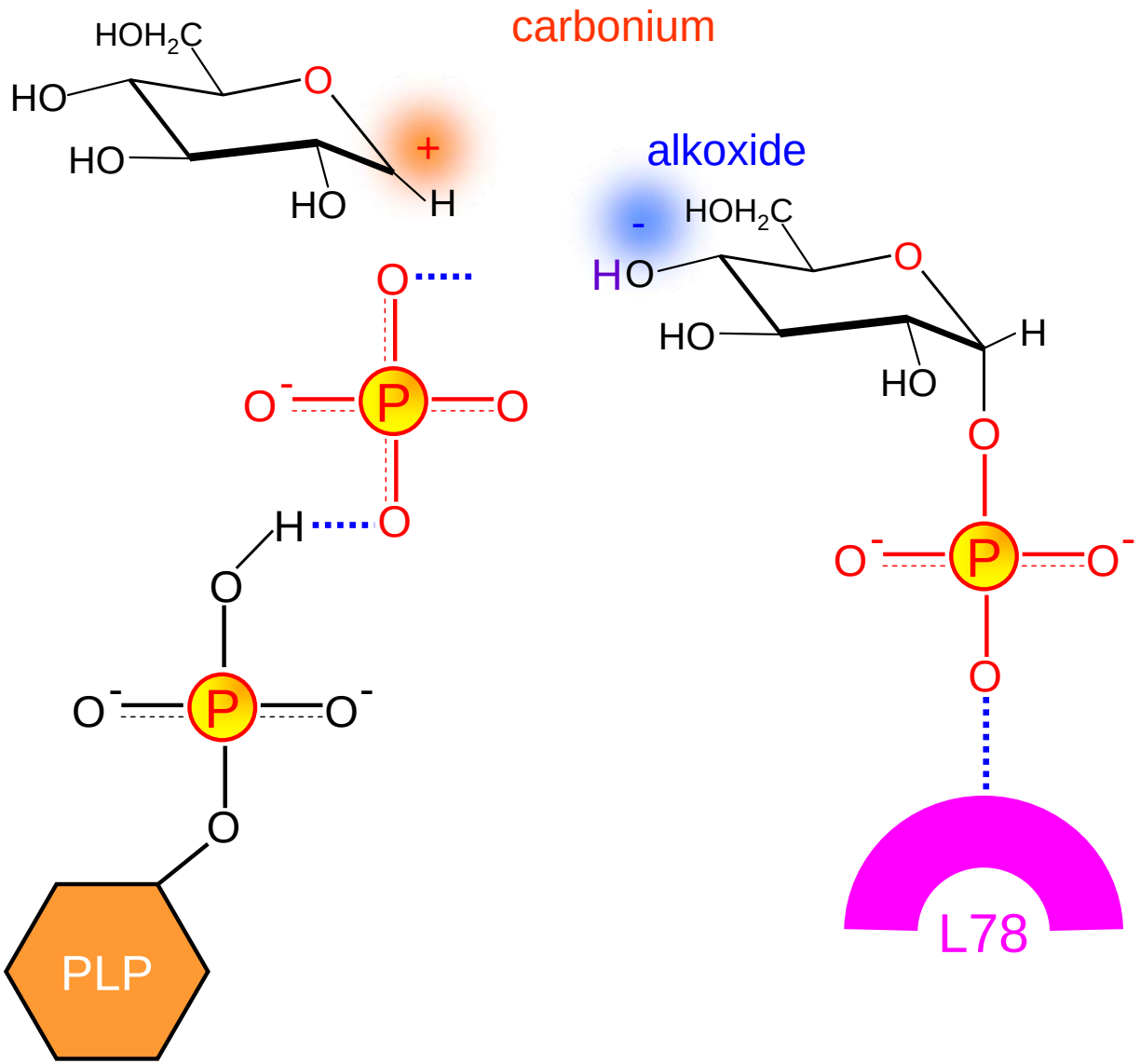
Unpublished



Glc-1-P on A site loses its phosphate as interacting with the phosphate on the cofactor PLP

Action mechanism for PI amylose synthesis (2)

Unpublished

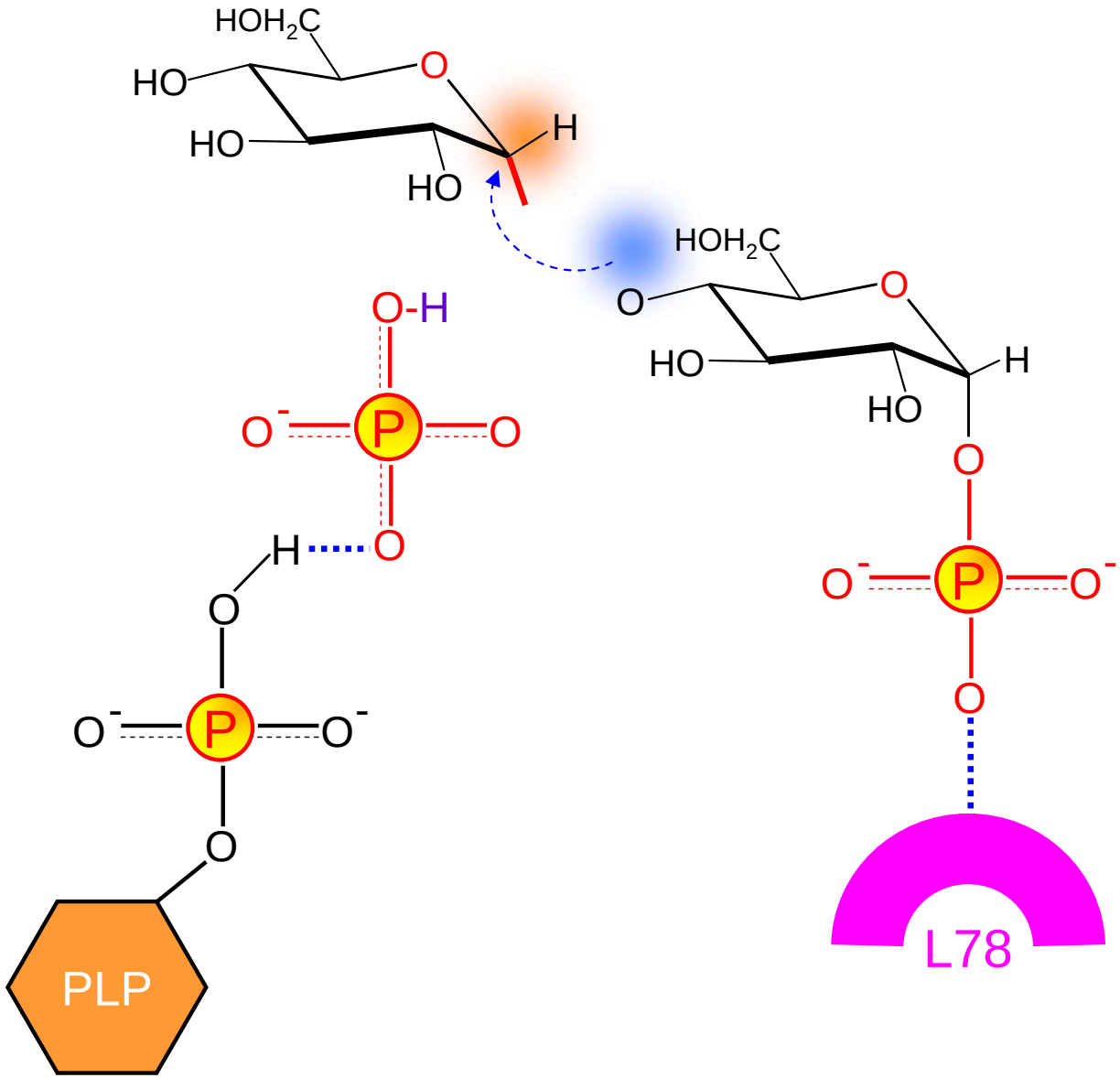


The C-1 on Glc (A site) becomes a carbonium ion after releasing the phosphate

The released phosphate attracted a proton from the hydroxyl group (C-4) of the B site Glc-1-P

Action mechanism for PI amylose synthesis (3)

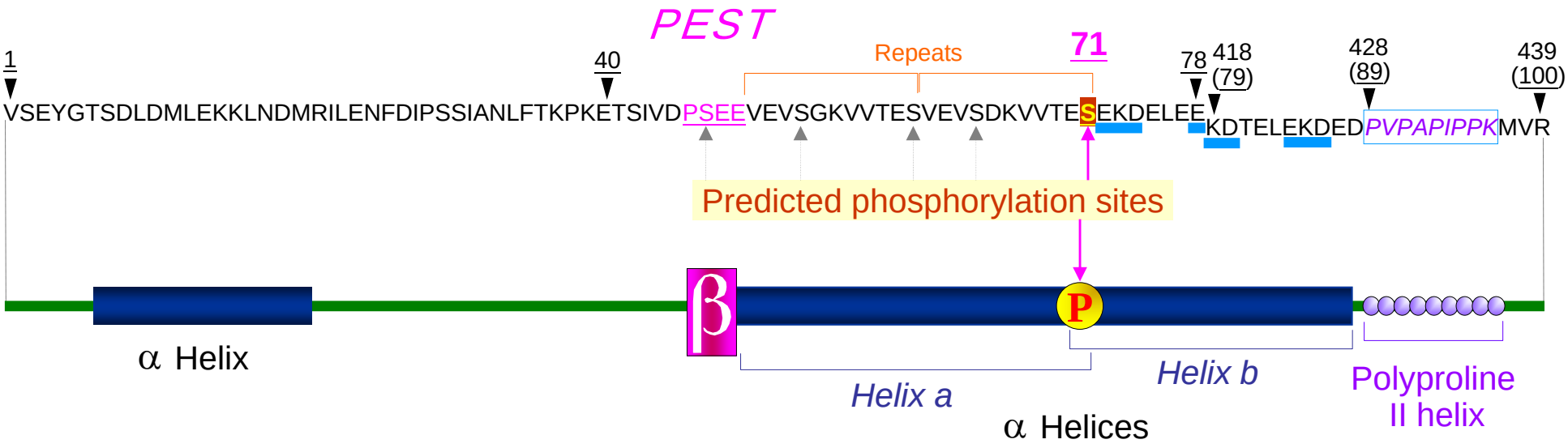
Unpublished

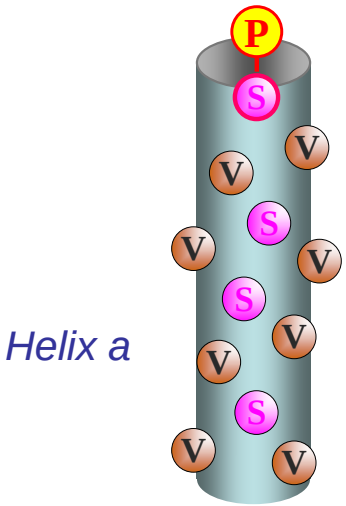


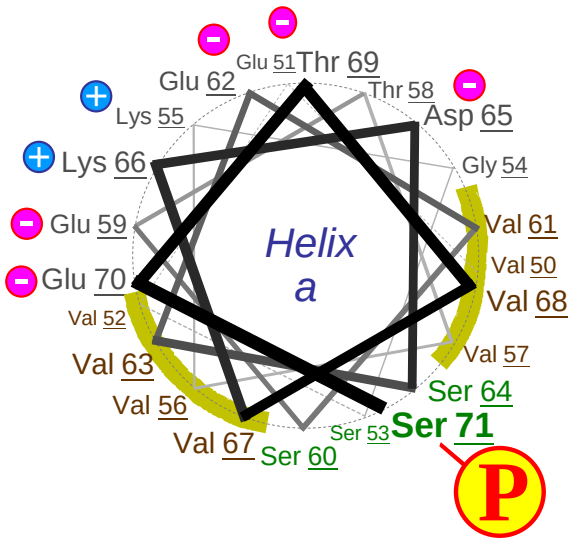
The negatively charged alkoxide attacks the carbonium ion producing a new glycosidic bond

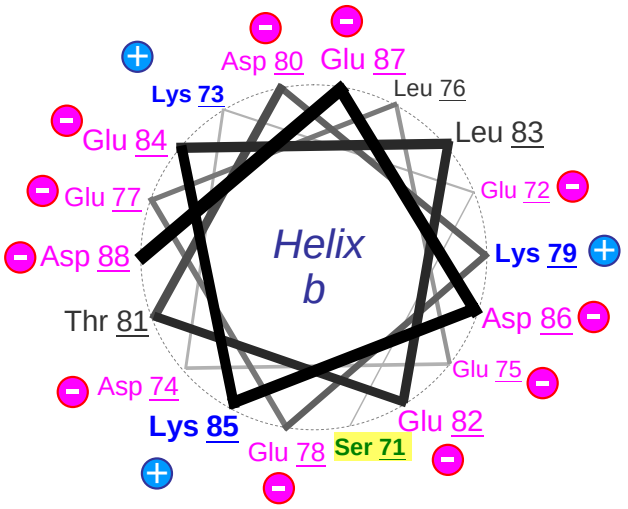
L-SP is predicted as phosphorylated

Chen et al, (2002) Physiologia Plantarum **114**:506-515





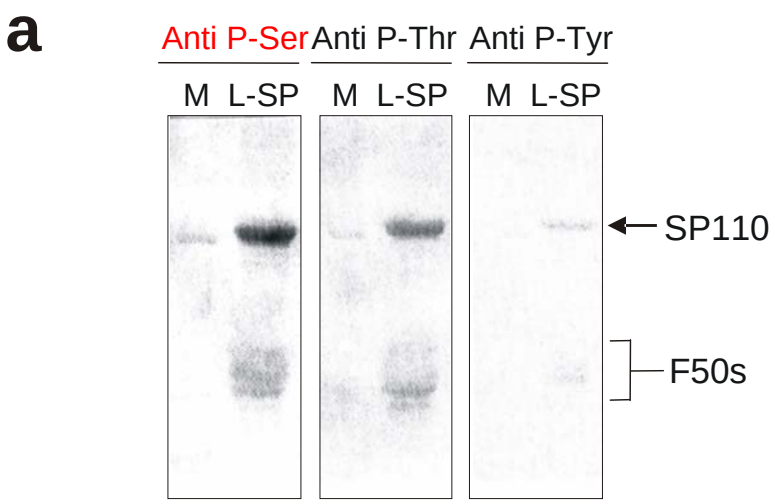




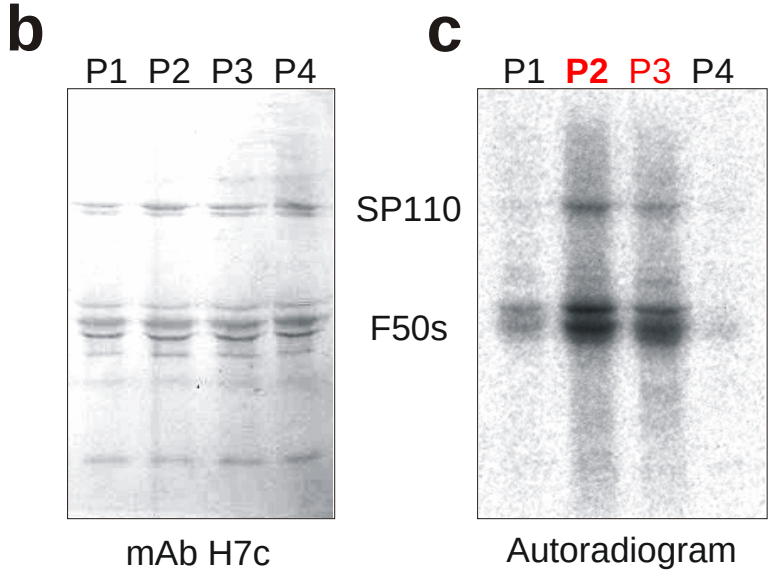
Several phosphorylation sites are predicted in the helices on L78

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

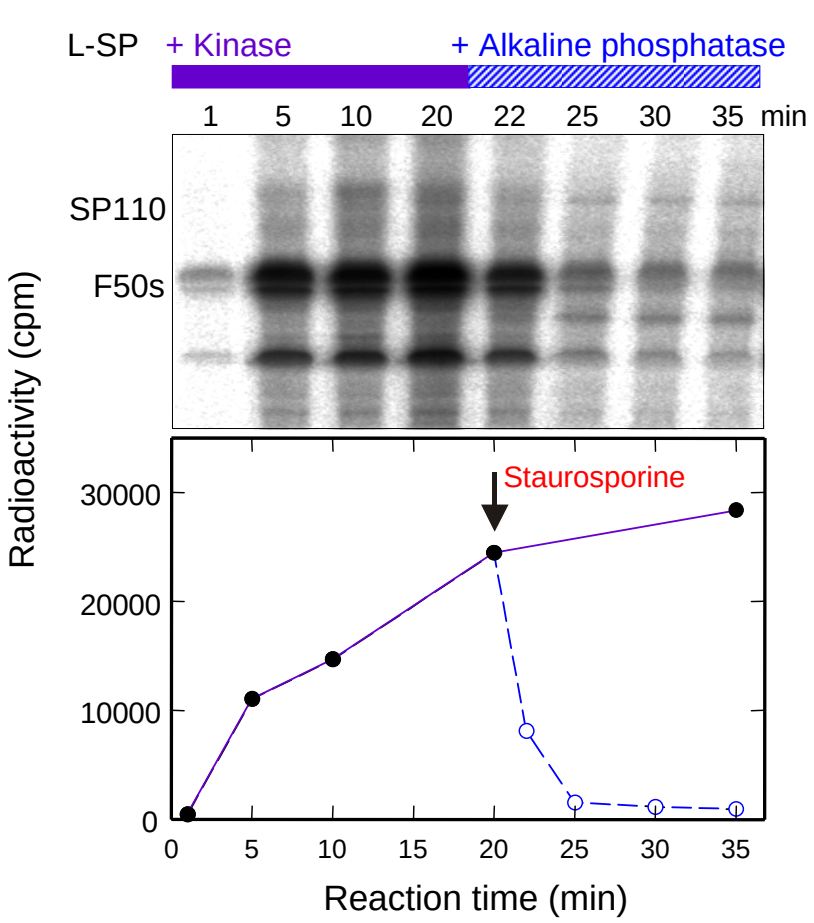
Young et al, (2006) Planta **223**: 468-478



Purified L-SP was phosphorylated in the plant



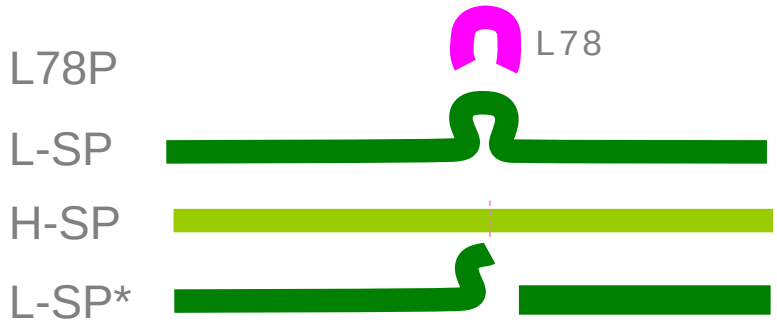
Ammonium sulfate fractions contained a kinase activity

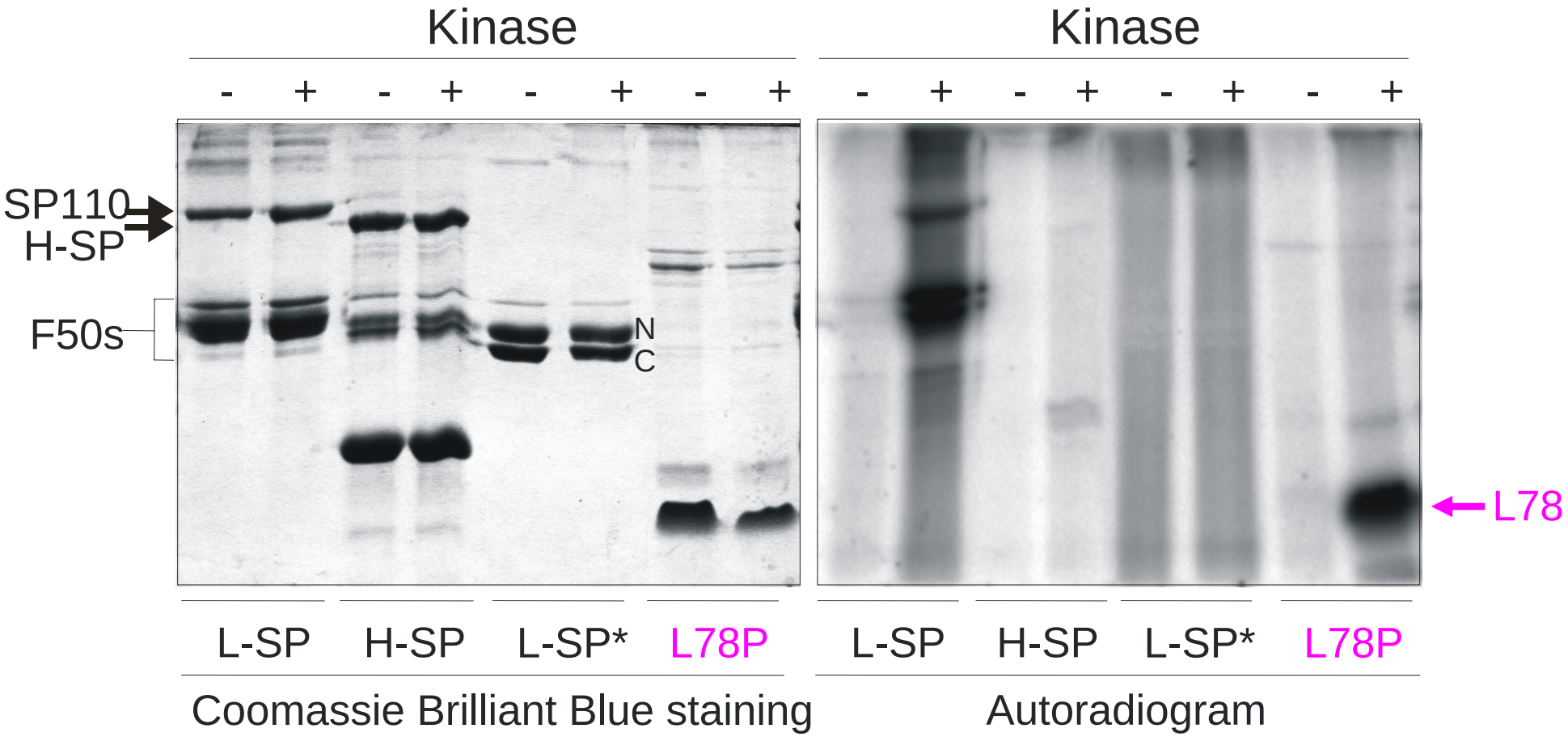


L-SP is found phosphorylated in sweet potato roots, or *in vitro* phosphorylated by a protein fraction from the root extract

L-SP is phosphorylated specifically on its L78 insertion

Young et al, (2006) Planta **223**: 468-478

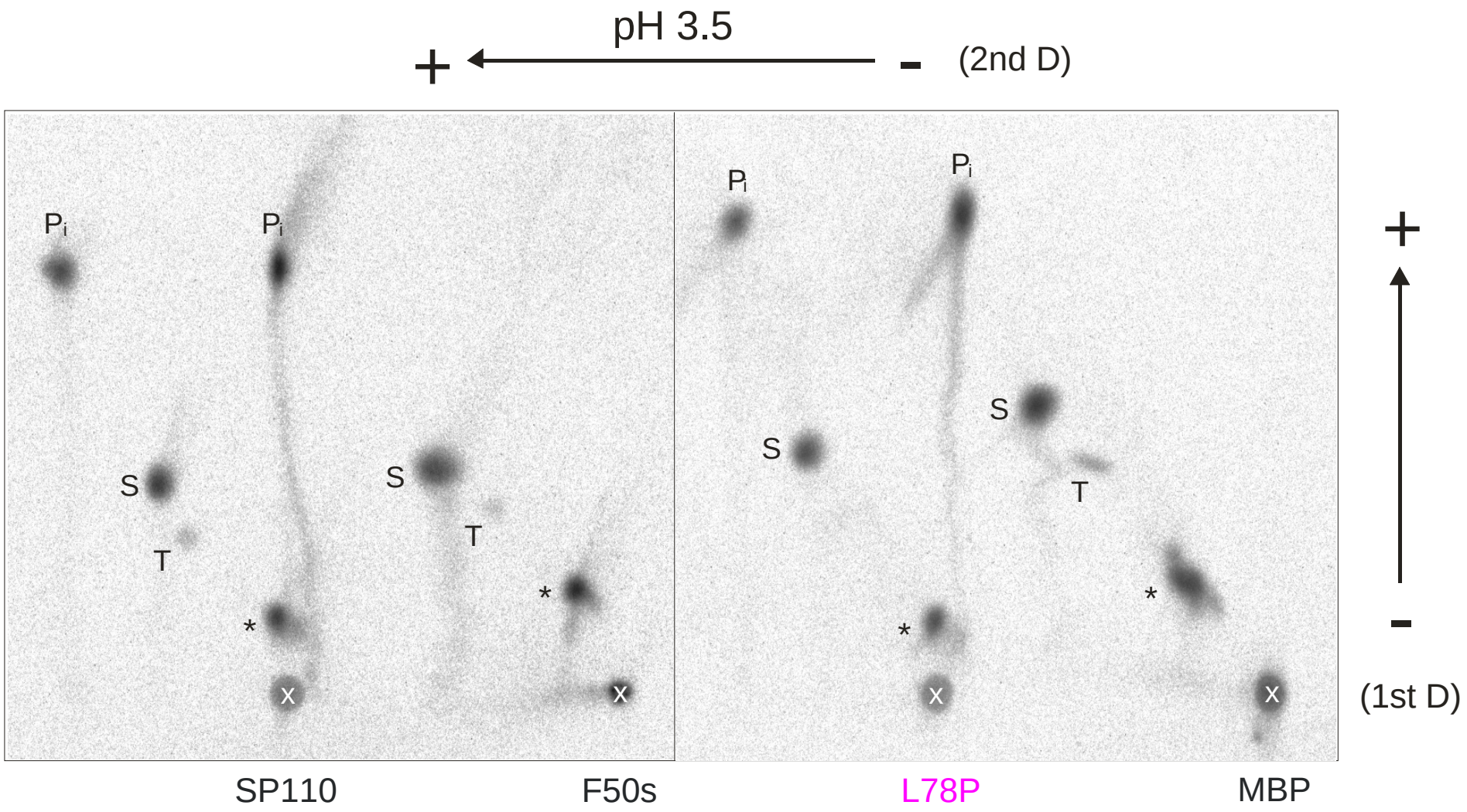




Phosphorylase molecules lacking L78 insertion can not been phosphorylated

Ser on L-SP is the target for the kinase

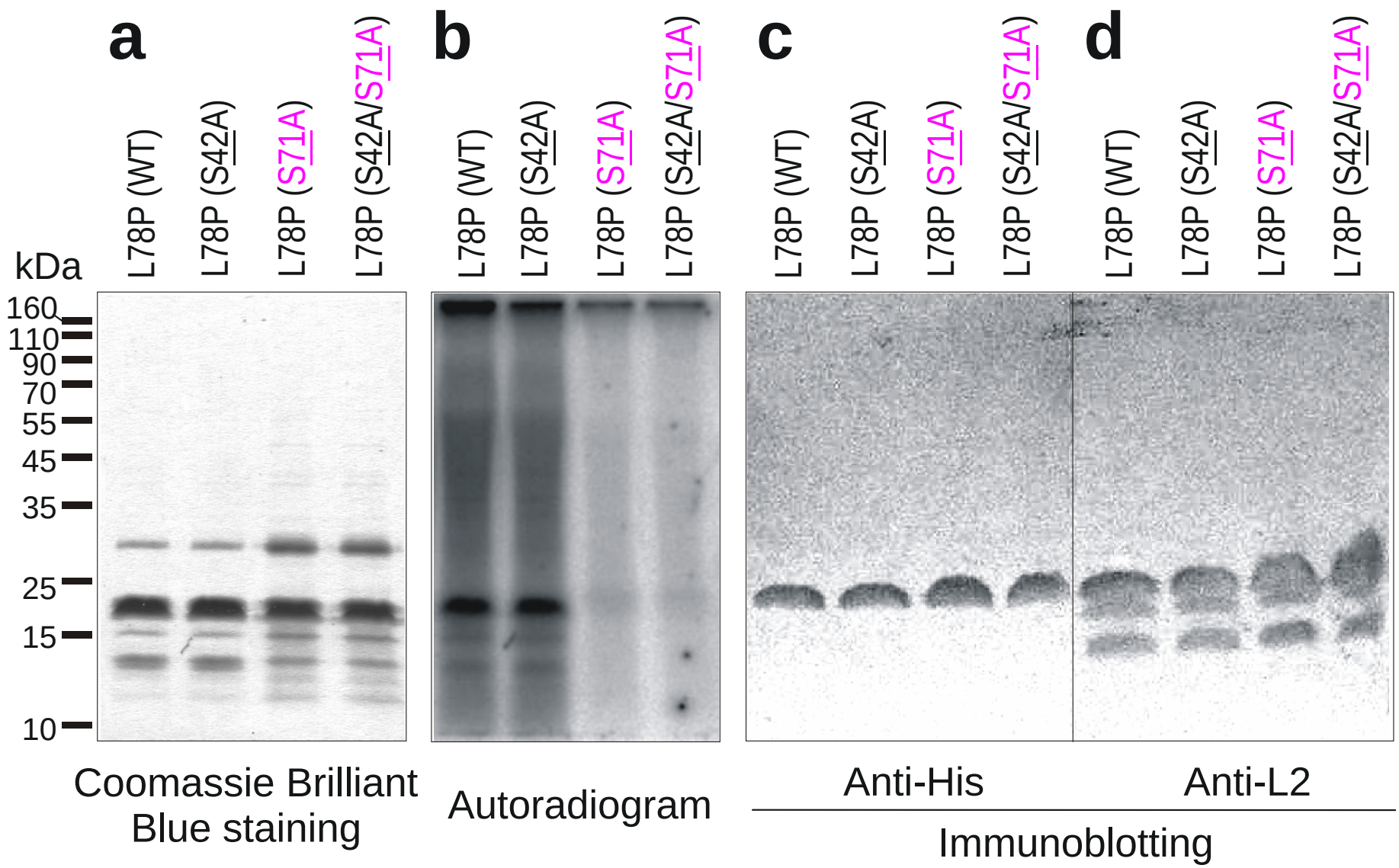
Young et al, (2006) Planta **223**: 468-478



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 71 of L78

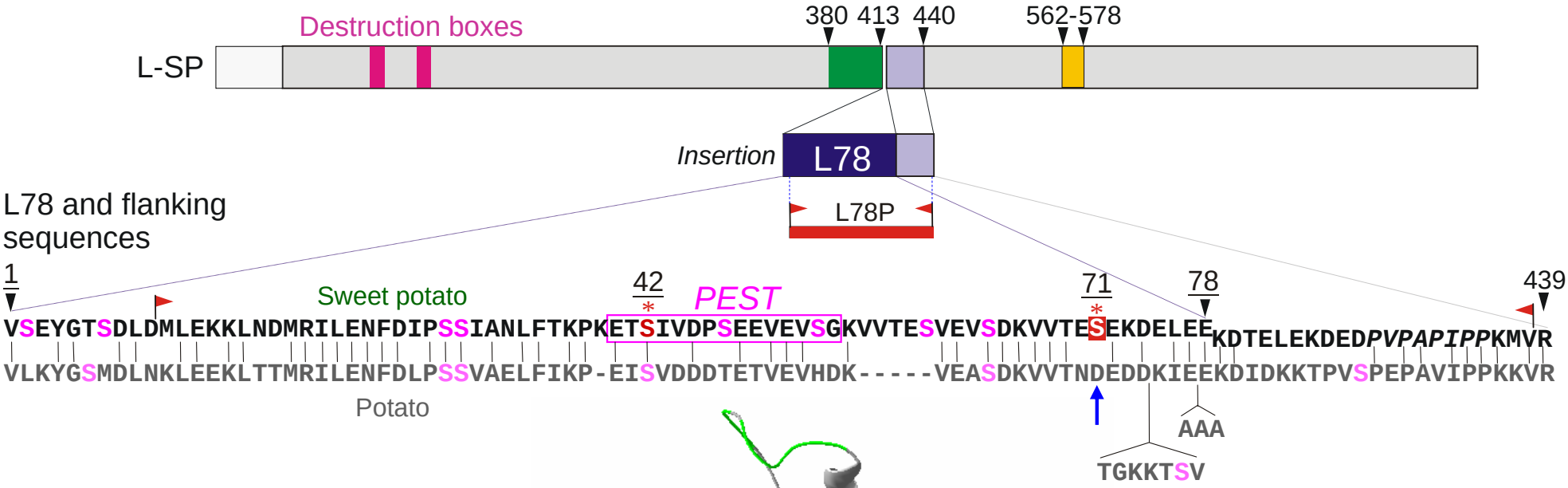
Young et al, (2006) Planta **223**: 468-478

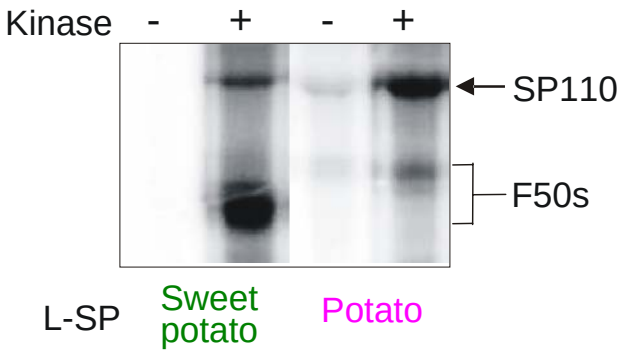


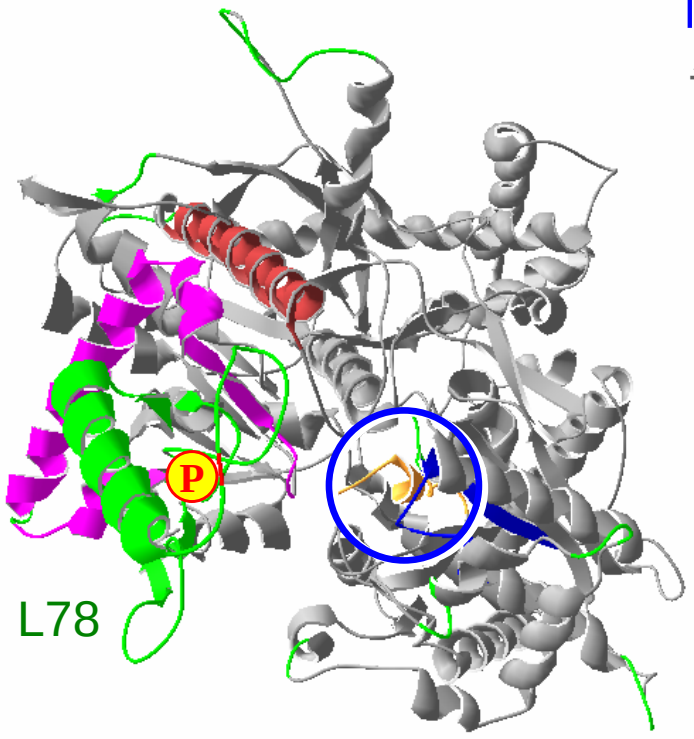
Ser 71 on L78 is the only phosphorylation site on L-SP by the kinase

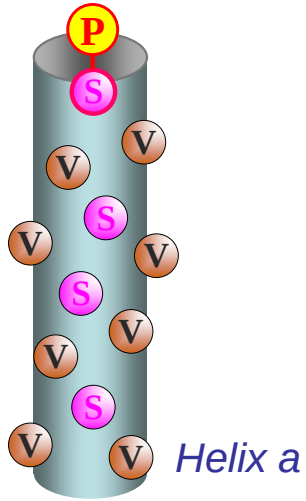
Specificity of the phosphorylation site

Young et al, (2006) Planta **223**: 468-478

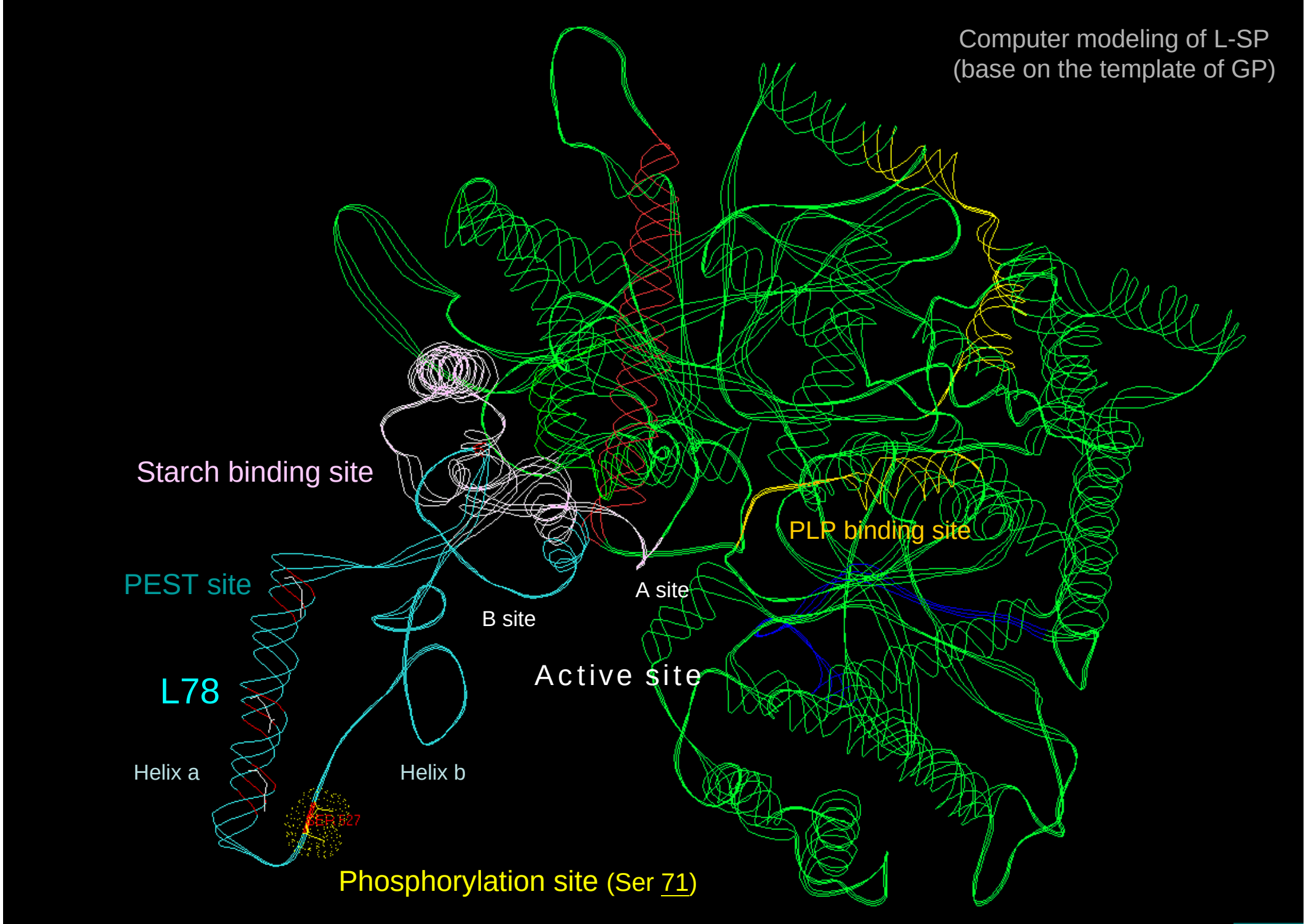








Although this kinase could also phosphorylate L-SP from potato, the exact phosphorylation site and mechanism are unclear



What is the possible physiological function for the phosphorylation of L-SP?

Phosphorylated L-SP has no change in its kinetic parameters

Young et al, (2006) Planta **223**: 468-478

$$\text{Starch}_{(n)} + P_i \rightleftharpoons \text{Starch}_{(n-1)} + \text{Glc-1-P}$$

Synthetic direction (Chen et al. 2002)

	K_m		K_{cat} (1/s)	
L-SP	Soluble starch (%, w/v)	Glc-1-P (mM)	Fixed [Glc-1-P] [a]	Fixed [soluble starch] [b]
Unmodified	0.077 ± 0.015	1.052 ± 0.311	100.1 ± 28.6	99.2 ± 5.8
Phosphorylated	0.070 ± 0.016	1.090 ± 0.320	98.6 ± 27.7	97.1 ± 4.5

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

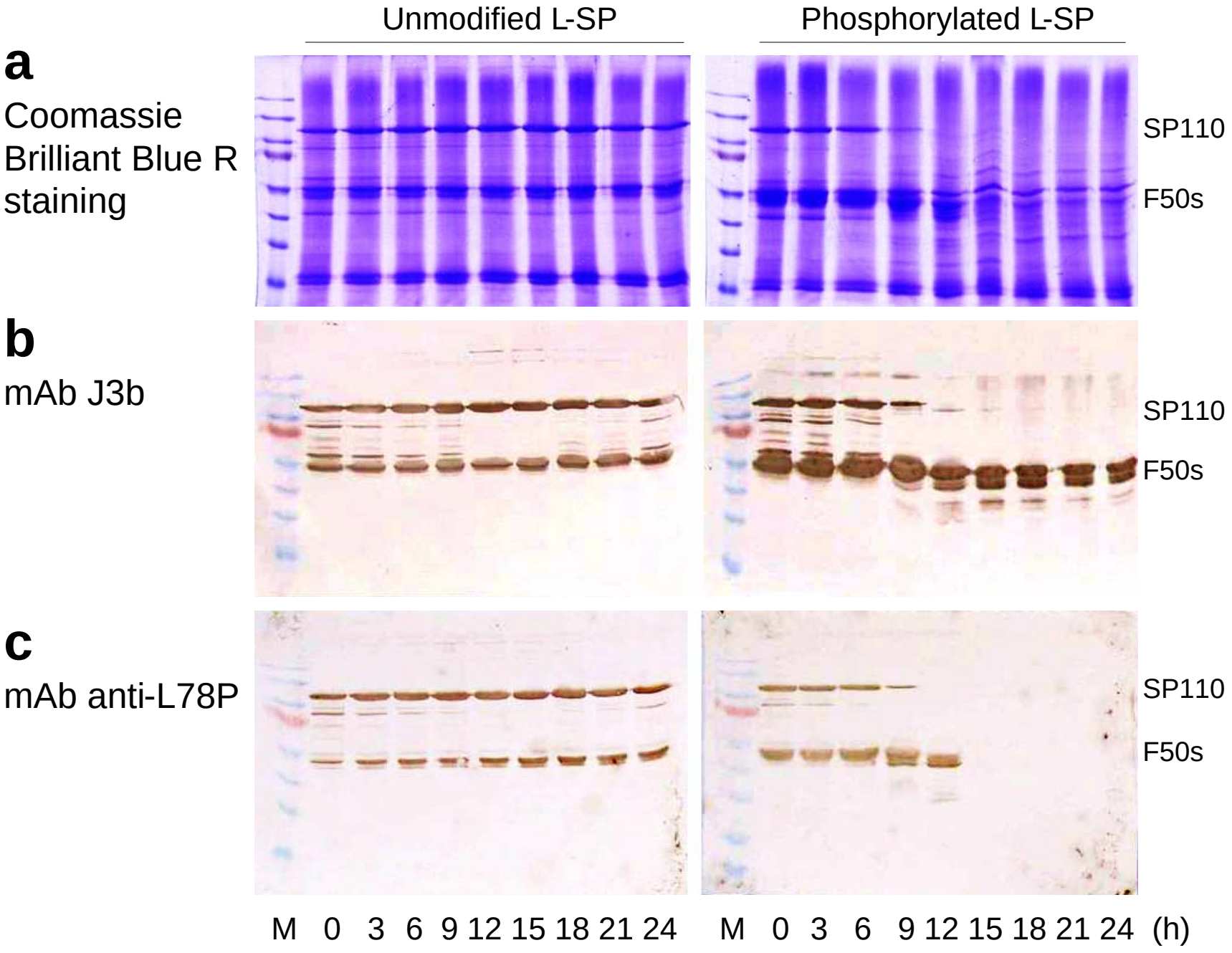
Phosphorolytic direction (Mori et al. 1993)

	K_m		K_{cat} (1/s)	
L-SP	Soluble starch (%, w/v)	P_i (mM)	Fixed [P_i] [c]	Fixed [soluble starch] [d]
Unmodified	0.115 ± 0.023	1.498 ± 0.562	22.39 ± 5.07	18.46 ± 4.08
Phosphorylated	0.108 ± 0.021	1.443 ± 0.568	21.92 ± 3.31	18.06 ± 3.40

[c] [P_i] = 5 mM; [d] [soluble starch] = 0.2%

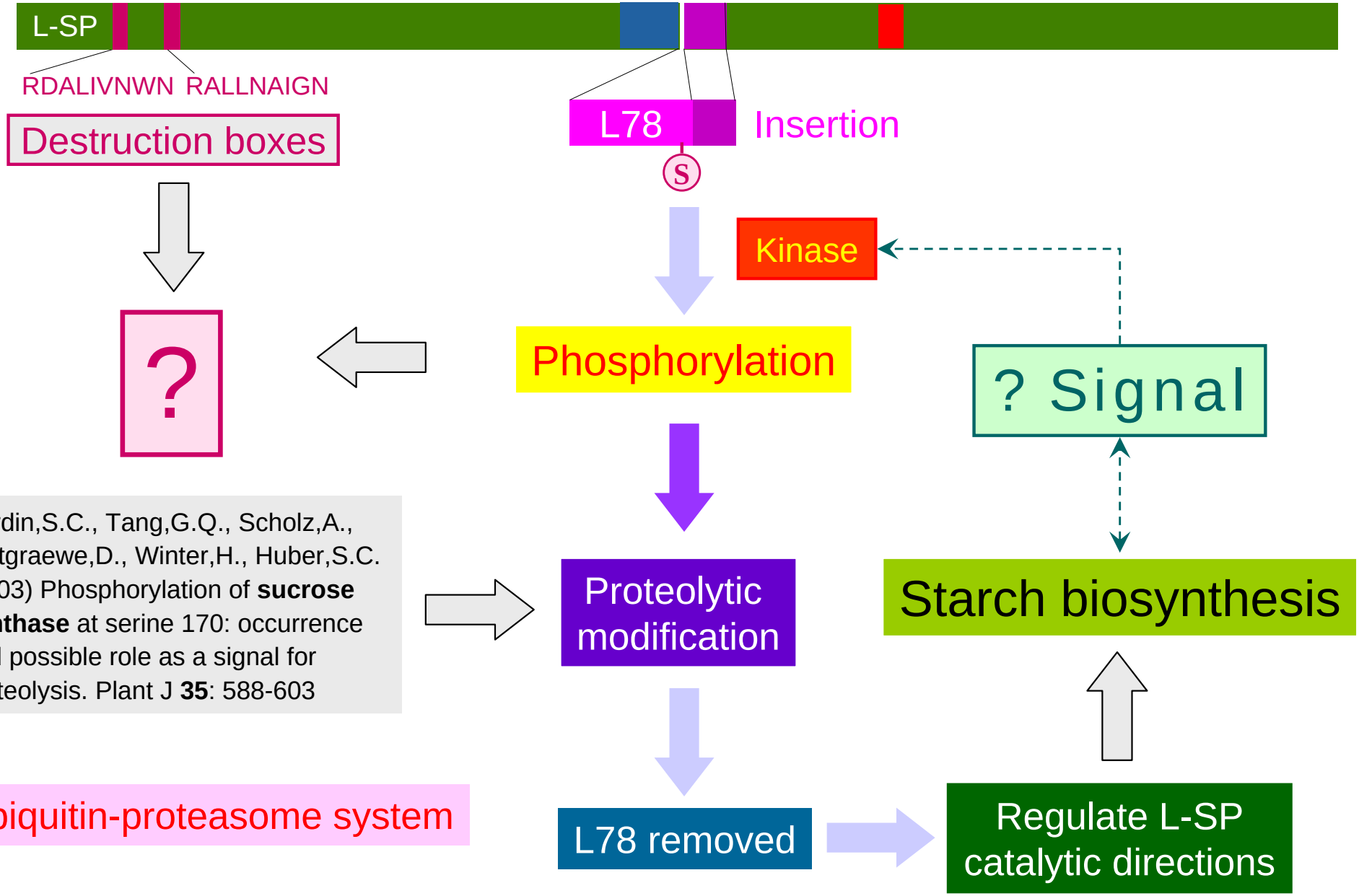
Phosphorylated L-SP is sensitive to proteolytic modification

Young et al, (2006) Planta **223**: 468-478



Is the phosphorylation of L-SP a signal for its proteolytic modification on L78?

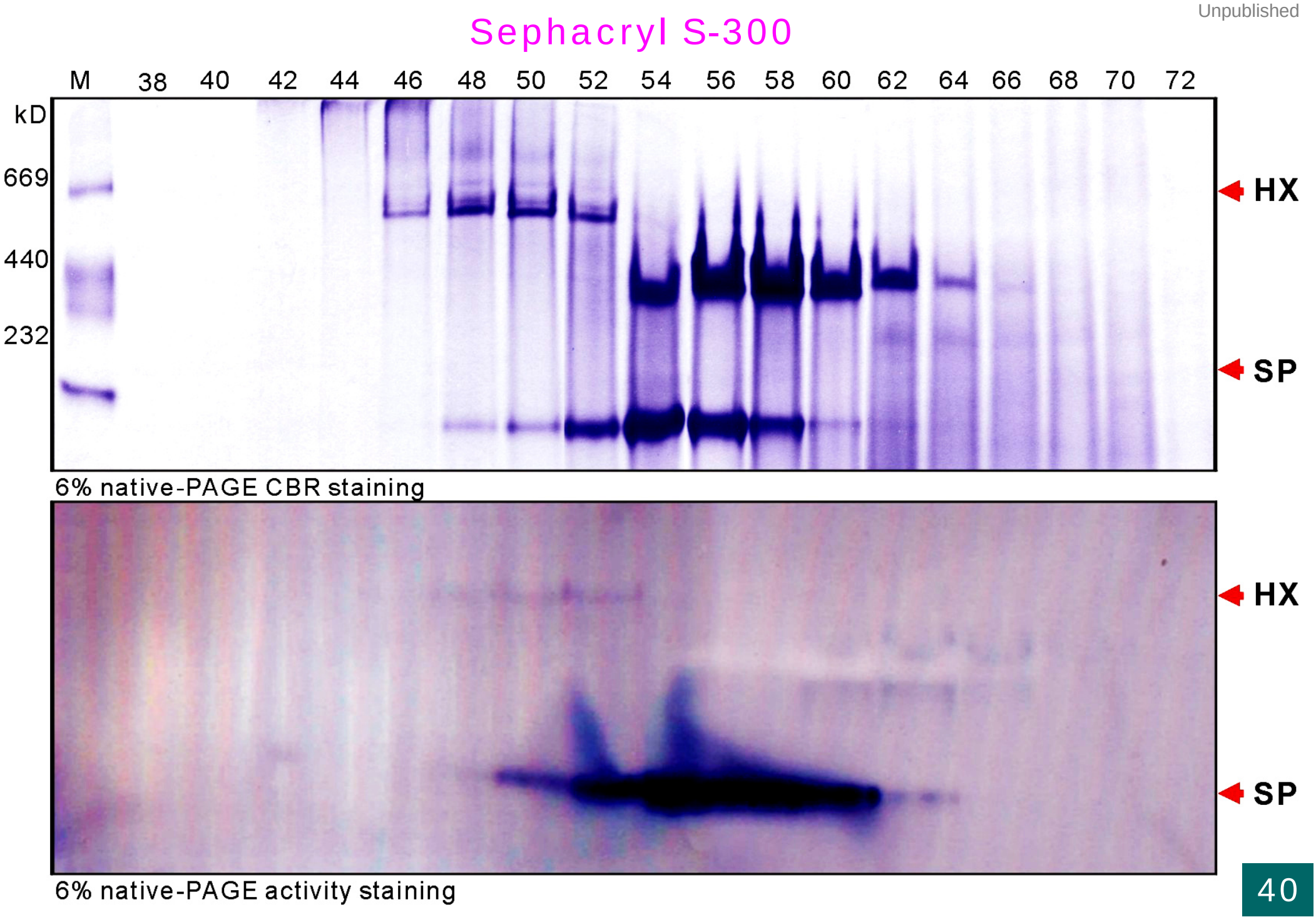
How is the phosphorylation connected to proteolysis?



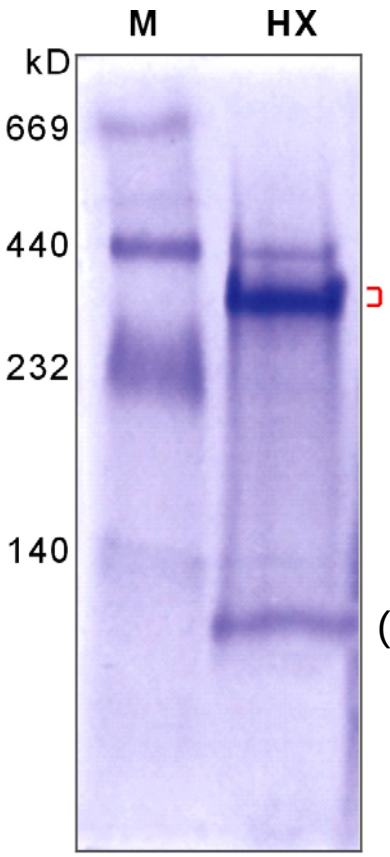
Phosphorylation of L78 might trigger the removal of the L78 insertion, and change the catalytic behavior of L-SP from starch synthesis to phosphorolysis

A high MW complex (HX) expressing SP activity is found

Unpublished



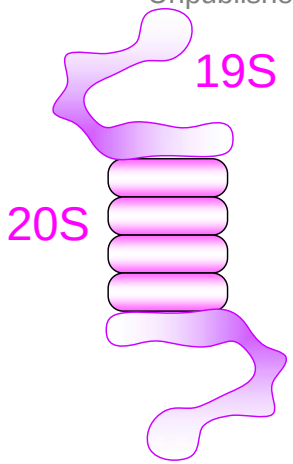
HX consists of L-SP and 20S proteasome



6 % native-PAGE
CBR staining

no.	Full protein name	Matched peptide	Sequence coverage (%)	Match score	Species	Accession number
1	20S proteasome α-subunit	8	29	333	*Lycopersicon esculentum*	CAA74725
	20S proteasome α-subunit	6	19	252	*Glycine max*	AAC28135
	20S proteasome α-subunit	6	21	145	*Arabidopsis thaliana*	CAA74025
	20S proteasome α2-subunit	6	16	236	*Arabidopsis thaliana*	AAG48830
	starch phosphorylase	4	5	228	*Ipomoea batatas*	1802404A
	20S proteasome α1-subunit	7	21	217	*Nicotiana tabacum*	CAB39975
	20S proteasome α1-subunit	4	13	174	*Oryza sativa*	XP_470540
	20S proteasome α5-subunit	6	17	190	*Glycine max*	AAF70292
	20S proteasome α3-subunit	5	17	184	*Euphorbia esula*	AAF34770
	20S proteasome α3-subunit	5	15	159	*Petunia x hybrida*	AAC35982

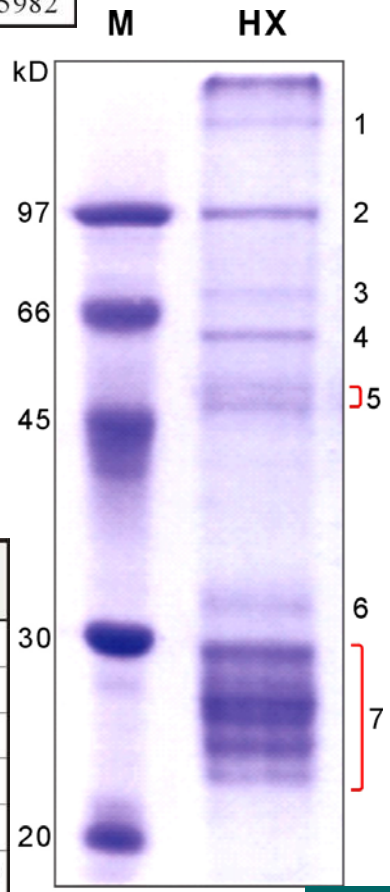
Unpublished



(L-SP)

HX → Native-PAGE → Band 1 → LC/MS/MS

HX → SDS-PAGE → Band 1~7 → LC/MS/MS

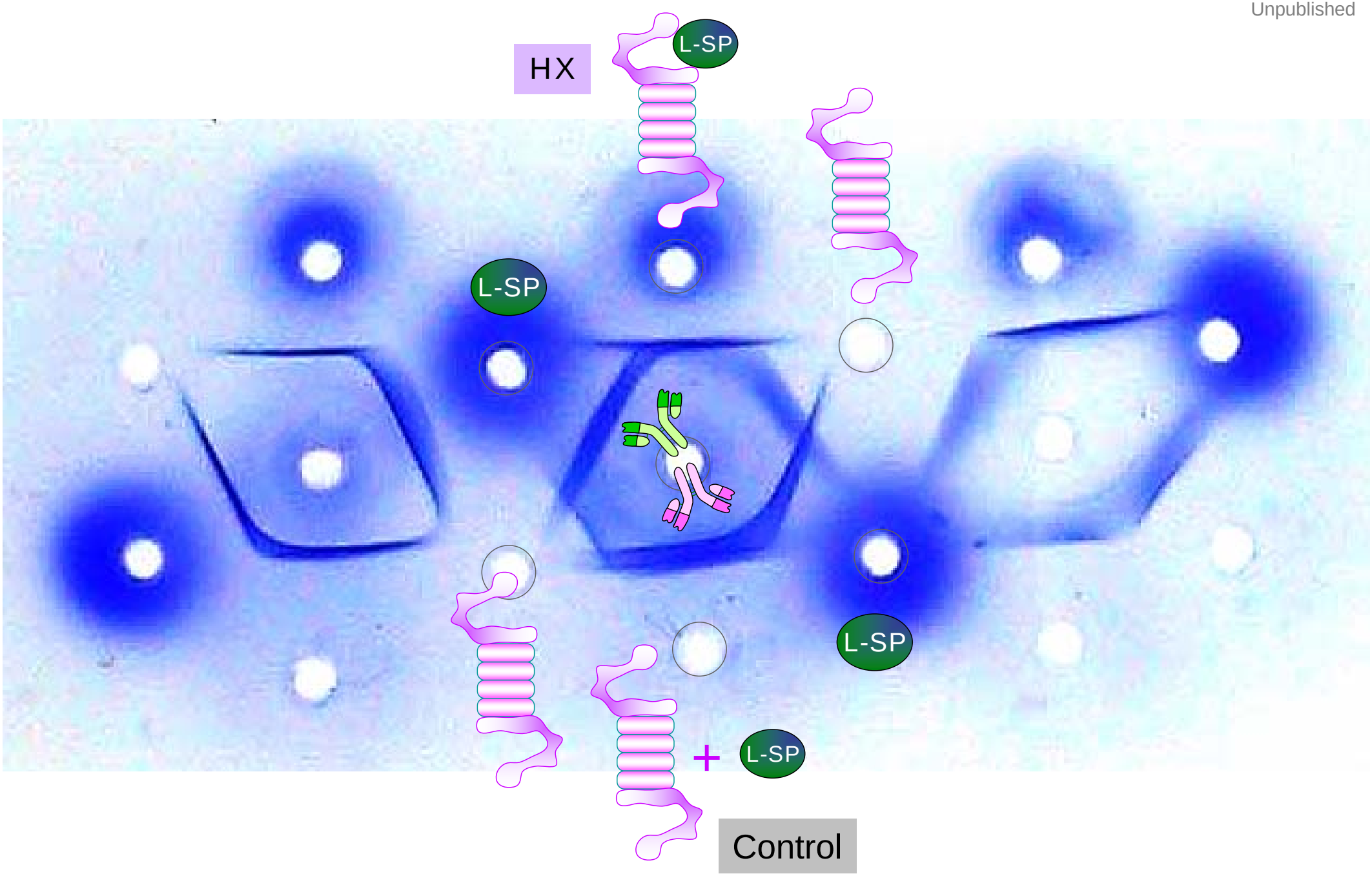


12.5 % SDS-PAGE
CBR Staining

no.	Full protein name	Matched peptide	Sequence coverage (%)	Match score	Species	Accession number
1	starch phosphorylase	10	14	220	*Ipomoea batatas*	1802404A
2	starch phosphorylase	86	51	1727	*Ipomoea batatas*	T10947
3	starch phosphorylase	18	19	428	*Ipomoea batatas*	1802404A
4	chaperonin 60	19	25	451	*Cucurbita*	CAA50218
5	starch phosphorylase	30	28	454	*Ipomoea batatas*	1802404A
6	20S proteasome α6-subunit	36	29	269	*Nicotiana benthamiana*	AAN07899
7	20S proteasome α-subunit	13	28	466	*Lycopersicon esculentum*	CAA74725

Double diffusion reveals the components of HX

Unpublished



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

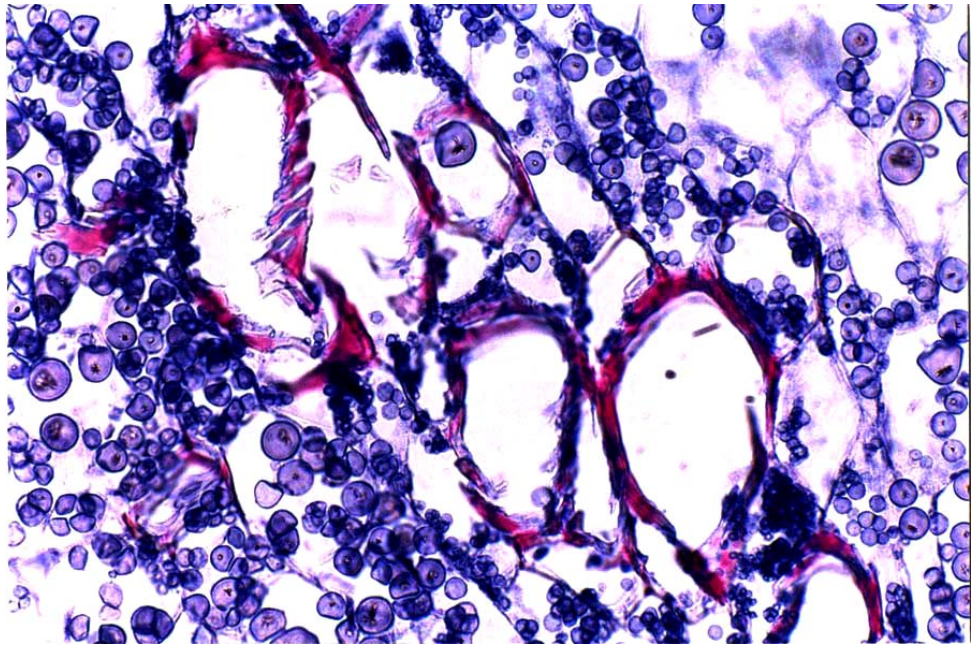
Unpublished

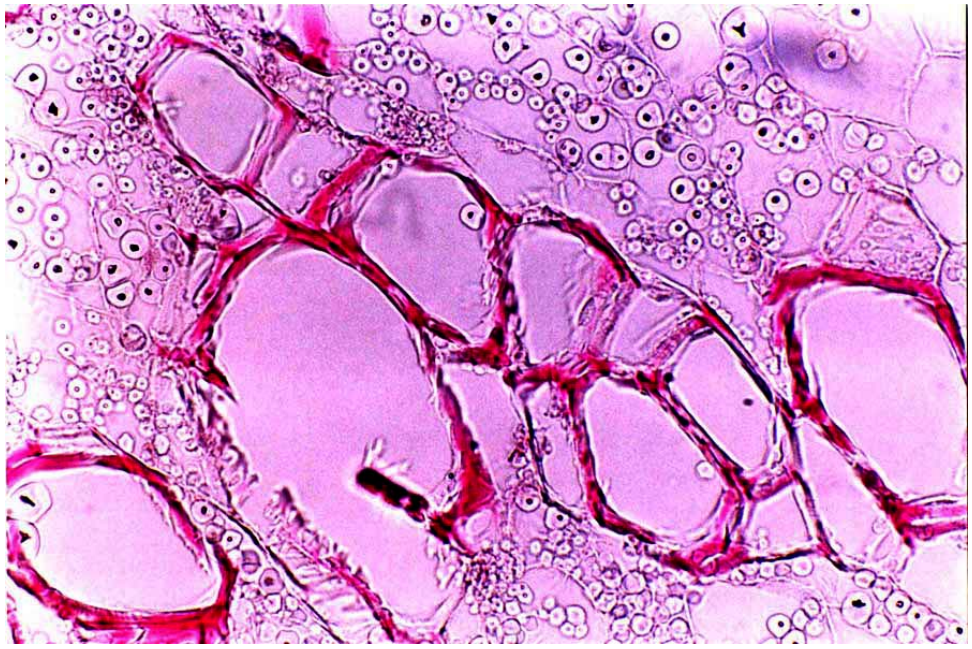
Immunostaining

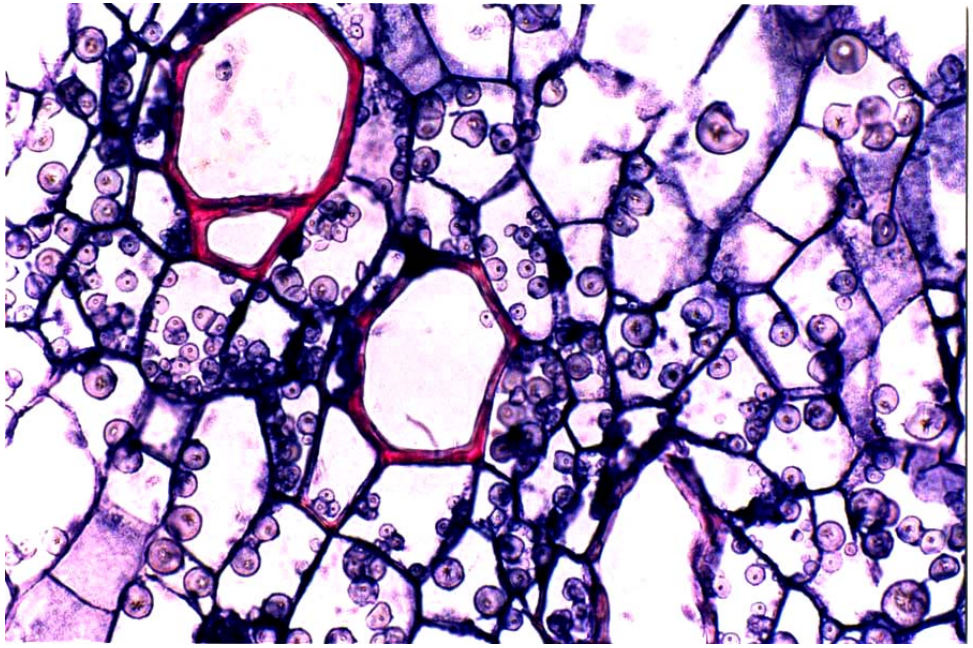
Control (no primary Ab)

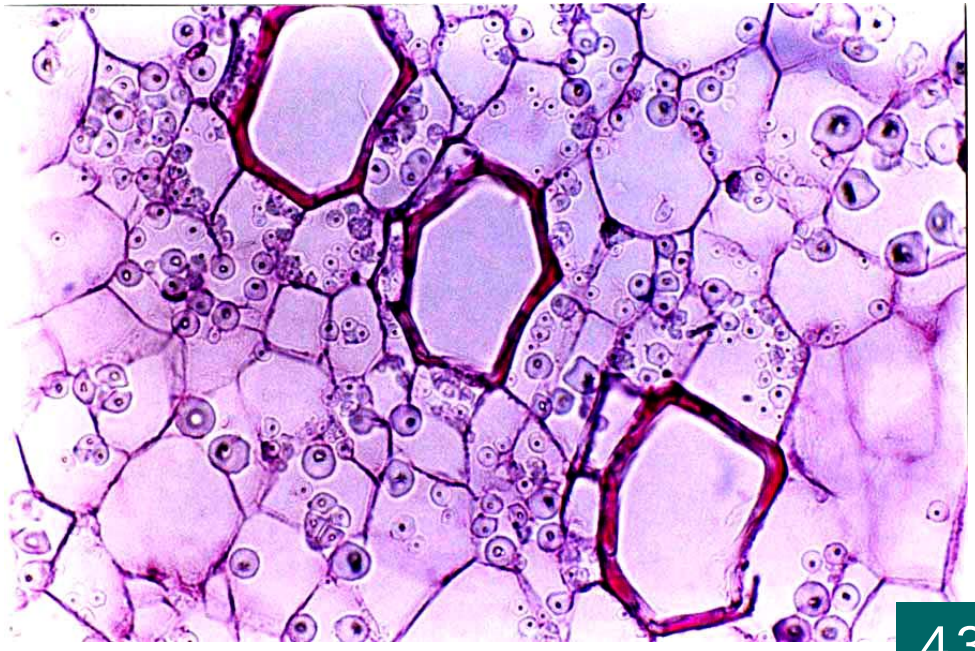
J3b (anti L-SP)

M71 (anti proteasome)



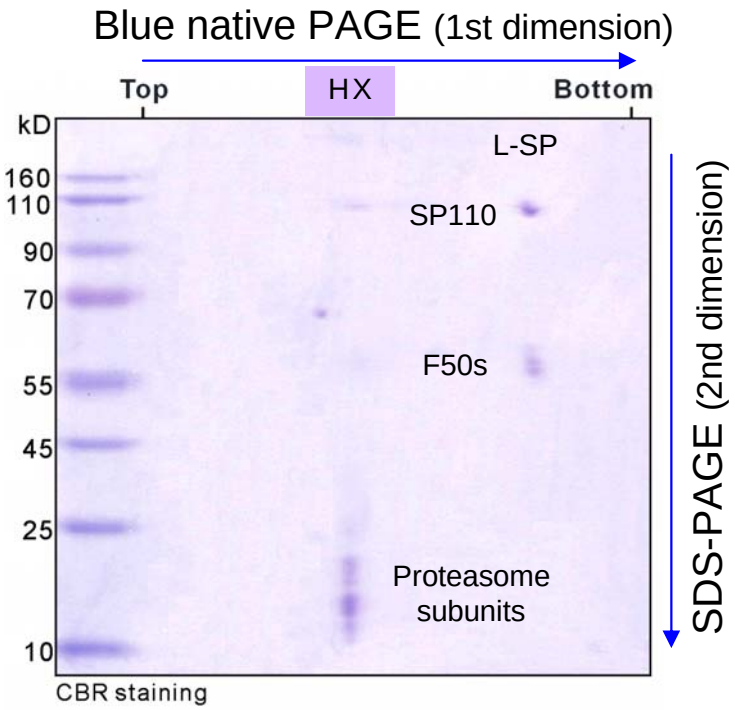


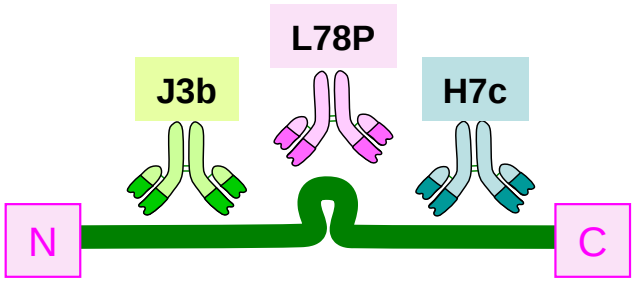




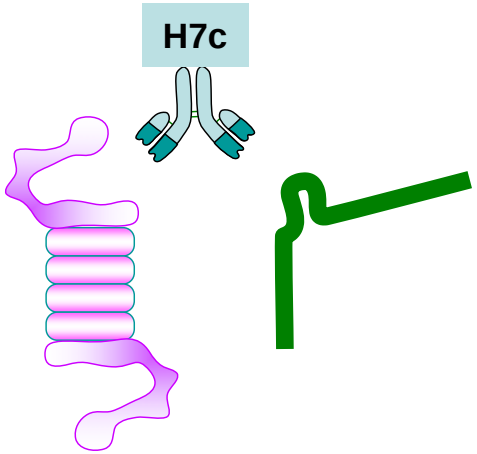
Blue-native 2D PAGE and immunostaining for HX

Unpublished

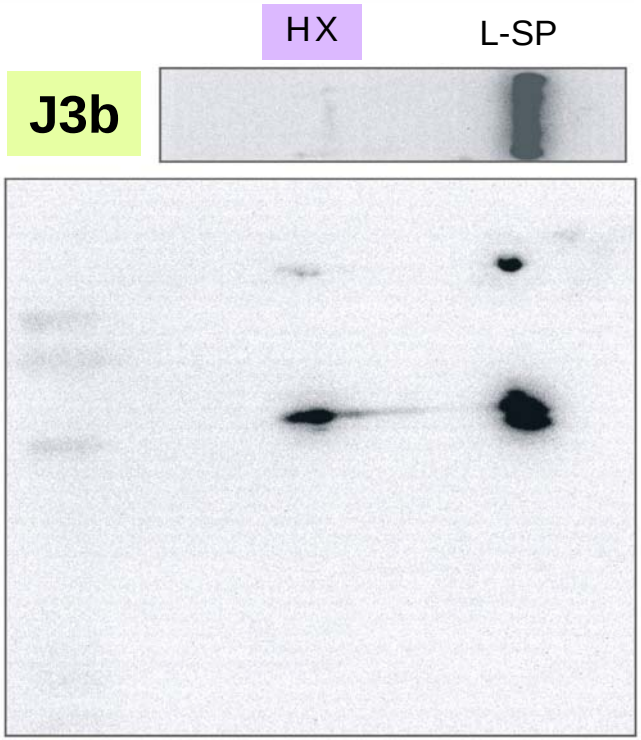


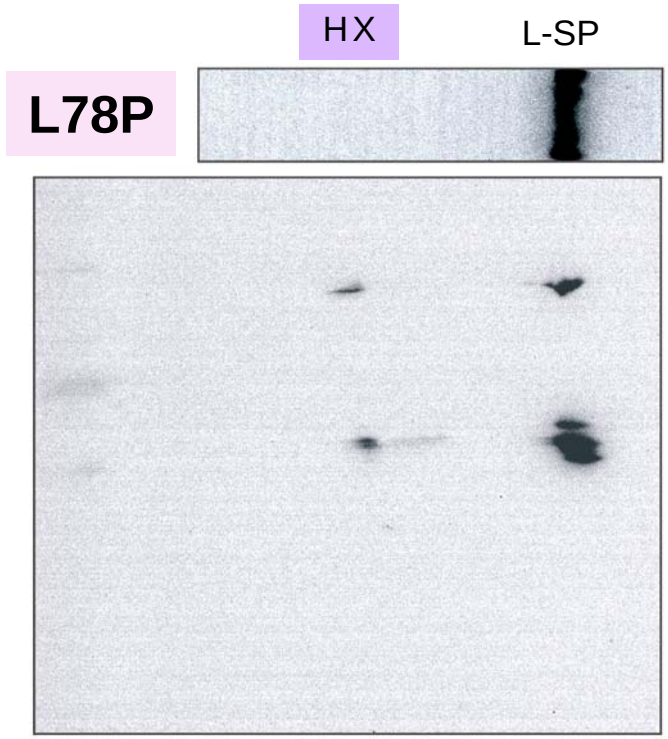


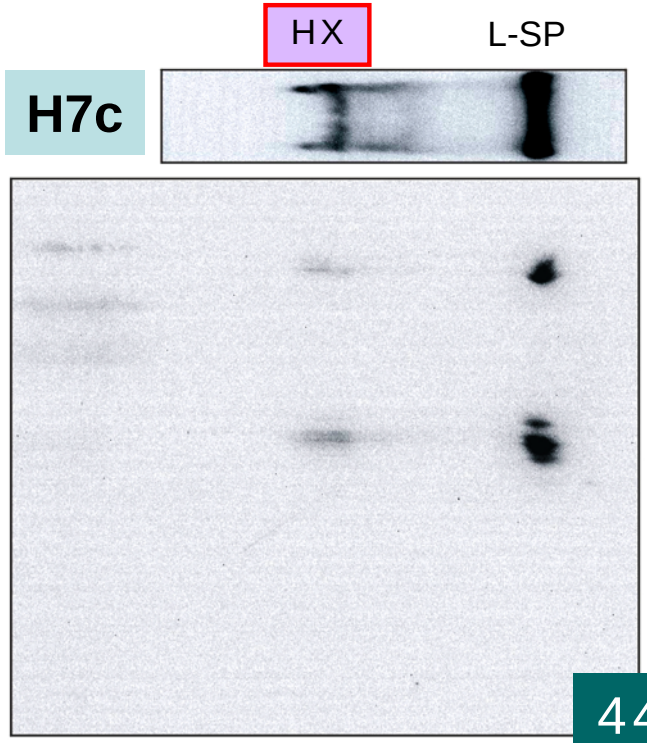
Only H7c could stain the HX band on the native PAGE



N-terminal half of L-SP including L78 is buried inside the proteasome



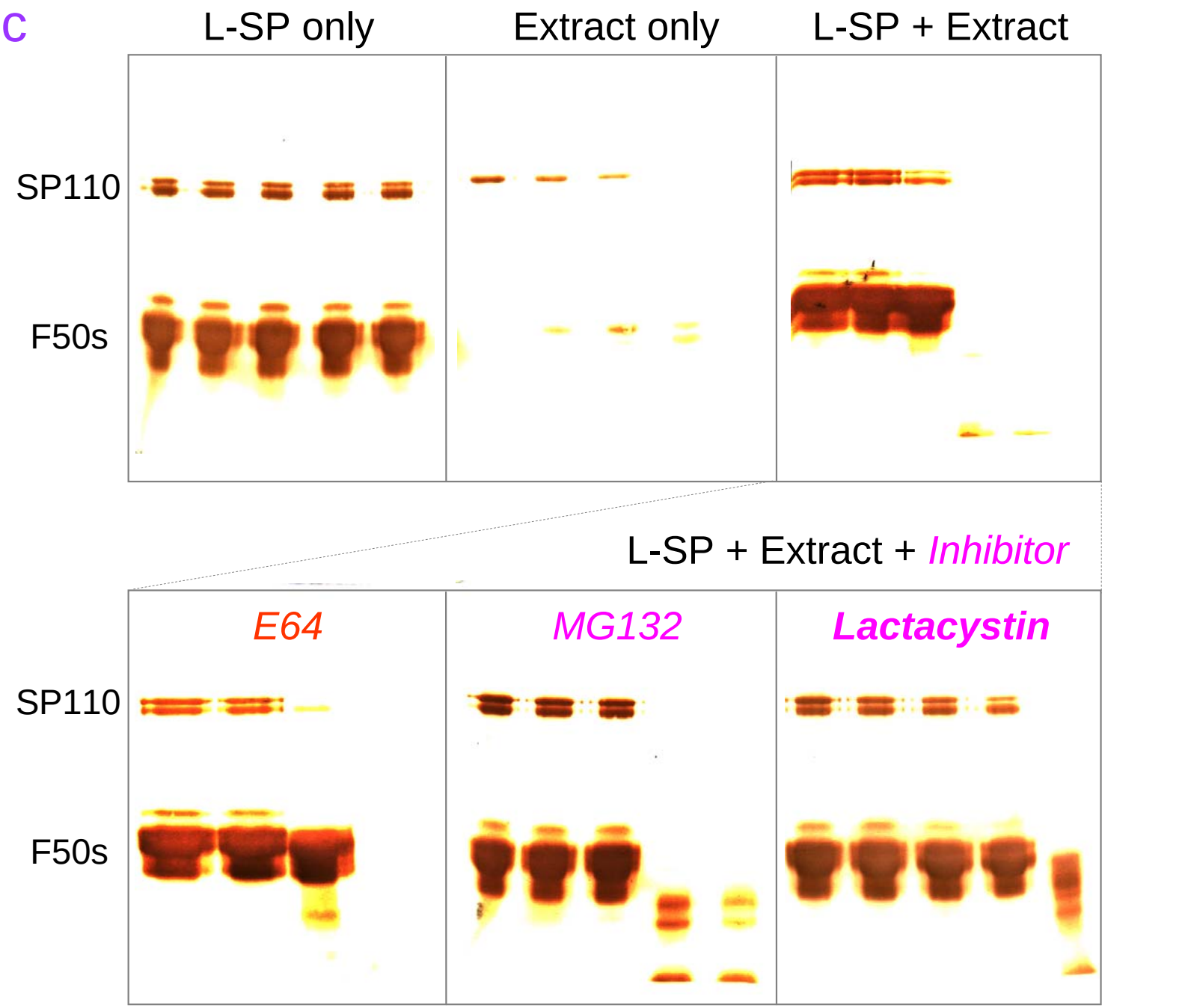




The degradation of L-SP is protected by proteasome inhibitor

Unpublished

mAb H7c

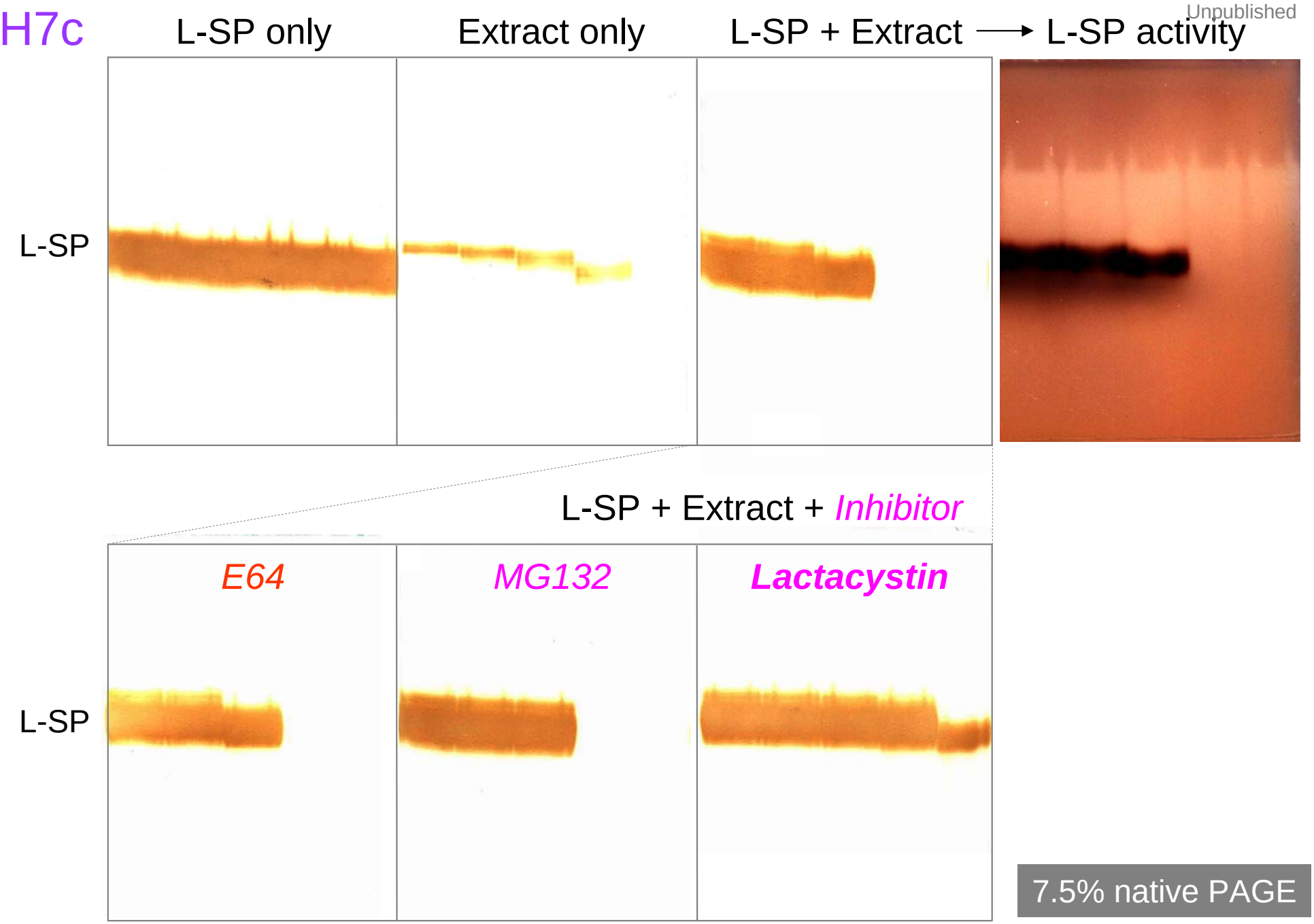


10% SDS-PAGE

The degradation of L-SP is protected by proteasome inhibitor

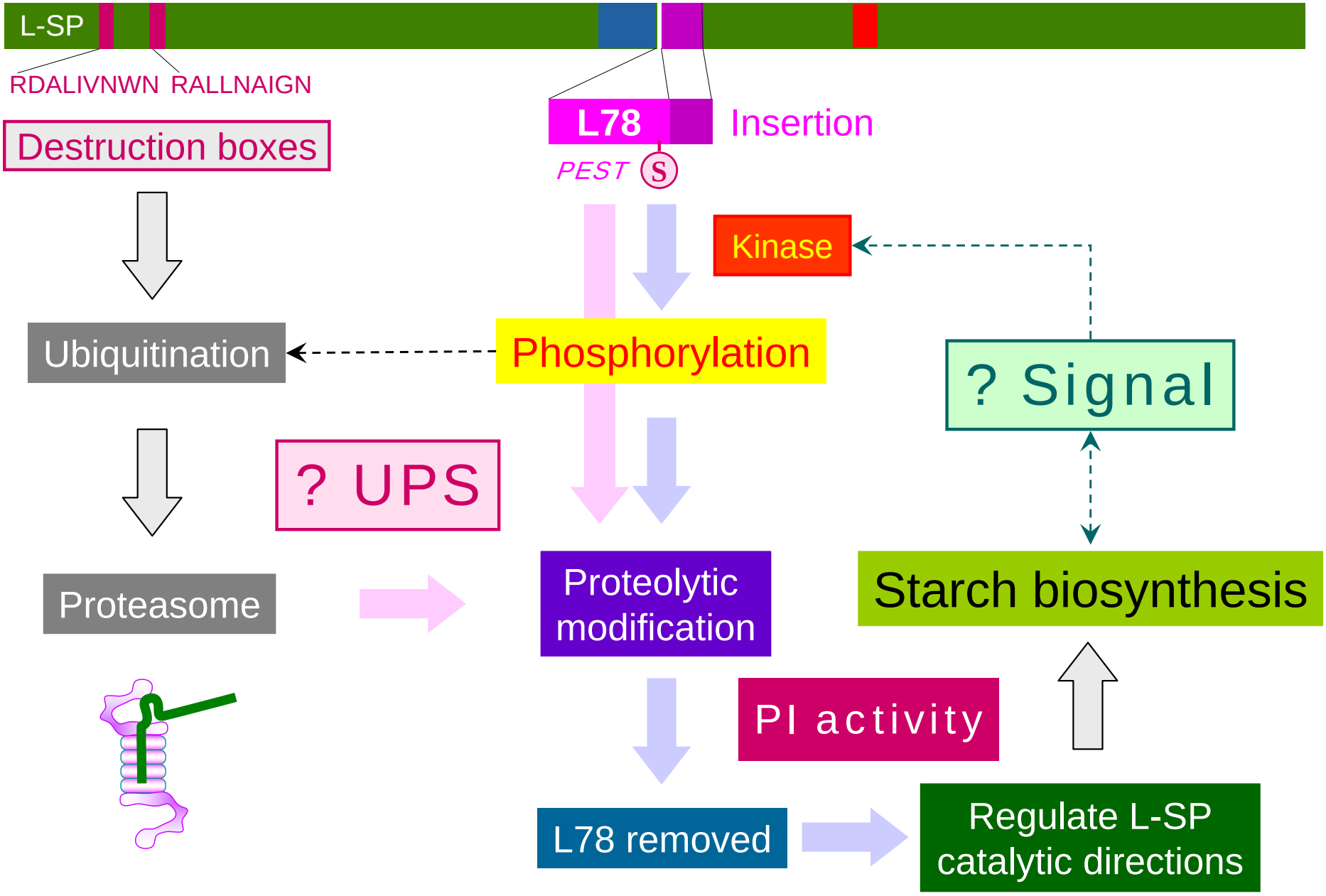
mAb H7c

Unpublished



7.5% native PAGE

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