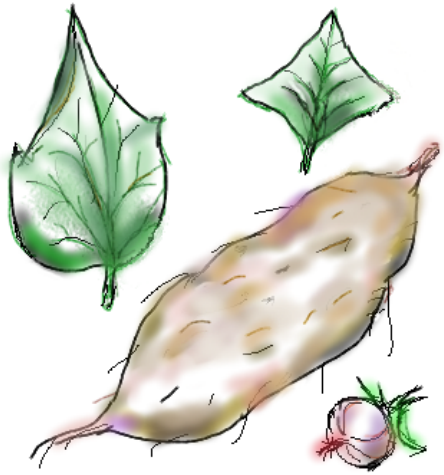
Structure and Physiological Function of Starch Phosphorylase from Sweet Potato Roots

- Regulation by Proteolytic Modification & Phosphorylation



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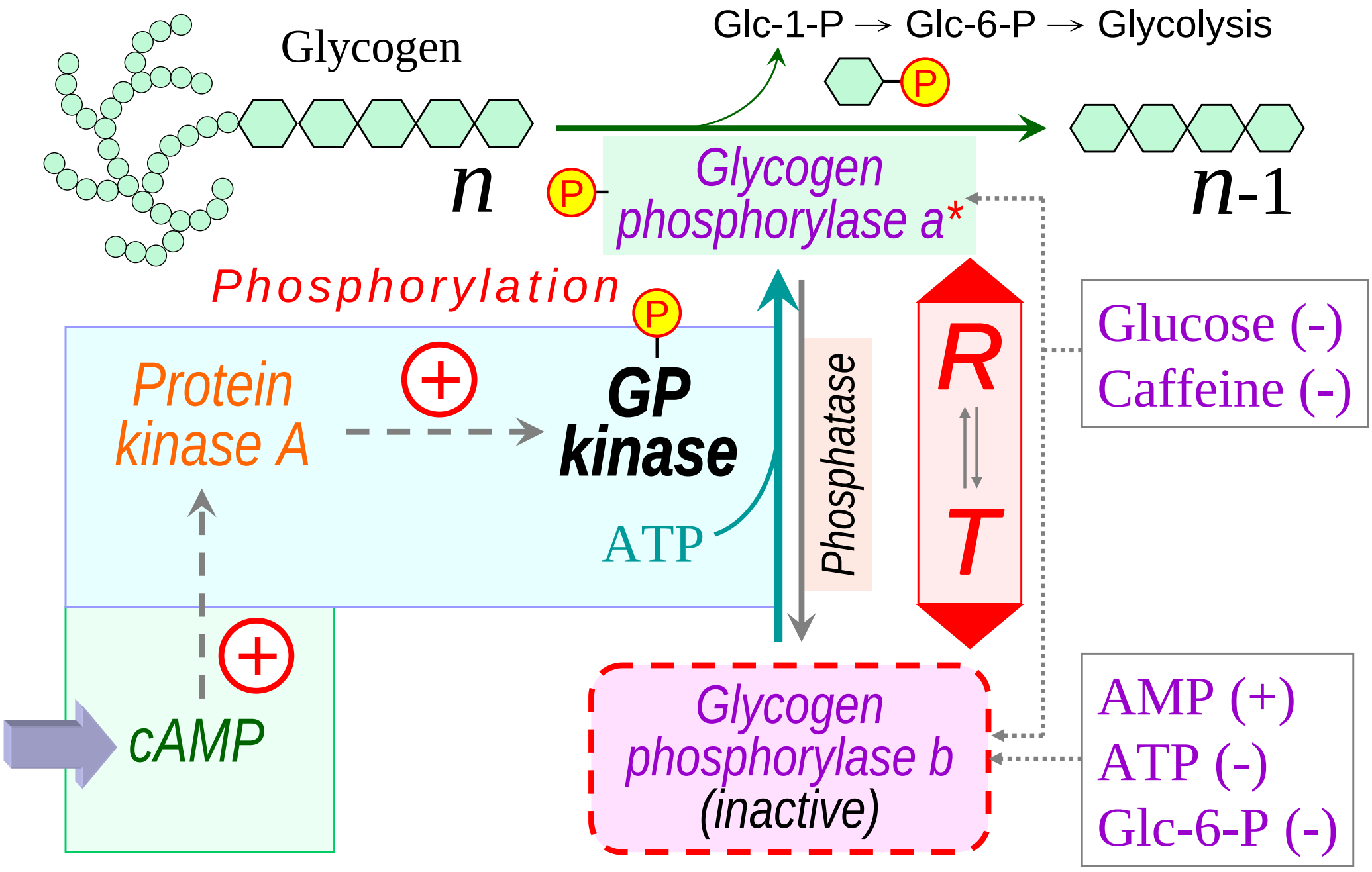
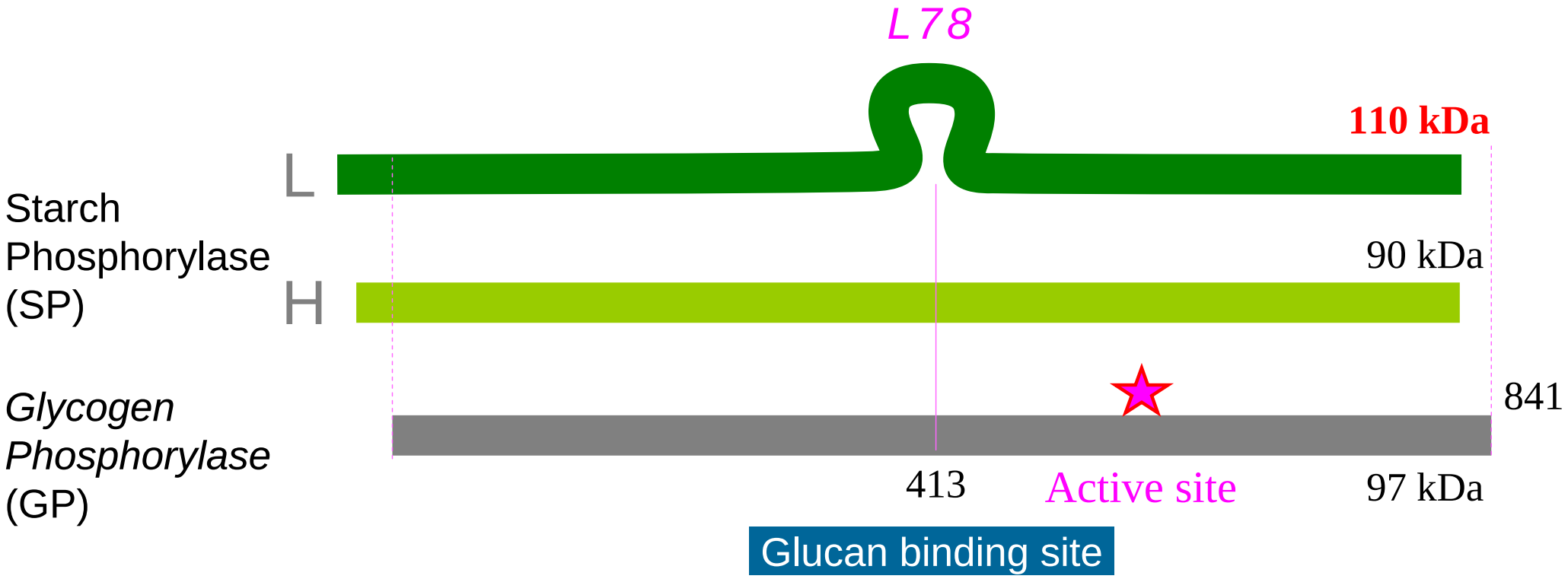
國立台灣大學 生化科技學系

Glycogen phosphorylase 肝糖磷解酶



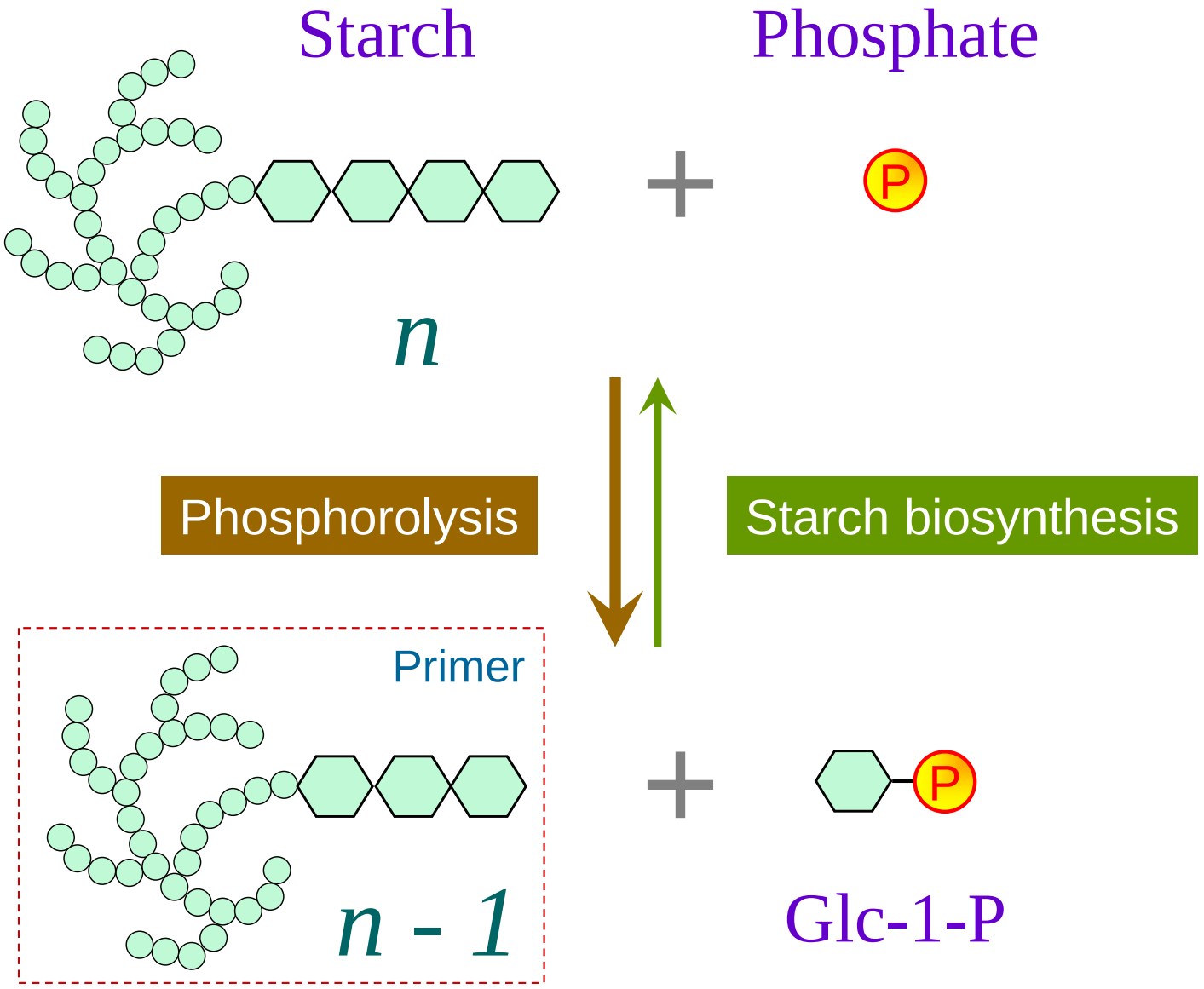
Glycogen phosphorylase is regulated extensively to control the carbohydrate metabolism

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

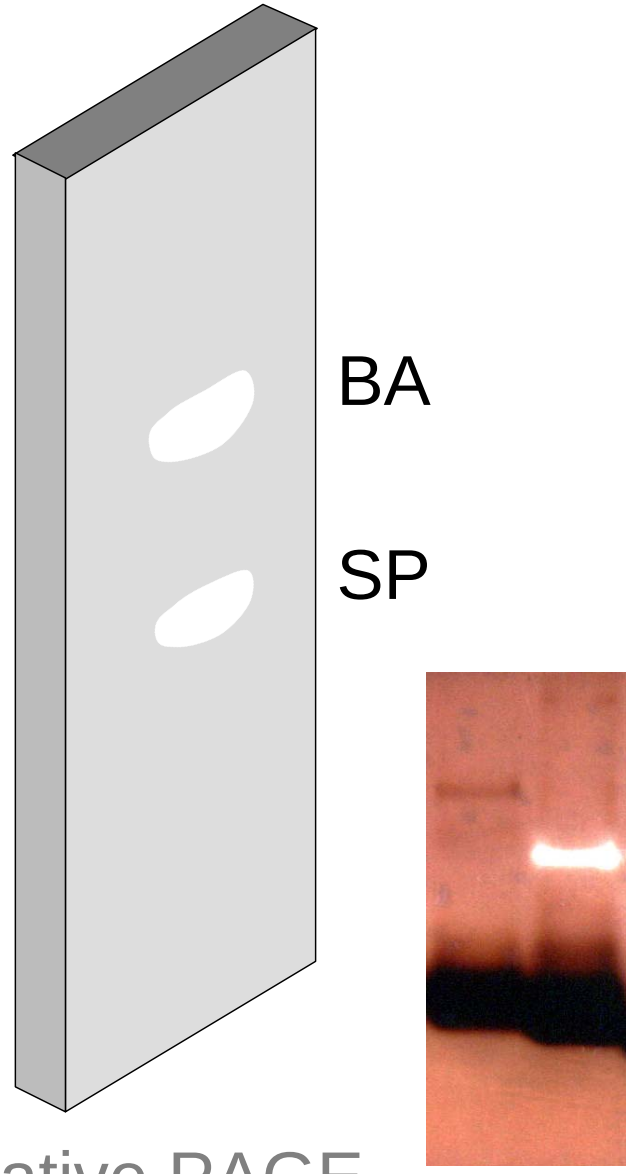
SP catalyzes the reversible phosphorolysis of starch



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

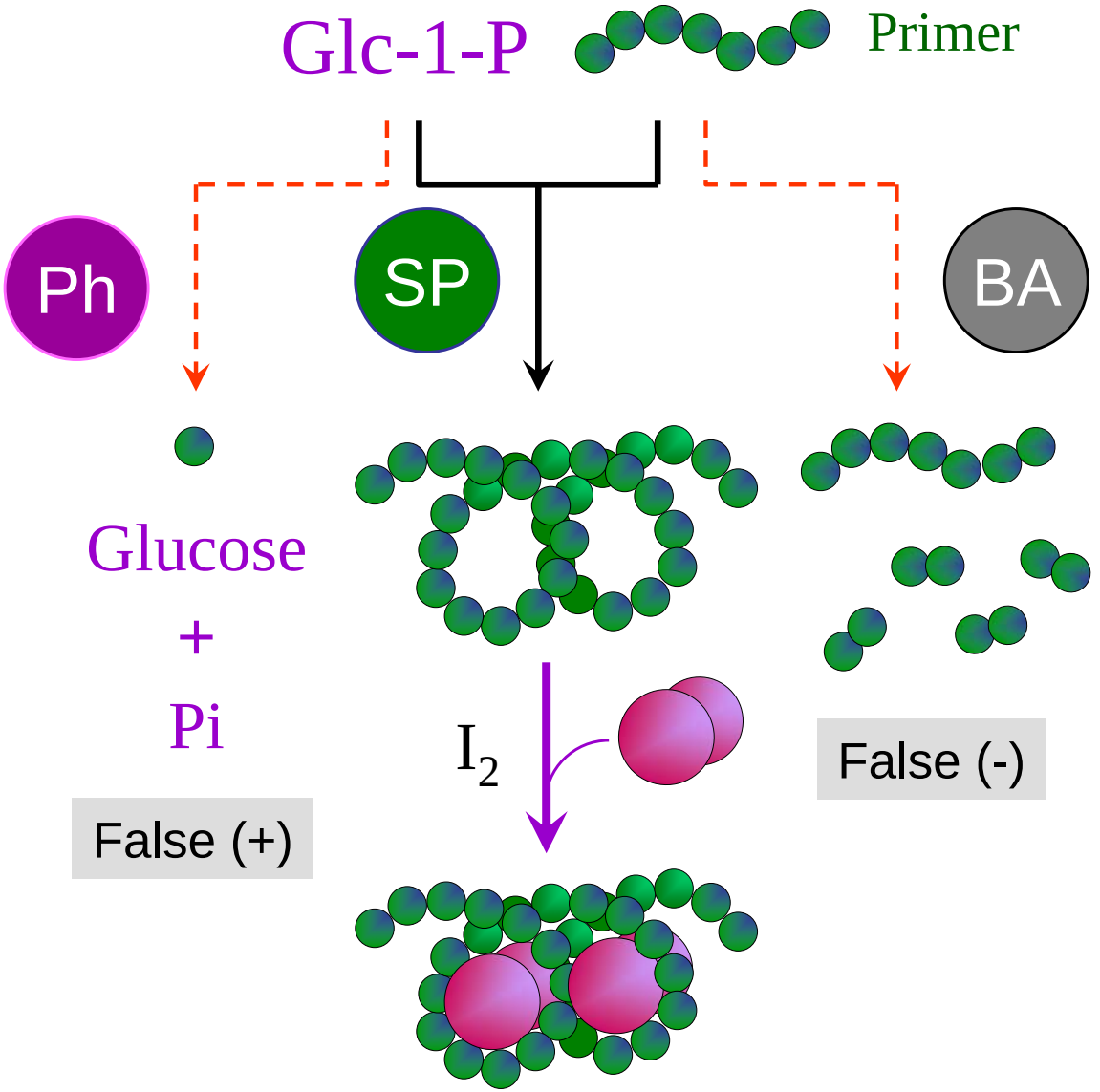
SP activity is interfered by other enzymes

A Activity staining



Native PAGE

B Activity assay and interference



Direct observation of color products

Some other enzymes might lead to false (+) or (-) result

The profiles of SP activity after gel filtration

甘藷全蛋白質

↓

膠體過濾分析

↓

蛋白質定量

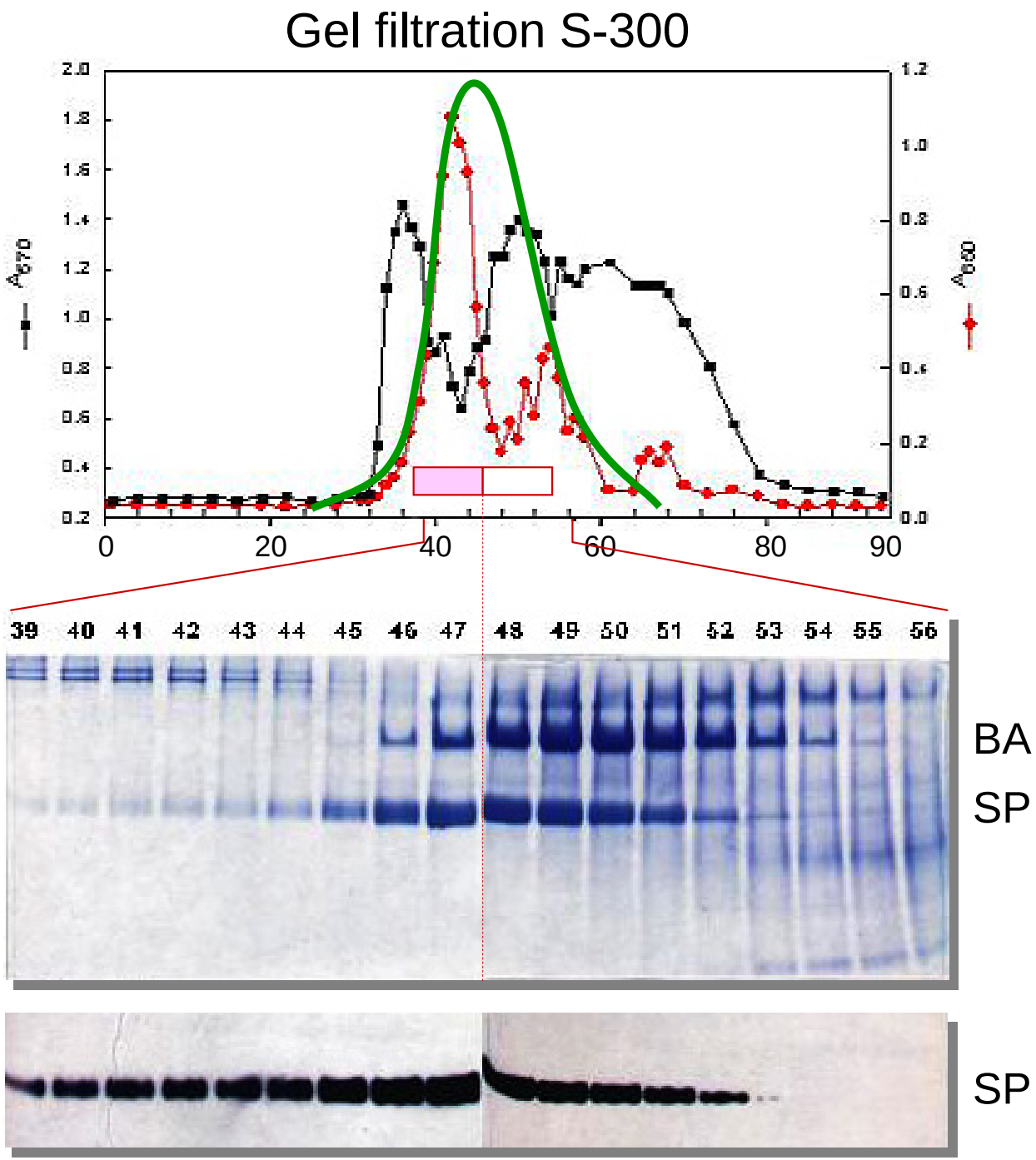
SP 活性分析

↓

SDS 膠體電泳

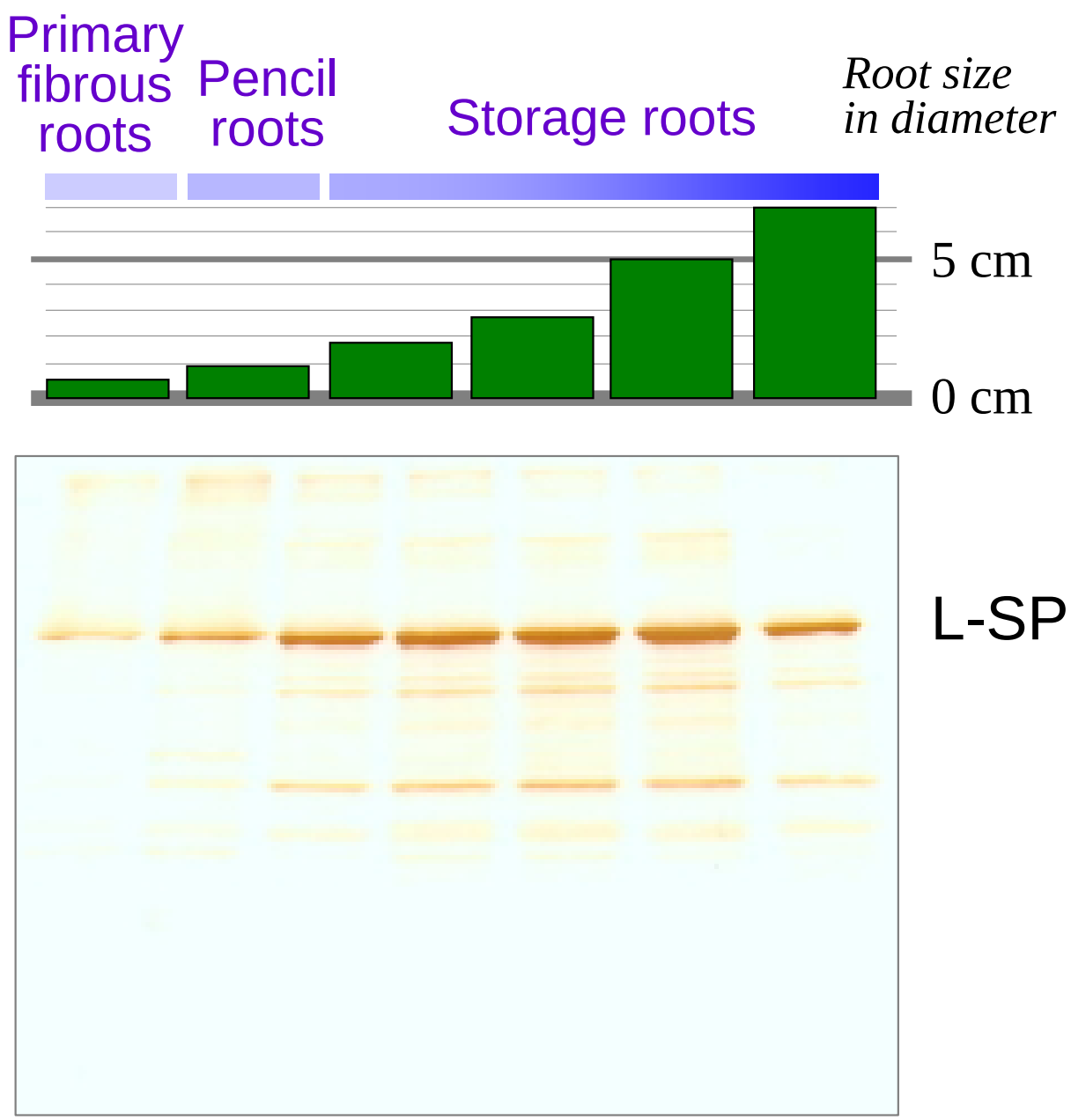
↓

免疫染色 Western



SP 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

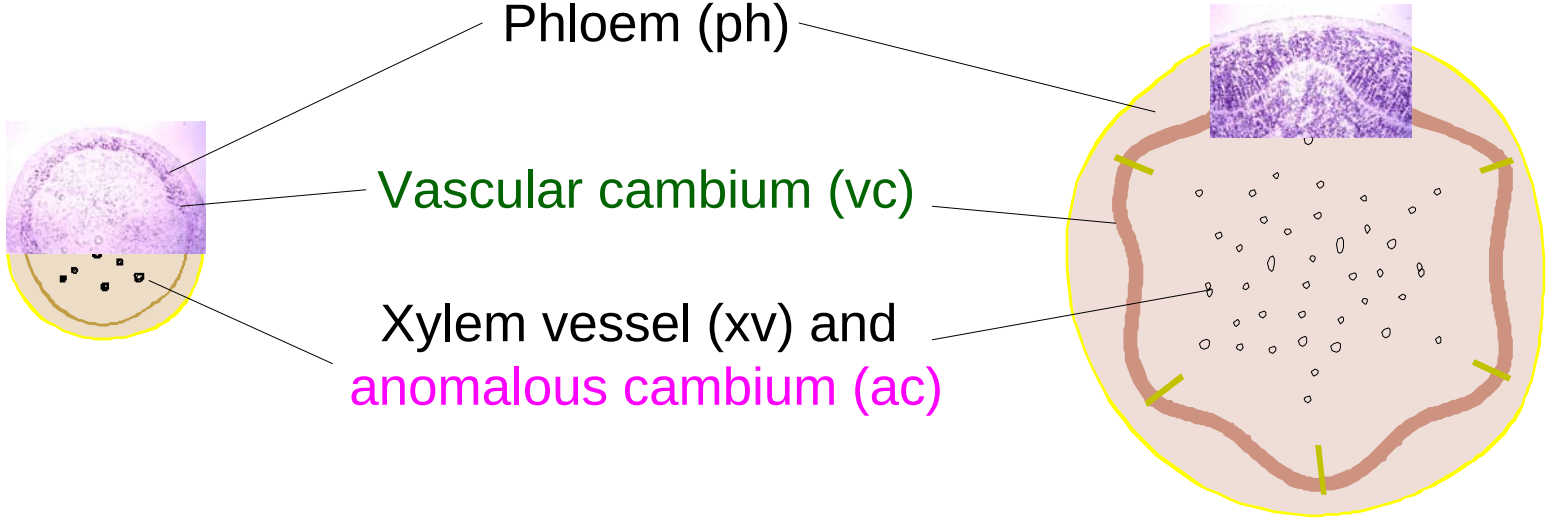
- Wheat
  - Tetlow et al. 2004

SP increases proportionally when the roots accumulate starch

Two growing stages of sweet potato roots

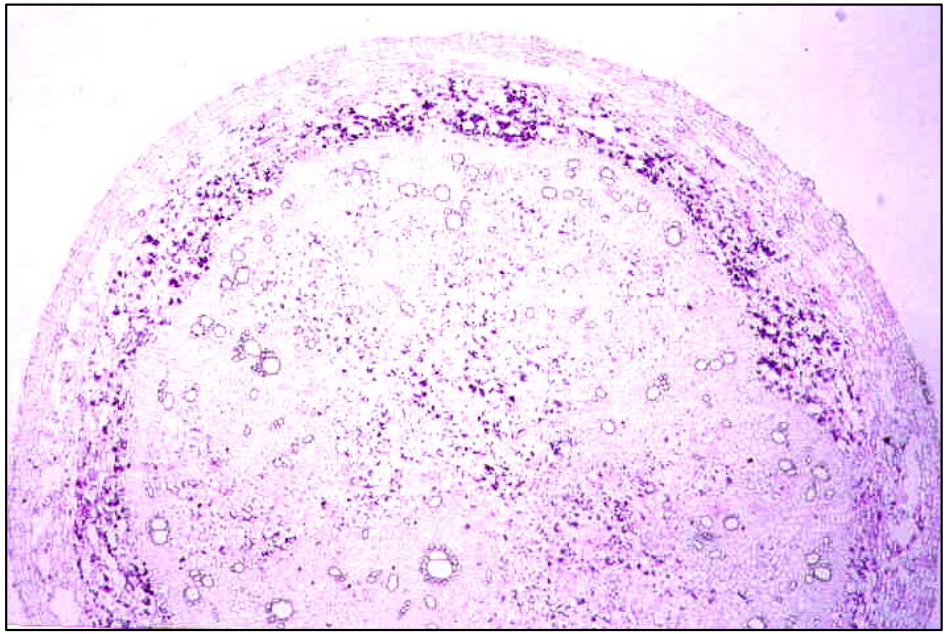
Primary fibrous root

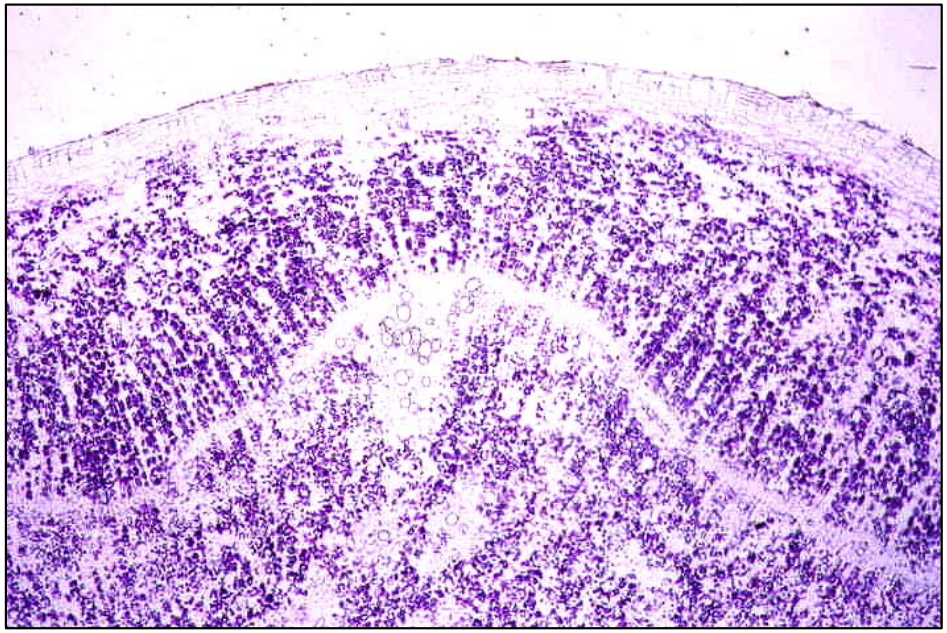
Storage root



< 5 mm diameter

> 15 mm diameter





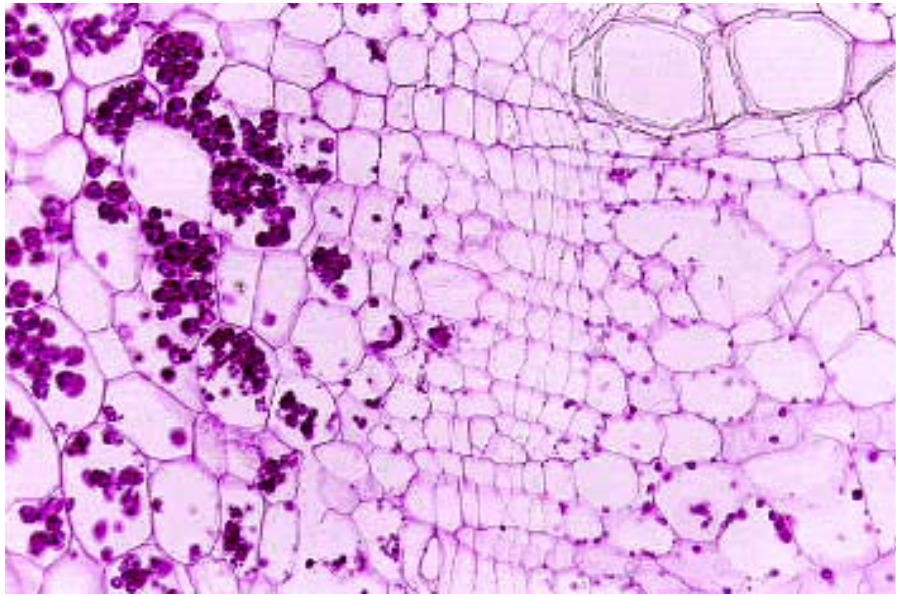
Phloem constantly accumulates starch granules and L-SP

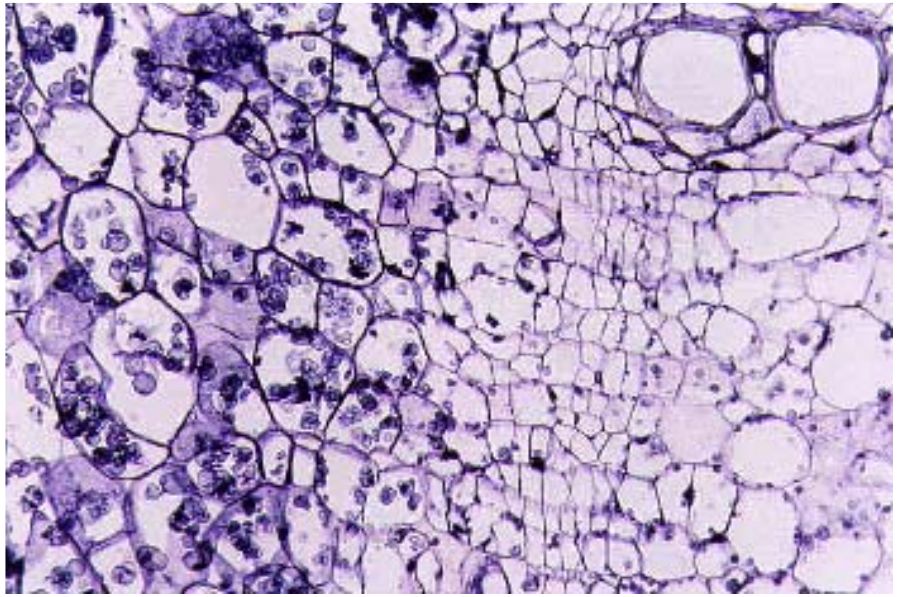
Vascular cambium

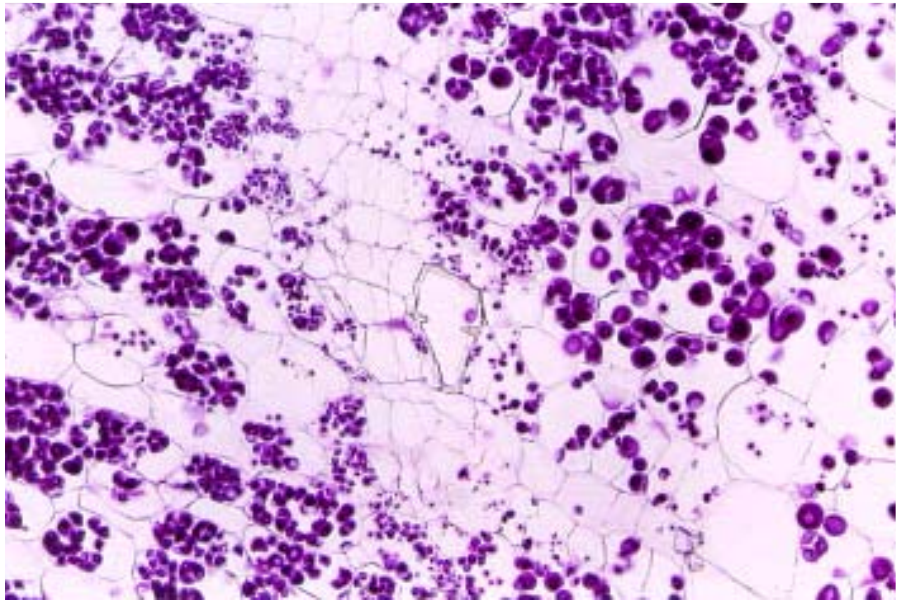
ph vc ph vc

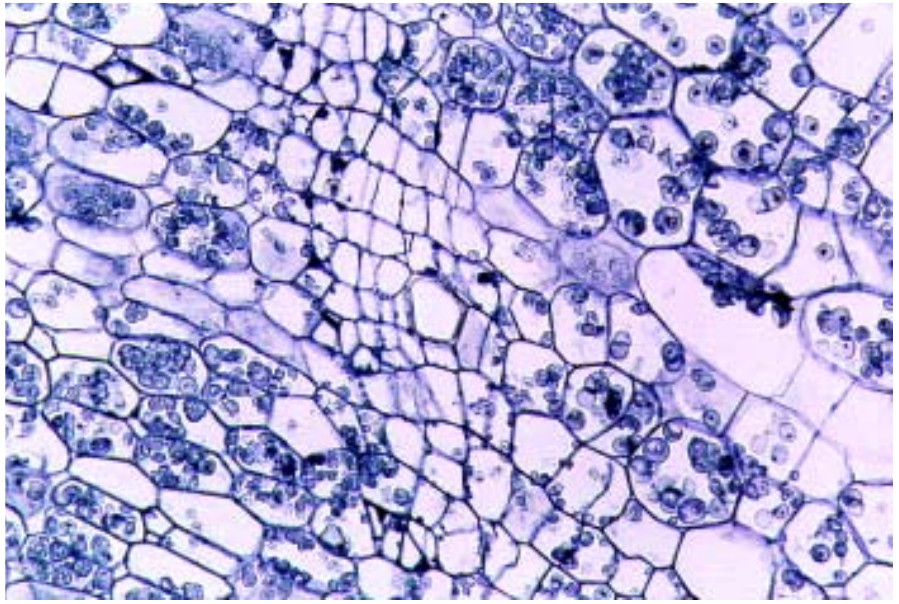
Primary fiberous roots

Storage roots









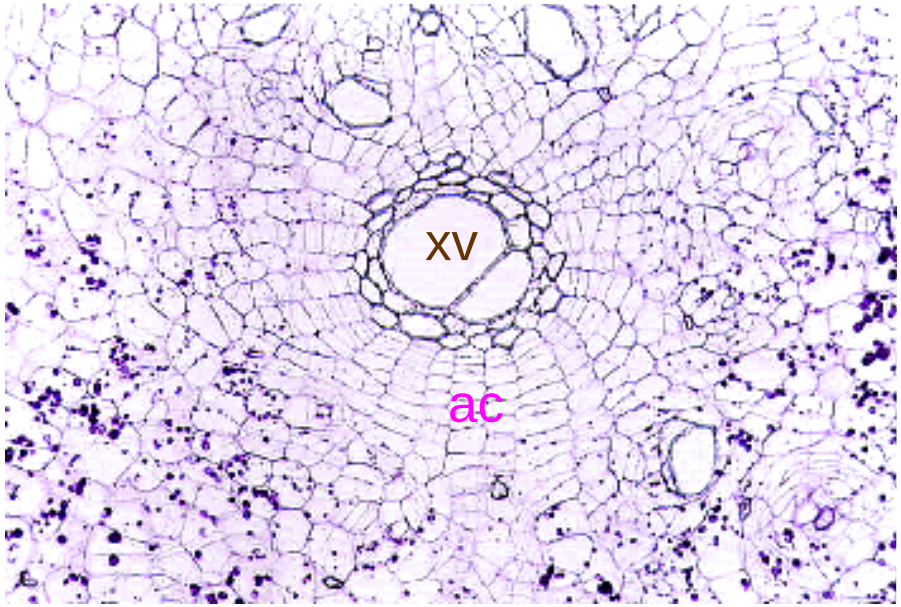
PAS

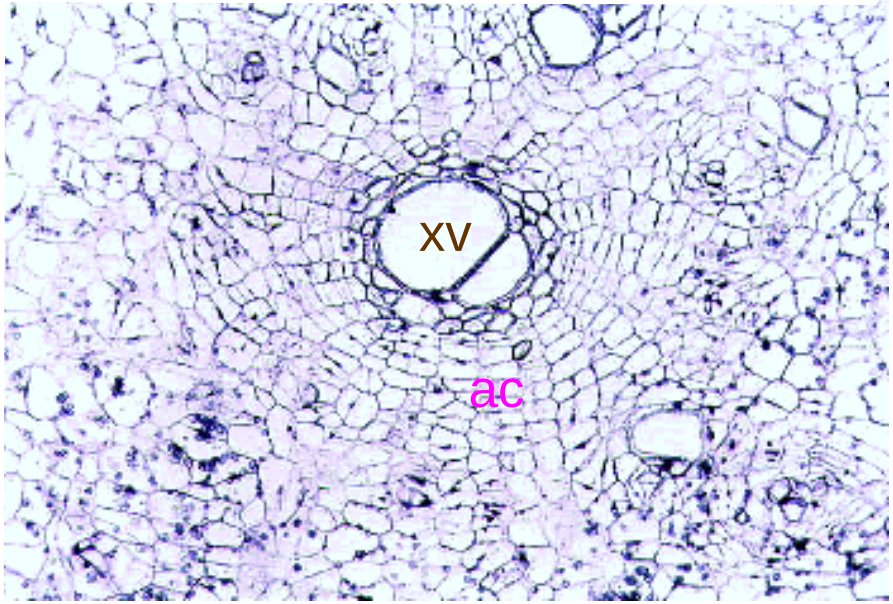
L-SP (immunostaining)

Anomalous cambium might initiate starch biosynthesis

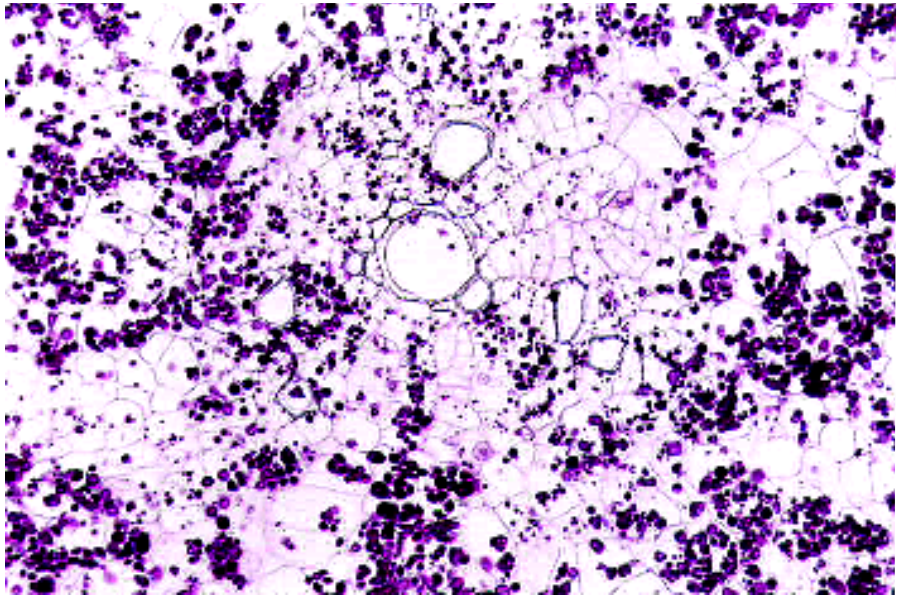
Anomalous cambium

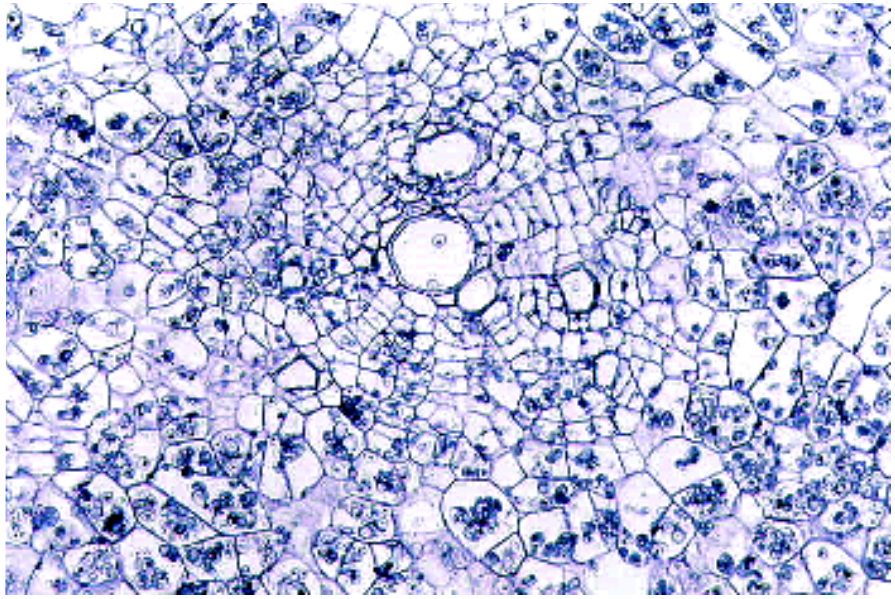
Primary fiberous roots





Storage roots



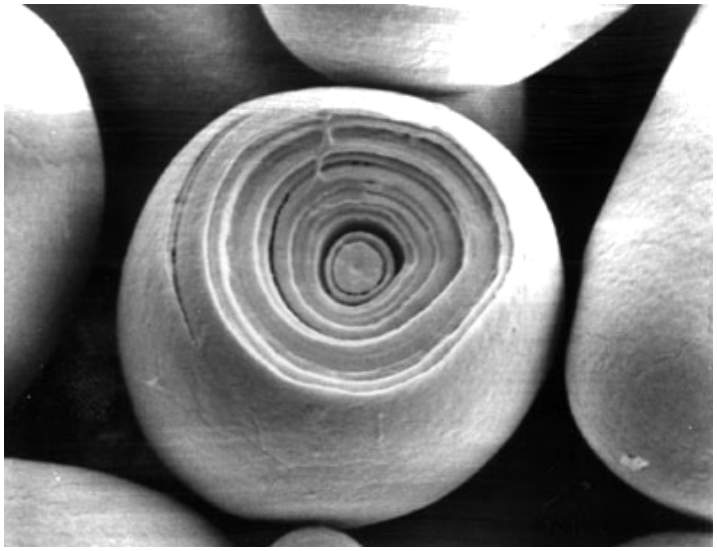


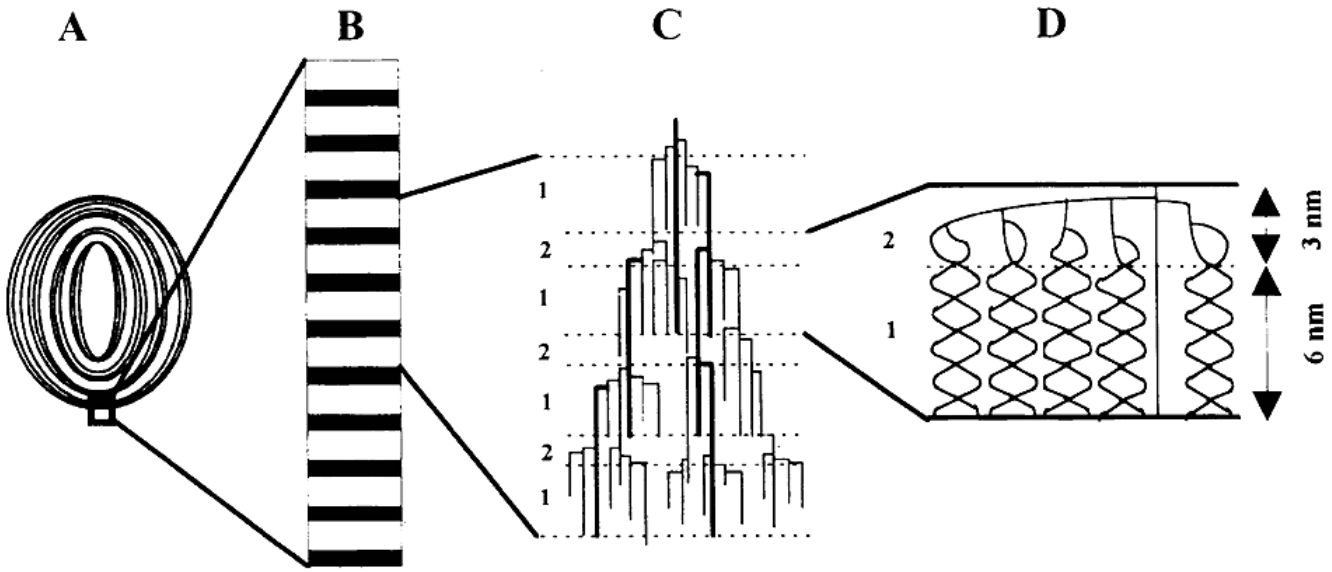
PAS

L-SP (immunostaining)

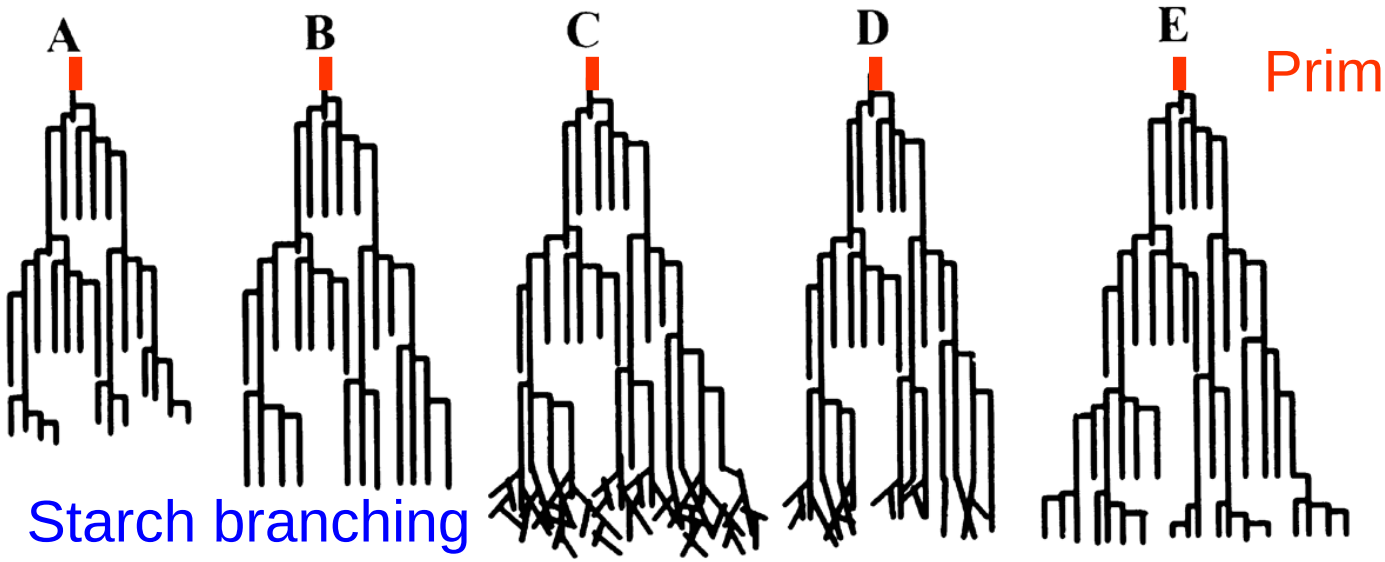
Starch is synthesized by elongation-branching-trimming cycles

http://www.starchmetnet.org/





Starch granule



Primer

Starch elongation

Starch synthase

Starch branching

Branching enzyme

Branch trimming

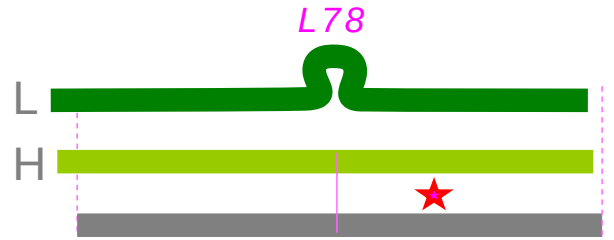
Debranching enzyme

Starch elongation

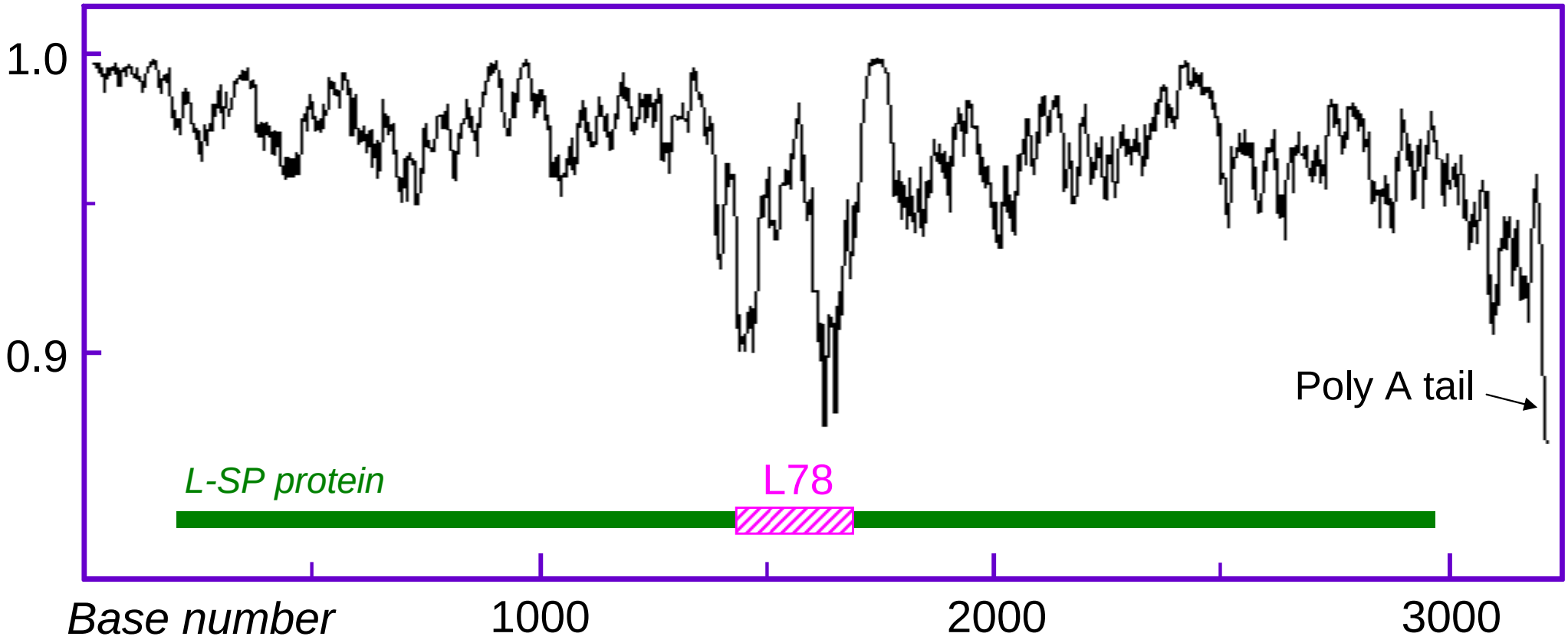
Ball S et al. (1996) From glycogen to amylopectin - a model for the biogenesis of the plant starch granule. Cell 86: 349-352

L78 might be evolved from an intron

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



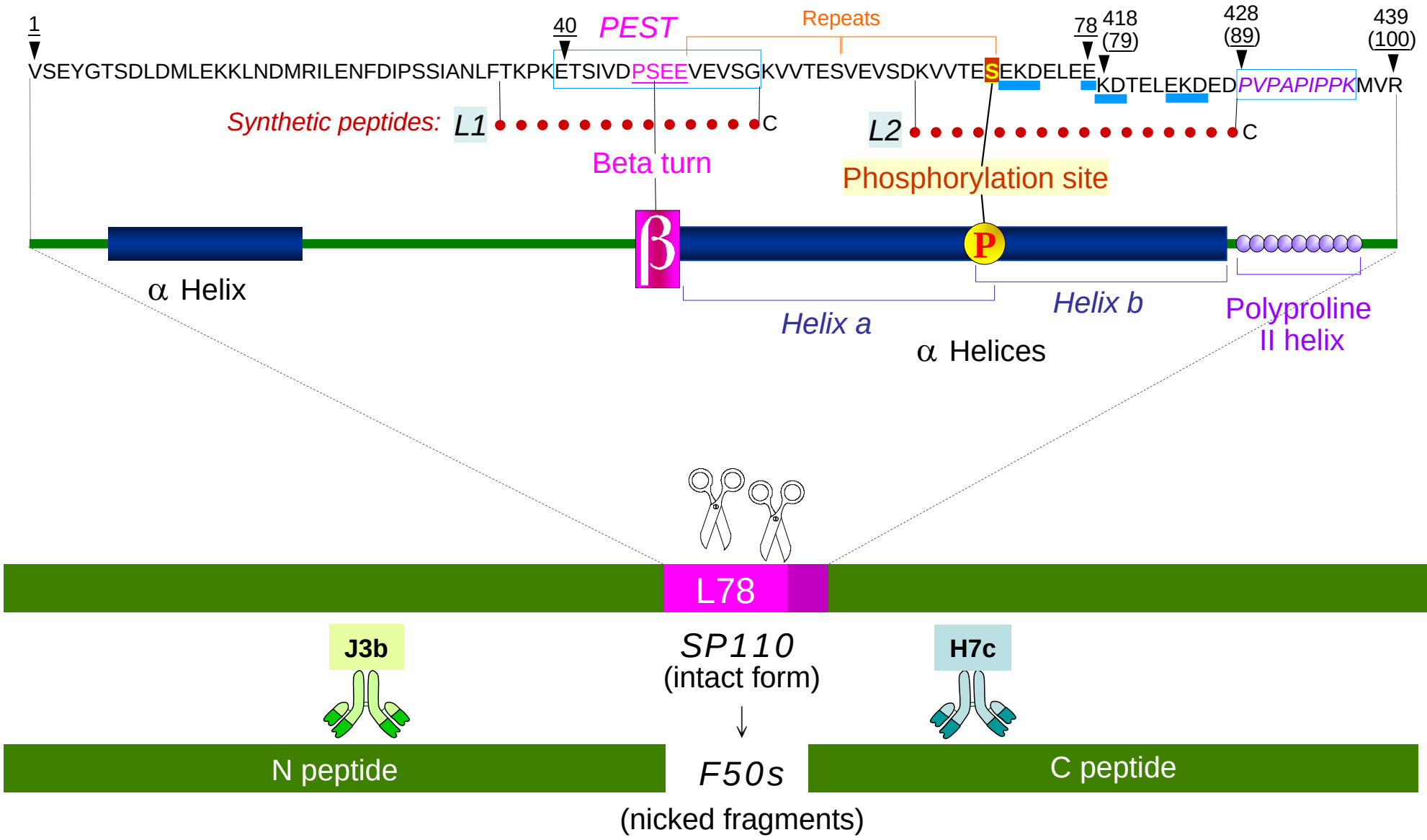
DNA complexity of L-SP cDNA



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

L78 sequence reveals unique structural features

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

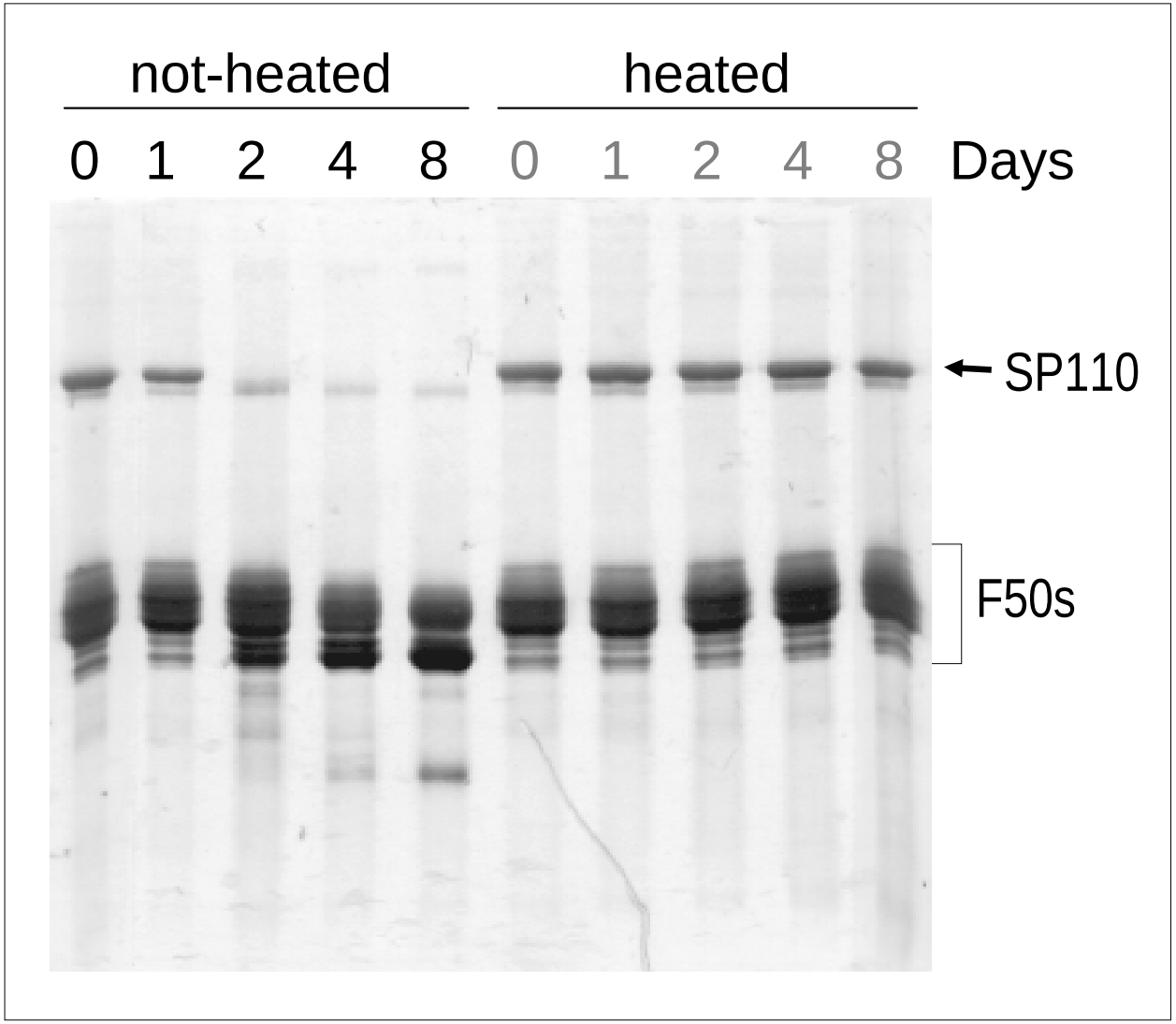


Analysis of the amino acid sequence on L78 and its C-terminal flanking residues shows several unique structural features. A "*PEST sequence*" is found in the middle of L78

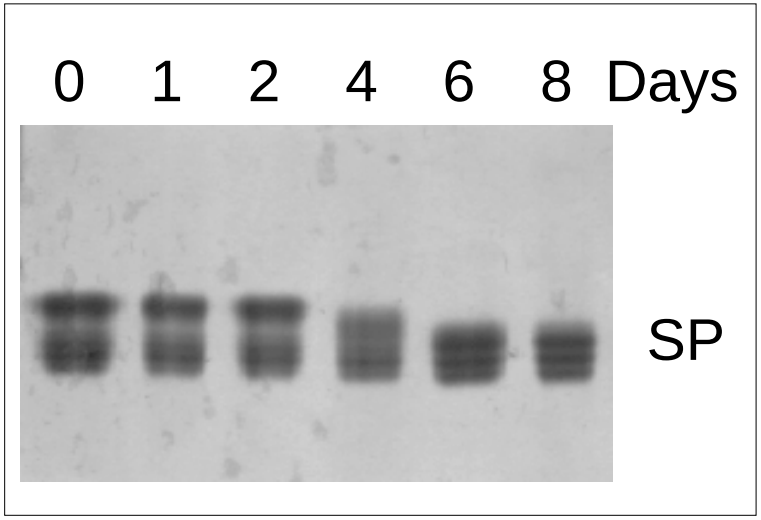
L-SP is proteolytic modified but still keeps its activity

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

A SDS-PAGE:



B Disc-PAGE:

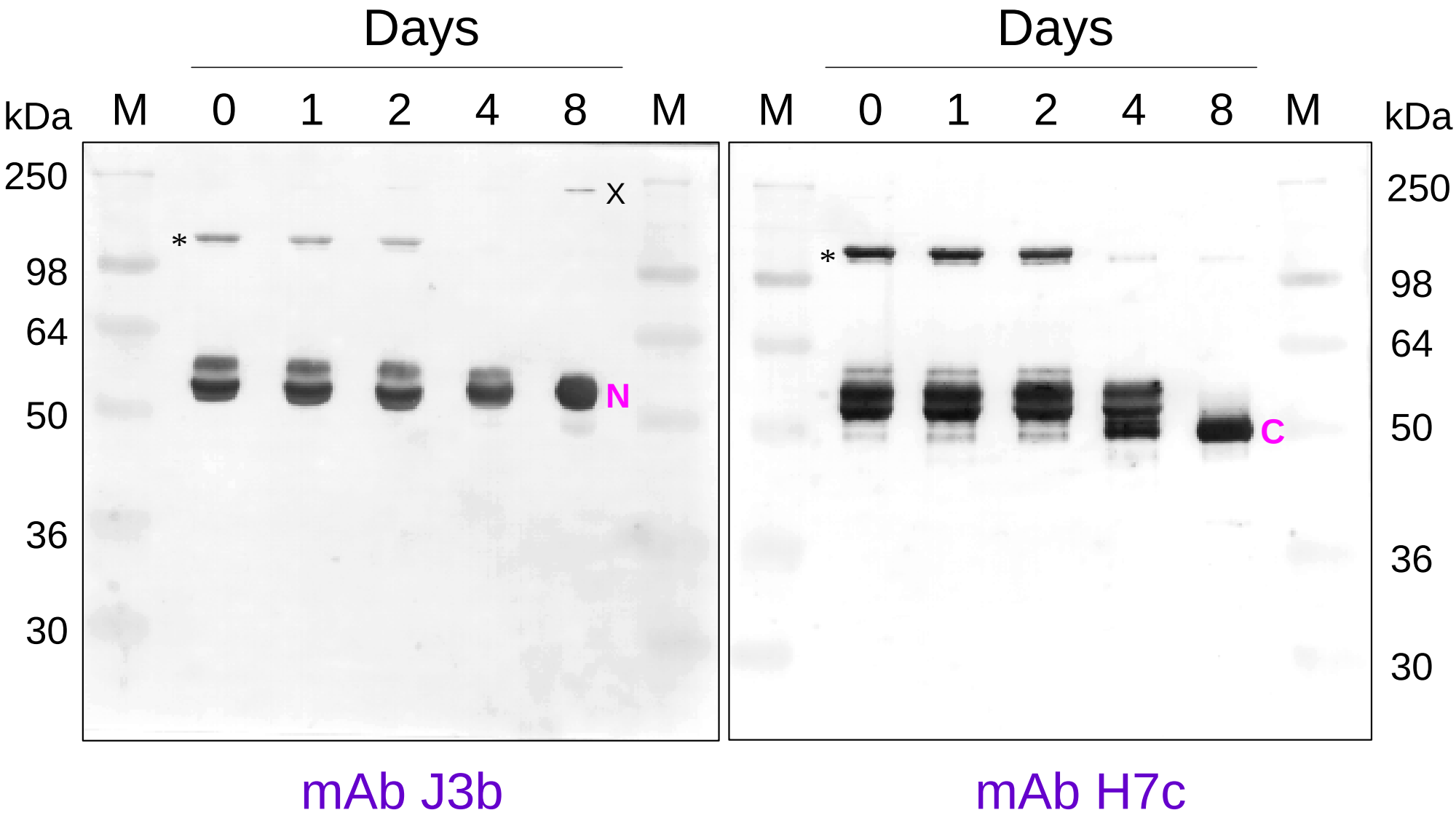


C Activity staining:

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 cleaved to form two final stable fragments

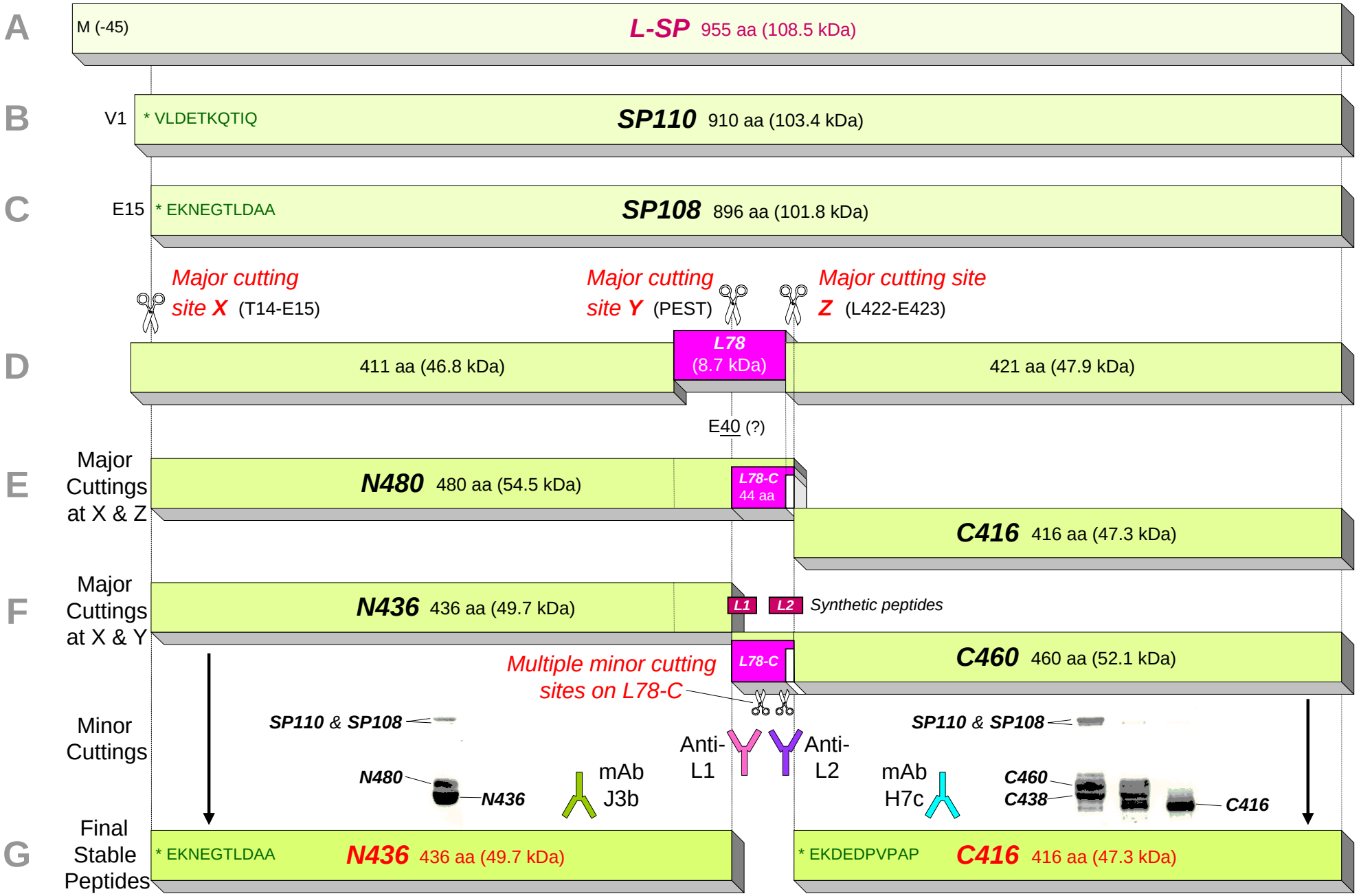
Chen et al, (2002) Physiologia Plantarum **114**:506-515



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



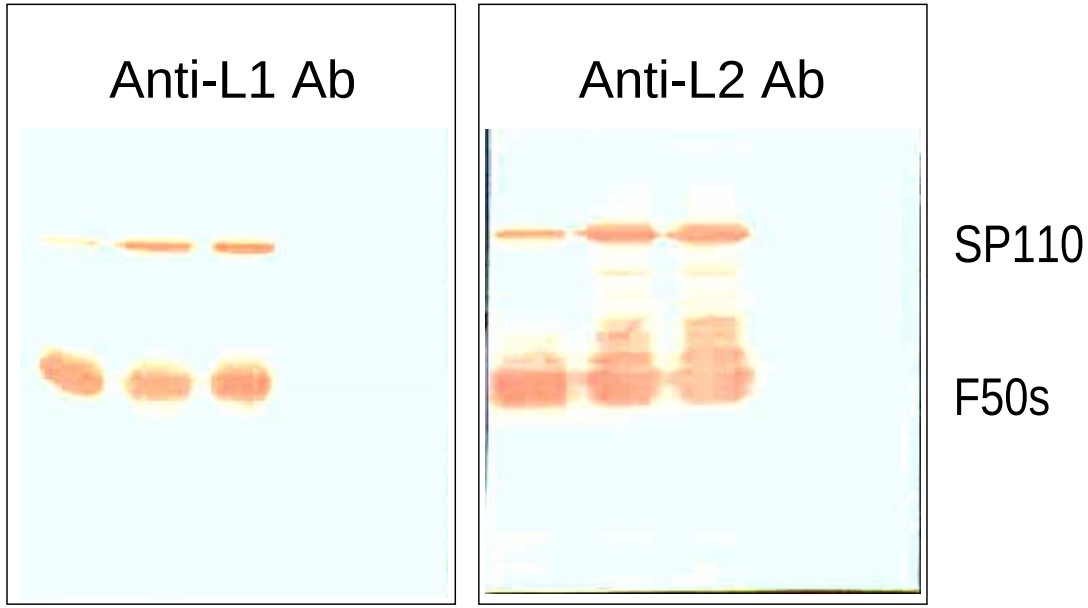
L1 and L2 peptides are completely removed from L78

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

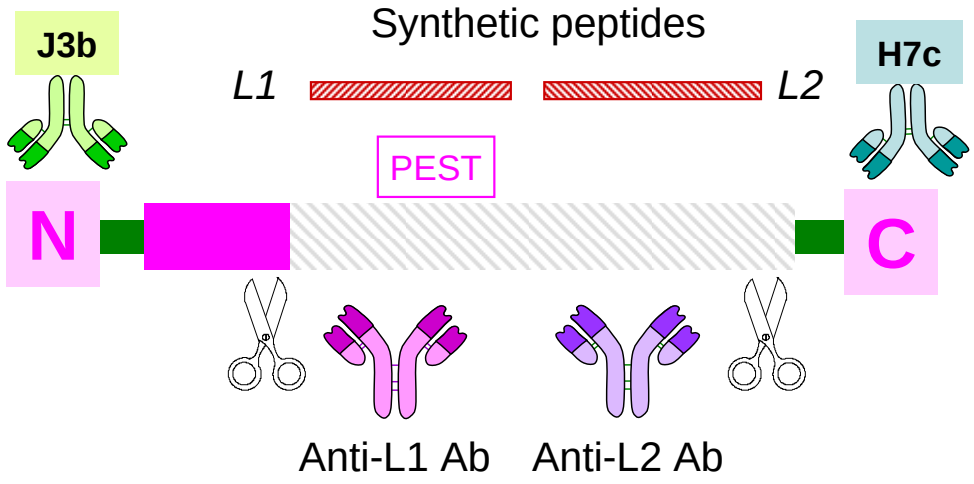
A

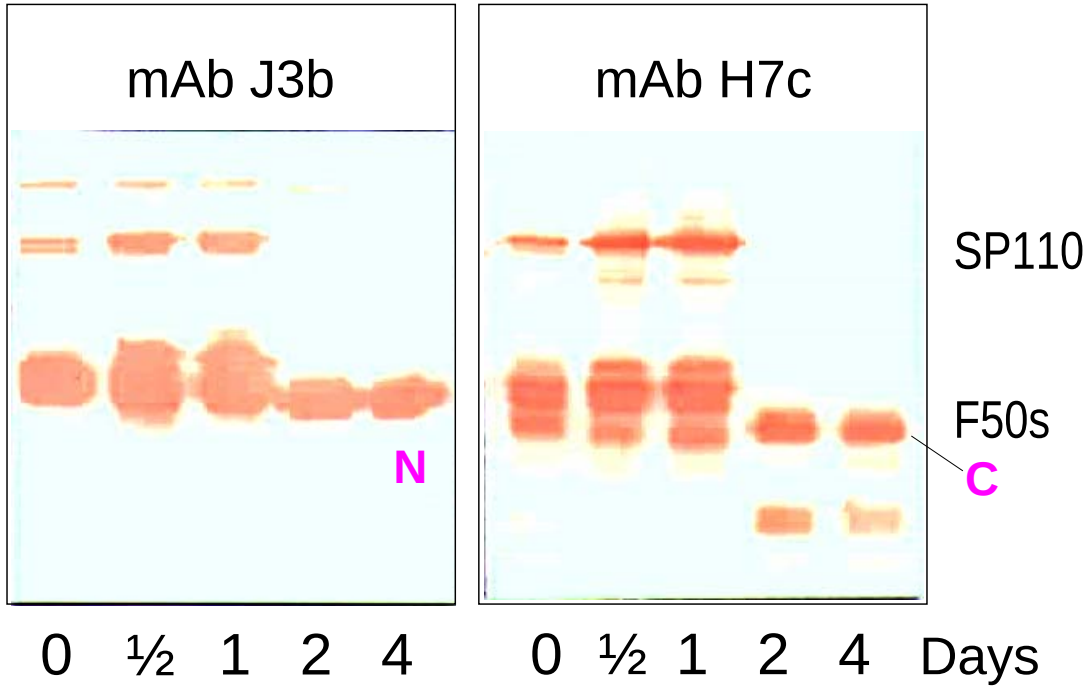
Reconstituted proteolysis

B



C





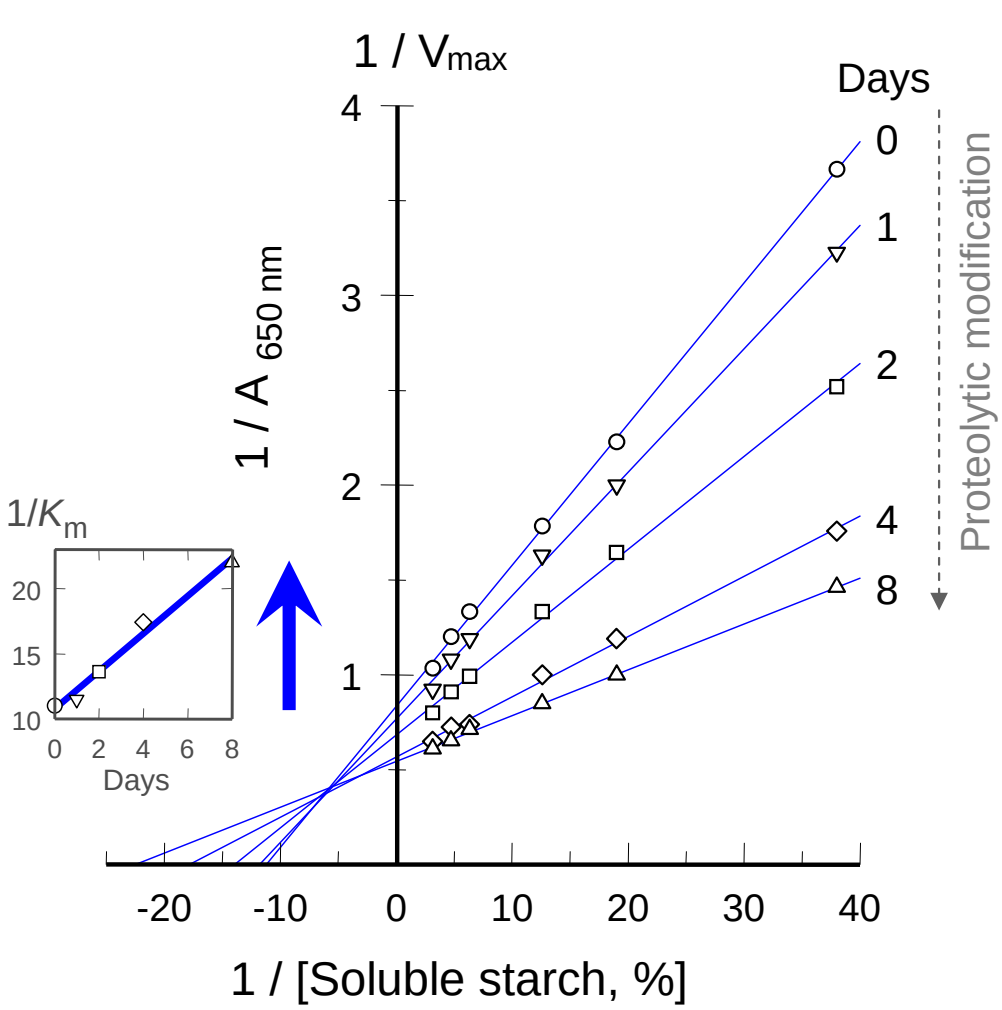
Why an intron was evolved to express, and then the expressed peptide was cut away?

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

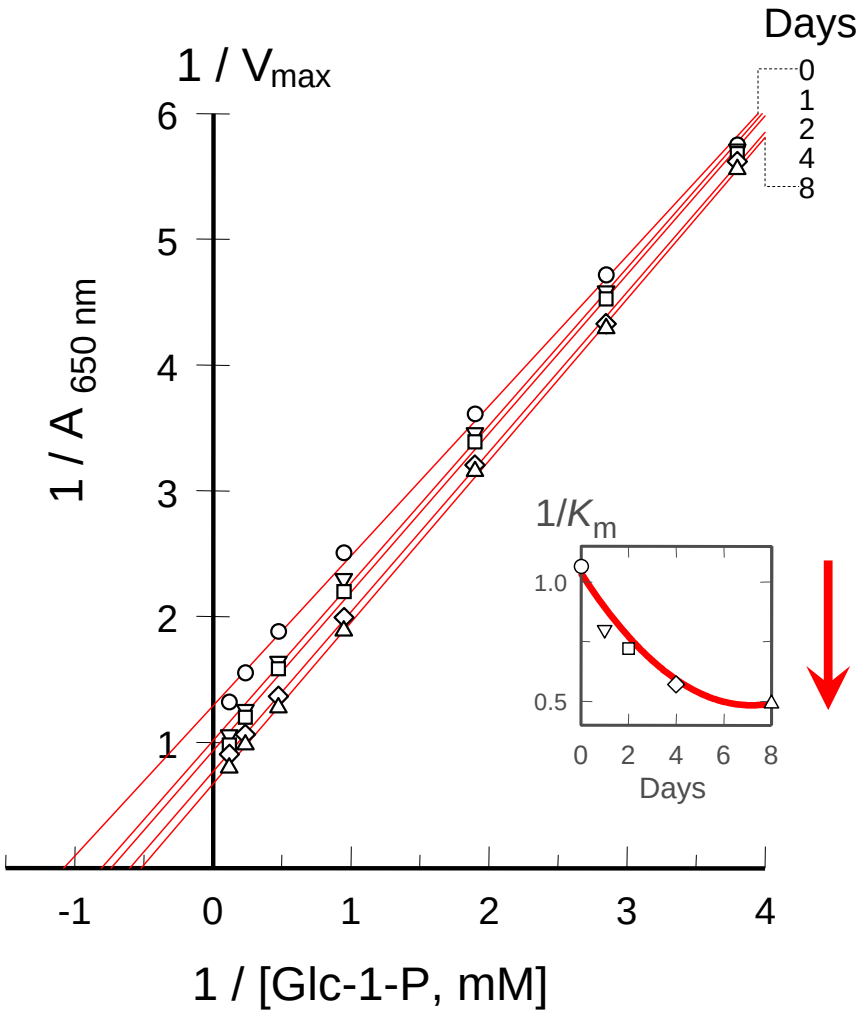
Chen et al, (2002) Physiologia Plantarum **114**:506-515

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

A Fixed [Glc-1-P]



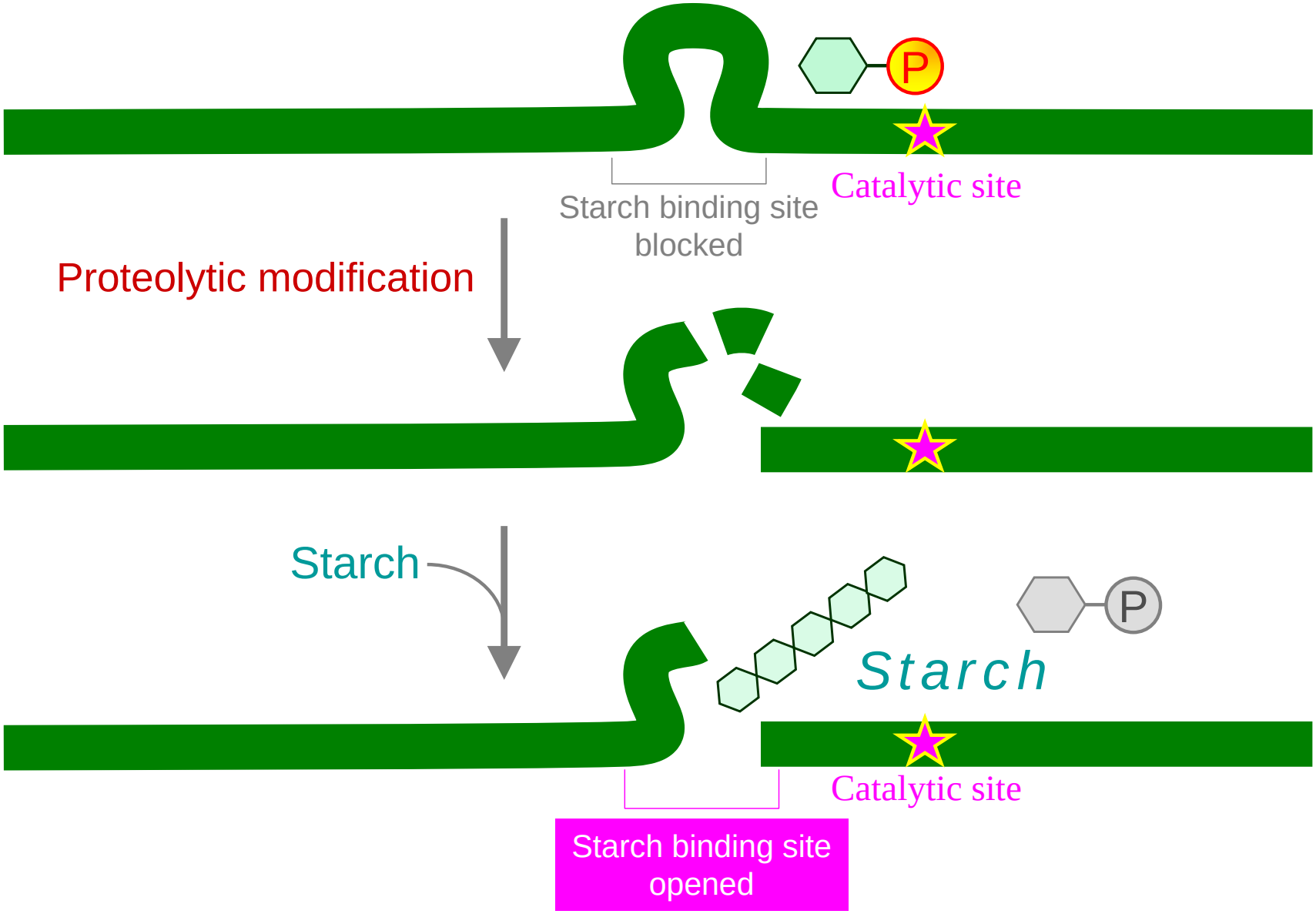
B Fixed [soluble starch]



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



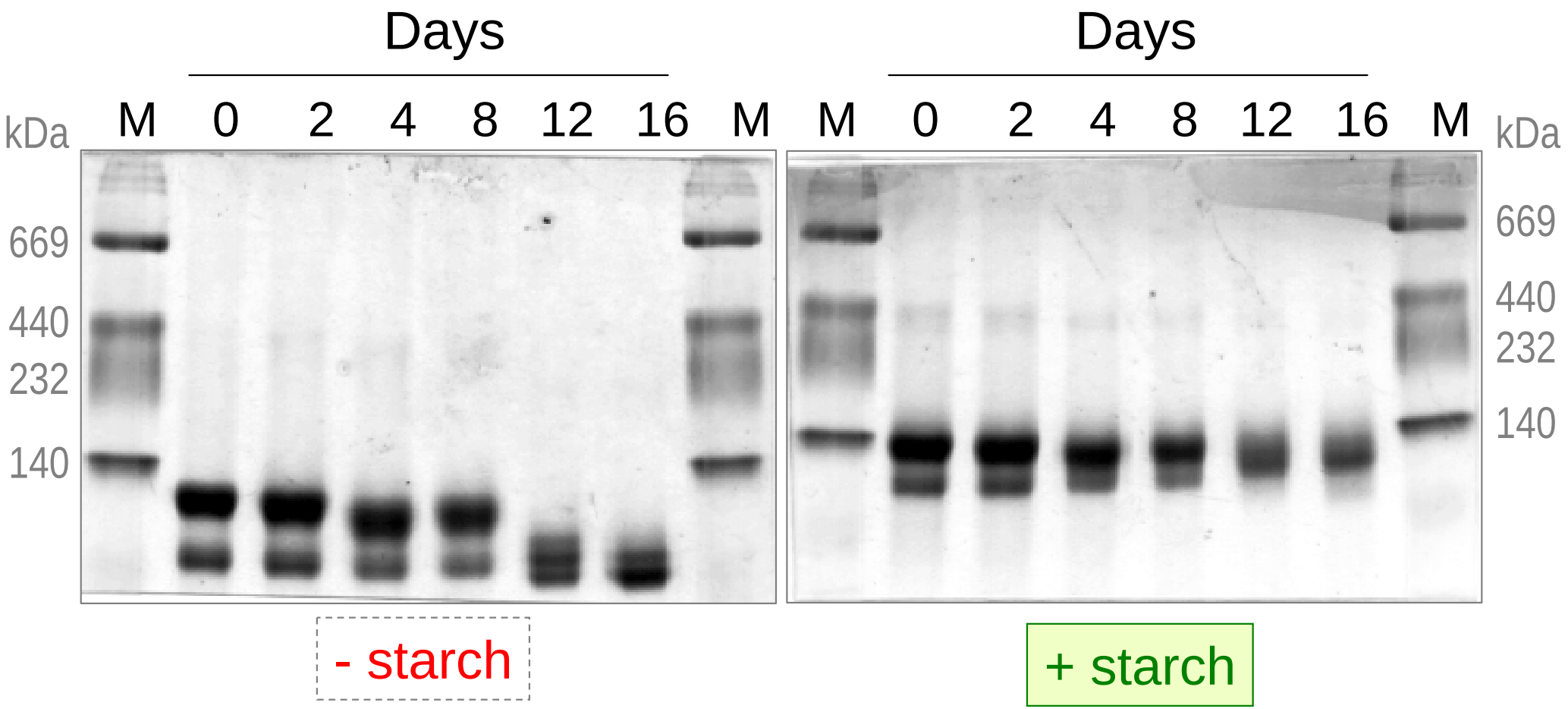
The starch binding site is opened by removing peptides on L78

The affinity to starch is increasing after modification

Native-PAGE

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

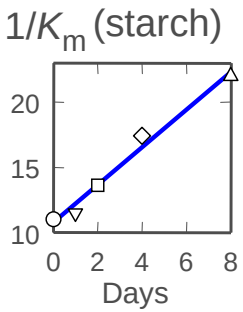
Proteolytic modification



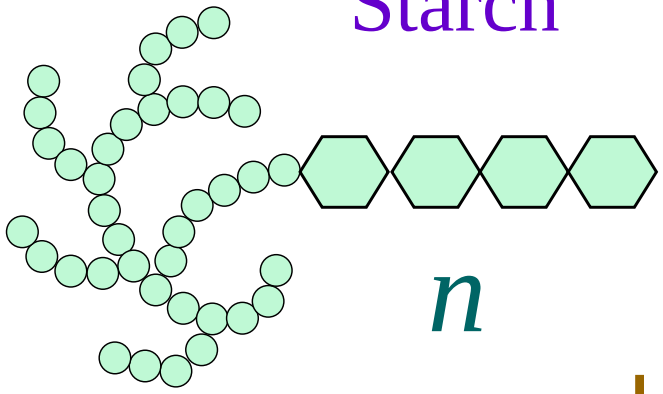
The modified L-SP is retarded in native electrophoresis gel containing soluble starch

L78 is a ***molecular switch*** regulating L-SP catalytic direction

L78 removed

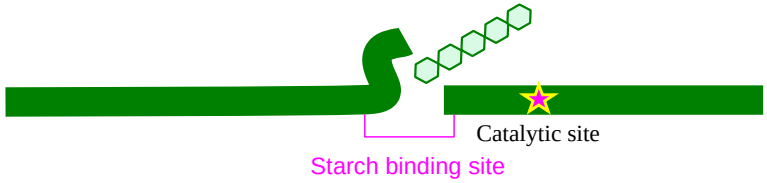


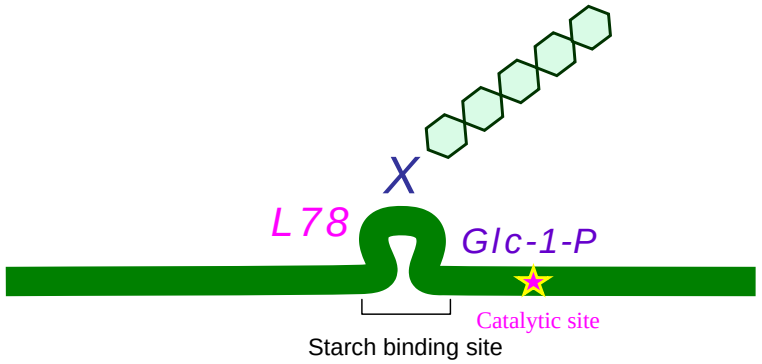
Starch



Phosphate

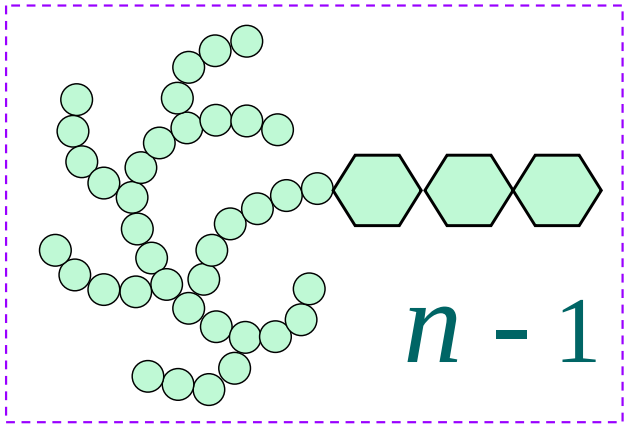
Phosphorolysis





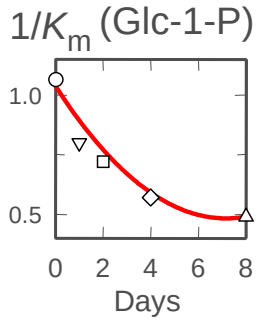
Starch biosynthesis

No primer ?
(primer-independent)



Glc-1-P

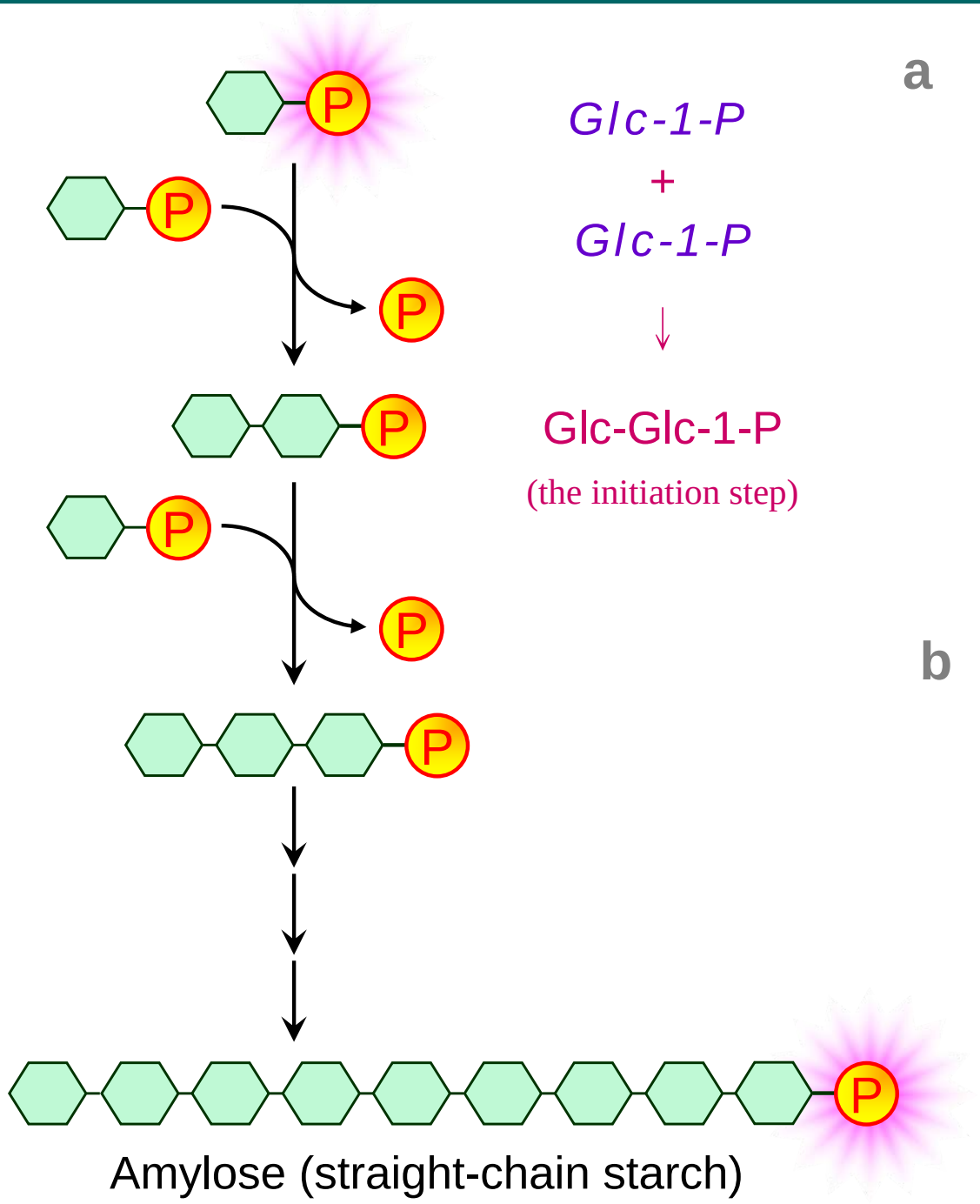
L78 intact



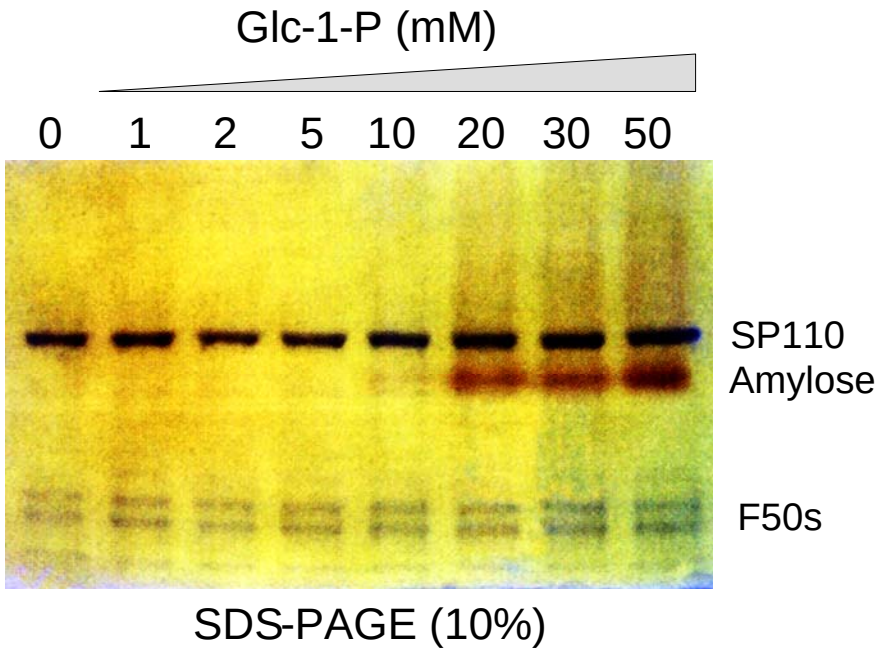
Intact L-SP binds Glc-1-P preferentially and the L78 blocks the starch binding site

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

Chen et al, (2007) *submitting*

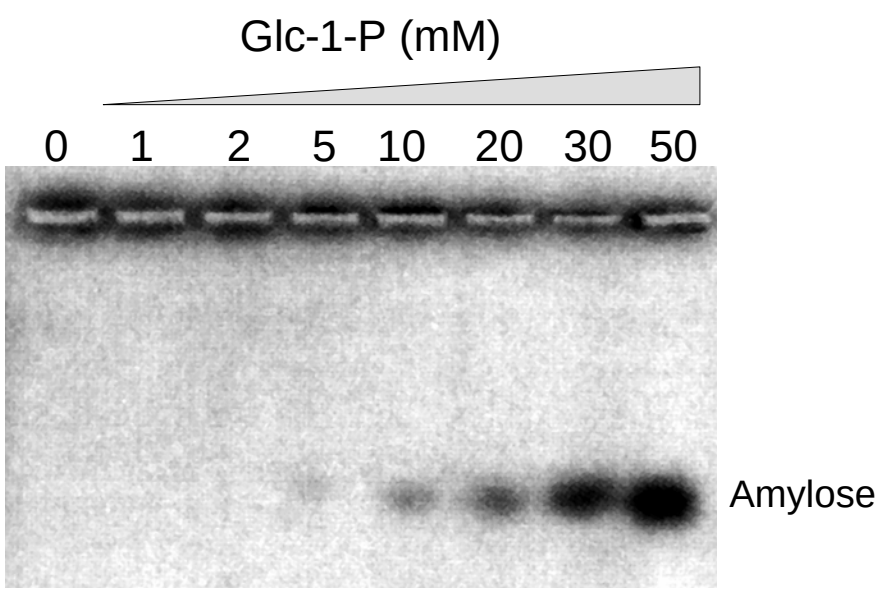


a



SDS-PAGE (10%)

b

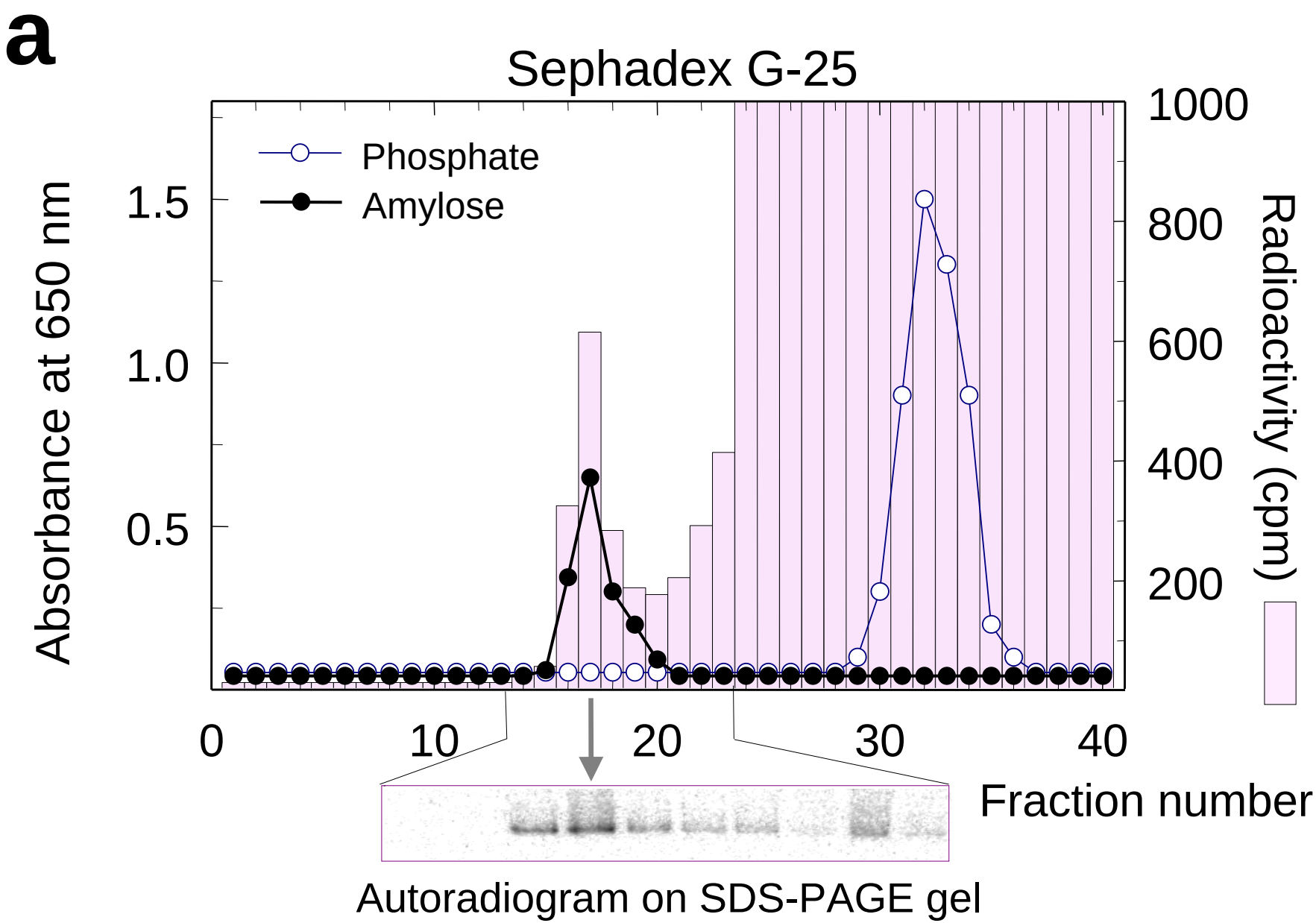


Agarose gel (0.5%)

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

The amylose synthesized is radioactive

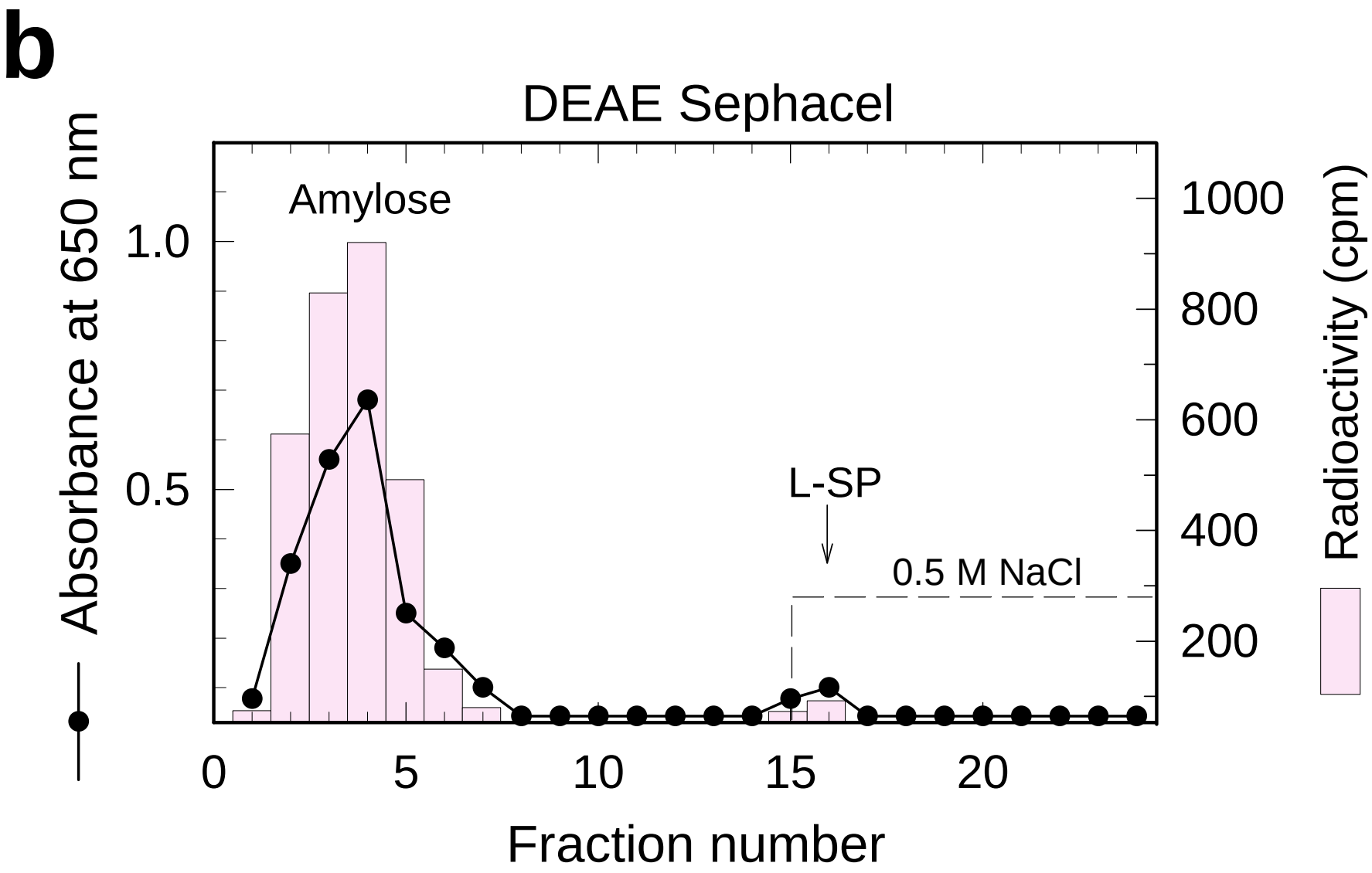
Chen et al, (2007) *submitting*



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

Further purification of amylose by ion exchange

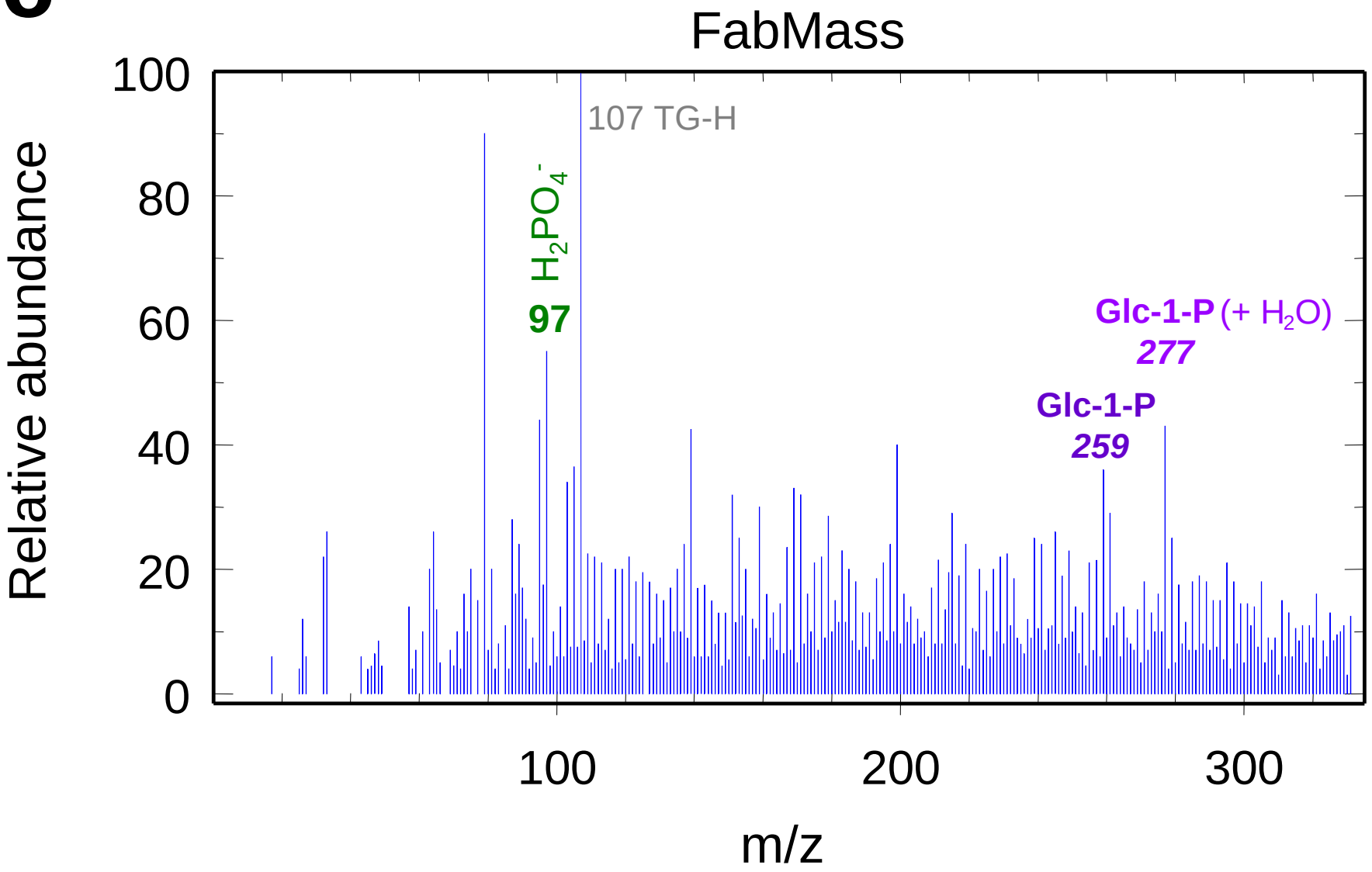
Chen et al, (2007) *submitting*



The amylose contains Glc-1-P moieties

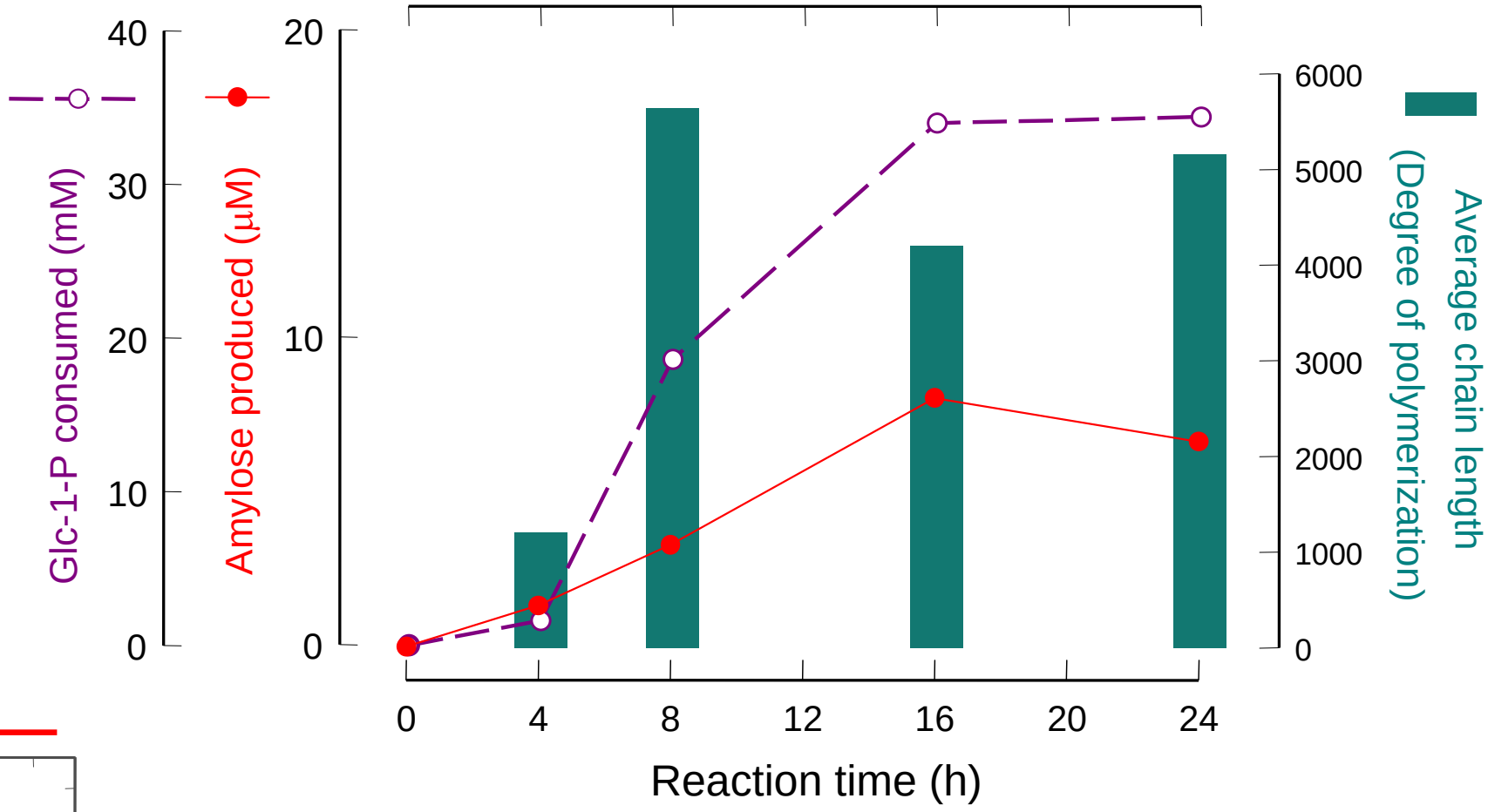
Chen et al, (2007) *submitting*

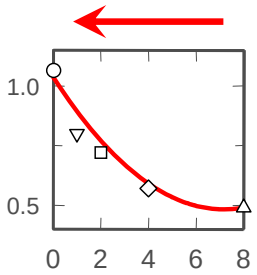
c



The process of polymerization shows three phases

Chen et al, (2007) *submitting*

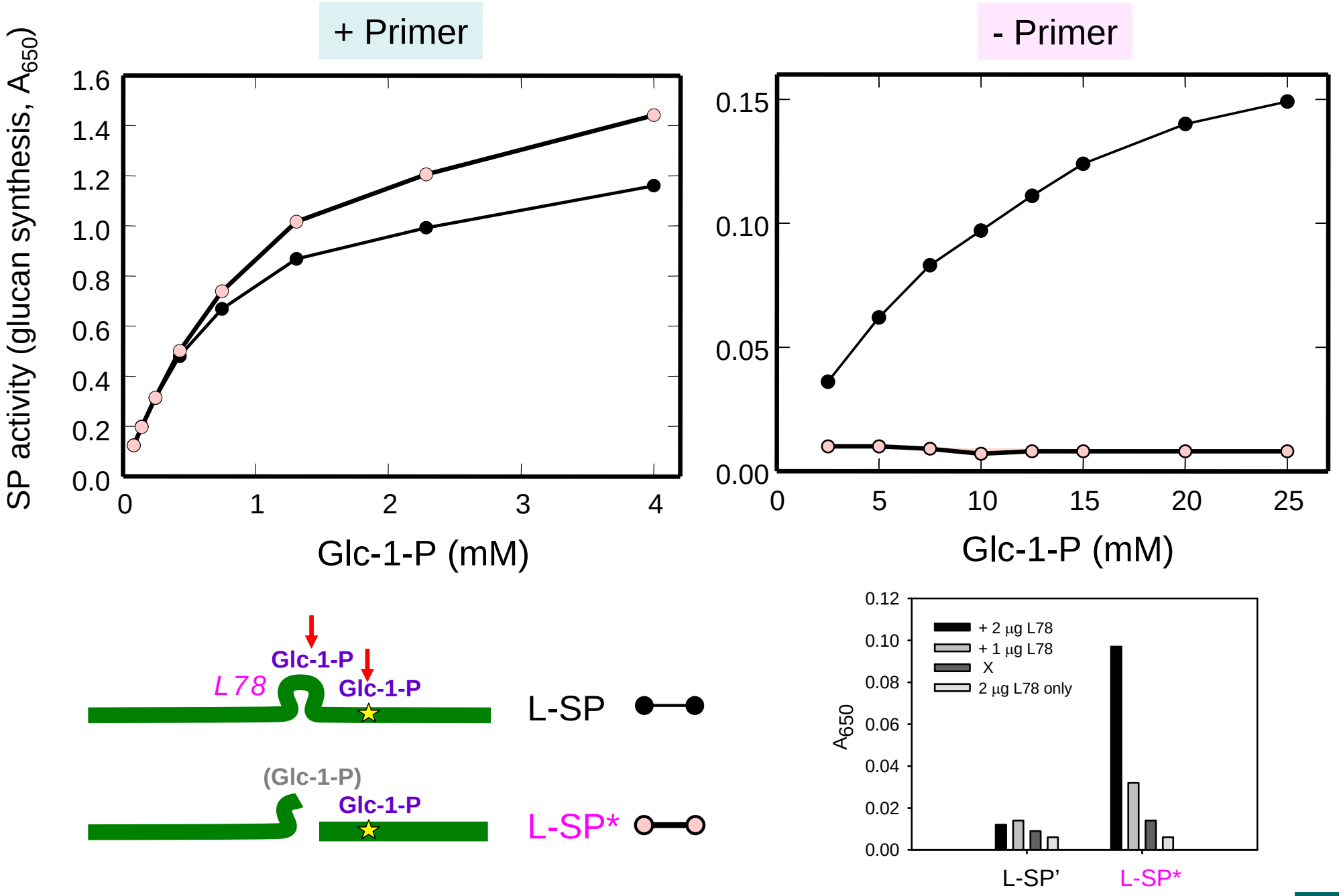




Glc-1-P consumption has three phases, suggesting a mechanism for glucan polymerization

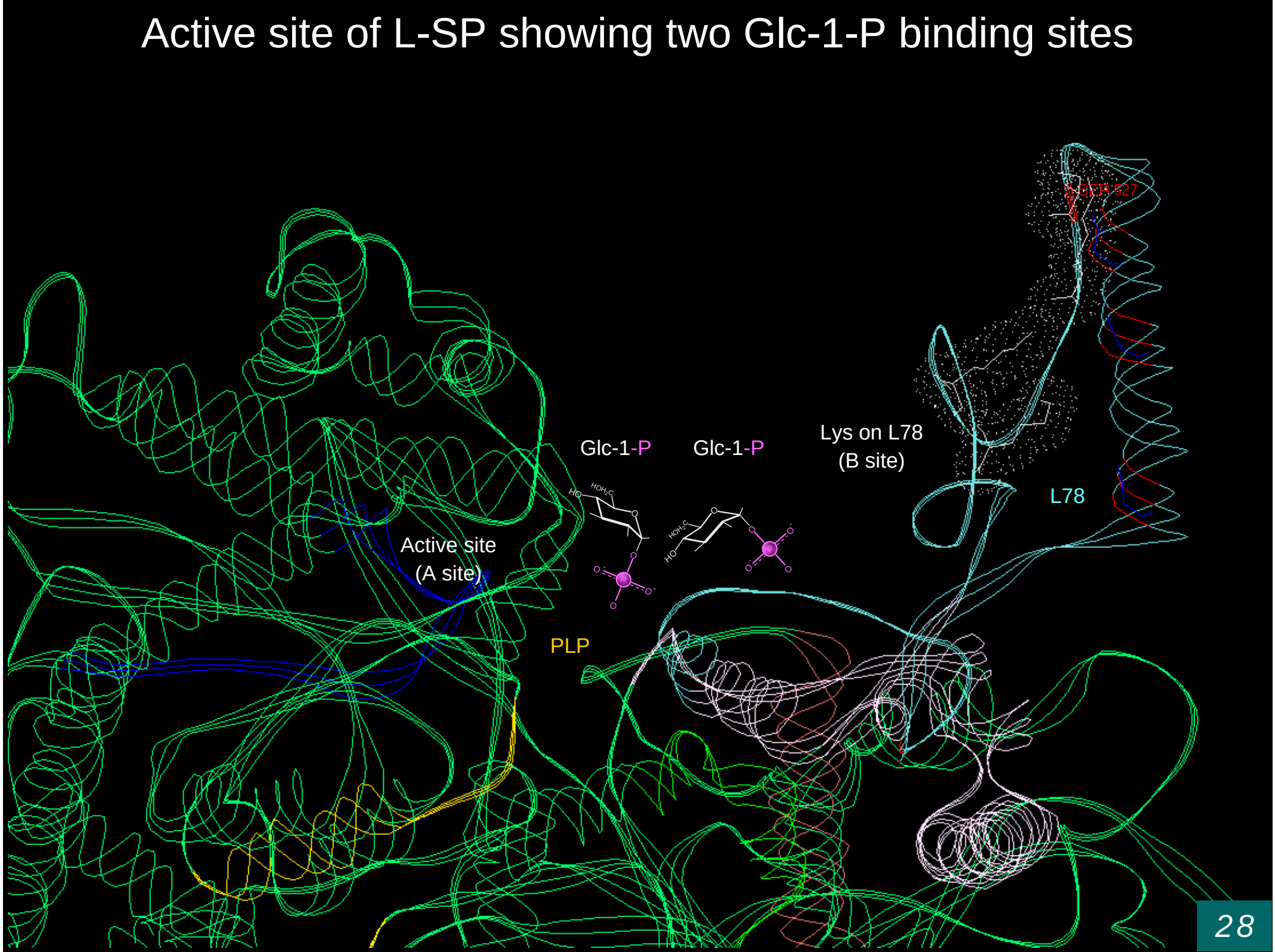
The PI activity of L-SP is lost if the L78 is removed

Chen et al, (2007) *submitting*



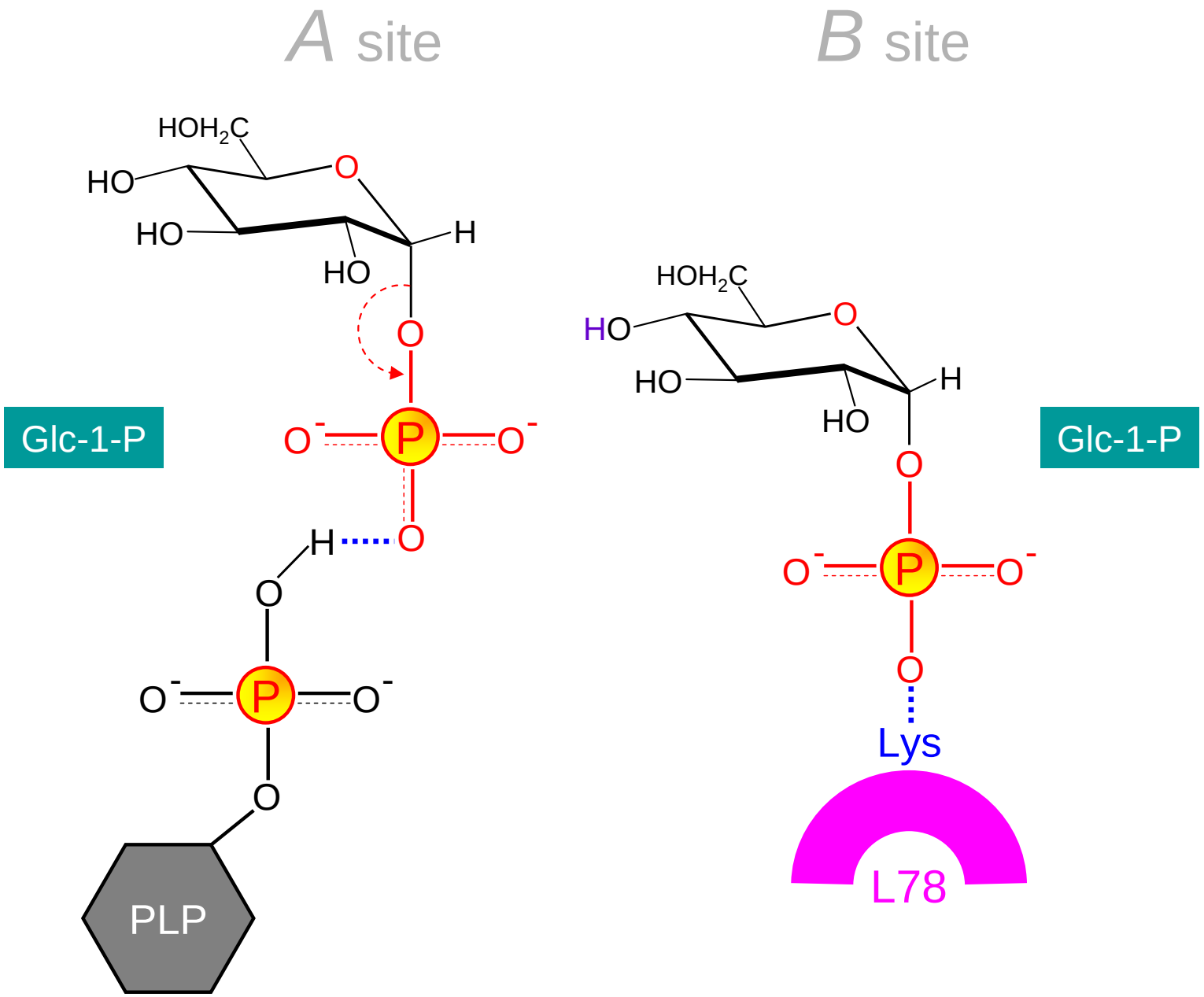
Does L78 serve as the “primer” for amylose synthesis ? Or an anchoring point for Glc-1-P?

Active site of L-SP showing two Glc-1-P binding sites



Action mechanism for PI amylose synthesis (1)

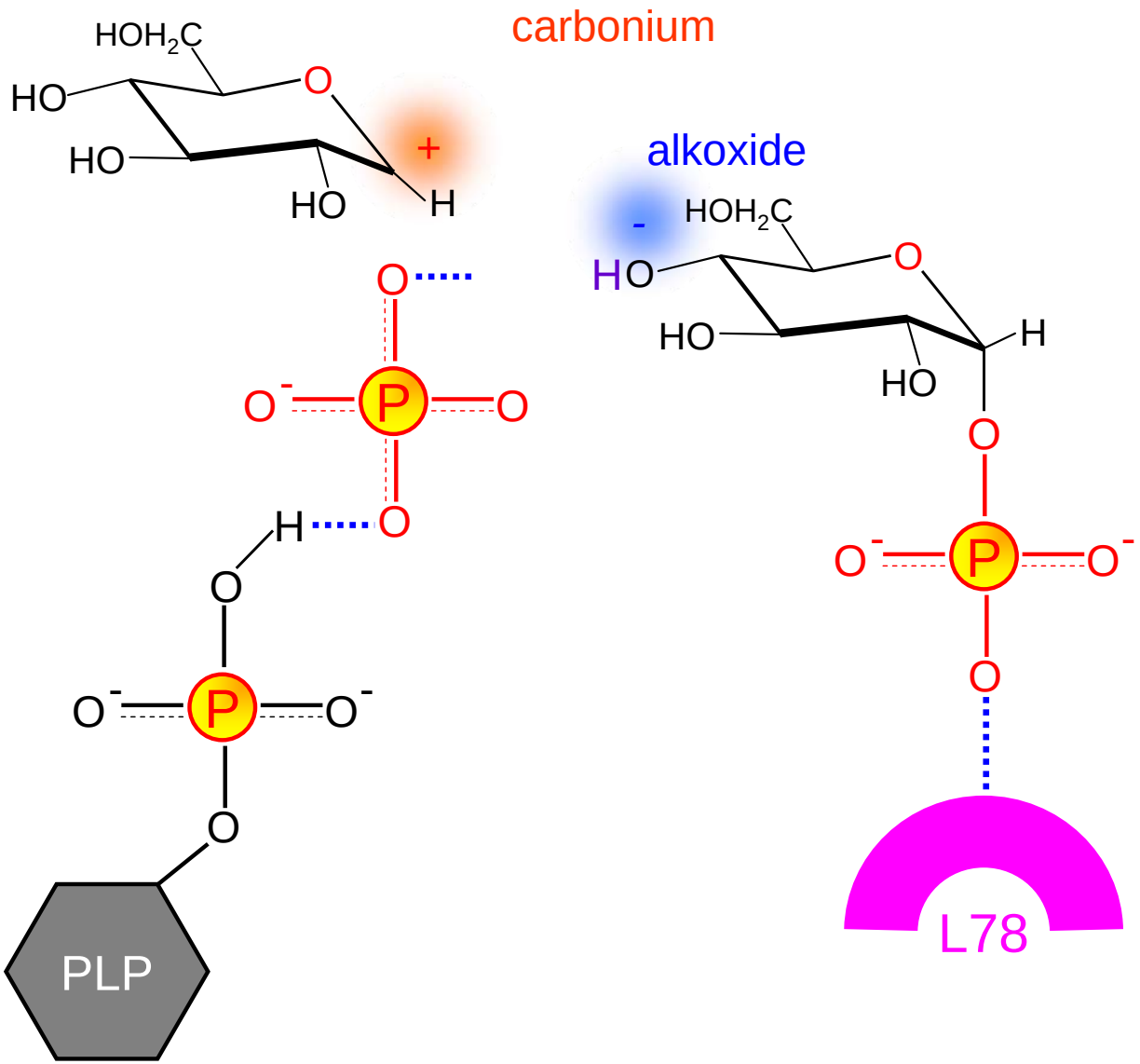
Unpublished



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

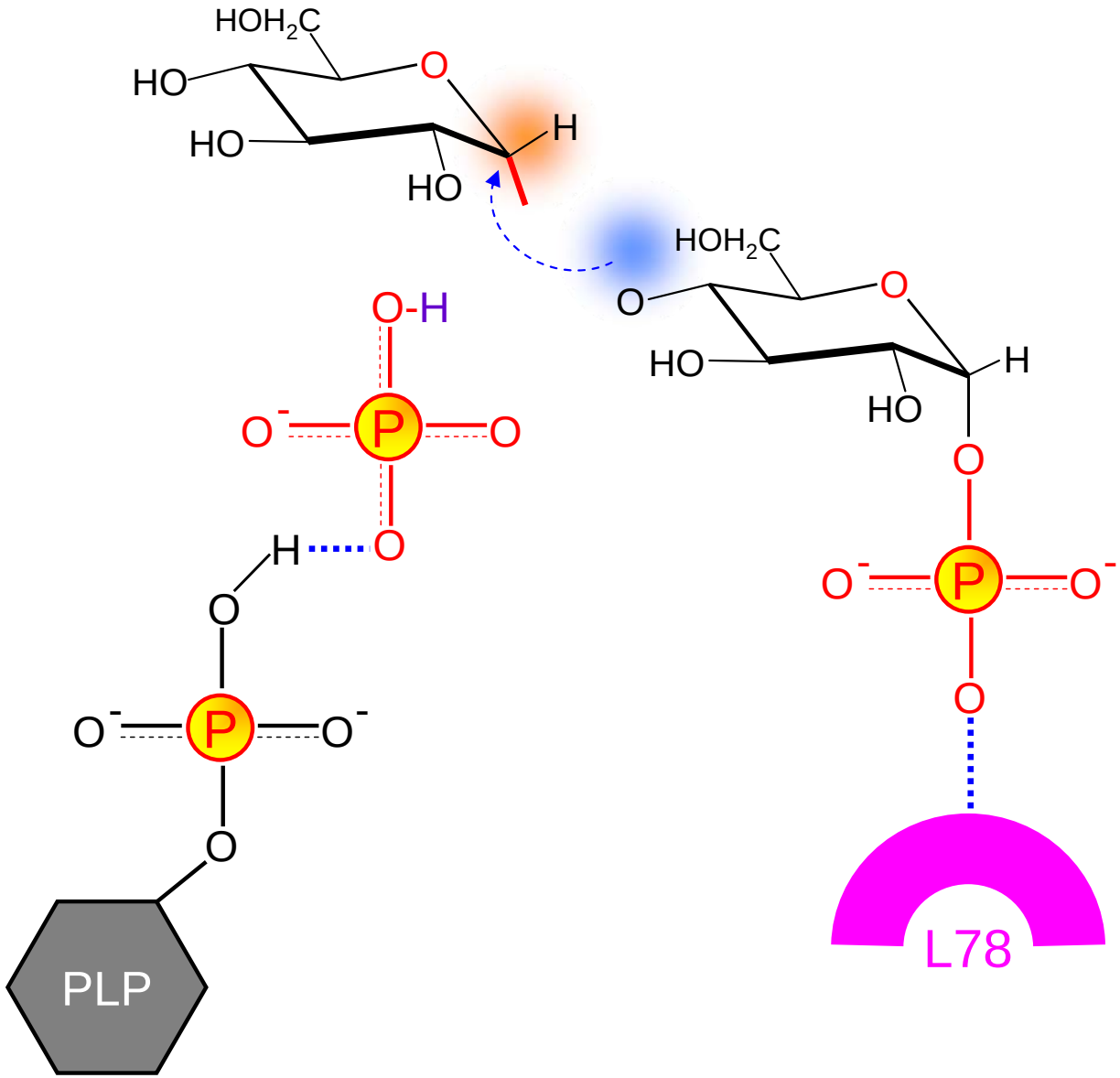


The C-1 on Glc (A site) becomes a carbonium ion after releasing the phosphate

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)

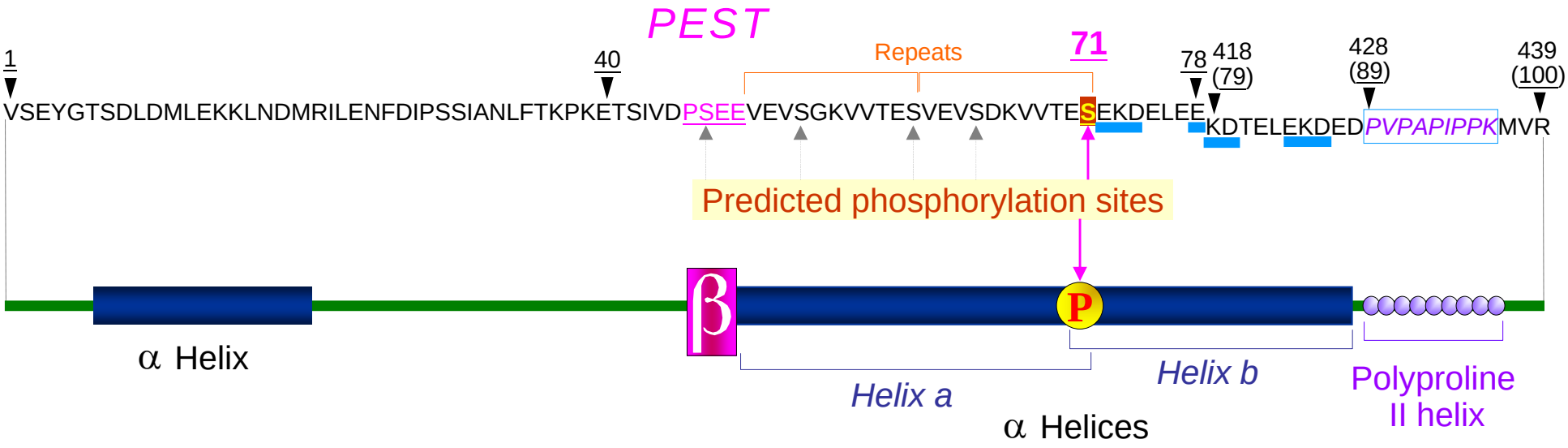
Unpublished

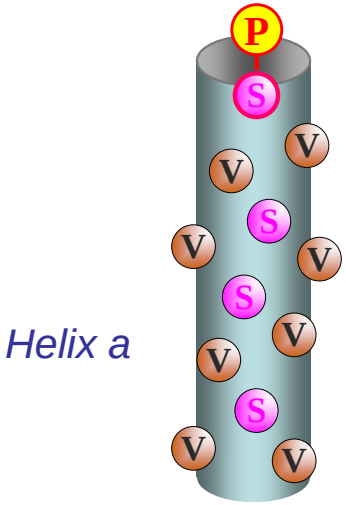


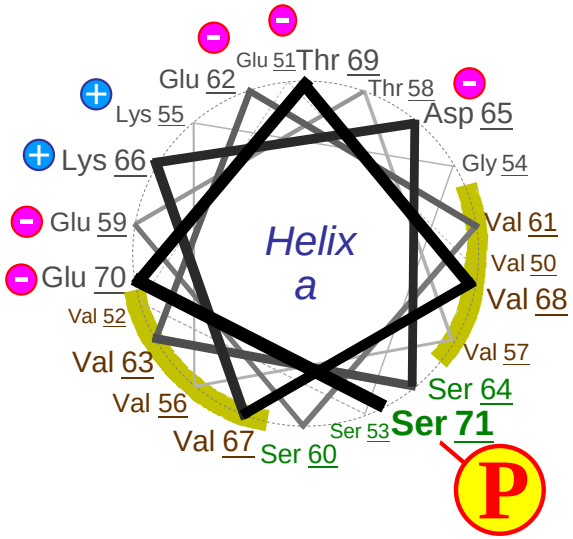
The negatively charged alkoxide attacks the carbonium ion producing a new glycosidic bond

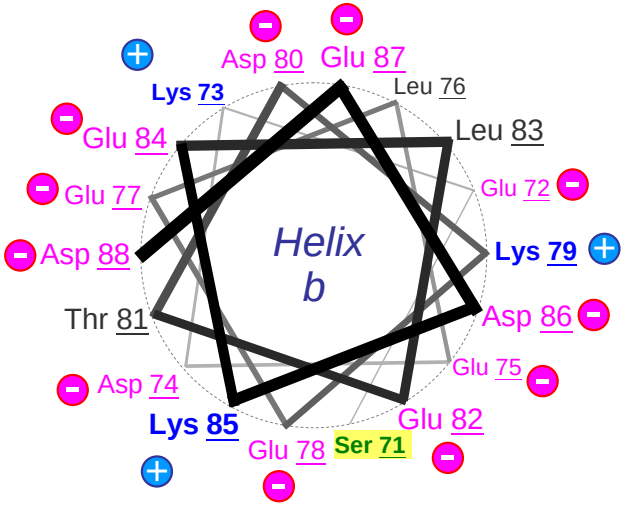
L-SP is predicted to be phosphorylated

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





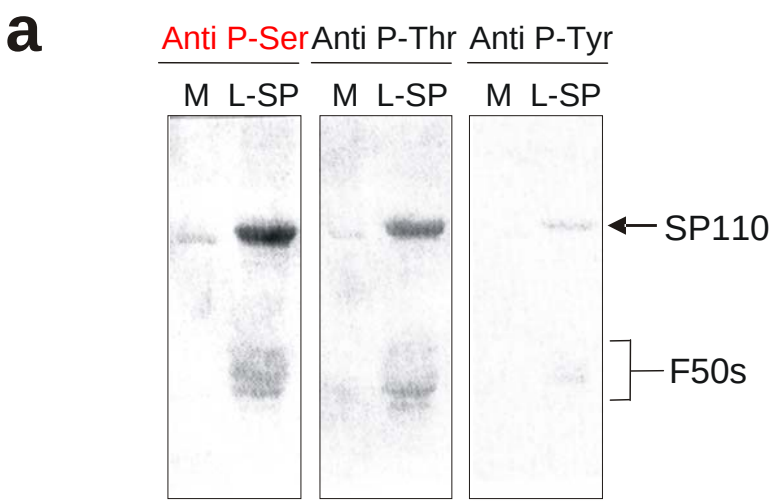




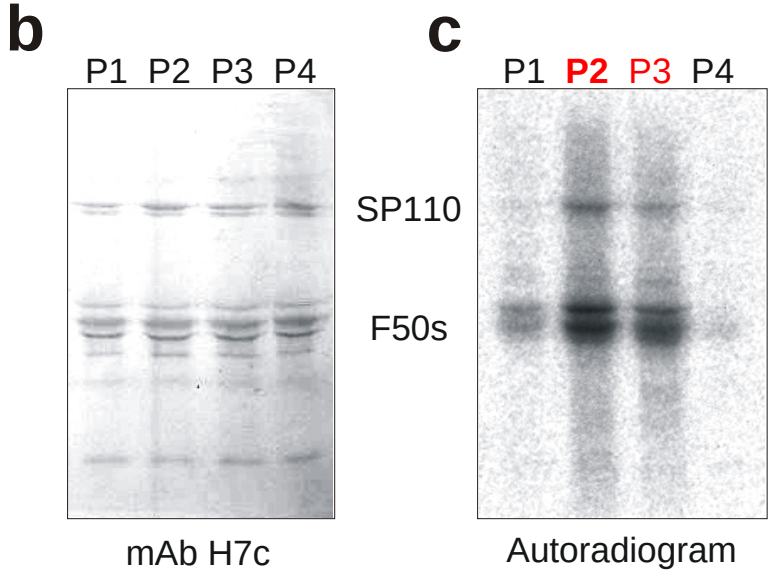
Several phosphorylation sites are predicted in the helices on L78

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

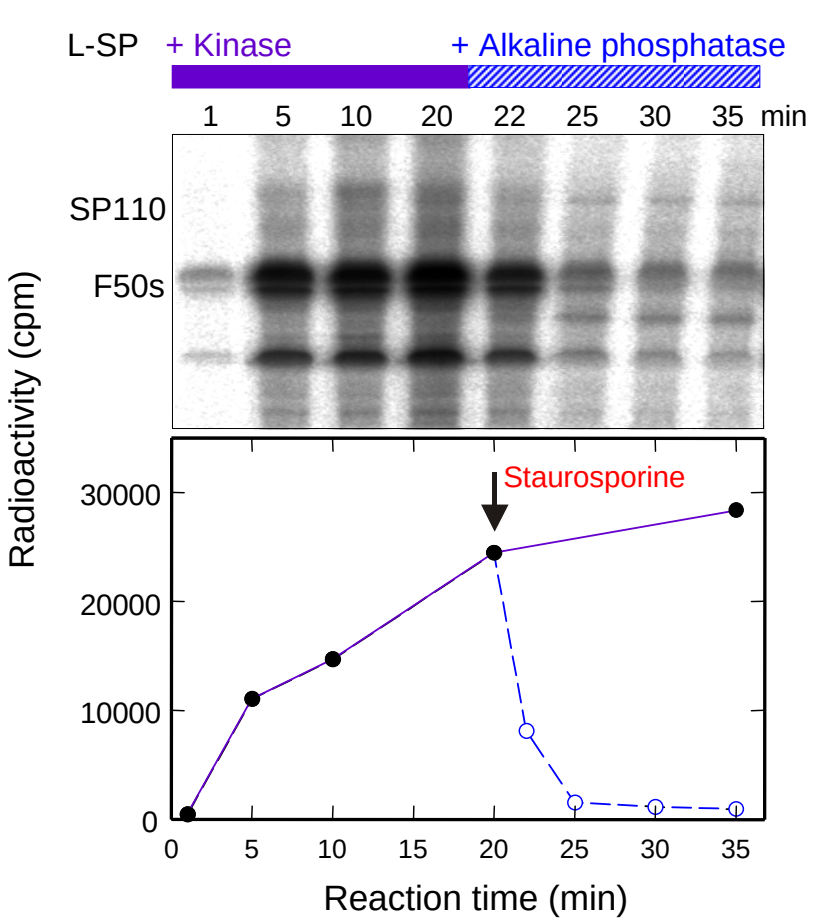
Young et al, (2006) Planta **223**: 468-478



Purified L-SP was phosphorylated in the plant



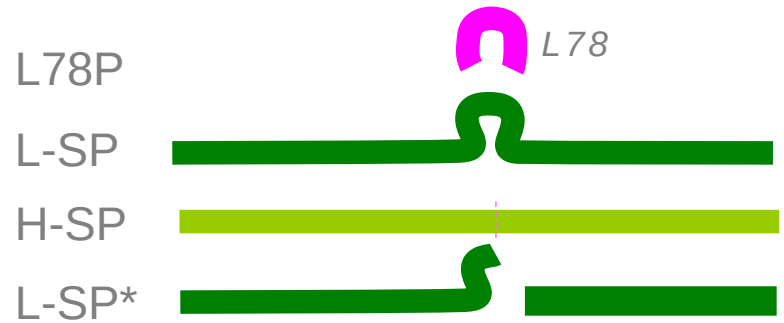
Ammonium sulfate fractions contained a kinase activity

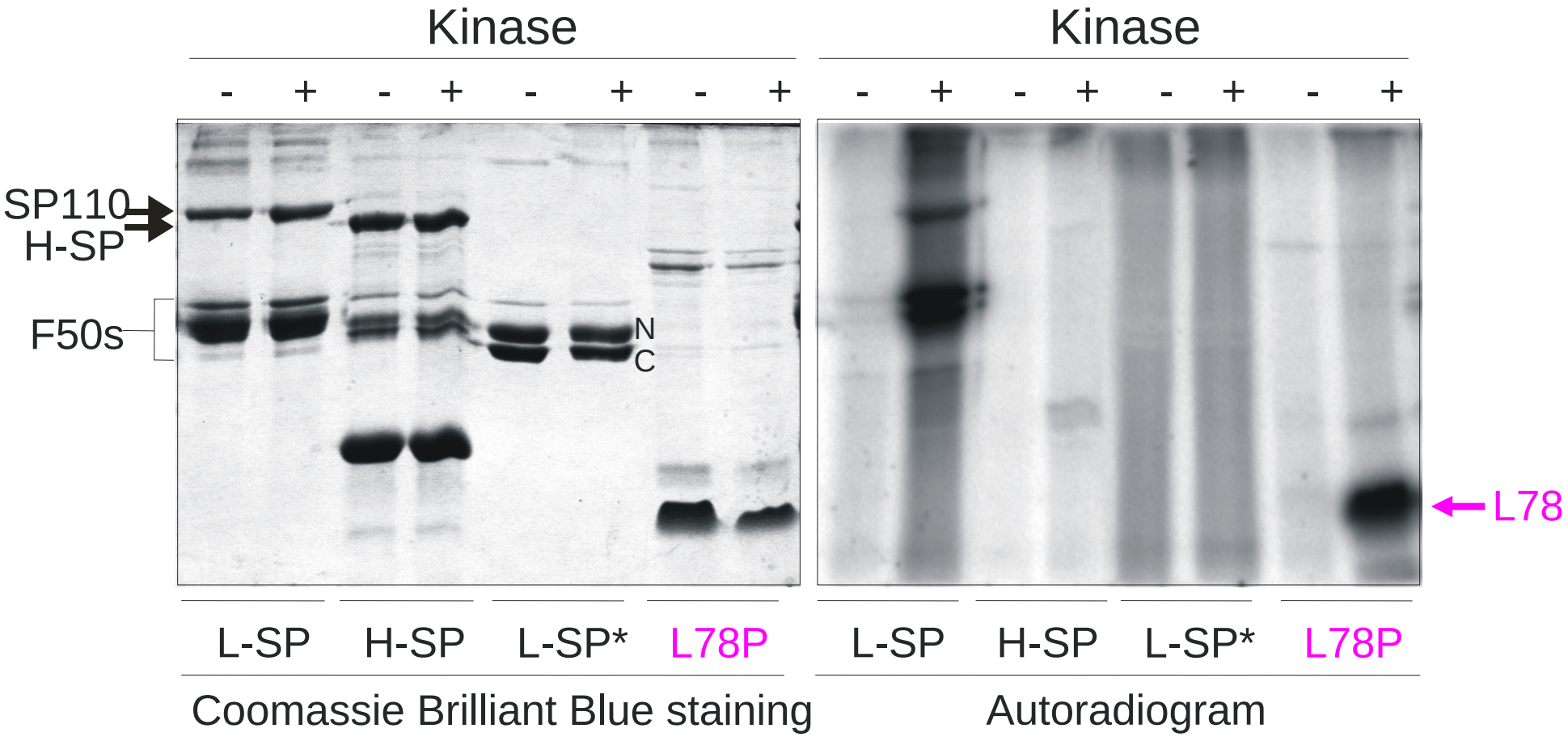


L-SP is found phosphorylated in sweet potato roots, or *in vitro* phosphorylated by a protein fraction from the root extract

L-SP is specifically phosphorylated on its L78 insertion

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

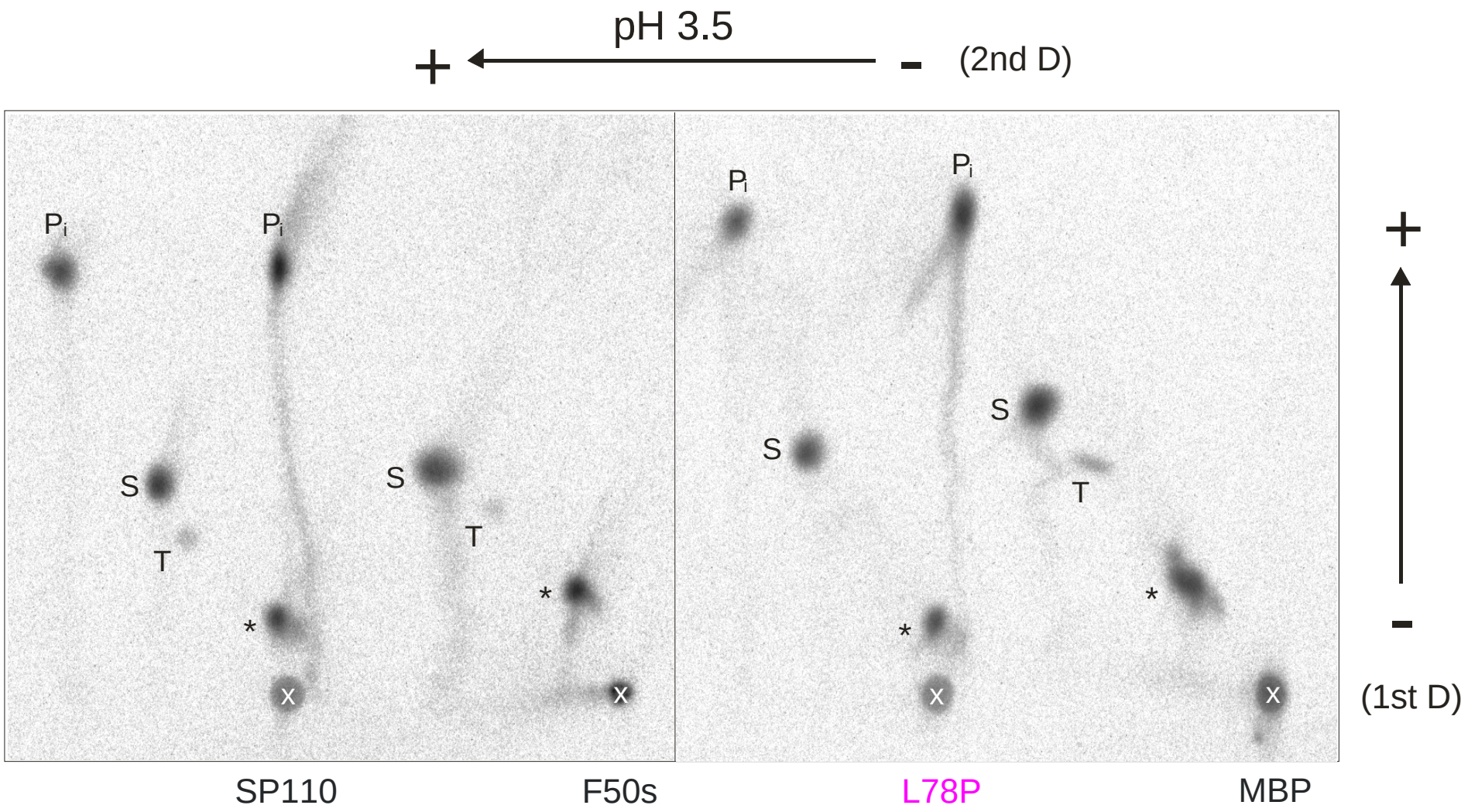




Phosphorylase molecules lacking L78 insertion can not been phosphorylated

Ser on L78 is the target for the kinase

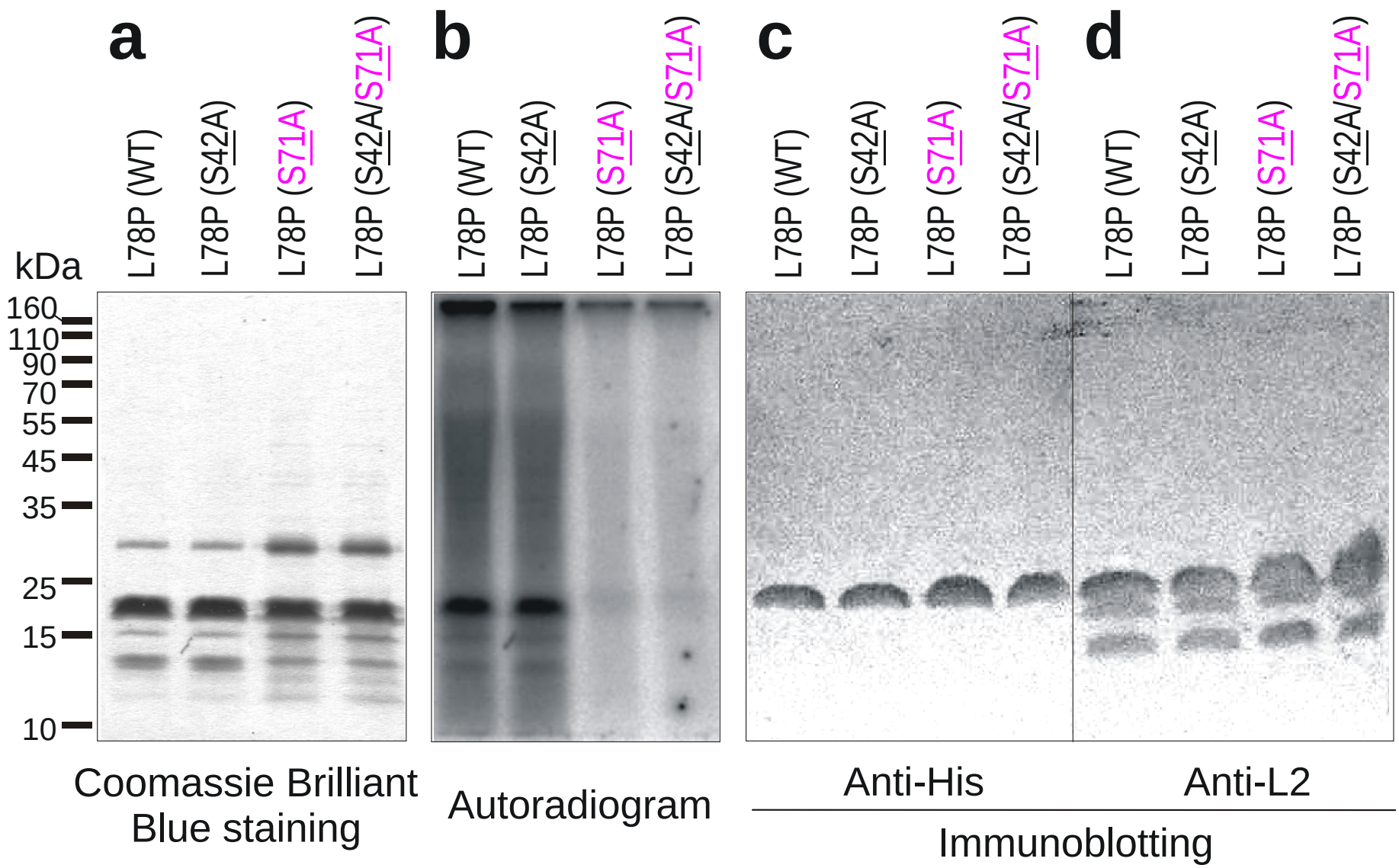
Young et al, (2006) Planta **223**: 468-478



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

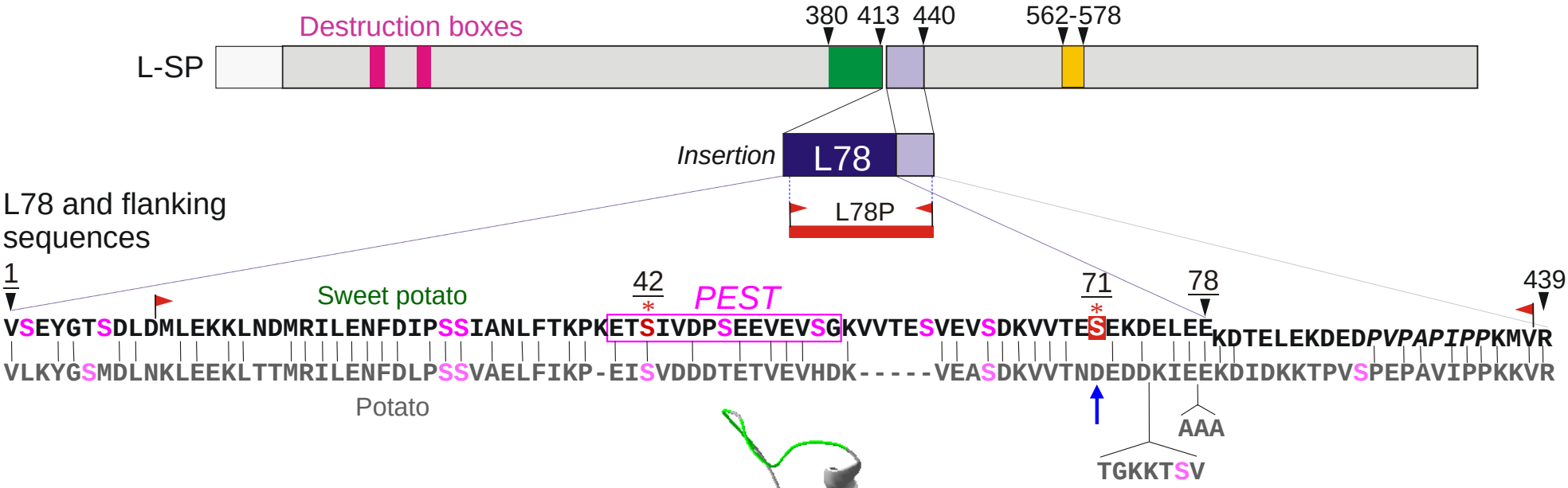
Young et al, (2006) Planta **223**: 468-478

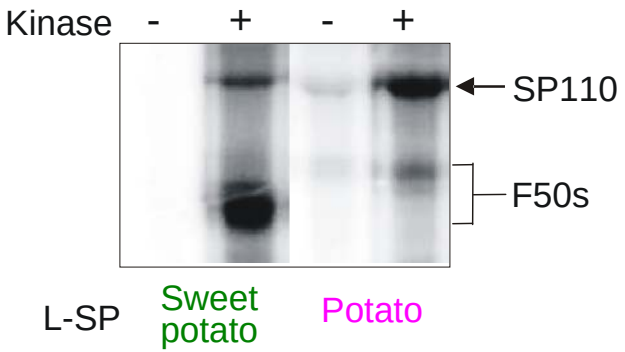


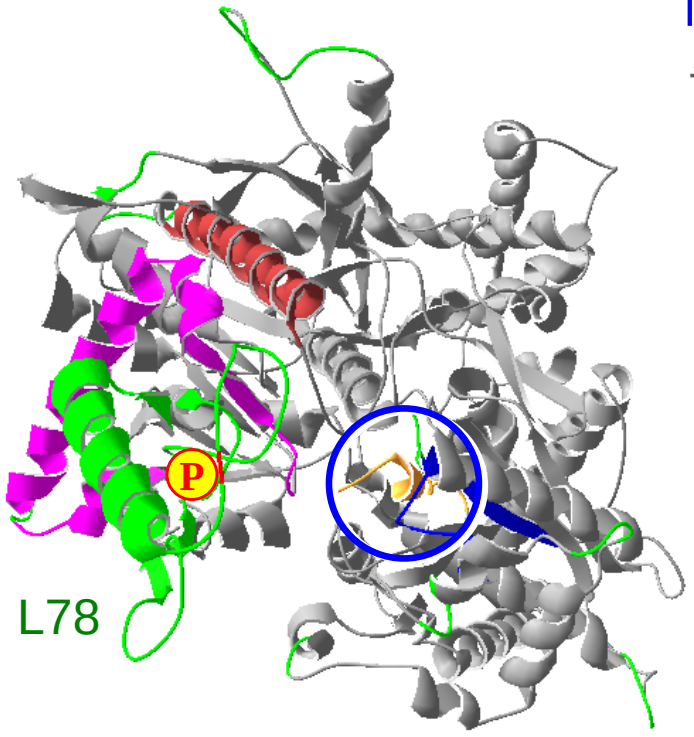
Ser 71 on L78 is the only phosphorylation site on L-SP by the kinase

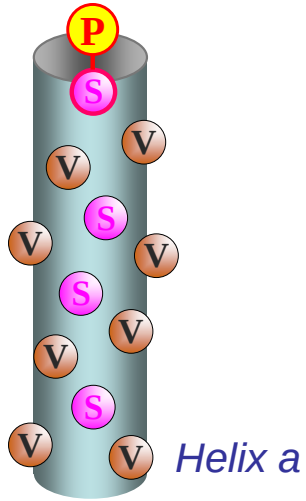
The phosphorylation site on L78 is specific

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



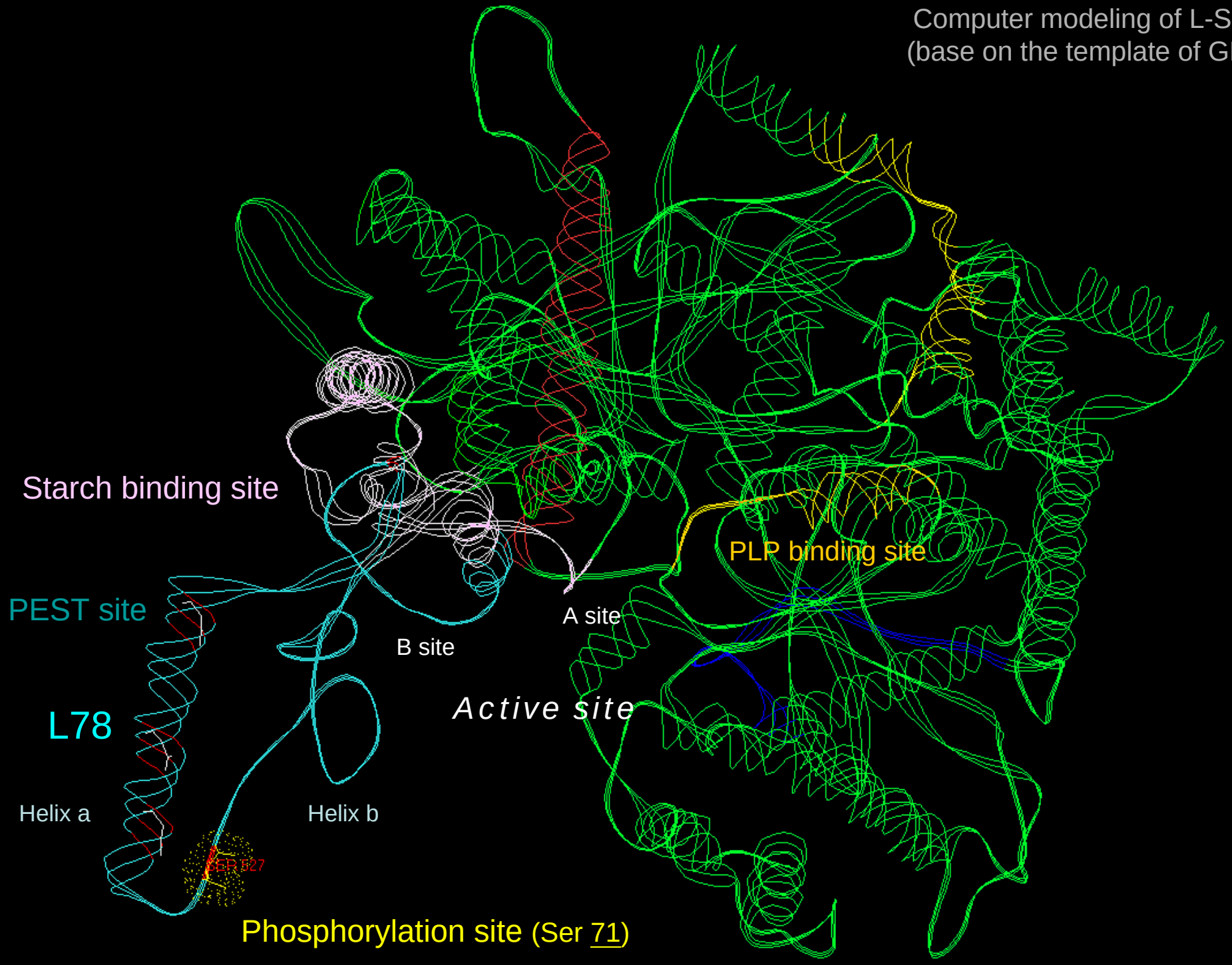






Although this kinase could also phosphorylate L-SP from potato, the exact phosphorylation site and mechanism are unclear

Computer modeling of L-SP
(base on the template of GP)



Phosphorylation site (Ser 71)

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%

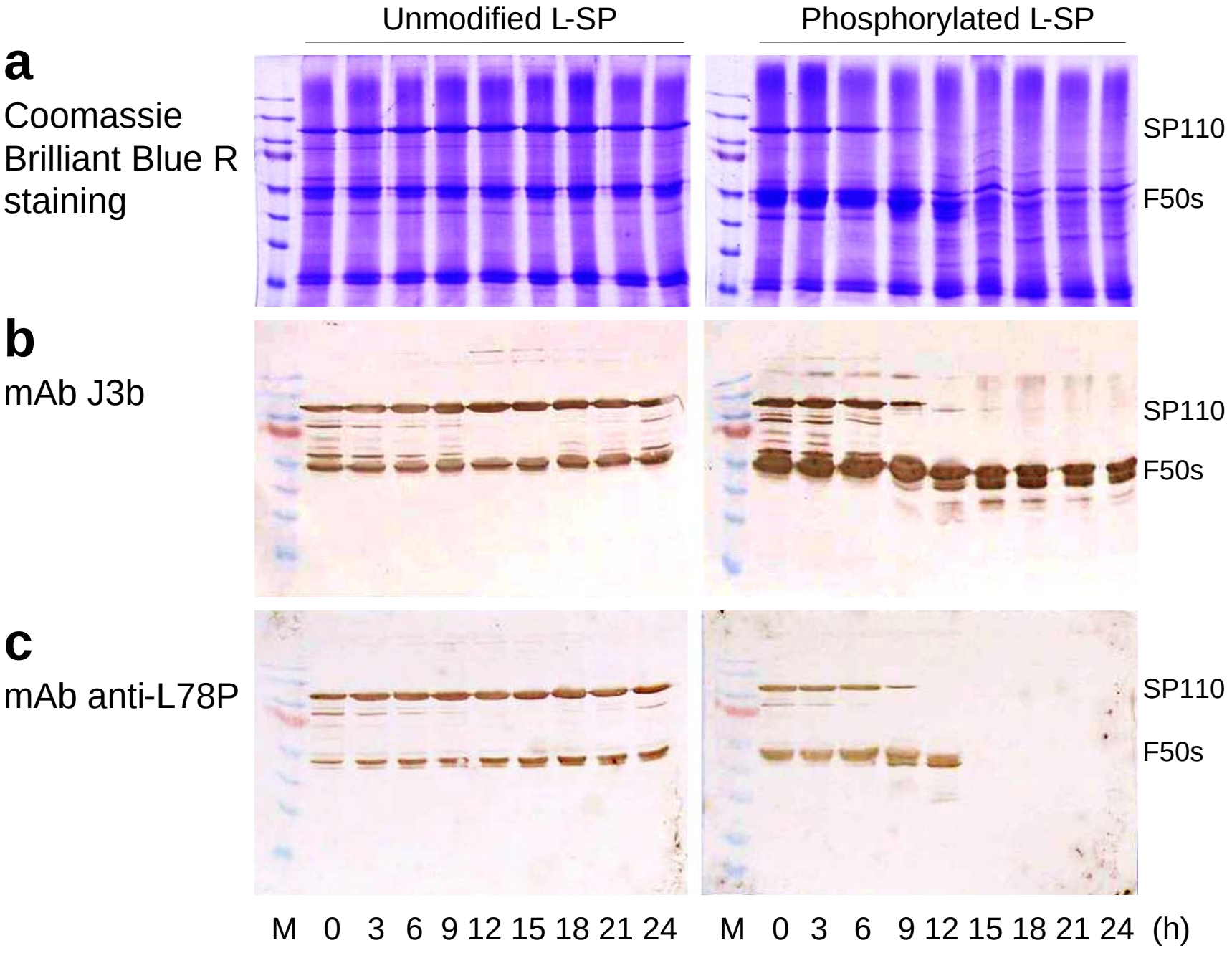
Phosphorolytic direction (Mori et al. 1993)

	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%

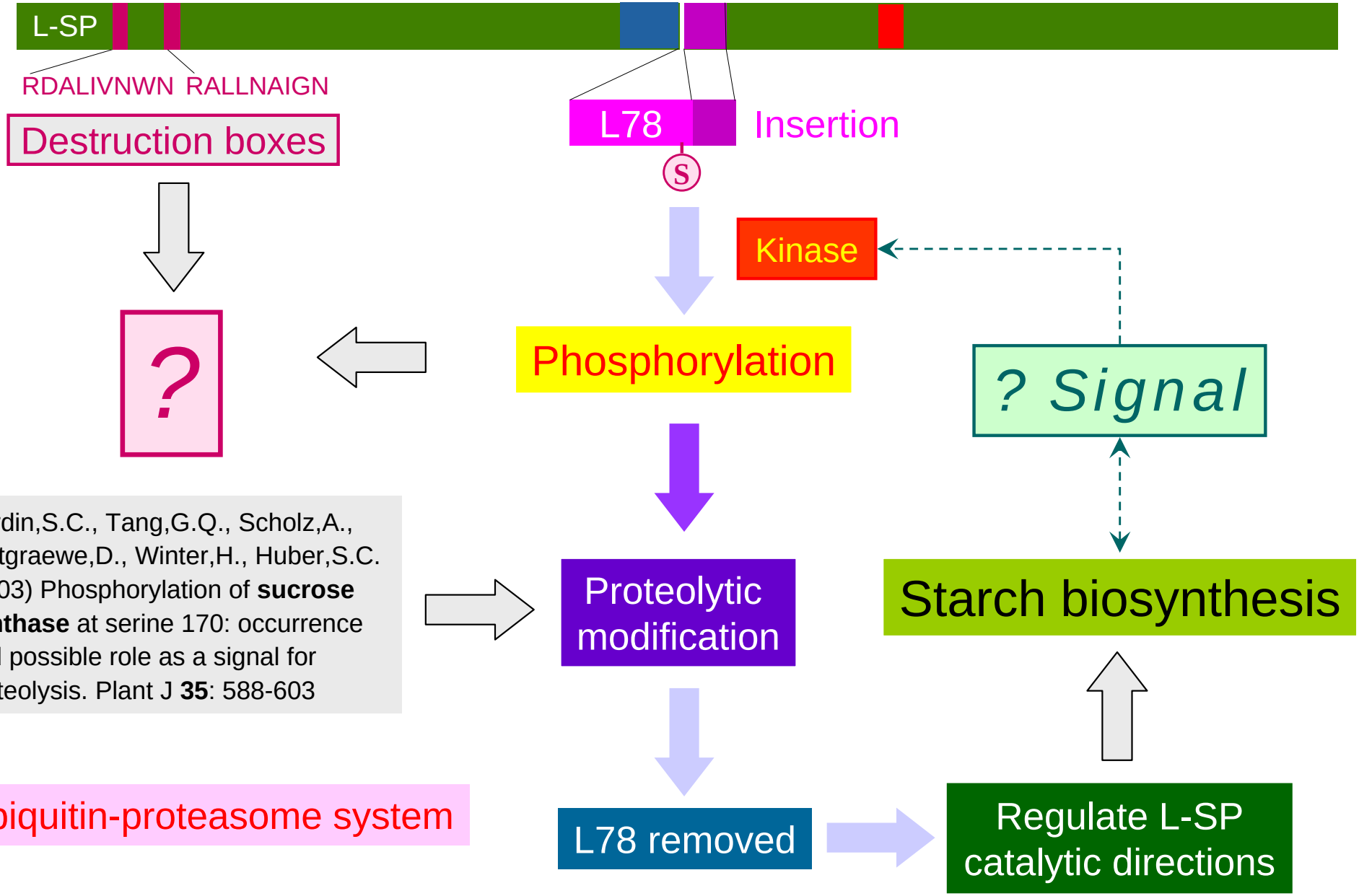
Phosphorylated L-SP is sensitive to proteolytic modification

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?

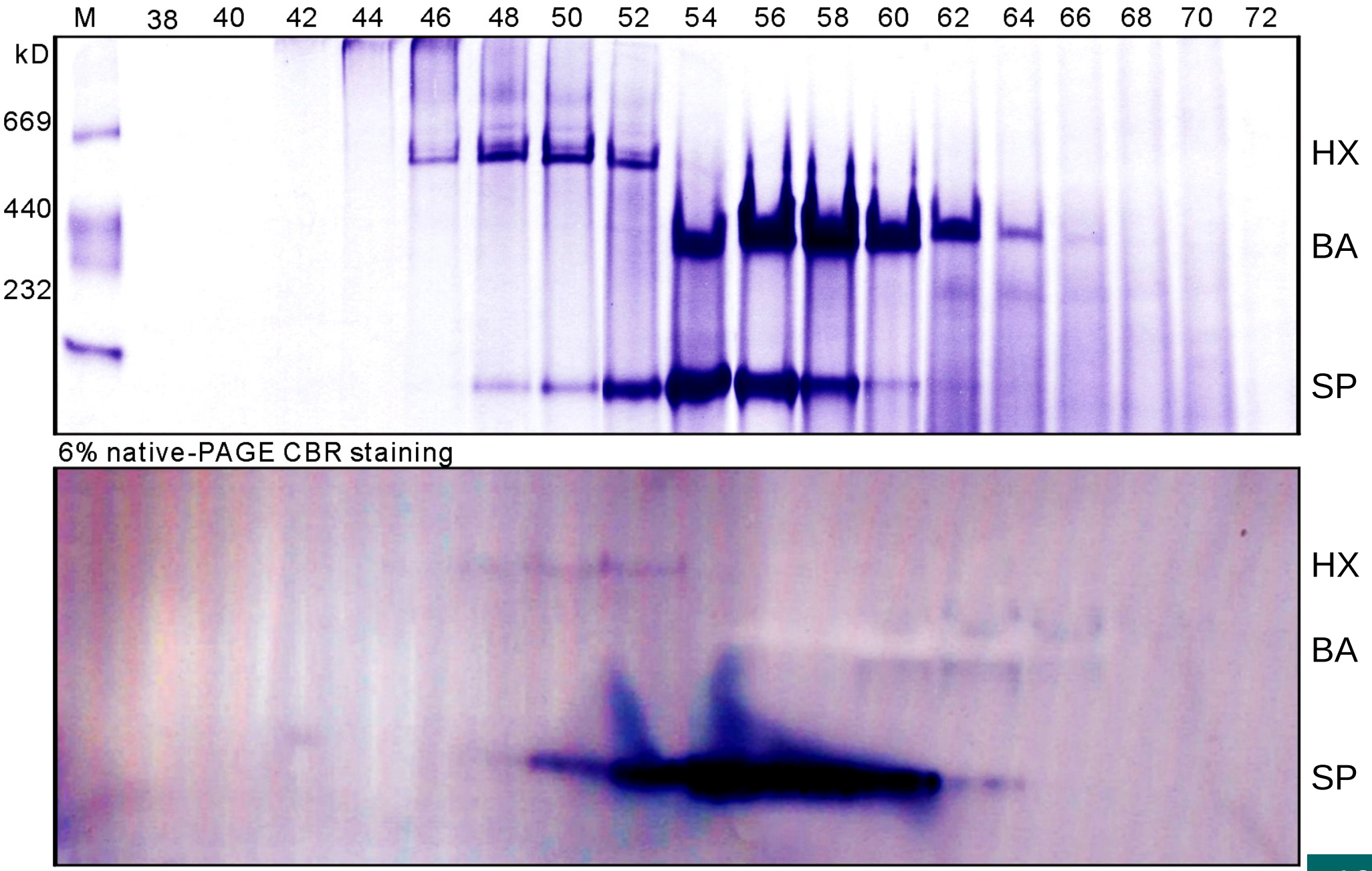


Phosphorylation of L78 might trigger the removal of the L78 insertion, and change the catalytic behavior of L-SP from starch synthesis to phosphorolysis

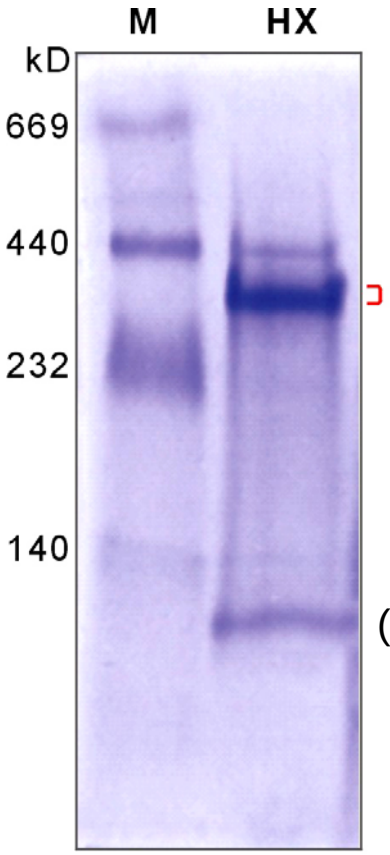
A high MW complex (HX) shows SP activity

Unpublished

Sephacryl S-300



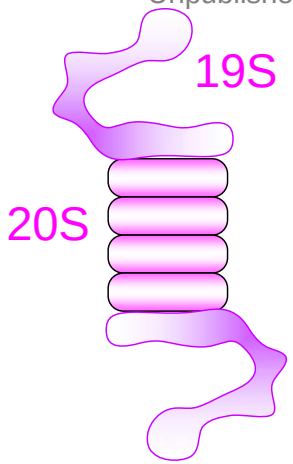
HX consists of L-SP and 20S proteasome



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

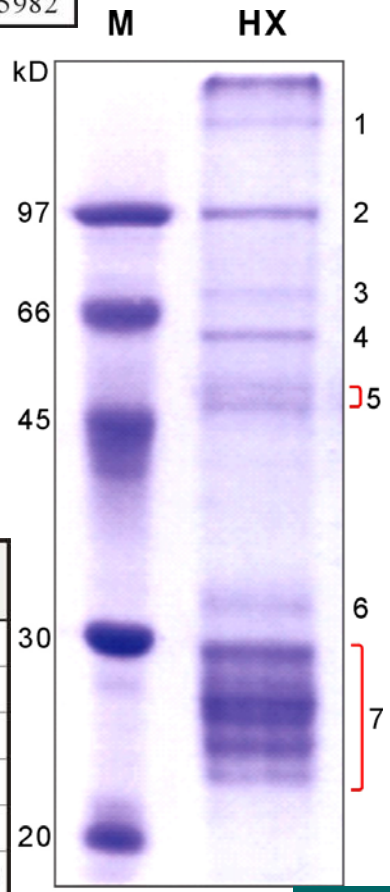


(L-SP)

HX

Native-PAGE → Band 1 → LC/MS/MS

SDS-PAGE → Band 1~7 → LC/MS/MS

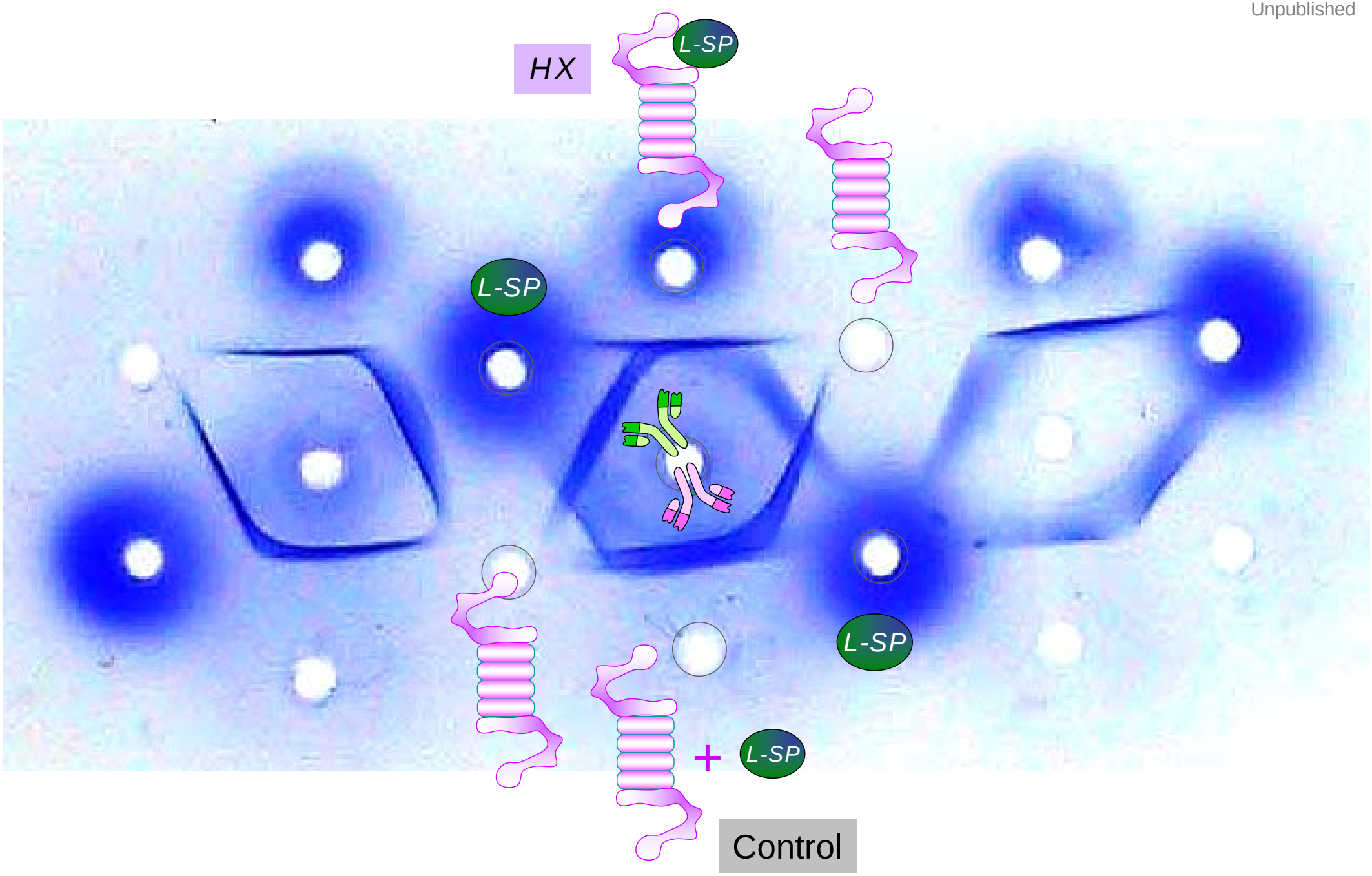


12.5 % SDS-PAGE
CBR Staining

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

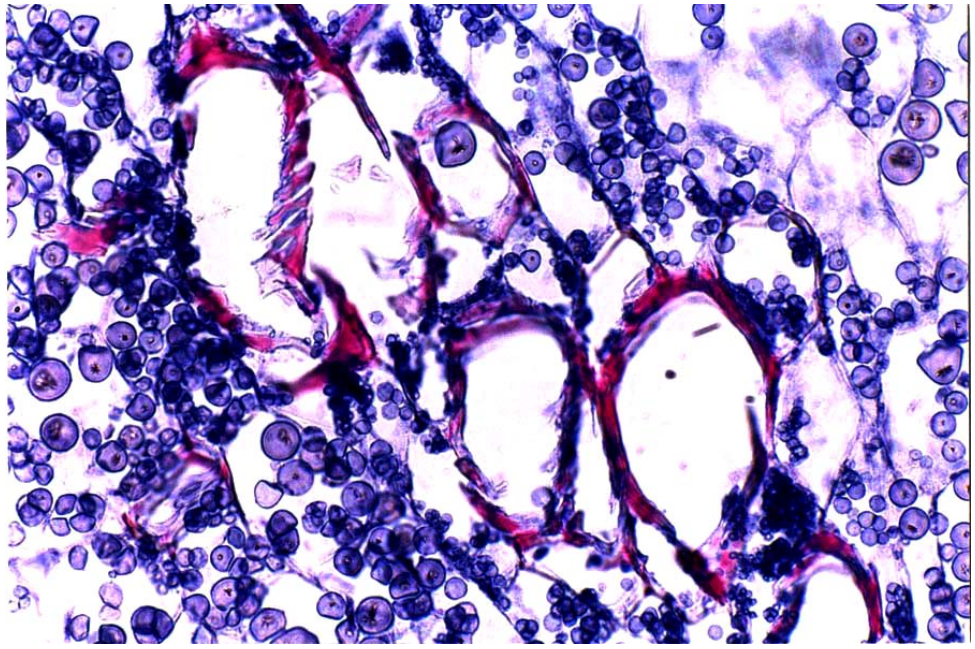
Unpublished

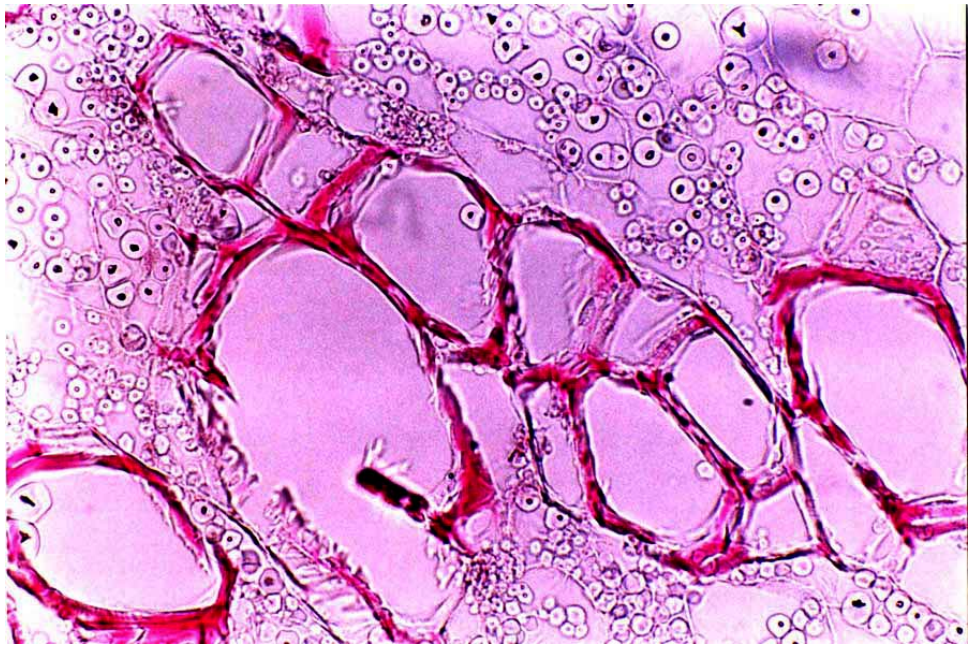
Immunostaining

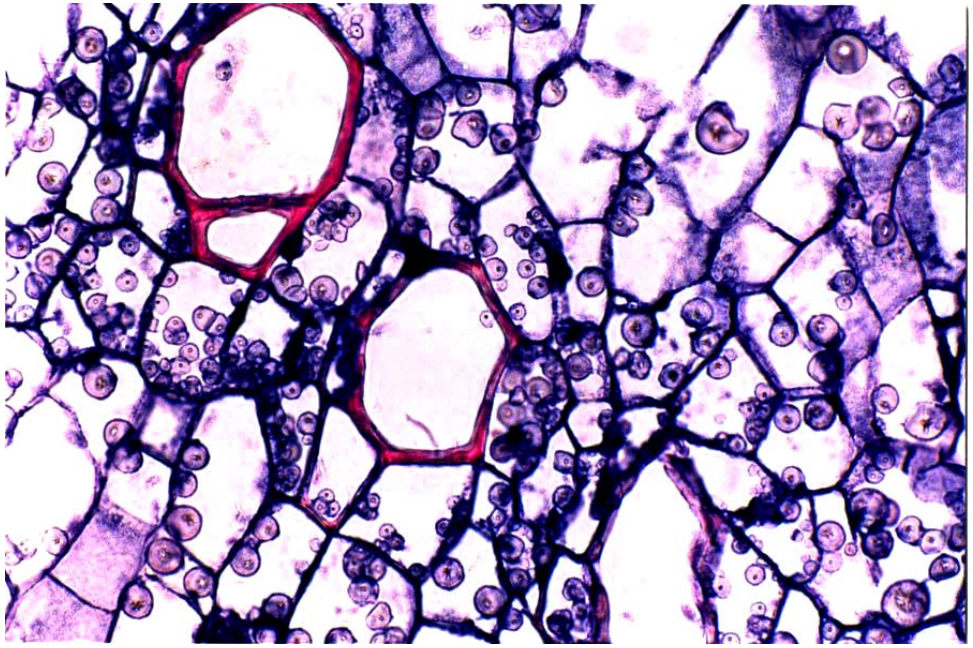
Control (no primary Ab)

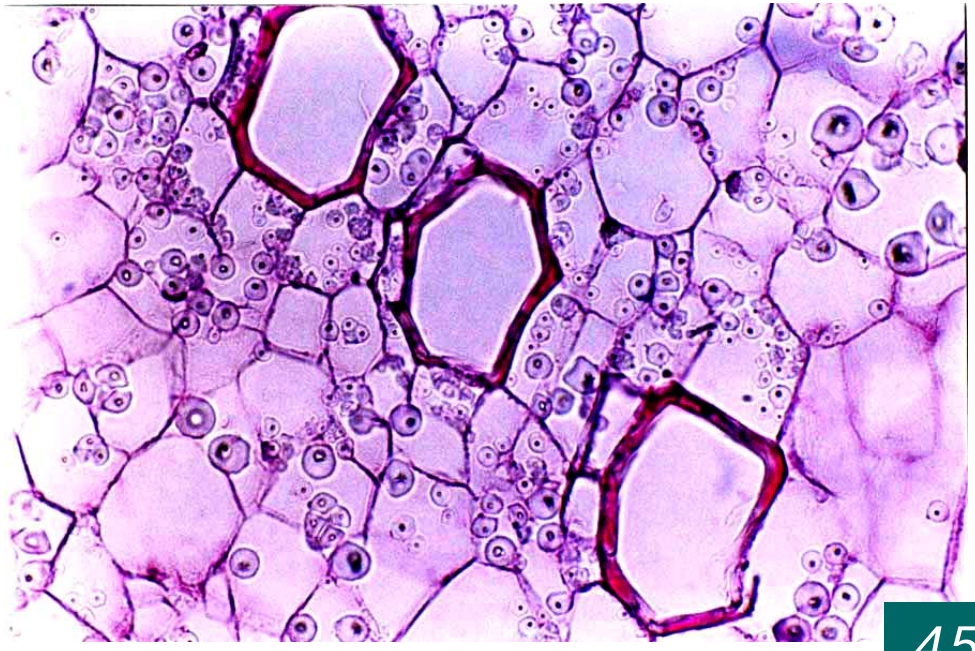
J3b (anti L-SP)

M71 (anti proteasome)

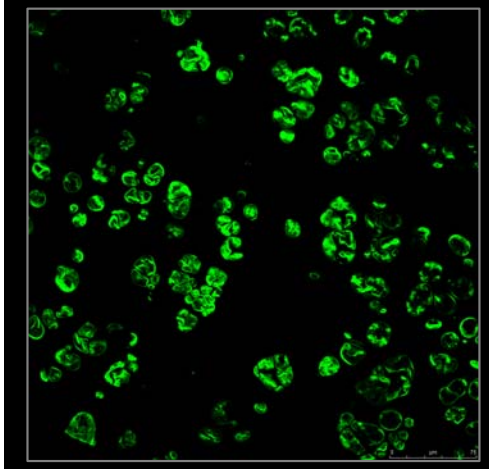




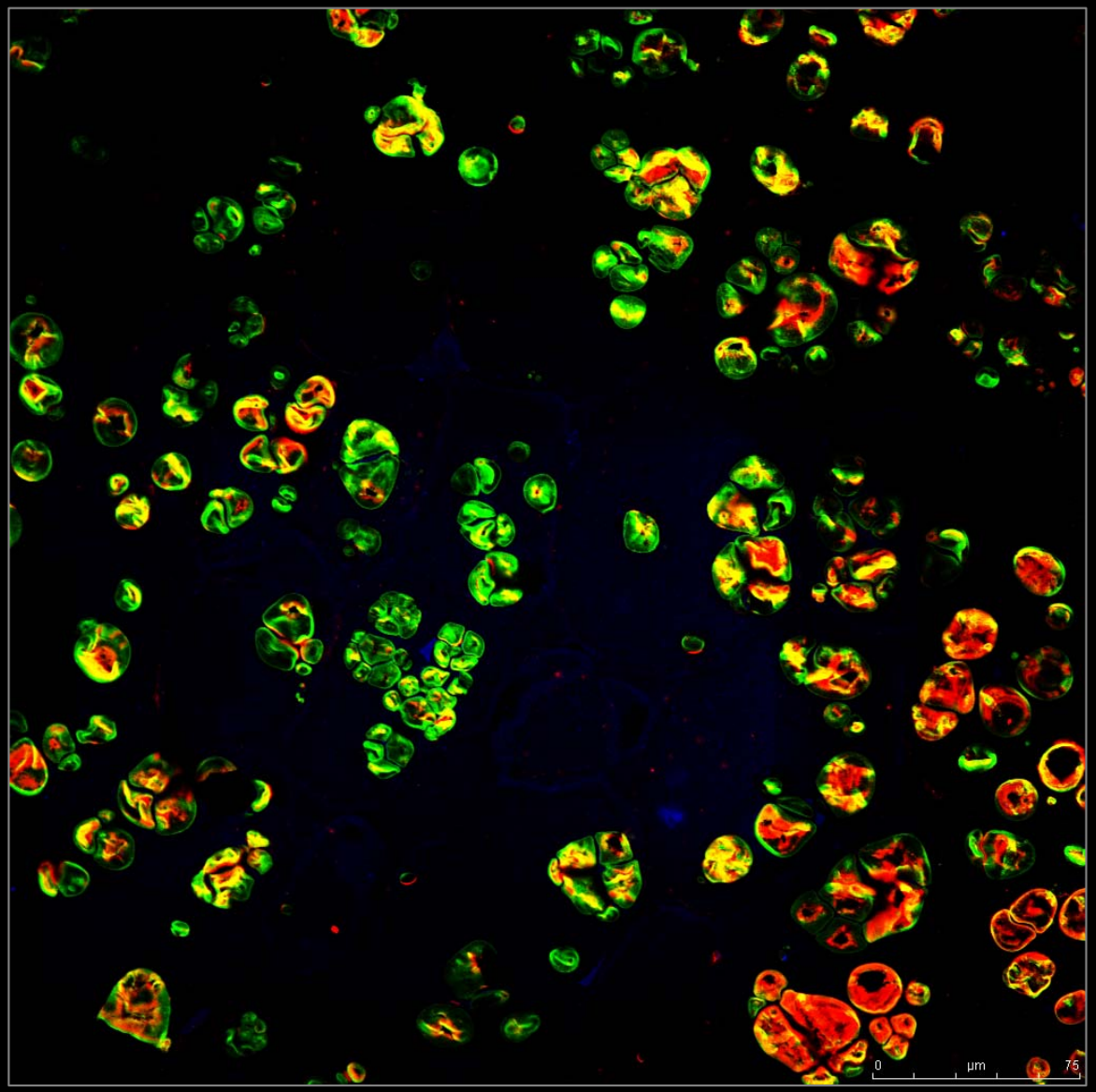


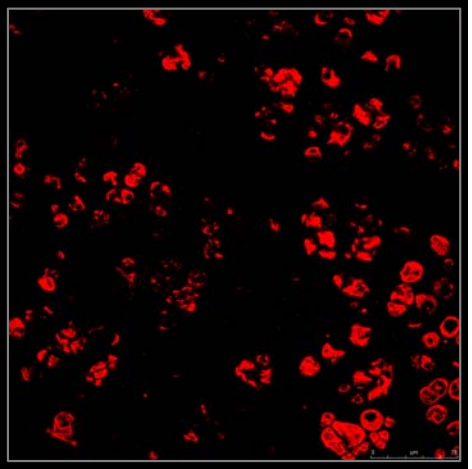


Merge

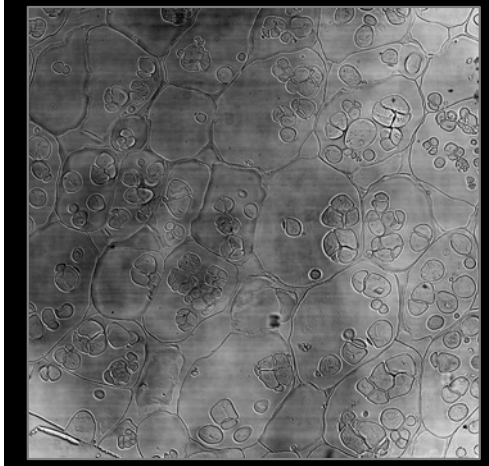


Anti-proteasome Ab (Cy3)

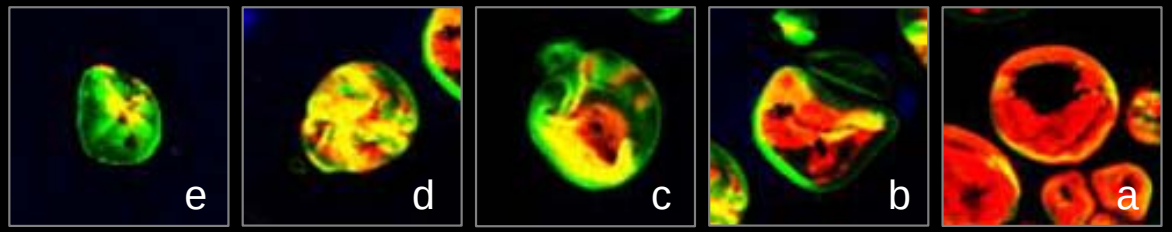


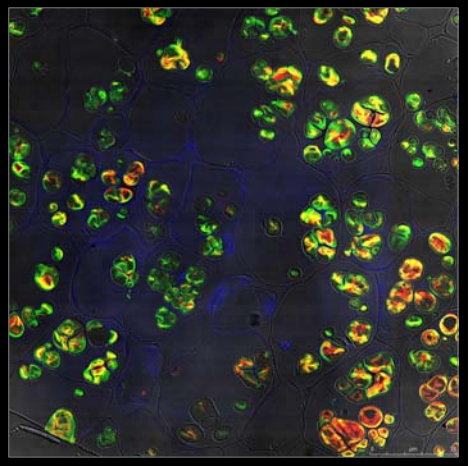


Anti-L-SP H7c (Cy5)



Light microscopy

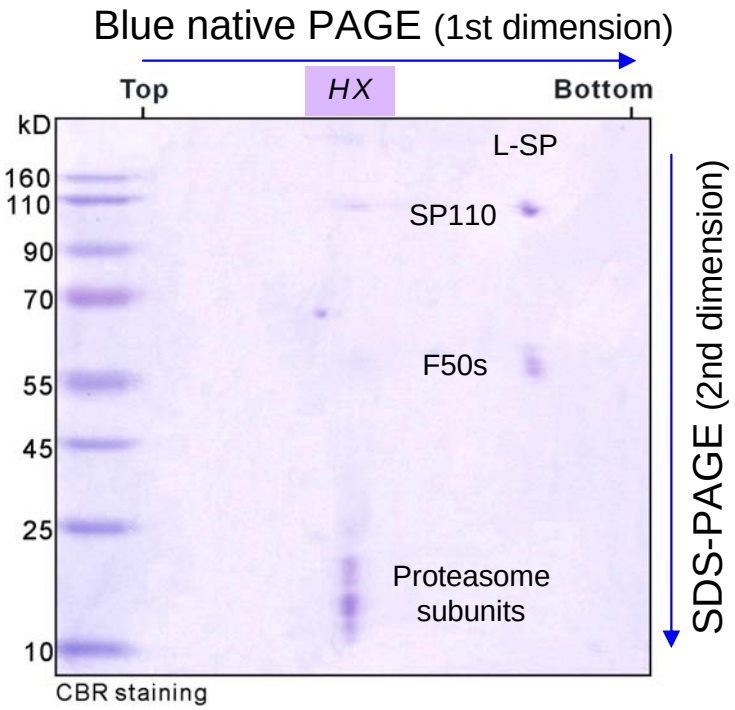


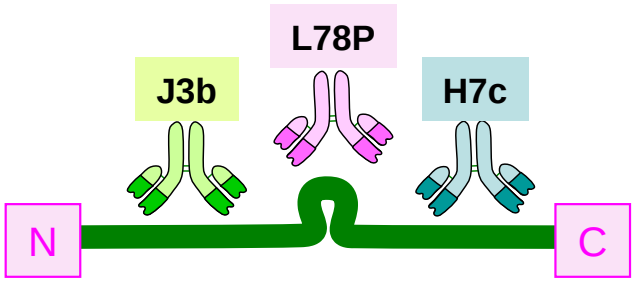


All merged

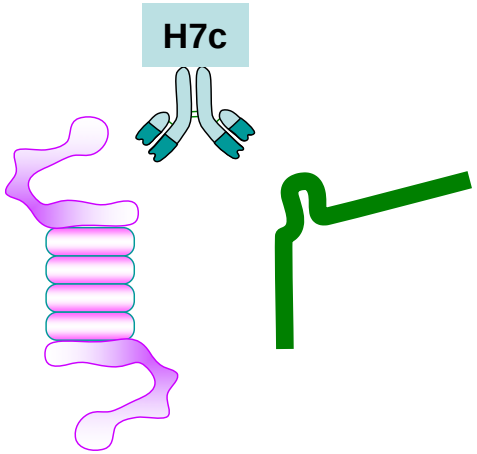
Blue-native 2D PAGE and immunostaining for HX

Unpublished

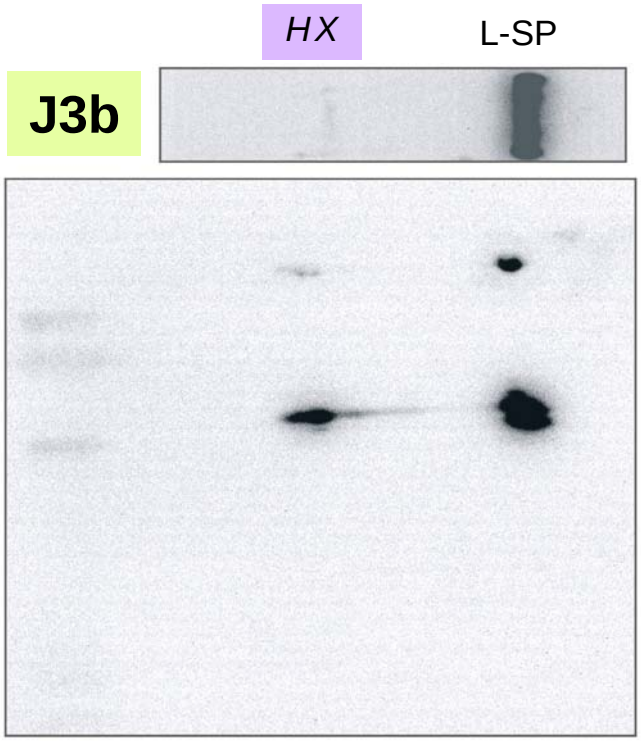


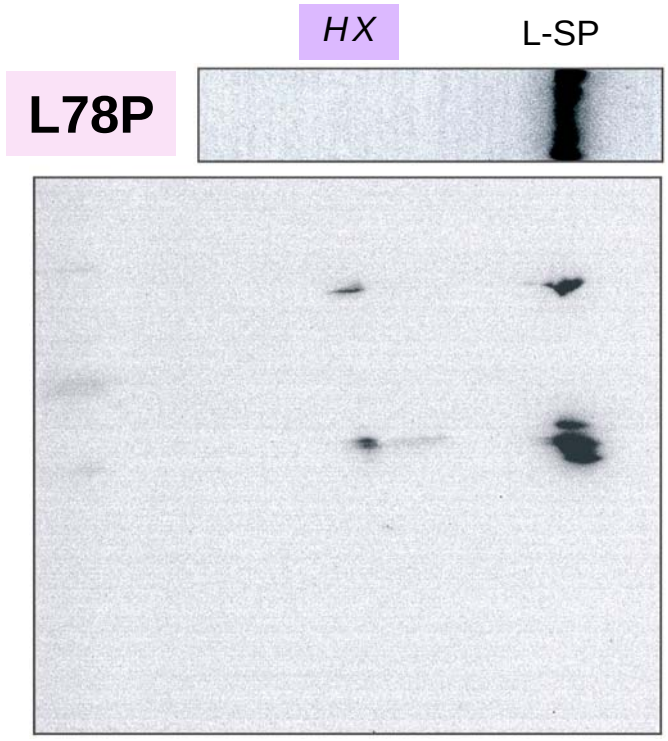


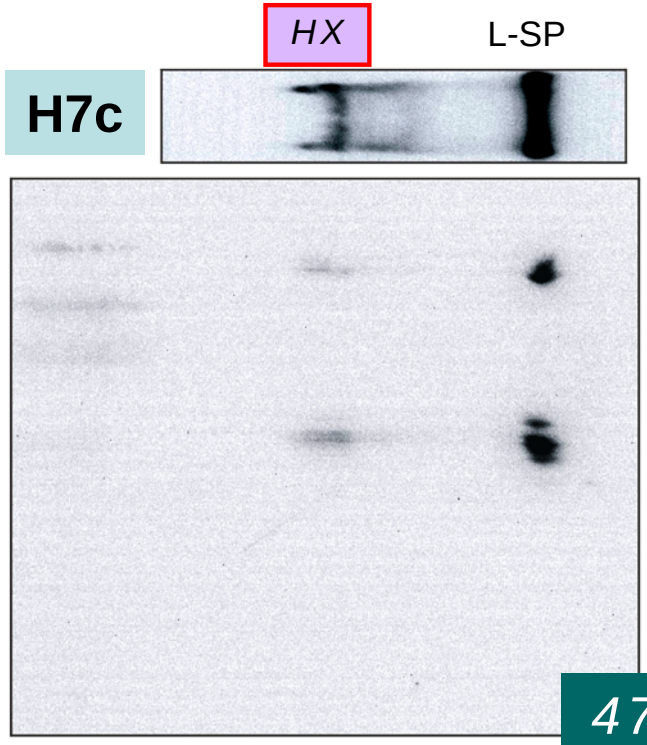
Only H7c could stain the HX band on the native PAGE



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



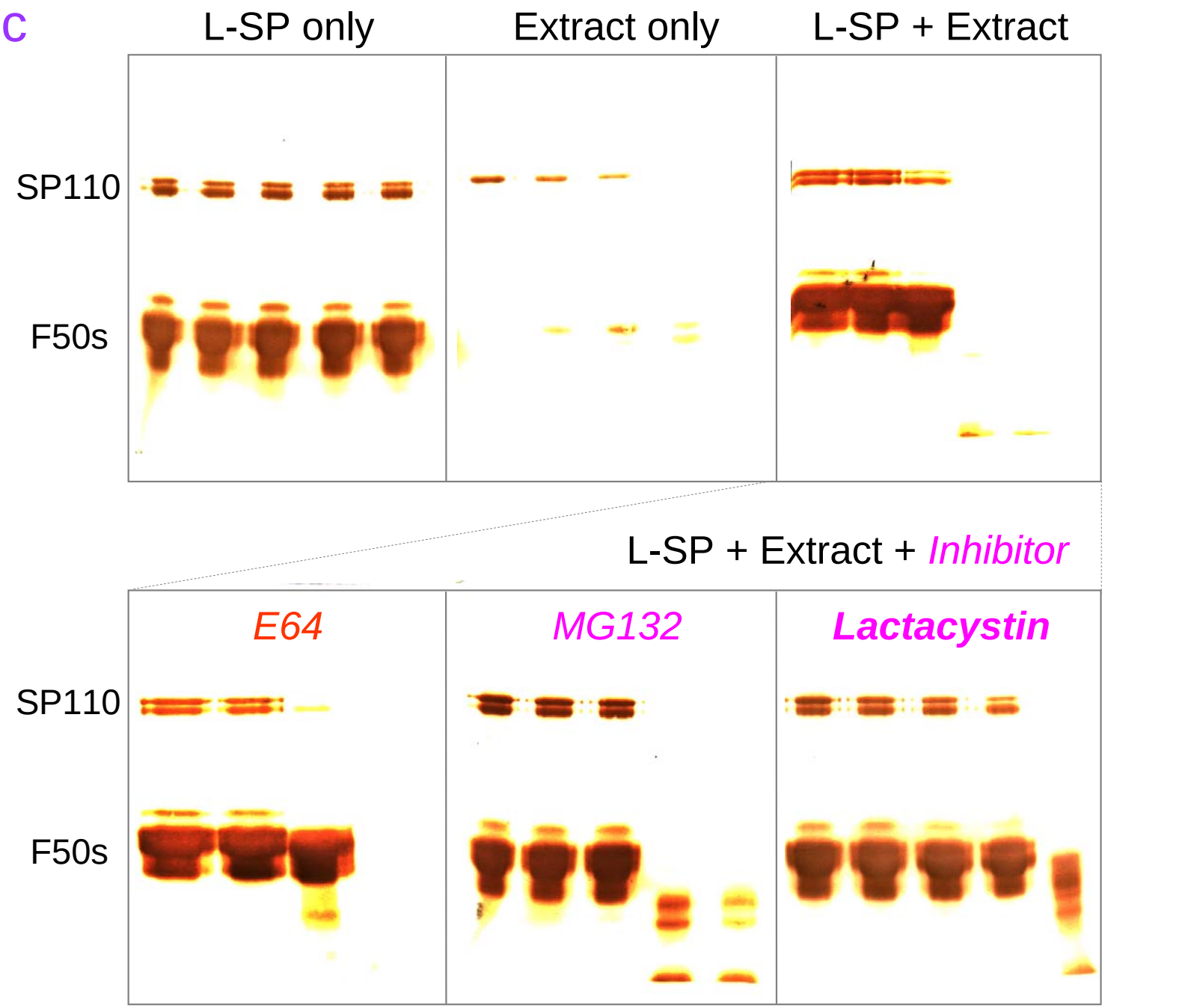




The degradation of L-SP is protected by proteasome inhibitor

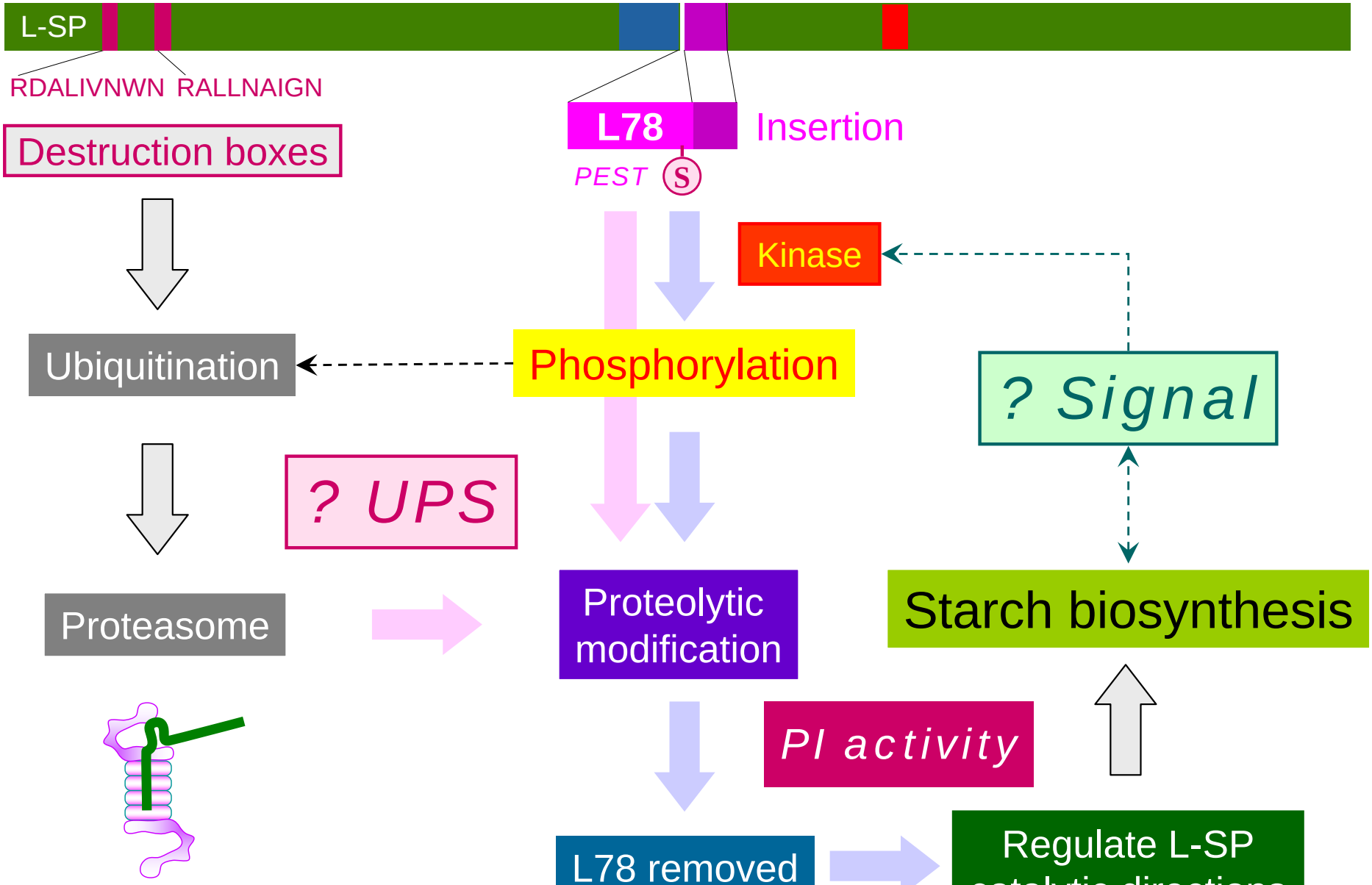
Unpublished

mAb H7c



10% SDS-PAGE

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