PERGAMON

Phytochemistry 61 (2002) 645–655

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Processing of *N*-glycans of two yellow lupin phosphohydrolases during seed maturation and dormancy

Mariusz Olczak, Wiesław Wątorek*

Wrocław University, Institute of Biochemistry and Molecular Biology, Tamka 2, 50-137 Wrocław, Poland

Received 16 April 2002; received in revised form 22 July 2002

Abstract

Acid phosphatase (AP) and diphosphonucleoside phosphatase/phosphodiesterase (PPD1) were purified from yellow lupin (*Lupinus luteus* L.) immature green seeds (40 days after blooming), dry seeds (40 days later) and dry seeds stored for 160 days. Both enzymes are known to differ in the type of *N*-glycosylation: the first has an *N*-glycosylation pattern typical for a vacuolar protein, while the second enzyme has a pattern typical for an extracellular or membrane-bound protein. *N*-Glycans were released from each of the enzyme preparations, fluorescence labeled, separated and identified by HPLC (GlycoSep N and GlycoSep H columns). Changes in the level of each *N*-glycan during seed maturation and dormancy were compared. The results show that *N*-glycan processing in the case of AP and PPD1—two proteins residing in the same plant organ, but possibly in different compartments—is not synchronized and performed not only in metabolically active maturing seeds, but also in metabolically inactive dormant seeds.

Keywords: *Lupinus luteus*; Papilionaceae; *N*-Glycan processing; Phosphohydrolases; Seed

1. Introduction

The last decade has seen great progress in research on the glycosylation of plant proteins (Lerouge et al., 1998). This is at least in part due to a rising interest in the expression of therapeutic glycoproteins in transgenic plants and the possible consequences of plant-specific glycosylation on a function of these proteins.

The major pathways of *N*-glycosylation are identical in plant, animal and fungal proteins; differences are found only in *N*-glycan processing in subcellular compartments (Sturm, 1995; Rayon et al., 1998). Plant complex *N*-glycans often terminate with *N*-acetylglucosamine and galactose (no sialic acid present as in the case of animals) and are fucosylated and xylosylated on proximal *N*-acetylglucosamine and β-mannose, respectively. Some complex-type plant *N*-glycans have additional α(1,4)-fucose and β(1,3)-galactose residues attached to the terminal *N*-acetylglucosamines, forming a sequence known as Lewis a (Le^a) antigen, usually found on mammalian cell-surface glycoconjugates. A characteristic feature of plant proteins is also the presence of paucimannosidic-type *N*-glycans with α(1,3)-fucose and/or a β(1,3)-galactose residue attached to the core $Man_{3–2}GlcNAc_2$ structures.

A strategy of plant *N*-glycosylation presented by recent reviewers (Sturm, 1995; Lerouge et al., 1998; Rayon et al., 1998) could be summarized as follows: *N*-glycan processing begins in the endoplasmatic reticulum (ER) by a removal of three glucoses and sometime one mannose (by a still unidentified plant ER-mannosidase) from $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor, resulting in a $Man_{8–9}GlcNAc_2$ structure. Further modifications are continued in the Golgi apparatus and start with the removal of one to four mannoses (by mannosidase I) leading to a high-mannose-type glycan $Man_5GlcNAc_2$. Addition of a β(1,2)-GlcNAc to the α(1,3)-mannose branch of the $Man_5GlcNAc_2$ opens the pathway to complex-type *N*-gly-

Abbreviations: 2-AB, 2-aminobenzamide; AP, acid phosphatase; PPD1, diphosphonucleoside phosphatase/phosphodiesterase; ER, endoplasmatic reticulum; Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; GU, glucose units; Le^a, Lewis a antigen

* Corresponding author. Tel.: +4871-3752-712; fax: +4871-3752-608.

E-mail address: watorek@bf.uni.wroc.pl (W. Wątorek).

0031-9422/02/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0031-9422(02)00360-6

cans. This step is followed by the action of mannosidase II, removing two mannoses, and by the addition of a β(1,2)- GlcNAc residue to the α(1,6)-mannose branch. The presence of at least one terminal *N*-acetylglucosamine allows for the subsequent attachment of β(1,2)-xylose to the core β-mannose and α(1,3)-fucose to the terminal β(1,4)- GlcNAc residue. As a result of these reactions, a paucimannosidic-type of *N*-glycan characteristic for plant vacuolar glycoproteins is formed. The final steps of plant complex-type *N*-glycans processing are terminal galactosylation and fucosylation leading to Le^a type structures.

Mature *N*-glycans undergo some trimming during transport and residency in their final destination. Terminal galactose, fucose and *N*-acetylglucosamine residues are removed from Lewis structures, resulting in paucimannosidic-type glycans. Finally, further trimming might release one mannose, fucose and/or xylose residues from these glycans.

A characteristic feature of plant *N*-glycosylation is the large heterogeneity among processed *N*-glycans. We have sequenced 8 different *N*-glycans in yellow lupin (*Lupinus luteus* L.) acid phosphatase (AP) and 24 different *N*-glycan structures in yellow lupin diphosphonucleotide phosphatase/phosphodiesterase (PPD1) (Olczak and Wątorek, 1998, 2000). In the present paper, two additional new glycan structure variants of the second enzyme are presented.

Most data on plant *N*-glycan biosynthesis and processing is based on the analysis of one specific glycoprotein (e.g. Hayashi et al., 1990; Sturm et al., 1987) or a total pool of *N*-glycans from a specific plant (e.g. Rayon et al., 1999; Kimura and Matsuo, 2000a; Makino et al., 2000). There is no comparative analysis of different glycoproteins from the same plant. Such a comparison would be very useful in elucidating some open questions about processing mechanisms and localization of glycoprotein processing enzymes (glycosyltransferases and glycosidases) in subcellular compartments and might indicate a possible function of carbohydrate moiety in plant glycoprotein targeting. For this reason we have decided to analyze and compare the *N*-glycosylation patterns of two differently located phosphatases during the maturation and dormancy of yellow lupin seeds.

2. Results and discussion

2.1. Purification of enzymes and separation of glycan pools

To compare *N*-glycosylation processes during seed maturation and dormancy, glycan pools were collected from AP and PPD1. Three batches of seeds (the first batch harvested one month after blooming, the second batch harvested 40 days later, and the third batch harvested at the same time as the second, but kept in the dark at +15 °C for an additional 160 days prior to freezing) frozen at −70 °C were processed without previous thawing and soaking. As a result, pure PPD1 and AP proteins were obtained (Fig. 1). Each enzyme preparation was fragmented subsequently with trypsin. Trypsin digests were further separated by reverse phase HPLC and collected peptides were deglycosylated enzymatically with *N*-glycosidase A. The yield and extent of enzymatic release of *N*-glycans were checked by a phenol method (Dubois et al., 1956) before and after deglycosylation. The yield was always higher than 90%. Protein denaturation and proteolytic fragmentation performed prior to deglycosylation made the preparation fully susceptible to endoglycosidase A activity. Glycan pools obtained in this way were fluorescence labeled, separated on a GlycoSep N column (Figs. 2 and 3) and identified by comparison of their retention times and GU values with AP and PPD1 reference glycans previously isolated and sequenced (Olczak and Wątorek, 1998, 2000) (Table 1). In the case of PPD1 glycans, an additional chromatography on a GlycoSep H column was necessary to separate glycans from peaks 3, 7 and 10 (Figs. 4, 5 and 6, respectively).

2.2. Structures of AP and PPD1 glycans

Structures of glycans AP and PPD1 were resolved previously in our laboratory by exoglycosidase sequencing and two-dimensional HPLC chromatography analysis (GlycoSep N and GlycoSep H columns) (Olczak and Wątorek, 1998, 2000), except for the two PPD1 glycans **3a** and **3d** from Fig. 4, which were discovered in maturing seeds and sequenced as below. Glycan **3a** was resistant to unspecific jack bean α-mannosidase. Appli-

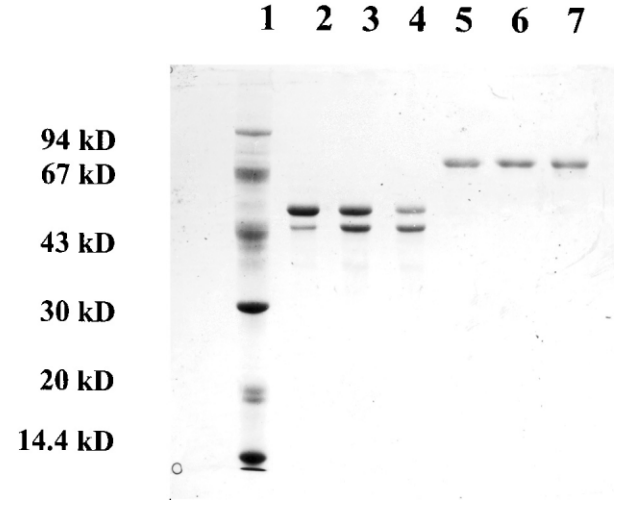


Fig. 1. SDS-PAGE of purified AP and PPD1. Line 1, protein molecular standards; lines 2, 3 and 4, monomers of AP from 0, 40 and 200 day preparations; lines 5, 6 and 7, PPD1 from 0, 40 and 200 day preparations.

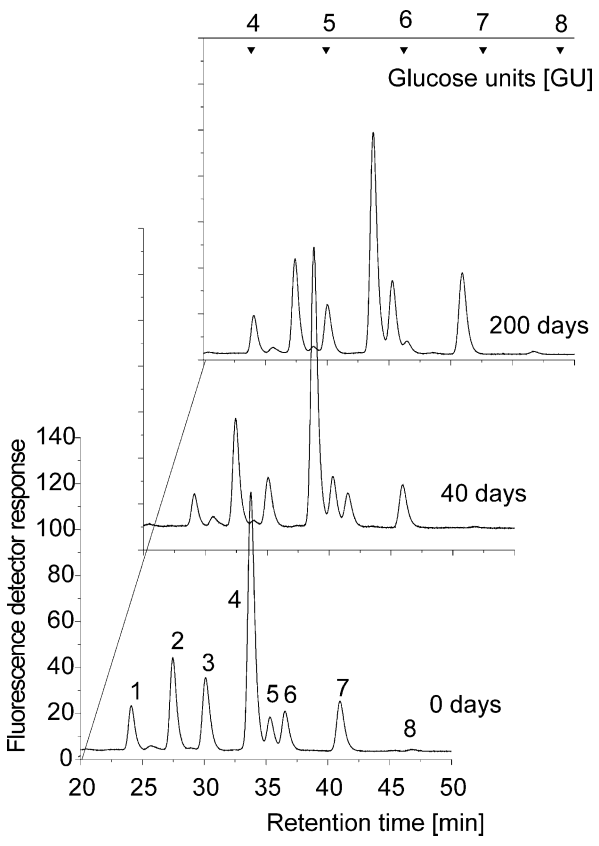


Fig. 2. Separation of fluorescence labeled *N*-glycans of AP on GlycoSep N column. Glycans were identified by comparison of their retention times and GU values with AP reference glycans sequenced previously (Olczak and Wątorek, 1998). 1, M3N2; 2, M3N2X; 3, M3N2F; 4, M3N2XF; 5, M5N2; 6, M3N2XFN; 7, M6N2; 8, M7N2-M9N2. Abbreviations refer to *N*-glycans listed in Table 1.

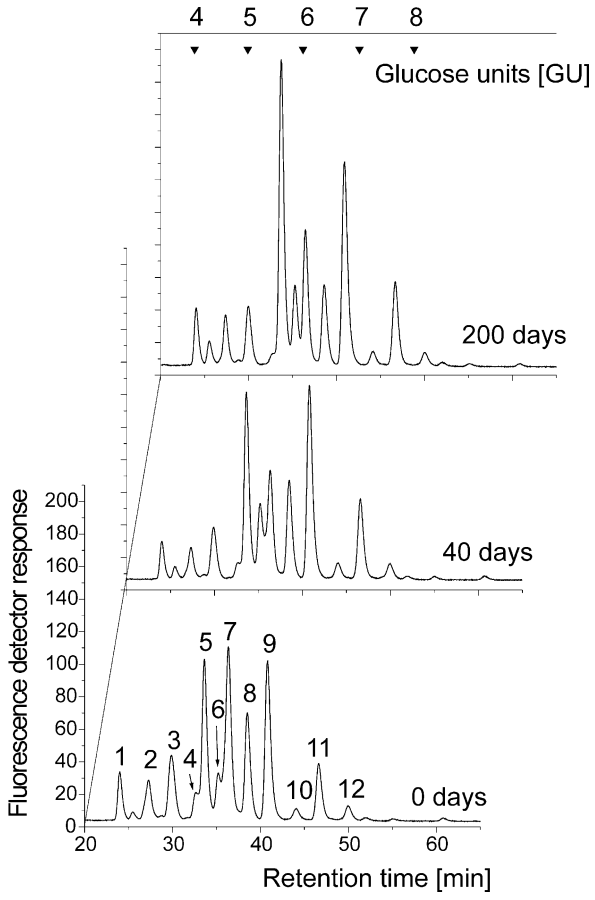


Fig. 3. Separation of fluorescence labeled *N*-glycans of PPD1 on GlycoSep N column. Glycans were identified by comparison of their retention times and GU values with PPD1 reference glycans sequenced previously (Olczak and Wątorek, 2000), except for two new glycans localized in peak 3 and sequenced in this paper. 1, M3N2; 2, M3N2X; 3, M3N2N2; M3N2F, M3N2XN (3) and M3N2XN(6); 4, M3N2XN2; 5, M3N2XF; 6, M5N2; 7, M3N2XFN(3) and M3N2XFN(6); 8, M3N2XFN2; 9, M6N2; 10, M3N2XFN2G(3) and M3N2XFN2G(6); 11, M7N2; 12, Le[a]. Abbreviations refer to *N*-glycans listed in Table 1.

cation of β(1,2)-hexosaminidase resulted in removal of two *N*-acetylglucosamine residues. Sequential digestion of the obtained product with unspecific α-mannosidase and β-mannosidase released two α-mannoses and one β-mannose residue, respectively, resulting in a chitobiose as a final product. One mannose residue was released after digestion of glycan **3d** with α(1,3)-mannosidase. Subsequent action by β(1,2)-hexosaminidase resulted in the removal of one *N*-acetylglucosamine. The resulting glycan after incubation with unspecific jack bean α-mannosidase/β-xylosidase mixture was converted to two products, one with α(1,6)-mannose removed and the second without α(1,6)-mannose and β(1,2)-xylose residues with hydrodynamic volume equal to mannochitobiose. The structures deduced from the results of the exoglycosidase sequencing are shown in Table 1.

2.3. Proteolytic processing of AP during seed maturation and dormancy

AP purified from dry seeds is composed of two homologous subunits differing in MW (44 and 50 kD). In our previous paper (Olczak et al., 1997) we have suggested that only one subunit in the AP dimer is proteolytically trimmed. PAGE of AP purified from seeds in different stages of maturation and dormancy clearly shows that both subunits undergo limited proteolysis and the amount of the 44 kD subunit increases with seed storage time (Fig. 1). Proteolysis occurs at the *N*-terminus (Olczak and Wątorek, 1998) and does not influence the *N*-glycosylation pattern because from the analysis of AP amino acid sequence it is known that the released *N*-terminal peptide/peptides have no potential *N*-glycosylation sites (Olczak and Wątorek, 1998; Olczak and Wątorek, unpublished). The composition of glycans is identical in both subunits (Olczak and Wątorek, 1998).

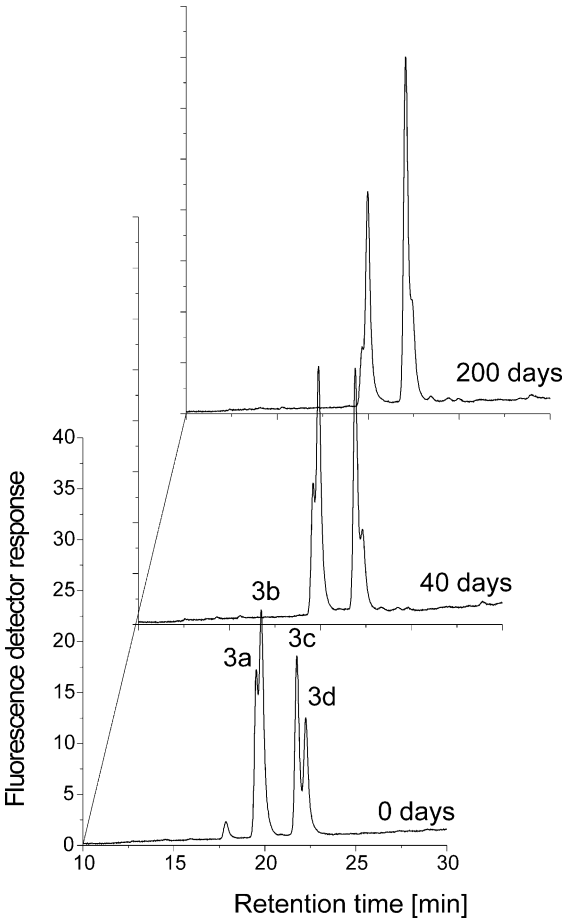


Fig. 4. Separation of PPD1 glycans from peak 3 (GlycoSep N column, Fig. 3) on GlycoSep H column. **3a**, M3N2N2; **3b**, M3N2XN(3); **3c**, M3N2F; **3d**, M3N2XN(6). Abbreviations refer to *N*-glycans listed in Table 1.

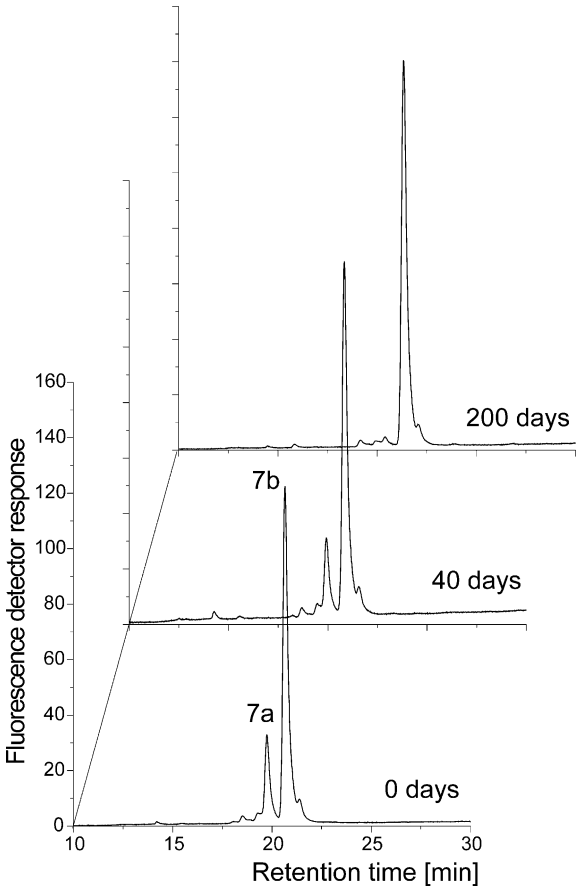


Fig. 5. Separation of PPD1 glycans from peak 7 (GlycoSep N column, Fig. 3) on GlycoSep H column. **7a**, M3N2XFN(3); **7b**, M3N2XFN(6). Abbreviations refer to *N*-glycans listed in Table 1.

2.4. Changes in N-glycosylation of AP and PPD1 during seed maturation and dormancy

The applied procedure allowed us to compare and analyze changes in the level of eight AP *N*-glycans and seventeen PPD1 *N*-glycans during yellow lupin seed maturation and dormancy from 40 to 200 days after blooming (Table 2).

The AP *N*-glycosylation pattern is typical for a plant vacuolar glycoprotein with paucimannosidic glycan M3N2XF (peak 2 from Fig. 2) as the major component. The most complex AP glycan M3N2XFN(3) (peak 6 from Fig. 3), a precursor of the paucimannosidic structure, disappears with time, showing that protein transport to vacuoles (protein bodies in seeds) occurs even during the dormancy period. It seems that AP transported to protein bodies is glycosylated with M3N2XFN(3) glycan. In the vacuolar compartment *N*-acetylhexosaminidase removes the terminal GlcNAc creating a paucimannosidic glycan.

The level of AP high-mannose M6N2 glycan (peak 7 from Fig. 2) decreases during the first 40 days of maturation, probably as a result of the prevalence of glycan trimming and modification over biosynthesis. A rise of AP high-mannose glycans after 200 days could be explained by the slow start of new glycan biosynthesis but the possibility of accumulation of these structures as the result of diminishing processing events cannot be excluded.

The cellular localization of PPD1 is unknown, but its *N*-glycosylation pattern and our unpublished data on amino acid sequence of this protein point to an extracellular localization. There are two major glycan components in PPD1: a paucimannosidic M3N2XF glycan (peak 5 from Fig. 3, probably a product of complex-type glycan degradation) and M6N2 high-mannose type glycan (peak 9 from Fig. 3), with six mannose units. A characteristic feature of PPD1 is also the presence of Lewis a (Le^a) type structures containing the Galβ(1,3)[Fucα(1,4)] GlcNAc epitope (peak 12 from Fig. 3) common for extracellular or membrane bound plant glycoproteins (Fitchette et al., 1999).

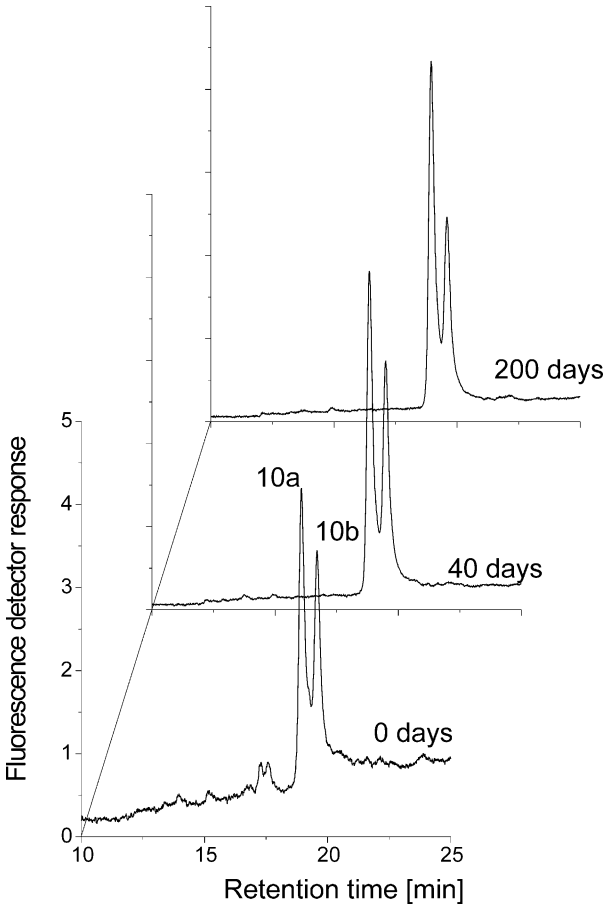


Fig. 6. Separation of PPD1 glycans from peak 10 (GlycoSep N column, Fig. 3) on GlycoSep H column. **10a**, M3N2XFN2G(3); **10b**, M3N2XFN2G(6). Abbreviations refer to *N*-glycans listed in Table 1.

M3N2XFN, a key intermediate of complex-type glycan biosynthesis, exists in two forms: as M3N2XFN(3) or M3N2XFN(6) with a terminal GlcNAc attached to either the Man (1,3) or Man (1,6) arm of the trimannosyl core, respectively. In PPD1 the N(3) form (peak 7a from Fig. 5) is depleted with time, while the level of the N(6) form (peak 7b from Fig. 5) goes down much slower, suggesting that M3N2XFN(3) is probably the major substrate for complex-type glycans and M3N2XFN(6) could be a slowly degraded intermediate or dead-end form.

The presence of terminal GlcNAc is a prerequisite for the action of fucosyl- and xylosyltransferases on M3N2N glycan (Johnson and Chrispeels, 1987). For this reason the paucimannosidic M3N2XF is not a precursor but rather the degradation product of M3N2XFN glycan. The amount of M3N2XF glycan increases in AP during the first forty days of seed maturation (Table 2). The increase is continued until 200 days in the case of PPD1 (Table 2). In AP after 200 days a decrease in the level of this glycan is observed together with an increase in glycosylation with a high mannose glycan M6N2 (Table 2). The increase of M3N2XF (peak 5 from Fig. 3) occurring in PPD1 during seed maturation and storage is probably caused by the fact that only a part of the M3N2XFN intermediate is used for complex-type glycan synthesis, while most of it is degraded to a paucimannosidic structure.

The presence of M3N2F glycan in AP and PPD1 (peak 3 from Fig. 2 and peak 3c from Fig. 4, respectively) is probably not caused by a xylose removal from M3N2XF because plant xylosidases are not able to release xylose residue from the fucosylated trimannosyl core (Tezuka et al., 1993). It could be the result of a fucosylation of M3N2N or M3N2N2 glycans. Plant fucosidases can remove a fucose residue from paucimannosidic glycans, leading to the M3N2X structure found in considerable amounts in AP (peak 2 from Fig. 2) and in much smaller quantities in PPD1 (peak 2 from Fig. 3).

Modifications leading to complex glycans M3N2XFN2G(3), M3N2XFN2G(6) and Le^a (peaks 10a and 10b from Fig. 6 and peak 12 from Fig. 3, respectively) are intensified in PPD1 during seed maturation and subsequently during the 160-day storage period a slow decrease in the level of these glycans is observed.

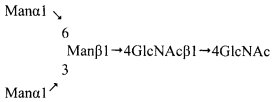
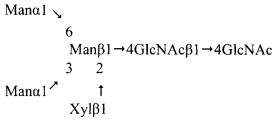
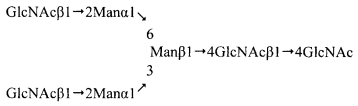
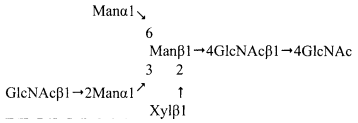
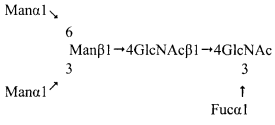
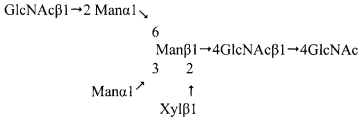
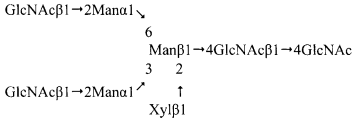
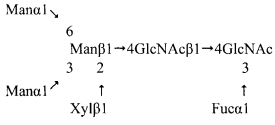
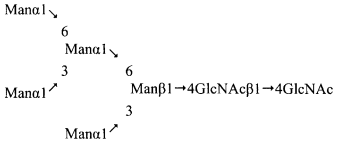
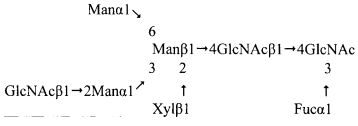
The level of high-mannose M5N2 and M7N2 glycans increases in PPD1 during seed maturation, showing the existence of active *N*-glycosylation processes. Later, during the dormancy period, the level of these glycans slowly drops as a result of a modification of existing glycans. A relatively high level of M6N2 glycan (peak 9 from Fig. 3; 20% of a total PPD1 glycan pool) suggests that probably one of the six *N*-glycosylation sites in PPD1 is (because of steric reasons) inaccessible for further processing, preserving the M6 high-mannose structure as a final product.

Analysis of changes in the AP and PPD1 glycan pools shows that *N*-glycosylation processes are not synchronized. The biosynthesis of new glycans starts during seed storage earlier in AP. One reason for this could be the need for a mobilization of phosphate reserves necessary for future germination. A processing of glycans in seed protein bodies was observed also in the red kidney bean (Stahl et al., 1994).

Differences in the level of glycan heterogeneity (8 and 26 different structures, respectively) (Olczak and Wątorek, 1998, 2000 and this paper) might be the result of different processing routes or might be related to the number of potential *N*-glycosylation sites: three in case of AP (Olczak and Wątorek, unpublished data) and six in PPD1 (Olczak and Olczak, 2002).

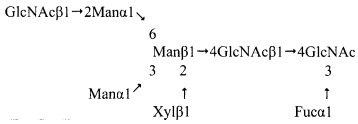
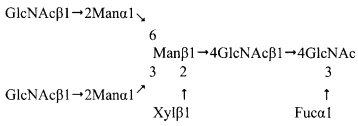
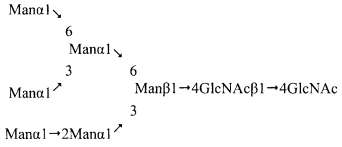
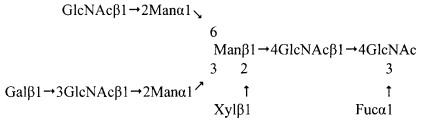
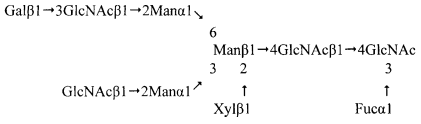
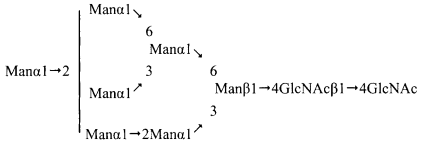
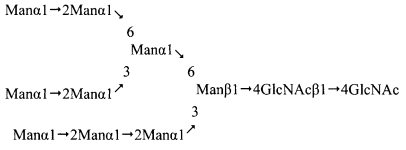
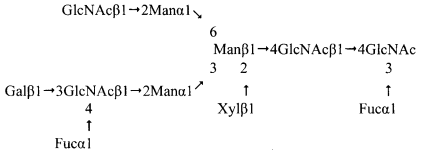
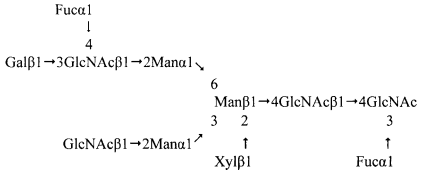
Continuous modification of glycan pools during seed maturation and dormancy could work as a sort of "biological clock," preparing a proper substrate for plant endoglucosidases to release free *N*-glycans necessary as regulating factors in developing seeds (Kimura and Matsuo, 2000b).

Table 1
Structures and abbreviations of AP and PPD1 glycans determined previously (Olczak and Wątorek, 1998; 2000) and in this paper

M3N2 PPD1 peak 3 (Fig. 3) AP peak 3 (Fig. 2)	
M3N2X AP peak 2 (Fig. 2) PPD1 peak 2 (Fig. 3)	
M3N2N2[a] PPD1 peak 3a (Fig. 4)	
M3N2XN(3) PPD1 peak 3b (Fig. 4)	
M3N2F AP peak 3 (Fig. 2) PPD1 peak 3c (Fig. 4)	
M3N2XN(6)[b] PPD1 peak 3d (Fig. 4)	
M3N2XN2 PPD1 peak 4 (Fig. 3)	
M3N2XF AP peak 4 (Fig. 2) PPD1 peak 5 (Fig. 3)	
M5N2 AP peak 5 (Fig. 2) PPD1 peak 6 (Fig. 3)	
M3N2XFN(3) AP peak 6 (Fig. 2) PPD1 peak 7a (Fig. 5)	

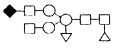
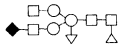
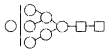
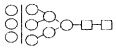
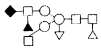
(*continued on next page*)

Table 1 (*continued*)

M3N2XFN(6) PPD1 peak 7b (Fig. 5)	
M3N2XFN2 PPD1 peak 8 (Fig. 3)	
M6N2 AP peak 7 (Fig. 2) PPD1 peak 9 (Fig. 3)	
M3N2XFN2G(3) PPD1 peak 10a (Fig. 6)	
M3N2XFN2G(6) PPD1 peak 10b (Fig. 6)	
M7N2 AP peak 8 (Fig. 2) PPD1 peak 11 (Fig. 3)	
M9N2 AP peak 8 (Fig. 2)	
Lewis a PPD1 peak 12 (Fig. 3)	
Lewis a PPD1 peak 12 (Fig. 3)	

[a] PPD1 glycan **3a** sequenced in this paper.
[b] PPD1 glycan **3d** sequenced in this paper.

Table 2
Changes in AP and PPD1 *N*-glycan content during yellow lupin seed maturation and dormancy

Symbols	Structure	% Of total carbohydrate		
		Time (days)	PPD1	AP
M3N2		0	4.2	6.6
		40	3.2	4.6
		200	4.2	5.8
M3N2X		0	4.4	14.6
		40	3.2	16.9
		200	3.9	15.5
M3N2N2		0	1.5	
		40	0.8	
		200	0.3	
M3N2XN(3)		0	2.7	
		40	2.3	
		200	1.9	
M3N2F		0	7.9	12.8
		40	5.9	7.7
		200	5.6	8.7
M3N2XN(6)		0	1.5	
		40	0.8	
		200	0.7	
M3N2XN2		0	2.5	
		40	1.3	
		200	0.8	
M3N2XF		0	16.5	43.5
		40	19.1	46.7
		200	26.5	37.6
M5N2		0	4.1	5.6
		40	8.1	8.9
		200	7.2	12.8
M3N2XFN(3)		0	4.0	7.0
		40	1.9	5.9
		200	0	2.1
M3N2XFN(6)		0	17.1	
		40	10.8	
		200	12.3	
M3N2XFN2		0	11.2	
		40	10.7	
		200	7.4	
M6N2		0	17.9	9.3
		40	22.0	8.8
		200	20.0	15.4
M3N2XFN2G(3)		0	1.2	
		40	1.7	
		200	1.3	
M3N2XFN2G(6)		0	0.6	
		40	0.8	
		200	0.5	
M7N2		0	6.2	
		40	9.0	
		200	7.9	
M7N2-M9N2[a]		0		0.1
		40		0.1
		200		0.6
Lewis a		0	2.4	
		40	2.8	
		200	1.8	

[a] AP only

□ *N*-Acetylglucosamine △ Fucose α 1,3 linked ▽ Xylose
○ Mannose ▲ Fucose α 1,4 linked ◆ Galactose

3. Experimental

3.1. Reagents

Formic acid and ammonia (aristar grade) for HPLC separation were from BDH, acetonitrile (gradient grade) from Roth, TFA (sequencing grade) from Fluka. All other chemicals were of the highest purity available from Sigma.

3.2. Plant material

Yellow lupin (*Lupinus luteus* L.) seeds were obtained from Centrala Nasienna in Poznan (Poland). Plants were grown outdoors. One month after blooming, a third of all pods (still green and soft) were collected (time 0) and frozen at −70 °C. Forty days later, the rest of the pods (fully developed and starting to break) were harvested. Half of this harvest was immediately frozen at −70 °C, while the rest were kept at +15 °C for 160 days before being frozen at −70 °C for one day before further handling.

Plant material was from one harvest, but *N*-glycans separations, labeling and profiling were repeated several times, always with very similar (quantitatively and qualitatively) results.

3.3. Enzyme purification

AP and PPD1 were purified separately from the each batch of seeds according to procedures described by the authors previously (Olczak et al., 1997, 2000), but modified in the first step. Instead of overnight soaking in distilled water, frozen immature seeds (0 time preparation) were homogenized directly in extraction buffer. Frozen dry mature seeds (40 and 200 days preparations) were pulverized and subsequently homogenized in extraction buffer. These modifications were introduced to prevent a mobilization of metabolic processes (including new *N*-glycosylation) induced by soaking.

3.4. Protein estimation

The concentration of purified enzymes was determined using absorbance coefficient $A^{280} = 23.6$ and 29.1 for AP and PPD1, respectively (Olczak et al., 1997, 2000).

3.5. Polyacrylamide gel electrophoresis (PAGE)

The homogeneity of purified enzymes was confirmed by SDS-PAGE (Laemmli, 1970) in 10% gels. Molecular mass standards (Pharmacia) were: phosphorylase *b* (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20 kD) and bovine milk α-lactalbumin (14.4 kD). Proteins were stained with Coomassie blue R-250.

3.6. N-Glycan pools preparation

The purified proteins were denatured in 8 M urea and after dilution (to obtain 2 M urea concentration) reduced at 55 °C with DTT (5 mM). Subsequently, sulfhydryl groups were blocked with iodoacetic acid. Unfolded polypeptides were cleaved with trypsin (1:25 w/w). The total peptide pool obtained after HPLC on reverse phase Eurosil Bioselect 300 C_{18} (5 μm) 250×4 mm (Knauer) column was deglycosylated with *N*-glycosidase A (Boehringer) (24 h, 37 °C) in 100 mM citrate/phosphate buffer, pH 5.0. The released *N*-glycans were purified on SPE GlycoClean H columns (Oxford GlycoSystems) and fluorescence labeled with 2-aminobenzamide (2AB) (Bigge et al., 1995).

3.7. Chromatographic separation of labeled N-glycans

Oligosaccharide fractionation was performed by HPLC on a Knauer apparatus with a Shimadzu RF-551 fluorescence detector. The 2-AB glycan derivatives were separated on a normal-phase amide column (GlycoSep N, 250×4.6 mm, Oxford GlycoSciences) with fluorescence detection (λ_{max} excitation = 330 nm and λ_{max} emission = 420 nm). A gradient was constructed with a low salt solvent system previously described (Guile et al., 1996). The total run time was 110 min and the column temperature was maintained at 30 °C. Partially hydrolyzed dextran (Oxford GlycoSciences), labeled with 2-AB, was used as an external standard. The values of the elution positions of separated AB-oligosaccharides were expressed in glucose units (GU) in reference to the AB-isomaltosooligosaccharides from dextran hydrolyzate. Fractions corresponding to fluorescence peaks were collected, dried, redissolved in a minimal volume (up to 6 μl) of water and injected onto a GlycoSep H column (150×4 mm, Oxford GlycoSciences). Glycans were eluted with a 10–30% acetonitrile gradient containing 0.1% TFA, following manufacturer protocol. Homogenous, desalted peaks were collected and dried. The amount of glycan in each peak was calculated after integration as a percent of a total glycan peaks area. Separated glycans were identified by comparison of their GU values (GlycoSep N column) and their retention times (GlycoSep H column) with AP and PPD1 glycans identified in previous papers (Olczak and Wątorek, 1998, 2000).

3.8. Exoglycosidase sequencing

Jack bean α-mannosidase specific towards α(1–2,3,6)-glycosidic bonds (10 U/ml), β-mannosidase from *Helix pomatia* specific towards β(1,4)-glycosidic bond (2.5 U/ml) and β-*N*-acetylhexosaminidase from *Streptococcus*

pneumoniae specific towards β(1,2)-glycosidic bond (10 mU/ml) were from Oxford GlycoSciences, α-mannosidase from *Xanthomonas manihotis* specific towards α(1–2,3)-glycosidic bonds (20 mU/ml) was from New England Biolabs. β-Xylosidase necessary for sequencing is not available commercially, but the jack bean α-mannosidase used in this study contains 0.003% β-xylosidase (very stable and active). β(1,2)-Xylose was completely removed after prolonged incubation (72 h) with this enzyme mixture. Shorter incubation times resulted in the partial release of xylose and two digestion products (xylose containing and xylose depleted). Comparative analysis of these products provided xylose location in the glycan structures. Before glycosidase treatment, purified and fluorescence-labeled glycans (0.08–1 nmol of each) were dissolved in 100 mM phosphate/citrate buffer pH 4.5 containing 100 mM NaCl and 0.2 mM $ZnCl_2$. The first exoglycosidase was added to obtain a final concentration as described above and a total volume of 15–25 μl. The incubation buffer, reaction vials, and pipette tips were autoclaved before the experiment. After 24–48 h of incubation at 37 °C, the reaction was stopped by filtration through a 5 kD membrane (Ultrafree MC, Millipore). The membrane was rinsed twice with 100 μl of water. Both filtrates were combined. A small aliquot from the filtrate (usually less than 1/8 of total volume) was dried on a rotary evaporator, redissolved in 7 μl of 70% acetonitrile in water (v/v) and injected onto a GlycoSep N column. The GU values of glycans were determined as described above. The rest of the filtrate was dried, dissolved in water and the next exoglycosidase added to obtain the required concentration of the enzyme in 15–25 μl of total volume.

In case of glycans making less than 1% of total glycan pool, the whole volume of the filtrate was dried and separated on GlycoSep N column. Fractions containing glycan peaks were collected, dried and dissolved in 15–25 μl of the incubation buffer and the next enzyme was added. The same procedure was performed prior to hydrolysis with α(1–3) mannosidase, when a different incubation buffer (100 mM citrate-phosphate pH 6.0 with 5 mM $CaCl_2$ and 0.1 mg/ml BSA) was used.

Acknowledgements

This work was supported by the State Committee for Scientific Research (KBN) through a research grant 6P04C 087 20.

References

Bigge, J.C., Patel, T.P, Bruce, J.A., Goulding, P.N., Charles, S.M., Parekh, R.B., 1995. Non-selective and efficient fluorescent labeling of glycans using 2-aminobenzamide and anthranilic acid. Anal. Biochem. 230, 229–238.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugar and related substances. Anal. Chem. 28, 350–356.

Fitchette, A.C., Cabanes-Macheteau, M., Marvin, L., Martin, B., Satiat-Jeunemaitre, B., Gomord, V., Crooks, K., Lerouge, P., Faye, L., Hawes, C., 1999. Biosynthesis and immunolocalization of Lewis a-containing *N*-glycans in the plant cell. Plant Physiol. 121, 333–343.

Guille, G.R., Rudd, P.M., Wing, D.R., Prime, S.B., Dwek, R.A., 1996. A rapid high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles. Anal. Biochem. 240, 210–226.

Hayashi, M., Tsuru, A., Mitsui, T., Takahashi, N., Hanzawa, H., Arata, Y., Akazawa, T., 1990. Structure and biosynthesis of the xylose-containing carbohydrate moiety of rice α-amylase. Eur. J. Biochem. 191, 287–295.

Johnson, K.D., Chrispeels, M.J., 1987. Substrate specificities of *N*-acetyglucosaminyl-, fucosyl- and xylosyltransferases that modify glycoproteins in the Golgi apparatus of bean cotyledons. Plant Physiol. 84, 1301–1308.

Kimura, Y., Matsuo, S., 2000a. Changes in *N*-linked oligosaccharides during seed development of *Ginkgo biloba*. Biosci. Biotechnol. Biochem. 64, 562–568.

Kimura, Y., Matsuo, S., 2000b. Free *N*-glycans already occur at an early stage of seed development. J. Biochem. 127, 1013–1019.

Laemmli, U., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lerouge, P., Cabanes-Macheteau, M., Rayon, C., Fischette-Laine, A.C., Gomord, V., Faye, L., 1998. *N*-Glycoprotein biosynthesis in plants: recent developments and future trends. Plant Mol. Biol. 38, 31–48.

Makino, Y., Shimazaki, A., Omichi, K., Odani, S., Hase, S., 2000. Processing pathway deduced from the structures of *N*-glycans in *Carica papaya*. J. Biochem. 127, 1121–1126.

Olczak, M., Olczak, T., 2002. Diphosphonucleotide phosphatase/phosphodiesterase from yellow lupin (*Lupinus luteus* L.) belongs to a novel group of specific metallophosphatases. FEBS Lett. 519, 159–163.

Olczak, M., Wątorek, W., Morawiecka, B., 1997. Purification and characterization of acid phosphatase from yellow lupin (*Lupinus luteus*) seeds. Biochim. Biophys. Acta 1341, 14–25.

Olczak, M., Wątorek, W., 1998. Oligosaccharide and polypeptide homology of lupin (*Lupinus luteus* L.) acid phosphatase subunits. Arch. Biochem. Biophys. 360, 85–92.

Olczak, M., Kobiałka, M., Wątorek, W., 2000. Characterization of diphosphonucleotide phosphatase/phosphodiesterase from yellow lupin (*Lupinus luteus* L.) seeds. Biochim. Biophys. Acta. 1478, 239–247.

Olczak, M., Wątorek, W., 2000. Structural analysis of *N*-glycans from yellow lupin (*Lupinus luteus*) seed diphosphonucleotide phosphatase/phosphodiesterase. Biochim. Biophys. Acta. 1523, 236–245.

Rayon, C., Cabanes-Macheteau, M., Loutelier-Bourhis, C., Salliot-Maire, I., Lemoine, J., Reite, W.D., Lerouge, P., Faye, L., 1999. Characterization of *N*-glycans from *Arabidopsis*. Application to a fucose-deficient mutant. Plant Physiol. 119, 725–733.

Rayon, C., Lerouge, P., Faye, L., 1998. The protein *N*-glycosylation in plants. J. Exp. Bot. 49, 1463–1472.

Stahl, B., Klabunde, T., Witzel, H., Krebs, B., Steup, M., Karas, M., Hillenkamp, F., 1994. The oligosaccharide of the Fe (III)–Zn (II) purple acid phosphatase of the red kidney bean. Determination of the structure by a combination of matrix-assisted laser desorption/ionization mass spectrometry and selective enzymic degradation. Eur. J. Biochem. 220, 321–330.

Sturm, A., 1995. Glycosylation of plant proteins. In: Montreuil, J.,

Vliegenthart, J.F.G., Schachter, H. (Eds.), Glycoproteins. Elsevier, Amsterdam, pp. 521–541.

Sturm, A., Van Kuik, J.A., Vliegenthart, J.F.G., Chrispeels, M.J., 1987. Structure, position, and biosynthesis of the high mannose and the complex oligosaccharide side chains of the bean storage protein phaseolin. J. Biol. Chem. 252, 13392–13403.

Tezuka, K., Hayashi, M., Ishihara, H., Nishimura, M., Onozaki, K., Takahashi, N., 1993. Purification and substrate specificity of β-xylosidase from sycamore cell (*Acer pseudoplatanus* L.): application for structural analysis of xylose-containing *N*-linked oligosaccharides. Anal. Biochem. 211, 205–209.