The activity of acid phosphatase and the level of storage proteins during the early stages of germination of *Lupinus luteus* L. and *Lupinus angustifolius* L. seeds

MARIUSZ OLCZAK, EWA NIZIOŁ, WIESŁAWA WIDŁAK,
BRONISŁAWA MORAWIECKA

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

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Abstract

Changes in the level of protein and in the activity of acid phosphatase in seeds of *Lupinus luteus* L. and *Lupinus angustifolius* L. during storage and germination were studied. The protein level decreased during germination, mainly as the result of a fall in the concentration of β -conglutin. After an initial decrease in the activity of acid phosphatase activity during the first five days of germination, the activity of this enzyme began to rise with the appearance of two new molecular forms. No significant effect of gibberelin A₃ on the specific activity of acid phosphatase was observed.

Key words: acid phosphatase, germination, lupin, storage protein, phytohormones

INTRODUCTION

The main storage material in the seeds of some dicotyledons consists of proteins which make up the pool of reduced nitrogen (J o u b e r t 1955a, b). In the seeds of all of the varieties of lupin, the main storage proteins are conglutins which belong to the class of globulins (B l a g r o v e and G i l l e s p i e 1975). Conglutins constitute about 80 to 90% of seed proteins, of which 40% are α - and 50% are β -conglutins, while only 10% are γ - and remaining conglutins. Recently, two new forms of conglutins have been isolated, δ_1 and δ_2 conglutins (L i l l e y 1986).

Abbreviations used: PBS — phosphate buffered saline, BSA — bovine serum albumin, pNPP — para-nitrophenylphosphate, SDS — sodium dodecylsulphate, PAGE — polyacrylamide gel electrophoresis, GA₃ — gibberellin A₃, AP-ase — acid phosphatase

Proteins which act as biocatalyzers occur among storage proteins that used for structural and energetic purposes. The total protein content drops during germination (Morawiecka 1961) while the activity of acid phosphatase (AP-ase), among others, increases. This enzyme is regulated by phytohormones (Uehara et al. 1974, Yamagata et al. 1980).

The purpose of this study was to analyse the changes in total protein content and acid phosphatase activity in dormant seeds and during the early stages of germination of yellow lupin (*Lupinus luteus*) and blue lupin (*Lupinus angustifolius*) in the presence and absence of the phytohormone, gibberelic acid (GA_3).

MATERIAL AND METHODS

The seeds of two varieties of lupin: *Lupinus luteus* L. (cv. Topaz) and *Lupinus angustifolius* L. (cv. Mirela) were from the 1985 harvest and were obtained from Centrala Nasienna in Poznań.

The seeds were sterilized in 95% acetone for 5 minutes and, after washing with water, were treated with 3% H_2O_2 for 15 minutes, rinsed with distilled water and then soaked in water at 25°C for 24 hours and sown on filter paper. After 5 days the seedlings were transferred to trays containing distilled water.

In the experiments involving GA_3 , hydroponic culture was conducted according to Akiyama and Suzuki (1980). Sterilized seed were soaked for 5 hours, then transferred to cheese cloth fixed over a Petri dish containing 0.2 mM acetate buffer, pH 5.1 (control) or containing the same buffer with 10 mM GA_3 (25°C, daylight). The collected material was washed twice with 0.05 M acetate buffer, pH 5.1. Protein and AP-ase activity were determined in the medium.

Proteins from dormant and germinating seeds and from young plants were extracted with 0.1 M borate buffer, pH 8.8 at a w/v proportion of 1:2.5 and with 0.1 M acetate buffer at a pH of 5.1 at a w/v proportion of 1:5. The dormant seeds with their shells removed were ground in a mixer, germinating seeds and young plants were homogenized in a Potter homogenizer. Proteins from ground seeds or homogenates were extracted for 1 hour, stirring occasionally. The extracts were centrifuged at $8000 \times g$ for 10 minutes. Protein and acid phosphatase (AP-ase) activity were determined in the supernatant. The extracts in acetate buffer were used for gel electrophoresis (Davis 1964, Laemli 1970). Conglutins from seeds were prepared according to Joubert (1955a, b). The conglutin pellets were then dissolved in PBS and protein and phosphatase activity determined in them. They were also analysed by electrophoresis (Davis 1964, Laemli 1970).

Protein was determined according to Lowry with bovine serum albumin (BSA) as the standard (Lowry et al. 1951).

Acid phosphatase activity was determined using p-nitrophenyl phosphate as the substrate. The reaction was conducted for 10 minutes in 0.1 M acetate buffer

at pH 5.1, 37°C with 2.5 mM sodium p-nitrophenyl phosphate (pNPP). The incubation mixture contained 250 µl of the enzyme dissolved in 0.1 M acetate buffer, pH 5.1 and 250 µl substrate (5mM) in the same buffer. The reaction was terminated by adding 1.5 ml of 0.25 M NaOH. The amount of liberated p-nitrophenol was measured at 410 nm in 1 cm quartz cuvettes using a VSU 2P spectrophotometer (Zeiss Jena). A unit of activity was taken to be the liberation of 1 µmole of p-nitrophenol from p-pNPP in 1 minute at 37°C. The specific activity of acid phosphatase was expressed in units of enzymatic activity per milligram of protein.

The effect of GA₃ on the activity of AP-ase was studied using 1 µM GA₃ per sample.

Proteins were separated by polyacrylamide gel electrophoresis (PAGE) under native conditions in a 7.5% gel with a 2.5% starter gel, at pH 8.4 (Davis 1964) and under reducing conditions in SDS in a 10% gel at pH 8.6 (Laemmli 1970). Ammonium persulphate was used as the polymerizing agent. About 20-200 µ protein and 0.6 units of phosphatase activity were used for separation. Bromophenol blue was used as a marker. The samples were concentrated in glycerol. A current of 2.5 mA per tube was applied for the first 15 minutes of electrophoresis, followed by 4 mA per tube. The protein in the gels was stained with a 0.4% solution of Coomassie Brilliant Blue R-250 in 5% acetic acid. Excess stain was removed by washing with 10% methanol in 5% acetic acid. Acid phosphatase activity was localized using sodium α-naphthyl phosphate as the substrate (1 mg/ml buffer) and Fast Blue B (1 mg/ml) as the coupling agent. The gels were incubated 3 times for 15 minutes (4°C) in 0.2 M acetate buffer, pH 5.1 in order to bring the gels to pH 5.1, which is the optimum of the enzymatic reaction.

In the case of SDS electrophoresis the gels were incubated for 2 hours on 0.2 M acetate buffer at pH 5.1 containing 2% Triton X-100. The gels were then incubated at 37°C in the same buffer containing 0.1% sodium 1-naphthyl phosphate in 0.1% Fast Blue B. Incubation was continued until bands appeared on the gels.

The electrophoretic protein standards were obtained from Pharmacia (Uppsala).

RESULTS AND DISCUSSION

Extraction to borate buffer was used in the determination of the protein levels and phosphatase activity in dormant and germinating seeds and in 5 and 12-day-old *L. luteus* and *L. angustifolius* seedlings. The results are presented in Tables 1 and 2. It was found that in both lupin species, the protein level during germination drops dramatically from its initial value of 365 mg/g weight in *L. angustifolius* and 370 mg/g in *L. luteus* to 134 mg/g and 188 mg/g, respectively, after 2 days of germination. The protein level remained further unchanged on day 5, while by day 12 of germination, it again dropped significantly and made up

only 22% (*L. angustifolius*) or 6% (*L. luteus*) of the protein level of dormant seeds. The significant decrease in the protein level between days 2 and 12 of germination was associated mainly with decreases in the protein content of cotyledons and the part above the cotyledons of the young seedling. In order to determine which of the forms of the storage protein undergoes degradation in the early stages of germination of both studied lupin species, the proteins of the cotyledons were extracted to acetate buffer at pH 5.1 and subjected to PAGE (Fig. 1) according to Davis (1964). In addition, conglutin α and β were isolated and partially purified according to Joubert (1955a and b) (Fig. 1A). The conglutin α preparation was slightly contaminated with one fraction of conglutin β , while that of conglutin β contained part of an α -conglutin fraction. Raw extracts of cotyledons contained at least 12 protein fractions, of which a fraction with conglutin β mobility gradually decreased, while that with α -conglutin mobility remained unchanged during a 5-day period of germination. It may be assumed that the dynamic degradation of conglutin β during the early stages of germination is due to the fact that this protein contains the amino acids methionine and tryptophan (Gerritsen 1955) which are precursors of methyl groups and phytohormones (IAA), required in the early stages of germination. The gradual disappearance of unidentified protein fractions was also observed.

Table 1

The protein level and acid phosphatase activity of dormant and germinating seeds and organs of young plants, extracted by borate buffer (*L. luteus*)

Material extracted into 0.1 M borate buffer pH 8.8	Day of germination	Protein*		AP-ase activity**			
		mg/g mass	%	units/g mass	%	units/mg protein	%
Dormant seeds	0	370	100	55.0	100	0.15	100
Germinating seeds	2	187	51	12.0	22	0.064	43
Cotyledons	5	123		104.6		0.85	
Part above cotyledons		30		100.0		3.30	
Part below cotyledons		11		25.0		2.23	
Roots		24		38.1		1.64	
Total	5	188	51	267.7	487	1.43	953
Cotyledons	12	10		30.0		3.00	
Part above cotyledons		5		14.4		3.00	
Part below cotyledons		4		6.5		1.63	
Roots		5		12.5		2.50	
Total	12	24	6	63.4	115	2.67	1780

x — protein was determined according to Lowry, xx — AP-ase activity was determined with pNPP as the substrate according to the method described in Material and Methods. The presented results are the arithmetic means of three determinations.

Table 2

The protein level and acid phosphatase activity of dormant and germinating seeds and organs of young plants, extracted by borate buffer (*L. angustifolius*)

Material (extracted into 0.1 M borate buffer pH 8.8)	Day of germination	Protein*		AP-ase activity**			
		mg/g mass	%	units/g mass	%	units/mg protein	%
Dormant seeds	0	365	100	7.0	100	0.021	100
Germinating seeds	2	134	37	1.73	22	0.013	62
Cotyledons	5	55		10.5		0.19	
Part above cotyledons		48		11.3		0.24	
Part below cotyledons		10		6.7		0.67	
Roots		12		4.7		0.40	
Total	5	125	34	33.2	424	0.27	1286
Cotyledons	12	21		31.9		1.52	
Part above cotyledons		44		20.2		0.45	
Part below cotyledons		9		9.7		1.12	
Roots		7		8.0		1.15	
Total	12	81	22	69.8	894	0.86	4095

x — protein was determined according to Lowry, xx — AP-ase activity was determined with pNPP as the substrate according to the method described in Material and Methods. The presented results are the arithmetic means of three determinations.

Fig. 1. Polyacrylamide gel electrophoresis according to Davis (see Material and Methods) — diagrams of proteinograms. A — conglutin α preparation: a — conglutin α , b — conglutin β , B — extracts to 0.1 M acetate buffer, pH 5.1: a — from one-day-old cotyledons, b — from two-day-old cotyledons, c — from five-day-old cotyledons

It was also interesting to trace concomitantly the changes in the protein level and in AP-ase activity as well as the effect of GA₃ during the early stages of seed germination of both species of lupin. In the first experiment the activity of acid phosphatase was determined in borate buffer extracts of seeds and seedlings (Tables 1 and 2). In the second variant, the activity of this enzyme was determined in acetate buffer extracts, pH 5.1, of seedling cotyledons, cultured in the presence and absence of GA₃ (Tables 3 and 4). The use of acetate buffer for extraction of AP-ase is supported by earlier studies (Lorenz-Kubis and Morawiecka 1973), which showed that the activity of this enzyme in mono- and dicotyledons is more efficiently extracted with acetate buffer, pH 5.1. It was shown that the specific activity of AP-ase in dormant *Lupinus luteus* seeds is about 7.5 times higher than in *Lupinus angustifolius*. On the second day of germination of both species of lupin, the specific activity of AP-ase decreased to 43% in *L. luteus* and 62% in *L. angustifolius*. On day 5 of germination, a 9 (*L. luteus*) and 15-fold (*L. angustifolius*) increase in specific activity in comparison with dormant seeds was observed. An increase in the activity of acid phosphatase was observed mainly in the cotyledons, a somewhat smaller increase was observed in the remaining parts of the seedling. The significant rise in activity takes place due to the lowering of the total protein content and, assumably, of the *de novo* synthesis of AP-ase in the remaining parts of the seedling.

Table 3

The protein level and acid phosphatase activity of dormant and germinating *L. angustifolius* seeds cultivated on a medium containing GA₃ (extraction by acetate buffer)

Material (extracted into 0.1M acetate buffer pH 5.1)	Day of germination	Protein		AP-ase activity			
		mg/g mass	%	units/g mass	%	units/mg protein	%
Dormant seeds with embryos removed	0	70.9 (+5.1)	100	8.8 (+0.32)	100	0.12	100
Cotyledons on medium with no GA ₃ (control) on GA ₃ -containing medium	1	42.5 (±1.6)	60	4.7 (±0.33)	54	0.11	92
		47.5 (±3.8)	67	5.0 (±0.17)	57	0.11	92
Cotyledons on medium with no GA ₃ (control) on GA ₃ -containing medium	2	33.3 (±0.8)	47	4.4 (±0.14)	50	0.13	108
		35.0 (±2.3)	49	4.4 (±0.20)	50	0.13	108
Cotyledons on medium with no GA ₃ (control) on GA ₃ -containing medium	5	17.5 (±1.9)	25	9.5 (±0.31)	108	0.54	450
		24.2 (±3.5)	34	9.4 (±0.18)	107	0.39	325

The presented results are the arithmetic means of three determinations. Standard deviations are given in brackets (±).

Table 4

The protein level and acid phosphatase activity of dormant and germinating *L. luteus* seeds cultivated on a medium containing GA₃ extraction by acetate buffer

Material (extracted into 0.1M acetate buffer pH 5.1)	Day of germination	Protein		AP-ase activity			
		mg/g mass	%	units/g mass	%	units/mg protein	%
Dormant seeds with embryos removed	0	90.9 (± 4.4)	100	31.3 (± 2.1)	100	0.34	100
Cotyledons on medium with no GA ₃ (control) on GA ₃ -containing medium	1	47.9 (± 3.1)	53	14.8 (± 1.3)	47	0.31	91
		51.3 (± 2.3)	56	16.9 (± 1.1)	54	0.33	97
Cotyledons on medium with no GA ₃ (control) on GA ₃ -containing medium	2	42.6 (± 2.1)	47	13.4 (± 0.9)	42	0.31	91
		43.3 (± 2.0)	48	12.5 (± 0.9)	40	0.29	85
Cotyledons on medium with no GA ₃ (control) on GA ₃ -containing medium	5	40.7 (± 3.1)	45	40.8 (± 2.0)	130	1.00	294
		34.4 (± 2.6)	38	34.0 (± 3.0)	109	0.99	291

The presented results are the arithmetic means of three determinations. Standard deviations are given in brackets ($\pm$).

The effect of gibberelic acid (GA₃) on acid phosphatase activity during germination of the seeds of both species of lupin is presented in Tables 3 and 4. The amount of protein extracted to acetate buffer from dormant seeds from which the embryos had been removed amounted to 24% (*L. luteus*) or 19% (*L. angustifolius*) of the protein extracted to borate buffer (Tables 1 and 2). The specific activity of acid phosphatase determined in acetate buffer extracts of dormant seeds was 227% of the specific activity of the enzyme extracted with borate buffer (*L. luteus*) and 580% in the case of *L. angustifolius*. The activity of AP-ase in 5-day-old cotyledons was proportionally higher and equalled for *L. luteus* 113% of the activity of the enzyme in the borate extract and 290% in *L. angustifolius*. Regardless of the differences shown in the specific activities of AP-ases in extracts from seeds and seedlings to acetate or borate buffers, it was shown that the general nature of the changes occurring during the 5-day period of germination of seeds is very similar. Comparison of the activities of AP-ase in developing leaves in seeds with no embryos, cultivated in mediums with and without GA₃ showed no significant effect of the phytohormone on either the behavior of phosphatase activity (during the 5 days of germination) or on changes in the protein level (Tables 3 and 4). A fall in the activity of acid

phosphatase was also observed in 5-day-old cotyledons of *L. angustifolius* seeds (Table 4). However, this decrease may have been caused by an increase in the amount of non-enzymic protein extracted by acetate buffer. It may also be assumed that the fall in the activity of this enzyme in cotyledons in response to GA_3 is caused by secretion of phosphatase to the medium. An approximately 2-fold higher AP-ase activity was found in the GA_3 -containing media from cultures being terminated after 12 days of germination of embryo-less *L. luteus* seeds. These results confirm those of Akya and Suzuki (1980) on the secretion of acid phosphatase into the medium during the early stages of germination of seed halves or seeds without embryos. This suggests that GA_3 increases the secretion of protein into the medium, although not specifically, but does not affect the activity of AP-ase by stimulating its synthesis *de novo*.

Increased activity of acid phosphatase during germination of seeds was found both upon extraction of this enzyme both by acetate as well as borate buffer. It was also interesting to see if changes in the activity of acid phosphatase were caused by *de novo* synthesis of the enzyme. For this purpose, acetate buffer extracts of dormant seeds and cotyledons of 1, 2 and 5-day-old germinating seeds were subjected to SDS PAGE. The results of these electrophoreses are illustrated on diagrams (Fig. 2). They were identical, regardless of the species of lupin. In dormant lupin seeds only one high molecular weight fraction having phosphatase activity was found, its level decreased during germination. Starting from day 2 of germination, initially one, then on day 5, a second new form of AP-ase appeared in the cotyledons. Both had high electrophoretic mobilities and masses of 35 and 45 KD. This result may point to the biosynthesis of new forms of acid phosphatase during germination.

Fig. 2. SDS-PAGE — diagram of electrophorograms (for conditions of electrophoresis see Material and Methods). A — Molecular weight standards: α -lactalbumin (14.4 kD), soybean trypsin inhibitor (20 kD), carbonic anhydrase (30 kD), ovalbumin (43 kD), BSA (67 kD), phosphorylase b (94 kD). B — AP-ase activity — a zymogram of 0.1 M acetate buffer, pH 5.1, extracts: a — from seeds, b — from one-day-old cotyledons, c — from two-day-old cotyledons, d — from five-day-old cotyledons

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Aktywność kwaśnej fosfatazy i poziom białek zapasowych we wczesnym okresie kiełkowania nasion Lupinus luteus L. i Lupinus angustifolius L.

Streszczenie

Zbadano zmiany poziomu białka i aktywności kwaśnej fosfatazy w nasionach spoczynkowych i podczas kiełkowania dwóch gatunków łubinu (*Lupinus luteus* i *Lupinus angustifolius*). Stwierdzono stopniowy spadek poziomu białka, związany m.in. ze spadkiem zawartości konglutyny β . Stwierdzono także początkowy spadek aktywności kwaśnej fosfatazy, a następnie, poczynając od 5 dnia kiełkowania, wzrost aktywności tego enzymu. Wykazano, że wzrostowi temu towarzyszy pojawienie się dwóch nowych form molekularnych AP-azy. Nie stwierdzono znaczącego wpływu GA₃ na aktywność właściwą kwaśnej fosfatazy.