5). Acidic subunits surround basic subunits in 11S globulin (A), and by heat treatment 11S globulin molecules are unfolded but still kept globular (B). Then they associate and form the aggregate whose molecular weight is 8000000 (C) (Nakamura et al., 1984). When the associate is treated with bromelain, its structure and characteristics change because cutting occurred in the strands or degradation products recombine (D). This association is believed to be due mainly to disulfide bond and hydrophobic interaction, because it dissociates into smaller fragments of 15000 molecular weight in the presence of SDS and 2-mercaptoethanol. As shown in Figure 4, these strands form a network structure and also aggregates by getting entangled (E).

This coagulum with a soft gel appearance had good hydration. Water absorption of the enzyme-treated products of acid-precipitated protein improved about 2 times more than that of the native and heated ones. But there was no significant improvement in hydration of heated and enzymatically treated 11S globulin. Water absorption of the lyophilized coagulum of 11S globulin was less than that before lyophilization. Lyophilization seems to be the cause for this decrease in water absorption of the coagulum. However, the improvement of water absorption of acid-precipitated protein is seen to be advantageous for food materials.

Registry No. Bromelain, 37189-34-7; water, 7732-18-5.

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Received for review October 10, 1983. Accepted January 10, 1984.

Changes in Composition and Subunits in the Storage Proteins of Germinating Lupin Seeds

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The modifications occurring during 16 days of germination in weight, length, lipids, total nitrogen, water-extracted and salt-extracted nitrogen, and amount and protomer composition of water-soluble proteins and of each separate salt-extracted protein were studied in *Lupinus albus* seeds and seedlings. As germination proceeds, nitrogen shifts from the storage globulins to other compounds. The rate of breakdown differs for each globulin. The two major vicilins are degraded first and at different rates, indicating a structural diversity of the two proteins. Legumins follow. Conglutin γ undergoes no change over the period studied. Extensive interruption of the covalent continuity in the subunits of vicilins (globulins 4 and 6) likely makes the breakdown easier, while interpeptide disulfide bonds in legumins (globulins 8 and 9a) and in conglutin γ slow down proteolysis.

Storage proteins in seeds are mobilized during germination with resulting formation of amino acids and peptides, which serve mainly as sources of nitrogen and carbon skeleton in molecules of the developing seedling.

Proteolytic activity and breakdown of storage proteins during germination have been studied in various legume seeds: peanut (Bagley et al., 1963), pumpkin (Khokhlova, 1971; Lott and Vollmer, 1973), broad bean (Briarty et al., 1970; Lichtenfeld et al., 1979), pea (Basha and Beevers, 1975; Konopska, 1978, 1979), soybean (Catsimpoolas et al., 1968), and mung bean (Baumgartner and Chrispeels, 1978).

The mobilization of total storage proteins in lupin seeds of different species germinated in the dark and in the light was studied and proteolysis was found to occur earlier in seeds kept in the light than in those grown in the dark (Prus-Glowialki, 1975). Vicilin and legumin breakdown in lupin seeds was described by Morawiecka (1961). However no information is available on the behavior of the various proteins in each fraction and of their constituent protomers during the germination of the seed. Indeed, in previous work we separated the storage proteins from lupin seeds into six major and six minor oligomeric proteins (Duranti et al., 1981; Restani et al., 1981). It therefore appeared worthwhile to study the degradation rates of each

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0021-8561/84/1432-0490$01.50/0

Table I. Analysis of Germinating Seeds or Seedlings[a]

	days of germination				
	0	6	9	12	16
dry weight, g	40.5	39.4	38.9	32.6	34.3
lipids, g	3.5	3.3	2.7	1.3	0.8
total N × 6.25, g	18.5	19.0	17.8	17.5	18.1
water-extracted N, % of total (A)	11.3	23.2	36.5	48.3	52.8
1 M NaCl extracted N, % of total (B)	80.7	68.5	54.6	37.5	25.6
A + B	92.0	91.7	91.1	85.8	78.4
gel filtration of NaCl-extracted compounds[b]					
excluded (M_r >30 000)	40	33	23	16	4
fractionated (M_r 24 000-26 000)	27	27	11	8	6
included (M_r <1500)	14	8	20	13	16
length of the seedlings, cm		6-7	10-12	18-20[c]	20-22[d]
av daily growth, cm day^{-1}	1.1	1.5	2.7	0.4	

[a] All determinations were on 100 seeds except for the gel filtration analyses on Sephadex G-50, which were done on single seeds as specified in the text. [b] The sum of elution areas at each day was related to the amount (B) of NaCl-extracted nitrogen at that day. The size of each peak is therefore given as percent of total nitrogen. [c] Very small leaves. [d] Larger leaves.

protein species by establishing its quantitative modification and the changes in its protomer pattern throughout the course of the seedling development. Results are given in this paper.

MATERIALS AND METHODS

Lupinus albus seeds of the sweet Multolupa variety were used. The proximate composition was determined for 100 seeds or seedlings after removing the seed coat, when present. They were frozen in liquid nitrogen and milled in a Moulinex grinder; the flour was dried under vacuum to constant weight. Lipid were assayed by loss of weight after pentane extraction in a Soxhlet apparatus. Nitrogen was assayed by the micro-Kjeldahl procedure (AOAC, 1970) on the flour and on the water and salt extracts. All other determinations were done on single frozen seeds or seedlings so as to approach the situation existing in each seed. Single seeds or seedlings were crushed in a mortar and dried as above. Other materials and methods used are described by Duranti et al. (1981), Restani et al. (1981), and Cerletti et al. (1978).

Germination of the Seeds. Seeds were germinated in the dark, according to most authors: the slower mobilization of the proteins under these conditions facilitates the study of their breakdown. Dormant seeds were gently shaken in tap water for 24 h at room temperature. They were then kept in the dark at 20 °C on sterilized cotton wool soaked with distilled water for 1, 2, 3, 6, 8, 9, 12, and 16 days.

Extraction and purification of proteins were performed according to Cerletti et al. (1978) with some modifications due to the very low amount of material available from single seeds and seedlings: desalting of the total globulin extract and separation from low molecular weight compounds was in a 270 × 21 mm column at a flow rate of 40 cm h^{-1}; ion-exchange chromatography of globulins on Whatman DEAE-cellulose was done in a column of 190 × 25 mm at a flow rate of 14.6 cm h^{-1}. Proteins loaded on it were 14.4 mg, as measured by the biuret reaction. Separated compounds, monitored at 280 nm, were quantified by comparing the areas of elution peaks. In the central tube of each peak of the ion-exchange column, proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in the presence of 2% 2-mercaptoethanol, as described by Restani et al. (1981). The same amounts, as measured by absorbance at 280 nm, were applied. The separated bands colored with Coomassie Blue were quantified by scanning at 637 nm with a laser densitometer, Ultroscan LKB, equipped with a HP 3390 integrator.

The nomenclature for each globulin fraction proposed by Duranti et al. (1981) is used in the present paper.

RESULTS

Changes in the composition of seeds and seedlings as germination proceeded are shown in Table I. The dry weight decreased moderately, because of a decrease of lipids and possibly also of carbohydrates, whereas total nitrogen did not change noticeably.

Water-extracted nitrogen, containing amino acids, peptides, albumins, and nucleotides, increased significantly during germination, while salt-extracted nitrogen, especially the high molecular weight components, i.e., globulins as shown by gel filtration, decreased, having been in part transferred to the water-soluble and to the insoluble forms.

The salt-extracted material of high molecular weight (>30 000), when submitted to ion-exchange chromatography did not permit the quantitative separation of globulins 4 and 6 from the minor proteins, namely, globulins 5 and 7, and globulins 8 from 9a and 9b, because of the limited amount of protein present in one seed. However, the electrophoretic patterns on SDS–PAGE illustrated the changes in protomer composition for each of the major proteins (Figure 1 and Tables II and III). The quantitative changes of globulins during germination are given in Table IV. With developing germination an increasing part of the material on the ion exchanger spread between peaks. This part was not considered in Table IV, which gives only the material contained in peaks. As shown in the table conglutin γ (globulin 1) was practically unchanged during the whole period. Its SDS–PAGE pattern was also unmodified both qualitatively and quantitatively (not shown). The other proteins decreased considerably, the most significant changes occurring between 6 and 9 germination days, and the decay pattern was typical for each globulin group.

As shown in Table II the vicilins, i.e., globulins 4 and 6, displayed only minor changes in their protomer pattern up to the sixth day. Both proteins showed a new band of apparent molecular weight of 17 000 and globulin 4 also one of 12 000, which in globulin 6 appeared at later times. Some protomers of intermediate molecular weight temporarily increased. After 8 days, the degradation of high molecular weight peptides was much more apparent for globulin 4 than for globulin 6.

In legumins, i.e., globulins 8 and 9a, the degradation was delayed as compared to the vicilins and new bands formed had different molecular weights (Table III). After 12 germination days, protomers of intermediate molecular weight were still present.

Table II. Protomer Composition of Major Vicilins at Progressive Stages of Germination[a]

$M_r \times 10^{-3}$	globulin 4, days of germination					globulin 6, days of germination				
	0	6	8	9	12	0	6	8	9	12
68-64	0.98	1.19	0.01	0	0	3.37	2.66	1.72	0	0
42	3.59	2.07	0.07	0	0	3.90	4.57	3.05	0	0
37	2.75	3.70	0.05	0	0	2.83	0.73	0.22	0	0
33	4.42	3.69	2.18	0	0	4.27	4.82	6.39	0	0
30	2.73	3.17	0.30	0	0	1.93	2.57	0.60	0	0
28	3.04	1.86	0.43	0	0	5.39	4.25	1.11	0	0
21	3.57	2.95	1.33	0.57	0	3.43	3.33	3.05	0	0
17	0	3.12	7.65	10.12	7.76	0	4.42	13.71	18.24	17.35
16	7.12	5.94	0.25	0	0	2.12	1.75	0	0	0
15	4.80	3.47	1.40	0	0	0.40	0.40	0	80	0
13	3.61	5.31	6.38	3.15	3.15	1.77	4.16	10.62	7.62	9.62
12	0	0.25	0.25	2.75	1.83	0	0	0	3.33	4.54
recovery of the applied amount as peptides, %	100	97	39	28	21	100	101	97	46	49

[a] Figures are in nanomoles and refer to 0.3 absorbance unit at 280 nm applied on the gel. They were calculated from the integrated densitometric patterns, according to the equation nanomoles = (area of peak/total measured area at $t = 0$)$(1/M_r)$.

Table III. Protein Composition of Globulin 8 at Progressive Stages of Germination[a]

$M_r \times 10^{-3}$	days of germination			
	0	6	9	12
55	2.82	2.78	2.69	0
44	2.66	0.91	1.15	0
39	5.00	2.08	3.23	0.45
33	4.21	4.69	4.61	2.56
22	1.77	2.64	4.00	2.36
18	12.33	10.83	10.50	13.44
14	0	4.14	5.00	7.93
13.5	9.56	0	0	0
13	0	4.85	5.31	7.92
12	0	0	0	0.75
total recovery of considered peptides, %	100.0	80.4	89.4	62.0

[a] Values are given as in Table II.

DISCUSSION

The modifications in proximate composition of lupin seeds observed during germination were consistent with those previously described for other legumes.

The decrease in soluble nitrogen compounds, with no change in total nitrogen, may have been due to synthesis of membrane proteins not extracted by aqueous solvents.

In early germination periods the total amount of each vicilin decreased but the protomer pattern was only slightly modified. This indicates that degraded molecules were separated from the unmodified ones during purification. However, as germination proceeded, the high molecular weight components that were eluted from the ion exchanger in volumes similar to those of the unmodified proteins displayed a highly modified protomer pattern. Associations of degraded protomers having ionic properties similar to those of the original proteins are likely present and become predominant in later germination days.

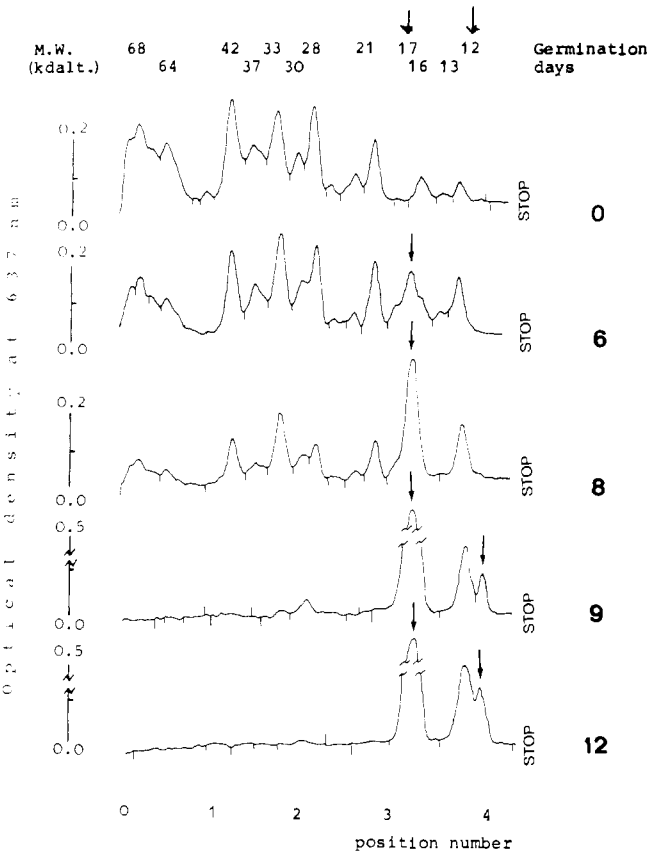

Figure 1. Densitometer tracing of globulin 6 at progressive stages of germination after SDS-PAGE in the presence of 2-mercaptoethanol.

Morawiecka (1961) showed that in germinating yellow lupin seeds conglutin β, i.e., the vicilins, was degraded before conglutin α, i.e., the legumins. Our work confirms

Table IV. Amount of Main Globulins at Progressive Stages of Germination[a]

days of germination	globulin fractions						recovery after ion-exchange chromatography, %
	1	3	4 + 5	6 + 7	8 + 9a + 9b	10 + 11	
0	1.9	5.6	19.5	37.3	21.9	13.8	100
6	1.9	5.6	16.1	20.3	25.1	12.4	81.4
9	2.3	4.3	5.4	9.9	7.2	6.2	35.3
12	2.3	2.9	3.7	6.2	6.6	3.7	25.4

[a] Compounds excluded in gel filtration on Sephadex G-50 (see Table I) were separated on a column of Whatman DE-52-cellulose. Elution areas, measured at 280 nm, are given as percent of the sum of areas obtained for the dormant seed. For other conditions see the text.

this observation and shows that the two major vicilins are degraded at a different rate. The different behavior of vicilins 4 and 6 likely relates to the association strength and to the oligomeric structure of these two proteins. Indeed, the vicilins are very similar in peptide and sugar composition, but they differ in molecular weight and surface hydrophobicity (Cerletti, 1982; Bonomi et al., 1983). This suggests that the peptides are differently assembled. Protomers appear to be more tightly associated in globulin 6 (Bonomi et al., 1983), and this protein undergoes proteolysis by trypsin less readily than globulin 4 (Semino et al., 1983). Our present data show that in early germination stages, although globulin 6 is quantitatively more degraded by endogenous proteases, it displays for a longer time its original protomer composition. This speaks for higher structural requirements in the molecule: once attacked, it breaks down and the products are separated to another fraction.

The later onset of breakdown in legumins is likely related to a more compact structure due to presence of interpeptide disulfide bonds that are not present in the vicilins and of less extensive interruption of covalent continuity in the peptides in the dormant seed (Restani et al., 1981; Cerletti, 1982).

Globulin 1, i.e., conglutin γ, is not degraded during the germination period investigated: this is probably related to its resistance to proteolysis by trypsin (Duranti et al., 1983) and by endogenous endopeptidases (Casero et al., 1983). This behavior may depend on the higher content of bound sugar and higher surface hydrophobicity of this protein (Duranti et al., 1981; Bonomi et al., 1983; Semino et al., 1983).

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Received for review May 19, 1983. Revised manuscript received November 10, 1983. Accepted January 24, 1984. This research was supported by C.N.R., Italy, Special Grant I.P.R.A., Subproject 3, Paper No. 123.

Interaction of Phytate with Mustard 12S Protein

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Binding of phytic acid with the mustard 12S protein was studied in the pH range 5.0–1.0 by the techniques of precipitation, turbidimetry, electrophoresis, and electrometric titration. The binding increased with decreasing pH. At pH 3.0, maximum binding of 170 mol of P/mol of protein was observed, which agreed with the basic amino acid content of the protein. Only one class of binding sites was indicated. Electrophoretic studies indicated that even at pH 3.0 some amount of protein was still left in solution. Electrometric titration studies indicated the possibility of the presence of soluble protein–phytate complexes in the system. At pH 3.0, calcium inhibited the formation of protein–phytate complex.

Phytic acid, the hexaphosphoric ester of myoinositol, is a constituent of many legumes and oilseeds including mustard seeds. It reduces the bioavailability of essential minerals such as calcium, zinc, magnesium, etc. by interacting with them (Cheryan, 1980; Cosgrove, 1980). The presence of phytic acid hampers peptic digestion of the protein in the alimentary canal (Barre, 1956). A number of proteins of animal and vegetable origin are known to form insoluble complexes with phytic acid below their isoelectric point (Posternak, 1965). The protein–phytate complex could also make zinc and other minerals biologically less available (Cheryan, 1980). Earlier studies on the interaction of phytic acid with proteins have shown that the interaction is dependent on the pH of the medium. At pH values below the isoelectric point of the protein, phytate binds directly to the protein cation, and at pH values above the isoelectric point, it binds to the protein through an alkaline-earth metal (Cheryan, 1980).

Mustard meal contains 5–7.5% phytic acid, depending upon the variety (Jones, 1976). Mustard seeds contain two

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0021-8561/84/1432-0493$01.50/0