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Changes in Nitrogen Fractions and Proteolytic Activities in the Cotyledons of Germinating Lentils

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Changes in ninhydrin positive material, free amino acids and protein content during germination of seeds of *Lens culinaris* Med. have been studied. Ninhydrin positive material and free amino acids reached their highest concentration at the fifth day of germination. Total protein which represents 21% of the total dry weight of the lentil cotyledons, suffers a degradation of only 24% in seven days of germination; in the same period of time, reserve proteins underwent a degradation of 69%, legumin being the more abundant at the start, and the more rapidly depleted. Five different classes of proteolytic activities have been reported in lentil cotyledons: caseinolytic, active against the reserve proteins of the lentil cotyledons themselves, aminopeptidase, peptidehydrolase, carboxypeptidase and dipeptidase. The removal of the axis did not seem to exert any significant influence on the enzymatic activity.

During seed germination the protein level decreases and the amino acids arising from the reserve tissues are translocated to the developing axis (3, 11, 13, 16, 21, 22). However, the mechanism by which the proteins are converted to amino acids is not completely understood (5). It is generally assumed that in the proteolysis which takes place in germinating seeds, the insoluble reserve proteins are hydrolysed in situ to soluble peptides by proteinases, after which the peptides are hydrolysed by some peptidases. This is shown by the accumulation of soluble amino acids in cotyledons or endosperm, and to a greater extent in the developing embryo axis.

The presence of proteolytic enzymes in germinating seeds is now well established. These are either pre-existent or synthesized de novo during germination, and their activity modulated by several control mechanisms, which are as yet still not fully understood (7, 10, 12, 19, 20). At present, however, their characterization and contribution to protein digestion is under intensive investigation (1, 4, 9).

In this paper we study the degradation of the storage proteins during germination of seeds of *Lens culinaris* Med. and

the characterization of some of the proteolytic enzymes involved in their digestion.

Materials and Methods

Lentil seeds (*Lens culinaris* Med. = *Lens esculenta* Moench) were soaked for 4 h in a disinfectant solution (0.1% Captan) at room temperature. The seeds were germinated in the dark at 25° C and 80% relative humidity on moist filter paper in Petri dishes for different periods of time, and then separated into cotyledons and embryos. Only the cotyledons were used for all subsequent assays. In the treatments with protein syntesis inhibitors, 25, 50 or 100 μg/ml of cycloheximide were added to the germination media.

The cotyledons from germinating lentil seeds were homogenized in a mortar with sand and 0.1 M sodium borate buffer pH 8.0 with 2 mM 2-mercaptoethanol. The homogenate was centrifuged at 12000 × *g* for 15 min. The supernatant, dialysed during 18 h against 25 mM sodium citrate-phosphate buffer pH 5.0 with 1 mM 2-mercaptoethanol, was used in the protease assays.

Free amino acids from the cotyledons were extracted three times with 80% boiling ethanol. Ethanol was removed at 37° C under vacuum and the extract was passed through an Amberlite IR 120 column (2 × 20 cm). Total ninhydrin-positive material was determined by the method of MOORE and STEIN (18). Quantitative determination of amino acids was performed in a Beckman 119 CL amino acid analyzer.

Total proteins were analyzed in the 80% ethanol insoluble material. This material was dried at 60° C and submitted to an alkaline hydrolysis as described by AZHAR *et al.* (2). Reserve proteins were fractionated from the intact cotyledons into albumin, and globulin, vicilin and legumin by the method of BASHA and BEEVERS (3). Proteins were determined by the method of LOWRY *et al.* (14).

Caseinolytic activity was assayed by the method of YOMO and VARNER (22). The proteolytic activity against the lentil seed proteins themselves was assayed by incubating the four proteic fractions obtained as previously described with the enzymatic extracts; the amino acids released were determined by the ninhydrin method (18). Peptidehydrolase activity, measured by hydrolysis of BAPA was assayed by the method of CALDWELL and SPARROW (6). Aminopeptidase activity was determined by the method of ELLEMAN (8) with LNA as the substrate. Carboxypeptidase and dipeptidase activities were assayed by the method of MIKOLA and KOLEHMAINEN(17) using z-Phe-Ala and Ala-Gly as respective substrates.

All the determinations were repeated at least three times with different samples, and the variation between parallel samples was less than 15%.

Results

The liberation of ninhydrin-positive material (not shown) increased rapidly during the first hours of germination, reaching the maximum value at 120 h (5 days). The quantitative determinations of amino acids (Table I) coincide with these results; all of them with exception of arginine reach their highest values at 120 h of germination, asparagine, serine and glutamate being the most abundant.

Table II shows the changes in total and reserve proteins during germination. Total protein, which represents 21% of the total dry weight of lentil cotyledons, un-

Abbreviations: BAPA, N-Benzoyl-DL-Arginin HCL; LNA, L-Leucyl-naphthylamide HCL: z-Phe-Ala, N-CBZ-L-Phenylalanyl-L-Alanine; Ala-Gly, DL-Alanyglycine; TCA, Trichloroacetic acid.

Table I. *Quantitative determination of free amino acids (nmol/seed) during germination of lentil seeds.*

Amino acid	Germination time (h)							
	0	12	24	48	72	96	120	168
Trypthophan	—	44.09	25.10	48.89	44.00	47.70	78.04	38.97
Aspartate	49.54	59.5	75.08	102.4	108.09	159.61	190.59	89.92
Threonine	1.28	7.35	20.62	29.32	41.75	215.82	501.2	114.02
Serine	24.55	31.76	126.33	204.6	238.17	435.6	734.17	501.09
Asparagine	39.62	88.87	99.33	258.89	307.27	875.3	1548.04	1201.82
Glutamate	24.19	85.99	68.00	80.00	67.01	203.15	256.86	222.95
Glutamine	5.83	17.93	19.47	24.54	10.36	48.59	53.93	44.39
Proline	14.98	17.13	24.99	61.07	54.69	82.58	93.39	27.08
Glycine	8.37	11.93	7.93	31.3	29.36	40.38	72.34	78.87
Alanine	14.43	33.95	30.76	29.36	44.76	99.05	160.01	162.54
Valine	7.88	18.54	20.40	34.19	60.56	74.89	131.30	119.93
Cysteine	7.51	6.85	8.91	8.76	11.80	20.17	18.17	11.61
Methionine	4.88	6.12	6.45	8.41	11.33	7.35	9.32	9.40
Isoleucine	5.24	8.73	7.25	12.80	10.86	58.14	66.40	62.35
Leucine	5.00	7.52	7.32	10.93	15.38	52.51	63.19	41.55
Tyrosine	4.18	5.02	6.63	10.64	8.17	12.34	21.21	16.30
Phenylalanine	5.74	12.10	10.60	15.51	15.90	32.50	61.69	59.31
Lysine	8.68	9.27	22.57	24.03	22.85	21.06	45.45	37.10
Histidine	5.63	13.21	16.76	25.24	19.10	44.47	107.42	36.80
Arginine	10.35	31.85	59.83	47.71	17.17	51.17	53.90	34.40
Total amino acids	247.88	517.71	707.10	1068.59	1138.49	2582.38	4266.62	2910.40

derwent a degradation of only 24% in 168 h (7 days) of germination. In the same period of time, reserve proteins, which represent about 50% of the total protein content, underwent a mean degradation of 69%, divided as follows: legumins which represented 57% of the reserve proteins, 68%, albumins which represented 36%, 65%, and vicilin which represented 7% of the reserve proteins, 83%. As shown in the Table II, the sums of albumin + legumin + vicilin fall far below the values given for total protein; unless that the methods used for the determination of total and reserve proteins had different sensitivity, and membrane-bound protein is not possible to explain these results.

In extracts prepared from cotyledons of germinating lentil seeds, there exist enzyme (s) capable of digestion of casein, releasing trichloroacetic-soluble material (Figure 1). This enzymatic system was

Table II. *Changes in total and reserve protein during germination of seeds of* Lens culinaris *Med.*

Results are expressed as mg of protein/seed. From five series of experiments.

Fraction	Germination (h)								Degradation (%) at 168 h
	0	12	24	48	72	96	120	168	
Total protein	12.0	11.8	11.5	11.1	10.6	9.7	9.5	9.1	24
Algumin	2.0	1.6	1.3	1.1	1.0	0.9	0.9	0.7	66
Legumin	4.7	4.1	3.8	3.5	2.6	2.1	1.7	1.5	68
Vicilin	0.8	0.6	0.6	0.5	0.3	0.2	0.1	0.1	83
Other *	4.5	5.5	5.8	6.1	6.7	6.5	6.8	6.8	

* Dialysate and insoluble proteins.

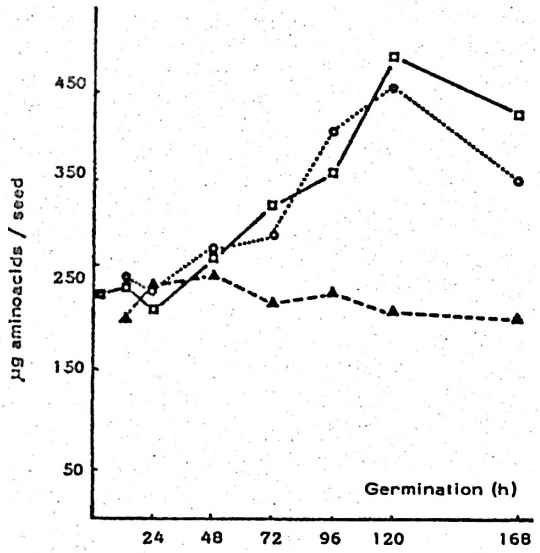

Fig. 1. *Changes in the caseinolytic activity (□—□) and the effect of the removal of the embryo (x····x) removed at the beginning of the incubation, and cycloheximide (Δ-----Δ) during germination of lentil seeds.*
pH of the assay 5.3. Each point is the average of at least three different experiments. The variation between parallel samples was less than 10%.

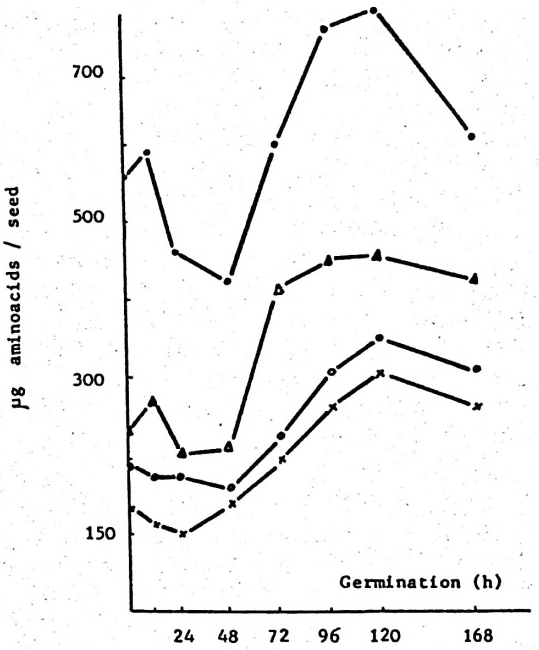

Fig. 2. *Changes in the proteolytic activity against the lentil seed reserve proteins during germination: legumin (●———●) vicilin (Δ———Δ); albumin (O———O); globulin (x———x) pH of the assay 5.3.* Otherwise as for figure 1.

present in dry seeds and its activity increased during germination reaching the maximum activity at 120 h of germination. When this enzymatic system was assayed against the reserve proteins of lentil seeds themselves, it could also release trichloroacetic acid soluble material (Figure 2) reaching its highest activity against legumins.

Figure 3 shows the activities of peptidehydrolase, aminopeptidase, carboxipeptidase and dipeptidase. All of these were present in dry seeds but only aminopeptidase exhibited a quantitatively considerable increase during germination.

The addition of cycloheximide to the incubation media (Figure 1) shows a decrease in the enzymatic activities from 48 h of germination onwards. The removal of the axis (Figure 1) did not seem to exert any significant influence on the enzymatic activities since in detached cotyle-

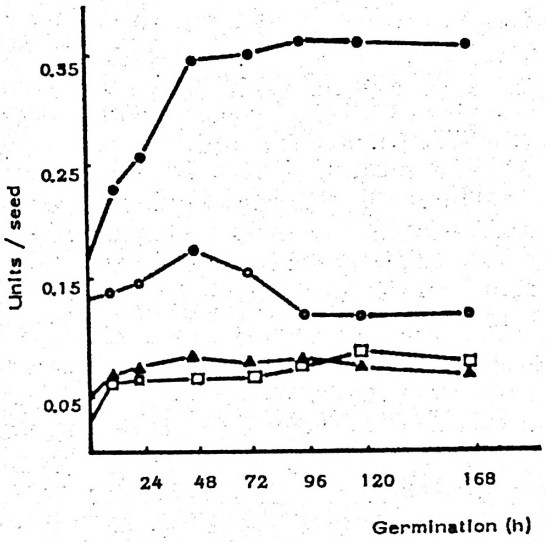

Fig. 3. *Changes in aminopepetidase (●———●), dipeptidase (O——O) peptidehydrolase (Δ——Δ) and carboxipeptidase (x———x) activities during germination of lentil seeds.*
Otherwise as for figure 1.

dons; the activities were similar to those obtained in complete seeds.

Discussion

During germination of lentil seeds there is a depletion of protein from the cotyledons accompanied by an accumulation of free amino acids. This result, together with the release of TCA-soluble materials from casein when incubated with an enzymatic extract obtained from cotyledons, is an indication of the existence of proteolytic enzymes. The caseinolytic activity (endoprotease) increases during germination reaching its maximal activity by the fifth day of germination, which coincides with the maximal accumulation of free amino acids, as has been found also in *Phaseolus aureus* (7) and in *Arachis hypogea* (15). Obviously, casein is not the normal substrate encountered by the lentil proteases in their *in vivo* activity. However, when this caseinolytic system was assayed against the reserve proteins of the lentil cotyledons, a similar pattern of protein degradation was obtained, the legumin being the more abundant reserve protein and the one more actively attacked by the proteolytic system.

Besides this caseinolytic system (unspecific endoprotease) we have found aminopeptidase, carboxypeptidase, peptidehydrolase and dipeptidase activities in lentil cotyledons. According to BEEVERS (5), these could be involved in the utilization of reserve proteins, while the caseinolytic enzyme could be involved in the turnover of the proteins. The legumin and vicilin proteins are the natural substrate for these enzymes in nature.

The behaviour of the proteolytic enzymes after cycloheximide treatment seems to indicate the presence in lentil seeds of two sets of enzymes: A) one pre-existent and obviously cycloheximide insensitive, whose activity is unchanged throughout germination, and B) another cycloheximide-sensitive set, which is synthesized de novo at about 48 h of germination (Figure 1). The lack of effect of the removal of the axis in proteolytic activity seems to indicate that the control of the enzyme activity in lentil seeds is not exerted, as has been suggested in other seeds (11), by the embryo. Further work is needed to determine the control of the proteolytic activity in germinating lentil seeds.

Resumen

Se estudia la variación del material soluble positivo a la ninhidrina, aminoácidos libres y contenido proteico durante la germinación de semillas de *Lens culinaris* Med. El material soluble positivo a la ninhidrina alcanza su máxima concentración en el 5.º día de germinación. Las proteínas totales, que representan el 21% del peso seco de los cotiledones de lenteja, sufren una degradación de sólo el 24% al cabo de 7 días y, en el mismo período, las proteínas de reserva se degradan en un 69% y de ellas las leguminas, que son las más abundantes, se degradan más rápidamente. Se han encontrado cinco clases de actividades proteolíticas: caseinolítica activa frente a las propias proteínas de reserva, aminopeptidasa, peptidohidrolasa, carboxipeptidasa y dipeptidasa. La eliminación del eje embrionario no parece tener ningún efecto significativo sobre las actividades enzimáticas.

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