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Autolysis of Protein Bodies in Germinating Lentil Seeds

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Protein bodies isolated from lentil (*Lens culinaris*, Medik) cotyledons exhibit autolytic activity which increases during seed germination. Such autolytic capacity is active across a broad pH range and shows a maximum at pH 6.5. Excision of the embryonic axis reduces autolytic capacity and application during incubation of the seeds without axis of both 6-benzylaminopurine and kinetin is able to replace it. On the other hand, the proteolytic activity in the protein body membrane, is located towards the proteinaceous matrix and is obviously partially responsible for this autolytic activity.

Key words: *Lens culinaris*, Cytokinins, Autolysis, Proteases.

The protein reserves of seeds are stored in special tissues and are contained in structures called protein bodies (16). During germination, protein bodies show different metabolic activities whose final purpose is to supply the elements necessary for seedling growth (4). Such metabolic activity is partly determined by the presence in these structures of certain hydrolases, as has been reported in different kind of seeds (6, 9, 13).

One of the few findings concerning the pH dependence of the hydrolytic activity in protein bodies can be inferred from a study on these structures in castor beans (21), when it was proposed a slightly acid pH for maximum hydrolytic activity to be reached (around 6.5), an *in vivo* value suggested for these organelles (20).

In lentil cotyledons different exo- and endoprotease activities have been reported (12); these would behave as an integral system in the solubilization of storage proteins. In previous reports (2, 3) we have been able to show that two such protease activities (of carboxypeptidase and caseinase) are compartmentalized within the protein bodies of two different forms: one in the proteinaceous matrix and the other one bound to the membrane of these structures; both are obviously responsible for autolysis within protein bodies.

The present work deals with the location of these two protease activities in the membrane of the protein body (internal or

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external) and the autolysis that these membrane activities, together with the protease activity in the proteinaceous matrix, confer upon protein bodies previously isolated from lentil cotyledons. The effect on this autolytic activity of both excision of the embryonic axis and the aplication of 6-benzylaminopurine (BA) and kinetin (KIN) is also examined.

Materials and Methods

Plant material.— Seeds of *Lens culinaris*, Medik cv Castellana were soaked for 20 min in a disinfectant solution (0.1 Captan) at room temperature. Seeds were allowed to germinate at 80 % relative humidity and 20 °C on glass plates, wrapped in a layer of moist filter paper in glass-covered seed trays for 3 and 7 days. The germination medium contained 7.5 Units/ml of acucilin to avoid possible bacterial contamination and, in some cases, 0.1 mM BA or KIN. When the embryonic axis was excised, the cotyledons with the testa were taken as starting material.

Protein bodies were extracted from cotyledons according to the method, slightly modified, of HUANG and BEEVERS (14) and described in a previous paper (2).

Globulins were extracted from ungerminated seeds (5) and from protein body extracts (22).

Assays.— Protein content was assayed by the Coomassie blue technique (8).

Alcohol dehydrogenase was used as the marker for soluble cytosol (24). Its activity was determined in the different steps of protein body extraction (homogenate of cotyledons, cytosolic-soluble material and protein body extracts) according to the method of RACKER (23).

Carboxypeptidase activity was evaluated using N-carbobenzoxy-phenylalanine as substrate (18). Enzyme activity was expressed as the increase in absorbance at 340 nm.

Aminopeptidase activity was determined with L-leu-β-naphthylamide as substrate (10).

Caseinolytic activity was determined by measuring the trichloroacetic acid (TCA) soluble material by the amount of peptides released at 37 °C in a reaction mixture containing 1 % W/V casein as substrate (15). Enzyme activity was expressed as units of activity; namely the amount of enzyme that would increase absorbance at 280 nm 0.001 per minute.

Autolytic activity was determined as follows: Sonicated protein body extracts were treated with 10 mM phosphate buffer, pH 7.0 containing 1 M NaCl and 1 % W/V Triton X-100 for one hour. The suspension was then dialyzed against the same buffer but free of Triton X-100. All these steps were carried out at 4 °C. A volume of 0.2 ml of the dialyzed solution was incubated for 4 hours at 37 °C at several pH values, using 50 mM citrate-phosphate buffer for the 3.0-7.0 range and 50 mM tris-HCl buffer for the 7.0-9.0 range. Undigested proteins were discarded as 8 % W/V TCA precipitate and the α-nitrogen released was measured by the ninhydrin reagent (19). Autolytic activity was expressed in μg of α-nitrogen released per seed at 37 °C for one hour by a straight glycine standard taken under the same conditions.

To study the location of the protease activities in the protein body membrane (internal or external), the following experiment was performed: one ml of the protein body extract (equivalent to 25 cotyledons) was suspended in the isotonic homogeneization medium (2) and then treated for 40 s with 300 μCi of ^{125}I in the presence of 10 μl of 0.2 % W/V chloramine T. The reaction was halted by adding 2 μl of 0.3 % W/V sodium metabisulphite and 5 μl of 16.6 % W/V potassium iodide.

The protein bodies were then immediately sonicated and dialyzed against water for 2 days at 4 °C. To identify casei-

nolytic activity, the dialyzate was passed through a Sepharose Cl 4B column (90 × 2 cm) previously equilibrated with 100 mM phosphate buffer, pH 7.5, containing 0.2 % W/V of bobine serum albumin and performing elution with the same buffer at a flow rate of 12 ml/h, determining both caseinolytic activity and the radioactivity present in the eluates. In order to localize the carboxypeptidase activity, electrophoresis was performed in 7 % polyacrylamide gel (7) using 25 mM Tris-borate buffer, pH 8.2, for the run. The gels were cut into 1 mM thick discs which were equilibrated in 50 mM acetate buffer, pH 5.2, containing 5 mM EDTA over 2 hours after which carboxypeptidase activity and radioactivity were measured in each of them. The results were compared with those obtained after performing labelling with previously sonicated protein bodies.

Results and Discussion

Isolation of protein bodies.— The study of the isolated protein bodies and the variations taking place within them during germination involves two important problems: the first lies in setting up a technique for the extraction of protein bodies whose recovery index does not have to vary when they are extracted at different germination times. The relationship between total globulins and globulins from the protein bodies could be an approximation to that recovery index (table I). Its value changes very little when protein bodies are isolated at 0, 3 and 7 days of germination. The second problem consists in obtaining protein body extracts that are uncontaminated by other cytoplasmic material. In the present work ethanol dehydrogenase activity was used as a marker for soluble cytosol material in cotyledon cell (23). The cytosolic contamination calculated according to this enzyme was less than 12 % (fig. 1).

Table I. *Relationship between total globulins and globulins present in protein bodies.*
Values are the means ± S.D. of three separate experiments with four determinations in each.

Germination (days)	Total globulins (A) ($\mu g \cdot seed^{-1}$)	Globulins from protein bodies (B) ($\mu g \cdot seed^{-1}$)	Index (A/B)
0	83.08 ± 2.51	13.23 ± 0.52	6.28
3	70.07 ± 0.17	9.87 ± 0.65	7.10
7	62.85 ± 2.15	8.53 ± 1.13	7.37

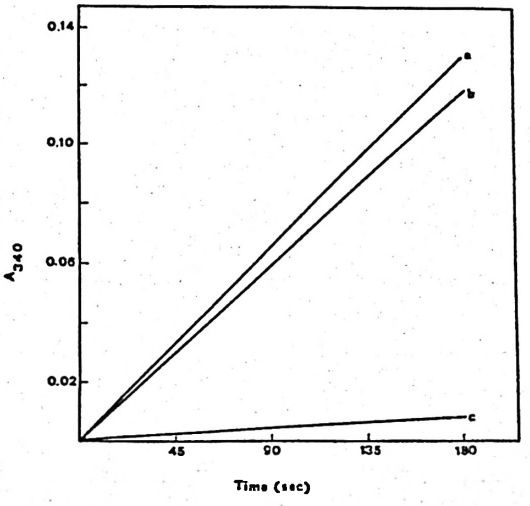

Fig. 1. *Alcohol dehydrogenase activity (kinetics of NADH formation).*
a: homogenate of cotyledons, b: cytoplasmic-soluble material and c: protein body extracts.

Autolysis of protein bodies.— Protein bodies isolated from ungerminated lentil seeds exhibited a low degree of autolytic activity which increased markedly as germination progressed (fig. 2). These changes were similar to those observed for *in vivo* degradation of reserve proteins in protein bodies from seeds of the same species and were easily detected with electronic microscopy (2). Autolysis occurred over a wide pH range, with a maximum at pH 6.5. A shoulder appeared at acid pH values, suggesting the presence of a pro-

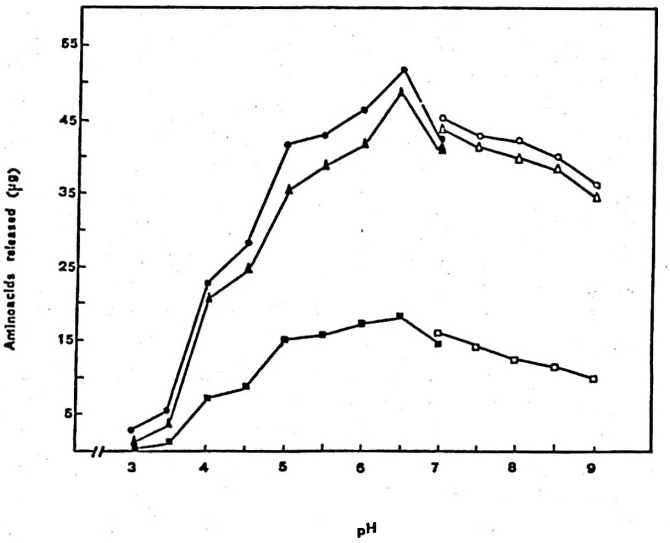

Fig. 2. *pH-dependent changes in autolytic levels in protein bodies during germination of lentil seeds.* (■, □) ungerminated seeds (●, ○), 3 day old seedlings and (▲, △), 7 day old seedlings. Buffer used: 60 mM citrate-phosphate (pH 3.0-7.0); 50 mM tris-HCl (pH 7.0-9.0). Values are the means of two separate experiments with five determinations in each. The standard deviation of the indicated values do not exceed 12 %.

teolytic activity with a maximum between 3.0 and 5.0. The proteases in lentil seed protein bodies have been purified and characterized (1) as showing carboxypeptidase activity with a maximum at pH 5.5 and caseinolytic activity peaking at pH 6.5. Both proteases show activity (ca 20 %) at basic pH values which would explain the autolysis found at this pH range. In the castor bean, NISHIMURA and BEEVERS (21) described a similar increase in the autolytic levels of protein bodies during seed germination. These authors proposed a pH range for autolysis from 3.5 to 6.5 with a maximum activity at pH 5. In this kind of seed no autolytic activity at basic pH values was detected. In our case, the autolysis at basic pH values cannot be due to contamination of basic and cytosolic aminopeptidases since this activity is not present in protein body extracts (1).

The extent of autolysis of protein bodies was partially reduced by excision of the embryonic axis. Application of both cytokinins (BA or KIN) during the incubation of seeds devoid of their embryonic axes was able to increase the level of autolysis inside the protein bodies. This increase varied between 32 and 50 % depending on the cytokinin used through the experiment (table II). Recently, it has

Table II. *Autolytic levels of protein bodies from lentil seeds.*

Results are expressed as µg aminoacids released to the media without application of exogenous substrates for one hour ± S.D. Values are the means of two separate experiments with five determinations in each.

Seeds	Germination	Activity		
		Standard	0.1 mM BA	0.1 mM KIN
With axis	3	53±1.50	69±1.75	66±1.75
	7	47±1.50	50±1.50	52±1.25
Without axis	3	48±1.75	72±1.75	70±2.50
	7	44±0.75	68±1.50	65±1.23

been observed that of the two protease activities detected in protein bodies (carboxypeptidase and endoprotease, the latter evaluated as caseinase) only the carboxypeptidase activity is negatively affected by excision of the embryonic axis (3). It is therefore possible that the effect of the embryonic axis on the degree of autolysis of the protein bodies could be mediated by carboxypeptidase activity and the normal development of this activity would be regulated by the endogenous level of cytokinins as has been previously suggested (3). Stimulation of autolysis in protein bodies by cytokinins, probably due to an enhanced biosynthesis of proteases (17), has also been described in excised watermelon cotyledons (11).

Location of proteases in the protein body membrane.— As reported above, autolysis in protein bodies is mainly due to the presence in these structures of proteolytic activity previously characterized as caseinolytic (2) and carboxypeptidase (3) activities. These activities can be found in both the proteinaceous matrix and protein body membrane. The latter represents 24 % of the carboxypeptidase activity and 60 % of the caseinolytic activity. In order to elucidate whether this latter activity also contributes to the degree of autolysis in protein bodies, its location (internal or external) in the protein body membrane was investigated by labelling intact and broken (sonicated) protein bodies with ^{125}I. The radioactivity peaks and enzyme activity were seen to coincide when the treatment was carried out after breakage, with respect to both the carboxypeptidase and caseinolytic activities (fig. 3). This would imply that both enzymes are located on the inner surface of the membrane, thus contributing to proteolysis inside the protein body.

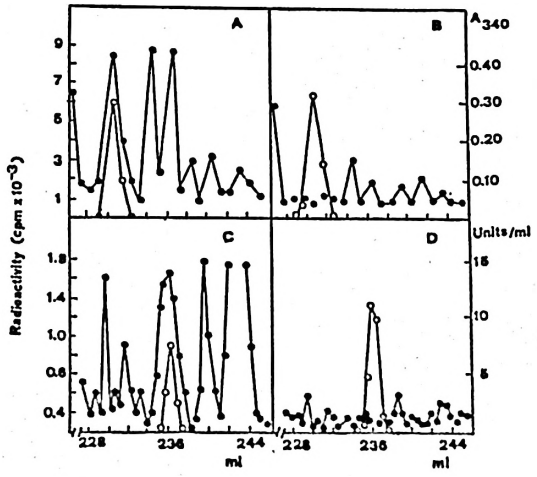

Fig. 3. *Localization of protease activities on protein body membrane.*
A and B (o — o) carboxypeptidase activity in 7 % acrylamide gel. C and D (o ___ o) caseinolytic activity in sepharose Cl 4B elution. A and C, labelling of broken protein bodies. B and D, labelling of intact protein bodies (• — •) radioactivity.

Resumen

Se aíslan cuerpos proteicos de semillas de lenteja (*Lens culinaris* Medik) con actividad autolítica que aumenta conforme avanza la germinación. Esta capacidad autolítica, activa en un amplio margen de pH, es máxima a un pH 6.5. La excisión del eje embrionario reduce parcialmente la capacidad autolítica de estos orgánulos y la aplicación de cualquiera de las citoquininas ensayadas, 6-bencilaminopurina y kinetina, incrementa los niveles de autolisis independientemente de la presencia o ausencia del eje embrionario. Además, se comprueba que la actividad proteolítica unida a la membrana del cuerpo proteico, se localiza hacia el interior del orgánulo, en contacto con el resto de la masa proteica, por lo que es parcialmente responsable de esta actividad autolítica.

Palabras clave: *Lens culinaris*, Citoquininas, Autolisis, Proteasas.

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