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Proteolytic Activities in Seeds of *Vigna unguiculata* (L.) WALP.

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Abstract. Proteolytic activities were studied in cotyledons of germinated and mature dry seed and axis of mature dry seed of *Vigna unguiculata* (L.) WALP. cv. Seridó using the following substrates: hemoglobin (pH 3.5), casein (pH 6.0), LPA (pH 7.0), and BAPA (pH 7.6). During the germination period (six days) examined, the total activities of LPA-ase and BAPA-ase decreased progressively in cotyledons corresponding to protein depletion. Consequently, the specific activities of the above proteases remain practically constant. In the case of caseinase and hemoglobinase, total activities increased in the cotyledons up to the third day of germination. Thereafter there was a decrease in these activities (total) but an increase in specific activities. Higher values than in the cotyledons for all the activities were obtained with the axis of mature dry seed. A 25 to 50 % ammonium sulfate fraction of a buffered extract from mature dry seed was used as source of enzyme for all the substrates used. Sephadex G-100 chromatography of the 25 to 50 % $(NH_4)_2SO_4$ fraction gave two main peaks which correspond to relative molecular mass of 100 000 and 60 000 and contained all the activities.

Protein bodies, storage proteins, and proteolytic enzymes are considered the three main components involved in the mobilization of reserve proteins of the seeds during germination (ASHTON 1976). The different levels of activity, and a very high complexity among the proteolytic enzymes studied, suggest that the data available presently are not satisfactory to ascribe the precise nature and function of proteolytic enzymes during germination (DECHARY 1970, RYAN 1973). Most seeds contain more than one proteolytic enzyme and although many of them have been considered as responsible for the breakdown of storage proteins this may be only the simplest function (MAYER and SHAIN 1974).

Experiments concerning proteolytic enzymes in leguminous seeds of the genus *Vigna* have been carried out mainly with *Vigna radiata* (CHRISPEELS and BOULTER 1975, HARRIS *et al.* 1975, BAUMGARTNER and CHRISPEELS 1977) and *Vigna unguiculata* (SEMETAITE 1962, ABEBONA 1973, ROYER *et al.* 1974, PRISCO *et al.* 1975, ROYER 1975).

The present paper shows the results of measuring the activities of some proteolytic enzymes in cotyledons and embryonic axis of mature dry seeds

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and cotyledons of germinating seeds of *Vigna unguiculata* (L.) WALP cv. Seridó (previously classified as *Vigna sinensis*). The preliminary objective of our work was to establish the conditions for further separation and purification of the enzymes from mature dry seeds.

MATERIAL AND METHODS

Seed Material

Seeds of *Vigna unguiculata* (L.) WALP. cv. Seridó provided by the seed bank of the Federal University of Ceará, Fortaleza, Ceará, Brazil, were used in all experiments.

Chemicals and Substrates

Bovine serum albumin (twice crystallized) was obtained from Nutritional Biochem. Corp., USA. Hemoglobin substrate powder OAA was from Worthington Biochem. Corp., USA. Casein (Hammarsten) was a preparation from E. Merck AG, Darmstadt. LPA and BAPA were from Sigma Chem. Co., USA. Sephadex was purchased from Pharmacia Fine Chemicals. All other reagents were of analytical grade.

Germination Conditions

Seeds were immersed for 5 min in commercial bleach (sodium hypochlorite solution containing 5.2% of active chlorine), rinsed several times with distilled water, planted in moist vermiculite (two volumes of vermiculite to one volume of water), and grown at a temperature of 28 °C in the dark. Cotyledons were removed at one-day intervals and used for protein extraction.

Protein Extraction

Dry seeds were milled in a Wiley mill through a 40-mesh screen. The proteins were extracted with aqueous 1% (w/v) NaCl in a meal solvent ratio 1 : 10 (w/v) for 3 h at 26 °C with occasional stirring. The mixture was centrifuged at 11 000 $\times g$ for 30 min at 4 °C. The precipitate was discarded and the supernatant was used as the enzyme source to establish the conditions for enzymatic assays in initial experiments. Extracts were also prepared with cotyledons of germinated and mature dry seeds. In this case seed coats and axis were removed and the cotyledons were ground in a mortar with cold 0.1% (w/v) NaCl in 0.02 M Na-K-phosphate buffer, pH 7.6, using 10 ml of medium per g of fresh tissue. The homogenate was centrifuged as described above, and the supernatant used in enzymatic assays. When the axes were used for protein extraction the volume of the buffer was 20 ml per g of tissue.

Protein content of the extracts was determined by the micro biuret method (GOA 1952) with bovine serum albumin as standard.

Ammonium Sulfate Fractionation

Extracts of mature dry seeds, prepared with phosphate buffer pH 7.6, in a meal solvent ratio 1 : 5 (w/v) was used for fractionation with ammonium

Abbreviations used: L-leucine-*p*-nitroanilide (LPA); α-N-benzoyl-DL-arginine-*p*-nitroanilide (BAPA); trichloroacetic acid (TCA).

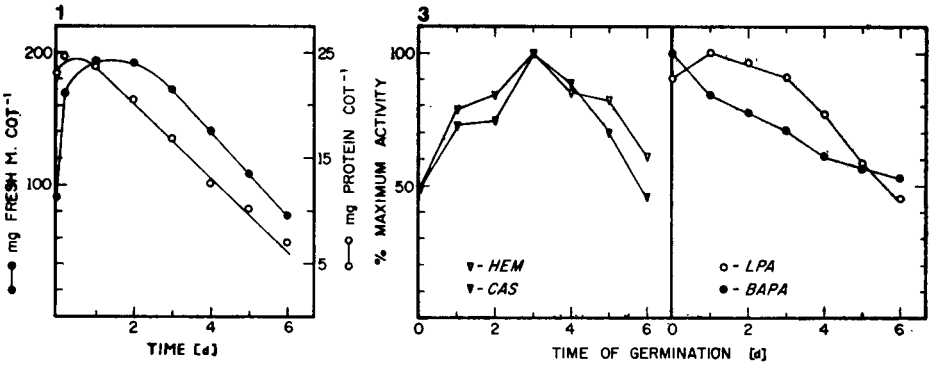

Fig. 1. Fresh matter (●) and extracted protein content (○) of cotyledons of germinated and mature dry seeds. Seeds were planted in moist vermiculite and grown in the dark at 28 °C.
Fig. 3. Total proteolytic activities in cotyledons of germinated and mature dry seeds. The activities were determined at pH 3.5 (hemoglobin), 6.0 (casein), 7.0 (LPA), and 7.6 (BAPA) according to the standard conditions described in the text.

sulfate. Solid ammonium sulfate was gradually added to the extract to 25%saturation and, after 3 h (26 °C), the precipitate was removed by centrifugation (11 000×g, 30 min, 4 °C). The resulting supernatant was used for the next precipitation to 50, 75, and 100% $(NH_4)_2SO_4$ saturation. Each of the precipitates obtained were resuspended and dialyzed against the same buffer and named F 0/25, F 25/50, F 50/75, and F 75/100. The protein content and the proteolytic activities were determined in all the ammonium sulfate fractions.

Caseolytic Activity

Heat denaturated 1% (w/v) casein in Na-K-phosphate buffer, pH 6.0 to 7.6, and Na-borate buffer, pH 8.0 and 9.0, was used as substrate. In the routine assay 1.0 ml of suitable diluted extract was incubated with 5.0 ml of 1% (w/v) casein at pH 6.0, for 30 min at 50 °C. The reaction was stopped with 1.0 ml 40% (w/v) TCA solution. The suspension was allowed to stand for 30 min and filtered. The filtrate was neutralized and reacted with Folin phenol reagent (0.5 ml), after alkaline copper treatment (5.0 ml) (Lowry

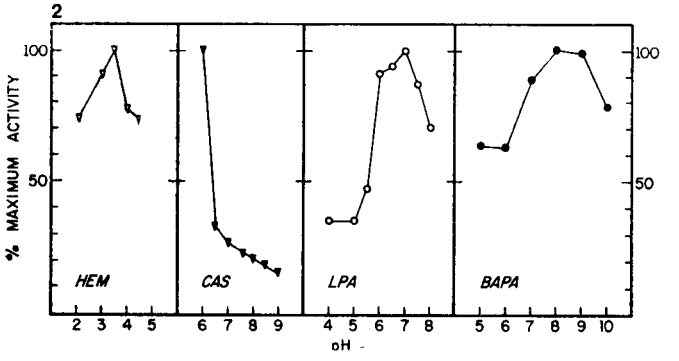

Fig. 2. pH profiles of hemoglobin (△), casein (▲), LPA (○), and BAPA (●) hydrolyzing activities present in extract of mature dry seed.

TABLE 1
Fresh matter, extracted protein content and proteolytic activities in cotyledons and axis of mature dry seeds

	Cotyledon	Axis
Fresh matter (mg/part)	98.9	3.9
Protein extracted (mg/part)	0.26	0.46
Caseinase	1.4	1.9
Hemoglobinase	1.0	4.3
LPA-ase	2.6	14.3
BAPA-ase	0.5	2.7

All the data are expressed on fresh matter basis. Caseinase and hemoglobinase are expressed in UA mg part^{-1} min^{-1} ($\times 10^3$). LPA-ase and BAPA-ase are expressed in μmol mg part^{-1} min^{-1} ($\times 10^3$).

et al. 1951). The absorbance at 750 nm was taken as a measure of the caseolytic activity. One unit of activity (UA) corresponds to the change in absorbance of 0.1.

Hemoglobin Splitting Activity

Hemoglobin was previously dissolved in 0.06 N HCl and made up to volume with Na-acetate buffer, pH 2.5 to 4.5. The ratio of HCl to buffer was controlled so that every substrate solution was at the desired pH. In the standard assay a mixture of 5.0 ml of 1% (w/v) hemoglobin in Na-acetate buffer, pH 3.5, and 1.0 ml of the extract suitably diluted was incubated for 60 min at 40 °C. The other conditions were the same as described for caseolytic activity.

LPA and BAPA Hydrolysing Activities

The method used was essentially that described by ERLANGER *et al.* (1961) and modified by BEEVERS (1968). The substrates were previously dissolved in 1.0 ml of dimethylsulfoxide and made up to volume (100 ml) with buffer to a final concentration of 3×10^{-4} M for LPA and 5×10^{-4} M for BAPA. The solutions were freshly prepared with Na-citrate buffer, pH 4.0 and 6.0, Na-K-phosphate buffer, pH 7.0 and 8.0, and Na-borate buffer, pH 9.0 and 10.0. The reaction mixture consisted of 2.5 ml of the substrate solutions plus an aliquot of the extract and buffer to make a total volume of 3.0 ml. The mixture was incubated for 15 min at 40 °C, and the reaction was stopped with 1.0 ml of 30% (w/v) acetic acid. If a precipitate was formed it was removed by centrifugation. The absorbance of the released dye was measured at 410 nm. The activity was expressed in μmol of substrate hydrolyzed using the absorption coefficient of 8 800.

Substrate and enzyme were incubated separately as control samples, in all enzyme assays, in order to correct for the autodigestive activity of the extract. All the proteolytic activities determinations were carried out using five different dilutions of the extract.

Gel Filtration

A Sephadex G-100 column (2.5 × 40 cm) was equilibrated with 0.1 NaCl in Na-K-phosphate buffer 0.02 M pH 7.6. The samples were applied in 10%

(w/v) sucrose in buffer and eluted with the same buffer. Fractions of 3.0 ml were collected at a flow rate of 30 ml h^{-1}. Protein content of the column effluent was determined by the absorbance at 280 nm. The molecular weights were estimated by the method of DETERMANN and MICHEL (1966).

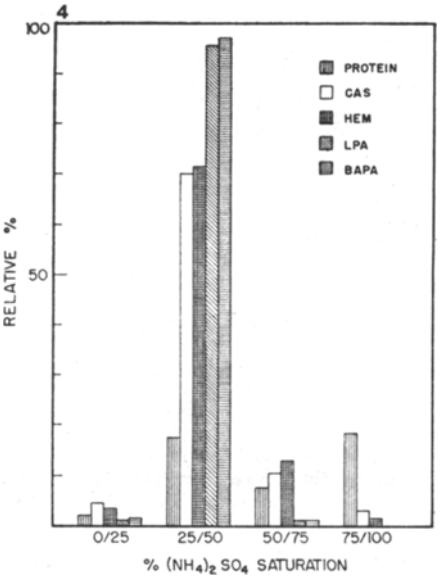

Fig. 4. Protein content and proteolytic activities in the fractions obtained by precipitation with ammonium sulfate of the extract of mature dry seed. The data are expressed considering the initial extract as 100 %.

RESULTS

The decrease in fresh weight of cotyledons and in Na-K-phosphate 0.02 M pH 7.6 soluble protein (Fig. 1) during germination are in agreement with previous data (PRISCO *et al.* 1975).

The pH optima found for the different substrates under the conditions used were: 3.5 for hemoglobin, 6.0 for casein, 7.0 for LPA, and 8.0 for BAPA (Fig. 2).

To determine the optimum assay conditions, crude extract of seed meal was used as the enzyme source. The temperature, time, substrate concentration, and dilution of the extract used in the assays were determined in the usual way. The optimum conditions are those described for the routine assays under material and methods. When the extracts were prepared using the relation 1 : 5 (tissue: extraction medium) the dilutions were: four times for caseinase and hemoglobinase activities, five times for BAPA-ase, and twenty-five times for LPA-ase. Absorbance above 0.300 at 750 nm was not taken into account for the estimation of the activity with 1% (w/v) casein solution. Above that value, the increase in protein in the reaction volume does not result in proportional increase in absorbance. The reason for this may be the presence of endogenous inhibitor. Different behaviour was found when LPA and BAPA were used as substrates.

Separate assays were carried out on axis and cotyledons from mature dry seeds. The activities under study were found in both parts (Table 1). The activities when expressed per mg of fresh matter tissue show higher values for the axis. The same was observed when the activities were expressed per mg of protein, except in the case of caseinase. LPA-ase activity

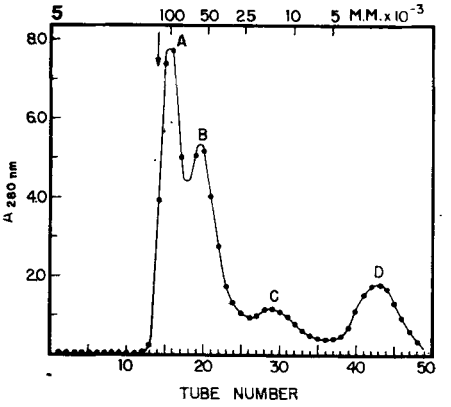

Fig. 5. Sephadex G-100 gel filtration of the fraction F 25/50 in Na-K-phosphate buffer (0.02 M pH 7.6). Three ml fractions were collected at a flow rate of 30 ml h^{-1}. All fractions were assayed for proteolytic activities by the standard procedures described in the text.

is about five times higher than BAPA-ase in the same part, and five times higher in the axis than in the cotyledons. The chromatography of the extracts from axis and cotyledons through Sephadex G-100 column are similar (results not shown).

The cotyledons from mature dry seeds and from a sample of seedlings at various stages of germination up to six days were used to study the changes in the levels of the activities. The BAPA-ase and LPA-ase activities (Fig. 3) decrease with germination time, thus following a similar pattern to protein content. The result is that the activities are practically constant when expressed per mg of protein. The patterns for caseinase and hemoglobinase activities (Fig. 3) are different from those obtained for BAPA-ase and LPA-ase.

An attempt to separate the enzymes responsible for each one of the activities detected, was made by submitting the extract of mature dry seeds to ammonium sulfate precipitation. It was possible to obtain a fraction precipitated between 25 to 50% saturation that corresponds to 17% of the total protein extracted and to 45% of the total protein precipitated and contains more than 70% of all the activities initially present in the extract (Fig. 4). The fraction, named F 25/50, when submitted to gel filtration on Sephadex G-100 at pH 7.6, shows four peaks (Fig. 5). Peak A (molecular mass *ca.* 100 000) contains the activity against casein and hemoglobin, and peak B (molecular mass *ca.* 60 000) with BAPA-ase and LPA-ase activities. Peaks C and D are free of the activities tested.

The fraction F 25/50 shows high stability when stored at −20 °C for more than one year. The protein fractions with activity against BAPA and hemoglobin were purified from F 25/50 (papers submitted for publication).

DISCUSSION

The results show that mature dry seeds of *Vigna unguiculata* (L.) WALP. cv. Seridó contain enzymes able to hydrolyze different substrates (casein, hemoglobin, BAPA, and LPA) commonly used in the study of the proteolytic enzymes. The levels of the activities, when expressed in terms of protein content, are higher in the embryonic axis than in cotyledons of mature dry seeds. This was observed by ROYER (1975) for caseinase and BAPA-ase activities in *Vigna unguiculata*.

During germination the behaviour of the activities suggests that at least four groups of proteolytic enzymes are present in cotyledons. Two groups of proteases, including those responsible for the hydrolysis of casein and hemoglobin, showing an increased activity up to the third day and a decrease until the sixth day. Two groups of peptidases, formed by enzymes presenting a gradual decline of activity during germination, able to hydrolyze BAPA and LPA. Although casein and BAPA have been used as substrates for trypsin-like enzymes, they seem to be hydrolyzed by different enzymes in plant tissue.

It is not clear whether the enzymes, responsible for the activities detected, are directly involved in the mobilization of the storage proteins, in the first stages of germination. The physiological role of these enzymes in mature dry seeds remains unknown.

Considering that the main objective of this work was to establish the conditions for further purification of the referred enzymes, we are led to conclude that the fraction precipitating between 25 to 50% saturation with ammonium sulfate, obtained from mature dry seed, presents a good recovery of the activities and can be used as a first stage of purification.

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BOOK REVIEW

KESSELL, S. R.: GRADIENT MODELING: RESOURCE AND FIRE MANAGEMENT. — Series on Environmental Management. Vol. 1. — Springer Verlag, New York—Heidelberg—Berlin 1979. 432 pp. 175 figs. DM 79.50; US $ 43.80.

The book reviewed is the first volume of the new Springer series on environmental management, dedicated to the solution of practical, serious environmental problems.

Gradient modeling is a new approach to scientific resource management, developed by the author. As he writes "it is nothing more than the linkage of multidimensional gradient analysis with a site-specific stand inventory and appropriate computer software". This method aims to construct a resource management computer information system for managerial use. Such a system was constructed in the Glacier National Park. It is possible in several minutes to describe the environmental, vegetational (*e.g.* species composition, diversity) and fuel conditions of arbitrary standpoint in the area under consideration and/or to predict fire behavior (*e.g.* rate of spread, flame length) and/or to predict the postfire successional development of the stand. The "conversation" with the computer is held in very simple language using common English words; knowledge of FORTRAN (in which the computer program is written) is not necessary; the system is acknowledged to be foolproof.

The development of this information system is described including basic data collection, data analysis, construction of gradient models of distribution of particular species and construction of succession model, of fire behavior models and the linkage of components. Related problems are discussed from both managerial and theoretical points of view. The methods used in field sampling, show some common features with the Braun-Blanquet methodology. Stands were selected to obtain representative samples from all possible combinations of environmental conditions (consequently non-randomly) and their area was stated according to the homogeneity of environment and of vegetation; on the other hand quadrates for undestory species composition sampling were located at random within the stand; herbs and shrubs were recorded by absolute cover for each quadrate on an eight-point scale, not much different from the Braun-Blanquet scale. Special attention is given to fuel properties. Furthermore, the data from shrub dimension analysis, fuel moisture and microclimate study, false color infrared aerial photography and fire behavior monitoring are employed. Properties of vegetation are related to the following gradients: elevation gradient, time since burn gradient (over 95% of Glacier forests below 1500 m have burned within the past 400 years), topographic-moisture gradient, drainage area gradient, primary succession gradient (Glacier vegetation was all developed during the past 10 000 years, since the last ice retreat) and Alpine wind-snow exposure gradient, and to categories of disturbance. It is surprising that the influence of the ground is ignored at all.

Special attention is paid to the role of fire and to the fire management. The total fire suppression policy is thoroughly critised. Fire is the most important and natural determinant of the structure, species composition and other community characteristics of the Glacier forests. Their total suppression hinders the maintenance of many tree species and community types (and consequently leads to the fall of ecological diversity).

In the last part of the book several subsequent applications of gradient modeling are described.

The book contains a great deal of useful information for both researchers and managers. It is written in a easily understandable manner and can serve as a textbook. It shows how to use some of the methods which have been used mostly in basic research so far — the ones in resource management.

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