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PROTEIN BREAKDOWN AND PROTEASE PROPERTIES OF GERMINATING MAIZE ENDOSPERM

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Key Word Index—*Zea mays*; Gramineae; maize; germination; protease; endosperm protein hydrolysis.

Abstract—Protein breakdown during germination of maize at 28° is closely correlated with the appearance of protease activity. In the first 2 days of germination, a slight disaggregation of only G_3 glutelins into more simple elements (albumin-globulins) can be observed. Between 2 and 2.5 days, there is extensive breakdown of all protein fractions, the rate of which coincides with the rate of appearance of proteolytic activity. After 2.5 days these phenomena slow down and the bulk of the endosperm proteins disappears. Three acid proteases in endosperm extracts of germinated grain (P_{11}, P_{21} and P_{22}) have been isolated by affinity chromatography and gel filtration, and partially characterized. P_{11} (MW 40000) which is present in the ungerminated grain, cannot hydrolyse prolamins and is insensitive to reducing agents. P_{21} (MW 36000) and P_{22} (MW 12000), which appear on day 3 of germination, can degrade prolamins *in vitro*. Reducing agents enhance their activity and prevent their aggregation or denaturation. Comparative assays with different substrates suggest our enzyme preparations are principally endotype proteases with little contaminating carboxypeptidase activity.

INTRODUCTION

The essential substances used for synthetic processes in the growing embryo of germinating cereal grains originate in the hydrolysed endosperm reserves. In particular, endogenous nitrogen results from the hydrolysis of storage proteins by proteolytic enzymes. Proteases break down proteins into various peptides which are further reduced to amino acids by peptidases and exopeptidases.

In recent years, only a few reports have been published on the relationship between protease appearance and hydrolysis of total protein [1, 2] or storage protein fractions [3] during the germination of cereal grain. Studies on protease properties in crude extracts [2, 4–6] and on partially purified preparations [7–10] have also been published for various species: barley [4, 5, 8], maize [2, 3, 6, 10, 11], oat [1], sorghum [7, 12] and wheat [9]. These studies refer to selected protease fractions which are generally the major ones and the easiest to isolate. In spite of their origin, these enzymes have several properties in common such as an optimum pH activity between 3 and 4 and similar sensitivities to oxidation and sulphhydryl blocking agents. On the other hand, the substrate specificities of these proteases have not been well characterized. Some authors have concluded that they are carboxypeptidases [9] and only Garg *et al.* [7] succeeded in purifying sorghum protease which specifically hydrolysed glutamyl and aspartyl peptide bonds.

In earlier investigations, we proposed a selective extraction method of maize grain proteins on the basis of their solubility, which permitted a classification of these proteins by amino acid composition [13], relative accumulation rate in developing grain and morphological location [14, 15]. Using this selective extraction, changes in well-defined endosperm protein fractions were followed during early germination. The present paper deals with correlation between protease activity and endosperm protein breakdown. We also describe the isolation and properties of protease components from germinated endosperm.

Table 1. Amounts of nitrogen in maize seedlings

Stages		Whole grain Nitrogen PN*	NPN	TN	Part of grain: Endosperm Nitrogen PN	NPN	TN	Embryo Nitrogen PN	NPN	TN
Ungerminated		5.40	0.47	5.87	4.72	0.27	4.99	0.68	0.20	0.88
Days germinated	2	5.30	0.65	5.95	4.61	0.35	4.96	0.69	0.30	0.99
	3	3.98	1.51	5.49	2.93	0.71	3.64	1.05	0.80	1.85
	5	2.83	2.05	4.88	1.47	0.79	2.26	1.36	1.26	2.62
	7	2.70	2.88	5.58	0.66	0.3	0.96	2.04	2.58	4.62

* PN: Protein nitrogen; NPN: non-protein nitrogen; TN: total nitrogen.

The values are expressed as mg N_2 per grain or grain part. The non-protein nitrogen was measured on salt-soluble extract after trichloroacetic acid precipitation of albumin-globulins.

RESULTS

Proteolytic activity and protein metabolism in the seedlings

Changes in total nitrogen (TN), protein nitrogen (PN) and non-protein nitrogen (NPN) in germinated grains and their two main morphological parts are summarized in Table 1. No important changes were observed before 2 days. The TN of the seedlings decreased from day 2 to 5, and then increased from day 5 to 7. These variations can be attributed to the NPN diffusion between seedlings and the water of the medium. In the endosperm, the amounts of TN and PN steadily diminished after 2 days and the NPN value remained low. In contrast, in the embryo all forms of nitrogen increased and the amount of NPN rose, attaining 56 % of its total nitrogen content by the 7th day.

These observations agree with our preliminary results on localization of proteases and peptidases, the latter being defined by their ability to hydrolyse synthetic substrates such as L-Leu-*p*-nitroanilide (LPA) and L-benzoyl L-Arg-*p*-nitroanilide (BAPA). Indeed most protease activity was located in the endosperm, contrary to the embryo localization of most of the peptidases defined as LPAase and BAPAase. The presence of these enzymes would explain the large amount of NPN in the embryo in spite of high levels of protein synthesis.

Proteolytic activity measured in a simple extract of the endosperm of ungerminated grain had a maximum activity at pH 3.2, whereas an extract obtained in the same experimental conditions, but from the endosperm of 3-day-old seedlings, had a maximum activity at pH 3.8. The sharply pointed and nearly symmetrical plot of activity as a function of pH suggests that the pH 3.2 activity is of secondary importance to that at pH 3.8 during germination. The latter seems to be newly synthesized as shown in Fig. 1, where changes in the level of proteases measured at pH 3.8 are plotted as a function of time. During the first 2 days, protease activity was low but constant. It increased 4- to 5-fold from day 2 to 4 with a maximum rate at 2.5 days and thereafter declined.

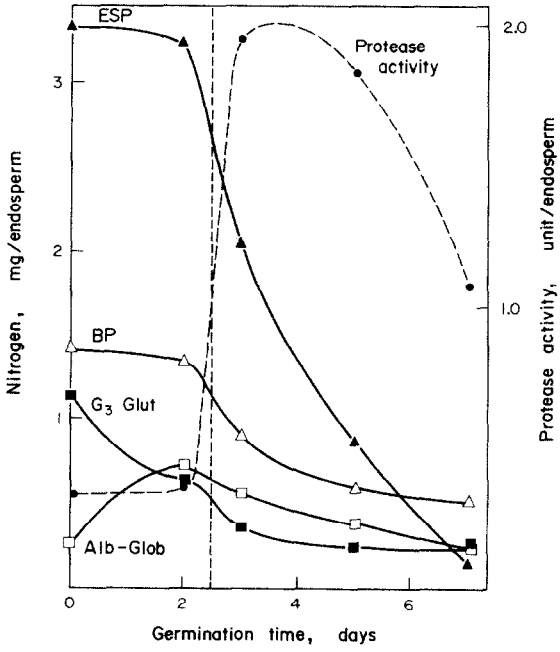

Fig. 1. Changes in protease activity measured at pH 3.8 according to Anson [18] and in protein fractions extracted according to Landry and Moureaux [13], in the endosperm during early germination. Protein fractions: Alb-Glob (albumin-globulins), G_3 Glut (G_3 glutelins), BP (basic proteins, i.e. Alb-Glob + G_3 Glut), ESP (endosperm specific proteins, i.e. zein + G_1 + G_2 Glut). The vertical line at 2.5 days indicates coincidence in time between the inflexion points of the different plots.

The PN of the endosperm has been fractionated by our improved selective extraction method [13]: albumin-globulins (Alb-Glob), zein, G_1, G_2 and G_3 glutelins (Glut). These fractions have been grouped according to the criteria defined above [13–15]. The first group, called the endosperm specific proteins (ESP), contains zein, G_1 and G_2 Glut, which are exclusively located in the endosperm; the second group, called basic proteins (BP), consists of Alb-Glob and G_3 Glut. The term 'basic' is used in the sense of fundamental as defined elsewhere [15]. Indeed these 'basic proteins' are located in all of the vegetative parts of plants.

In Fig. 1, changes in the amounts of these fractions or groups of fractions in the endosperm have been plotted as a function of germination time. ESP and BP exhibited the same behaviour as total protein (Table 1), i.e. their amounts did not change during the first 2 days, but thereafter decreased. The separately plotted changes in zein, G_1 and G_2 Glut were similar to that of their sum, i.e. ESP, and so were not represented. In contrast, in the group BP, Alb-Glob and G_3 Glut varied inversely during the first 2 days, i.e. a decline in G_3 Glut accompanied an identical increase of Alb-Glob. This temporary increase suggests either a new synthesis of enzyme in aleurone cells, or a disaggregation of G_3 Glut into Alb-Glob during grain hydration. We do not think that *de novo* enzyme synthesis can explain such a high increase of Alb-Glob. Furthermore, the appearance of Alb-Glob and protease activity are non-synchronous, therefore disaggregation of G_3 Glut is more probable (Fig. 1). The disaggregation recorded during the hydration of grain presumably corresponds to the aggregation of Alb-Glob observed during the dehydration of ripening grain [14, 15]. After 2 days germination, the two fractions Alb-Glob and G_3 Glut gradually disappeared.

Fig. 1 also shows that the plots of the breakdown of all the protein fractions or groups of fractions have an inflexion point (expression as a maximum hydrolysis speed) between the 2nd and 3rd day. This point coincides with the maximum rate of appearance of protease activity, indicating a good correlation between high protease activity and high non-specific protein degradation.

Protease studies

The properties of proteases responsible for protein breakdown were studied in endosperm extracts from 5-day-old seedlings. At this stage, there is high enzyme activity in relatively few buffer soluble proteins, which constitute the major part of Alb-Glob (Fig. 1). The 40–80% $(NH_4)_2SO_4$ protease fraction (containing 100 mg protein extracted from 54 g of endosperm power) was separated by affinity chromatography on hemoglobin–Sepharose column into unadsorbed (P_1) and fixed material (P_2). P_1 contained 75 and 20%, P_2 15 and 45–60% of proteins and protease activities of the sample, respectively. Sephadex G100 gel filtration and enzymatic

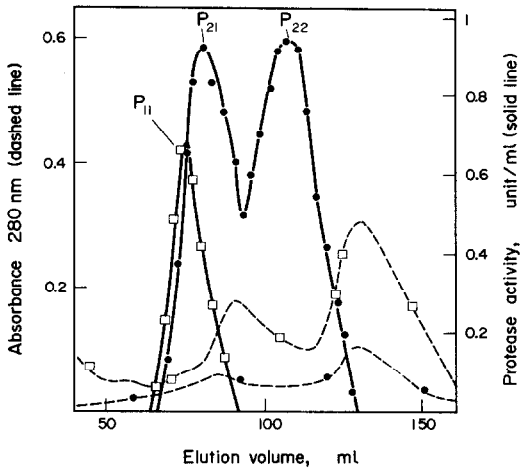

Fig. 2. Gel filtration on a 1.6 × 70 cm Sephadex G100 column of the P_1 and P_2 fractions from affinity chromatography. Protease activities were assayed at pH 3.8 by the Anson procedure [13].

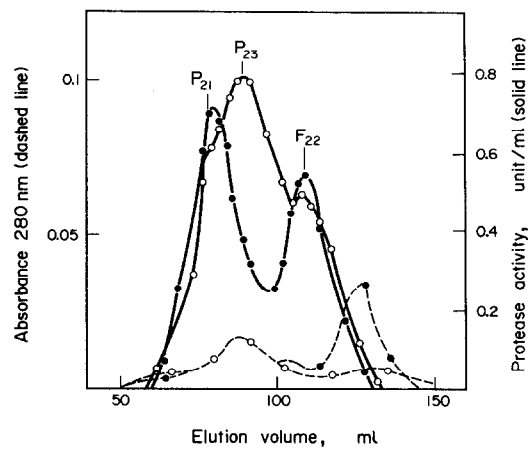

Fig. 3. Gel filtration on a 1.6 × 70 cm Sephadex G100 column of equal quantities of P_2 fraction in the presence (●—●) and in the absence (○—○) of DTT in the buffer. Protease activities were assayed at pH 3.8 by the Anson procedure [13].

assays on the effluents indicated one peak P_{11} for P_1, having a MW of 40000, identical to the proteases detected in ungerminated grain and two peaks (P_{21} and P_{22}) for P_2 with MWs of 36000 and 12000, respectively (Fig. 2).

Disc electrophoresis of these components was performed in acrylamide gels containing dispersed gliadin as substrate to identify their location after electrophoretic migration. P_{11} could not be detected because it lacked hydrolysing prolamins; P_{21} and P_{22} appeared as two active bands with R_f values of *ca* 0.18 and 0.8, respectively.

Activity assays of 40–80% $(NH_4)_2SO_4$ fractions incubated for 15 hr at 4° in buffers at various pH (3 to 8) showed that the proteases had a maximum stability in the range 4.5–6.5 pH.

The presence of dithiothreitol (DTT) in extracting buffer increases protease activity of the crude extracts. The effect of DTT on protease activity was therefore studied quantitatively and qualitatively. Removal of DTT by dialysis from the 40–80% $(NH_4)_2SO_4$ fraction (originally containing 0.5 mM DTT) resulted in a 50% reduction in the original activity, which was restored to 95% by the addition of DTT to 1 mM. P_1 and P_2 fractions were chromatographed on Sephadex G100 in the absence of DTT in the samples and in the gel. It appears that the elution profile of P_1 is not quantitatively and qualitatively changed as compared to the results obtained in the presence of DTT (Fig. 2). In contrast, the pattern of P_2 shows a new component, P_{23}, in addition to earlier isolated P_{21} and P_{22} fractions (Fig. 3). P_{23} has a MW of 24000; its existence is correlated with the presence of additional bands in the electrophoresis gels having R_f values between P_{21} and P_{22}.

Various substrates have been tested to qualitatively define the activity of P_1 and P_2, e.g. proteins (zein, gliadin and hemoglobin) and some synthetic substrates (poly and dipeptides). P_1 and P_2 fractions could not hydrolyse BAPA and LPA, and therefore contain no trypsin-like and aminopeptidase activities. Both P_1 and P_2 broke down hemoglobin, poly-Asp (MW 26000), poly-Glu (MW 48700) and all of the *N*-carbobenzoxy (*Z*)-dipeptides used (*Z*-Glu-Tyr, *Z*-Glu-Phe and *Z*-Phe-Ala) with 10 times higher activity for P_2 than for P_1. However P_2 differed from P_1 by the fact that only P_2 could hydrolyse zein and gliadin.

DISCUSSION

Our observations on the physiological processes taking place in endosperm during germination agree to some extent with those of Harvey and Oaks [3, 6]. Their experimental conditions, although differing by a daily cycle of 16 hr illumination, do not produce differences in the general breakdown of total nitrogen and protein fractions. Nevertheless, during the initial 2 days of germination these authors observed some hydrolysis of zein and glutelins (defined according to the Osborne procedure) that they interpreted as being caused by a low but unidentified protease activity. In fact, with the help of a more selective extraction method, our results suggest that during this period, a disaggregation process of G_3 Glut into Alb-Glob takes place. However the low protease activity we have detected from the onset of germination also suggests the presence of weak protein hydrolysis. This protease system presumably corresponds to the P_{11} fraction which has some properties similar to those of the proteases we isolated from ungerminated grains, e.g. a MW of 40000, insensitivity to DTT and inability to degrade prolamins.

After two days germination, Harvey and Oaks [3] also report the appearance of a protease activity, at first low, thereafter reaching a maximum at 8 days when 80% of the TN are hydrolysed. On the contrary, our results indicate a much faster accumulation of proteases reaching a maximum at 3.5 days when only 40% of TN are degraded. This difference can be attributed to differences in experimental procedures. Indeed, Harvey and Oaks [6] carry out their enzymatic assays in crude extracts isolated at pH 3.8, whereas in our work the protease activity is defined from pH 6.5 extracts dialysed at this same pH. The extraction at pH 3.8 probably causes partial denaturation of proteases. This assumption is supported by the fact that this pH value is out of the range of optimum pH stability, as we have measured it. The high correlation which is seen between the maximum rates of protease appearance and of protein hydrolysis also emphasizes the validity of our experimental conditions.

By the use of cycloheximide, Harvey and Oaks [3] conclude that after 70 hr the grain contains sufficient proteases to account for the total protein breakdown, although this amount is *ca* 50% of the total possible hydrolase (as defined by Harvey and Oaks [3]). Fig. 1 indeed shows that the amount of protease activity, reaching a maximum between 3 (72 hr) and 4 (96 hr) days, is sufficient to hydrolyse the bulk of proteins, after this period there is no more synthesis of proteases in our experimental conditions.

These proteases, which from a quantitative point of view must be assumed to belong to the P_2 fraction, have the same general properties as those reported by Abe *et al.* [10] and Fujimaki *et al.* [11], i.e. optimum pH activity at 3.8, ability to degrade prolamins *in vitro*, activity enhanced by DTT and existence of a fraction with a MW of 21 000 close to the value estimated for the P_{23} fraction. These authors, however, did not detect P_{21} and P_{22}, probably because they did not use DTT in any stage of their isolation procedure. It is probable that the absence of DTT in all the steps of protease preparation would prevent P_{21} and P_{22} from being detected because P_{21} would be denatured and P_{22} aggregated into P_{23}. In contrast, in the absence of reducing agents during gel filtration only, as shown in the Fig. 3, a similar but incomplete phenomenon would take place.

Preliminary assays with various proteins and synthetic peptides as substrates show that the protease fractions P_1 and P_2 do not have well-defined specificities. These results demonstrate that P_2 fractions, which can break down zein and gliadin trapped into gels, are the main endotype proteases responsible for prolamin and presumably ESP degradation. In contrast, P_1, which hydrolyses hemoglobin and is unable to degrade prolamins, is assumed to degrade the other protein fractions, i.e. BP. P_1 and P_2 can also hydrolyse the 5 synthetic substrates tested, which contain Glu or Asp in their molecule. At the present time we cannot be certain that maize proteases like sorghum protease [7] possess specificity towards the peptide bonds containing these amino acids. Among these synthetic substrates, 3 are *Z*-dipeptides which are used not only to study the specificity of an enzyme towards a given peptide bond, as in Garg *et al.* [7], but more often to identify carboxypeptidase activities. The degradation of the 3 dipeptides, as detected by sensitive methods [16] after 15 hr of hydrolysis with our enzymic preparations, suggests the presence of some contaminating carboxypeptidase activities. These conclusions agree with those of Burger [8] working with barley endoproteases but differ from those of Preston and Kruger [9, 17]. The latter found that their wheat protease preparation had carboxypeptidase properties. In conclusion, we agree with other studies in maize [2, 3, 6] and other cereals [4, 7, 8], that our enzymic preparations principally consist of endotype proteases with broad specificities.

EXPERIMENTAL

Plant material. Zea mays INRA 260 hybrid ($F_7 \times F_2 \times$ W 182 E) was used. Seeds were surface-sterilized in 10% $Ca(OCl)_2$ soln, imbibed for 15 hr at 10° in sterilized H_2O, then germinated at 28° in the dark on filter paper imbibed with sterilized H_2O. From seedlings harvested at different times, endosperm and germ were hand-separated, frozen in liquid N_2 and lyophilized. The finely ground material was then defatted and stored at −20°.

Reagents. Hemoglobin substrate for protease according to ref. [18] was purchased from Merck. Hemoglobin–Sepharose was prepared according to ref. [19] with modifications as described in ref. [20] from twice-crystallized beef hemoglobin (Sigma) and Sepharose 4B (Pharmacia). Gliadin and zein were purified in the laboratory.

Protein measurements. Protein content of the column eluates was monitored by measuring the *A* at 280 nm. Protein was quantitated by the method of ref. [21] using BSA as standard.

Peptidase essays. BAPAase and LPAase activities were determined by the method of ref. [22].

Proteolytic assays. The modified procedure of ref. [18] was used on the different steps of enzyme prepn with denatured hemoglobin as substrate: the reaction mixture (4 ml) contained 20 mg hemoglobin, 0.1 M citrate–Pi buffer, pH 3.2 or 3.8, and suitable dilution of enzyme. After 1 hr at 40°, 1.5 ml of 10% TCA was added and the mixture was allowed to stand for 18 hr at 4°. After centrifugation, 1 ml of neutralized supernatant was estimated for amino acids and peptides released from the *A* values at 340 nm obtained by the reaction of TNBS (2,4,6-trinitrobenzene sulphonic acid) [23]. One unit of protease activity was defined as the amount which released 1 μM of leucine equivalent per hr under standard conditions.

Protein extraction at different stages of germination. The endosperm protein fractions were extracted and quantified according to the method of ref. [13]. Amounts of protein fractions are expressed in mg N_2/part.

Protease extraction at different stages of germination. Endosperm powder (1 g) of seedlings harvested at different times was stirred for 30 min at 4° with 10 ml of Pi buffer (pH 6.5, μ0.05) 0.5 mM DTT and 5 mM di Na EDTA. The suspension was centrifuged at 40000 *g* for 15 min; the supernatant collected was then dialysed against the buffer and assayed for proteolytic activity according to ref. [18].

Preparation of proteases. Crude extract was prepared as before at 4° from endosperm of 5-day-old seedlings. Satd $(NH_4)_2SO_4$ soln containing 0.5 mM DTT was added to the extract to 40% satn and the resulting ppt. removed after 4 hr by centrifugation. The supernatant was brought to 80% satn by addition of solid $(NH_4)_2SO_4$. The ppt. was collected after 18 hr, dissolved, then dialysed for 2 hr with acetate buffer (pH 5.5, μ0.05) 0.5 mM DTT in a Bio-Fiber 50 device from Bio-Rad laboratories. Affinity chromatography was performed with a hemoglobin–Sepharose column (2.3 × 22 cm) according to the procedure of ref. [19] except that 0.5 mM DTT was added in all the buffers and that the acidic eluate was collected as described by ref. [8] into 4 M acetate buffer, pH 4.5, to increase pH and avoid enzyme denaturation. Chromatographic effluents were concd on a PM10 membrane Amicon. Fractionation was carried out by gel filtration. A column of Sephadex G100 (1.6 × 70 cm) equilibrated with 50 mM NaOAc buffer (pH 4.5), 0.1 M NaCl and 0.5 mM DTT was eluted at 20 ml/hr. MWs were estimated on a column calibrated with serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c* according to ref. [24].

Disc electrophoresis and protease detection. Disc electrophoresis and protease detection in the gel were carried out with the modified method of ref. [25]. Final composition of gel was 0.37 M Tris–HCl buffer (pH 8.9), 7.5% acrylamide and 0.05% dispersed gliadin as substrate. Electrophoresis was performed with 2 mA/tube for 2 hr. Areas of proteolytic activities were detected by 5–15 hr incubation of the gels at 37° in 0.1 M citrate–Pi buffer, pH 3.5, changed twice during the incubation period. The areas of proteolytic activities appeared clear while the background containing unhydrolysed gliadin remained diffuse.

Differences could be enhanced by light colouration of the gels. After fixation in 3% TCA, gels were stained with 0.5% amido black in 0.5 M acetate buffer, pH 4.8.

Assays on substrates. Semi-quantitative assays for enzyme activity on Z-dipeptides (Z-Glu-Tyr, Z-Glu-Phe, Z-Phe-Ala) and on polyamino acids (poly-Asp MW 26000, poly-Glu MW 48700) were performed according to ref. [16]. Enzymatic hydrolysis was conducted at 35° for 15 hr with Z-dipeptides, 2 mM at pH 5, according to ref. [26] or with polyamino acids, 0.5–1% at pH 3.8 in 0.1 M citrate–Pi buffer. Hydrolysis products were observed by TLC. Agar gel assay [6] was employed to test proteolytic activity on prolamins (gliadin and zein).

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