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MAJOR PROTEINASE HYDROLYSING GLIADIN DURING WHEAT GERMINATION

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Key Word Index—*Triticum durum*; Gramineae; wheat; purification and characterization; proteinases; cysteine proteinase.

Abstract—A proteinase, representing the bulk of the enzyme activity for the hydrolysis of gliadin, was extracted from endosperms isolated from germinated seeds (four days) and was purified by ion-exchange chromatography and preparative isoelectric focusing. The optimal pH for gliadin hydrolysis was 4.25. The M_r, determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, was 30 000; the isoelectric point was 4.5. The enzyme activity was totally inhibited by E-64 and cystatin, while inhibitors of other classes of proteinases were barely effective or ineffective. The activity was stimulated by sulphhydryl compounds. The proteinase hydrolysed to small peptides the gliadins from durum and soft wheat seeds. Other protein substrates were weakly degraded or not degraded. The proteinase appears to belong to the cysteine class and to play a key role in the initial mobilization of the main reserve protein in the starchy endosperm. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The mobilization of seed storage proteins represents one of the most important post-germinative events in growth and seedling development. In fact, hydrolysis of these reserves to soluble products supports seedling growth prior to the development of autotrophy.

In a wheat grain most of the nitrogen is found in the storage proteins, known as gliadins, which are soluble in alcohol solution. They are deposited in the starchy endosperm where they constitute up to 50% of the total proteins [1]. It is known that cereal storage proteins are degraded during early seedling growth by endo- and exopeptidases, although the cooperative role of those enzymes in protein breakdown still remains to be elucidated in many of the plant materials studied [2]. Carboxypeptidases and aspartic proteinases are already present in dry wheat seeds [3–5]. In *in vitro* experiments they showed a weak hydrolytic action on the gliadin, which increased by about 25% when both enzymes were added simultaneously [4]. However, the *in vitro* hydrolysis of the gliadin started after the first day of germination of wheat seeds, in association with the appearance of proteinase activity against this storage protein [6]. Preliminary studies conducted with crude extracts from germinated (4 days) wheat seeds in the presence of proteinase inhibitors showed that about 89% of the proteinase activity responsible for the increase in gliadin hydrolysis was of cysteine-type (EC 3.4.22) [6]. Although cysteine proteinases have been previously found in germinating wheat seeds [4, 7], they have not been isolated to a sufficiently high degree of purification for the study of their characteristics and physiological role. The present work describes the isolation and the partial characterization of a cysteine proteinase that is probably involved in the initial cleavage of gliadin to polypeptides in germinated *Triticum durum* seeds.

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RESULTS AND DISCUSSION

Purification of the proteinase

The purification of the proteinase from endosperms of germinating durum wheat is summarized in Table 1. Endosperms of 4-day-old seedlings were used as the starting material for the purification of the enzyme, since our previous work [6] showed that gliadin mobilization and gliadin activity were most prominent during this period. The fractionation of the proteins on DEAE-cellulose allowed us to eliminate most of the contaminating proteins. Initial attempts to fractionate the crude protein extract with the Rotofor cell, either by using ampholytes with a broad pH range (3–10), or with a narrow pH range (4–6) were unsuccessful. In both cases there was considerable precipitation, which started in the end compartments and then spread inward during the run. Use of glycerol and pre-focus of the pH gradient before loading the extract did not alleviate the problem. On the other hand, protein precipitation did not happen when samples fractionated on DEAE-cellulose were applied to the Rotofor cell. However, the

Table 1. Purification of cysteine proteinase from endosperms of germinating wheat

Step	Total protein (mg)	Total activity* (U)	Yield (%)	Purification (-fold)
1. Crude extract	403	2570	100	1
2. $(NH_4)_2SO_4$	225	1990	77.6	3.56
3. DEAE	61.7	1300	50.7	8.5
4. IEF	0.15	53	2.1	144

*Azocasein degrading activity was measured as described in the Experimental.

pre-focus of the ampholytes was always necessary to avoid subjecting the proteins to rapid shifts in pH, as the pH gradient was established. If this step was omitted the proteinase activity was lost both with a broad and narrow range of ampholytes. The use of the broad pH range 3–10 allowed the recovery of all the azocaseinase activity which was separated into two distinct peaks, the former with pI *ca* 4.35 and the latter with pI *ca* 5.15 (data not shown). However, many contaminants were still present in the two isolated active fractions. The use of the narrow pH range 4–6 enhanced the separation of the most acidic peak of activity (Fig. 1). The present work does not further investigate the fractions with less acidic pI after the Rotofor separation. Thus all the following data refer to the most acidic peak alone. Fig. 2 shows the SDS-PAGE profiles following crude extraction, dialysis, DEAE-cellulose and Rotofor steps while the purification of the proteinase achieved is shown in Table 1. The enzyme was purified 144-fold with a yield of 2.1%. However, the purification factor obtained was underestimated, since a substantial part of the proteinase, present in the crude extract and revealed by azocasein degrading activity, was eliminated after the Rotofor separation. It must be emphasized that several classes of endoproteinases contributed to the overall azocaseinase activity. Furthermore, the enzyme was more labile during the purification and this contributed to the high losses of activity and to the consequent low yield of the purification procedure. These characteristics of lability appear to be common to other similar enzymes isolated from cereals [8, 9].

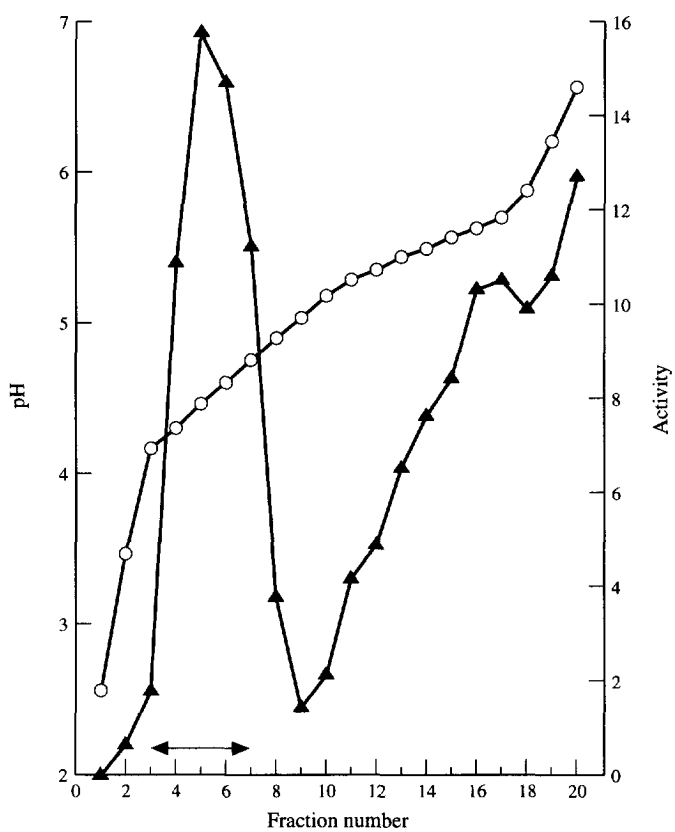

Fig. 1. Preparative isoelectric focusing of germinating wheat proteinase. pH (○) was measured immediately after collecting fractions; the enzyme activity ($Uml^{-1}\ 10^{-3}$; ▲) was measured with azocasein. The double arrow shows collected fractions.

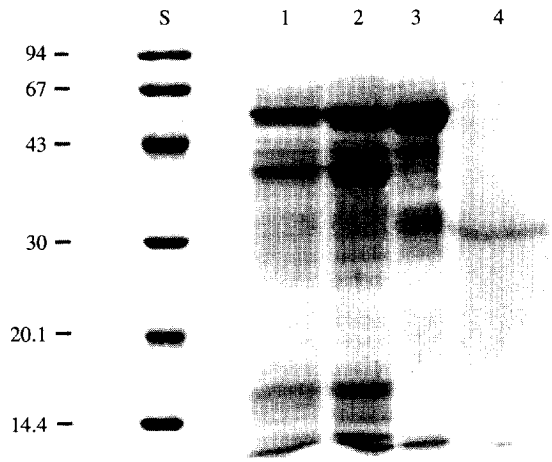

Fig. 2. SDS-PAGE of the sequential purification of proteinase from germinating wheat seeds. S represents M_r standards; lane 1, crude extract; lane 2, following ammonium sulphate precipitation and dialysis; lane 3, pooled fractions from DEAE-cellulose column; lane 4, pooled fractions following IEF separation.

Characterization of the proteinase

SDS-PAGE of the purified proteinase revealed a single polypeptide with a M_r *ca* 30 000 (Fig. 2). A closely related M_r value was obtained by submitting the enzyme to SDS-PAGE under weakly denaturing conditions, and renaturing it with Triton-X-100 (Fig. 3). Once the gel had been stained with Coomassie Brilliant Blue, the active protein appeared as a clear zone. It was completely inhibited by incubating the gel in the presence of E-64. The pI of the proteinase was acidic and was calculated to be *ca* 4.5.

The proteinase could be stored at −20° for one month. If the storage of the enzyme solution was performed at 4° after one week most of the activity was lost. Although the enzyme was stable to freezing, it could not be submitted to lyophilization. Furthermore, the activity was completely lost by exposing the enzyme to temperatures above 60°, although some lower losses could be detected at 40°. The pH stability was maximal between pH 3 and 6, while below pH 2 and above pH 7 the activity was lost. The optimum pH, assayed over a pH range from 2.5 to 5.5 with gliadin as a substrate, was 4.25 (Fig. 4). No activity was obtained

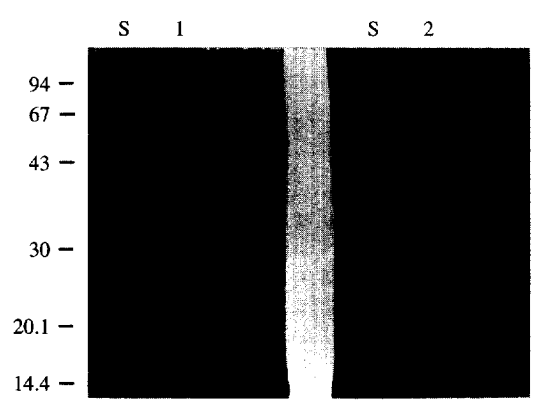

Fig. 3. Electropherogram showing the gelatinolytic activity of germinating wheat proteinase after weakly-denaturing SDS-PAGE. (1), Control; (2), in the presence of E-64. The 10% polyacrylamide gel was copolymerized with 0.1% gelatin. Incubation time for proteolysis was 2 hr.

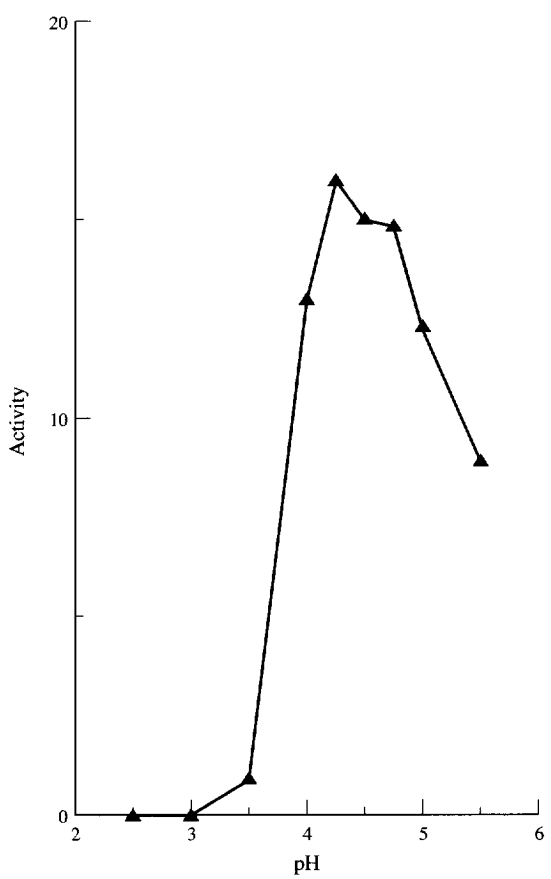

Fig. 4. pH optimum of the germinating wheat proteinase using gliadin as a substrate.

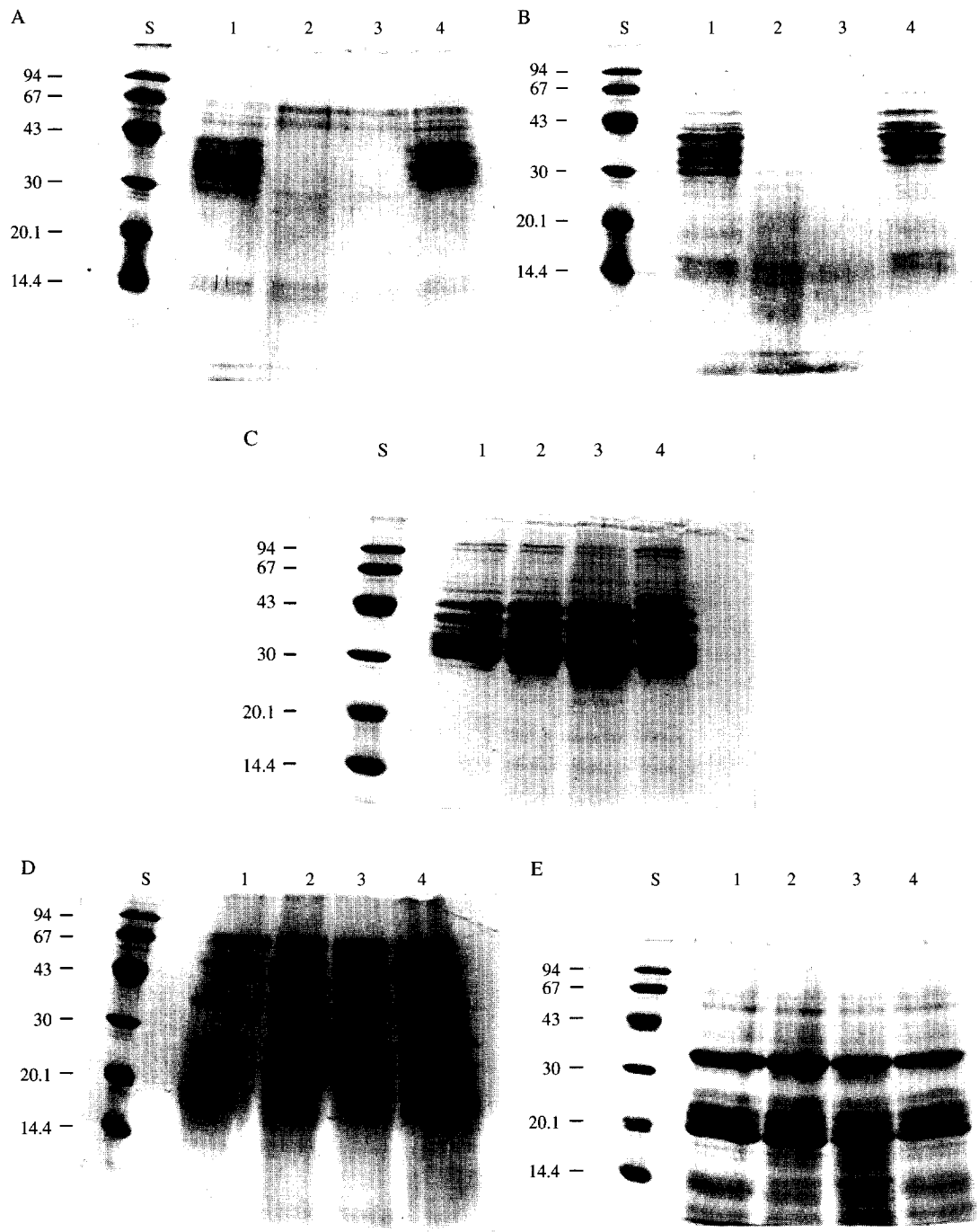

Fig. 5. SDS-PAGE of *in vitro* digestion of various substrates by the germinating wheat proteinase. The substrates were: (A) gliadin from *T. durum*; (B) gliadin from *T. aestivum*; (C) prolamin from ×*H. sardoum*; (D) globulin from *T. aestivum*; (E) edestin from *C. sativa*. S represents M_r standards. The reactions were incubated at 30° for 0 hr (lane 1), 4 hr (lane 2), 20 hr (lane 3). The control sample (lane 4) was incubated for 20 hr, omitting the enzyme.

with *N*-carbobenzoxy(Cbz)-Phe-Ala, a substrate of carboxypeptidases, and with benzoyl-Asn-*p*-nitroanilide, a substrate of asparaginyl(Asn)-endopeptidases [10]. Asn-endopeptidases have been involved in the mobilization of storage proteins during the germination of dicot seeds [11]. They are inactive toward storage proteins from ungerminated seeds and can hydrolyse the same proteins after being modified during germination [11]. Although the proteinase purified from germinating wheat is not an Asn-endopeptidase, crude extracts from germinating wheat seeds have been shown to contain Asn-endopeptidase activity (L. Galleschi, personal communication).

The most effective inhibitors for the wheat proteinase

Table 2. Effect of various inhibitors on the enzyme activity

Compound	Concentration (M)	Relative activity (%)
Control	—	100
E-64	15×10^{-8}	0
Cystatin	50×10^{-8}	0
PMSF	1×10^{-3}	85.3
Pepstatin A	2×10^{-6}	99
EDTA	1×10^{-3}	89
−2 ME	—	70

The purified proteinase was pre-incubated at pH 4.25 with each of the effectors for 10 min at 30°. The reaction was then initiated by adding gliadin as a substrate. All the assays, with the exception of that −ME, contained 2.5×10^{-6} M ME.

activity were E-64 and hen-egg-white cystatin, both inhibitors of several cysteine proteinases (Table 2). PMSF, an inhibitor of serine and cysteine proteinases, had a limited effect. Na_2EDTA, a metalloproteinase inhibitor, slightly inhibited the enzyme activity. Finally pepstatin A, an aspartic proteinase inhibitor, had no effect on the enzyme activity. When the assay was performed in the absence of 2-ME (2-mercapto-ethanol), the activity decreased more than 30% in comparison with the control performed in the presence of 2-ME. Furthermore, prolonged dialysis without 2-ME caused the loss of the bulk of the enzyme activity, which was partially (20%) recovered by pre-incubating the enzyme with the sulphhydryl agent.

These results are consistent with the enzyme being a cysteine proteinase. It shows a M_r in close agreement with that of the cysteine proteinases isolated from barley [8, 9] and maize [12] and with that of similar enzymes isolated from some leguminous species [13]. A preliminary tissue localization, performed by dissecting the endosperm from seeds germinated for four days, revealed about 50% of the hydrolysing activity against gliadin in the starchy endosperm, and the other 50% in the external tissues (aleurone plus seed coat and pericarp). It is likely that the wheat enzyme is synthesized in the aleurone layer and secreted into the starchy endosperm, like the cysteine proteinase of barley [14, 15].

In vitro *digestion of substrates*

The digestion of several protein substrates was followed by SDS-PAGE of the hydrolysis products. Figure 5a shows that the enzyme cleaves to small peptides the gliadin extracted from *T. durum* endosperms. A similar pattern of hydrolysis was shown when gliadin from *T. aestivum* was used as a substrate (Fig. 5b). On the other hand, the prolamin extracted from ×*Haynaldoticum sardoum* was scarcely degraded, since only two high M_r components were cleaved after prolonged incubation (Fig. 5c, lane 3). Globulins from wheat (Fig. 5d) and from hemp (Fig. 5e) seeds appeared to be resistant or scarcely susceptible, respectively, to the proteolytic attack.

These results show that the proteinase from germinating wheat shows a high degree of specificity for the hydrolysis of its natural substrate, i.e. the gliadin from dry seeds. A proteinase with a similar specificity has been isolated from germinating *Vicia faba* seeds [13]. The cysteine proteinase from wheat appears to belong to the proteinase A-class according to [11]. Since the cysteine proteinase represents the bulk of the activity of the germinating wheat seeds and is able to cleave the gliadin to small peptides, it appears to play a key role in the initial degradation of the storage proteins present in the starchy endosperm.

EXPERIMENTAL

Plant material and germination of seeds. Wheat seeds (*T. durum* L., cv. Creso) were utilized. Similar sized seeds were surface-sterilized with 1% NaOCl soln and allowed to germinate in the dark at 23° four days. After this period the hand-isolated endosperms were frozen and lyophilized.

Enzyme extraction and purification. 1000 endosperms were reduced in a blender to fine powder. This powder was extracted for 1 hr at 4° with a 200 mM NaOAc buffer pH 5 containing 5 mM 2-ME at a ratio of 1:3 (w/v). The extract was centrifuged at 40 000*g* and 4° for 10 min. The supernatant was brought to 80% satn with solid $(NH_4)_2SO_4$ and centrifuged as above. The ppt. was redissolved in 6 ml of 2.5 mM K-Pi buffer (pH 7.3) containing 1 mM 2-ME and dialysed at 4° against 3 l of the same buffer for 18 hr. The dialysate was cleared by centrifugation at 40 000*g* for 10 min. The supernatant was applied to a DEAE-cellulose column (23 SS, Serva, Heidelberg, Germany; 20 cm long, 2 cm i.d.) equilibrated with a 2.5 mM K-Pi buffer (pH 7.3) containing 1 mM 2-ME. The column was washed with the same buffer until the A_{280} decreased and maintained a constant value. The proteinase was eluted by a 200 mM NaOAc buffer (pH 4) containing 1 mM 2-ME. Frs of 2.5 ml were collected and those with maximum azocasein-degrading activity were pooled and concd up to 3 ml with solid PEG 6000. The concd sample was sepd by prep. isoelectric focusing in the Rotofor cell. 45 ml of bidistd H_2O containing 15% glycerol and 2.5% ampholytes (pH range 4–6; 40% w/v) was prefocused for 40 min at 12 W constant power and at 5°. The protein soln was loaded into the focusing chamber of the Rotofor cell through the port in the sixth compartment from the anode. Focusing proceeded for 4 hr at 12 W constant power. 20 frs were collected and their pH values measured. Each fr. was assayed for azocasein degrading activity and those frs with a maximum activity between pH 4.2 and 4.6 were pooled, concd with PEG 6000, frozen and stored at −20°.

Proteinase activity. The enzyme was routinely measured with azocasein. 0.2 ml of enzyme soln was mixed with 2-ME (2.5 mM final concn) and 200 mM NaOAc buffer (pH 5.4) up to 1 ml. The reaction was started by

adding 1 ml of 0.2% (w/v) azocasein in a 50 mM NaOAc buffer (pH 5.4). The reaction was incubated at 30° for 1 hr and stopped by adding 1 ml of 24% (w/v) TCA and vortexing. After standing at 4° for 10 min, the soln was cleared in a bench centrifuge. Soluble peptides in the supernatant were determined by A_{330}. Controls for each sample were similarly treated, but without incubation. One unit of proteinase activity was defined as the amount of enzyme required to produce an A change of 1 in a 1 cm cuvette, under the conditions of the assay, compared with the blank. The activity of the purified enzyme was measured with gliadin. 0.2 ml of enzyme were mixed with 2-ME (2.5 mM final concn) and 200 mM NaOAc buffer (pH 4.25) up to 1 ml. The reaction was started by adding 1 ml of 1% (w/v) gliadin purified from dry durum wheat endosperms. After 1 hr incubation at 30°, the reaction was stopped by adding 1 ml of 24% TCA. The suspension was centrifuged and 0.5 ml of the supernatant were utilized to determine the TCA-soluble products by the ninhydrin method [16]. Controls for each sample were similarly treated, but without incubation. One unit is equivalent to 1 μmol α-amino N released hr^{-1}.

Preparation of substrates. Gliadin was extracted from endosperms isolated from dry seeds of *T. durum*, *T. aestivum* and ×*H. sardoum*. The meals were pre-extracted ×3 with 0.5 M NaCl for 1 hr at room temp. and then the gliadin was extracted ×3 with 70% EtOH. After centrifugation, the supernatants were collected, pooled, frozen and lyophilized. Globulin from *T. aestivum* was extracted according to [5]. Edestin from *Cannabis sativa* was obtained from Sigma.

In vitro *digestion of protein substrates.* Gliadin extracted from *T. durum* and *T. aestivum*, prolamin from ×*H. sardoum*, globulin from *T. aestivum* and *C. sativa* were utilized as substrates. For each treatment, 5 mg of enzyme was mixed with 200 mM NaOAc (pH 4.25) containing 2-ME to a final concn of 2.5 mM. Then the reaction was started by adding 1 ml of protein substrate (0.1% w/v). After incubation at 30° for 4 and 20 h, 400-μl samples were taken from the mixture, frozen in liquid N_2 and lyophilized. The samples were solubilized with a SDS-sample buffer and heated at 100° for 3 min. Control reactions were performed without adding the enzyme and incubated for the same periods. Digestion patterns were analysed by SDS-PAGE.

Electrophoretic activity assay. This was performed according to [17], modified by [18]. After the electrophoretic run, each gel was renatured with Triton X-100 and incubated in 200 mM NaOAc buffer (pH 4.25) containing 2.5 mM 2-ME. When proteinase inhibitor E-64 was utilized, it was added to the incubation buffer. After incubation, the gels were stained overnight with Coomassie Brilliant blue R-250 [19] and destained with 7.5% HOAc.

SDS-PAGE. The protein solns were analysed by SDS-PAGE according to [20].

Protein estimation. This was performed using bovine serum albumin as the standard [21].

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