Proteases of germinating winged-bean (*Psophocarpus tetragonolobus*) seeds: purification and characterization of an acidic protease

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Two major classes of protease are shown to occur in germinating winged-bean (*Psophocarpus tetragonolobus*) seeds, by assaying extracts at pH 8.0 and pH 5.1 with [¹⁴C]gelatin as substrate. At pH 8.0, the activity profile of the enzyme shows a steady rise throughout the period of germination, whereas the activity at the acidic pH is very low up to day 5 and then increases sharply reaching a peak on day 11, followed by an equally sharp decline. The winged-bean acidic protease (WbAP) has been purified to apparent homogeneity, as attested by a single protein band on both PAGE and SDS/PAGE. WbAP is a monomeric enzyme with a molecular mass of 35 kDa and a pH optimum of 6.0. It is

a thiol protease that does not belong to the papain family and it has tightly bound Ca²⁺ as shown by ⁴⁵Ca²⁺-exchange studies. Besides gelatin and casein, it hydrolyses a 29 kDa winged-bean protein, indicating a prospective physiological role for it in storage-protein mobilization. Immunoblot analysis shows that it occurs only in the seeds and sprouting tubers of this plant and also that it is synthesized in developing seeds just before desiccation. It appears that the newly synthesized enzyme is inactive, and activation takes place around day 6 of germination. However, neither the mechanism of activation nor the signal that triggers it is clearly understood.

INTRODUCTION

Germination of seeds is an important event in the life cycle of plants and it initiates numerous physiological and biochemical activities when the apparent metabolic dormancy of desiccated seeds is disrupted by imbibition. It leads to extensive breakdown of stored carbohydrates, lipids and proteins in the storage organs of seeds to provide energy and other nutritional requirements of the growing embryo. Several hydrolytic enzymes participate in this process and to understand the mechanism of storage-protein mobilization, a variety of germinating seeds has been examined [1–7]. These studies have led to the realization that proteases and peptidases act in a concerted manner to hydrolyse storage proteins during this period, and the combination of enzymes varies from seed to seed [8]. Recent studies have also revealed the existence of more than one system of proteases in the same seed for the storage-protein mobilization process, as there is a multiplicity of storage proteins in many seeds [3,8–10]. However, in spite of the significant advances in the characterization of a large number of proteases of plant origin [1,6,9–13], our understanding of the protease systems responsible for the mobilization of storage proteins remains unclear.

Of the legumes, the winged bean (*Psophocarpus tetragonolobus*) is considered to be a potentially important crop because of its high protein and oil contents. With the realization that this crop could be as important as soya bean, particularly in the humid tropics [14], extensive studies were carried out to characterize the storage proteins, lectins and protease inhibitors of this plant by various groups, including ours [15–22]. We have previously presented the protein profile of the germinating seeds, but nothing is known about the proteases or the mechanism of storage-protein mobilization. In the present paper we report the identification of two major classes of protease in germinating winged-bean seeds, the alkaline metalloprotease and the acidic thiol protease. We also report the purification and characterization of the acidic protease and look at its possible involvement in the mobilization of storage proteins.

MATERIALS AND METHODS

Chemicals

Protein A–horseradish peroxidase, 4-chloro-1-naphthol, bovine brain calmodulin, casein and gelatin from swine skin were obtained from Sigma Chemical Co. Sepharose CL-4B, Sephacryl S-200 and Sephadex G-25 were purchased from Pharmacia Fine Chemicals. CM-cellulose and DEAE-cellulose were from Pierce Chemical Company. Nitrocellulose paper (BA-85), Bio-Gel P-60 and [¹⁴C]formaldehyde were products of Schleicher and Schuell, Bio-Rad and Amersham International respectively. Na¹²⁵I and ⁴⁵CaCl₂ were purchased from Bhaba Atomic Research Centre. Gelatin was radiolabelled by reductive methylation using [¹⁴C]formaldehyde (50 µCi/15 µl) and sodium cyanoborohydride [23]. The [¹⁴C]gelatin was diluted with unlabelled gelatin to obtain a working substrate solution with a constant specific radioactivity of 2600 c.p.m./µg. Casein and winged-bean-seed proteins were iodinated using Na¹²⁵I and chloramine- τ [24].

Plant materials

Dry winged-bean seeds were obtained from local farms or the Institute garden. They were germinated on moist sterile blotting paper as described by Punjabi and Basu [25]. The day on which the seed coat cracked was taken as day 1 of germination. Epicotyls and hypocotyls were collected from 15-day-old winged-bean seedlings; leaves and sprouting tubers were from the garden plants.

Preparation of cotyledonary extracts

When small amounts of extract were needed, cotyledons free from seed coats and embryonic axes were homogenized in 5 vol. of ice-cold 25 mM Tris/HCl buffer, pH 6.8, using a chilled mortar and pestle and the homogenate was centrifuged at 16000 g

Abbreviations used: pCMB, *p*-chloromercuribenzoic acid; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; WbAP, winged-bean acidic protease.

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for 30 min at 4 °C. The supernatant was used as the extract for the preliminary studies.

Protease assay and definition of enzyme unit

Proteolytic activity was routinely assayed using [14 C]gelatin as the substrate. The reaction mixture contained, in a total volume of 100 μ l, buffer (50–200 mM and pH as desired), 6.5 μ g of [14 C]gelatin (2600 c.p.m./ μ g) and the enzyme. Incubation was carried out at 37 °C for a fixed period during which the rate was linear. It was terminated by the addition of cold trichloroacetic acid to a final concentration of 25 % and 250 μ g of BSA, kept on ice for 5 min. After centrifugation, an aliquot of the supernatant was counted for 14 C radioactivity in a liquid-scintillation counter.

One enzyme unit is defined as the amount of enzyme that yields 1000 c.p.m. in the trichloroacetic acid-soluble supernatant/min, under the conditions of the assay.

Proteolysis of 125 I-labelled winged-bean-seed proteins (3×10^4 c.p.m./ μ g) and 125 I-casein (4.8×10^4 c.p.m./ μ g) by the purified thiol protease was studied by incubating the proteins with the enzyme at pH 6.0 in the presence of 1 mM dithiothreitol (DTT). Equal aliquots were taken at intervals, boiled with SDS sample buffer and subjected to SDS/PAGE followed by autoradiography.

Gelatin/PAGE and analysis of protease activity

The proteins in the extracts were resolved in polyacrylamide gel impregnated with 0.1 % gelatin in the presence of SDS by a procedure similar to that described by Heussen and Dowdle [26]. Samples were treated with 2 % SDS (without boiling), and electrophoresis was carried out at 4 °C. Gels were washed 3 times with different buffers of the desired pH containing 1 % Triton X-100 to remove SDS. They were then washed in the same buffer without Triton X-100 and finally incubated for 24 h in the respective buffers to allow hydrolysis of the entrapped gelatin. The unhydrolysed proteins were stained with Coomassie Blue and, on destaining, clear bands corresponding to the gelatin-hydrolysis zones, indicating the presence of protease activity, were detectable.

Other methods

Protein was determined by the Coomassie Blue-dye-binding method of Bradford, with BSA as standard [27]. It was also monitored by measuring its A_{280} . The molecular mass of the purified protease was determined by FPLC gel filtration on Sephacryl S-100. SDS/PAGE was performed as described by Laemmli [28].

Raising of polyclonal antibody against the purified winged-bean protease

Polyclonal antibodies were raised in rabbits against the protease by injecting highly purified enzyme (a total of 0.3 mg of protein), and these were used for immunodiffusion, immunoelectrophoresis [29] and immunoblot analysis [30,31].

Purification of an acidic winged-bean protease

A procedure for the purification of an acidic protease from the winged-bean seeds has been standardized. Starting with seeds that had been germinated for 8–9 days, the following steps were carried out at 4 °C. (i) Cotyledons (40 g) were homogenized in 5 vol. of ice-cold 10 mM sodium acetate buffer, pH 5.0, containing 1 mM CaCl_2 in a Sorvall Omnimixer, a few drops of Antifoam-

C being added to prevent foaming. A clear homogenate was obtained after centrifugation at 16000 *g* for 30 min. (ii) The extract was concentrated by $(\text{NH}_4)_2\text{SO}_4$ fractionation and the 25–70 % -satd. fraction, dissolved in a minimum volume of the above buffer, was subjected to gel filtration on a Sephacryl S-200 column (1.5 cm $\times$ 95 cm), pre-equilibrated with the same buffer. Fractions containing enzyme activity were pooled. (iii) The pooled fractions were passed through a CM-cellulose column (3 cm $\times$ 25 cm), previously equilibrated with the same buffer. The breakthrough and wash fractions representing all the protease activity were pooled. (iv) The pH of the pooled fractions was raised to 5.4 by adding 0.1 M NaOH; the fractions were then extensively dialysed against 10 mM sodium acetate, pH 5.4, containing 1 mM CaCl_2 and applied to a DEAE-cellulose column (2 cm $\times$ 10 cm) equilibrated with the same buffer. After extensive washing with the buffer, the bound enzyme was eluted by a linear gradient of NaCl (0–300 mM) in the same buffer. Fractions containing enzyme with high specific activity were pooled. (v) The final purified enzyme preparation was obtained by gel filtration on a Bio-Gel P-60 column (0.6 cm $\times$ 83 cm) pre-equilibrated with the above buffer (pH 5.4).

RESULTS

Identification of two major classes of protease in germinating winged-bean seeds

In order to standardize a suitable assay system for the proteases, preliminary studies were carried out using a mid-stage-germinated (7 days) winged-bean-seed cotyledon extract as the source of enzyme. Various substances, such as radiolabelled proteins, azocasein and synthetic chromogenic peptides, were tested as possible proteolytic enzyme substrates. [14 C]Gelatin was found to be a versatile and sensitive substrate, suitable for routine assays under various pH conditions.

With this assay system, the pH-activity relationship was mapped out for the proteases from mid-stage-germinated winged-bean seeds. Two major classes of proteolytic activity can be distinguished: one having an acidic pH optimum (between 4.0 and 6.0) and the other being maximally active in the alkaline range (around pH 8.0). DTT and 2-mercaptoethanol both enhance the acidic protease activity severalfold, but *p*-chloromercuribenzoate (pCMB) dramatically reduces it, the inhibition being partially reversed by both DTT and 2-mercaptoethanol. This activity in the acidic pH is not affected by preincubation with Mg^{2+} , Ca^{2+} , EDTA or EGTA. On the other hand, similar studies show drastic inhibition of the alkaline protease activity by EGTA and to a lesser extent by EDTA, although there is no significant enhancement of the activity on preincubation with Ca^{2+} or Mg^{2+} .

Zymograms obtained by electrophoretic resolution of the proteins of cotyledonary extracts (samples being treated with SDS, without boiling) in gelatin-impregnated polyacrylamide gels in the presence of SDS followed by activity staining under different incubation conditions also clearly reveal the presence of these two major classes of protease in the germinating winged-bean seeds (results not shown).

Changes in acidic and alkaline proteases during seed germination

The profiles of the two major classes of protease in the germinating winged-bean seeds have been mapped out by assaying their activities at pH 5.1 and pH 8.0 in the presence of 1 mM DTT and 1 mM CaCl_2 respectively. The activity of the acidic protease is very low at the early stages of germination but increases sharply from day 5 up to around day 11; this is

Figure 1 Proteolytic activity and protein profile during winged-bean-seed germination

Cotyledonary extracts from seedlings after different lengths of germination were assayed for proteolytic activity at pH 5.1 in the presence of 1 mM DTT (●) and at pH 8.0 in the presence of 1 mM CaCl_2 (○). Total protein content of the seeds (△) was determined by the method of Bradford [27] with BSA as standard. The values are means $\pm$ S.E.M. of five independent assays. Enzyme activity is presented as units/g fresh weight of tissue.

Table 1 Summary of the purification of an acidic thiol protease from germinating winged-bean seeds

Cotyledons (40 g) obtained from winged-bean seeds germinated for 8–9 days, free from seed coats and embryonic axes, were used.

Steps	Total protein (mg)	Enzyme activity (units)	Specific activity (units/mg)	Yield (%)
1. Crude extract (16000 g homogenate)	850.0	332	0.4	100
2. Sephacryl S-200	353.0	281	0.8	85
3. CM-cellulose	9.3	256	27.5	77
4. DEAE-cellulose	1.4	99	70.7	30
5. Bio-Gel P-60	0.3	51	170.0	15

followed by an equally sharp decline (Figure 1). In the case of the alkaline protease, the activity is higher than that of the acidic enzyme at the early stages and, unlike the latter, it increases steadily until the late stages of germination. Figure 1 also presents the decrease in total protein content of the cotyledon with germination. To investigate any possible relationship between the germination profile of the acidic protease and the process of storage-protein mobilization, we purified and characterized the acidic protease of the germinating winged-bean seed.

Purification of an acidic protease from germinating winged-bean seed

The results of the purification of the winged-bean acid protease (WbAP) are summarized in Table 1. The crude extract contains a total of about 330 enzyme units with a specific activity of 0.4 unit/mg. The combined $(\text{NH}_4)_2\text{SO}_4$ and Sephacryl S-200 steps result in only a slight enrichment of the enzyme, but they are critically important for the next step, to ensure fine adjustment of pH and ionic strength of the solution. The bulk of the protein binds to CM-cellulose, and WbAP is recovered in the breakthrough fraction with substantial purification and high yield. In

Figure 2 Purification of the acidic protease

(a) DEAE-cellulose column chromatography. The breakthrough fraction from the CM-cellulose column was subjected to anion-exchange chromatography on a DEAE-cellulose column; bound proteins were eluted with a linear gradient of NaCl (0–300 mM) and 3 ml fractions were collected. Those fractions containing high enzyme activity (44–61) were pooled and concentrated using poly(ethylene glycol). ●, A_{280} (protein concentration); ○, enzyme activity; ---, NaCl concentration. (b) Gel filtration on Bio-Gel P-60. The concentrated pooled fractions from (a) were loaded on a Bio-Gel P-60 column and 1.5 ml fractions were collected. ●, A_{280} ; ○, enzyme activity.

the next step, the enzyme binds to DEAE-cellulose and is eluted by the NaCl gradient in the trailing side of the major protein peak (Figure 2a). As it is not completely resolved from con-

Figure 3 Homogeneity of the purified protease

(a) SDS/PAGE. The purified protease was subjected to alkaline PAGE under denaturing conditions (12% acrylamide), followed by staining with Coomassie Blue R-250. Lanes: 1, molecular-mass markers; 2, the purified protease (3 μ g). (b) PAGE. The purified protease (18 μ g) was analysed by PAGE (7.5% acrylamide) under non-denaturing conditions. (c) Immunoelectrophoresis of crude extract and purified WbAP. Immunoelectrophoresis was carried out on 1.5% agarose in Tris/glycine buffer, pH 8.3, as described by Grabar and Williams [29]. Well A, seed extract; well B, purified enzyme; the longitudinal slot, immune serum.

Figure 4 pH optimum of the purified enzyme

Enzyme activity was assayed at various pH values with [14 C]gelatin as substrate. Buffers used were sodium acetate (pH 4.0–5.5), sodium citrate/sodium phosphate (pH 6–7) and Tris/HCl (pH 7.5–8.5).

taminating proteins, high yield has been sacrificed in order to recover fractions with high specific activity. In the Bio-Gel P-60 gel-filtration step (Figure 2b), the specific activity of the enzyme across the protein/activity peak is high and more or less constant, and these fractions (18–30) are pooled.

SDS/PAGE analysis of the proteins at each step of WbAP purification (results not shown) shows that there is no substantial qualitative difference between the protein profiles of the crude extract and the Sephacryl S-200 eluate. The presence of only two prominent protein bands is seen after the CM-cellulose step; one

Table 2 Effects of thiols and bivalent metal ions on WbAP

Enzyme assays were carried out at 37 °C for 20 min in a reaction mixture containing 200 mM sodium citrate/sodium phosphate buffer, pH 6.0 and [14 C]gelatin (6.5 μ g), in a total volume of 100 μ l, as described in the Materials and methods section. The enzyme was added to start the reaction after preincubation for 5 min with various reagents as indicated.

Additions (mM)	Protease activity (c.p.m.)	Relative activity (%)	Fold increase
1. None	200	6	1.0
2. 2-Mercaptoethanol (0.7)	2930	90	14.6
3. DTT (0.5)	3270	100	16.3
4. DTT (0.5) + MgCl ₂ (1.0)	3430	105	17.1
5. DTT (0.5) + CaCl ₂ (1.0)	3340	102	16.7
6. DTT (0.5) + MnCl ₂ (1.0)	2850	86	14.2
7. DTT (0.5) + FeSO ₄ (1.0)	2630	80	13.1
8. DTT (0.5) + ZnSO ₄ (1.0)	1720	53	8.6
9. DTT (0.5) + CuSO ₄ (1.0)	430	13	2.1

of them is removed in the next step and the minor bands are eliminated in the final Bio-Gel P-60 step.

Homogeneity and size of the purified WbAP

The purified enzyme preparation has been judged to be homogeneous by the detection of single protein bands by alkaline PAGE, both in the presence and absence of SDS (Figures 3a and 3b). This is further supported by the occurrence of a single precipitin band on immunoelectrophoresis of the crude seed extract (Figure 3c), attesting to the apparent absence of contaminating antigenic proteins in the WbAP preparation. At the same time, Figure 3(c) also affirms the monoreactivity of the antiserum. A molecular mass of 35 kDa, as assessed by SDS/PAGE (Figure 3a) as well as by FPLC gel filtration on Sephacryl S-100 (results not shown), indicates WbAP to be a single-polypeptide enzyme.

pH-dependence and thiol and bivalent metal ion requirements of WbAP

Although preliminary studies with cotyledonary extracts indicated maximal activity of the acidic protease to occur around pH 5.0, the purified enzyme has a sharp pH optimum of 6.0 (Figure 4). The thiol nature of WbAP is clearly indicated by the enormous activation by both 2-mercaptoethanol and DTT (Table 2) and this prompted us to include DTT (0.5 mM) in all routine assays and consider activation or inhibition of the enzyme in this background. The other results presented in Table 2 do not show any obvious metal ion requirements of the purified enzyme even though strong inhibition by Cu²⁺ and some inhibition by Zn²⁺ are indicated.

Effect of various inhibitors on WbAP

The purified enzyme is strongly inhibited by thiol-blocking agents such as pCMB, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and HgCl₂ and to a lesser extent by iodoacetate, and partial reversal by DTT has been shown in the case of pCMB and DTNB (Table

Table 3 Effect of various inhibitors on WbAP

Enzyme assays were carried out as in Table 2, with the reaction mixture containing an additional 0.5 mM DTT. Sequential preincubations were performed when two reagents were added. WbCl, winged-bean chymotrypsin inhibitor; SbTI, soya-bean trypsin inhibitor.

Reagents (mM)	Inhibition (%)
1. pCMB (5.0)	100
2. pCMB (5.0) + DTT (10)	59
3. DTNB (5.0)	94
4. DTNB (5) + DTT (10)	38
5. HgCl ₂ (5.0)	95
6. Iodoacetate (5.0)	53
7. E64 (0.5)	0
8. EDTA (1.0)	76
9. EGTA (1.0)	100
10. Fluphenazine (1.0)	0
11. PMSF (2.0)	10
12. Benzamidine (2.0)	0
13. Pepstatin (2.0)	0
14. Leupeptin (2.0)	0
15. WbCl (0.0025)	0
16. SbTI (0.01)	0

Figure 5 Binding of $^{45}\text{Ca}^{2+}$

WbAP and bovine brain calmodulin were subjected to SDS/PAGE (12% acrylamide) and transferred to nitrocellulose paper. Ca^{2+} binding of the blot was assessed using $^{45}\text{CaCl}_2$ as described by Maruyama et al [34]. Lane 1, calmodulin; lane 2, WbAP.

3). This attests to the thiol nature of WbAP, and the lack of any effect by E64, a specific inhibitor of cysteine proteases of the papain family, may be significant.

Complete inhibition by EGTA, strong inhibition by EDTA and no inhibition by fluphenazine (Table 3) in spite of the lack of significant stimulation by exogenous Mg^{2+} or Ca^{2+} (Table 2) indicate an interesting feature of the metal ion requirements of WbAP. Table 3 also shows that WbAP is not affected by some common broad-spectrum as well as specific protease inhibitors, the only exception being a 10% inhibition by PMSF.

Tightly bound Ca^{2+} in WbAP

Specific binding of Ca^{2+} by the purified enzyme is shown by $^{45}\text{Ca}^{2+}$ -exchange studies carried out after SDS/PAGE and Western transfer (Figure 5). This clearly indicates the likely presence of tightly bound Ca^{2+} in the enzyme and accounts for the observation of lack of stimulation by exogenous Ca^{2+} (Table 2) and complete inhibition by EGTA (Table 3).

Figure 6 *In vitro* hydrolysis of casein, winged-bean-seed proteins and winged-bean chymotrypsin inhibitor by the purified protease

The purified enzyme was incubated with ^{125}I -casein, ^{125}I -labelled winged-bean proteins or the chymotrypsin inhibitor under the optimal conditions of enzyme activity. At different time intervals, aliquots were taken, boiled in SDS sample buffer and subjected to SDS/PAGE, followed by autoradiography or Coomassie Blue staining in the case of winged-bean chymotrypsin inhibitor. (a) Time course of casein hydrolysis; (b) time course of winged-bean-seed protein hydrolysis; (c) time course of hydrolysis of winged-bean chymotrypsin inhibitor. The positions of the standard molecular-mass marker proteins are indicated. Appropriate controls were carried out in all cases.

Substrate specificity of WbAP and hydrolysis of winged-bean storage protein

Gelatin is a good substrate of the enzyme and [^{14}C]gelatin has been almost exclusively used for the routine assays. Other common protease substrates, e.g. *N*- α -*p*-tosyl-L-arginine methyl ester, *N*-benzoyl-L-tyrosine ethyl ester and azocasein, have been found to be unsuitable for assaying the enzyme. However, slow hydrolysis of most of the components of casein other than the small 18.5 kDa component takes place when ^{125}I -casein is incubated with the purified enzyme yielding smaller polypeptides (Figure 6a).

In an attempt to assess the role of the enzyme in the mobilization of storage protein(s) during germination, we examined winged-bean-seed proteins as possible substrates for the purified enzyme. As shown in Figure 6(b), one of the major components of winged-bean-seed protein, with a molecular mass of 29 kDa, is rapidly hydrolysed when ^{125}I -proteins (a mixture prepared from the seed) are incubated with WbAP, whereas most of the other polypeptides remain unaffected. The chymotrypsin inhibitor, a major constituent of the seed, is not degraded even

Figure 7 Distribution of WbAP and its profile in developing and germinating seeds

Immunoblot experiments were carried out with various tissue extracts using rabbit immune serum as described in the text. (a) Distribution of the enzyme. Various tissue extracts were examined for the presence of the enzyme: E, purified enzyme; S, seed; T₁ and T₂, tuber at early and late stages of sprouting respectively. (b) Developing winged-bean seeds. Cotyledonary extracts at different stages of development were examined for the presence of WbAP by immunoblot analysis. E, purified enzyme. (c) Germination of winged-bean seeds. Immunoblot analysis of the cotyledonary extracts of winged-bean seeds germinated for different lengths of time. E, purified enzyme.

on prolonged incubation with the enzyme (Figure 6c). In all these studies, incubation without WbAP shows that the iodinated proteins do not undergo degradation under the conditions of the assay (results not shown).

Distribution of WbAP and its profile in developing and germinating seeds

The occurrence of the protease in other germinating seeds, such as soya bean, jack bean and pea, as well as in different tissues of the winged-bean plant has been examined by Western blotting followed by immunodecoration. In the latter, it is present in the sprouting tuber as well as the seeds (Figure 7a). It is not detectable in the epicotyl, hypocotyl, leaf and developing tuber of the plant or in the three other leguminous seeds examined (results not shown). As shown in Figure 7(b), it starts to appear in the late stages of winged-bean-seed maturation, before desiccation and the protease activity of the seeds (about 37 days after pod formation) assayed at pH 5.1 is 0.81 unit/g. The results of immunoblot analysis showing the abundance of WbAP in the imbibing seeds up to the mid-stages of germination and the gradual decrease between day 7 and day 15 are presented in Figure 7(c).

DISCUSSION

In the life cycle of a plant, protein degradation has many essential functions, one of which is mobilization of storage proteins during seed germination. To study the mechanism of this physiologically vital process, many have undertaken the task of purifying and characterizing a variety of proteases and

peptidases, some of which occur only transiently in germinating seeds [1,3,6,8–12]. However, the main drawback in these studies is the lack of reliable assay methods for these enzymes, since seed extracts usually contain protease inhibitors as well as other proteins that compete with the added substrate. Synthetic substrates are also often used in the assay of these enzymes, but such assays further minimize the relevance of the studies to the storage-protein mobilization process [8,32]. For exploratory purposes and assays of protease are not fully characterized, [¹⁴C]gelatin is preferred to casein derivatives, as the latter tend to have serious limitations at low pH.

In the present study, some zymograms show the presence of more than one acidic protease (results not shown); however, this apparent resolution of the enzyme has to be treated with some caution. As the protein samples are not boiled after SDS treatment, there are some inherent limitations in the methodology [26]. Unlike in the Laemmli procedure [28], the milder conditions of treatment may give rise to protein populations with various electrophoretic mobilities from the same sample as a result of differential binding of SDS. Moreover, the self-folding of the polypeptide after removal of SDS may not be accurate.

The present purification procedure yields an essentially homogeneous preparation with an overall recovery of 15%. Similar or even lower yields are common for acidic proteases from other germinating seeds [1,2,6,9,11]. As quantification of individual enzymes has not been possible by the assay used, the yield of 15% does not accurately reflect the efficiency or otherwise of the present procedure. It is for similar reasons that the pH for apparent optimal activity of the acidic proteases in the crude extract is around 5.0 whereas that of the purified enzyme is 6.0 (Figure 4).

The molecular mass of WbAP has been estimated to be around 35 kDa, with a single polypeptide chain. In this respect, it is similar to acidic thiol proteases from other germinating seeds [1,2,9,33]. Several lines of evidence clearly establish the thiol nature of the enzyme and the lack of inhibition by E64 indicates that, like some other thiol proteases [9,12], it does not belong to the papain family. The present finding of ⁴⁵Ca²⁺ binding on Western-blot analysis (Figure 5) and complete inhibition by EGTA but without any stimulation by exogenous Ca²⁺ are consistent with the presence of tightly bound Ca²⁺ at or near the active site of the enzyme. The involvement of a calmodulin-like Ca²⁺-binding domain is not indicated, as fluphenazine does not have any effect on the activity of the enzyme. Its high activity in the mid-stage of germination suggests a role in the germination process, which would be consistent with the rapid hydrolysis *in vitro* of the 29 kDa winged-bean-seed protein by WbAP (Figure 6b) as well as the lack of inhibition by the winged-bean chymotrypsin inhibitor (Table 3).

Western-blot analysis reveals the appearance of WbAP in the late stages of seed development and its abundance during the initial periods of germination (Figure 7); however, the enzyme assay shows only very low activities until day 6 of germination (Figure 1) as well as in maturing seeds. This implies the synthesis of an inactive WbAP during the later stages of seed development and suggests that it is stored as such in desiccated seeds. The Western-blot analysis also makes it clear that there is no significant change in the apparent molecular mass of the enzyme during germination. This rules out any extensive proteolytic processing of WbAP, unlike other thiol proteases such as the *Vigna mungo* enzyme which are known to undergo multistep proteolytic processing [33]. As extensive proteolytic processing in the activation of WbAP is ruled out, removal of a few amino acid residues from the N- or C-terminus and/or reversible ligand attachment are attractive alternatives; but it is not possible to

favour any of these mechanisms at this stage. Nevertheless, it offers an interesting area of enquiry about the nature of the signals that initiate and regulate the activation of the protease that may be involved in the process of storage-protein mobilization.

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