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DETECTION OF THE ISOENZYMES OF WHEAT GRAIN PROTEINASE A

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; seeds; cysteine proteinase A; purification; isoenzymes.

Abstract—A new method for the purification of wheat cysteine proteinase A which plays a key role in the mobilization of seed storage proteins during germination has been developed. It consists of $(NH_4)_2SO_4$ fractionation, gel filtration, and both ion-exchange and hydrophobic chromatography. Constancy of the specific activity of chromatographic fractions and their SDS-electrophoretic pattern indicates the homogeneity of the final enzyme preparation. However, electrophoresis in nondenaturing conditions revealed three protein bands of similar intensity, each showing proteolytic activity. The N-terminal sequences of all three electrophoretic components are identical. They are also identical to a segment of the amino acid sequence deduced from one of several cDNA clones derived from closely related, but non-identical mRNAs that accumulate in the aleurone layer of gibberellic acid-treated wheat [1]. It is very likely that the three electrophoretic components found are isoenzymes encoded by cDNA clones described by these authors.

INTRODUCTION

Seed cysteine proteinases A are of key importance in the mobilization of storage proteins during germination. They trigger the proteolysis of the storage proteins and cause also their extensive degradation [2]. In wheat and in other cereals cysteine proteinases are synthesized in the aleurone layer of germinated seeds, and their synthesis is induced by gibberellic acid [1, 3, 4]. Homologous proteinases were found also in other plant organs during their senescence and massive protein degradation [5–7]. All these proteinases make up part of the papain family that includes the cysteine cathepsins B, H and L of mammals [8].

Several proteinases of this family, detected in seeds, were purified and partially characterized [2, 9, 10]. Others were identified by sequencing the corresponding cDNA [3, 4, 11, 12]. In some cases closely related clones indicative of isoenzymes were found [4, 12–14]. Multiple forms of two cysteine proteinases isolated from germinated barley grains (EP-A [15] and EP-B [16]) were detected. Those of EP-B were proved to be isoenzymes [13]. Multiple forms of purified proteinase A, presumably isoenzymes, were also detected in germinated maize grains [17].

In germinated wheat grains a proteinase belonging to proteinase A group was found, purified and partially characterized [18, 19]. However, no indications of the existence of multiple forms of proteinase A are given in these reports. On the other hand, six clones of cDNA were derived from closely related, but not identical, mRNAs that accumulate in the aleurone layer of the giberellic acid-treated wheat grain [1]. The amino acid sequence deduced from one of these clones (clone 2529) is homologous to that of proteinases of the papain family. These results indicate the existence of a multigenic family encoding a wheat proteinase isoenzymes.

In the present work we describe the isolation of a highly purified wheat proteinase A and show, at the protein level, the existence of its isoenzymes. We have also established the correspondence between the isolated proteinase A and the proteinase whose existence was shown only by the homology of the amino acid sequence deduced from cDNA [1].

RESULTS AND DISCUSSION

Purification of the proteinase

Proteinase A was extracted from wheat caryopses after 4 days of germination. At this time its maximum

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Table 1. Purification of wheat proteinase A from 25 g lyophilized caryopses

Step	Volume, ml	Protein, mg	Activity nkat	Specific activity, nkat mg^{-1}	Yield, %	Purification -fold
1. Crude extract	105	395	1270	3.0	100	1
2. $(NH_4)_2SO_4$	13.0	159	834	4.8	66.2	1.6
3. Sephacryl S-200	66.0	62.3	386	6.0	30.6	1.9
4. DEAE-Sepharose	60.3	5.61	88.8	15.6	7.0	5.2
5. Phenyl-agarose pH 6.6	30.9	0.66	62.4	94.2	4.9	31.4
6. Phenyl-agarose pH 4.8	11.0	0.44	54.6	126	4.3	42.0

activity is attained [18]. Vetch vicilin was used as substrate. It was shown earlier that *in vitro* this protein is a better substrate for wheat proteinase A than are wheat storage proteins, due to a low solubility of the latter [18].

Representative results of proteinase A purification are shown in Table 1. These results were obtained with dithiothreitol added to all solutions used. In the absence of dithiothreitol, complete inactivation occurred during the first stages of purification. The sharp fall of the total activity during the first stages of purification was caused mainly by the removal of other proteinases, especially, the highly active wheat grain carboxypeptidases [20]. The latter, although not acting on native storage proteins, attack them after their modification by proteinase A [2], producing a considerable overestimation of the total and specific activities. Accordingly, the degree of purification and the yield of the final preparation are considerably understated.

The purification of proteinases A is hampered by their tendency to form relatively stable complexes with some proteins [18]. The separation of the latter may be achieved in conditions inducing their dissociation, namely low protein concentration and/or increased ionic strength. That is why we used salting-out in the first stage of purification, although only a rather low degree of purification was achieved by this method.

Most of the inactive proteins passed unabsorbed through the DEAE-Sepharose column at pH 6.6 and initial ionic strength 0.1. The peak of activity was usually eluted at ionic strength 0.24. In a number of experiments a second peak of activity was detected with a maximum at ionic strength 0.33. When rechromatographed in the same conditions the first peak was invariably eluted in the position of the second one. Nevertheless, when two peaks were formed, their subsequent purification on phenyl agarose was carried out separately. In these cases both peaks were eluted in the same interval of NaCl concentration both at pH 6.6 and 4.8, and showed identical electrophoretic patterns after the last step of purification. If a lower initial ionic strength (0.01) was used during the ion-exchange chromatography a third peak of activity eluting earlier at ionic strength 0.17 appeared. Evidently, the strong interaction of proteinase A with some proteins still persists at this stage of purification and causes its artificial separation into two or three fractions depending on the conditions of the experiment.

On phenyl-agarose the maximum activity was eluted by 1 M NaCl, whereas the contaminating proteins were firmly bound and later eluted by water. The contaminants remaining in the enzyme preparation were removed by rechromatography on the same adsorbent at pH 4.8 (Fig. 1). In this final stage of purification the specific activity of the fractions across the active peak was constant within the limits of the error of protein determination (standard deviation $\pm 4\%$).

SDS electrophoresis of the purified preparation detected only one distinct band of protein with $M_r = 33\,500$ [Fig. 2(a)]. This value is close to those obtained by this method for several other seed cysteine proteinases (30–34 k) [16, 21–23]. The exact M_r of the same proteinases of determined primary structure are in the range from 24 000 to 25 000 [5, 11, 13, 24]. A similar value for the M_r of wheat proteinase A (22 000) was obtained by gel filtration [18]. Evidently, SDS electrophoresis overestimates the M_r of proteinases A, probably because of low binding of SDS by these acid proteins (Senyuk V.I, personal communication).

The proteinase isolated was inactivated by iodoacetate, the inhibitor of cysteine proteinases [25], and its maximum activity was in the pH range 4.0–4.5. These properties, its high activity in germinating grains, action on an storage protein, and the value of M_r together indicate the identity of the proteinase isolated and of wheat proteinase A isolated earlier [18, 19]. Given the presence of only one band during SDS electrophoresis and given the constant specific activity of the fractions across the single activity peak found during the last stage of purification, the preparation obtained seems to be homogeneous.

Evidence for isoenzymes

Electrophoresis of the preparation in non-denaturing conditions showed three protein bands of similar intensity [Fig. 2(b)]. Zymograms obtained in gels containing protein substrates also showed three bands of proteolytic activity coinciding with protein bands [Fig. 2(b)]. All three active bands were detected in gels containing both gelatin and vicilin and their activities

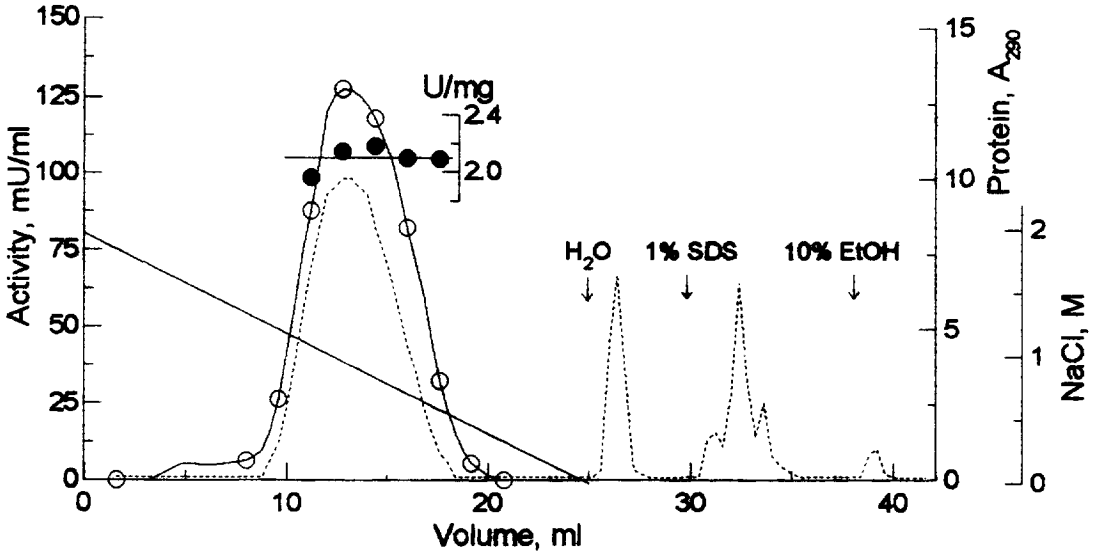

Fig. 1. Last step of purification of proteinase A. Chromatography on phenyl agarose. –○– -activity; ----- -protein; –●– -specific activity; – – – , -NaCl concentration. Arrows mark the points of application of the indicated eluents.

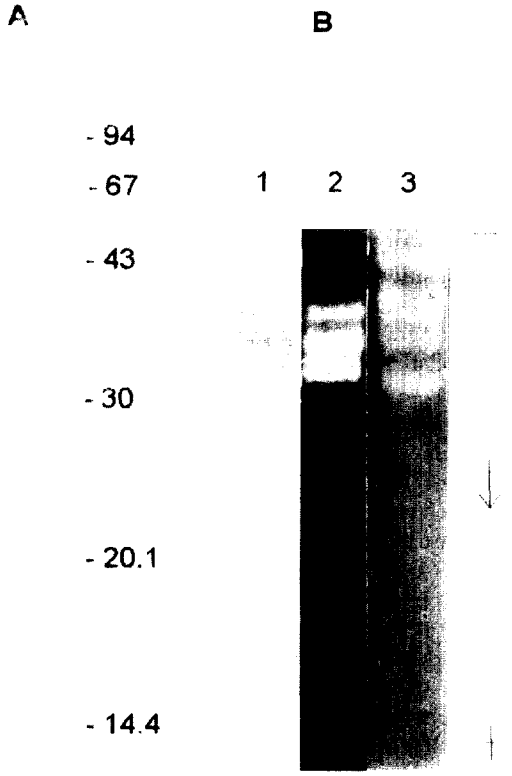

Fig. 2. Electrophoresis of proteinase A. (A) SDS-electrophoresis of purified preparation; 15 μg protein loaded. (B) Non-denaturing electrophoresis; lanes 1 and 2, purified preparation; lane 3, crude extract; lane 1, gel stained for protein after washing out the gelatin; lanes 2 and 3, zymograms.

disappeared when the enzyme preparation was treated with iodoacetate before electrophoresis. Identical zymograms were observed for preparations derived from peaks eluted both at ionic strength 0.24 and 0.33 during chromatography on DEAE-Sepharose.

All three electrophoretic components have the same

Proteinase A Isoenzyme 1	NH_2-SEQLPKVFDARXKXX
Proteinase A Isoenzyme 2	NH_2-SEQLPKVFDARSKXS (XQLPKV)
Proteinase A Isoenzyme 3	NH_2-SEQLP (EQLPK)
Proteinase 2529	54-SEQLPKVFDARSKWS

Fig. 3. NH_2-terminal amino acid sequence of the electrophoretically separated isozymes of wheat proteinase A compared with the corresponding sequence deduced from the nucleotide sequence of 2529 proteinase cDNA [1]. In parentheses are shown amino acids found in the same positions in minor quantities; X designates unidentified amino acids.

N-terminal sequence (Fig. 3). The N-terminal sequence of the isoenzymes of EP-B, the cysteine proteinase of barley seeds is also identical, and all differences in the amino acid sequence of the isoenzymes are located in their C-terminal regions [13]. A smaller content of other amino acid residues was detected during sequencing of components 2 and 3 indicating the presence of species shortened by one amino acid residue. The determined N-terminal sequence coincides with the corresponding sequence deduced from the nucleotide sequence of 2529 proteinase cDNA (Fig. 3) and differs from those of other seed proteinases of papain family [1]. Therefore, the wheat proteinase A and 2529 proteinase are closely related. The position of the identical segment in the deduced sequence of 2529 proteinase indicates that wheat proteinase A, like other proteinases of papain family [13, 21], is synthesized with large prosequences that are post-translationally cleaved to form mature protein.

Separation of proteinase A preparations into three electrophoretic bands was not the result of any changes during their isolation. The zymograms of the crude extracts already show 4–5 bands active towards vicilin [Fig. 2(b)] and inhibited by iodoacetate. The greater number of zones detected, as well as the artificial separation during chromatography, was due in all prob-

ability, to the binding of proteinase A to protein substrates. The identical value of M_r, and the similarity to the proteinase encoded by 2529 cDNA, which belongs to a small multigene family [1] suggest that the three electrophoretic components of wheat proteinase A are true isoenzymes and are encoded by 2529 cDNA and/or the closely related members of this family. Possibly, one of them is identical to proteinase 2529. As to the appearance of components with N-terminal sequence shortened by one amino acid residue, their presence may be due to post-translational processes.

The expression of mRNA corresponding to proteinase 2529 in different tissues was studied [1]. On the basis of these studies it was assumed that this proteinase is non-secreted and is not implicated in the mobilization of storage proteins. Its close similarity to proteinase A is at variance with this assumption.

Sequence alignment of proteinase 2529 with other proteinases of papain family showed a best match with cathepsin B sequence [1]. These two proteinases form a group clearly differing from seed proteinases of other plants including cereals. The close relationship between proteinase 2529 and proteinase A suggests that the latter also belongs to this group.

EXPERIMENTAL

Materials. The seeds of winter wheat (*Triticum aestivum* L., cv. Odesskaya 51) were soaked for 8 hr in H_2O and then germinated on wet filter paper in the dark for 4 days at 24 . Caryopses were sepd, lyophilized and stored at −18 .

Vetch (*Vicia sativa* L. cv. Flora) vicilin was isolated by the method of Shutov A. D. (personal communication). The milled seeds were extracted by half-satd $(NH_4)_2SO_4$, and the extracted proteins were pptd by adding $(NH_4)_2SO_4$ up to 90% satn. The ppt. was dialysed against H_2O, the dialysate was adjusted to pH 6.5, and the pptd protein was extracted ×3 with 0.2 M NaOAc buffer pH 4.8 containing 0.13 M NaCl. After dialysis against H_2O acidified to pH 4.5 the combined extracts were lyophilized.

Isolation of the proteinase. The lyophilized caryopses (25 g) were homogenized and extracted during 10–15 min with H_2O (1:5). The extract after centrifugation was fractionated with $(NH_4)_2SO_4$. Most of proteolytic activity was concd in the fr. salted out at 35–65% satn. The ppt. was dissolved in 13 ml of H_2O and loaded onto a column (3.2 × 48 cm) of Sephacryl S-200 HR equilibrated with 6 mM NaPi buffer pH 6.6 containing NaCl to give an ionic strength 0.1 (buffer A). The column was then eluted with the same buffer at 66 ml hr $^{-1}$. The frs showing proteolytic activity were loaded onto a column (1.5 × 20 cm) of DEAE-Sepharose CL 6B equilibrated with buffer A and eluted with a linear gradient of NaCl (5 mM cm^2 ml). The elution rate was 33 ml hr^{-1}. NaCl was added to the active frs to a final 2 M concn, and these were then loaded onto a column (1.5 × 5 cm) of phenyl-agarose equilibrated with buffer A containing 2 M NaCl. Elution was performed by a linear gradient of decreasing NaCl concn. The elution rate was 33 ml hr^{-1}. Finally, the enzyme prepn obtained was rechromatographed on phenyl-agarose at pH 4.8. The dimensions of the column were 0.8 × 5.3 cm, and the elution rate was halved. For electrophoresis the enzyme soln after the last stage of purification was concd to a final vol. of about 100 μl, at first by ultrafiltration through the acetylcellulose membrane UF-40 and then in the microconcentrator Centricon 10.

All solns contained 1 mM EDTA, 2 mM dithiothreitol (DTT) and 0.02% NaN_3, and the isolation was performed at 4–5°.

Determination of proteolytic activity. An equal vol. of 1% soln of vetch vicilin in 0.2 M NaOAc buffer pH 4.15 containing 1 mM EDTA and 2 mM DTT was added to 0.15 ml of the enzyme prepn and incubated at 30°. The incubation time varied from 1 to 2 hr depending on the activity of the enzyme prepn. The reaction was stopped by adding TCA up to 4%. The increase of the amino groups in the supernatant was determined as described earlier using 2,4,6-trinitrobenzenesulphonic acid [26]. In control experiments H_2O was substituted for the enzyme prepn. The enzyme activity was expressed in nkat (the amount of enzyme releasing 1 nmol of amino groups in the TCA-soluble fr. sec^{-1}).

Protein determination. Performed using a dye-binding method [27] with BSA as standard. Three replicate determinations were made for each sample with standard deviation within 0.01 *A* units. In the course of chromatographic purification the protein concn was recorded at 290 nm using the flow-photometer PUM-2.

Gel electrophoresis. A vertical flat-bed gel of 1 mm thickness was used. The run length was 9 cm. SDS-electrophoresis was carried out according to [28] in a 15% gel using the standard kit of markers: Phosphorylase b (94 k), bovine serum albumin (67 k), ovalbumin (43 k), carbonic anhydrase (30 k), Kunitz soybean trypsin inhibitor (20.1 k), and α-lactalbumin (14.4 k).

Non-denaturing electrophoresis was performed at 4–5° in 10% gel using the buffer system [29] where electrophoresis proceeds at pH 8.0. Proteinase A is more stable at this pH than at that of the usual system. The gel polymerization was initiated by riboflavin to avoid the inactivation of the enzyme by the persulfate remaining in the gel. The salt content in the enzyme prepn concd in the microconcentrator was reduced in the same place by repeated washing with H_2O.

Proteolytic activity was detected in gels containing 0.1% gelatin [30] or 0.05% vicilin. After electrophoresis the gels were equilibrated with 0.2 M NaOAc buffer pH 4.15 containing 1 mM EDTA and 2 mM DTT, and incubated for 6–12 hr at 35°. Then the gels were stained by the soln of Coomassie brilliant-blue G-250 in $HClO_4$. The proteolytic activity was manifested as colourless bands on a blue back-

ground. Protein bands were detected in a section of the same gel block after washing out the gelatin by 7% HOAc immediately after electrophoresis.

To study the action of ICH_2COOH on the proteolytic components detected on electrophoregrams the enzyme prepn was incubated before electrophoresis with 10 mM ICH_2COOH during 40 min at room temp.

N-terminal sequencing. The components of purified enzyme sepd by nondenaturing electrophoresis were blotted on PVDF membranes, and sequenced according to a special procedure (Beckman protocols) using the Beckman sequencer LF3400.

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