Phytochemistry, 1980, Vol. 19, pp. 2535–2540. © Pergamon Press Ltd. Printed in England. 0031-9422/80/1201-2535 $02.00/0

ROLE OF PROTEOLYTIC ENZYMES IN THE DEVELOPMENT OF AMINO ACID IMBALANCE IN WHEAT ENDOSPERM PROTEINS*

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(*Received* 4 *January* 1980)

Key Word Index–*Triticum aestivum*; Gramineae; wheat; lysine-rich and leucine-rich endosperm proteins; proteases; esterolytic activity; amino acyl ethyl esters; poly-L-lysine; poly-L-aspartic acid; poly-L-glutamic acid.

Abstract—Three proteases with caseinolytic activity have been isolated from the developing wheat endosperm. Two have been purified. The activity of protease A, the one that appears early in endosperm development, is inhibited by –SH inhibitors. Protease C, the one that appears late in endosperm development, is not affected. Protease A cleaves polyaspartic acid and polyglutamic acid but not polylysine. Protease C, on the other hand, cleaves polylysine but not polyaspartic acid and polyglutamic acid. Protease C degrades lysine-rich proteins isolated from wheat endosperm more efficiently than protease A.

INTRODUCTION

Proteins from mature endosperms of cereals like wheat are not considered to be of good nutritional quality as they contain limited amounts of certain amino acids. It has been reported earlier that lysine exists in relatively high proportion in the proteins of immature wheat endosperm, but this declines gradually as the grains mature [1]. This phenomenon is due to two processes that take place *in vivo* during endosperm maturation: (i) diminished synthesis of some proteins that are rich in lysine and (ii) selective degradation of the lysine-rich proteins [2]. We report here the purification and characterization of two proteolytic enzymes, one present at the early stage of endosperm development and the other at a late stage. The latter has been observed to bring about a preferential degradation of lysine-rich proteins.

RESULTS

Existence of multiple forms of protease in crude extract of wheat endosperm

It has been observed, by using endosperm proteins labelled with [^{14}C]-leucine and [^{3}H]-lysine as substrates for proteolysis, that the extent of release of lysine radioactivity compared to that of leucine, by the action of extracts from endosperms 20 days after anthesis, is more pronounced than by the action of extracts from endosperms 8 days after anthesis [2]. We have now sought multiple species of proteolytic enzymes in the extracts of endosperm by carrying out polyacrylamide disc electrophoresis of the extract. The presence of proteolytic activity in the gel was located by using azocasein, as mentioned in the Experimental. At least three different forms of proteolytic enzymes could indeed by detected.

Separation of the proteases

DEAE-cellulose column chromatography. A typical set of chromatrographic elution profiles obtained with extracts of wheat endosperms at various stages of development is shown in Fig. 1. The first peak with protease activity (protease C) eluted with 0.06 M KPi buffer pH 7.5. This protease was present only in the more mature endosperms (16 days onward). The second peak with protease activity (protease B) eluted with the main bulk of proteins with the same eluting buffer and was present only in endosperms 12 days after anthesis and onwards. The last peak with protease activity (protease A) was eluted with 0.07 M KPi buffer pH 7.5. This protease was present only in endosperms 8 and 12 days after anthesis. Thus the fractionation procedure serves to remove the bulk of the wheat proteins from the proteases A and C. The efficiency of separation was greatly influenced by temperature. Fractionation at room temperature yielded an elution profile quite different from that obtained by chromatography at 4°. Chromatography of different batches showed good reproducibility.

Rechromatography of form C resulted in the emergence of the enzyme at the same position as it appeared in the previous elution profile. Due to the instability of the other form (A), rechromatography could not be carried out. The fractionation of the mixture of extracts from 8-day- and 20-day-old endosperms under identical conditions showed the three protease activity peaks at the expected positions. Table 1 shows the specific activities of the enzymes A and C after DEAE-cellulose fractionation.

* Part 3 in the series "Development of Amino Acid Imbalance in Wheat Proteins". For Part 2 see Gupta R. K., Tiwari O. P., Gupta A. K. and Das H. K. (1976) *Phytochemistry* **15**, 1101.

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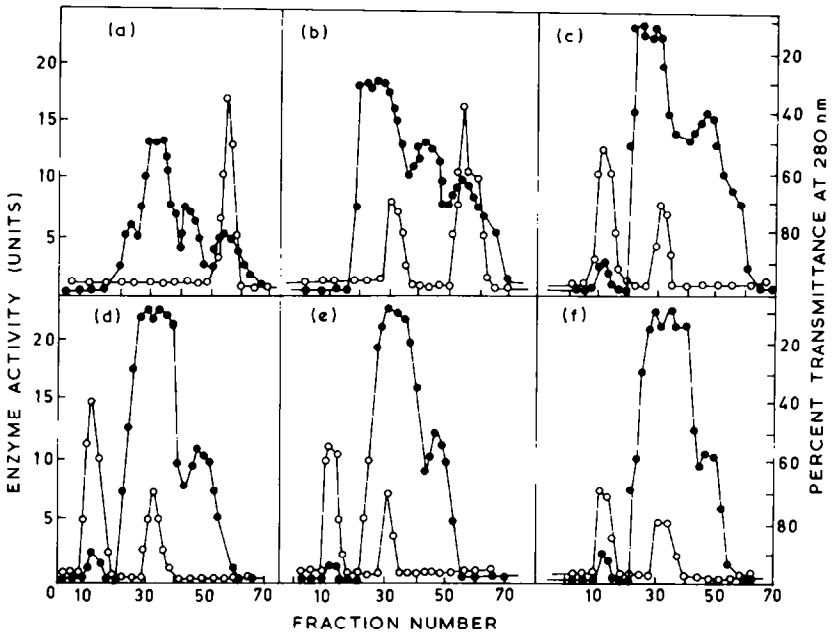

Fig. 1. DEAE-cellulose fractionation of crude extract of wheat endosperm. (●——●) Protein measured by absorbance at 280 nm; (○—- ○) protease activity. Extract from (a) 8-day-old endosperm; (b) 12-day-old endosperm; (c) 16-day-old endosperm; (d) 20-day-old endosperm; (e) 24-day-old endosperm and (f) 28-day-old endosperm.

Properties of proteases A and C

Stability on storage. Enzyme A was found to be very unstable. Enzyme C could be stored at 4° for a week and sometimes even longer without appreciable loss of activity. However, enzyme A lost activity very fast under similar storage conditions. Besides, reduced glutathione (4×10^{-3} M) was found to be essential for the maintenance of activity of enzyme A at 37° after 90 min incubation. Reduced glutathione had no effect on enzyme C.

Other properties. The pH optimum for the enzyme A was found to be 7.9 and for C around 7.4. MW determined by the gel filtration procedure [3] was around 10000 for A and 22000 for C. The activity of enzyme A was completely inhibited in the presence of 0.02 M *p*-hydroxymercury benzoate while the activity of C was unaffected (Table 2.). The soybean trypsin inhibitor was found to inhibit both enzymes (Table 3), the effect being more pronounced on enzyme C. Enzyme A (in KPi buffer, 0.07 M, pH 7.5) could withstand a temperature as high as 65° for 2 min (treatment

Table 1. Purification of enzymes A and C

Preparation	Total activity (Units)	Total protein (mg)	Specific activity	Purification (fold)
Enzyme A				
Crude extract from 8 day endosperms	806	45.8	17	0
Pooled DEAE fractions (50 to 60)	916	2	458	26
Enzyme C				
Crude extract from 20 day endosperms	1005	48.5	20	0
Pooled DEAE fractions (10 to 15)	349	0.5	698	34

Table 2. Effect of *p*-hydroxymercury benzoate on the activity of purified enzymes. The enzymes (100 μg protein for A, 90 μg protein for C) were preincubated with or without the inhibitor for 15 min at 37° before the addition of casein

	p-Hydroxymercury benzoate	Activity units	Inhibition (%)
A (enzyme from endosperms 8 days after anthesis)			
	None	18	
	0.0044 M	6	66
	0.02 M	1	95
C (enzyme from endosperms 20 days after enthesis)			
	None	11	
	0.0044 M	11	0
	0.02 M	20	0

Table 3. Effect of soybean trypsin inhibitor on the activity of purified enzymes

	Soybean trypsin inhibitor	Activity units	Inhibition (%)
A (enzyme from endosperms 8 days after anthesis)			
	None	15	
	1.8×10^{-5} M	4	73
C (enzyme from endosperms 20 days after anthesis)			
	None	24	
	1.8×10^{-5} M	0	100

terminated by plunging into crushed ice) and retain about 67% of its activity. On the other hand, enzyme C was totally inactivated by this treatment.

Specificity. The proteolytic specificities of the two enzymes have been investigated. As shown in Fig. 2, protease C was unable to cleave poly-L-aspartic and poly-L-glutamic acid, but was effective against poly-L-lysine. In contrast, protease A cleaved these two acidic homopolypeptides, but had very little activity towards the basic homopolypeptides. Trypsin (not shown in figure) was active only on poly-L-lysine. It was also found (Table 4A) that protease C preferentially attacked lysine-rich proteins of wheat endosperm, while protease A was more active towards the lysine-poor (leucine-rich) proteins. The isolation of the proteins used as substrate here was done by DEAE-cellulose fractionation on the basis of content of radioactive lysine and leucine. Moreover, protease C was more active on the albumin fraction of wheat endosperm, while activity of protease A was more pronounced on the globulin fraction (Table 4B). The albumin fraction is known to be lysine-rich and the globulin fraction lysine-poor. These fractions were isolated on the basis of solubility.

However, no definite preference could be seen with respect to size and charge of the substrate in the experiments using other natural proteins (Table 5). On the other hand, no esterolytic activity of enzyme A was detected towards LEE, though TAME and BTEE were cleaved. However, enzyme C acted on all three synthetic substrates including LEE (Table 6).

Acrylamide gel electrophoresis. Both the purified enzymes migrated in basic gel as a main protein band with shoulders, but in acidic gel a distinct minor band was also observed. For both enzymes only one active peak was observed even in acidic gel.

DISCUSSION

The existence of proteolytic enzymes in mature wheat grain and flour had been established quite early [4–8] but there has been no suggestion of their involvement in the regulation of the quality of proteins in the endosperm tissue. The results of our experiments establish the sequential appearance of at least three distinct proteases in wheat endosperm during the course of tissue development. One enzyme can be detected only at the very early stage of maturation, (A) while another specifically appears at the late stage (C). A third form (B) makes its appearance at an intermediate stage and is associated with a broad protein peak on DEAE-cellulose fractionation. Form B has not been worked upon.

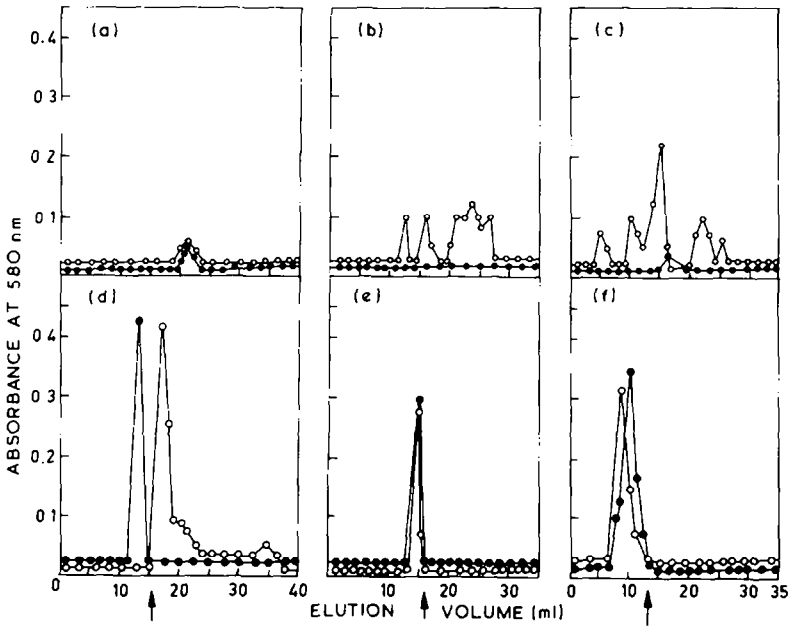

Fig. 2. Action of the proteolytic enzymes on homopolypeptides. The homopolypeptides were incubated at 37 with the purified enzymes. The reaction was terminated, the denatured enzyme was removed by centrifugation and the supernatant solution was layered on appropriate Sephadex columns. The presence of the polypeptides or their digestion products in the eluted fractions was determined by absorbance at 580 nm after reaction with ninhydrin. The cleavage of the homopolypeptides was ascertained by the disappearance or reduction in their size. Enzyme used: A, 75 μg protein/ml; C, 45 μg protein/ml. (●— ●) Homopolypeptide treated with inactivated enzyme; (○ — ○) treated with active anzyme. (a) Protease A on poly-L-lysine; (b) protease A on poly-L-glutamic acid; (c) protease A on poly-L-aspartic acid; (d) protease C on poly-L-lysine; (e) protease C on poly-L-glutamic acid; (f) protease C on poly-L-aspartic acid.

Table 4A. Lysine-rich and leucine-rich endosperm proteins as substrates for proteases A and C

Enzyme	Substrate (activity units)	
	[^{3}H]-Lysine-rich protein from wheat endosperm	[^{14}C]-Leucine-rich protein from wheat endosperm
Wheat protease A (50 μg protein/ml, from 8-day-old endosperm)	1	4
Wheat protease C (48 μg protein/ml, from 20-day-old endosperm)	7	4

Table 4B. Wheat endosperm albumin and globulin as substrates for proteases A and C

	Substrate(activity units)		
	Casein (300 μg/ml)	Endosperm albumin (160 μg/ml)	Endosperm globulin (140 μg/ml)
Trypsin (2.5 μg protein/ml)	6	6	2
Wheat protease A (from 8-day-old endosperm, 75 μg protein/ml	10	5	10
Wheat protease C (60 μg protein/ml, from 20-day-old endosperm)	8	4	1

Table 5. Natural proteins as substrates for proteases A and C

Substrate	MW	P_t value	Nature	Concn of substrate used (mg/ml)	Proteolytic enzyme (activity units) Trypsin (15 μg/ml)	A (75 μg/ml)	C (50 μg/ml)
Deoxyribonuclease	31000	4.70	Glycoprotein	1.5	30	41	45
				0.3	10	6	10
Catalase	250000	5.4	Contains haemin	1.5	45	30	28
Glucose oxidase	150000	4.2	Glycoprotein containing FAD	1.5	17	12	9
Lipoxidase	102400	5.4		1.5	0	15	16
Carbonic anhydrase	30000	5.3	Zn containing protein	1.5	13	0	0
Lysozyme	14200	11.0	Basic protein	1.5	45	8	10
Casein	375000	4.8	Phosphoprotein	3	200	10	10
Haemoglobin	68000	6.09	Iron containing protein	1.5	41	4	4

Table 6. Activity of wheat proteases A and C on synthetic substrates

Enzyme	Substrate (activity units) TAME (5×10^{-4} M)	BTEE (5×10^{-4} M)	LEE (1×10^{-2} M)
Trypsin (5 μg protein/ml)	52	5	3.5
Protease A (50 μg protein/ml, from 8-day-old endosperm)	15	28	0
Protease C (40 μg protein/ml, from 20-day-old endosperm)	15	7	2.0

The characteristics of enzymes A and C are very different. The important point is that the synthetic substrate lysine ethyl ester was cleaved by C, but not by A. Similarly, poly-L-lysine was cleaved only by C. On the other hand, poly-L-aspartic acid and poly L-glutamic acid were hydrolysed only by A. Form C was found to be more active towards lysine-rich proteins isolated from wheat endosperm while A was more so towards lysine-poor ones.

The relative lysine content of wheat endosperm proteins decreases during the course of seed development. The appearance of the proteolytic enzyme C towards the late stage, thus is indicative of its involvement in the regulation of the nature of proteins that would constitute the mature endosperm. Some evidence of specific degradation of lysine-rich proteins *in vivo* has been presented earlier [2].

EXPERIMENTAL

Plants. A large number of plants (*Triticum aestivum* L. var. Kalyan Sona) which had anthesis on the same day were chosen at random and tagged. Spikes were collected at 8, 12, 16, 20, 24 and 28 days after anthesis and stored at −20°.

Homogenization. The grains were dissected and the endosperms were collected. Homogenization of the endosperms was carried out at 4° in a Waring Blendor (mini-jar, 37 ml capacity) with 12 ml 0.05 M KPi buffer (pH 7.5) containing cysteine HCl (0.00 1 M) and EDTA (0.035 M). The number of endosperms used was 400 for 8-day-old, 270 for 12-day-old, 95 for both 16- and 20-day-old, 70 for 24-day-old and 55 for 28-day-old. The days were counted from the day of anthesis. The homogenate was centrifuged at 20000 *g* for 15 min at 4°. The supernatant solution was used and has been referred to as crude extract.

DEAE-cellulose column chromatography. The crude extract was subjected to chromatography on a DEAE-cellulose column (22 × 1.5 cm) equilibrated with 0.05 M KPi buffer, pH 7.5 at 4°. Crude extract (10 ml) was applied, followed by 2 ml of buffer. Elution was initiated with 80 ml 0.06 M KPi buffer, pH 7.5 and was subsequently followed with 92 ml 0.07 M KPi buffer, pH 7.5. The effluent fractions (2.3 ml) collected at a flow rate of 30 ml/hr were analysed for enzyme activity. The eluate from the column was monitored for absorbance at 280 nm just prior to collection of fractions.

Determination of protein. Protein was determined according to Lowry *et al.* [9]. Bovine serum albumin was used as standard and corrections were made for interference from glutathione and cysteine in the protein determination.

Preparation of substrate for enzyme assay. Casein (1%) was prepared in citrate phosphate buffer (8.5 mM citric acid, 16.5 mM NaPi) pH 7.5 by treating in a boiling water bath for *ca* 15 min. The solution was dialysed against the same buffer for 18 hr at 4°.

Enzyme assay. The activity of protease was assayed in a system containing 0.5 ml of dialysed solution of casein or other proteins and 0.5 ml of crude extract or column eluate or trypsin solution in 0.05–0.07 M KPi buffer, pH 7.5. The mixture was incubated at 37° for 2 hr. The incubation was terminated by adding 0.1 ml 55% TCA and cooling in an ice bath for 1 hr. The ppt. was removed by centrifugation or by filtration (Whatman, GF/C). The clear soln (0.8 ml) was neutralized with NaOH and the volume was made up to 0.9 ml. The product of proteolysis was estimated in 0.5 ml of this solution by the method of Lowry *et al.* [9] (2.5 ml Lowry C and 0.25 ml Folin reagent). Control samples were treated with TCA before incubation.

Enzyme unit. One unit of activity was defined as the amount of enzyme which caused on assay, as described above, an absorbance change of 0.01 measured at 610 nm.

Sephadex column chromatography. (i) *For MW determination.* The column used for MW determination was Sephadex G-100 (30 × 1.6 cm). It was equilibrated and eluted with 0.05 M KPi buffer, pH 7.5. The flow rate was 10 ml/hr and 1.2 ml fractions were collected. The location of marker proteins was determined either by their enzymatic activity or by protein estimation. (ii) *For study of cleavage of homopolypeptides.* The column used for poly-L-lysine (26 000) and poly-L-glutamic acid (19 700) was of Sephadex G-25 (40 × 0.9 cm). The buffer used was 0.05 M KPi, pH 7.5 and the flow rate was 7.5 ml/hr, vol. of each fraction being 0.9 ml. In the case of poly-L-aspartic acid (4460), a Sephadex G-10 (40 × 0.9 cm) column was used with the same buffer at a flow rate of 8–9 ml/hr. Vol. of each fraction here was 1.0 ml.

Hydrolysis of homopolypeptides by the proteases. The assay mixture containing 0.5 ml of the enzyme solution in buffer and 0.5 ml of the homopolypeptide solution (1 mg/ml) was incubated at 37° for 2.5 hr. The reaction was arrested by treatment of the mixture in a boiling water bath for 15 min. After centrifugation, the supernatant soln (0.6 ml) was layered on respective Sephadex columns. Similarly, a control was run with the boiled enzyme. However, this procedure was found not suitable for enzyme A, as the heat treatment degraded the enzyme itself. In this case, the reaction was stopped by the addition of 0.2 N PCA and excess acid was subsequently neutralized with KOH. Precipitated potassium perchlorate was removed by centrifugation. Since the reduced glutathione, present in the preparation of the form A, was also eluted with the degraded products, it was removed with a saturated solution of lead acetate [10] prior to gel filtration. The excess lead acetate was removed by treatment with sodium carbonate and centrifugation. The proteolysed products in the eluted fractions were estimated by ninhydrin reaction [11].

Preparation of wheat proteins. (i) *Fractionation of endosperm proteins.* Wheat endosperm proteins were fractionated by the method of Osborne as adopted by Harvey and Oaks [12]. The albumin fraction (water soluble) and the globulin fraction (extracted with 5% NaCl) were saved. (ii) *Preparation of lysine-rich proteins and leucine-rich proteins from the radioactive wheat endosperm.* A mixture of two labelled amino acids ([^{14}C]-L-leucine and [^{3}H]-L-lysine) was injected into the wheat plants at the base of the spikes 16 days after anthesis. The spikes were collected 24 hr later and endosperms from the radioactive seeds were removed. The endosperms (100) were homogenized in a Potter–Elvehjem homogenizer with 5 ml 0.05 M KPi buffer, pH 7.5 and centrifuged at 4°. The supernatant soln (7 ml) was applied to a DEAE-cellulose column (22 × 1.5 cm) which was equilibrated with the same buffer. After washing in the sample with 2 ml buffer, elution was done at 30 ml/hr with 0.06 M KPi buffer, pH 7.5. The first 35 ml of the eluate was rejected and the subsequent 33 ml was collected. This portion was found to be free from proteases A and C. The remaining eluate was made 70% saturated with respect to $(NH_4)_2SO_4$ and left at 4° for 18 hr. The ppt was collected by centrifugation and dialysed against 0.01 M Tris–HCl buffer, pH 9.2. Finally fractionation was achieved by second chromatography of the dialysed solution on a DEAE-cellulose column (10 × 1 cm). Stepwise elution was done with 0.01 M Tris–HCl buffer, pH 9.2 containing 0.02, 0.05, 0.1 and 0.15 M NaCl. Vol. of liquid per step was 25 ml and flow rate was 44 ml/hr. The first 25 ml eluate was discarded and the next 12.5 ml was collected. This portion contained the leucine-rich proteins as was evident from the high ratio of ^{14}C/^{3}H radioactivity. Then 42.5 ml of the eluate was again discarded and the subsequent 10 ml which contained the lysine-rich proteins (low ^{14}C/^{3}H ratio) was collected. Radioactivity was determined using Bray's [13] scintillation mixture.

Esterolytic activity. (i) *Hydrolysis of p-tosyl-L-arginine methyl ester and N-benzoyl-L-tyrosine ethyl ester.* BTEE (0.75 ml, 0.001 M) or TAME (0.75 ml, 0.001 M) was added to a mixture of 0.2 ml of enzyme soln and 0.55 ml Tris–HCl buffer (0.04 M), pH 7.9. The cleavage was determined from absorbance at 256 or 247 nm respectively against a control [14]. One unit of protease activity was arbitrarily defined as the amount of enzyme which produces an increase in absorbance of 0.1 through 1 cm light path. (ii) *Hydrolysis of L-lysine ethyl ester.* Hydrolysis was determined by the pH stat method [15]. For each assay, 1 ml of 0.1 M LEE was added to a mixture of 8 ml 0.005 M Tris–HCl buffer, pH 7.9 and 1 ml of enzyme soln.

Polyacrylamide disc electrophoresis. Electrophoresis was carried out by the method of Davis [16] for basic gels and by the method of Reisfeld *et al.* [17] for acidic gels. The enzymatic activity was detected by the method of Andary and Dabich [18] using azocasein instead of casein. Electrophoresis was carried out at 4 mA/tube for the first 1 hr and 5 mA/tube for the next 2 hr. The gels were scanned at 410 nm.

Acknowledgements—The project was financed by the Indian Council of Agricultural Research. We are grateful to Dr. Rajat De of the Division of Agronomy, Indian Agricultural Research Institute for kindly permitting us to use the plants from his cultivated field.

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