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Purification and characterization of proteolytic enzymes from normal and opaque-2 *Zea mays* L. developing endosperms*

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Abstract. Purification and characterization of proteases from developing normal maize endosperm and high lysine opaque-2 maize endosperm have been carried out with a view to understand their role in storage protein modification. At day 15, normal maize endosperm had two types of proteolytic enzymes, namely, protease I and protease II, while at day 25 protease II disappeared and in place protease III appeared. However, in opaque-2 maize endosperm at both the stages only one type of enzyme (protease I) was present. These proteases had many properties in common—optimum pH and temperature were respectively, 5·7and 40°C; their activity was inhibited to the extent of 75 –93 % by *p*-chloromercuribenzoate; trypsin inhibitor inhibited the activity more at early stages of endosperm development; all proteases cleaved synthetic substrates *p*-tosyl-L-arginine methylesler and N-benzoyl-L-tyrosine ethyl ester and poly-L-glutamic acid. The *Km* values of day 15 and 25 normal maize endosperm proteases ranged from 2·73–3·30, while for opaque-2 maize endosperm protease I it was 3·33 mg azocasein per ml assay medium. These enzymes, however, differed with respect to proteolytic activity towards poly-L-lysine. Only normal maize endosperm protease III at day 25 followed by protease II at day 15 showed high activity towards this homopolypeptide suggesting thereby their role in determining the quality of normal maize endosperm protein.

Keywords. *Zea Mays;* Gramineae; proteases; esterolytic activity; homopolypeptides.

Introduction

Maize endosperm protein is considered to be nutritionally poor because of its deficiency in lysine and tryptophan. In the presence of opaque-2 gene, nutritional quality of maize protein is improved mainly because of suppressed synthesis of zein (Murphy and Dalby, 1971; Lodha *et al.,* 1978), which is extremely deficient in lysine and tryptophan. However, as the grain matures, nutritional quality of not only normal maize protein but also of opaque-2 maize deteriorates (Gupta *et al.,* 1978). Decrease in lysine per endosperm towards maturity has been reported in normal and opaque-2 maize (Gupta *et al.,* 1977; Lodha *et al.,* 1978), which may be due to protein turnover or selective degradation of lysine-rich proteins. Sodek and Wilson (1970) reported conversion of injected lysine to glutamic acid and proline more in normal maize endosperms than in opaque-2 maize endosperms during development. In our earlier

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Abbreviations used: DTT, Dithiothreitol; PCA, perchloric acid; M., molecular weight; TAME, *p*-tosyl-L-arginine methylester; BTEE, N-benzoyl-tyrosine ethyl ester; PCMB, *p*-chloromercuribenzoate.

study (Ram *et al.,* 1986) based on degradation of [^{3}H]-lysine and [^{14}C]-leucine labelled protein *in vitro* by proteases from developing normal and opaque-2 maize endosperms, it was suggested that lysine-specific proteases may be present in both the normal and opaque-2 developing maize endosperms. In the present investigation, purification and characterization of proteolytic enzymes from developing normal and opaque-2 maize endosperms have been carried out.

Materials and methods

A normal maize inbred line Fla 3H94 and its opaque-2 isogenic line were obtained from the All India Coordinated Maize Improvement Project, IARI, New Delhi. Self-pollinated ears were harvested at 15, 20, 25 and 30 days after pollination and endosperms were collected after removing the pericarp and embryo from the kernels at 0–4°C. The endosperms were stored in deep freeze till further use.

Extraction of proteases

Endosperms were hand ground in liquid nitrogen in a pestle and mortar using sterilized sand with medium (1:2, W/V) containing 50 mM potassium phosphate buffer (pH 7·5), 35 mM EDTA, 10 mM dithiothreitol (DTT). The homogenate was centrifuged for 15 min at 20,000 *g* and the supernatant was used as crude enzyme. All the operations unless otherwise stated were carried out at 4°C.

Assay of acid protease activity

Acid protease activity was assayed according to Miller and Huffaker (1981) with slight modifications. The reaction mixture in a final volume of 2·2 ml contained: 50 mM potassium phosphate buffer (pH 5·7), 1·0 ml, azocasein (4 mg/ml of 50 mM phosphate buffer, pH 5·7), 1·0 ml, and enzyme preparation, 0·1–0·2 ml. After incubation at 40°C for 1 h, 1 ·0 ml of 14 % perchloric acid (PCA) was added to stop the reaction. Tubes were kept for 1 h at 0°C for ageing and then centrifuged. Hydrolysis was measured as an increase in absorbance of supernatant at 340 nm. In the blank 1·0 ml 14% PCA was added before adding the enzyme.

Enzyme unit

One unit of activity was defined as the amount of enzyme which caused an absorbance change of 0·01/h measured at 340 nm. The specific activity of enzyme was expressed as enzyme units per mg protein.

Purification of proteases

Ammonium sulphate fractionation: Solid ammonium sulphate was added slowly to the crude extract with constant stirring till 70 % saturation was obtained. The extract was allowed to stand for 1 h in deep freeze. The pellet recovered by centrifugation at 20,000 *g* for 15 min was resuspended in extraction buffer. It was dialysed for 16 h against the extraction buffer at 4°C. Two changes of the buffer were made. The recovery of protease enzyme was 80 %.

DEAE-cellulose chromatography: Dialysed supernatant (18–20 ml) from the above step was layered on DEAE-cellulose column (2·5 × 15 cm) equilibrated with 0·05 M potassium phosphate buffer (pH 7·5). After washing the sample with 2 ml of buffer, elution was first done with 110 ml of 0·06 M phosphate buffer (pH 7·5) and then with 120 ml of 0·07 M phosphate buffer (pH 7·5). The effluent was monitored at 280 nm using LKB uvicord-II and 2·2 ml fractions were collected. Flow rate was maintained at 30 ml per h. Each fraction was assayed for protease activity.

Characterization of purified proteases

Two forms of proteases from normal endosperm and one from opaque-2 endosperm at 15 and 25 day stages obtained as above were characterized. For determining the optimum pH, temperature, incubation time and substrate concentration, assay mixture and other conditions of the assay were the same as used for the assay of crude enzyme.

Hydrolysis of homopoly peptides by proteases

The hydrolysis of homopolypeptides such as poly-L-lysine [molecular weight (M_r) 6000] and poly-L-glutamic acid (M_r 10,000), was studied using an assay mixture which contained: purified enzyme, 0·1ml, 50 mM phosphate buffer (pH 5·7) containing 1 mM DTT, 0·4 ml; and homopolypeptide solution (1 mg homopolypeptide/ml phosphate buffer), 0·5 ml. The reaction mixture was incubated at 40°C for 1 h. The reaction was stopped by adding 1 ml of 14% PCA. In control, 1 ml 14% PCA was added at zero time. After centrifugation, 1 ml of supernatant solution was layered on Sephadex G-50 column (1 × 30 cm) equilibrated with phosphate buffer (pH 7·5). The elution buffer used was 50 mM phosphate buffer (pH 7·5) and the flow rate was 10 ml per h. Two ml fractions were collected. The concentration of proteolysed products in the eluted fractions was estimated by ninhydrin reaction as described by Moore and Stein (1954).

Esterolytic activity

Esterolytic activity of the purified enzymes towards synthetic substrates such as *p*-tosyl-L-arginine methyl ester (TAME) and N-benzoyl-L-tyrosine ethyl ester (BTEE) was determined as follows: TAME (1 mM, 1·0 ml) was added to a mixture of 0·1 ml of purified enzyme solution and 0·9 ml of 50 mM phosphate buffer (pH 5·7) containing 1 mM DTT. The cleavage was determined from the change in absorbance at 247 nm against a control. Similarly, cleavage of BTEE was determined from the change in absorbance at 256 nm.

Effect of inhibitors

In order to study the effect of PCMB, 0·1 ml purified enzyme preparation was pre-incubated with 1·0 ml of 0·063 M *p*-chloromercuribenzoate (PCMB) in 50 mM potassium phosphate buffer (pH 5·7) for 15 min at 40°C. Then it was incubated with 1 ml azocasein (4 mg/ml) solution and protease activity with and without PCMB, was determined as described earlier.

In the case of trypsin inhibitor study, the assay mixture in a final volume of 2 ml

contained: 1 ml azocasein solution, 1 ml inhibitor solution (Sigma Type II-S soybean trypsin inhibitor, 250 μg/ml of 50 mM potassium phosphate buffer, pH 5·7) and 0·1 ml enzyme preparation. Protease activity with and without the inhibitor, was determined as described earlier.

The results presented are an average of at least two experiments, each having been carried out in triplicate.

Results

Protease activity

Acid protease activity per endosperm with azocasein as substrate remained constant till day 20, increased at day 25 and decreased thereafter at day 30 in normal maize, while in opaque-2 endosperms it increased linearly upto day 25 and then declined at day 30. The activity was higher at days 15 and 25 and lower at days 20 and 30 in normal maize than in opaque-2 maize endosperm (figure 1).

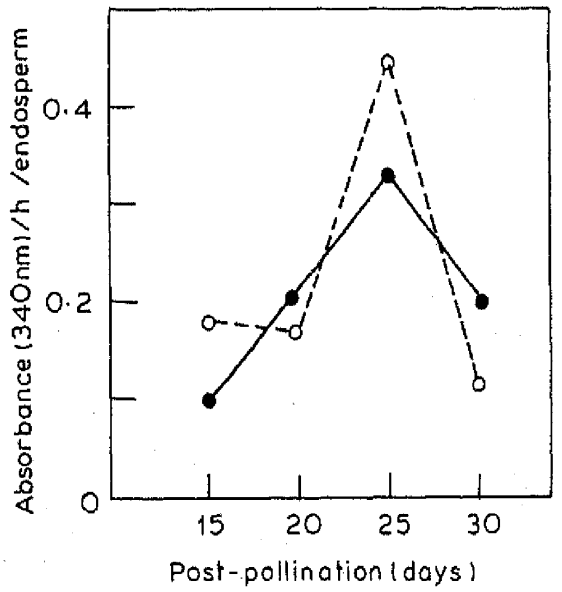

Figure 1. Acid protease activity with azocasein in the developing endosperm of normal (O) and opaque-2 (●) maize.

Purification of proteases

Proteases from normal and opaque-2 endosperms of days 15 and 25 post-pollination stages were purified using ammonium sulphate fractionation and DEAE-cellulose column chromatography. The results are presented in table 1 and figure 2.

Twenty six to eighty five fold purification was obtained for different proteases. Three forms of proteases, namely, protease I, II and III were isolated. Protease III (figure 2C) was present only at day 25 and protease II (figure 2A) at day 15 in normal maize. Opaque-2 endosperm at both the stages had only one major peak of protease I

Table 1. Purification of proteases.

Preparation	Total activity (units)	Total protein (mg)	Specific activity	Purification (fold)
	Day 15 normal maize endosperm			
Crude extract	3120	190	16	0
Ammonium sulphate fraction	2800	82	34	2
Pooled DEAE fractions (42–46)—Protease II	1100	2·6	423	26
Pooled DEAE fractions (84–88)—Protease I	1615	1·3	1242	78
	Day 15 opaque-2 endosperm			
Crude extract	2720	178	15	0
Ammonium sulphate fraction	2070	57·2	36	2·4
Pooled DEAE fractions (81–85)—Protease I	1540	1·2	1283	85
	Day 25 normal maize endosperm			
Crude extract	3510	184	19	0
Ammonium sulphate fraction	2755	60·8	45	2·4
Pooled DEAE fractions (12–16)—Protease III	1870	1·3	1438	76
Pooled DEAE fractions (71–75)—Protease I	770	1·4	550	29
	Day 25 opaque-2 endosperm			
Crude extract	2613	171·6	15	0
Ammonium sulphate fraction	2070	55·8	37	2·5
Pooled DEAE fractions (87–91)—Protease I	1540	1·3	1184	79

Enzyme unit: One unit of enzyme activity caused an absorbance change of 0·01/h at 340 nm.

(figure 2B, D). Protease I was also present at both the stages in normal maize endosperms. Protease II and III were eluted with 0·06 M phosphate buffer (p H 7·5), while protease I with 0·07 M phosphate buffer (pH 7·5).

Characterization of proteases

The purified fractions of proteases obtained from DEAE-cellulose column were characterized with respect to optimum pH, temperature, incubation time, substrate concentration, esterolytic activity, hydrolysis of homopolypeptides, stability and the effect of inhibitors. The results are presented in tables 2 and 3 and in figures 3 and 4.

The activity of proteases from both normal and opaque-2 endosperms was linear upto 90 min with an optimum of pH 5·7 and optimum temperature of 40°C. Therefore, in all subsequent studies, an incubation time of 60 min was used and assay done at the optimum pH and temperature.

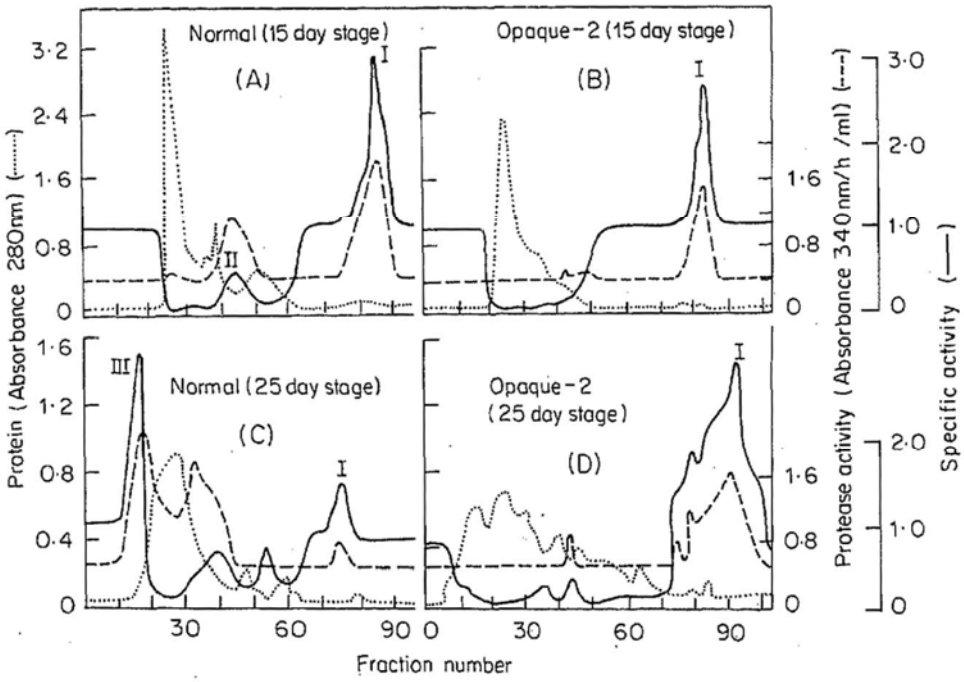

Figure 2. Elution profile of normal and opaque-2 maize endosperm proteases from DEAE-cellulose column.

Table 2. Activity of normal and opaque-2 maize endosperm proteases on synthetic substrates.

	Activity (Δabsorbance/h/ml)	
Enzyme	TAME	BTEE
	15 day stage	
Normal maize protease II	0·60	0·55
Normal maize protease I	0·60	0·60
Opaque-2 protease I	0·63	0·70
	25 day stage	
Normal maize protease III	0·45	0·50
Normal maize protease I	0·50	0·46
Opaque-2 protease I	0·45	0·66

Substrate concentration and K_m: The effect of substrate (azocasein) concentration on the velocity of the reaction was characterized by a hyperbolic curve. The K_m for each protease was calculated from the Lineweaver-Burk plot. The *Km* values for 15 day stage normal protease II, normal protease I and opaque-2 protease I were 3·30, 2·82 and

Table 3. Effect of different storage temperatures on the activity of normal and opaque-2 maize endosperm proteases.

Enzyme	Initial protease activity (Δ absorbance/h/ml)	Protease activity after 10 day storage (Δ absorbance/h/ml)		
		4°C	−20°C	−196°C
	15 day stage			
Normal maize protease II	1·0	0·70	0·90	0·90
Normal maize protease I	1·55	1·0	1·30	1·28
Opaque-2 protease I	1·40	0·90	1·18	1·15
	25 day stage			
Normal maize protease III	1·60	1·05	1·35	1·30
Normal maize protease I	0·80	0·65	0·75	0·73
Opaque-2 protease I	1·40	0·85	1·20	1·15

3·33 mg azocasein per ml, respectively. The K_m values for 25 day stage normal protease III, normal protease I and opaque-2 protease I were 3·0, 2·73 and 3·33 mg azocasein per ml, respectively.

Esterolytic activity: The esterolytic activities of $_{all}$ proteases, except day 25 opaque-2 protease I, with TAME and BTEE as substrate were similar. Day 25 opaque-2 protease I, however, preferred BTEE over TAME as substrate (table 2).

Specificity: The substrate specificity of normal and opaque-2 purified proteases was investigated using poly-L-lysine and poly-L-glutamic acid as substrates and the results are illustrated in figures 3 and 4.

All proteases cleaved poly-L-glutamic acid (figures 3 and 4). However, poly-L-lysine was a good substrate only for normal protease III from day 25 (figure 4C) and normal protease II from day 15 (figure 3C) endosperms. Day 15 normal protease I (figure 3A) and day 25 opaque-2 protease I (figure 4E) hydrolysed poly-L-lysine at a slower rate. No activity was observed with 25 day normal protease I (figure 4A) and 15 day opaque-2 protease I (figure 3E).

Enzyme stability: The enzymes were more stable when stored at – 20°C and – 196°C than at 4°C (table 3). Normal maize protease II at 15 day stage and protease I at 25 day stage were relatively more stable.

Inhibitors: PCMB at 0·03 M concentration inhibited all proteases to the extent of 75–93 %. Trypsin inhibitor also inhibited the activity of normal and opaque-2 maize proteases. At the trypsin inhibitor concentration of 250 μg per 2·1 ml assay medium, the activities of day 15 normal maize protease I and II were inhibited to the extent of 70 % as against 46 % inhibition of 25 day stage normal maize protease I and III. At the same concentration of trypsin inhibitor the activity of opaque-2 protease I of 15 and 25 day stages was inhibited by 64% and 34%, respectively.

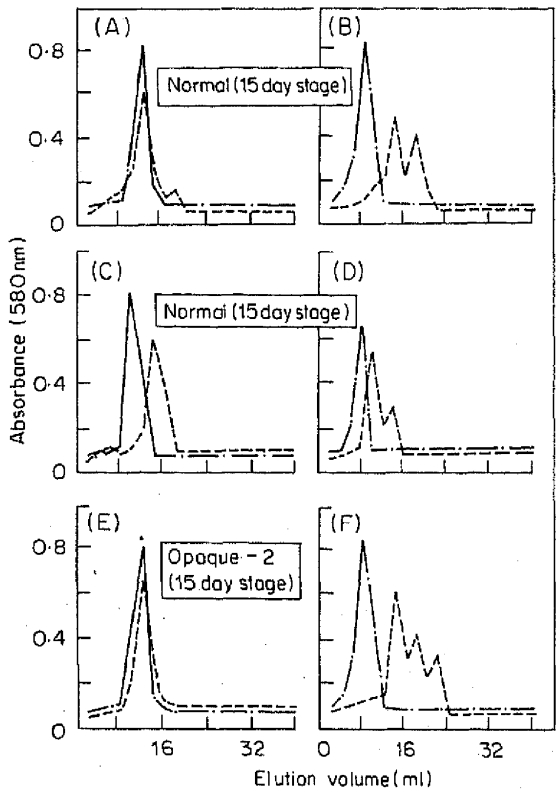

Figure 3. Action of proteolytic enzymes from 15-day old normal and opaque-2 maize endosperms on homopolypeptides. After the reaction was terminated, the supernatant solution was layered on Sephadex G-50 column. The presence of the polypeptides or their digestion products in the eluted fraction was determined by absorbance at 580 nm after reaction with ninhydrin. The cleavage of the homopolypeptides was ascertained by the disappearance or reduction in their size. (——), Homopolypeptides treated with inactive enzyme and (------), with active enzyme. **A.** Normal maize protease I on poly-L-lysine. **B.** Poly-L-glutamic acid. **C.** Normal maize protease II on poly-L-lysine. **D.** Poly-L-glutamic acid. **E.** Opaque-2 maize protease I on poly-L-lysine. **F.** Poly-L-glutamic acid.

Discussion

Proteases are known to play an important role in the breakdown of proteins and in protein turnover in germinating cereal seeds. The acid protease from maize endosperm has been shown to be active against native proteins zein and glutelin (Harvey and Oaks, 1974). Our earlier study has suggested the presence of lysine-specific proteases in both normal and opaque-2 developing endosperms (Ram *et al.,* 1986). The present study showed the presence of two proteases, protease I and II in 15-day old normal maize endosperm, and appearance of protease III in place of protease II at 25 day stage. In case of opaque-2 maize endosperm only one type of protease (protease I) has been found to be present at both 15 and 25-day stages. All the proteases cleaved poly-L-

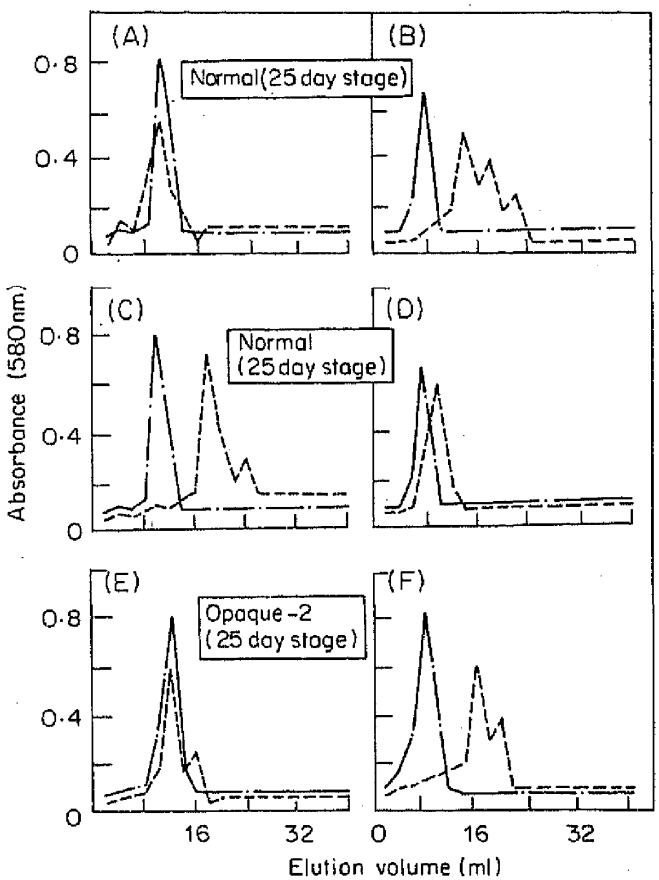

Figure 4. Action of proteolytic enzymes from 25-day old normal and opaque-2 maize endosperms on homopolypeptides. Experimental details are same as in figure 3. **A.** Normal maize protease I on poly-L-lysine. **B.** Poly-L-glutamic acid. **C.** Normal maize protease III on poly-L-lysine. **D.** Poly-L-glutamic acid. **E.** Opaque-2 protease I on poly-L-lysine. **F.** Poly-L-glutamic acid.

glutamic acid. As regards proteolytic specificity towards poly-L-lysine, only 25-day normal protease III followed by 15-day normal protease II showed high activity, while 15 day normal maize protease I and 25 day opaque-2 protease I showed only a minor activity. Esterolytic activity with synthetic substrates TAME and BTEE suggested that all the proteases had this activity like trypsin and chymotrypsin.

The biological value of normal maize proteins deteriorates more as compared to opaque-2 maize protein during the later stages of grain maturation (Gupta *et al.,* 1978). This is mainly due to the increased synthesis of lysine-poor proteins during later stages of endosperm development (Murphy and Dalby, 1971; Lodha *et al.,* 1978), protein turnover and/or removal of lysine from performed proteins and its conversion to other amino acids (Sodek and Wilson, 1970). Synthesis of protease III with high activity for poly-L-lysine in the later stages of normal maize endosperm development would favour degradation of lysine-rich proteins or will result in post-translational modifications where lysine-rich residues are removed. This is consistent with the observation that during this period inspite of substantial accumulation of storage protein in endosperm,

the absolute amount of lysine per endosperm decreases (Gupta *etal*.,1977; Lodha *et al.,* 1978). This has also been confirmed by rat feeding trials, wherein the protein quality of normal maize kernals at day 25 has been shown to be equal to that of opaque-2 maize at maturity (Gupta *et al.,* 1978). The absence of protease III in opaque-2 maize endosperm would favour lack of such modifications as may perhaps occur in normal maize endosperm. Ghosh and Das (1980) have also reported that in wheat, protease C, which appears late in endosperm development, cleaves poly-L-lysine. Therefore, the results of this study along with the results obtained in an earlier study (Ram *et al.,* 1986) are suggestive of the role of proteases in protein turnover/post-translational modifications of storage proteins in normal maize endosperm.

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