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Storage Protein Characteristics of Proline-requiring Mutants of *Zea mays* (L.)

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Summary. Protein and amino acid composition of mature karnels from three allelic proline-requiring mutants in maize, pro_{1-1}, pro_{1-2}, and pro_{1-3} were analyzed and compared to kernels of the stock A 188 containing the wild type allele. The amount of free proline was specifically reduced in the embryos of all three mutants, while in the endosperm such a reduction was only found for pro_{1-2} and pro_{1-3}. Accumulation of the proline-rich zeins was strongly reduced in the mutants, but in contrast to opaque-2 the reduction affected all major zein polypeptides to the same extent, possibly as a consequence of the defective proline metabolism. Albumins and globulins as well as free amino acids were more abundant in the endosperms of the mutants than in the wild type. Analysis of the albumins and globulins by SDS-PAGE revealed specific increases as well as reductions of certain polypeptides in the endosperms and embryos of the mutants.

Key words: *Pro* mutants of maize – Amino acids – Proteins of endosperm and embryos – SDS-PAGE (sodium dodecylsulfate containing polyacrylamide gel electrophoresis)

Introduction

Mature karnels of homozygous *pro* mutants of maize exhibit a collapsed and dull endosperm. They germinate into seedlings which require proline for further growth (Gavazzi et al. 1975). Other amino acids cannot substitute for proline (Racchi et al. 1978). Clarification of the biochemical lesion resulting in this requirement is still wanting. Recent investigations have demonstrated that proline synthesis as such is not deficient in pro_{1-1} seedlings (Bertani et al. 1980; Dierks-Ventling and Tonelli 1981). Proline catabolism appears to be enhanced over proline biosynthesis (Dierks-Ventling and Tonelli 1981) and this may be a cause for the requirement. Such an alteration in general metabolism is expected to affect the amount of free amino acids available for protein synthesis and could alter the types of protein synthesized in the cells. As reserve proteins such as zeins (and also albumins and globulins) are rich in proline (Sodek and Wilson 1971; Gianezza et al. 1977), a specific depression in the synthesis of these storage proteins might be expected. I have therefore studied the amount and types of reserve proteins present in the mature endosperm and embryos, including free amino acids of three allelic *pro* mutants (Racchi et al. 1981) and compared them with those present in sibling stock A 188 kernels containing the wild type *PRO* allele.

Materials and Methods

Plant Material

The genetic background of the mutant stocks pro_{1-1}, pro_{1-2}, and pro_{1-3} was relatively homogenous, as the mutants were obtained by selfing after three (in the case of pro_{1-2}) and two (in the case of pro_{1-1} and pro_{1-3}) backcrosses to the inbred line A 188. Seeds were harvested in 1980. Analysis was done on mutants and their normal siblings (A 188) containing the *PRO* allele from the same cob. All seeds were produced and kindly supplied by G. Gavazzi (University of Milano, Italy).

Quantitative Analysis of Seed Proteins

For an analysis of endosperm proteins mature seeds were deembryonated by hand and the endosperms were ground to a fine flour using an UDY cyclon mill (Tekator, Sweden). For an analysis of embryo proteins, the seeds were soaked for 4 hours in distilled water at room temperature which facilitated removal of the entire embryo with scutellum and axis (referred to in the text as embryo for reasons of brevity). Embryos were homogenized quickly in ice-cold acetone (10 ml per 5 embryos) by means of a Polytron PT 10S power-driven homogenizer (Kinematica, Kriens/Luzern, Switzerland). Endosperms were also first treated with ice-cold acetone (10 ml per 1 g flour). Defatting was done with shaking at –20 °C for a minimum of 2 h. After removal of the acetone phase, the sedi-

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ment was resuspended in 10 ml dry ether, centrifuged and the ether removed to leave a dry sample. This was then either used for a quantitative extraction of proteins or for amino acid analysis. Proteins were quantitatively extracted according to Landry and Moureaux (1970). The procedure was modified so as to include in all extraction phases 0.1 mM phenylmethylsulfonyl-fluoride, 0,01% ethylmercuri-thiosalicylic acid, Na salt (both from Serva, Heidelberg) and 0.6% β-mercaptoethanol. Extractions were done at room temperature and centrifugations at 16,000×g for 20 min in a Sorvall RC-5 centrifuge. The extractions of each series were pooled. The combined saline extracts were dialyzed against a large volume of ice-cold distilled water for at least 3 h. Globulins which precipitated during the dialysis were separated from the soluble albumins by centrifugation as described above; they were quantitatively determined by difference before and after dialysis. Zein and glutelin extractions were done according to the original procedure.

Protein Determinations

In all aqueous solutions proteins were quantitatively determined according to Bradford (1976). Zeins were measured as Kjeldahl nitrogen and converted to protein quantities by multiplication with the factor of 6.25. Proteins are expressed as mg protein/endosperm or embryo.

Free Amino Acid Analysis

After defatting, endosperms or embryos were extracted twice with distilled water (5 ml per 1 g flour or 5 embryos) for 30 min at room temperature, the samples were centrifuged as above and the supernatant fluid was brought to pH 2.0 before being subjected to amino acid analysis on a Technicon semiautomatic amino acid analyzer according to Von Arx et al. (1974).

Gel Electrophoresis

After quantitative protein determinations, albumins were diluted with modified Laemmli' sample buffer (Thomas et al. 1975) to a concentration of 1 µg/µl. Globulins were dissolved directly in this sample buffer at the same concentration. Electrophoresis was done in vertical slab gels of 15% polyacrylamide-1% sodium dodecylsulfate containing 6 M urea with up to 100 µg protein per track for 16 h at room temperature at a constant 50 V (Thomas et al. 1975). Gels were stained with Coomassie Blue R 250 for 4 h at room temperature and destained electrophoretically for 15 min at 3 amp in 5% methanol – 7.5% acetic acid.

Results

Proline and Glutamate Content in pro Mutant Endosperms and Embryos

The total free amino acid content of mutant and normal endosperm and embryos were analyzed; however, only lysine, proline and glutamate are listed in Table 1 because of their relative importance for the synthesis of storage proteins and because all the other amino acids did not show striking differences among mutant tissues. In comparison to the normal, all three *pro* mutants exhibited an increased amount of free amino acids in endosperms, the largest being that of pro_{1-3}. The increase in the total amount of free amino acids was not reflected in an increase in the individual amino acids listed. On a mole per cent basis, all three mutants contained less lysine and pro_{1-2} and pro_{1-3} also less proline; the latter amounted to only about 25% of the amino acid content in normal stocks. Mutant pro_{1-1} on the other hand contained more than three times the amount of free proline as the wild type endosperm. The relative amount of free glutamate was increased in pro_{1-1} whereas the other three genotypes had about the same proportion of this amino acid.

The total free amino acid content of *pro* embryos was higher than of *pro* endosperms but about the same in the three mutants. Lysine was somewhat increased, mostly in pro_{1-3} embryos. Proline concentrations were significantly reduced in all three mutants. Mole percentages of glutamate were the same in pro_{1-2}, pro_{1-3}, and *PRO*, whereas pro_{1-1} showed an increase comparable to that in the endosperm.

Quantitative Estimation of Protein Subfraction in Endosperm and Embryos of pro Mutants

Endosperm and embryo proteins from *pro* mutants and *PRO* sibling seeds were fractionated according to their differential solubility. Endosperm proteins are listed in

Table 1. Free lysine, proline and glutamate content in endosperm and embryos from mature kernels of *pro* mutants and stock A 188 containing the *PRO* wild type allele

Genotype	Endosperm				Embryo			
	Total free amino acids µmol endosperm	Lysine µmol %	Proline µmol %	Glutamate µmol %	Total free amino acids µmol/embryo	Lysine µmol %	Proline µmol %	Glutamate µmol %
PRO (A 188)	0.087	4.3	17.7	11.0	0.497	4.6	51.8	12.9
pro_{1-1}	0.300	3.6	18.9	17.5	0.553	5.4	22.6	19.4
pro_{1-2}	0.185	3.3	4.1	11.3	0.455	5.7	6.8	14.5
pro_{1-3}	0.628	1.5	4.5	9.5	0.546	8.2	10.1	11.0

Table 2. Content of storage proteins in endosperms of *pro* mutants and stock A 188 containing the *PRO* wild type allele

	PRO (A 188)		pro_{1-1}		pro_{1-2}		pro_{1-3}	
	mg/ endosperm	% of total	mg/ endosperm	% of total	mg/ endosperm	% of total	mg/ endosperm	% of total
Albumins	1.31±0.27	6.5	1.77	17.2	4.21	31.5	2.33	16.4
Globulins	1.11±0.10	5.5	1.13	11.0	1.66	12.4	1.53	10.8
Zeins	1 393±2.43	68.8	5.15	50.0	4.28	32.1	7.52	52.0
Glutelins	3.90±0.29	19.3	2.26	21.9	3.20	23.9	2.81	19.8
Total protein	20.25	100	10.31	100	13.35	100	14.19	100
Average wt. of 1 seed (g)	0.247		0.155		0.242		0.197	

For A 188 the average value from 6 determinations is given ± 1 SD. For *pro* mutants the average value from 2 duplicate determinations is given. For details see the section of "Materials and Methods". Zeins are given as the sum of the pooled fractions; similarly, glutelin-2 and glutelin-3 fractions, although analyzed separately, were pooled in the Table

Table 3. Content of proteins in embryos of *pro* mutants and stock A 188 containing the *pro* wild type allele

	Pro (A 188)		pro_{1-1}		pro_{1-2}		pro_{1-3}	
	mg/ embryo	% of total	mg/ embryo	% of total	mg/ embryo	% of total	mg/ embryo	% of total
Albumins	3.50±0.18	58.6	0.86	36.4	2.96	50.0	3.79	58.2
Globulins	1.54±0.27	25.8	0.57	24.2	1.34	22.6	1.41	21.7
Glutelins	0.93±0.16	15.6	0.93	39.4	1.62	27.4	1.31	20.1
Total Protein	5.97	100	2.36	100	5.92	100	6.51	100

For A 188 the average value from 6 determinations is given ± 1 SD. For *pro* mutants the average value from 2 duplicate determinations is given. For details see the section of "Materials and Methods". Glutelin-2 and glutelin-3 fractions, although analyzed separatedly, were pooled in the table

Table 2 and embryo proteins in Table 3. Among the endosperm proteins, zeins are dominating, accounting for 32 to 69% of the proteins. All three mutants accumulated less zeins than the *PRO* kernels. Albumins, on the other hand, were increased, as were globulins, both in absolute amounts and in relative amounts considering the decreased protein content. Absolute amounts of glutelins were decreased in the mutants, but accounted for about 20% of the total proteins in all four genotypes. There was considerable variation in seed size: pro_{1-1} and pro_{1-3} seeds were smaller than pro_{1-2} and *PRO* seeds.

The majority of the proteins (about 60%) of the embryo, scutellum and axis in stock A 188 were water soluble and are therefore listed as albumins in Table 3. Globulins and glutelins comprise about 26 and 16% respectively whereas zeins could not be detected. In pro_{1-1} and pro_{1-2}, but not in pro_{1-3} mutants, albumins were decreased. The relative content of globulins was fairly constant in the three mutants and the wild type, but the percentage of glutelins was increased in all mutants relative to *PRO*, which resulted in a high proportion of this protein class in the *pro* mutants. Compared to *PRO* the overall protein content of embryos was reduced in pro_{1-1}, the same in pro_{1-2} and increased in pro_{1-3}.

Qualitative Analysis of Endosperm and Embryo Proteins

a) Zeins

Since *pro* mutant endosperms contain reduced quantities of zein compared to those found in *PRO* endosperm it was of interest to find out whether the mutation affected a single or all zein components. Zeins were analyzed by SDS-PAGE (see Fig. 1) and in all four cases the typical polypeptide pattern with two major bands was obtained.

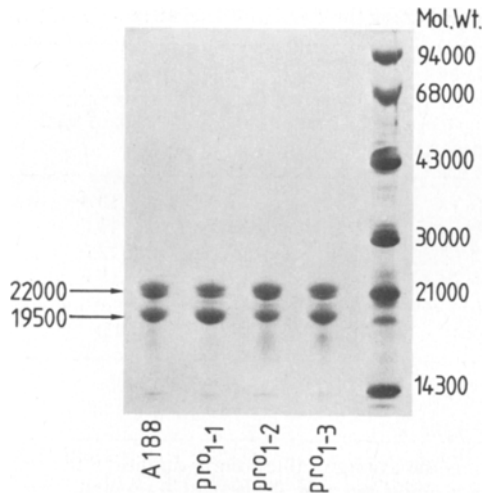

Fig. 1. Gel electrophoresis of zeins: Zeins (20 µg per track) extracted from endosperms of *pro* mutants and stock A 188 containing the *PRO* wild type alle were analyzed on SDS-PAGE. Molecular weight markers were run in the last track on the right and comprise phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000) and lysozyme (14,300). The specific zein bands are marked 22,000 and 19,500 respectively

b) Albumins

The polypeptide pattern of the water soluble proteins as revealed by SDS-PAGE is shown in Figure 2. As expected the albumins consisted of a large population of polypeptides ranging in molecular weights from less than 21,000 to over 70,000. There are marked differences between the polypeptide patterns of endosperm and embryo albumins. In *PRO* endosperm (A 188) the 40,000, 38,500, 36,500 and 35,500 bands were prevalent over the others, the 40,000 and 36,500 polypeptides were preferentially reduced in mutants examined whereas the 35,500 component was missing alltogether. In embryos, the higher molecular weight polypeptides (40,000 to 60,000) were dominating over lower molecular weight polypeptides. A 58,000 polypeptide was very much decreased and a 15,000 band strongly enhanced in pro_{1-1}. There were no specific differences seen in pro_{1-2} albumins compared to *PRO,* but in pro_{1-3} albumins a 56,000 polypeptide was increased concomittant with a reduction in the 52,000 band. In general, changes in albumin pattern were more pronounced in all mutant endosperms than in the embryos.

c) Globulins

A qualitative analysis of globulins was carried out by SDS-PAGE as for albumins and is shown on Figure 3. The patterns of globulins reveal considerable similarity between endosperm and embryos and consisted of fewer components than the albumins. A 64,000 molecular weight polypeptide absent in *PRO* endosperm and embryo was visible in all *pro* mutants examined. The strong 56,000 and 47,000 component of the mutants was weakly represented or absent in the *PRO* endosperms. A group consisting of globulins in the 25,000 to 31,000 molecular weight range was increased in the mutant endosperms. Pro_{1-2} embryo globulins were very similar to those of *PRO.* The pro_{1-1} pattern was characterized by a decrease in a 52,000 and 47,000 polypeptide while pro_{1-3} embryo globulins contained a 36,500 component only very weakly present in normal embryos.

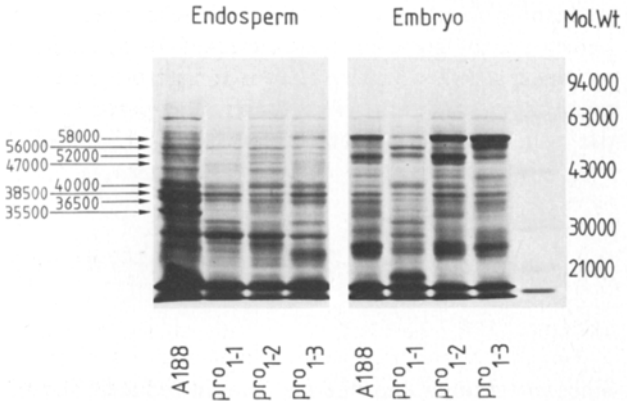

Fig. 2. Gel electrophoresis of albumins: Albumins (100 µg per track) extracted from *pro* mutant and *PRO* wild type endosperms we well as embryos were analyzed on SDS-PAGE as described in the section on Materials and Methods. Molecular weight markers were as described for Fig. 1. Molecular weights of specific albumin polypeptides are indicated and range from 35,500 to 58,000

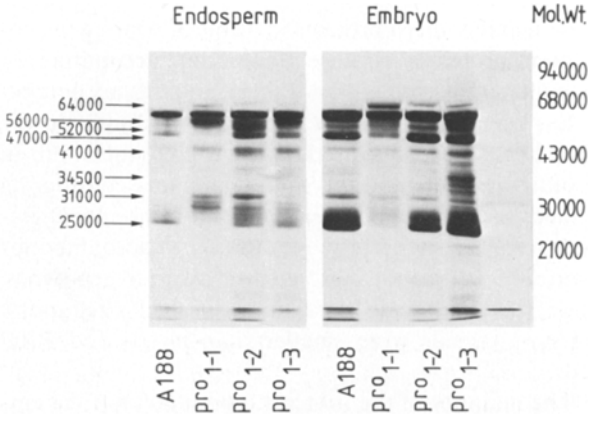

Fig. 3. Gel electrophoresis of globulins: Globulins (100 µg per track) extracted from *pro* mutant and *PRO* wild type endosperms as well as embryos were analyzed on SDS-PAGE as described in the section on Materials and Methods. Molecular weight markers were as described for Fig. 1. Molecular weights of specific globulins are indicated and range from 25,000 to 64,000

Discussion

Zygotes homozygous for the recessive *pro* mutant alleles develop into kernels on plants which are heterozygous for the *pro* allele and thus do not require proline. Since the homozygous *pro/pro* zygotes can form an embryo and an endosperm the proline requirements of these tissues are satisfied either by the supply from maternal tissue or by suppression of the abberrant proline metabolism. It has been recently shown that proline is synthesized by pro_{1-1} mutant root tips but that the catabolism apparently is enhanced (Dierks-Ventling and Tonelli 1981). That the defective proline metabolism is operative in the *pro/pro* endosperms is evidenced by the following observations: the mutant endosperms contain several-fold higher amounts of free amino acids. In two of the mutants (pro_{1-2}, pro_{1-3}) the relative content of proline among the free amino acids was drastically reduced. A strong depression in the synthesis of zein polypeptides is observed for all three mutants whether analyzed in absolute amounts per endosperm or in relation to the total amount of endosperm protein. Depression of zein synthesis is also characteristic for opaque-2 mutants (Jones et al. 1977). In the latter the reduction is limited to the 22,000 component, while in the *pro* mutants all zein polypeptides are equally affected. Considering that zeins contain 12% proline and 20% glutamate (Sodek and Wilson 1971; Gianezza et al. 1977), this provides support to the notion that the depression in zein synthesis in the *pro* mutants actually is due to a limited supply of proline. Albumins and globulins contain about half as much proline residues as zeins (and half as much glutamate) and their synthesis is favoured in the mutants compared to the *PRO* endosperms. Whether the observed changes in the amounts of individual polypeptides of the albumin and globulins in the mutants compared to wild type are correlated to differences in proline content remains to be investigated.

The embryos of the *pro* mutants do not accumulate more free amino acids than the *PRO* containing embryos. A dramatic reduction in the amount of the free proline content was observed in all three mutants, revealing that proline accumulation is hampered in the mutant embryos. Systematic changes in the amount of water-soluble, salt-soluble and alkali-soluble proteins were not observed in comparisons between the mutants and the wild type embryos. Differences in the polypeptide patterns of the albumins and globulins of the embryo were allele-specific rather than characteristic for the *pro* versus *PRO* genes.

This paper demonstrates that the embryo and endosperms of *pro* mutants show alterations in protein composition which appear to be a direct consequence of a defective proline metabolism. The mutants will provide in situ as well as in embryo and endosperm cultures a valuable tool in exploring proline metabolism in these organs.

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