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SYNTHESIS AND DEGRADATION OF PROTEINS DURING WHEAT ENDOSPERM DEVELOPMENT*

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Abstract—Synthesis of proteins rich in lysine declines progressively with endosperm development and these proteins appear to be degraded preferentially at later stages. The proteolytic enzymes in extracts of endosperms at a late stage of development release considerably more lysine radioactivity from labelled endosperm proteins as compared with the enzymes in endosperms at an early stage.

INTRODUCTION

The proportion of lysine relative to the other amino acids in the proteins of mature wheat endosperm is not adequate from the point of view of human nutrition. Lysine is relatively high in protein at the early stages of endosperm formation. However, a fall occurs progressively as the endosperms undergo development [1–4]. This progressive amino acid imbalance might be the result of two basic phenomena, (i) decreased synthesis of certain classes of proteins and/or (ii) preferential degradation of these proteins. We report here results of experiments on the incorporation of labelled amino acids into the proteins of developing wheat endosperm and also on the degradation of these proteins.

RESULTS AND DISCUSSION

Incorporation of lysine[^3H] into the proteins of wheat endosperm in vivo

Incorporation of lysine into the proteins of wheat endosperm at different stages of grain development would be a direct measure of synthesis of proteins containing appreciable amount of lysine at those stages. Preliminary experiments had revealed that there was considerable variation in the transport of the injected radioactive lysine to the grains at different stages of development. Free lysine in the internal pool, which dilutes the labelled lysine, also changes to a great extent. These variables would thus have to be taken into consideration. Specific radioactivity of lysine in the tissue has therefore been determined by estimating the free lysine in the internal pool and the radioactivity of the free lysine actually present in the endosperm tissue at the different stages of development. The data have been reported in terms of nmol lysine incorporated rather than cpm. We have assumed that the radioactive lysine is in equilibrium with the non-radioactive lysine detected in the endosperm tissues. This assumption is probably valid because, (i) the radioactivity has been injected at the base of the stalk and not directly into the endosperm, (ii) most of the amino acids present in the endosperm are known to be transported to this tissue through the stalk and (iii) 24 hr elapse between injection and determination. It has also been found in preliminary experiments that protein synthetic efficiency was not uniform with all plants, even at the same stage of development. Hence, the lysine incorporation data, by itself would not probably be a reliable measure. It was therefore decided to study also the incorporation of labelled leucine, to serve as an internal standard. Leucine[^{14}C] and lysine[^3H] were mixed and injected into the experimental plants. Leucine was chosen because it was present in the endosperm proteins in largest quantities among those amino acids whose relative content remained more or less unaltered throughout the maturation process [4].

The rate of incorporation of lysine into endosperm proteins relative to that of leucine declines progressively with development from 8 days after anthesis to 28 days (Table 1)‡ The data have been obtained by averaging the results of 8–10 experiments. It was not possible to record any data beyond 28 days, as very little radioactivity was recovered in the endosperms when the labelled amino acids were injected 32 days after anthesis. The synthesis of some proteins comparatively rich in lysine apparently takes place at the early stages of endosperm formation, but not at the later stages of development (Table 1). We have assumed here that ^3H radioactivity in the endosperm tissue was primarily due to lysine and likewise ^{14}C radioactivity was due to leucine, since the time elapsed after injection was only 24 hr. This has been checked for one stage, that with 4 spikes after 16 days

* Part 2 in the series "Development of amino acid imbalance in wheat proteins". For part 1 see ref. [4].

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‡ Protein content of endosperms per spike (12 grains) varied between 2.6–4.5 mg (average 4.0 mg) for spikes collected 9 days after anthesis; between 8.2–11.7 mg (average 9.3 mg) for spikes collected after 13 days; between 13.9–19.9 mg (average 17.8 mg) for spikes collected after 17 days; between 21.0–29.0 mg (average 24.1 mg) for spikes collected after 21 days; between 33.8–46.2 mg (average 38.5 mg) for spikes collected after 25 days; and between 35.1–45.8 mg (average 39.1 mg) for spikes collected after 29 days.

PHYTO 15/7-A

Table 1. Rate of incorporation of lysine and leucine into the proteins of wheat endosperm at different stages of development

Stage of development of endosperms (days after anthesis)	Amino acid incorporation into endosperm proteins (nmol/grain/24 hr) Lysine	Leucine	Ratio of lysine/leucine incorporation in 24 hr
8	8.7 (1.7)	25.7 (5.0)	0.34
12	4.0 (1.3)	21.0 (5.8)	0.19
16	3.7 (1.0)	18.0 (3.6)	0.20
20	2.1 (0.4)	19.6 (2.2)	0.11
24	2.4 (0.4)	16.6 (3.7)	0.14
28	1.0 (0.3)	10.8 (1.0)	0.09

A mixture of lysine [^{3}H] (10 μCi, 10 μl) and leucine [^{14}C] (1 μCi, 10 μl) was injected into the plants at the base of the spikes at intervals after anthesis mentioned in the table. Spikes were removed after 24 hr and 12 grains were sampled from one spike as described. Radioactivity in the free amino acids of the internal pool (counting efficiency, 14% for ^{14}C and 3% for ^{3}H) and that incorporated into proteins (counting efficiency, 30% for ^{14}C and 7% for ^{3}H) were assayed for the grains of each individual spike. Contents of free amino acids were also determined. Data presented have been calculated on the basis of specific radioactivity of the amino acids in the endosperm tissue. Figures in parentheses represent standard deviations from the mean.

of anthesis. Proteins were hydrolysed and amino acids were fractionated by paper chromatography as described in details under Experimental. For both lysine and leucine, more than 80% of radioactivity was detected in regions specified for these two amino acids. This possibly represents an under-estimate, in view of the spreading always observed in paper chromatograms.

In vivo *degradation of labelled proteins during endosperm development*

The fate during development, of endosperm proteins labelled early with both lysine [^{3}H] and leucine [^{14}C] was next studied. At 8 days after anthesis, a mixture of the two labelled amino acids was injected into the plants. A mixture of the amino acids in non-radioactive form (400-fold excess for each) was injected again after 24 hr to stop incorporation of the radioactive amino acids into the proteins. However, as is evident from the results, this objective was not achieved fully. Spikes were then plucked periodically for the determination of radioactivity in the endosperm proteins. The chances of amino acid interconversion would possibly be greater here since this experiment was extended. Total ^{3}H or ^{14}C radioactivity in the proteins was, therefore, not considered a proper measure of incorporation of lysine or leucine. The amino acids separated chromatographically from protein hydrolysates would offer a more appropriate estimate. The average of the results of 5–6 experiments have been presented in Table 2. Radioactivity in both lysine and leucine increased till the 16th day after injection i.e. 24th day after anthesis. The rates of average increase of radioactivity in the proteins due to both lysine and leucine, have also been recorded by estimating from best fit curves, the increase over a period of 24 hr at the points representing the stages of development mentioned (Table 2). The observed preferential decline in the rate of increase of lysine radioactivity was not the result of non-availability of radioactive free lysine in the endosperm at the later stages. The radioactivity (cpm, average of 8 experiments) in the free amino acids per endosperm were—24 hr after injection, 9 days after anthesis: lysine [^{3}H] 2120, leucine [^{14}C] 330; 16 days after injection, 24 days after anthesis: lysine [^{3}H] 1800, leucine [^{14}C] 350.

Table 2. Radioactivity in endosperm proteins at different stages of development after injection of labelled lysine and leucine on day 8 after anthesis

Stage of development at which endosperms were sampled (days after anthesis)	Radioactivity in proteins (dpm./endosperm) Lysine [^{3}H]	Leucine [^{14}C]	Average increase in radioactivity over a period of 24 hr (dpm./endosperm) Lysine [^{3}H]	Leucine [^{14}C]	Ratio of increase in lysine/leucine radioactivity over a period of 24 hr
9	13900 (6250)	880 (430)	8700	1050	8.3
12	39100 (7700)	4230 (1480)	5200	800	6.5
16	49400 (12100)	6370 (1670)	3800	750	5.0
20	67000 (7020)	9870 (1810)	2100	875	2.4
24	72700 (4570)	13380 (1890)	500	875	0.6

A mixture of lysine [^{3}H] (10 μCi, 10 μl) and leucine [^{14}C] (1 μCi, 10 μl) was injected into the plants at the base of the spikes 8 days after anthesis. A mixture of the amino acids in non-radioactive form (400-fold excess for each) was injected after 9 days and 12 grains from each spike were sampled periodically as described. The hydrolysates of the washed endosperm proteins were chromatographed and radioactivity in lysine and leucine residues were determined (counting efficiency, 65% for ^{14}C and 1% for ^{3}H). Figures in parentheses represent standard deviations from the mean.

Table 3. Degradation of casein by enzymes in endosperm extract

Extract from endosperms after anthesis (days)	Protein content of extract (mg/ml)	Extent of degradation of casein (*A* at 610 nm)	
		(per endosperm)	(per mg protein in extract)
8	1.6	0.04	0.28
12	3.1	0.01	0.03
16	3.3	0.04	0.10
20	2.6	0.08	0.26
24	4.5	0.04	0.08

Endosperm extract (0.5 ml) was incubated with 0.5 ml dialysed casein soln (0.6% in 8.5 mM citric acid, 16.5 mM NaPi buffer, pH 6.2) at 37° for 2 hr. Peptides in the supernatant soln after addition of TCA were assayed colorimetrically (ref. [6]).

It can be seen that the ratio of 24 hr-incorporation of lysine/leucine (last column, Table 1) decline at a considerably slower rate during endosperm development compared to the computed values of increase of lysine–leucine radioactivity from the continuous incorporation experiment (last column, Table 2). This strongly suggests that degradation of proteins takes place in the endosperm concomitant with synthesis. More importantly, it indicates that this degradation is not uniform for all proteins, and the ones preferentially affected are comparatively rich in lysine.

The failure to dilute effectively the radioactive amino acids by a second injection of the non-radioactive form of the same amino acids indicates that all of the injected amino acids do not go directly to the endosperms. A large proportion may equilibrate through the plant before being available to the endosperm. However, it seems reasonable to assume that the extent of dilution of both lysine and leucine would be about the same.

Degradation of casein by enzymes in endosperm extracts

Extracts from endosperms contain proteolytic activity as measured by the degradation of casein (Table 3). This activity was maximum in grains after 8 days and 20 days of anthesis. The result presented is the average of two experiments.

Degradation in vitro *of endosperm proteins labelled with leucine [^{14}C] and lysine [^{3}H]*

Proteins labelled with leucine [^{14}C] and lysine [^{3}H] were next obtained from endosperms of spikes (15 days after anthesis) injected with the amino acids. Degradation of the labelled proteins by the enzymes from endosperms at 8 and 20 days of anthesis was then studied. The average of the results of four experiments has been recorded in Table 4.* The extent of release of lysine radioactivity compared to that of leucine, by the action of proteolytic enzymes from endosperms 20 days after anthesis, is considerably more than that with enzymes from endosperms 8 days after anthesis. This suggests that lysine rich proteins are degraded in proportionately greater amounts by enzymes present at about 20 days after anthesis and confirms the indication obtained from the *in vivo* experiments.

We conclude that though the decreased rate of synthesis of some proteins that are rich in lysine is important during wheat endosperm development, selective degradation of such proteins is also a factor in producing a lower relative amount of lysine in the proteins of developed endosperm.

* Amount of proteins in the incubation mixture: labelled proteins, 2.12 mg; protein in extract 8 days after anthesis, 0.49 mg; protein in extract after 20 days, 1.24 mg.

EXPERIMENTAL

Plants. Triticum aestivum L. var. Kalyan Sona was used. Plants which had anthesis on the same day were chosen at random and tagged. Spikes were collected at different stages of development and stored at −20°. Each spike had about 50 grains. However, only 12 grains per spike were used for our studies, 6 outer florets from each side, half way from the bottom. These were of nearly equal development. Complete maturation of the grains took place about 40 days after anthesis.

Radiochemicals. L-Leucine-U-[^{14}C] (130 Ci/mol) and L-lysine-G-[^{3}H] (1500 Ci/mol) were obtained from Bhabha Atomic Research Centre, Trombay, India. A mixture of these two labelled amino acids (20 μl) was injected at the base of the spike. When a chase was desired, a mixture of the two non-radioactive amino acids in 400-fold excess was injected after 24 hr.

Determination of free amino acid pool in the endosperm tissue. Spikes of wheat which had not been injected with any amino acid were removed at intervals after anthesis and 360 grains

Table 4. Degradation *in vitro* of endosperm proteins labelled with leucine [^{14}C] and lysine [^{3}H]

Radioactivity in protein		Extracts from endosperm after anthesis (days)	Release of radioactivity after 2 hr			Percentage release	
Lysine [^{3}H] (dpm)	Leucine [^{14}C] (dpm)		Lysine [^{3}H] (dpm)	Leucine [^{14}C] (dpm)	lys/leu	Lys (%)	Leu (%)
18700	2800	8	1310 (168)	334 (44)	3.9	7.0	12.0
18700	2800	20	1910 (189)	157 (44)	12.2	10.2	5.6

Labelled proteins (0.75 ml) were incubated with endosperm extracts (0.75 ml) at 37° for 2 hr. Release of radioactivity has been determined in supernatant soln after addition of TCA. Radioactivity in ^{3}H and ^{14}C have been taken as due to lysine and leucine respectively. Counting efficiency: protein on glass-fibre filter, 80% for ^{14}C and 11% for ^{3}H; amino acids and peptides in soln, 63% for ^{14}C and 10% for ^{3}H. Figures in parentheses represent standard deviations from the mean.

were sampled as described earlier. The grains were dissected, the endosperms removed and homogenized in batches with TCA (5%). The homogenates were pooled, heated for 30 min at 80°, allowed to cool to room temp., centrifuged at 3000 *g* for 30 min and the supernatant soln saved. This treatment solubilized negligible amounts of proteins in confirmation of ref. [5]. An aliquot of the supernatant soln was evaporated at 100°, the evaporation was repeated 3× after addition of H_2O and residue was dissolved in a suitable amount of citrate buffer (0.1 M), pH 2 containing 1% thiodiglycol. Traces of residual TCA were removed with 3 extractions of buffer-satd Et_2O. Expts showed no loss of amino acids occurs during this step. Limits of reproducibility in the amino acid analyzer varied between 2–10%.

Determination of endosperm proteins. Protein content of the endosperm tissue was determined by the method of ref. [6]. Bovine serum albumin was used as standard.

Determination of radioactivity in the free amino acid pool and of that incorporated into the proteins of endosperm. Grains (12) sampled in the usual way from a spike that had received injection of radioactive amino acids were used for one experiment. Endosperms were dissected out, homogenized with TCA and heated at 80° as described before. The pptd proteins were separated by centrifugation and an aliquot of the supernatant soln (1 ml) was counted for radioactivity with Bray's [7] mixture. This is a measure of the radioactivity in the free amino acid pool in the endosperm. The sedimented protein ppt was washed 3× by resuspension in TCA and recentrifugation. Proteins were then dissolved in M NaOH, undissolved materials removed by filtration through a glass-fibre filter and the proteins reprecipitated with TCA. The ppt was collected on a glass-fibre filter the filter placed in a glass counting vial, dried at 105° for 2 hr and counted for radioactivity with a toluene based scintillation mixture.

Determination of radioactivity in the lysine and leucine residues of endosperm proteins. Proteins were obtained from the endosperms (12 grains/spike) by the usual digestion with TCA, centrifugation, washing, dissolution in NaOH and reprecipitation. The proteins were then hydrolysed in vacuum sealed tubes with 5.7 M HCl for 24 hr at 110°. Hydrolysate was evaporated at 100° and HCl removed by repeated (5×) addition of H_2O and evaporation. Residue was dissolved in a suitable amount of *iso*PrOH soln (10%) and undissolved matter removed by centrifugation. Descending PC was done for 72 hr on Whatman No. 1 using *iso*BuOH, 3% NH_4OH (3:1) as the developing solvent [8]. This method was chosen because it allowed separation of both lysine and leucine from other amino acids including *iso*leucine. Positions of radioactive lysine and leucine were determined by ninhydrin spray of cut strips containing the markers. Radioactivity in the dried paper strips was determined with the toluene based scintillation mixture.

Preparation of extract from endosperms for the estimation of proteolytic enzymes. Spikes which had not been injected with any amino acid were sampled after anthesis. Twenty-four grains were taken in the usual way from 2 spikes for each expt. The endosperms were dissected out and then homogenized at 0° with 2 ml of a medium containing 50 mM KPi, 35 mM EDTA, 10 mM cysteine adjusted with KOH to pH 7.5. Composition of the medium has been adopted from ref. [9]. The homogenate was centrifuged for 15 min at 20000 *g* at 4°C and the supernatant soln was tested for proteolytic activity.

Assay of proteolytic enzymes. The procedure was adopted from ref. [10]. The assay mixture contained in a total vol of 1 ml, dialysed casein soln (0.6% in 8.5 mM citric acid, 16.5 mM NaPi buffer, pH 6.2), 0.5 ml and endosperm extract, 0.5 ml. Incubation was carried out for 2 hr at 37° and terminated by the addition of 0.1 ml 55% TCA. The pptd protein was centrifuged and the peptides in the supernatant soln were assayed by the method of ref. [6]. NaOH (7%), 0.1 ml was added to 0.5 ml of the supernatant soln from the TCA step. This mixture (0.5 ml) was treated with Lowry C reagent (with alkali) (2.5 ml) and then with phenol reagent (0.25 ml). A was measured at 610 nm. Endogenous and zero time controls were always run and the data corrected.

Preparation of labelled proteins from endosperms for use as substrate for proteolysis. A mixture of lysine [^{3}H] (10 μCi, 10 μl) and leucine [^{14}C] (1 μCi, 10 μl) was injected into the plants at the base of the spikes 15 days from anthesis. The spikes were removed after 24 hr, stored at −20° and thawed just prior to use. All the grains from a spike were taken. They were heterogeneous in development, conforming to grains of *ca* 12–16 days after anthesis according to the sampling procedure described before. The grains from one spike were dissected, the endosperms removed and homogenized with 5 ml Na pyrophosphate buffer (50 mM, pH 8.3). Homogenate was centrifuged at 20000 *g* for 10 min and the supernatant soln dialysed for 16 hr against the same buffer. All operations were done at 0–4°. The dialysed soln was stored at −20° and was used as the substrate for proteolysis. The native conformation of the labelled proteins was retained as far as possible.

Degradation in vitro of labelled proteins from endosperms. For the study of degradation of the labelled proteins, 0.75 ml of this soln was incubated at 37° for 2 hr with the same vol of extracts of endosperms having proteolytic activity. The reaction was terminated with TCA, the proteins centrifuged down and radioactivity released in the supernatant soln was determined by Bray's [7] mixture. Controls were included for release of radioactivity in the absence of extracts of endosperms and the data have been corrected accordingly.

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