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Synthesis of the major storage protein, hordein, in barley

Pulse-labeling study of grain filling in liquid-cultured detached spikes

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Abstract. A liquid culture system for culturing detached spikes of barley (*Hordeum vulgare* L.) at different nutritional levels was established. The synthesis of hordein polypeptides was studied by pulse-labeling with [^{14}C]sucrose at different stages of development and nitrogen (N) nutrition. All polypeptides were synthesised at 10 d after anthesis and hereafter an increase was observed for all polypeptides. A fivefold increase in total hordein was observed within the N range tested. Hordein-1 increased considerably more than hordein-2 with increased N nutrition, and hordein-1 synthesis exceeded that of hordein-2 at the highest N level 20 and 25 d after anthesis. Hordein-1 thus appears to act as the main N sink at high N levels. The synthesis of the major groups of hordein-2 polypeptides responded differently to increasing N in that the slower-migrating polypeptides increased more with increasing N than the faster-migrating polypeptides.

Key words: Grain filling – Hordein – *Hordeum* (storage protein) – Nitrogen nutrition – Seed protein synthesis.

Introduction

The major storage protein in the barley endosperm, hordein, which is low in essential amino acids, has been extensively studied in attempts to improve the nutritional quality of barley protein. The hordein belongs to the prolamin group of plant proteins, as defined by Osborne (1885) as soluble in 70% ethanol. The hordein protein fraction can be separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) into two major groups of polypeptides called hordein-1 and hordein-2. These polypeptides are coded by genes in two linked loci *Hor 1* and *Hor 2*, respectively (Doll and Brown 1979; Jensen et al. 1980; Shewry et al. 1980a).

Bishop (1928, 1929, 1930) reported on the responsiveness of hordein to increasing nitrogen (N) and the relatively late occurrence of these proteins during endosperm development. Køie et al. (1976) studied the response of the two fractions to increasing N and found that hordein-1 responded more than hordein-2. In a recent study by Kirkman et al. (1982) this was confirmed and no relative changes within each hordein group was observed with increasing N. Brandt (1976) and Shewry et al. (1979) found that hordein is detectable two weeks after anthesis. Shewry also found that there appears to be relatively more hordein-1 in young grains than in mature grains, but observed no changes within the hordein-1 or hordein-2 polypeptide groups. Rahman et al. (1982), however, observed a relative increase of the faster-migrating hordein-2 polypeptides in SDS-PAGE separation, late in endosperm development.

The variation in the relative amounts of hordein-1 and hordein-2 with nutrition and stage of endosperm development led Faulks et al. (1981) to suggest that the syntheses of hordein-1 and hordein-2, at least under certain conditions, are under separate regulatory control. It now seems that individual hordein-2 polypeptides may also be under a separate control (Rahman et al. 1982).

The present study was carried out to investigate this hypothesis by looking at the effect of different N levels on the synthesis of hordein polypeptides at different stages during endosperm development.

In order to do this, a method of growing detached barley spikes in liquid culture was designed, as described for wheat by Donovan and Lee (1977). This method allows the control of N nutri-

Abbreviation: SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

tion and the performance of pulse-labeling experiments at different stages during endosperm development. In this way it is possible, using electrophoresis followed by fluorography, to get an accurate estimate of the relative amounts of polypeptides synthesised during a specific period at a given N level.

Material and methods

Plant material. Seeds of barley (*Hordeum vulgare* L. cv. Bomi) were grown in a growth chamber under a diurnal cycle of 18 h light, 18° C, and 6 h darkness, 12° C. The relative humidity was about 70 and 90%, respectively. Natural light in the growth chamber was supplemented with Osram HQIL, 400-W lamps at a rate of approx. 20,000 lx 1 m below the lamps. Plants were grown, two plants per pot, in 13-cm pots containing a standard peat-clay soil mixture with added nutrients (Jensen 1979). Three weeks after sowing, 5 g of a fertilizer containing 25% N, 3% P and 6% K was given.

Spikes of the two main tillers of each plant were tagged at anthesis. Two spikes were harvested at days 8, 10, 12, 15, 20, 25, 30 and 35 after anthesis. The endosperm was dissected out, frozen in liquid N_2, freeze-dried and kept in a desiccator at −20° C until use.

Liquid culture of detached spikes. The technique developed by Donovan and Lee (1977, 1978) for growing detached wheat spikes in liquid culture was adjusted to barley. Barley cv. Bomi was grown in a growth chamber as described above. At 8 d after anthesis the stems were cut as described by Donovan and Lee (1977). The detached barley spike was fitted through a radiation-sterilized polystyrene plug into a sterilized test tube (20 cm long; 2.5 cm diameter) containing 50 ml of culture medium. The media were sterilized by passage through 0.22-μm Millipore filters (Bedford, Mass., USA).

The test tubes containing the detached spikes were placed in waterbaths with circulating water at 4° C in a Conviron E8H (Winnipeg, Canada) growth cabinet. This was programmed to keep a diurnal cycle of 18 h light, 18° C, and 6 h darkness, 12° C.

The stock solutions used in the culture media were the same as described by Donovan and Lee (1977). Preliminary studies showed that spikes cultured in a media containing 40 g l^{-1} of sucrose and 0.5 g l^{-1} of N (given as NH_4NO_3) had a grain filling corresponding to that in intact plants when spikes were cultured from 8 d after anthesis until 22 d after anthesis. In the present experiment, spikes were cultured with 40 g l^{-1} of sucrose in the media and the following N levels: 0, 0.5, 1.0 and 2.0 g l^{-1} of N. Five spikes of each N treatment were cultured for radioactive labeling at different stages of development.

Pulse-labeling with ^{14}C. Detached spikes of barley growing in liquid culture were transferred to a small vessel containing 250 μl of culture medium in which part of the sucrose consisted of ^{14}C-labeled sucrose (I.C.N. (Irvine, Calif., USA), chemical & radioisotope division, 496 MBq $mmol^{-1}$). In this way a pulse of 3.7 MBq was administered to separate spikes at days 9, 14, 19 and 24 after anthesis, respectively, from each of the N treatments. After about 3 h the 250 μl were taken up and the spikes returned to their original test tubes. Twenty-four hours later the grains were harvested as described above. One spike from each of the N treatments was labeled continuously between days eight and 25 after anthesis by adding 2 MBq [^{14}C]sucrose to the 50 ml of culture media.

Protein extraction. Ten endosperms from each treatment were ground in a mortar and dry weights determined. Endosperms which were 10 and 15 d old, were extracted with 1.25 ml of solvent and those which were 20 and 25 d old were extracted with 2.5 ml of solvent. All extractions were carried out in 15-ml screw-capped centrifuge tubes under continuous stirring. The flour was extracted twice for 1 h at 20° C with 0.1 M phosphate buffer (pH 8), 5 mM dithiothreitol (DTT) to remove the salt-soluble proteins and non-protein N. These extracts were kept for a separate study. A further three 1-h extractions were carried out with 50% (v/v) propan-2-ol, 4 mM DTT at 60° C to extract hordein.

The combined extracts containing hordein were divided and one part dialysed against distilled water followed by Kjeldahl N determination. The other part was further reduced and alkylated at 50° C as described by Doll and Andersen (1981). The extracts were then dialysed against distilled water and freeze-dried before electrophoresis.

Electrophoresis and fluorography. Separation of hordein polypeptides by SDS-PAGE and staining with Coomassie brilliant blue, R-250, was carried out as described by Doll and Andersen (1981). The gels were prepared for fluorography by leaving them for 20 min in a solution of 1 M sodium salicylate in 20% methanol before drying (Chamberlain 1979). The dried gel was immediately exposed to a preflashed Kodak RP Royal X-Omat film and kept at −70° C for 4–5 weeks as described by Laskey and Bonner (1975). Densitometer readings of the gel-film were carried out as described by Doll (1980).

Results

Hordein, nitrogen content. Kjeldahl N determination of hordein in the endosperm at days 10, 15, 20 and 25 after anthesis at N levels of 0, 0.5, 1.0 and 2.0 g l^{-1} are presented in Fig. 1. At 10 d after anthesis there is no measurable amount of hordein N at any of the N levels examined. Fifteen days after anthesis there is a very low level of hordein where no N was given, but a clear increase in hordein at the higher levels of N is apparent. In the 20- and 25-d-old endosperms there is an approximate doubling of hordein from 0–0.5 g l^{-1} N given and from 0.5–1.0 g l^{-1} N given in the medium whereas the increase from 1.0–2.0 g l^{-1} gave relatively little response. The hordein content in grains harvested from intact plants show a pattern of accumulation (data not presented) similar to that of grains cultured at 0.5 g l^{-1} N.

Hordein composition. Hordein polypeptides were separated by SDS-PAGE on a kernel basis and stained with Coomassie blue to illustrate the changes in amount of polypeptides with time of development (Fig. 2).There is a clear increase in all polypeptide bands with time of development and there does not appear to be any notable change with

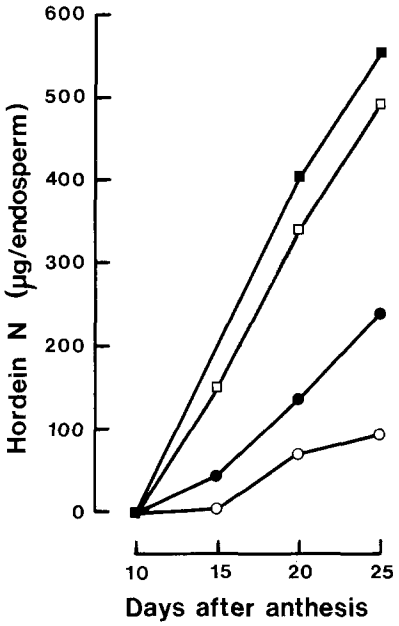

Fig. 1. Increase in hordein N during grain filling at different N levels. ○, 0.0 g l^{-1}; ●, 0.5 g l^{-1}; □, 1.0 g l^{-1}; ■, 2.0 g l^{-1}

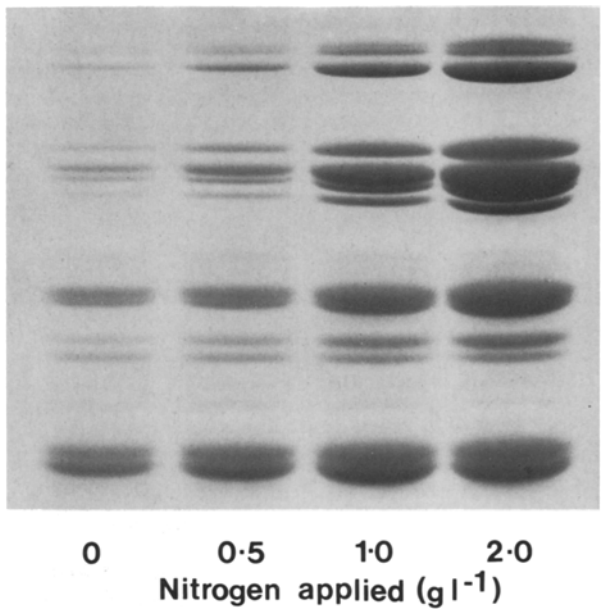

Fig. 3. The effect of increasing N on hordein polypeptide pattern in 20-d-old grain revealed by SDS-PAGE and Coomassie-blue staining

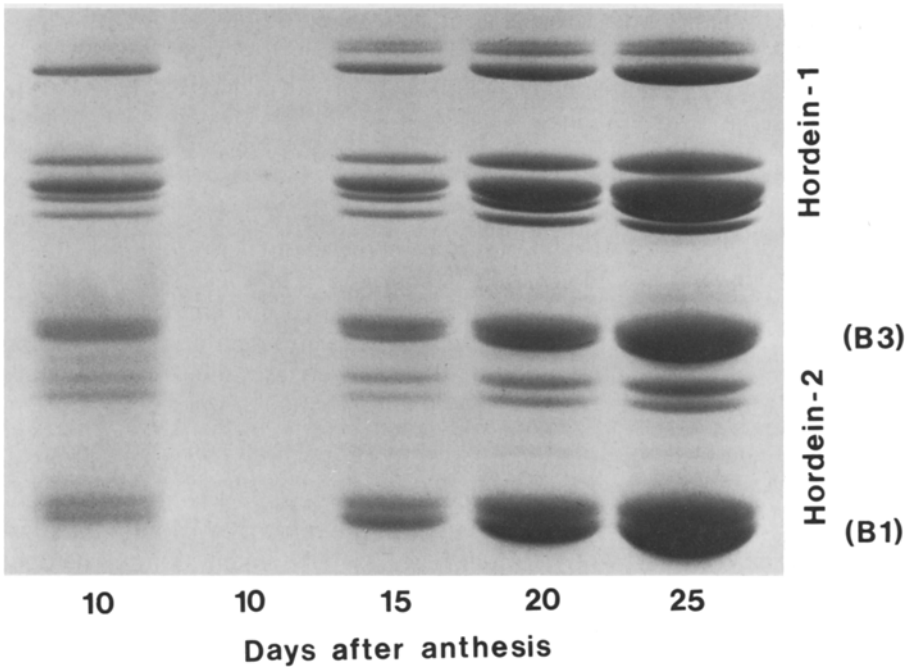

Fig. 2. Developmental pattern of hordein polypeptides from spikes cultured at 1.0 g l^{-1} N. Protein was separated by SDS-PAGE on a grain basis and detected by Coomassie-blue staining. The outer-left track shows a 30-times-concentrated sample from 10-d-old grains

time in the relative amounts of individual polypeptide bands. No protein bands could be seen at 10 d with the standard loading, but electrophoresis of an extract 30 times more concentrated (Fig. 2) shows that all hordein bands except the slowest-migrating hordein-1 bands are present.

The hordein polypeptide composition in grain harvested from intact plants at 20 d after anthesis was compared by SDS-PAGE separation with that of grains from liquid culture at 20 d and at N levels of 0.5 g l^{-1} N in the medium. No difference in polypeptide-band pattern was observed.

The response to increase in N levels of 0, 0.5, 1.0 and 2.0 g l^{-1} in 20-d-old kernels is shown in Fig. 3. It is clear that the content of hordein-1 increases more with increasing N levels than that of hordein-2.

Whereas the Coomassie-blue-stained gels show

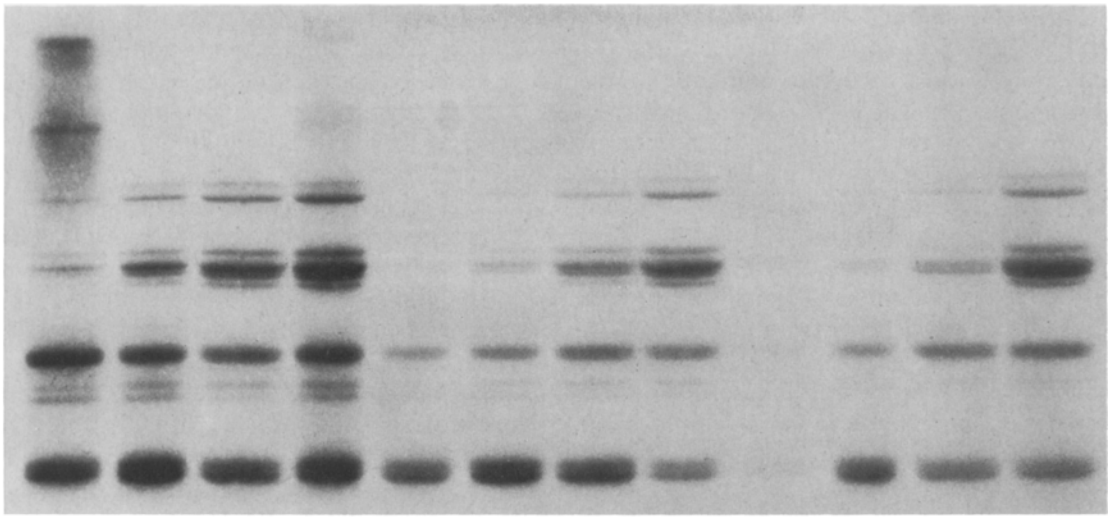

Fig. 4. Developmental pattern of ^{14}C-labeled hordein polypeptides, cultured at N levels of 0, 0.5, 1.0 and 2.0 g l^{-1}, separated by SDS-PAGE and detected by fluorography

Table 1. The effect of different N levels on the relative amounts of hordein-1 and hordein-2 and of B1 and B3 at different stages of barley endosperm development as determined by densitometric scanning of fluorographs

Days after anthesis	Hordein-2:hordein-1 N (g l^{-1})			Hordein-2 (B1:B3) N (g l^{-1})		
	0.5	1.0	2.0	0.5	1.0	2.0
15	3.82	1.44	1.33	2.07	1.51	1.67
20	10.20	2.46	0.69	4.15	2.20	1.38
25	15.00	2.71	0.60	3.33	1.40	1.00

the presence of total hordein polypeptides up to the day of harvest, the fluorography reveals the actual synthesis of polypeptides on a specific day during grain development. Figure 4 shows the results from a separation of 1 µg of hordein N per slot from kernels cultured at 0, 0.5, 1.0 and 2.0 g l^{-1} N and harvested at days 15, 20 and 25 after anthesis. The bands get weaker with the stage of development as the labeled hordein is diluted with an increasing amount of cold hordein.

Densitometer readings of the film, expressed as hordein-2/hordein-1 and for the hordein-2 group as B1/B3 (Table 1), show differences in the synthesis of these different fractions with developmental stage and N nutrition. The B1 double band represents the hordein 2 polypeptides with the highest migration rate and the B3 double band the hordein-2 polypeptides with the slowest migration rate. At the level of 0.5 g l^{-1} N in the medium, there is a relative increase with time of development in the synthesis of hordein-2 compared with hordein-1, but at the highest level of 2.0 g l^{-1} in the medium there is a relative decrease. The same is the case of the synthesis of B1 polypeptides compared with B3 polypeptides. The results show that the synthesis of hordein-1 increases more with increasing N than does hordein-2, and that more hordein-1 than hordein-2 is synthesised at the highest N level at 20 and 25 d after anthesis. Within the hordein-2 group, there are indications that B3 responds more to increasing N than does B1.

Densitometer reading of a Coomassie-blue-stained gel and the corresponding fluorograph, of hordein from continuously labeled kernels, indicate no significant differences in the relative intensity of hordein bands. The Coomassie-blue-stained protein bands thus presents a realisitc picture of the hordein composition.

Discussion

The Kjeldahl N determination of hordein content in the endosperm obtained from liquid culture shows that at 25 d after anthesis a greater hordein content has been obtained than previously recorded for pot experiments. The two highest N levels of 1 and 2 g l^{-1} resulted in a hordein-N content of about 500 µg/endosperm, whereas grains harvested from intact plants at 25 d after anthesis

in the same experiment had hordein content of about 250 μg/endosperm. At maturity, grains harvested directly had hordein contents of about 700 μg/endosperm which corresponds to that obtained by Kirkman et al. (1982) at the highest N level in a pot experiment.

The pulse-labeling study shows that where no N is given it is predominantly hordein-2 polypeptides that are synthesised at 15 and 20 d after anthesis; at 25 d after anthesis hardly any synthesis of hordein occurs (Fig. 4) presumably because of lack of N. At N levels of 0.5 $g\,l^{-1}$, which is that considered to give a grain filling corresponding to that in intact plants, there is a clear increase in the synthesis of hordein-2 relative to the synthesis of hordein-1 with time of development (Table 1). However, at high N levels the synthesis of hordein-2, in contrast to that of hordein-1, appears to become saturated, resulting in a higher absolute synthesis of hordein-1. This tendency apparently becomes more pronounced with time of development. This finding confirms the results obtained by Køie et al. (1976) and Kirkman et al. (1982). Hordein-2 usually constitutes about 80% of the total hordein content in mature field-grown grain (Kirkman et al. 1982), but in this experiment the synthesis of hordein-1 exceeds that of hordein-2 at the highest N level at 20 and 25 d after anthesis (Table 1). Hordein-2 is rich in S amino acids whereas hordein-1 is low in S amino acids (Shewry et al. 1980b). It is possible that the low hordein-2 content at high N levels is the result of a lack of S. However, an experiment in which the S/N ratio was doubled gave the same result. Furthermore, as the S/N ratio in the media with the highest N content was 0.05 and the ratio in hordein-2 is 0.03 (Shewry et al. 1980b) it is considered unlikely that S was the limiting factor.

Within the hordein-2 group there is a relative increase in the synthesis of the hordein-2 double band, B1, compared with the double band, B3, from 15 to 20 d after anthesis at N levels of 0.5 and 1.0 $g\,l^{-1}$ in the medium. This is in agreement with Rahman et al. (1982) who studied a variety with the same hordein-2 pattern and found a relative increase in B1 with time of endosperm development. However, the synthesis of B3 polypeptides increases more with increasing N than does the synthesis of B1 polypeptides, cancelling the developmental effect of the increased synthesis of B1 at high N levels.

The present study supports the hypothesis put forward by Faulks et al. (1981) that there are differences in the expression of genes coding for the different hordein fractions with regard to the stage of endosperm development and N nutrition. It appears that hordein-2 polypeptides may be preferentially synthesised at restricting N levels and that a saturation level is reached around an N level of 1–2 $g\,l^{-1}$ in the liquid culture system. The synthesis of hordein-1 does not stabilise at this N level and it seems that it is particularly hordein-1 that acts as an N sink at high N levels. During endosperm development, the synthesis of hordein-1 and the highest-molecular-weight hordein-2 band (B3) appears to be stable, whereas there is a relative increase in the synthesis of the lowest-molecular-weight hordein-2 band (B1). There is also a difference in response to N as the synthesis of B3 polypeptides responds more to increasing N than the synthesis of B1 polypeptides.

The pulse-labeling system proved effective in revealing, at specific stages of endosperm development, differences in protein synthesis which would otherwise have been obscured by the previously synthesised proteins.

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