Localization of Carboxypeptidase I in Germinating Barley Grain

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ABSTRACT

Activity measurements and Northern blot hybridizations were used to study the temporal and spatial expression of carboxypeptidase I in germinating grains of barley (Hordeum vulgare L. cv Himalaya). In the resting grain no carboxypeptidase I activity was found in the aleurone layer, scutellum, or starchy endosperm. During germination high levels of enzyme activity appeared in the scutellum and in the starchy endosperm but only low activity was found in the aleurone layer. No mRNA for carboxypeptidase I was observed in the resting grain. By day 1 of germination the mRNA appeared in the scutellum where its level remained high for several days. In contrast, little mRNA was observed in the aleurone layer. These results indicate that the scutellum plays an important role in the production of carboxypeptidase I in germinating barley grain.

In the starchy endosperm of a germinating barley grain, the storage proteins are degraded into a mixture of free amino acids and small peptides by a concerted action of proteases and carboxypeptidases (10). Careful chromatographic studies by Mikola (12) have shown that the germinating grain contains five different carboxypeptidases with partially complementary substrate specificities. Three of these (I-III) act, although at different rates, on almost any C-terminal amino acid including proline, but do not act if there is a proline residue in the penultimate position. Such substrates are hydrolyzed by carboxypeptidases IV and V.

Carboxypeptidase I accounts for the majority of the total carboxypeptidase activity toward a standard substrate, Z-Phe-Ala in a germinating grain (12). Carboxypeptidase I has been purified and is known to be composed of two identical subunits, each of which consists of two disulfide-linked polypeptides (3, 20). The nucleotide sequence of the cDNA for carboxypeptidase I indicates that the two polypeptides are encoded by the same mRNA (5).

The tissue distribution of carboxypeptidases in the barley grain has been studied earlier when the number and specificity of these enzymes were not known (11). The carboxypeptidase activities were found to increase during germination, the bulk of the increase taking place in the starchy endosperm. Since the starchy endosperm contains no living cells, the increase of the enzyme activity must be due to activation of precursors or to secretion from the surrounding living tissues, the aleurone layer, or the scutellum.

The aim of the present study is to determine whether carboxypeptidase I is synthesized during germination and—if so—whether the synthesis takes place in the scutellum or in the aleurone layer. In addition to enzyme activity, mRNA for this enzyme was also localized.

MATERIALS AND METHODS

Plant Material

Barley (Hordeum vulgare L. cv Himalaya) grains were obtained from the Agronomy Club, Washington State University, Pullman, WA. The grains were surface-sterilized and allowed to germinate aseptically in the dark at 20°C (16). In these conditions the coleoptile was about 1.5 cm long after 3 d.

Separation of Parts of Grain and Extraction of Carboxypeptidase

Grains were allowed to germinate for 0 to 8 d and duplicate samples of 10 grains were taken for the extractions. Scutella were separated as described before (16). To approximate carboxypeptidase activity in the aleurone layer (see "Results"), the level of the endosperm was carved off with a scalpel and the carvings were bathed in 10 mL of 0.32 M NaOH (pH 10.5) for 30 min at 20°C and thereafter rinsed with 50 mL of 50 mM sodium phosphate buffer (pH 6.5) before extraction. The separated whole endosperms, aleurone layers, or scutella were homogenized at room temperature in a mortar with quartz sand in 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM DTT. After centrifugation, the supernatants were dialyzed overnight at 4°C against the extraction buffer containing 0.1 mM DTT.

Assay of Carboxypeptidase I Activity

Z-Phe-Ala was used to assay carboxypeptidase I although it is also hydrolyzed by carboxypeptidases II and III (13). The contribution of carboxypeptidase II to the hydrolysis of Z-Phe-Ala can be estimated by multiplying by 1.3 (calculated from Fig. 1 in ref. 12) the activity on Z-Phe-Glu. The contribution of carboxypeptidase III to the hydrolysis of Z-Phe-Ala in a germinating grain is only 10%. Therefore it has been neglected. The activity on Z-Phe-Ala minus 1.3 times the activity on Z-Phe-Glu has been taken as a reasonably good

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estimate of the activity of carboxypeptidase I. The activities were assayed as described before (12) except that pH was 5 for Z-Phe-Glu.

RNA Analysis

Samples of 30 grains were germinated for the indicated periods after which the grain parts were detached as described above. The separated parts of grain were frozen with liquid nitrogen, homogenized, and thawed in 0.1 M NaCl and 0.01 M EDTA in 0.1 M Tris-HCl (pH 9) (2). After adding SDS to a final concentration of 1%, the suspension was extracted twice with phenol-chloroform and once with chloroform. The nucleic acids in the aqueous-phase were precipitated overnight at -20°C with 0.1 volume 3 M sodium acetate-acetic acid and 2.5 volumes ethanol. The precipitate was collected by centrifugation and redissolved in water whereafter RNA was specifically precipitated with 2.5 M LiCl for 16 h at 4°C and redissolved in water. The amount of RNA was determined spectrophotometrically.

Northern blot analysis with the cDNA probes and with the oligonucleotide probe was done as has been described previously (25 and 26, respectively) except that Hybond-N (Amer sham, UK) nylon membranes were used for the RNA-blottings.

Probes

A 28-base oligonucleotide (5'GGC ATG GAT TGC AGA TCT GAC GGC AGC G'-3') was synthesized and end-labeled with [32P]dCTP by terminal transferase (9). This sequence, corresponds to the bases 840 to 867 of the cDNA for carboxypeptidase I of barley (5). The cDNA probe for soybean ribosomal-RNA was obtained from Dr. L. Zimmer, Department of Biochemistry, Louisiana State University, Baton Rouge, LA. The cDNA probe for high pI α-amylase (pM/C insert) was obtained from Dr. J. C. Rogers, Washington University Medical Schools.

RESULTS

Determination of Carboxypeptidase I Activity in the Aleurone Layer

Because the aleurone layer could not be separated free from the adhering starchy endosperm during the first 3 d of germination, a special method was used to estimate the carboxypeptidase activity in these tissues. Susceptibility to low pH has previously been used to distinguish intracellular from extracellular α-amylase (16, 23). Since carboxypeptidases are sensitive to high pH (24) their inactivation by NaOH was tested. The preliminary results showed that at pH 10.5 the activities on Z-Phe-Ala and Z-Phe-Glu in an extract made of starchy endosperm were completely destroyed. To test the effect of high pH on intracellular enzymes the aleurone layers were cut off from grains germinated for 1 d. The samples which contained a variable amount of the starchy endospermal tissue were incubated at pH 10.5 (0.32 mM NaOH) for 0 to 30 min at 20°C whereafter they were extracted. The treatment had no effect on aminopeptidase activity on leucyl-β-
naphthylamide which is due to two aminopeptidases localized in the aleurone layer but not in the starchy endosperm (11) and which is inactivated at pH values above 10 (19). The treatment, therefore, does not destroy intracellular enzymes. The activities on Z-Phe-Ala and Z-Phe-Glu in the samples decreased by about 50% during the first 5 min of NaOH treatment but, thereafter, remained unchanged for at least 25 min (Fig. 1). The decrease is evidently due to inactivation of the extracellular carboxypeptidases in the contaminating cells of the starchy endosperm, the remaining activity being due to intracellular carboxypeptidases in the aleurone layers. After 4 d of germination, aleurone layers essentially free from the starchy endosperm could be obtained; the activities in these after the NaOH treatment were the same as in untreated controls. This result gives further support for the conclusion that the treatment in 0.32 mM NaOH inactivates carboxypeptidases outside but not inside the aleurone cells.

Changes of Carboxypeptidase I Activity

In a resting grain, carboxypeptidase activities on Z-Phe-Ala and Z-Phe-Glu were relatively high (data not presented). However, calculations (see "Materials and Methods") showed that these activities were due to carboxypeptidase II. Thus, no activity of carboxypeptidase I was found in the resting grain (Fig. 2). During germination there was a lag of more than 12 h before the activity of carboxypeptidase I could be found in the starchy endosperm and in the scutellum. In the starchy endosperm the activity increased linearly until d 3, after which it began to decrease. In the scutellum, the decrease began after d 2. In the aleurone layer a low activity appeared

![Figure 1](https://example.com/figure1.png)

Figure 1. Effect of treatment in 0.32 mM NaOH (pH 10.5) on carboxypeptidase I and aminopeptidase activities in the carved aleurone layer samples. After germination for 1 d, the surface of the endosperms was carved off and samples corresponding to 10 aleurone layers were incubated for 5 to 30 min in 10 mL of 0.32 mM NaOH at 20°C. Then the tissues were rinsed with 50 mM Na-phosphate buffer (pH 6.5) containing 1 mM DTT, extracted, and the carboxypeptidase and aminopeptidase activity was assayed.
during the first day, but thereafter the activity remained unchanged for at least 7 d.

Changes in mRNA for Carboxypeptidase I

The oligonucleotide probe for carboxypeptidase I did not hybridize with RNA from scutella in resting grains (Fig. 3). The scutella separated after germination of 1 to 5 d contained RNA of about 1.8 kb which hybridized with the probe. The size of the transcript is of the right order when compared to the size of the incomplete cDNA, 1.443 kb (5) when the missing sequences at the 5′ end are taken into account. These should include the untranslated region and sequences encoding the putative signal sequence and the 57 amino acids in the aminoterminal end of the mature protein. To study the expression of the carboxypeptidase I mRNA in the aleurone layer, RNA was isolated from grains germinated for 2 d, when the activity in the starchy endosperm was rapidly increasing (cf. Fig. 2). Very little of carboxypeptidase I mRNA was found in the aleurone layer which is consistent with the low amount of the enzyme activity in this tissue. The spatial and temporal pattern of mRNA expression was in good agreement with the activity pattern of carboxypeptidase I.

To control the tissue distribution of carboxypeptidase I mRNA, the filter was hybridized with a probe for rRNA. The amount of rRNA was then determined with densitometer. Comparable rRNA intensities (28S + 18S) were found in lanes a2 and s2. Because lane a2 contains very low levels of carboxypeptidase I mRNA compared to lane s2, the scutellum specific expression of carboxypeptidase I is apparent. As an additional control, the filter was hybridized with a probe for high pl α-amylase, which in germinating grain is predominantly expressed in the aleurone layer (7, 8). As expected, the bulk of the α-amylase label of predicted size (17) was localized in the aleurone sample.

DISCUSSION

Although barley carboxypeptidases I and II have some sequence homology and their specificities partially overlap (12, 20, 21), the expression of these enzymes is different. All carboxypeptidase I is synthesized during germination, whereas at least a considerable portion of carboxypeptidase II is synthesized during grain development. This indicates that the genes for these two enzymes are regulated separately. Results suggesting separate regulation of individual carboxypeptidases have been presented earlier, using less specific assays. The results of Mikola and Kolehmainen (11) show that activity on Z-Phe-Ala in a germinating grain develops differently from that on Z-Phe-Phe. Later, it was shown that the latter activity, but not the former one, is increased by gibberellic acid during malting (4).

The high amount of carboxypeptidase I mRNA, together with the high enzyme activity in the scutellum, in contrast to the low amount of mRNA and low activity in the aleurone layer suggest that the bulk of the carboxypeptidase I activity appearing in the starchy endosperm during germination is secreted by the scutellum. This conclusion is supported by

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**Figure 2.** Activity of carboxypeptidase I in various parts of grain during germination. Grains were allowed to germinate at 20°C, and after various periods the activities were assayed in different parts of the grain. The activities in the aleurone layers were estimated with the use of the NaOH treatment inactivation method. The activities in the starchy endosperm were obtained by subtracting the activities in the aleurone layers from those in the whole endosperm which were determined separately.

**Figure 3.** Localization of carboxypeptidase I mRNA in the scutellum and aleurone layer of germinating grain. Grains were allowed to germinate at 20°C, and after various periods the RNA was isolated. Samples of 25 μg of total RNA from each isolation were fractioned by electrophoresis and blotted on filter to be probed with the oligonucleotide probe for carboxypeptidase I (CPase I) mRNA, the cDNA probe for ribosomal RNA (rRNA), and with the cDNA probe for high pl α-amylase (AMY). Lanes s0 to s5: total RNA from the scutellum after germination of 0, 1, 2, 3 or 5 d. Lane a2: total RNA isolated from the aleurone layer after 2 d of germination. The mol wt for the carboxypeptidase I mRNA was estimated by comparison to Hind III-cleaved bacteriophage lambda (λ) fragments.
our preliminary results which show that scutella separated from germinating grains can secrete carboxypeptidase I at a rate which could account for most of the increase of the activity in the starchy endosperm. Consistent with this is the fact that after prolonged germination about 90% of the activity on Z-Phe-Ala (mostly hydrolyzed by carboxypeptidase I in a germinating grain) is in the proximal half of the endosperm (11). Mundy et al. (14) showed that the scutellum contains the bulk of the mRNA encoding for a protein which is precipitated by an antiserum prepared against purified carboxypeptidase I. This result also suggests that carboxypeptidase I is mainly synthesized in the scutellum. However, later it was pointed out (5) that the size of the translation product (35,000) is smaller than the size of the precursor polypeptide predicted from the cDNA sequence (at least 50,000). The isolation by Doan and Fincher (5) of the cDNA clone based on scutellar mRNA also suggests that there is some carboxypeptidase mRNA in the scutellum.

Although the majority of hydrolases of the starchy endosperm are secreted by the aleurone layer (see refs. in 1, 6, 7), the scutellum also has secretory activity. Besides carboxypeptidase I, the bulk of two other enzymes, (1 → 3, 1 → 4)-β-D-glucan endohydrolase isoenzyme I, and an immunologically related protein designated isoenzyme III have been reported to be secreted by the scutellum (22). Scutellum also secretes little α-amylase (8, 16). In addition, some RNAses and proteinases may originate in the scutellum (15).

At present, an interesting discrepancy exists: When ‘half-grains’ (which lack the embryo and the proximal endosperm) are incubated, after 1 d the activities on Z-Phe-Ala and Z-Phe-Phe start to increase to attain a level roughly comparable to that in the whole grain (18). The high activity on Z-Phe-Ala relative to that on Z-Phe-Phe in the half-grains suggests that the bulk of this increase is due to carboxypeptidase I. This result could mean that in a whole grain the synthesis of carboxypeptidase I in the aleurone layer is suppressed by some factor originating in the embryo, while in the half-grain the lack of such a factor promotes the synthesis. We have not studied carboxypeptidase I synthesis in the half-grains but we separated endosperms after 1 and 2 d of germination; in these endosperms no increase of carboxypeptidase I was observed during further incubation. Therefore, it is evident that the scutellum plays an important role in the secretion of hydrolytic enzymes into the starchy endosperm.

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LITERATURE CITED


