Localization of major endopeptidase activities in maize endosperms

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Abstract

Using a tissue print method, major endopeptidase activities were observed in the aleurone layer and along parts of scutellum surface 1 d after imbibition. By day 2 the zone of activity had spread into the subaleurone and starchy parenchyma cells of the endosperm. Three days later, activity was detected throughout the endosperm tissue, but not in the embryo. Endosperm tissues, aleurone layers and scutella were dissected from the seedlings at different stages after imbibition and endopeptidase activity was analysed by an activity stain after native PAGE. At least ten different endopeptidase activities were detected in the endosperm tissues during the initial 5 d. Activities similar to these ten enzymes were also detected in aleurone layers. These results suggest that the main source of these endopeptidases in the endosperm is the aleurone layer. The scutellum had a different spectrum of endopeptidases. One of these alternative endopeptidases, which was detected on the first day after the addition of water, was a metalloenzyme with electrophoretic properties similar to an activity found in endosperm tissue shortly after imbibition.

Key words: Zea mays, endopeptidase localization, seed germination.

Introduction

During early growth of maize seedlings γ-, β-, α-, and δ-zein are degraded sequentially in endosperm tissue (Mitsuhashi and Oaks, 1994). The order of the zein degradation reflects both the localization of the zein proteins in protein bodies and the development of specific endopeptidase activities. For example, γ-zein, which disappears early in the sequence after imbibition, is found in the peripheral layers of the protein bodies (Ludevid et al., 1984; Lending et al., 1988; Lending and Larkins, 1989). It should, therefore, be exposed to endopeptidase activities which appear in the endosperm. In addition, the endopeptidase which appears 1 or 2 d after the addition of water has the capacity to degrade γ-zein (Mitsuhashi and Oaks, 1994, and unpublished results), a requirement of those endopeptidases involved in the hydrolysis of γ-zein.

The results of many workers (Linderstrom-Lang and Engel, 1938; Chrispeels and Varner, 1967; Ho and Varner, 1974; Higgins et al., 1976; Jones and Chen, 1976; Gibbons, 1981; MacGregor et al., 1984; Ranki and Sopanen, 1984; Martilla et al., 1993) have suggested an important role of the aleurone cells in cereal seeds in the synthesis and secretion of many hydrolytic enzymes which are involved in the degradation of starch and protein reserves. For example, in the case of α-amylase in barley, the enzyme is synthesized de novo in the aleurone cells, and is subsequently secreted into the medium (Jacobsen and Chandler, 1987). Miyata et al. (1981) were also able to demonstrate the actual secretion of α-amylase from the scutellum by showing that an enzyme synthesized de novo in excised rice scutellum was secreted into the medium. The situation is less well defined in maize. Some reports (Dure, 1960; Okamoto et al., 1980) suggested that the scutellum was one of the major sources in α-amylase production shortly after the addition of water.

Although the synthesis and distribution of the amylases has been extensively studied and reviewed (Jacobsen and Chandler, 1987; Fincher, 1989), there are only a few reports describing the distribution of specific endopeptidases in the cereal grain (Engel and Bretschneider, 1947;
Engel and Heins, 1947; Okamoto et al., 1980; Ranki et al., 1994) and none defining the function of these enzymes. Using a gelatin film technique Okamoto et al. (1980) found endopeptidase activities in the region of the scutellar epithelial cells shortly after the addition of water in several cereal species. Subsequently, the activities diffused into the entire region of the endosperm. Wrobel and Jones (1992) have also detected two neutral endopeptidases in the scutellum and one of these enzymes was also found in aleurone layers and starchy endosperm tissues in germinated barley caryopsis. Observations by Winspear et al. (1984) indicated that carboxypeptidases were different in barley and maize and a comparison of the results of Wrobel and Jones (1992) and Mitsuhashi and Oaks (1994) shows that this is also true of endopeptidases.

In this paper, the distribution of endopeptidase activities which develop in maize scutellum, endosperm and aleurone tissues at specific times after germination is examined, using a tissue print method in order to define specific locations of the various endopeptidases. In addition, the aleurone, scutellum, and starchy endosperm tissues were dissected from the kernel and analysed for endopeptidase activities by using an activity staining after native polyacrylamide gel electrophoresis. Unlike barley which expresses an aspartate-endopeptidase in the scutellar tissues (Sarkkinen et al., 1992; Wrobel and Jones, 1992) our results show that the metallo-endopeptidases which appear soon after imbibition could originate in the scutellum and that most of the 17 SH-endopeptidases identified by Mitsuhashi and Oaks (1994) probably originate in the aleurone layer.

Materials and methods

Plant materials
Hybrid maize kernels (Zea mays, W64A x W182E) purchased from Wisconsin Seed Foundation (Madison, WI, USA) were germinated on clay (Turface, Applied Industrial Materials Corp., Deerfield, IL, USA) at 28 °C with 16 h light (225 μmol m⁻² s⁻¹) and 8 h dark. Deionized water was supplied to the kernels for the initial 2 d after sowing. After that time a one-tenth strength Hoagland solution which contained no nitrogen was supplied. Endosperm tissues, aleurone layers and scutella were harvested from the seedlings at designated times and were stored at −20 °C until required, but for no longer than 3 weeks. Because most of the aleurone cells were not yet swollen and hence were tightly bound to the endosperm only one-third of the total aleurone layer was collected from day 2 or day 3 kernels. About one-half of the aleurone layer was collected from day 4 samples. At later stages the complete aleurone layer was collected. To collect endosperm free from aleurone cells, the half portion of endosperm distil from the aleurone was used for day 2 and 3 samples.

Tissue print method
Freshly harvested whole kernels were cut transversely at three different points, and these slices were placed on a 1% (w/v) gelatin–8% (w/v) polyacrylamide gel plate (Mitsuhashi and Oaks, 1994). The substrate plate consisted of 1% (w/v) swine skin gelatin (type I from Sigma), 8% (w/v) polyacrylamide, 0.1% (v/v) N,N',N'-tetramethylenediamine, and 0.1% ammonium persulphate. The slices were pressed against the plate and fixed in position with tape. Sections prepared from 1, 2, 3 or 4-d-old kernels were incubated at 37 °C for times suitable for detection of the endopeptidase activities (e.g. 1 d after imbibition for 3 h, 2 d for 2 h, and 3 and 4 d after imbibition for 10 min). The plates were then stained with 1% (w/v) amido black 10B in 7% (v/v) acetic acid overnight, followed by destaining with 7% (v/v) acetic acid. Clear zones in the blue background indicated the presence of endopeptidase activity (Plate 1).

Activity staining for endopeptidases after native PAGE
The method described by Mitsuhashi and Oaks (1994) was used. Five starchy endosperms or ten scutella were extracted with 2 volumes of sodium phosphate buffer, (50 mM, pH 7.0) containing 10 mM 2-mercaptoethanol. For aleurone layers, 8 volumes of extraction buffer were used. After centrifugation, the proteins in the supernatant were separated by 10% (w/v) native PAGE, and the PAGE gels were rinsed for a few minutes in 1 M sodium acetate buffer (pH 4.8) containing 30 mM 2-mercaptoethanol before being placed on 1% (w/v) gelatin–8% (w/v) polyacrylamide gel plates, as described by Mitsuhashi and Oaks (1994). These sandwiches were incubated for the appropriate time at 37 °C. The substrate plates were then stained with 1% (w/v) amido black 10B dissolved in 7% acetic acid. After destaining with 7% (v/v) acetic acid, clear zones which appeared after the destaining indicated the presence of endopeptidase activities.

Native polyacrylamide gel electrophoresis
Native PAGE was carried out according to the method of Hedric and Smith (1968). Ten per cent (w/v) gels made up with Tris–HCl buffer (0.5 M, pH 7.9) were used for the protein separations. A TRIS-asparagine buffer (45 mM, pH 7.3) was used for the cathode buffer, and a TRIS–HCl buffer (62.5 mM, pH 8.0) was used for the anode buffer. Forty μl of sample solution containing 16% (v/v) glycerol were applied to the stacking gel. The gels were run at a constant voltage of 100 V at 4 °C.

Results and discussion

Change of endopeptidase distribution in endosperm tissue during early seedling growth
In a previous publication it was established that there were up to 17 different protease activities, representing different proteins based on sensitivities to different endopeptidase inhibitors. To characterize these activities further the distribution of the various endopeptidases in the various tissues were examined in situ. To achieve this, whole kernels were cut transversely at three different points (designated as top, middle and bottom panels), and activity zones for endopeptidase were visualized by placing the sections on gelatin–polyacrylamide gel plates (Plates 1, 2). One day after imbibition, endopeptidase activity was detected in the aleurone layer and in cells adjacent to the scutellum (Plate 2, lane 1). The aleurone layer seemed to be a major source for the activity in the top part of the kernel (lane 1, top panel). However, high activity zones were observed in the scutellar region adj-
Plate 1. Tissue blot technique for localizing endopeptidase activities in situ. Whole kernels were cut transversely and the sliced sections were then placed on a plate overlaid with a gelatin-polyacrylamide gel for 10–120 min. The gels were then stained for protein as described in the Materials and methods. Activity zones where the gelatin was digested are seen as clear areas against a blue background of unhydrolysed gelatin. Samples from left to right are 1, 2, and 3 d after the addition of water.

Plate 2. Distribution of endopeptidase in endosperm tissue. Whole kernels harvested from day 1 (lanes 1 and 2), 2 (lane 3), 3 (lane 4), and 4 (lane 5) were cut transversely at three different points. These sliced sections were then treated as described in Plate 1 and in the Materials and methods. After incubation for the appropriate time, the endopeptidase activity zones were visualized by staining for protein.
cent to the starchy endosperm in the middle and bottom sections of the starchy endosperm. Similar activity patterns were observed in many kernels, however, in some kernels high activities were observed in the middle section by day 1 (lane 2, middle panel). These results indicate that, as with the amylases, maize kernels could have two sources of endopeptidase activity, a scutellar source and an aleurone source. By day 2 the activity zones had spread from the aleurone itself to the subaleurone and cells of the starchy endosperm (Plate 2, lane 3, top and middle panels). At this stage, a very active zone surrounding the scutellum is also observed (lane 3, top and middle panels). By day 3, all of the starchy endosperm showed high levels of endopeptidase activity (lane 4, top and middle panels). New activity zones were also observed in the scutellum by day 4 (lane 5). From these observations, it seems likely that the basal part of the kernel was the first to show good endopeptidase activity. Thus it could be that the epithelium cells of the scutellum are involved in the synthesis and release of some of the endopeptidase activities detected in the starchy endosperm. In the top part of the kernel (Plate 2, lanes 1 and 3, top panels) which is relatively distant from the scutellum, the aleurone layer also appears to be producing endopeptidase activity shortly after imbibition. Two inhibitors were used in connection with these tissue blots: leupeptin to inhibit SH-endopeptidases and 1,10-phenanthroline to inhibit the metallo-endopeptidase activity (unpublished observations). Leupeptin inhibited activities around the aleurone layer. There was a much weaker inhibition detected around the scutellum. Results with phenanthroline, where a clear inhibition of the metallo-endopeptidases had been expected, were not so clear, perhaps because of contamination with aleurone endopeptidases. When both inhibitors were added together most of the activities disappeared in the scutellar regions as well as in the aleurone layer. Thus as with our previous investigation most of the endopeptidase activities are associated with SH-endopeptidase-mediated activities, but the early activities seen adjacent to the scutellum may be metallo-endopeptidases.

Change of sequential development of endopeptidase activity in endosperm, aleurone layer and scutellum

Results from the tissue print experiment (Plates 1, 2) indicated that the scutellum may produce and secrete...
endopeptidase protein shortly after the addition of water. To extend this observation, the developmental changes of endopeptidase activity in the starchy endosperm, aleurone layer and scutellum were examined (Plate 3). At least ten different enzymes were observed on gels prepared from extracts from starchy endosperm plus aleurone tissues (Plate 3, panel B). These activities fell into two different groups based on time of appearance after the addition of water, groups II and III as defined by Mitsuhashi and Oaks (1994). Although all of these activities were detected in both the aleurone layer (panel C) and in the starchy endosperm (panel D), their appearance was not synchronized in the two tissues. For example, one of the group III activities which had the highest mobility in this group on the gel showed the strongest activity from days 2 to 5 in the starchy endosperm (Plate 3D), whereas at no time did this enzyme have a major activity in the aleurone (Plate 3C). The second group of activities which reached the highest values in the starchy endosperm by day 2 or day 3 before they disappeared remained fairly constant in the aleurone during the entire experimental period. Group IV enzymes were detected only in the aleurone layer (Plate 3, panels B, C). The mobilities of the group IV enzymes were slightly lower than that of the group II enzymes. These activity bands had a relatively high specificity to a-zein (Mitsuhashi and Oaks, 1994). The similarities in the activity distributions in the aleurone and endosperm tissues indicate that the aleurone is a major source of the group II and III enzymes found in the endosperm. The scutellum, on the other hand, had a different set of endopeptidase activities (Plate 3A). One major activity which appeared on day 1 (Plate 3A, at arrow). A similar activity was also detected in the day 1 starchy endosperm tissue with aleurone when crude extracts were concentrated four times (Mitsuhashi and Oaks, 1994). These particular scutellar and endospermic enzymes appear to be metallo-endopeptidases based on their sensitivity to EDTA (data not shown). Feller et al. (1978) also described unique endopeptidase activities which were present in the maize scutellum. The time-course for the appearance of this activity is similar to that of our metallo-enzymes. Wrobel and Jones (1992) and Sarkkinen et al. (1992) found aspartic endopeptidase activities in barley which were high initially and then decreased to a low level 2 d after the addition of water. Activities of two neutral endopeptidases increased on the first day of steeping. Martilla et al. (1993) also demonstrated the secretion of a 30 kDa cysteine-endopeptidase into the starchy endosperm from scutellar epithelium at the end of the first day of imbibition, and later (1–3 d) this activity developed along the aleurone to the distal end of the grain. A similar pattern was described for the expression of the two isozymes of (1→3, 1→4)-β-D-glucanohydrolases again in barley (McFadden et al., 1988). These observations are more extensive than our observations with maize, but they do suggest that the hydrolysis of storage products might be quite different in the endosperm tissues of maize and barley.

Differences in the endopeptidase spectrum among these three maize tissues were clearly defined by day 6 after imbibition (Plate 4). In endosperm tissues, all of the group III enzymes are present (lanes 1 and 4), whereas group IV enzymes and one of the group III enzymes had major activities in aleurone layers (lane 2). There were very weak activities in scutellum at this stage. In contrast to this observation, Wrobel and Jones (1992) found a very similar spectrum of four cysteine-endopeptidases in aleurone layers, scutellum and endosperm tissues. These results suggest that the scutellum and aleurone layers produce a different spectrum of endopeptidases and that some of these endopeptidases are transferred to the endosperm. Recent evidence from this laboratory also indicates that not all the activity bands are unique proteins. For example, by dialysing crude extracts prepared from day 2 endosperms at low pH (4.5) a shift in activity from group II to group III bands after native PAGE was found (Wallace and Oaks, unpublished observation). Those endopeptidases which develop in the earliest times after imbibition are currently being characterized in order to determine more precisely their site of production and their specificity for particular zein substrates.

Plate 4. Comparison of endopeptidase spectra in endosperm, aleurone layer and scutellum 6 d after imbibition. Whole endosperm tissue, scutellum and aleurone layers were harvested from 6-d-old seedlings. Aleurone layers and scutellum were washed with water to remove endospermic enzymes. Proteins in these three tissues were separated by 10% (w/v) polyacrylamide native PAGE, and then endopeptidase activities were detected by activity staining as described. Lane 1, endosperm tissues; lane 2, aleurone layers; lane 3, scutellum; lane 4, endosperm tissues with aleurone layers.
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References


