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Development of Endopeptidase Activities in Maize (*Zea mays* L.) Endosperms[1]

Wataru Mitsuhashi[2] and Ann Oaks*

Department of Botany, University of Guelph, Guelph, Ontario, Canada N1G 2W1

An activity stain was used after native polyacrylamide gel electrophoresis, and at least 17 different endopeptidase activities were detected in maize (*Zea mays* L.) endosperm extracts prepared during the first 6 d after imbibition. The enzymes detected were classified into four groups based on their time of appearance and on their mobility in polyacrylamide gels. The first group, which included two enzymes present in dry endosperms, disappeared soon after imbibition. The second group, comprising five activity bands, appeared during the first 2 to 3 d after imbibition and then disappeared. The third set of enzymes increased continuously throughout the experimental period. The fourth group appeared after d 3 and remained at a constant level after that time. The endopeptidase activities were characterized by the effect of specific inhibitors on their activities. The two enzymes of the first group are metalloendopeptidases based on their sensitivity to ethylenediaminetetracetate (EDTA). Enzymes of the second, third, and fourth groups are sulfhydryl-endopeptidases as judged by their sensitivity to antipain, chymostatin, leupeptin, and E-64 and by their requirement for 2-mercaptoethanol. Pepstatin, phenylmethylsulfonyl fluoride, or EDTA had no effect on these enzymes. Many of the second, third, and fourth group enzymes cleaved α-zein-rich proteins as well as such easily obtained proteins as gelatin (used in our standard assay) and hemoglobin. The second group had a high affinity for γ-zein, whereas none of the bands in the fourth group of enzymes cleaved this type of zein. The two metalloenzymes of the first group cleaved neither α- nor γ-zeins.

Storage protein and carbohydrates are degraded to supply the amino acids and sugars required for the growth of the young seedling. In maize (*Zea mays* L.) endosperms the alcohol-soluble prolamins, the zeins, can represent up to 50% of the total protein (Harvey and Oaks, 1974b; Tsai et al., 1980). The zeins are designated by subclass (α-, β-, γ-, and δ-zein) according to solubility and mol wt (Esen, 1986; Wilson, 1991). They are found in protein bodies (Burr and Burr, 1976; Larkins et al., 1976) with γ-zein localized in the peripheral part of the protein core and the α- and β-zeins in the matrix (Ludevid et al., 1984; Lending et al., 1988; Lending and Larkins, 1989). δ-Zein is found well embedded in the core of the protein body (Esen and Stetler, 1992). Many reports have shown that zein proteins are preferentially degraded after germination. In addition, within the zein group there is a sequential order of degradation. For example, γ-zein is the first to be degraded, followed by β-, α-, and δ-zein (Ludevid et al., 1984; Torrent et al., 1989; Mohammad and Esen, 1990). This pattern of degradation could reflect the localization of each of the zein proteins within the protein bodies, with the peripheral proteins being the first to be degraded. However, specific endopeptidases could also be important in determining the sequence of these hydrolytic events.

Although total endopeptidase activities have been measured, only a few attempts have been made to determine how many endopeptidases are actually involved in the hydrolysis of the zeins. Recently, for example, many endopeptidase activities have been detected in maize endosperms after PAGE (De Barros and Larkins, 1990; Segundo et al., 1990; Hay et al., 1991). De Barros and Larkins (1990) identified six different endopeptidase activities with activity gels, and of these, four were partially purified and characterized. They were SH-proteinases. Segundo et al. (1990) found that at least eight different endopeptidase activity bands appeared in an easy-to-determine sequence after germination and that some of these bands responded to treatments with GA_3 and ABA. Hay et al. (1991) also detected at least three major and four minor proteinase activities in maize endosperms. Some of these endopeptidases have been isolated and characterized. For example, Abe et al. (1977) identified a 21-kD SH-endopeptidase (P-Ia), Moureaux (1979) identified a 36- and 12-kD SH-endopeptidases, and De Barros and Larkins (1990) identified four SH-endopeptidases that have molecular masses of between 26 and 33 kD. Because these enzymes were purified from endosperms that were harvested at relatively late stages (5 d after germination), they may be involved only in the terminal stages of degradation of zein. We have also reported high levels of carboxypeptidase activities in endosperm tissues in several cereals (Winspear et al., 1984). These enzymes, too, may be contributing to the final stages of prolamin degradation.

In this paper, we show that in our system many of the zein proteins are degraded during the first 6 d after germination. For detection of endopeptidase activity on PAGE gels, we used native PAGE and a sensitive activity stain described by Mitsuhashi et al. (1986). We found more than 17 activity bands. Some of these activity bands, the second group, reached peak activities soon after imbibition (2 and 3 d), and of these, two activity bands have the capacity to cleave γ-zein. The third and fourth groups of endopeptidase activities

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[2] Present address: Plant Hormone Function Team, Frontire Research Program RIKEN, Wako-shi, Saitama 351-01 Japan.

* Corresponding author; fax 1-519-767-1991.

Abbreviation: SH, sulfhydryl.

appeared at later times. Our third group of enzymes seems to be similar to those enzymes described by De Barros and Larkins (1990), because antibodies prepared against their endopeptidases recognized only the group 3 endopeptidases in our system. A major endopeptidase activity band that appeared at d 5 (group 4) had a high degree of specificity for α-zein.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays* L., W64A × W182E) kernels purchased from Wisconsin Seed Foundation (Madison, WI) were grown in clay (Turface; Applied Industrial Materials Corp., Deerfield, IL) at 28°C. A 16-/8-h light/dark period was used. The light intensity was 225 μmol m^{-2} s^{-1}. The seeds were well watered with deionized water at zero time and subsequently were watered with a one-tenth-strength Hoagland solution that contained no nitrogen (Winspear et al., 1984). Endosperms with aleurone layers were dissected from seedlings at daily intervals and were stored at −20°C until required. Each experiment described in the text was repeated at least three times.

Zein Extraction

The extraction was performed according to the method described by De Barros and Larkins (1990). Five endosperms at each stage were extracted with 70% (v/v) ethanol containing 1% (v/v) 2-mercaptoethanol and 0.1% (v/v) Triton X-100 at 65°C for 60 min. Three millimeters of the ethanol solution were used per g of tissue. The supernatant solution was used as the total zein fraction. For the SDS-PAGE analysis, equal amounts of protein (4 μg) were analyzed using 16% (w/v) SDS-PAGE in a minigel system (Bio-Rad, Richmond, CA). In these extracts about 90% of the total zein is α-zein. γ-Zein was purified according to the method described by Esen (1986).

Albumin, Globulin, and Glutelin Extractions

For albumin and globulin proteins, five endosperms were ground with a mortar and pestle. Two milliliters of distilled water were used per g of tissue. The extracts were shaken for 12 h at 4°C. The supernatant solutions obtained after centrifugation were designated as the albumin fraction. The precipitates were washed two times with distilled water and after centrifugation were resuspended in a 5% (w/v) NaCl solution. The suspensions were shaken for 12 h at 4°C. The supernatant solutions obtained after centrifugation were designated as the globulin fraction. Another five endosperms were extracted with 50 mM sodium borate buffer (pH 8.0) containing 0.2 M NaCl. Two milliliters of the solution were used per g tissue. The supernatant obtained after centrifugation was designated as the glutelin fraction. Changes of protein pattern in each fraction were analyzed using a 20% polyacrylamide gel in an SDS-PAGE system.

Activity Staining for Endopeptidase after Native PAGE

Five endosperms were extracted with a mortar and pestle with 50 mM sodium phosphate buffer (pH 7.0), containing 10 mM 2-mercaptoethanol. Two milliliters of the buffer solution were used per g of tissue. The proteins in the supernatant fractions were separated by native PAGE after centrifugation. The gels were rinsed for a few minutes in 1 M sodium acetate buffer (pH 4.8) containing 30 mM 2-mercaptoethanol, and then the gels were overlaid on gelatin plates containing 1% (w/v) swine skin gelatin (type I from Sigma), 8% (w/v) polyacrylamide, 0.1% (v/v) *N,N,N′,N′*-tetramethylethylenediamine, and 0.1% (w/v) ammonium persulfate. When required, zein, casein, or hemoglobin proteins were also co-polymerized with the polyacrylamide. The gel-substrate plates were incubated with the native PAGE gels for 3 h at 37°C. Subsequently, the substrate plates were soaked in 1% (w/v) amido black 10B in 7% (v/v) acetic acid. Clear bands where the substrate had been digested by the endopeptidases were detected after destaining the plates with 7% (v/v) acetic acid. When zein was used as substrate, it was stained with 0.5% Coomassie brilliant blue R in 40% (v/v) methanol in 10% (v/v) acetic acid, followed by destaining with 5% (v/v) methanol in 10% (v/v) acetic acid. For the inhibitor studies, the native PAGE gels were immersed in inhibitor solutions (50 μM leupeptin, 50 μM chymostatin, 50 μM antipain, 20 μM pepstatin, 10 mM Na_2-EDTA, 5 mM PMSF, or 50 μM E-64 dissolved in the 1 M sodium acetate buffer [pH 4.8], containing 30 mM 2-mercaptoethanol) for 20 min at room temperature. The gels so treated were then placed on the gelatin plates as described above.

Native PAGE

PAGE was carried out according to the method of Hedrick and Smith (1968). The stacking gel consisted of 2.8% (w/v) polyacrylamide, 0.25 M imidazole-HCl buffer (pH 5.7); riboflavin (0.0005% [w/v]) and potassium persulfate (0.015% [w/v]) were used as a photosensitizer to polymerize the acrylamide gel. Polyacrylamide gels (9 or 10% [w/v]) containing 0.5 M Tris-HCl buffer (pH 7.9) were used in the separations. Tris-Asn buffer (0.045 M, pH 7.3) was used as the cathode electrode solution, and Tris-HCl buffer (0.0625 M, pH 8.0) was used as the anode electrode solution. Enzyme solutions were mixed with glycerol to give a final concentration of 16% (w/v), and a 40-μL aliquot was loaded on each lane of stacking gel. The gels were usually run at 100 V and 4°C. If prerunning was required, the gels were run for 30 min at 30 mA before the samples were loaded onto the stacking gel.

SDS-PAGE

SDS-PAGE was done according to the method described by Laemmli (1970). Gels (16 and 20% [w/v]) were used for the analysis of prolamin (zein) and other protein fractions (albumin, globulin, or glutelin). The protein fractions were mixed with equal volumes of SDS sample buffer consisting of Tris-HCl buffer (50 mM, pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, and 0.01% (w/v) bromphenol blue. Samples were boiled for 2 min before being added to the gels.

Western Blotting

Proteins were separated by SDS-PAGE or native gels (followed by soaking the gel in 2% SDS, 5% mercaptoethanol, 10% glycerol in 375 mM Tris-HCl [pH 8.8] for 30 min) and transferred to nitrocellulose membrane (BioBlot; Costar, Cambridge, MA), according to the method of Towbin et al. (1979). Development of protein bands was accomplished with rabbit polyclonal antibodies specific to zein or a Cys-endopeptidase, and the presence of IgG was indicated by use of goat anti-rabbit antibodies conjugated to alkaline phosphatase.

Silver Staining of Proteins after SDS-PAGE

Silver staining was carried out by the method of Wray et al. (1981). Protein gels were soaked in 50% (v/v) methanol to fix the proteins and to remove gel buffer from the gels. After the gels were rinsed two times with deionized water, they were immersed for 20 min in a silver solution that contained 0.075% (w/v) NaOH, 0.4 M ammonium hydroxide, and 0.8% (w/v) $AgNO_3$. The silvered proteins were developed by adding 0.019% (v/v) formaldehyde in 0.005% (w/v) citrate.

Protein Measurement

Protein contents in the solutions were determined by using the Bio-Rad protein assay system. The assay procedure was done according to the manufacturer's guide book.

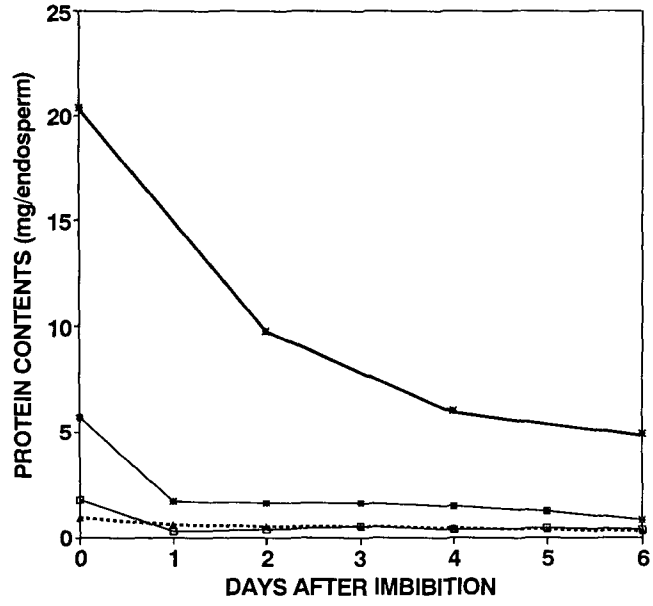

Figure 1. Change in protein contents in maize endosperm tissue. Endosperms were harvested from seedlings each day and stored at −20°C until required. Endosperms (five per sample) were extracted as described in "Materials and Methods." After the samples were centrifuged, the protein contents in supernatant solutions were measured. ●, Zein; ■, glutelin; ▲, albumin; □, globulin. With glutelin, albumin, and globulin the SE values were smaller than the symbols.

RESULTS

Change in Endosperm Protein during Early Seedling Growth

In preliminary experiments, we grew the seedlings at 20 or 28°C. Although 20°C is probably closer to the normal temperature experienced by these seedlings in the field, the growth was uneven and the development of endopeptidase activity in the endosperms was not synchronous. As a result we used 28°C for our standard growth conditions.

The prolamin-type proteins, the zeins, are the initial target proteins hydrolyzed in endosperms after germination (Ingle et al., 1964; Harvey and Oaks, 1974b; Fujimaki et al., 1977). In our growth conditions, about 50% of original zein was consumed by d 2 (Fig. 1). About 70% of the total glutelin and a major portion of the globulin fraction had also disappeared within the 1st d (Fig. 1). On the other hand, the albumin fraction was basically unchanged during the 6-d growing period (Fig. 1). These observations basically confirm the earlier observations of Moureaux (1979).

There were only minor changes in the actual protein bands of the albumin (Fig. 2A) and globulin (Fig. 2B) fractions during the 6 d after germination. For instance, there were two major polypeptide bands, at 62 and 65 kD, in the albumin fraction of the dry endosperm, which disappeared soon after germination (arrow in Fig. 2A). Other peptides with molecular masses of 30 and 19.5 kD appeared at d 1 and 2. A 58-kD band accumulated 2 d after imbibition. Certain polypeptides that have molecular masses of 40 and 25.5 kD accumulated gradually throughout the growing period. In the globulin fraction, a few small peptides with molecular masses of 13, 15, 16, and 47 kD accumulated during the 6-d period (arrows in Fig. 2B). Polypeptides with molecular masses of 44 and 25.5 kD were seen in dry seeds and at d 1 after imbibition and then disappeared. Again, in the glutelin fraction, there were only minor changes in the composition of polypeptide (arrows in Fig. 2C). For example, a few small peptides with molecular masses of 12, 14, and 16 kD accumulated during the 6 d after the addition of water. A 33-kD peptide was present in endosperms only in the d 1 samples. In addition, a 23.5-kD polypeptide was present only in the d 5 extracts. Although there were many transient changes in the albumin, globulin, and glutelin fractions, none of these bands cross-reacted with a zein antiserum that recognizes α-, β-, and δ-zeins (data not shown).

The reduction of total zein shown in Figure 1 reflects an absolute loss in the α-zein bands (22 and 19 kD). When similar amounts (4 μg) of total zein were analyzed using a 16% SDS-PAGE, it was apparent that γ-zein bands (γ_1, 27 kD; γ_2, 16 kD) disappeared within the first 2 d after imbibition (Fig. 3). β-Zein (14 kD) or δ-zein (10 kD) bands also disappeared before changes in the α-zein band were detected.

Change in Endopeptidase Activities after Imbibition

Preliminary tests showed that the hemoglobin hydrolytic activity increased from very low levels in the dry seed and reached a peak at 4 d after imbibition (our unpublished data).

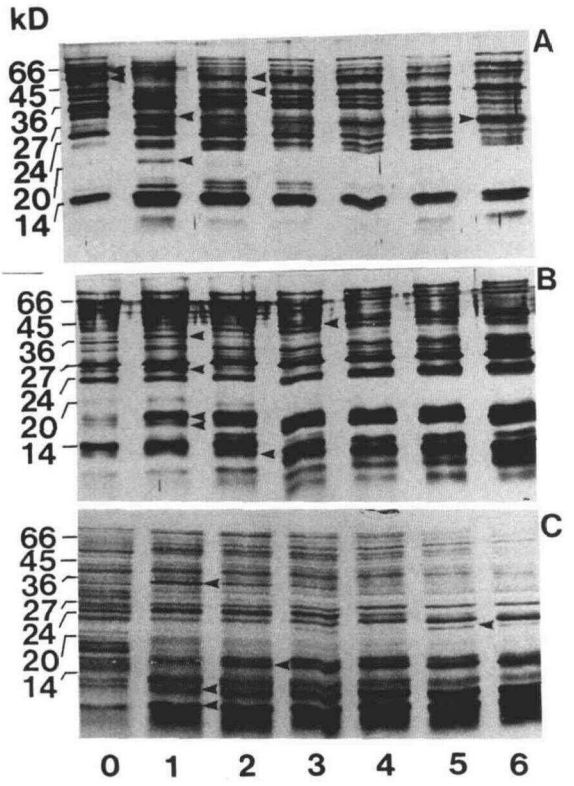

Figure 2. Change of protein (albumin, globulin, and glutelin) patterns in maize endosperm tissues. Each protein fraction was treated with an equal volume of SDS sample buffer and boiled for 3 min. These proteins were separated by SDS-PAGE using a 20% (w/v) gel. Similar amounts of protein (1.5 μg) were loaded to compare the polypeptide pattern in endosperms. After SDS-PAGE, these gels were stained with silver nitrate. A, Albumin fractions; B, globulin fractions; C, glutelin fractions. Lane numbers show days after the addition of water. The changes of protein bands indicated by arrows are described in the text.

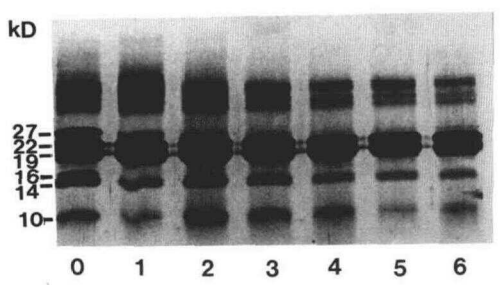

Figure 3. Change of zein proteins in maize endosperm tissues. Total zein proteins extracted from the endosperms were mixed with the same volume of SDS sample buffer and boiled for 2 min. These proteins (4 μg) were analyzed by 16% SDS-PAGE. Zein proteins were stained with silver nitrate. Lane numbers show days after the addition of water.

Previously, Harvey and Oaks (1974a) found similar results with hemoglobin, gliadin (wheat prolamin), and total zein proteins as substrates. To examine whether there was a sequential development of endopeptidase in the endosperms, we introduced a mild native PAGE as reported by Hedrick and Smith (1968) coupled with an activity stain to detect endopeptidase activities after PAGE (Mitsuhashi et al., 1986). We used several types of substrate proteins (hemoglobin, casein, gelatin, γ-zein, and total zein), and we found that gelatin gave the best resolution for each endopeptidase activity (data not shown). In the literature, native PAGE separations with fresh gels and with gels pretreated with standard running buffer have been described (De Barros and Larkins, 1990). As shown in Figure 4, there were at least two endopeptidase activity bands in endosperms from dry seeds (Fig. 4, d 0). The two activities had disappeared 1 d after imbibition. The intensity of these activity bands was not affected by prerunning the gels with buffer only (data not shown). During the next 6 d, 15 different endopeptidase activities were detected (Fig. 4). In this case the sensitivity of detection for some activities, especially for group III activities, was enhanced by prerunning the gels with buffer (Fig. 4B). This indicates that impurities in the polyacrylamide were present in the original system that inhibited some of the proteinase activities. De Barros and Larkins (1990) also reported some enhancement of endopeptidase activities in a gel after prerunning with buffer.

In our experiments the endopeptidase activities appear to fall into four groups based on their time of appearance. The first group in dry endosperms comprises two enzymes that disappear within 1 d. The second group contains at least five endopeptidase activities that appear temporarily at d 2 and 3 (Fig. 4). The third group, which also contains at least five endopeptidase activities, is easily detected by d 5 (Fig. 4). This group increases in activity throughout the experimental period. The fourth group is observed after d 3, and the activities remained fairly constant after that time.

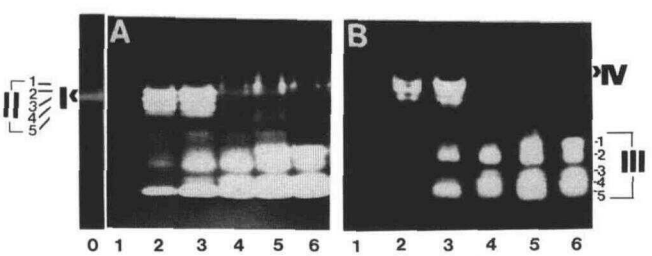

Figure 4. Change of endopeptidase spectrum in endosperms during early seedling growth. Five endosperms at each stage were extracted with 50 mM sodium phosphate buffer (pH 7.0), containing 10 mM 2-mercaptoethanol. Equal volumes of the supernatants were mixed with glycerol. The final concentration of glycerol was 16% (v/v). For 0-d endosperms, crude extracts were concentrated to one-fourth of the original volume by use of a Centricon-10 (Amicon, Beverly, MA). Ten-microliter portions were loaded on the gel, and native PAGE (9% gels) was carried out at 4°C. The endopeptidase activities were visualized by the gelatin plate method (see "Materials and Methods"). Lane numbers show days after imbibition. A, PAGE was done by our standard procedure. B, PAGE gel was prerun with buffer before sample loading.

Effects of Exogenous Inhibitors on Endopeptidase Activity

After native PAGE, the gels were soaked in several inhibitor solutions and the effects of these inhibitors were observed by the disappearance of activity bands on the activity staining gel (Fig. 5). In dry endosperms, there were two endopeptidase activities (group I enzymes, Figs. 4 and 5). These activities were detected with acidic pH (pH 4.8) but not with neutral conditions (pH 6.8) (data not shown). These activities were not affected by 2-mercaptoethanol, PMSF, or leupeptin (Fig. 5, column I). E-64, pepstatin, chymostatin, and antipain inhibited these activities slightly. However, addition of EDTA (column I-3) did result in a strong inhibition, which suggests that these activities are due to metalloendopeptidases.

Groups II and III enzymes showed higher activities with acidic (pH 4.8) than with neutral (pH 6.8) conditions (data not shown). At least five of the enzymes in group II required a reductant such as 2-mercaptoethanol to be detected. If the gel was rinsed with a reductant-free solution, these activities were not seen (Fig. 5, lane 2). These five activities have a high sensitivity to leupeptin, chymostatin, and E-64, minor sensitivity to pepstatin, but no sensitivity to EDTA and PMSF (Fig. 6, columns II, III and III'). Although sensitive to antipain, all enzymes in group II retained some of their activities after treatment with this inhibitor (lane 9). However, E-64 (lane 5), leupeptin (lane 8), and chymostatin (lane 7) were potent inhibitors. These results indicate that these five activity bands are Cys-endopeptidases. The sensitivity to these inhibitors was not affected by the prerunning treatment (data not shown).

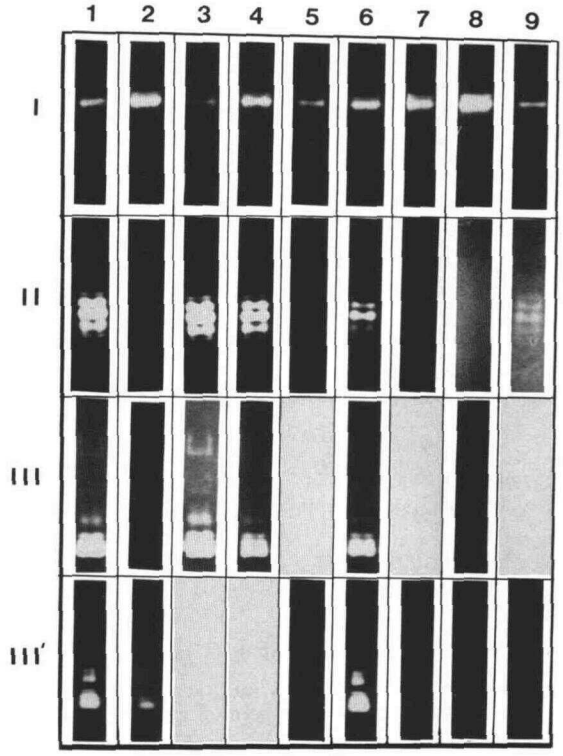

Figure 5. Effects of inhibitors on endopeptidase activities in endosperms from d-2 and d-5 seedlings. Four endosperms were harvested from d-2 or d-5 seedlings and were extracted with 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol. After the samples were centrifuged, the supernatant solutions were mixed with glycerol and loaded on a 9% gel for d-2 extracts or on a 10% gel for d-5 samples. The gels were soaked in different types of inhibitors before incubation with the gelatin plates. The endopeptidase activities were visualized by the gelatin plate method (see "Materials and Methods"). Lane 1, Control with mercaptoethanol (30 mM); lane 2, control without mercaptoethanol; lane 3, EDTA (10 mM); lane 4, PMSF (5 mM); lane 5, E-64 (50 μM); lane 6, pepstatin (20 μM); lane 7, chymostatin (50 μM); lane 8, leupeptin (50 μM); lane 9, antipain (50 μM) for the first (I), second (II), and third (III) groups enzymes. For the third group, the PAGE gel was also prerun with buffer (III').

Group III enzymes also required 2-mercaptoethanol for their activities (lanes 1 and 2). However, the activity was detectable even under 2-mercaptoethanol-free conditions when the gels had been prerun with buffer before adding the extract (column III', lane 2). Pepstatin, EDTA, and PMSF did not affect these activities, whereas addition of leupeptin, antipain, chymostatin, and E-64 did result in clear inhibition. These results show that the five late-appearing enzymes are also Cys-endopeptidases. Thus, the properties of group III activity bands were quite similar to the enzymes A_1 to A_4 described by De Barros and Larkins (1990). Two activity bands in the fourth group that were observed after d 3 (Fig. 4) were also inhibited by E-64, chymostatin, leupeptin, and antipain (data not shown).

Substrate Specificity for the Enzymes after Native PAGE

When we examined the substrate specificities of the enzymes in the various groups we found that group I enzymes in dry endosperms have no affinity to zein proteins but did have the ability to degrade the glutelin-type proteins (our unpublished data). Preliminary experiments showed that many of the group II and III enzymes had the capacity to degrade both γ- and α-zeins. The group IV enzymes cleaved α-zein but not γ-zein. The substrate specificities for the enzymes of group II and III are shown in Figure 6. After native PAGE, endopeptidase activities from d-2 or -5 endosperms were visualized against gelatin (lane A), total zein without γ-zein (lane B), or γ-zein, a 22-kD protein purified as described in "Materials and Methods" (lane C). Many of the second, third, and fourth group enzymes cleaved our total zein, the major component of which is α-zein (lane B); however, two enzymes of the fourth group had the highest affinity for this zein protein (arrow in Fig. 6B). As with the other group II and III activities, these two activities in group IV appear to be Cys-endopeptidases because of their requirement of 2-mercaptoethanol and inhibition by leupeptin, chymostatin, antipain, and E-64 (data not shown). Enzymes in groups II (faint band) and III (strong band) had the capacity to degrade γ-zein. These activities are indicated by arrows in Figure 6C. By the time group III enzymes appear there is very little γ-zein left in the endosperm. Thus, there is probably no physiological role of the group III enzymes in the degradation of γ-zein. We are currently in the process of optimizing the activities of the enzymes in group II, which have the capacity to degrade zein.

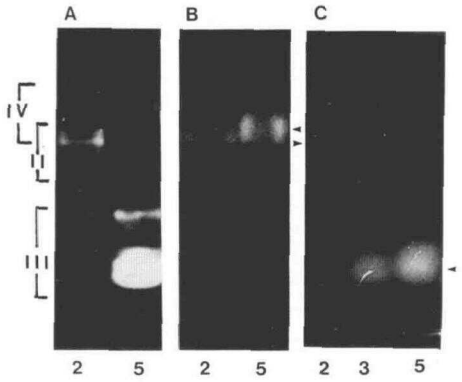

Figure 6. Substrate specificity for the enzymes after native PAGE. Four endosperms from d 2, 3, and 5 after imbibition were extracted and the proteins were separated by native PAGE. The gels were overlaid on the substrate plates that contained 1% (w/v) gelatin (A), 1.5% (w/v) zein (B), or 0.75% (w/v) enriched γ-zein (C). They were incubated at 37°C for 4 h (A) or 18 h (B and C), respectively. Endopeptidase activities were visualized after protein staining (1% amido black 10B for the gelatin plate or 1.5% Coomassie brilliant blue R for these zein plates), followed by destaining. Arrows indicate endopeptidase bands with a high specificity for α-zein (B) or γ-zein (C).

DISCUSSION

Using an activity stain De Barros and Larkins (1990) found four major endopeptidase proteins in crude extracts separated by native gel electrophoresis in a gel containing gelatin as substrate for endopeptidase activity. The gels were incubated in a buffer solution, and the bands digested by proteinases were detected on the gel after protein staining. The major activities, A_3 and A_4, reached maxima 4 and 6 d after imbibition, respectively. Two other activities increased at least until d 10 under their growth conditions (25°C). Because these endopeptidase activities (A_1-A_4) appear relatively late after imbibition and because all are characterized as Cys-endopeptidases, we consider them to be the same as our group III enzymes. When we tested antibodies prepared against A_1 to A_4 Cys-endopeptidases by De Barros and Larkins, they reacted only with our group III enzymes (data not shown). Thus, although the group II and IV activities are Cys-endopeptidases, they are sufficiently different from the group III enzymes not to be recognized by these antibodies. A number of other workers have used a system similar to that of De Barros and Larkins and have also found multiple endopeptidase activities in cereal endosperms.

In the system of De Barrows and Larkins there were no activities similar to enzymes of our group II, which appear at d 2. One possible difference between the extraction procedures is a difference in the concentration of 2-mercaptoethanol. When we reduced the concentration of 2-mercaptoethanol from 10 to 1 mM, as was used by De Barros and Larkins, we found group III, but not group II, enzymes (data not shown).

When the reduction of different zein proteins was analyzed using SDS-PAGE, a sequential disappearance of these zeins was observed during early seedling growth (Fig. 3). γ-Zeins disappeared within the first 2 d after imbibition, and then β- or δ-zein disappeared. Finally, degradation of α-zein was detected. Because the endosperm is not a living tissue, we do not envisage a sequential transport of these endopeptidases into protein bodies. Rather, we think that they are either made sequentially in the aleurone or scutella tissue or are activated from endosperm zymogens.

There are two possible explanations for this sequential disappearance of the zeins: (a) the major endopeptidase(s) can degrade the different types of zein proteins at different rates because of the special distribution of the zeins in protein bodies (for a discussion of this point, see Esen and Stetler, 1992) or (b) there are different sets of endopeptidase(s) that have different specificities for each of the zein-type proteins. The first possibility is supported by many observations at the level of the electron microscope (Burr and Burr, 1976; Larkins et al., 1976; Ludevid et al., 1984; Lending et al., 1988; Lending and Larkins, 1989; Esen and Stetler, 1992). In contrast, our activity-staining patterns (Fig. 4) for endopeptidase activities and the substrate specificity for the various activities (Fig. 6) strongly support the contention that substrate specificity of the various endopeptidases could be at least in part responsible for the sequence in the loss of specific zeins. In our system the group II and III enzymes have a capacity to degrade both γ- and α-zeins. However, the γ-zeins are probably degraded in situ by group II enzymes because the appearance of these activities coincides with the loss of γ-zein. The group IV enzymes, a late-stage group of activities, cleave α-zein but not γ-zein (Fig. 6). Thus, in addition to structural organization, the sequential development of different groups of proteolytic enzymes is probably also required for differential degradation of the zein proteins.

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