

# A Sensitive Diffusion Plate Assay for Screening Inhibitors of Protease Activity in Plant Cell Fractions<sup>1</sup>

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## ABSTRACT

Proteolytic activity was detected, using a sensitive radial diffusion plate assay, in the plasma membrane fractions of corn (*Zea mays* L.) roots and from roots of several other plant species. The proteases could be effectively inhibited in corn with phenylmethane sulfonyl fluoride or chymostatin. Protease activity of oat roots, however, was not significantly reduced by these inhibitors. The results of diffusion plate assay were confirmed with the less sensitive azocasein assay using crude cell homogenates. Chymostatin and phenylmethane sulfonyl fluoride were effective in preventing protease degradation of polypeptides as revealed by electrophoresis. The diffusion plate assay uses a permanent support for a 0.75 millimeter thick agarose slab containing 200 micrograms per milliliter casein. By staining the fixed and dried gel with Coomassie blue R-250, proteolytic activity was visualized as a cleared area around the sample well with a detection limit of about 0.3 nanograms trypsin. The diffusion plate assay should prove useful for screening inhibitors of proteases where limited amounts of material are available, such as with plant cell fractions or highly purified proteins.

During the process of cell fractionation and protein purification, proteolytic enzymes released from the vacuole and present in various organelle fractions can degrade proteins and cause a loss of enzyme activity (2, 12). This endogenous protease activity can also lead to artifacts during SDS-PAGE, such as a general smearing of the protein staining pattern on the gel, a loss of high mol wt polypeptides, and subtle shifts in electrophoretic mobility of specific polypeptides (1, 8, 12, 18).

Proteolytic enzymes are classified into two broad categories; the exopeptidases, or peptidases, which cleave peptide bonds at the N or C terminal positions producing very subtle modifications of the protein that are difficult to detect during protein purification, and the endopeptidases, or proteinases, which cleave internal peptide bonds producing dramatic effects on native protein structure and activity that are readily detected during protein purification (3, 13). In this paper, we will use the term protease to refer to this later category of proteolytic enzyme.

Based on catalytic mechanism, proteases can be separated into various classes which are usually distinguished by the use of specific inhibitors, rather than by activity with a particular substrate. Chemical inhibitors covalently modify a critical amino

acid in the active site of the protease. For example, PMSF<sup>3</sup> alkylates a reactive serine of the serine type of protease (13). However, chemical inhibitors are not always specific, and PMSF will also react with sulfhydryl proteases or with an important sulfhydryl on the enzyme being purified. Competitive inhibitors compete at the active site of the protease and are usually very specific inhibitors (17). The microbial product, chymostatin, is an example of a competitive type of protease inhibitor.

There are several approaches that can be taken to reduce protease activity during plant cell fractionation (12). One of the simplest approaches is to choose a tissue that has a low protease content. Because this is usually not possible, fractionation must be conducted under conditions which limit protease activity. This includes adjusting pH above or below the optimum for protease activity, lowering temperature to near 0°C, controlling the concentration of divalent cation activators of proteases, keeping the total time for fractionation as short as possible, removal of proteases from the homogenate by, for example, affinity chromatography, and/or adding an effective inhibitor of proteases. The addition of an inhibitor is by far the simplest and most effective way to control the activity of proteases. However, the effectiveness of a particular inhibitor in reducing protease activity will depend on the type of protease present in a given plant cell fraction or plant species. It is desirable to have a convenient and sensitive protease assay for screening inhibitors for their effectiveness in reducing protease activity in a particular plant cell fraction or plant species.

In this paper, we describe a sensitive diffusion plate assay for detecting protease activity in small quantities of plant cell fractions. The assay also provides a simple way to screen inhibitors for their effectiveness in reducing protease activity.

## MATERIALS AND METHODS

**Plant Material.** Corn seeds (*Zea mays* L. WF9 × MO17) were germinated on blotter paper saturated with 1 mM CaCl<sub>2</sub> for 3 d in low light at 27°C. The seedlings were then transferred to aeroponic tanks for further growth (6). Seeds of other plant tissues were germinated in vermiculite-filled containers suspended over the aeroponic chamber. After 14 to 21 d, roots were excised, rinsed with cold distilled H<sub>2</sub>O, and excess water removed with the aid of a lettuce drier.

**Preparation of Cell Fractions.** The plasma membrane fraction was isolated as previously described (6). Briefly, the roots were homogenized with a mortar and pestle in 0.25 M sucrose, 3 mM EDTA, 2.5 mM DTT, and 25 mM Tris-Mes (pH 7.7) (2.4 ml medium/g tissue). The filtered homogenate was centrifuged at

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<sup>3</sup>Abbreviations: PMSF, phenylmethane sulfonyl fluoride; TX 114, Triton X-114, octylphenoxy polyethoxyethanol; %T, % w/v of total monomer (acrylamide and *N,N'*-methylenebis acrylamide).

13,000g for 20 min, followed by 80,000g for 30 min. The 13,000 to 80,000g pellet was suspended in 10 ml of suspension buffer (0.25 M sucrose, 1 mM DTT, and 1 mM Tris-Mes [pH 7.2]), and applied to four 36 ml-34/45% (w/w, 8/28 ml, respectively) sucrose step gradients buffered with 1 mM Tris-Mes (pH 7.2) in 1 mM DTT. After centrifugation at 82,500g for 2 h, the plasma membrane fraction was collected at the 34/45% sucrose interface, diluted with suspension buffer, and centrifuged at 80,000g for 1 h. The final pellet was adjusted to 15 to 20 mg protein/ml with suspension buffer and stored in 0.25 ml aliquots under liquid N<sub>2</sub>.

For preparation of a homogenate fraction, the roots were homogenized as described above, but with 1 ml homogenization medium/g tissue, and the homogenate was filtered through four layers of cheese cloth. The homogenate was used without further treatment.

**Azocasein Assay.** The method of Shannon and Wallace (15) was modified as follows. In a microfuge tube, 0.3 ml of homogenate was added to 0.33 ml of 0.3 M Tris-Mes (pH 7.5), 0.07 ml distilled H<sub>2</sub>O and 0.1 ml of stock inhibitor solution to achieve the stated concentrations. After a 30-min incubation at room temperature, the assay was started by addition of 0.2 ml azocasein stock (10 mg/ml). The assay was allowed to proceed for 2 h at room temperature before termination by the addition of 0.1 ml protein precipitant (3 M acetic acid, 1 M TCA, and 2 M sodium acetate) placed on ice for 10 min and then centrifuged for 3 min in a Beckman Microfuge. Next, 0.5 ml aliquots of the supernatant solution were placed in a spectrophotometer tube, followed by 0.1 ml of 10 M NaOH and 2.4 ml water. After mixing, the absorbance was read at 440 nm. One unit of activity was defined as the amount of homogenate needed to cause an increase of 1.0 A<sub>440</sub> unit per min at room temperature.

**Diffusion Plate Assay.** This procedure is a radial diffusion technique similar to one previously described (4, 5, 10, 12), but the procedure has been modified for analysis of nonspecific proteases. Glass plates were cut to fit Gel-Fix sheets for agarose (12.5 × 12.5 cm; Serva, Garden City Park, NY). The glass plates were cleaned with hot soapy water and then rinsed with 95% methanol. A small amount of water was applied to the glass plate to provide good contact between the Gel-Fix sheet and the surface of the glass. The Gel-Fix sheet was aligned with the edges of the glass plate and the excess water squeezed out by rolling a brayer (*i.e.* ink roller) several times over the surface of the Gel-Fix sheet while blotting the edges with tissue paper. Teflon spacers (0.75 mm thick by 0.5 cm wide and 13 cm long) were positioned on two opposite edges of the Gel-Fix sheet, and the mold was assembled by clamping a second, identical, glass plate on top of the spacers with edges flush with the lower glass plate. The mold was heated to 70°C in an oven.

A solution containing 1% agarose (Serva Standard EEO), 200 µg/ml boiled casein (Sigma, sodium salt), and 0.1 M Tris-Mes (pH 7.5) was heated to melting (approximately 90°C) and then cooled to 70°C. The solution was injected (20 ml syringe) into the diffusion plate mold with care taken to avoid air bubbles. The mold was tilted slightly (10° from horizontal) to allow the agarose to flow smoothly between the plates. Agarose was held in the mold by capillary action.

After filling, the mold was laid flat and the agarose allowed to solidify at room temperature, and then was incubated at 4°C in a refrigerator. The chilled mold was dismantled by removing the clamps, spacers, and the top plate was removed by carefully sliding the plate over the surface of the agarose (simply pulling the plates apart dislodged the agarose). For all manipulations, excluding fixation and staining, the Gel-Fix sheet was left on the lower glass plate.

Wells were cut in the agarose by using a 3 mm (*i.d.*) blunt end syringe needle. The end of the needle was pushed into the agarose,

carefully removed, and the remaining agarose disc aspirated with a pasteur pipette. A paper template, with appropriately spaced marks, was placed under the glass plate with the Gel-Fix as a guide for punching holes.

Aliquots (2.5 µl) of either homogenate prepared as stipulated above, or plasma membrane fraction diluted to 1 to 2 µg protein/µl with suspension medium were added to each well. Inhibitors were mixed with the sample prior to application. Trypsin (302 units/mg, Millipore Corp.) was prepared in 1 mM H<sub>2</sub>SO<sub>4</sub> and then diluted into 0.1 M Tris-Mes (pH 7.5) buffer immediately prior to application to the diffusion plate. Generally, the protease diffusion plates were allowed to develop 24 h in a water-saturated chamber at room temperature.

Before staining the plate, the unhydrolyzed protein substrate was fixed in place while allowing the hydrolyzed protein to diffuse out of the agar. All fixation and staining steps were performed with gentle shaking. The diffusion plate was fixed by immersion in 400 ml of 10% TCA for 10 min in a plastic freezer storage container. The TCA was replaced with 400 ml 95% ethanol for 10 min to dehydrate the agarose. The fixed and dehydrated agarose gel was covered with alcohol-saturated Whatman No. 1 filter paper, inverted, and placed on several layers of filter paper with the Gel-Fix sheet facing up. A glass plate was removed and the agarose side of the Gel-Fix was peeled off the filter paper. The flattened but still damp agarose was dried onto the Gel-Fix sheet with hot air from a hair drier.

The dried diffusion plate was stained by placing in 100 ml of 0.025% Coomassie blue R-250, 40% methanol, and 7% acetic acid until a sufficient background was evident (10–20 min). The plate was destained in 100 ml of 40% methanol and 7% acetic acid until the diffusion spots were clearly visible. The plate was transferred to 5% methanol, 7% acetic acid for several hours to complete the destaining. The gel surface was carefully wiped with cotton saturated with the final destain solution and then dried with a hot air dryer.

**SDS-PAGE.** SDS-PAGE was performed essentially as described (8, 9). Sample treatments are described in the Figure legends.

**Proteins.** Proteins were determined by the method of Lowry as modified by Peterson (11).

## RESULTS

A diffusion plate assay was developed which detects the activity of proteases as a clearing of Coomassie blue positive protein (casein) around a sample well which was cut in agarose. The agarose was mixed with the protein substrate and poured as a thin film on a plastic Gel-Fix sheet. The sample to be tested, along with any additions, was added to the sample well and allowed to diffuse through the agarose to hydrolyze protein. Ease of handling was also greatly improved by the use of a thin (0.75 mm) agarose film on the plastic support. The agarose was dried onto the plastic support to provide a permanent record that was easily stored.

To compare the sensitivity of the Coomassie blue stained diffusion plates with that of previous methods that used protein precipitation to visualize the zones of hydrolysis (4, 5, 10, 14), a diffusion plate was prepared assaying 0.3 ng to 2.5 µg purified trypsin (Fig. 1). Between 39 ng and 2.5 µg trypsin, the diameter of the cleared area around the well shows a linear relationship with the logarithm of the amount of trypsin in each well (Fig. 1). A similar relationship has been reported using the protein precipitation procedure (4, 5, 10, 14). Below 39 ng, the standard curve deviates from linearity making quantitative interpretations of such low levels of proteolytic activity difficult even though a proteolytic clearing was evident around the well (Fig. 1).

The diffusion plate assay shown in Figure 2 indicates that protease activity was detected with small amounts (5 µg of

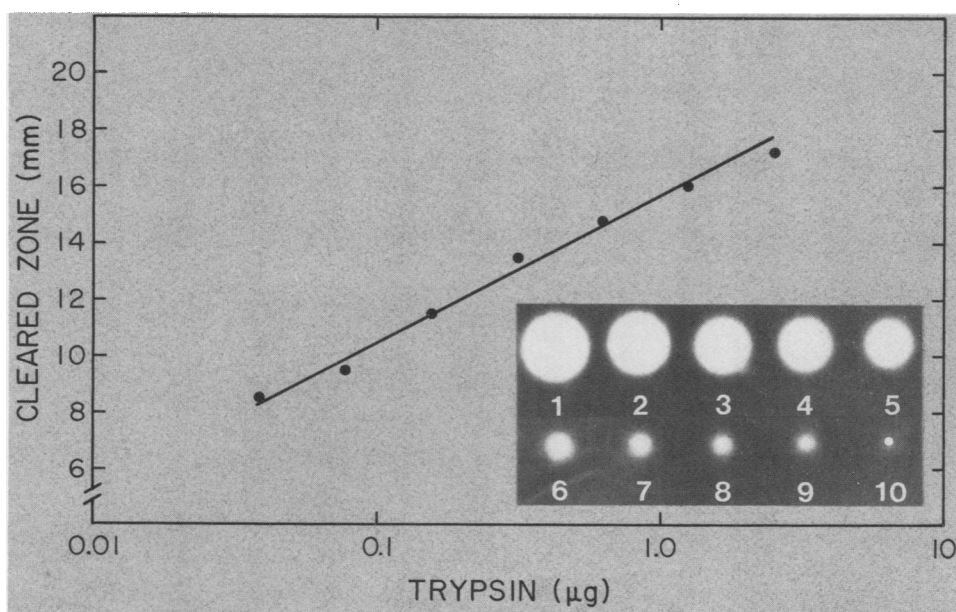


FIG. 1. Relationship between amount of trypsin and size of cleared zone in the casein diffusion plate assay. Each point represents the average zone diameter from duplicate diffusion plates. Inset shows the appearance of the diffusion plate treated with trypsin at the following concentrations. Well 1, 2500 ng; 2, 1250 ng; 3, 625 ng; 4, 313 ng; 5, 156 ng; 6, 2.44 ng; 7, 1.22 ng; 8, 0.61 ng; 9, 0.31 ng; 10, none.

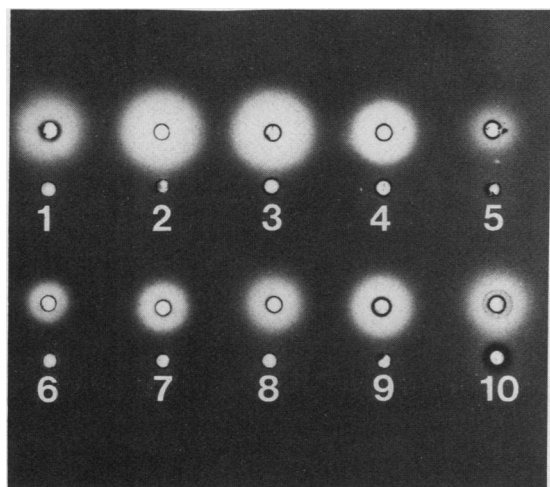


FIG. 2. Casein diffusion plate assay for proteases in plasma membrane fractions from roots of several plant species. The wells contained 5  $\mu$ g of protein from the following plasma membrane preparations: 1, corn (*Zea mays*); 2, tomato (*Lycopersicon cheesmanii*); 3, tomato (*L. esculentum*); 4, oat (*Avena sativa*); 5, *Amaranthus* species; 6, barley (*Hordeum vulgare* cv Arivat); 7, rye (*Triticum aestivum*); 8, barley (*H. vulgare* cv CM-72); 9, barley (*H. vulgare* cv Briggs); 10, *Limonium taratica*. Lower wells contained boiled (3 min) samples of the respective plasma membrane preparations.

protein) of plasma membrane fractions from corn, oat, rye, *Limonium*, *Amaranthus*, two species of tomato, and three strains of barley. Various protease inhibitors were screened using the radial diffusion plate assay for these plant species. The results for the plasma membrane fractions from corn and oat roots are shown in Figure 3. Of the 12 inhibitors tested, only PMSF and chymostatin were found to be effective against proteases in the corn root plasma membrane fraction, and it appeared that chymostatin was a more potent inhibitor than PMSF (Fig. 3A). None of the inhibitors significantly reduced protease activity in the plasma membrane fraction from oat roots (Fig. 2B). However, PMSF or chymostatin inhibited protease activity in plasma membrane preparations of the other plant species listed above (not shown). Diffusion plate analysis of the corn root protease associated with both the homogenate and plasma membrane

fractions showed that  $\text{HgCl}_2$  (not shown) and  $\text{CuCl}_2$  (Fig. 4) were also inhibitors.

Quantitation of inhibition of homogenate proteolytic activity by  $\text{CuCl}_2$ , PMSF, and chymostatin using the diffusion plate (Fig. 4) indicates that complete inhibition was achieved by 0.1 M  $\text{CuCl}_2$ , 10 mM PMSF, and 10  $\mu$ g/ml chymostatin. This compares with 5  $\mu$ g/ml chymostatin and 1 mM PMSF based on the azocasein assay discussed below and indicates the diffusion plate assay can be used as a semiquantitative assay for protease inhibition.

The homogenate from corn roots had the same inhibitor specificity as that observed for the plasma membrane fraction. Because large quantities of protein are required, the homogenate was used to verify the diffusion plate assay using the more traditional and quantitative azocasein assay (15). Chymostatin ( $I_{50} = 0.5$   $\mu$ g/ml) and PMSF ( $I_{50} = 0.06$  mM) were potent inhibitors of protease activity in corn root homogenates (Fig. 5). Leupeptin, which was not an effective inhibitor of corn root protease as judged by the diffusion plate assay, was likewise not effective based on the azocasein assay. The results demonstrate that the diffusion plate assay gives qualitatively identical results to the azocasein assay.

An experiment was conducted with the plasma membrane fraction (Fig. 6) and the TX 114 extracted plasma membrane fraction (Fig. 7) from corn roots to determine if protease inhibitors shown to be effective in the diffusion plate assay would also be effective in reducing alterations in polypeptides separated by SDS-PAGE. Proteases are active in SDS and can markedly hydrolyze proteins because of increased availability of cleavage sites produced by SDS treatment (12, 18). Incubation of the plasma membrane fraction in SDS-PAGE solubilization buffer for 90 min prior to electrophoresis produced the expected loss of polypeptide bands as compared to the boiled control (Fig. 6, lanes 2 and 7). Inclusion of PMSF in the solubilization buffer did not reduce degradation of polypeptides (Fig. 6, lane 3), while chymostatin was an effective inhibitor under these conditions (Fig. 6, lane 4). When both PMSF and chymostatin were included in the solubilization buffer, further protection against protease degradation was observed (Fig. 6, lane 5). These results are in agreement with those with the diffusion plate assay which indicated that, for corn root proteases, chymostatin was a more effective inhibitor than PMSF (Fig. 3A).

The SDS-PAGE gel in Figure 6 had poor resolution even in

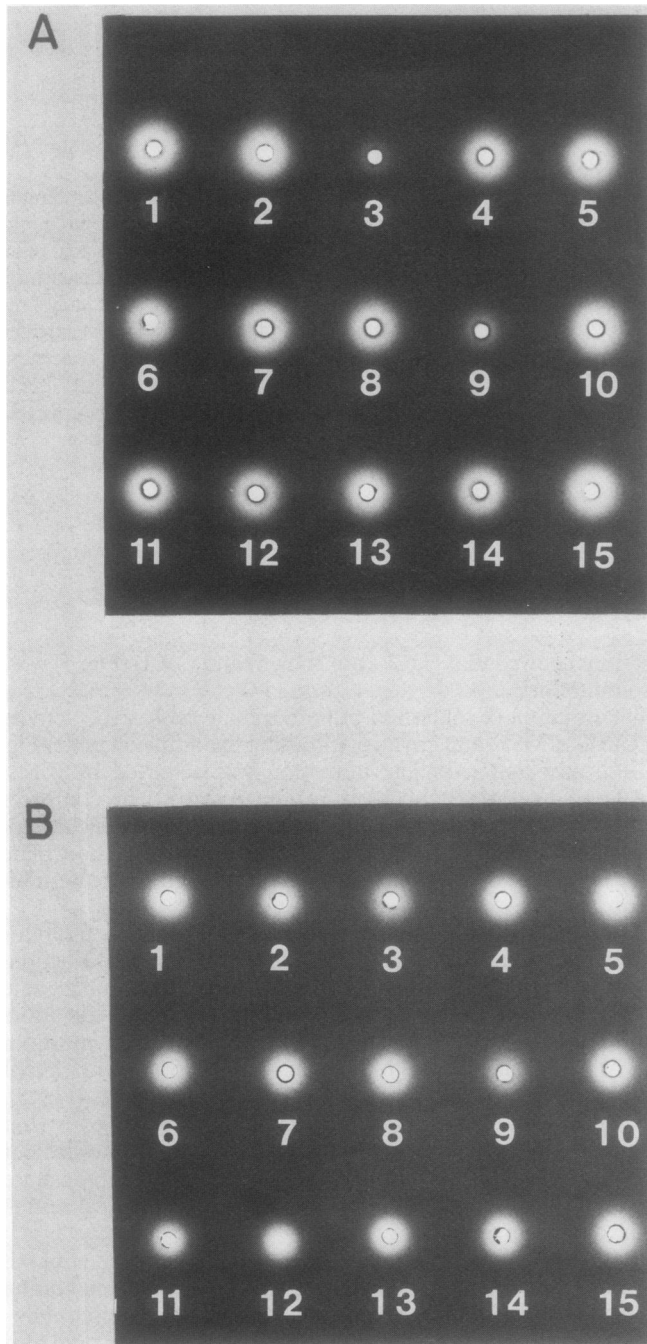


FIG. 3. Casein diffusion plate assay for proteases associated with the plasma membrane fractions from corn (A) and oat (B) roots in the presence of various protease inhibitors. The  $2.5 \mu\text{l}$  membrane samples containing  $5 \mu\text{g}$  protein were mixed with  $2.5 \mu\text{l}$  inhibitor to give an inhibitor concentration of  $200 \mu\text{g/ml}$  (2–8) and  $10 \text{ mM}$  (9–13): 1, water control; 2, antipain; 3, chymostatin; 4, elastinal; 5, leupeptin; 6, phosphoramidon; 7, pepstatin; 8, aprotinin; 9, PMSF; 10, *N*-tosyl-L-lysine chloromethyl ketone; 11, *N*-tosyl-L-phenylalanine chloromethyl ketone; 12, *N*-CBZ-L-phenylalanine chloromethyl ketone; 13, benzamidine; 14, DMSO control; 15, ethanol control.

the boiled control (lane 7). Polypeptides in the plasma membrane fraction are not well resolved by SDS-PAGE because of protease degradation during cell fractionation and because of heat-induced aggregation (7). The TX 114 extracted plasma membrane fraction from corn roots was used to determine if PMSF and chymostatin were effective in preventing subtle changes in mol

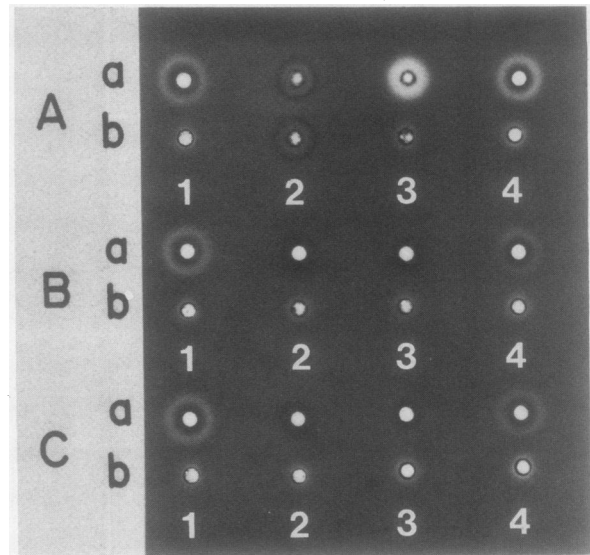


FIG. 4. Casein diffusion plate assay for proteases in corn root homogenates in the presence of  $\text{CuCl}_2$ , PMSF, and chymostatin. (a) Contains inhibitors and (b) represents boiled controls. Homogenate was mixed with inhibitors to give the following concentrations for lanes 2 to 4 (lane 1 is the untreated control), respectively; (A)  $0.1 \text{ M}$ ,  $0.01 \text{ M}$ ,  $0.001 \text{ M}$   $\text{CuCl}_2$ ; (B)  $0.1$ ,  $0.01$ ,  $0.001 \text{ M}$  PMSF; (C)  $100$ ,  $10$ ,  $1 \mu\text{g/ml}$  chymostatin. Plate was developed for 4 h.

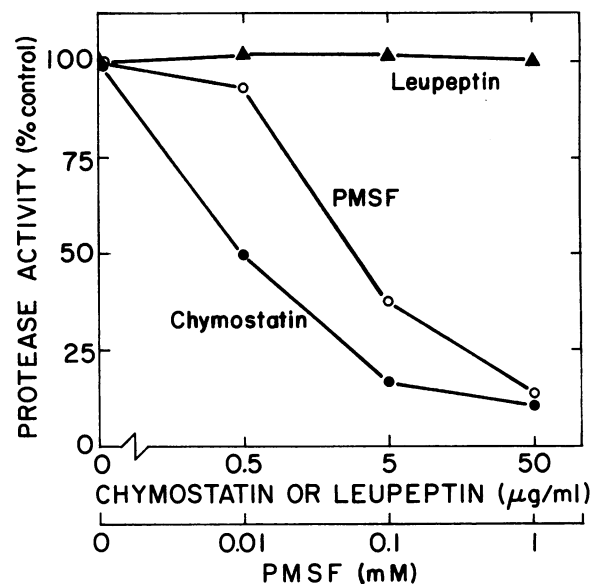


FIG. 5. Azocasein assay for protease activity in corn root homogenates. Chymostatin and PMSF assays contained  $0.5\%$  DMSO and  $1\%$  ethanol, respectively, in the  $1 \text{ ml}$  assay volume. Control values (–background) were  $0.216$ ,  $0.235$ , and  $0.232$  absorbance units per 2 h assay for leupeptin, chymostatin, and PMSF, respectively.

wt of major polypeptides because this fraction has prominent polypeptides that are readily resolved by SDS-PAGE (Fig. 7). Preparation of the plasma membrane fraction and extraction with TX 114 without addition of PMSF and chymostatin resulted in a marked loss of resolution (e.g.  $43$  and  $24 \text{ kD}$  polypeptides appear more diffuse) and a shift in the apparent molecular mass of several of the major polypeptides. For example, two polypeptide bands were observed in the  $100,000 \text{ D}$  region of the gel when inhibitors of protease activity were not included at any stage of preparation of the plasma membrane fraction for SDS-PAGE (Fig. 7, lanes 3 and 4). However, when PMSF and chymostatin

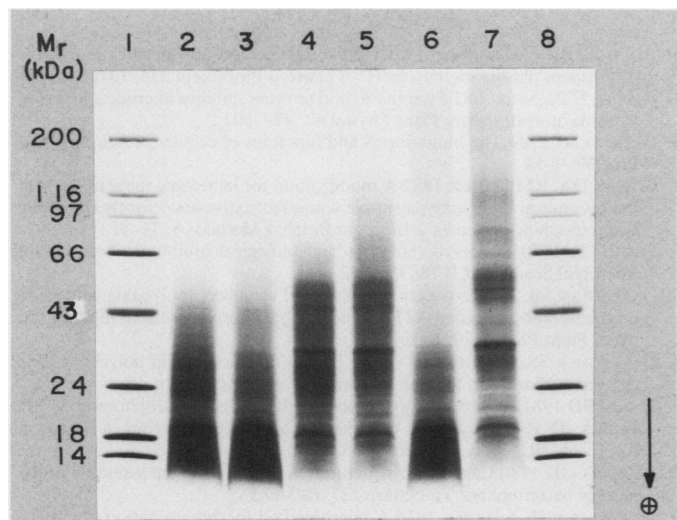


FIG. 6. Effect of PMSF and chymostatin on protease activity during solubilization of the plasma membrane fraction from corn roots in SDS-PAGE buffer. The plasma membrane fraction was isolated in the absence of protease inhibitors and 60  $\mu\text{g}$  of protein was applied to lanes 2 to 7. For lanes 2 to 6, the sample was mixed with 5% SDS-PAGE solubilization buffer in the absence or presence of protease inhibitors and incubated for 90 min at 38°C. For lane 7, the sample was mixed with SDS solubilization buffer, immediately boiled for 3 min, and then incubated for 90 min at 38°C. Lane 2 was incubated without inhibitor; lane 3, plus 5 mM PMSF; lane 4, plus 500  $\mu\text{g}/\text{ml}$  chymostatin; lane 5, plus 5 mM PMSF and 500  $\mu\text{g}/\text{ml}$  chymostatin; lane 6, boiled for 3 min after incubation without protease inhibitor. Lanes 1 and 8 contained boiled and reduced mol wt standards which were: myosin (200 kD), B-galactosidase (116 kD), phosphorylase a (97 kD), BSA (66 kD), ovalbumin (43 kD), trypsinogen (24 kD), B-lactoglobulin (18 kD), and lysozyme (14 kD). All samples were reduced by the addition of 0.2 M DTT to the solubilization buffer. The resolving gel consisted of a 5.1 to 20.5% T gradient of polyacrylamide.

were included during isolation and preparation for electrophoresis, the lower band in this region was markedly reduced in intensity, suggesting that it resulted in part from protease degradation of the upper band (Fig. 7, lanes 7 and 8). Inclusion of protease inhibitors and chemical reduction of the sample resulted in the best resolution of the polypeptides in SDS-PAGE (Fig. 7, lane 7). The inhibitors which were effective against protease activity in the diffusion plate assay were also effective in preventing protease degradation during cell fractionation.

## DISCUSSION

The results of this study demonstrate the utility of a sensitive diffusion plate assay for detecting the presence of proteases in small quantities of plant cell fractions and for determining the effectiveness of substances which are presumed to be inhibitors of the protease activity (Figs. 2-4). Results with inhibitors in the diffusion plate assay were confirmed by the more traditional azocasein assay (Fig. 5) and by prevention of polypeptide degradation as detected by SDS-PAGE (Figs. 6 and 7).

The limit of detection of the Coomassie blue stained diffusion plate was about 0.3 ng trypsin. This compares with a 2.4 ng (5) to a 10 ng (14) limit of detection for trypsin using standard diffusion plate assays that depend on protein precipitation. Sensitivity of the casein precipitation method to thermostable proteases can be increased to 0.3 ng trypsin through use of elevated temperatures (>45°C; 4). A combination of high temperatures and Coomassie blue staining may further increase the sensitivity of the diffusion plate assay presented here, but this variation was

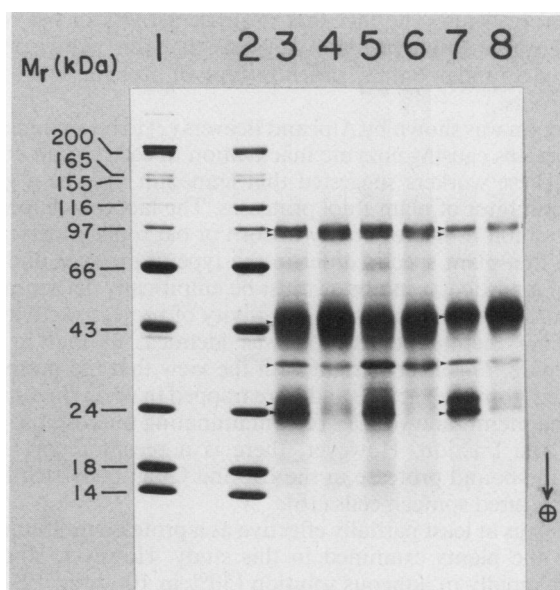


FIG. 7. Effect of protease inhibitors on SDS-PAGE of a detergent extracted plasma membrane fraction from corn roots. The plasma membrane fractions were extracted with 1% (w/v) Triton X-114 (TX 114) and centrifuged at 100,000g for 30 min. The pellet was suspended in 10 mM imidazole, 2 mM EDTA, 10% (w/v) glycerol, and 1 mM DTT (pH 7.5) and centrifuged again to obtain the membrane preparation used for electrophoresis. The sample applied to lane 2 was boiled for 3 min before application, while those in lanes 1, and 3 to 8 were not boiled. The samples applied to lanes 1, 2, 3, 5 and 7 were chemically reduced by addition of 0.2 M DTT prior to electrophoresis, while those applied to the other lanes were not reduced. Lanes 1 and 2 contained the mol wt standards described in Figure 6, except for the addition of RNA polymerase (155 and 165 kD). For lanes 3 and 4, the plasma membrane fraction was isolated and extracted with TX 114 without addition of protease inhibitors. For lanes 5 and 6, 25  $\mu\text{g}/\text{ml}$  chymostatin and 1 mM PMSF were added only during TX 114 extraction. For lanes 7 and 8, chymostatin and PMSF were included during the isolation of the plasma membrane fraction and during TX 114 extraction. The SDS sample buffer contained 500  $\mu\text{g}/\text{ml}$  chymostatin and 1 mM PMSF in all samples. The resolving gel consisted of a 5.1 to 20.5% T gradient of polyacrylamide. Approximately 50  $\mu\text{g}$  of protein from the plasma membrane preparation was applied per lane.

not tried.

Chymostatin, and to a lesser extent PMSF, was a potent inhibitor of proteases in cell fractions from corn roots. However, these inhibitors were not uniformly effective against proteases of all the plant species studied. Hence, it is essential to screen inhibitors for their ability to reduce protease activity to justify their inclusion during plant cell fractionation. The diffusion plate assay described in this report should be useful for that purpose.

For corn roots, inhibition by chymostatin and PMSF, both potent inhibitors of chymotrypsin, indicates the presence of a protease with an important serine at the active site. However, chymostatin and PMSF selectivity is not absolute within types of proteases because papain (a nonserrine type) is also sensitive to these inhibitors (12, 17). Yeast proteinase B, like corn root protease, is inhibited by PMSF, Cu, and chymostatin which is consistent with the conclusion that the predominant protease in corn roots has a similar serine type mechanism of action (12). Shannon and Wallace (15) reported that a corn root protease (proteinase I) had similar properties to yeast proteinase B and to alkaline proteinase of *Neurospora crassa*. All three enzymes had neutral to alkaline pH optima and were sensitive to PMSF, although the corn root proteinase I was relatively insensitive to



mercurial reagents. The fact that protease activity of oat roots was insensitive to chymostatin suggests that this plant species may have a fundamentally different type of protease from that in corn roots.

Leupeptin was shown by Alpi and Beevers (2) to be an inhibitor of proteinases causing enzyme inactivation in castor bean endosperm. These workers suggested that leupeptin may be a good general inhibitor of plant thiol proteases. The fact that leupeptin did not inhibit protease activity in corn or oat roots underscores the idea that plant species differ in the type of protease and the utility of a particular inhibitor must be empirically determined.

For corn roots, the inhibitor sensitivity of protease activity in the plasma membrane fraction was identical to that in the homogenate. This is consistent with the view that the proteases originated from the vacuole and were trapped in or on the surface of plasma membrane vesicles or contaminating microsomal vesicles in the fraction. However, there is a recent report of a membrane-bound protease in the ER and Golgi body-rich fraction of cultured spinach cells (16).

PMSF was at least partially effective as a protease inhibitor for most of the plants examined in this study. However, since it degrades rapidly in aqueous solution (50% in 100 min; 12) and can inhibit any enzyme that has an important serine or sulfhydryl group, its use should be carefully evaluated. In contrast, chymostatin is specific for proteases and it is relatively stable in aqueous solution (17). However, the high cost of microbially derived products like chymostatin may restrict their use in cell fractionation procedures that involve large volumes. For corn roots, we suggest the use of PMSF during homogenization, and then chymostatin during the later stages of cell fractionation which involve small volumes to overcome the disadvantages of both inhibitors.

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