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Purification and Characterization of Ethylene Inducing Proteins from Cellulysin

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YORAM FUCHS[1] AND JAMES D. ANDERSON*
United States Department of Agriculture, Agricultural Research Service, Plant Hormone Laboratory, Beltsville Agricultural Research Center-West, Beltsville, Maryland 20705

ABSTRACT

Ethylene inducing proteins were partially purified and characterized from the cell wall digesting enzyme mixture, Cellulysin. Purification included binding to Sephacryl S-200, isoelectric focusing, molecular sieving on Sephadex G-75, agarose electrophoresis, and sizing using a Superose 12 column. At least three active proteins were obtained from the Sephadex G-75 fraction that move towards the cathode during nondenaturing agarose electrophoresis. These three protein fractions separated by preparative agarose electrophoresis contain polypeptide patterns that are very similar on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fractions contain three main Coomassie blue stained bands of about 10, 14, and 18 kilodaltons. Gel filtration of the major fraction on a Superose 12 column yields an active peak with an apparent molecular weight of 27,000. Proteolytic enzymes, in the presence of urea, destroy the ethylene inducing activity. We conclude that the ethylene inducing factor (EIF) that we have isolated from Cellulysin is protein. Similar ethylene inducing factors are present in Cellulase RS. Ethylene inducing components from pectinase, Pectolyase, and Rhozyme do not bind to Sephacryl like EIF from Cellulysin. Thus, the components responsible for the ethylene inducing activity in these latter enzyme preparations differ from that of EIF.

It has been reported in recent years that freshly prepared protoplasts produce ethylene (3, 6, 11, 12) and that some cell wall degrading enzymes, commonly used for protoplast isolation, are probably responsible for inducing ethylene production (1, 2, 4). An EIF[2] was partially purified from Cellulysin[3] using methods of membrane-ultrafiltration, gel filtration, and isoelectric focusing (1, 2). It has been shown that EIF has many characteristics of proteins in that it is heat labile, sensitive to low concentrations of SDS, and moves and behaves as a protein in various chromatographic and electrophoretic systems. However, it is quite stable to protease attack. In this report we describe our studies on further purification and characterization of this factor, showing its proteinaceous nature and its sensitivity to protease activity in the presence of urea.

[1] On leave from the Department of Fruit and Vegetable Storage, Institute for Technology and Storage of Agricultural Products, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel.

[2] Abbreviations: EIF, ethylene inducing factor from Cellulysin.

[3] The mention of specific instruments, trade names, or manufacturers is for the purpose of identification and does not imply any endorsement by the United States Government.

MATERIALS AND METHODS

Cellulysin was obtained from Calbiochem; cellulase "Onozuka" RS from Yakult Pharmaceutical Industry Co., LTD; Cellulase Grade PB from Worthington; antibotics, purified pectinase, β-chymotypsin, trypsin, and papain from Sigma; Rhozyme from Rohm and Hass; Pectolyase from Kyowa Hakho Kogyo Co., LTD; protease of *Staphylococcus aureus* V_8 from Miles Laboratories; Sephadex, Sepharyl S-200, and the Superose 12 column was from Pharmacia; protein mol wt markers and agarose from Bethesda Research Laboratories (BRL), ampholines from LKB, and other electrophoretic reagents from BioRad. Tobacco (*Nicotiana tabacum* L. cv Xanthi) leaf discs (1 cm in diameter) used for induction of ethylene biosynthesis were obtained from leaves which were pretreated for 16 h with 100 μl/L ethylene (7). Six discs were incubated, for 4 h, in 25 ml Erlenmeyer flasks with 1 ml basal medium containing 700 mM sorbitol, 10 mM Mes-NaOH (pH 6.0), 10 mM $CaCl_2$, 50 μg/ml streptomycin sulfate, and 50 units/ml penicillin together with the sample to be bioassayed. Ethylene was quantified by gas chromatography.

Purification Techniques. Desalting and concentrating were carried out using Amicon PM-10 membranes, Centricon 10 microconcentrators, Sephadex G-25 minicolumns (6-ml disposable syringes), or dialysis tubing. The procedure used for purification of EIF is depicted in Figure 1. Sephacryl S-200 was used as an affinity support to adsorb and purify the ethylene inducing factor. Desalted Cellulysin (50–75 mg protein) in water was applied to Sephacryl S-200 columns (14 × 2.4 cm). The bulk of the protein, which was not retained, was eluted with H_2O, collected, and saved. After extensive washing with water, the column was washed with 1 M NaCl. The salt fraction was previously shown to contain the EIF (1). The salt washes from about 20 columns were combined for further purification (Fig. 1). All $(NH_4)_2SO_4$ precipitations were done at 90% saturation. After dialysis to remove ammonium sulfate, the EIF was purified using preparative isoelectric focusing column electrophoresis (pH 3.0–10.0), as described by Baker (5). The active fractions, which were found in the 6.7 to 9.8 pH range, were combined and concentrated by $(NH_4)_2SO_4$ precipitation for separation on Sephadex G-75 columns equilibrated with 0.5 M NaCl in 10 mM Mes-NaOH (pH 6.0). The active fractions which were eluted near the total column bed volume were combined, concentrated, and used for agarose electrophoresis. A horizontal mini-gel (7.5 × 4.5 cm) composed of 1% agarose in 20 ml 0.05 M Mes-NaOH buffer was used. Samples were applied in the middle of the agarose gel between the anode and cathode; the gel was run at 40 mamp. In some experiments gels were sliced and placed in bioassay media to determine the location of the active components. After it was determined how the active components migrated in min-gels, a preparative 1% agarose gel (7 × 1 cm) was used for continuous elution (BRL) of electrophoretically sepa-

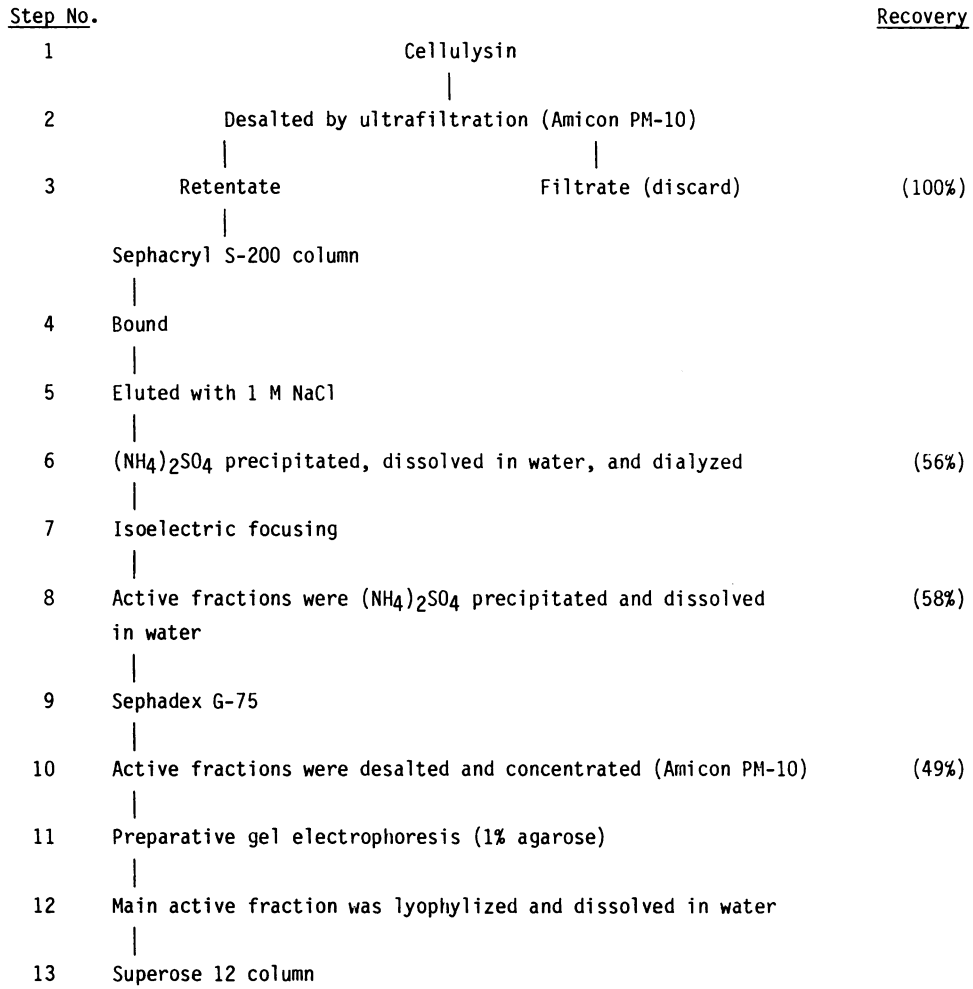

FIG. 1. Purification scheme of EIF from Cellulysin. Numbers in parentheses represent the percentage of ethylene inducing activity recovered at each step.

rated samples. One mg of protein obtained in step 10 of the purification scheme (Fig. 1) was used in each preparative run. Fractions (1 ml) were collected by continuously eluting the base of the column. A constant current of 7.5 mamp was applied using 0.05 M Mes-NaOH buffer (pH 6.0) in both upper and lower tanks as well as for elution. The major active fraction which contained at least two active components, was concentrated by freeze drying and applied to a Superose 12 column. This sizing column was eluted with 50 mM Mes-NaOH buffer at pH 6.0 containing 0.15 M NaCl at a pressure of 150 p.s.i. Fractions of 0.5 ml were collected.

Steps 2, 6, 7, 8, 10, and 13 of the purification scheme were carried out at 2 to 4°C while all other steps were done at 22°C. The EIF is quite stable at room temperature and can be frozen and thawed many times without significant loss of activity.

Analytical SDS-PAGE Electrophoresis. Gradient SDS-PAGE (12–20%) was run at 25 mamp per gel in a Protean II cell (Bio-Rad). Gels were stained with Coomassie blue after electrophoresis. In some experiments, gels were also silver stained (13). Mol wt markers were run with each gel.

Comparison of Other Enzymes. Mini-columns (6 ml in volume) of Sephacryl S-200 were used to determine if the ethylene inducing component of other enzyme mixtures known to induce ethylene (1) would behave similarly to those in Cellulysin. Two fractions from each enzyme were saved (*i.e.* the material that did not bind and the material that bound to Sephacryl S-200 and were subsequently eluted with 1 M NaCl). Individual fractions were then bioassayed.

Samples of each of the enzyme mixtures used in the Sephacryl minicolumns were also subjected to electrophoresis on 1% agar to determine if all contained a similar protein that moved towards the cathode. After electrophoresis the gels were stained with Coomassie blue.

Protease and Urea Treatments. The active fraction after Sephadex G-75 filtration (35 μg) was used in these studies. Urea (1 mg/μl) was dissolved in EIF (5 μg/ml). Proteases (15–30 μg in 5 μl) were incubated with 10 μl EIF ± urea for 17 h at 30°C. After incubation, reaction mixtures were sampled and bioassayed for ethylene inducing activity.

RESULTS AND DISCUSSION

The EIF from Cellulysin has a high affinity for Sephacryl S-200 (1). This affinity seems to be ionic because salt was able to elute it once bound. The EIF is precipitated with $(NH_4)_2$ SO_4 and migrates in an isoelectric focusing column as a basic protein with an apparent mol wt of 27,000 as determined by Sephadex G-75 and Superose 12 column chromatography. We consider the mol wt of the native protein as tentative because it might be interacting with the Superose 12 column material. This would tend to slow the migration of the protein through the column, increasing its elution volume, and decreasing the apparent mol wt estimate. We have tried to counteract this possibility by including 0.15 M NaCl in the eluting buffer. In the methods employed to purify EIF, approximately 50% of the original activity is accounted for after Sephadex G-75 fractionation. The recovery of activity after agarose electrophoresis and Superose 12 chromatography was at least 80%. The determination of precise recovery is difficult because of bioassay differences. Most of the ethylene inducing activity was lost in the initial Sephacryl

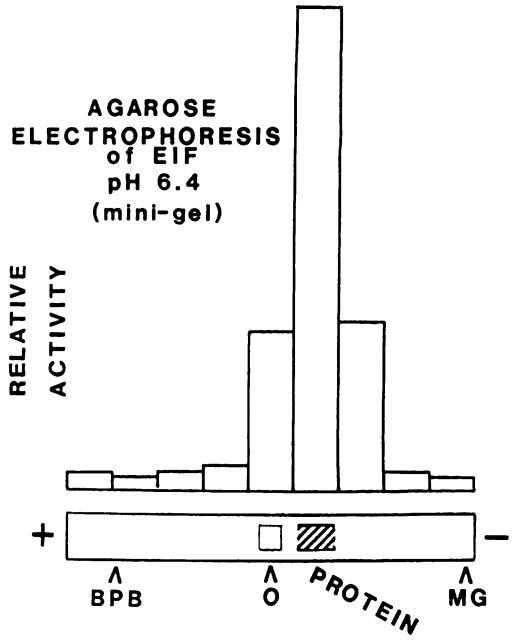

FIG. 2. Electrophoresis and subsequent bioassay of EIF in 1% agarose (pH 6.0); 50 μg protein were applied per well. BPB, Bromophenol blue (negatively charged); O, origin; MG, methylgreen (positively charged).

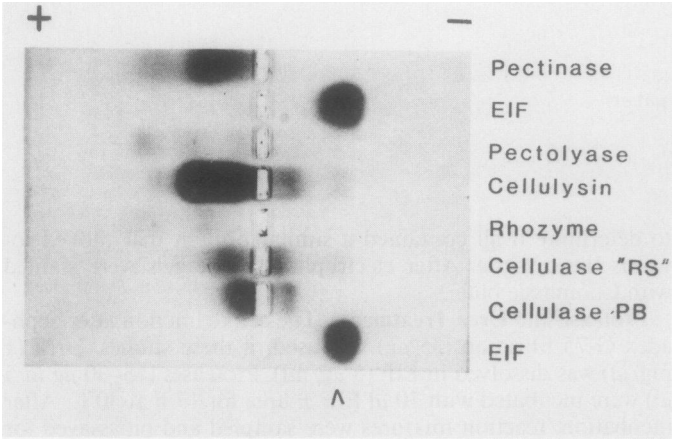

FIG. 3. Electrophoretic patterns of hydrolytic enzyme mixtures and partially purified EIF separated in 1% agarose at pH 6.0 (Mes 0.05 M). The gel was stained with Coomassie blue. The + and − represent the anode and cathode, respectively. The Λ indicates area the EIF migrates to.

step. Some of this could have been due to material not binding to Sephacryl. It is also possible that some EIF was not removed from Sephacryl S-200 with NaCl. In some experiments we reapplied the unbound Cellulysin protein and found that 1 M NaCl did not remove further EIF activity. The ethylene inducing activity that remains in Cellulysin after passing through Sephacryl S-200 is probably structurally different than the fraction we were working with.

Electrophoresis, in 1% agarose at pH 6.0, and subsequent bioassay of the protein showed that the biological activity migrated towards the cathode (Fig. 2). Similar gels were run with this same active fraction (step 10), together with six different commercial hydrolytic enzyme preparations used to produce protoplasts (Fig. 3). It can be seen that Cellulysin and Cellulase RS, which were reported to have ethylene-inducing activity (1), have a protein band similar to that of purified protein from step 10. No protein was detected in this region for Cellulase PB, which was reported to lack the activity. Very little if any detectable protein moves to the same position as EIF for pectinase, Pectolyase, and Rhozyme. These later enzyme mixtures do induce ethylene (1); however, the ethylene inducing component(s) did not bind to Sephacryl S-200 as does EIF (Table I). Instead, the activity passes through. The migration towards the cathode and the wide range of activity between pH 6.7 and 9.8, upon isoelectric focusing suggested that Cellulysin has several active proteins containing a relatively high percentage of basic amino acids or is a protein that is highly modified. The protein after Superose 12 column chromatography (step 13) is not a lectin (did not coagulate red blood cells) (9) and is devoid of carbohydrate (as determined by a modified periodic acid-Schiff technique) (16) (data not presented). The data obtained with the preparative agarose gel showed at least three regions of activity (Fig. 4). Thus, we now have evidence that there are multiple forms of the ethylene inducing protein. The SDS-PAGE polypeptide pattern of the three active agarose fractions are very similar, if not identical (Fig. 5), when stained with Coomassie blue. Silver staining only brought out a few additional bands. The multiple bands in SDS-PAGE probably indicate that these agarose fractions contain more than one protein or that the active component is composed of subunits.

Previously it was reported that proteolytic enzymes were unable to destroy EIF activity (1). However, if urea is present in the reaction mixture (Table II), papain, trypsin, and protease from *S. aureus* V_8 are effective in destroying EIF activity. This provides additional evidence of the proteinaceous nature of the EIF which we have found in Cellulysin. We suspect that EIF in solution is a tightly coiled protein because it is not affected to any extent by proteases in the absence of urea. Urea probably unfolds EIF and allows the proteases to act.

The mechanism by which EIF and other nonpurified hydrolytic enzyme systems induces ethylene biosynthesis is not known. The work of Tong *et al.* (14) suggests that a heat stable cell wall

Table I. *Differential Binding to Sephacryl S 200 of Ethylene Inducing Activity of Several Hydrolytic Enzyme Preparations Used to Produce Protoplasts*

Enzyme Preparation	Protein		Ethylene Inducing Activity		Total Recovered	
	Not bound	Bound	Not bound	Bound	Protein	EIF
	% of recovered			*% of applied*		
Cellulysin	95	5	10	98	70	108
Cellulase RS	83	17	100	38	110	147
Pectinase	100	0	91	4	100	96
Pectolyase	76	24	102	2	65	103
Rhozyme	98	2	34	3	102	37

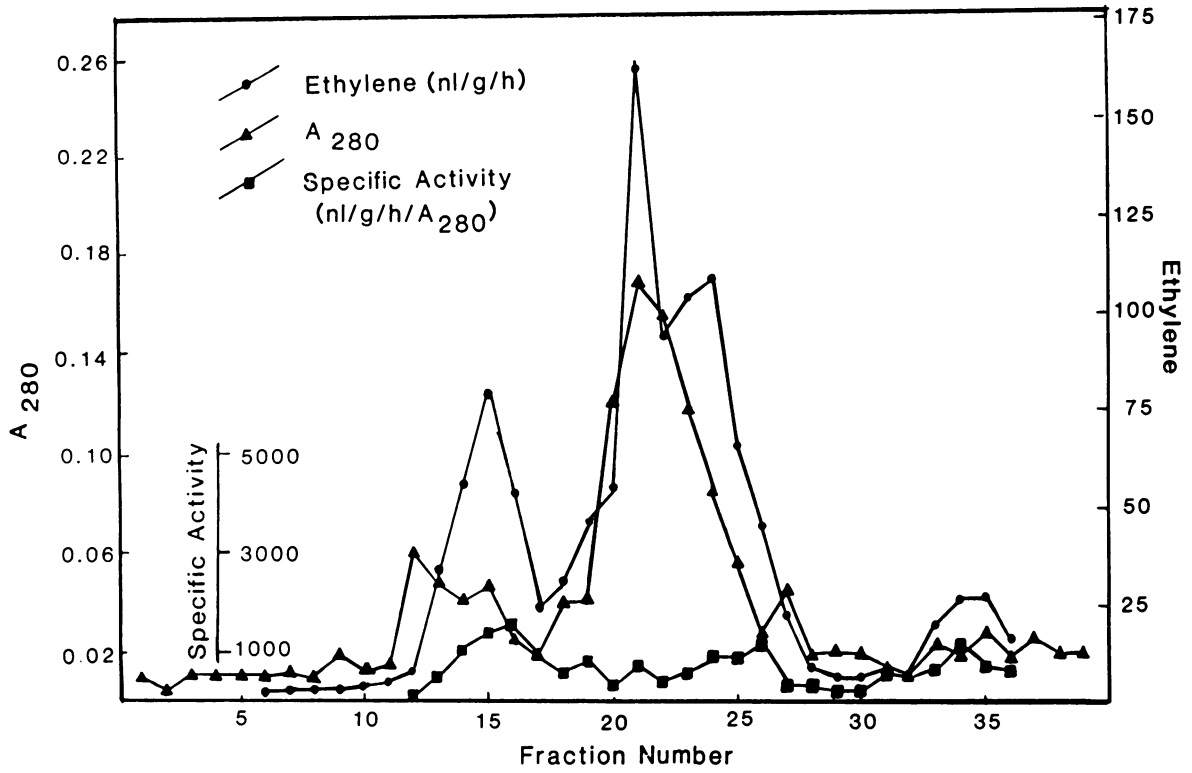

FIG. 4. Preparative agarose electrophoresis of the active material from step 10. Those active fractions were pooled as follows: fraction 1, tubes 13 to 16; fraction 2, tubes 20 to 26; and fraction 3, tubes 33 to 35.

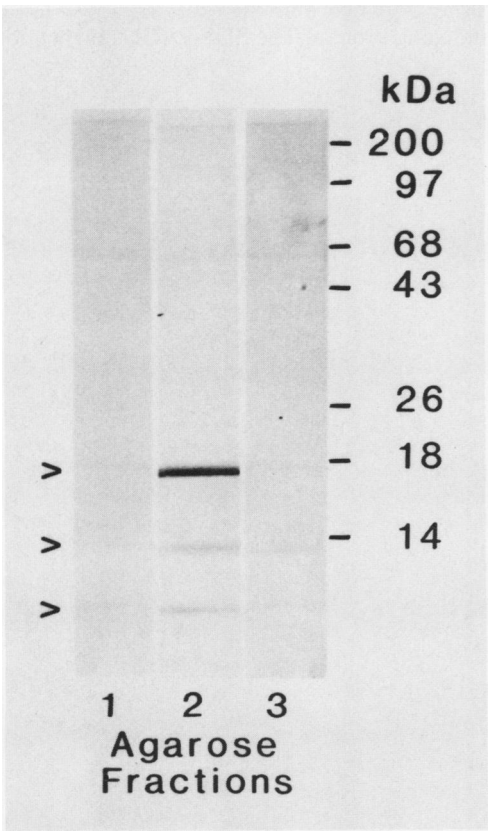

FIG. 5. SDS polyacrylamide gel (gradient 12–20%, 25 mamp/gel, Coomassie blue stained) showing the electrophoretic separation pattern of three different active fractions collected from preparative agarose gel electrophoresis from Figure 4.

Table II. *Effect of Proteolytic Enzymes and Urea on the Ethylene Inducing Activity of EIF in Tobacco Leaf Discs*

Urea was dissolved in EIF (5 μg protein/μl) solution (1 mg/μl) and 10 μl of it was incubated with 5 μl of proteolytic enzyme solution (3–6 mg/ml) for 17 h at 30°C. At the end of the incubation each sample was diluted with 0.5 ml basal medium and a 0.2 ml aliquot was bioassayed.

Treatment	Ethylene Production	
	(−) Urea	(+) Urea
	nl/g·h	
EIF	87	42
EIF + β-chymoytypsin	85	30
EIF + *S. aureus*	89	13
EIF + trypsin	89	9
EIF + papain	81	4
No EIF or any one of the above enzymes	4	4

fragment produced by Macerase action on isolated cell walls of cultured cell induces ethylene in cultured pear cells. Furthermore, chitin fragments (8) as well as other types of elicitors (10), are known to induce ethylene biosynthesis. The data showing that Macerase releases cell wall fragments that induces ethylene (14), compliment the findings that oligosaccharins can have pronounced effects on morphogenesis and development of tobacco tissue cultures (15). The results we have to date have been negative as far as finding heat stable components of cell walls that induce ethylene after EIF treatment. However, our data show that the ethylene inducing activity of various enzymes (*i.e.* pectinase, Pectolyase, and Rhozyme) are different than that of the EIF which we have isolated (Table I) because the activity does not bind to Sephacyl S-200. Of the enzymes tested, only Cellulysin and Cellulase RS contained ethylene inducing factors that bound to Sephacryl S-200. This clearly shows that these enzyme mixtures contain ethylene inducing components that

differ in their physical or chemical properties. These different enzyme mixtures may also have different mechanisms by which they induce ethylene.

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LITERATURE CITED

1. ANDERSON JD, E CHALUTZ, AK MATTOO 1984 Purification and properties of the ethylene-inducing factor from the cell wall digesting mixture, Cellulysin. *In* Y Fuchs, E Chalutz, eds, Ethylene: Biochemical Physiological and Applied Aspects. Martinus Nijhoff/Dr. W. Junk, The Hague, pp 189–193
2. ANDERSON JD, E CHAULTZ, AK MATTOO 1985 Induction of ethylene biosynthesis by cell wall digesting enzymes. *In* JL Key, T Kosuge, eds, Cellular and Molecular Biology of Plant Stress, New Series, Vol 22. Alan R. Liss, New York, pp 263–273
3. ANDERSON JD, M LIEBERMAN, RW STEWART 1979 Ethylene production by apple protoplasts. Plant Physiol 63: 931–935
4. ANDERSON JD, AK MATTOO, M LIEBERMAN 1982 Induction of ethylene biosynthesis in tobacco leaf discs by cell wall digesting enzymes. Biochem Biopys Res Commun 107: 588–596
5. BAKER JE 1976 Superoxide dismutase in ripening fruits. Plant Physiol 58: 644–647
6. CASSELLS AC, FM COCKER, S AUSTIN 1980 Ethylene release during tobacco protoplast isolation and subsequent protoplast survival. Plant Sci Lett 19: 169–173
7. CHALUTZ E, AK MATTOO, T SOLOMOS, JD ANDERSON 1984 Enhancement by ethylene of Cellulysin induced ethylene production by tobacco leaf discs. Plant Physiol 74: 99–103
8. CHAPPELL J, K HAHLBROCK, T BOLLER 1984 Rapid induction of ethylene biosynthesis in cultured parsley cells by fungal elicitor and its relationship to the induction of phenylalanine ammonia-lyase. Planta 161: 475–480
9. CLARKE AE, RM HOGGAT 1982 The use of lectins in the study of glycoproteins. *In* JJ Marchalonis, GW Warr, eds, Antibodies as a Tool, The Applications of Immunochemistry. John Wiley & Sons, Chichester, pp 384–385
10. ESQUERRE-TUGAYE MT, D MAZAU, B PELISSIU, D ROBY, D RUMEAU, A TOPPAN 1985 Induction by elicitors and ethylene of proteins associated to the defense of plants. *In* JL Key, T Kosuge eds, Cellular and Molecular Biology of Plant Stress, New Series, Vol 22. Alan R. Liss, New York, pp 459–473
11. FACCIOTTI D, PE PILET 1981 Ethylene release during haploid and diploid protoplast isolation and viability. Z Pflanzenphysiol 104: 401–407
12. GUY M, H KENDE 1984 Ethylene formation in *Pisum sativum* and *Vicia faba* protoplasts. Planta 160: 276–280
13. MOLINE HE, KS JOHNSON, JD ANDERSON 1983 Evaluation of two-dimensional polyacrylamide gel electrophoresis of acidic proteins of ribosome preparations for identifying plant pathogenic soft-rotting bacteria. Phytopathology 73: 224–227
14. TONG CB, JM LABAVITCH, SF YANG 1986 The induction of ethylene production from pear cell culture by cell wall fragments. Plant Physiol 81: 929–930
15. TRAN THANH VAN K, P TOUBART, A COUSSON, AG DARVILL, DJ GOLLIN, P CHELF, P ALBERSHEIM 1985 Manipulation of the morphogenic pathways of tobacco explants by oligosaccharins. Nature 314: 615–617
16. ZACHARIUS RM, TE ZELL, JH MORRISON, JJ WOODLOCK 1969 Glycoprotein staining following electrophoresis on acrylamide gels. Analyt Biochem 30: 148–152