Effects of Purified Cutin Esterase Upon the Permeability and Mechanical Strength of Cutin Membranes

C. Jacyn Baker, Susan L. McCormick, and Durward F. Bateman

Department of Plant Pathology, Cornell University, Ithaca, NY 14853.
Present addresses: U.S. Department of Agriculture, Applied Plant Pathology Laboratory, Beltsville, MD 20705; Department of Zoology, University of Georgia, Athens 30602; and North Carolina Agricultural Research Service, North Carolina State University, Raleigh 27650, respectively.
Accepted for publication 6 July 1981.

ABSTRACT

Baker, C. J., McCormick, S. L., and Bateman, D. F. 1982. Effects of purified cutin esterase upon the permeability and mechanical strength of cutin membranes. Phytopathology 72:420-423.

Cutin esterase was purified from 5-day-old cultures of *Fusarium solani* f. sp. *pisi* grown on V-8 juice medium supplemented with 0.5% apple cutin. Cutin membranes were prepared from skins of grape berries by dewaxing in organic solvents followed by treatment with pectinase and cellulase. Intact cutin membranes in the absence of cutin esterase failed to permit the passage of glucose-U-^{14}C between the cells of a double cell diffusion apparatus, whereas in the presence of 3–4 units of cutin esterase (1 unit of enzyme at 25 C hydrolyzed 1 μmole of *p*-nitrophenyl acetate per min at pH 8.5), glucose as well as cutin esterase readily traversed the cutin membranes. Cutin membranes exposed to cutin esterase for 72 hr permitted the passage of three enzymes that degrade cell walls: β-1,4-xylanase (mol wt 15,000), endo-α-1,4-pectate lyase (mol wt 30,000), and α-L-arabinofuranosidase (mol wt 60,000); such membranes were ruptured by a hydrostatic pressure of 0–15 cm, whereas control membranes were ruptured by a hydrostatic pressure of 48–107 cm.

The mechanism of fungal ingress through intact plant cuticles has been a controversy for many years (7,14). Two hypotheses have predominated: 1) cuticular penetration results from mechanical force exerted by the pathogen, and 2) penetration is facilitated by enzymatic digestion of the cuticle. The only continuous, covalently linked structure in the plant cuticle appears to be the cutin membrane (10). This membrane is regarded as the primary barrier to microbial ingress through plant surfaces. Over the past two decades much evidence has been obtained that indicates that a broad range of plant pathogenic fungi can produce cutinolytic enzymes (3,9,18,21). The objective of this study was to determine the influence of a highly purified fungal cutin esterase upon the permeability and mechanical strength of cutin membranes.

Fusarium solani f. sp. *pisi* was selected as the cutin esterase source (18). Grape berries were selected as a source of relatively uniform cutin membranes with few or no natural openings such as stomata (17). The results reported in this article provide experimental proof that cutin esterase drastically alters the permeability of cutin membranes and destroys their mechanical strength. The hypothesis favoring the involvement of enzymatic mechanisms of fungal ingress is supported and a role for cutin esterase in plant pathogenesis is indicated.

MATERIALS AND METHODS

Tritiated apple cutin, β-1,4-xylanase, α-L-arabinofuranosidase, and endo-α-1,4-pectate lyase were prepared as previously described (2–5). D-Glucose-U-^{14}C was purchased from Calatomic (Los Angeles, CA 90054); *p*-nitrophenyl (PNP)-acetate, pectinase, and cellulase were obtained from Sigma Chemical Co. (St. Louis, MO 63178); and Paraoxon (diethyl *p*-nitrophenyl phosphate) was purchased from Chem Service (West Chester, PA 19380).

The diffusion cells (Fig. 1) used in this study were similar to the one designed by Reed (19).

Enzyme assays. Esterase activity was assayed at 25 C in reaction mixtures containing: 0.5 ml of 0.1% PNP-acetate in 0.01% Triton X-100, 0.5 ml of 500 m*M* Tris-HCl buffer, pH 8.5, and sufficient enzyme plus water to give a 3.0-ml reaction mixture. Activity was determined by measuring increase in absorbance at 400 nm in a recording spectrophotometer. Initial reaction rates were corrected against those of control reaction mixtures containing enzyme inactivated by autoclaving for 1 hr or enzyme preincubated for 10 min with 1 m*M* Paraoxon. A standard curve was prepared using PNP. One unit of esterase activity was defined as the amount of enzyme that hydrolyzed 1 μmole of PNP-acetate per minute under the above conditions.

During the purification of cutin esterase, cutinolytic activity was

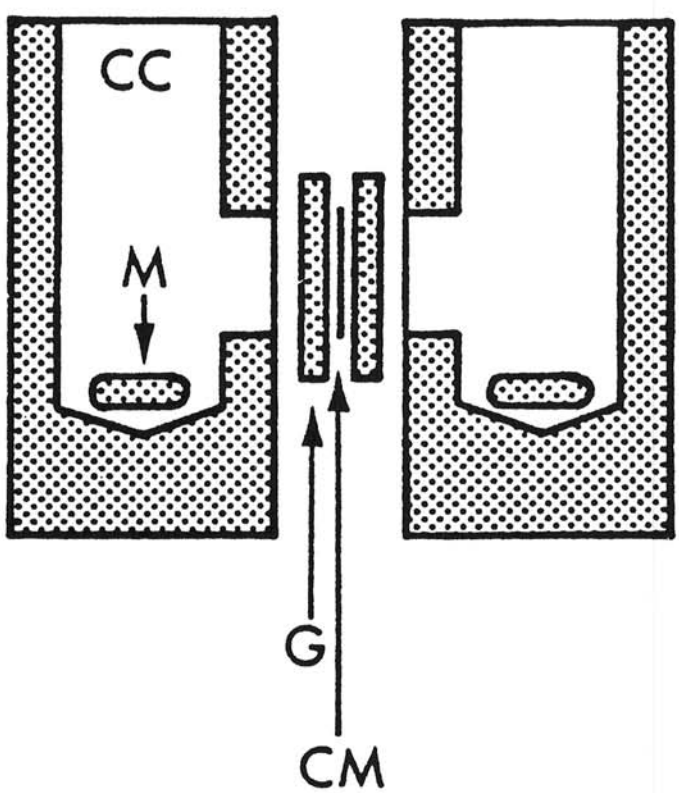

Fig. 1. Diffusion cell. CC = cell chamber, CM = cutin membrane, G = gasket, M = magnetic stirring bar.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

0031-949X/82/04042004/$03.00/0

measured by determining the amount of ether-soluble radioactivity released from ^{3}H-apple cutin using procedures previously described (3). Endo-α-1,4-pectate lyase, β-1,4-xylanase, and α-L-arabinofuranosidase activities were measured as previously described (2,4,5).

Purification of cutin esterase. This enzyme was purified from culture filtrates of *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (F. R. Jones) Snyd. and Hans. by a modification of the procedure of Purdy and Kolattukudy (18). Culture filtrates were obtained from 6-day-old cultures grown in V-8 juice medium supplemented with 0.5% apple cutin (3).

All steps in the purification were carried out at 4 C. Two liters of culture filtrate were dialyzed against 10 m*M* Tris-HCl buffer, pH 8.5. Dialyzed culture filtrate was passed through a diethylaminoethyl (DEAE)-Sephadex (A-25) column (2.5 cm diameter $\times$40 cm) equilibrated with 10 m*M* Tris-HCl buffer. Eluate from this column contained the cutin esterase activity and was dialyzed against 50 m*M* sodium citrate-10 m*M* potassium phosphate buffer, pH 5.0. This dialysate was applied to a column (1.5 cm diameter $\times$ 26 cm) of SP-Sephadex (C-25) equilibrated with citrate-phosphate buffer. The cutin esterase bound to this column and was eluted with a 0–200 m*M* NaCl gradient in 500 ml of buffer. Protein content was measured by the method of Lowry et al (11). Enzyme was stored in column eluate at −20 C for periods up to 1 yr without significant loss in activity. The purified enzyme was subjected to electrofocusing and gel electrophoresis by procedures previously described (1,4).

Preparation of cutin membranes. Grape berries (*Vitis vinifera* var. *calmeria*) were hand-peeled and the skins washed with methanol (100 ml of methanol per gram of skins) for 2 hr at 27 C on a rotary shaker. The methanol was decanted, and the skins were washed with chloroform in a similar manner. The chloroform was then decanted and the skins were shaken in acetone overnight. Dewaxed skins were individually placed between two rubber gaskets used in the diffusion cell, placed in a mixture of 0.5% pectinase and 1.5% cellulase in 50 m*M* sodium acetate buffer (pH 4.5) and 0.02% sodium azide, and incubated for 24–48 hr at 25 C. After incubation, any cellular debris remaining on the inner surface of the skin (cutin membrane) was gently removed with tissue paper. The prepared membranes were then thoroughly washed with distilled water and inserted between the chambers of diffusion cells for use in experiments.

Estimate of the permeability and mechanical strength of isolated cutin membranes. Each chamber of the diffusion cell apparatus was filled with 5.0 ml of 100 m*M* Tris-HCl buffer, pH 8.5, containing 0.02% sodium azide. The integrity of each membrane was tested before use by checking its impermeability to radiolabeled glucose. The radioactive glucose (0.1 μC) was placed in the chamber facing the outer surface of the cutin membrane. After 18 hr of incubation at 25 C, the radioactivity in 100-μl aliquots from both chambers of the diffusion cell was determined with a scintillation counter (3). Only membranes that failed to permit passage of radiolabeled glucose in the above test were considered intact and used in experiments.

Experiments to determine the effects of cutin esterase on cutin membrane permeability involved the addition of 3–4 units of cutin esterase to the chamber facing the outer surface of intact cutin membranes. Radiolabeled glucose or other molecules (enzymes) added to the chamber containing cutin esterase were assayed in 100-μl aliquots removed from both chambers after given periods of incubation. In each experiment, six replicates were used for each treatment, including six controls that contained inactivated enzyme. The mechanical strength of cutin membranes after various treatments was estimated by the amount of hydrostatic pressure each could withstand before rupture. An open-ended glass tube (1.1 cm in diameter) was attached via a rubber stopper to the top of the cell chamber facing the outer surface of the cutin membrane. This tube was filled with water containing a blue dye at a rate of 30 cm/min, and the height of the water column achieved before membrane rupture was recorded.

RESULTS

Cutin esterase purification. The cutin esterase activity in dialyzed culture filtrate of *F. solani* f. sp. *pisi* failed to bind to diethylaminoethyl-Sephadex, but considerable extraneous protein was removed by passing the crude enzyme through a column of this anion exchanger at pH 8.5. The partially purified enzyme was retained by a column of SP-Sephadex and was eluted with a NaCl gradient as two peaks designated SP-1 and SP-2 at 100 and 130 m*M* NaCl, respectively (Table 1). Both enzyme fractions exhibited a pH optimum of about 8.5 for hydrolysis of apple cutin and were highly resistant to heat. After autoclaving for 15 min, both preparations retained 30% of their esterase activity.

Electrophoresis of SP-1 and SP-2 in low pH, 12% polyacrylamide gels indicated that both fractions were highly purified. Sodium dodecylsulfate-gel electrophoresis of these fractions revealed that each contained one major and two minor protein bands that migrated very close to each other. The estimated molecular weight of the major polypeptide was about 15,000 for each fraction. Upon isoelectric focusing of SP-1 and SP-2 in pH 7–11 ampholytes, both preparations were seen to contain a mixture of isozymes; those of SP-1 occurred at pH 7.5, 8.1, and 9.3, and those of SP-2 occurred between pH 7.2 and 8.4 and at pH 8.9 and 9.6. All of these isozymes have been shown to release fatty acids from apple cutin (1).

Effect of cutin esterase on cutin membrane permeability. The purified cutin esterase isozymes in fraction SP-1 were used to examine the effect of cutin esterase on the permeability of isolated cutin membranes. When 3–4 units of cutin esterase were added to the diffusion cell chamber exposing the exterior surface of cutin membranes, radiolabeled glucose diffused from the chamber containing the enzymes across the membrane into the opposite chamber within 12 hr (Fig. 2). Equilibrium of radiolabeled glucose

TABLE 1. Purification of cutinase from culture filtrates of *F. solani* f. sp. *pisi* grown for 6 days on V-8 juice broth medium supplemented with 0.5% purified apple cutin

Fraction	Volume (ml)	Total protein (mg)	^{3}H-cutin assay: Specific activity[a] (10^3 DPM/hr/mg of protein)	^{3}H-cutin assay: Recovery (%)	*P*-Nitrophenyl (PNP)-acetate assay: Specific activity[b] (mole/min/mg of protein)	*P*-Nitrophenyl (PNP)-acetate assay: Recovery (%)
Culture filtrate	2,000	...	(10.6/ml)[c]	100	(10.5/ml)[c]	100
Dialyzed culture filtrate	2,070	1,190	12.9	72	17.7	100
Diethylaminoethyl Sephadex void	2,052	213	55.4	55.7	91.1	92
Dialyzed diethylaminoethyl-Sephadex void	1,996	179	46.7	39.5	76.8	65
SP-Sephadex	46	63	121.6	36.2	156.2	57
Peak 1	17	24	158.8	18.0	162.6	19
Peak 2	14	22	87.2	9.0	147.7	15

[a] Cutinase activity was assayed at 28 C by measuring ether-soluble radioactivity released from ^{3}H-cutin at pH 8.5 (3).
[b] Esterase activity was assayed at 25 C by spectrophotometrically (400 nm) measuring the rate of release of PNP in reaction mixtures containing 0.5 ml of 0.1% PNP-acetate in 0.01% Triton X-100, 0.5 ml of 500 mM Tris buffer (pH 8.5), and sufficient amount of enzyme and water to bring the volume to 3 ml.
[c] Accurate protein measurements of culture filtrate could not be made.

between the chambers was usually reached within 24–72 hr in the presence of cutin esterase, whereas no radioactivity above background could be detected in the opposite chamber of diffusion cells when cutin membranes were exposed to heat-inactivated cutin esterase or to cutin esterase in the presence of 1 m*M* Paraoxon.

The diffusion of cutin esterase itself across the cutin membranes was examined. Esterase activity was usually detected in the opposite chamber of diffusion cells containing active enzyme within 12 hr. Esterase activity usually equilibrated between the chambers within 72 hr.

In view of these results, experiments were conducted to determine whether cutin membranes exposed to cutin esterase for 72 hr would permit the passage of three enzymes that degrade cell walls. One to two units (2,4,5) each of β-1,4-xylanase (mol wt 15,000), endo-α-1,4-pectate lyase (mol wt 30,000), and α-L-arabinofuranosidase (mol wt 60,000) was added to chambers of diffusion cells facing the exterior surface of cutin membranes treated with active or inactive cutin esterase. Cutin membranes treated with inactive cutin esterase failed to permit the passage of any of the cell wall degrading enzymes; membranes treated with active cutin esterase permitted all three cell wall degrading enzymes to diffuse across to the opposite chamber of the diffusion cells. In fact, all three enzymes usually reached equilibrium in the chambers of diffusion cells within a period of 12 hr.

Effect of cutin esterase on the mechanical strength of cutin membranes. The hydrostatic pressure required to disrupt cutin membranes in diffusion cells was estimated from the column height of water necessary to cause physical disruption. Cutin membranes exposed to 2–4 units of cutin esterase for 72 hr were ruptured by a hydrostatic pressure between 0 and 15 cm, whereas membranes exposed to inactive enzyme were ruptured by hydrostatic pressure between 48 and 107 cm.

DISCUSSION

The cutin esterase fractions designated SP-1 and SP-2 purified and characterized from the culture filtrate of *F. solani* f. sp. *pisi* in this study appear to be comparable to cutinase I and cutinase II described by Purdy and Kolattakudy (18). Grape skins were shown to contain relatively uniform, thin (about 5 μm thick) cutin membranes free of pores that would permit the passage of small molecules such as glucose. Purified cutin esterase and isolated cutin membranes were employed in experiments using double-chambered diffusion cells to demonstrate that cutin esterase per se is capable of altering cutin membranes so that both small and large molecules (enzymes) can readily transverse these membranes. The action of cutin esterase on cutin membranes also drastically reduced their mechanical strength. The demonstrated effects of cutin esterase action on cutin membranes has significant implications for a possible role for cutin esterase in fungal penetration of intact plant surfaces and plant pathogenesis.

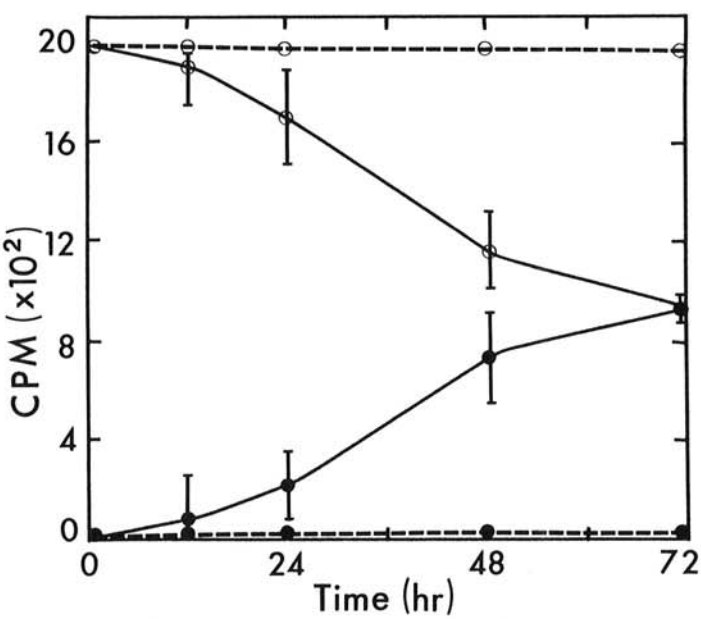

Fig. 2. The diffusion of glucose across isolated cutin membranes upon exposure to cutin esterase. Radiolabeled glucose (0.2 μC) was added to the diffusion cell chamber exposing the exterior surface of the cutin membrane. Radioactivity in chambers exposed to exterior (o) and interior (•) sides of the membrane was measured at intervals after exposure to active (—) or inactive (---) cutin esterase.

The weakening of the cutin membrane by cutinolytic enzymes during fungal penetration of plant surfaces has long been implied by those who support the enzymatic hypothesis of fungal ingress. During the past two decades, numerous ultrastructural studies have provided evidence to suggest that the cuticle is degraded enzymatically during fungal penetration (8,13,16). Penetration pegs from appressoria or other infection structures are not necessarily cone-shaped as described by Brown and Harvey (7); rather, the tips of infection pegs may be blunt or uneven and without a cell wall (8,13,16). For example, the penetration of barley leaves by *Erysiphe graminis* f. sp. *hordei* reveals no evidence of mechanical stress or force during cuticular penetration; instead, the cuticle appears to be dissolved during penetration (8). Also, during penetration of broadbean by *Botrytis cinerea*, infection pegs of the pathogen pass through the cuticle without causing any indentation, break, or tear (13).

A broad range of phytopathogenic fungi has been reported to produce cutinolytic enzymes (1,3,9,18,21). A number of investigators have provided evidence that cutin esterase is produced by pathogens at penetration sites (12,13,15,20). The results reported in this article demonstrate that cutin esterase can alter the cutin membrane to the extent that its mechanical strength is lost. Thus, cutin esterase action may be expected to facilitate fungal ingress into plant tissue.

In the current study we demonstrated that cutin esterase action permitted a number of plant cell wall degrading enzymes to traverse cutin membranes. This indicates that the action of cutin esterase on cutin membranes before fungal ingress could have significant influence upon the early phases of pathogenesis. For example, cutin esterase action on cutin membranes could facilitate the passage of pectic enzymes, known to be toxic to plant tissues (6), and of other fungal enzymes and metabolites into host tissue before pathogen ingress.

The effects of cutin esterase on intact cutin membranes by purified cutin esterase demonstrated in this study, coupled with the histological and histochemical evidence in the literature concerning the involvement of cutinolytic enzymes in fungal penetration of plants, leaves little doubt that cutin digestion by cutin esterase or other enyzmes is a significant factor in pathogen ingress and plant pathogenesis.

LITERATURE CITED

1. Baker, C. J. 1978. Cutin degradation by plant pathogenic fungi. Ph.D. thesis. Cornell University, Ithaca, NY. 127 pp.
2. Baker, C. J., Aist, J. R., and Bateman, D. F. 1980. Ultrastructural and biochemical effects of endopectate lyase on cell walls from cell suspension cultures of bean and rice. Can. J. Bot. 58:867-880.
3. Baker, C. J., and Bateman, D. F. 1978. Cutin degradation by plant pathogenic fungi. Phytopathology 68:1577-1584.
4. Baker, C. J., Whalen, C. H., and Bateman, D. F. 1977. Xylanase from *Trichoderma pseudokoningii*: Purification, characterization, and effects on isolated plant cell walls. Phytopathology 67:1250-1258.
5. Baker, C. J., Whalen, C. H., Korman, R. Z., and Bateman, D. F. 1979. α-L-Arabinosidase from *Sclerotinia sclerotiorum*: Purification, characterization, and effects on plant cell walls and tissue. Phytopathology 69:789-793.
6. Bateman, D. F., and Basham, H. G. 1976. Degradation of plant cell walls and membranes by microbial enzymes. Pages 316–355 in: R. Heitefuss and P. Williams, eds. Encyclopedia of Plant Physiology. Vol. 4. Physiological Plant Pathology. Springer-Verlag, Berlin. 820 pp.
7. Brown, W., and Harvey, C. C. 1927. Studies in the physiology of parasitism. X. On the entrance of parasitic fungi into the host plant. Ann. Bot. 41:643-662.
8. Edwards, H. H., and Allen, P. J. 1970. A fine-structure study of the

primary infection process during infection of barley by *Erysiphe graminis* f. sp. *hordei*. Phytopathology 60:1504-1509.
9. Heinen, W. 1960. Uber den enzymatischen cutin-abbau. I. Metteilung: Nachweis eines "cutinase"-systems. Acta Bot. Neerl. 9:167-190.
10. Kolattukudy, P. E. 1980. Biopolymer membranes of plants: Cutin and suberin. Science 208:990-1000.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
12. Maiti, I. B., and Kolattukudy, P. E. 1979. Prevention of fungal infection of plants by specific inhibition of cutinase. Science 205:507-508.
13. McKeen, W. E. 1974. Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea*. Phytopathology 64:461-467.
14. Miyoshi, M. 1895. Die durchbohrung von membranen durch pilzfaden. Jahrb. Wiss. Bot. 28:269-289.
15. Nicholson, R. L., Kuć, J., and Williams, E. B. 1972. Histochemical demonstration of transitory esterase activity in *Venturia inaequalis*. Phytopathology 62:1242-1247.
16. Politis, D. J., and Wheeler, H. 1973. Ultrastructural study of penetration of maize leaves by *Colletotrichum graminicola*. Physiol. Plant Pathol. 3:465-471.
17. Pratt, C. 1971. Reproductive anatomy in cultivated grapes—A review. Am. J. Enol. Vitic. 22:92-109.
18. Purdy, R. E., and Kolattukudy, P. E. 1975. Hydrolysis of plant cuticle by plant pathogens. Purification, amino acid composition, and molecular weight of two isozymes of cutinase and a nonspecific esterase from *Fusarium solani* f. sp. *pisi*. Biochemistry 14:2824-2831.
19. Reed, O. W. 1979. Ultrastructural studies of plant cuticles: Environmental effects, permeability, electron microscopy preparation and specific staining. Ph.D. thesis. Cornell University, Ithaca, NY. 345 pp.
20. Shaykh, M., Soliday, C., and Kolattukudy, P. E. 1977. Proof for the production of cutinase by *Fusarium solani* f. sp. *pisi* during penetration into its host, *Pisum sativum*. Plant Physiol. 60:170-172.
21. Shishiyama, J., Araki, F., and Akai, S. 1970. Studies on cutin-esterase. II. Characteristics of cutin-esterase from *Botrytis cinerea* and its activity on tomato cutin. Plant Cell Physiol. 11:937-945.