Detection of Polygalacturonase Enzymes in Fruits of Both a Normal Tomato and its Nonripening *Nor* Mutant Infected with *Rhizopus stolonifer*

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ABSTRACT

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Rhizopus stolonifer growing on autoclaved tomato fruit homogenate produces at least seven forms of polygalacturonase separable by polyacrylamide gel electrophoresis. Mature-green fruit from normally ripening lines of tomato (*Lycopersicon esculentum* 'Rutgers') and also from its nonripening *nor* mutant line contain no polygalacturonase. After infection with *R. stolonifer*, the mature-green fruit contain polygalacturonase enzymes that correspond by gel electrophoresis analysis with the fungal enzymes and are not precipitated by antiserum to tomato polygalacturonase. Infected ripe fruit contains both fruit and fungal enzymes; rocket immunoelectrophoresis showed that the infected ripe fruit contained more tomato enzyme than the uninfected fruit of similar maturity. In mature *nor* fruit, there was no enhancement of plant enzyme by fungal infection. It is concluded that infection by *R. stolonifer* advanced the ripening process in genetically and developmentally competent tissue but did not induce ripening in the mutant fruits which lack an active ripening system.

Fruits of the two nonripening mutants of tomato, *nor* and *rin*, are devoid of pectolytic enzymes during all stages of development and maturation except that trace levels have sometimes been recorded for the mature *nor* mutant (7,10). However, following infection by *Rhizopus stolonifer* (Ehrenb. ex. Fr.) Lind, polygalacturonase (PG) activity was recorded in the two mutant fruits and activity increased with disease development (3). Fruits of normal lines of tomato exhibited PG activity but fungal infection resulted in increased activity, the effect being most pronounced at the early stages of maturity when pectolytic activity in noninfected samples was low (3).

However, the source of PG activity in infected enzyme-free mutant tomato tissues is not clear. Enzyme activity following infection may be of fungal origin (8,12), but it may also be the result of induction of otherwise unexpressed tomato enzymes as was suggested by Barash and Khazzam (2) in anthracnose disease of avocado.

Three molecular forms of tomato PG, termed PG1, PG2A, and PG2B, have been described (1,6). No information is available on the molecular forms of PG produced by *R. stolonifer*.

The purpose of the work reported here was to study the origin of pectolytic enzymes in infected tomato fruit by using electrophoretic and immunological tests. A host-pathogen system composed of the pectolytic fungus *R. stolonifer* and the enzyme-free *nor* tomato mutant was used to study pectolytic enzyme activity of infected tomato fruit.

MATERIALS AND METHODS

A spore suspension of 10^7 spores per milliliter was prepared on potato-dextrose agar (PDA) from a 5-day, single-spore culture of *R. stolonifer*. The culture was isolated from a postharvest infected tomato rot. Aliquots of 1 ml of this spore suspension were used to inoculate 30 ml of a tomato-homogenate (TH) medium prepared by blending a mature Rutgers tomato fruit for 2 min. The natural pectolytic enzymes of the tomato were denatured during autoclaving for 20 min at a pressure of 1.2 atmospheres. After 3 days at 23 C, the cultures were filtered through several layers of cheesecloth, and the supernatant was lyophilized.

For in vivo studies, plants of tomato cultivar Rutgers and the nonripening *nor* mutant, which was partially isogenic to Rutgers (third backcross) were grown in glasshouses and harvested at two stages of maturation—"mature green" and "mature." For the *nor* mutant fruit, 14 days after the "turning point" (the stage of changing color from green to yellow) was referred to as its "mature stage." The harvested fruit were inoculated by injecting 0.05 ml of spore suspension into the pericarp tissue. After 3 days of incubation at 23 C, the fruit was frozen at −20 C for 24 hr, cut into small pieces, lyophilized to 9−10% of the fresh fruit weight, and ground to a powder. Extracts were prepared by adding 0.35 g of each of the powder samples into 1.0 ml of 0.1 M sodium acetate, 1.3 M NaCl, 40 mM β-mercaptoethanol, pH 6. The slurry was agitated for 16 hr at 0 C on a roller-mixer. Debris was removed by centrifuging at 12,000 *g* for 30 min, and the extracts were used for enzyme assays.

PG activity in the in vitro and in vivo extracts was determined by using the viscometric method described by Ali and Brady (1). Units of activity were calculated as the initial rate of change of the reciprocal of the specific viscosity per minute per gram fresh weight. Identification of the molecular forms of PG in the various extracts was by gel electrophoresis. Extracts (25−100 μl) were separated by polyacrylamide gel electrophoresis using the buffer system described by Reisfeld et al (11). PG activity within the gels was detected by staining pectic acid substrate with ruthenium red as specified in the method of Lisker and Retig (9).

The presence and the amounts of PG protein in the various samples were compared by using rocket immunoelectrophoresis with antiserum prepared against PG2A in rabbits as previously described by Brady et al (5).

RESULTS

Viscometric assay. Table 1 shows that while no PG activity was detected in the autoclaved sample TH, 0.60 units were recorded in samples of TH inoculated with *Rhizopus*. Following infection by *Rhizopus*, PG activities of 2.21 and 1.62 units were recorded in mature-green samples of normal and *nor* fruits, respectively; these fruits were devoid of activity if not infected.

In the mature Rutgers fruit, fungal infection increased PG activity to about three times that of the uninfected fruit. In the mature *nor* fruit, where no enzyme activity was detected prior to

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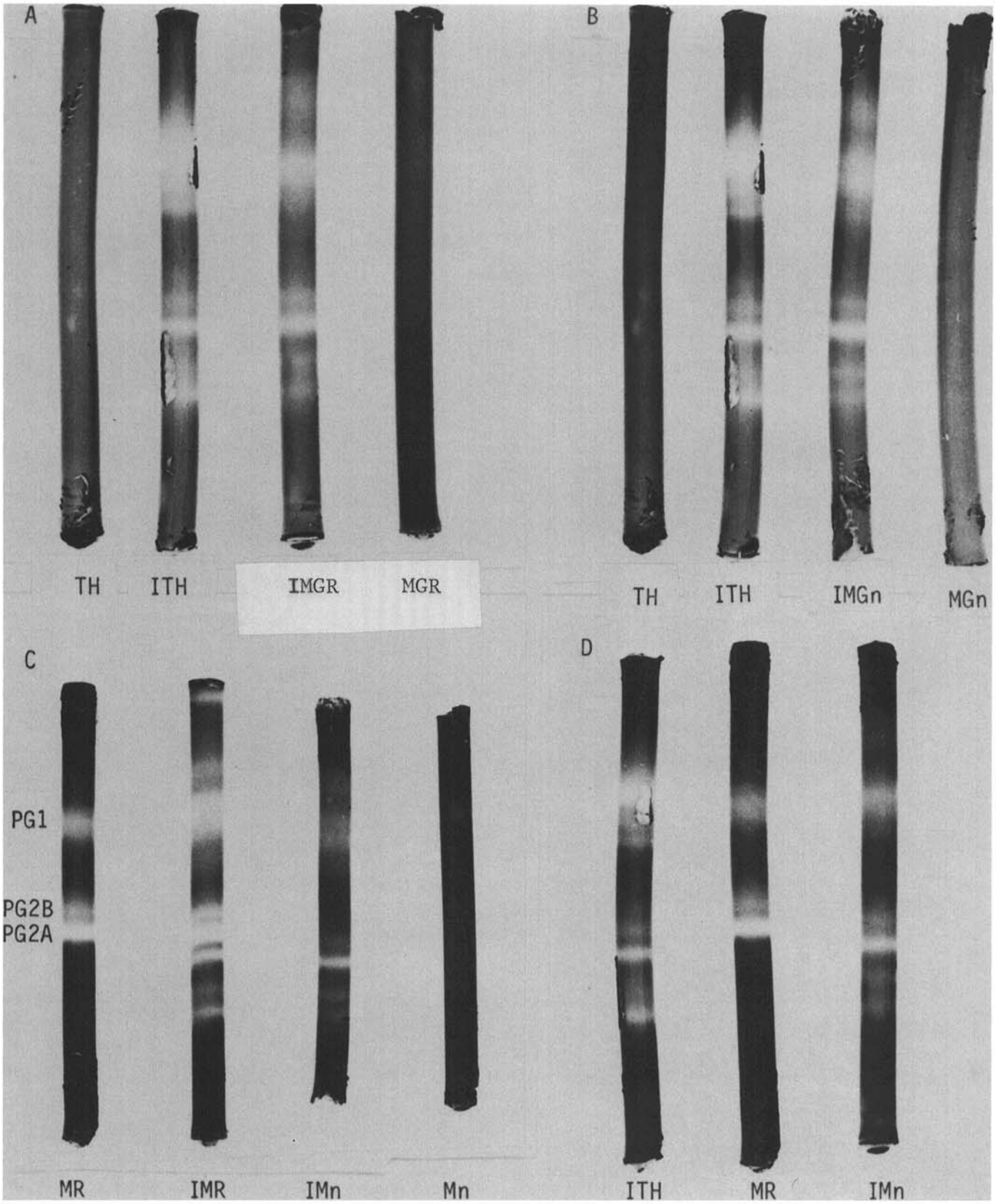

Fig.1. Polyacrylamide gel analysis of polygalacturonase (PG) enzymes in filtrates of cultures of *Rhizopus stolonifer* following in vitro growth on tomato homogenate (TH) and in normal cultivar Rutgers and *nor* (nonripening) mutant tomato fruits infected by *R. stolonifer* at two stages of maturity. **A,** Isozyme patterns following inoculation of TH medium with *R. stolonifer* (ITH) and of mature-green Rutgers fruit (MGR) before and following infection (IMGR). **B,** Isozyme patterns of TH medium inoculated with *R. stolonifer* (ITH) and of mature-green *nor* fruit (MGn) before and following infection (IMGn). **C,** Isozyme patterns of mature Rutgers fruit (MR), infected mature Rutgers fruit (IMR), mature *nor* (Mn) fruit, and infected mature *nor* fruit (IMn). **D,** Isozyme patterns of TH medium inoculated with *R. stolonifer* (ITH), mature Rutgers fruit (MR), and infected mature *nor* fruit (IMn).

TABLE 1. Polygalacturonase (PG) activity in culture filtrates of *Rhizopus stolonifer* and in normal and *nor* tomato fruits infected by *Rhizopus stolonifer* as rated by viscosity assay[a] at two stages of maturation

Source of enzyme	PG activity (units)	
	Uninoculated	Inoculated
Tomato homogenate	0.00	0.60
Tomato fruit (cultivar Rutgers)		
Mature-green	0.00	2.21
Mature	2.18	6.43
Nonripening (*nor*) mutant fruit		
Mature-green	0.00	1.62
Mature	0.00	1.53

[a]Viscosity assay units are $(1/\text{min}^{-1} \cdot \text{g}^{-1})$, the initial rate of change of the reciprocal of the specific viscosity per minute (min) per gram fresh weight (g).

infection, activity reached about the same level as that of the *nor* mature-green fruit following infection by *Rhizopus* (Table 1).

Analysis by polyacrylamide gel electrophoresis. An analysis of filtrates derived from in vitro growth of *Rhizopus* on TH revealed that the fungal PG is composed of a number of molecular forms, at least seven bands of activity being apparent on polyacrylamide gels (Fig. 1). After infection by *Rhizopus*, samples of normal as well as mutant mature-green fruit, which did not show PG activity prior to infection, exhibited a number of PG bands, all of which corresponded to the fungal bands detected in the in vitro samples of inoculated, incubated TH (Fig. 1A and B).

Fig. 1C compares the enzymes in mature normal and mature *nor* fruit prior to and after infection by *Rhizopus*. Gels of the mature normal fruit showed the typical pattern of tomato PG composed of the three bands—PG1, PG2A, and PG2B; gels of the uninfected mature *nor* fruit showed only a trace of activity in the PG1 position. Samples of the two infected mature fruits, the normal and the mutant, showed enzyme bands corresponding to both the fungus and the fruit enzymes. Gels of infected mature *nor* fruit were, however, devoid of bands corresponding to PG2A and PG2B, which existed in gels of the infected mature normal fruit (Fig. 1C).

The enzymes from infected *nor* fruit consisted of bands which migrated further into the gel than the major ripe-fruit enzyme PG2A, as well as a band in the same region as PG1 (Fig. 1D).

Rocket immunoelectrophoresis. Neither the TH medium nor the inoculated medium showed any immunoreactive material after immunoelectrophoresis with antiserum to PG2 (Fig. 2). Thus, the fungal enzymes did not cross react with tomato PG-specific serum. The analysis also shows that the mature-green fruit, both the normal and the *nor* mutant, had no tomato PG, whether infected or not. Fig. 2 demonstrates that in the mature normal fruit, infection by *Rhizopus* increased the amount of tomato PG present. In the uninfected mature *nor* fruit a small amount of PG antigen was found by this method, and this amount was not increased by infection.

The last three tracks in Fig. 2 confirm that both purified PG1 and PG2 react with the antisera to PG2 and that the peak heights are concentration dependent.

DISCUSSION

Viscometric studies showed the presence of active PG in all mature-green fruit infected by *Rhizopus* of the normal and the mutant, as well as in the infected mature *nor* fruit. None of these showed any activity prior to infection; the only uninfected fruit with detectable activity were the normal mature fruits.

The study of fungal and fruit PG enzymes by polyacrylamide gel electrophoresis showed that all the molecular forms of the enzyme which were found in fungal PG after in vitro growth on heat-treated tissue were apparent in infected mature-green fruits. This analysis clearly demonstrated that the enzyme bands from infected mature-green samples which migrated faster in the gels than tomato PG2A corresponded to the enzymes of *Rhizopus* and are thus of fungal origin. However, the electrophoretic analysis was unable to provide

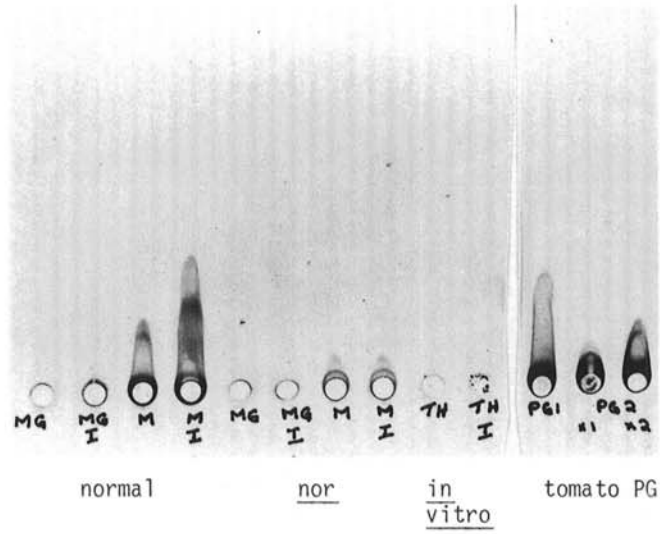

Fig. 2. Amounts of polygalacturonase (PG) protein estimated by rocket immunoelectrophoresis in normal cultivar Rutgers and *nor* mutant tomato fruits at the mature-green (MG) and mature (M) stages, prior to and after infection by *Rhizopus stolonifer* (IMG and IM). Comparison is made with PG in filtrates of in vitro cultured *R. stolonifer* following inoculation of tomato homogenate medium (ITH), and with PG1 and PG2 of tomato at two concentrations, ×1 and ×2.

a clear differentiation between the slower-migrating fungal and fruit enzymes which migrated to nearly identical positions.

The differentiation between these enzymes was completed by the immunological analysis with antiserum against PG2A, which reacts also with PG1 (1). This analysis established that the fungal enzyme does not react with the fruit-specific antiserum and that the active enzyme in the infected mature-green fruit, normal as well as mutant, is not tomato PG.

The viscometric assay showed that the level of the pectolytic activity of the infected normal mature fruit was three times that of the uninfected fruit. Since the rocket immunoelectrophoresis revealed that the amount of tomato PG in the normal mature fruit was increased about twofold when infected, the total pectolytic activity in the normal infected fruit is about two-third tomato PG and one-third fungal PG.

The immunological analysis showed the presence of a small amount of PG antigen which was not detected by the viscosity assay of the uninfected *nor* fruit. This was confirmed by gel electrophoresis where a trace of activity in the PG1 position was found.

In contrast to the normal mature fruit, no enhancement of fruit PG was recorded in the mature *nor* fruit following infection by *Rhizopus*. The difference in the response of the two fruits to fungal infection may lie in the possibility that the fungus affects PG production indirectly, through the whole ripening process. This assumption is supported by the finding that infection by *Rhizopus* also induced ethylene and CO_2 production in both the normal and the nonripening genotypes (4).

However, in the mutant fruit, which lacks an active ripening system, no fruit ripening follows exogenic ethylene treatment (13) and no acceleration in the production of fruit PG is induced by fungal infection. We may conclude that fungal infection does not induce expression of tomato PG where it would otherwise not be expressed, but it does seem to enhance, or advance, PG production in the normally ripening fruit.

LITERATURE CITED

1. Ali, Z. M., and Brady, C. J. 1982. Purification and characterization of the polygalacturonases of tomato fruits. Aust. J. Plant Physiol. 9:155-169.
2. Barash, I., and Khazzam, S. 1970. The relationship and properties of

pectic glycosidases produced by host and pathogen during anthracnose disease of avocado. Phytochemistry 9:1189-1197.
3. Barkai-Golan, R., and Kopeliovitch, E. 1983. Pectolytic and cellulolytic activity in *Rhizopus*-infected *rin* and *nor* tomato mutants. Mycopathologia 82:89-93.
4. Barkai-Golan, R., and Kopeliovitch, E. 1983. Induced ethylene evolution and climacteric-like respiration in *Rhizopus*-infected *rin* and *nor* tomato mutants. Physiol. Plant Pathol. 22:357-362.
5. Brady, C. J., MacAlpine, G., McGlasson, W. B., and Ueda, Y. 1982. Polygalacturonase in tomato fruits and the induction of ripening. Aust. J. Plant Physiol. 9:171-178.
6. Brady, C. J., Meldrum, S. K., McGlasson, W. B., and Ali, Z. M. 1983. Differential accumulation of the molecular forms of polygalacturonase in tomato mutants. J. Food Biochem. 7:7-14.
7. Buescher, R. W., and Tigchelaar, E. C. 1975. Pectinesterase, polygalacturonase, C_x-cellulase activities and softening of the *rin* tomato mutant. HortScience 10:624-625.
8. Cappelini, R. W. 1966. Growth and polygalacturonase production by *Rhizopus stolonifer*. Phytopathology 56:734-737.
9. Lisker, N., and Retig, N. 1974. Detection of polygalacturonase and pection lyase isoenzymes in polyacrylamide gels. J. Chromatogr. 96:245-249.
10. Ng, T. J., and Tigchelaar, E. C. 1977. Action of the non-ripening (nor) mutant on fruit ripening of tomato. J. Am. Soc. Hortic. Sci. 102:504-509.
11. Reisfeld, R. A., Lewis, U. J., and Williams, D. E. 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195:281-283.
12. Spalding, D. H. 1963. Production of pectinolytic and cellulolytic enzymes by *Rhizopus stolonifer*. Phytopathology 63:929-931.
13. Tigchelaar, E. C., McGlasson, W. B., and Buescher, R. W. 1978. Genetic regulation of tomato fruit ripening. HortScience 13:508-513.