Susceptibility of Phytopathogenic Bacteria to Wheat Purothionins In Vitro

R. FERNANDEZ DE CALEYA, BLANCA GONZALEZ-PASCUAL, F. GARCÍA-OLMEDO, AND PILAR CARBONERO

Departamento de Bioquímica, E.T.S. de Ingenieros Agrónomos, Universidad Politécnica, Madrid-3, Spain, and Departamento de Bioquímica, Microbiología y Fisiología, Instituto Nacional de Investigaciones Agrarias, Madrid-3, Spain

Purothionins are basic polypeptides with antimicrobial properties that are present in the endosperm of wheat and other cereal species. Susceptibility to wheat purothionins among phytopathogenic bacteria of the genera *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, *Erwinia*, and *Corynebacterium* has been investigated. Sensitive strains have been found in all of these genera except *Agrobacterium* (the only strain of *A. tumefaciens* available proved to be resistant). Minimal inhibitory concentrations (MIC) with partially purified crude purothionins ranged from 1 μg/ml for *C. sepedonicum* (C.5) to 540 μg/ml for *E. amylovora* (E.3). Minimal bactericidal concentrations (MBC) were not higher than twice the MIC value, except for *C. poinsettiae* (C.4) (MBC/MIC = 8). Purothionins α and β, obtained by carboxymethyl-cellulose column chromatography, were tested against *P. solanacearum* (P.2) and *X. phaseoli* (X.2); α purothionin was more active than β against X.2, and β more active than α against P.2. This suggests a relationship between polypeptide sequence and specificity of action.

Several kinds of antimicrobial substances have been isolated from higher plants. Some of them inhibit the growth of phytopathogenic microorganisms and have been occasionally implicated in plant immunity (6, 11).

Balls et al. (1) crystallized the protein moiety of a proteolipid from the endosperm of common wheat (*Triticum aestivum* L.). This material, which was named purothionin, has been recently shown (4, 5, 7) to be a mixture of at least two forms: purothionins α and β. Proteins electrophoretically similar to α and β purothionins have been found in barley (10) and in 22 species of the Aegilops-Triticum group (2). Both biochemical (9) and genetic (2) evidence points to heterogeneity within the α and β classes.

The crystalline purothionin of Balls et al. (1) was found to possess antimicrobial (12) and uterus-contracting (3) properties. Positive results were obtained when purothionin was tested in vitro against some human pathogenic bacteria and against yeast, but not against mycelial fungi. No in vivo activity could be demonstrated when purothionin was tested in mice against pneumococci and streptococci (12).

The activity of purothionin against plant phytopathogens has not been investigated. The interest of such study derives not only from the biological implications of the recently established diversity of purothionins but also from our observation (5) that certain common wheat varieties can easily yield up to 800 mg of crude purothionin per kg of flour. We report here in vitro tests of wheat purothionins against several bacterial phytopathogens.

MATERIALS AND METHODS

Bacteria. Strains of phytopathogenic bacteria tested against wheat purothionins appear in Table 1. *Agrobacterium tumefaciens* (Ag.6) was kindly supplied by J. Santa Maria and the remaining strains were supplied by A. Alfaro.

Purothionins. Crude purothionins (CP) were obtained from *Triticum aestivum* L. flour as previously described (5).

Filter-sterilized crude purothionins (FP) were prepared by filtering through a Sartorius membrane (0.2 μm) a 10 mg/ml solution of CP in acetate buffer (0.02 M acetate, 0.032 M NaCl, *p*H 6.5) previously centrifuged at 20,000 × *g* for 15 min.

Purification and quantitation of α and β purothionins in CP and FP were achieved by carboxymethyl (CM)-cellulose column chromatography as

described by Redman and Fisher (9). Concentrations were determined spectrophotometrically at 280 nm, with CP used as standard. CP contained 25 to 30% α and β purothionins in a 2:1 ratio. A twofold purification with an 80% yield results from filtering through the Sartorius membrane.

Chromatography fractions were filter-sterilized as above before use in sensitivity tests.

Media. The basal medium used was a nutrient broth containing 0.1% beef extract (Difco), 0.2% yeast extract (Oxoid), 0.5% peptone, and 0.5% sodium chloride; final *p*H was adjusted at 6.5.

Nutrient agar for diffusion tests was prepared by adding 1% Ionagar no. 2 (Oxoid) to the above medium.

Maintenance of phytopathogenic bacteria was on nutrient agar slants with 2% agar.

Sensitivity tests. The preliminary screening for susceptibility was performed with FP by the agar disc diffusion technique. Nutrient agar plates were surface-inoculated with a standardized bacterial broth culture. Whatman AA discs (13 mm) were impregnated with 75 μliters of a 6 mg/ml FP solution. Incubation at 25 or 30 C, depending on the strain, was carried out for 48 hr.

Sensitive bacteria were further tested in liquid medium. Minimal inhibitory concentrations (MIC) were determined in nutrient broth. Inocula were standardized to yield approximately 10^7 bacteria/ml. Concentrations of FP ranged from 900 μg/ml to 1 μg/ml. Incubation was performed at 25 C with shaking until inoculated controls without FP reached the end of log phase (48 to 72 hr). The MIC was taken as the lowest concentration of FP completely inhibiting growth as judged turbidimetrically at 600 nm. The minimal bactericidal concentration (MBC) was determined through subculture of a loopful of inhibited broth into fresh nutrient medium and also by streaking into quarter sectors of prepoured nutrient agar plates (2% agar) which were further incubated for 48 hr. The MBC was defined as the lowest concentration of FP yielding no growth after subculture.

Sensitivity tests with purified fractions of purothionin were performed in a similar manner.

RESULTS

Results of disc diffusion tests are summarized in Table 1. Bacteria inhibited by FP in solid media include both gram-positive and gram-negative strains. Nine of the 17 strains tested showed susceptibility. Enlargement of inhibition zones by lysis of bacterial colonies was observed upon cold storage for several weeks. This was particularly marked for *C. sepedonicum* (C.5) and *P. solanacearum* (P.2).

MIC and MBC data for the susceptible strains are presented in Table 2. A wide range of sensitivity was observed. In all these strains, except in *C. poinsettiae* (C.4), the MBC is no higher than twice the MIC.

TABLE 1. *Inhibition of phytopathogenic bacteria by filter-sterilized crude purothionin: disc diffusion test*

Microorganism	Inhibition
Pseudomonas savastanoi (P.1)	-[a]
Pseudomonas solanacearum (P.2)	+
Pseudomonas lachrymans (P.3)	-
Pseudomonas syringae (P.4)	-
Pseudomonas morsprunorum (P.5)	-
Pseudomonas tomato (P.6)	-
Pseudomonas marginalis (P.8)	-
Xanthomonas phaseoli (X.2)	+
Xanthomonas campestris (X.4)	+
Agrobacterium tumefaciens (Ag.6)	-
Erwinia carotovora (E.2)	-
Erwinia amylovora (E.3)	+
Corynebacterium flaccumfaciens (C.1)	+
Corynebacterium michiganense (C.3)	+
Corynebacterium poinsettiae (C.4)	+
Corynebacterium sepedonicum (C.5)	+
Corynebacterium fascians (C.6)	+

[a] -, No inhibition observed with Whatman AA, 13-mm disc impregnated with 75 μliters of a 6 mg/ml FP solution.

Fractions obtained by CM-cellulose ion exchange chromatography were tested against *P. solanacearum* (P.2) and *X. phaseoli* (X.2). Only those corresponding to the α and β purothionin peaks showed antibacterial activity. MIC and MBC of α and β purothionins against the two strains are compared with those of FP in Table 3. The activity of FP, which contained 50 to 60% α plus β purothionins in a 2:1 ratio, is consistent within experimental error with the measured activity of the α and β fractions. It is to be noted that α purothionin is less active than β against *P. solanacearum* (P.2), the opposite being true for *X. phaseoli* (X.2).

DISCUSSION

Stuart and Harris (12) concluded that purothionin was active in vitro mainly against gram-positive organisms, but also to some extent against gram-negative ones. Accordingly, all insensitive strains found in our studies were gram-negative bacteria.

Woolley and Krampitz (14) found that the action of purothionin was antagonized by phosphatides and suggested that its mode of action could be similar to that of other known polypeptide antibiotics. Indeed, later structural work on purothionins, although contradictory, shows some features that could relate purothionins to the basic cyclic polypeptide antibiotics. Molecular weight of purothionins is at least of the order of 6,000, which is considerably above that of the mentioned antibiotics. In any case, approximately 25 amino acid residues out of 100 are basic (lysine and

TABLE 2. *Inhibition of phytopathogenic bacteria by filter-sterilized crude purothionin: broth dilution test*

Microorganism	MIC[a]	MBC[a]	MBC/MIC
Pseudomonas solanacearum (P.2)	5	5	1
Xanthomonas phaseoli (X.2)	27	56	2
Xanthomonas campestris (X.4)	56	110	2
Erwinia amylovora (E.3)	540	540	1
Corynebacterium flaccumfaciens (C.1)	110	230	2
Corynebacterium michiganense (C.3)	450	450	1
Corynebacterium poinsettiae (C.4)	56	450	8
Corynebacterium sepedonicum (C.5)	1	1	1
Corynebacterium fascians (C.6)	680[b]		

[a] Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) expressed as micrograms per milliliter.
[b] Only partially inhibited.

TABLE 3. *Susceptibility of Pseudomonas solanacearum (P.2) and Xanthomonas phaseoli (X.2) to the purified α and β purothionin fractions*

Purothionins	*P. solanacearum* (P.2)		*X. phaseoli* (X.2)	
	MIC[a]	MBC[a]	MIC	MBC
α Purothionin fraction	3	6	6	12
β Purothionin fraction	1.5	1.5	12	25
Filter-sterilized crude purothionin	5	5	27	56

[a] MIC and MBC expressed as micrograms per milliliter.

arginine), and purothionins themselves are highly basic. Disulfide bridges, an average of 1 per 10 to 12 amino acids, probably fold the polypeptide into cycle-like loops.

Species-dependent differences in activity between α and β purothionins (Table 3) suggest that primary structure modifications may result in specificity changes. This matter merits further research when homogeneous polypeptides can be obtained.

Purothionin was considered to be bactericidal by Stuart and Harris (12) on the basis of survival curves. However, they observed growth at 48 hr in thioglycolate broth subcultures of cultures inhibited by purothionin concentrations up to 500 times higher than the MIC. This has not been the case with our test organisms under our experimental conditions (Table 2). A possible explanation of the discrepancy could be that the thioglycolate broth releases strongly bound purothionin from the cell surface.

It is premature at this stage to offer predictions concerning agronomical applications of purothionins. However such possibility should be explored, considering that seed-borne bacteria of the genera *Corynebacterium*, *Pseudomonas*, and *Xanthomonas* (8), as well as *Erwinia amylovora*, have been successfully controlled with antibiotics (13).

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