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EXPERIMENTAL ARTICLES

The Extracellular Proteases of the Phytopathogenic Bacterium *Xanthomonas campestris*

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Abstract—The culture liquids of three *Xanthomonas campestris* pv. *campestris* strains were found to possess proteolytic activity. The culture liquid of strain B611 with the highest proteolytic activity was fractionated by salting-out with ammonium sulfate, gel filtration, and ion-exchange chromatography. The electrophoretic analysis of active fractions showed the presence of two proteases in the culture liquid of strain B611, the major of which was serine protease. The treatment of cabbage seedlings with the proteases augmented the activity of peroxidase in the cabbage roots by 28%.

Key words: *Xanthomonas campestris*, extracellular proteases.

The crucifer-attacking black rot pathogen *X. campestris* pv. *campestris* is one of the most agriculturally important representatives of more than 120 *X. campestris* subspecies [1]. It is believed that the extracellular enzymes of xanthomonads (endoglucanases, polygalacturonate lyases, amylases, and proteases) and their exopolysaccharides play an important part in phytopathogenesis by promoting the colonization of host plants and the parasitism of xanthomonads in infected plants [2]. The role of individual enzymes in pathogenesis remains, however, unknown.

The wild-type strain of *X. campestris* causing turnip black rot was found to synthesize serine protease and metalloprotease [3]. Mutants lacking these enzymes were considerably less virulent to turnip than was the parent strain. *X. campestris* pv. *campestris*, which infects the conducting tissues of plants, clearly differs from *X. campestris* pv. *armoraceae* and *X. campestris* pv. *raphani*, which infect leaf mesophyll, in the range of extracellular proteases [4]. This confirms the suggestion that the extracellular proteases of phytopathogens are essential in pathogenesis. On the other hand, these enzymes may play the role of elicitors signalling to plants about the presence of phytopathogens.

This work was undertaken to gain more insight into the composition of the proteolytic complex of *X. campestris* and its role in phytopathogenesis. We present here the results of investigation of the proteolytic activity of the culture liquid of *X. campestris* pv. *campestris*, the extracellular proteases of this bacterium, and their effect on the response of cabbage plants to phytopathogen invasion.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. Experiments were carried out with three strains of *Xanthomonas campestris* pv. *campestris*, 8183a (obtained from UMB, Kiev, Ukraine), B610, and B611 (both obtained from VKM, the All-Russia Collection of Microorganisms). Cells for inoculation were grown for 24 h at 30°C on a solid medium containing (g/l) yeast extract, 2.0; glucose, 10.0; $CaCO_3$, 3.0; and agar, 15.0 [5]. Cells were washed off from the agar plates with a sterile 0.15 M solution of NaCl and then washed and suspended in the same saline solution. After measuring the concentration of cells in the suspension by the turbidimetric method [6], it was used for the inoculation (10^8 cells/ml) of nutrient medium 1159 (ATCC), from which glucose was omitted. The strains were grown at 30°C for 42–46 h. The culture liquids were obtained by centrifugation at 2500 *g* for 20 min.

Plant experiments. Seeds of the *Brassica oleraceae* cv. Gribovskii no. 1 (obtained from the Sortsemovoshch seed-producing company, Saratov, Russia) were washed with a detergent solution, sterilized in 2% calcium hypochlorite for 20 min, washed with sterile water, kept at 4°C for 18 h, and additionally sterilized in the hypochlorite solution for 5 min. Then the seeds were germinated on LB agar (which allows the absence of contaminating microorganisms to be checked) at 24°C for 2–3 days. Cabbage seedlings were aseptically transferred into 20-ml test tubes containing, as the growth substrate, 2 cm^3 of a coarse river sand and

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0.5 ml of a mineral medium of the following composition (g/l): K_2HPO_4, 0.7; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $Na_2MoO_4 \cdot 2H_2O$, 0.02; Na_2SO_4, 0.1; and NH_4Cl, 0.134 (pH 7.0). The seedlings were incubated for 1 week. Then 0.5-ml aliquots of proteases were added to the growth substrate, and the incubation was continued for one week.

Peroxidase assay. The activity of peroxidase in the seedling roots was determined as described earlier [8].

Determination of proteolytic activity. The proteolytic activity of the culture liquids was determined by the method of radial diffusion [9] using a solid medium containing a 50 mM Tris–HCl buffer (pH 8.0), 2% skim milk, and 2% agar. Aliquots (20 μl) of the culture liquids were placed in 4-mm-diameter wells made in agar plates, and the plates were incubated at 30°C for 24 h. The proteolytic activity of the culture liquids was assessed from the diameter of the zone of milk hydrolysis around the wells, using a solution of 10 μg/ml trypsin as the protease-positive control.

The proteolytic activity of protease preparations was determined with azocasein [9]. Aliquots (150 μl) of protease preparations were mixed with the same volume of a 0.4% solution of azocasein in a 50 mM Tris–HCl buffer (pH 8.0) and incubated at 37°C for 120 min. The reaction was stopped by adding 1.2 ml of 10% trichloroacetic acid. After incubation for 15 min, the mixture was centrifuged at 8000 *g* for 5 min. The supernatant (1.2 ml) was neutralized by adding 1.4 ml of 1 M NaOH, and its absorbance was measured at 440 nm. One unit of proteolytic activity (U) was defined as the amount of enzyme that increases the absorbance of the supernatant by unity. The effect of inhibitors (EDTA as an inhibitor of metalloproteases and phenylmethylsulfonyl fluoride (PMSF) as an inhibitor of serine proteases) on the activity of proteases was studied by preincubating protease–inhibitor mixtures at 30 min and determining proteolytic activity as described above. The concentrations of PMSF and EDTA in these experiments were 5 and 10 mM, respectively.

Fractionation of proteases. The proteins of the supernatant of the 42-h-old *X. campestris* pv. *campestris* B611 culture were precipitated by adding ammonium sulfate to 90% saturation. The precipitate was dissolved in a minimum volume of distilled water. The solution was dialyzed against water for 18 h at 4°C and purified first by gel filtration on a column with Sephadex G-25 and then by ion-exchange chromatography on a (1.5 × 7 cm) column with DEAE-Toyopearl 650M equilibrated with a 25 mM Tris–HCl buffer (pH 7.0). Adsorbed proteins were eluted from the column with a linear gradient (0–0.5 M) of NaCl in the same buffer (the total volume 50 ml) at a flow rate of 30 ml/h. Fractions with a volume of 3 ml were analyzed for protein content and proteolytic activity.

Electrophoresis. Proteases were analyzed by SDS-electrophoresis in PAAG with covalently bound casein [9]. The electrophoresis was carried out by the method of Laemmli [10] with ovalbumin (10 μg) as the molecular marker. Samples for analysis were prepared by mixing active fractions after ion-exchange chromatography with a sample buffer in a volume proportion of 1 : 1 and incubating the mixtures (100 μl) at 40°C for 30 min. After electrophoresis, the gel slab was washed with distilled water and incubated first in 1% Triton X-100 for 60 min and then overnight (with a single exchange of the buffer after 20 min) in a 10 mM Tris–HCl buffer (pH 8.0) containing 0.2 M NaCl, 10 mM $CaCl_2$, and 0.02% Brij. Then the gel slab was stained in a 0.5% Coomassie Brilliant Blue R-250 solution in 40% ethanol containing 10% acetic acid and then destained in 10% acetic acid. Zones with proteolytic activity were seen as transparent bands on a blue background.

Protein was quantified by the Bradford method [11].

Experimental results were statistically processed as described in handbook [12].

RESULTS AND DISCUSSION

Analysis by the radial-diffusion method showed that the culture liquids of all three *Xanthomonas* strains tested had proteolytic activity, the activities of the virulent strains B610 and B611 being about twofold higher than the activity of the avirulent strain 8183a (Fig. 1). Further experiments were carried out with strain B611, whose proteolytic activity was maximum. The culture liquid was obtained from a culture of this strain grown for 42 h, when its proteolytic activity was at a maximum. Optimal values of temperature and pH for the measurement of the extracellular proteases of *X. campestris* B611 were found to be 37°C and pH 7.5–8.0 (Fig. 2). All further measurements of proteolytic activity were carried out under these conditions.

During ion-exchange chromatography on DEAE-Toyopearl 650M, the adsorbed proteases of *X. campestris* B611 were eluted from the column at NaCl concentrations of 140 to 260 mM (fractions 6 through 9) (Fig. 3). The total yield of proteolytic activity was 8% with a maximum degree of purification in fraction 8 equal to 32 (Table 1). The electrophoretic analysis of the four active fractions (Fig. 4) showed the presence of zones of azocasein hydrolysis corresponding to a protein with a molecular mass of 45 kDa. In addition, fraction 6 showed the presence of a zone of azocasein hydrolysis corresponding to a 30-kDa protein. It should be noted that these estimations of the molecular mass are rough, since the conditions that were used for sample preparation (30-min incubation at 40°C) are insufficient for the complete defolding of proteases; however, more severe treatment conditions caused the irreversible denaturation of the proteases.

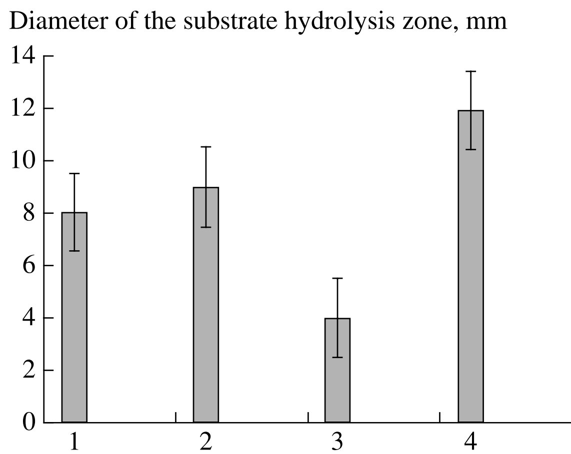

Fig. 1. The comparative proteolytic activity of the culture liquids of three *X. campestris* pv. *campestris* strains, (1) B610, (2) B611, and (3) 8183a, and (4) a solution containing 10 µg/ml trypsin. The error bars represent standard deviations.

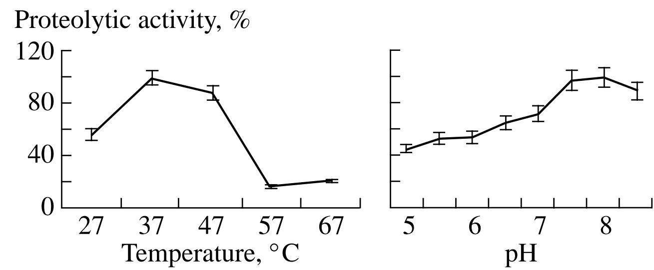

Fig. 2. The effect of temperature and pH on the activity of the extracellular proteases of *X. campestris* B611. Protease samples were incubated under specified conditions for 30 min, and then their proteolytic activity was determined as described in the Materials and Methods section. The effect of pH was studied using a 50 mM Tris–maleate buffer (pH 5.0–7.0) and a 50 mM Tris–HCl buffer (pH 7.5–8.5). The data are the mean values of triplicate experiments. The maximum proteolytic activity in the experiment was taken to be 100%.

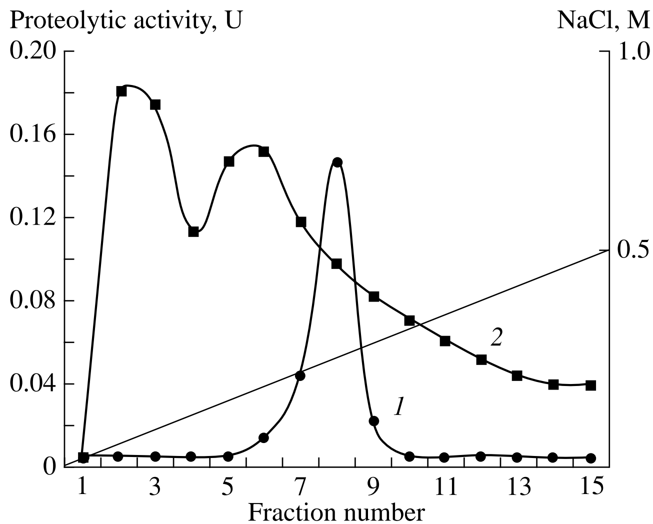

Fig. 3. The ion-exchange chromatography on DEAE-Toyopearl 650M of the culture liquid preparation of *X. campestris* B611: (*1*) the proteolytic activity of fractions (3 ml) determined with azocasein; (*2*) the protein elution profile.

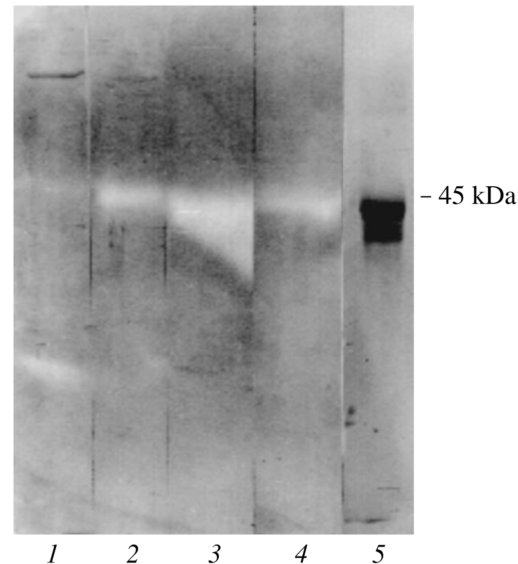

Fig. 4. The electrophoresis of (*1*) fraction 6, (*2*) fraction 7, (*3*) fraction 8, and (*4*) fraction 9 obtained by ion-exchange chromatography of *X. campestris* proteases and (*5*) ovalbumin. Fractions 6, 7, 8, and 9 contained 2.2, 1.1, 0.8, and 0.4 µg of protein, respectively.

Further experiments were carried out with the major 45-kDa protease present in fractions 7 through 9. The inhibitor analysis of this enzyme (Fig. 5) showed that it was inhibited by PMSF by 84–97%, whereas it was resistant to the action of EDTA. These data suggest the 45-kDa enzyme is serine protease, which is in agreement with the earlier observation of Dow *et al.* [3] that *X. campestris* produces serine proteases.

In the next set of experiments, the extracellular proteases of *X. campestris* B611 were tested for the ability to induce protective response in cabbage plants. For this, we used the biochemical method [8] based on the observation that the increased activity of peroxidase is one of the primary responses of plants to the invasion of phytopathogenic microorganisms [13]. The tests were carried out with 7-day-old cabbage seedlings, whose growth substrates were supplemented with one of the three preparations: the supernatant of the culture liquid of *X. campestris* B611 with proteolytic activity 0.061 U/ml, the preparation obtained by salting-out the culture liquid with ammonium sulfate (proteolytic activity 0.38 U/ml), and the serine protease–containing fraction

Table 1. Purification of extracellular proteases from the culture liquid of *X. campestris* B611

Preparation, procedure		Volume, ml	Proteolytic activity, U/ml	Total activity, U	Protein concentration, mg/ml	Specific activity, U/mg	Yield, %	Purification index
Culture liquid		260	0.028	7.280	0.100	0.28	100.0	1.0
Precipitation with ammonium sulfate (90% saturation)		9	0.250	2.250	0.300	0.84	28.0	3.0
Gel filtration on Sephadex G-25		25	0.050	1.250	0.100	0.50	15.6	1.8
Ion-exchange chromatography on DEAE-Toyopearl 650M	Fraction 6	3	0.014	0.042	0.045	0.33	0.5	1.2
	Fraction 7	3	0.045	0.135	0.022	2.00	1.7	7.1
	Fraction 8	3	0.147	0.441	0.016	9.00	5.5	32.0
	Fraction 9	3	0.022	0.066	0.007	3.00	0.8	10.7

obtained by ion-exchange chromatography with proteolytic activity 0.123 U/ml. In the control, the growth substrate was supplemented with an equivalent volume of the mineral medium. The data presented in Table 2 show that all the tested preparations with proteolytic activity augmented the activity of peroxidase in the cabbage roots. The more pronounced peroxidase-inducing effect of the culture liquid, whose proteolytic activity (0.061 U/ml) was lower than the activities of the two other preparations, can be accounted for by the fact that the intact culture liquid may contain other extracellular enzymes (as pectinases and endoglucanases) necessary for the induction of a protective response in plants [2]. The 28% increase in the activity of peroxidase in the cabbage roots under the action of the serine protease–containing preparation suggests that this protease is involved in black rot pathogenesis and in the induction of protective responses in the infected plants.

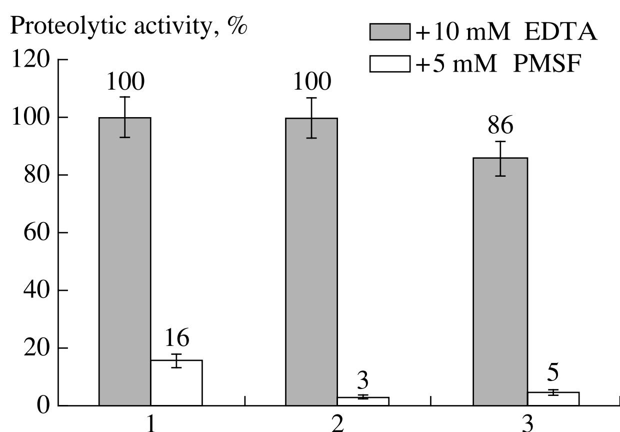

Fig. 5. The effect of EDTA and PMSF on the proteolytic activity of (1) fraction 7, (2) fraction 8, and (3) fraction 9 obtained by the ion-exchange chromatography of *X. campestris* proteases. Proteolytic activity was determined with azocasein. Proteolytic activity in the absence of inhibitors was taken to be 100%. The data are the means of triplicate experiments.

It is known that the proteolytic enzymes of many phytopathogens are important factors in their pathogenicity [3, 4]. For instance, the protease-negative mutants of the oilseed rape–infecting spot blotch phytopathogen *Pirenopeziza brassicae* were found to be avirent, but they restored their virulence as the synthesis of proteases was restored [14].

The role of proteolytic enzymes in phytopathogenesis is still the subject of debate. On the one hand, extracellular proteases may serve nutritive functions to producing microorganisms. On the other hand, they may promote the invasion of phytopathogens into plant tissues and destroy the protective proteins of plants. For instance, one of the proteases of *Erwinia carotovora* causing late blight of potato was found to be able to degrade potato lectin in vitro [15]. The metalloprotease of *X. campestris* can specifically degrade some hydroxyproline-rich glycoproteins related to the turnip extracellular matrix [16]. Taking into account the fact that these glycoproteins are involved in plant protection against diseases, it can be suggested that their degradation by *X. campestris* proteases may considerably facilitate the pathogen invasion.

Thus, the serine protease isolated from the culture liquid of the virulent *X. campestris* strain B611 has a putative molecular mass of 45 kDa, an optimum temperature of 37°C, and an optimum pH equal to 8.0. When added to the growth substrate of cabbage seed-

Table 2. The effect of the extracellular proteases of *X. campestris* B611 on the activity of peroxidase in the cabbage roots

Preparation	Peroxidase content, μg/ml
Control	1.4 ± 0.07 (100%)
Culture liquid with proteolytic activity 0.061 U/ml	2.2 ± 0.11 (157%)
Culture liquid precipitate with proteolytic activity 0.38 U/ml	1.9 ± 0.09 (135%)
Crude serine protease with proteolytic activity 0.123 U/ml	1.8 ± 0.07 (128%)

Note: Parenthesized are changes in the peroxidase content expressed as a percent of the control.

lings, this enzyme enhanced the activity of peroxidase in the cabbage roots by 28%, suggesting that it may be responsible for some aspects of black rot pathogenesis.

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