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Antimicrobial activity of glycosidase inhibitory protein isolated from *Cyphomandra betacea* Sendt. fruit

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ABSTRACT

Broad-spectrum antimicrobial activity of an invertase inhibitory protein (IIP) isolated from *Cyphomandra betacea* ripe fruits is documented. Minimal inhibitory concentration (MIC) values were determined by agar macrodilution and broth microdilution assays. This IIP inhibited the growth of xylophagous and phytopatogenic fungi (*Ganoderma applanatum*, *Schizophyllum commune*, *Lenzites elegans*, *Pycnoporus sanguineous*, *Penicillium notatum*, *Aspergillus niger*, *Phomopsis sojae* and *Fusarium mango*) and phytopathogenic bacteria (*Xanthomonas campestris* pvar vesicatoria CECT 792, *Pseudomonas solanacearum* CECT 125, *Pseudomonas corrugata* CECT 124, *Pseudomonas syringae* pv. *syringae* and *Erwinia carotovora* var carotovora). The IIP concentration required to completely inhibit the growth of all studied fungi ranged from 7.8 to 62.5 μg/ml. Phytopatogenic bacteria were the most sensitive, with MIC values between 7.8 and 31.25 μg/ml. Antifungal and antibacterial activities can be associated with their ability to inhibit hydrolytic enzymes. Our results indicate the possible participation of IIP in the plant defense mechanism and its potential application as a biocontrol agent against phytopathogenic fungi and bacteria.

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1. Introduction

In response to microbial attack, plants activate a complex series of responses that lead to the local and systemic induction of a broad spectrum of antimicrobial defences [8]. Accumulation of pathogenesis related protein (PRP) represents a major quantitative change in protein composition that occurs during the hypersensitive response and it includes the synthesis of low molecular weight compounds, proteins and peptides that have antimicrobial activity [4]. To date, several proteins with antibacterial and/or antifungal properties have been isolated and characterized from different plant species and tissue [19]. Some of these proteins are classified as thionins, lipid transfers proteins, plant defensins, chitinases, ribosome inactivating proteins and others [26–27,21,5,20]. Among crop plants, fungal and bacterial diseases are some of the major biotic stresses that contribute substantially to the overall yield loss. The agricultural industry employs a wide variety of synthetic antimicrobial agents, but they are associated with several drawbacks: lack of specificity, increasing incidence of resistance upon prolonged application and the environmental hazard inherent in residual toxicity. Antimicrobial proteins are currently receiving increased attention as defence compounds because of their dual action against phytopathogenic bacteria and fungi. In this sense, enzymes and enzyme inhibitory proteins involved in defence mechanisms that exhibit in vitro antifungal activity have been described [25,12,28]. We have recently isolated and character-

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ized inhibitory proteins from *Cyphomandra betacea* mature fruit and *Solanum tuberosum* tubers. These proteins inhibit in vitro invertase activity from different species, genera and even plant family [7,15]. Furthermore, these proteinaceous inhibitors are not invertase specific: fungal, bacterial and higher plant hydrolases are also inhibited [6]. Consequently, a possible participation of the proteinaceous inhibitor in the plant defence mechanism is proposed. The present paper reports the antifungal and antibacterial activity of the invertase inhibitory protein (IIP) isolated from *C. betacea* mature fruits.

2. Materials and methods

2.1. Plant material

C. betacea Sendt. plants were grown in a garden at San Miguel de Tucumán. Ripe fruits were collected and frozen at −20 °C until use.

2.2. Reagents

All chemicals used were of analytical grade and purchased from *Sigma–Aldrich Company* and *Merck, Argentina*.

2.3. Inhibitory protein purification

Inhibitory protein was isolated and purified according to Ordóñez et al. [15]. The protein concentration obtained was 4 μg of IIP/g fresh weight.

2.4. Protein determination

Protein concentration was determined by the method of Lowry et al. [10] using bovine serum albumin as standard.

2.5. Fungal strains

Wild-type strains of *Pycnosporus sanguineous*, *Ganoderma applanatum*, *Schizophyllum commune* and *Lenzytes elegans* (xylophagous fungi) were used. The strains were obtained from the Botanical Institute ''Miguel Lillo'', Tucumán, Argentina and were originally isolated from the local decaying wood. *Penicillium notatum*, *Phomopsis sojae* and *Fusarium mango* were provided by Instituto Nacional de Tecnología Agropecuaria (INTA, Tucumán). *Aspergillus niger* was provided by Cátedra de Micología, Facultad de Bioquímica, Química y Farmacia.

All strains were maintained in a solid culture medium (SM: 15 g/l malt extract, 5 g/l peptone and 15 g/l agar). Other media used were soft medium (SSM: 15 g/l malt extract, 5 g/l peptone and 6 g/l agar) and liquid medium (LM: 15 g/l malt extract and 5 g/l peptone).

2.6. Bacterial strains

The bacterial species tested were obtained from the Spanish Type Culture Collection (Department of Microbiology, Faculty of Biological Sciences, University of Valencia, Burjasot, Valencia, Spain). *Xanthomonas campestris* pvar vesicatoria, *Pseudomonas solanacearum*, *Pseudomonas corrugata*, *Pseudomonas syringae* pv. *syringae* and *Erwinia carotovora* var carotovora were used.

All strains were maintained in a solid culture medium (MHA: 15 g/l Müller Hinton agar, *Britania*). Other media used were soft medium and liquid medium (MHB: 22 g/l Müller Hinton broth).

2.7. Antimicrobial activity assay: minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) determination

2.7.1. Agar macrodilution method

The test was performed in MHA for bacteria or SM for mycelial fungi. A serial two-fold dilution of inhibitory protein was added to an equal volume of medium to get a concentration of 500 μg/ml and it was serially diluted by double technique to achieve 250–7.8 μg/ml. Control dishes containing the same volume of distilled water were prepared. After cooling and drying the plates were inoculated with 2 μl of each fungi spores (10^4 spores/ml) or bacterial suspension (10^4 colony forming units, CFU) and incubated aerobically at 27 °C for 16–20 h for bacteria or 72–96 h for mycelial fungi. A growth control of each tested strain was included.

2.7.2. Broth microdilution method

This test was used to determine MIC and MBC or MFC of the proteinaceous inhibitor against the test organism as recommended by the National Committee for Clinical Laboratory Standard [13,17]. This test was performed in sterile 96-well microplates. The inhibitory protein was properly prepared and transferred to each microplate well in order to obtain a two-fold serial dilution of the original sample. The inocula (100 μl) containing 10^4 CFU of bacteria or 10^4 spore/ml were added to each well. A number of wells were reserved in each plate for sterile control (no inoculum added) and inoculum viability (no inhibitory protein added). Plates were aerobically incubated at 27 °C for 16–20 h for bacteria or 72–96 h for mycelial fungi. After incubation, microorganism growth was determined by absorbance measurement at 625 nm using an automatic microplate reader Model 550 (BioRad Laboratories, Richmond, USA).

MIC was defined as the lowest concentration of inhibitory protein at which no growth was observed after incubation.

To determine MBC or MFC values, 10 μl of each culture medium with no visible growth were removed of each well and inoculated in MHA or SM plates, respectively. After aerobic incubation at 27 °C during 16–20 h for bacteria or 72–96 h for mycelial fungi the number of surviving organisms was determined.

MBC and MFC were defined as the lowest protein concentrations at which 99.9% of the microorganisms were killed. Each assay of this experiment was repeated thrice.

Micelial growth was observed both directly and by analysis of the samples under an OLYMPUS Bx50 microscope.

2.7.3. Bioautographic agar overlay method

The assay was carried out according to Nieva Moreno et al. [14] on Silica gel 60 plates by dot blot of IIP. After plates

(4 cm × 4 cm) were dried under sterile conditions, they were covered with 2 ml of brain–heart infusion (BHI) with 0.6% agar containing 10^5 CFU/ml or 2 ml of SSM containing 0.3 ml of a spore suspension (10^4 spores per ml). Fungitoxic activity was macroscopically visualized after incubation at 27 °C in a moist chamber for 96 h. Bactericidal activity was visualized when the plates were sprayed with a 2.5 mg/ml MTT solution (3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium) in PBS buffer (10 mM sodium phosphate buffer, pH 7, with 0.15 M NaCl). Plates were incubated at 27 °C for 1 h in the dark for colour development.

2.8. Sulfhydryl groups determination

The concentration of sulfydryl groups was determined by Ellman's reagent, 5′,5′-dithio-bis-2-nitrobenzoic acid (DTNB) according to the method of Welch and Norvell [29] with minor modifications. Bacteria suspensions were treated with different concentrations of inhibitory protein (around the MIC values) in NaCl 0.9% during 6 h at room temperature. Then, 100 μl of the mixture was added in 1.9 ml of 0.2 M Tris–HCl buffer pH 8.2 containing 0.2 M Na-EDTA. Then, 0.5 ml of 10 mM DTNB was added to the reaction. After 15 min at room temperature, the reaction mixture was centrifuged at 600 × *g* and the supernatant was assayed for reactive sulfhydryl groups at 412 nm on a Beckman DU 650 spectrophotometer.

2.9. Cytotoxicity test

The protein was tested using initial concentrations of 10, 100 and 1000 μg/ml in vials containing 5 ml of NaCl 0.15 M and 10 brine shrimp larvae in each of three replicates, using the method of the McLaughlin et al. [11]. Survivors were counted after 24 h. The data were processed using a Finney program on a simple computer and LD_{50} values were obtained.

3. Results

The antifungal activity of the purified invertase inhibitory protein of *C. betacea* fruit was evaluated in vitro against *P. sanguineous*, *G. applanatum*, *Schyzophyllum commune* and *Lenzites elegans* (xylophagous fungi), *P. notatum*, *Ph. sojae*, *F. mango* (phytopthogenic fungi) and *A. niger*. The IIP exerted prominent antifungal activity against *G. applanatum* and *S. commune* and moderate activity against *L. elegans*, *P. notatum*, *Ph. sojae* and *F. mango* (Table 1). *A. niger* did not show crescents of retarded growth even with a high dose of protein (500 μg/ml). The MFC values obtained were around 60–125 μg/ml. *G. applanatum* was the most sensitive to inhibition requiring less than 20 μg/ml as a minimum inhibitory dose (Fig. 1A) while *P. notatum* needed 31.25 μg/ml (Fig. 1B). Purified IIP mantained at 95 °C for 10 min showed no inhibition on fungi strains tested.

Antibacterial activity was determined by macrodilution, microdilution and bioautographic assay. The MIC values obtained by macrodilution and microdilution techniques indicate that bacterial strains were more susceptible to the IIP than fungi strains. MIC values for *P. syringae*, *X. campestris* and *E. carotovora* were around 10 μg/ml (Table 2). The bioautographic technique may be considered one of the most effective assays for the detection of antimicrobial compounds and bacteria growth inhibition can be visualized at a lower concentration than with other methods. Fig. 2 shows the *P. syringae* growth inhibition at the smallest concentration (7.8 μg/ml) of IIP. This IIP was cytotoxically effective against

Table 1 – Antimicrobial activity of proteinaceous inhibitor from *C. betaceae* against phytopathogenic and xylophagous fungi

Fungi strains	MIC and MFC values from Inhibitory protein (μg/ml)	
	MIC	MFC
G. applanatum	15.60 ± 0.5	31.25 ± 1.0
S. commune	31.25 ± 1.0	31.25 ± 1.0
P. sanguineous	31.25 ± 1.0	62.5 ± 1.5
L. elegans	62.50 ± 1.5	250 ± 10
F. mango	31.25 ± 1.5	125 ± 5
P. notatum	31.25 ± 1.5	125 ± 5
Ph. sojae	62.50 ± 1.5	125 ± 5

MIC and MFC values obtained for each microorganism were calculated according to described in Section 2.

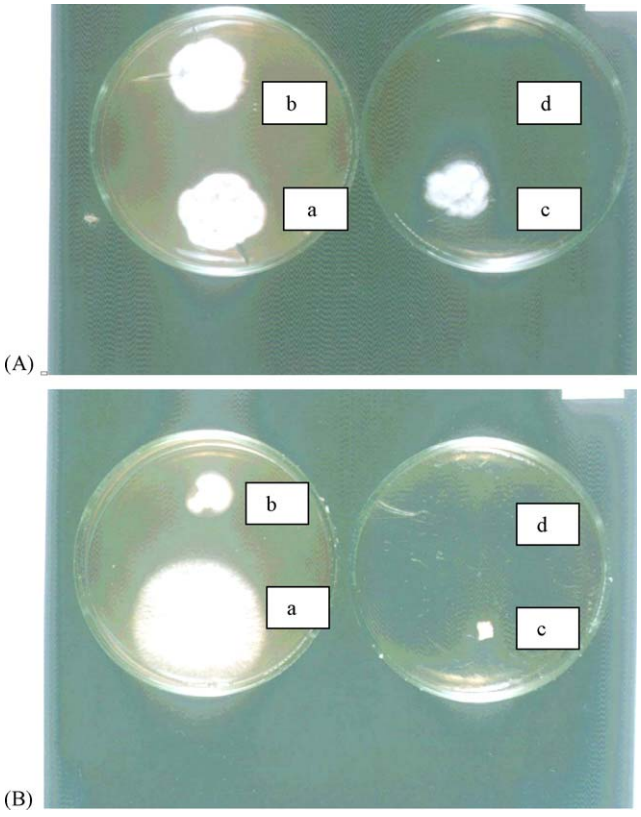

Fig. 1 – Antifungal activity of IIP. (A) Growth inhibition of *G. applanatum* by different concentrations of protein: (a) control; (b) 3.90; (c) 7.80; (d) 15.60 μg/ml of protein. (B) Growth inhibition of *P. notatum* by different concentrations of protein: (a) control; (b) 15.60; (c) 31.25; (d) 62.5 μg/ml of protein.

Table 2 – Antimicrobial activity of proteinaceous inhibitor from *C. betaceae* against phytopathogenic bacteria

Bacteria strains	MIC and MBC values from Inhibitory protein (μg/ml)	
	MIC	MBC
X. campestris	7.80 ± 0.1	15.6 ± 0.5
P. syringae	7.80 ± 0.1	31.2 ± 1.0
E. carotovora	15.60 ± 0.5	15.60 ± 0.5
P. corrugata	60.20 ± 1.5	60.2 ± 1.5

MIC and MFC values obtained for each microorganism were calculated according to described in Section 2.

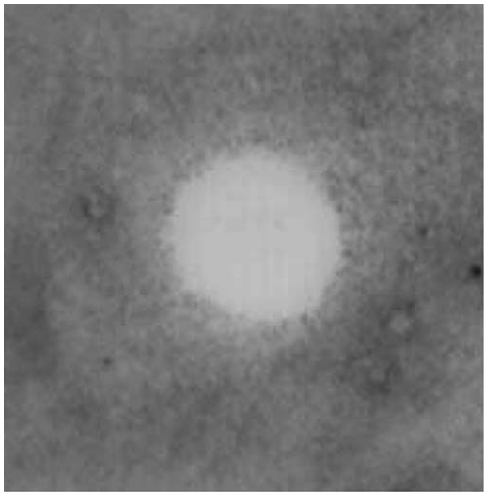

Fig. 2 – Bioautography assay. IIP (7.8 μg) were loaded on silica gel plates and the growth inhibition was detected against *Pseudomonas syrinage*. The plates were revealed according to Section 2.

assayed microorganisms at concentrations smaller than 250 μg/ml. The protein was found to be relatively non-toxic for brine shrimp larvae *Artemia salina* with LD_{50} value greater than 350 μg/ml.

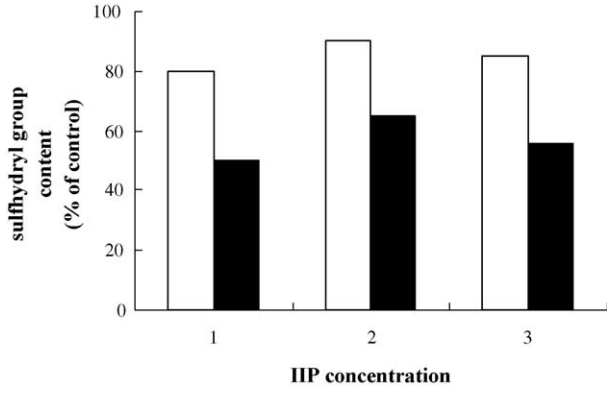

Fig. 3 – Analysis of sulfhydryl groups contents in different bacteria suspensions after 6 h of treatment with (□) 30 μg/ml and (■) 100 μg/ml of IIP. 1, *P. syringae*; 2, *E. carotovora*; 3, *X. campestris*.

In order to elucidate the probable action site of IIP, we examined the influence of the IIP on sulfhydryl groups in bacteria. Bacterial suspensions treated with different concentrations of IIP during 6 h, contained lower concentrations of sulfhydryl groups than control (Fig. 3).

4. Discussion

The current studies were an extension of a previous report where isolation and characterization of invertase inhibitory protein (IIP) from *C. betacea* fruit are described. The purification of the invertase inhibitor from *C. betacea* involved precipitation at pH 3, fractionation with ammonium sulfate, gel filtration on Sephadex G-100 and ion exchange cromatography on DEAE Sepharose CL-4B. This IIP had a monomeric structure with a Mr of 19,000, high temperature stability, and it retained its biological activity against invertase after incubation at 90 °C during 5 min, the same as several proteinase inhibitors [15,24]. IIP were signed as invertase activity modulator in other systems [18]. Although, *C. betaceae* IIP has a subcellular localization in the cell wall while the soluble acid invertase was localized in isolated vacuoles. These results suggest that the putative invertase inhibitor lacks a role in acid soluble invertase activity regulation in these tissues. In a previous work, we demonstrated an inhibitory activity on cell wall degrading enzymes isolated from fungi and bacteria and the inhibitory effect of *C. betaceae* IIP on the growth of phytopathogenic bacteria by the agar diffusion method [6,16]. Thus, the putative invertase proteinaceous inhibitor is likely related to the known pathogenesis related proteins (PRP) and to the antimicrobial proteins.

According to the MIC values obtained in this work, the IIP from *C. betacea* is one the most effective antibacterial and antifungal proteins reported until present. Several proteins isolated from other plant species present MIC and MBC values from approximately 50 μg/ml to 10 mg/ml [19,26,21]. Protease inhibitors have recently been described that are considered important components of the plant defense mechanism for their inhibition of fungi protease, with in vitro antimicrobial activity [12,9,22,3]. These protease inhibitors present MIC values from 40 to 1000 μg/ml.

Proteins exert their antimicrobial activity by means of different mechanisms such as inhibition of several microorganism-secreted enzymes [25], or they can interfere directly with carrier proteins [1,23]. Some peptide molecules form a channel on cell membranes and cells die because of cellular content loss [2]. The IIP inhibit in vitro fungal or bacterial hydrolases [6]. Otherwise, since the bacterial plasmatic membrane is relatively impermeable to the DTNB, there is evidence that IIP may be affecting essential sulfhydryl groups of carrier protein located at the plasmatic membrane.

5. Conclusions

The potency exhibited by IIP against all bacteria and fungi strains tested, suggest that it can play an important role in the defense of plants against agronomically important phytopathogens. Furthermore, its low molecular mass, coupled with

its high antibacterial potency, should make it a strong candidate for the exploitation of its biological activities.

The simultaneous accumulation of the antimicrobial protein and sugar during tomato ripening was correlated with the characteristic development of pathogen resistance that occurs in fruits during ripening.

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