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Inhibition of Hydrolytic Enzyme Activities and Plant Pathogen Growth by Invertase Inhibitors

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The invertase inhibitory protein isolated from *Cyphomandra betacea* Sendt and *Solanum tuberosum* inhibited the invertase activity from different species, genera and even plant family. Furthermore, proteinaceous inhibitors are not invertase specific; fungal, bacterial and higher plant enzymes including polygalacturonase, pectinase, pectin lyase, α-L-arabinofuranosidase and β-glucosidase are also shown to be inhibited.

Both inhibitors exhibited an *in vitro* antibacterial action against phytopathogenics strains of *Xanthomonas campestris* pvar vesicatoria CECT 792, *Pseudomonas solanacearum* CECT 125, *Pseudomonas corrugata* CECT 124, *Pseudomonas syringae* and *Erwinia carotovora* var carotovora.

Keywords: *Cyphomandra betacea* Sendt; Invertase; Pathogenesis related protein; Plant defense mechanism; Invertase proteinaceous inhibitor; *Solanum tuberosum*

INTRODUCTION

Potato tubers are known to possess a soluble vacuolar invertase. Schwimmer *et al.*[1] reported preliminary evidence for the existence of a proteinaceous endogenous inhibitor of the soluble acid invertase activity. Pressey[2–4] further investigated the inhibitor, and proposed that it was a soluble acid invertase biochemical modulator. Pressey and Shaw[5] reported that the inhibitor level decreased when the potato tubers were stored at low temperatures and increased at higher ones. Invertase levels also responded to temperature changes, but exhibited the opposite behavior. This led to the theory that the inhibitor concentration regulates the hydrolysis of sucrose to hexoses. Since this original proposal, many communications have followed concerning several plant species,[6–10] examining the putative regulatory model of the invertase activity by proteinaceous inhibitors. Isla *et al.*[11] found that the soluble invertase was inhibited to a greater extent by the known potato lectin than by the previously identified proteinaceous inhibitor. It was concluded that the binding of the potato lectin and of the endogenous inhibitor to the invertase are mutually exclusive. The soluble invertase (acid β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) together with its substrate (sucrose) and products (fructose and glucose) were found to be located within the vacuole.[12] Neither the proteinaceous inhibitor nor *Solanum tuberosum* agglutinin were at a detectable concentration in vacuolar or protoplast preparations, indicating the lack of a role for the *in vivo* regulation of the soluble invertase activity. The aim of this study was to show the lack of specificity of the putative proteinaceous inhibitor of the potato invertase. The inhibition of several plant and microbial enzymes by the proteinaceous inhibitor are reported here together with its inhibitory action on the growth of several plant pathogenic bacteria. Consequently, a possible participation of the proteinaceous

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inhibitor in the plant defence mechanism is discussed.

MATERIALS AND METHODS

Plant Material

Mature *Solanum tuberosum* L var Kennebec tubers maintained at 4 or 25°C for 25 d were used. Tubers were obtained from the Estación Experimental Agroindustrial "Obispo Colombres", Tucumán, Argentina. Ripe fruits of *Cyphomandra betacea* Sendt were collected in a garden at San Miguel de Tucumán, Argentina, and frozen at −20°C until use.

Reagents

All chemicals used were of analytical grade. *p*-Nitrophenyl-α-L-arabinofuranoside (*p*NPA) was from Sigma, Silica gel G60 F_{254} plates and MTT (3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide) were from Merck.

Inhibitor Preparation

Extraction and purification of the proteinaceous inhibitor from *S. tuberosum* tubers were performed according to Isla *et al.*[13] with slight modifications. Briefly, the juice of potato tubers (500 g) was extracted with a juicer and solid Na_2SO_3 was added up to 0.16 M. The mixture was centrifuged at 26,000*g* for 10 min, the supernatant adjusted to pH 4 and re-centrifuged at 26,000*g* for 10 min. The pellet was suspended in 0.2 M NaCl and adjusted to pH 6. The preparation was stirred for 1 h at 4°C, centrifuged at 26,000*g* for 10 min and the supernatant was then adjusted to pH 5.5. Solid ammonium sulfate was added up to 25% saturation. After 10 min at 4°C and re-centrifugation, the supernatant was adjusted to pH 5 and solid ammonium sulfate added up to 35% saturation. The precipitate was collected by centrifugation, dissolved in 0.2 M NaCl and adjusted to pH 6. The preparation was dialyzed against 0.2 M NaCl for 90 min and clarified by centrifugation. Further inhibitor purification was made by gel filtration on a Sephadex G-100 column (90 × 1.2 cm) equilibrated and eluted with 0.2 M NaCl. Fractions of 3 ml were collected. Proteins were detected by absorbance at 280 nm. Agglutination was checked with human red blood cells of groups A, B and O from healthy donors. The inhibitory effect on invertase activity was assayed in each purification step and on all tubes. The inhibitor preparation was stored at −20°C until use. The invertase inhibitor concentration obtained was 2.8 μg inhibitory protein/g fresh weight.

The proteinaceous inhibitor from *Cyphomandra betacea* Sendt fruit was purified according to Ordóñez *et al.*[18] The invertase inhibitor concentration obtained was 4 μg inhibitory protein/g fresh weight.

Invertase Preparations

Acid soluble invertases from *S. tuberosum* tubers, *Ricinus communis* and *Carica papaya* leaves, were prepared according to Isla *et al.*,[14] Prado *et al.*[15] and Rojo *et al.*,[16] respectively. Acid soluble invertase from *Equisetum giganteum* and *Cyphomandra betacea* Sendt was prepared according to Leal *et al.*[17] and Ordóñez *et al.*,[18] respectively.

Estimation of Molecular Mass

The molecular mass of the native proteinaceous inhibitors was estimated by the method of Andrews[19] on a calibrated column of Sephadex G-75 (60 × 1.5 cm). The standards used were: bovine serum albumin (67000), ovalbumin (43000), chymotrypsinogen A (25000) and ribonuclease A (13700).

SDS-polyacrylamide Gel Electrophoresis

Samples (2 μg of protein) were treated and analyzed by electrophoresis as described by Laemmli.[20] The Mr markers used were: lysozyme (14300), β-lactoglobulin (18400), pepsin (34700), ovalbumin (45000) and bovine serum albumin (67000). Proteins were detected by $AgNO_3$ impregnation.[21]

Isoelectric Focusing

The isoelectric point was determined in the pH range 4–7 on a Bio Rad Mini-Protean II gel apparatus.

Protein Determination

Protein concentration was determined by the method of Lowry *et al.*[22] using bovine serum albumin as standard. Protein concentration in the column fractions was determined by absorption at 280 nm.

Fungus Strains

Wild-type strains of *Pycnoporus sanguineus* and *Lenzites elegans* (xylophagous fungi) were used. The strains were obtained from the Botanical Institute "Miguel Lillo", Tucumán, Argentina, and were originally isolated from local decaying wood. An isolate of *Penicillium notatum* pathogenic to citrus were provided by Instituto Nacional de Tecnología Agrícola (INTA).

Fungal Culture Conditions

Fungi were grown in 250 ml of liquid medium, containing 1% (w/v) sodium polygalacturonate, citrus pectin or sucrose; 0.3% (w/v) yeast extract and 1% (w/v) peptone at 30°C. After strong growth was obtained (10–20 days), the culture was filtered through Whatman No. 4 paper and centrifuged at 21,000*g* for 10 min. Solid ammonium sulfate was added to the supernatant. The precipitate obtained between 30 and 80% of saturation was collected by centrifugation, dissolved in 10 mM sodium acetate buffer, pH 4.5 containing 1 mM 2-mercaptoethanol (buffer A) and was dialyzed against the same buffer for 2 h. The sample was then applied to a Sephadex G-150 column (37 × 2.5 cm) previously equilibrated with buffer A. Fractions with enzyme activity (polygalacturonase, invertase, pectinase, pectin-lyase, arabinofuranosidase or xylopyranosidase) were collected, pooled and kept at −20°C until use.

Enzyme Assays

Invertase activity. Reaction mixtures consisted of 25 µl (2 EU) of invertase preparation (xylophagous fungi or higher plant invertase), 40 µl of 0.2 M sodium acetate buffer pH 4.5, 15 µl of 0.6 M sucrose and water to a final volume of 150 µl. Incubations were performed at 37°C for 30 min. Reactions were stopped by a cupric-alkaline reagent. Reducing power release was measured by the method of Somogyi[23] as described by Nelson.[24]

α-L-Arabinofuranosidase activity. 40 µl of 1 mM *p*NPA in 20 mM sodium acetate buffer pH 4.65, was incubated with 40 µl (1.5 EU) of the enzyme from xylophagous or phytopathogenic fungi or with 100 µl of the *S. tuberosum* tubers enzyme at 37°C for 60 min and distilled water to a final volume of 200 µl. The reaction was stopped by addition of 1.6 ml of 0.2 M Na_2CO_3 and then 250 µl of distilled water. The amount of *p*-nitrophenol liberated was determined at 405 nm. One enzyme unit is defined as the amount of the enzyme that releases 1 µmol of the aglycon per min at 37°C, at the optimum pH.

Pectin lyase activity. It was determined in a Beckman spectrophotometer by monitoring the absorption increase at 235 nm as described by Albersheim and Killias.[25]

Polygalacturonase activity. Enzyme reactions were done using 50 µl of 1% sodium polygalacturonate, 100 µl of enzyme (1.5 EU), 40 µl of 0.2 M sodium acetate buffer pH 4.65 and distilled water to a final volume of 250 µl. The mixture was incubated at 37°C for up to 60 min. The activity was assayed by the method of Somogyi-Nelson.[23,24]

Pectinase activity. The reaction mixture contained of 50µl of 1% (w/v) citrus pectin in 50 mM Na_2HPO_4/KH_2PO_4 buffer pH 6 with 1.5 M NaCl, 100 µl of culture filtrate medium or *C. betacea* enzyme and 40 µl of 0.2 M sodium acetate buffer pH 4.65. The final volume was 250 µl. The mixture was incubated at 37°C for up to 60 min. The activity was assayed by measuring sugar-reducing release by the method of Somogyi-Nelson.[23,24] One unit of the pectinase, poligalacturonase and invertase activity was defined as the amount of the enzyme that liberates 1 µmol of reducing sugars per min at 37°C.

Inhibition assays. Invertase inhibitors were assayed by measuring the decrease in enzyme activity after addition of the inhibitor to the reaction mixture using the experimental methods previously described. Purified *C. betacea* inhibitor (0–100 µg) or *S. tuberosum* inhibitor (0–120 µg) with and without heating (5 min at 100°C) was used in all studies. Samples containing enzyme-proteinaceous inhibitor and buffer were incubated at 4°C during different times (0–30 min). Reactions were started by substrate addition and were incubated at 37°C up to 60 min.

Antibacterial Assays

Microorganisms. Bacterial strains were obtained from the Spanish Type Culture Collection (Burjasot, Valencia, Spain). *Xanthomonas campestris* pvar vesicatoria CECT 792, *Pseudomonas solanacearum* CECT 125, *Pseudomonas corrugata* CECT 124, *Pseudomonas syringae* and *Erwinia carotovora* var carotovora were used.

Agar diffusion method. The agar well technique was carried out in Petri dishes containing a fixed volume of potato-agar medium supplemented with 2% glucose. After the media had solidified 2 ml of the soft agar medium containing 10^5 CFU (colony forming units)/ml was added and evenly spread over the whole plate. Small wells (5 mm diam.) were cut in the agar using a cork borer. A fixed volume (50 µl) of different inhibitor concentrations (0–1.2 mg/ml in PBS buffer) and PBS buffer as control were loaded in each well. Petri dishes were incubated at 37°C for 24 h. The diameter of the inhibition zones was measured. The minimum inhibitory concentrations (MICs) were determined experimentally and confirmed with software based on the Michaelis-Menten equation.[26]

Bioautographic Agar Overlay Method

Inoculum for the assay. 2 ml of BHI medium (brain–heart infusion) with 0.6% agar was used as the solid media for the overlays. The media was maintained in a water bath at 45°C and was added with a bacterial suspension. The final bacterial concentration in the solid medium was approximately 10^5 CFU/ml. These suspensions were prepared immediately before the test was carried out.

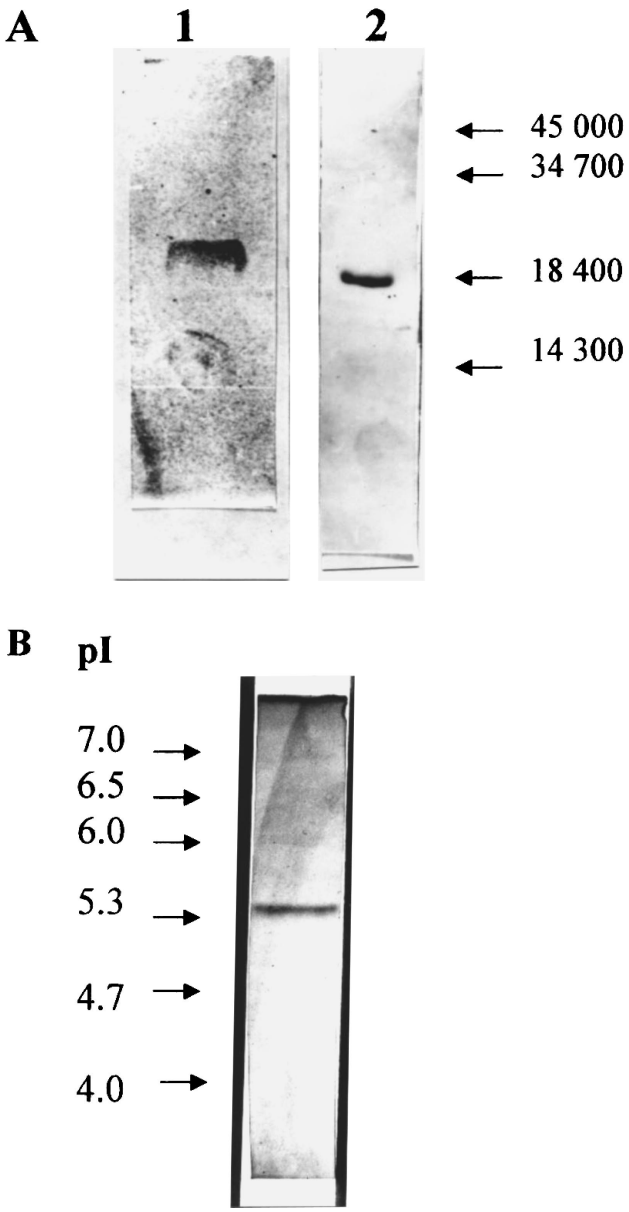

FIGURE 1 (A) SDS-PAGE of: *Lane 1*: purified invertase proteinaceus inhibitor of *C. betacea* fruits. *Lane 2*: purified invertase proteinaceous inhibitor of *S. tuberosum*. The arrows show the position of the *Mr* markers. Gels were stained with $AgNO_3$. (B) An IEF-PAGE of potato proteinaceous inhibitor after purification on Sephadex G-100 shows only one protein (pI 5.3). The values for pI were determined from standard protein (Pharmacia).

Bioautography. Silica gel G60 F_{254} plates (7 × 3 cm) were used. Proteinaceous inhibitor dilution (1–50 μg) from *S. tuberosum* tubers was applied on the plates and these were dried under sterile conditions. The inoculum (2 ml) was rapidly distributed over the TLC plate with a sterile pipette. After solidification of the medium, TLC plates were incubated at 37°C for 24 h. The bioautograms were sprayed with a solution of MTT (2.5 mg/ml in PBS buffer) and incubated at 30°C for 2 h. Clear inhibition zones were observed against a purple background indicating growth inhibition.

RESULTS

The procedure applied to the *S. tuberosum* invertase inhibitor purification was a modification of that used by Isla *et al.*[13] However, 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 chromatography on DEAE Sepharose CL-4B. Molecular exclusion chromatography indicated an M_r of about 18,000 for *S. tuberosum* and 19,000 for *C. betacea* invertase inhibitors, respectively (data not shown). Both invertase inhibitors were purified to electrophoretic homogeneity. A single band appeared in all of our experiments after $AgNO_3$ staining (Fig. 1A, lanes 1 and 2). The pI for *S. tuberosum* invertase inhibitor was 5.3 (Fig. 1B).

A study of the effect of proteinaceous inhibitors from different sources on higher plant and xylophagous fungal invertases revealed that the action of these inhibitors is not limited to the invertase from the same source. Both invertase inhibitors partially inhibit other plant or fungal invertases. This effect was not a function of the preincubation time. The inhibition was pH-dependent with maximal inhibition at approximately pH 4.75. The amount of inhibitor required for the maximal invertase inhibition varied considerably with the enzyme source (Table I). All these experiments were performed at pH 4.75 at which the highest inhibitory effect was observed.

The effect of invertase proteinaceous inhibitors on other hydrolytic activities from *C. betacea* fruits, *S. tuberosum* tubers and xylophagous fungi (Table II) were analyzed. The inhibitory activity of the proteinaceous inhibitors was observed in all cases indicating that these inhibitors are not specific for invertases but are, at least, general inhibitors of glycosidases and polysaccharases. Inhibitor levels needed to inhibit these enzymes suggests the stochiometric ratio of inhibitor to enzyme is not 1:1. Percentage inhibition increases with increasing preincubation time between enzyme and inhibitor until it plateaus at 10 min All experiments were therefore carried out with a preincubation time of 10 min.

The next question examined was the inhibitory capacity of the proteinaceous inhibitor on enzymes produced by phytopathogenic fungi.

Our experiments show that the proteinaceous inhibitors from *S. tuberosum* and *C. betacea* inhibit the α-L-arabinofuranosidase, pectinase and polygalacturonase activities from *P. notatum* (Table II). The protein concentration necessary to cause the

TABLE I Effect of invertase proteinaceous inhibitors on higher plant and fungal invertases. The minimal amount of proteinaceous inhibitor that produces the maximal inhibition is expressed as the inhibitor amount (μg of protein) in the incubation mixture (150 μl). The reaction mixture contained 2 invertase units/ml in 0.2 M sodium acetate buffer, pH 4.75. Inhibitor preparations of *C. betacea* and *S. tuberosum* were heated for 5 min at 100°C, retaining their inhibitory action[11,18]

	S. tuberosum inhibitor		*C. betacea* inhibitor	
Invertase source	Amount (μg)	Inhibition (%)	Amount (μg)	Inhibition (%)
S. tuberosum tubers	9.50	87	1.61	36
C. betacea fruits	21.08	72	3.20	74
Oryza sativa leaves	12.23	39	1.20	76
Carica papaya leaves	7.40	33	12.70	53
Pycnoporus sanguineus	21.02	97	11.60	52
Lenzites elegans	52.57	47	47.50	35
Ricinus communis	15	56	15	68
Equisetum giganteum	24.05	45	24.05	68

Mean values (for $n = 5$) are shown with a spread of ±3%.

maximal inhibition was lower for *C. betacea* than for *S. tuberosum* proteinaceous inhibitors.

Antimicrobial activity. Using an agar well technique, antibacterial activity was detected in all samples tested against with plant pathogenic strains (Fig. 2). The minimal inhibitory concentration (MIC) of the proteinaceous inhibitor isolated from *C. betacea* fruits against *Erwinia carotovora* and *Pseudomonas syringae* was 102.16 and 67.12 μg/ml, respectively.

Bioautography may be considered one of the most effective assays for the detection of antimicrobial compounds. The inhibition zones of bacterial growth (Fig. 3) were visualized at far lower concentrations (2 μg of protein) than that used in the agar diffusion technique.

DISCUSSION

Invertase activity regulation by products has been found in some plants.[11,15,17,27–29] This modulation has been confirmed working with vacuolar suspensions from potato tubers.[30] However, Pressey[2] proposed a regulation of the potato soluble acid invertase by a proteinaceous inhibitor. Nevertheless, the physiological reasons for the existence of an endogenous complex between invertase and the proteinaceous inhibitor is not clear, specially when a large excess of non functional invertase is considered.

In our laboratory, it has been shown that the soluble acid invertase and most of the protoplast sucrose have a vacuolar localization, while the proteinaceous inhibitor was found in the cell wall of potato tubers.[12] On other hand, some papers have reported inhibition of soluble invertases by peptides in several species[6,8,31,32] and in some instances a regulatory role towards cell wall invertases has been attributed to the inhibitory protein.[9,10,33,34] The tobacco apoplasmic invertase inhibitor has been cloned and the specificity of the recombinant inhibitor analyzed.[35] It was reported that the recombinant inhibitor inhibited plant cell wall and soluble acid invertases but fungal invertases from *S. cerevisiae* and *C. utilis* were not affected. In addition, it was demonstrated that after ectopic expression of a

TABLE II Effect of invertase proteinaceous inhibitors on hydrolytic activities from higher plants and xylophagous fungi. The maximal inhibition of enzymatic activity obtained with 10 μg of proteinaceous inhibitor is shown

		Maximal inhibition (%)	
Enzyme activity assayed	Enzyme source	*S. tuberosum* inhibitor	*C. betacea* inhibitor
Pectinase	*C. betacea*	15	59
	P. notatum	20	40
	L. elegans	48	48
	P. sanguineous	79	43
Polygalacturonase	*C. betacea*	44	27
	P. notatum	36	46
	L. elegans	49	47
	P. sanguineous	10	50
Pectinlyase	L. elegans	45	43
	P. sanguineous	43	55
β-Glucosidase	L. elegans	20	25
	P. sanguineous	40	66
α-L-arabinofuranosidase	*S. tuberosum*	10	23
	P. notatum	48	23

Mean values are (for $m = 5$) shown ±3%.

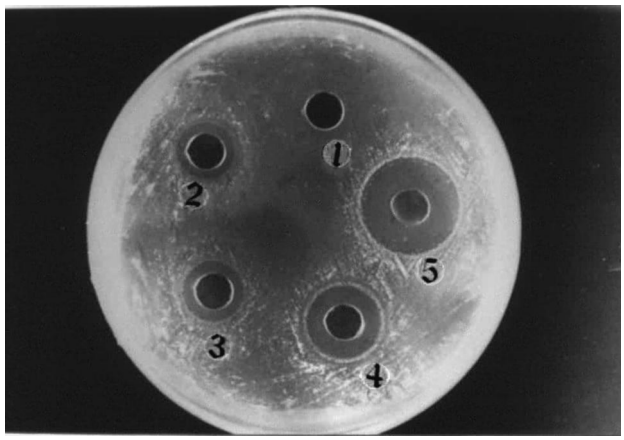

FIGURE 2 Growth inhibition of *Erwinia carotovora* var carotovora with increasing concentrations of *C. betacea* inhibitor. (1) Control; (2) 7.5 μg of protein; (3) 15 μg of protein; (4) 30 μg of protein; (5) 60 μg of protein in 50 μl of PBS buffer (10 mM sodium phospate buffer, pH 7, with 0.15 M NaCl).

vacuolar invertase inhibitor homologue in potato, the activity of vacuolar invertase was reduced.[36]

Under our experimental conditions, the proteinaceous inhibitor from potato tubers and *C. betacea* fruits were found to be inhibitors of xylophagous fungus and higher plant invertases. This result is coincident with that reported for *Dioscorea rotundata* inhibitor.[7]

Plant pectic substances have a major function in cell-wall cementing in the middle lamellae between adjacent primary cell walls and can be regarded as filler substances within the primary cell wall.[37] Accordingly, depolymerization of pectin components causes cell separation, release of cell wall bound proteins and leakage of electrolytes from protoplasts resulting in cell lysis. In fact, tissue maceration is caused by enzymes that degrade the α (1-4)-glycosidic bonds found in uronic polymers of pectic substances, i.e. pectate lyase (PAL), pectin lyase (PL) and polygalacturonase (PG). Many phytopathogenic bacteria and fungi produce pectolytic enzymes that digest plant tissues.[38] PAL and PL cleave the pectic polymers by β-elimination, creating a double bond between C-4 and C-5, while PG catalyses a hydrolytic cleavage. PAL and PG prefer galacturonic acid polymers of low C-6 methylation (pectate) as substrate, while PL only cleaves galacturonic acid polymers in which a high proportion of C-6 carboxyl groups are methylated (pectin).[39] We have shown that the putative invertase inhibitor of potato and *C. betacea* inhibited this entire group of enzymes, including those of plant origin. The proteinaceous inhibitors inhibited all the tested cell wall degrading enzymes. The results of the present paper show that the invertase proteinaceous inhibitor from potato and *C. betacea* has antibacterial activity against some plant pathogenic bacteria. In addition, these inhibitors have activity against polygalacturonases from fungal source (PGs) similar to the polygalacturonase-inhibiting proteins (PGIPs) found in dicotyledonous[40] and monocotyledonous plants.[41] These proteins are located in the cell wall[12] consistent with other PGIPs.[42,44] The PGIPs role *in vivo* is not clear but it is usually assumed that these proteins are involved in the host-parasite interactions during plant tissue infection by pathogens.[42–44]

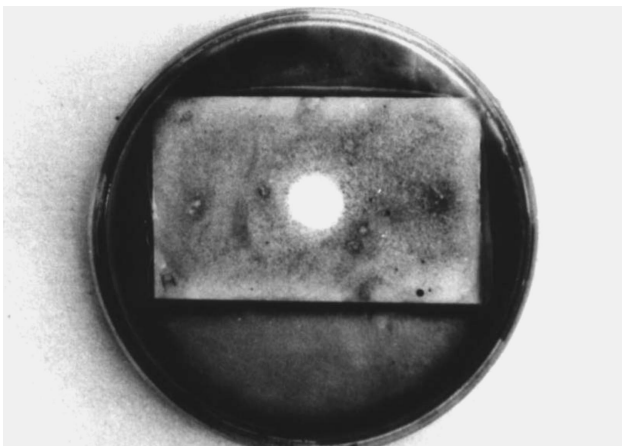

FIGURE 3 Effect of putative invertase inhibitor from *S. tuberosum* tubers on *Pseudomonas solanacearum* CECT 125 growth. A dilution in PBS buffer of the proteinaceous inhibitor containing 2 μg of protein was spotted in the center of a Silica gel plate ($7 \times 3\,cm^2$). After the plate was dried in sterile conditions, the bioautographic agar overlay method was applied. Briefly, soft BHI media containing 10^5 CFU/ml was overlayed on the plate and incubated at 37°C for 24 h. Then, the plate was sprayed with a 2.5 mg/ml solution of MTT in PBS. After a new incubation at 30°C for 2 h microbial growth inhibition was visualized by a clear circular zone on a purple background.

Our results also show a total lack of specificity of potato and *C. betacea* proteinaceous inhibitors, an unsustainable condition for an enzyme modulator. Instead, these putative proteinaceous inhibitors of the invertases are more likely related to the known pathogenesis related proteins (PRP), and to the antimicrobial proteins.

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