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Occurrence of Antimicrobial Serin-Proteinases in Sunflower Seeds

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Abstract

Using an experimental approach directed to the isolation of antimicrobial proteins, we have detected the presence of a trypsin inhibitor (TI) with associated antifungal activity in sunflower seeds. Purification of the isolated protein by affinity chromatography on a trypsin-agarose matrix confirmed that a trypsin inhibitor was responsible for the inhibition of spore germination of the fungal pathogen *Sclerotinia sclerotiorum*. The protein is a potent antifungal compound as it can completely inhibit the germination of *S. sclerotiorum* ascospores at a concentration of 14 μg/ml. The putative contribution of this TI to control fungal invasion is discussed.

Introduction

Proteinase inhibitors (PI) are considered important components of the plant defence response to insect predation (Koiwa et al., 1997) and are part of the pathogenesis-related (PR) protein family (Van Loon and Van Strien, 1999). At least 12 types of PI have been characterized according to their amino acid sequence and the type of proteinase that they inhibit (Shewry, 1999). Among them are the serin proteinase inhibitors, polypeptides that can inhibit enzymes of animal and microbial origin *in vitro*, such as trypsin, chymotrypsin, proteinase K and subtilisin. However, little information is available concerning their putative substrates or their alternative roles in plants. The fact that they are frequently found in plant seeds has led to speculate that they may regulate endogenous proteinases during seed dormancy and also serve as storage source of protein (Ryan, 1990). In addition to these functions, it has also been described that certain serin proteinase inhibitors isolated from different plant sources can display antifungal activity *in vitro*. Among these are the trypsin inhibitors (TI) from barley (Terras et al., 1993), TI from maize seeds (Huynh et al., 1992) inhibitors of trypsin and chymotrypsin from *Brassica oleracea* (Lorito et al., 1994), and TI from *Helianthus annuus* flowers (Giudici et al., 2000). Purified fractions of these inhibitors have been shown to have a direct effect on fungi leading to their growth inhibition. According to these experimental evidences it has been proposed that some TI may be involved in plant defence towards the attack of pathogenic micro-organisms and should then be considered a new family of antifungal compounds (Lorito et al., 1994).

Several studies demonstrated the existence of TI in sunflower seeds, although originally a low content was reported (Roy and Bhat, 1974). Konarev et al. (2000) detected several low molecular weight TI and a detailed analysis of serin proteinase inhibitors from compositae including their distribution has recently been published (Konarev et al., 2002). This report includes the description in seeds from the genus *Helianthus* of several TIs with molecular masses ranging from 1500 to 7600 Da, in addition to inhibitors of subtilisin and chymotrypsin. According to evidences obtained in other species, it has been proposed that sunflower proteinase inhibitors could contribute to plant resistance towards invading fungi, although no evidence has been presented to support such role. In this context, we decided to explore whether sunflower seeds contain serin proteinase inhibitors which would be able to block or reduce the growth of phytopathogenic fungi.

Materials and Methods

Protein purification

Commercially available sunflower hybrid seeds were decoated and ground in extraction buffer: 50 mM Tris-HCl pH 7.5, 0.1% (v/v) beta-mercaptoethanol, 0.6 M NaCl, 0.13% (v/v) Tween 20. Proteins were extracted by stirring during 16 h at 4°C and then submitted to heating at 80°C for 15 min. The extract was clarified

by centrifugation at 10 000 × ***g*** for 20 min and dialysed against water. The resultant extract was concentrated and fractionated by FPLC gel filtration on a Superdex 75 HR 10/30 column equilibrated with 40 mM sodium acetate pH 5.2. Samples were run at a flow rate of 0.6 ml/min. Molecular mass calibration of the column was performed with BSA (67 kDa), ovoalbumin (43 kDa), C cytochrome (12.4 kDa) and ubiquitin (9 kDa). Void volume and total volume were determined with blue dextran (2000 kDa) and cytidine (240 Da), respectively. The molecular weight of elution peaks was estimated by calculating the Kav parameter.

In another experimental approach, an affinity chromatography was performed. In this case, a thermoresistent extract prepared as described above was incubated overnight on a trypsin-agarose matrix (ICN) equilibrated with 10 mM Tris HCl pH 8, 0.1 M KCl. The suspension was loaded on a column and extensively washed at a flow rate of 0.6 ml/min with the same buffer in order to eliminate the unbound proteins. The process was monitored by Abs 280 nm and the elution of bound proteins was made with 0.5 M NaCl, pH 2. The protein fraction eluted at pH 2 was pooled, dialysed and concentrated. This pool was used for the determination of trypsin inhibition and antifungal activity and then submitted to gel filtration on Superdex 75 HR 13/30 as described above. Five elution peaks were obtained from this chromatography and protein concentration of each one was determined using the bicinchoninic acid assay (Smith et al., 1985) with bovine serum albumin (BSA) as standard.

Test for antifungal activity

Sclerotinia sclerotiorum ascospores were a kind gift of Dr M. E. Bazzalo (Advanta Semillas SAIC, Argentina) and were obtained from naturally infected sunflower plants collected in Balcarce, Argentina during 1999.

Spores from *Fusarium solani* f. sp. *eumartii*, isolate 3122 (EEA-INTA, Balcarce, Argentina) were collected from 8-day-old cultures grown on potato dextrose agar supplemented with 100 μg/ml ampicilin. Spores were obtained by the addition of sterile water on the plate followed by gentle suspension and counting.

The antifungal activity was evaluated as the inhibition of *S. sclerotiorum* or *F. solani* spores germination. The test was developed on microslides in 4% sucrose as previously described (Regente and de la Canal, 2000). After overnight incubation at 25°C and 100% relative humidity, samples were evaluated for the presence of germinated spores and hyphae using an optical microscope.

Serin proteinase inhibitor activity

Trypsin inhibitor activity was measured as previously described by Giudici et al. (2000). Each sample was preincubated for 20 min at 37°C with 0.25 μg of the serin proteinase to be tested (trypsin, chymotrypsin, subtilisin or proteinase K). Azocasein was then added as substrate (0.5% final concentration) and the mixture was incubated for 1 h at 37°C. The reaction was stopped by adding the same volume of TCA 10% and the mixture was centrifuged for 15 min at 1200 × ***g***. Finally, the absorbance of the supernatant at 350 nm was determined as an indicator of the residual proteinase activity.

Results

In order to analyse the presence of antifungal serin proteinase inhibitors in sunflower seeds, protein extracts were obtained using a protocol frequently used for the preparation of plant antimicrobial proteins (Regente and de la Canal, 2000). This procedure includes an extraction step in the presence of a high-salt buffer to favour the solubilization of cell wall associated proteins. In addition, as compact serin proteinases can be heat resistant (Richardson, 1976), we have submitted the extracted protein fraction to 80°C for 15 min. This heat resistant protein preparation was finally fractionated by gel filtration on Superdex 75 (FPLC) and the screening for serin proteinase inhibitors was performed using four different substrates: trypsin, chymotrypsin, subtilisin and proteinase K. The protein profile was tested for the inhibition of the four proteinases but only trypsin was inhibited. Fig. 1a presents the elution profile on Superdex 75 showing that only the fractions 28 and 29 display a clear activity of trypsin inhibition in the assay conditions used. The estimated molecular weight of these fractions according to the calibration of the column was around 10 kDa.

Having verified the presence of fractions behaving as trypsin inhibitors (TIs), we tested whether these fractions also displayed antifungal activity. To that aim we have analysed the ability of fractions 28 and 29 to inhibit the germination of spores of two phytopathogenic fungi: *Sclerotinia sclerotiorum* and *Fusarium solani*. As shown in Fig. 1b, both fractions inhibit the germination of fungal spores. Although fractions 28 and 29 exhibit both trypsin inhibition and fungal growth inhibition, the procedure used for the isolation do not allow to strictly assign the antifungal activity to a TI. In fact, PAGE-SDS controls revealed the presence of several protein bands in fraction 28 (date not shown). To confirm that the antifungal activity belongs to a TI, we have applied a different isolation protocol including a binding step to an agarose-trypsin matrix, a procedure used for the specific isolation of TIs. Hence, a heat resistant protein fraction obtained from decoated sunflower seeds was submitted to affinity chromatography and eluted with pH changes. It can be seen in Fig. 2a that the extract contains proteins capable of binding to trypsin which could be recovered by an elution step at pH 2. The protein pool recovered was neutralized and tested for TI activity and antifungal activity, revealing that both activities were present (data not shown). As it has been described that several proteinase inhibitors are present in sunflower seeds, we have submitted the protein pool eluted at pH 2 to gel filtration in order to separate

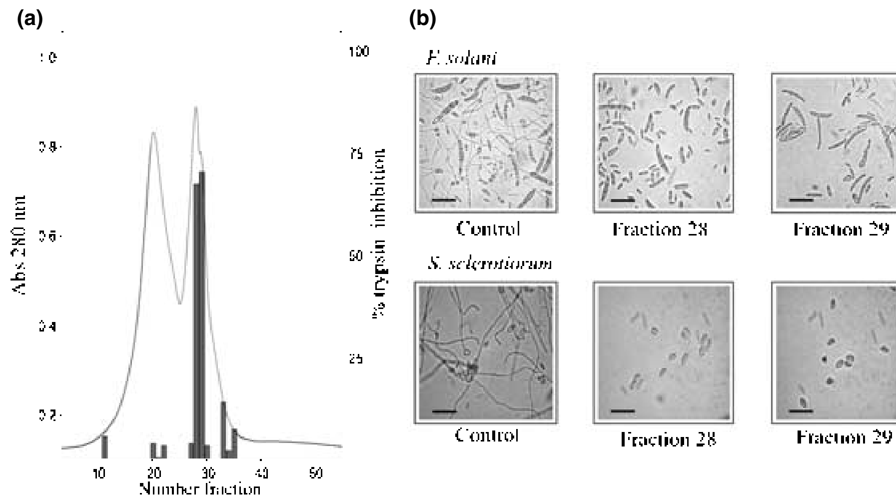

Fig. 1 Detection of trypsin inhibitors with antifungal activity in sunflower seeds. (a) Gel filtration chromatography on Superdex 75 FPLC. The column was equilibrated with sodium acetate 40 mM pH 5.2, 0.1 M KCl and the elution was monitored by Abs 280 nm. The per cent of trypsin inhibition was tested for each fraction (bars) using azocasein as substrate. (b) Antifungal activity of fractions 28 and 29. The test for inhibition of *Fusarium solani* and *Sclerotinia sclerotiorum* spores germination was carried out on microslides containing water (control) or protein sample (fraction 28 or 29 from Fig. 1a). After 16 h of incubation, the germination of the spores was evaluated under a light microscope. Bars: 80 μm

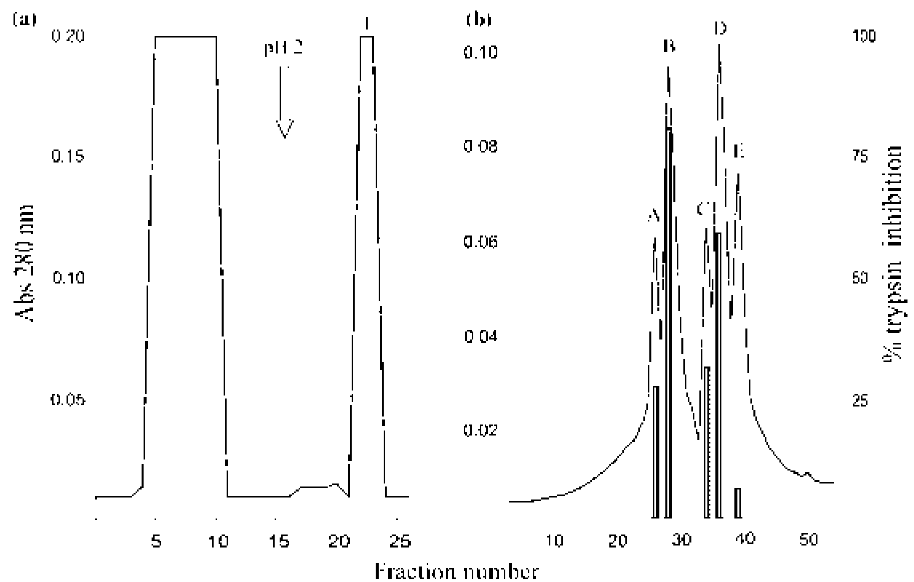

Fig. 2 Purification of trypsin inhibitors detected in sunflower seeds. (a) Trypsin affinity chromatography. After loading, the column was extensively washed with 10 mM Tris HCl pH 8, 0.1 M KCl. The elution with 0.5 M NaCl, pH 2 is indicated. Fractions 21 to 24 containing the proteins bound to the trypsin matrix were pooled (I). (b) Gel filtration chromatography on Superdex 75 FPLC. Peak I from Fig. 2a was submitted to gel filtration chromatography as described in Fig. 1a. The protein peaks were separately pooled (A, B, C, D and E) and trypsin inhibition was tested for each one (bars)

potential different inhibitors according to their molecular masses. Fig. 2b shows the elution profile of a Superdex 75 column loaded with the fraction retained in the affinity chromatography. Five protein peaks were detected and fractions were pooled in five groups, named A to E, respectively. As shown in Fig. 2b, each pool exhibit trypsin inhibitory activity and was also able to inhibit the germination of *S. sclerotiorum* ascospores (data not shown). As the protein mass recovered do not allow to characterize each protein pool, we decided to continue the analysis with pools C and D which displayed the strongest antifungal activities.

The protein concentration required to inhibit fungal germination is a relevant parameter to infer whether an antifungal protein could be active *in planta*. In fact, many basic proteins can behave as 'antifungal' *in vitro* but require protein doses much higher than those antimicrobial proteins whose role in plant defence has been demonstrated (Regente and de la Canal, 2001). To analyse this point, we have determined the antifungal potency of the isolated TIs. Fig. 3 shows that pool C and D can completely block the germination of *S. sclerotiorum* ascospores at a concentration of 14 μg/ml.

Discussion

The role of serin proteinase inhibitors in plant defence towards insect attack has largely been documented. However, the ability of some of them to inhibit fungal growth has not been studied in detail. Particularly,

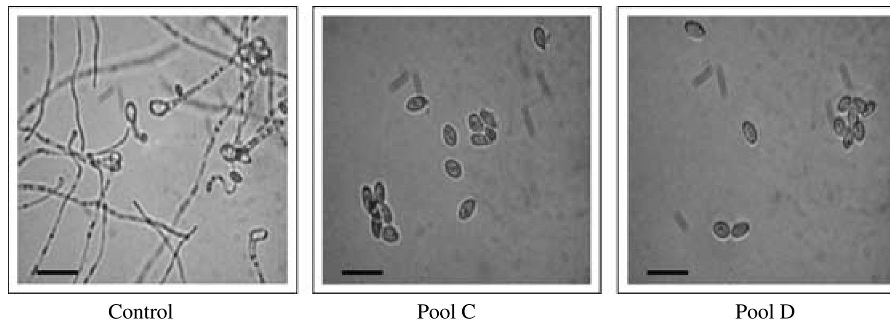

Fig. 3 Antifungal activity of trypsin inhibitors from sunflower seeds. Proteins bound to trypsin agarose and fractionated by gel filtration were tested for the inhibition of *S. sclerotioirum* ascospore germination. Photomicrographs were taken after 16 h of incubation of the ascospores in the presence of water (control) or 14 μg/ml of protein sample (pools C and D from Fig. 2b). Bars: 80 μm

antifungal TIs constitute a barely known family of antimicrobial proteins, although they have been proposed as components of the plant defence mechanism (Terras et al., 1993; Lorito et al., 1994). Several serin proteinase inhibitors have already been detected in sunflower seeds (Roy and Bhat, 1974; Konarev et al., 2000; Konarev et al., 2002) but their ability to control microbial growth has not been demonstrated previously. Using a purification protocol aimed at the detection of antifungal proteins, we show that sunflower seeds contain TI that can inhibit the germination of spores of phytopathogenic fungi. The fact that the antifungal activity was detected in a protein fraction retained in a trypsin-affinity matrix clearly indicates that it is a trypsin-like inhibitor.

Our experimental evidence do not allow to confirm if one or more antifungal TIs are originally present in the seeds. The fact that five protein peaks are detected on gel filtration after affinity chromatography could be indicative of the presence of distinct proteins. However, it must be mentioned that in the first experiment aimed at the detection of serin proteinase inhibitors in total extracts (Fig. 1a), TIs were detected in a narrow range of the Superdex 75 elution profile (fractions 28 and 29), while under the same chromatographic conditions a larger distribution of TIs was detected after affinity chromatography (fractions 27 to 38, Fig. 2b). This observation opens the possibility that the peaks corresponding to lower molecular masses could be originated by protein cleavage from a unique protein (fraction 28 and 29).

A relevant observation in this work is the potent activity displayed by the antifungal TIs isolated. We show that a complete inhibition of the germination of fungal spores is seen in the presence of 14 μg/ml of TIs. This value represents a potent activity compared with other antimicrobial serin proteinases. For example, those isolated from cabbage required a concentration of 600 μg/ml to produce the inhibition of *F. solani* spores (Lorito et al., 1994), while TIs from barley (Terras et al., 1993) and maize (Chen et al., 1998; Chen et al., 1999) inhibit several fungi at concentrations between 40 and 1000 μg/ml. Interestingly, another antifungal TI isolated from *H. annuus* flowers also showed a potent activity leading to a complete inhibition of *S. sclerotiorum* ascospore germination at a concentration of 5 μg/ml (Giudici et al., 2000).

We demonstrate that antifungal TIs are present in *H. annuus* seeds. However, a more detailed characterization of this inhibitor is required to determine whether it is one of the TIs already described. Seeds are particularly resistant to the attack of microbial pathogens compared with other plant parts or organs and this correlates with high levels of expression of antifungal proteins (Broekaert et al., 1997). Although the contribution of this antifungal TI to a direct inhibition of fungal growth *in vivo* remains to be demonstrated, the potent activity shown in this work suggests its potential role in seed protection towards the attack of fungi.

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