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The Detection of Inhibitors of the *Sclerotinia sclerotiorum* (Lib.) de Bary Extracellular Proteinases in Sunflower

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With 2 figures

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Abstract

Water-soluble protein fractions from leaves, seeds and heads of sunflower were shown to contain inhibitors of trypsin, chymotrypsin and extracellular proteinases from *Sclerotinia sclerotiorum*, a pathogen of sunflower, and *Colletotrichum lindemuthianum*. These included bifunctional inhibitors of trypsin and subtilisin. Comparison with the patterns of inhibition of standard proteinases indicated that the major extracellular proteinases of *S. sclerotiorum* are subtilisin-like. It is speculated that the sunflower inhibitors play a role in conferring resistance to fungal infection.

Zusammenfassung

Feststellung von Inhibitoren von extrazellulären Proteinasen aus *Sclerotinia sclerotiorum* (Lib.) de Bary in der Sonnenblume

Es konnte ermittelt werden, daß wasserlösliche Proteinfraktionen aus Blättern, Samen und Blütentellern der Sonnenblume Inhibitoren von Trypsin, Chymotrypsin sowie den extrazellulären Proteinasen von sowohl *Sclerotinia sclerotiorum*, einem Pathogen der Sonnenblume, als auch *Colletotrichum lindemuthianum* enthielten. Diese umfaßte auch bifunktionale Inhibitoren von Trypsin und Subtilisin. Der Vergleich mit den Inhibitionsmustern von Standardproteinasen zeigte, daß die bedeutendesten extrazellulären Proteinasen von *S. sclerotiorum* Subtilisin ähnlich sind. Es werden Vermutungen angestellt, daß diese Sonnenblumeninhibitoren eine Rolle in der Resistenz gegenüber pilzlichen Pathogenen spielen.

Introduction

Protein inhibitors of the proteinases of insects and microorganisms are of widespread occurrence in plants with seeds and other storage tissues, being particularly rich sources (Garcia-Olmedo et al., 1987; Ryan, 1990; Richardson, 1991). Some 11 families of inhibitors have been described, based on their amino acid sequence relationships and inhibitory properties (Kreis et al., 1985; Garcia-Olmedo et al., 1987; Richardson, 1991). However, the inhibitors of sunflower seeds, and their possible role in resistance to pathogenic fungi, have not been studied in detail, although a preliminary report (Levitskii and Pogoreletskaya, 1985) described the presence of proteins inhibitory to growth of *Botrytis cinerea* Pers. (grey foot rot). We have therefore analysed seeds of sunflower for protein inhibitors of proteinases, using enzymes from *Colletotrichum lindemuthianum* (a non-pathogen of sunflower) and *Sclerotinia sclerotiorum* (a sunflower pathogen of wide distribution) as target proteinases.

Materials and Methods

Sunflower seeds were obtained from N.I. Vavilov, All-Russian Institute of Plant Industry and All-Russian Institute for Plant Protection (St. Petersburg, Russia) and grown in the glasshouse at Long Ashton (UK). Leaves were collected during flowering, and head tissue just before seed maturity. The material was frozen and lyophilized.

The culture of C. *lindemuthianum* was from the collection at IACR-Long Ashton Research Station and the culture of *S. sclerotiorum* was supplied by Dr E.L. Slusar (Institute of Oil Crops, Krasnodar). *C. lindemuthianum* was cultivated on modified liquid nutrient medium (Dunaevskii et al., 1995) on a rotating shaker at 25°C. *S. sclerotiorum* was cultivated on Czapek Dox medium under stationary conditions. Casein 2% (w/v) was added to both media for induction of proteinase production. After 3 days of incubation the culture filtrate was centrifuged and ammonium sulphate added to a concentration of 40% (w/v). The precipitate was collected by centrifugation and was stored as a suspension in ammonium sulphate with 30% (w/v) glycerol at −20°C. Ammonium sulphate was removed by dialysis against water. As a result, the proteins in the cultural filtrate were concentrated 10-fold, allowing the proteinases to be

partly purified by micropreparative isoelectric focusing (see below).

The seed proteins were isolated from ground seeds by stirring with water (1:4 w/v) for 1 h at 20°C followed by centrifugation at 12 000 × ***g*** for 5 min. Proteins were isolated from leaves and other tissues by homogenization in water (1:1 w/v) and centrifugation.

Isoelectric focusing (IEF) of proteins was carried out in Servalyt Precote pH 3–10 gels (Serva Feinbiochimica GmbH, Heidelberg, Germany) on the Multiphor II (Amersham Pharmacia Biotech, Uppsala, Sweden) with 50 mm between electrodes. Anode fluid 10 and cathode fluid 3 diluted with water (1:3) (Serva) were used as electrode buffers. The protein samples (water extracts of sunflower or dialyzed culture filtrate at 0.5–2 mg protein ml^{-1}) were applied to the gel in paper strips (15 × 1 mm) in volumes of 2–15 μl at 0.5 cm from the anode. IEF was conducted in 62 × 125 mm gels for 40 min at power 2 W and a final voltage 1200 V. Cytochrome c (pI 10.65), horse myoglobin (pI 7.3), and whale myoglobin (pI 8.3) were used as pI markers.

For the purification of proteinases, 10 × 50 mm paper strips moistened with culture filtrate were applied to the gel at 10 mm from the anode. After separation, three 1 × 40 mm strips of photo film 'Foto 65' (Tasma, Kazan, Russia) were placed on the gel along the pH gradient (Konarev and Fomicheva, 1991). The gel was covered with plastic film in order to protect it from drying and incubated for 5 min at 40°C. The zones of hydrolyzed gelatin corresponding to proteinases were cut out, immersed in 200 μl of 25% (w/v) sucrose for 1 h and the solution used for detection of proteinase inhibitors. The sensitivities of proteinase components to various inhibitors were estimated in the IEF gel directly by the 'cross' method (Konarev and Fomicheva, 1991).

The improved 'gelatin replicas method' (Konarev, 1986a) was used for the detection of proteinase inhibitors. After IEF of proteins, photographic film 'Foto 65' was superimposed onto a gel for 40 min at 20°C. The film with the separated proteins imprinted was then laid on a plate of 0.8% agarose gel (Serva, gel-point = 33°) containing the proteinase and 0.1 M Na_2HPO_4, pH 9.0, and incubated at 40°C for 30 min on the Multiphor II plate. The gelatin of the film was not hydrolyzed in the zones containing inhibitors. The concentration of trypsin (Sigma-Aldrich Company Ltd, Poole, UK) was 1.5 μg ml^{-1} and of subtilisin (Novabiochem, San Diego, USA) 5 μg ml^{-1}. The amounts of fungal proteinases used corresponded to trypsin based on activity. In order to determine the optimum volume of culture filtrate for addition to the agarose solution, a series of two-fold dilutions in Na_2HPO_4 was prepared (1:2–1:32) using strips moistened with trypsin solution (1, 1.5 and 2 μg ml^{-1}) as controls. The paper strips (2 × 10 mm) were moistened with the proteinase solutions and placed onto the gelatin layer of the film. The film was then incubated in a humid chamber for 30 min at 40°C, washed with water and blotted dry. The zones of gelatin hydrolyzed by trypsin and the tested proteinase were compared and the proteinase concentration which gave activity close to that of trypsin at 1.5 μg ml^{-1} was chosen for the detection of inhibitors.

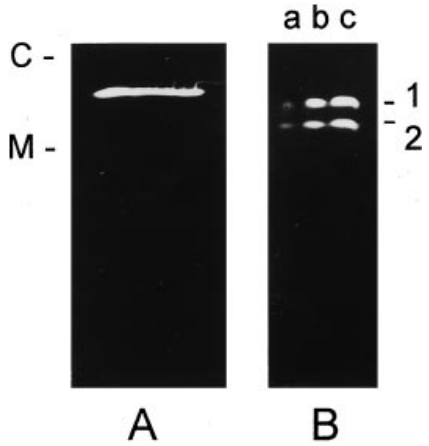

Fig. 1 Isoelectric focusing (pH range 3–10) of extracellular proteinases of *Colletotrichum lindemuthianum* (A) and *Sclerotinia sclerotiorum* (B). Proteinases were detected by overlaying the gels with gelatin-coated photographic films followed by washing the films to reveal areas of protein digestion. The *Sclerotinia* proteinase was applied at three concentrations (corresponding to 2, 5 and 10 μl of concentrated culture filtrate). The positions of marker proteins of pI 7.3 (horse myoglobin) and 10.65 (cytochrome c) are indicated

Results and Discussion

Analytical isoelectric focusing of the extracellular proteinases from *C. lindemuthianum and S. sclerotiorum* was carried out in the pH range 3–10 using a gelatin layer as substrate. This showed one and two major bands, respectively, with pI values in the range 8–9.5 (Fig. 1). Preliminary studies, in which proteinase inhibitors were absorbed onto gelatin layers and placed onto the separated proteinases prior to incubation, demonstrated that the major proteinases of both fungi were inhibited by various inhibitors of subtilisin. These were the wheat α-amylase/subtilisin inhibitor (WASI) (Konarev, 1982, 1986b), the wheat chymotrypsin/subtilisin inhibitor (C/SI) (Konarev, 1986b, 1996) and an inhibitor fraction prepared from sunflower seeds by affinity chromatography on trypsin-Sepharose, all at concentrations of about 1 mg ml^{-1}. In contrast, no inhibition was observed with the soybean trypsin inhibitor or with a trypsin inhibitor from wheat leaves (Konarev, 1987, 1993). Specific inhibitors of *C. lindemuthianum* proteinases were previously found in kidney bean seeds (Mosolov et al., 1979).

In order to study the inhibitors of sunflower in more detail, the major proteinases from *S. sclerotiorum* were extracted from the IEF gel and incorporated into a 0.8% agarose gel. Water-soluble proteins were then extracted from seeds and other tissues of three lines of sunflower, separated by IEF, transferred to a gelatin layer and laid onto the agarose containing the fungal proteinases. Figure 2C shows the results obtained with the proteinase (pI ≅ 9) labelled 1 in Fig. 1B. Similar separations of the sunflower proteins were laid onto agarose containing trypsin (Fig. 2A) and subtilisin (Fig. 2B). Incubation revealed areas of undigested gelatin, due to the presence of inhibitors of the three proteinases.

The trypsin inhibitors present in the three lines could be divided into three broad groups, with high pI (≅8.5–

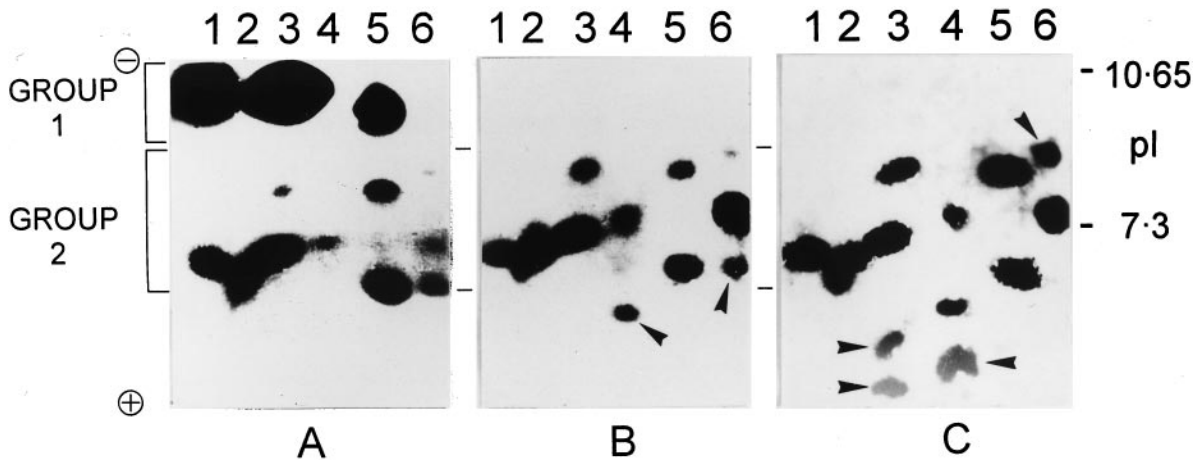

Fig. 2 Detection of proteinase inhibitors in water-soluble protein fractions from three lines of sunflower.The fractions were separated by isoelectric focusing (pH range 3–10), transferred to a gelatin layer on photographic film and then laid on an agarose gel containing A, trypsin; B, subtilisin and C, proteinase 1 from *S. sclerotiorum* (See Fig. 1B). Tracks 1 and 2, sunflower variety Omskii (1, seeds; 2, tissue from the back of the head); tracks 3 and 4, line VIR 130 (1, seeds; 2, leaves); tracks 5 and 6, line VIR 648b (5, seeds; 6, leaves).

The groups of inhibitors I and 2 and the components indicated by arrows are discussed in the text. The position of marker proteins of pI 7.3 (horse myoglobin) and pI 10.65 (cytochrome C) are indicated

10) inhibitors (group 1 in Fig. 2A) present only in the seeds and low pI ($\cong$6.5–8) inhibitors present in the seeds and also vegetative tissues (group 2 in Fig. 2A). The group 1 inhibitors did not show any inhibition of subtilisin (cf Fig. 2B) and corresponded to trypsin inhibitors. In contrast, the group 2 inhibitors also inhibited subtilisin (Fig. 2B) and were therefore bifunctional trypsin/subtilisin inhibitors. The leaves of line VIR 130 also contained an additional inhibitor which appeared to inhibit subtilisin but not trypsin (see arrow in Fig. 2B, track 4). This may correspond to a specific subtilisin inhibitor.

The pattern of inhibition of the *S. sclerotiorum* proteinase (Fig. 2C) was similar to that of subtilisin, confirming the preliminary identification of the proteinase as subtilisin-like. The results obtained with proteinase 2 in Fig. 1B are not shown but similar inhibitory properties were observed. However, several additional low pI inhibitors were detected in seed and leaf extracts of line VIR 130 (arrowed in Fig. 2C tracks 3 and 4) while leaves of line VIR 648b showed a different inhibitory spectrum, with an additional high pI inhibitor (arrowed in Fig. 2C, track 6) and little or no activity displayed by the low pI subtilisin inhibitor (arrowed in Fig. 2B, track 6).

It is clear therefore that seeds, heads and leaves of sunflower contain protein inhibitors of the major subtilisin-like extracellular proteinases of *Sclerotinia sclerotiorum*, an important pathogen infecting shoots, stems, developing flower heads and seeds in Russia, Turkey and many European countries. These inhibitors may play a role in providing a wide spectrum resistance to *Sclerotinia* and other pathogens (see Shewry and Lucas, 1997). In addition, they could also be attractive targets for the genetic engineering of sunflower for improved pathogen resistance.

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Literature

Dunaevskii, Ya. E., G. A. Belyakova, E. B. Pavlukova, M. A. Belozersky (1995): Influence of cultivation conditions on synthesis and secretion of proteases by the fungi *Alternaria alternata* and *Fusarium oxysporum*. Microbiol. (Moscow) **64,** 327–330.

Garcia-Olmedo, F., G. Salcedo, R. Sanchez-Monge, L. Gomez, J. Royo, P. Carbonero, (1987): Plant proteinaceous inhibitors of proteinases and α-amylases. Oxford Surveys Plant Mol. Cell. Biol. **4,** 275–334.

Konarev, AL. V. (1982): Identification of inhibitors of endogenous and exogenous alpha-amylases among wheat proteins. Bull. VIR **118,** 11–12. (In Russian).

Konarev, AL. V. (1986a): Analysis of proteinase inhibitors from wheat grain by gelatin replicas method. Biochem. (Moscow) **51,** 195–201.

Konarev, AL. V. (1986b): Relationship and variability of proteinase and amylase inhibitors in wheat and related cereals. Selkochozajstvennaya biologia **3,** 46–52. (In Russian).

Konarev, AL. V. (1993): Component composition, genome and chromosome control. of trypsin inhibitors from bread wheat leaves, roots and germs. Selkochozajstvennaya biologia **1,** 43–52. (In Russian).

Konarev, AL. V. (1996): Interaction of insect digestive enzymes with protein inhibitors from plants and host-parasite coevolution. Euphytica **92,** 89–94.

Konarev, AL. V. (1987): Variability of trypsin-like proteinase inhibitors in wheat and related cereals. Selkochozajstvennaya biologia **5,** 17–24. (In Russian).

Konarev, AL. V., Yu. V. Fomicheva (1991): Cross analysis of the interaction of alpha-amylase and proteinase components of insects with protein inhibitors from wheat endosperm. Biochem. (Moscow) **56,** 628–638.

Kreis, M., P. R. Shewry, E. G. Forde, J. Forde, B. J. Miflin (1985):

Structure and evolution of seed storage proteins and their genes with particular reference to those of wheat, barley and rye. Oxford Surveys Plant Cell Mol. Biol. **2,** 253–317.

Levitskii, A. P., B. B. Pogoreletskaya (1985): Proteinase inhibitors from sunflower seeds. Physiol. Biochem. Cultivated Plants (Kiev) **17,** 153–157.

Mosolov, V. V., M. D. Loginova, E. L. Malova, I. I. Benken (1979): A specific inhibitor of *Colletotrichum lindemuthianum* protease from kidney bean (*Phaselous vulgaris*) seeds. Planta **144,** 265–269.

Richardson, M. (1991): Seed storage proteins: the enzyme inhibitors. Methods Plant Biochem. **5,** 259–305.

Ryan, C. A. (1990): Protease inhibitors in plants: gens for improving defenses against insects and pathogens. Annu. Rev. Phytopathol. **28,** 425–449.

Shewry, P. R., J. A. Lucas (1997): Plant proteins that confer resistance to pests and pathogens. Adv. Bot. Res. **26,** 135–192.