Theor Appl Genet (2000) 100:82–88

Al.V. Konarev · I.N. Anisimova · V.A. Gavrilova
V.T. Rozhkova · R. Fido · A.S. Tatham · P.R. Shewry

Novel proteinase inhibitors in seeds of sunflower (*Helianthus annuus* L.) : polymorphism, inheritance and properties

Received: 7 March 1999 / Accepted: 18 March 1999

Abstract A highly sensitive gelatin overlay procedure was used to identify inhibitors of serine proteinases and of the cysteine proteinase ficin in seeds and leaves of sunflower. One major and two minor groups of trypsin inhibitors were identified in seeds, the former having a high pI (≅10) and also inhibiting chymotrypsin. Three groups of trypsin/subtilisin inhibitors were also present in seeds, together with three inhibitors of ficin. All groups showed polymorphism between lines of *Helianthus annuus*, while the trypsin and trypsin/subtilisin inhibitors also varied between wild species of *Helianthus*, with no apparent relationship to growth type (annual or perennial), genome constitution or ploidy level. Genetic analysis showed that the major trypsin inhibitor and three groups of trypsin/subtilisin inhibitors are each controlled by single Mendelian loci, with the three loci for trypsin/subtilisin inhibitors showing recombination values of 0.23–0.40. Purification by RP-HPLC allowed the M_r of two trypsin inhibitors to be determined by SDS-PAGE to be about 1,500 and 2,500, while the three trypsin/subtilisin inhibitors varied in M_r from about 1,500 to 6,000.

Key words Sunflower · Seeds · Proteinase inhibitors · Polymorphism · Genetic control

Communicated by H.F. Linskens

Al.V. Konarev
All-Russian Institute of Plant Protection, St. Petersburg, Russia

I.N. Anisimova · V.A. Gavrilova · V.T. Rozhkova
N.I. Vavilov All-Russian Institute of Plant Industry (VIR),
St. Petersburg, Russia

R. Fido · A.S. Tatham · P.R. Shewry (✉)
IACR-Long Ashton Research Station,
Department of Agricultural Sciences, University of Bristol,
Long Ashton, Bristol BS41 9AF, UK
e-mail: Peter.Shewry@bbsrc.ac.uk

Introduction

Protein inhibitors of proteolytic and amylolytic enzymes have been reported in many plant species and organs and are particularly abundant in storage organs such as seeds and tubers where they may account for a significant proportion of the water-soluble protein fractions. Comparisons of amino acid sequences and other properties indicate the existence of at least 12 families of proteinase inhibitors, some of which include inhibitors of α-amylase and/or bifunctional proteinase/α-amylase inhibitors, and 4 families which inhibit α-amylases but not proteinases (Richardson 1991; Shewry 1999). Although some of these proteins inhibit endogenous enzymes and may play a role in metabolic regulation, most inhibit exogenous enzymes from insect pests and/or microbial pathogens. They are therefore considered to play a role in plant protection, forming part of a broad-spectrum defence response which may also include the production of PR (pathogenesis-related) proteins (Ryan 1990; Bowles 1990; Richardson 1991; Konarev 1996). The inhibitors have therefore been of particular interest in relation to engineering crop plants in order to confer protection to pests and pathogens, as discussed by Shewry and Lucas (1997).

Sunflower is a major oil crop in Europe, the former Soviet Union, Asia and S. America. Although the developing head is susceptible to fungal infections (notably *Botrytis* and *Sclerotinia*), there have been no systematic attempts to identify inhibitors which may contribute natural resistance to these or other pests and pathogens. We therefore report here a detailed study of the inhibitors of sunflowers using a sensitive gelatin replica technique for micro-scale identification and characterization.

Materials and Methods

Seeds

Sunflower seeds were obtained from the world collection of the N.I.Vavilov Institute of Plant Industry (VIR, St. Petersburg) and

multiplied in the glasshouse as necessary. The material included 12 cultivars of different origins and 70 inbred lines selected on the basis of various morphological and other features for 7–18 generations. Artificial pollination was carried out to produce F_1 and F_2 seeds. The varieties 'Rodeo' and 'Alfasol' were obtained from Zeneca Agrochemicals. Wild species included annual [*H. petiolaris* Nutt. (2n=34), *H. debilis* Nutt. (2n=34), *H laetiflorus* Pers., *H. nuttallii* Torr. ex A. Gray., *H. praecox ssp. praecox* (2n=34)] and perennial [*H mollis* L. (genome A 2n=34), *H. angustifolius* L. (genome A, 2n=34), *H. hirsutus* (genomes AA, tetraploid 2n=68), *H. rigidus* (Cass.) Desf. (genomes AAA, hexaploid, 2n=102), *H. tuberosus* L. (AAB, 2n=102) and *H. tomentosus* Michx (2n=68)] types. The leaves were harvested from lines grown in a glasshouse just before flowering and stored at –18°C.

Extraction of proteins

Seed proteins were isolated by grinding with water (1:4 w/v) or with other extractants [30%, 50% and 70% (v/v) aqueous ethanol, 0.5 *M* ammonium acetate, 0.2 *M* acetic acid, 0.1 *M* HC1 and 2% (w/v) NH_4OH]. Defatting did not affect inhibitor banding patterns by isoelectric focusing but was carried out before any chromatographic separation. Supernatants were stored at –18°C after addition of glycerol to 40% (w/v). Leaf proteins were extracted by homogenization in water (1:1, w/v) followed by centrifugation.

Gel filtration

Sunflower meal was defatted with petroleum ether, and 1 g was then extracted with 4 ml water. After centrifugation at 20,000 *g*, ammonium acetate was added to the supernatant to 0.2 *M*. A 100-µl aliquot of a 2 mg/ml protein solution was applied to a column of Superose 12 HR 10/30 (Pharmacia) and fractionated by FPLC (Pharmacia), eluting with 0.2 *M* ammonium acetate, pH 6.0. Fractions (0.4 ml) were collected, lyophilized, dissolved in 0.2 ml water and separated by isoelectric focusing. Bovine serum albumin (M_r: 66,000), ovalbumin (M_r: 45,000), horse myoglobin (M_r: 17,800) and horse cytochrome c (M_r: 12,400) were used as marker proteins to calibrate the column. Similar conditions were used for the separation of proteinase inhibitors obtained by affinity chromatography.

Affinity chromatography

Eight milligrams of trypsin (Sigma) was incubated with 10 ml of CNBr-Sepharose 4B gel (Pharmacia) according to the manufacturer's instructions. One hundred grams of defatted sunflower flour (cvs 'Trudovik' and 'Alfasol') was extracted with 500 ml of water; after centrifugation at 20,000 *g*, ammonium acetate was added to 0.2 *M*, the pH of the solution being about 6.0. Ten milliliters of the trypsin-Sepharose gel was then added to the extract and incubated for 1 h on a rotary shaker. The gel was separated by filtration and washed first with 200 ml of 0.2 *M* ammonium acetate, followed by 200 ml of TRIS-HC1 buffer, pH 8.0, containing 0.5 *M* NaCl and finally by 200 ml of water. The gel was packed into a column and inhibitors were eluted with 0.015 *M* HCl (Konarev, 1987). The protein peak detected by UV absorbence was lyophilized.

Reversed-phase (RP)-high-pressure liquid chromatography (HPLC)

RP-HPLC of proteins purified by affinity chromatography was carried out on a Vydac analytical C_{18} HPLC column with a 0–60% (v/v) gradient of acetonitrile in 0.1% trifluoroacetic acid. A 1-mg aliquot of lyophilized protein was injected onto the column and separated with a flow rate of 1 ml/min.

Isoelectric focusing (IEF)

IEF of proteins was carried out using Servalyt Precotes pH 3–10 gels (Serva) on an LKB Multiphor II apparatus with 100 mm between electrodes or employing a Phast System (Pharmacia) with 50 mm between electrodes. Anode fluid 10 and cathode fluid 3 (Serva) were diluted 1:3 with water. Protein samples (0.5–2 mg protein/ml water) were applied on paper strips (1×1 mm - 20×1 mm) in a volume of 1–20 µl at 0.5 cm from the anode. Focusing was for 60 min. at 4 W with a final voltage of 1,700 V for a 125×125–mm gel. Cytochrome c (pI 10.65), horse myoglobin (pI 7.3) and whale myoglobin (pI 8.3) (Serva) were used as markers.

For micropreparative IEF, 0.5–1 mg of trypsin inhibitors eluted from the trypsin-Sepharose column was applied in 0.4 ml water on a 10 × 60-mm paper strip across the pH gradient of a Servalyt Precotes gel, and IEF was carried out using a Multiphor with a distance of 10 cm between electrodes. The gel was then placed in 40% (w/v) ammonium sulphate for 5 min and the opalescent protein bands excised and placed in 0.5 ml of water for 1 h to elute inhibitors.

Transfer of proteins onto PVDF-membrane

After micropreparative IEF the gel was placed in 40% (w/v) ammonium sulphate for 2 h in order to elute the Servalytes and fix the inhibitors as opalescent bands. PVDF-membrane (Pro-Blott membrane, Applied Biosystems, USA) was moistened with methanol, applied to the gel and covered with ten layers of filter paper moistened with 10 m*M* CAPS buffer, pH 10, containing 10% (v/v) methanol. The filter paper was changed after 1 and 2 h. Protein bands were detected by staining with Ponceau S.

Sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of proteins reduced by 2-mercaptoethanol was carried out using the TRIS-Tricine system at pH 8.45 (Schagger and von Jagow 1987). Marker proteins (M_r range 2,512–16,949) were purchased from BDH. After separation, the gel was incubated in 10% (w/v) glutaraldehyde for 30 min and stained with 0.1% Coomassie BBR-250 in 10% (w/v) TCA and 40% (v/v) methanol.

Detection of proteinase inhibitors

The improved "gelatin replicas method" (Konarev 1986) was used to detect the proteinase inhibitors. After IEF, photographic film "Foto 65" (Russia) was superimposed on the gel for 10–60 min at 20°C. The photographic film with the separated proteins imprinted was then laid onto a plate of 0.8% (w/v) agarose gel (Serva, melting point 33°C) containing the proteinase and an appropriate buffer and incubated at 40°C for 30 min on the LKB Multiphor II. The gelatin of the film was not hydrolyzed in the zones containing the inhibitors. To prepare the gel, we mixed the proteinases with agarose solution cooled to 45°C. The mixture was then poured on Gel Bond film for agarose (LKB), laid on the Multiphor apparatus at 45°C and allowed to cool. Proteinase concentrations were 1.5 µg/ml trypsin (Sigma), 10 µg/ml chymotrypsin (Sigma), 5 µg/ml subtilisin (Calbiochem) and 7 µg/ml ficin (Calbiochem); 0.1 *M* Na_2HPO_4, pH 9.0, was used for serine proteinases and 0.1 *M* Na phosphate buffer, pH 6.5, containing 0.005 *M* dithioerythritol for ficin.

Genetic analysis

Genetic analysis of inhibitors was carried out on F_2 seeds of crosses between lines with contrasting inhibitor patterns. χ^2 and recombination values were calculated using standard methods.

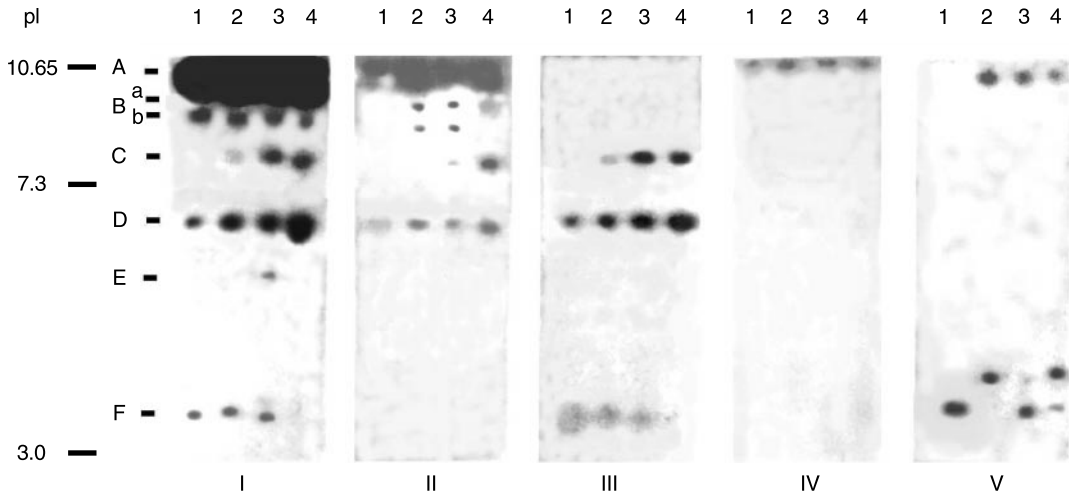

Fig. 1 Patterns of inhibitors of serine and cysteine proteinases in seeds of cultivated sunflower separated by IEF. Water-soluble fractions extracted from samples of 10 seeds were separated by IEF, transferred to gelatin film and then placed onto agarose containing trypsin (*I*), chymotrypsin (*II*), subtilisin (*III*), elastase (*IV*) and ficin (*V*). Staining of the gelatin film revealed the presence of inhibitors as zones of undigested protein. The main groups of serine proteinase inhibitors are labelled A-F. *Track 1* cv 'Sunbred', *2* line VIR104, *3* F_1 of VIR130 x VIR104, *4* line VIR130

Table 1 Characteristics of the groups of proteinase inhibitors present in seeds of sunflower

Group	pI	Approximate M_r[a]	Inhibitory spectrum[b]
A	9.9–10.0	1,500	T/C
B	9.0	2,500	T/C
C	7.7	1,500–6,000	T/C/S
D (Da)	6.8	1,500–6,000	T/C/S
E			T
F	6.4		T/S

[a] Determined by SDS-PAGE
[b] T, trypsin; C, chymotrypsin; S, subtilisin

Results

Groups of proteinase inhibitors in leaves and seeds

Separation of the water-soluble (albumin) protein fraction from sunflower seeds by isoelectric focusing followed by transfer to gelatin-coated photographic film and overlaying onto agarose containing proteinase allowed the identification of inhibitory proteins as zones of undigested gelatin. This analysis was carried out with albumin fractions from 3 lines and one F_1 hybrid of sunflower and five commercially available proteinases, including four serine proteinases (trypsin, chymotrypsin, subtilisin and elastase) and one cysteine proteinase (ficin) (Fig. 1). Six groups of trypsin inhibitors were resolved (A-F in Fig. 1, part I) two of which (A, B) comprised several components of similar pI. Groups A, B, C and D also appeared to inhibit chymotrypsin (Fig. 1, part II) and groups C, D and F subtilisin (Fig. 1, part III). No inhibitors of elastase were detected (Fig. 1, part IV), but three groups of ficin inhibitors were present (Fig. 1, part V), none of which corresponded in mobility to the inhibitors of serine proteinases (A-F). The co-migration of the inhibitors of serine proteinases suggests that groups C, D and F comprise bifunctional trypsin/subtilisin inhibitors, while groups A and B comprise bifunctional trypsin/chymotrypsin inhibitors. The properties of the groups of serine proteinase inhibitors, based on studies described below, are summarized in Table 1. Comparison of the patterns of inhibitors in the 3 lines showed variation in the presence or absence of the different groups. This is investigated further below.

Analysis of the leaves showed the presence of trypsin/subtilisin inhibitors with a similar pI to groups C and D present in seeds, but there was no evidence of high pI trypsin inhibitors corresponding to groups A and B (results not shown).

Extraction of seed meals with a range of solvents showed that the major trypsin inhibitor group (A) was efficiently extracted with water, 30–70% (v/v) ethanol, 0.5 *M* ammonium acetate, 0.2 *M* acetic acid, 0.1 *M* HCl or 2% (w/v) ammonium hydroxide, while all solvents except 70% ethanol also gave efficient extraction of the major trypsin/subtilisin inhibitors (groups C and D).

Polymorphism of inhibitors

IEF of water-soluble seed protein fractions from 70 inbred lines and 10 cultivars revealed polymorphism in the patterns of inhibitors both within and between cultivars. This is illustrated in Fig. 2, which shows the patterns of trypsin inhibitors in five seeds each of 5 inbred lines. The patterns of all the groups of inhibitors varied between lines, and some also varied within lines (e.g. VIR387 and VIR649, Fig. 2 parts I and IV, respectively). The major trypsin inhibitors, group A, were absent from 5 of the 70 inbred lines including VIR369 and VIR648b shown in Fig. 2, parts II and V, respectively. The absence

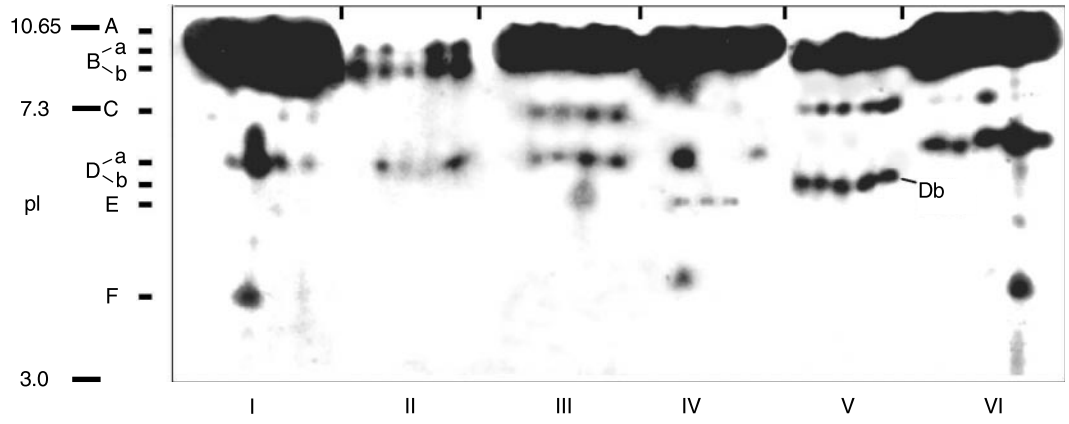

Fig. 2 Polymorphism of trypsin inhibitors in single seeds of 6 genotypes of cultivated sunflower. Water-soluble fractions from 5 individual seeds were separated by IEF (pH range 3–10) and assayed for inhibitors of trypsin as described in the legend to Fig. 1. *I* line VIR387, *II* line VIR369, *III* line VIR340, *IV* line VIR649, *V* line VIR648b (k-3195), *VI*, line VIR648a (k-2961). *A*, *Ba*, *Bb*, *C*, *Da*, *Db*, *E* and *F* indicate groups of inhibitors

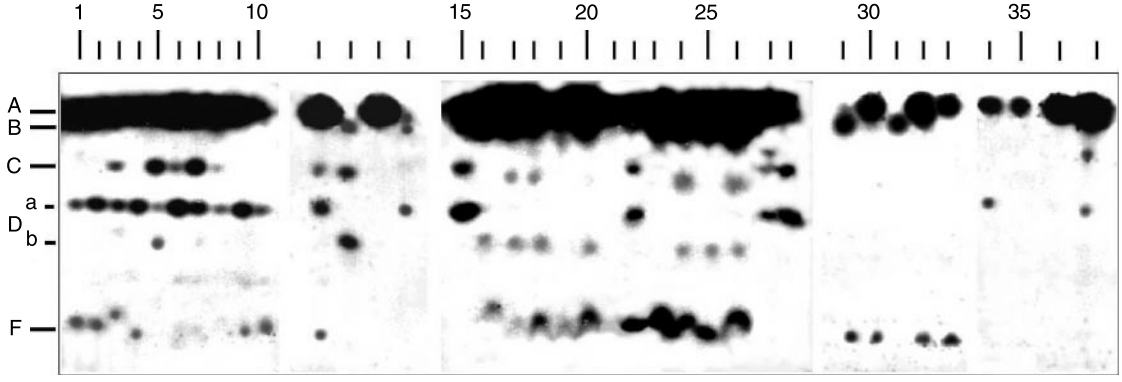

Fig. 3 Polymorphism of trypsin inhibitors in seeds of cultivated and wild species of sunflower. Water-soluble fractions were separated by IEF (pH range 3–10) and assayed for inhibition of trypsin as described in the legend to Fig. 1. *Tracks 1–15* are cultivated sunflower (*H. annuus*): *1* VIR262, *2* VIR273, *3* VIR296, *4* VIR328, *5* VIR340, *6* VIR403, *7* VIR434, *8* VIR676, *9* VIR278, *10* VIR675, *11* VIR648A, *12* VIR648B, *13* VIR649, *14* VIR369, *15* VIR130. *Tracks 16–37* are various species of *Helianthus*: *16 H. tomentosus*, *17 H. lactiflorus*, *18 H. angustifolius*, *19 H. mollis*, *20 H. rigidus*, *21 H. rigidus* x *H. annuus*, *22 H. annuus*, *23 H. rigidus*, *24 H. tuberosus*, *25 H. nuttalii*, *26 H. hirsutus*, *27 H. praecox* ssp *praecox*, *28 H. annuus*, *29–33* single seeds of *H. hirsutus*, *34–35* single seeds of *H. petiolaris*, *36–37*, single seeds of *H. debilis*

of the group A inhibitors in these lines allowed the group B components to be resolved into two sub-groups, called Ba and Bb in Fig. 2.

The trypsin/subtilisin inhibitors, groups C, D and F, were more variable than the major trypsin inhibitors (groups A and B). All three groups varied between single seeds of some inbred lines, with the group D inhibitors in VIR648b (Fig. 2, part V) having slightly lower pI than in the other lines. This variant is called Db in Fig. 2, and the normal type protein, Da.

Analysis of seed proteins from a range of cultivated and wild species of *Helianthus* showed that all contained major high pI trypsin inhibitors corresponding to the groups A and B inhibitors of *H. annuus* (cf. Fig. 3, tracks 16–37 and 1–15). Some also had components with a similar, but not always identical, pI to the group C, D and F trypsin/subtilisin inhibitors, although the levels of activity were often low (the group E trypsin inhibitors are not visible in this figure).

Whereas Fig. 3 tracks 1–28 show extracts from bulked seeds, tracks 29–37 show extracts from single seeds of 3 wild species, *H. hirsutum* (tracks 29–33), *H. petiolaris* (34, 35) and *H. debilis* (36, 37), with the loadings reduced to give better resolution of the group A and B inhibitors. Seeds 29 and 31 of *H. hirsutum* can be seen to lack group A inhibitors, similar to the *H. annuus* lines VIR648b and VIR369 (tracks 12 and 14, respectively). The single seeds also vary in the absence or presence of the group C (*H. debilis*), D (*H. petiolaris*, *H. debilis*) and F (*H. hirsutus*) trypsin/subtilisin inhibitors (Fig. 3, tracks 29–37).

No distinctions were found between the patterns of inhibitors in annual or perennial species, or between species differing in their genome composition or ploidy level. For example, the hexaploids *H. tuberosus* (2n=102, AAB) (Fig. 3, track 24) and *H. rigidus* (2n=102, AAA) (Fig. 3, track 20) had similar patterns of inhibitors to the tetraploid *H. hirsutus* (2n=68, AA) (Fig. 3, track 26) and the diploid *H. angustifolius* (2n=34, A) (Fig. 3, track 18).

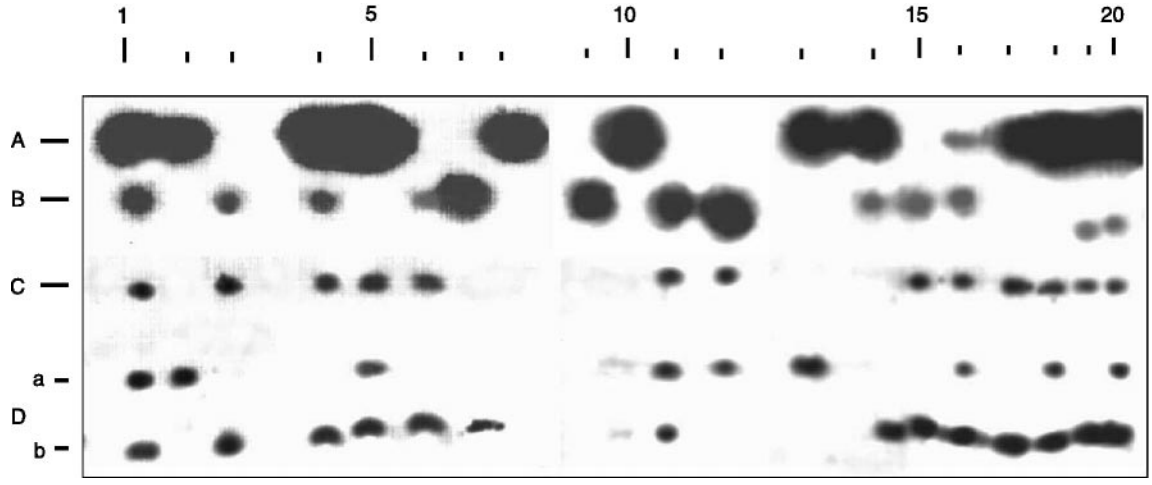

Fig. 4 Genetic analysis of trypsin inhibitors from cultivated sunflower. Water-soluble fractions from single seeds were separated by IEF (pH range 3–10) and assayed for inhibition of trypsin as described in the legend to Fig. 1. *1* F_1 hybrid VIR670 x VIR648b, *2* parental line VIR670, *3* parental line VIR648b, *4–20* F_2 seeds of the cross VIR670 x VIR648b. *A-D* indicates groups of inhibitors

Table 2 Segregation for pairs of loci encoding proteinase inhibitors in F_2 seeds from the cross VIR670 x VIR648

Pair of loci[a]	Phenotype frequency				χ^2	P (df=1)	$r \pm S_r$
	A-B-	A-bb	aaB-	aabb			
T/SI-C – T/SI-Da	68	41	31	5	8.130	< 0.05	0.32 ± 0.039
T/SI-Da – T/SI-Db	55	33	45	3	23.124	< 0.05	0.23 ± 0.036
T/SI-C – T/SI-Db	83	23	25	14	8.407	< 0.05	0.40 ± 0.050
TI-A – T/SI-C	91	20	29	6	4.020	0.025–0.05	> 0.050
TI-A – T/SI-Da	66	44	25	11	10.288	< 0.05	0.44 ± 0.041
TI-A – T/SI-Db	85	26	22	12	1.971	0.10–0.25	> 0.50

[a] T/SI, Trypsin/subtilisin inhibitors; TI, trypsin inhibitors

The only consistent difference between the patterns of inhibitors in cultivated *H. annuus* and wild *Helianthus* species was that the latter tended to have lower activities of trypsin/subtilisin inhibitors, which also tended to have lower pI than in *H. annuus*.

Inheritance of inhibitors

A series of F_1 and F_2 lines, generated by crossing lines of *H. annuus* differing in inhibitor composition, were analyzed in order to determine the inheritance and linkage relationships of the groups of inhibitors.

Lines VIR104 and VIR369 differ in that the latter lacks the major group A trypsin inhibitors, presumably a result of silencing or the absence of the encoding genes. Analysis of four F_2 families, each comprising 25 seeds, from a cross between these lines showed that 73 seeds contained group A inhibitors and 27 seeds did not. The segregation ratio of 2.7:1 corresponds to a Mendelian ratio of 3:1 ($\chi^2 = 0.212$), indicating monogenic control.

A second cross was made between line VIR670, which has the major trypsin inhibitor group A and trypsin/subtilisin inhibitor band, Da, and line VIR648b, which has trypsin inhibitor group B and trypsin/subtilisin inhibitor bands C and Db (Fig. 4). Analysis of F_1 seeds showed that the inhibitor groups present in the two parents were expressed co-dominantly. Three F_2 families, of 50, 51 and 54 seeds, were analyzed. The A, C, Da and Db inhibitors were inherited in a 3:1 ratio (Table 2), which is consistent with monogenic inheritance. The three loci encoding trypsin/subtilisin inhibitors were also linked, with the locus for the group Da inhibitors being located between those for the group C and group Db inhibitors.

The major group A trypsin inhibitors showed no linkage to the group C and Db trypsin/subtilisin inhibitors, and the significance of the weak linkage of this group with the Da inhibitors (44 ± 4%) is unclear in view of the low population of F_2 seeds analyzed.

It can be concluded, therefore, that the group A trypsin inhibitors and the group C, Da and Db trypsin/subtilisin inhibitors are each controlled by single Mendelian loci, with the three loci for trypsin/subtilisin inhibitors being linked.

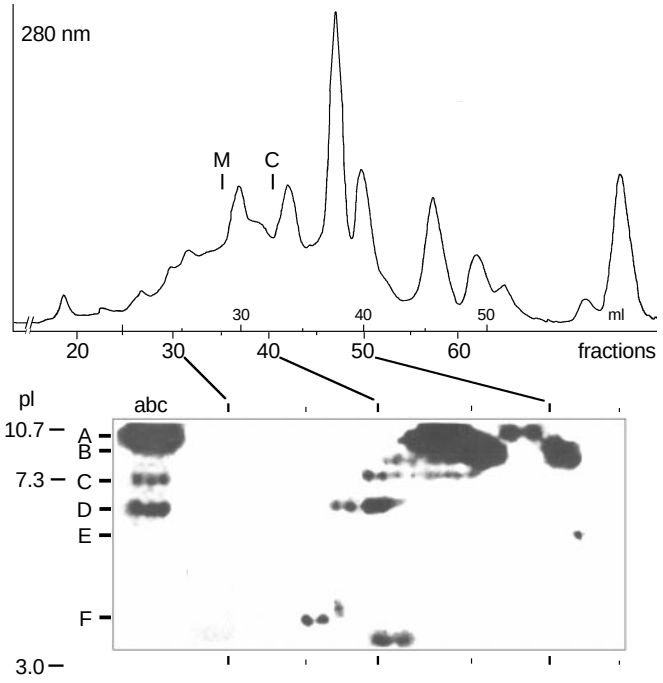

Fig. 5 Distribution of proteinase inhibitors in the water-soluble fraction from cultivated sunflower (line VIR130) separated by gel filtration on Superose 12 HR. **Top panel** Gel filtration profile. The positions of the marker proteins horse myoglobin (M_r:17,800) and horse cytochrome c (M_r: 12,400) are indicated by *M* and *C*, respectively. **Bottom panel** Separation of gel filtration fractions by IEF followed by assay for inhibition of trypsin, as described in the legend to Fig. 1. *a, b, c* are replicate samples of the fraction applied to the gel filtration column; other tracks correspond to fractions 30–65. *A-F* indicate groups of inhibitors

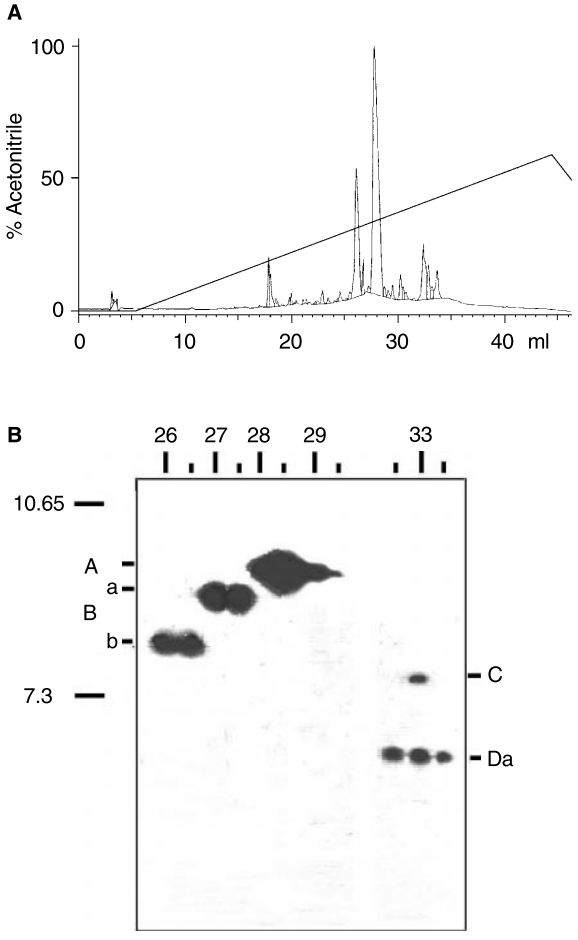

Fig. 6A, B Reversed-phase HPLC separation of sunflower (cv 'Trudovic') seed inhibitors prepared by trypsin affinity chromatography. **A** RP-HPLC profile on a Vydac C18 column, measuring the absorbance at 225 nm. **B** IEF of fractions 26–34 from RP-HPLC followed by assay for inhibition of trypsin, as described in the legend to Fig. 1. *A-D* indicates groups of inhibitors

Purification of the proteinase inhibitors

Gel filtration of a water-soluble fraction from seeds of line VIR130 on a column of Superose 12 HR 10/30 revealed a number of peaks based on absorbance at 280 nm (Fig. 5, top panel). Analysis of the individual fractions for inhibition of trypsin (Fig. 5, bottom panel), chymotrypsin and subtilisin (not shown) showed that the group C, D and F trypsin/subtilisin inhibitors were eluted earlier than the group A, B and E trypsin inhibitors, in fractions 35–42 and 41–52, respectively. It is of interest that the group A and B inhibitors in this line were each separated into several components eluting in separate peaks. The major group A components were present in fractions 43–45 and were shown to also exhibit activity against chymotrypsin.

In order to purify the individual inhibitors in sufficient amounts to characterize, we initially separated the water-soluble seed protein fraction from the cv 'Trudovic' by affinity chromatography on a trypsin-Sepharose column. A 1-mg aliquot of the fraction, which bound to the column, was then separated by RP-HPLC using a Vydac C18 column (Fig 6 A) and the fractions monitored for inhibitory activity (Fig. 6B).

Three trypsin inhibitors were initially eluted, one of which corresponded to group A inhibitors and two to group B inhibitors (labeled Ba and Bb in Fig. 6). These were followed by fractions containing the C and Da groups of trypsin/subtilisin inhibitors.

IEF of the purified inhibitors with protein markers of known pI allowed a more precise determination of their pI, as given in Table 1. The purified fractions were also analyzed by one-dimensional SDS-PAGE using a TRIS/Tricine gel system to resolve low M_r components. This showed that the group A and B trypsin inhibitors had a low M_r, of about 1,500 and about 2,500, respectively, while three bands corresponding to trypsin/subtilisin inhibitors had a M_r ranging from about 1,500 to 6,000 (Fig. 7). It is no table that the group A trypsin inhibitors were only fixed in the gel by treatment with 15% glutaraldehyde, which is consistent with their low M_r. A similar low M_r was demonstrated for the group A trypsin inhibitors prepared by micro-preparative IEF rather than RP-HPLC (results not shown).

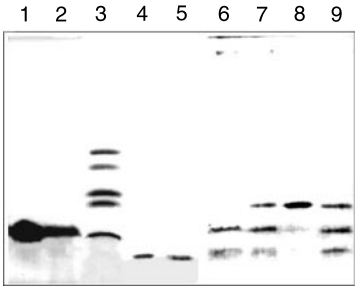

Fig. 7 SDS-PAGE of inhibitor fractions prepared from sunflower (cv 'Trudovic') by affinity chromatography on trypsin-Sepharose followed by RP-HPLC (**Fig. 6**). *Lane 1* Fraction 26 (component Bb), *2* fraction 27 (Ba), *3* M_r marker proteins, *4* fraction 28 (group A), *5* fraction 28.5 (group A), *6–9* fractions 32, 32.5, 33, 33.5 (groups C and D). The M_r marker proteins in track 3 are, in order of decreasing M_r, 16,949; 14,404; 8,159; 6,214 and 2,512.

Discussion

We have described the identification, genetic control and characterization of novel low M_r trypsin and trypsin/subtilisin inhibitors from seeds of sunflower using a combination of small-scale gelatin replica overlays and conventional chromatographic procedures. The trypsin inhibitors are basic proteins (pI 9.0–10.0) of M_r 1,500 and 2,500. They are considerably smaller than other trypsin inhibitors previously characterized from plant sources (Richardson 1991; Shewry 1999), which may relate to the methods of preparation rather than the absence of low M_r inhibitors from other plant species. These methods avoided precipitation or dialysis, procedures which may lead to the loss of low M_r components. Similarly, the three trypsin/subtilisin inhibitors which were partially purified had a low M_r (1,500–6,000) when compared with other characterized proteinase inhibitors, most of which have a M_r above about 8,000 (Richardson 1991; Shewry 1999). An exception is the squash family of serine proteinase inhibitors which are only present in seeds of the Cucurbitaceae and comprise 27–33 residues (Otlewski and Krowarsch 1996). These were the smallest known plant protein inhibitors of serine proteinases known before the present study.

Further studies of the sunflower trypsin and chymotrypsin inhibitors are in progress in order to determine their interactions with the target proteinases and structures. Konarev et al. (1999) found trypsin/subtilisin inhibitor groups C, Da and Db to be active against extracellular proteinases of the sunflower pathogen *Sclerotinia sclerotiorum.*

The major group A trypsin inhibitor (SFTI-1) has also been purified from the cultivar 'Sunbred 246' and its structure determined in complex with trypsin (Luckett *et al.* 1999). This has revealed an unusual cyclic structure comprising 14 amino acid residues with an M_r of 1,513. Detailed studies of the other inhibitor groups have not been carried out, but *N*-terminal sequencing of the group D trypsin/subtilisin inhibitors has revealed sequence heterogenity with no indication of sequence similarly with previously characterized groups of inhibitors (authors' unpublished results).

It is clear, therefore, that sunflower seeds, and probably also other plant tissues, contain novel groups of low-M_r proteinase inhibitors which have not been detected by previous studies. These may have unusual structures and properties with potential applications in crop protection and medicine.

Acknowledgements IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom. The study was supported by the Royal Society Joint Project "Characterization of Sunflower Seed Proteins" (683072.P810).

References

Bowles DJ (1990) Defence-related proteins in higher plants. Annu Rev Biochem 59: 873–907

Konarev AlV (1986) Analysis of proteinase inhibitors from wheat grain by gelatin replicas method. Biokhimiya 51: 195–201

Konarev AlV (1987) Variability of trypsin-like proteinase inhibitors in wheat and related cereals (in Russian). Skh Biol 5: 17–24

Konarev AlV 1996. Interaction of insect digestive enzymes with protein inhibitors from plants and host-parasite coevolution. Euphytica 92: 89–94

Konarev AlV, Kochetkov VV, Bailey JA, Shewry PR (1999) The detection of inhibitors of the *Sclerotinia sclerotiorum* (Lib) de Bary extracellular proteinases in sunflower. J Phytopathol 147: 105–108

Luckett S, Garcia RS, Barker JJ, Konarev AlV, Shewry PR, Clarke AR, Brady RL (1999) High-resolution structure of a potent, cyclic protease inhibitor from sunflower seeds. J Mol Biol 290:525–533

Otlewski, J, Krowarsch D (1996) Squash inhibitor family of serine proteinases. Acta Biochim Pol 43: 431–444

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

Ryan CA (1990) Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu Rev Phytopathol 28: 425–449

Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulphate-poly-acrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166: 368–379

Shewry PR (1999) Enzyme inhibitors of seeds: types and properties. In: Shewry PR, Casey R (eds) Seed proteins. Kluwer Academic Publ Dordrecht, the Netherlands 587–615

Shewry PR, Lucas JA (1997) Plant proteins that confer resistance to pests and pathogens. Adv Bot Res 26: 135–192