The structure and sites of biochemical interaction of cotton defensive proteins and secondary metabolites

CRDC number: ULA5C

Principal investigator:
Dr Roland Chung
Present address: Victorian College of Pharmacy, Department of Medicinal Chemistry, Monash University, 381 Royal Parade, Parkville, Victoria 3052
Telephone: (03) 9903 9000
Fax: (03) 9903 9582

Sponsor/team leader/co-investigator:

Dr Gideon Polya
School of Biochemistry, La Trobe University, Bundoora, Victoria, 3083
Telephone: (03) 9479 2157
Fax: (03) 9479 2467
e-mail: g.polya@latrobe.edu.au

Plain English summary:

A variety of proteins were purified from cotton seeds and were analysed by amino acid sequence determination (automated Edman degradation) and by state-of-the-art electrospray ionization mass spectrometry (ESMS). The amino acid sequences of about 40 vicilin-related cotton proteins were precisely defined. The molecular masses of the deduced sequences were in agreement with the masses observed by ESMS to within about 1-2 Da (i.e. to within the mass of 1-2 hydrogen atoms). A further major cotton seed protein was purified and shown to be a γ-conglutin-related protein with 2 component subunits and a molecular mass of 46250.3 ± 1.3 Da. The vicilin fractions and the γ-conglutin variously have anti-fungal activity against a wide range of fungi tested including the cotton pathogens *Fusarium oxysporum* and *Verticilium dahliae*. This precise work is potentially useful for classical and transgenic approaches to cotton plant breeding for pest resistance. Cotton leaves also contain anti-fungal components and a defensive component with larvicidal activity against the blowfly *Lucilia cuprina* and the mosquito *Aedes camptorhynchus*. Large-scale purifcation procedures were developed to enable further biological testing. Protein and non-protein fractions from cotton are variously active as inhibitors of animal cyclic AMP-dependent protein kinase and of the proteases trypsin and chymotrypsin.

Background to the project:

Cotton (*Gossypium hirsutum*) is subject to attack at particular developmental stages from a variety of insect herbivores (most notably *Helicoverpa punctigera*) and fungal pathogens such as *Fusarium oxysporum* and *Verticilium dahliae*. Plants, including cotton, defend themselves against microbial pathogens and insects by elaborating a variety of defensive proteins and defensive secondary metabolites. Such defensive molecules include small to medium-sized anti-fungal and/or anti-insect proteins and relatively much smaller bioactive secondary metabolites. This project was directed to detecting and isolating such defensive molecules from cotton, defining the structure of such molecules and determining molecular sites of action i.e. possible biochemical sites of interaction in the insects and fungi targeted by these defensive molecules.

The project built on a large body of recent published work from Dr Polya's laboratory defining the structure and biochemical interactions of a variety of plant defensive proteins and secondary metabolites (for review see refs. 1', 2', 1, 7 & 8 at the end of this report). This work also included determination of high affinity biochemical sites of action of the cotton defensive metabolite gossypol that is a potent protein kinase inhibitor (refs. 1', 2'). We have shown that representatives of about a dozen major classes of plant defensive metabolites are also potent and selective protein kinase inhibitors (refs. 1, 7) and indeed many of the plant defensive proteins we have studied interact with a protein kinase (ref. 8). Protease inhibitors can interfere with insect digestion of proteins and can have an anti-feedant anti-insect effect. Many of the plant defensive proteins we have studied in recent years are anti-fungal proteins and also have protease inhibitory properties, suggesting a potential dual anti-fungal and anti-insect function for these proteins.

Accordingly it was considered useful to attempt to detect, purify and define the structure and biochemical targets of defensive proteins and metabolites elaborated by cotton. The precise objectives of the project are outlined below.

The objectives of the project and the extent to which they have been achieved:

The objectives of the project are outlined below with the extent to which they have been achieved included in parentheses:

1. Purification and structural characterization of cotton defensive proteins. [Over 40 such proteins - including precisely defined processing variants - have been resolved by cation exchange and RP HPLC. The amino acid sequences of most of these have been completely or near-completely deduced such that calculated masses are in precise agreement with those observed by ESMS within experimental error. The study targeted cotton seeds and leaves].

2. Determination of anti-fungal biological targets of cotton defensive proteins. [The anti-fungal activity of the major vicilin-related proteins and the cotton γ-conglutin purified and characterized in (1) was demonstrated in a variety of systems including growth assays involving cotton pathogens. The same type of analysis was applied to cotton leaf fractions].

3. Determination of anti-insect activity of cotton proteins and metabolites. [No activity against *Helicoverpa punctigera* was demonstrated - in a sense not surprising in view of the tagreting of cotton by this organism. However some non-protein and highly-purified protein fractions from cotton were found to be variously active against *Lucilia cuprina* and *Aedes camptorhynchus*].

4. Determination of biochemical targets of cotton (seed and leaf) proteins and secondary metabolites. [Potent non-protein protein kinase A, trypsin and chymotrypsin inhibitory activity was detected and resolved in various systems. Extensive purification of cotton seed trypsin and chymotrypsin inhibitory proteins was achieved].

5. Large-scale purifcation of bioactive proteins and metabolites from cotton with a view to additional value-added by-products. [Large-scale purification to homogeneity of 2 specific processed vicilin-related gene products and of cotton seed γ-conglutin was achieved. Purification of potent bioactive secondary metabolites is advanced but not yet concluded].

Results of the project

The results of the project are outlined below. Sections 1 and 2 of the report detail the work of Dr Roland Chung that also involved inputs from myself, Dr Greg Neumann (ESMS analysis) and Mrs Rosemary Condron (Edman sequencing). Sections 3 and 4 of the report deal with work largely carried out by Dr Gideon Polya with contributions specifically acknowledged in relation to purification of γ-conglutin (Dr Roland Chung), anti-fungal testing (Dr Roland Chung, Ms Vinochani Pillay), protease and PKA inhibitory activities and isolation of vicilin-related proteins (Ms Donna Grey), protease inhibitory activity of a cotton protein fraction and rapid large-scale isolation of vicilins (Aaaron Uschakov), *Lucilia cuprina* and *Helicoverpa punctigera* anti-insect assays (Antonio Rajic), ESMS analysis (Dr Greg Neumann) and Edman sequencing (Mrs Rosemary Condron).

The methodologies employed are largely set out in the major research publication so far deriving from the project (ref. 4) but succinct descriptions of key processes are also given in the detailed report below.

1. Proteins from cotton seeds with *in vitro* antifungal activity.

Introduction.

Antifungal proteins (AFPs) are elaborated by plants as a defence against fungal pathogens[1,2]. Some of these are enzymes, like chitinases, which catalyses the hydrolyses of fungal cell wall components[1]. However, there are others which are categorised as storage proteins or having other non-defence related functions that nonetheless exhibits defence functionality[3,4]. This section summarizes the results presented in the paper[5] concerning proteins isolated from cotton seeds which exhibited *in vitro* antifungal activity and also presents additional antifungal data not discussed in the paper.

Experimental.

Cotton seeds or leaves were ground up in liquid N_2 and the soluble protein extracted into sodium phosphate (pH 6) buffer by homogenizing with an Ultra Turrax blender, followed by filtration and then centrifugation. The supernatant was filtered and the basic component isolated by binding onto and elution from carboxymethylcellulose (Whatman CM52) and subsequently by C_8 reversed phase HPLC. The relatively insoluble "wall" component was extracted from the residue of this initial extraction with a high salt-high pH buffer, usually 1 M NaCl pH 8 Tris buffer or 2 M NaCl pH 9.5 glycine buffer. The extract was then filtered and centrifuged before it was diluted 20- to 40- fold (for 1 M and 2 M NaCl, respectively) with pH 6 phosphate buffer. The diluted extract was then bound onto and eluted from CM52 matrix as described. The CM52-binding basic protein solution was concentrated by pressure filtration (Amicon YM3 membrane), desalted by dilution with water and then reconcentrated to around 2-3 ml. This concentrate was then filtered through a 0.22 μm filter (Phenomenex 4 mm nylon) and then injected onto a Baker Bond C_8 reversed phase HPLC. The bound components were then eluted with an ascending gradient of acetonitrile in water. The acidic components of the extract were isolated by binding onto and elution from Diethylaminoethyl (DEAE) cellulose.

Results.

In the HPLC profile of a standard extraction (Figure 1), 4 major peaks were observed. The first 3 peaks eluted at between 25-27% acetonitrile. The fourth peak eluted much later at 50% acetonitrile. Peaks 1 and 2 encompasses a family of proteins with masses of between 9 to 11 kDa. Peak 3 was found to comprise a major component with a mass of 16318 Da, structurally related 15-16 kDa entities and minor 9-11 kDa entities with similar masses to those in peaks 1 and 2. Peak 4 has the largest mass of around 46250 Da.

The 16.3 kDa and 46.3 kDa fractions were successfully subjected to extensive N-terminal sequence analyses (Table 1). The 16.3 kDa fraction was found to be nearly identical with a cotton vicilin (α-globulin) preprotein while the 46.3 kDa fraction was found to have a 61% identity and 75% similarity with a γ-conglutin from *Lupinus angustifolius*.

Detailed analysis of peaks 1 to 3 by electrospray ionization mass spectrometry (ESMS), N-terminal Edman sequencing and cDNA-based sequencing data revealed that the 9-11 kDa proteins in peaks 1 and 2 are probably processing variants from the16.3 kDa vicilin preprotein[5].

Table 1. N-terminal sequences of HPLC fractions.

Protein/ HPLC fractions	N-terminal Edman sequence
16.3 kDa fraction	1 KDFPGRRGDDDPSKRYE 17
alpha-globulin	26 KDFPGRRGDDDPPKRYE 42
46.3 kDa fraction	1 VDITRPLSYTPLIISPQGEYYMEVKSIR 28
gamma-conglutin	251 LDVLHDLVYTPLTISKQGEYFIQVNAIR 278

The HPLC profile of the "wall" extraction shows a major peak which eluted at around 50% CH_3CN and was determined by ESMS to be the same as the 46.3 kDa protein from the standard extraction described previously. Very small quantities of lower molecular mass entities were detected. The high salt-high pH extraction is therefore a good method for isolating from plant sources the less soluble components, like the 46.3 kDa protein, in substantially higher quantities.

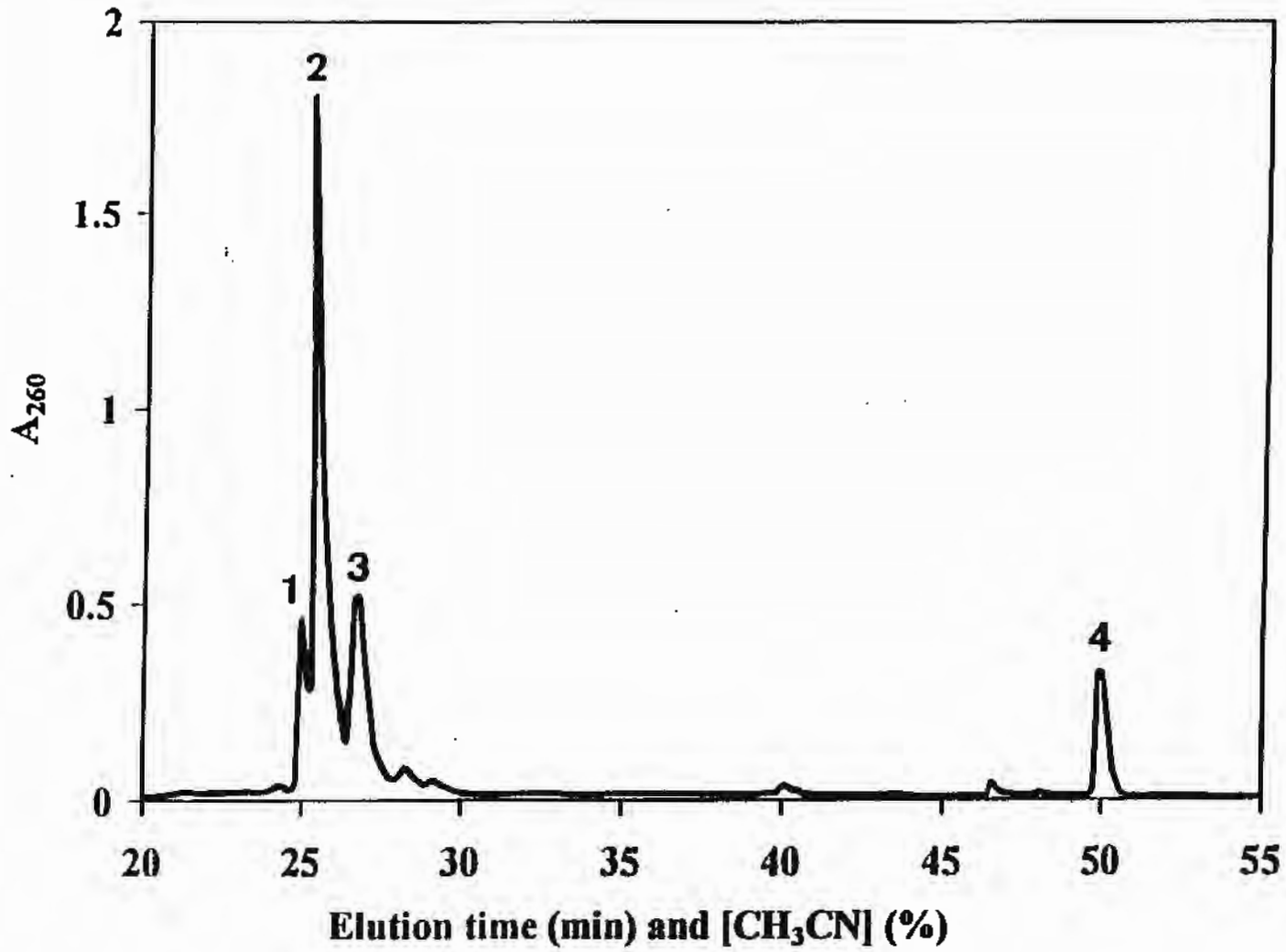


Figure 1. Purification of cotton basic proteins by C_8 reversed phase HPLC, with an increasing CH_3CN concentration in aqueous-0.1% TFA.

Antifungal testing.

Antifungal testing were carried out against the following fungi: Alternaria brassicicola (Schw.) Wiltsh isolated from *Brassica oleracea* L. var gemmifera DC., *Botrytis cinerea* (apple fruit and rosemary leaves), *Chalara elegans* (tobacco roots), *Fusarium oxysporum* Schlecht isolated from *Atriplex* sp., *Rhizotonia solani* Kuhn isolated from *Chrysanthenum* sp., *Alternaria macrospora* and *Verticillium dahliae* isolated from cotton (Narrabri), *Fusarium oxysporum* f.sp. vasinfectum (*Fov*) isolated from cotton (Cecil Plains, Queensland) and *Fusarium proliferatum* (Matsushima) Nirenberg isolated from *Asparagus officinalis* L.. The following fungi were all obtained from the Institute for Horticulture Development, Agriculture Victoria, Department of Natural Resources and Environment and were also tested: *Penicillium italicum*, *Penicillium digitatum*, *Alternaria tenuis*, *Botrytis cinerea* (pear and grape), *Geotrichum candium* and *Penicillium expansum*.

In most cases, spore producing fungi were tested in 96-well flat-bottomed microtitre plates with ½ strength potato dextrose broth (PDB) as the culture media while the non-spore producing fungi, like Rhizoctonia solani, and ones where there are great difficulty in inducing spore production, like Alternaria macrospora, were tested on potato dextrose agar (PDA) plates. In the microtitre plate method, 80 µl of media containing 10^4 spores/ml were tested with with the addition of 20 µl of test sample or appropriate control. The growth of the fungi hyphae were monitored photometrically at 540 nm or 595 nm in a Spectra Max 250 spectrophotometer (Molecular Devices). The microtitre plates were incubated at 24°C and were monitored at various intervals over several days. In the PDA petridish method, 15 µl of test sample or controls were placed separately onto sterile 1 cm diameter filter paper disks (Whatman no. 1) which were positioned at the outer edge of the plate, equidistant from the centre, where a PDA block containing the mycellium was aseptically positioned. The growth of fungal hyphae, or absence of it, over each of the paper disks were monitored visually.

In a typical graphical profile of an antifungal assay, in this case the effect of the cotton seed basic proteins on the growth of *Botrytis cinerea* isolated from rosemary is highlighted (Figure 2). It shows, by the retardation of hyphae growth, that fraction 3 (16.3 kDa) and fraction 2 (9-11 kDa proteins) and to a lesser extent fraction 4 (46.3 kDa) have antifungal activity against this fungi. The growth of various filamentous fungi was measured in the presence or absence of various concentrations of cotton seed basic proteins (fractions 1-4), as shown in Table 2.

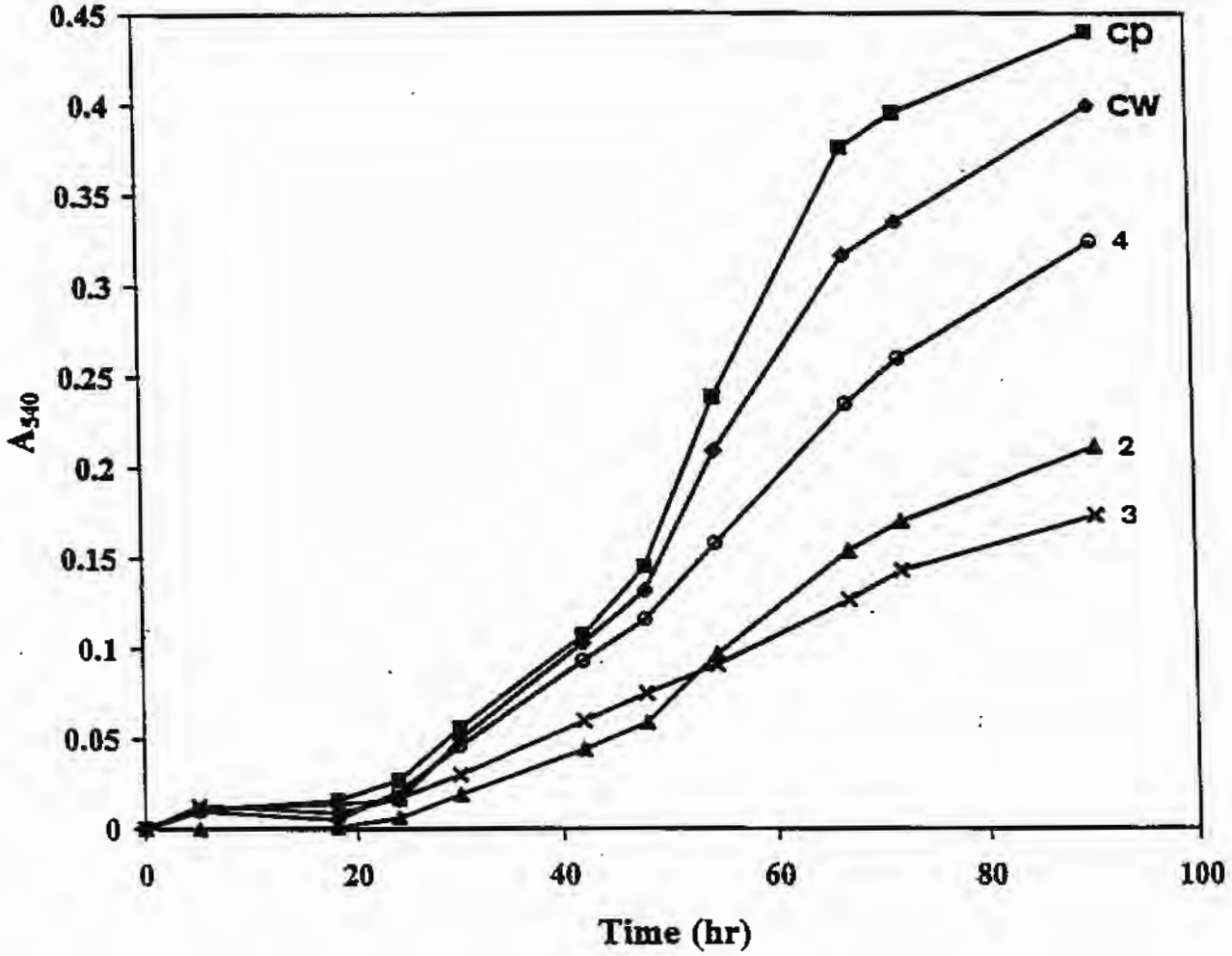


Figure 2. Effect of the various HPLC fractions on *Botrytis cinerea*.(■-■), cp, protein control, 200 µg/ml BSA; (◆-◆), cw, water control; (o-o), HPLC peak fraction 4, 46.3 kDa , 10 µg/ml; (x-x), HPLC peak fraction 3, 16.3kDa, 9.2 µg/ml; (▲-▲), HPLC peak fraction 2, 9-11 kDa proteins, 1 µg/ml.

Table 2. Inhibition of fungal growth by cotton basic protein fractions 1-4.

Fungus	Fraction	Concentration(μg/ml) and [% Inhibition]	IC_{50} (μg/ml)
Botrytis cinerea (rosemary)	2	1 [52 %]	-
	3	9 [53 %]	-
	4	10 [21 %]	-
Botrytis cinerea (apple)	1	3 [45 %]	-
	2	1 [29 %]	-
	3	6 [36 %]	-
	4	41 [44 %]	-
Alternaria brassicicola	2	6 [36 %]	-
(*Brassica oleracea*)	3	12 [57 %]	9
	4	67 [36 %]	-
Chalara elegans	2	2 [57 %]	2
(tobacco root)	3	7 [95 %]	2
	4	13 [0 %]	-
Fusarium oxysporum	2	10 [86 %]	6
(*Atriplex* sp.)	3	29 [27 %]	-
	4	20 [0 %]	-
Fusarium proliferatum	2	2 [34 %]	-
	3	6 [22 %]	-
	4	9 [53 %]	-
Fusarium oxysporum Fov	2	100 [45 %]	-
(cotton)	3	100 [27 %]	-
	3	286 [44 %]	-
	4	62 [27 %]	-
	4	124 [51 %]	-
Verticillium dahliae	2	148 [33 %]	-
(cotton)	3	100 [24 %]	286
	4	124 [54 %]	-

Fractions 1 and 2 contain overlapping sets of 9-11 kDa cysteine-rich vicilin-derived proteins, fraction 3 contains minor amounts of these proteins along with a major 16.3 kDa cysteine-rich small vicilin protein and fraction 4 contains only 46.3 kDa γ-conglutin-like protein. Percent inhibition of fungal growth at the indicated concentrations of inhibitory proteins was determined relative to the water controls after about 50-70 hr of incubation is presented in square brackets. Also presented are IC_{50} values (concentrations for 50 % inhibition of fungal growth) where determined, based on plots of the concentration dependence of fungal growth inhibition.

The crude CM52-binding and DEAE-binding fractions were also used in tests against various other fungi obtained from the Institute for Horticultural Development. The results are summarised in Table 3.

Table 3. Inhibition of fungal growth by cotton basic CM52-binding (CM+) and acidic DEAE-binding (DE+) protein fractions.

Fungus	Fraction	Concentration(μg/ml) and [% Inhibition]
Penicillium expansum	CM+	17 [0 %]
	DE+	33 [0 %]
Geotrichum candium	CM+	17 [0 %]
	DE+	33 [91 %]
Botrytis cinerea (grape)	CM+	17 [59 %]
	DE+	33 [32 %]
Botrytis cinerea (pear)	CM+	40 [31 %]
	DE+	15 [20 %]
Alternaria tenuis	CM+	40 [73 %]
	DE+	15 [72 %]
Penicillium digitatum	CM+	40 [38 %]
	DE+	15 [21 %]
Penicillium italicum	CM+	40 [35 %]
	DE+	15 [31 %]

Such tests undertaken with crude protein extracts represents a rapid path to screening for effectiveness against a large number of fungal or other microbial pathogens.

In tests against the mycellium of *Rhizoctonia solani*, as carried out on PDA plate, no inhibition was detected with 1-3 μg of fractions 1-4 proteins in the test area. For *Alternaria macrospora*, no inhibition was detected with 10 μg of the same proteins in the test area.

Other Biological Assays.

Anti-insect assays, carried out by Dr Ray Akhurst of the CSIRO Division of Entomology (Canberra), involves mixing ng-scale of Bt toxin with 1-10 μg of the basic protein fractions 1-4 in 24-well plate at 50°C. No synergism with these proteins were detected.

Cytotoxicity assays was carried out in a 96-well microtitre plate with each well containing 100 μl of leukemia K562 cells (10000 cells/ ml in 10 % foetal blood serum FBS) and 10 μl of a protein sample. The plate was then incubated at 37°C for 5 days and then subjected to a MTT assay. It was found that 2 μg/ ml of the basic 9-11 kDa protein fractions 1-2 gave a 17 % inhibition while the 16 kDa fraction 3 and the 46 kDa fraction 4 proteins were less effective.

The basic protein fractions 1-4 were found to be neither substrates for nor significant inhibitors of wheat germ calcium-dependent protein kinase (CDPK), avian gizzard myosin light chain kinase (MLCK), rat liver protein kinase A catalytic subunit (cAK) and rat brain protein kinase C (PKC). No inhibitions were detected against potato phosphatases, bovine pancreas chymotrypsin, bovine pancreas trypsin, porcine pancreas elastase and *Bacillus* a-amylase.

In tests against blowfly (*Lucilia* sp.) larvae, undertaken by Antonio Rajic of the Department of Biochemistry (La Trobe), preliminary results show some inhibition of the growth of these larvae with 500 μg/ ml of the 46 kDa basic protein fraction but not 1 mg/ ml of the 16 kDa or 500 μg/ ml of the 9-11 kDa basic protein fractions.

Summary.

The cotton seed basic protein fraction was separated into 4 peaks by C_8 reversed phase HPLC. The structures of the constituent proteins were mainly determined by the use of ESMS, Edman N-terminal sequencing and cDNA-based

database information. Peaks 1-3 comprise about 40 vicilin-derived 9-11 kDa proteins deriving from four main vicilin variants, namely AG1, AG2 and two AG1 variants. For each variant, there are families of proteins with differences at the C and/or N terminals of the sequence. The major component of peak 3 are 16 kDa vicilin-related proteins and a family of 7 proteins deriving from different truncation at the N- and C-terminal of a closely related protein. Peak 4 contains a 46250 Da protein which comprises 22441 Da and 22821 Da disulphide-linked subunits. This protein has a 61 % identity with a γ-conglutin from *Lupinus angustifolius*.

The basic protein fractions have *in vitro* antifungal activity against a variety of fungi. The IC_{50} values of the 9-11 kDa protein peak 2 fraction for *Botrytis cinerea* (from rosemary), *Chalara elegans* and *Fusarium oxysporum* (from tobacco root) are around 1, 2 and 6 µg/ ml, respectively. The IC_{50} value of the 16.3 kDa peak 3 fraction for *Alternaria brassicicola* is 9 µg/ ml. None of the resolved cotton seed basic proteins are substrates for or inhibitors of PKA, PKC, MLCK or CDPK and nor are they inhibitors of chymotrypsin, trypsin, elastase or cyclic nucleotide-binding phosphatase.

References.

[1] D.J. Bowles, Defence-related proteins in higher plants. Annu. Rev. Biochem.,59 (1990) 873-907.

[2] H. Bohlmann and K. Apel, Thionins. Annu. Rev. Plant Physiol. Mol. Biol., 42 (1991) 227-240.

[3] F.R.G. Terras, H.M.E. Schoofs, K. Thevissen, R.W. Osborn, J. Vanderleyden, B.P.A. Cammue and W.F. Broekaert, Synergistic enhancement of the antifungal activity of wheat and barley thionins by radish and oil-seed rape 2S albumins and by barley trypsin inhibitors. Plant Physiol., 103 (1993) 1311-1319.

[4] F.R.G. Terras, H.M.E. Schoofs, M.F.C. De Bolle, F. Van Leuven, S.B. Rees, J. Vanderleyden, B.P.A. Cammue and W.F. Broekaert, Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. J. Biol. Chem., 267 (1992) 15301-15309.

[5] R.P.-T. Chung, G.M. Neumann and G.M. Polya, Purification and characterization of basic proteins with *in vitro* antifungal activity from seeds of cotton, *Gossypium hirsutum*. Plant Science, 127 (1997) 1-16.

2. Proteins from leaves of cotton.

Introduction.

The predominant proteins in the seeds are the storage proteins, some of which have already been established in the previous section to be potential antifungal proteins. The processed proteins in the leaves would serve vastly different functions. It is important to screen these proteins for antifungal and anti-microbial activities. This information may then be used by the molecular biologists to find the genes coding for such proteins and then incorporating them in breeding programs to find more fungi-tolerant plants. The efforts to isolate antifungal proteins were again being directed primarily at the basic proteins because of the relative small number of basic proteins and also the characteristics of antifungal proteins in other plant species being small cysteine-rich basic proteins. Nevertheless, other fractions that showed biological activity were also examined.

Experimental.

A typical extraction of proteins from cotton leaves is similar to that performed on seeds. Leaves (65 g) from 2-4 week old plants, grown in a temperature-controlled laboratory with light from both tungsten and fluorescent sources, were harvested. These were either used immediately or stored at –25°C. The leaves were ground up in liquid nitrogen to a powder, suspended in 300 ml of 10 mM sodium phosphate buffer (pH 6) and then homogenized in an ultraturrax blender. The homogenate was filtered through 2 layers of gauze and miracloth and then centrifuged at 16000 rpm for 15 min in a SS34 rotor (Sorvall). The supernatant was filtered through miracloth and the basic proteins then bound to CM52 matrix. Unbound proteins were removed by repeated washings with the extraction buffer and the basic proteins then eluted with 1 M NaCl in 10 mM Tris buffer (Cl^-, pH 8). Less than 100 µg of basic protein was obtained by this procedure from more than 0.5 g of protein in the supernatant.

An alternative protocol involves initially two methanolic extractions (200 ml and 140 ml, respectively) of 91 g of powdered leaves, afterwhich the resulting residue was then extracted successively with water (2 x 200 ml) by homogenization in a blender and with 2 M NaCl in 10 mM glycine (pH 9.5) (150 ml) by stirring for 16 hr at 4°C. The water extracts were filtered, treated with CM52 cellulose and the basic proteins eluted with 1 M NaCl in 10 mM Tris (Cl^-, pH 8) buffer. The high

salt-high pH (2 M NaCl pH 9.5 glycine buffer) extract, termed the "wall" fraction, was filtered and centrifuged at 16000 rpm for 15 min before the basic proteins were bound onto and eluted from CM52 matrix. By using this alternative method, 1.2 mg, 0.2 mg and 1.5 mg of CM52-binding basic proteins were isolated from the two water and the one salt-glycine extractions, respectively.

The CM52-binding fraction from the first water extraction was concentrated by pressure filtration (Amicon YM3 membrane), desalted by dilution with water and then reconcentrated to a 5 ml solution containing 0.1 mg/ml of protein. This concentrate was then filtered through a 0.22 μm filter (Phenomenex 4 mm nylon) and then injected onto a Vydac C_8 reversed phase HPLC. The bound components were eluted with an ascending gradient of acetonitrile in water.

Non-CM52 binding eluate from both the above methods were treated with DEAE cellulose from which the DEAE-binding acidic proteins were eluted with 1 M NaCl in 10 mM Tris (Cl^-, pH 8) buffer. Approximately 0.3-0.4 g of acidic protein was obtained from the former method. A low yield (0.15 mg) of water-eluted protein was obtained from the binding onto phenyl-sepharose (CL-4B) matrix. Similarly, treatment of the DEAE-binding protein (31 mg) with concanavalin A agarose resulted in the elution (with 0.2 M NaCl, 0.5 M a-methyl glucoside in 50 mM Tris pH 8 buffer) of only 1.4 mg of protein. The initial methanolic extract was evaporated *in vacuo* and the residue reconstituted in water.

Results.

Two main components were eluted from the C_8 reversed phase HPLC of the CM52-binding water extract, as shown in the HPLC profile (Figure 3). The broad tailing peak that eluted at 21-27 % acetonitrile (in a uniform gradient of 1 % per min increase from water) has a yellow colouration and contains very little protein as determined by the Bradford method (ref). This observation was confirmed by the absence of any protein signals in an ESMS spectrum. Thin-layered chromatography (TLC) of this fraction on a silica plate revealed a lower R_f value than for Gossypol indicating a more polar compound. The second component in the HPLC profile is a smaller, sharper peak that eluted at 46 % acetonitrile. An ESMS spectrum of this peak shows a single species with a molecular mass of 10654 ± 1 Da. An Edman N-terminal sequencing of this fraction revealed the following sequence: IEVLLGSDDGGLAFIPKEFS. Search of the protein databases gave a 85 % identity and a 90 % similarity with plastocyanin of *Solanum crispum* and *Nicotiana tabacum*.

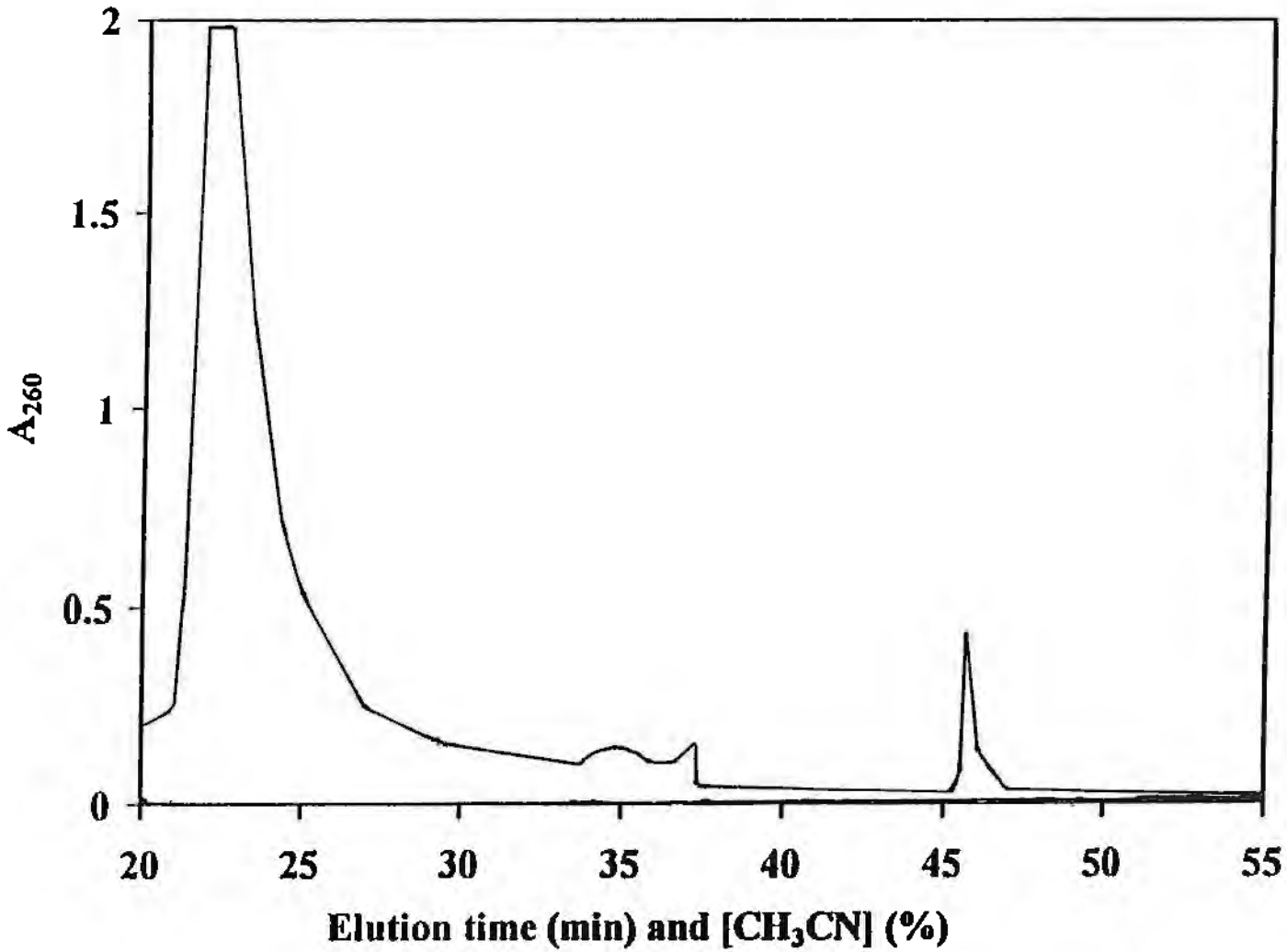


Figure 3. Purification of cotton leaves basic proteins by C_8 reversed phase HPLC, with an increasing CH_3CN concentration in aqueous-0.1% TFA.

The DEAE-binding fraction was eluted with a stepwise gradient of increasing concentrations of sodium chloride in pH 8 Tris buffer. A 200-300 mM salt concentration gave the optimum elution of protein from the DEAE matrix which corresponded to phosphatase activity with p-nitrophenyl phosphate (PNP) as substrate. Phosphatase activity was determined photometrically at 400 nm in 100 mM acetate buffer (Na^+, pH 5.0) containing 4 mM $MgCl_2$ and 1 mM PNP.

Antifungal assays.

The protocols employed were the same as those used in testing for antifungal activity in seed proteins. The following table summarises the effect on the growth of some filamentous fungi, as measured in the presence or absence of various concentrations of various cotton leaf protein fractions (Table 4).

Inhibition of fungal growth is observed with both the DE-binding and concanavalin A agarose-binding fractions for *Verticillium dahliae* (cotton), *Botrytis cinerea* (apple), *Penicillium italicum* and *Fusarium oxysporum* (*Atriplex* sp.). Phosphatase activity was detected in these fractions with PNP as substrate, therefore it is possible that the antifungal activity arose from this phosphatase. This postulation is supported by the significant loss of inhibitory effect when the DE-binding fraction was heated at 100 °C for 5 min. Complete obliteration of activity was not observed, either because of incomplete heating or that there is also a heat stable component with antifungal activity in this fraction.

Some inhibition of the growth of fungal hyphae of *Penicillium italicum* and *Fusarium oxysporum* was also observed with the water reconstituted methanolic extract. This is a useful result as the methanolic extract contains substantial amount of proteins.

For *Alternaria macrospora*, no inhibition was detected with 2.2 µg and 0.3 µg, of the respective CM-binding and DE-binding protein fractions of the first water extraction, in the test area.

Summary.

The preliminary testing of the leaf-derived proteins showed some *in vitro* antifungal activity. Different proteins to those isolated in the seed were obtained, including a phosphatase and a plastocyanine. Substantial amount of protein were also present in some of the other fractions, for example the methanolic extract. There are therefore scope for discovering potential antifungal proteins in leaves.

Table 4. Inhibition of fungal growth by cotton leaf protein fractions. The fractions tested were: The DE-binding fraction (DE+), the concanavalin A agarose-binding fraction (Con A Agarose+), the methanolic extract that was evaporated and then redissolved in water (Methanol+ {H_2O}) and the high salt-high pH "wall" extract that had been bound onto and eluted from CM52 matrix ("wall" CM+). Percent inhibition of fungal growth at the indicated concentrations of inhibitory proteins was determined relative to the water controls after about 50-70 hr of incubation is presented.

Fungus	Fraction	Concentration (µg/ml)	[% Inhibition]
Botrytis cinerea	Con A Agarose+	4	14
(apple)	"wall" CM+	10	NI
	Methanol+ {H_2O}	20	NI
Chalara elegans	Con A Agarose+	4	NI
(tobacco root)	"wall" CM+	10	NI
	Methanol+ {H_2O}	20	NI
Penicillium italicum	Con A Agarose+	4	11
	"wall" CM+	10	26
	Methanol+ {H_2O}	20	32
Fusarium oxysporum	Con A Agarose+	4	11
(*Atriplex* sp.)	"wall" CM+	10	25
	Methanol+ {H_2O}	20	58
Verticillium dahliae	DE+	44	64
(cotton)	DE+ heat treated	44	18
	Con A Agarose+	8	6

Acknowledgements.

This project was supported by a research fellowship and grant to R. Chung from the Cotton Research and Development Corporation (CRDC). We are grateful to Ian Pascoe and Bronwyn Clarke (Institute for Horticulture Development, Agriculture Victoria), Dr Joe Kochman (Queensland Department of Primary Industries), Dr Steve Allen (Narrabri) and Dr Marilyn Anderson (School of Biochemistry, La Trobe University) for supplying fungi, Harman Hamidson and Mery Hasmeda for assistance with antifungal assays, Rosemary Condron for Edman sequencing and particularly Dr Gregory Neumann for ESMS and invaluable assistance in sequence analysis. Thanks also to the Cotton Seed Distributors (Wee Waa, NSW) for cotton seeds and the other personnel in the laboratory for their participation in discussion and experiments. Finally, much appreciation to the project supervisor Dr Gideon Polya.

Report sections 3 & 4

As indicated above, studies on cotton defensive components were conducted in parallel to Dr Chung's work outlined in sections 1 and 2. Most of the work described in parts 3 and 4 below was done by Dr Gideon Polya but the contributions of others are as follows: γ-conglutin purification and *Verticilium dahliae* and *Fusarium oxysporum* growth inhibition assays (Dr Roland Chung), PKIA activity in cotton seed vicilin-containing fractions (Ms Donna Gray, an honours student in 1997), cotton protease inhibitor studies on the cotton seed wall CM+/∅+ fraction and rapid large-scale purification of cotton vicilin and vicilin-related proteins from the same fraction (Aaron Uschakov, an honours student in 1998), *Helicoverpa punctigera* and *Lucilia cuprina* larva growth assays applied to cotton seed and leaf fractions (Antonio Rajic, graduate student) and *Leptosphaeria maculans* anti-fungal assays (Ms Vinochani Pillay, graduate student). ESMS analysis in parts 1-4 of the project was conducted by Dr Greg Neumann and Edman sequencing by Mrs Rosemary Condron.

3. Protease inhibitors, protein kinase inhibitors and antifungal proteins in cotton

Plant protease inhibitors have a major anti-insect function and can in a number of cases act also as anti-fungal agents. It was accordingly of major interest to detect and resolve protease inhibitory proteins and secondary metabolites from cotton. Further, we have shown that representatives of some major classes of plant defensive secondary metabolites are potent inhibitors of eukaryote protein kinases and notably of cyclic AMP-dependent protein kinase A (PKA) (refs. 1', 2'). In particular the cotton defensive compound gossypol inhibits various eukaryote protein kinases (refs. 1', 2'). Accordingly such components were sought in cotton seeds and leaves.

Initial experiments indicated the presence of chymotrypsin, trypsin and PKA inhibitory activity (CI, TI and PKAI activity, respectively) in both aqueous and methanol extracts of cotton seeds (Sicala 33 and Sicala V2) that were routinely pulverized after addition of liquid nitrogen. **Nearly all of the experiments were conducted using Sicala V2 seeds.**

Trypsin activity was routinely measured at 30°C over 1 hr in a 1 ml reaction medium containing 0.1 M Tris (Cl^-, pH 8.0), 0.05 mM BAPNA (N-α-benzoyl-D,L-arginine-p-nitroanilide) and 0.01 mg/ml TPCK-treated trypsin (Worthington). Liberated p-nitroaniline was measured from the absorbance at 405 nm. Chymotrypsin activity was measured in the same fashion but with 0.05 mM BYPNA (N-benzoyl-L-tyrosine-p-nitroanilide) as the chromogenic substrate and 0.01 mg/ml TLCK-treated bovine chymotrypsin present. Protein kinase activity was measured radiochemically using highly puriifed eukaryote protein kinases and specific synthetic peptide substrates as described previously (refs. 1', 2').

(a) Methanol-soluble trypsin inhibitors

Methanol-soluble trypsin inhibitory activity was found in a variety of fractions prepared as outlined below:

i. Pulverized seeds were extracted in 10 mM sodium phosphate buffer (pH 6.0) (buffer A), the homogenate filtered through Miracloth, centrifuged at 50,000 g for 15 min and the resulting supernatant passed through carboxymethylcellulose (CM52). After elution of the vicilin-rich protein fraction in 2M NaCl- 10 mM sodium phosphate (pH 9.5) (buffer B) the eluate was passed through phenylsepharose CL4B and retained protein eluted in H_2O. However a yellow fraction remained bound to this hydrophobic matrix and could be eluted in methanol (MeOH). The MeOH soluble fraction was rotevaporated to dryness and the residue re-dissolved in H_2O and thence MeOH. Both of these fractions contain trypsin inhibitory (TI) activity. MeOH elution of the CM52 matrix from application of this procedure also yielded some TI activity.

ii. Seeds were extracted in MeOH. The MeOH solution (dark brown) was concentrated by rotevaporation and applied to a Waters C18 Sep-Pak cartridge which was subsequently eluted with H_2O, MeOH and absolute ethanol (EtOH). The MeOH and EtOH eluates contain TI activity (as well as PKA inhibitory (PKI) activity).

iii. A seed MeOH extract was diluted in buffer A and applied to CM52 which was successively eluted with buffer A, buffer B, H_2O, MeOH, EtOH and EtOH-0.1 M HCl, the last fraction containing PKAI activity. The same procedure was also applied using DEAE-cellulose (DE52). The MeOH extract was diluted in buffer A, then brought to 20 mM Tris (Cl^-, pH 8.0) and applied to DE52 which was then successively eluted with 20 mM NaCl- 20 mM Tris (Cl^-, pH 8.0), H_2O and MeOH-0.1 M HCl., the last fraction containing TI activity (as well as PKAI activity).

iv. After seed was extracted in buffer A and centrifuged, the resultant pellet was extracted in a number of solvents. An EtOH-soluble red oil from the pellet extraction with 70% CH_3CN has TI activity as does the MeOH extract of this pellet fraction.

v. Material retained on Miracloth after MeOH extraction of seeds but removed (as a cloudy suspension) from the crude cell wall material by extensive washing with H_2O was passed through CM52 in buffer A. The material subsequently eluted in buffer C has TI and CI activity as described in 3b(vii) below. However coloured (possibly secondary metabolite) material retained on the CM52 and eluted in MeOH has TI activity.

We conclude that ethanol-soluble and methanol-soluble trypsin inhibitory (TI) activity is present in cotton seeds. It should be noted that the initial MeOH extract also contains chymotrypsin inhibitory (CI) activity and PKA (PKAI) inhibitory activity. No α-amylasae inhibitory activity was found in any of the MeOH or H_2O soluble cotton seed fractions examined. The active components have not yet been purified and characterized although a number of chromatographic approaches to large-scale isolation have been explored.

(b) Trypsin inhibitory (TI) activity in aqueous fractions

TI activity was found in aqueous fractions from subfractionation of cotton seed extracts and attempts were made to purify the active component.

i. Cotton seed was extracted in MeOH and the wall fraction (after extensive washing with H_2O) was extracted in 2M NaCl-10 mM glycine pH 9.5 (buffer C). The MeOH fraction contains PKAI, TI and CI activity (as described above). However TI activity (and also some weaker CI activity) is present in the aqueous buffer C extract. The non-wall, MeOH-insoluble fraction that dissolves at high ionic strength also contains TI and CI activity. The aqueous washings of the wall fraction contain TI activity that binds to CM52 at pH 6.

ii. The cotton seed wall fraction TI activity extracted in buffer C (as in (i) above) binds to phenylsepharose CL4B and is eluted in H_2O. This activity binds to a Sep-Pak C18 matrix in the presence of 0.1% TFA and is eluted by 70% CH_3CN in 0.1% TFA (as does the non-wall soluble fraction TI activity) but also binds to CM52.

iii. Attempts were made to purify the non-wall, buffer C-soluble TI activity. One progression involved MeOH seed extract → non-wall, MeOH insoluble fraction → buffer C extract (43 mg protein) → C18 reverse phase → passage through CM52 (TI does not bind) (0.04 mg) → C8 RP HPLC (activity finally lost or too dilute to measure).

iv. An attempt was made to purify the wall-associated TI. The progression involved MeOH seed extract → buffer C extract of wall (no prior H_2O washing of the wall since TI activity was found in the H_2O washings in previous preparations) → binding to CM52 (eluted in buffer C) → gel filtration on Ultrogel AcA 44 (low molecular weight TI activity) → reverse phase chromatography on C18 (activity finally lost).

v. A further attempt was made to purify the wall-associated TI. The progression involved MeOH seed extract → cell wall washed well with H_2O and then extracted in buffer C → off phenylsepharose CL4B in H_2O → off CM52 in buffer C → RP HPLC on a C8 column → activity lost (inactivated, lost or too dilute).

vi. A possible explanation for the result in (v) above was given by the finding that when the wall fraction extract in buffer C (isolated as described in (v)) was acidified with 0.2% TFA prior to C18 chromatography on bulk C18 matrix, the resultant pellet fraction when re-solubilized (yield about 2.6 mg/10 g seed) had TI (and weak CI) activity. As described in part 4(b) below, this fraction had larvicidal activity against *Lucilia cuprina* larvae.

vii. The following modified MeOH procedure was applied to 50 g Sicala V2 seeds: pulverized in liquid N_2 → extracted twice in MeOH → MeOH insoluble material not retained by Miracloth dissolved in buffer A (14 mg) and the MeOH insoluble and H_2O-insoluble material dissolving in buffer C (43 mg) → water washing of the cell wall fraction on Miracloth (300 mg) → cell wall fraction extracted in buffer C (noting that the pH was adjusted back to pH 9.5 in this procedure) (29 mg). The water washings of the

wall were applied to CM52 (on in buffer A and off in buffer C) to yield a CM52-binding (CM+) fraction (163 mg) and subsequent fractionation of the CM– fraction on DE52 (on in 10 mM Tris (Cl^-, pH 8.0), off in 1M NaCl-10 mM Tris (Cl^-, pH 8.0) (buffer D) yielded a DE52-binding (DE+) fraction (42 mg).

These various fractions were subfractionated using C18 and CM52 and analyzed for CI and TI activity. The material washed off the cell wall in H_2O and binding to CM52 has TI and CI activity. TI activity (but not CI activity) is retained after centrifugation and after binding to C18. The non-wall, MeOH-insoluble and H_2O-isoluble material that is solubilized by buffer C has both TI and CI activity that is retained after binding to C18.

It should be noted that material retained on Miracloth after MeOH extraction but removed (as a cloudy suspension) from the crude cell wall material by extensive washing with H_2O was passed through CM52 in buffer A. The material subsequently eluted in buffer C has TI and CI activity as described above. However coloured (possibly secondary metabolite) material retained on the CM52 and eluted in MeOH has TI actvity.

viii. Finally, 50 g pulverized cotton seed (Sicala V2) was extracted in MeOH and the wall fraction retained on Miracloth and the MeOH-insoluble fraction that passed through Miracloth was recovered by centrifugation. The wall fraction extracted with buffer C (without prior washing of the wall with H_2O) (968 mg) contains both TI and CI activity. The MeOH-insoluble fraction that passed through Miracloth and which was insoluble in buffer A but soluble in buffer C contains TI activity.

We conclude that in addition to the TI/CI activity in the MeOH fraction, such activity associated with proteins is present in the cell wall fraction and in the non-wall fraction. Procedures for extensive puriifcation of these entities have been established and scaled up purification procedures should permit eventual purification and structural characterization of these defensive protease inhibitory components.

(c) Protein kinase inhibitors in cotton

We found that the cotton bioactive secondary metabolite gossypol is an inhibitor of cyclic AMP-dependent protein kinase (PKA) (IC_{50} 10 μM) as well as of Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK) (IC_{50} 144 μM) and of plant Ca^{2+}-dependent protein kinase (CDPK) (IC_{50} 17 μM) (refs. 1', 2'). Gossypol also inhibits Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) (Caulfield & Bolander, 1986). However other PKA-inhibitory (PKAI) components are likely to be present.

As described in section 3(a) above, MeOH-soluble components in a number of cotton seed fractions are potent inhibitors of PKA. Attempts were made to resolve these components from raw cotton seed oil and from MeOH extracts of pulverised cotton seeds. These investigations are succinctly outlined below.

i. Both the MeOH extract of cotton seeds and raw cotton oil contain potent PKA inhibitory (PKAI) activity. Thus even a million-fold dilution of raw cotton seed oil in the standard PKA assay produces about 80% inhibition of PKA.

ii. An extensive fractionation of cotton seed proteins described in section 3(a)i above yielded a multiplicity of fractions from either the soluble or cell wall-derived fractions and which in turn gave protein fractions which variously bound to CM52 (or did not) and bound to phenylsepharose CL4B (or did not). A number of these fractions inhibit PKA in the standard assay conditions. However in many cases the inhibition was trivial and could be attributed to protein kinase inhibition by phosphatase activity in the fraction.

However a soluble, CM52-binding fraction was found to inhibit PKA after a reverse phase step on a C18 matrix in conditions (acid pH and high CH_3CN concentration) that would be expected to inactivate enzymic inhibitors of the net protein phosphorylation reaction. This inhibitory activity could be shown to co-purify on reverse phase HPLC on a C8 column with vicilin-related proteins (for details see section 3(d) below). However after a large number of careful experiments we concluded that this effect was due to co-purification with these proteins of residual amounts of the extraordinarily potent PKAIs found in the MeOH extract and oil fraction from cotton seeds i.e. that we were not dealing with PKAIs that were proteins.

This conclusion led to a modification of our procedures for the isolation of bioactive proteins from cotton - we inserted an initial step involving successive extractions with MeOH in order to avoid any contamination with potent PKAI metabolites.

iii. A large number of experiments were conducted in attempt to resolve the presumed secondary metabolite PKAIs from cotton seed oil. PKAI activity was shown to partition from cotton oil into various solvents including H_2O and MeOH. Such partitioning experiments were conducted using centrifugation in Eppendorf tubes or by use of a separating funnel. In a major progression, cotton seed oil was extracted with H_2O and then the H_2O-extracted oil was extracted in MeOH. PKAI activity was present in the MeOH fraction and the residual oil fraction. The cotton seed oil material dispersed in H_2O from the procedure was subjected to reverse phase chromatography on a C18 SepPak in 0.1% TFA. Material eluting in 0.1% TFA-70% CH_3CN and 0.1% TFA-100% CH_3CN contained PKAI activity. The PKAI material eluting in 0.1% TFA-70% CH_3CN was soluble in 70% CH_3CN but not in H_2O after rotoevaporation.

(iv) Successive extraction of 50 g Sicala V2 seeds with *circa* 70 ml of the following solvents yielded the following apparent protein yields (on the basis of Coomassie binding): MeOH 1 (434 mg) → MeOH (2) (28 mg) → EtOH (1358 mg) → BuOH (9 mg) → H_2O (329 mg) →) 0.1% TFA- 80% CH_3CN (80 mg) → 0.1% TFA-100 %CH_3CN (6 mg). PKAI activity is present in the MeOH, EtOH and BuOH fractions but not in the subsequent extracts.

(d) Large-scale fractionation of cotton seed protein constituents

Following on our successful resolution and structural characterization of a multiplicity of cotton antifungal proteins, a major effort was made to scale up the purification procedures. High yield preparations are needed for anti-insect screening experiments and

for bioactivity screening against a variety of fungal pathogens. Ultimately high yield preparations will be required for 3-dimensional structural studies if these are warranted.

Because gossypol is biologically active, a sensible early strategy for relatively large-scale isolation involved seed powder extraction with relatively non-polar solvents to remove gossypol and other secondary metabolites soluble in such solvents. Methanol was chosen as a compromise non-polar solvent to minimize protein denaturation while maximizing secondary metabolite, phospholipid and oil extraction. A variety of basic protocols were successively employed and refined and these are summarized below in order of development.

Procedure 1. Initial procedure.

The procedure initially employed to isolate and characterize cotton seed defensive involved the following protocol: pulverized seed → 10 mM phosphate (Na^+, pH 6.0) (buffer A) extraction → filtration and centrifugation → batchwise carboxymethylcellulose (CM52) chromatography with elution in 1M NaCl in 10 mM Tris (Cl^-, pH 8.0) (buffer D) → RP HPLC on a C8 column in 0.1 % TFA (elution in a gradient of increasing CH_3CN in 0.1% TFA) → *circa* 0.1 mg amounts of γ-conglutin and vicilin-related defensive proteins.

The need to have much larger amounts of such proteins for anti-fungal testing, anti-insect testing and for further structural studies led to extensive revision and refinement of the above procedure as outlined below.

Procedure 2. Cell wall as well as soluble proteins extracted. Since the major proteins found in the first part of the project (section 1 above) were basic, it was considered likely that some of these would bind to cell walls and accordingly the cell wall fraction was retained and extracted at high ionic strength. The first progression was as follows: 50 g pulverised cotton seed (Sicala 33) → extraction in buffer A → filtration (to recover cell wall) & centrifugation of the filtered homogenate (to obtain the soluble fraction;). The cell wall fraction was extracted in buffer B → cell wall fraction. Both the cell fraction and the soluble fraction were subjected to chromatography on CM52 (on at low ionic strength in buffer A and elution in buffer B), on phenylsepharose CL4B (on at high ionic strength and elution in H_2O) and on a C18 matrix (on in 0.1% TFA and off in 70% CH_3CN-0.1% TFA). The fractions were concentrated by pressure filtration using a YM3 membrane (cut-off 3000 Da) The fractions from this (and indeed further preparations) were routinely designated wall or soluble (wall-derived or soluble), CM+ or CM– (binding to CM52 or not), Ø+ or Ø– (binding to phenylsepharose CL4B or not), C18 + or C18– (binding to C18 or not), DE+ or DE– (binding to DE52 DEAE-cellulose or not) and YM3+ or YM3– (retained by a YM3 membrane on pressure filtration or not).

This procedure yielded a much greater amount of protein as follows: soluble CM+ (175 mg), soluble CM+/Ø– (57 mg), soluble CM+/Ø+ (33 mg), wall CM+ (677 mg), wall CM+/Ø– (139 mg) , wall CM+/Ø+ (9 mg), wall DE+ (294 mg), wall DE+/Ø– (170 mg) and wall CM+/Ø+ (7 mg).

RP HPLC and biochemical activity/structure analysis:

RP HPLC analysis of the various fractions from this procedure was conducted and of particular interest was such analysis applied to the "soluble CM+/∅–/YM3+" fraction (i.e. the cotton seed soluble fraction binding to CM52 but not to phenylsepharose CL4B and concentrated by pressure filtration on a YM3 membrane). This fraction was of interest because it was most similar to the soluble fraction dealt with in part 1 of this report and because it contained PKAI activity after fractionation on C18. (It should be noted that some of the fractions from this procedure contained phosphatase activity and heat-labile PKAI activity in these fractions could be attributed to this enzymic activity).
RP HPLC of the "soluble CM+/∅–/YM3+" fraction in the standard system on a C8 column (increasing gradient of CH_3CN concentration in 0.1% TFA) initially indicated 3 components eluting with PKAI activity (Fig. 3.1). Better resolution was achieved by flattening the CH_3CN gradient (Fig. 3.2), the PKIA activity in this instance going with the trailing edge of the profile. However this latter analysis also revealed a further peak of PKAI activity not associated with a protein peak. As discussed in section 3(c), from a large amount of experimentation relating to possible PKAI proteins from cotton seed, we concluded that the PKAI activity associated with purified cotton proteins might be due to residual PKAI secondary metabolites associated with such proteins.

RP HPLC was also carried out on other cotton seed protein fractions from application of this procedure.

Structural analysis of "soluble CM+" cotton seed proteins:

The well-resolved peak 3 material from RP HPLC ("Cot 3") (Fig. 3.2) was analyzed by electrospray ionization mass spectrometry (ESMS) (Fig. 3.3 A & B), SDS-PAGE (Fig. 3.4) and Edman N-terminal amino acid sequencing (Fig. 3.5). C-terminal processing (a "C-terminal ladder") (Fig. 3.3 A) revealed that there were 2 families of vicilin-related entities present that could be very precisely defined with respect to amino acid sequence.

One family of components derive from the cotton vicilin-related protein AG1 (R^{77}-YQEN^{155}) (calculated average molecular mass 10266.2 Da; observed mass 10267.0 Da) through progressive deletion of C-terminal residues thus: AG1 (R^{77}-E^{154}) (calculated mass 10152.1 Da; observed mass 10152.0 Da), AG1 (R^{77}-Q^{153}) (calculated mass 10023.0 Da; observed mass 10024.0 Da) and AG1 (R^{77}-Y^{152}) (calculated mass 9894.8 Da; observed mass 9896.2 Da).

A second family of vicilin-related proteins in peak 3 correspond to processing products of a vicilin-related protein AG1' (D^{73}QQQR^{77}-YQEN^{155}) (calculated mass 10749.7 Da; observed 10749.6 Da) (that is related to AG1 simply by being 17 kDa lower in mass in the common D^{73}-N^{155} region (see Chung et al., 1997) (Fig. 3.6), thus: AG1' (D^{73}-E^{154}) (calculated mass 10635.6 Da; observed mass 10634.2 Da), AG1' (D^{73}-Q^{153}) (calculated mass 10506.5 Da; observed mass 10506.6 Da) and AG1' (D^{73}-Y^{152}) (calculated mass 10378.3 Da; observed mass 10378.4 Da).

RP HPLC on a C8 column of the cotton seed wall CM+ fraction revealed components eluting in the same region as the vicilin-related proteins described above as

Fig. 3.1 RP HPLC of the cotton seed "soluble CM+/∅–/YM3+" fraction.

The cotton seed "soluble CM+/∅–/YM3+" fraction was subjected to RP HPLC on a C8 column eluted in a gradient of increasing CH_3CN concentration in 0.1% TFA. Continuous trace, A_{230} ; histogram, PKA activity (% control with 25 µl of the indicated fraction in the standard assay).

Fig. 3.2 RP HPLC of the cotton seed "soluble CM+/∅–/YM3+" fraction.

The cotton seed "soluble CM+/∅–/YM3+" fraction was subjected to RP HPLC on a C8 column eluted in a gradient of increasing CH_3CN concentration in 0.1% TFA (graident more extended than in Fig. 3.1). Continuous trace, A_{230} ; histogram, PKA activity (% control with 25 µl of the indicated fraction in the standard assay).

Fig. 3.3 A ESMS of cotton seed proteins (fraction Cot3 from Fig. 3.2).

The Cot 3 fraction from Fig. 3.2 was subjected to ESMS analysis.

Fig. 3.3 B ESMS of cotton seed proteins (fraction Cot3 from Fig. 3.2).

The Cot 3 fraction from Fig. 3.2 was subjected to ESMS analysis with analysis of the higher molecular weight zone.

Fig. 3.4 SDS PAGE of cotton seed fraction Cot 3 (Fig. 3.2).

Fig. 3.5 Edman N-terminal sequencing of Cot 3 (Fig. 3.2).

Fig. 3.6 Average molecular masses and inferred amino acid sequences of Cot3 proteins.

Fig. 3.1 RP HPLC of the cotton seed "soluble CM+/∅–/YM3+" fraction.

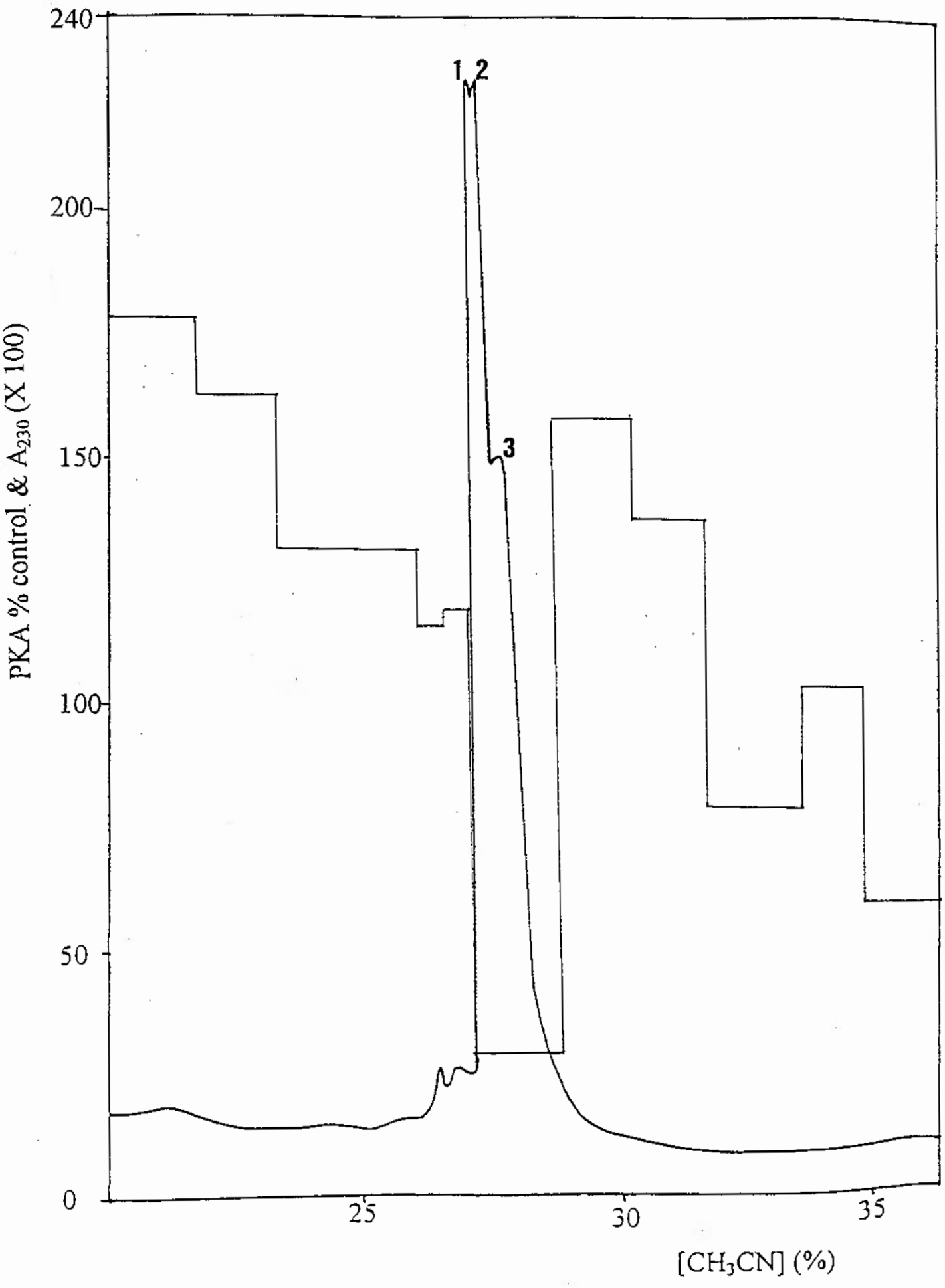
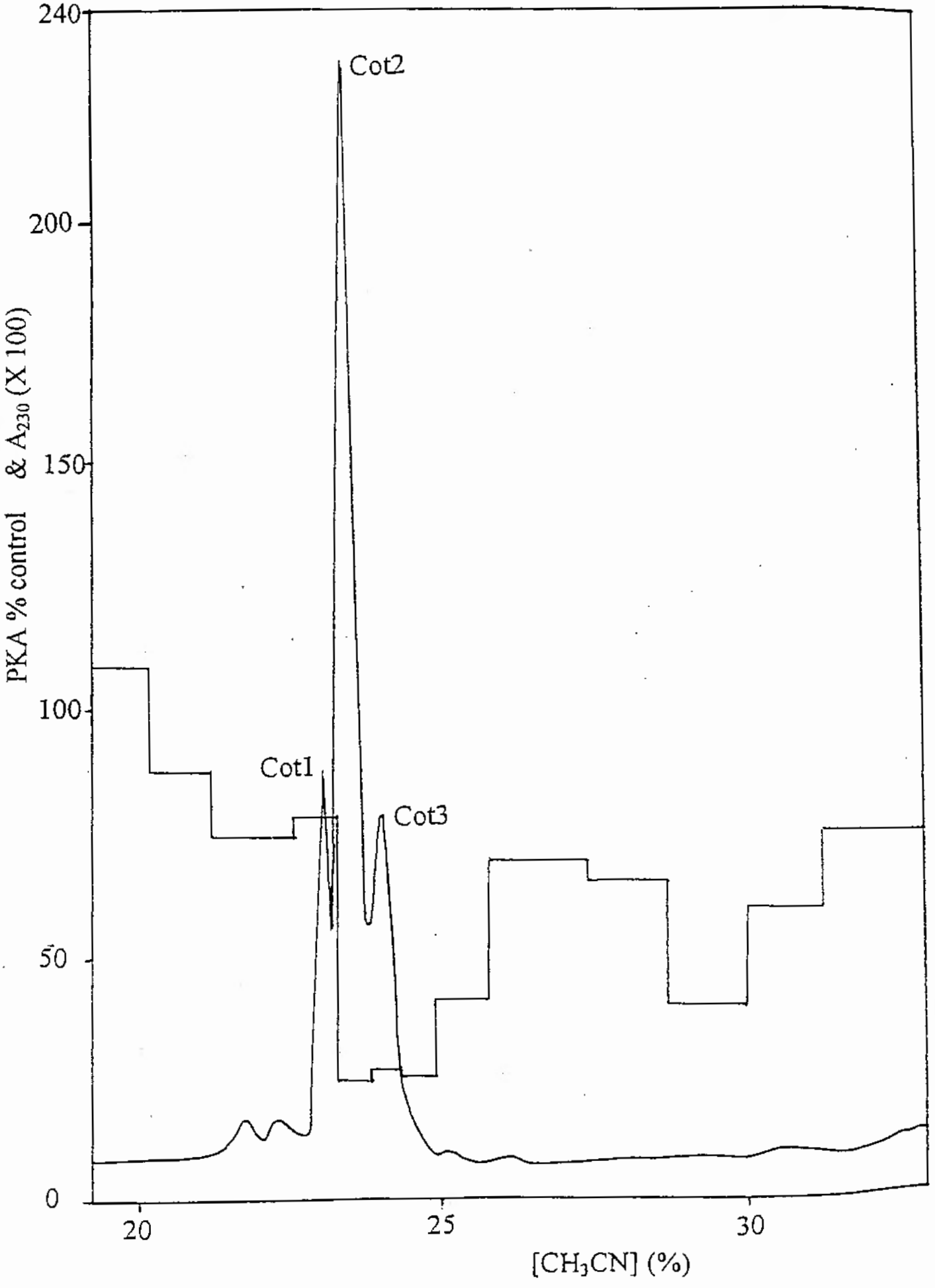


Fig. 3.2 RP HPLC of the cotton seed "soluble CM+/∅–/YM3+" fraction.



CS13221: No Title

BioSpec Reconstruct for Spectrum from 2.40 min (55 scans) from CS13221

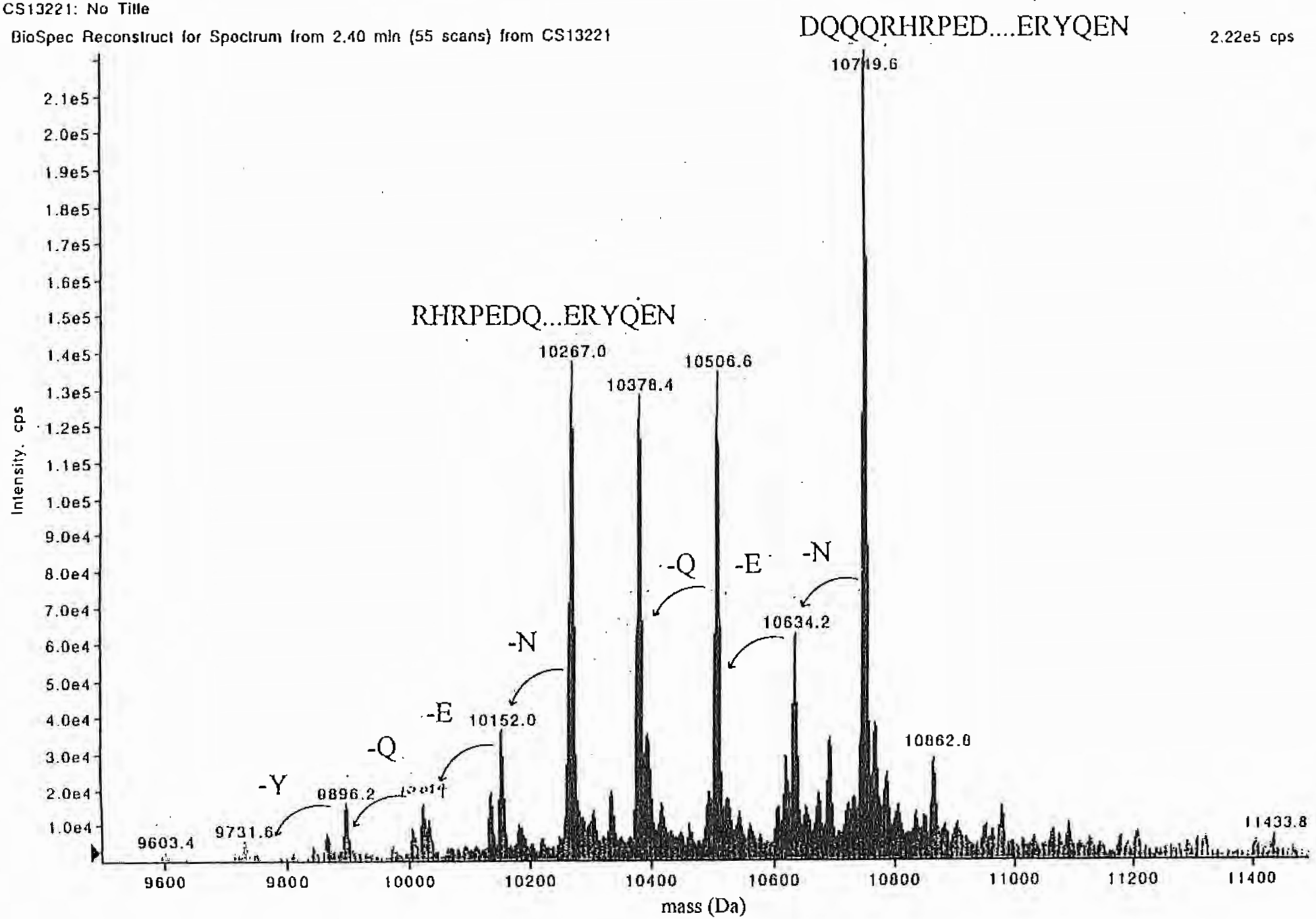


Fig. 3.3 A ESMS of cotton seed proteins (fraction Cot3 from Fig. 3.2).

BioSpec Reconstruct for Spectrum from 4.45 min (99 scans) from CS13221hor200

1.37e5 cps

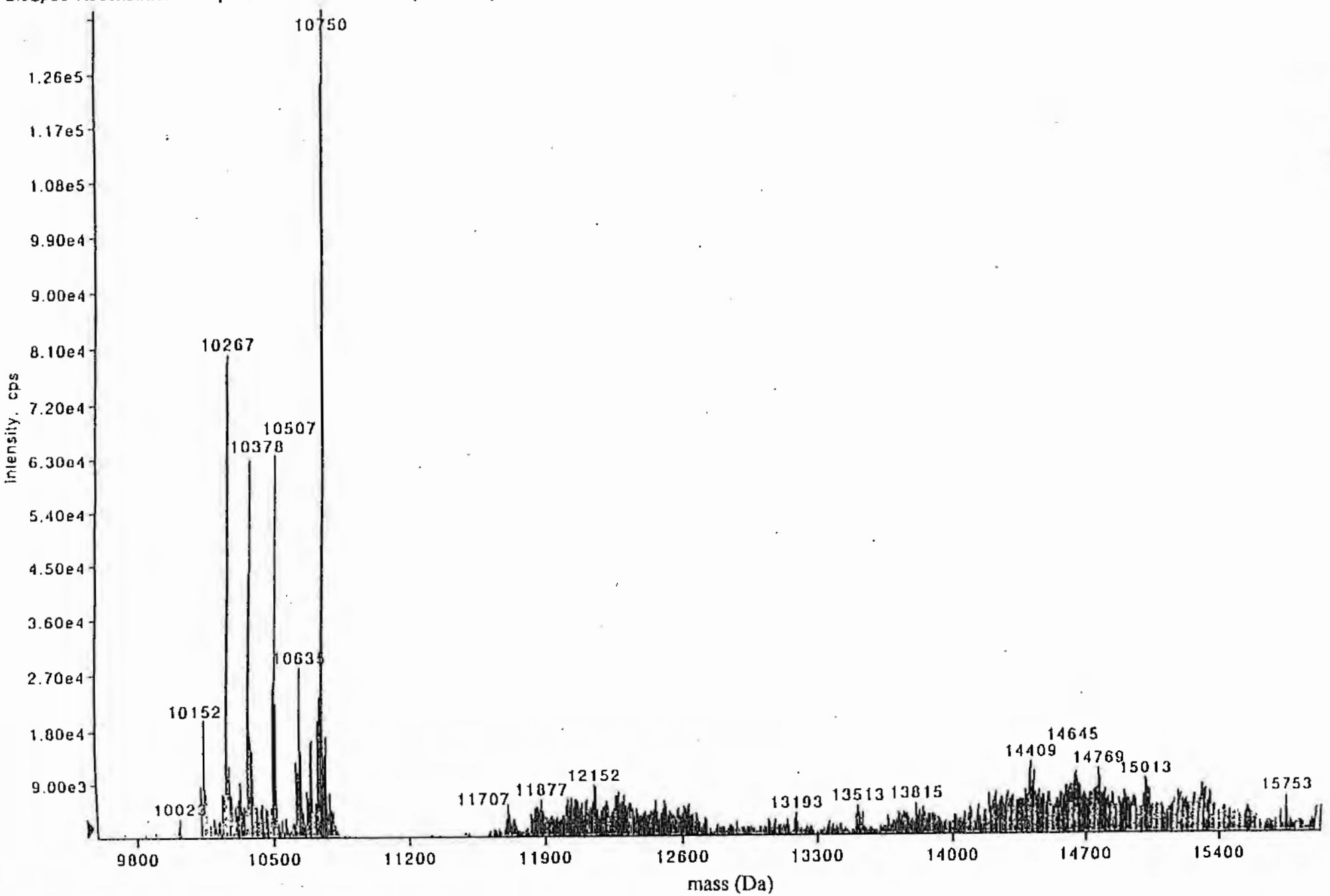


Fig. 3.3 B ESMS of cotton seed proteins (fraction Cot3 from Fig. 3.2).

Fig. 3.4 SDS PAGE of cotton seed fraction Cot 3 (Fig. 3.2).

Electrophoresis of of the unreduced protein was performed on a 15% (glycine) gel. The gel was stained with Coomassie blue after fixing with 15% glyceraldehyde. In Lane 2, two bands are apparent, one with apparent molecular mass 14kDa and the other with molecular mass 9kDa. (MW markers (lane 1): Myoglobin III MW 2,512, Myoglobin II MW 6,214, Myoglobin I MW 8,159, Myoglobin I & II MW 14,404, myoglobin MW 16,949)

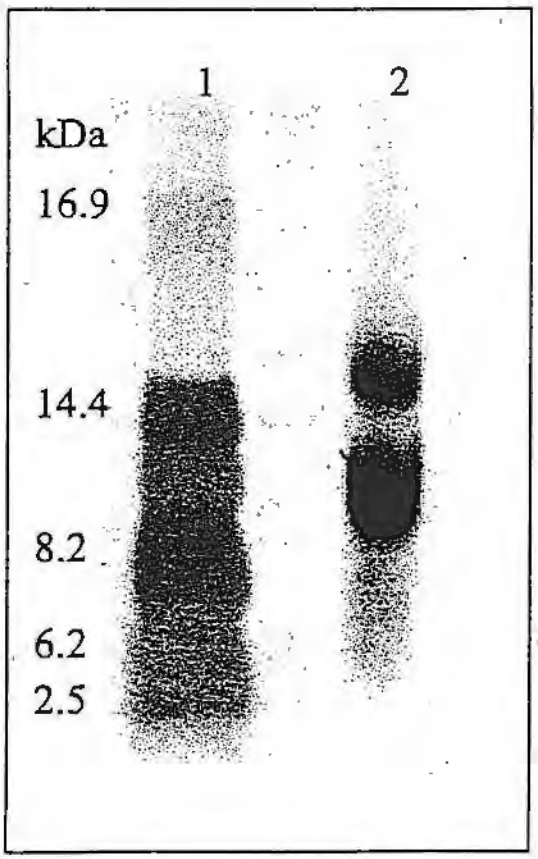


Fig. 3.5 Edman N-terminal sequencing of Cot 3 (Fig. 3.2).

Residue number	1	2	3	4	5	6	7	8	9	10	11	12
	G	H	D	P	Q	R	R	Y	Q	D	Y	E
	R	E	R	Q	E	D	P	Q		(R)		
	S	D										
observed	R	H	R	P	E	D	P	Q		(R)	Y	E
AG1	^{77}R	H	R	P	E	D	P	Q	R	R	Y	E
observed	G	E	D	P	Q	R	R	Y	Q	D	(C)	
AG2	^{77}G	E	D-	P	Q	R	R	Y	Q	D	C	Q
observed	S	D		Q								
AG1		^{82}D	P	Q								
AG2		^{78}D	P	Q								

Edman N-terminal sequencing data for the components of Cot3 (Fig 3.) is presented compared to homologous cDNA-derived sequences of *Gossypol hirsutum* vicilins (Chalan *et. al.*1986; Chalan *et. al.* 1987; Chung *et. al.* 1997).

Fig. 3.6 Average molecular masses and inferred amino acid sequences of Cot3 proteins.

Vicilin	Assigned sequences	Average molecular mass (Da)	
		Observed	Calculated
AG1	$RHRPEDPQRRY^{87}(E^{88}$------$R^{149})ERYQEN^{155}$	10267.0 ± 1.4	10266.2
	$ERYQE^{154}$	10152.0 ± 1.4	10152.1
	$ERYQ^{153}$	10024.0 ± 1.3	10023.0
	ERY^{152}	9896.2 ± 1.3	9894.8
AG1'	$DQQQRHRPEDPQRRY^{87}(E^{88}$------$R^{149})ERYQEN^{155}$	10749.6 ± 1.3	10749.7
	$ERYQE^{154}$	10634.2 ± 1.6	10635.6
	$ERYQ^{153}$	10506.6 ± 1.8	10506.5
	ERY^{152}	10378.4 ± 1.4	10378.3

Observed masses with 95% confidence limits were obtained from ESMS of peak 3 (Fig 3.2). Calculated masses are average molecular masses calculated from inferred sequences. Inferred sequences are based on Edman N-terminal sequencing (Fig 3.5), the ESMS masses shown and published cotton seed α-globulin (vicilin) precursor cDNA-base sequences denoted as AG1 (Chlan *et. al.* 1986; Chung *et. al.* 1997) and AG2 (Chlan *et. al.* 1987; Chung *et. al.* 1997). Amino acids are numbered according to the translated cDNA sequences.

well as 2 components eluting at about 40% CH_3CN in the standard system and corresponding in elution to the γ-conglutin-type protein described in part 1 of this report.

Purification and ESMS characterization of anti-fungal γ-conglutin-related protein from the cotton seed wall CM+ fraction:

After extraction of 100g of pulverized Sicala V2 seeds in buffer A and subsequent recovery of the cell wall fraction on Miracloth, the cell wall fraction was washed with 13l H_2O before extraction in 1M NaCl- 10 mM Tris (Cl^-, pH 8.0) (buffer D). This eluate was diluted in buffer A and applied to CM52 (at pH 6.0) and eluted from the CM52 in 1M NaCl- 10 mM Tris (Cl^-, pH 8.0). Subsequent RP HPLC on C8 in the standard conditions yielded a major peak eluting at 49% CH_3CN in 0.1% TFA. ESMS indicated a major component with a mass of 46246.3 ± 3.2 Da, corresponding to the 46250.3 ± 1.3 Da γ-conglutin-related heterodimeric anti-fungal protein previously resolved from the soluble fraction (Chung et al., 1997). The only additional entities detected by ESMS were barely detectable entities with average molecular masses of 44575.8 Da and 47053.0 Da, respectively. These barely detected entities are likely to be processing variants of the major 46246 Da γ-conglutin. The yield of this highly purified anti-fungal protein from this procedure was about 0.6 mg/100g seeds.

Procedure 3. Methanol extracted seeds:

Because of the copurification of PKAI activity with highly purified vicilin-related proteins and the likelihood that the PKAI activity was due to copurifying non-protein metabolites (e.g. see Figs. 3.1, 3.2), it was considered sensible to insert a MeOH extraction step into the protocol. Indeed after such MeOH extraction no non-enzymic PKAI activity was found associated with the subsequently derived protein fractions.

(a) Successive organic solvent extraction: As described in 2 (iv) above, successive extraction of 50 g pulverized cotton seeds with MeOH (twice), EtOH, BuOH, H_2O, 0.1% TFA- 80% CH_3CN and 0.1% TFA-100 %CH_3CN yielded 329, 80 and 6 mg protein in the last 3 fractions, respectively, but no PKAI activity. PKAI activity was only present in the MeOH, EtOH and BuOH fractions.

(b) MeOH extraction followed by gel filtration chromatography, ion exchange and C8 RP HPLC: The H_2O- and 0.1% TFA- 80% CH_3CN-soluble fractions from organic solvent-extracted seeds were concentrated and chromatographed on an Ultrogel AcA44 gel filtration column in 0.2 M NaCl- 50 mM Tris (Cl^-, pH 8.0) to yield high and low molecular weight fractions in high yield. A subsequent progression involving ion exchange chromatography of the H_2O- and 0.1% TFA- 80% CH_3CN-soluble fractions on CM52 and DE52 was followed by RP HPLC on a C8 column. The DE+ (DE52-binding) and CM+ (CM52-binding) fractions both yielded multiple peaks in the *circa* 25% CH_3CN region and single and double peaks, respectively, eluting at about 40 % CH_3CN in good yield..

(c) Large-scale purification of the major cotton 16318 Da vicilin and related processing products from the wall fraction extracted in buffer C:

Pulverized cotton seed (Sicala V2) (10 g) was extracted in MeOH and the cell wall fraction recovered on Miracloth and washed extensively with H_2O before extraction in buffer C. The extract was applplied to phenylsepharose CL4B which was washed with buffer C before elution of the wall ∅+ fraction in H_2O. This fraction was applied to CM52 in buffer A and the cotton wall CM+/ ∅+ fraction eluted in buffer C.

Subsequent RP HPLC on a C8 column in the standard conditions resolved 4 major components 1-4 (Fig. 3.7). ESMS of peaks 1-4 (Figs. 3.8A, 3.8B, 3.8C and 3.8D) revealed a multiplicity of vicilin-related proteins purified in high yield by this rapid procedure. The primary structure assignments for these components are summarized in Fig. 3.9. The major component in peak 1 is a 10632 Da AG1' (AG1 - 17 Da) vicilin processing product D^{73}- E^{154}. Peaks 2 and 3 contain a multiplicity of circa 10 kDa AG1, AG' and AG2 vicilin processing products. The major component of peak 4 is the 16318 Da vicilin AG1X K^{26}-N^{155}. The AG1 vicilin K^{26}-N^{155} (calculated mass 16468.8 Da, observed mass 16317.6 Da) is a barely detected entity in this preparation. The yield of this highly-purified major vicilin-related protein is about 0.5 mg/10g.

(d) MeOH extraction to yield soluble and H_2O-washed wall CM52-binding fractions: 50 g pulverized Sicala V2 seeds were extracted twice in MeOH and the wall fraction recovered on Miracloth, washed with 3 l H_2O and extracted in 2 M NaCl-10 mM phosphate (K^+, pH 9.5) (buffer B). The MeOH-insoluble material passing through Miracloth was recovered by centrifugation and successively solubilized in H_2O and in 0.1% TFA- 70% CH_3CN. The H_2O-soluble and wall-derived material was bound to CM52 in buffer A and eluted in buffer B. The protein yields from this MeOH extraction procedure were: soluble CM+ (cms fraction) (30 mg) and wall CM+ (cmw fraction) (117 mg).

When the same procedure was applied on the same scale to seeds extracted in buffer A (and without any MeOH extraction) the yields were: soluble CM+ (cs fraction) (156 mg) and wall CM+ (cw fraction) (37 mg). (It should be noted that other fractions were generated in this procedure through a variety of procedures but the CM52-binding fractions were the main focus of our attention in view of the preceding experiments in sections 1-3 above).

Antifnngal testing of the H_2O (cs & cw) and MeOH (cms & cmw) extraction procedure soluble and wall CM+ fractions: The key CM52-binding fractions from the above procedure were tested for anti-fungal activity against *Verticilium dahliae* and *Fusarium oxysporum* vasinfectum O111 using the procedures described in part 1 (Figs. 3.10 & 3.11). In the quasi-linear growth range between 60 and 90 hr, the various preparations gave the following % inhibition of growth of *V. dahliae*: cs (310 µg/ml; 43%), cw (60 µg/ml; 23%), cms (77 µg/ml; 16%) and cmw (96 µg/ml; 25%). In the same growth range between 60 and 90 hr, the various preparations gave the following % inhibition of growth of *F. oxysporum*: cs (310 µg/ml; 69%), cw (60 µg/ml; 65%), cms (77 µg/ml; 51%) and cmw (96 µg/ml; 37%). We conclude that these key fractions were more

Fig. 3.7 RP HPLC of the cotton wall "CM+/∅+" fraction on a C8 column.

The cotton seed wall "CM+/∅+" fraction was subjected to RP HPLC on a C8 column eluted in a gradient of increasing CH_3CN concentration in 0.1% TFA.

Fig. 3.8A ESMS of peak 1 (from Fig. 3.7).

Fig. 3.8B ESMS of peak 2 (from Fig. 3.7).

Fig. 3.8C ESMS of peak 3 (from Fig. 3.7).

Fig. 3.8D ESMS of peak 4 (from Fig. 3.7).

Fig. 3.9 Average molecular masses and inferred sequences of cotton seed wall proteins.

Gossypium hirsutum (Sicala V2) RP-HPLC trace following phenyl sepharose & CM protocols

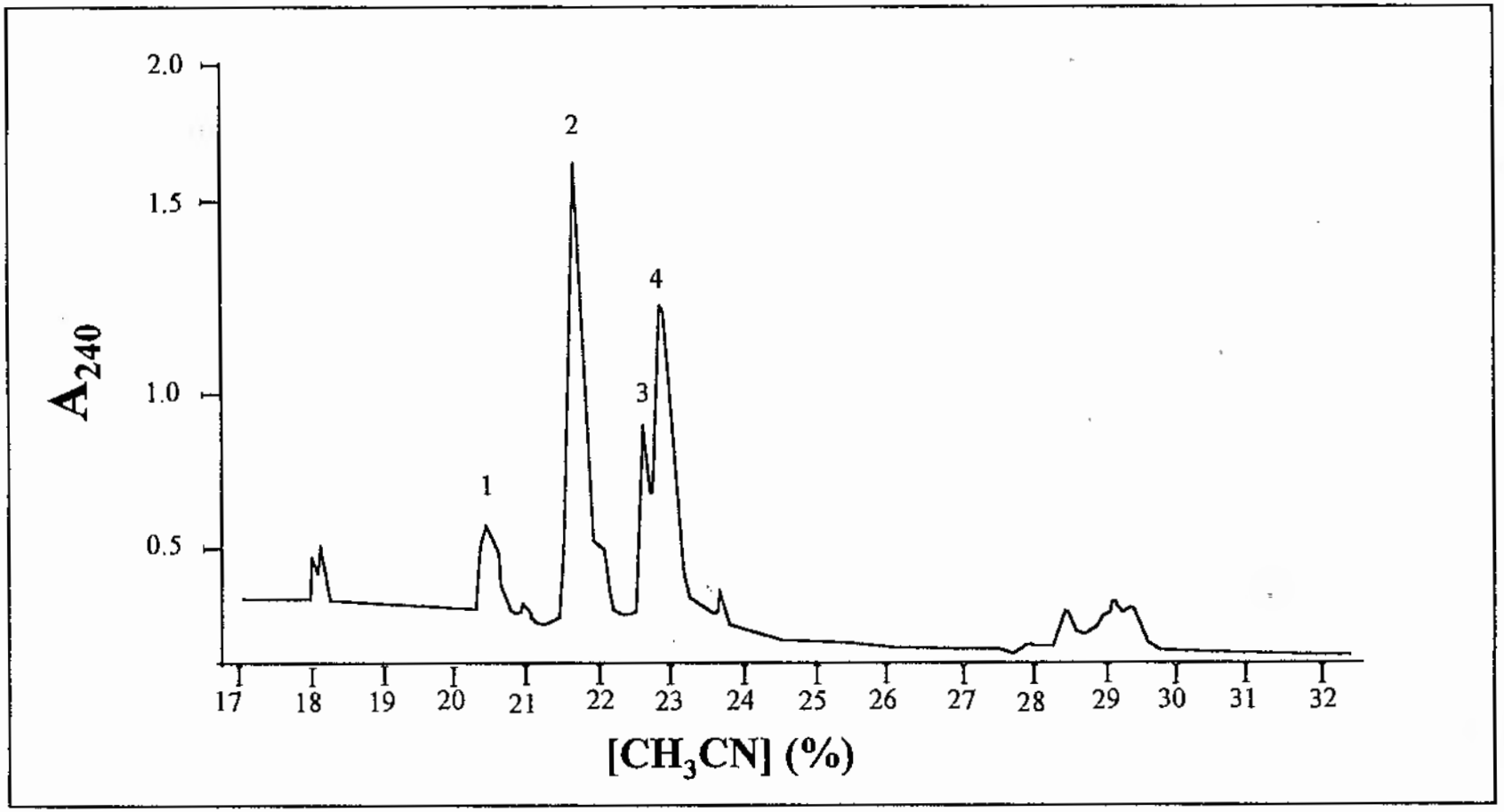


Fig. 3.7 RP HPLC of the cotton wall "CM+/∅+" fraction on a C8 column.

BioSpec Reconstruct for Spectrum from 3.50 min (39 scans) from CW1#31

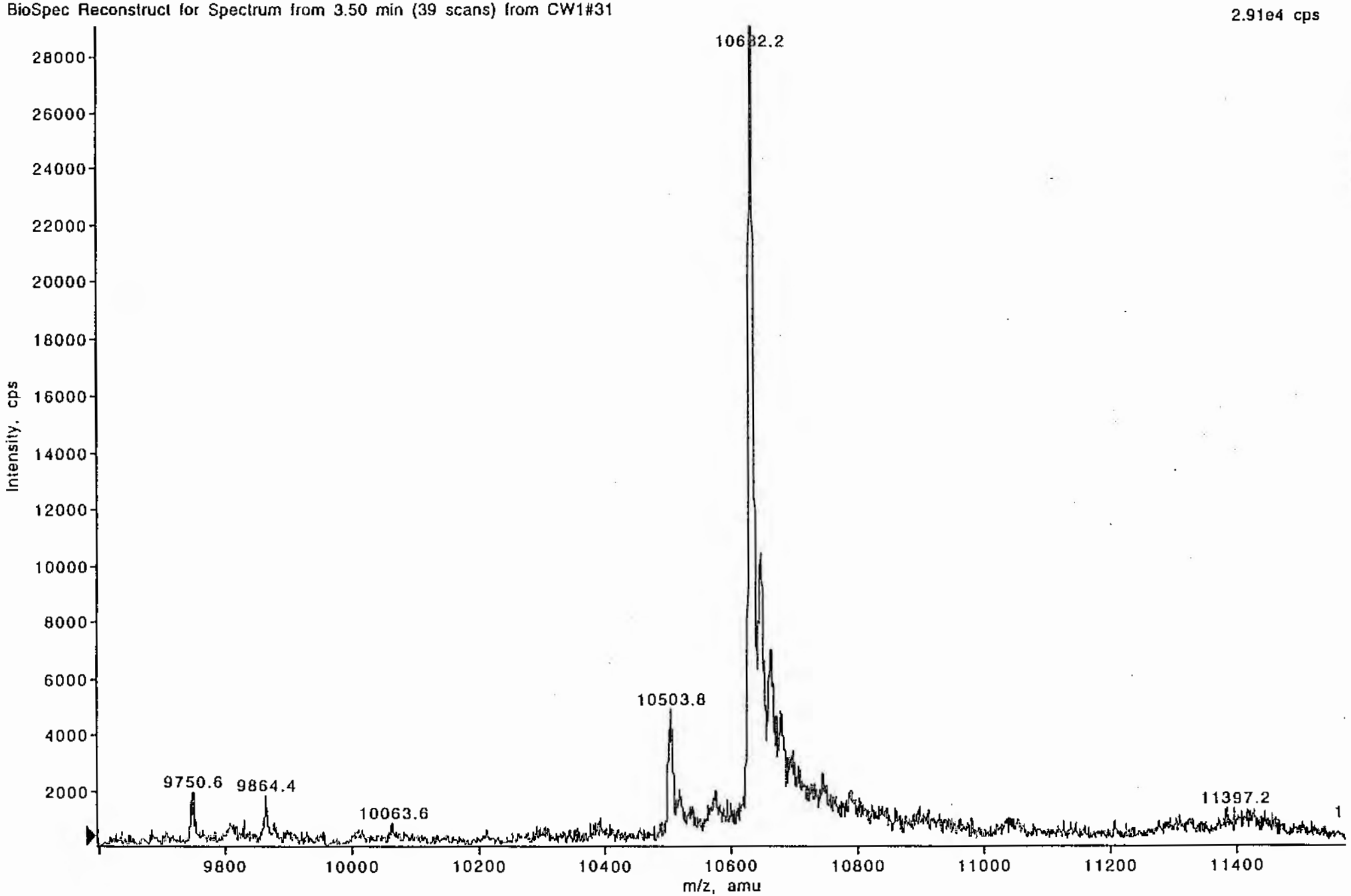


Fig. 3.8A ESMS of peak 1 (from Fig. 3.7).

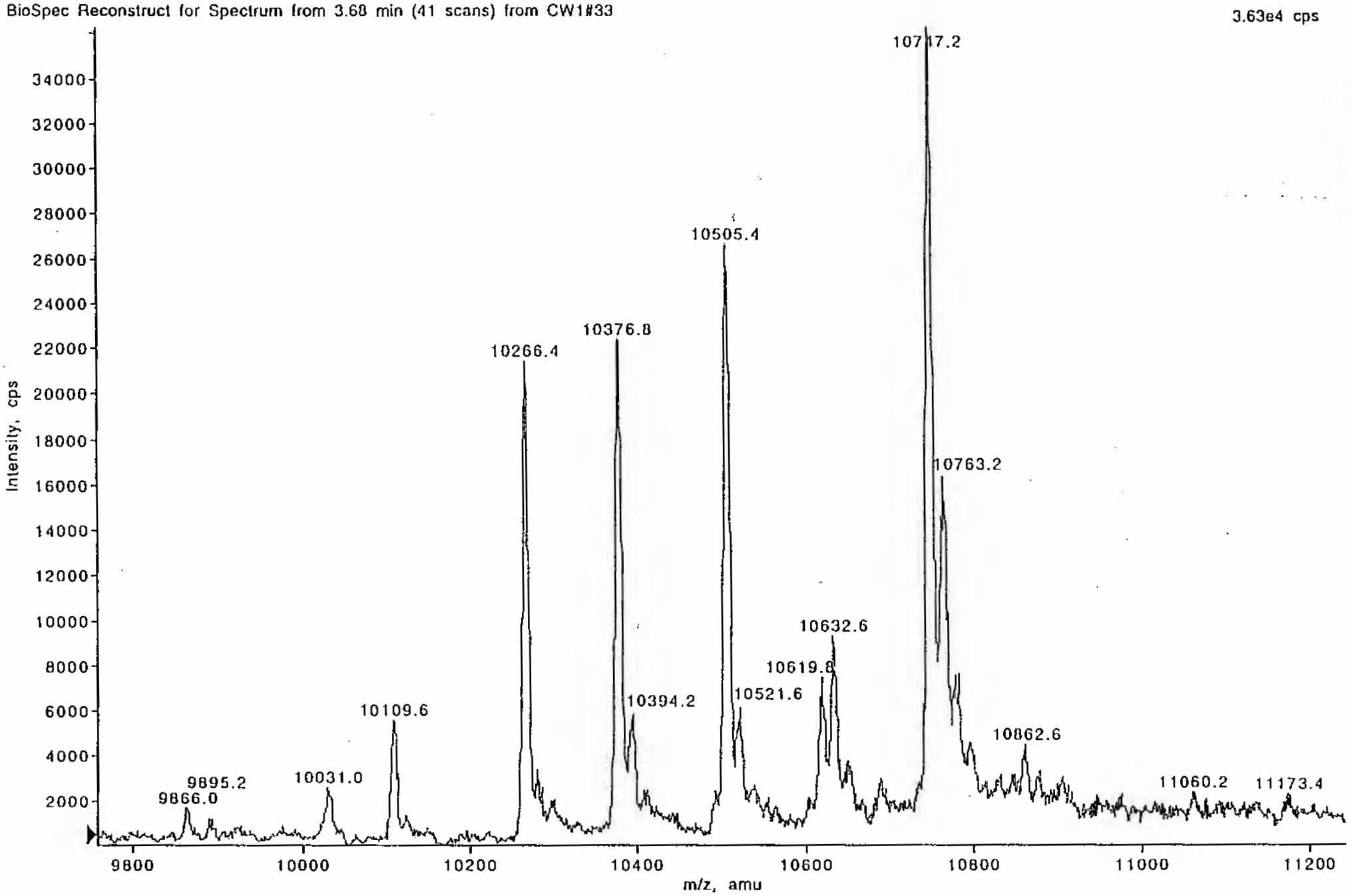


Fig. 3.8B ESMS of peak 2 (from Fig. 3.7).

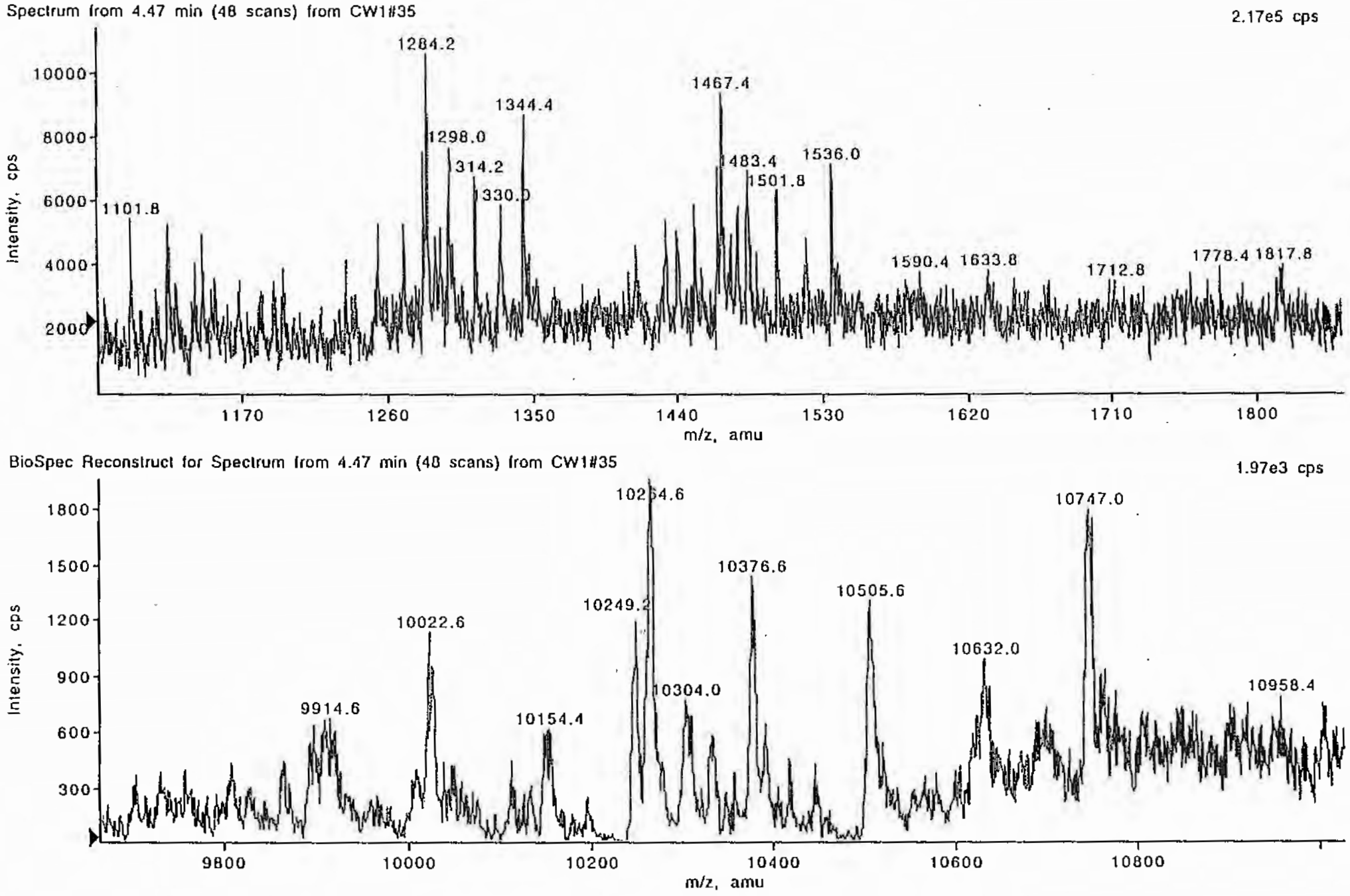
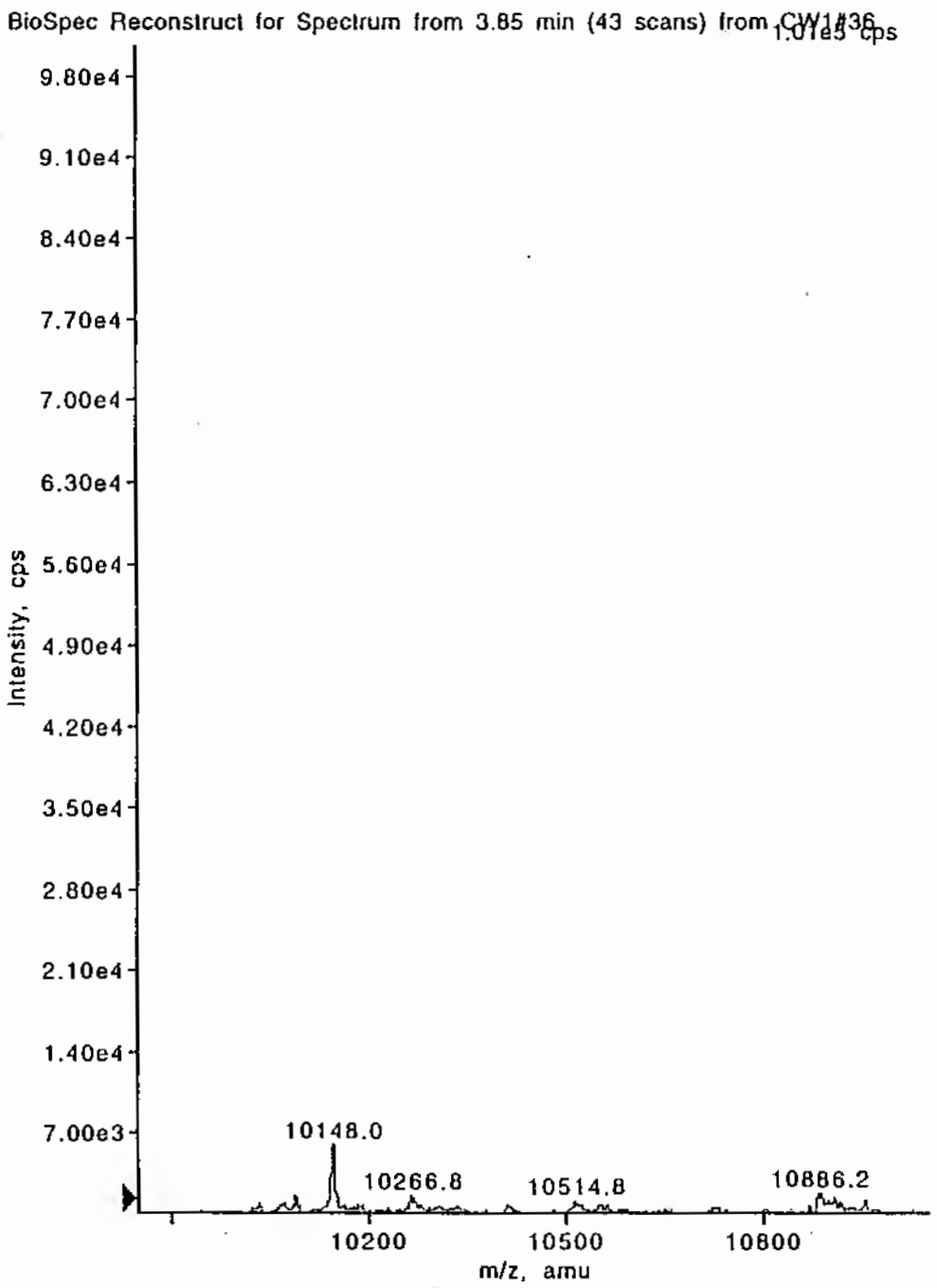


Fig. 3.8C ESMS of peak 3 (from Fig. 3.7).



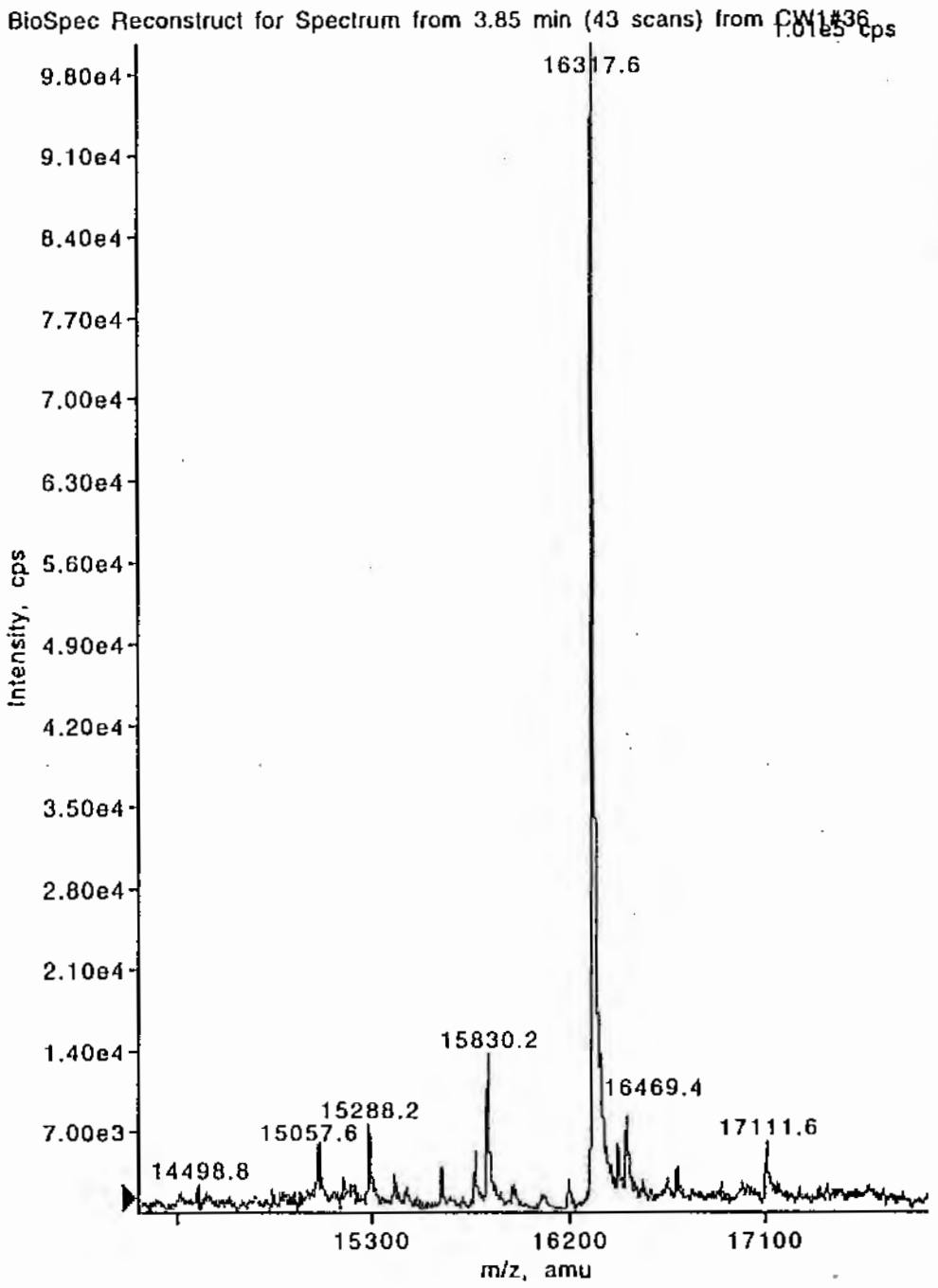


Fig. 3.8D ESMS of peak 4 (from Fig. 3.7).

Fig. 3.9 Average molecular masses and inferred sequences of cotton seed wall proteins.

Average molecular masses of the major constituents of the cotton basic protein fractions 1-4

Peak Fraction	Vicilin related proteins	Assigned sequence	Calculated (Da)	Observed (Da)
	AG1	(26-155) K^{26}DFPGRRGDDDPP^{38}-YQEN^{155}	16 468.8	
4	AG1X	K^{26}DFPGRRGDDDPS^{38}-X-YQEN^{155}	16 318.3	16 317.6
2 & 3	Series B (AG1-17 Da)	D^{73}QQQRHRPEDPQRRY^{87}(E^{88}-R^{149})ERYQEN^{155}	10 749.7	10 747.2
1, 2 & 3		-ERYQE^{154}	10 635.6	10 632.6
1, 2 & 3		-ERYQ^{153}	10 506.5	10 505.4
2 & 3		-ERY^{152}	10 378.3	10 376.8
2 & 3	Series C (AG1)	R^{77} RPEDPQRRY^{87}(E^{88}-R^{149})ERYQEN^{155}	10 266.2	10 266.4
3 & 4		-ERYQE^{154}	10 152.1	10 154.4
3 & 4		-ERYQ^{153}	10 023.0	10 022.6
2		-ERY^{152}	9894.8	9895.2
1 & 2	Series D (AG2)	G^{77}EDPQRRY^{84}(Q^{85}-R^{147})EQYQED^{153}	9864.8	9864.4
1		-EQYQE^{152}	9749.7	9750.6

Peak fractions were referenced as per the RP-HPLC profile Fig. 3.7. AG1 and AG2 sequence and mass data was acquired from published cDNA precursor sequences of cotton alpha-globulin A (AG1) clone C-71 (Chlan et al 1986) and alpha globulin B (AG2) (Chlan et al 1987). AG1X is a variant of AG1 where there is a S^{38} for P^{38} substitution and further substitutions within the sequence element denoted X. Calculated masses are average molecular masses calculated from the inferred sequences with allowance for oxidation of 8 cysteines. Amino acids are numbered according to the translated cDNA sequences. Additional sequence and mass data is as described by Chung et al 1997.

Fig. 3.10 Effect of cotton soluble and wall fractions on the growth of *Verticilium dahliae.*

V. dahliae was grown on PDB medium and growth monitored over 120 hr in the presence or absence of cotton seed soluble (s) and wall (w) CM52-binding fractions. The concentrations of the various fractions were as follows: cs=c1, 310 µg/ml; cw=c2, 60 µg/ml; cms=cm1, 77 µg/ml; cmw=cm2, 96 µg/ml. The cs & cw (soluble- and wall-derived, respectively) fractions derived from an extraction procedure not involving prior MeOH extraction; the cms & cmw fractions are the corresponding fractions from a procedure involving initial MeOH extraction of the cotton seed.

Fig. 3.11 Effect of cotton soluble and wall fractions on the growth of *Fusarium oxysporum* vasinfectum O111.

F. oxysporum vasinfectum O111 was grown on PDB medium and growth monitored over 120 hr in the presence or absence of cotton seed soluble (s) and wall (w) CM52-binding fractions. The concentrations of the various fractions were as follows: cs=c1, 310 µg/ml; cw=c2, 60 µg/ml; cms=cm1, 77 µg/ml; cmw=cm2, 96 µg/ml. The cs & cw (soluble- and wall-derived, respectively) fractions derived from an extraction procedure not involving prior MeOH extraction; the cms & cmw fractions are the corresponding fractions from a procedure involving initial MeOH extraction of the cotton seed.

Fig. 3.10 Effect of cotton soluble and wall fractions on the growth of *Verticilium dahliae*.

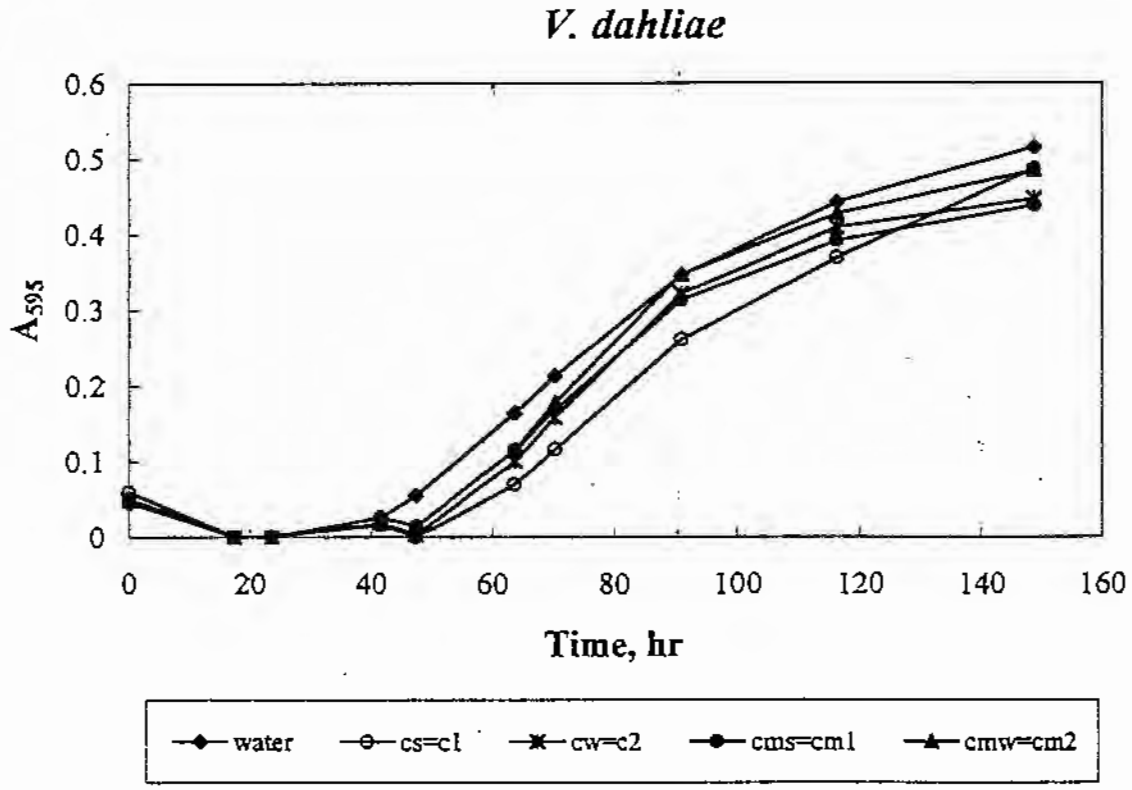
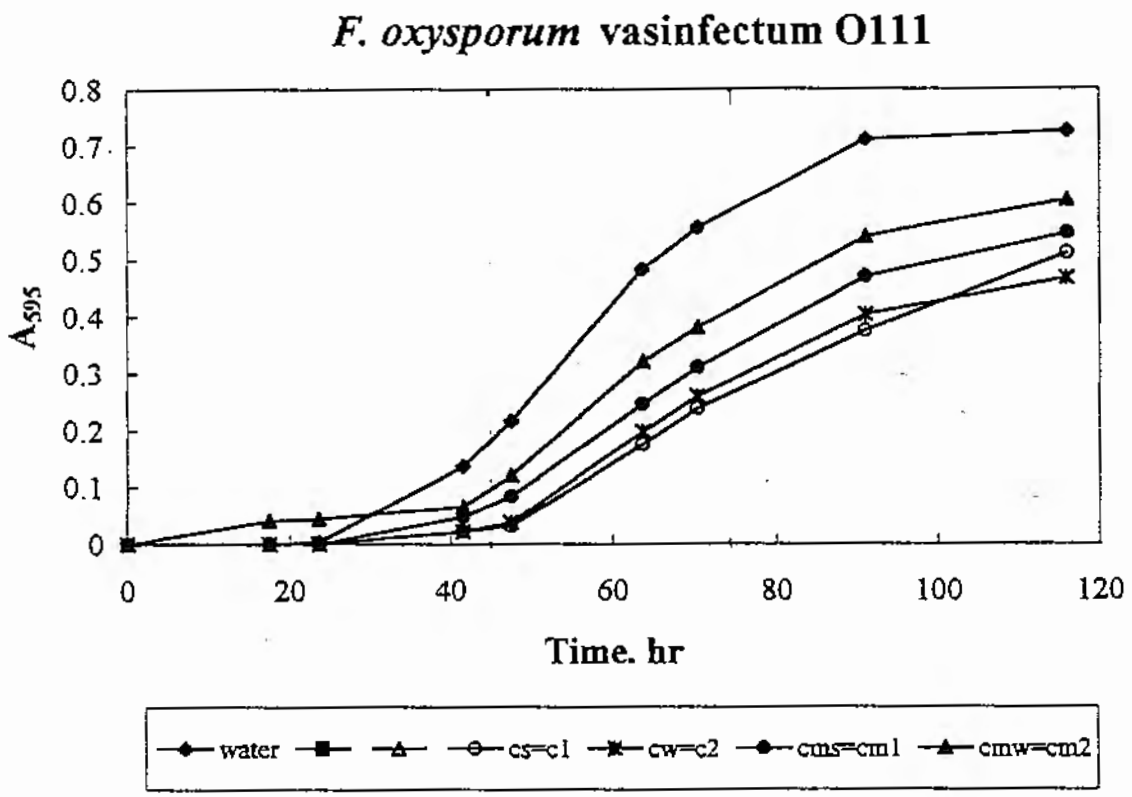


Fig. 3.11 Effect of cotton soluble and wall fractions on the growth of *Fusarium oxysporum* vasinfectum O111.



effective against *F. oxysporum* and that the MeOH procedure only slightly reduced effectiveness against this virulent cotton pathogen.

Possible biochemical targets of the H_2O extraction procedure soluble and wall CM+ fractions (cs & cw) and the soluble CM–/ ∅+ fraction: The cs, cw and soluble CM–/ ∅+ fractions were tested for possible CDPK , PKA, tyrosine kinase and PKG activity in radiochemical protein kinase assays involving histone III-S (+/- Ca^{2+}), kemptide + cAMP, insulin receptor kinase substrate and PKG peptide substrate + cGMP, respectively. No such activities were found However the soluble CM–/ ∅+ fraction contains a protein kinase and its endogenous substrate and phosphorylation of this protein is inhibited by Ca^{2+}.

Procedure 4. MeOH extraction and direct RP HPLC of the water-washed wall extract:

The cotton wall fraction that had been extracted in buffer C in a MeOH-extraction protocol was subfractionated into a fraction insoluble in 0.2% TFA but resolubilized in buffer A and a 0.2% TFA-soluble fraction that was retained on C18, eluted in 0.1% TFA-70% CH_3CN and dissolved in buffer A after rotevaporation. Neither of these fractions (at 16 and 34 µg/ml, respectively) were active as growth inhibitors of *Leptosphaeria maculans* (blackleg that infects canola) in the standard anti-fungal assay conducted in V8 broth at 23^0C. [These anti-fungal assays were conducted by Ms Vinochani Pillay, a graduate student in the laboratory).

However both of these fractions gave some inhibition of *Lucilia cuprina* larval growth (the TFA-insoluble and soluble fractions giving 61.6% and 21.4% inhibition respectively, at final concentrations of 66 and 30 µg/ml, respectively). The TFA-insoluble fraction had TI activity (giving 52.3% inhibition at 37 µg/ml). However further experiments with these fractions gave equivocal results in this assay system (see section 4 below).

RP HPLC of the 0.2% TFA-soluble wall fraction on a C8 column yielded the pattern of 4 major peaks eluting at 16-18% CH_3CN and a single peak eluting at 43% CH_3CN - components shown to be vicilin-related and γ-conglutin-related, respectively, in repeated analyses reported elsewhere in this report. These major cotton seed components may have anti-insect activity (see section 4 below).

Procedure 5. Low-ionic strength insoluble wall-associated protein and elimination of the water-washing step from the MeOH procedure.

It was found that a large amount of protein is lost during the water washing of the wall fraction and that a major wall and soluble fraction component is insoluble in H_2O or low ionic stength media but dissolves at low pH or at very high ionic strength.

The following modified MeOH procedure was applied to 50 g Sicala V2 seeds: pulverized in liquid N_2 → extracted twice in MeOH → MeOH insoluble material not

retained by Miracloth dissolved in buffer A (14 mg) → water washing of the cell wall fraction on Miracloth (300 mg) → cell wall fraction extracted in buffer C (noting that the pH was adjusted back to pH 9.5 in this procedure) (29 mg). The water washings of the wall were applied to CM52 (on in buffer A and off in buffer C) to yield a CM+ fraction (163 mg) and subsequent fractionation of the CM– fraction on DE52 (on in 10 mM Tris (Cl^-, pH 8.0), off in 1M NaCl-10 mM Tris (Cl^-, pH 8.0)) (42 mg). The water-washings CM+ fraction material becomes insoluble when diluted 10-fold in H_2O but re-dissolves at high ionic strength (2M NaCl) or at low pH (below pH 3.5). [It should be noted that the CM52 became very dark on direct application of the water-washings fractions but the coloured material could be eluted in MeOH and was shown to contain TI activity].

The "non-wall" MeOH-insoluble material passing through Miracloth in this procedure could be recovered by centrifugation and separated into 2 fractions - material dissolving in buffer A (14 mg) and material insoluble in buffer A but soluble in buffer C (43 mg). The latter fraction contains material that precipitates at low ionic strength conditions but re-dissolves at high ionic strength. This fraction also contains a TI that does not bind to CM52 (see 3b(iii) above).

Finally, 50 g pulverized cotton seed (Sicala V2) was extracted in MeOH and the wall fraction retained on Miracloth and the MeOH-insoluble fraction that passed through Miracloth was recovered by centrifugation. The wall fraction extracted with buffer C (without prior washing of the wall with H_2O) (968 mg) contains both TI and CI activity. The MeOH-insoluble fraction that passed through Miracloth and which was insoluble in buffer A but soluble in buffer C (310 mg) contains TI activity. The MeOH-insoluble fraction that is soluble in buffer A (34 mg) has anti-mosquito activity as described in section 4(c) below.

In summary, building on the work of Dr Chung in parts 1 and 2, a variety of protocols have been progressively refined enabling large scale isolation of highly purified proteins from cotton seed for further biological testing. Major vicilin- and γ-conglutin-related components were found associated with the "cell wall" material which thence became a major target of investigation.The recognition of the existence of potent MeOH-extractable PKAIs, TIs and CIs in cotton led to introduction of initial MeOH extraction steps. Recognition of a major CM52-binding and H_2O-insoluble (but water-leachable) protein fraction in the "cell wall" material led to progressively higher yield procedures for purification of cotton seed defensive proteins. In particular rapid procedures were developed for purifying a major 10632 Da AG1' (AG1 - 17 Da) vicilin processing product AG1' D^{73}- E^{154} and the 16318 Da vicilin AG1X K^{26}-N^{155}. In addition a high yield procedure was developed by Dr Chung for purification of γ-conglutin from the cotton seed cell wall fraction.

4. Anti-insect cotton constituents

Cotton is a targeted by a variety of insect pests of which the bollworm *Helicoverpa punctigera* is a major insect herbivore that damages cotton (CRDC, 1995). It was of interest to resolve fractions from cotton seeds and leaves that might impair the growth of

Helicoverpa punctigera larvae. Further, since cotton represents a major source of agricultural biomass, it was of interest to see if particular cotton seed and leaf components might have bioactivity against other insects having an impact on agricultural production or human health.

(a) ***Helicoverpa punctigera***

A variety of protein and non-protein fractions from sub-fractionation of cotton seed (Sicala V2) and mature leaves of cotton (Sicala V2) were examined for activity against *Heliocverpa punctigera* larvae. Second instar larvae were fed at 27°C over 3 days on a broad bean-based meal incorporating the test components. After 3 days the control and test larvae were weighed. Control and test growth determinations were conducted in triplicate. Unfortunately no protein or secondary metabolite-containing cotton fractions were found to inhibit larval growth at the concentrations employed and the conditions used. However, as shown below, we have found that cotton elaborates some components that are active against some other insects of economic importance. Indeed one might have expected a result of this kind in view of the susceptibility of cotton to *Helicoverpa punctigera.*

(b) ***Lucilia cuprina***

The larvae of the Australian green bottle blowfly (*Lucilia cuprina*) are responsible for blowfly strike in sheep. Blowfly strike occurs on the tail region, the shoulder and elsewhere on the sheep body. The annual cost is about $200 million and some 3 million sheep deaths as well as acute animal distress. In addition to shearing and mulesing, protocols using organophosphates, organochlorines and vetrazine are employed as control measures. Cheap, safe, biodegradable and specific larvicides or larval growth inhibitors would be desirable and it would be particularly useful if these could be derived as cheap by-products of major agricultural crop plant production. Accordingly a number of cotton seed and leaf fractions were examined as ***Lucilia cuprina*** larval growth inhibitors.

Larvae were grown in sterile conditions at 37°C on a 2.3% agar-based growth medium that also contained 10% sodium caseinate, 5% baker's yeast and 0.1% sucrose. First instar larvae were tested for effects of potential growth inhibitory cotton fractions in bioassays conducted at 37°C in subdued light over 24 hr. Test preparations were incorporated into the growth medium. Controls (containing the appropriate solvent addition) and test growth assays (containing test cotton component added in the same solvent) were conducted in triplicate. After 24 hr larvae were collected, frozen at -70°C and then weighed.

A variety of protein fractions from cotton seeds were examined as potential inhibitors of *Lucilia cuprina* larval growth with equivocal results that may derive from the purity of the preparations and the concentrations of the active proteins used. These experiments will continue using higher concentrations of highly-purified proteins from the relevant fractions. In marked contrast, in a series of experiments a cotton leaf fraction that is ethanol-soluble and also water-soluble was consistently shown to cause substantial inhibition of the growth of *Lucilia cuprina* larvae. The major cotton secondary

metabolite gossypol did not inhibit larval growth in the conditions used. The nature of this bioactive leaf larval growth inhibitor has yet to be determined. However the same fraction is a potent larvicide in relation to particular mosquito larvae and this further anti-insect assay system (to be described below) may assist in the purification of the bioactive component.

(c) *Aedes camptorhynchus*

Insect-borne disease infects about 0.4 billion people world-wide. About 200 million are infected with malaria and several million die each year from this disease. Anopheles species mosquitoes are vectors for malaria. *Aedes* species can act as vectors for dengue fever and Japanese encephalitis. A local Victorian mosquito *Aedes camptorhynchus* can carry Murray Valley encephalitis and Ross River fever. Cheap, safe, biodegradable anti-mosquito agents would be of immense utility for application in areas of significant human habitation (noting that adult mosquitoes and the larval stages can be major ecological components as, for example, food for bats and fish, respectively). Further, such anti-insect components from a major agricultural crop source would be extremely useful by-products. We chose to use the local, robust *Aedes camptorhynchus* mosquito for our studies on potential anti-insect components from cotton.

Aedes camptorhynchus larvae were collected (by the Victorian Department of Agriculture Animal Health division at Atwood) from Lake Wellington, Gippsland, Victoria and kept at 24^0C with a 12hr/12hr light/dark cycle in 500 ml 0.35% NaCl. Larvae were fed by addition of 50 mg finely ground tropical fish food every 3 days. Instar 3 and instar 4 larvae were sorted and 0.9 ml of medium containing about 5 larvae was pipetted into a glass test tube for each test. Test compounds were added in 0.1 ml and control assays (in which 0.1 ml of appropriate solvent was added) were also conducted. Larvae were kept at 24^0C with a 12hr/12hr light/dark cycle. The number and state of the instar 3 and instar 4 larvae in each situation were monitored daily over about 10 days.

Raw cotton seed oil (final concentration 1% in 1% methanol) caused 100% death of both instar 3 and instar 4 larvae within 1 day. The oil extracted from Sicala V2 seeds in methanol and which separates out at low temperature is similarly toxic - at 1% total death occurs after 38 hr. The cotton leaf fraction soluble in both ethanol and water applied in water at 10% gives 100% death of instar 3 and instar 4 larvae within 1 day. The bioactive component(s) responsible have not yet been resolved. No lethality was observed with 10 μM gossypolone-1% methanol or with 10 μM gossypol-1% methanol.

A large number of water-soluble fractions deriving from large-scale fractionation of Sicala V2 seeds (see section 4, procedure 5)were tested for larvicidal activity against *Aedes camptorhynchus* instar 3 larvae. Two fractions showed strong larvicidal activity, namely (1) the methanol-insoluble but water-soluble fraction and (2) the fraction extracted from cell walls at high NaCl concentration and high pH and which binds to a C18 matrix in acid conditions. RP- HPLC of these fractions and subsequent ESMS/Edman analysis has shown that they contain vicilin-related processing products. The larvicidal assays will now be conducted on a more extensive scale to determine which of the RP-HPLC-purified fractions have the best activity. The primary structures of the active proteins will be determined by a combination of ESMS and Edman sequencing

analyzed in relation to previously-reported protein and cDNA sequence information as described above. The data obtained to date certainly vindicate the utility of this approach. These anti-mosquito studies are now being extended to include local *Culex* and *Anopheles* species.

Overall discussion of results

Research outcomes:

The overall research outcome was as follows:

1. the purification and mass spectrometry-, Edman- and cDNA sequence-based primary structural characterization of a large number of defensive vicilin proteins (and their differential processing products) (about 40 in all) present in cotton seed;

2. the purification and characterization of a major γ-conglutin-related defensive protein from cotton seed;

3. the precise mass spectrometry-based primary structure determinations has defined the processing of the products of the corresponding defensive protein precursors;

4. the anti-fungal properties of these preparations was defined in relation to a variety of fungal pathogens and in particular in relation to the cotton pathogens *Fusarium oxysporum* and *Verticilium dahliae*;

5. a variety of rapid protocols for large-scale isolation of these defensive proteins were developed and the major products defined structurally;

6. this purification and structure/function analysis was also extended to cotton leaf components;

7. cotton seed protein components having protease inhibitory activity were detected and extensively purified;

8. methanol-soluble non-proteins fractions having trypsin-, chymotrypsin- or PKA-inhibiting activity were detected and extensively purified;

9. while no fractions inhibitory to the growth of *Helicoverpa punctigera* were found (a not unsurprising result), a cotton leaf, non-protein fraction is a potent larvicide with respect to *Lucilia cuprina* (responsible for sheep blowfly strike) and *Aedes camptorhynchus* (the mosquito growing in brackish water that can carry Ross River fever). Some highly-resolved protein preparations were also larvicidal.

Work on parts 5, 6, 7, 8 and 9 is still proceeding.

Likely impact of the results:

1. The protein chemical work very precisely defines the primary structures of a large number of cotton seed defensive components and should therefore be very useful in a plant breeding sense (the masses are accurate to within the mass of 1 or 2 hydrogen atoms).

2. The demonstration of anti-fungal (and possibly anti-insect) actvity of a number of cotton seed proteins is novel and opens up future transgenic crop protection possibilities. (Indeed I understand that a vicilin gene has now been introduced into a plant in an attempt to enhance anti-fungal protection).

3. The rapid, large-scale isolation of defensive proteins from cotton seed points to potential cotton seed anti-fungal protein applications.

4. The protease- and PKA-inhibitory activities have yet to be molecularly characterized but indicate potentially useful value-added defensive products from cotton seed.

5. The larvicidal components from cotton seed and especially from cotton leaves indicate potentially very useful anti-insect components from a major agricultural crop. The anti-mosquito component may be particularly useful in view of the transmission of a variety of viral diseases by *Aedes* species (Dengue fever, Japanese encephalitis, Ross River Fever, Murray Valley encephalitis), the increasing threat to Australia due to southward movement of the first 2 from the Torres Straits and irrigation- and salination-promoted *Aedes* establishment.

Project technology:

No patents have been appplied for but as described above there may well be useful practical and commercial cotton industry "value-added" possibilities arising from this project.

Technology developments and its dissemination:

1. Major new technical developments arising from the project relate to purification of anti-fungal proteins from cotton seed (and indeed the demonstration of anti-fungal activity against a variety of fungi pathogenic to cotton and other plants). Information on this has been disseminated in various conference presentations and in a major publication (ref. 4).

2. Methodologies relating to larger scale purifcation of cotton seed defensive proteins has not yet been published (see section 4 of the above research report).

3. The anti-insect aspects of the project is particularly promising and we are indeed proceeding with this study directed to purification and structural characterization of larvicidal and/or growth retarding cotton leaf and seed components.

Publications

Publications relating to cotton defensive components published before the CRDC funding period.

1′. W. Jinsart, B. Ternai and G.M. Polya (1991). Inhibition of embryo calcium-dependent protein kinase and avian myosin light chain kinase by flavonoids and related compounds. Biol. Chem. Hoppe-Seyler, 372, 819-827.

2′. W. Jinsart, B. Ternai and G.M. Polya (1992) Inhibition of rat liver cyclic AMP-dependent protein kinase by flavonoids. Biol. Chem. Hoppe-Seyler, 373, 205-211.

Publications relating to cotton defensive components published during the funding period (1996-1998)

1. G.M. Polya and B.H. Wang (1996). Plants, metabolites and medicine. Today's Life Science , March 1996, 18-24.

2. G.M.Polya, G.M.Neumann, R.Condron, A.Vassiliou, S.Christov, R.Chung, Z.X.Lu and M.Hasmeda (1996). Structure and function of plant defensive proteins and interactions with calmodulin and calcium-dependent protein kinase. Proc. Joint ASBMB/ASPP Annual Conference, Canberra, September/October 1996, POS-134-02.

3. R. P-T. Chung and G.M. Polya (1996). The structure and sites of biochemical action of cotton defensive proteins and secondary metabolites. Proceedings of 8th Australian Cotton Conference, 671-676.

4. R.P-T. Chung, G.M. Neumann and G.M Polya. (1997). Purification and characterization of basic proteins with antifungal activity from seeds of cotton (*Gossypium hirsutum*). Plant Science 127, 1-16.

5. R.P-T. Chung, G.M. Neumann and G.M. Polya (1997). Purification and mass spectrometry-based characterization of basic proteins with antifungal activity from cotton seeds. Proceedings 41st Annual ASBMB & 37th Annual ASPP Conferences, Poster P1-95.

6. G.M. Polya, G.M. Neumann, R. Condron, A. Vassiliou, S. Christov, R. Chung, Z.X. Lu and M. Hasmeda (1997). Structure and function correlations for interaction of antifungal proteins with protein kinases and calmodulin. Abstracts of 22nd Annual Lorne Conference on Protein Structure and Function, A9.

7. G.M. Polya and B.H. Wang (1997). Inhibition of eukaryote protein kinases by plant defensive secondary metabolites *in* Recent Research Developments in Phytochemistry (S.G. Pandalai, editor), Research Signpost, Trivandrum, volume 1,77-94.

8. G.M. Polya (1997). The structure and sites of action of plant defensive proteins *in* Recent Research Developments in Phytochemistry (S.G. Pandalai, editor), Research Signpost, Trivandrum, volume 1, 95-110.

9. R.P-T. Chung, D. Gray, G.M. Neumann and G.M. Polya (1998). Purification and mass spectrometry-based characterization of cotton seed antifungal proteins. Abstracts of 23rd Annual Lorne Conference on Protein Structure and Function, B-6.

Other references cited:

Caulfield, J.J. and Bolander, F.F. (1986) Involvement of protein kinase C in mouse mammary gland development. J. Endocrinol., 109, 29-34.

Chlan, C.A., Pyle, J.B., Legocki, A.B.., Dure, L. (1986) Developmental biochemistry of cottonseed embryogenesis and germination. XVIII. cDNA and amino acid sequences of memebers of the storage protein families. Plant Mol. Biol. 7, 475-489.

Chlan, C.A., Borroto, K., Kamalay, J.A., Dure, L. (1987). Developmental biochemistry of cottonseed embryogenesis and germination. XIX. Sequences and genomic organization of the α-globulin (vicilin) genes of cottonseed. Plant Mol. Biol. 9, 533-546.

Cotton Research and Development Corporation (1995). The Australian cotton industry: an economic assessment.

Acknowledgements:

The financial support of the CRDC over the period of 1996-1998 for the salary of the principal investigator research fellow Dr Roland Chung and attendant research expenses is gratefully acknowledged. We must also gratefully acknowledge the contributions of Dr Greg Neumann (mass spectrometry), Mrs Rosemary Condron (Edman sequencing), Ms Donna Gray and Aaron Uschakov (honours students involved with cotton seed protein purification and characterization), Antonio Rajic (*Helicoverpa punctigera* and *Lucilia cuprina* larval growth assays), Ms Vinochani Pillay (*Leptosphaeria maculans* anti-fungal assays), Ms Elwyn Wisharte (Agriculture Victoria, Animal Health, Atwood, Victoria) (provision of *Aedes camptorhynchus* larvae) and Mrs Ratna Wijaya (assistance with separation of different larval instar stages).