The structure and sites of biochemical action of cotton defensive proteins and secondary metabolites.

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Introduction.

Plants exhibit defence mechanisms against microorganisms (e.g. fungi) and herbivores (e.g. insects) by the deployment of defensive proteins and secondary metabolites. Many of these plant defensive compounds have been shown to target elements of the signal transduction pathways of higher organisms (Jinsart, 1991, 1992). A number of these are also important pharmaceutical agents (Rang and Dale, 1991).

In cotton, the best known of the secondary metabolites isolated, Gossypol, have been found to have antifungal and antitumour activity (Harborne and Baxter, 1993) and to target enzymes in the signal transduction system, notably protein kinase A and myosin light chain kinase (Jinsart, 1991, 1992).

This project aims to define the chemical structure, high affinity biochemical sites of action and biological activities (especially anti-insect and antifungal activities) of cotton defensive proteins and secondary metabolites.

Experimental.

Cotton seeds were ground up in liquid N_2 and the protein extracted into 10mM sodium phosphate (pH6) buffer by homogenizing with an Ultra Turrax blender. The extract was filtered successively through gauze and miracloth, then centrifuged at 39000xg for 15min. The supernatant was filtered through miracloth and the basic component bound onto a CM-52 matrix; this fraction was then eluted

with 1M sodium chloride-10mM Tris (pH8) buffer. The solution was concentrated by pressure filtration (Amicon YM3 membrane), diluted 200-fold with milli-Q water and reconcentrated to about 2ml. This solution was loaded onto a C_8 reversed phase column and eluted with a linear gradient of increasing acetonitrile concentration in 0.1% aqueous TFA. The protein fractions, as detected by UV absorbance at 240nm, were collected and the solvent removed *in vacuo*. The fractions were then taken up in milli-Q water and separately characterized (by electrospray ionization mass spectrometry and N-terminal Edman sequencing) and assayed for activity. The amount of protein was determined using Coomassie Blue and measurement of absorbance at 595nm. Antifungal tests were carried out with fungal spores in potato broth (80µl) (10^4spores/ml) with 20µl of the protein solution for testing filter-sterilized through a sterile 0.22µm Durapore-polypropylene filter unit. Aseptic conditions were observed. The growth of the hyphae was monitored as increased absorbance at 540nm.

Results.

In the HPLC profile (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. Peak 1 encompasses a family of proteins with masses between 9.2-9.8kDa. Peak 2 was also found to consist of a family with a mass of around 10.6kDa. Peak 3 was found to be a single peak with a mass of 16321Da (SD 1.40Da). Peak 4 has the largest mass of around 46250Da (SD 2.14). The mass spectrum of the 10.6kDa fraction also provided C-terminal sequence information from the differences in mass between successive peaks in the same spectrum, deriving from progressive C-terminal residue cleavage (Table 1).

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

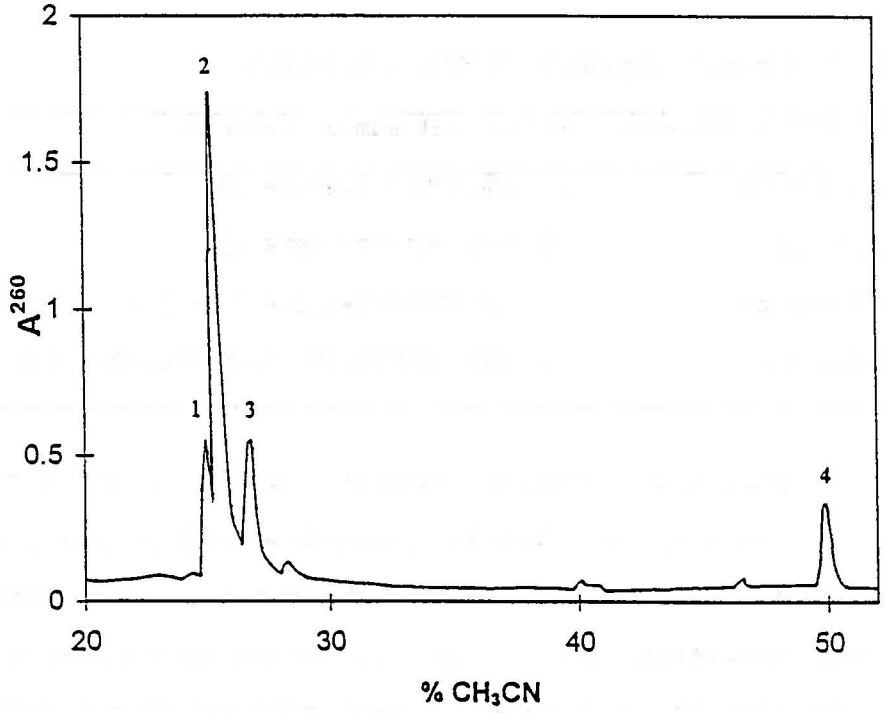


Table 1. Average molecular masses of HPLC peak fraction #2.

observed masses, Da	standard deviation, Da
10748.65	0.95
10633.42	0.83
10505.98	1.11
10378.30	0.89
10266.99	0.56
10111.03	0.80

The N-terminal sequencing of the 10.6kDa fraction was complicated by the presence of contaminant proteins. The 16.3kDa and 46.2kDa fractions were successfully subjected to extensive N-terminal sequence analyses (Table 2). The 16.3kDa fraction was found to have a very high sequence similarity with an alpha-globulin type

B precursor while the 46.2kDa fraction was found to have a high sequence similarity with a gamma conglutin.

Table 2. N-terminal sequences of HPLC fractions.

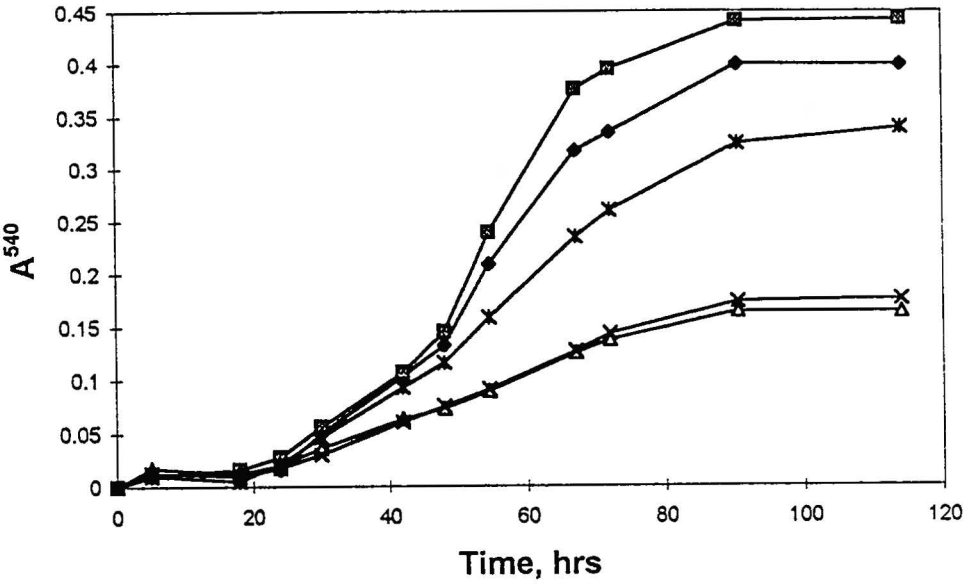
Protein/ HPLC fractions	N-terminal Edman sequence
16.3kDa fraction	1 KDFPGRRGDDDPSKRYE 17
alpha-globulin	26 KDFPGRRGDDDPPKRYE 42
46.2kDa fraction	1 VDITRPLSYTPLIISPQGEYYMEVKSIR 28
gamma-conglutin	251 LDVLHDLVYTPLTISKQGEYFIQVNAIR 278

Antifungal testing against spores from Botrytis cinerea, Chalara elegans, Fusarium oxysporum and Rhizotonia solani, obtained from the Knoxfield Herbarium's National Collection of Fungi, were undertaken. The 16.3kDa fraction and to a lesser extent the 46.2kDa fraction were shown to have some antifungal activity against Botrytis cinerea (figure 2). The 10.6kDa fraction showed good inhibition of hyphae growth of Fusarium oxysporum for up to 50 hours after which subsequent monitoring showed parallel growth to that of the controls. A possible explanation for this could be the delayed action of protease from the fungus on the inhibitory protein. The 16.3kDa and 46.2kDa fractions do not appear to have significant activities against Fusarium oxysporum.

Conclusions.

Four major basic protein types were partially purified. The 9.8kDa and 10.6kDa series appears to be comprised of families of proteins with a difference of a single amino acid between adjacent members. The 16.3kDa protein appears to be a single species and found to have a high similarity with alpha-globulin or vicillin. The 46.2kDa protein has a high similarity with gamma-conglutin.

Figure 2. Effect of the various HPLC fractions on Botrytis cinerea. (■-■), protein control, 200µg/ml BSA; (◆-◆), water control; (✱-✱), HPLC peak4, 46.2kDa, 10µg/ml; (x-x), HPLC peak3, 16.3kDa, 9.2µg/ml; (Δ-Δ), carboxymethyl cellulose binding fraction, 50µg/ml.



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