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# Comparative Characteristic Components of Plant Protective System by Example of Three Varieties of Cotton Plants

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## ABSTRACT

High-resistant cotton varieties contain a large number of components of plant protective system against the pathogen and insect pests. The content of gossypol, its enantiomers and sum of cation peptides were determined in three cotton varieties with different resistance to pathogens. The most resistant cotton variety Namangan-77 contains the highest level of total gossypol, (+)-gossypol and total sum of cation peptides in cotton seeds have been detected a correlation. From sum of cation peptides were isolated low molecular proteins by a combination of different chromatographic procedures. It was established that all peptide fractions contain of low molecular protein with molecular mass 10,635 Da, relevant to class of 2S-albumin. Antifungal and antimicrobial activities were tested in sum of low molecular proteins. Cottonseed proteins exhibit high antifungal and antimicrobial activities inhibiting growth of hyphae and causing spore destruction in a number of fungal and microbial pathogens.

**Keywords:** antifungal activity, antimicrobial activity, gossypol, *Fusarium oxysporum,* HPLC, LC/MS, storage proteins, varieties of cotton, *Xanthomonas malvacearum*

## INTRODUCTION

Plant seeds are able to survive and resist environmental damage for several months. Essential to this resting state is low water content and thus a low metabolic activity, which resumes after rehydration and germination of the seeds. Germination also involves the mobilization of storage compounds, e.g. storage proteins that are sequestered in specialized seed tissues such as cotyledons and endosperm. Much of our current knowledge on seed proteins stems from studies on storage proteins, i.e. proteins that accumulate to high levels during seed maturation and are rapidly degraded during germination (Ferreira *et al.* 2007). Well-known examples of such storage proteins are the 2S albumins, which have been detected in seeds of many species (Monsalve *et al.* 2003).

Other seed proteins are thought to play a role in the protection of resting seeds or seedlings against insect attacks or microbial infection. Examples of such proteins are chitinases and glucanases (Carstens *et al.* 2003), ribosome-inactivating proteins (Zhu *et al.* 2005), chitin-binding lectins (Raikhel *et al*. 1993; Mosolov *et al.* 2005), or more recently discovered antimicrobial proteins (Banzet *et al.* 2002; Choon *et al.* 2002; Jeandet *et al.* 2002; Sawada *et al.* 2004; Castro *et al.* 2005; Silverstein *et al.* 2005, 2007). In a number of cases, however, the distinction between storage function and defense function is vague, and many authors have proposed a dual role for storage proteins.

Earlier a series of storage proteins consisting of 2 and 3 subunits belonging to the class 7S and 11S globulins were extracted from cotton seeds (Asatov *et al*. 1977; Marcus *et al.* 1999; Yadgarov *et al*. 1978).

In Uzbekistan, cotton seed is a main source of feed protein, but the high content of toxic gossypol in cotton seed limits it to be used as a feed for ruminant animals. Therefore, Uzbek scientists have developed new varieties and lines of cotton plants with low gossypol content as well as glandless lines.

American scientists observed that glandless plants in the field were completely damaged by insects whereas adjacent glanded plants showed little or no damage. Even insects that are not considered as pests of cotton attacked the glandless plants. Bottger *et al.* (1964) showed that gossypol was toxic to cotton aphids, lygus bugs, salt-marsh caterpillars, thurberia weevils, and bollworms.

However, glandless and low gossypol Uzbek varieties of cotton plants possess some resistance to insects and pathogens. This resistance may be due to induced defense proteins and peptides. Herein we report our initial comparative study of peptide profiles in 3 cotton varieties, and outlines our primarily research using proteomic analysis.

## MATERIALS AND METHODS

### Cotton varieties

Cotton varieties used in this study were Namangan-77 (normal gossypol content), MG-02 (minor gossypol content), GB (glandless).

### Determination of total gossypol and enantiomers

Twenty seeds from a cotton plant was manually dehulled, and then grounded into meal with an agate mortar and pestle. The meal was then stored at –20°C until the analysis were carried out. The gossypol derivatization procedure was carried out (Dowd *et al.* 2005) using 20 mg of meal in a screw-cap test tube. The reaction mixture was diluted with 5.0 ml of acetonitrile and centrifuged (3000 rpm, 3 min). A portion of the resulting clear supernatant was transferred to a vial for HPLC analysis.

HPLC analysis was performed within 8 h of obtaining the supernatant samples from either seed meal. The column was Zorbax Eclipse XDB $C_{18}$ (3.0 mm × 100 mm). The mobile phase was an 80:20 mixture of acetonitrile and 10 mM $KH_2PO_4$ (the latter adjusted to pH 3.0 with concentrated $H_3PO_4$) and is run at a flow rate of 0.600 mL/min. The chromatogram signal was collected at

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254 nm while UV spectra were obtained over 220-400 nm. The injection volume was 5 or 10 µL depending on the concentration of gossypol in the sample. The gossypol-amino-propanol complexes appear at 3.2 and 4.8 min for the (+)- and (-)-enantiomers, respectively. These peaks were integrated and the peak area percentages calculated. As the UV spectra for the (+)- and (-)-gossypol adducts are identical, the ratio of the peak area percentages of these compounds are considered to be equivalent to the enantiomeric ratio.

## Isolation of peptides from cotton seeds

The dehulled cotton seeds (100 g) were ground and defatted for 72 h by hexane. The seeds were homogenized using 10 N acetic acid in 1:10 (w/v) ratio. The protein–peptide fraction was extracted for 1 h at room temperature under continuous stirring. The supernatant obtained after centrifugation (6000 rpm, 30 min, 4°C) was filtered through a filter paper B, neutralized with 10 N NaOH, and kept at 4°C for 12 h. The precipitate was isolated by centrifugation (6000 rpm, 30 min, 4°C) and the supernatant was dialyzed.

## Ion-exchange chromatography

The dialyzed extract was adjusted to pH 9 with ammonium acetate (0.050 M) and passed over a column of Servacel DEAE-23SN (2.0 × 10 cm, Reanal) equilibrated with ammonium acetate (0.050 M, pH 9) at flow rate 0.5 mL/min. Fractions of non-bonded proteins were mainly thermally stable proteins. The resulting eluate was adjusted to pH 6 with acetic acid and applied on a column of CM-TSK-650M (2.0 × 5 cm, Tosoh Bioscience) equilibrated with ammonium acetate (0.50 M, pH 6). Proteins bound to the sorbent were eluted with a linear gradient of NaCl (from 0 M to 1 M, 400 mL) in ammonium acetate (0.050 M, pH 6) at flow rate 0.5 mL/min. Proteins were detected at 280 nm.

## Reversed-phase HPLC

Peptides eluted from the cation-exchange column were dialyzed against distilled water and separated in a chromatograph (DuPont 8800) using a 250/8/4 Protein@Peptide C18 column. Solution A: TFA (0.1%); B: $CH_3CN$; flow rate 1 mL/min; absorption at 280 nm; gradient (%/min): 0%/3 min, 0-70%/25-30 min; 70-0%/35-40 min.

## Protein concentration

Protein concentration was determined by the Lowry method using albumin as the standard protein (Darbre 1989).

## Analysis of antifungal activity

The antifungal activity of the peptides was tested against several fungi using 96-well micro titer-plate as described previously (Broekaert *et al*. 1990). Wells were filled with 10 µl of twofold serial dilutions of the peptide and mixed with 90 µl half-strength potato-glucose broth containing $10^4$ spores/ml. The inhibition of spore germination was evaluated by measuring the absorbance at 490 nm using an automated micro plate reader (Bio-Rad, Model 3550). $IC_{50}$ values showing protein concentration required for 50% growth inhibition were calculated. Experiments were performed at least in three replicates.

## Analysis of antimicrobial activity

The antimicrobial activity of peptides was assayed against the strain of *Xanthomonas malvacearum* using radial diffusion assay. Petri dishes with Luria-Bertani agar were seeded with test bacteria. The peptide solutions (50 µl) were applied to the wells (5 mm in diameter) punched into the agar, and incubated at room temperature for 48 h. The inhibition of spore germination was evaluated by measuring the absorbance at 490 nm using an automated micro plate reader (Bio-Rad, Model 3550). $IC_{50}$ values showing protein concentration required for 50% growth inhibition were calculated. Experiments were performed at least in three replicates.

## Statistical analysis

Values are mean ±SD (standard deviation) of three replicates. All experiments were performed at least, three times (unless indicated otherwise) and were highly reproducible. Therefore, data from one replicate is presented below.

## MALDI mass spectrometry

The molecular masses of proteins and peptides were measured on an Ultraflex time-of-flight Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS in the regime of positive ions. 2,5-Dihydroxybenzoic acid was used as a matrix. The accuracy of measurement was 0.015%.

# RESULTS AND DISCUSSION

The seeds of the three cotton varieties [Namangan-77 (normal gossypol content), MG-02 (minor gossypol content) and GB (glandless)] were analyzed for total gossypol and percentage of enantiomers.

Evidence varieties of cotton have different content of total gossypol and its enantiomers.

It may be suggested that the level of biocide peptides in different varieties of cotton may correlate with their resistance for fungal disease. We carried out isolation of cationic peptides from seeds of three cotton varieties and compared their molecular masses and antifungal and antimicrobial activities (**Table 1**).

## Purification of peptides from cotton seeds included several chromatographic procedures

Cotton seeds were ground in a coffee mil1 and the resulting meal was extracted for 1 h at 4°C with 1000 mL of 10N acetic acid. After extraction the slurry was mixed in a Waring blender and subsequently squeezed through a jam mincer to separate the extract from the solid residue. The product of extraction was purified by following steps.

The total fraction of thermally stable peptides was applied on an anion-exchange column of Servacel DEAE-23SN. The main peptide fraction that was not bound to the anion-exchange column was placed on a CM-TSK-650M cation-exchange column. The peptide fraction bound to the CM-TSK-650M column was eluted by a NaCl gradient (0-1 M) and cationic peptides fraction were collected, desalted by dialysis, and used for further characterization.

All fractionated peaks were used for antifungal activity assay. The fractions which possessed antifungal activity were collected and re-purified with RP-HPLC.

*In vitro* antifungal susceptibility tests of the isolated peptides' fractions were assayed against the pathogenic strain of *Fusarium oxysporum* and antimicrobial susceptibility tests against the strain of *Xanthomonas malvacearum* (**Table 2**).

The rest of fractions did not inhibit the growth of the fungus. The highest antifungal activity was observed in CM-2 fraction from MG-02 variety. The highest antimicrobial activity was observed in CM-6 fraction from var. 'Namangan 77'.

Molecular masses infractions after cation-exchange chromatography were determined using the method of Accurate-Mass Q-TOF LC/MS. It has shown a presence of

**Table 1** Amount of gossypol, its enantiomers and total sum of cation peptides.

| Cotton variety | Gossypol | | | Total sum of cation peptides, mg/g (M±m) |
|---|---|---|---|---|
| | Content of gossypol, % | Ratio of enantiomer of gossypol, % | | |
| | | (+) | (–) | |
| Namangan-77 | 2.79 | 68 | 32 | 57 ± 1 |
| Mg-02 | 1.84 | 53 | 47 | 43 ± 0.866 |
| Glandless | 1.41 | 63 | 37 | 55 ± 1.73 |

**Table 2** Antifungal and antimicrobial activity of peptide fractions after separation on CM-TSK-650M.

| № | Cotton variety | Fraction | $IC_{50}$, µg/ml (*Fusarium oxysporum*) (M±m) | $IC_{50}$, µg/ml (*Xanthomonas malvacearum*) (M±m) |
|---|---|---|---|---|
| 1 | Namangan 77 | CM-2 | 2.9 ± 0.1 | 3.3 ± 0.1 |
| | | CM-3 | 87.0 ± 0 | 61.88 ± 0.01 |
| | | CM-4 | 19.7 ± 0.1 | 49.8 ± 0.1 |
| | | CM-5 | 6.06 ± 0.01 | 4.63 ± 0 |
| | | CM-6 | 3.0 ± 0.26 | 0.86 ± 0.01 |
| 2 | MG-02 | CM-1 | - | 7.55 ± 0.01 |
| | | CM-2 | 1.55 ± 0.017 | 3.25 ± 0.01 |
| | | CM-3 | 18.1 ± 0.1 | 37.34 ± 0.005 |
| | | CM-4 | 2.8 ± 0.1 | 7.3 ± 0 |
| 3 | Glandless | CM-2 | 2.09 ± 0.01 | 96.1 ± 0.1 |
| | | CM-3 | - | 142.8 ± 0.1 |
| | | CM-4 | - | 185.2 ± 0 |
| | | CM-5 | 42.8 ± 0.2 | 44.6 ± 0 |

$IC_{50}$ - concentration of peptides at µg, which inhibit growth of fungus at 50%.

low molecular protein (molecular mass 10,635 Da) in all studied fractions.

It is proposed that the observed protein belongs to class of 2S albumins – cystein-rich proteins, present in seeds. Several studies have demonstrated that 2S albumins possess antimicrobial and antifungal activities. Our results also support these reports.

## CONCLUSION

From the results obtained in our work, it can be concluded, that between the resistance to pathogens and low molecular proteins in different varieties of cotton seeds have been detected a correlation. The highest amount low molecular proteins concentration was observed in 'Namangan 77' seeds and the lowest concentration was observed in var. 'Glandless' seeds. The results have shown a presence of low molecular protein (molecular mass 10 635 Da) in all cotton varieties. The protein isolated from MG-02 CM-2 possessed the highest antifungal activity and protein isolated from 'Namangan 77' CM-6 possessed the highest antimicrobial activity. These data support the view that 2S albumins may protect seeds from microbial infestation in soil during the germination.

## REFERENCES


**Asatov SI, Yunusov TS** (1977) The globulins of cotton seeds. The structure of the chymotryptic peptide of the 7S globulin. *Chemistry of Natural Compound* **2**, 291-292

**Banzet N, Latorse M-P, Bulet P, Francois E, Derpierre C, Dubald M** (2002) Expression of insect cystein-rich antifungal peptides in transgenic tobacco enhances resistance to a fungal disease. *Plant Science* **162**, 995-1006

**Bottger GT, Sheehan ET, Lukefahr MJ** (1964) Relation of gossypol content of cotton plants to insect resistance. *Journal of Economic Entomology* **57**, 283-285

**Broekaert WF, Terras FRG, Cammue BPA, Vanderleyden J** (1990) An automated quantitative assay for fungal growth inhibition. *FEMS Microbiology Letter* **69**, 55-59

**Carstens M, Vivier MA, Pretorius IS** (2003) The *Saccharomyces cerevisiae* chitinase, encoded by the *CTS1-2* gene, confers antifungal activity to transgenic tobacco. *Transgenic Research* **12**, 497-508

**Castro MS, Fontes W** (2005) Plant defense and antimicrobial peptides. *Protein Peptide Letters* **12**, 13-18

**Choon KJ, Jin CH, Cheol PH, Chul KM, Duck KY, Cheol KS, Mi OH, Jeong PS, Lee SH, Yun DJ, Oh LC, Dong BJ, Yeol LS, Cho MJ** (2002) Over-expression of a seed specific hevein-like antimicrobial peptide from *Pharbitis nil* enhances resistance to a fungal pathogen in transgenic tobacco plants. *Plant Molecular Biology* **50**, 441-449

**Darbre A** (1989) *Practical Chemistry of Proteins*, Moscow, 623 pp

**Dowd MK, Pelitire SM** (2005) Separation and purification of gossypol, 6-methoxy-gossypol and 6,6'-dimethoxy-gossypol from sea island *Gossypium barbadense*. In: *Book of Abstracts, 96th American Oil Chemists' Society Meeting*, May 1-4, Salt Lake City, UT, pp 98-99

**Dillen L, Claeys M, Rees SB, Vanderleyden J, Cammue BPA** (1992) Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine/glycinerich domain of chitin-binding proteins. *Biochemistry* **31**, 4308-4314

**Ferreira RB, Monteiro S, Freitas R, Santos CN, Chen Z, Batista LM, Duarte J, Borges A, Teixeira AR** (2007) The role of plant defence proteins in fungal pathogenesis. *Molecular Plant Pathology* **8**, 677-700

**Jeandet P, Douillet-Breuil AC, Bessis R, Debord S, Sbaghi M, Adrian M** (2002) Phytoalexins from the Vitaceae: Biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. *Journal Agriculture Food Chemistry* **50**, 2731-2741

**Marcus JP, Green JL, Goulter KC, Manners JM** (1999) A family of antimicrobial peptides is produced by processing of a 7S globulin protein in *Macadamia integrifolia* kernels. *The Plant Journal* **19**, 699-710

**Monsalve RI, Villalba M, Rico M, Shewry PR, Rodríguez R** (2003) The 2S albumin proteins. *Plant Food Allergens* **103**, 42-56

**Mosolov V, Valueva TA** (2005) Proteinase inhibitors and their functions in plants: A review. *Biochemistry and Microbiology* **41**, 227-246

**Raikhel NV, Lee H-I, Broekaert WF** (1993) Structure and function of chitin-binding proteins. *Annual Review Plant Physiology and Plant Mo1ecular Biology* **44**, 591-615

**Sawada K, Hasegawa M, Tokuda L, Kameyama J, Kodama O, Kohchi T, Yoshida K, Shinmyo A** (2004) Enhanced resistance to blast fungus and bacterial blight in transgenic rice constitutively expressing *OsSBP*, a rice homologue of mammalian selenium-binding proteins. *Bioscience, Biotechnology and Biochemistry* **68**, 873-880

**Silverstein KA, Graham MA, Paape TD, Van den Bosch KA** (2005) Genome organization of more than 300 defensin-like genes in *Arabidopsis*. *Plant Physiology* **138**, 600-610

**Silverstein KAT, Moskal WA, Wu HC, Underwood BA, Graham MA, Town CD, Van den Bosch KA** (2007) Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants. *Plant Journal* **51**, 262-280

**Yadgarov AG, Yunusov TS** (1978) A study of the structure of the 11S globulin from cotton seeds. *Chemistry of Natural Compounds* **2**, 231-232

**Zhu S, Gao B, Tytgat J** (2005) Phylogenetic distribution, functional epitopes and evolution of the CSab superfamily. *Cellular and Molecular Life Sciences* **62**, 2257-2269