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Screening antimicrobial activities of basic protein fractions from dry and germinated wheat seeds

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Abstract

Two small cationic peptide fractions (5 kDa) were isolated from dry and germinated seeds of wheat, named WAP and GWAP, respectively. The antifungal and antibacterial activities of the peptides were analyzed using disk diffusion and turbidity measurement assays. The peptides *in vitro* exhibited effective antifungal activity against four plant pathogenic fungi at minimum concentration of 15 μg(protein) cm^{-3}. Their antimicrobial activity was negatively affected by the presence of 5 mM $CaCl_2$. The peptides were less effective against Gram-negative bacterium *Erwinia carotovora* subsp. *carotovora*, but they demonstrated inhibitory activity against Gram-positive bacterium *Clavibacter michiganensis* subsp. *sepedonicus.* The antimicrobial activity of GWAP was more effective than WAP.

Additional key words: antifungal/antibacterial activity, *Triticum durum*.

Introduction

During the past 15 years, a large number of antimicrobial proteins (AMPs) have been identified in different plants (Broekaert *et al.* 1997). AMPs constitute a heterogenous class of low molecular mass proteins, which are recognized as important components of defense system. They directly interfere with the growth, multiplication and spread of microbial organisms (Lehrer and Ganz 1999). Different proteins with antibacterial and/or antifungal activity have been isolated from seeds, where they accumulate to high levels and may also function as storage proteins. Homologous of the seed proteins have also been identified at very low concentrations in floral and vegetative tissues (Terras *et al.* 1995). There are several classes of proteins having antimicrobial properties which include thionins, lipid transfer proteins, plant defensins, chitinases, glucanases, 2S albumins, ribosome-inactivating proteins and lectin (Ye *et al.* 2002, Zhang and Halaweish 2003).

Transgenic plants constitutively expressing anti-microbial proteins have shown enhanced resistance to a number of microbial pathogen (De Bolle *et al.* 1996, Epple *et al.* 1997). Similarly, plant extracts having antimicrobial activity have been of great interest not only in phytopathological but also in medicinal research. This paper reports the purification of water soluble, low molecular mass, cationic proteins from dry and germinated wheat seeds and measurements of their antimicrobial properties against a number of pathogenic fungi and bacteria.

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Abbreviations: AMPs - antimicrobial proteins; WAP - wheat dry seed antimicrobial protein; GWAP - germinated wheat seed antimicrobial protein.

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Materials and methods

Purification of small basic proteins of wheat seeds: Wheat (*Triticum durum* L. cv. Altintoprak-98) seeds were obtained from the Tekirdağ Agricultural Faculty, Department of Field Crops, the University of Trakya, Turkey. The wheat seeds were surface sterilized and germinated between sterile wet filter papers for 48 h in the dark. The dry and germinated wheat seeds were ground finely and the seed meal (60 g) was extracted with four volumes of cold extraction buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 1.5 mM EDTA) with constant stirring for 2 h and followed by centrifugation at 15 000 *g* for 30 min. During extraction phenylmethylsulfonyl fluoride (PMSF) was added at a level of 1 mM to inhibit proteolysis. The clear supernatant (total crude extract) was subjected to ammonium sulphate precipitation to obtain a final 30 % saturation and was gently stirred for 2 h. The mixture was centrifuged at 15 000 *g* for 30 min, and the supernatant was adjusted to 70 % relative ammonium sulphate saturation. The slurry was stirred for 2 h and centrifuged as previously done. The pellet was resuspended in minimal amount of distilled water and the protein suspension was kept at 80 ºC for 10 min. The soluble fraction obtained after a final centrifugation at 13 000 *g* for 15 min and dialyzed against distilled water for 20 h using a dialysis tubing with a molecular cut-off 1 kDa. Elimination of the protein samples greater than 10 kDa was performed using *Centricon YM-10* filter (*Millipore Corporation*, Bedford, USA). The sample was applied directly to a diethylaminoethylcellulose (DEAE-52) column (1.0 × 10 cm) which had been previously equilibrated with 20 mM Tris-HCl buffer (pH 8.0). Unadsorbed protein fractions were collected with the equilibration buffer while adsorbed proteins were eluted by addition of 0.5 M NaCl in the buffer. The protein eluates were collected at a flow rate of 22.5 cm^3 h^{-1} and the elution profile was monitored at 280 nm (A_{280}). All steps were performed at 4 °C.

The eluates were sterilized with 0.22 μm syringe-filter and submitted to bioassay for detection of antimicrobial activity. The active fractions of peak D1 were combined and dried in a vacuum concentrator. The dried material were dissolved in water and further submitted to HPLC-gel filtration chromatography on a *Bio-Sil Sec 250* (*Bio-Rad*, Hercules, USA) 300 × 7.8 mm column. The column was equilibrated in 150 mM NaCl, 50 mM potassium phosphate buffer (pH 8.0) and calibrated with several proteins of known molecular masses (M_r; 24 000, 14 200, 6 500, 1 350) for the estimation of M_r of the peptides. Absorbance at 280 nm was also used to monitor elution profiles during chromatography and to determine the protein content of the column eluates. The absolute protein concentrations of the eluates were determined as described by Bradford (1976) using bovine serum albumin as a standard. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was carried on 15 % acrylamide separating gel (Laemmli 1970) and the protein bands were stained with silver reagents (Nesterenko *et al.* 1994).

Microorganisms and antimicrobial activity assays: Four strains of fungi, *Botrytis cinerea*, *Fusarium oxysporum, Rhizoctonia solani, Verticillium dahliae* and two bacterial strains *Clavibacter michiganensis* subsp. *sepedonicus* NCPPB 2137 (Davis *et al.* 1984), and *Erwinia carotovora* subsp. *carotovora* NCPPB 929 (Hauben *et al.* 1998) were used in the bioassays. The fungal cultures were kindly provided by University of Cukurova, Department of Plant Protection, Turkey. The bacterial cultures used were from stock cultures of RIGEB.

The fungi were maintained on potato dextrose agar (PDA, *Oxoid CM139*, Basingstoke, UK). For the preparation of spores, sterile distilled water (5 cm^3) was added to the dishes of fungal culture and these were gently agitated for 1 min for spore liberation. The spore densities were determined microscopically by using a Thoma counting chamber. Paper disk diffusion and microtiterplate based turbidity assays were performed to determine the antifungal/ antibacterial activities of the small, basic peptides of the dry and germinated wheat seeds.

The assay of antifungal activity towards fungal species was assessed on basis of hypal extension assay of filamentous fungi by paper disk diffusion assay. An agar plug containing mycelia of the test fungus was harvested from actively growing fungal plates and placed in the center of 9.1 mm Petri plate containing 15 cm^3 PDA. The plates were incubated at 26 ºC for 7 days in the dark until the mycelial colony reached a diameter of 3 cm. Sterile filter paper disks (*Whatman No.3*; 6 mm in diameter) were placed on the agar surface at a distance of 0.5 cm away from the growing front of the mycelial colony and then various amounts of the protein solution was applied to each disk in 0.01 cm^3. Control disks were prepared by replacing the protein sample with the same volume of 10 mM Tris-HCl (pH 7.5). The plates were incubated at room temperature and the diameter of the inhibition zone around each disk was measured after 72 h. If the material being tested had antifungal activity, a transparent zone was observed around each disk. The fungal concentration of 2×10^4 spores cm^{-3} was used for the antifungal assays.

In vitro inhibitory effect of the basic peptides of wheat seeds were quantified using flat bottom 96 well microtiterplate based turbidity measurement assay and the percentage of growth inhibition was determined. The growth medium (medium I) for the antifungal assay was either half-strength Saboroud dextrose broth (SDB) containing 40 g dm^3 glucose, 10 g dm^3 peptone (*BD Biosciences 211677*, Franklin Lakes, USA) or

supplemented with 5 mM $CaCl_2$ (medium II) The turbidity assay for bacteria was carried out in two media; half strength Luria-Bertani (LB) broth (*Difco 244620*) (medium I) and medium I supplemented with 5 mM $CaCl_2$ (medium II). Each well contained 0.02 cm^3 of various concentrations of filter sterilized protein solution and 0.110 cm^3 of appropriate growth medium containing 2×10^4 fungal spores cm^{-3} or 2×10^5 colony forming units cm^{-3} of bacterial suspension in a total of volume 0.130 cm^3. In control wells, the protein solution was replaced by an equal volume of sterile distilled water. The microplate was incubated at 26 ºC and the degree of growth inhibition was determined by measuring culture turbidity over time intervals (24 h for bacteria and 48 h for fungi in the dark) at 595 nm using an automatic microplate reader *Model 3550* (*Bio-Rad*). All assays were performed in triplicate.

Results and discussion

Purification of the antimicrobial proteins: The purification of low M_r, soluble, basic antimicrobial peptides from *Triticum durum* seeds was performed basically by chromatographic method. Anion-exchange chromatography of the *DEAE-52* column was initially used for the separation of low M_r peptides of the seeds. The flow-through from the *DEAE-52* column represented the basic protein fractions (peak D1) and adsorbed protein fractions (peak D2) were eluted with 0.5 M NaCl wash of the column (Fig. 1). Protein fractionations from the dry and germinated wheat seeds showed similar chromatographic profiles on the column. The overall purification stages of dry and germinated seed proteins were summarized in Table 1.

The purity and apparent M_r of the peptides of the dry and germinated seed of wheat were analyzed by SDS-PAGE (15 % acrylamide gel) under reducing conditions and they gave approximately 5 kDa of protein bands (Fig. 2) which are similar to the M_r of known AMPs. The active cationic peptides of the dry and germinated seeds were named WAP and GWAP,

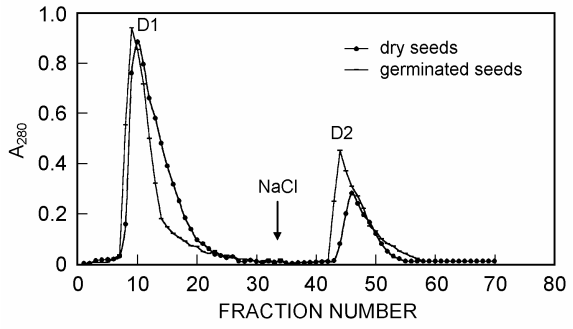

Fig. 1. Elution profile of an ion-exchange column to fractionate basic proteins of dry and germinated wheat seeds. Ammonium sulphate precipitated proteins of the seeds were heat denatured, fractionated with *YM-10* filter and applied onto column of *DEAE-52*. The protein eluates were monitored spectrophotometrically at 280 nm. The *arrow* indicates addition of 0.5 M NaCl to the column.

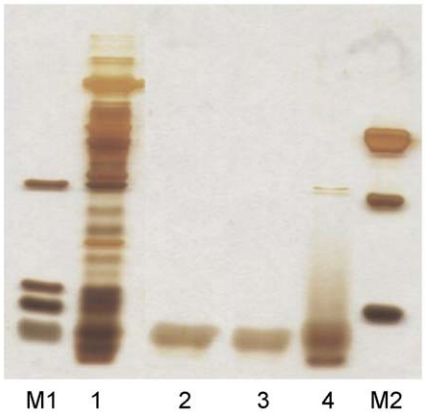

Fig. 2. SDS-PAGE of different protein fractions from the dry and germinated wheat seeds. *Lane 1*: dialyzed protein fraction of the dry seeds. *Lanes 2 and 3*: 10 µg WAP and GWAP. *Lane 4*: *YM-10* filtrate of the dry seeds. M1 shows molecular mass standards (M_r 26 600, 17 000, 14 200, 6 500, and 3 496). M2 shows the molecular mass marker proteins (bovine serum albumin 6 700; trypsinogen 24 000; α-lactalbumin 14 200).

Table 1. Purification steps of small protein fractions from the germinated and non-germinated wheat seed meal. The inhibition concentrations (IC_{50}) of the fractions were determined against *Verticillium dahliae.*

Purification step	Dry seeds			Germinated seeds		
	Total protein [mg]	Yield [%]	IC_{50}	Total protein [mg]	Yield [%]	IC_{50}
Crude seed extract	320.0	100.0	400	300.0	100.0	400
$(NH_4)_2SO_4$ precipitation (70 %)	68.5	21.4	360	62.0	20.8	350
80 ºC denaturation	54.0	16.8	250	49.5	16.5	250
Dialysis	49.0	15.3	220	42.0	14.0	210
YM-10 filtration	21.0	6.5	180	18.0	6.0	160

respectively. Molecular mass estimation of the WAP and GWAP yielded two closest peaks by gel filtration chromatography with a value of 5 kDa. The majority of the antimicrobial proteins isolated from other plants is relatively small (≤ 10 kDa) cysteine-rich basic peptides (Garcia-Olmedo *et al.* 1998, Koo *et al.* 2002,). Their low M_r, coupled with their high antifungal potency, should make them strong candidates for exploitation of its biological activities.

Antimicrobial properties of the wheat seed proteins: *In vitro* antimicrobial susceptibility tests of the wheat proteins were performed against four agronomically important phytopathogenic fungi and two bacterial strains by turbidity measurement and paper disk diffusion assay (Cole 1994). The increase in absorbance is proportional to microbial growth and the cell turbidity is frequently used as a parameter for the growth of microorganisms. Microtiterplate turbidity assays were used to monitor the inhibitory effect of the wheat peptides on the growth of the fungi and bacteria. The data of the assay was used to obtain the dose response curves (percent growth inhibition versus protein concentration) where effective protein concentrations required for 50 % inhibition of bacterial growth (IC_{50}: inhibition concentration) were determined.

Table 2. Antifungal activity of WAP and GWAP measured as protein concentrations required for 50 % growth inhibition (IC_{50}) after 24 h of incubation. Half strength growth medium (Medium I) for fungi and bacteria were supplemented with 5 mM $CaCl_2$ (Medium II). For media compositions see Materials and methods.

Test organism	Medium I		Medium II	
	WAP	GWAP	WAP	GWAP
B. cinerea	20	20	200	200
F. oxysporum	30	20	200	200
R.. solani	20	15	200	200
V. dahliae	40	30	200	200
C. michiganensis	>200	180	>200	>200
E. carotovora	>200	>200	>200	>200

The basic protein fractions of *DEAE-52* (peak D1) showed effective antimicrobial activity against the tested microorganisms (Table 2) while no such activity was detected with the protein fractions of second peak (D2). The IC_{50} values of WAP and GWAP against the four fungi ranged from 20 to 40 μg cm^{-3} and 15 to 30 μg cm^{-3}, respectively. Generally, the peptide (GWAP) from the germinated wheat seed seems to be more effective antimicrobial protein than the peptide (WAP) of the dry wheat seed. The minimum IC_{50} value of the peptides towards fungi was 15 μg protein cm^{-3}, so GWAP affected growth of *R. solani* at this concentration in the medium I. The basic peptides of the wheat seed did not influence the growth of the Gram-negative bacteria *E. carotovora* subsp. *carotovora* when added in the growth media at up to 200 μg cm^{-3}. Likewise, WAP did not affect the growth of *C. michiganensis* subsp. *sepedonicus* at this concentration but GWAP inhibited the growth of this bacterium with 180 μg protein cm^{-3}. Generally Gram-positive bacteria are more sensitive to antimicrobial agents because they lack an outer membrane that serves as an effective barrier for penetration of large molecules (Zhang and Lewis 1997). The antimicrobial spectrum of the WAP and GWAP is more effective against the tested fungi than the tested bacteria when assayed in the growth medium I. Inhibition concentrations (IC_{50}) of the seed protein meal during purification steps were also determined against *V. dahliae* (Table 1) and resulted with increasing antifungal activity at each step.

The antagonistic effect of calcium ion was examined on the activity of the peptides by adding 5 mM $CaCl_2$ to the growth media. Addition of the $CaCl_2$ to the growth media completely reduced the activity of the WAP and GWAP at least 13 fold and growth inhibition of microorganisms was barely detectable at concentration of 200 μg protein cm^{-3}. This phenomenon has been identified for a number of small antimicrobial peptides

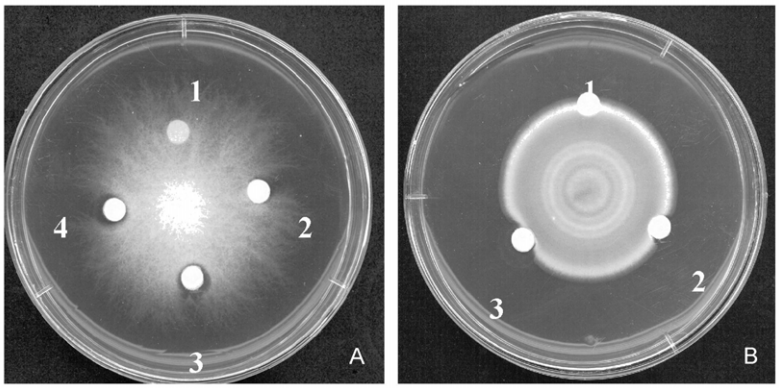

Fig. 3. Inhibitory activity of GWAP on the growth of *Fusarium oxysporum* (A) and *Verticillium dahliae* (B) by paper disk diffusion assay. A1 and B1 contain 10 mM Tris-HCl buffer (pH 7.5) as control. A2, A3 and A4; 20, 40 and 60 μg GWAP. B2 and B3; 30 and 60 μg GWAP, respectively.

and reported previously (Cammue *et al.* 1992).

The antimicrobial activity of the peptides was also determined by paper disk diffusion assay and the inhibitory activity of GWAP against *V. dahliae* and *F. oxysporum* was photographed (Fig. 3). The mycelial growth had enveloped disks containing the control and had formed transparent zone of inhibition around disks containing protein samples with antifungal activity. *In vitro* antimicrobial screening allows the selection of plant extracts with potentially useful properties to be used for further studies for plant protection. The bioassays showed that WAP and GWAP as well as other identified antimicrobial peptides (Koo *et al.* 2002) exhibited effective inhibitory activity as antimicrobial agents especially against the tested pathogenic fungi including *B. cinerea*, *F. oxysporum, R. solani* and *V. dahliae.* The mechanisms of action of the antimicrobial peptides are not known in detail however, it is assumed that the antimicrobial proteins are interacting directly with microbial membranes or they have a protein/receptor target. They are believed to generate pores in the microbial membranes resulting in leakage of the cytoplasmic material which then leads to the death of microorganisms (Giudici *et al.* 2000).

In this study, effect of germination on the antimicrobial activity of the wheat proteins was also examined. During germination period of plant seed, there is a drastic onset of antimicrobial activities that are not present in dormant phase of the seeds. It is believed that there is a mechanism in the seed to generate an array of antimicrobial proteins for the protection of embryo and young seedlings during earlier stage of development in highly microbic environment of soil (De Bolle *et al.* 1996). Purification and antifungal properties of barley seed chitinases released during early stages of imbibition have also been reported by Swegle *et al.* (1992). The study revealed that the small, heat stable, basic protein fractions of the dry and germinated seeds of wheat contained potential antimicrobial activity profiles on the growth of some agronomically important phytopathogenic bacteria and fungi. The detailed analysis of the peptides should be clarified by amino acid analysis and protein database searches to find out identity with the known antimicrobial proteins and further studies are being conducted to elucidate the properties of the peptides for antimicrobial activity. Isolation of active antimicrobial proteins from wheat seed provides a tool for the use of gene transformation studies aimed to obtain transgenic cotton plants displaying enhanced tolerance to fungi especially for *Verticillium dahliae.*

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