DIFFERENTIAL AND DEVELOPMENTAL STAGE SELECTIVE TOXICITY OF A PENTAPEPTIDE DERIVATIVE TO SUNFLOWER DOWNY MILDEW PATHOGEN

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SUMMARY

The activity of 2-Amino-7-fluorenyl-succinamoyl-FTPRL-NH_2 (PPD) against sunflower downy mildew pathogen was investigated with special regard to host independent ontogenetic stages. The size of the cells as well as she presence and structure of the cell wall apparently influenced the performance of PPD activity as the fungus showed a marked developmental stage dependent response to it: the cell-walled zoosporangia were more tolerant (MIC>1μM) than the zoospores (MIC<1μM), while the smallest cytospores MIC values were 0.03 μM. The early stages of zoosporogenesis were inhibited suggesting that PPD permeates the cell wall of zoosporangia. Functions regulating plasmalemma semipermeability were less sensitive (MIC≈1 μM) than the motility apparatus (MIC≈0.1 μM) of zoospores to PPD and Cu^{2+} ions. These cells reacted rapidly, as the first observable changes occurred within 5-10 seconds after contact with the compound asserting a target site in the cell membrane. The inhibitory effect was manifested at the nanomolar level, indicating a highly specific binding affinity to the target site and highlighting the importance of the inhibited function for normal operation of the cell membrane.
The overall lesson of our experiment is that the combination of microchemical methods with micro-techniques in screening lead to valuable toxicological data on the membrane-function disturbing activity of synthetic peptide analogues.

INTRODUCTION

Short chain peptides play an important role in the regulation of diverse biochemical activities. Hundreds of them exert antibiotic properties, so animals, plants and microbes have antibiotic peptides as their first defense system against infections by pathogens[1,2] or help pathogens to attack their hosts[3,4] and may act as ammensalistic moderators.[5] The intensive research in this field is motivated by the need to introduce new compounds into various human practices. Substitutions altering physicochemical properties are frequently linked to peptide antibiotics moreover they may contain rare amino acids that are not typically presented in proteins (tabtoxin, coronatine etc.). Some of the well-characterized antibiotic peptides and their derivatives have been developed as antibiotic drugs (bacitracin, vancomycin, polymixin B, amoxycillin etc.) and kills microorganisms by destroying their cell membranes or by inhibiting biosynthetic processes in a short span of time. Hydroxymethylphosphinylated

tripeptide bilanafos got introduction into agricultural use as a herbicide[6] and herbicidal activity of alanine tripeptide and of pentapeptide (Leu-Ser-Pro-Ala-Gln) was also reported.[7,8] However the most of research has been focused on medical[9] and veterinary[10] as well as entomological aspects[11] and the agronomically important microbial pathogens receive less attention, data on facultative plant pathogens were reported. An ergosterol complexing synthetic peptide inhibited conidia of *Aspergillus* and *Fusarium* species at 3-25 μM level [12,13] the membrane destroying effect of penta- and hexa-peptides on cells of *Fusarium oxysporum*, *Ceratocystis fagarcearum, Rhizoctonia solani* and *Pythium ultimum* was also demonstrated,[14] an other hexapeptide inhibited *Botrytis cinerea* and *Penicillium digitatum.*[15] The fungicidal activity of some plant extracts can also be linked to their peptide content.[16,17]

The aim of the present study was to characterize the activity of a new peptide pentapeptide derivative 2A7bf-PK against obligatory parasitic fungi. High toxicity of cupric ions serving as a reference substance to asexual spores of downy mildew fungus is well known since discovery of Bordeaux mixture[18] and large number of various copper containing mixtures are widely used in pest control. Short chain peptides besides of diverse biochemical activities have important role in regulation of the level of physiologically essential d-field elements of animals as well. By this reason the interaction of PPD with copper ions to control oomycota in host independent stages was also tested.

MATERIALS AND METHODS

The pentapeptide derivative (N-(7-Br-fluorenyl)-succinamoil-Phe-Tre-Pro-Lys-Arg-NH_2) was synthesised by previous line.[11] Tween 20 and copper sulphate was purchased from SIGMA (StLouis, USA). The analytical grade carboxin was the gift of the manufacturer (Uniroyal, Evesham, UK).

Sunflower downy mildew pathogen, *Plasmopara halstedii* (Farl.) Berlese et de Toni (Oomycota) race 1 was maintained on sunflower plants (*Helianthus annuus* L. cv GK-70). The suspensions of asexual spores ($2*10^5$ cell per ml) were prepared in sterile distilled water as described by OROS and UJVARY.[19]

Determination of biological activities Two-fold dilution series were prepared in sterile distilled water where the drug concentration ranged from 10^{-10} to 10^{-3} M. To assess the effect of **PPD** on asexual spores of *P. halstedii* following parameters were recorded: viability of zoosporangia, germination of zoosporangia, viability of zoospores (motion and plasmalemma semipermeability) and cystospore germination. All these events were observed microscop-

ically in suspensions of spores mixed (1:1 by volume) with solutions of drugs with appropriate concentrations. Lethal effects of the compounds were determined by adding an aqueous solution of Rose Bengal (Fluka, Buchs) (0.1 mg per ml) to an aliquot (1:2, by volume) of the zoosporangium suspension. Non-viable cells stained deep purple while dormant (viable) ones remained unstained. Watery solution of copper sulphate was used as a reference. These methods were described in detail previously by OROS et al.[20]

Data analyses At list five samples were taken for microscopic observations, and each test was made at least in two series. The activity of compounds was characterized by either maximum tolerated or minimum inhibitory concentration values (MTC and MIC, respectively) both expressed in microM. For these values a concentration range was given where they could be approximated. The ED_{50} values were calculated using a curve-fitting method based on log/probit function a similar way to that described by SVÁB[21]. The synergetic interaction was evaluated according to SUN[22].

RESULTS

Asexual spores of *P. halstedii* showed well-marked developmental stage dependent response to PPD and its inhibitory effect was commensurable to that of copper ions (Table 1). The presence and structure of cell wall seemingly influences on the performance of PPD activity. The sensitivity of cell- wall bearing zoosporangia and cystospores was greatly different.

There were revealed differences by means of microscopic survey in performance of zoosporangium killing effect as well. The fact, that the zoosporangia were lethally inhibited in early stages of zoosporegenesis evidently demonstrate, that **PPD** permeates their cell wall, contrarily to copper ions which kill cells next to opening of the operculum in the ultimate stage of zoospore differentiation. The plasmalemma semipermeability regulating functions were less sensitive than the motility apparatus of zoospores to both **PPD** and copper ions.

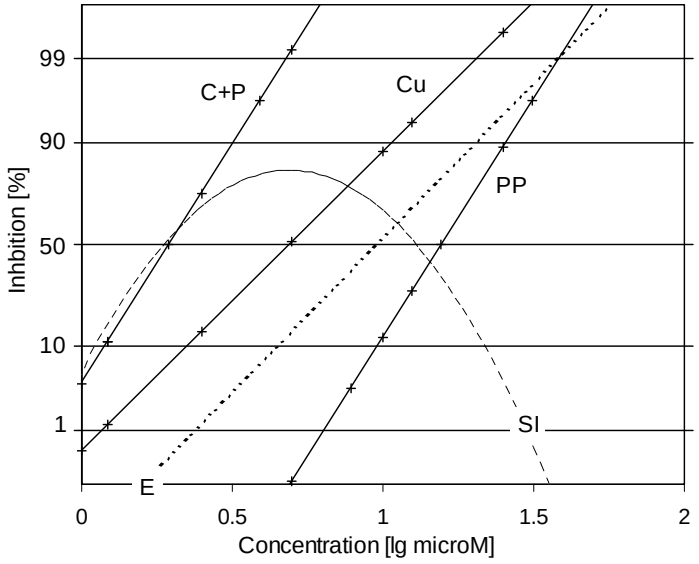
Test results with mixture of **PPD**+Cu^{2+} (1:1) revealed synergetic joint action against *P. halstedii* asexual spores (Figure 1). The character of this interaction is determined by the physiological status of the target cells having an evident developmental stage dependent character that was particularly expressed in killing effect on zoosporangia. The slope of dose-response curve of the mixture is similar to that of **PPD.**

Table 1. Sensitivity of *Plasmopara halstedii* to pentapeptide derivative.

	PPD		Copper	
Type of cells (events)[a]	MTC	MIC	MTC	MIC
Zoosporangia				
Survival	0.24-0.49	7.8 - 16	0.61-1.2	20 - 39
Germination	0.031-0.061	7.8 - 16	0.31-0.61	2.4 - 4.9
Zoospores				
Plasmalemma semipermeability	1.9-3.8	7.8-15.6	0.61-1.22	1.95-3.9
Motion	0.12-0.24	1.95-3.9	0.031-0.061	0.12-0.24
Cystospores				
Germination	0.015-0.031	0.12 - 0.24	0.15-0.31	63 - 125

a= The concentration limits are given in microM, where MTC = maximum tolerated concentration, under this limit no difference could be seen as compared to the watery control, MIC = minimum inhibitory concentration, over this limit all cells were killed or the event concerned completely was inhibited.

Figure 1. Synergetic interaction between **PPD** and copper in killing effect on zoosporangia of *Plasmopara halstedii*.



Treatments		MIC (μM)
No.	Compounds	ED_{50}
1	**PPD**	12.9
2	$CuSO_4$	4.90
3	1+2 (1:1)	1.93
Expected according to SUN[22]		
	1+2 (1:1)	7.10
Comparative Toxicity Index		3.68

Dose response lines (DRL) are as follows: PP=pentapeptide derivative, Cu= copper sulfate, C+P= combination of peptide and copper (1:1), E=the expected DRL calculated according to COLBY[23], SI= the synergetic increase.

DISCUSSION

The new pentapeptide derivative (2A7bf-PK) strictly differs with the fungicides used in everyday practices. Having unique physicochemical and structural properties this compound probably has an oligo-site action in the target cell. One part it acts as a cationic surfactant

molecule interacting with negatively charged moieties in plasmalemma. Among the possible candidates we assume sulfo- and phospholipids taking part in regulation of the membrane fluidity, which could explain the high effectiveness in disturbing of motility apparatus of diverse species. By means of microscopic observations a lag-phase with irregular movement preceded the complete inhibition of motility apparatus. The other site is probably situated in cell wall, which bind the positively charged **PPD** and do not allow the contact with fungal plasmalemma. There can be assumed participation in promoting permeation of ions of *d*-elements through biomembranes leading to unfavourable consequences as well.

The *P. halstedii* spores are not particularly sensitive to antibiotics, and among 32 antibiotics tested no one enriched the effectiveness of PPD to asexual spores.[24] Some of them were able to penetrate throughout the cell wall of zoosporangia (streptomycin, cycloheximide) but only caused a delay in zoosporogenesis contrary to PPD, which lethally inhibited the differentiation of zoospores. Interestingly an oligo-peptide produced by *Bacillus subtilis* acted also on organogenesis of *P. halstedii*, namely inhibited the transmission of cell nuclei into the zoosporangiophores resulting stop of the formation of zoosporangia.[25]

The activity of new PPD on asexual spores on peronosporaceous fungi and influences on cell differentiation might be evaluated as a finding of theoretical interest. These results lead us one step closer to the development of biorational peptide-like chemicals that will be effective in controlling certain microbial pests in an environmentally friendly fashion.

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