Developmental and Comparative Immunology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Functional role of charged residues in drosomycin, a *Drosophila* antifungal peptide

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ARTICLE INFO

Article history:
Received 15 March 2010
Received in revised form 17 April 2010
Accepted 17 April 2010
Available online xxx

Keywords:
Antifungal defensin
Site-directed mutagenesis
Functional site
Insect

ABSTRACT

The functional importance of positively charged residues in antibacterial peptides (ABPs) has been well documented. However, their role in antifungal peptides (AFPs) has not been extensively evaluated. To address this question, we investigated the functional role of charged residues in drosomycin, a specific *Drosophila* antifungal peptide, using site-directed mutagenesis in combination with circular dichroism (CD) analysis and antifungal assays. As a result, we firstly identified five cationic residues (R6, K8, R20, R21 and K38) whose alterations significantly affected the antifungal activity. Intriguingly, two negatively charged residues (D1 and E25) are also recognized as functional determinants of drosomycin. This indicates that it is the location of these charged residues other than net charges is crucial for activity. These functional sites are, respectively, located in different secondary structure elements, including the N-loop, α-helix and γ-core regions, all highly exposed on the molecular surface, suggesting that drosomycin may bind to fungal targets through electrostatic interactions. Our work has implications for further modification of drosomycin to obtain new antifungal peptides with enhanced activity.

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1. Introduction

Antimicrobial peptides (AMPs), the first line of host defense of multicellular organisms, play crucial roles in eliminating infection from various bacteria, fungi, viruses, and protozoa (Zasloff, 2002; Bulet et al., 2004). In general, these peptides are some small and cationic molecules with 12–50 amino acids in length and most of them have a positive net charge at physiological pH, due to the presence of a highly content in Arg and Lys. They usually adopt an amphipathic structure in which the positively charged domains are separated from the hydrophobic domains (Hancock and Diamond, 2000; Hancock, 2001). Such structure associated with the cationic character is critical for antibacterial activity of AMPs. In fact, electrostatic interaction between cationic AMPs and anionic bacterial membranes induces the initial step of antibacterial action and finally leads to membrane disruption (Zasloff, 2002; Matsuzaki, 2001; Yount et al., 2006). Researchers have proposed different pore-forming models to explain membrane permeabilization of AMPs with such a design (Brogden, 2005). The functional importance of positively charged residues in antibacterial activity is further strengthened by charge reversal or charge neutralization mutations in two AMPs (i.e. cryptdin-4 and thionin) (Tanabe et al., 2004; Vila-Perello et al., 2003). Results from three C-terminal (R36-K45) analogues of human β-defensin-3 also demonstrated that the peptide charge density, mediated by corresponding three-dimensional (3D) structures, was directly correlated with the antibacterial activity (Bai et al., 2009).

For antifungal peptides (AFPs), site-directed mutational analysis of three mutants of a plant defensin—Rs-AFP2 (Gly9Arg, Val39Arg and Lys44Glu) highlighted the contribution of cationic amino acids to its antifungal activity (De Samblanx et al., 1997). Another example is derived from ARD1, an analogue of the insect AFP—heliomicin, both differing by only two residues. A mutation of ARD1 from Asn to Arg was found to improve its function (Landon et al., 2004).

To evaluate the role of positively charged residues in AFPs, we investigated the charged sites of drosomycin, an antifungal peptide from *Drosophila melanogaster* (Fehlbaum et al., 1994). It is a small protein of 44 amino acids belonging to the scorpion toxin-like superfamily according to the structural classification of proteins in the SCOP database (http://scop.mrc-lmb.cam.ac.uk/scop/). All members in this subfamily adopt a cysteine-stabilized α-helix and β-sheet (CSαβ) fold (Landon et al., 1997). Drosomycin lacks activity on bacteria but can inhibit the growth of filamentous fungi and the yeast *Saccharomyces cerevisiae* (Landon et al., 1997; Tian et al., 2008). It has seven positively charged residues and six of them are highly exposed on the molecular surface and thus selected for mutational analysis. As a control, two negatively charged residues were also chosen. We found that five cationic residues mutated here are functionally important because their alterations significantly affected the antifungal activity of drosomycin. Two negatively

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0145-305X/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.dci.2010.04.003

charged residues are also recognized as functional determinants of drosomycin. Based on these findings, we compared functional sites of drosomycin and plant defensin and discussed the possible role of charged residues.

2. Materials and methods

2.1. Site-directed mutagenesis

Inverse PCR was used to generate all mutants using the constructed vector pGEX-6P-1 carrying GST-tag and wild-type drosomycin gene as template (Yuan et al., 2007; Zhu et al., 2008). Primers used are listed in Table 1. Phosphorylation of the 5′-end of primers was performed using polynucleotide kinase (TOYOBO, Osaka, Japan) and ATP (Takara, Dalian, China). Reaction conditions were as follows: 30 cycles of 45 s at 94 °C and 8 min at 68 °C with ExTaq polymerase (Takara, Dalian, China). Subsequently, linear PCR products were circularized by T4 DNA ligase (Takara, Dalian, China) after end polishing using Pfu polymerase (newProbe, Beijing, China). Circularized products were transformed into *E. coli* DH5α competent cells. Positive clones were confirmed by DNA sequencing.

2.2. Expression and purification

Expression and purification of drosomycin and its mutants were performed according to the methods previously described (Gao and Zhu, 2008). In brief, the expression of GST-drosomycin mutants in *E. coli* BL21 (DE3) was induced by 0.5 mM IPTG and fusion protein was acquired in supernatant after sonication, followed by affinity chromatography with glutathione–Sepharose 4B beads from Phamacia. Then the fusion protein was cleaved with enterkinase (Sinobio Biotech Co. Ltd., Shanghai, China) at 23 °C overnight. Reverse-phase HPLC was applied to obtain final pure mutant proteins.

2.3. Molecular mass determination and circular dichroism (CD) analysis

Molecular weights of purified products were determined by MALDI-TOF mass spectra on a Kratos PC Axima CFR plus (Shimadzu Co., Ltd., Kyoto). For CD analysis, peptide was dissolved in phosphate buffer (5 mM NaH_2PO_4, 5 mM Na_2HPO_4) at a concentration of 0.3 mg/ml. Spectra were recorded on a JASCO J-720 spectropolarimeter (Jasco, Tokyo, Japan) at 25 °C from 260 to 190 nm using a

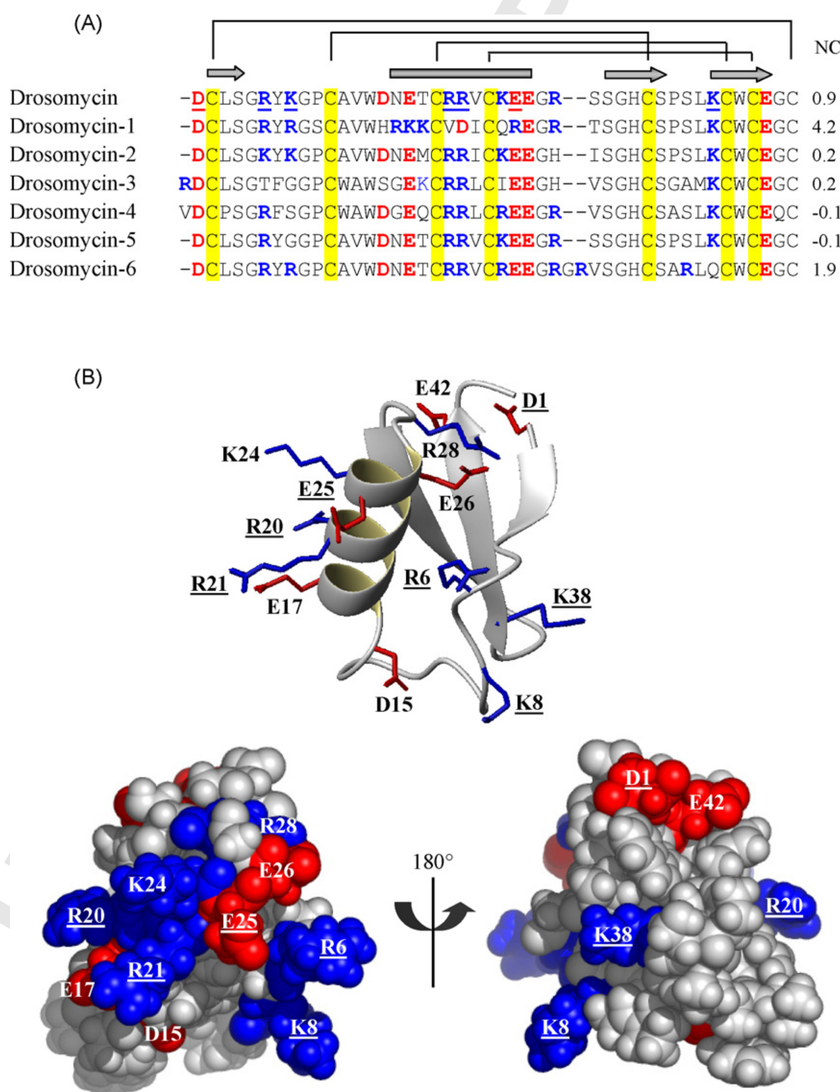

Fig. 1. Sequence alignment and 3D structure of drosomycin. (A) Multiple sequence alignment. Net charge (NC) was estimated at pH 7.0 using protein calculationV3.3 (http://www.scripps.edu/~cdputnam/protcalc.html); (B) ribbon and sphere models of drosomycin (PDB: 1MYN) with indication of charged residues. Residues underlined once are subjected to mutational analysis. Positively and negatively charged residues are shown in blue and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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Table 1
Primers used in this study.

Mutants	Name	Sequence
D1A	D1A-FP	5′-TGCCTGTCCGGAAGATACAAGGGTC-3′
	D1A-RP	5′-G**G**CCTTGTCATCGTCATCGGATCCCAG-3′
R6A	R6A-FP	5′-**GC**ATACAAGGGTCCCTGTGCCGTCTGG-3′
	R6A-RP	5′-TCCGGACAGGCAGTCCTTGTCATCGT-3′
K8A	K8A-FP	5′-**GC**GGGTCCCTGTGCCGTCTGGGACAACGA-3′
	K8A-RP	5′-GTATCTTCCGGACAGGCAGTCCTTGTCA-3′
R20A	R20A-FP	5′-**GC**TCGTGTGTGCAAGGAGGAGGGACGCT-3′
	R20A-RP	5′-ACAGGTCTCGTTGTCCCAGACGGCAC-3′
R21A	R21A-FP	5′-**GC**TGTGTGCAAGGAGGAGGGACGCTCCAGT-3′
	R21A-RP	5′-ACGACAGGTCTCGTTGTCCCAGACGGCA-3′
K24A	K24A-FP	5′-**GC**GGAGGAGGGACGCTCCAGTGGCCACTGC-3′
	K24A-RP	5′-GCACACACGACGACAGGTCTCGTTG-3′
E25A	E25A-FP	5′-G**C**GGAGGGACGCTCCAGTGGCCACTGCA-3′
	E25A-RP	5′-CTTGCACACACGACGACAGGTCTCGTT-3′
K38A	K38A-FP	5′-**GC**GTGCTGGTGCGAAGGATGCTAAGTC-3′
	K38A-RP	5′-CAGACTGGGGCTGCAGTGGCCACTGGA-3′
K38E	K38E-FP	5′-**G**AGTGCTGGTGCGAAGGATGCTAAGTC-3′
	K38E-RP	5′-CAGACTGGGGCTGCAGTGGCCACTGGA-3′

Note: Mutated nucleotides are boldfaced. All primers listed here were synthesized in SBS Genetech (Beijing, China).

quartz cell 1.0 mm in length. Data were collected at 0.5-nm intervals with a scan rate of 50 nm/min.

2.4. Antifungal assays

Liquid growth inhibition assay was performed according to the previously described method (Yuan et al., 2007). Briefly, spores of *Neurospora crassa* suspended in 1× MEA with the OD_{595} of 0.1 were dispensed in aliquots of 80 μl into wells of a 96-well microplate. 20 μl series of diluted samples were added to the spore suspension to a final concentration of 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6 and 51.2 μM and each concentration had triplicate wells. After incubation at 30 °C for 24 h in dark, the growth of *N. crassa* was evaluated by measuring the culture absorbance at 595 nm using Microplate Reader (Pelong tech, Beijing). OD_{595} of each concentration was the average of three wells and was used to calculate IC_{50}, a peptide concentration that gives 50% of growth inhibition, which were calculated at the server (http://chiryo.phar.nagoya-cu.ac.jp/javastat/JavaStat-e.htm).

Inhibition zone assay was performed according to the previously described method (Gao and Zhu, 2008). *Geotrichum candidum* and *Aspergillus niger* were incubated at 30 °C 1 week on 1× MEA containing 1.5% agar. Spores were harvested and suspended in 1× MEA with an OD_{595} of 0.5. 6 ml of 1× MEA containing 0.8% agarose was mixed with 50 μl spores suspension and poured into one Petri dish of 9.0 cm in diameter. Holes with a diameter of 2 mm were punched into the medium, filled with 2 μl of sample each hole. Concentration used here was 1 mM. Fungi were incubated in dark at 30 °C for 24 h and then zone of inhibition was measured. Every experiment was repeated three times.

3. Results

As shown in Fig. 1A, drosomycin is a charged amino acid-rich AFP comprising seven positively and six negatively charged residues. To investigate possible functional significance of these positively charged residues, we selected six highly exposed sites (>30% accessibility) (R6, K8, R20, R21, K24 and K38) for mutational analysis. Only one excluded is R28 that is buried in the molecular interior with <20% surface accessibility. As a control, we also mutated two

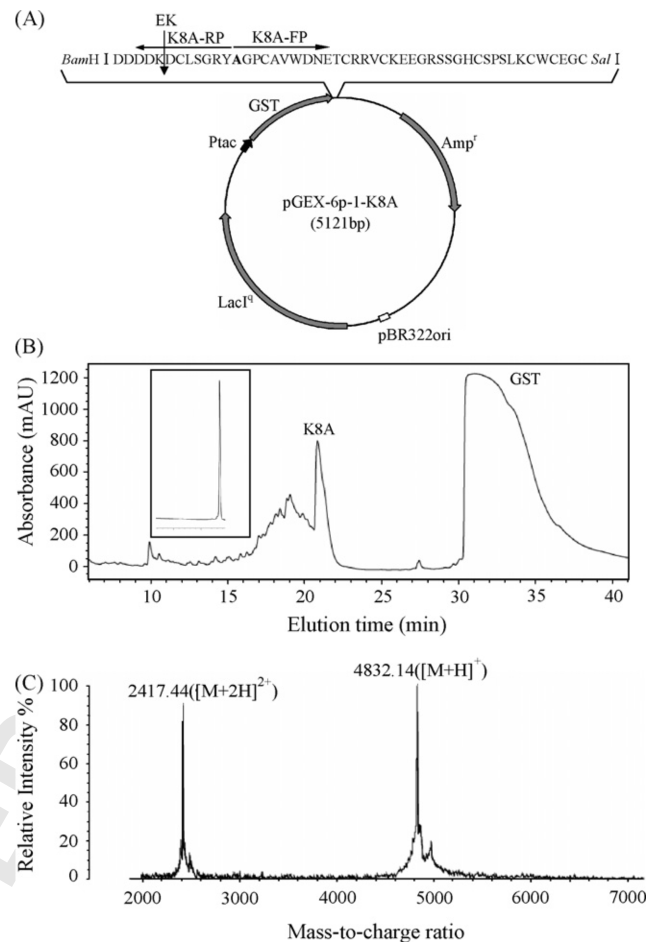

Fig. 2. Expression, purification and identification of drosomycin mutants. K8A is presented here as an example. (A) Construction of pGEX-6P-1-K8A expression vector by inverse PCR; (B) RP-HPLC. The re-purification is provided in inset; (C) MALDI-TOF. The spectrum has two main peaks, respectively, corresponding to the singly and doubly protonated forms of the peptide.

negatively charged sites (E25 and D1) for the same analysis (Fig. 1B). All these residues were substituted by Ala, a small amino acid that can be accommodated in most types of secondary structure. This minimizes the chance that the point mutation will induce major structural perturbations. Considering the putative importance of K38, as predicted by both structural comparison and evolutionary tracing analysis (Tian et al., 2008; Landon et al., 2000), we also designed a charge reversal mutation—K38E.

Using inverse PCR to amplify the drosomycin plasmid, we successfully constructed all the mutants mentioned above with the exception of K24. Recombinant peptides were expressed as fusion proteins with a GST-tag in the *E. coli* system and purified by glutathione affinity chromatography. The fusion protein was digested with EK and the resultant product was applied on Reverse-phase HPLC to separate the peptide from GST (Fig. 2). MWs and purity of all HPLC-purified mutants were confirmed by MALDI-TOF (Table 2).

Subsequently, we compared activities of each mutant with that of drosomycin on *N. crassa*, a highly sensitive fungus species to drosomycin. As presented in Table 2 and Fig. 3A, wild-type drosomycin showed potent antifungal activity with an IC_{50} of 0.83 μM, comparable with the native peptide (0.6 μM) (Fehlbaum et al., 1994). By contrast, four substitutions (e.g. D1A, R20A, R21A, and K38E) resulted in a complete loss of antifungal activity of drosomycin, even at the concentration >50 μM. Other mutants (e.g. R6A, K8A, E25A, and K38A) exhibited significantly decreased antifungal potency relative to the wild-type drosomycin. Evaluated by

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Table 2
Antifungal activity of drosomycin and its mutants against *N. crassa*.

Peptides	Theoretical MW (Da)	Experimental MW (Da)	IC_{50} (μM)[a] (95% confidence limit)	Fold reduction[b]
Drosomycin	4889.54	4889.00	0.83 (0.39–1.58)	
drsD1A	4845.53	4846.65	N.A.	
drsR6A	4804.43	4804.40	>51.2	>61.7
drsK8A	4832.44	4832.14	8.78 (3.47–32.57)	10.60
drsR20A	4804.43	4806.47	N.A.	
drsR21A	4804.43	4803.60	N.A.	
drsE25A	4831.50	4834.10	12.40 (8.36–20.68)	14.90
drsK38A	4832.44	4832.60	>51.2	>61.7
drsK38E	4890.48	4890.00	N.A.	

[a] IC_{50} means the concentration for 50% inhibition of growth. ">51.2" means the IC_{50} is larger than 51.2 μM (weak activity). "N.A." means no activity even at the concentration of 51.2 μM.
[b] Fold reduction was calculated as IC_{50} of a mutant/IC_{50} of drosomycin.

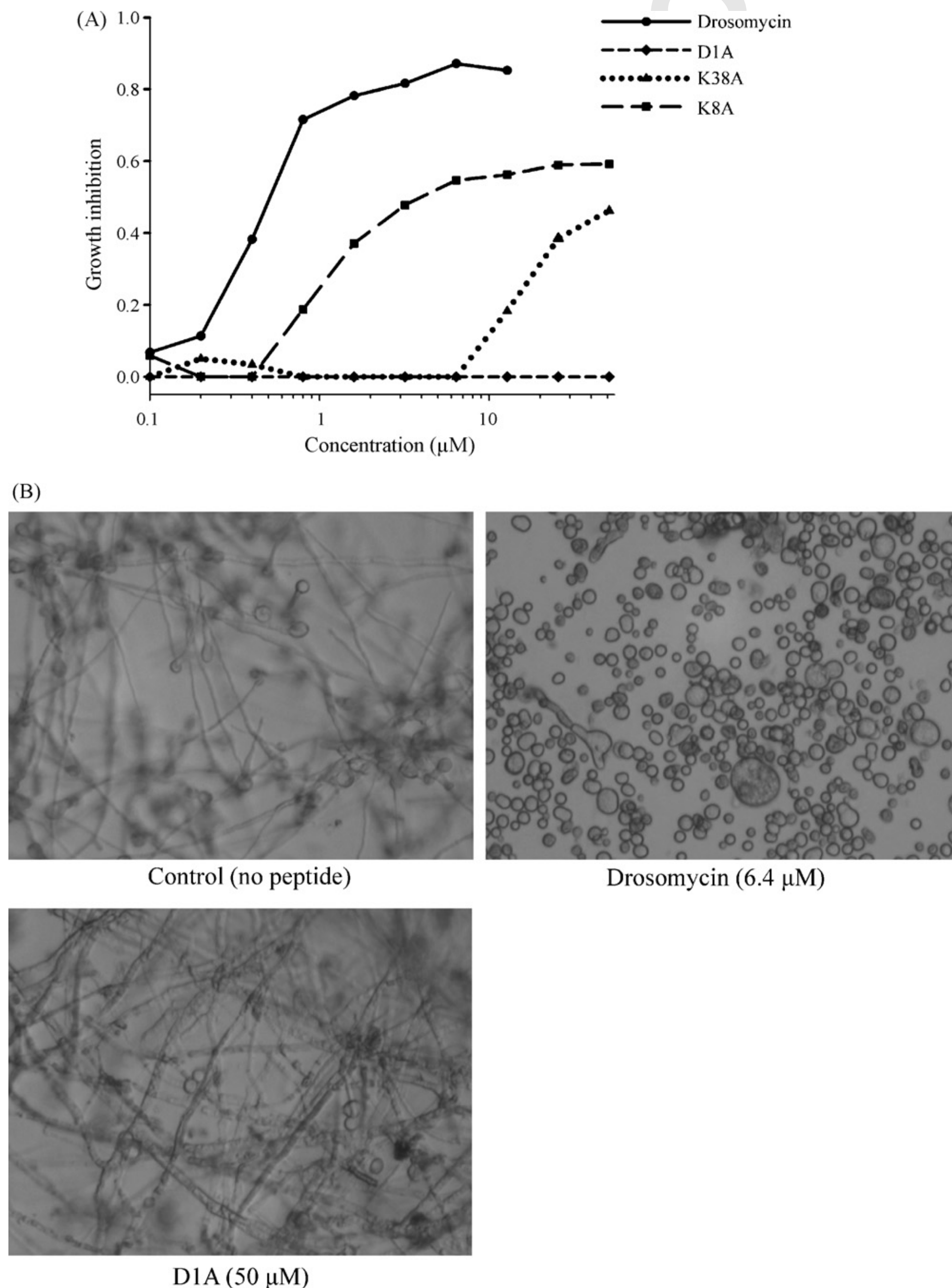

Fig. 3. Antifungal activity of drosomycin and mutants against *N. crassa*. (A) Dose–response relationship of drosomycin and three representative mutants evaluated by liquid growth inhibition assays which were performed in triplicate in one experiment; (B) hyphal lysis of *N. crassa* by drosomycin but not by D1A.

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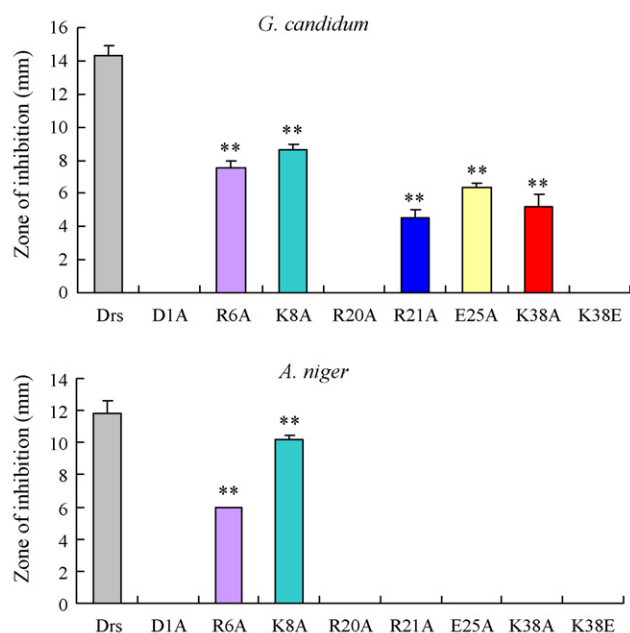

Fig. 4. Growth inhibition of drosmycin and its mutants against *G. candidum* and *A. niger*. Peptide amount used in each well is 2 nmol. Inhibition zone diameters are shown as mean ± SD ($n = 3$). Results were analyzed by Student Neuman–Keuls test. $^{**}P < 0.01$ indicates statistical difference compared to drosomycin.

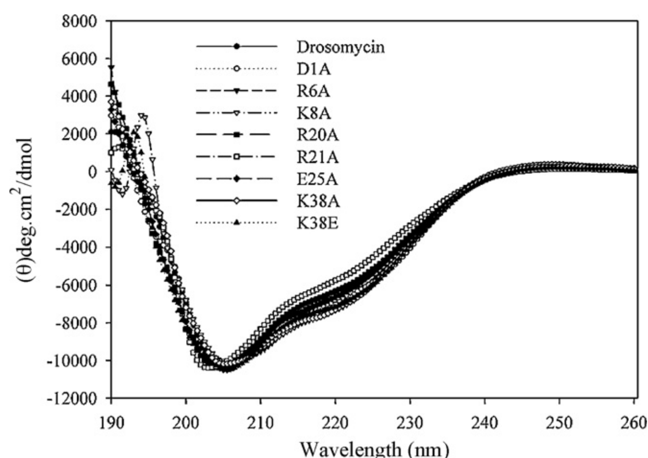

Fig. 5. CD spectra of drosomycin and its mutants. Spectra were taken at a peptide concentration of 0.3 mg/ml and were performed by averaging three scans.

167 IC_{50} values, we found that the reduced folds are 10.6 for K8A, 14.9 for E25, and >61.7 for R6A and K38A. Fungal growth inhibition was also monitored microscopically, in which 50 μM D1A had no effect on the hyphal growth of *N. crass*, whereas 6.4 μM drosomycin caused severe hyphal lysis (Fig. 3B).

172 To investigate whether the functional residues against *N. crassa* have the same role towards other fungal species, we evaluated the antifungal effect of these mutants on *G. candidum* and *A. niger* by inhibition zone assays (Fig. 4). Results showed that three mutants (D1A, R20A and K38E) had no effect on *G. candidum*, while five (R6A, K8A, R21A, E25A and K38A) displayed significantly decreased antifungal potency. For *A. niger*, all mutants lose activity except R6A and K8A that remained some weak potency. Overall, these observations recognized D1, R20 and K38 as the most important residues involved in the interaction with diverse fungal species, whose mutations led to a complete loss of activity.

183 To distinguish whether the functional loss or reduction of these mutants is associated with structural alterations originated from point mutations or amino acid changes in functional sites, we compared structural features of drosomycin and its mutants by CD analysis, which confirmed all these recombinant peptides present a typical curve of CSαβ peptides with a positive maximum around 190 nm and a negative minimum at 205 nm (Fig. 5). These results show that the structures of all mutants are not significantly different from those of drosomycin and exclude structural impact on the function of these mutants. This study implies that drosomyicn, like the plant defensin (Yang et al., 2009), is highly tolerant to amino acid substitutions and thus represents a candidate scaffold for protein engineering.

196 4. Discussion

197 Drosomycin is the first inducible AFP initially isolated from bacteria-challenged *D. melanogaster*. Despite structural, functional and evolutionary data are available at present, little progress has been made in understanding its functional surface involved in antifungal activity (Fehlbaum et al., 1994; Landon et al., 1997; Tian et al., 2008; Gao and Zhu, 2008). Some researchers have made efforts to predict the putative functional sites of drosomycin through structural comparison and evolutionary tracing analysis (Tian et al., 2008; Landon et al., 2000). However, experimental data are lacking. As the first step towards this task, we performed evaluation of the importance of charged residues in antifungal activity of drosomycin and found two most important cationic residues (R20 and K38) whose alterations resulted in functional loss towards different fungal species (*N. crassa*, *G. candidum* and *A. niger*). K38 is the only cationic residue located in the γ-core, an important functional motif proposed by Yount and Yeaman (Yount and Yeaman, 2004), and was also predicted as a putative antifungal site by structural and evolutionary analysis (Tian et al., 2008; Landon et al., 2000). Our mutational experiments verified the predictions. When the basic side-chain of K38 was replaced by a small hydrophobic residue (A38), the mutant K38A exhibited a significantly decreased antifungal potency, whereas the reversal charge mutation from K38 to E38 eliminated the antifungal activity, suggesting electrostatic repulsion is a possible reason for the loss of activity. We thus suspect that K38 makes an ionic interaction with a negatively charged group on the fungal targets. R21 is also an essential site since the mutant R21A only slightly inhibited the growth of *G. candidum* and had no activity on *N. crass* and *A. niger*. R6 and K8 also contribute to the antifungal potency of drosomycin. Mutations of these two sites differentially affected the antifungal activity of drosomycin to different fungal species. These observations agree with the results of an α-defensin—cryptdin-4 (Crp4), in which all mutations from an Arg to an Asp attenuate or eliminate microbicidal activity regardless of the Arg residue position (Tanabe et al., 2004).

231 In addition, we also highlighted the significance of negatively charged residues in antifungal activity. It is attractive that substitution of D1 with an Ala completely abolished the antifungal activity of drosomycin against all three fungi used here. Mutation of E25 also reduced the antifungal activity. These observations provide the first experimental evidence for the important role of negatively charge sites in antifungal activity. A previous study has demonstrated that net positive charge is a major factor for antimicrobial activity of decapeptides designed based on human β-defensin-3 (Papanastasiou et al., 2009). The discovery of functional roles of two negatively charged residues hints the difference in antimicrobial action mode between these peptides. Our opinion is further strengthened by drosomycin-2 that is a neutral molecule but has potent antifungal activity (Tian et al., 2008). Supported by these observations, it is reasonable to infer that the distribution of charged residues rather than net positive charges could be a major factor of drosomycin to mediate specific electrostatic interaction between these residues and fungal targets by charge

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complementarity, a proposed mechanism for specific high-affinity binding between proteins (Sinha and Smith-Gill, 2002).

Rs-AFP2 is the first antifungal defensin whose functional sites are known. This radish seed-derived peptide shares similar 3D structure and antifungal spectrum to drosomycin (Landon et al., 2000). Rs-AFP2 possesses two adjacent functional sites: one being in the γ-core, the other being formed by residues on the N-loop and contiguous sites on the α-helix and β strand 3 (De Samblanx et al., 1997). In drosomycin, R20, R21 and E25 are located on the α-helix, D1, R6 and K8 in the N-terminus and N-loop, and K38 in the γ-core. As shown in Fig. 1B, these sites form one main patch comprising residues located in the N-loop and α-helix. Two separated sites K38 and D1 are scattered in the opposite side of the main patch. Overall, the distribution of functional sites between Rs-AFP2 and drosomycin has some similarity despite their side-chain types are rather different due to only charged amino acids investigated here. This observation is of significance if we consider functional commonality between drosomycin and plant defensins (Gao and Zhu, 2008).

In conclusion, our work firstly highlights the functional role of charged residues in antifungal drosomycin and demonstrates that this molecule is a promising framework with high tolerance to residue substitutions. These observations have implications for rational modification of drosomycin to improve its functions. Investigation of antifungal role of other types of residues in drosomycin using the strategy developed here is under way.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (30730015 and 90608009) and the 973 Program from the Ministry of Science and Technology of China (2010CB945304).

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