Inhibition of melanization by a *Nasonia* defensin-like peptide: Implications for host immune suppression

Caihuan Tian [a], Lei Wang [b], Gongyin Ye [b], Shunyi Zhu [a,*]

[a] *Group of Animal Innate Immunity, State Key Laboratory of Integrated Management of Pest Insects & Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, PR China*
[b] *State Key Laboratory of Rice Biology, Institute of Insect Sciences, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310029, PR China*

A B S T R A C T

The parasitic wasp *Nasonia vitripennis* suppresses host immune mechanisms that include melanization reactions. Melanization is an important immune response of hosts induced by wasp infection and thus its inhibition represents a successful strategy for parasitism. However, the molecular basis associated with such inhibition is largely unknown in *N. vitripennis*. Here, we report recombinant expression, structural and functional characterization of a *Nasonia*-derived defensin-like peptide (called nasonin-3) whose recombinant product exerts inhibitory effect on host melanization. The possible role of nasonin-3 in immune suppression is also discussed.

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1. Introduction

The melanization reaction involves multiple host defense mechanisms in insects. In this process, phenols are oxidized to quinines which are subsequently polymerized to form melanin (Tang, 2009). Phenoloxidase (PO), usually synthesized as an inactive zymogen called pro-phenoloxidase (PPO), is a key enzyme in melanin biosynthesis. Recognition of pathogens leads to the activation of a serine protease cascade that culminates in proteolytic cleavage of inactive PPO to active PO (Jiravanichpaisal et al., 2006; Marmaras and Lampropoulou, 2009; Tang, 2009). Previous studies have indicated that melanization is an immediate immune response observed at the site of cuticular injury or on the surface of parasites invading the haemocoel. It can promote coagulation after physical breakage of the insect cuticle and also help to blacken the capsule after encapsulation (Lemaitre and Hoffmann, 2007), mechanisms that are particularly important in defense against parasitoids. Given such crucial roles for melanization, suppression of the activity of POs in hosts likely represents an ideal strategy for successful parasitism. Some examples have been observed in *Drosophila melanogaster* and its endoparasite wasp *Leptopilina boulardi* (Nappi et al., 2005) and in *Manduca sexta* and its parasitoid *Microplitis demolitor* (Beck and Strand, 2007).

Nasonia vitripennis (Hymenoptera: Pteromalidae) is a small parasitic wasp which stings and lays eggs in pupae of various fly species, such as blow flies and flesh flies. Recently, Werren et al. (2010) reported several protease inhibitor-like peptides in *N. vitripennis* which include cysteine-rich venom peptides of the Kunitz type motif, the trypsin inhibitor-like motif which typically contains 10 cysteines that form five disulfide bridges, and also some Kazal-type inhibitors (de Graaf et al., 2010; Werren et al., 2010). However, functional data on these peptides is lacking.

Here, we describe a new *N. vitripennis* defensin-like peptide (nasonin-3) that has a completely different amino acid sequence from those mentioned above. Nasonin-3 adopts a typical insect defensin-like structure with a conserved cysteine-stabilized α-helical and β-sheet (CSαβ) motif. Recombinant nasonin-3 inhibits the melanization of its host's haemocytes through decreasing PO activity. Sequence and structure comparison allows us to identify a putatively functional motif that is possibly associated with the protease inhibitory activity of nasonin-3.

2. Materials and methods

2.1. Construction of recombinant expression vector

N. vitripennis was maintained as a laboratory culture on pupae of the house fly *Musca domestica*. They were reared under a 14:10 light–dark cycle at 25 °C in glass containers fed on 20% (v/v) honey solution.

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* Corresponding author. Tel.: +86 010 64807112; fax: +86 010 64807099.
  E-mail address: Zhusy@ioz.ac.cn (S. Zhu).

For isolating *N. vitripennis* total RNA, 50 adult wasps were grounded into fine power in liquid nitrogen. The Trizol reagent (SBS Genetech, Beijing) was used to prepare total RNA according to the supplier's instructions. Reverse transcription of total RNAs was performed using RT PreMix kit (TransGen, Beijing) and a universal oligo(dT)-containing adaptor primer dT3AP (Zhu and Gao, 2006; Tian et al., 2010).

To construct pGEX-6P-1-nasonin-3 expression vector, we amplified nasonin-3 cDNA using primers FP and RP (FP: AT<u>GGATCC</u>**GATGACGATGACAAG**TCTGCTATTCCACACTGCAGCCCG; RP: AT<u>GTCGAC</u>**TTA**TGGCCGGATGCCTCCAAT. BamHI and SalI sites are underlined once and the EK site-coding region and the stop codon are bolded). To facilitate correct in-frame with the vector, we introduced a BamHI site and codons of enterokinase (EK) cleavage site at 5′ end of the forward primer FP and a SalI site and a stop codon at 5′ end of the reverse primer RP. The PCR product was digested by BamHI and SalI and ligated into pGEX-6P-1. Finally, the recombinant plasmid was transformed into *E. coli* DH5α and positive clones were confirmed by DNA sequencing using pGEX 5′.

2.2. Expression, purification and characterization of nasonin-3

Expression of GST-nasonin-3 in *E. coli* BL21 (DE3) was induced by 0.5 mM IPTG and fusion protein was acquired in the supernatant after sonication, followed by affinity chromatography with glutathione-Sepharose 4B beads from GE Healthcare (Shanghai, China). The fusion protein in Tris–HCl buffer was then digested with EK (Sinobio Biotech Co. Ltd., Shanghai, China) at 22 °C overnight. Reverse phase HPLC (RP-HPLC) was applied to separate nasonin-3 from GST. The molecular weight of the peak at 23 min was determined by matrix-assisted laser desorption/ionization time of flight mass spectrum (MALDI-TOF MS) on a Kratos PC Axima CFR plus (Shimadzu Co. Ltd., Kyoto).

2.3. CD spectroscopy

CD spectra of nasonin-3 were recorded on a JASCO J-720 spectropolarimeter (Jasco, Tokyo, Japan) at a protein concentration of 0.3 mg/ml dissolved in 5 mM sodium phosphate buffer, pH 7.0. Spectra were measured at 25 °C from 260 to 190 nm by using a quartz cell of 1.0 mm thickness. Data were collected at 0.5 nm intervals with a scan rate of 50 nm/min. The CD spectra were measured by averaging three scans. Data are expressed as mean residue molar ellipticity ($[\theta]$), calculated as follows: $[\theta] = \theta \times (0.1 \times MRW)/(L \times C)$, where θ is the ellipticity (in millidegrees), C is the concentration (in mg/ml), L is the path length (in cm), and MRW is the mean residue weight (in dalton). Percentages of peptide secondary structure elements were calculated with the DICHROWEB software, an online server for protein secondary structure analyses from CD data (http://dichroweb.cryst.bbk.ac.uk).

2.4. Antimicrobial assays

The inhibition zone assay was performed according to previously described methods (Gao and Zhu, 2008). Microorganisms used in this assay include three Gram-positive bacteria, ten Gram-negative bacteria, two yeasts and five fungi (Table S1).

2.5. Melanization of haemolymph in vitro

The flesh fly *Boettcherisca peregrina* (Diptera: Sarcophagidae) was maintained as a laboratory culture under a 14:10 light–dark cycle at 25 °C with a relative humidity of 75%. Larvae were reared on an artificial diet (wheat powder:water:porcine liver = 3:5:6) (Aoki and Suzuki, 1984) and adults were fed with dry milk and sugar.

Pupae of *B. peregrina* were surface sterilized by 15 min soaking treatments of each pupa in successive baths of a 10% (v/v) bleach solution containing 1% (v/v) Triton X-100, 70% (v/v) ethanol, and sterile distilled water (Rivers et al., 2002). Following sterilization, pupae were bled by puncturing the dorsal surface of the head with an insect pin. Haemolymph from individual pupae was then collected into sterile Eppendorf tubes on ice. Ten microliters of undiluted haemolymph was then added to 100 μl phosphate-buffered saline (PBS, pH 7.4) without or with different treatments of BSA (0.5 mg/ml), nasonin-3 (0.5 mg/ml) and phenylthiourea (PTU, 1%, a PO inhibitor, Sigma, USA) in the wells of a sterile, flat-bottomed, 96-well microtitre plate, and then incubated. Darkening of the haemolymph (indicating that melanization was occurring) was assessed by eye (Richards and Edwards, 2000).

2.6. Phenoloxidase assays

Phenoloxidase activity was assayed using the method of Wilson et al. (2001). In brief, the whole haemolymph (i.e., plasma and haemocytes) was extracted from pupae of *B. peregrina* (12 h after pupariation at 25 °C). Ten microliters of such haemolymph was placed in 490 μl PBS containing recombinant protein with different concentrations pre-cooled on ice and mixed in plastic Eppendorf tubes. PBS with BSA/PTU was treated as a negative/positive control. The sample was frozen to disrupt haemocyte membranes and PO activity in the defrosted sample was assayed spectrophotometrically using L-DOPA as a substrate. This involved pipetting triplicate 100 μl samples of the buffered haemolymph into a microtitre plate, adding 100 μl of 20 mM L-DOPA to each and incubating the mixture at 25 °C. The absorbance was read at 492 nm on a temperature-controlled microplate reader (Bio-Tek Instruments) over 60 min with 1 min interval. Data for final analysis were selected from the linear phase of the reaction. Using 10 μl of the haemolymph/PBS mixture, the amount of protein in the sample was also measured (calibrated using a standard curve created on the same microtitre plate using a BSA standard). Phenoloxidase activity is expressed as PO units per mg protein, where one unit is the amount of enzyme required to increase the absorbance by 0.001 min^{-1}.

2.7. Structural modeling

To construct a structural model, the mature peptide sequence of nasonin-3 was submitted to the protein structure prediction server (http://bioserv.cbs.cnrs.fr/) by comparative modeling (Douguet and Labesse, 2001). Models were visualized using MOLMOL (http://hugin.ethz.ch/wuthrich/software/molmol/index.html) in ribbon and Pymol (http://www.pymol.org/) in spheres. The modeled structure of nasonin-3 has been deposited in the Protein Model Database (http://mi.caspur.it/PMDB/) under the id number of PM0075698.

3. Results

We employed a GST system to efficiently produce recombinant nasonin-3 for structural and functional characterization (Fig. 1A–C). The molecular weight of purified nasonin-3 determined by MALDI-TOF MS is 4662.30 Da (Fig. 1D), which perfectly matches its theoretical value (4662.54 Da). The yield of nasonin-3 is 2 mg per litre culture.

The CD spectra of nasonin-3 shows a typical curve of CSαβ conformation, as identified by a positive maximum at 193 nm and a negative minimum at 209 nm. The spectrum quality is appropriate for calculating the secondary structure content of nasonin-3 because it is nearly identical with the calculated spectrum by the CDSSTR method and reference data set 4
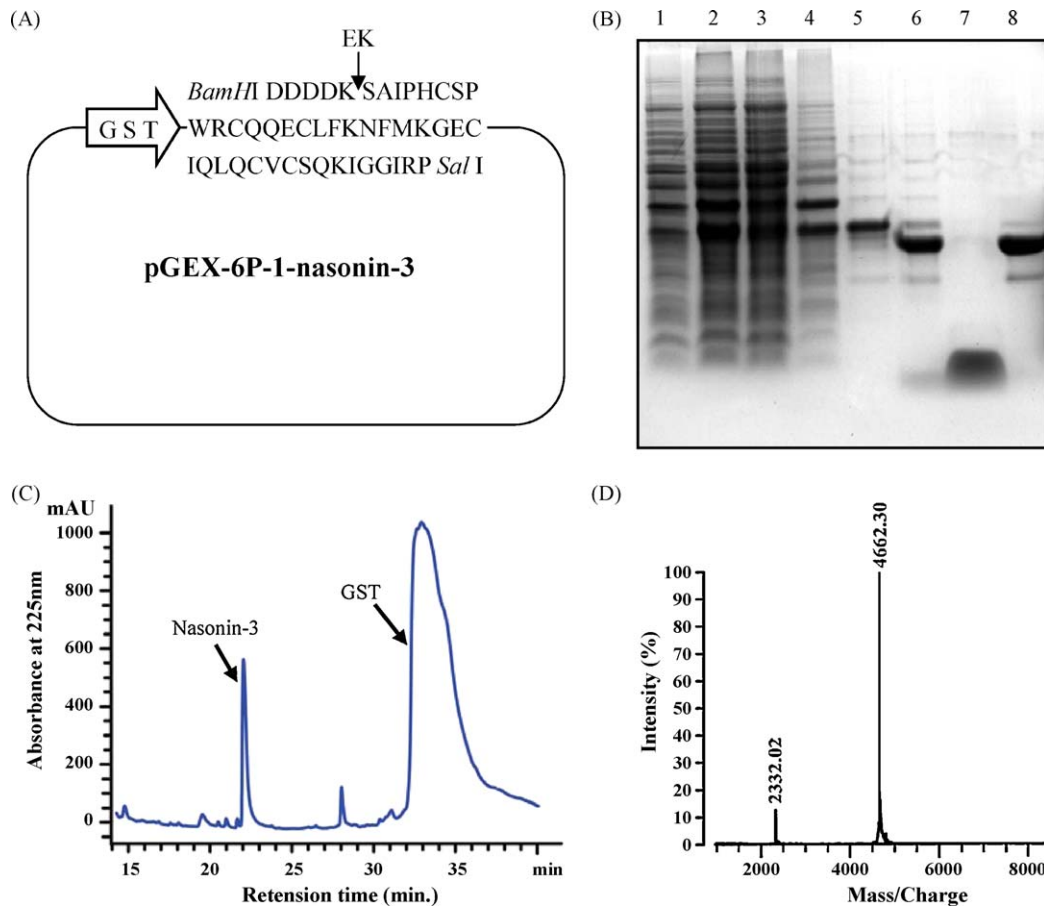
Fig. 1. Expression, purification and characterization of nasonin-3. (A) Construction of pGEX-6P-1-nasonin-3 expression vector. The amplified DNA sequence of nasonin-3 was inserted into BamHI and SalI sites of pGEX-6P-1 with an EK cleavage site at the 5′ end; (B) SDS-PAGE of the expression and purification of nasonin-3. Lane 1: total protein of non-induced *E. coli*; Lane 2: total protein of induced *E. coli*; Lane 3: supernatant of total protein after sonication; Lane 4: precipitation after sonication; Lane 5: fusion protein by affinity chromatography; Lane 6: fusion protein cleaved by EK; Lane 7: purified nasonin-3 by HPLC; Lane 8: GST tag by HPLC; (C) RP-HPLC showing the purification of nasonin-3 from GST tag. C18 column was equilibrated with 0.05% TFA and the purified proteins were eluted from the column with a linear gradient from 0% to 60% acetonitrile in 0.05% TFA within 40 min; (D) determination of the molecular weight of nasonin-3 by MALDI-TOF. The spectrum has two main peaks, corresponding to the singly and doubly protonated forms of the peptide.

(Fig. 2A). As estimated by DICHROWEB (Whitmore and Wallace, 2004, 2008) based on the CD data, the recombinant peptide contains 17% α-helix and 27% β-sheet, compatible with some structurally known defensins and scorpion potassium channel toxins (e.g. defensin A (pdb entry 1ICA), termicin (pdb entry 1MM0) and cobatoxin (pdb entry 1PJV)).

To obtain a reliable structure model with compatible secondary structure contents with the CD results, we applied comparative
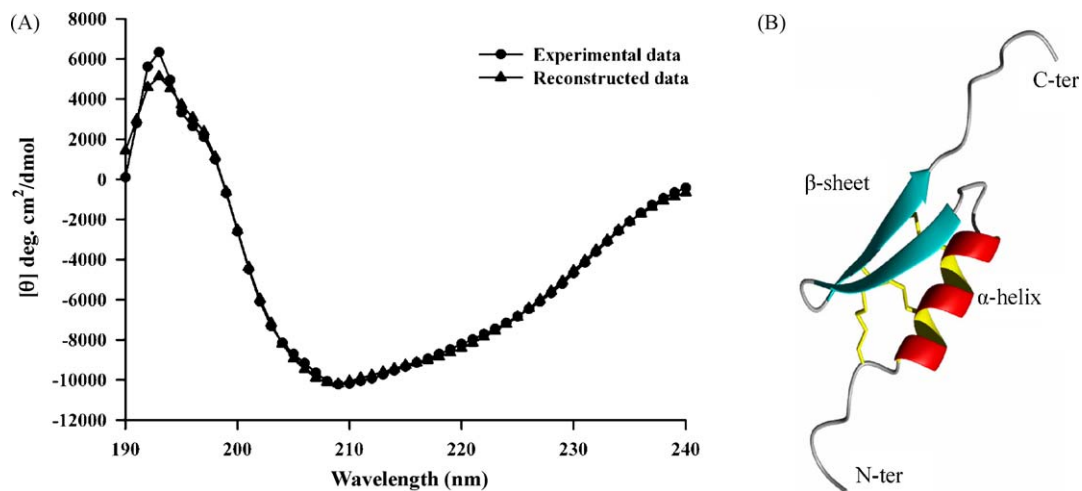
Fig. 2. Structural analysis of nasonin-3 by CD and computational prediction. (A) The CD spectrum of nasonin-3; (B) the structure of nasonin-3 predicted by comparative model. Ribbon is shown by MOLMOL, in which disulfide bridges are represented as yellow sticks. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)
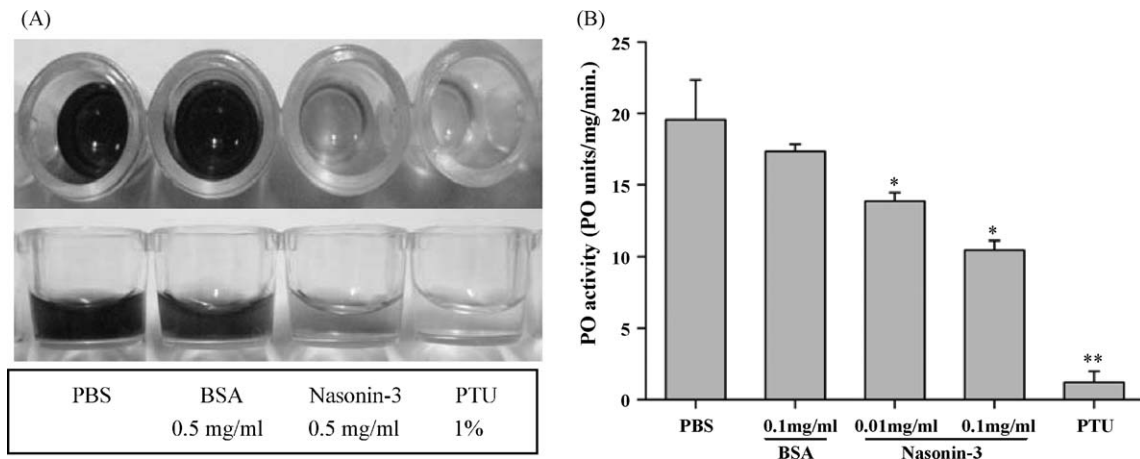
Fig. 3. Melanization of haemolymph and phenoloxidase activity. (A) Melanization of haemolymph from *B. peregrina* in vitro. (B) Phenoloxidase activity (PO units/mg protein per min). Statistical significances at 5% and 1% levels are indicated by * and **.

modeling to construct the structure of nasonin-3, in which the scorpion potassium channel toxin cobatoxin (Jouirou et al., 2004) was used as a template. The generated model has a Verify 3D value of 0.295, indicating that nasonin-3 is a typical CSαβ peptide, as identified by CD analysis (Fig. 2B).

As indicated in the parasitoid *Eulophus pennicornis* (Hymenoptera: Eulophidae), parasitism on its host *Lacanobia oleracea*

decreased the melanization of host haemolymph and reduced the PO activity (Richards and Edwards, 2000). Melanization and clotting of haemolymph neither occur in flies parasitized by *N. vitripennis* (Rivers et al., 2002), indicating the presence of proteins inhibiting such reactions (de Graaf et al., 2010), as in *Pimpla hypochondriaca* (Parkinson et al., 2002). In the melanization assay in vitro, pupal haemolymph from *B. peregrina* incubated with PBS
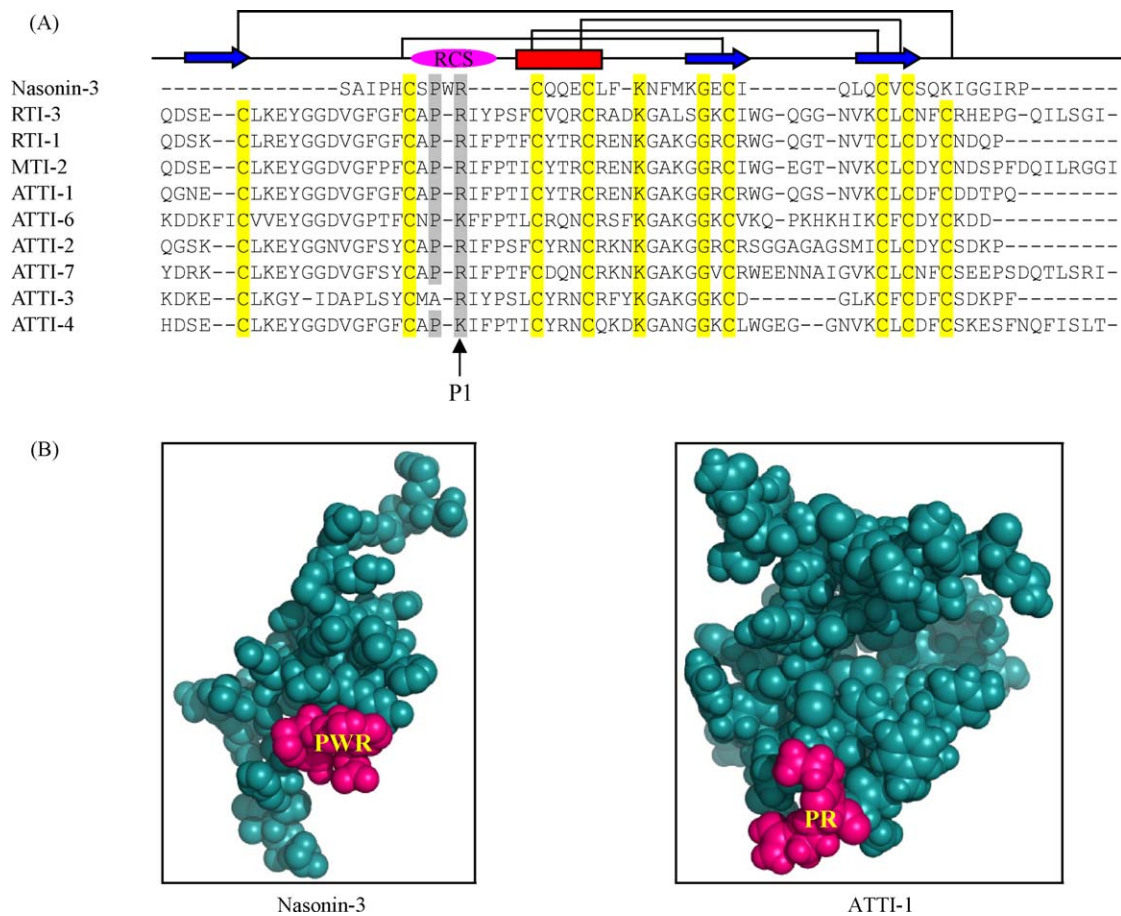
Fig. 4. Structural motif involved in protease inhibitory activity. (A) Sequence alignment of nasonin-3 with plant protease inhibitors. Identical residues are shadowed in yellow and PR motif in the reactive center site (RCS) in gray, in which P1 site is indicated by an arrow. Secondary structure elements (α-helix: cylinder; β-strand: arrow) and disulfide bridge connectivity are shown above the alignment. (B) Functional motif displayed on the structure. Structures of nasonin-3 and ATTI-1 (PDB entry: 1JXC) are shown in sphere by Pymol, on which the functional motifs (PWR for nasonin-3 and PR for ATTI-1) are displayed in pink. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

or BSA darkened progressively with time once exposed to air. This darkening was completely inhibited by PTU, a PO inhibitor. All these observations indicate that when exposed to air, the haemolymph PO cascade can be activated to generate products reacting with endogenous substrate which results in the production of melanin. In our study, haemolymph with nasonin-3 darkened more slowly and to a lesser extent than that with PBS or BSA (Fig. 3A). The reduction in melanization occurring in haemolymph could be due to lack of activation of PO and/or the absence of an appropriate substrate (Richards and Edwards, 2000). To verify these possibilities, we assayed the haemolymph PO activity in the presence of nasonin-3 and an excess of exogenous substrate L-DOPA. BSA and PTU were used as control. Results show that this recombinant peptide significantly inhibited the PO activity by 29.1% and 49.5% at 0.01 and 0.1 mg/ml concentrations (Fig. 3B).

4. Discussion

Nasonin-3 was identified as a member of the nasonin subfamily of defensin-like peptides due to its cysteine arrangement pattern and structural feature (Tian et al., 2010). Members in this subfamily have a conserved CSαβ motif shared by an array of peptides with divergent functions (Zhu et al., 2005). Nasonin-1 has been shown to be active on two Gram-negative bacteria (Tian et al., 2010). However, nasonin-3 lacks such activities against bacteria, yeasts and fungi used here. Amphiphilic architecture with a positive potential in the γ-core region of many classical insect defensins is a key antibacterial element (Gao and Zhu, 2010), which is obviously absent in nasonin-3 (data not shown) and this may account for the lack of its antimicrobial activity.

Strategies for successful parasitism by parasitic wasps involve suppression on the host immune response (Libersat et al., 2009). The inhibition of melanization may therefore represent a successful strategy for parasitism, as suggested here by nasonin-3. In fact, such strategy was also observed in several other parasitic insects, such as M. demolitor (Beck and Strand, 2007) and Cotesia rubecula (Asgari et al., 2003). Inhibitors that directly suppress the enzymatic activity of PO (called phenoloxidase inhibitor, POI) have been reported in M. domestica, M. sexta, and Anopheles gambiae (Daquinag et al., 1995; Shi et al., 2006; Lu and Jiang, 2007). In addition, a 43-kDa melanization-inhibiting protein (MIP) was found to specifically inhibit melanin synthesis from Tenebrio molitor (Zhao et al., 2005; Jiravanichpaisal et al., 2006).

Although nasonin-3 shares no similarity to the inhibitors mentioned above, database search revealed this peptide is distantly related to several plant protease inhibitors (PIs) (e.g. ATTIs, MTI-2 and RTIs) (Volpicella et al., 2009) which also adopt a CSαβ architecture (Fig. 4) in spite of the fact that there is an additional disulfide bridge to link their N-terminus to C-terminus. The reactive center site (RCS) of plant PIs is located preceding the α-helix, in which the P1 position is occupied by a positively charged residue, especially arginine (Clauss and Mitchell-Olds, 2004; Volpicella et al., 2009). It lies in an exposed loop, surrounded by amino acids which are important for the stability of the complex between P1 and protease. A proline before the P1 position was also conserved in other PIs. Mutational experiments have highlighted the importance of these two residues in MTI-2 inhibiting trypsin. Intriguingly, a similar sequence motif (PWR) was also found in the corresponding position of nasonin-3 (Fig. 4), which could explain its inhibitory effect on PO. The functional role of this motif is further strengthened by the observation that the mutation from PR to PWR changes the inhibitory activity of MTI-2 from trypsin to chymotrypsin (Ceci et al., 2003).

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2010.08.004.

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