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# Immunosuppressive properties of a protein (rVPr1) from the venom of the endoparasitic wasp, *Pimpla hypochondriaca*: Mechanism of action and potential use for improving biological control strategies

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ABSTRACT

Previously, it was determined that the presence of rVPr1 (a recombinant *Pimpla hypochondriaca* venom protein), in the haemocoel of two lepidopteran larvae, significantly increases their susceptibility to the biological control agents (BCAs), *Bacillus thuringiensis* (*Bt*) and *Beauveria bassiana* (Richards and Dani, 2010; Richards et al., 2011). The current work examines the mechanism of action of rVPr1 and demonstrates that it binds to the surface of some haemocytes and disrupts the organization of the haemocyte cytoskeleton. This binding is associated with a reduction in the ability of haemocytes to extend pseudopods, and to move and form aggregates *in vitro* over an 18 h period. Moreover, rVPr1 exerts these effects after a relatively short incubation period (1.5 h) and the haemocytes do not recover their ability to form aggregates after rVPr1 has been removed. In addition, rVPr1 significantly reduces haemocyte-mediated phagocytosis of *Bt* and *B. bassiana in vitro* ($p < 0.05$) and, following injection into the insect haemocoel, rVPr1 reduces the number of circulating haemocytes per ml of haemolymph (this being significantly different to the controls 3 h after injection [$p = 0.05$]). The finding that rVPr1 has an adverse effect on haemocyte function and number *in vivo*, supports the hypothesis that this wasp protein significantly increases the susceptibility of lepidopteran larvae to *Bt* and *B. bassiana*, by suppressing haemocyte-mediated immune responses in the insects which otherwise would be directed against these BCAs.



## 1. Introduction

At the time of oviposition, female endoparasitic wasps typically introduce venom into the haemocoel of their host insect that, in conjunction with other maternal factors, serves to condition the host in order to enhance survival of wasp offspring. Furthermore, for endoparasitoids which develop in the haemocoel of their host, a major role of venom is to suppress host immune responses directed against the developing wasp egg and/or larva (see reviews by Vinson and Iwantsch, 1980; Vinson, 1990; Strand and Pech, 1995; Beckage, 1998; Pennachio and Strand, 2006; Asgari and Rivers, 2011). In some cases, the venom acts synergistically with other maternal components such as polydnaviruses (PDV; *e.g.* Kitano, 1986; Tanaka, 1987; Wago and Tanaka, 1989). However, for endoparasitoid wasps that lack PDV (*e.g. Pimpla hypochondriaca* and *Pteromalus puparum*), venom proteins alone appear to be responsible for exerting adverse effects on host haemocytes and haemocyte-mediated immune responses (Richards and Parkinson, 2000; Parkinson et al., 2002; Cai et al., 2004; Zhang et al., 2004; Wu et al., 2008).

Typically, endoparasitoid venoms are relatively complex in nature, being composed of a variety of proteinaceous and non-proteinaceous components (Pimenta and De Lima, 2005; Moreau and Guillot, 2005; Wang and Yang, 2008; Asgari and Rivers, 2011). More recently, utilization of a variety of molecular and proteomic techniques has allowed for significant advances in our understanding of the composition of endoparasitoid venoms and the nature and function(s) of individual components (Moreau and Guillot, 2005; Asgari and Rivers, 2011). For example, at least seventeen proteins/polypeptides have been identified in *P. hypochondriaca* venom (Parkinson et al., 2002, 2004; Dani et al., 2003, 2005; Richards and Dani, 2008; Dani and Richards, 2010), twelve proteins were identified in venom from *P. puparum* (Zhu et al., 2010), whilst 29 proteins were identified in *Chelonus inanitus* venom (Vincent et al., 2010).

Without doubt, this increase in information will lead to a much better understanding of the role of venom components in the conditioning of host insects. However, in addition to this, there is increasing interest in the potential use of venom components within the agronomic and biomedical sectors. For example, small venom components with a range of biological activities (including vasoactive, antimicrobial, enzymatic activity, and others), may be utilized within the biomedical and pharmacological sectors

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(Kuhn-Nentwig, 2003; Pimenta and De Lima, 2005; Moreau and Guillot, 2005; Wang and Yang, 2008). Another area where endoparasitoid venom components may be put to practical/commercial use is in the control of agricultural and horticultural insect pests (Beckage and Gelman, 2004). This is particularly important since the majority of insecticides in use today are broad-spectrum neurotoxins and there are increasing concerns about their deleterious effects on the environment, on non-target organisms, and on the emergence of resistant pest insects (Wilson and Tisdall, 2001; Oerke and Dehne, 2004; ffrench-Constant et al., 2004). In view of this and the imminent withdrawal of certain pesticides, it is accepted that alternatives to these neurotoxins are urgently needed. Biological control agents (BCAs; including certain bacteria and fungi), offer environmentally friendly and sustainable alternatives to chemical pesticides; however, producers and end users agree that their efficacy needs to be considerably increased. In some cases, this may be achieved by utilizing immunosuppressive proteins from endoparasitoid venoms to inhibit the immune responses that a pest insect would mount against a BCA (*i.e.* to tip the outcome of the struggle for survival between the pest insect and the BCA in favour of the latter).

In support of this, *P. hypochondriaca* venom has been determined to increase the susceptibility of *Lacanobia oleracea* larvae to *Escherichia coli* and *B. bassiana* (Dani et al., 2004). More recently, two *P. hypochondriaca* venom proteins (VPr3 and VPr1), with anti-haemocyte and immunosuppressive properties were identified (Richards and Dani, 2008; Dani and Richards, 2009, 2010). Also, rVPr1 was determined to increase the susceptibility of two pest Lepidoptera to the bacterial and fungal BCAs, *Bt* and *B. bassiana*, respectively, and it was hypothesized that rVPr1 does this by suppressing immune responses in the pest insects (Richards and Dani, 2010; Richards et al., 2011). In view of these results, the main aim of the current work was to test this hypothesis by gaining information on the functions and mechanism of action of rVPr1. The results are discussed within the context of utilizing rVPr1 to increase the efficacy of BCAs.

## 2. Materials and methods

### 2.1. Chemicals, solutions and preparation of plastics and glassware

Unless otherwise stated, all chemicals were obtained from Sigma Aldrich (UK). All procedures involving haemocytes were performed inside a laminar flow cabinet using sterile conditions. Solutions were prepared using sterile, pyrogen-free water (Baxters, UK) and solutions were made iso-osmotic to insect plasma.

### 2.2. Insects

*L. oleracea* and *Mamestra brassicae* larvae were taken from laboratory populations reared (at Fera) in a constant environment room under standard conditions of 22 °C, 70% relative humidity, and a light: dark cycle of 16 h: 8 h. Larvae were fed on an artificial diet (Bioserve, USA).

### 2.3. Preparation of rVPr1

The gene for rVPr1 was cloned and protein expressed in *E. coli* as described previously (Dani and Richards, 2010). Briefly, *vpr*1 was directionally cloned and expressed with an N-terminal His-tag using a Champion™ pET Directional TOPO expression kit (Invitrogen). Recombinant VPr1 was purified using a MagneHis Protein Purification System (Promega), the protein being eluted in Dulbecco's phosphate buffered saline (DPBS; Sigma–Aldrich, D8602) containing 100 mM L-histidine, pH 7.7. Routinely, the purity of rVPr1 was assessed by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using a 12.5% resolving gel and Coomassie blue staining. Gels were calibrated using PageRuler™ Prestained Protein Ladder Plus (Fermentas Life Sciences). In addition, a haemocyte anti-aggregation assay (see below) was used to estimate the bioactivity of each batch of rVPr1 purified; the elution buffer (EB) used to purify rVPr1 served as a control (this has no adverse effect on haemocyte aggregation *in vitro*; Dani and Richards, 2010). For assays performed in the current work (unless stated otherwise), batches of rVPr1 with an average protein concentration of 160 ng/μl, and a haemocyte anti-aggregation end point (see below) of 1:1024 were utilised.

### 2.4. In vitro haemocyte anti-aggregation assay

The ability of different batches of rVPr1 to damage insect haemocytes and inhibit aggregation was determined using a standard *in vitro* haemocyte anti-aggregation assay utilising haemocytes from *L. oleracea* larvae (Richards and Dani, 2008, 2010). Basically, 50 μl of rVPr1 were serially titrated in 50 μl of TC-100 (containing 20 μM phenylthiocarbamide, 0.004% (w/v) methylparaben and 100 μg/ml ampicillin; dilutions performed in 96-well plates with flat-bottomed wells [Costar, Fisher Scientific, UK]). The controls consisted of 50 μl of TC-100, or 50 μl of EB titrated in place of rVPr1, or 50 μl of heat-treated rVPr1 (60 °C for 20 min). Fifty μl of TC-100 containing $1.5 \times 10^5$ haemocytes were then added to each well and the plates incubated in a moist chamber at RT for 18 h. Haemocyte monolayers were fixed and stained in 0.125% (w/v) Coomassie blue G250 (Bio-Rad) in 10% (v/v) acetic acid, 40% (v/v) methanol. Following destaining (in 10% (v/v) acetic acid, 40% (v/v) methanol), each monolayer was covered in PBS, then 6 separate fields of view were examined using an inverted microscope (Leitz Labovert FS) and the lowest concentration of rVPr1 that inhibited haemocyte aggregate formation determined. Note, a haemocyte aggregate is defined as a closely associated group of approximately ten or more haemocytes making contact with each other, whilst the sample end point is defined as the dilution of sample in the well before the one that most resembles the control (Richards and Dani, 2008). The effect on haemocyte aggregation of incubating rVPr1 with haemocytes for shorter time periods (1.5 h and 3 h, as opposed to 18 h) was also determined. For these assays, monolayers were prepared as described above except that rVPr1 was removed after 1.5 h or 3 h. Haemocytes were then washed five times in TC-100, overlaid with fresh TC-100 and incubated for a further 18.5 h or 17 h, as appropriate. Finally, monolayers were stained, destained and assessed as described above.

### 2.5. Assessment of enzyme activity

To determine if rVPr1 possesses enzyme activity three assays were utilised (i) a phenoloxidase assay to determine if rVPr1 possesses phenoloxidase activity, (ii) a zone of clearance assay (to detect protease activity), and (iii) a colourimetric API ZYM assay (to detect a variety of hydrolases.(i) To determine if rVPr1 possesses phenoloxidase activity, a standard assay was utilised (Richards and Edwards, 2000a). Basically, 10 μl of rVPr1, EB, or DPBS were diluted in 10 μl of sodium cacodylate buffer (NaCac/$Ca^{2+}$; 0.01 M sodium cacodylate, 10 mM $CaCl_2$, NaCl to 309 mmol/kg, pH 6.9), and then added to 20 μl of PO activator (0.3 mg/ml trypsin in NaCac/$Ca^{2+}$). Additional controls consisted of reaction mixes with buffer containing PTC at a final concentration of 50 μM. Blanks consisted of 20 μl of NaCac pH 7.0 (instead of the test solution) added to 20 μl NaCac/$Ca^{2+}$ with or without 0.3 mg/ml trypsin and PTC (50 mM final concentration) as appropriate. After 10 min at RT, 60 μl of 4 mg/ml L-DOPA (in NaCac/$Ca^{2+}$) were added and absorbance readings were taken at 490 nm every 5 min for the first