The effects of immune challenge on phenoloxidase activity in locust salivary glands in vitro

BioscienceHorizons Volume 1 • Number 2 • June 2008 10.1093/biohorizons/hzn015

Research article

The effects of immune challenge on phenoloxidase activity in locust salivary glands *in vitro*

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Homogenates of salivary glands from *Locusta migratoria* possess phenoloxidase (PO) activity. This study investigates the activation of prophenoloxidase (PPO) in these glands *in vitro*. When freshly dissected salivary glands from *L. migratoria* are incubated with the immunogen laminarin, and then homogenized, a ~4-fold increase in PO activity (expressed as a percentage of the total PO) can be measured within 20 min. Addition of laminarin to the incubation medium is best made prior to addition of salivary gland tissue, because when laminarin is added 10 min after the addition of tissue, the response to laminarin is reduced by ~50%. When salivary glands are incubated in Ca^{2+}-free Ringer, the response to laminarin cannot be demonstrated. Addition of a calcium ionophore to the incubation in normal Ringer does not initiate a response on its own, but does augment the response to laminarin. Addition of phorbol ester to an incubation containing normal Ringer has no effect on PO activity, and does not augment the response to laminarin. In contrast, addition of okadaic acid to normal Ringer has no effect on its own, but does augment the response to laminarin. Activation of PPO in response to laminarin is therefore calcium-dependant, possibly involving an influx of extracellular Ca^{2+}, and modulated by protein phosphatases. Future work should aim to clarify the function of salivary glands in the immune defence of the locust and to investigate the exact source of the PO associated with the glands.

Key words: *Locusta migratoria*, phenoloxidase, salivary glands, laminarin.

Introduction

Insects possess a highly efficient immune system.[1] An understanding of this system of immune defence is important not only in terms of potential applications to the biocontrol of pest insects, but also because insects are useful models for investigating immune systems. Therefore insect immunity is increasingly well-studied, especially in *Drosophila*, which has become a particularly valuable model for studying innate immunity over the past 10 years due to the power of molecular genetics.[2] Locusts are also commonly used as a model in studies of insect immunity due to their prevalence as a pest in parts of the world and their relatively large size.

Invertebrates do not show adaptive immunity and do not produce antibodies in response to infection. Instead, they rely on a system of innate immunity to protect them from pathogens.[1] The insect immune system consists of rapid, non-specific responses to infection[3] and can be said to comprise three types of defence: physical, cellular and humoral.[1]

The cuticle is an insect's first line of defence against invading microorganisms and it presents an impenetrable physical barrier to many potential pathogens.[4, 5] However, if a pathogen is able to overcome cuticular defences, or cross the gut wall, coordinated responses of immune cells in the haemolymph are initiated in response to pathogen detection.[1] This involves detection by pattern recognition proteins (PRPs) present both in the haemolymph and on the surfaces of haemocytes in the haemolymph. Haemocytes are cells involved in haemolymph clotting and defence. They are able to bind pathogens, initiating signalling pathways leading to the encapsulation or phagocytosis of the foreign body by the haemocyte.[1] Haemocytes can respond to infection in a coordinated manner, trapping foreign

bodies within aggregates of many haemocytes called nodules.[6] Nodules are often visible to the naked eye as blackened spots along the internal body wall of an infected insect that has undergone dissection.

Insects also synthesize a variety of extracellular humoral compounds in response to immune challenge. Some of the various signal transduction pathways initiated as a result of pathogen detection culminate in an alteration of gene expression in specialist cells, primarily in the fat body, causing these cells to produce and secrete infection-fighting substances into the haemolymph.[1] For example, lysozymes are synthesized in the fat body of lepidopterans in response to bacterial injection; broad-spectrum antibacterial peptides (cecropins) are synthesized in the fat body, cuticle and endothelium of lepidopterans and dipterans in response to bacterial challenge; and the antifungal drosomycin from *Drosophila melanogaster* also has antibacterial activity.[1] These examples represent a small fraction of the many antibacterial and antifungal compounds that have been identified and sequenced from many different insect species.

One of the most important and ubiquitous compounds released by insects in response to immune challenge, and of particular relevance to this study, is the enzyme phenoloxidase (PO). PO is activated in the cuticle or the haemolymph of many invertebrates in response to immune challenge or wounding and is activated via the prophenoloxidase (PPO) cascade, PPO being the inactive zymogen of PO. PPO is present in the haemolymph of *Locusta migratoria*[7] and is activated in response to immune challenge.[8]

Detection of a pathogen or immunogen by a PRP leads to a Ca^{2+}-dependant cascade of serine proteases, the final component of which, the PPO activating enzyme, is thought to be a clip domain serine protease that cleaves PPO to PO.[9] PO, an oxidoreductase, catalyses the oxidation of phenols present in the haemolymph to cytotoxic quinones.[9] These quinones polymerize non-enzymatically to melanin. Both quinones and melanin are toxic to microorganisms. The deposition of melanin causes parasites to become blackened in the haemolymph, and in nodules due to melanization of haemocytes encapsulating foreign bodies.[10]

Although the cuticle is an effective barrier to most pathogens, many isolates of entomopathogenic fungi are able to penetrate the insect cuticle.[11] As a result, over the past two decades there has been considerable interest in these fungi as biocontrol agents. After a short delay, topically applied conidia of *Metarhizium*, for example, send out apressoria that can penetrate the cuticle. To circumvent the delay and produce synchronized infection, Mullen and Goldsworthy[12] studied PO activity in haemolymph of *L. migratoria* after injection of *Metarhizium* blastospores. During the course of these experiments, Mullen and Goldsworthy[12] observed that salivary glands of 5th instar *L. migratoria* nymphs melanized in response to injection of blastospores or high doses of laminarin (a β1–3 glucan that is representative of polysaccharides found in fungal cell walls) into the haemolymph: the acini of the salivary glands in dissected locusts became intensely black. It was thought that this phenomenon did not occur in adult locusts, however, it has been shown subsequently that the salivary glands of adult locusts, while showing a less pronounced melanization response to injected laminarin, do exhibit a strong response in terms of increased PO activity in the glands (G.J. Goldsworthy, personal communication).

This previously unreported phenomenon raises a number of questions. For example, what are the characteristics of the PO response in locust salivary glands? What are the signalling pathways mediating the activation of PPO in locust salivary glands, and how is the immunogen being detected by the salivary glands? This study of PO activity in salivary glands of adult locusts addresses these questions.

Materials and methods

Insects

L. migratoria migratorioides were reared under crowded conditions at 30°C in a light:dark 12:12 h photocycle, and fed daily with fresh grass and wheat seedlings supplemented with bran. Adult male locusts between 15 and 25 days after the final moult were used in all experiments.

Materials

Locust Ringer (168 mM NaCl, 6.4 mM KCl, 3.6 mM $MgCl_2$, 6 mM $NaH_2PO_42H_2O$, 2.1 mM $NaHCO_3$, 20 mM Hepes, 2.1 mM $CaCl_2$, 4% Sucrose, pH 7.0) was prepared, autoclaved and stored at 5°C until used as incubation medium. A Ca^{2+}-free Ringer was used in some experiments (no $CaCl_2$, 1 mM EGTA added).

All chemicals were purchased from Sigma Chemical Company. Laminarin was dissolved in locust Ringer to give a 10 mg mL^{-1} solution. A protease inhibitor, phenylmethanesulphonylfluoride (PMSF), was dissolved in propanol to give a 0.5% solution. Immediately prior to use, dopamine was dissolved in 0.01 M phosphate buffer (pH 5.9) to give a 3 mg mL^{-1} solution. Absolute methanol was used to activate PPO. Calcimycin, a calcium ionophore (A23187) was dissolved in DMSO to give a 10 μM stock solution. Phorbol 12-myristate 13-acetate (PMA) and okadaic acid were each dissolved in DMSO to give separate 100 nM stock solutions.

Dissection

The head of the insect was removed using sharp scissors and a mid-dorsal cut made along the full length of the body. The body was pinned 'dry' onto a corkboard with the inner ventral surface of the body wall uppermost. The salivary glands were viewed under a binocular microscope and removed using two pairs of fine forceps. Each insect

usually yielded sufficient salivary gland tissue for two incubations.

Measurement of PO activity

Salivary gland tissue was incubated in 1 ml plastic centrifuge tubes containing 150 μl of locust Ringer (and other incubation constituents as appropriate) at room temperature for 60 min unless otherwise stated. The room temperature did not vary appreciably between different experiments, but control incubations were always included to allow for possible differences due to any variations in room temperature. Incubations were stopped by the addition of 1 μl of PMSF to prevent further serine protease activity. After homogenization by sonication (Cole-Parmer for 10 s) and centrifugation (13 000 rpm (10 000*g*) for 2 min), two 50 μl samples of supernatant were taken from each tube and pipetted into separate wells of a microtitre plate. Total PPO was activated in one of the wells by adding 50 μl of methanol, mixing and discarding 50 μl of the mixture to leave 50 μl in the well. After addition of 150 μl of dopamine solution to both wells, PO activity was assessed by determining the initial linear increase in absorbance at 492 nm. Absorbance values were read every minute for 15 min in a Labsystems Multiskan Bichromatic plate reader. Appropriate allowance was made for the fact that the total PO activity related to half the amount of salivary gland homogenate than was present in the sample not reacted with methanol.

Data analysis

Data were initially evaluated using Microsoft Excel to calculate the slope of a line of best fit for the absorbance measurements taken from each microtitre-plate well between 0 and 15 min. The PO activity in each well that had not been activated with methanol was expressed as a percentage of the PO activity in its corresponding well in which total PPO had been activated with methanol. Data were analysed statistically using a one-way ANOVA followed by Fisher's one-way multiple comparison tests. All statistical tests were undertaken using Minitab.

Results

PO activity in response to addition of laminarin and the effect of time of incubation

When laminarin was present in the incubation medium at the start of the incubations, PO activity in the homogenates of the glands did not increase significantly within the first 10 min of incubation, but subsequently it increased to reach a maximal level by about 20 min that persisted for up to 60 min (Fig. 1). Delaying the addition of laminarin to the incubations of salivary glands by 10 min reduced PO activity by ~ 50% in comparison with the levels when laminarin was added before the glands. In comparison with

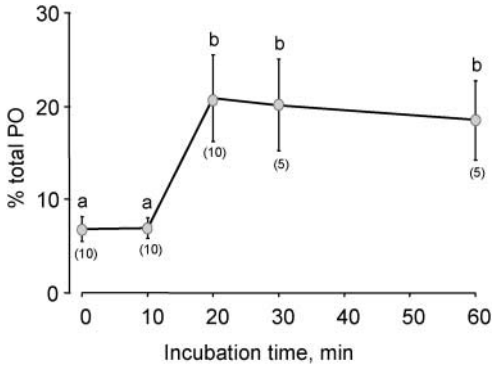

Figure 1. Percentage of total PO activated in salivary gland tissue incubated with 4 μl laminarin (added to the incubation prior to tissue) for various time periods. A control group of salivary glands was incubated for 60 min in a mixture to which 1 μl PMSF had been added before tissue (0 min incubation time). Data points and vertical lines represent mean % total PO ± SE, respectively. Data points with different letters are significantly different from each other taking the level of significance as $P \leq 0.05$. Numbers in parentheses refer to the number of observations per group.

incubations that did not have any addition of laminarin, there was no statistically significance increase in PO activity when laminarin was added to the incubation 10 min after the tissue (Fig. 2).

Calcium dependency

When salivary gland tissue samples were incubated with laminarin in Ca^{2+}-free Ringer, PO activity was reduced by ~ 80% in comparison with incubations in normal Ringer and was similar to that in either Ca^{2+}-free or standard Ringer in the absence of laminarin (Fig. 3A).

Calcium ionophore

When salivary glands were incubated in normal Ringer with calcimycin only (no laminarin) PO activity did not

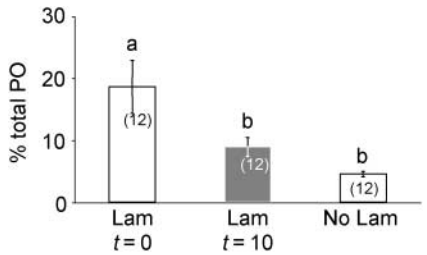

Figure 2. Percentage of total PO activated in salivary gland tissue incubated with 4 μl laminarin which had been added to the incubation before tissue (open bar, time = 0) and 10 min after tissue (shaded bar). A control group of salivary glands was incubated without laminarin (open bar, No Lam). Bars and vertical lines represent mean % total PO ± SE, respectively. Bars with different letters are significantly different from each other taking the level of significance as $P \leq 0.05$. Numbers in parentheses refer to the number of observations per group.

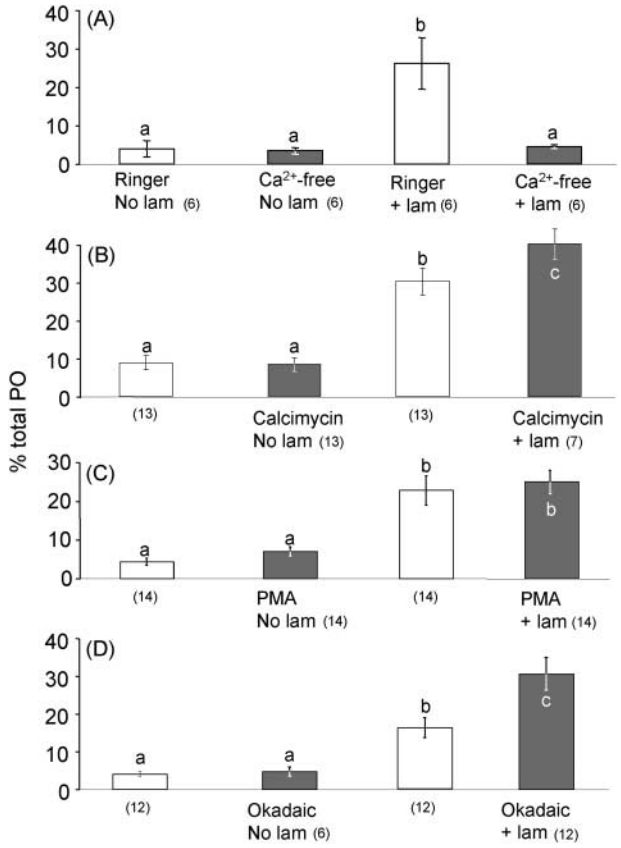

Figure 3. Percentage of total PO activated in salivary gland tissue in various incubation mixtures. Control groups of salivary glands (open bars) were incubated with 4 μl laminarin added to the incubation prior to tissue (the first bar on the left of each panel), and without laminarin (the third bar from the left of each panel). Bars and vertical lines represent mean % total PO ± SE, respectively. Bars with different letters are significantly different from each other taking the level of significance as $P \leq 0.05$. Numbers in parentheses refer to the number of observations per group. (**A**) Salivary glands incubated in calcium-free ringer with and without laminarin. (**B**) Salivary glands incubated in normal Ringer with and without laminarin, with 3 μl of calcimycin added to the incubation before salivary gland tissue. (**C**) Salivary glands incubated in normal Ringer with and without laminarin, with 3 μl of PMA added to the incubation before salivary gland tissue. (**D**) Salivary glands incubated in normal Ringer with and without laminarin, with 15 μl of okadaic acid added to the incubation before salivary gland tissue.

change significantly. When salivary glands were incubated with calcimycin and laminarin there was a statistically significant greater increase in PO activity in comparison with incubations of laminarin alone (Fig. 3B).

Phorbol ester

When salivary glands were incubated with PMA only (no laminarin), there was no statistically significant increase in PO. However, when salivary gland tissue was incubated with PMA and laminarin together, although the PO activity increased ~4-fold in comparison with incubations in the absence of laminarin, there was no indication that the PMA had enhanced the increase in PO activity due to the laminarin (Fig. 3C).

Okadaic acid

Addition of okadaic acid on its own did not change the PO activity significantly. When salivary glands were incubated with okadaic acid and laminarin together, PO activity increased ~6-fold in comparison with incubations in which laminarin was absent, which represented a statistically significant ~2-fold enhancement of the increase in PO activity in comparison with incubations with laminarin on its own (Fig. 3D).

Discussion

Locust salivary glands comprise secretory acini bundled together in a grape-like arrangement.[13] Associated with each acinus of *L. migratoria* there are three or four nephrocytes.[14] These are pericardial cells and, like haemocytes, originate from the mesoderm, but unlike haemocytes are static cells associated with tissues.[15] They are involved in clearance of substances from the haemolymph and are able to take up material by endocytosis and release it by exocytosis.[16] In *L. migratoria*, nephrocytes are involved in immunogen uptake leading to haemocyte recruitment and nodule formation.[12, 17]

It seems likely that salivary gland melanization in locusts *in vivo* as described by Mullen and Goldsworthy[12] is a two-phase process, whereby nephrocytes recognize foreign material and then themselves become a focus for 'attack' by haemocytes. This leads to a very focused formation of melanized nodules associated with the acini of the salivary glands. The present study concerns only the first phase of this response because dissected salivary gland tissue incubated *in vitro* will contain few, if any, of the haemocytes that circulate normally in the haemolymph. Therefore it is doubtful that the PO activity observed in salivary glands *in vitro* originates from haemocytes, especially because an increase in PO activity has been observed in locust salivary gland tissue that has been rinsed in Ringer before being incubated with laminarin *in vitro* (G.J. Goldsworthy, personal communication). Therefore it is thought that the nephrocytes associated with the acini are the source of the PO activity measured in this study. Since haemocytes and nephrocytes share the same embryological origin, this hypothesis seems logical, but remains to be demonstrated.

Effect of incubation time

The laminarin-induced increase in PO activity in salivary gland tissue *in vitro* occurs within 20 min. In terms of the speed of the response, this is consistent with findings by Goldsworthy *et al.*[8] who measured changes in PO activity *in vivo* in samples of haemolymph taken from adult locusts

at various times after injection with laminarin, but the response in the haemolymph is more prolonged. In the present study, incubations need only have been left for 20 min, but were in fact allowed to run for 60 min because this allowed time for completion of all dissections before the addition of protease inhibitor. Note that PO activity increases from minimal to maximal levels between 10 and 20 min, suggesting that signalling activity leading to PPO activation takes place during the first 10 min after tissue comes into contact with immunogen. In terms of magnitude of response, the percentage activation of the total PPO in the salivary glands is greater (at around 20%) than that observed in the haemolymph (around 10%) under the most favourable conditions by Goldsworthy *et al.*[8]

Interestingly, the PO response to laminarin in salivary glands is lost when the addition of immunogen to the incubation mixture is delayed for 10 min after the addition of the salivary gland tissue. The reason for this is unclear, but it could be that the cells responsible for PPO activation become refractory soon after being extracted from the animal and therefore need to come into contact with immunogen directly after dissection for a response to take place. This loss of sensitivity to laminarin *in vitro* may explain partly why the response to laminarin increases for only 20 min in the salivary gland *in vitro* (this study), and for up to 60 min in the haemolymph *in vivo*.[8]

Calcium dependency

When salivary glands are incubated in Ca^{2+}-free Ringer (with EGTA), the increase in PO activity in response to laminarin is lost. The loss in PO activation by laminarin in the absence of calcium is not caused by toxicity of the EGTA because salivary glands incubated in a Ca^{2+}-free Ringer without EGTA do not show an increase in PO activity in response to laminarin. This suggests strongly that activation of the PPO cascade in locust salivary glands is calcium-dependent. This is not surprising because PPO in insect haemolymph and cuticle is activated by a calcium-dependant serine protease cascade.[7] Further, the addition of PMSF (a non-competitive inhibitor of serine proteases) to the incubation prior to salivary gland tissue prevents any increase in PO activity in response to laminarin. Therefore, at least in this respect the evidence suggests that PPO in the salivary glands is activated by a mechanism similar to that operating elsewhere in the animal.

A rise in intracellular calcium concentration is a ubiquitous signal in biological systems and can be achieved either by the release of calcium from intracellular stores, or the influx of extracellular calcium due to the opening of Ca^{2+}-specific ion channels in the plasma membrane. Calcimycin is a carrier of calcium ions, allowing calcium to cross the cell membrane. Therefore if the action of laminarin is only to trigger the opening of plasma membrane Ca^{2+} channels, in theory, laminarin could be replaced in the incubation with calcimycin with the same effect on PO activity. However, calcimycin alone does not bring about PPO activation in the salivary glands but, interestingly, there is significant augmentation of the rise of PO activity in response to laminarin when salivary glands are incubated with calcimycin and laminarin together. The reasons for this are unclear, but the results suggest that an influx of extracellular calcium is involved in the PO response, but that other cellular events triggered by laminarin need to take place for PPO to become activated.

Other second messengers

The possible role of cAMP as a second messenger in PPO activation in the salivary glands was not examined in this study because the cAMP activator forskolin is ineffective when added to incubations of salivary glands *in vitro* (G.J. Goldsworthy, personal communication). However, the results of this study suggest that protein kinase C (PKC) is also not involved in the activation cascade for PPO induced by laminarin. Phorbol esters such as PMA activate PKC, and many (although not all) kinases in this family are calcium-dependent and it could therefore have been possible that the calcium-dependency of the PO response is due to the presence of calcium-dependent kinases in the PPO activation cascade. However, addition of PMA, either in the presence or absence of laminarin, does not influence PO activity in the salivary glands *in vitro*.

Okadaic acid is an inhibitor of phosphatases that remove phosphate groups from proteins, often returning proteins to an 'inactive' state. Their presence in the PPO cascade would modulate PPO activation. Thus, if the laminarin-sensitive pathway for PPO activation is constitutively active, incubating salivary glands with a phosphatase inhibitor could mimic the PPO activating effect of laminarin. Incubation with okadaic acid alone showed that this is not the case, but the response to laminarin is augmented markedly when salivary glands are incubated with okadaic acid and laminarin together, suggesting that the PO response to laminarin in the salivary glands is modulated by protein phosphatases. However, other cellular events triggered by laminarin are required to initiate PPO activation. The final concentration of okadaic acid in the incubation in these studies was 10 nM. According to Schönthal,[18] a minimum okadaic acid concentration of 20 nM is required for 50% inhibition of PP1, and a maximum 1 nM concentration for 50% inhibition of PP2A. It may therefore be informative to test different concentrations of okadaic acid in incubations of salivary glands.

Physiological role of PO activity associated with insect salivary glands

The present study is not the first in which PO activity has been detected in the salivary glands of insects. Miles[19] stained the salivary glands of aphids with dopamine and provided evidence of the presence of PO. More recently, Hattori

et al.[20] used SDS–PAGE to reveal the presence of two types of PO (a laccase and a PO) in salivary gland homogenates from the green rice leafhopper. This raises a question: what is the physiological role of PO activity in insect salivary glands? One hypothesis is that PO is secreted into the saliva with the purpose of attacking pathogens or oxidizing toxins in the mouth or gut. For example, Hattori *et al.*[20] suggested that a possible function of the leafhopper salivary laccase may be to rapidly oxidize toxins while feeding on plants. It seems possible that PO may be secreted in locust saliva for the purpose of oxidizing toxins in the food, or it may even be transferred to the cuticle via grooming behaviour where it could have a protective effect against infection. However, there is as yet no evidence that the PO detected in locust salivary glands *in vitro* is actually secreted into the saliva *in vivo*, although the presence of PO in the saliva has been demonstrated in other insects. For example, an immunohistochemical study on the salivary proteins of aphids by Cherqui and Tjallingii[21] confirmed the presence of PO in the saliva. Hattori *et al.*[20] analysed leafhopper saliva that had been deposited on the food while feeding and showed that the laccase found in the salivary gland homogenates of the leafhoppers is secreted in the watery saliva.

Conclusions

This study has shown that adult locust salivary glands possess PPO activity and that this can be activated in response to laminarin *in vitro*. The response is calcium-dependent, possibly involving an influx of extracellular Ca^{2+}, and is modulated by protein phosphatases. Future research should aim to analyse locust saliva to establish whether PO is present, and if so whether the levels change during immune challenge. It is hoped that such work would help to clarify the function of salivary glands in the immune defence of the locust and to shed further light on this intriguing aspect of insect immunity.

Acknowledgements

I am grateful to Professor Graham Goldsworthy, Birkbeck College, University of London, for supervision and encouragement, and Mary Lightfoot, Birkbeck College, University of London, for general technical guidance and for providing live insects for my research.

Funding

This work was funded by the School of Biological and Chemical Sciences, Birkbeck College, University of London.

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Submitted on 26 September 2007; accepted on 17 December 2007; advance access publication 17 April 2008