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Adipokinetic hormone enhances laminarin and bacterial lipopolysaccharide-induced activation of the prophenoloxidase cascade in the African migratory locust, *Locusta migratoria*.

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Abstract

Lom-AKH-I enhances the activation *in vivo* of prophenoloxidase in the haemolymph of the African migratory locust, *Locusta migratoria*, in response to challenge with laminarin. AKH does not influence the speed or initial magnitude of the phenoloxidase response to laminarin, but prolongs the period of activation of the enzyme in a dose-dependent manner. Injections of preparations of bacterial lipopolysaccharide (LPS) do not activate prophenoloxidase *in vivo*, but co-injection of *Lom*-AKH-I with commercial preparations of LPS from *Klebsiella pneumoniae*, *Escherichia coli*, or *Shigella flexneri* (but not one from *Pseudomonas aeroginosa*) results in dose-dependent increases in the levels of phenoloxidase that persist in the haemolymph for several hours. It is argued that the effects of AKH on phenoloxidase activation in locusts described here are, at least in part, related directly to changes in lipid metabolism brought about by the hormone.

Keywords: *In vivo*; adipokinetic hormone; immune response; phenoloxidase; lipopolysaccharide; laminarin; *Locusta migratoria*

1. Introduction

Proteins present in the plasma or haemocytes of insects bind to polysaccharides of bacteria or fungi and form the basis of the recognition of 'non-self' material, thus activating the immune system. Haemocytes adhere to invading organisms or foreign particles and may either engulf them or aggregate to surround and trap them in nodules. These responses are usually accompanied by activation of a prophenoloxidase in the haemolymph. Later responses to infection include the synthesis and release into the haemolymph, mainly from the fat body, of a variety of antibacterial and/or antifungal peptides (Gillespie *et al*., 1997).

In mammals, prostaglandins (or eicosanoids) and hormones such as corticosterone regulate aspects of the immune response and, in insects, eicosanoids are an important component of the cellular response; injection of biogenic amines, ecdysteroids, and opiate peptides exert effects on insect immune responses (Gillespie *et al*., 1997). However, there are several arguments to support the idea that the regulation of lipid transport mechanisms in insects could be an additional important point of interaction between the endocrine and immune signalling systems. Lipophorins that carry lipids in the haemolymph are in many insects the major component of the clot that forms after wounding, and purified lipophorin from *Periplaneta* (Coodin and Caveney,

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1992) and *Galleria* (Mandato *et al*., 1996) inhibits haemocyte adhesion, suggesting that localised changes in the concentration of lipophorin may influence wound healing. Further, one component of the lipid transport system is an apolipophorin (ApoLp-III) which in *Galleria* has immune-stimulating activity (Wiesner *et al*., 1997; Halwani *et al*., 1999, 2000; Dettloff *et al*., 2001). Thus, because adipokinetic hormones (AKHs) regulate lipid transport in locusts (Goldsworthy, 1983; Goldsworthy *et al*., 1997; Goldsworthy and Joyce, 2001) the potential of these neurohormones to regulate immune responses triggered by polysaccharides from bacteria or fungi has been tested in locusts. Evidence is presented for the first time that AKHs enhance the activation *in vivo* of prophenoloxidase in the haemolymph of the African migratory locust, *Locusta migratoria*, in response to challenges with laminarin, a β-1,3-glucan (with some interstrand β-1,6 linkages and branch points) typical of glucans in fungal cell walls, and with bacterial lipopolysaccharide (LPS).

2. Materials and methods

2.1. Insects

Adult male *Locusta migratoria migratorioides* (R. & F.) >12 days after adult emergence were taken from laboratory colonies reared under crowded conditions at 30°C in a LD 12:12h photocycle, and fed daily with fresh grass and wheat seedlings supplemented with bran.

2.2. Injection of materials into the haemolymph of locusts and sampling of haemolymph

Injections of AKHs, and preparations of laminarin or LPS were made using specially adapted plastic pipette tips within the bore of which a short length of stainless steel needle was held by friction. Using these, small volumes (usually 20 μl) of test materials could be taken up accurately and injected into the haemocoel by inserting the needle between two abdominal terga and expelling the sample using an automatic pippettor. AKH-I and AKH-II were purchased from Novabiochem and Sigma Chemical Co. respectively, and stock solutions were made up in 80% methanol (usually *c.* 20 pmol/μl) and quantified by measuring the tryptophan fluorescence in an LS50B Fluorimeter (Ex, 280 nm; Em, 348 nm) and calibrating against a standard solution of tryptophan. Laminarin and various preparations of LPS (purified by phenol extraction with the exception of that from *Pseudomonas*, which was prepared by trichloroacetic acid extraction) were purchased from Sigma Chemical Co.

Samples of haemolymph were taken from locusts without cooling or anaesthesia, by making a small puncture in the arthrodial membrane at the base of a hind leg. A calibrated capillary tube was used to take up 5 μl of haemolymph. Haemolymph samples were taken immediately before injection and then at different times after administration of test material.

2.3. Measurement of phenoloxidase activity

Phenoloxidase activity was measured by blowing 5 μl of whole haemolymph into 95 μl of 10 mM phosphate buffer (pH 5.9). After centrifugation (10000 x g, at 4°C for 5 min), 40 μl of this haemolymph/buffer supernatant was pipetted into a well of a microtitre plate: the washed pellet contained no measurable phenoloxidase or prophenoloxidase activity (before or after injection with either LPS or laminarin) and was discarded. After addition of 160 μl of *L*-dopa (3mg/ml phosphate buffer) as substrate, phenoloxidase activity was assessed by determining the initial linear increase in absorbance at 492 nm over 30 min using a Labsystems Multiskan Bichromatic plate reader. Enzyme activity is expressed here in absorbance units (au)

Table 1. The phenoloxidase activity and protein content (Mean ± SE, *n*=10) of the haemolymph of adult male *Locusta* after injection of 15 μg of laminarin in the presence and absence of 20 pmol of *Lom*-AKH-I.

Injection treatment	**Saline**	**Laminarin**	**Laminarin + *Lom*-AKH-I**
Phenoloxidase activity at 3 h: Au_{492}/min/μl x10^3	1.54 ∀ 0.47	5.28 ∀ 1.33	17.7 ∀ 0.89
Protein in haemolymph ∀1.3 At time 0: μg/μl	26.4 ∀ 1.7	27.5 ∀ 1.8	31.1
Protein in haemolymph 1.4 At time 3 h: μg/μl	24.6 ∀ 1.8	25.0 ∀ 1.8	32.3 ∀
Phenoloxidase activity at 3 h: Au_{492}/min/μl/mg protein	0.062 ∀ 0.019	0.211 ∀ 0.042	0.546 ∀ 0.033

at 492 nm per minute per microlitre of haemolymph. Prophenoloxidase activity was determined after activation *in vitro* by treatment with methanol: 40 μl of the haemolymph/buffer supernatant was mixed with 40 μl of absolute methanol and 10 μl of the resulting solution was mixed with 190 μl of substrate in a microtitre plate well and enzyme activity recorded as described above. In some experiments, prophenoloxidase activity was determined by measuring phenoloxidase activity after incubation of the haemolymph/buffer supernatant for 30 min with an equal volume of chymotrypsin (Sigma Chemical Co.: 1 mg/ml in phosphate buffer, pH 5.9). Enzyme activity as defined here was completely inhibited (in samples of haemolymph from resting locusts and those injected with laminarin or LPS, with or without AKH-I) by the addition of kojic acid (3mM final concentration), showing that the activity meaured could be attributed to phenoloxidase.

Protein in the haemolymph was measured using a modification of the Coomassie-blue dye binding method as described by Zor and Seliger (1996). An aliquot (1 μl) of the haemolymph/buffer solution was pipetted into a microtitre plate well and mixed with 49 μl of distilled water and 200 μl of dye reagent. The ratio of the absorbancies at 590 nm and 450 nm was determined. Protein concentrations were calculated by calibration against a range of concentrations of bovine serum albumen (Fraction V; Pierce Chemical Co.) from 0 - 4 μg, in which range the relationship was linear.

Fig 1. Changes in the levels of phenoloxidase and prophenoloxidase in the haemolymph of *Locusta* after injection with 20 µl of saline (solid squares) or 20 µl of saline containing 125 µg of laminarin (open diamonds). Vertical lines represent ± SEM, *n* = 10.

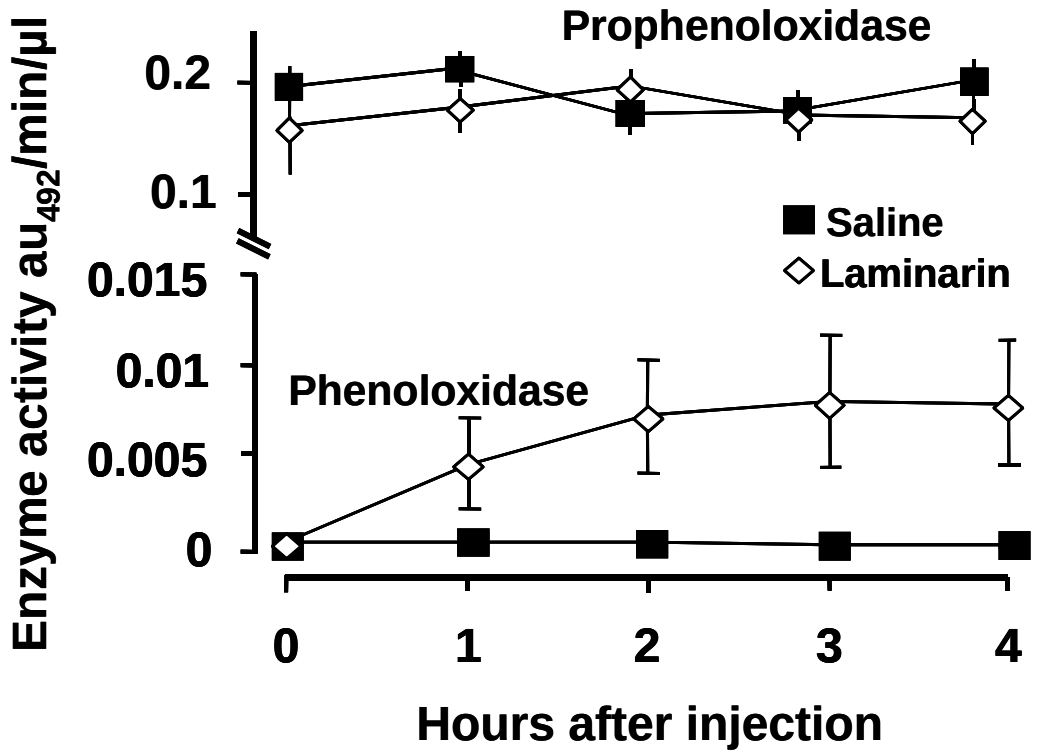
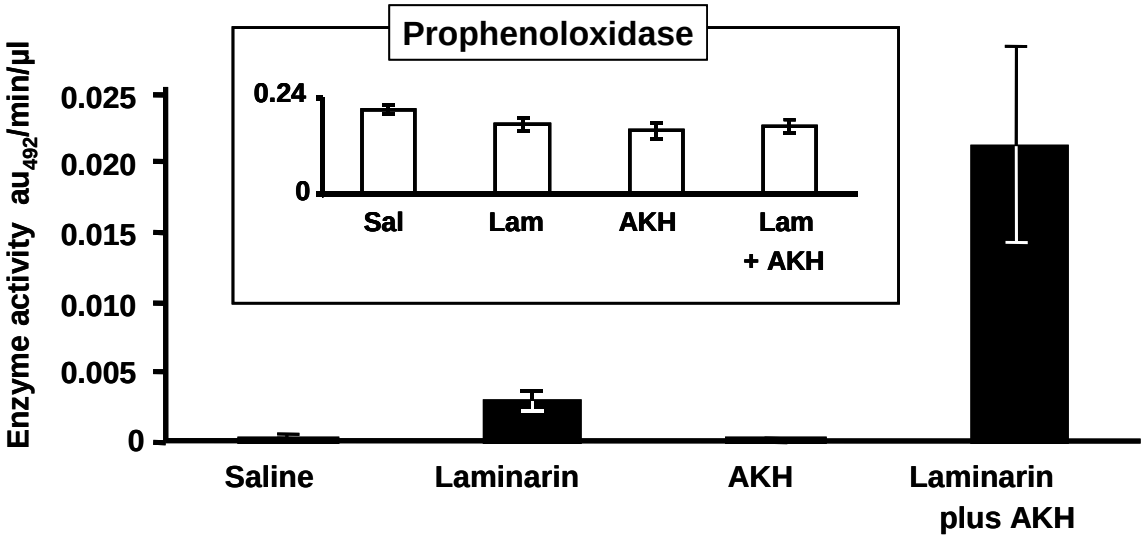


Fig. 2 The effect of co-injection of 20 pmol of *Lom*-AKH-I with 125 µg of laminarin on the phenoloxidase and prophenoloxidase activity in the haemolymph of *Locusta* at 3 h after injection. Vertical lines represent ± SEM, *n* = 10.



3. Results

Levels of phenoloxidase in resting locusts were always extremely low, but treatment of the haemolymph/buffer solution with methanol or incubation with chymotrypsin brought about a marked increase in measurable enzyme activity. For example, in one group of adult male *Locusta* the phenoloxidase activity of the haemolymph was 0.505 ∀ 0.016 x 10^{-3} au_{492}/min/μl (*n*=5). Methanol treatment of haemolymph from the same group of locusts brought about an increase in phenoloxidase activity (to 0.32 ∀ 0.04 au_{492}/min/μl, *n*=5) that was similar to that after treatment with chymotrypsin (to 0.26 ∀ 0.03 au_{492}/min/μl, *n*=5). However, methanol treatment of the haemolymph/buffer solution was more rapid and easily performed routinely than incubation with chymotrypsin, and was therefore adopted in this study as a standard method for measuring prophenoloxidase (total phenoloxidase activity after treatment with methanol *in vitro*). In the anticipation that phenoloxidase activity measured in the whole haemolymph might be related to the protein content of the samples, levels of protein were initially measured routinely alongside phenoloxidase activity. The levels of protein in the haemolymph/buffer solution did not, however, change significantly with any of the treatments studied (Table 1). Thus, expressing phenoloxidase activity in terms of the amounts of protein in the haemolymph had little impact compared with standardisation against volume of haemolymph used (Table 1) and, for simplicity, phenoloxidase activity was subsequently expressed routinely only in terms of rates of change of absorbance at 492 nm per unit volume of haemolymph. Because the levels of phenoloxidase in resting locusts at time 0 were extremely low in comparison with levels recorded after challenge with laminarin or LPS (in the presence of AKH), they could be regarded as virtually zero, and most of the data given here are only for the levels of enzyme activity at a given time after injection.

3.1. Injection of laminarin

In an initial experiment, adult male *Locusta* were injected with 125 μg of laminarin in 20 μl of saline, and the phenoloxidase and prophenoloxidase activity in

Fig. 3. The effect of the amount of laminarin injected on the phenoloxidase activity in the haemolymph of *Locusta* in the presence (solid circles) and absence of 20 pmol of *Lom*-AKH-I (open circles) 3 h after injection. Curve fitting and estimation of ED_{50} values were undertaken as Hill-plots in FigP (Biosoft). Vertical lines represent ± SEM, $n \geq 10$.

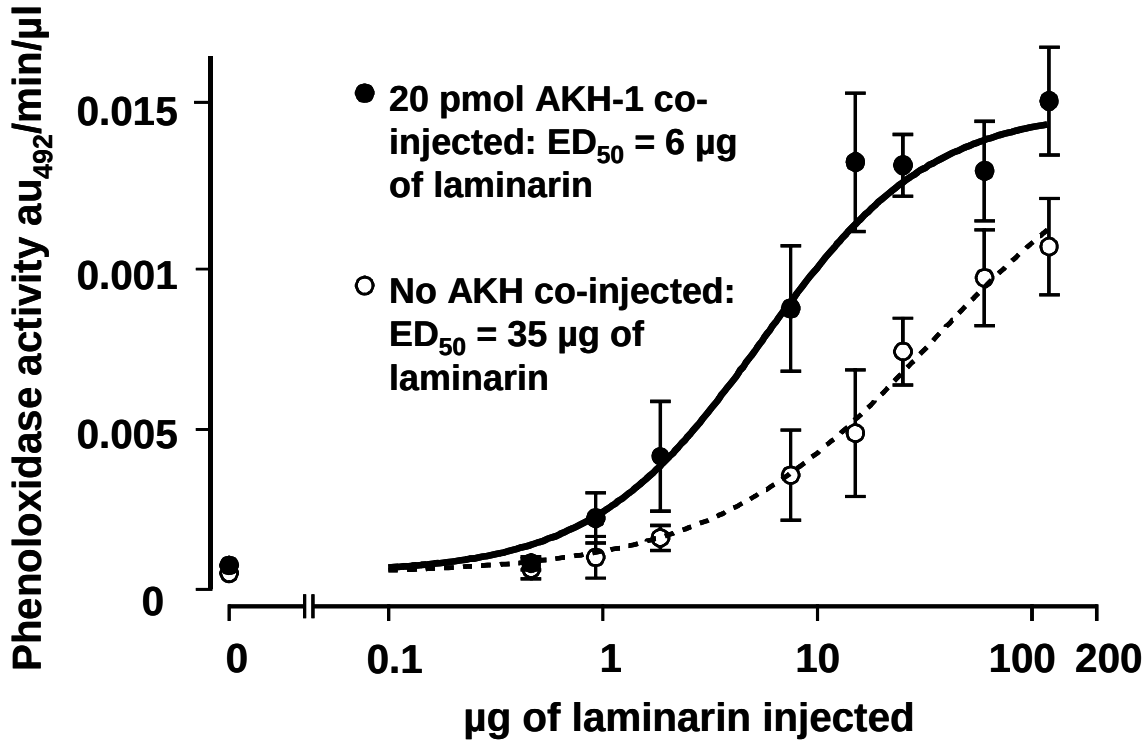
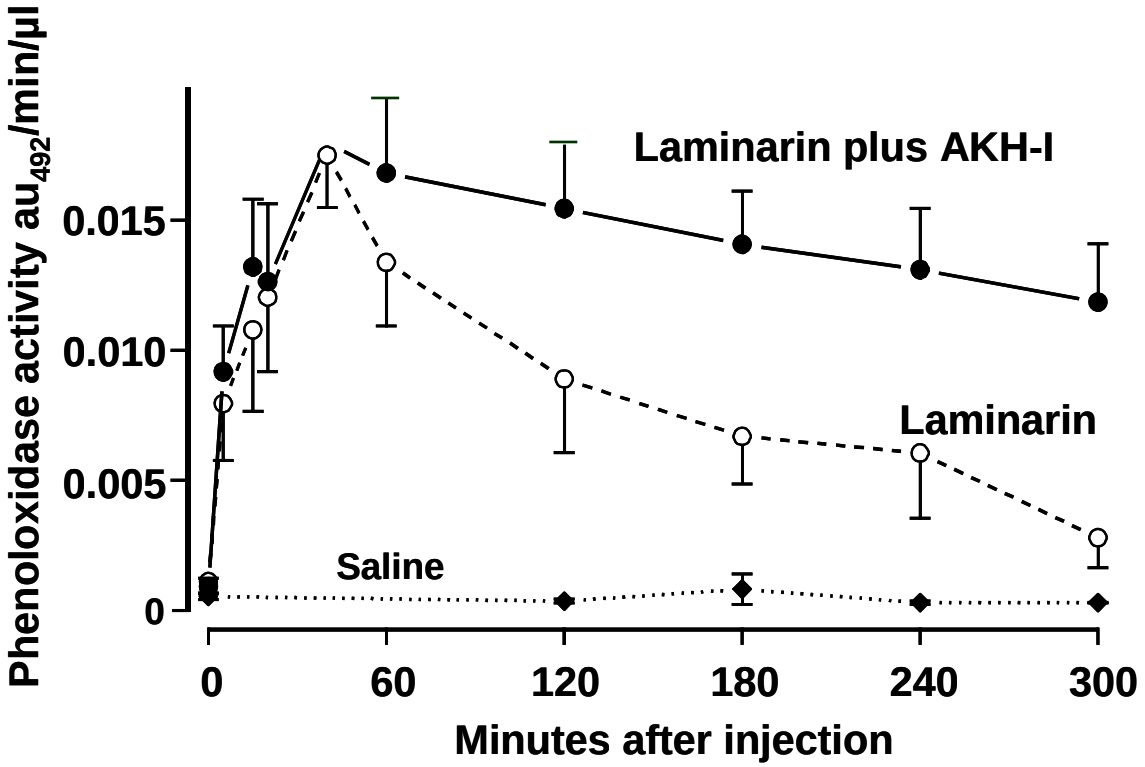


Fig. 4. Changes in the phenoloxidase activity of the haemolymph of *Locusta* with time after injection with saline (solid diamonds), saline containing 15 µg of laminarin (open circles), or saline containing 15 µg of laminarin and 20 pmol of *Lom*-AKH-I (closed circles). Vertical lines represent ± SEM, $n \geq 10$.



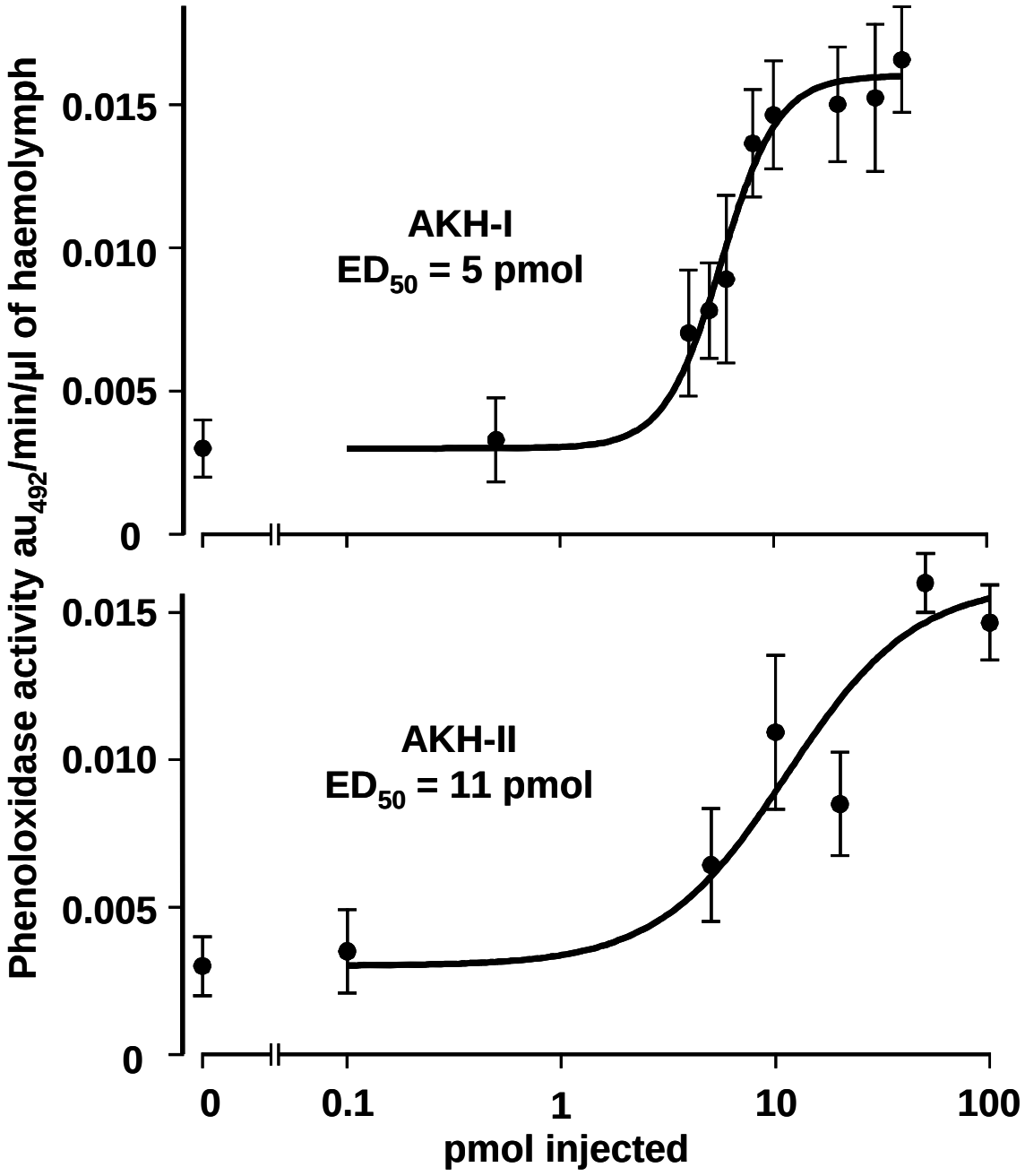
whole haemolymph was measured at 1, 2, 3 and 4 h after injection (Fig. 1). There were no significant changes in prophenoloxidase activity during this period, but the levels of phenoloxidase activity increased, reaching a maximum at 3 h. To test the possible effects of *Lom*-AKH-I, adult male *Locusta* were injected with 125 μg of laminarin in the presence and absence of 20 pmol of AKH-I, and the phenoloxidase activity in whole haemolymph measured 3 h after injection. As can be seen in Figure 2, the AKH appeared to augment the response to laminarin in that there was a significantly higher level of phenoloxidase activity in the presence of the hormone. Because the stock solutions of AKH used in this study were made in 80% methanol, when locusts were injected with 20 pmol of *Lom*-AKH-I, for example, each locust received the equivalent of 1μl of 80% methanol, so the possibility was examined that this methanol was activating the prophenoloxidase in the haemolymph. However, when the equivalent of 5 μl of 80% methanol was injected into locusts there was no increase in phenoloxidase activity in the haemolymph, nor did this amount of methanol enhance the phenoloxidase response to laminarin (data not shown). Further, injection of *Lom*-AKH-I in the absence of laminarin (or LPS) had no effect on phenoloxidase activity in the haemolymph (see later and Fig. 6). There were no significant changes in the levels of prophenoloxidase activity with or without the addition of AKH to the laminarin injection solution (Fig. 2). Although the levels of prophenoloxidase were determined in most experiments described here, only a limited exemplary set of data is presented because no significant changes were observed.

The effect of varying the amount of laminarin injected on the increase in phenoloxidase activity was determined 3 h after injection in both the presence and absence of AKH-I. As shown in Figure 3, co-injection of the hormone influenced the ED_{50} for laminarin markedly. Although the highest amount of laminarin injected (150 μg) failed to elicit as great a response as when AKH was co-injected, the ED_{50} was calculated on the assumption that higher doses would have proved equally effective as laminarin in the presence of AKH (see fig. 3). The time course for the activation of phenoloxidase was re-examined (Fig. 4) using only 15 μg of laminarin. Surprisingly, AKH had little effect on the initial increase in phenoloxidase, which reached a maximum within about 30 min of injection, but the hormone prolonged the response (Fig. 4). In an attempt to quantify in an indirect way the relationship between this prolongation effect and the amount of hormone, varying amounts of AKH-I and II were injected and the levels of phenoloxidase activity determined 3 h later. As can be seen in Figure 5, the peptides differed in their potencies in enhancing the response to laminarin, and ED_{50} values of 5 and 11 pmol were obtained for AKH-I and II respectively.

3.2. Injection of bacterial lipopolysaccharides (LPS)

A range of readily available commercial preparations of LPS from different gram-negative bacteria was tested by injection into adult male locusts as described above for laminarin: doses of LPS from 1-150 μg per locust were used: the low solubility of these preparations in aqueous media meant that, without increasing the volume injected, greater amounts could not be tested. Injected on their own, even the highest doses of these preparations of LPS failed to activate prophenoloxidase in the haemolymph significantly within 3 h ($P>0.09$, one way ANOVA) compared with

Fig. 5. Dose response relationships for the effects of *Lom*-AKH-I (upper graph) and II (lower graph) on the phenoloxidase activity in the haemolymph of *Locusta* 3 h after co-injection with 15 µg of laminarin. Curve fitting and estimation of ED_{50} values were undertaken as Hill-plots in FigP (Biosoft). Vertical lines represent ± SEM, $n \geq 10$.



injections of saline or AKH (see Fig. 6), or even up to 24 h after administration (data for times other than 3 h not shown).

Injection of 20 pmol of AKH-I together with preparations of LPS (100 μg) from *Klebsiella pneumoniae*, *Escherichia coli* (Serotype 0111:B4), or *Shigella flexneri* 1A did, however, elicit a pronounced activation of haemolymph prophenoloxidase, but a preparation of LPS from *Pseudomonas aeroginosa* (Serotype 10) remained inactive in this respect (see Figure 6).

Using a preparation of LPS derived from *E. coli*, the effects of varying the dose of LPS on the activation of phenoloxidase in the presence of AKH were determined. It can be seen from Figure 7 that an ED_{50} value of 40 μg of LPS was obtained, and that a dose of 100 μg of LPS from *E. coli* was just supramaximal. For this reason, a constant dose of 100 μg of LPS was used to test the effects of varying the dose of AKH-I on phenoloxidase activity 3 h after injection, and an ED_{50} value of 3 pmol of AKH-I obtained (Fig. 8). The time course for activation of prophenoloxidase by LPS in the presence of *Lom*-AKH-1 (Fig. 9) was broadly similar to that of the response to laminarin in the presence of AKH (*cf*. Fig.4), with a rapid increase in phenoloxidase activity that remained high for several hours.

3.3. The appearance of the haemolymph and mortality of the injected locusts

After injection of laminarin, haemolymph samples were often noticeably grey in appearance. When AKH was co-injected with laminarin or LPS, the haemolymph was commonly dark grey or even black. It was clear that these changes in appearance of the haemolymph correlated broadly with the increased levels of phenoloxidase measured. Although there appeared to be no activation of phenoloxidase after injection of LPS in the absence of AKH, even low doses of LPS caused some mortality between 24 and 48 h after injection, and doses >100 μg killed c. 90% of the locusts after an initial delay of *c*. 24 h. Generally, all locusts survived for 24 h whether injected with LPS or laminarin but, as with the injection of LPS, large doses of laminarin (>25 μg) also resulted in high levels of mortality between 24 and 48 h. Injection with AKH alone did not increase mortality, and the hormone appeared not to influence survival after treatment with either microbial substance.

4. Discussion

There appear to have been many studies on the activation of phenoloxidase in locust haemolymph *in vitro*, but few studies *in vivo*. Direct evidence for the interaction of an AKH with the immune system is presented for the first time in any insect. It is demonstrated here that co-injection of *Lom*-AKH-I with preparations of LPS from some species of gram negative bacteria can activate the phenoloxidase cascade in locusts *in vivo*.

It is clear that although methanol activates prophenoloxidase *in vitro*, it is not the residual methanol in the injected solutions of AKH that is responsible for the effects described here *in vivo*, nor do injections of AKH on its own activate the phenoloxidase cascade.

The failure of the preparation of LPS from *Pseudomonas* to activate the phenoloxidase cascade even when co-injected with AKH is intriguing. Further, LPS preparations from *Pseudomonas* but not from *E. coli*, are reported to be effective *in vitro* in activating prophenoloxidase in haemolymph from *Schistocerca* (Ratcliffe *et al*., 1991), and injections of live or heat-killed *Pseudomonas* cause the appearance of

Fig. 6. Phenoloxidase activity in the haemolymph of *Locusta* injected with 100 µg of different preparations of LPS. The vertical bars represent the enzyme activity 3 h after injection of LPS with (solid bars) or without (open bars) 20 pmol of *Lom*-AKH-I. Vertical lines represent ± SEM, $n \geq 10$.

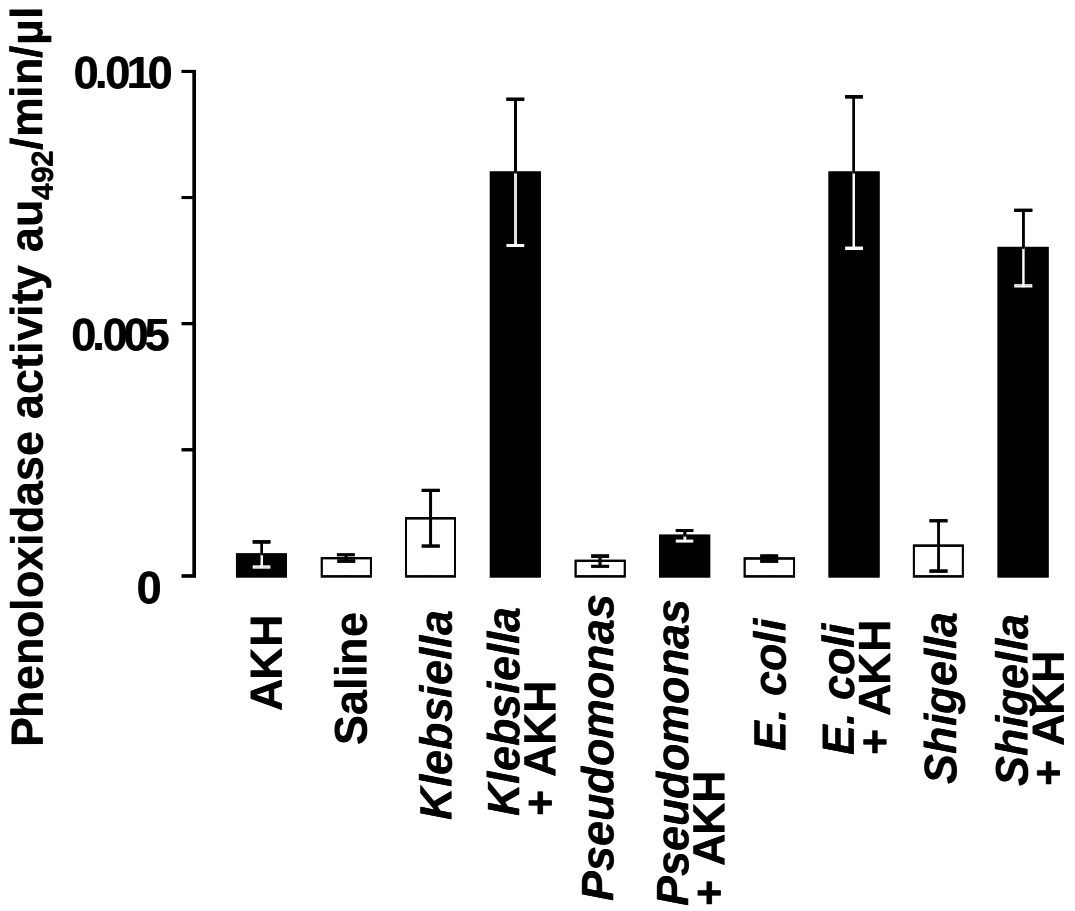
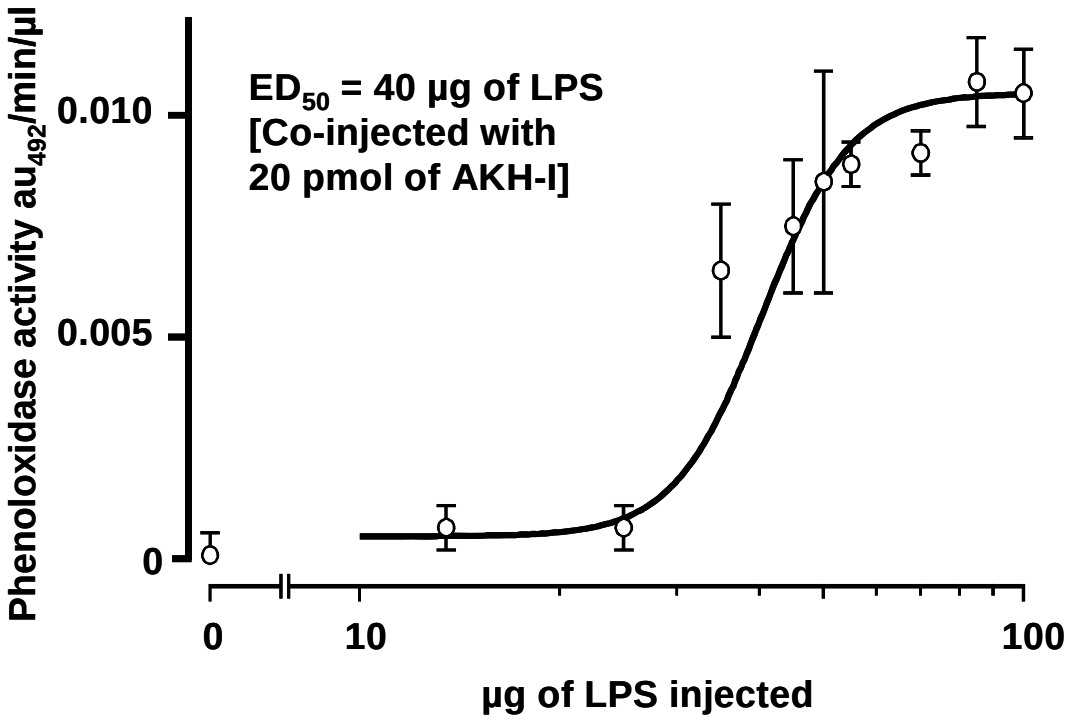


Fig. 7. The effect of varying the amount of LPS injected on the phenoloxidase activity in the haemolymph of *Locusta* 3 h after co-injection of 20 pmol of *Lom*-AKH-I. Curve fitting and estimation of ED_{50} values were undertaken as Hill-plots in FigP (Biosoft). Vertical lines represent ± SEM, $n \geq 10$.



antibacterial activity in the haemolymph *in vivo* in *Locusta* (Hoffman, 1980). We have yet to establish whether a commercial preparation of LPS from *Pseudomonas* extracted by a different chemical procedure might be more effective than the preparation tested here.

The differences between the responses of the phenoloxidase system *in vivo* to preparations of LPS and laminarin are intriguing. Why does injection of LPS from *E. coli*, for example, activate the phenoloxidase cascade *in vivo* only when AKH is co-injected, whereas injections of laminarin are effective in this respect without the hormone? It is possible that the hyperlipaemia induced by AKH affects the rate of degradation or excretion of the LPS, and this is being investigated. Nevertheless, although laminarin is effective in inducing a phenoloxidase response when injected into locusts on its own, co-injection of AKH-I or II does prolong the phenoloxidase response to this glucan.

In *Locusta*, injection of 15 μg of laminarin per locust causes a transient increase in phenoloxidase activity in the haemolymph, peaking at *c*. 30 min and returning towards pre-injection levels within 5 h. Co-injection of *Lom*-AKH-I prolongs the period of prophenoloxidase activation rather than affecting the initial speed or magnitude of the response. Qualitatively similar results are obtained after injection of either laminarin or LPS into adult desert locusts, *Schistocerca gregaria* (unpublished observations).

Gunnarsson (1988) demonstrated that injection of 250 μg of Zymosan (a source of β-1,3-glucans) into adult male *Schistocerca* causes a dramatic decrease in the total number of circulating haemocytes within 10 min. Further, when laminarin is added *in vitro* to plasma from *Locusta*, two proteins that appear to be important in activation of phenoloxidase are precipitated (Duvic and Brehelin, 1998). Thus it might be expected that the phenoloxidase activity in the haemolymph could decrease in response to injection of laminarin rather than increase in the manner described here. However, it is not known whether laminarin precipitates haemolymph proteins *in vivo*, or whether the relatively small amounts of β-1,3-glucan used in the present study reduce haemocyte numbers sufficiently quickly or with sufficient magnitude to impact on phenoloxidase activity. We agree with Gunnarsson and Lackie (1985) that injections of laminarin or LPS into locusts cause significant numbers of nodules to be formed, but under the conditions employed in this study nodule formation is not complete until about 8 h after injection (S. Chandrakant and G.J. Goldsworthy, unpublished observations).

The time course for activation of prophenoloxidase by LPS in the presence of *Lom*-AKH-I *in vivo* is perhaps slightly slower, but broadly similar to that of the response to laminarin in the presence of AKH, with high levels of phenoloxidase activity persisting for some hours. On the other hand, after co-injection with AKH-I the levels of phenoloxidase activity induced by injection of LPS are consistently lower than those induced by laminarin. We are investigating currently whether these differences are related in part to differences in the functioning of the binding proteins for bacterial LPS and for β-1,3-glucan in the haemolymph, and whether the two substances activate prophenoloxidase by independent mechanisms, as has been suggested previously (see Brehelin *et al*., 1989; Ratcliffe *et al*., 1981). It should also be noted that the maximum levels of activation of prophenoloxidase recorded here for either LPS or laminarin in the presence of AKH *in vivo* represent less than 10% of the prophenoloxidase activity determined after treatment of the haemolymph/buffer

Fig. 8. Dose response relationships for the effects of *Lom*-AKH-I on the phenoloxidase activity in the haemolymph of *Locusta* 3 h after co-injection with 100 µl of LPS. Curve fitting and estimation of ED_{50} values were undertaken as Hill-plots in FigP (Biosoft). Vertical lines represent ± SEM, $n \geq 10$.

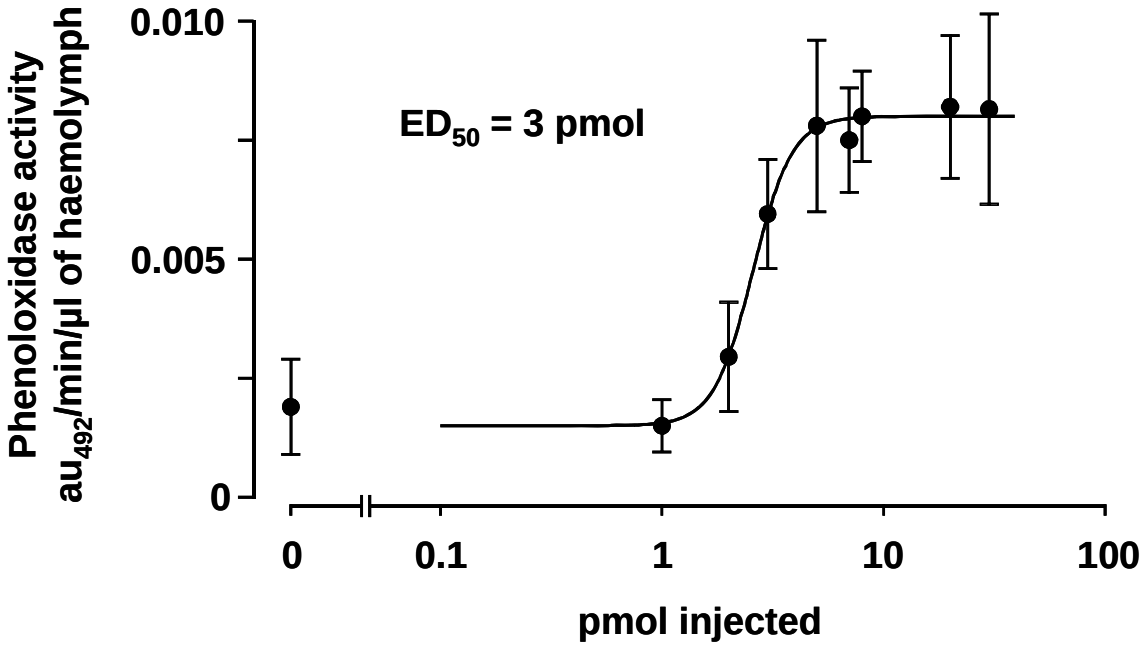
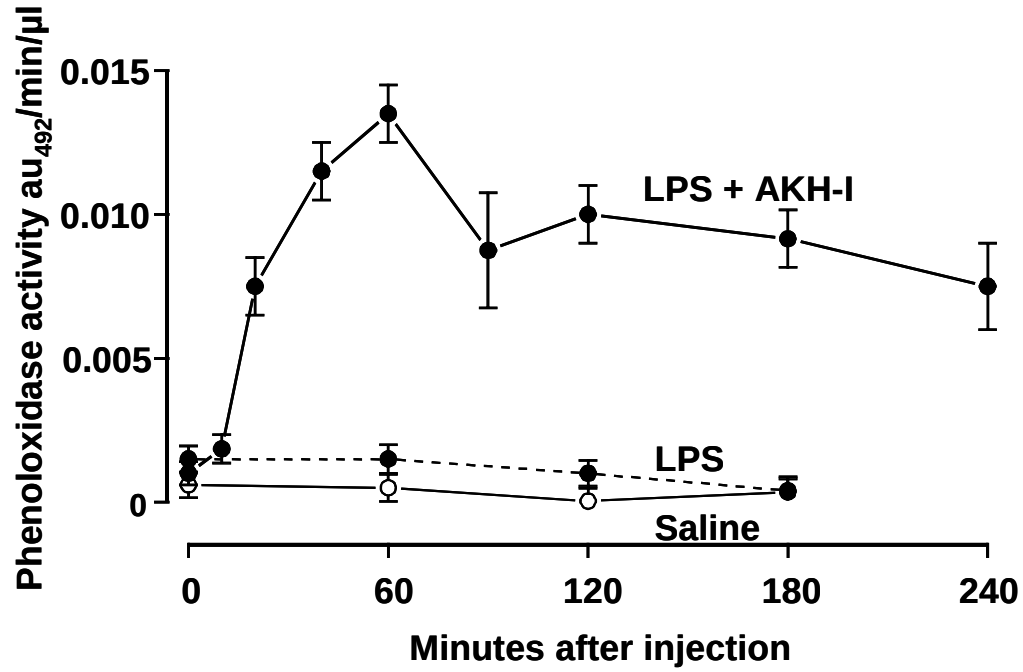


Fig. 9. Changes in the phenoloxidase activity of the haemolymph of *Locusta* with time after injection with saline (open circles, solid line), saline containing 100 µg of LPS (closed circles, dashed line), or saline containing 100 µg of LPS and 20 pmol of *Lom*-AKH-I (closed circles, solid line). Vertical lines represent ± SEM, $n \geq 10$.



solution with methanol or chymotrypsin compared with values of *c*.80% determined by Brehelin *et al*. (1989) *in vitro*.

Both laminarin and LPS proved toxic to locusts at high doses. This was true, even when LPS preparations were injected without AKH and failed to activate the phenoloxidase cascade. In mammals, LPS can also be lethal, with animals dying from +septic shock. In the locusts studied here, mortality generally occurred after about 24 h, the insects appearing unaffected in their behaviour up to this time, but subsequently often being found dead while still clinging to the underside of horizontal surfaces (unpublished observations). Presumably, the locusts die from an insect version of 'septic shock'. On dissection, the internal organs appear normal but many black nodules are present (unpublished observation) even in locusts receiving LPS in the absence of AKH. Again, in mammals, injected LPS can bind to high-density lipoprotein (HDL) and this prevents some of the toxic effects of LPS (see Kato *et al*., 1994b; Kitchens *et al*., 1999). Injected LPS associates with HDL in insect haemolymph (see Kato *et al*., 1994a) and, in resting fed locusts, the haemolymph contains mainly HDL (Goldsworthy, 1983). It may be that, in the absence of AKH in the locust, the HDL provides a pool of binding sites for LPS, thus preventing some of the actions of LPS, such as activation of the phenoloxidase cascade, although nodule formation still occurs (see also Gunnarsson and Lackie, 1985; Ratcliffe *et al*., 1991).

Adipokinetic hormone causes the mobilisation of diacylglycerols into the blood and a major shift towards the predominance of low-density lipoprotein (LDL) in locust haemolymph (Goldsworthy, 1983). In *Galleria*, two LPS-binding proteins have been identified, and are thought to modulate the LPS-induced inhibition of phenoloxidase in this insect (Dunphy and Halwani, 1997). Binding of LPS to LDL or other haemolymph proteins does not appear to have been investigated in locusts, but it may be that it is its effect on lipid metabolism that is important in the enabling action of AKH on phenoloxidase activation by LPS, but the precise mechanism requires further elucidation.

Prolongation or elicitation of the phenoloxidase response is AKH-I dose-dependent for laminarin and LPS respectively. When injected alongside laminarin, *Lom*-AKH-II is less potent than *Lom*-AKH-I in enhancing the phenoloxidase response to this glucan. The ED_{50} values quoted here for the effects of AKHs on the activation of the prophenoloxidase system are somewhat higher than those for their effects on lipid mobilisation (Goldsworthy *et al*., 1997), but such a direct quantitative comparison may not be valid. Certainly, in the case of laminarin the responses were measured indirectly as an effect (3 h after the injection) on reducing the decay of the phenoloxidase response. Nevertheless, the different potencies of *Lom*-AKH-I and II in the phenoloxidase assay described here are consistent with their different potencies in the locust lipid-mobilisation assay (Goldsworthy *et al*., 1997), suggesting a direct link between the immune and lipid mobilisation responses. In fact, the effect of AKH on the activation of prophenoloxidase in the haemolymph of locusts described here is consistent with the suggestion by Wiesner *et al*. (1997), Halwani *et al*. (1999, 2000) and Dettloff *et al*. (2001) that in *Galleria* a component of the lipid mobilisation system, ApoLp-III, is involved in anti-bacterial defence. One of the LPS-binding proteins in *Galleria* has a molecular mass of 17.2 kDa (Dunphy and Halwani, 1997), which is similar to that of ApoLp-III in this moth (Wiesner et al., 1997). The exact involvement of ApoLp-III in the immune system of locusts remains to be determined, but it seems likely that AKH could be exerting its effect at least partly through its effects on ApoLp-III metabolism. A lipid-associated form of ApoLp-III is induced by the action of

AKHs, and this may be necessary for the activation of prophenoloxidase (in the haemocytes?) by LPS (see Dettloff *et al*., 2001). This might help to explain the responses to preparations of LPS in the presence of AKH, but the fact that laminarin induces a rapid phenoloxidase response in the absence of AKH, and the effect of AKH is to delay the return of phenoloxidase activity to pre-injection levels, suggests that AKH may exert more than one effect on this system, and requires further investigation.

The physiological significance of the actions of AKHs reported here remains to be determined. AKHs can be regarded as typical stress hormones, but whether they are released and are functionally significant during infection is not known.

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