SCIENTIFIC NOTE

Activation of Prophenoloxidase and Removal of *Bacillus subtilis* from the Hemolymph of *Acheta domesticus* (L.) (Orthoptera: Gryllidae)

CLEONOR C.A. DA SILVA

Embrapa Recursos Genéticos e Biotecnologia, C. postal 02372, 70770-900, Brasilia, DF

Ativação da Profenoloxidase e Remoção de *Bacillus subtilis* da Hemolinfa de *Acheta domesticus* (L.) (Orthoptera: Gryllidae)

RESUMO - Fenoloxidase é considerada um importante mediador no sistema de defesa dos insetos contra patógenos e parasitóides. Essa enzima está presente na hemolinfa da maioria dos insetos estudados como uma proenzima inativa chamada profenoloxidase. Em *Acheta domesticus* (L.) a atividade da enzima foi estudada na presença de *Bacillus subtilis*. Injeções de bactéria na hemocele de ninfas desse inseto elevaram o nível de atividade da phenoloxidase. A taxa de aumento foi semelhante à observada nos insetos injetados com laminarin, um ativador da proenzima. A incubação de células de *B. subtilis* em soro com fenoloxidase ativada *in vitro*, e posterior injeção nos insetos, acelerou a remoção das bactérias da hemolinfa. A concentração das bactérias foi menor do que a observada nas ninfas injetadas apenas com bactéria em solução tampão. Por outro lado, ninfas injetadas com suspensão de bactéria incubada em soro com fenoloxidase inativada *in vitro* apresentaram alta concentração de bactéria na hemolinfa. Esses resultados indicam que *B. subtilis* é sensível à cascata de profenoloxidase de *A. domesticus*. Sugere-se que a fenoloxidase ativada adere à superfície das bactérias e aumenta a fixação dos hemócitos em torno das bactérias. Isso contribui para acelerar sua remoção da hemolinfa através da formação de nódulos.

PALAVRAS-CHAVE: Hemolinfa, fenoloxidase, hemócito, reação de defesa.

ABSTRACT - Insect phenoloxidase is considered an important mediator in defense reaction against pathogens and parasites. It is present in the hemolymph of most insects as an inactive proenzyme, called prophenoloxidase. In *Acheta domesticus* (L.) the phenoloxidase activity was studied in the presence of *Bacillus subtilis*. The bacteria induced the activation of the prophenoloxidase and increased the level of phenoloxidase activity when injected into the hemocoel of *A. domesticus* nymphs. The level of increase was comparable to those observed in nymphs injected with laminarin, an activator of the proenzyme. Incubating *B. subtilis* in serum with phenoloxidase activated *in vitro* and then injecting into the insects accelerated the removal of the bacteria from the hemolymph *in vivo*. The concentration of bacteria was lower than those observed in nymphs injected with Mes buffer alone. In control insects, injection of soybean trypsin inhibitor lowered phenoloxidase activity and the removal of bacteria from the hemolymph. These results indicate that *B. subtilis* is sensitive to the prophenoloxidase cascade of *A. domesticus*. It is suggested that activated phenoloxidase could bind to the surface of bacteria and increase the adhesion of hemocytes to bacteria, and thus accelerating their removal by nodule formation.

KEY WORS: Hemolynph, phenoloxidase, hemocyte, defense reaction.

It has become evident that insects possess an effective defense mechanism designed to resist the various microorganisms that use them as hosts. Certain events associated with these antimicrobial defenses are generally common to many insect species when exposed to different microbial attack and they include: recognition of nonself, changes in circulating hemocytes, antigen adhesion to hemocytes, enzyme activities and synthesis of antibacterial peptides in fat body (a functional equivalent of the vertebrate liver) (Brookman *et al*. 1989b, Gillespie *et al*. 1997). The change in the total and differential hemocyte counts, in parasitized or infected insects, is one of the first indication of a defense reaction (Wang *et al*. 1994, Gillespie *et al*. 2000, Silva *et al*. 2000b). Plasmatocytes and granular cells are the two types of hemocytes most often observed in hemocyte-mediated defenses such as phagocytosis, encapsulation and nodule formation (Gillespie *et al* 2000, Togo *et al*. 2000). Nodule formation consists of microaggregates of hemocyte entrapping large numbers of bacteria within a mucopolysaccharide matrix. The process ends with the

melanization, which leaves darkened nodules attached to various organs of the insect. In orthopteran insects the immediate response to entomopathogenic fungus is nodule formation (Bidochka & Kachatourians 1987, Brookman *et al*. 1989a, Gillespie *et al. 2*000). In A. *domesticus* nodule formation around *Xenorhabdus nematophilus*, an intestinal symbiotic bacterium of the entomopathogenic nematode *Steinernema carpocapsae*, starts with a decrease in the total hemocyte counts during the first hour of exposure to the bacteria; the amount of bacteria in the hemolymph also declines during this time. Later the bacterium suppresses phenoloxidase activity (Silva *et al*. 2000a,b). Phenoloxidase participates in three important physiological processes: cuticular sclerotization, defense reaction and wound healing (Ratcliffe 1993, Sugumaran *et al*. 2000). The activation of prophenoloxidase through serine proteases produces phenoloxidase, the enzyme responsible for the synthesis of melanin (Sugumaran & Saul 1987, Brookman, *et al*. 1989b).

It has been demonstrated that insect parasitism and infection suppress phenoloxidase activity *in vivo* and *in vitro* (Richards & Edwards 2000, Silva *et al*. 2000b).

Pathogenic and non-pathogenic bacteria interact with the insect antimicrobial defenses in different ways. Studies *in vitro* and *in vivo* with the pathogenic *X. nematophilus* demonstrated that bacteria overcome the antimicrobial defenses of *A. domesticus,* by initially reducing the number of hemocytes, mainly granular cells, and later reducing phenoloxidase activity (Silva *et al*. 2000 a,b).

The interaction of *Bacillus subtilis*, a non-pathogenic Gram-positive bacterium, with the antibacterial system of insects is known for a few numbers of species. In *Locusta migratoria* (L.) and *Galleria mellonella* (L.) live and dead *B. subtilis* induced serum melanization and phenoloxidase activation *in vitro*, respectively (Brehélin *et al*. 1989, Dunphy & Bouchier 1992) and in locust *Euprepocnemis shirakii* (Bolivar), induced phagocytosis by both plasmatocytes and granular cells (Chang *et al*. 1992).

The objective of this study was the activation of the phenoloxidase of *A. domesticus in vivo* with *B. subtilis* and its mediation in removing the bacteria from the hemolymph. Fifth instar females and males of A. *domesticus*, weighing 200 ± 10 mg, were used. The insects were reared on rabbit pellet chow at 30°C and 55 ± 5% RH with a 14:10h light:dark photoperiod (McFarlane 1985). Gram-positive *B. subtilis* (ATCC nº 6051) was maintained on tryptic soy agar. For experimental purposes *B. subtilis* was grown in 10 µl of tryptic soy broth in 50-ml Erlenmeyer flasks at 27°C on a horizontal, gyratory shaker (150 rpm) for 12h. Upon achieving early stationary phase, (OD_{669} = 2.0), the bacteria were washed three times by centrifugation (12,000 g, 3 min., 27°C) in N-morpholino-2-ethansulforic acid (Mes buffer, pH 6.5). Bacterial density was adjusted to 1.6 x 10^7 cells/µl for the experiments.

For phenoloxidase activation, twenty chilled (-20ºC, 10 min.) nymphs were injected dorsolaterally between the third and fourth abdominal segments with 10 µl of bacterial suspension in Mes buffer. A positive control group was injected with 10µl of the phenoloxidase activator laminarin (1 mg/ml in Mes buffer), and the negative controls were injected with soybean trypsin inhibitor (500 µg/ml) and Mes buffer alone. At 5, 10, 20 and 30 min. post-injection the insects were bled by cutting ventral abdominal entersegmental membrane, the hemolymph was collected with micropipettes and centrifuged (12,000 g, 27°C, 10 min.) to remove cell debris. Phenoloxidase activity was measured spectrophotometrically (OD_{490}) 30 min. after the addition of 2 mg/ml of L-dihydroxyphenylalanine (L-DOPA; Sigma Chemical Co., St Louis, MO) in 10 µl of serum as the formation of dopachrome (Silva *et al. 2*000a). The use of serum more closely approximate the *in vivo* bacterial infection during which the hemocytes discharge cytoplasmic contents during the formation of nodules and eventually lyse releasing enzymes of the prophenoloxidase system into the plasma (Dunphy and Bourchier 1992). One enzyme unit (U) was defined as a change of 0.001 OD_{490} units/min. Phenoloxidase activity was expressed as units per mg total protein.

Phenoloxidase of *A. domesticus* bind to the surface of *B. subtillis* and increase the binding of hemocytes to the bacteria to *in vitro* (Silva *et al*. 2000a). To determine if components of the prophenoloxidase system accelerate the removal of the bacteria from the hemocoel, *B. subtilis* was incubated for 30 min. in: (a) serum with phenoloxidase activated by laminarin (1 mg/ml in Mes buffer); (b) serum with phenoloxidase inhibited with 500 µg of soybean trypsin inhibitor (Sigma) and (c) in Mes buffer alone. After the incubation time the bacteria were centrifuged and washed (12,000 g, 27°C, 10 min.) three times in Mes buffer and 10 µl of each treatment was injected into the insects. At 5, 10, 20 and 30 min. post-injection the insects were bled, and the number of unattached bacteria was counted on a hemocytometer using phase contrast microscopy. Total serum protein was assayed by the Bradford method (1976) using bovine albumin as the standard.

Data are expressed as the mean ± standard error of the mean ($n \geq 10$ samples) and the percentage data are expressed as the decode mean of the arcsine $\sqrt{P}$ transformed data (with 95% of confidence limits of the transformed data). The rate of bacterial removal was analyzed using one-way analyses of variance. Correlation coefficients were calculated for the relationship between phenoloxidase and activity bacterial removal.

B. subtilis activates the prophenolxidase of *A. domesticus*. Injection of *B. subtilis* into the hemocoel stimulated the activation of the proenzyme and increased the level of the phenoloxidase activity. By 30 min. post-injection the enzyme activity reached 0.83 units and was higher than the concentration in control insects injected with Mes buffer (Fig. 1). The increase of phenoloxidase activity may be an indication of an immune response toward the bacteria. There was no larval death during the period of study. These results agree with Clarke and Dowds (1995) who found that injection of viable cells of *B. subtilis* into *G. mellonela* did not kill the larvae. However, injections of outer membrane fractions of the bacteria shown to be toxic and killed the larvae. Dead insects turned black into a few hours post-injection, probably due to melanization produced by phenoloxidase.

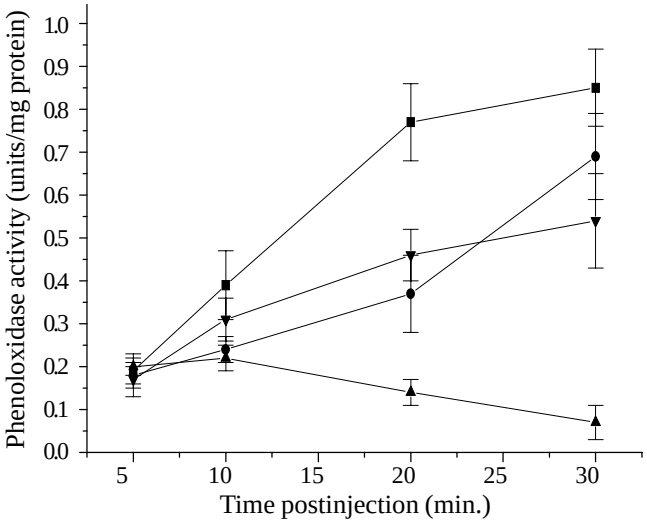

Figura 1. Changes in the level of phenoloxidase activity in the hemolymph of fifth instar *A. domesticus* in response to injection of *B. subtilis* (■), laminarin (●), STI (▲) and MES buffer (▼). Each point represents the mean ± SE (n= 20). Specific activity was calculated with 1.92 mg protein.

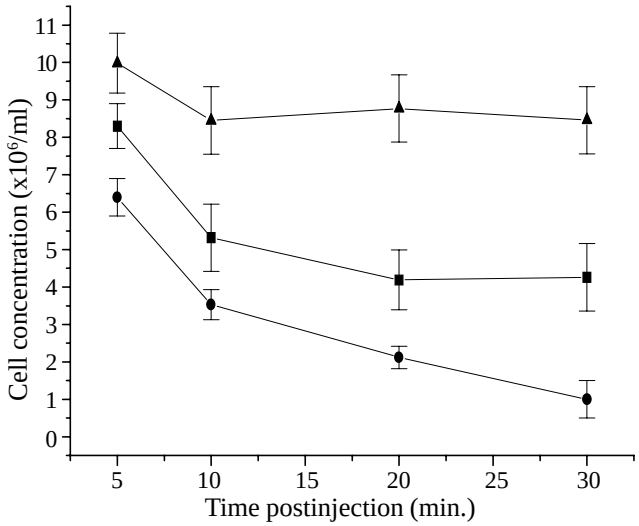

Figura 2. Removal of *B. subtilis* previously incubated in phenoloxidase activated hemolymph by laminarin (●), inhibited by STI (▲) and Mes buffer (■) from the hemolymph of *A. domesticus*. Each point represents the mean ± SE (n= 20 insects).

Injections of laminarin, a soluble b-1,3 glucan, into *A. domesticus* activated phenoloxidase throughout the 30-min. study when compared with the control. Phenoloxidase activity increased concomitant with a decrease in total hemocyte counts (5 min. post-injection, $1.5 \pm 0.3 \times 10^6$ hemocytes/ml; 30 min. postinjection, $0.4 \pm 0.2 \times 10^6$ hemocytes/ml). This decrease could be a result of hemocyte aggregation (nodule formation) induced by the presense of the bacteria. In a previous study using monolayers of *A. domesticus* hemocytes was found that *B. subtilis* adhere to more granular cells than plasmatocytes and serine proteases of the prophenoloxidase cascade had opsonic properties for *B. subtilis*. Also, phenoloxidase was present in greater quantities in the hemocytes than in the serum (Silva *et al.* 2000a).

Laminarin was also found to be a strong activator of prophenoloxidase *in vivo* in other orthopteran insects such as *L. migratoria, Schistocerca gregaria* (Forskal) and *Poecilocerus pictus* (F.) (Leonard *et al*. 1985, Bidochka *et al.* 1989, Rowley *et al*. 1990, Nellaiappan 1992) but it is failed to activate the proenzime of *S. gregaria in vitro* (Gillespie *et al*. 2000).

Phenoloxidase activity was greatly reduced by soybean trypsin inhibitor. This may suggest the presence of serine proteases in the prophenoloxidase cascade as reported for other insects (Brehelin *et al*. 1991, Boigegrain *et al*. 1992).

Incubating *B. subtilis in vitro* with phenoloxidase activated serum, and then injecting into the insects, accelerated its removal ($6.4 \times 0.7 \times 10^6$ bacteria/ml/min.) from the hemolymph when compared with control group injected with Mes buffer ($8.3 \times 0.6 \times 10^6$ bacteria/ml/min.) (Fig.2). The enhanced clearance of the bacteria may represents the binding of components of the prophenoloxidase cascade to the bacteria (Silva *et al.* 2000a) and thus removing bacteria by nodulation or bacterial surface modification by serine proteases (Dunphy & Webster 1991).

Inhibiting the serine proteases in phenoloxidase activated serum with soybean trypsin inhibitor lowered the bacterial removal from the hemocoel ($10.1 \times 0.6 \times 10^6$).

B. subtilis was sensitive to phenoloxidase of *A. domesticus*. It is known that in this insect granular cells/ hemolymph are the source of prophenoloxidase prior to the formation of phenoloxidase active enzyme (Silva *et al.* 2000a). The insect eliminated most of the bacteria from the circulation. During the 30 min. of the study the phenoloxidase titer was correlated ($r = 0.85$ $P<0.05$) with the removal of bacteria from the hemolymph. As the level of PO activity

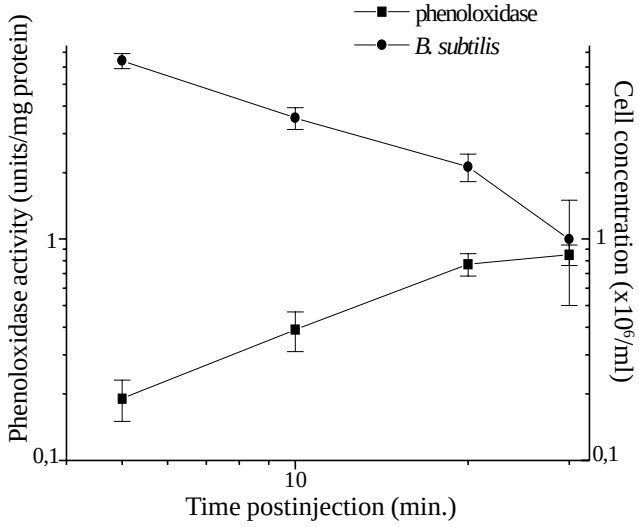

Figura 3. Correlation between phenoloxidase active enzyme and the removel of *B. subtilis* from the hemolymph of fifth instar *A. domesticus* (r= 0.85 P<0.05).

increased the number of bacteria removed increased (Fig. 3).

Activation of the prophenoloxidase cascade is one of the early events of an immune response to bacteria. In *A. domesticus* the strategy of *B. subtilis*, which activates this proenzyme, differs for the pathogenic bacterium *X. nematophilus* that has lipopolysaccharide in the outer membrane, which damages hemocytes and reduce phenoloxidase activity and later kill the insect. The understanding of insect immune response to bacterial infection has potential to improve efforts at microbiological control of pest insect.

Acknowledgments

The author are thankful to EMBRAPA for the financial suport and to Garry B. Dunphy for his valuable comments on this paper.

Literature Cited

Bidochka, M.J. & G.C. Kachatourians. 1987. Haemocytic defense response to the entomopathogenic fungus *Beauveria bassiana* in the migratory grasshopper, *Melanoplus sanguinipes*. Entomol. Exp. Appl. 45: 151-156.

Bidochka, M.J., J.P. Gillespie & G.C. Kachatourians. 1989. Phenoloxidase activity of acridid grasshoppers from the subfamilies Melanoplinae and Oedipodinae. Comp. Biochem. Physiol. 94 B: 117-124

Boigegrain, R.A., H. Mattras, M. Brehélin, P. Proutand & M.A. Coletti-Previero. 1992. Insect immunity: two proteinase inhibitors from hemolymph of *Locusta migratoria*. Biochem. Biophys. Res. Commun. 189: 790-793.

Bradford, M.N. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Brehélin, M., L. Drif & H. Boemare. 1989. Insect haemolymph: cooperation between humoral and cellular factors in *Locusta migratoria*. Insect Biochem. 19: 301-307.

Brehélin, M., R.A. Bergegrain, L. Drif. & M.A. Coletti-Previere. 1991. Purification of a protease inhibitor which controls prophenoloxidase activation in hemolymph of *Locusta migratoria* (Insecta). Biochem. Biophy. Res. Commun. 179: 841-849.

Brookman, J.L., A.F. Rowley & N.A. Ratcliffe. 1989a. Studies on nodule formation in locusts following injection of microbial products. J. Invertebr. Pathol. 53: 315-323.

Brookman, J.L., N.A. Ratcliffe, & A.F. Rowley. 1989b. Studies on the activation of the prophenoloxidase system on insects by bacterial cell wall components. Insect Biochem. 19: 47-57.

Chang, B.S., M.J. Moon, S.S. Han & S.M. Yoe. 1992. Cellular immune response of the grasshopper, *Euprepocnemis shirakii*, Bolivar following injection of *Bacillus subtilis*. Korean J. Zool. 35: 383-393.

Clarke, D.J. & B.C.A. Dowds. 1995. Virulence mechanism of *Photorhabdus* sp strain K 122 toward wax moth larvae. J. Invertebr. Pathol. 66: 149-155.

Dunphy, G.B. & J.M. Webster. 1991. Antihemocytic surface and the components of *Xenorhabdus nematophilus* var. dutki and their modification by serum of nonimmune larvae of *Galleria mellonella*. J. Invertebr. Pathol. 58: 40-51.

Dunphy, G.B. & R.S. Bouchier. 1992. Cellular and humoral defense reaction of non-immune larvae of the gypsy moth, *Lymantria dispar* to bacteria and the influence of tanic acid. J. Invertebr. Pathol. 60: 26-32.

Gillespie, J.P., C. Burnett & A.K. Charnley. 2000. The immune response of the desert locust *Schistocerca gregaria* during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae* var *acridum*. J. Insect Physiol. 46: 429-437.

Gillespie, J.P., M.R. Kanost & T. Trenczek. 1997. Biological mediators of insect immunity. Ann. Rev. Entomol. 42: 611-643.

Leonard, C., N.A. Ratcliffe & A.F. Rowley. 1985. The role of prophenoloxidase activation in non-self recognition and phagocytosis by insect blood cells. J. Insect Physiol. 31: 789-799.

McFarlane, J.E. 1985. *Acheta domesticus*, p. 891-896. In P. Singh and R.E. Moore (eds.), Hand book of insect rearing, vol. 1. New York, Elsevier, 900p.

Nellaiappan, K. 1992. On the latency of blood phenoloxidase of painted locust *Poecilocerus pictus*. Arch. Inter. Physiol. Bioch. Biophy. 100: 223-229.

Ratcliffe, N.A. 1993. Cellular defense responses of insects: unresolved problems, p. 267-304. In N.E. Beckage, S.N. Thompson, B.A. Federici (eds.), Parasites and pathogens of insects. Academic Press, San Diego, 515p.

Richards, E. & J.P. Eswards. 2000. Parasitism of *Lacanobia oleracea* (Lepidoptera) by the ectoparasitoid, *Eulophus pennicornis* is associated with a reduction in host hemolymph phenoloxidase activity. Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol. 127: 289-298.

Rowley, A.F., J.L. Brookman & N.A. Ratcliffe. 1990. Possible involvement of the prophrnoloxidase system of *Locusta migratoria* in antimicrobial activity. J. Invertebr. Pathol. 56: 31-38.

Silva, C.C.A., G.B. Dunphy & M.E. Rau. 2000a. Interaction of hemocytes and prophenoloxidase system of fifth instar nymphs of *Acheta domesticus* with bacteria. Dev. Comp. Immu. 24: 367-379.

Silva, C.C.A., G.B. Dunphy & M.E. Rau. 2000b. Interaction of *Xenorhabdus nematophilus* (Enterobacteriaceae) with the antimicrobial defenses of the house cricket, *Acheta domesticus* J. Invertebr. Pathol. 76: 285-292.

Sugumaran, M., K. Nellaiappan & K. Valivittan. 2000. A new mechanism for the control of phenoloxidase activity: Inhibition and complex formation with quinone isomerase. Arch. Biochem. Biophy. 379: 252-260.

Sugumaran, M. & S.J. Saul. 1987. Protease mediate prophenoloxidase activation in the hemolymph of tobacco hornworm, *Manduca sexta*. Arch. Insect Biochem. Physiol. 5: 1-11.

Togo, S., F. Naganuma, K. Arakawa & S. Yokoo. 2000. Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, *Galleria mellonella*. J. Insect Physiol. 46: 1129-1135.

Wang, Y., R. Gaugler & C. Cui. 1994. Variation in immune response of *Popilla japonica* and *Acheta domesticus* to *Heterorhabditis bacteriophora* and *Steinernema* species. J. Nematol. 26: 11-18.

Received 21/08/01. Accepted10/07/02.