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Effect of *Pseudomonas aeruginosa* Crude Proteolytic Fraction on Antibacterial Activity of *Galleria mellonella* Haemolymph

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The antibacterial activity of immune haemolymph *Galleria mellonella* directed against *Escherichia coli* D31 was destroyed by *Pseudomonas aeruginosa* crude proteolytic fraction. This was demonstrated by diffusion well assay and acid gel electrophoresis and subsequent bioautography. On the contrary, lysozyme activity appeared to be insensitive to extracellular proteases of *P. aeruginosa* when activity was determined using the bioautography method. In addition, no change in lysozyme protein level was observed by immunoblotting with specific antibodies directed against *G. mellonella* lysozyme, which confirmed that lysozyme was not degraded by the crude proteolytic fraction of *P. aeruginosa*. However, a significant decrease of lysozyme activity in naive and immune haemolymph exposed to the action of *P. aeruginosa* proteins determined by using diffusion well assay was observed. Mechanisms of the observed inhibition require further studies.

Key words: *Pseudomonas aeruginosa*, extracellular proteases, *Galleria mellonella*, antimicrobial peptides, lysozyme.

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Insects have a defense mechanisms consisting of cellular and humoral immune response systems. Several cell-mediated immune responses such as phagocytosis (SALT 1970), encapsulation (POINAR *et al*. 1968), and nodule formation (GAGEN & RATCLIFFE 1976) have well documented as defense mechanisms in insects. One of the most important mechanisms of humoral defense in insects is induction of antimicrobial peptides and proteins in response to infection with bacteria or inoculation of bacteria products (lipopolisaccharide-LPS, peptidoglycan) into the body cavity. Cecropins, insect defensins, glicine-rich and proline-rich peptides, lysozyme, hemolin and others have been identified among inducible peptides/proteins in challenged insects. (HETRU *et al*. 1998; BULET *et al*. 1999).

From earlier studies, it is known that the insect-pathogenic strain *Pseudomonas aeruginosa* impaired cellular defenses of the greater wax moth *Galleria mellonella* larvae by hemocyte breakdown (MADZIARA-BORUSEWICZ & LYSENKO 1971). It was also demonstrated that antibacterial peptides from immune larvae were degraded during infection by *P. aeruginosa*, while lysozyme appeared to be resistant for degradation (JAROSZ 1995; JAROSZ 1997; ANDREJKO 1999). There are suggestions that proteases secreted by *P. aeruginosa* are responsible for bacterial virulence, but their exact role in insect pathology has not been defined.

It is also known that *P. aeruginosa* is an opportunistic human pathogen responsible for many types of infections. Bacteria secrete several proteases that have been implicated as virulance factors (ENGEL *et al*. 1998). This group of enzymes includes at least four proteases: alkaline protease, two elastases (A and B) and protease IV (CABALLERO *et al*. 2001).

In this paper *in vitro* studies are presented on the effects of *P. aeruginosa* culture supernatant on antibacterial activity of *G. mellonella* immune haemolymph. Long-term observations showed that when *P. aeruginosa* was grown in nutrient broth, proteolytic activity appeared rapidly in the medium. Such samples of supernatant, tentatively named crude proteolytic fraction, were used as a source of extracellular proteases in our studies.

Material and Methods

Bacterial strain

P. aeruginosa clinical isolate designated H3 was used. The pyocyanin-producing strain H3 is an

isolate of moderate virulence to the 7th instar larvae of *G. mellonella* (LD_{50} = 17 cells). Bacteria were inoculated into 50 ml of nutrient broth in a 250 ml Erlenmeyer flask. The culture was incubated on a rotary shaker (90 rpm) at 38°C for 24 h and centrifuged (20 000 g, 60 min) to pellet the cells. The supernatant was filtered through a 0.30 μm-pore-size filter (Millipore) to remove any remaining bacteria. Supernatant was stored at -20°C in sterile containers until needed.

Insects

Larvae of the greater wax moth *G. mellonella* L. (Lepidoptera, Pyralidae) were reared on an artificial diet (12.5% wheat meal, 25% corn meal, 12.5% wheat germ, 12.5% dried yeast) at 30°C in darkness, as described by SEHNAL (1966) but without the addition of bee wax. Larvae selected for the study were in the last instar (seventh).

Insect immune challenge and haemolymph collection

Immune challenge was performed with *Escherichia coli* LPS (Serotype 026.B6, Sigma) diluted in sterile water (2.5 μg LPS in 5 μl water). LPS was injected at the base of the last proleg of larvae using a Hamilton syringe. The insects were kept at 30°C in darkness and the haemolymph (20 μl per larva) was collected 24 hours after immunization. Prior to haemolymph collection, the insects were chilled for 15 min at 4°C. Haemolymph samples were obtained by puncturing the larval abdomen with a sterile needle. Out-flowing haemolymph was immediately transferred into sterile and chilled Eppendorf tubes containing a few crystals of phenylthiourea (PTU) to prevent melanisation. The hemocyte-free haemolymph was obtained by centrifugation at 200 g for 5 min and subsequently at 20 000 g for 15 min at 4°C. Pooled supernatants were used immediately or stored at -20°C until needed.

Acidic/methanol extraction of small proteins and peptides

Peptides of antibacterial activity were partially purified from the hemocyte-free haemolymph by an acidic/methanol extraction method adapted from SCHOOFS *et al*. (1990). Haemolymph was diluted ten times with the extraction solution consisting of methanol/acetic acid glacial/water (90:1:9) and mixed thoroughly. Precipitated proteins were pelleted by centrifugation at 20000 g for 30 min at 4°C. The obtained supernatant containing polipeptides mainly of M_r below 30 kDa was collected, vacuum dried and the pellet was stored at -20°C until needed. It was dissolved before use in an appropriate volume of water (usually the water volume was 2/3 of the initial hemocyte - free haemolymph volume). The protein concentration was 1.5-2 mg/ml.

Protein determination

The protein concentration was measured by the Bradford method using bovine serum albumin (BSA) as a standard (BRADFORD 1976).

Determination of proteolytic activity

Proteolytic activity was assayed by the azocasein method, where azocasein was hydrolysed by the protease, releasing red-coloured azopeptide, according to EL-SISSI *et al*. (1982), with some modifications. The assay mixture contained 250 μl azocasein in water (5 mg/ml) and 250 μl of crude proteolytic fraction containing a protein concentration of about 0.153 mg/ml. After incubation for 60 min at 37°C, the reaction was stopped by adding 500 μl of 5% (w/v) trichloroacetic acid. Precipited azocasein was then centrifuged at 20 000 g for 10 min. Supernatant (750 μl) was mixed with 375 μl of 0.5 M NaOH solution. Proteolytic activity was calculated as units ml^{-1} (U/ml). One unit was defined as an increase in A_{450} of 0.02 above the level of the control sample. Average proteolytic activity in crude *P. aeruginosa* proteolytic fraction from a 24 h culture was about 318 U/ml.

Estimation of antibacterial activity by diffusion well assay

For the antibacterial activity test, a lipopolysaccharide (LPS) defective, streptomycin and ampicilin resistant mutant of *E. coli* K12 , strain D31 was used (BOMAN *et al*. 1974).

The presence of antibacterial activity in the haemolymph and acidic/methanol extracts of haemolymph was detected by a growth inhibition zone assay using solid agar plates containing viable *E. coli* cells as described in HOFFMANN *et al*. (1981). To improve the sensitivity of the method, chicken egg white lysozyme (EWL) in a concentration of 5 mg/ml of medium was added (CHALK *et al*. 1994; CYTRYŃSKA *et al*. 2001). The level of anti-*E. coli* activity was calculated using the algorithm described by HULTMARK *et al*. (1982). For evaluation of antibacterial activity, synthetic cecropin B of *Hyalophora cecropia* (Sigma) was used as standard. Lysozyme activity in samples was detected in an inhibition zone assay using freeze-dried *Micrococcus luteus* (Sigma) accord-

ing to MOHRIG and MESSNER (1968). The activity of lysozyme was calculated from a calibration curve made with chicken egg white lysozyme (EC 3.2.1.17) and expressed in μg/ml EWL. Each well on the Petri dish was filled with 5 μl of samples. The diameter of *E. coli* or *M. luteus* lytic zones was measured after 24 h of incubation at 37°C.

Estimation of antibacterial activity by electrophoresis and subsequent bioautography

Protein samples were electrophoresed on 15% polyacrylamide gels at pH 4.3 using a discontinuous non-denaturing buffer system by the method of GOLDENBERG (1997). To localize bands with anti-*E. coli* activity, the gels were overlaid with a soft nutrient agar inoculated with an exponential phase of *E. coli* D31 or with *E. coli* D31 suspended in nutrient agar containing 5 mg/ml of EWL. For detection of lysozyme activity, the gels were covered with 0.7% agarose containing freeze-dried *M. luteus* in Sörensen buffer (pH 6.4). The gels were incubated at 37°C for 24 h, then bacterial growth inhibition zones were measured.

Immunoblotting

Samples were subjected to 15% polyacrylamide gels at pH 4.3 using a discontinous non-denaturing buffer system and electroblotted onto Immobilon P membranes for 90 minutes at 350 mA. For identification of lysozyme, rabbit anti-*G. mellonella* lysozyme antibodies (kindly provided by Prof. Kyung Hyun Yu, University Hoseo, South Korea) were used. As second antibodies, alkaline phosphatase-conjugated goat anti-rabbit IgGs were used. Immunoreactive bands were visualized by incubation with p-nitroblue tetrazolium chloride and 5-bromo-chloro-3-indolyl phosphate.

Results and Discussion

The effect of *P. aeruginosa* crude proteolytic fraction on *G. mellonella* antibacterial peptides activity

In order to obtain immune haemolymph, larvae of *G. mellonella* were challanged with *E. coli* lipopolisaccharide (LPS) to induce synthesis of antibacterial proteins and peptides. Samples of immune haemolymph containing about 1000 μg protein were mixed with *P. aeruginosa* crude proteolytic fraction and after 1 h incubation at 37°C they were tested for antibacterial activity. The activity directed against indicator strain *E. coli* D31 was determined.

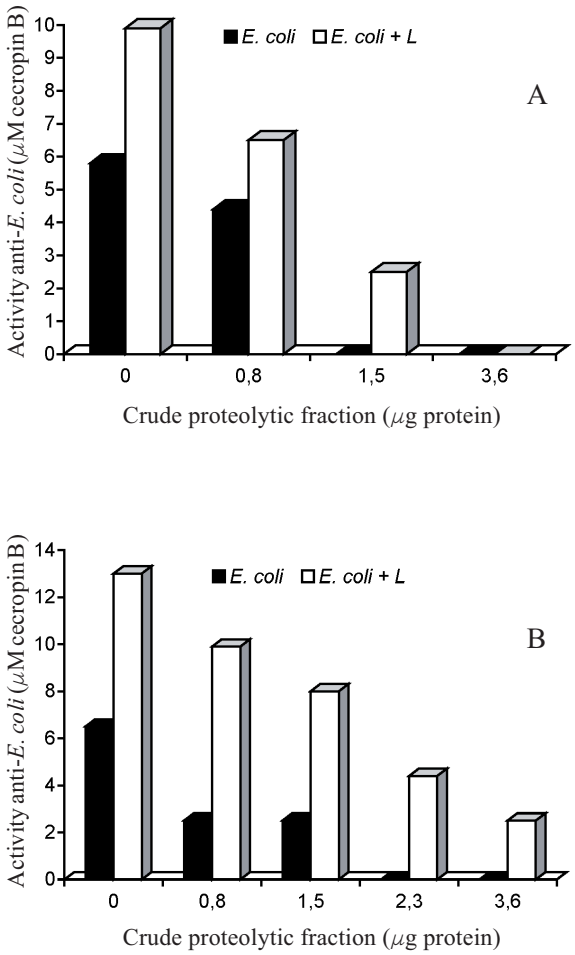

Fig. 1. Effect of *Pseudomonas aeruginosa* crude proteolytic fraction on anti-*Escherichia coli* activity of *Galleria mellonella* larvae. A: immune haemolymph. B: immune haemolymph extract. ■ – nutrient agar with *E. coli*. □ – nutrient agar with *E. coli* and egg white lysozyme (L).

From the data summarized in Fig. 1A it appeared that antibacterial activity measured by diffusion well assay was significantly reduced in samples preincubated with *Pseudomonas* proteins. Crude proteolytic fraction containing 1.5 μg total protein completely abolished bactericidal activity of haemolymph.

Similar results were obtained in the case of acidic/methanol haemolymph extracts. It is known from unpublished data (CYTRYŃSKA *et al.*) that haemolymph extracts contain proteins of M_r below 30 kDa, among which immune polypeptides are present. As can be seen in Fig. 1B, antibacterial activity of haemolymph extracts was completely inhibited by culture supernatant containing 2.3 μg protein.

Synergy exists between lysozyme and the bactericidal effect of cecropins and other antibacterial peptides (CHALK *et al.* 1994; CYTRYŃSKA *et al.* 2001). Therefore, in the case of experiments performed with the addition of lysozyme, higher pep-

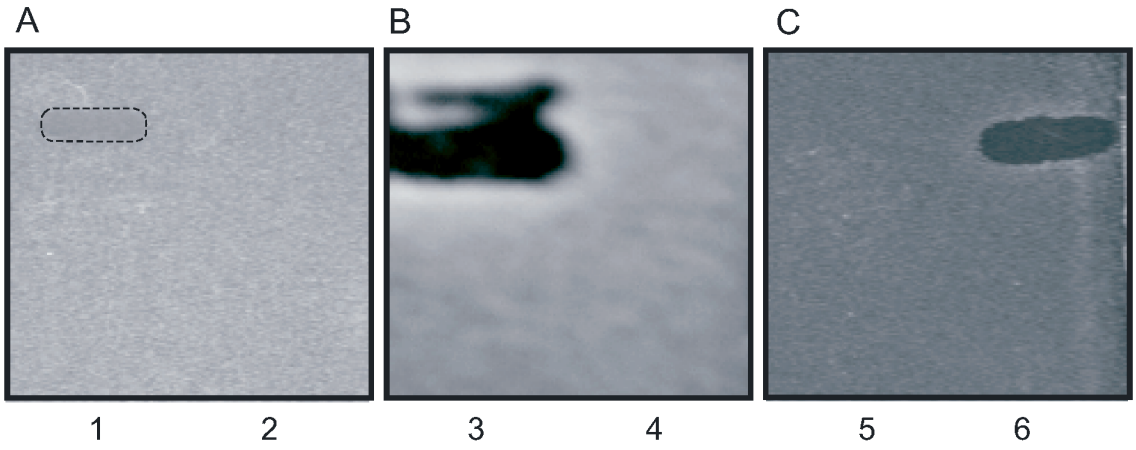

Fig. 2. Bioautography of antibacterial polypeptides of *Galleria mellonella* larvae exposed to *Pseudomonas aeruginosa* crude proteolytic fraction. Samples containing 1000 μg of immune haemolymph proteins, 45 μg of haemolymph extract proteins or 2 μg of cecropin B (Sigma) from *Hyalophora cecropia* used as standard were incubated with *P. aeruginosa* crude proteolytic fraction (4.6 μg protein) at 37°C for 1 h. Then samples were separated by acid electrophoresis in 15% polyacrylamide gels (Material and Methods). Afterwards, electrophoresis gels were overlaid with *E. coli* D31 from exponential growth phase in nutrient agar. A: immune haemolymph extract (lane 1) and immune haemolymph extract treated with crude proteolytic fraction (lane 2). B: immune haemolymph (lane 3) and immune haemolymph treated with crude proteolytic fraction (lane 4). C: cecropin B treated with crude proteolytic fraction (lane 5) and cecropin B (lane 6). For gel image acquisition and documentation, the video image analyzer Gel Doc 2000 (Bio Rad) with Quantity One software (Bio Rad) was used.

tides activity was noted in control haemolymph and haemolymph extract samples (Fig. 1A and 1B). The concentration of antibacterial peptides was calculated as 9.9 μM in terms of cecropin B activity in the case of immune haemolymph and 13 μM in the case of immune haemolymph extract. When *P. aeruginosa* crude proteolytic fraction was added to haemolymph at a concentration of 3. 6 μg, complete inactivation of peptide antibacterial activity was observed. But in the case of the haemolymph extract, trace antimicrobial activity was noted in the same concentration of *Pseudomonas* proteins.

Additionally, antibacterial activity was tested by the bioautography procedure after electrophoretic resolution of proteins in polyacrylamide gels (Material and Methods). The data presented in Fig. 2 clearly showed *E. coli* growth inhibition zones in the case of control samples of cecropin B, immune haemolymph and a smaller intesity zone in the case of immune haemolymph extracts. No growth inhibition zones were observed in the case of the same samples preincubated with *P. aeruginosa* culture supernatant.

To improve the sensitivity of the bioautography method, gels were overlaid with *E. coli* D31 in nutrient agar containing EWL (5 mg/ml). The results presented in Fig. 3 showed that in the presence of lysozyme, growth inhibition zones of bacterial cells were significantly larger in control samples in comparison to those observed without lysozyme addition. Lytic zones around peptides in haemolymph (lane 3 in Fig. 3A) and in extracts (lane 1 in Fig. 3A) were localized on the gel minimally higher than zones caused by lytic activity of synthetic cecropin B (lane 2 in Fig. 3A). Lytic activity against *E. coli* was significantly (not completely) reduced in samples containing immune peptides preincubated with proteases (both in the case of extract and whole haemolymph samples) (lane 1 and 3 in Fig. 3B). Synthetic cecropin B exposed to the *Pseudomonas* proteases entirely lost its antibacterial activity by proteolytic digestion of the molecule (lane 2 in Fig. 3B).

Altogether, the obtained results seem to indicate that antibacterial peptides of immune haemo-

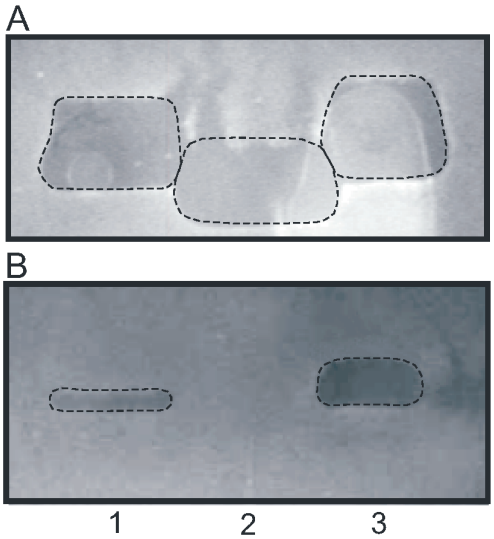

Fig. 3. Bioautography of antibacterial polypeptides of *Galleria mellonella* larvae exposed to *Pseudomonas aeruginosa* crude proteolytic fraction in the presence of lysozyme. After acid electrophoresis, gels were overlaid with *E. coli* D31 from exponential growth phase suspended in nutrient agar containing chicken egg white lysozyme (EWL). Other conditions as described in Fig. 2. A: immune haemolymph extract (lane 1), cecropin B (lane 2), immune haemolymph (lane 3). B: immune haemolymph extract treated with crude proteolytic fraction (lane 1), cecropin B treated with crude proteolytic fraction (lane 2), immune haemolymph treated with crude proteolytic fraction (lane 3).

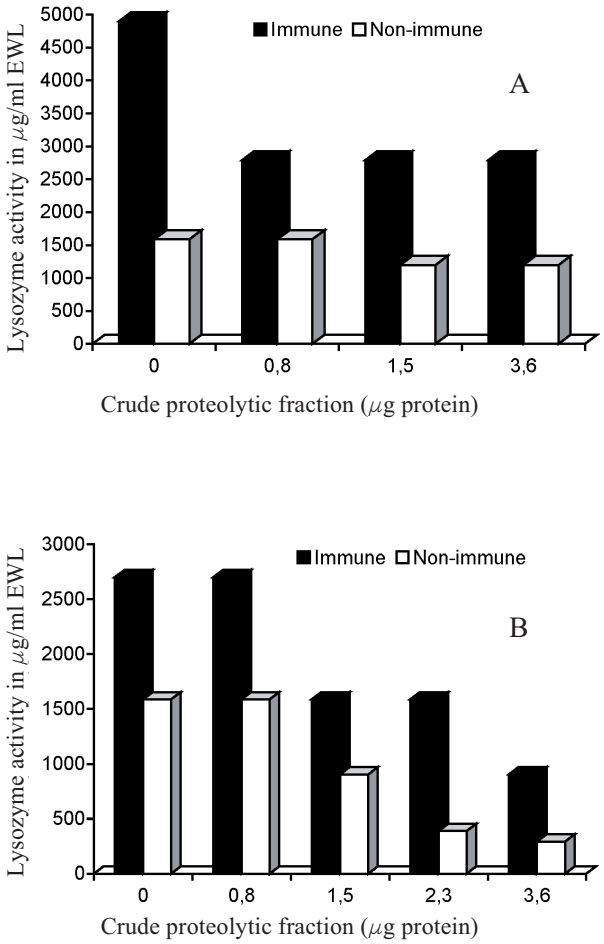

Fig. 4. Effect of *Pseudomonas aeruginosa* crude proteolytic fraction on lysozyme activity of non-immunised and immunised *Galleria mellonella* larvae. A: haemolymph. B: haemolymph extract.

lymph with activity directed against *E. coli* are degraded by exoproteases of *P. aeruginosa*. Other studies have shown that four different proteases, namely elastase A, elastase B, alkaline protease and protease IV *P. aeruginosa*, are secreted into the medium (ENGEL *et al*. 1998; CABALLERO *et al*. 2001). It would be interesting to determine which of them plays a major role in insect pathogenicity. This requires further studies on individual enzyme preparations.

The effect of *P. aeruginosa* crude proteolytic fraction on lysozyme activity and expression on protein level

Lysozyme plays an important role in humoral defense of *G. mellonella*. The activity of haemolymph lysozyme in non-immunised larvae is relatively high (about 1600 μg/ml EWL). The innate level of lysozyme increased about 3-fold 24 h after injection of LPS *E. coli* The activity of lysozyme was significantly reduced in haemolymph exposed to the action of *P. aeruginosa* culture supernatant (Fig. 4A). A nearly 25% decrease of lysozyme activity in the case of haemolymph from non-immunised and 43% in the case of haemolymph from immunised insects was observed.

The lysozyme concentration in haemolymph is low in comparison to other proteins, among which lypophorins of M_r 60-80 kDa constitute about 80% of total haemolymph proteins. Therefore, in further studies haemolymph acidic/methanol extracts were used in which proteins of M_r above 30 kDa were excluded. When haemolymph extracts were incubated with *P. aeruginosa* culture supernatant, a nearly 80% decrease of lysozyme activity in the case of extract from non-immunised insects and 70% in the case of extract from *Galleria* immunised larvae was noted in samples containing 3,6 μg *Pseudomonas* proteins (Fig. 4B). The obtained results clearly indicate that lysozyme activity is inhibited by *P. aeruginosa* culture supernatant, which is especially noticeable in the case of haemolymph extracts.

In order to answer the question if a decrease of lysozyme activity is connected with proteolytic degradation, lysozyme protein level were tested by immunoblotting. Specific antibodies directed against *G. mellonella* lysozyme were used. As can be seen in Fig. 5, a single protein band was recognised by the antibodies. The expression level of lysozyme in the immune haemolymph was much higher in comparison to non-immune haemolymph samples. Surprisingly, in haemolymph samples incubated with *P. aeruginosa* culture supernatants, no change in the lysozyme-protein level was observed. This data seemed to indicate that lysozyme was not degraded under the experimental conditions used.

In addition, when lysozyme activity was determined using the bioautography method on the gels after acid gel electrophoresis, the lytic *M. luteus* zones were observed in both control and haemo-

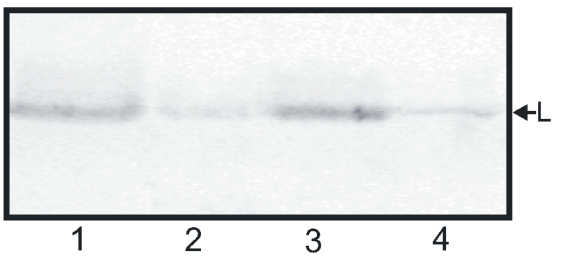

Fig. 5. Immunoblotting of non-immune and immune haemolymph extract with rabbit anti-*G. mellonella* lysozyme antibodies. Samples of immune haemolymph extract (45 μg protein) were incubated with *P. aeruginosa* crude proteolytic fraction (4.6 μg protein) at 37°C for 1 h. Then samples were separated on 15% polyacrylamide gels according to the Goldenberg (1997) procedure and electroblotted onto Immobilon P membranes (Material and Methods). 1: immune haemolymph extract treated with crude proteolytic fraction; 2: non-immune haemolymph extract treated crude proteolytic fraction; 3: immune haemolymph extract; 4: non-immune haemolymph extract; L, lysozyme.

lymph samples preincubated with *P. aeruginosa* culture supernatants (Fig. 6).

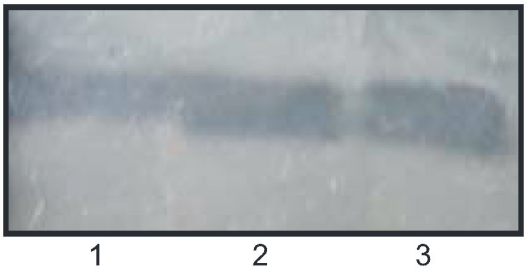

Fig. 6. Detection of lysozyme activity by the bioautography method. Gel after acid electrophoresis was covered with freeze-dried *M. luteus* cells. 1: chicken egg white lysozyme (2 μg). 2: immune haemolymph (1000 μg protein) treated with *P. aeruginosa* crude proteolytic fraction (4.6 μg protein). 3: immune haemolymph (1000 μg protein). Samples of immune haemolymph were incubated with crude proteolytic fraction for 1 h at 37°C.

The obtained results indicate that insect lysozyme appeared to be insensitive to extracellular proteases of *P. aeruginosa*. In the light of these results, a decrease of lysozyme activity observed in Fig. 4 using the diffusion well assay might suggest the presence of unidentified inhibitors of lysozyme activity in the crude proteolytic fraction of *P. aeruginosa*, which might play some role in *P. aeruginosa* virulence. Further studies are required to solve this problem.

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