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Synergistic action of *Galleria mellonella* apolipophorin III and lysozyme against Gram-negative bacteria

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ABSTRACT

Insect immune response relies on the humoral and cellular mechanisms of innate immunity. The key factors are the antimicrobial polypeptides that act in concert against invading pathogens. Several such components, e.g. apolipophorin III (apoLp-III), lysozyme, and anionic peptide 2, are present constitutively in the hemolymph of non-challenged *Galleria mellonella* larvae. In the present study, we demonstrate an evidence for a synergistic action of *G. mellonella* lysozyme and apoLp-III against Gram-negative bacteria, providing novel insights into the mode of action of these proteins in insect antimicrobial defense. It was found that the muramidase activity of *G. mellonella* lysozyme considerably increased in the presence of apoLp-III. Moreover, apoLp-III enhanced the permeabilizing activity of lysozyme toward *Escherichia coli* cells. As shown using non-denaturing PAGE, the proteins did not form intermolecular complexes in vivo and in vitro, indicating that the effect observed was not connected with the intermolecular interactions between the proteins. Analysis of AFM images of *E. coli* cells exposed to *G. mellonella* lysozyme and/or apoLp-III revealed evident alterations in the bacterial surface structure accompanied by the changes in their biophysical properties. The bacterial cells demonstrated significant differences in elasticity, reflected by Young's modulus, as well as in adhesive forces and roughness values in comparison to the control ones. The constitutive presence of these two defense molecules in *G. mellonella* hemolymph and the fact that apoLp-III enhances lysozyme muramidase and perforating activities indicate that they can be regarded as important antibacterial factors acting at the early stage of infection against Gram-negative as well as Gram-positive bacteria.

1. Introduction

Insect immune response relies on the humoral and cellular mechanisms of innate immunity. The key factors in fighting against invading pathogens are the defense peptides and proteins synthesized mainly in the fat body (functional analog of the mammalian liver) and released into the hemolymph (insect blood). Recognition of pathogens inside the insect hemocoel leads to rapid induction of antimicrobial peptide synthesis and a considerable increase in their concentration in the hemolymph. However, the hemolymph of naive individuals exhibits a certain level of peptides and proteins with antimicrobial activity, which are most probably involved in the early steps of fighting infection [1–6]. Several such components, e.g. apolipophorin III (apoLp-III), lysozyme, anionic peptide 2, and cecropin D-like peptide were detected in the hemolymph of non-challenged larvae of a lepidopteran insect, the greater wax moth *Galleria mellonella* [7, 8].

Apolipophorin III (apoLp-III) is a well-known abundant hemolymph protein functioning in the lipid transport and immune response in insects. ApoLp-III is involved in the recognition of pathogens as it binds components of bacterial and fungal cell walls, e.g. lipopolysaccharide, lipoteichoic acids, and β-1,3-glucan. In addition, the protein is engaged in the detoxification of microbial cell envelope components. Participation of apoLp-III in the signaling network between hemolymph, hemocytes, and fat body cells has also been postulated [9–17]. Recently, apoLp-III antimicrobial activity against selected Gram-positive and Gram-negative bacteria has been demonstrated [18, 19].

Similar to most insect lysozymes, *G. mellonella* lysozyme belongs to a c-type lysozyme family [20–22]. The activity of lysozyme against Gram-positive bacteria based on its muramidase enzymatic activity is well documented [21, 23]. However, a relatively low lysozyme activity against Gram-negative bacteria was also demonstrated [22]. This activity can be attributed to the non-enzymatic action of lysozyme related to the cationic properties of the protein molecule or to its particular cationic peptide fragments [24, 25].

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It was reported that in the presence of egg white lysozyme (EWL) *Escherichia coli* became more sensitive to defense peptides such as attacins, cecropins, and insect defensins [26, 27]. It was also suggested that EWL could act synergistically with certain *G. mellonella* antimicrobial peptides and proteins against Gram-negative bacteria in vitro [28]. Halwani and Dunphy [29] demonstrated an increase in EWL activity against *Micrococcus luteus* in the presence of apoLp-III; however, the mechanism of the synergistic action of these compounds was not explained.

It is generally believed that antimicrobial peptides and proteins present simultaneously in the insect hemolymph act in concert against invading pathogens. Recently, results confirming this statement have been obtained in our laboratory. It has been found that the activity of *G. mellonella* lysozyme against Gram-negative bacteria considerably increased in the presence of Gm anionic peptide 2, suggesting a synergistic antimicrobial action of these two defense factors [30]. Since the muramidase activity of *G. mellonella* lysozyme was not affected in the presence of anionic peptide 2, the peptide most probably enhanced the non-enzymatic mode of lysozyme action. In addition to these two defense compounds, also apoLp-III occurs constitutively in the *G. mellonella* hemolymph. Taking this into account, research on the impact of apoLp-III on the antibacterial activity of *G. mellonella* lysozyme has been undertaken. In the present study, we demonstrate an evidence and an explanation for the synergistic action of *G. mellonella* lysozyme and apoLp-III against Gram-negative bacteria. Moreover, our results provide novel insights into the mode of action of both these proteins in insect antimicrobial defense.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Gram-positive *M. luteus* ATCC 10240 and Gram-negative bacteria *E. coli* D31 and JM83 were grown at 28 °C and 37 °C, respectively, in 2.5% Luria–Bertani (LB) medium. Bacteria in the logarithmic phase of growth were used during the experiments.

2.2. Insect immune challenge, hemolymph collection, and purification of *G. mellonella* lysozyme and apolipophorin III

The larvae of *G. mellonella* (Lepidoptera: Pyralidae) were reared on honeybee nest debris (a natural diet). The hemolymph was collected 24 h after immunization of the larvae with live *E. coli* D31 and *M. luteus* and then methanolic extracts were prepared from the hemocyte-free hemolymph as described earlier [30, 31].

For the purification of *G. mellonella* lysozyme and apoLp-III, the hemolymph extract dissolved in 0.1% trifluoroacetic acid (TFA) was subjected to HPLC using a Discovery Bio Wide Pore C18 4.6 mm × 250 mm column (Sigma-Aldrich, USA) and two buffer sets: A: 0.1% TFA (v/v), and B: 0.07% TFA, 80% acetonitrile (v/v). A 1 ml/min flow rate and a linear gradient from 30 to 70% of buffer B were applied over 35 min. The homogenous fraction containing apoLp-III eluted as a broad main peak at ca. 31–33 min. The fraction containing lysozyme eluting at 15 min was collected, freeze-dried, redissolved in 0.1% TFA, and additionally purified on the same column and a buffer set as above, using a linear gradient from 37 to 42% B over 15 min. The homogenous peak of lysozyme eluted at 12 min. The fractions containing proteins were freeze-dried and stored at −80 °C until use. The quantity of apoLp-III was determined by weighing the freeze-dried protein powder while the concentration of lysozyme in the solution was determined by amino acid analysis [32]. The homogeneity and identity of both proteins were confirmed by SDS-PAGE electrophoresis [33] and by N-terminal sequencing on an automatic protein sequencer (Procise 491, Applied Biosystems).

2.3. Interaction between molecules of *G. mellonella* lysozyme and apoLp-III

To elucidate the formation of a possible intermolecular complex in vivo, non-immune and immune hemolymph samples (50 μg of total protein) were resolved in non-denaturing conditions [34] and electrotransferred onto PVDF membranes; the membranes were probed with antibodies against *G. mellonella* apoLp-III or against *G. mellonella* lysozyme (see below).

For testing the formation of the complex in vitro, the solutions of purified lysozyme and apoLp-III in 20 mM sodium phosphate pH 6.8 were mixed together at 1:1 or 1:10 molar proportions. After incubation for 1 h at 28 °C or 37 °C, an appropriate volume of a sample buffer (50% glycerol; 0.2% methyl green) was added, and the mixtures were then analyzed by PAGE in non-denaturing conditions [34].

2.4. Immunoblotting

The protein samples subjected to non-denaturing PAGE were transferred onto PVDF membranes (Millipore) as described previously [30]. For lysozyme and apoLp-III detection, the membranes were probed with rabbit polyclonal antibodies to *G. mellonella* lysozyme (1:1000) and to *G. mellonella* apoLp-III (1:2500), respectively. Alkaline phosphatase-conjugated goat anti-rabbit IgG (1:30000) was used as a second antibody. Anti-*G. mellonella* lysozyme antibodies were kindly provided by Prof. I.H. Lee (Department of Life Science, Hoseo University, South Korea). Anti-*G. mellonella* apoLp-III antibodies were custom ordered in Agrisera (Sweden).

2.5. Antibacterial activity of *G. mellonella* lysozyme

2.5.1. Muramidase activity

The enzymatic activity of lysozyme was determined by measuring the decrease in the optical density of the lyophilized *M. luteus* suspension at 450 nm (0.2 mg/ml in 150 mM phosphate buffer pH 6.4) during incubation with lysozyme (0.015 μM), apoLp-III as well as the mixtures of both compounds. Prior to incubation, lysozyme, apoLp-III, and the combinations of lysozyme with apoLp-III were prepared in 20 mM phosphate buffer pH 6.8 (final volume 30 μl) in the wells of a 96-well polypropylene plate. The final molar ratios of lysozyme and apoLp-III were 1:0, 1:0.1, 1:0.2, 1:1, 1:5, 1:10, 1:25, and 0:1. After 15 min preincubation at 28 °C, 100 μl of *M. luteus* suspension was added to the mixtures and the plate was incubated for 3 h at 28 °C with shaking. The optical density of the suspensions was measured every 30 min at 450 nm.

2.5.2. Bacterial membrane permeabilization assay

The membrane perforation ability of lysozyme and apoLp-III was determined by a colorimetric method using an *E. coli* JM83 strain containing plasmid pCH110 (Pharmacia-Amersham, Piscatway, NJ, USA). The plasmid encodes ampicillin resistance and constitutively synthesized cytoplasmic β-galactosidase [35]. The conditions of the assay were described in detail in our previous paper [30]. Briefly, after preincubation of *G. mellonella* lysozyme, apoLp-III, and the mixtures of both compounds in 20 mM phosphate buffer pH 6.8, a suspension containing live mid-logarithmic-phase bacterial cells (5×10^5 CFU) was added and the samples were incubated for 45 min at 37 °C (the final concentrations of the proteins studied were 0.5 μM and 5 μM). Then, a β-galactosidase colorimetric substrate was added and, after an additional 90 min of incubation at 37 °C, absorbance was measured at 405 nm. All the assays were performed in triplicate.

2.6. AFM imaging of bacterial cells

Preparation of bacterial samples and the conditions of cell imaging by AFM were described in detail in our previous paper [30]. Briefly, log-phase *E. coli* JM83 cells were incubated for 1.5 h without (control)

and in the presence of purified *G. mellonella* lysozyme, apoLp-III, or both compounds (final concentrations of 0.5 μM and 5 μM) at 28 °C. After the treatment, the bacteria were suspended in apyrogenic water (5 μl); the samples were applied on the surface of mica disks and allowed to dry overnight at 28 °C before imaging.

AFM imaging of the bacterial cells was carried out using a NanoScope V AFM equipped with NanoScope 8.10 software (Veeco, USA). Three fields were imaged on each mica disk. WSxM 5.0 software (Nanotec, Spain) was used for the analysis of the obtained data. The roughness values were measured on 600×600 nm areas of the entire bacterial cells. For the calculation of the average surface root-mean-square (RMS) roughness, forty fields from two independent experiments were estimated. The elasticity and adhesion properties of the bacterial cells were analyzed with NanoScope Analysis ver. 1.40 software, after force measurements had been performed in the "PeakForce QNM" operation mode using a silicon tip SCANASYST-AIR with a spring constant of 0.4 N/m (Veeco, USA).

2.7. Other methods

The protein concentration in the hemolymph and methanolic extracts was estimated by the Bradford method using BSA as a standard [36].

The data are presented as means ± standard deviation (SD) of at least three experiments. In order to compare two means, statistical analysis was performed by the Student's *t*-test.

3. Results

3.1. The effect of apoLp-III on the level of lysozyme enzymatic activity

The influence of apoLp-III on the lysozyme muramidase activity was examined using a suspension containing *M. luteus* cell wall components as a substrate for lysozyme. The suspension was incubated in the presence of lysozyme or apoLp-III or a mixture of both proteins in different molar ratios, including 1:5, 1:10, and 1:25 (lysozyme: apoLp-III). One-hour incubation with lysozyme (0.015 μM) decreased the optical density of the suspension by ca. 42%, whereas the presence of apoLp-III did not affect its optical density at all the concentrations used (Fig. 1). However, when the *M. luteus* suspension was treated with the mixtures of both compounds, further reduction of the optical density was noticed. At the molar ratios 1:10 and 1:25, the decreases were calculated as ca. 58% and 61%, respectively (Fig. 1). The results obtained suggest that the muramidase activity of *G. mellonella* lysozyme is considerably increased in the presence of apoLp-III.

3.2. The effect of apoLp-III on the level of E. coli perforation by lysozyme

As it was demonstrated in our previous paper, *G. mellonella* lysozyme exhibited a bacterial membrane permeabilization activity when tested against *E. coli* JM83 cells [30]. We were interested in whether the increased muramidase activity of lysozyme detected in the presence of apoLp-III could enhance the above-mentioned lysozyme permeabilizing activity. ApoLp-III used alone at the concentration range of 0.5–5 μM (up to 50 μM) did not perforate *E. coli* cells. However, it increased significantly the perforating activity of *G. mellonella* lysozyme (Fig. 2). Lysozyme used alone at the concentrations of 0.5 μM and 5 μM perforated bacterial cells in ca. 10% and 44%, respectively. When the bacteria were incubated in the presence of 0.5 μM lysozyme, the addition of apoLp-III at the concentrations of 0.5 μM and 5 μM caused two- and three-fold increases in the lysozyme permeabilizing activity, respectively. Treatment of the bacteria with both compounds used at the concentration of 5 μM led to an increase in bacterial membrane perforation from ca. 44% to 60% (Fig. 2). The results presented indicate clearly that the perforation activity of *G. mellonella* lysozyme is enhanced by apoLp-III.

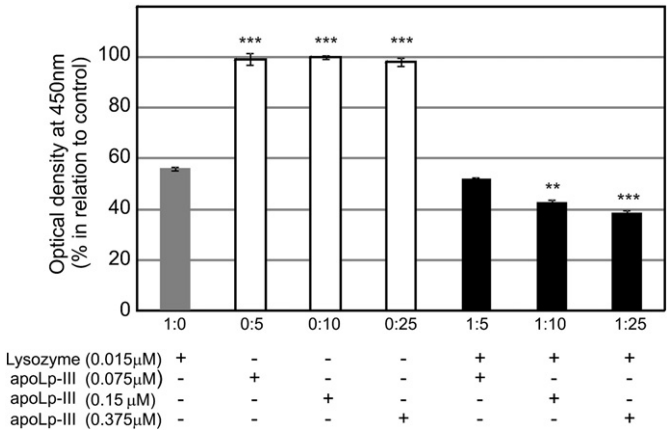


Fig. 1. The effect of apolipophorin III on the muramidase activity of *G. mellonella* lysozyme. The suspension of lyophilized *M. luteus* was incubated without (control) and in the presence of lysozyme (0.015 μM) or apoLp-III or with both compounds at the indicated molar ratios (lysozyme:apoLp-III) as described in Materials and methods. The absorbance at 450 nm was measured every 30 min during a period of 3 h. The diagram shows representative results obtained after 1 h incubation. The optical density of the bacterial suspension incubated without any addition was assumed as 100%. The data are presented as ±SD of three independent experiments. Statistical significance in comparison to optical density of the bacterial suspension treated with lysozyme: $^{**}p<0.01$; $^{***}p\leq0.001$.

3.3. AFM analysis of E. coli cell surface alterations caused by lysozyme and apoLp-III

E. coli JM83 cells treated with *G. mellonella* lysozyme or/and apoLp-III were analyzed by AFM. The analysis revealed that the incubation of the bacterial cells in the presence of the insect defense factors studied caused considerable alterations in the cell surface (Figs. 3, 4). The evident changes in the surface structure were accompanied by the changes in the biophysical properties (Table 1). Despite the fact that apoLp-III did not perforate *E. coli* cells, it induced alterations in the bacterial cell surface and modified, to some extent, the morphology of the bacteria. When the bacterial cells were treated with apoLp-III used at a concentration of 0.5 μM, the cell surface became rougher in comparison to the control cells (Fig. 3). The RMS

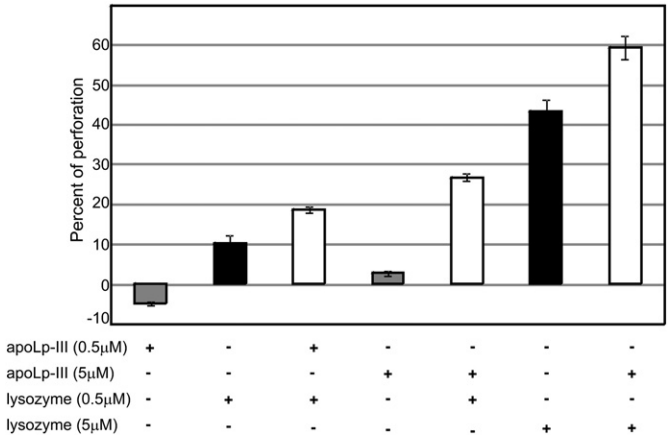


Fig. 2. The effect of apolipophorin III on the bacterial membrane permeabilizing activity of *G. mellonella* lysozyme. *E. coli* JM83 cells were incubated without (control) and in the presence of lysozyme or apoLp-III (0.5 μM and 5 μM) or both compounds as described in Materials and methods. Then β-galactosidase substrate was added and the absorbance was measured at 405 nm. The perforation level of the dead bacteria was assumed as 100%. The data are presented as ±SD of three independent experiments.

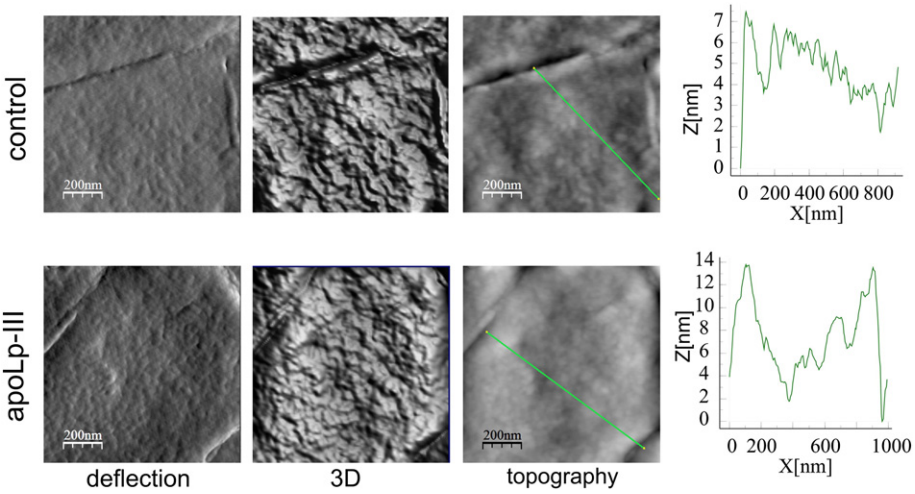


Fig. 3. AFM image of *E. coli* JM83 cell surface alterations after treatment with *G. mellonella* apolipophorin III. The bacteria were incubated without (control) or in the presence of apoLp-III (0.5 μM) as described in Materials and methods; then, the microscopic analysis was performed. The deflection, 3D, and topography images are presented. The diagrams show the section profiles corresponding to the green lines visible in the topography images.

roughness values for the control and apoLp-III-exposed bacteria were calculated as 7.653 nm (±2.71) and 11.564 nm (±4.92; $p = 0.01$), respectively. Upon treatment of *E. coli* with apoLp-III used at a concentration of 5 μM, further cell surface alterations appeared and some changes in the bacterial cell morphology were observed (Fig. 4A). Some blurred cell contours were noticed especially at the poles. Although the cells incubated in the presence of *G. mellonella* lysozyme (0.5 μM) retained their shape, numerous cavities and altered surface texture of the cells were detected. In addition, the flagella, well visible in the control cell images, were no longer noticed (Fig. 4A). The *E. coli* cells treated with both compounds presented a different picture. The cells were still rod-shaped, but their poles were not round. They also exhibited a distinct surface texture (Fig. 4A). The cells treated with each compound, however, especially those exposed to lysozyme, were much higher than the control ones, probably indicating swelling symptoms (Fig. 4B). The bacterial cells exposed to *G. mellonella* lysozyme and/or apoLp-III demonstrated significant differences in elasticity, reflected by Young's modulus, as well as in adhesive forces and roughness values in comparison to the control ones (Table 1). Treatment of *E. coli* with lysozyme or apoLp-III alone led to a significant, ca. 2.7–3.1 fold, decrease in the bacterial cell surface elasticity, which was accompanied by a ca. 1.8–2.3 fold increase in adhesive forces to the AFM tip. In addition, an increase in the RMS roughness values was detected. However, although the treatment of the bacteria with apoLp-III or lysozyme alone induced apparent changes of all the cell surface properties tested, only adhesion force was altered considerably after exposing the bacteria to the mixture of both compounds. In comparison to the control cells, a 2.9-fold increase in this parameter value was noticed, indicating that the treatment of *E. coli* with both compounds had a stronger impact on adhesion forces than the exposure to apoLp-III or lysozyme alone (Table 1).

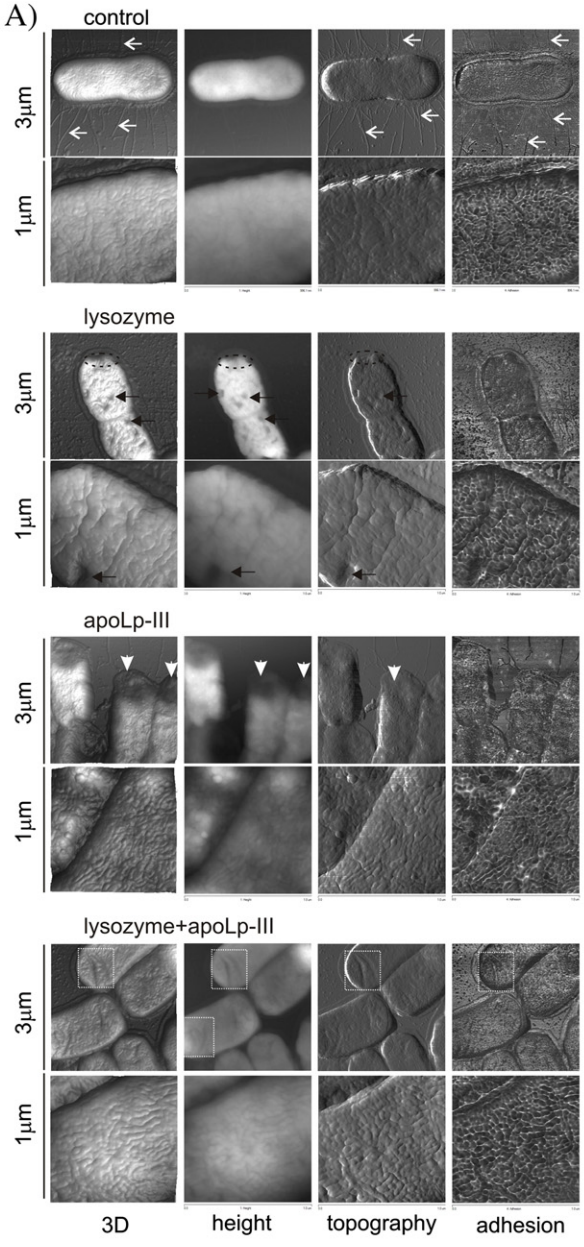
3.4. Analysis of intermolecular complex formation between G. mellonella lysozyme and apoLp-III

In order to provide more insight into the mode of the synergistic action of the proteins studied, the possibility of intermolecular complex formation between apoLp-III and lysozyme in vivo and in vitro was investigated. For evaluating whether the proteins form a complex in vivo, the samples of *G. mellonella* non-immune and immune hemolymphs collected at different time points after the challenge were resolved in non-denaturing conditions and electrotransferred onto PVDF membranes. Then the membranes were probed with antibodies against *G. mellonella* apoLp-III and *G. mellonella* lysozyme. Several bands reflecting apoLp-III were recognized by anti-apoLp-III antibodies, suggesting formation of complexes with other hemolymph proteins or the presence of apoLp-III isoforms in the hemolymph. The multiple apoLp-III bands could also be explained by the occurrence of the lipid-free and lipid-bound populations of this protein in *G. mellonella* hemolymph. In contrast, only one band was recognized by anti-lysozyme antibodies in all the hemolymph samples tested (Fig. 5). The location of the apoLp-III and lysozyme bands on the membranes indicated that the proteins did not form intermolecular complexes in vivo in the hemolymph of the naive as well as immune-challenged insects. These findings were confirmed by in vitro experiments in which potential complex formation between purified apoLp-III and lysozyme was investigated. Incubation of the proteins in different molar ratios at two different temperatures (28 °C and 37 °C) did not lead to the formation of a complex as revealed by electrophoresis in non-denaturing conditions (data not shown). All these results demonstrate clearly that the observed apoLp-III-induced enhancement of the permeabilizing activity of *G. mellonella* lysozyme was not connected with the intermolecular interactions between the proteins.

4. Discussion

Binding of *G. mellonella* lysozyme and apoLp-III to the cell surface of different Gram-positive and Gram-negative bacteria, including *E. coli*, was demonstrated in our previous reports [18, 30]. In addition, the synergistic action of two constitutive components of *G. mellonella* hemolymph, lysozyme and Gm anionic peptide 2, against Gram-negative bacteria was documented [30]. The present study provides further evidence on the synergistic action of *G. mellonella* lysozyme and other hemolymph defense factors against this class of bacteria. The permeabilizing activity of *G. mellonella* lysozyme toward *E. coli* cells was significantly enhanced in the presence of apoLp-III, which did not

Fig. 4. AFM image of *E. coli* JM83 cell surface alterations after treatment with *G. mellonella* lysozyme and apolipophorin III. The bacteria were incubated without (control) or in the presence of lysozyme (0.5 μM) or apoLp-III (5 μM) or both compounds as described in Materials and methods; then, the microscopic analysis was performed. (A) 3D, height and topography images, as well as the image of the adhesion analysis are presented. The white arrows mark flagella, well visible in the control images. The black arrows and black ellipses indicate the cavities and pole damages, respectively, appearing after the treatment of cells with lysozyme. The white arrowheads indicate blurred contours at the poles of the cells treated with apoLp-III. The white squares mark the altered poles of the cells treated with lysozyme and apoLp-III. (B) The height images and diagrams showing the section profiles corresponding to the green lines in the images are presented.


A)
control
lysozyme
apoLp-III
lysozyme+apoLp-III
3µm
1µm
3D
height
topography
adhesion

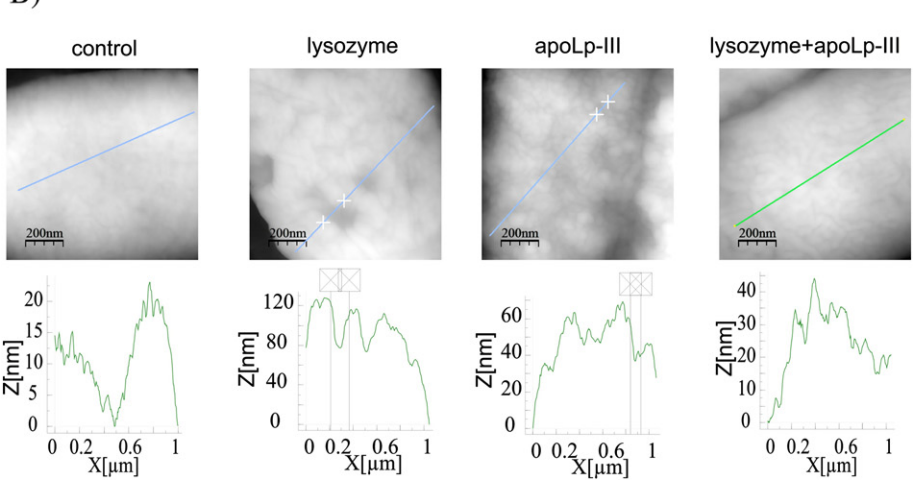

B)
control
lysozyme
apoLp-III
lysozyme+apoLp-III
200nm
Z[nm]
X[µm]

Table 1
The effect of *G. mellonella* lysozyme and apolipophorin III on the biophysical properties of the *E. coli* JM83 cell surface.

	Control	Lysozyme (0.5 μM)	apoLp-III (5 μM)	Lysozyme + apoLp-III (0.5 μM + 5 μM)
Young's modulus (GPa)	2.118 (±0.364)	0.681*** (±0.197)	0.796*** (±0.264)	1.856 (±1.158)
Adhesion force (nN)	0.357 (±0.182)	0.813*** (±0.175)	0.634*** (±0.11)	1.053*** (±0.233)
RMS roughness (nm)	7.653 (±2.71)	10.718*** (±4.524)	12.218*** (±5.29)	7.584 (±2.0)

The bacteria were incubated without (control) or in the presence of lysozyme, apoLp-III or both compounds. Analysis of the biophysical properties of the bacterial cell surface was performed using AFM. The data are presented as means ± SD. Statistical significance: *** $p \leq 0.001$.

perforate the bacteria when used alone. To elucidate this question, the possibility of complex formation between apoLp-III and lysozyme molecules was evaluated. Our results demonstrated that the proteins did not form intermolecular complexes either in vivo or in vitro, as revealed by PAGE in non-denaturing conditions. Similar results were described by Halwani and Dunphy [29]; however, instead of *G. mellonella* lysozyme, a commercial egg white lysozyme was used in their study. Another explanation of the observed phenomenon might be the induction of alterations in the bacterial cell surface upon the binding of apoLp-III. The changes caused by apoLp-III in the cell surface of different bacteria were described in our previous paper [19]. Evidence supporting such prediction has been demonstrated recently by Oztug et al. [17], who have reported the formation of stable complexes consisting of four *G. mellonella* apoLp-III molecules and 24 molecules of *E. coli* LPS. Moreover, as found by Zakarian et al. [37], the binding of *G. mellonella* apoLp-III increased hydrophobicity as well as the negative charge of the bacterial cell surface. Such alterations in the properties could enhance the interaction of cationic lysozyme molecules with bacterial cell surface and facilitate more effective peptidoglycan digestion, finally leading to a cell death. The alterations in the biophysical properties of the bacterial cell surface detected by us using AFM analysis indicated that the presence of both apoLp-III and lysozyme altered the surface characteristics considerably. However, apparent changes in the properties analyzed, such as elasticity, adhesion forces, and roughness, were detected in *E. coli* cells exposed to apoLp-III or lysozyme alone. Although the treatment of the bacteria with a mixture of both compounds led to a further increase in adhesion forces, surprisingly, the other parameters were not altered in comparison to the control cells. Logically thinking, one would expect that the increased

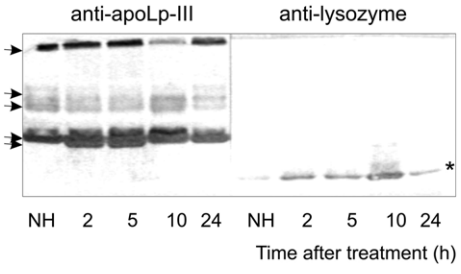


Fig. 5. Analysis of complex formation between *G. mellonella* apolipophorin III and lysozyme in vivo. The non-immune (NH) and immune hemolymph samples (50 μg of total protein) collected at different time points after the challenge were resolved by PAGE in non-denaturing conditions as described in Materials and methods. Then the proteins were electrotransferred onto PVDF membranes and the membranes were probed with antibodies against *G. mellonella* apoLp-III or *G. mellonella* lysozyme. The arrows and the asterisk indicate several bands and a single protein band recognized by anti-apoLp-III and anti-lysozyme antibodies, respectively.

permeabilizing effect of the simultaneous action of both factors studied against *E. coli* should be reflected by considerable changes in the parameters describing the biophysical properties of the surface of the treated cells. However, since the Gram-negative bacterial cell envelope is a complex structure consisting of the extracellular polymeric substance (EPS) and outer membrane components, this relationship may be much more complicated. The interactions of one compound (e.g. apoLp-III) with the *E. coli* cell surface could certainly affect the binding of the other one (e.g. lysozyme). In addition, this could provoke unusual interactions between the cell envelope molecules. Moreover, the biophysical properties of the cell surface of *E. coli* treated with both apoLp-III and lysozyme could change continuously during the treatment, however in a different manner and to a different extent than while exposing the bacteria to a single defense protein. Therefore, the final effect, determined at a certain time point, might not be reflected by the changes in each biophysical parameter of the surface.

Taking the above into consideration, *G. mellonella* apoLp-III seems to potentiate the anti-*E. coli* activity of lysozyme in two steps. Initial binding of apoLp-III to the bacterial cell surface (most probably to LPS) alters cell surface texture and properties in a way that facilitates and/or accelerates lysozyme binding to the bacteria. Simultaneous binding of both proteins to the bacterial cell surface could also take place. After the interaction of lysozyme with the altered cell surface, apoLp-III, still surface-bound, enhances the muramidase activity of lysozyme by a yet unexplained mechanism. In our study, *G. mellonella* apoLp-III increased the muramidase activity in a standard assay, in which dead *M. luteus* cells were used. This fact, together with the observation that apoLp-III and lysozyme do not form intermolecular complexes, suggests that apoLp-III enables a more efficient action of lysozyme by influencing the properties of certain bacterial cell wall components. Possibly, the changes caused by apoLp-III interaction with the *E. coli* cell envelope are sufficient to facilitate accessibility of the peptidoglycan layer to the muramidase activity of lysozyme. Considerably, the enhancing effect of apoLp-III on both perforating and muramidase lysozyme activities was observed at the same molar excess of apoLp-III (lysozyme:apoLp-III — 1:10), supporting the idea that the apoLp-III-induced increment in the muramidase activity could explain the enhanced perforating activity of lysozyme toward *E. coli*. This is noticeable because the lysozyme concentration in *G. mellonella* hemolymph is much lower in comparison to the apoLp-III concentration. In the hemolymph of *G. mellonella* naive larvae, this protein is present constitutively at a concentration of ca. 0.76 μM (±0.17 μM); however, in bacteria-challenged insects a ca. 10-fold increase in the lysozyme concentration was reported [8]. In contrast, the hemolymph titer of apoLp-III in *G. mellonella* larvae was estimated at 0.7 mg/ml (ca. 40 μM) [38] and even 8.7 mg/ml (ca. 480 μM) [29]. On the basis of our study, the concentration of this protein in *G. mellonella* non-immune hemolymph was calculated as ca. 350–400 μM (unpublished data). A simple mathematical calculation reveals at least 50-fold (or even 600-fold) molar excess of apoLp-III in comparison to lysozyme in the hemolymph of the naive larvae. However, one should keep in mind that apoLp-III exists in hemolymph in a free and lipophorin-bound form [13]. Interaction with lipophorin particles decreases the concentration of free apoLp-III, which would bind to a bacterial cell surface and act synergistically with other defense factors.

It can be postulated that apoLp-III plays an analogous role in an insect immune response to that played by bactericidal/permeability-increasing protein (BPI) in a mammalian innate immunity. BPI is a 55 kDa cationic antimicrobial polypeptide exhibiting, likewise apoLp-III, high affinity to the lipid A region common to all LPS of Gram-negative bacteria. After binding to LPS in the bacterial outer membrane, BPI molecules penetrate the bacterial inner membrane, which leads to the loss of membrane integrity, electrochemical gradient impairment, and death of the bacterium [39]. Moreover, the BPI's antibacterial activity is synergistically enhanced by defense peptides, such as defensins and cathelicidins [40].

In contrast to our previous study, in which the synergistic action of *G. mellonella* lysozyme and anionic peptide 2 against *E. coli* led to the formation of sodium chloride crystals suggesting the destabilization of the osmotic balance of the bacteria [30], no crystals were detected after the treatment of *E. coli* with a mixture of lysozyme and apoLp-III. Moreover, anionic peptide 2 enhanced the antibacterial activity of *G. mellonella* lysozyme without affecting its enzymatic activity [30]. In contrast, as presented in this study, apoLp-III increased the muramidase activity of *G. mellonella* lysozyme. These findings clearly indicate a distinct mode of the synergistic antibacterial action of apoLp-III and anionic peptide 2 with lysozyme in *G. mellonella* hemolymph. Our results suggest that the effect of the action of *G. mellonella* lysozyme against Gram-negative bacteria could be amplified through increasing its enzymatic and non-enzymatic activities by apoLp-III and anionic peptide 2, respectively.

As demonstrated previously, the growth of *E. coli* was not inhibited by *G. mellonella* apoLp-III, whereas other Gram-negative bacteria, such as *Salmonella Typhimurium* and *Klebsiella pneumoniae*, were highly sensitive to apoLp-III action [18]. The causes of the different antibacterial activity of apoLp-III remain unclear. During binding to LPS, apoLp-III interacts with lipid A as well as a carbohydrate core of the LPS molecule [16]. Since lipid A represents the least heterogeneous region of the LPS molecule [41, 42], the differences in the apoLp-III activity toward Gram-negative bacteria could be related to the differences in the structure and composition of the carbohydrate core regions. However, irrespective of this, it is possible that an effective fight against particular bacteria, e.g. *E. coli*, requires cooperation between certain *G. mellonella* hemolymph defense factors. So far, the results of our research on *G. mellonella* antimicrobial peptides and proteins provided two examples of such cooperation: lysozyme with anionic peptide 2 and lysozyme with apoLp-III. Taking into consideration the constitutive presence of these three defense molecules in *G. mellonella* hemolymph and the fact that apoLp-III enhances lysozyme muramidase activity, they can be assumed as important antibacterial factors acting at the early stage of infection with Gram-negative as well as Gram-positive bacteria.

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