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Galleria mellonella apolipophorin III – an apolipoprotein with anti-*Legionella pneumophila* activity

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ABSTRACT

The greater wax moth *Galleria mellonella* has been exploited worldwide as an alternative model host for studying pathogenicity and virulence factors of different pathogens, including *Legionella pneumophila*, a causative agent of a severe form of pneumonia called Legionnaires' disease. An important role in the insect immune response against invading pathogens is played by apolipophorin III (apoLp-III), a lipid- and pathogen associated molecular pattern-binding protein able to inhibit growth of some Gram-negative bacteria, including *Legionella dumoffii*. In the present study, anti-*L. pneumophila* activity of *G. mellonella* apoLp-III and the effects of the interaction of this protein with *L. pneumophila* cells are demonstrated. Alterations in the bacteria cell surface occurring upon apoLp-III treatment, revealed by Fourier transform infrared (FTIR) spectroscopy and atomic force microscopy, are also documented. ApoLp-III interactions with purified *L. pneumophila* LPS, an essential virulence factor of the bacteria, were analysed using electrophoresis and immunoblotting with anti-apoLp-III antibodies. Moreover, FTIR spectroscopy was used to gain detailed information on the type of conformational changes in *L. pneumophila* LPS and *G. mellonella* apoLp-III induced by their mutual interactions. The results indicate that apoLp-III binding to components of bacterial cell envelope, including LPS, may be responsible for anti-*L. pneumophila* activity of *G. mellonella* apoLp-III.

1. Introduction

Legionellae are Gram-negative bacteria found in moist natural environments as intracellular parasites of protozoa. In humans, the virulence mechanisms evolved by these bacteria enable attachment, survival, and replication inside macrophages. Infection of alveolar macrophages can cause a severe form of pneumonia—Legionnaires' disease [1]. The highly specific interactions of *Legionella* with host organisms, which allow them to generate a replication-permissive niche, depend on many factors. A crucial role in the generation of a replication-permissive environment is played by the Dot/Icm type IV secretion system (T4SS). This system secretes and translocates into the host cells the bacterial proteins that modulate phagosome biogenesis to evade endocytic fusion [2]. Additionally, pathogenicity of intracellular parasites, such as *Legionella* spp., largely depends on the characteristics and components of the cell envelope: lipopolysaccharide (LPS), lipids, proteins, and peptidoglycan [3]. Despite its similar general architecture, *Legionella pneumophila* LPS differs considerably from LPS of other Gram-negative bacteria. The O-chain of *L. pneumophila* LPS is a homopolymer of an unusual sugar, i.e. legionaminic acid. The outer core is a seven-sugar oligosaccharide composed of rhamnose (Rha), mannose (Man), acetylquinovosamine (QuiNAc), and acetylglucosamine (GlcNAc) in the molar ratio of 2.1:1.1:1:1.4. The oligosaccharide outer core is hydrophobic, likewise the O-specific chain. Its hydrophobicity is a consequence of the presence of *N*-acetyl aminosaccharide groups (QuiNAc and GlcNAc) as well as methyl groups of 6-deoxy sugars, Rha and QuiNAc [4]. The hydrophobic nature of the O-chain and the outer core of *L. pneumophila* LPS facilitates concentration of the bacterial cells in aerosols and enables the pathogen to reach alveolar macrophages and to initiate pulmonary infection in humans. Moreover, LPS containing vesicles released from the outer membrane of growing *L. pneumophila* inhibit phagosome–lysosome fusion in infected macrophages independently of the Dot/Icm secretion system [5]. Compared to other Gram-negative bacteria, the *Legionella* LPS has a high content of non-hydroxy fatty acids (ester-linked) and long-chain (ω-1)-oxo, (ω-1)-hydroxy as well as (1, ω)-dioic fatty acids (ester-linked) [6]. In *L. pneumophila* lipid A, there are eight non-hydroxylated acids, which contain from 14 to 20

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carbon atoms in the molecule, and five long-chain fatty acids [28:0(27-OH), 28:0(27-oxo), 30:0(29-oxo), 27-dioic, and 29-dioic]. The 27-oxo-octacosanoic acid is generally present in all *Legionella* studied, and thus, serves as a useful marker of this group [7]. Mature LPS molecules are located in the outer membrane of the *Legionella* cell envelope and are components of its lipid bilayer, besides phospholipids, lipoproteins and proteins.

Recently, the greater wax moth *Galleria mellonella* has been exploited as an alternative model host for studying *L. pneumophila* virulence [8–10]. In the search of potential anti-*Legionella* agents, we examined defence peptides and proteins—the well-known and effective antibacterial and antifungal components of *G. mellonella* hemolymph. Susceptibility of *Legionella dumoffii* to defensin and apolipophorin III (apoLp-III) purified from *G. mellonella* hemolymph was reported in our previous paper [11]. ApoLp-III is a lipid-binding protein occurring abundantly in hemolymph of *G. mellonella* larvae. This protein is involved in lipid transport as well as insect immune response. The apoLp-III molecule is composed of five antiparallel amphipathic α-helices forming a bundle in which hydrophobic surfaces are directed inwards and the hydrophilic regions outwards the bundle. The protein is an exchangeable component of insect lipoproteins, i.e. lipophorins, and exists in hemolymph in a lipid-free as well as lipid-bound state. Upon lipid binding, apoLp-III undergoes considerable conformational reorganization leading to bundle opening [12,13]. The protein is classified as a pattern recognition receptor, as it binds pathogen associated molecular patterns (PAMPs), e.g. bacterial LPS and lipoteichoic acid (LTA), and fungal β-1,3-glucan [14–17]. Binding of *G. mellonella* apoLp-III to the cell surface of different bacteria, yeasts, and filamentous fungi has been demonstrated [18,19]. In *G. mellonella* hemolymph, apoLp-III acts synergistically with lysozyme and other defence peptides [20–25]. It has been documented that *G. mellonella* apoLp-III significantly inhibited growth of Gram-negative bacteria, including *L. dumoffii* [11,18,26]. Interestingly, susceptibility of *L. dumoffii* to apoLp-III was dependent on the composition of the medium used for bacterial culture. The bacteria grown on a choline-enriched medium were three times more susceptible to apoLp-III than the ones growing on a non-supplemented medium, suggesting an important role of phosphatidylcholine (PC) in apoLp-III binding to *L. dumoffii* cells [11].

In the present study, anti-*L. pneumophila* activity of *G. mellonella* apoLp-III as well as the effects of the interaction of this protein with *L. pneumophila* cell surface are demonstrated. Alterations in the bacteria cell surface topography occurring upon apoLp-III treatment, revealed by an atomic force microscopy (AFM) analysis, are documented. Moreover, a Fourier transform infrared (FTIR) spectroscopy analysis confirming changes in *L. pneumophila* cell surface resulting from apoLp-III binding as well as the interactions of apoLp-III with isolated *L. pneumophila* LPS is presented. The results indicate that apoLp-III binding to components of bacterial cell envelope, including LPS, may be responsible for anti-*L. pneumophila* activity of *G. mellonella* apoLp-III.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. pneumophila ATCC 33152 was cultured for 3 days at 37 °C in humid atmosphere and 5% CO_2 on buffered charcoal yeast extract (BCYE) agar plates supplemented with the Growth Supplement SR110 (Oxoid, Basingstoke, Hampshire, UK). For the growth of the bacteria on the choline-enriched medium, BCYE agar was supplied with 100 μg/ml choline chloride (Sigma–Aldrich, Chemical Co., St. Louis, MO, USA). The bacteria collected from this medium were washed for three times with pyrogen-free water by intensive vortexing and centrifugation at 8000 ×*g* for 10 min. *Escherichia coli* D31 and *Micrococcus luteus* ATCC 10240 were grown in 2.5% LB medium at 37 °C or 28 °C, respectively.

2.2. Immunisation of insects and hemolymph collection

The larvae of the greater wax moth *G. mellonella* (Lepidoptera: Pyralidae) were reared on a natural diet, honey bee nest debris, at 30 °C in darkness. Last instar larvae were immune-challenged by puncturing the last proleg with a needle dipped into a pellet containing live *E. coli* and *M. luteus* cells [27]. An acidic/methanolic extract, rich in peptides and proteins below 30 kDa, was prepared from the hemolymph collected 24 h later. Then the extract was deprived of lipids, freeze-dried, and stored at −80 °C [28]. The protein concentration in the hemolymph and methanolic extract was determined according to Bradford [29].

2.3. Purification of G. mellonella apoLp-III

ApoLp-III was purified from the methanolic extract of *G. mellonella* immune hemolymph using a modified technique described in our previous study [25]. Briefly, the hemolymph extract was dissolved in 0.1% trifluoroacetic acid (TFA) and subjected to the HPLC chromatography using a Discovery Bio Wide Pore C18 4.6 mm × 250 mm column (Sigma–Aldrich, USA). Two buffers, A: 0.1% TFA (v/v) and B: 0.07% TFA, 80% acetonitrile (v/v), a linear gradient from 30 to 70% of buffer B over 35 min, and a 1 ml/min flow rate were used. The homogenous fraction containing apoLp-III, eluting as a broad main peak at ca. 31–33 min, was freeze-dried and stored at −80 °C until use. The quantity of apoLp-III was determined by weighing of the freeze-dried protein powder, whilst its homogeneity and identity was confirmed by SDS-PAGE electrophoresis [30] and by N-terminal sequencing on an automatic protein sequencer (Procise 491, Applied Biosystems).

2.4. Fluorescent labelling of apoLp-III

Two milligrams of apoLp-III were dissolved in 200 μl of sodium borate pH 9.0 and to this solution an equimolar amount of dimethylsulfoxide solution of fluorescein isothiocyanate (FITC, isomer 1, Sigma) was added. The solution was mixed, incubated in darkness for 60 min at room temperature and then the unreacted FITC was quenched by the addition of 50 μl of 1 M glycine solution in water. After successive 30 min in darkness the mixture was acidified to pH 2.0 using TFA and subjected to HPLC chromatography on a C18 column (the details concerning the column and buffers used were identical to those ones presented above in subsection 2.3.). A linear gradient from 55 to 70% of buffer B over 20 min and 1 ml/min flow rate was applied. The first and the most hydrophilic peak containing monosubstituted FITC-apoLp-III was manually collected and freeze-dried. After quantitation by weight the labelled protein was additionally checked by SDS-PAGE electrophoresis (a single yellow band at 18 kDa was obtained). Before use the FITC-apoLp-III was dissolved in sterile water.

2.5. Antibacterial activity assay

The activity of *G. mellonella* apoLp-III against *L. pneumophila* was determined using a colony-counting assay. A bacterial suspension of OD_{620} of 0.1 was diluted 10^{-5}-fold in pyrogen-free water. 5 μl of the final dilution of the bacterial culture was transferred into a sterile Eppendorf tube, mixed with 5 μl of pyrogen-free water (control) or apoLp-III solution (final concentrations 0.1–1.6 mg/ml), and incubated for 1 h at 37 °C. Next, the mixtures were transferred onto BCYE plates and the colonies were counted after 3 days of incubation at 37 °C and 5% CO_2. The experiments with the bacteria cultured on the choline-enriched medium were performed in the same way. The controls defined the total (100%) survival of *L. pneumophila* cells incubated under identical conditions but without apoLp-III addition. Results from the antimicrobial assay represent the mean of three independent experiments, with three replicates per experiment.

2.6. Binding of G. mellonella apoLp-III to L. pneumophila cells

2.6.1. Binding of unlabelled apoLp-III

40 μl of suspensions containing *L. pneumophila* cells ($OD_{620} = 0.2$) cultured on the BCYE medium were incubated without (control) and in the presence of *G. mellonella* apoLp-III (final concentrations 0.4 mg/ml and 0.1 mg/ml for AFM and FTIR-ATR analysis, respectively) at 37 °C for 1 h. Then the bacterial suspensions for FTIR-ATR analysis were immediately applied to the crystal and analyzed (see subsection 2.11 for details). The bacterial suspensions for AFM analysis were centrifuged at 8000 ×*g* for 10 min at 4 °C and prepared on the mica discs for imaging as described previously [26].

2.6.2. Binding of FITC-labelled apoLp-III

40 μl of suspension containing *L. pneumophila* cells ($OD_{620} = 0.2$) cultured on the BCYE medium was incubated with FITC-apoLp-III (final concentration 0.1 mg/ml) at 37 °C for 15 min. Then the bacterial suspension was centrifuged (2000 ×*g*, 10 min, 4 °C) and washed twice with 100 μl of 20 mM phosphate buffer pH 7.4 containing 0.9% NaCl. After final centrifugation, the bacterial cells were suspended in 10 μl of 20 mM phosphate buffer pH 7.4, and imaged using a confocal laser scanning microscope LSM 5 PASCAL (Carl Zeiss, Jena, Germany) (excitation and emission wavelengths were 470 nm and 520 nm, respectively; excitation time was 600 ms).

2.7. AFM imaging

The *L. pneumophila* cell surface structure and nanomechanical properties were imaged and analysed using a NanoScope V AFM (Veeco, USA) (Analytical Laboratory, Faculty of Chemistry, UMCS, Lublin, Poland). All measurements were performed in the "Peak Force QNM" operation mode using a silicon tip with a spring constant of 20 N/m (NSG30, NT-MDT, Russia). The data were analysed with Nanoscope Analysis ver. 1.40 software (Veeco, USA). Three fields on each mica disc were imaged. Roughness values were measured over the entire bacterial cell surface in 3 μm × 3 μm areas. The average surface root-mean-square (RMS) roughness of the cells was calculated from forty fields (270 nm × 270 nm) estimated during two independent experiments. The Student's *t* test was used to establish differences between two mean values. Three dimensional (3D) images and section profiles of the cells were generated using WSxM 5.0 software (Nanotec, Spain) [31].

2.8. Isolation and purification of L. pneumophila LPS

After the culture on the BCYE medium, *L. pneumophila* cells were harvested by centrifugation (8 000 ×*g*, 30 min). The bacterial pellet was washed twice with 0.5 M saline and once with distilled water. Lyophilised dried mass of the cells was delipidated with a chloroform: methanol mixture (1:2; v/v) according to the Bligh and Dyer method [32], suspended in water and lyophilised again. The bacterial cells were digested with lysozyme, DNAse, RNAse (1 mg/ml, 24 h, 37 °C; Sigma–Aldrich, USA), and then with proteinase K (1 mg/ml, 36 h, 37 °C; Sigma–Aldrich, USA) in 50 mM phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$. The suspension was dialysed against distilled water. LPS from the digested cells was extracted two times with the hot 45% phenol/water procedure [33,34]. The two phases (water and phenol) and the interphase obtained after the extraction were dialysed extensively against tap, and then distilled water. The crude material from both phases and the interphase was purified by repeated ultracentrifugation at 105 000 ×*g* for 4 h (Beckman Coulter, SW 32Ti rotor), suspended in water, lyophilised, and weighed. In all the phases, the composition of sugars with reference to the standards (inositol for neutral sugars and mannosamine for amino sugars) and of fatty acids (tricosanoic acid) was determined as described by Sonesson et al. [35] (data not shown). Fatty acids in the form of their methyl esters and sugars in the form of their alditol acetates were identified by gas–liquid chromatography–mass spectrometry (GLC–MS; see subsection 2.7). In agreement with literature data, material with the composition characteristic of smooth *L. pneumophila* LPS was recovered from the phenol phase [35].

L. pneumophila LPS recovered from the phenol phase was electrodialysed in order to remove cations [36]. Approximately 16 mg of LPS was dissolved in 7 ml of deionised water. The LPS was placed in the dialysis sac (10 kDa), which was put into the inner chamber of the electrophoresis apparatus (BioRad). Electrodialysis was performed for 24 h at 500 mA, 200 V and 40 W. The water in the chambers was changed every 5 h. LPS from the dialysis sac was then lyophilised. Prior to further analyses, LPS dissolved in water was sonicated in a water bath at a room temperature for 10 min (Elmasonic S100H, Elma, Germany). To be sure that the LPS preparation is free from any proteolytic activity, gelatin and casein zymography as well as colorimetric methods were used [37]. No proteolytic activity was detected in the preparation of purified *L. pneumophila* LPS.

2.9. GLC–MS analysis of fatty acids and sugars of L. pneumophila LPS

GLC–MS was carried out on an Agilent 7890A-5975C instrument equipped with an HP-5 ms (SLB-5 ms) capillary column (30 m × 0.25 mm; Supelco, St. Louis, MO, USA). Helium was the carrier gas and the temperature programme was initially 150 °C for 5 min, then it was raised to 310 °C at a ramp rate of 3 °C min^{-1} and final time of 20 min. The structures of fatty acids and sugars were elucidated as described previously [35,38].

2.10. Binding of G. mellonella apoLp-III to L. pneumophila LPS

The *L. pneumophila* LPS (4 μg and 50 μg for SDS-PAGE and FTIR analysis, respectively) and apoLp-III were incubated for 1 h at 37 °C alone (the controls) and in combination in the ratio 1:1 for SDS-PAGE and 5:1 for the FTIR analysis. The temperature conditions used were adapted from Oztug et al. [15], who demonstrated optimal binding of *G. mellonella* apoLp-III with *E. coli* LPS at 37–40 °C, close to the LPS phase transition temperature.

2.11. FTIR analysis of apoLp-III interactions with L. pneumophila cell surface and with purified LPS

Infrared absorption spectra were recorded using the Fourier-transform infrared absorption spectrometer equipped with the attenuated total reflection set up (FTIR-ATR). The IR absorption spectra were recorded with a Vector 33 spectrometer (Bruker, Germany). The internal reflection element was a ZnSe crystal (45° cut) yielding 10 internal reflections. Typically, 20 scans were collected, Fourier-transformed, and averaged for each measurement. Absorption spectra were collected in the region between 4000 and 600 cm^{-1} at a resolution of one data point every 2 cm^{-1}. The spectrum of a clean crystal was used as the background. The samples were deposited on the ZnSe crystal via partial evaporation from water solutions. The instrument was purged with dry air for 40 min, before and continuously during the measurements. The ATR crystal was cleaned with organic solvents. All measurements were done at 21 °C. Spectral analysis was performed with OPUS (Bruker, Germany) and Grams AI (Thermo Galactic, USA) software. The spectra were corrected by subtraction of the water vapour contributions and smoothing procedure (Golay–Savitzky on 21 points). More details of the measurements were presented previously [39].

2.12. SDS-polyacrylamide gel electrophoresis

In order to obtain proper separation of proteins and LPS in polyacrylamide gels (12.5%), the samples examined for apoLp-III or LPS were anayzed, respectively, by SDS-PAGE according to Laemmli [40]

or Tris-tricine SDS-PAGE [41]. For this reason, the samples incubated as described above (subsection 2.10) were prepared in appropriate sample buffers. The buffer containing 175 mM Tris–HCl pH 6.8, 0.25% SDS, 2% glycerol, and 0.04% bromophenol blue was used for LPS analysis, whilst for apoLp-III analysis the buffer consisting of 30 mM Tris–HCl pH 6.8, 1% SDS, 50 mM DTT, 5% glycerol, and 0.04% bromophenol blue was applied. Electrophoresis of the samples examined for LPS was performed at constant current, initially at 15 mA and then at 35 mA, whereas those examined for apoLp-III at a constant voltage of 100 V. After resolution, the apoLp-III and LPS were visualised by Coomassie Brilliant Blue and silver staining, respectively [42].

2.13. Immunoblotting

For apoLp-III detection, the samples resolved by SDS-PAGE were electrotransferred onto PVDF membranes (Millipore) for 90 min at 350 mA. Then the membranes were probed with rabbit polyclonal antibodies to *G. mellonella* apoLp-III (1:2500; Agrisera, Sweden; custom ordered) and alkaline phosphatase-conjugated goat anti-rabbit IgGs (1:30000; Sigma–Aldrich, USA) as described previously [18].

3. Results

3.1. The effect of G. mellonella apoLp-III on the L. pneumophila survival rate

L. pneumophila susceptibility to apoLp-III was tested by a colony-counting assay. ApoLp-III used at the concentrations from 0.1 to 1.6 mg/ml reduced the bacterial survival rate in a dose-dependent manner. As presented in Table 1, apoLp-III at the concentration of 0.1 mg/ml killed ca. 55% of *L. pneumophila* cells, whereas less than 10% of the cells survived in the presence of 0.8 mg/ml of apoLp-III. No bacterial growth was detected after the exposure of the bacteria to the 1.6 mg/ml concentration of apoLp-III (Table 1).

Our previous study showed that an increased PC content in *L. dumoffii* cells enhanced the antimicrobial activity of *G. mellonella* apoLp-III against these bacteria. In the presence of apoLp-III, a 3-fold decrease in the *L. dumoffii* survival rate was detected when the bacteria were grown on a choline-enriched medium in comparison with the non-supplemented culture, suggesting that PC increased apoLp-III binding to *L. dumoffii* cells [11]. To test if choline supplementation affects *L. pneumophila* susceptibility to *G. mellonella* apoLp-III in a similar manner, the effect of the apoLp-III treatment on the survival rate of *L. pneumophila* cells grown on the choline-supplemented medium was assessed. When apoLp-III was used at the concentration of 0.1 mg/ml, the bacterial survival rate was calculated as ca. 37.3% (32%–41.3%). In contrast to *L. dumoffii*, the choline enrichment did not affect significantly the susceptibility of *L. pneumophila* cells to *G. mellonella* apoLp-III.

3.2. The effects of G. mellonella apoLp-III treatment on L. pneumophila cell surface topography and properties

In order to determine if the above described effect could result from apoLp-III binding to *L. pneumophila* cells, FITC-labelled apoLp-III was used. Fluorescence microscopy imaging of the bacteria after incubation with FITC-apoLp-III revealed the presence of green fluorescing bacterial cells, clearly indicating interaction of the protein with *L. pneumophila* (Fig. 1).

Upon exposure to apoLp-III (0.4 mg/ml), the cell surface topography of the bacteria underwent slight alterations in comparison with the control ones (Fig. 2). AFM images of the *L. pneumophila* control cells revealed small protuberances covering the cell surface. In addition, regular-shaped cavities, most probably reflecting vacuoles, were detected (Fig. 2A). The whole cell surface of apoLp-III-treated *L. pneumophila* cells was equally decorated with numerous tiny granules and resembled a sand-covered surface. Between the granules, very shallow furrows, ca. 5 nm in depth, were also noticed (Fig. 2B). The section profiles presented in Fig. 2B indicated more regular topography of the cell surface of apoLp-III-exposed bacteria in comparison with the control ones, however the RMS roughness value was not affected (8.538 ± 3.765 and 7.834 ± 2.743, for the control and apoLp-III-treated cells, respectively).

Table 1
The effect of *G. mellonella* apoLp-III on the *L. pneumophila* survival rate.

ApoLp-III concentration (mg/ml)	*L. pneumophila* survival rate (%)
0 (control)	100
0.1	43.7 ± 6.8
0.2	36.6 ± 3.8
0.4	18.9 ± 1.5
0.8	7.7 ± 2.2
1.6	0

After incubation without (control) or in the presence of apoLp-III, the bacteria were plated onto BCYE plates and grown colonies were counted. The results are presented as ± SD of three independent experiments.

3.3. FTIR analysis of interactions of apoLp-III with L. pneumophila cell surface

The results presented in the previous sections suggested interaction of apoLp-III with *L. pneumophila* cell surface. To determine which components of the bacterial cell envelope might be involved in apoLp-III binding, FTIR-ATR analysis of *L. pneumophila* cells exposed to *G. mellonella* apoLp-III was performed. Fig. 3 presents the IR absorption spectra recorded from intact *Legionella* cells and cells incubated with purified apoLp-III. Difference of these spectra represents the spectrum of apoLp-III incubated with bacteria cells combined with the difference spectrum of bacteria due to interaction with the protein. As can be seen, an interaction with apoLp-III results in a shift of the spectral band attributed to the ester carbonyl stretching vibrations of lipids in the bacterial membranes toward lower wavenumbers, represented by the negative band between 1720 and 1760 cm^{-1}. Such a shift can be attributed to a hydrogen bond formation by the carbonyl groups in the membranes of the bacteria with the protein molecules added to the system. More detailed analysis of the negative band in the difference spectrum, representing stretching vibrations of ester carbonyl groups, reveals relatively complex substructure and therefore can be interpreted as an indication of interaction of apoLp-III with different classes of lipids (see Fig. 4). As can be seen, the negative band contains at least two components with the minima at 1739 and 1723 cm^{-1} (Fig. 4). Precise localization of these minima was possible thanks to the calculation of a second derivative of the absorption spectrum. The component with the minimum at 1723 cm^{-1} can be interpreted as representing the membrane-bound LPS (see description of isolated *L. pneumophila* LPS spectra in subsection 3.5.) and the other component with the minimum at 1739 cm^{-1} represents another lipid constituent of the *L. pneumophila* membrane, interacting with apoLp-III [43,44].

3.4. Analysis of G. mellonella apoLp-III–L. pneumophila LPS interactions

In order to confirm interaction of apoLp-III with *L. pneumophila* LPS, an analysis of the binding of the protein with LPS purified from *L. pneumophila* cells was performed. Phenol-soluble S-form LPS of *L. pneumophila* (4 μg) was incubated with apoLp-III (4 μg) and then analysed by SDS-PAGE electrophoresis. Silver staining demonstrated that LPS mobility decreased slightly upon the apoLp-III treatment (Fig. 5A). The change in LPS mobility probably resulted from changes in the charge and size of LPS caused by the interaction with the protein. In addition, Coomassie Brilliant Blue staining of the gels and immunoblotting with anti-apoLp-III antibodies performed after SDS-PAGE revealed a considerable decrease in apoLp-III mobility after incubation with LPS (Fig. 5B, C). In these samples, the apoLp-III band, recognized by the antibodies, had an appearance characteristic of LPS-bound apoLp-III [16]. Assuming the denaturing conditions of the electrophoresis,

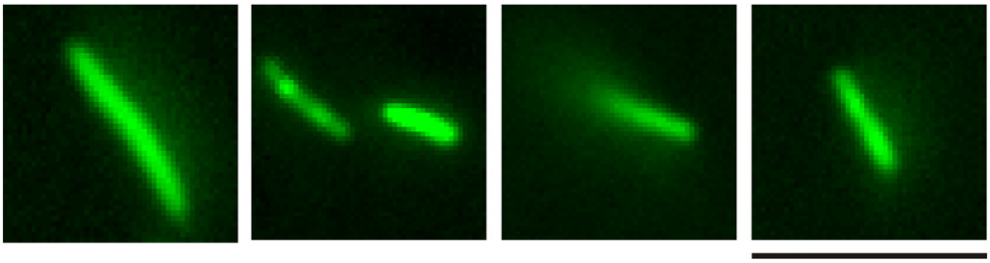

Fig. 1. Binding of *G. mellonella* apoLp-III to *L. pneumophila* cells. The bacteria were incubated in the presence of FITC-labelled apoLp-III (0.1 mg/ml) and imaged using a confocal laser scanning microscope (excitation and emission wavelengths were 470 nm and 520 nm, respectively; excitation time was 600 ms). Bar = 5 μm.

the results indicated a strong interaction between apoLp-III and *L. pneumophila* LPS and suggested formation of tight apoLp-III-LPS complexes.

3.5. FTIR analysis of the apoLp-III-LPS interaction

FTIR spectroscopy was used to gain detailed information on the type of conformational changes in LPS and apoLp-III induced by their mutual interactions. Fig. 6 presents the IR absorption spectra of LPS, apoLp-III, and the two-component system composed of LPS + apoLp-III. The high-wavenumber spectral region represents O–H stretching vibrations, which give rise to the band between 3000 and 3500 cm^{-1}. The fact that this band is relatively broad is an indication that hydroxyl groups of all the components are involved in hydrogen bond formation. It is highly probable that water molecules are trapped via hydrogen bonds by the examined molecules and involved in molecular organization of both LPS and the protein in the samples. The relatively sharp bands corresponding to the C–H stretching vibrations are also visible in the high-wavenumber spectral region: representing the symmetric (2853 cm^{-1}) and antisymmetric (2922 cm^{-1}) stretching in CH_2 groups. Several IR absorption bands are also visible in the low-wavenumber spectral region. In the case of apoLp-III, the principal band between 1600 and 1700 cm^{-1} is referred to as Amide I band. C═O stretching in peptide bonds gives a major contribution to this band [45]. Analysis of the Amide I band can provide information on the protein secondary structure and molecular organization. The maximum of the Amide I spectral band of apoLp-III, visible at 1654 cm^{-1}, corresponds to the α-helical secondary structure of the protein. Several relatively intensive IR absorption bands are also visible in the LPS spectrum. The major bands center in the region of 1640 cm^{-1} and can be assigned to C═O stretching vibrations, in the 1380 cm^{-1} region, which can be assigned to deformation (umbrella) vibrations in CH_3 groups, at 1255 cm^{-1}, which can be assigned to PO_2 antisymmetric stretching, and at 1073 cm^{-1}, which can be assigned to the stretching vibrations of the C–O–P–O fragments of LPS. In order to analyse the LPS-protein interaction, the spectra of the two component system (apoLp-III + LPS) and

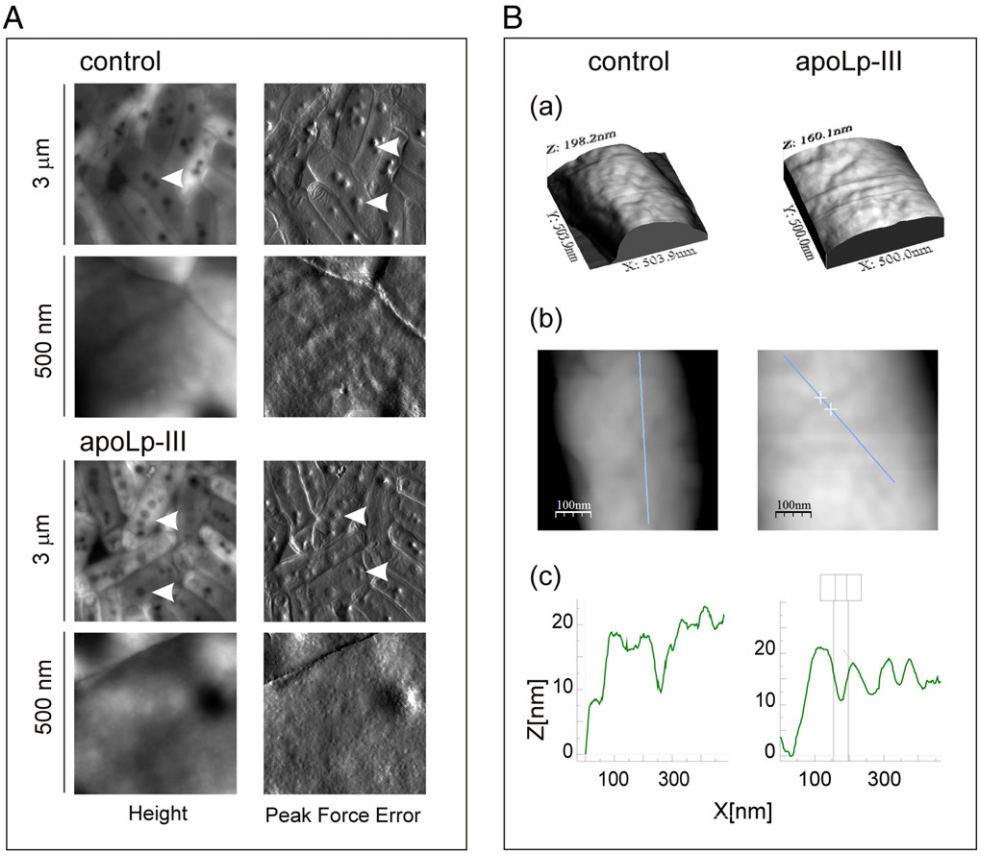

Fig. 2. AFM imaging of *L. pneumophila* cell surface after treatment with *G. mellonella* apoLp-III. The bacteria were incubated without (control) or in the presence of apoLp-III (0.4 mg/ml) and examined by AFM. In (A) the height and peak force error images are presented. The white arrowheads indicate regularly shaped cavities. In (B) the 3D images (a), height images (b), and section profiles corresponding to the lines in the height images (c) are demonstrated.

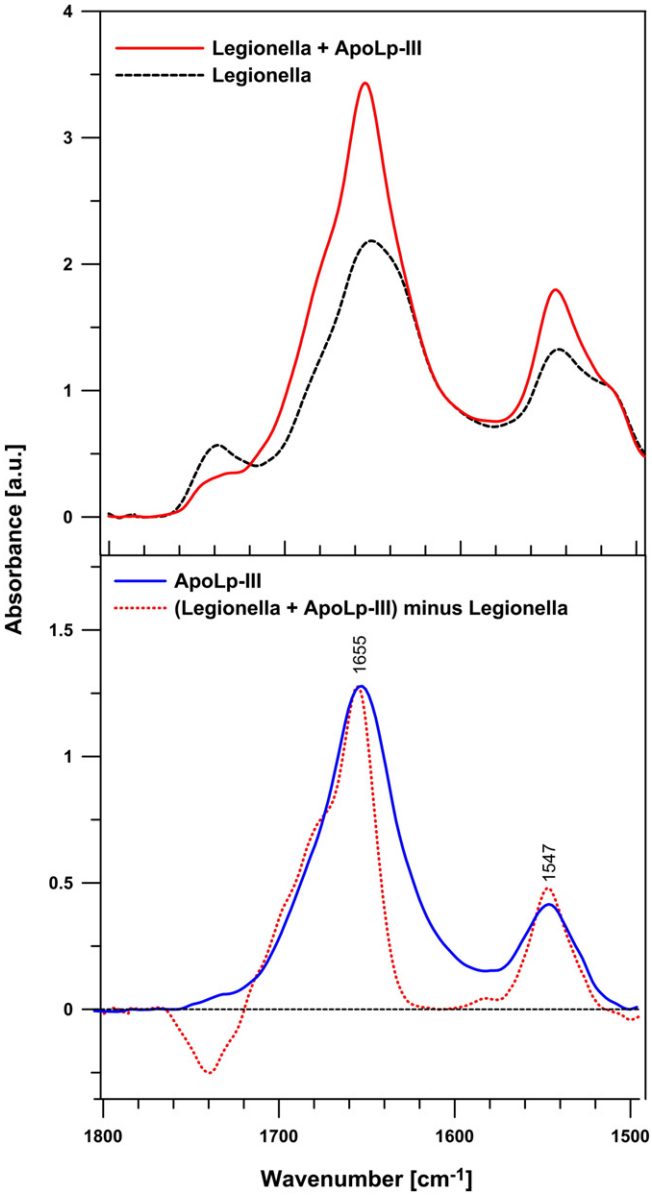

Fig. 3. IR absorption spectra, in the Amide I-Amide II spectral region, of intact *L. pneumophila* cells and cells incubated with *G. mellonella* apoLp-III. Top panel shows the FTIR spectrum of intact *Legionella* cells (dashed line) and the cells incubated with apoLp-III (solid line). The spectra were normalized at 1513 cm^{-1}, at the maximum of the band absent in apoLp-III. Bottom panel shows the difference spectrum (dotted line), calculated by subtracting the spectra presented in the upper panel (the spectrum of bacteria cells incubated with apoLp-III minus the spectrum of bacteria cells) superimposed on the spectrum of pure apoLp-III (solid line). The spectrum of apoLp-III was scaled at the maximum of the difference spectrum.

pure LPS were normalised at the 1395 cm^{-1} maximum and the difference spectrum has been calculated: apoLp-III + LPS minus LPS (Fig. 7). In principle, the difference spectrum should follow the pure apoLp-III spectrum, assuming the lack of a protein–LPS interaction. As can be seen from the comparison of the pure protein spectrum and the difference spectrum, this is not the case. The spectra are distinctly different in the Amide I spectral region. The main maximum in the amide I band remains at 1654 cm^{-1}, which shows that the α-helical structure of apoLp-III is not affected upon the interaction with LPS. Interestingly, an additional band at 1610 cm^{-1} appears in the Amide I spectral region in the difference spectrum. Formally, a band in this spectral region can be assigned to protein aggregated structures [45]. On the other hand, it is very likely that this band in the difference spectrum originates from the LPS carbonyl stretching, shifted from 1627 cm^{-1}

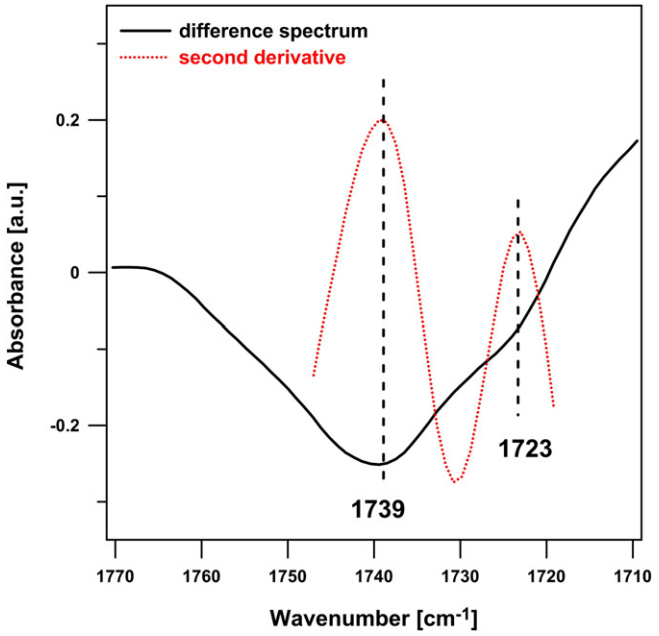

Fig. 4. The difference spectrum from Fig. 3. presented in the ester carbonyl stretching spectral region. The spectrum of the second derivative is superimposed on the difference spectrum (dotted line). The maxima of the second derivative spectrum point into the minima of the spectral components.

toward lower frequencies due to hydrogen bond formation with LPS. Also, the shoulder at 1718 cm^{-1} (Fig. 7) assigned to ester carbonyl vibrations appears in the difference spectrum, most probably due to the fact that owing to hydrogen bond formation with the involvement of these groups, the oscillator strength of C═O stretching is higher and this is manifested by the increased band intensity. Very similar effects of increased band intensity in the LPS upon interaction with apoLp-III can be observed at 1255 cm^{-1} and at 1073 cm^{-1} (see Fig. 6). This is an indication that also the PO_2 and C–O–P–O groups of LPS are involved in the interaction with the protein via hydrogen bonds.

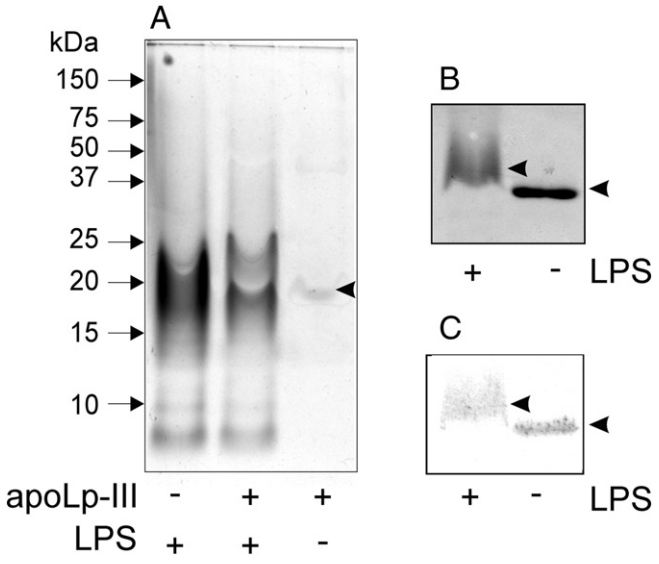

Fig. 5. Electrophoretic and immunoblotting analysis of *G. mellonella* apoLp-III interaction with *L. pneumophila* LPS. *L. pneumophila* LPS (4 μg) and apoLp-III (4 μg) were incubated alone or in combination, resolved by SDS-PAGE and electrotransferred onto PVDF membranes. LPS (A) and apoLp-III (B) were visualised by silver and Coomassie Brilliant Blue staining, respectively. (C) shows a fragment of the PVDF membrane after immunoblotting with anti-apoLp-III antibodies, corresponding to the gel fragment in (B). The apoLp-III bands are indicated by the arrowheads.

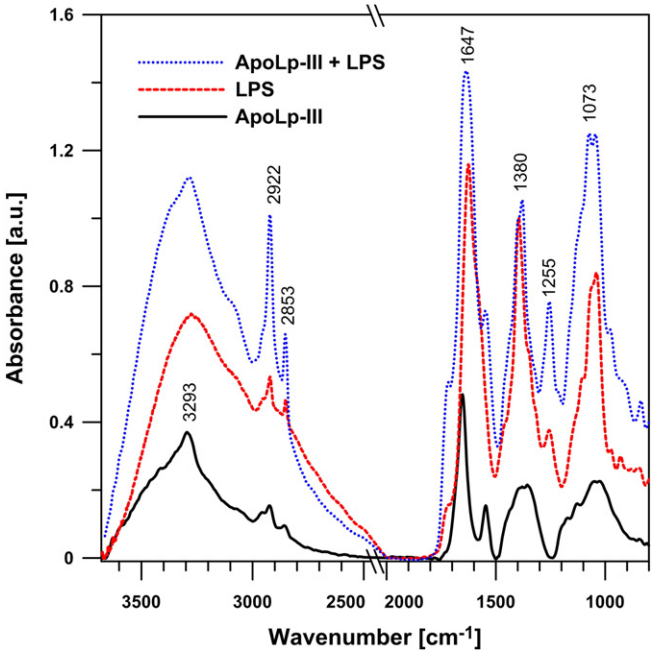

Fig. 6. FTIR spectra of *G. mellonella* apoLp-III, *L. pneumophila* LPS, and the two-component mixture of apoLp-III and LPS. *L. pneumophila* LPS (50 μg) and apoLp-III (10 μg) were incubated alone and in combination. After deposition of the samples on the ZnSe crystal, the IR absorption spectra were recorded as described in Materials and methods. The apoLp-III + LPS and LPS spectra were normalised at 1395 cm^{-1}.

4. Discussion

In the present study, we provide evidence that *G. mellonella* apoLp-III exhibits anti-*L. pneumophila* activity, in addition to anti-*L. dumoffii* activity reported by us previously [11]. Interaction of *G. mellonella* apoLp-III with *L. pneumophila* cells was confirmed by fluorescence microscopy imaging of the bacteria incubated in the presence of FITC-labelled apoLp-III. As revealed by AFM analysis, binding of apoLp-III was accompanied by alterations in *L. pneumophila* cell surface topography, suggesting an interaction of the protein with components of the bacteria cell envelope. Furthermore, the results of the FTIR spectroscopy analysis of *L. pneumophila* cells exposed to apoLp-III demonstrated interaction of apoLp-III with the membrane-bound LPS and another lipid constituent of *L. pneumophila* membrane. Such a spectroscopic result goes along with the interpretation that apoLp-III interactions to lipid components of the bacteria cell envelope can be responsible for the changes in the cell surface topography monitored by AFM technique.

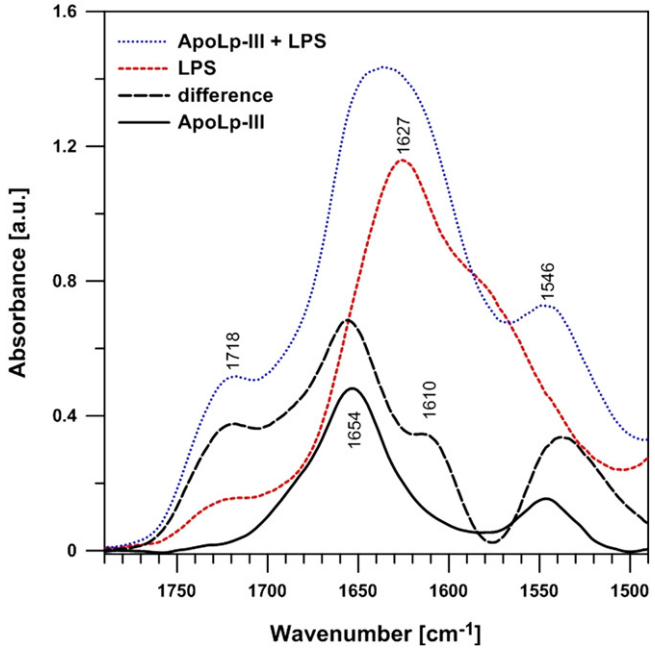

Fig. 7. FTIR spectra in the Amide I and Amide II regions of *G. mellonella* apoLp-III, pure and admixed to *L. pneumophila* LPS. The pure LPS and the difference spectra (apoLp-III + LPS minus apoLp-III) are also shown. The LPS and apoLp-III spectra were normalised at 1395 cm^{-1} (see Fig. 6).

Antibacterial activity of apoLp-III and binding of the protein to the cell surface of various Gram-positive and Gram-negative bacteria have been demonstrated [18,26]. It has been documented that apoLp-III can interact with LPS and different phospholipids, including these constituting essential components of the cell envelopes of Gram-negative bacteria. Analysis of *Manduca sexta* apoLp-III interactions with different lipid bilayers revealed that binding to phospholipids is mediated primarily through polar and/or ionic interactions at the bilayer surface, whilst hydrophobic forces may play a bigger role in interactions between apoLp-III and nonionic lipid bilayers [46]. In turn, in their studies on *G. mellonella* apoLp-III, Leon et al. [47] found no evidence that charge is a factor in the apoLp-III-*E. coli* LPS binding. Investigating a requirement of apoLp-III helix bundle opening for LPS binding, they demonstrated that when the bundle is locked by disulphide bond formation, the protein is unable to interact with LPS. According to these authors, the findings implied direct binding of the protein hydrophobic interior with LPS and underlined the essential role of hydrophobic interactions in apoLp-III-LPS binding [48]. As demonstrated using *E. coli* LPS, binding of apoLp-III with LPS is probably initiated by ionic interactions with exposed carbohydrate moiety. However, the lipid A region is required for a more stable LPS binding, indicating that the second step is driven by hydrophobic interactions [15,47]. It was demonstrated in our previous paper that *L. dumoffii* cells cultured on a choline-enriched medium were 3 times more sensitive to apoLp-III in comparison with the bacteria grown on a non-supplemented medium, suggesting that an increased PC content (and/or different composition of the PC species) in the *L. dumoffii* cell envelope facilitated a stronger interaction of apoLp-III with the bacterial cell surface [11]. In contrast, cultivation of *L. pneumophila* cells on the choline-supplemented medium did not cause such an effect. Interestingly, the susceptibility level of *L. dumoffii* grown on the choline-supplemented medium to apoLp-III became similar to the *L. pneumophila* sensitivity. The relatively high sensitivity of *L. pneumophila* to apoLp-III action, LPS-binding ability of apoLp-III, strong hydrophobic properties of the bacteria LPS, as well as a similar survival rate of *L. pneumophila* cells cultured on the choline-supplemented and non-supplemented media also imply the participation of surface structures other than PC in apoLp-III binding by the *L. pneumophila* cell envelope. Since *L. pneumophila* susceptibility to apoLp-III was not affected by choline supplementation, one can postulate that in this case rather an extremely hydrophobic LPS is involved in apoLp-III binding. Given the results reported previously [11], one can also assume that LPS and phospholipids (especially PC) might be essential in the interaction of *G. mellonella* apoLp-III with *L. pneumophila* and *L. dumoffii*, respectively. This could also explain the considerable difference in susceptibility of both *Legionella* species grown without choline supplementation to this insect defence protein, as *L. pneumophila* appeared to be much more sensitive to apoLp-III than *L. dumoffii*. However, when the results of FTIR-ATR analysis of *L. pneumophila* cells treated with apoLp-III are taken into consideration, the more complicated picture arises. Given the lipid and LPS binding properties of apoLp-III and demonstrated interaction of the protein with LPS as well as other lipid component in the bacteria cell surface, the anti-*L. pneumophila* activity of *G. mellonella* apoLp-III could be a result of interaction with different cell envelope components.

The results of the SDS-PAGE analysis and immunoblotting with anti-apoLp-III antibodies revealed that incubation of apoLp-III with LPS affected the electrophoretic mobility of the protein as well as the LPS,

pointing toward an apoLp-III interaction with LPS molecules in the *L. pneumophila* cell envelope. Moreover, the apoLp-III band had the characteristic appearance for LPS-bound apoLp-III reported previously [16], which could result from conformational changes (confirmed by FTIR analysis) induced by the interaction of apoLp-III with LPS. Pratt and Weers [16] also demonstrated that *E. coli* LPS affected electrophoretic properties of apoLp-III under denaturing conditions, indicating a possibility of forming SDS-stable complexes between these molecules. Given the denaturing electrophoretic conditions, our results point toward a very strong interaction of *G. mellonella* apoLp-III with *L. pneumophila* LPS. This is in agreement with the results presented by Oztug et al. [15], who reported high resistance to guanidine-induced denaturation of the LPS-bound form of apoLp-III in comparison with unbound protein. Similarly, compared to the apoLp-III lipid-free state, the apoLp-III-dimyristoylphosphatidylcholine (DMPC) complexes were relatively resistant to denaturation by guanidine-HCl [48]. The particular composition of *L. pneumophila* LPS, conferring very strong hydrophobic properties of the molecule [4], may be responsible for the high *L. pneumophila* susceptibility to apoLp-III described in this study. ApoLp-III binding with such an unusual LPS has been investigated for the first time. So far, interactions of apoLp-III with LPS of *E. coli* O55:B5 have been analysed [16,47,48]. Recently, it has been demonstrated that *G. mellonella* apoLp-III can disaggregate large *E. coli* LPS complexes by binding of four apoLp-III molecules to 24 molecules of LPS. Analysis of the complexes formed by *G. mellonella* apoLp-III and *Klebsiella pneumoniae* LPS revealed the presence of three apoLp-III molecules and nine molecules of LPS [15]. Our results together with those reported by other authors suggest that apoLp-III interaction with LPS in bacterial outer membrane could be considered as a prerequisite for *G. mellonella* apoLp-III antibacterial action against some Gram-negative bacteria, e.g. *L. pneumophila*, *K. pneumoniae* and *Salmonella typhimurium* [15,18,26].

The interaction of *G. mellonella* apoLp-III with LPS isolated from *L. pneumophila* was examined using FTIR spectroscopy. The analysis of the difference spectrum revealed that upon the interaction, the apoLp-III as well as *L. pneumophila* LPS molecules underwent conformational changes. Although the α-helical structure of apoLp-III seemed not to be affected, the presence of aggregated protein structures after incubation with *L. pneumophila* LPS could be postulated. Interestingly, as revealed FTIR spectroscopy analysis of *L. pneumophila* cells treated with apoLp-III, the shape of the Amide I band of apoLp-III interacting with the bacterial membranes is distinctly different that the shape of this band recorded from the isolated protein. The most striking difference is associated with narrowing of the spectrum in the low wavenumber edge of the Amide I band. Intensity in this spectral region (near 1640 cm^{-1}) can be attributed to either β-sheet structure or to the pseudo-β-structure related to formation of aggregated strands. It is possible that during deposition of purified apoLp-III to the ATR crystal the protein becomes partially aggregated and such aggregated structures are not formed upon interaction of the protein molecules to the surface of the bacterial membranes.

It has been documented that upon binding to *E. coli* LPS, apoLp-III needs to rearrange its helices, similar to remodelling occurring during interactions with lipid surfaces of lipophorin particles [48]. When interacting with model phospholipid vesicles composed of DMPC, apoLp-III transforms them into discoidal particles. Although the tertiary structure of the protein is dramatically altered, the secondary structure is maintained under such conditions [49–55]. On the other hand, the difference spectrum calculated by us indicated the involvement of ester carbonyl groups, PO_2 and C–O–P–O groups of LPS in the interaction with apoLp-III via hydrogen bonds. These results provide further evidence on the strong interaction occurring between *G. mellonella* apoLp-III and *L. pneumophila* LPS.

Both *Legionella* species studied by us so far, *L. dumoffii* [11] and *L. pneumophila*, were sensitive to *G. mellonella* apoLp-III. However, the differences in the susceptibility of the bacteria cultured on the choline-enriched medium suggested that these species may display different types of response to apoLp-III, implying participation of different bacterial cell surface structures in apoLp-III binding. Both LPS and PC are cell envelope components essential for *Legionella* virulence enabling the bacteria to bind efficiently to human macrophages [56–58]. Blocking of these molecules by an interaction with adequately designed agents would impede binding of the bacteria to the host cells and would be a strategy against infections caused by *Legionella*.

It has been documented that apoLp-III is homologous to the N-terminal part of human apolipoprotein E (apoE) [12]. Involvement of apoE in innate immune response against the extracellular pathogenic bacteria, e.g. *K. pneumoniae*, as well as intracellular pathogen, *Listeria monocytogenes*, has been reported [59–61]. Taking this into consideration, one cannot exclude that apoE might participate in immune response toward *Legionella* in humans.

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