Carbohydrate Polymers 78 (2009) 481–487

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Binding and biological properties of lipopolysaccharide *Proteus vulgaris* O25 (48/57)–chitosan complexes

Michał Arabski [a,*], Viktoria N. Davydova [b], Sławomir Wąsik [c], Anatoliy V. Reunov [b], Larisa A. Lapshina [b], Irina M. Yermak [b], Wiesław Kaca [a]

[a] Institute of Biology, Jan Kochanowski University, Kielce, Świętokrzyska 15, 25-406, Kielce, Poland
[b] Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences, 159, Pr.100-letiya, 690022 Vladivostok, Russia
[c] Institute of Physics, Jan Kochanowski University in Kielce, Świętokrzyska 15, 25-406 Kielce, Poland

ARTICLE INFO

Article history:
Received 15 January 2009
Received in revised form 4 March 2009
Accepted 11 May 2009
Available online 18 May 2009

Keywords:
Lipopolysaccharide
Chitosan
Toxicity
Proteus vulgaris
Laser interferometry

ABSTRACT

In this study the negatively charged *Proteus vulgaris* O25 LPS was chosen for studying interaction with polycationic chitosan. The complex formation of LPS with chitosan was demonstrated using gradient centrifugation and laser interferometry method. The presented results have shown that laser interferometry method is sensitive enough for LPS–chitosan interaction studies. The changing in the ultra structure of LPS during binding with chitosan was observed by electronic microscope. The interaction of *P. vulgaris* O25 LPS with chitosan was shown to modulate significantly the biological activities of LPS. The toxicity of *P. vulgaris* O25 LPS decreased 10-fold after forming complexes with chitosan at injection to mice in the similar concentration of endotoxin. The complex LPS–chitosan was less effective than LPS alone in Limulus amabocyte lysate assay. Induction of TNF biosynthesis by LPS–chitosan complex was found to be 65% lower than that by parent LPS at concentration of 100 ng/ml.

1. Introduction

Lipopolysaccharides (LPSs) are unique cell wall components of Gram-negative bacteria. They represent amphiphilic biopolymeric compounds combining, in a single molecule, hydrophilic (O-specific chains, core oligosaccharide, etc.) and hydrophobic (lipid A) entities. LPS play a crucial role in various interactions between micro- and macro-organisms and display a broad range of biological activities including toxic activity and ability to activate immune cells (Vanhaecke, Pijck, & Vuye, 1987). The last may be beneficial at low concentrations of LPS, but pathophysiological at higher endotoxin levels due to an overproduction of cytokine in immune cells, such as interleukins and tumor necrosis factor alpha (TNF-α).

Due to a high negative charge, the LPS macromolecules represented an important target for polycationic antibacterial peptides which are discussed as new potential antiseptic drugs. Furthermore, as lipid A is anionic and amphiphilic, it has been suggested that cationic molecules represented potentially ideal ligands for sequestering LPS and may be useful in a therapy of septic shock caused by Gram-negative bacteria (David, Bechtel, Annaiah, Mathan, & Balaram, 1994). The most popular cationic antibiotics often exert side effects on the host organism; therefore, a search for compounds neutralizing toxic effects of endotoxin by binding to them without any detriment for the organism is a task of paramount importance. Natural polysaccharides in particular chitosan, meet this requirement by virtue of their safety.

Chitosan (CH), a linear polymer consisting of 2-amino-2-deoxy-D-glucopyranose and 2-acetylamido-2-deoxy-D-glucopyranose residues linked through a β-1,4 glycoside bond is manifests a broad range of physiological activities (antibacterial, antiviral, antitumor, anticholesterol, etc.), which makes this compound a promising tool for medicine and pharmacology. The ability of chitosan to form specific complexes with polyanions opens up broad opportunities for its application as a drug and gene delivery vector as well as a constituent component of biospecific adsorbents and composites (Fernandez-Saiz, Lagaron, & Ocio, 2009; Kumar, Muzzarelli, Muzzarelli, Sashiva, & Domb, 2004; Singla & Chawla, 2001; Xing et al., 2009).

We showed earlier that LPSs from *Yersinia pseudotuberculosis* and *Escherichia coli* interact with the chitosan and produce stable complex (Davydova, Yermak, Gorbach, Krasikova, & Solov'eva, 2000). The interaction of chitosan with LPS was shown to modulate significantly the biological activity of these LPS (Polyakova et al., 1995, Yermak et al., 2004, 2006).

Gram-negative bacteria genus *Proteus* cause nosocomial, wound and urinary tract infections (O'Hara et al., 2000). In this connection for the present genus of bacteria, together with the therapy with using of antibiotics, the approach connected to using positively charged neutralizing agents, in particular chitosan, can be especially successful. In our recent studies a comparative study of electrokinetic aspects of the interaction of LPS from three different

* Corresponding author. Tel./fax: +48 41 349 63 07.
E-mail address: arabski@ujk.kielce.pl (M. Arabski).

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.05.004

microorganism *Y. pseudotuberculosis*, *E. coli* and *Proteus vulgaris* with chitosan was carried out (Davydova et al., 2008). These LPSs possess different chemical structures: the number of negatively chares groups in the lipid A-core moiety, the degree of acylation of lipid A and various lengths of the 0-specific chains that were shown to be important factors of binding LPS to CH.

However some questions remained undetermined. In particular there is questions about complete binding LPS *P. vulgaris* with chitosan. It is important to determinate whether can binding of LPS with chitosan change of biological activity of endotoxin from *P. vulgaris*. In this connection the aims of the present study were as follows (1) to study of process of binding of LPS with chitosan by a method of direct registration of a complex-velocity sedimentation; (2) to test if biological activities of *P. vulgaris* O25 LPS is different in presence of chitosan; (3) It is known, that biological activity of LPS depends on the supramolecular organizations of endotoxin. So the method of electronic microscopy was used for studying the influence of chitosan to the LPS ultrastructure; (4) to find out if laser interferometry method might be used to study interaction of lipopolysaccharide with polycationic chitosan and colistin.

2. Materials and methods

2.1. Isolation of lipopolysaccharide and chitosan

P. vulgaris O25 (48/57) was from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). The bacteria were cultivated under aerobic conditions in nutrient broth (BTL, Poland) under controlled conditions (37 °C, pH 7.4–7.6). Cells were harvested at the end of the logarithmic growth phase, centrifuged (5000×*g*, 30 min), washed with distilled water, and lyophilized. The LPS was isolated by the phenol–water procedure (Westphal & Jann, 1965) and purified by treatment with DNase and RNase (Boehringer Mannheim, Germany). The LPS preparation thus obtained was practically free of nucleic acid and contained <2.5% proteins.

A chitosan sample of molecular mass 130 kDa and a 4% degree of *N*-acetylation was obtained by alkaline treatment of crab chitin according to a published protocol (Wolfrom & Shen-Han, 1959). The degree of *N*-acetylation of the chitosan sample was calculated according to IR-spectroscopic data (Domszy & Roberts, 1985). The molecular weight of the chitosan was determined by the Archibald method at 12,000 rpm (Archibald, 1947).

2.2. Preparation of LPS–chitosan complexes

LPS (1 mg) and CH (1 mg) were dissolved separately in 0.5 ml of 0.1 M sodium phosphate buffer (pH 5.0). The solutions were stored for 48 h at 37 °C, then mixed and incubated for 18 h at 37 °C. The LPS and chitosan solutions were decontaminated of bacteria by filtration using "Millex GS" (Millipore, Ireland).

2.3. Sucrose centrifugation

The solutions of CH (1 mg/ml), LPS (1 mg/ml), or LPS–CH mixture obtained as described above were layered (5 ml) on a linear sucrose gradient (15–30%). Centrifugation was performed in the SW-45 ×3 bucket-rotor of a K-32 ultracentrifuge at 40,000 rpm for 90 min at 18 °C. The tube contents were withdrawn from the top drop-wise; the volume of each fraction was 0.3 ml. The amounts in each fraction of LPS were estimated spectrophotometrically by the reaction with Teylor's blue at 535 nm (Keler & Nowotny, 1986) and those of chitosan by determining the content of the amino groups of its glucosamine residues at 475 nm (Inman & Dintzins, 1969).

2.4. Electron microscopy of LPS and LPS–chitosan complexes

The solutions of CH (0.5 mg/ml), LPS (0.5 mg/ml), or LPS–CH mixture were obtained as described above. Negative staining was performed with 2% phosphatotungstic acid at pH 7.0 on parlodoon-filmed carbon-coated grids. Briefly, a drop of LPS, chitosan, or LPS–CH mixture obtained as described above were placed on the grid for approximately 30 s. The drop was then drawn with a Pasteur pipette and excess material was removed from the grid surface with Wharman filter paper. A drop of 2% phosphatotungstic acid was immediately added and after 15 s contact time, excess fluid was again removed and the grid was allowed to air dry. The grids were examined and photographed in a JEM-7A electron microscope (Electron Optics Laboratory Ltd., Japan). Electron microscopy was used for study probable modification LPS morphology as results of binding with chitosan.

2.5. Lethal toxicity test

The toxic properties of the complexes of LPS–CH (in comparison with LPS) were tested in outbred D-galactosamin-sensitized mice of the CD line (16–18 g) by the method of Galanos et al. (Galanos, Freudenberg, & Reutter, 1979). D-Galactosamin-hydrochloric acid (15 mg per animal) and different amounts of LPS–chitosan complex or parent LPS (0.004–4 μg) as mixtures in 0.2 ml of saline solution were administered intraperitoneally to groups of six animals. A control group of six mice was injected with saline solution only. Death of animals was monitored for 48 h. Toxicity was expressed as LD_{50} and calculated by Kurber's formula (Novotny, 1979). The experiments were performed in accordance with the Pacific Institute of Bioorganic Chemistry Policy on the Humane Care and Use of Laboratory Animals.

2.6. TNF-inducing activity

Blood processing was performed using the procedure of Bienvenu et al. (Bienvenu, Doche, Gutowski, Lenoble, & Pedrix, 1995). Human peripheral blood was collected by venapuncture into sterile siliconized tubes containing 30 IU of lithium heparinate per 5 ml tube diluted 1:5 in sterile Medium 199 (Sigma, USA) containing 300 mg/l of glutamine (Gibco, Life Technology, Germany) and 50 μg/ml of gentamicin. The diluted blood (0.1 ml) was transferred into sterile polypropylene plates and then incubated with the corresponding LPS, CH, or LPS–CH complex (1:1 w/w) (37 °C, 5% CO_2). A control incubation with 10 μg/ml of LPS from *E. coli* (strain 055:B5) was performed for each experiment. After 24 h the supernatants were collected and frozen, followed by cytokine determination using specific ELISA (DuoSet developing system, Genzyme, Boston, USA).

2.7. Limulus amebocyte lysate (LAL) assay

The chromogenic LAL test was purchased from Cambrex Bio Science (Walkersville, USA). The reaction mixtures contained LPS, chitosan, or LPS/chitosan complex at concentrations of 0.025, 0.05, 0.1, 50, 100, 200, or 400 ng/ml. All procedures were done in accordance with the test instruction.

2.8. LPS/colistin/chitosan precipitation assay

Stock solutions of *P. vulgaris* O25 (48/57) LPS, chitosan, or LPS/chitosan complexes were diluted with appropriate amounts of 0.1 M sodium phosphate buffer (pH 5.0) prior to the addition of colistin. The reaction mixtures contained LPS, chitosan, or LPS/chitosan complexes at concentrations of 12, 25, 50, 100, 200, or 400 μg/ml and of colistin 2 mg/ml in each probe in final volumes

of 200 μl. Controls were corresponding solutions without colistin. Precipitation proceeded for 18 h at 37 °C. Aggregates were detected by spectroscopic measurement at 630 nm using EL 340 Microplate Readers (MTX Lab Systems, USA).

2.9. Laser interferometric analysis of colistin transport

The amount of aggregate-free colistin, $N(t)$, which diffuses during time t from a gel solution to phosphate buffer (pH 5.0) was calculated by integrating the concentration profile according to:

$$N(t) = S\int_0^{\delta} C_1(x,t)dx,$$

where $C_1(x,t)$ denotes the concentration of colistin at a point situated at a distance of x from the gel–water interface, S the surface of the gel–water interface ($S = 35 \times 10^{-6}$ m^2), and δ the concentration boundary layer (CBL) thickness.

The values $C_1(x,t)$ and δ were determined experimentally by means of laser interferometry. The measurement set-up for the interferometric investigations of the substance transport was presented previously (Arabski, Wąsik, Dworecki, & Kaca, 2007; Dworecki, Wąsik, & Ślęzak, 2003; Dworecki, Ślęzak, Drabik, Ornal-Wąsik, & Wąsik, 2006). It consists of a Mach–Zehnder interferometer with an He–Ne laser, a system of two measurement cuvettes, a TV-CCD camera, and a computer with a system for the acquisition and processing of interference images. The computer program used to analyze these images allows, among other things, ascertaining the concentration profiles and CBL thicknesses. The interferograms, which appear due to the interference of two laser beams, are determined by the refraction coefficient of the solute, which in turn depends on the concentration of the substance. When the solute is uniform, the interference fringes are straight and they bend when a concentration gradient appears. The concentration profile $C(x,t)$ is determined by the deviation of the fringes from a straight course. Since the concentration C and the refraction coefficient are assumed to be linear, we have:

$$C(x,t) = C_0 + a\frac{\lambda d(x,t)}{hf},$$

where C_0 is the initial substance concentration, a the proportionality constant between the concentration and the refraction index ($a = 2.92 \times 10^5$ mol/m^3 for the colistin aqueous solution), λ the wavelength of the laser light, h the distance between the fringes in the field where they are straight lines, and f the thickness of the solution layer in the measurement cuvette. The CBL thickness δ was defined as the distance from the gel–water interface to the point at which the deviation d of the interference fringe from its straight line course is 10% of the fringe thickness. By recording the interferograms at a given time interval, one can reconstruct the concentration profiles at different times. Such profiles were used to calculate the membrane permeability coefficient. All experiments were performed at a temperature of 295 ± 0.3 K. The description of new, modified measurement cuvettes system are presented in Section 3.

3. Results

3.1. Sucrose gradient centrifugation of the LPS, chitosan, and LPS–CH complexes

Using velocity sedimentation in sucrose gradient, the formation of complexes of the *P. vulgaris* LPS O25 (48/57) with chitosan was investigated. The results of the sucrose gradient centrifugation of mixtures of LPS and chitosan are presented in Fig. 1. As may be seen, free LPS is separated into two main fractions of different molecular mass which are sedimentated into the bottom and top parts of the gradient (Fig. 1A). Chitosan is localized in the top part of the gradient with a low sedimentation velocity. The distribution of the sedimentation profiles along the sucrose gradient of LPS and chitosan mixtures (Fig. 1C) differed from those of the initial components (for comparison, see Fig. 1A and B). These data illustrate the completely superimposed overlapping of LPS *P. vulgaris* O25 and chitosan in the top, middle, and bottom levels of the sucrose gradient (Fig. 1C). This pattern of migration suggests the formation of three types of *P. vulgaris* O25 (48/57) LPS–chitosan complexes, differing in molecular weight.

3.2. Electron microscopy of LPS and LPS–chitosan mixtures

Negative-stained preparations of LPS existed in a number of highly aggregated forms, including different freely branching ribbon-like structures (thickness: 5–13 nm), as may see in Fig. 2A and B when stained with phosphatotungstic acid. Chitosan alone (Fig. 2C) appeared as small, uniform discs with diameters of approximately 6–13 nm combined in a chain-like structure. The LPS–CH mixture consisted of a number of particles with various forms. There were heterogeneous particles of irregular form (Fig. 2D) and/or membrane-like structures (Fig. 2E).

3.3. The modulation of the biological activity of LPSs by chitosan

As was shown earlier, the interaction of chitosan with LPS modulates the biological activity of the LPSs of *Y. pseudotuberculosis* and

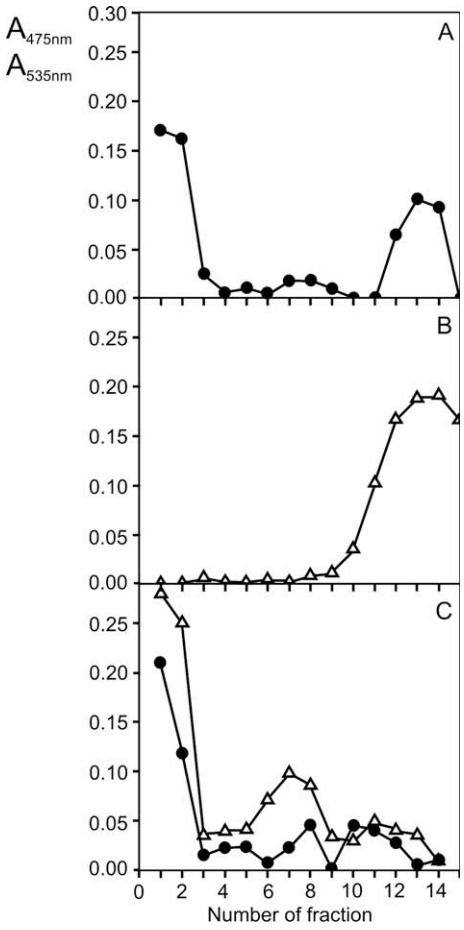

Fig. 1. Sucrose gradient centrifugation of LPS (A), chitosan (B), and mixtures of LPS and chitosan (C). NH_2 (A_{475}) content (open triangles); LPS (A_{535}) content (solid circles).

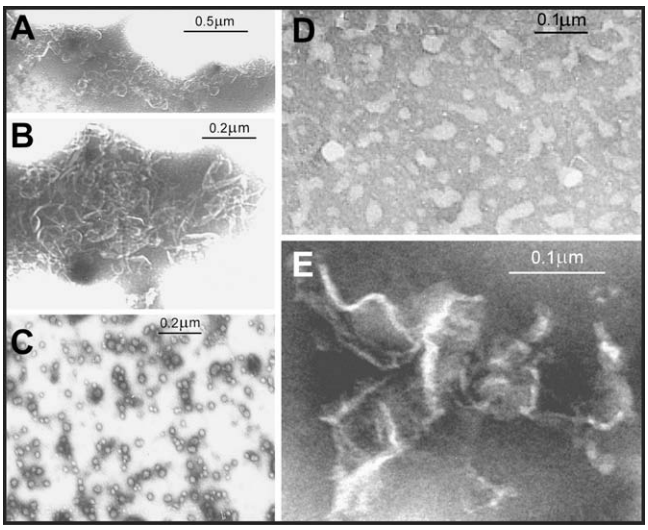

Fig. 2. Electron microscopy 2% phosphatotungstic acid negatively contrasted preparation LPS (A and B), chitosan (C), and LPS–CH mixture (D and E).

E. coli and decreases their toxicity (Yermak et al., 2004, 2006). In the presented study the acute toxicity of *P. vulgaris* O25 LPS alone and its complex with CH were assayed. Simultaneous injection of endotoxin with galactosamine has been shown to increase the LPS's toxicity substantially (10^4–10^5 times) and appears to be the most convenient procedure for toxicity assay (Galanos et al., 1979).

The LD_{50} values of O25 LPS and the complex of O25 LPS–CH at a ratio of 1:1 (w/w) were calculated according to Kurber's formula (Novotny, 1979) using mice of the CD line. We observed that in the experiments with galactosamine the toxicity of *P. vulgaris* O25 LPS (LD_{50} = 0.126) decreased 10-fold after forming complexes with chitosan (LD_{50} = 1.26).

LPSs are known to stimulate the production of cytokines by cells. Experiments were performed to determine in human mononuclear blood cells the effects of LPS from *P. vulgaris*, CH and the LPS–CH (1:1 w/w) complex on the production of TNF-α by human mononuclear blood cells. Fig. 3 shows that all the components examined stimulate the induction of TNF-α. However, the activity of the LPS–CH complex to induce TNF-α was 65 % lower than that of 100 ng of *P. vulgaris* O25 LPS alone. At a dose 1 ng of LPS, the ability of chitosan to reduce TNF-α release was not significant.

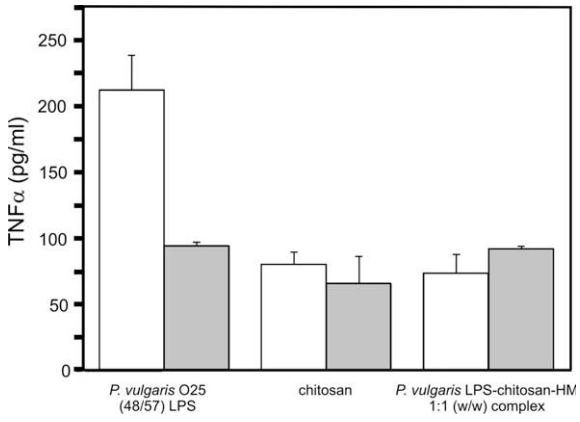

Fig. 3. The influence of *P. vulgaris* O25 (48/57) LPS, chitosan and *P. vulgaris* LPS–chitosan–HM 1:1 (w/w) complex on the induction of TNF-α. White columns: content of LPS equal to 100 ng/ml, content of chitosan equal to 100 ng/ml; gray columns: content of LPS equal to 1 ng/ml, content of chitosan equal to 1 ng/ml.

3.4. LAL assay

The chromogenic limulus amebocyte lysate (LAL) test is commonly used to detect biologically active endotoxins in the nanogram range in human blood and pharmaceutical samples (Gorbet & Sefton, 2005). Fig. 4 presents LAL test with LPS O25 and chitosan alone and in complexes. As expected, *P. vulgaris* O25 LPS alone activated LAL in a dose-dependent manner. Chitosan alone also induced LAL reactions, not in a dose-dependent way. Chitosan was 60–70% less effective than LPS alone. The complexes of LPS–chitosan in the range of doses used were less effective in LAL activation than the sum of the two components alone.

3.5. Colistin/LPS/chitosan precipitation assay

The interaction of negatively charged endotoxin with polycationic molecules resulted in aggregate formation and increases in absorption values. The best exemplar of this reaction is the precipitation of LPS by polycationic antibiotics-polymyxins. As expected, colistin (polymyxin antibiotic type)-precipitated O25 LPS depends on the amount used (Fig. 5). Interestingly, chitosan alone also increases the turbidity of solution with colistin. A dose-dependent interaction was observed up to 25 μg/ml of chitosan. In general, in complexes of chitosan–LPS the amount of precipitate was lower than that of the sum of each heteropolymer alone. This was similar to the LAL test (Fig. 4). It is worth stressing that in the highest amounts used (400 μg/ml LPS O25) chitosan–LPS precipitates are significantly lower than in LPS alone.

3.6. Laser interferometric analysis of colistin transport in presence of LPS–chitosan complexes

In order to used the laser interferometric method for turbid solutions following apparatus was established. The system under study consists of two glass cuvettes (internal dimensions: 70 mm high, 10 mm wide, 7 mm optical path length). The measurement cuvettes are made of optical glass of high uniformity. The amounts of LPS and colistin produced turbidity significant enough to disturb the laser interferometric analysis. We therefore modified this method to evaluate the concentration boundary layers of antibiotic release from the agarose gel to phosphate buffer (pH 5.0). The concentrations of LPS *P. vulgaris* O25 (48/57), LPS/chitosan complexes, and colistin used in laser interferometry were establish on the basic of a precipitation assay (Fig. 5). We filled the lower cuvette with an aqueous 1% agarose solution (in phosphate buffer, pH 5.0) with

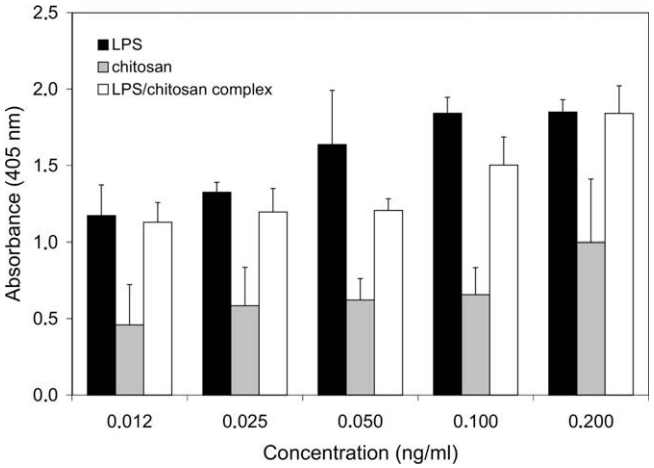

Fig. 4. The LAL activities of LPS *P. vulgaris* O25 (48/57), chitosan, and LPS/chitosan complex 1:1 (w/w).

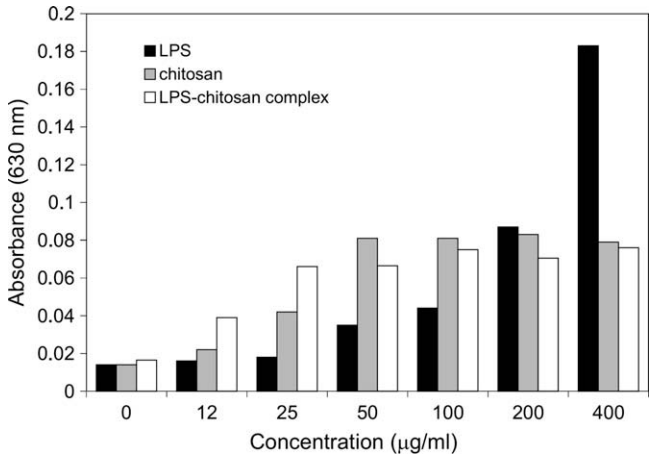

Fig. 5. Precipitation of colistin (2 mg/ml) with *P. vulgaris* O25 (48/57), chitosan, or LPS–chitosan complex after 18 h at 37 °C.

the colistin or chitosan alone or complexes: LPS/colistin or LPS/chitosan/colistin. The upper cuvette was phosphate buffer (pH 5.0) only. With such a configuration of the measurement system the solution in the upper cuvette remains undisturbed and stable CBLs are created (Fig. 6). The aggregate-free colistin concentration is uniform in the planes parallel to the gel–solution interface and gradients occur only in the vertical direction.

The experiment shows the time-dependent colistin release from immobilized agarose gel. The presence of chitosan reduced the speed of colistin diffusion from the gel. That may indicates on interaction of both positively charged molecules. The preformed complex of LPS–chitosan did not affect colistin diffusion, which was identical to that of the LPS–colistin complex (Fig. 6). It may be due to much stronger interaction of *P. vulgaris* O25 LPS with colistin than with chitosan.

4. Discussion

In our previous study electrokinetic properties of complexes of chitosan (Ch) with lipopolysaccharides (LPSs) from *E. coli* O55:B5, *Y. pseudotuberculosis* 1B 598, and *P. vulgaris* O25 (48/57) and their size distribution were investigated using ζ-potential distribution assay and quasi-elastic light scattering (Davydova et al., 2008). The interaction of LPS from different microorganisms with chitosan resulted in the formation of complexes in which the negative charge of LPS was neutralized (LPS from *E. coli*) or overcompensated (*Y. pseudotuberculosis* and *P. vulgaris*). The difference observed in the electrokinetic properties of the complexes suggests

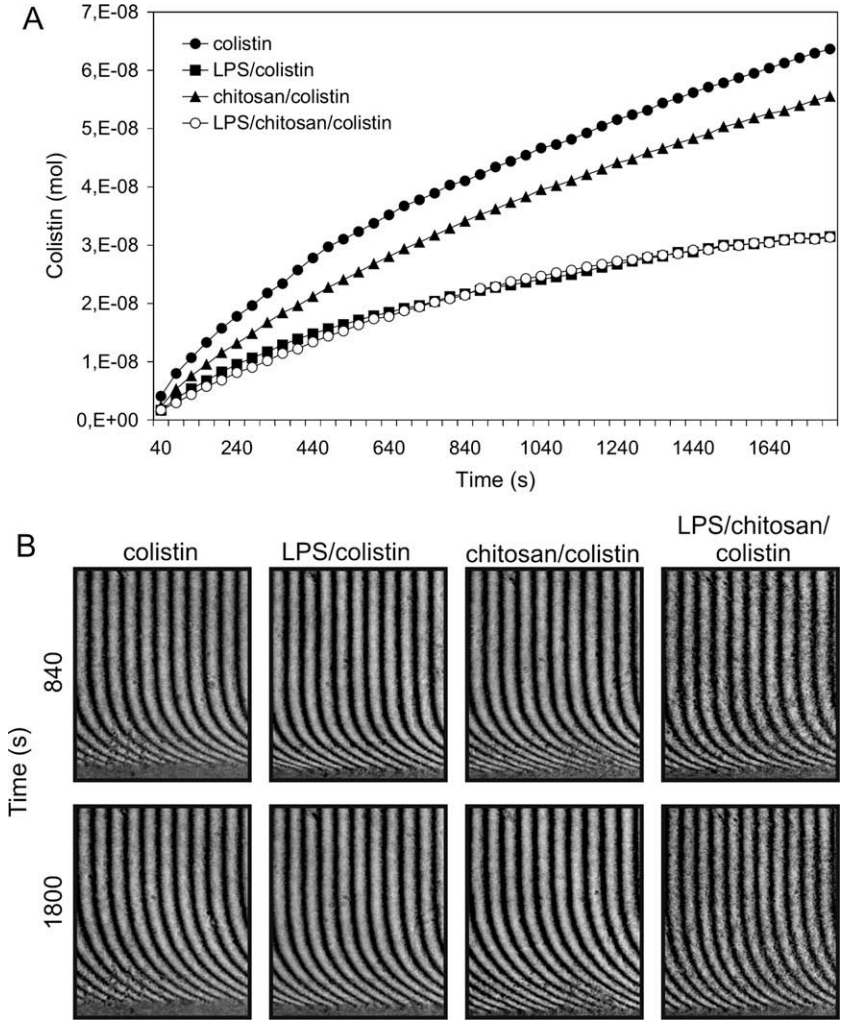

Fig. 6. The concentration profiles of aggregate-free colistin released from 1% agarose gel in the presence of *P. vulgaris* LPS O25 at 250 μg/ml or LPS–chitosan complex 1:1 (w/w) after 30 min of culture at room temperature measured by the laser interferometric system (A). Panel B presents interferograms for 840 and 1800 s of the experiment LPS or LPS–chitosan complex were incubated with colistin at 8 mg/ml for 18 h at 37 °C.

that the availability of negatively charged groups on LPS molecules is completely different and depends on the aggregate structure of the endotoxins.

The present paper demonstrates the complex formation of *P. vulgaris* LPS with CH by using centrifugation in sucrose gradient, laser interferometric analysis and the effects of the CH on the ultra structure and some biological properties of *P. vulgaris* O25 LPS.

Our earlier data showed that stable complexes are formed only after incubation of *Y. pseudotuberculosis* 1B LPS with the polycation at 37 °C, but in the case of *E. coli* at 25 °C (Davydova, Yermak, Gorbach, & Solovieva, 1999; Davydova et al., 2008). In the case of LPS from *P. vulgaris* O25, we also registered complex formation only after incubation of the LPS–polycation mixture at 37 °C. This may be interpreted in light of the change in the supramolecular structure of LPS aggregates, which occurs at increasing temperature (Zhang, Mann, & Tsai, 1999). As is known, rising temperature results in melting of the acyl chains of lipid A of the endotoxin aggregates, which is accompanied by changes in shape and size of the aggregates (Brandenburg & Wiese, 2004), which probably provides the availability of negative groups for interaction.

According to sucrose gradient sedimentation, *P. vulgaris* LPS forms aggregates of different molecular weight in aqueous solutions (Fig. 1A). These data are in agreement with those obtained by electron microscopy (Fig. 2A and B). Negative-stained preparations of *P. vulgaris* O25 LPS exhibited different freely branching ribbon-like structures, similarly to the of *E. coli* and *S. minnesota* 595 LPSs (Roth, Wong, & Hamilton, 1996).

The differences in the size distributions of the LPS and LPS–CH complexes were observed by the sedimentation velocity in a sucrose gradient. Several populations of particles having different velocities of sedimentation were registered after complex formation (Fig. 1C) and they was different from the sedimentation velocity of LPS alone. It was shown that the interaction of LPS and chitosan resulted in the formation of several complexes of different molecular mass from that of the initial LPS. As shown DLS results obtained by us earlier, the complex of *P. vulgaris* LPS with chitosan has a multimodal particle size distribution with very high dispersion. The main fraction of the complex contains particles with sizes in the range of 60–1100 nm (Davydova et al., 2008).

Such data may be a result of the transformation of the supramolecular structure of LPS during complex formation. Electron microscopy was used to check this assumption. Electron microscopy of negatively stained preparations of LPS, chitosan, and their complex allows elucidating the essential differences in their complexes morphologies. After incubation of LPS with chitosan, none of the large ribbon-like structures remained. The heterogeneity of the structures: membrane-like sheets, heterogeneous oblong particles were exhibited by LPS and chitosan complexes (Fig. 2D and E). Polymyxin B, which is known to bind to LPS with very high affinity and to neutralize LPS's biological activity (Lopes & Innis, 1969), has been reported to disaggregate LPS, as seen by electron microscopy. Characterization of the LPS disaggregation induced by human hemoglobin at the ultrastructural level was demonstrated by Roth et al. (Roth et al., 1996). Our results obtained by electron microscopy may indicate on the disaggregation of LPS by chitosan.

The laser interferometric experiment provided a suggestion about the interaction of chitosan with O25 LPS. The lack of differences in colistin diffusion from LPS alone and the LPS–CH complexes indicates that chitosan did not disturb colistin binding to the lipid A part of LPS. This may suggest that chitosan's binding to LPS is weaker than that of colistin. It may be connect with the presence of fatty acid residues as colistin components make those molecules partially hydrophobic. The essential role of hydrophobic interaction between LPS and polymyxin B and its analogs has been reported (Brandenburg, Arraiza, Lehwark-Ivetot, Moriyon, Zähringer, 2002). We can propose that hydrophobic interaction involved in the binding of 025 LPS with colistin. In additional to in the case of *P. vulgaris* LPS, chitosan is perhaps bound with carboxylic residues in the O-specific chains of the endotoxin and the binding of *P. vulgaris* LPS with chitosan takes place on the surfaces of LPS aggregates as was suggest us earlier (Davydova et al., 2008; Knirel et al., 1997).

The solubilities of the lipopolysaccharides might be one important factor in complex formation with biological macromolecules, including chitosan. In our published experiments (Arabski et al., 2007) we showed that native, highly aggregated *P. vulgaris* O25 LPS bound the polymyxin antibiotic colistin less effectively than deacylated *P. vulgaris* O25 LPS, which was well soluble in water. In the present studies we have shown that chitosan, at least partially and in a dose-dependent manner, reduces the precipitation of O25 LPS by colistin. One may assume that this is a result of the partial screening of the lipid A portion of O25 LPS by chitosan. This resulted in blocking the access of colistin to that part of LPS.

The LAL test indicated that LPS–CH complexes are less biologically active than each heteropolymer alone. LAL activation by chitosan alone is probably due to environmental endotoxin contamination of the chitosan preparation and should not affect the presented experiments.

As is known, the binding of the polycations to LPS is a necessary but not a sufficient condition for modification of the biological activity of LPS. Interaction of LPS with CH appeared to be connected with the lipid A region represented a toxic center of endotoxin. LPS–CH complex was shown to possess much lower toxicity in a comparison with parent LPS at injection to mice in the similar concentration. It is interesting to note the tenfold decrease in toxicity of the complex *P. vulgaris* LPS–chitosan in ratio 1:1 w/w. However, in the case of LPSs from *Y. pseudotuberculosis* and *E. coli*, a similar decrease in toxicity of LPS after complexation with chitosan requires a five-times greater amount of polycation (Yermak et al., 2006). This can probably be connected with an increase in the number of linkage sites on *P. vulgaris* O25 LPS to chitosan, which is defined by the chemical structures of the O25 LPS; its O-polysaccharides and lipid A parts as was shown us earlier (Davydova et al., 2008).

It is known that endotoxins stimulate a biosynthesis of different mediators of immune system such as interleukins, TNF and other. In our case, the O25 LPS–CH complexes were shown to maintain an ability to induce TNF-α production. Induction of TNF biosynthesis by LPS–CH complex was found to be 65% lower than that by parent LPS at concentration of 100 ng/ml whereas at 1 ng/ml there was no appreciable change in the biological properties of the endotoxin after binding to chitosan. Some result were obtained by Chou et al. (Chou, Fu, & Shen, 2003). They showed that CH added simultaneously with LPS inhibited the proinflammatory cytokine formation in RAW 264.7 cell.

5. Conclusion

In summary, our result shown that an additional of chitosan to *P. vulgaris* O25 LPS solution to result in formation LPS–CH complexes. The interaction of CH with LPS was shown change of ultra structure of LPS and modulate significantly the biological activity of endotoxin. These CH–LPS complexes possess much lower toxicity than LPS alone. A substation reduction of the LPS toxicity in the complex and in stimulation of cytokines secretion may be explained due to CH as a constituent of the complex my prevent attachment of the LPS to specific cellular receptors or directly neutralize LPS toxicity alternating the molecular charge and/or structures of the aggregates of LPS. This problem requires future investigation.

Acknowledgment

This study was supported by Grant No. NN304 4114 33 from the Ministry of Sciences and Higher Education, Poland, Grant No. 09-11-YO-05-001 FEB RAN from the Russian Academy of Sciences, and by the Leading Scientific Scholl grant to Academician Ovodov Yu.S. -5796.2006.4.

References

Arabski, M., Wąsik, S., Dworecki, K., & Kaca, W. (2007). Laser interferometric determination of ampicillin and colistin transfer through cellulose biomembrane in the presence of *Proteus vulgaris* O25 lipopolysaccharide. *Journal of Membrane Science, 299*, 268–275.

Archibald, W. J. (1947). A demonstration of some new methods of determining molecular weights from the data of the ultracentrifuge. *Journal of Physical Chemistry, 51*, 1204–1214.

Bienvenu, J., Doche, C., Gutowski, M., Lenoble, M., & Pedrix, J. (1995). Production of proinflammatory cytokines and cytokines involved in the TH1/TH2 balance is modulated by pentoxifylline. *Journal of Cardiovascular Pharmacology, 25*, 80–84.

Brandenburg, K., Arraiza, M. D., Lehwark-Ivetot, G., Moriyon, I., & Zähringer, U. (2002). The interaction of rough and smooth form lipopolysaccharides with polymyxins as studied by titration calorimetry. *Thermochimica Acta, 394*, 53–61.

Brandenburg, K., & Wiese, A. (2004). Endotoxins: Relationships between structure, function and activity. *Current Topics in Medicinal Chemistry, 4*, 1127–1146.

Chou, T. C., Fu, E., & Shen, E. C. (2003). Chitosan inhibits prostaglandin E_2 formation and cyclooxygenase-2 induction in lipopolysaccharide-treated RAW 264.7 macrophages. *Biochemical and Biophysical Research Communications, 308*, 403–407.

David, S. A., Bechtel, B., Annaiah, C., Mathan, V. I., & Balaram, P. (1994). Interaction of cationic amphiphilic drugs with lipid A: Implication for development of endotoxin antagonists. *Biochemical and Biophysical, 12*, 167–175.

Davydova, V. N., Bratskaya, S. Y., Gorbach, V. I., Solov'eva, T. F., Kaca, W., & Yermak, I. M. (2008). Comparative study of electrokinetic potentials and binding affinity of lipopolysaccharides–chitosan complexes. *Biophysical Chemistry, 136*, 1–6.

Davydova, V. N., Yermak, I. M., Gorbach, V. J., Krasikova, I. N., & Solov'eva, T. F. (2000). Interaction of bacterial endotoxins with chitosan. Effect of endotoxin structure, chitosan molecular mass, and ionic strength of the solution on the formation of the complex. *Biochemistry (Mosc), 65*, 1082–1090.

Davydova, V. N., Yermak, I. M., Gorbach, V. I., & Solovieva, T. F. (1999). The effect of temperature on the interaction of *Yersinia pseudotuberculosis* lipopolysaccharide with chitosan. *Membrane & Cell Biology, 13*, 49–58.

Domszy, J. G., & Roberts, G. A. F. (1985). Evaluation of infrared spectroscopic techniques for analyzing chitosan. *Macromolecular Chemistry, 186*, 1671–1677.

Dworecki, K., Ślęzak, A., Drabik, M., Ornal-Wąsik, B., & Wąsik, S. (2006). Determination of the membrane permeability coefficient under concentration polarisation conditions. *Desalination, 198*, 326–334.

Dworecki, K., Wąsik, S., & Ślęzak, A. (2003). Temporal and spatial structure of the concentration boundary layers in a membrane system. *Physica A, 326*, 360–369.

Fernandez-Saiz, P., Lagaron, J. M., & Ocio, M. J. (2009). Optimization of the biocide properties of chitosan for its application in the design of active films of interest in the food area. *Food Hydrocolloids, 3*, 913–921.

Galanos, C., Freudenberg, M. A., & Reutter, W. (1979). Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proceedings of the National Academy of Science, 76*, 5939–5943.

Gorbet, M. B., & Sefton, M. V. (2005). Endotoxin: The uninvited guest. *Biomaterials, 26*, 6811–6817.

Inman, J., & Dintzins, H. (1969). Analytical determination of NH_2-groups. *Biochemistry, 8*, 4074–4082.

Keler, T., & Nowotny, A. (1986). Metachromatic assay for the quantitative determination of bacterial endotoxins. *Analytical Biochemistry, 156*, 189–193.

Knirel, Y. A., Kaca, W., Paramonov, N. A., Cedzyński, M., Vinogradov, E. V., Shashkov, A. S., et al. (1997). Structure of the O-specific polysaccharide of *Proteus vulgaris* O25 containing 3-O-[(*R*)-1-carboxyethyl]-D-glucose. *European Journal of Biochemistry, 247*, 951–954.

Kumar, M. N., Muzzarelli, R. A., Muzzarelli, C., Sashiva, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspective. *Chemical Reviews, 104*, 6017–6084.

Lopes, L., & Innis, W. E. (1969). Electron microscopy of effect of polymyxin on *Escherichia coli* lipopolysaccharide. *Journal of Bacteriology, 5*, 1128–1130.

Novotny, A. (1979). Immunologic and other biologic assays. In Basic exercises in immunochemistry: A laboratory manual (pp. 303–305). New-York: Springer-Verlag.

O'Hara, C. M., Brenner, F. V., & Miller, J. W. (2000). Classification, identification and clinical significance of *Proteus*, *Providencia*, and *Morganella*. *Clinical Microbiology Reviews, 13*, 534–546.

Polyakova, A. M., Kravchenko, A. V., Ermak, I. M., Gorbach, V. I., Astrina, O. S., Luk'yanov, P. A., et al. (1995). Effects of chitosan on the biological properties of gram-negative bacteria endotoxins. *Bulletin of Experimental Biology and Medicine, 120*, 814–817.

Roth, R., Wong, J. S., & Hamilton, R. J. (1996). Ultrastructural changes in bacterial lipopolysaccharide induced by human hemoglobin. *Journal of Endotoxin Research, 3*, 361–366.

Singla, A. K., & Chawla, M. (2001). Chitosan: Some pharmaceutical and biological aspects an update. *Journal of Pharmacy and Pharmacology, 53*, 1047–1067.

Vanhaecke, E., Pijck, J., & Vuye, A. (1987). Endotoxin testing. *Journal of Clinical Pharmacy & Therapeutics, 12*, 223–235.

Westphal, O., & Jann, K. (1965). Bacterial lipopolysaccharides: extraction with phenol-water and further application of the procedure. In R. C. Whistler (Ed.), *Methods in Carbohydrate Chemistry* (pp. 5–83). New York: Academic Press Inc.

Wolfrom, M. L., & Shen-Han, T. M. (1959). The sulfonation of chitosan. *Journal of the American Chemical Society, 8*, 1764–1766.

Xing, K., Chen, G.X., Kong, M., Liu, C.S., Cha, D.S., & Park, H.J. (2009). Effect of oleoyl-chitosan nanoparticles as a novel antibacterial dispersion system on viability, membrane permeability and cell morphology of *Escherichia coli* and *Staphylococcus aureus*. *Carbohydrate Polymers, 76*, 17-22.

Yermak, I. M., Davidova, V. N., Gorbach, V. J., Luk'yanov, P. A., Solov'eva, T. F., & Ulmer, A. J. (2006). Forming and immunological properties of some lipopolysaccharide–chitosan complexes. *Biochimie, 88*, 23–30.

Yermak, J. M., Davydova, V. N., Gorbach, V. I., Berdyshev, E. L., Kuznetsova, T. A., & Ivanushko, I. A. (2004). Modification of biological activity of lipopolysaccharide in the complex with chitosan. *Bulletin of Experimental Biology and Medicine, 137*, 379–381.

Zhang, G., Mann, D., & Tsai, C. (1999). Neutralization of endotoxin in vitro and in vivo by a human lactoferrin-derived peptide. *Infection and Immunity, 67*, 1353–1358.