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Interaction of Chitosans and Their N-Acylated Derivatives with Lipopolysaccharide of Gram-Negative Bacteria

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Abstract—The interactions of lipopolysaccharide (LPS) with the natural polycation chitosan and its derivatives—high molecular weight chitosans (80 kD) with different degree of acetylation, low molecular weight chitosan (15 kD), acylated oligochitosan (5.5 kD) and chitooligosaccharides (biose, triose, and tetraose)—were studied using ligand–enzyme solid-phase assay. The LPS-binding activity of chitosans (80 kD) decreased with increase in acetylation degree. Affinity of LPS interaction with chitosans increased after introduction of a fatty acid residue at the reducing end of chitosan. Activity of N-monoacylated chitooligosaccharides decreased in the order: oligochitosan → tetra- → tri- → disaccharides. The three-dimensional structures of complexes of R-LPS and chitosans with different degree of acetylation, chitooligosaccharides, and their N-monoacylated derivatives were generated by molecular modeling. The number of bonds stabilizing the complexes and the energy of LPS binding with chitosans decreased with increase in acetate group content in chitosans and resulted in changing of binding sites. It was shown that binding sites of chitooligosaccharides on R-LPS overlapped and chitooligosaccharide binding energies increased with increase in number of monosaccharide residues in chitosan molecules. The input of the hydrophobic fragment in complex formation energy is most prominent for complexes in water phase and is due to the hydrophobic interaction of chitooligosaccharide acyl fragment with fatty acid residues of LPS.

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Key words: lipopolysaccharide, chitosan, binding, computer simulation, molecular docking

Lipopolysaccharide (LPS, endotoxin), one of the main components of the outer membrane of Gram-negative bacteria, plays the key role in appearance of a complex clinical syndrome—septic shock. LPS is an amphiphilic biopolymer composed of three structurally different parts: O-specific polysaccharide, core oligosaccharide, and lipid A (Fig. 1) [1, 2]. The toxic center of LPS is its glycolipid fragment, lipid A, whose structure is similar in many Gram-negative bacteria [3]. Lipid A consists of the diphosphorylated disaccharide glucosamine acylated by varied amounts of fatty acids. Sufficient amounts of anionic groups—phosphate, pyrophosphate, and carboxyl—are also present in the inner area of core oligosaccharide. The negative charge and amphiphilic nature of the LPS molecule makes it capable of binding with ligands whose molecules are positively charged and amphiphilic, and lipid A is considered as a potential target for substances neutralizing the endotoxic activity of LPS [4].

Many cationic proteins, peptides, and polyamines, which can bind to LPS and modify its endotoxic properties, have been isolated from natural sources [4-6]. The amphiphilic polycationic peptide polymyxin B, one of the most effective neutralizers of LPS, possesses considerable toxicity [4]. Several research groups are searching for synthetic non-toxic polyamines capable of high-affinity binding of LPS and applicable to therapy of septic shock [6, 7]. Mono- and diacylated polyamines containing 14-16 carbon atoms in the acyl residue have demonstrated the most effective binding with LPS [8, 9]. Computer simulation of the interaction between lipopolyamines and lipid A has shown that affinity of the binding strongly depends on both number of charged groups in the polycations and distance between them [10].

We have shown previously that the cationic polyelectrolyte chitosan, a linear polysaccharide composed of β-1,4-bound glucosamine residues, interacts with LPS to

Abbreviations: B-LC) biotinylated low molecular weight chitosan; COL) oligochitosan; COS) chitooligosaccharide; HC) high molecular weight chitosan; LESPA) ligand–enzyme solid-phase assay; LC) low molecular weight chitosan; LPS) lipopolysaccharide.

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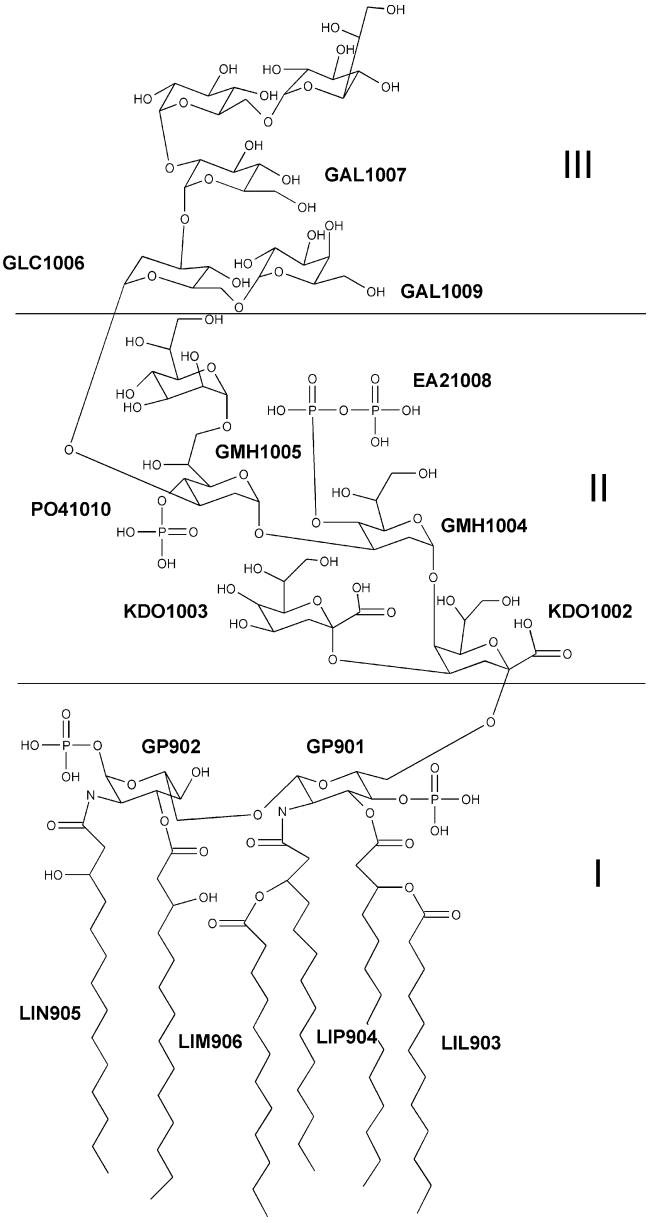

Fig. 1. Structure of lipopolysaccharide from *E. coli* K12. I) Lipid A; II) core oligosaccharide; III) O-specific polysaccharide. Denotations of structural elements are given in accordance with the crystal structure of lipopolysaccharide (code PDB 2FCP) [18].

form stable complexes of various stoichiometry [11]. It might be supposed from the literature data on the interaction of lipopolyamines with LPS [6, 9] that water-soluble hydrophobic derivatives of chitosan are promising for high-affinity LPS binding. In this study, we examined approaches allowing enhanced efficiency of interaction between chitosan and LPS. With this object in mind, we have prepared chitosans with different degree of N-acetylation and chitooligosaccharides N-acylated with long-chain fatty acid and have explored the LPS-binding activity of these compounds.

MATERIALS AND METHODS

LPS from *Escherichia coli* 055:B5, biotinamidohexanoic acid N-hydroxysuccinimide ester, and streptavidin–horseradish peroxidase conjugate were purchased from Sigma (USA) and ultrafiltration membranes from Millipore (USA). All other chemicals were of chemical purity grade (Reakhim, Russia) and were used without additional purification.

^{1}H- and ^{13}C-NMR spectra were recorded on a DPX-300 spectrometer (Bruker Physik, USA) at 300 MHz. D_2O was used as a solvent, and acetone (δ_H = 2.225 and δ_C = 31.45 ppm) served as an internal standard.

The following solutions were used for buffer systems: 0.01 M CH_3COONa/CH_3COOH, pH 5.5 (buffer A) and 0.01 M Tris-HCl, pH 7.2, containing 0.5% gelatin and 0.25% Tween 20 (buffer B).

Preparation of high molecular weight chitosan (HC). Commercially available chitosan (40 g) with N-acetylation degree of 17% obtained after alkaline treatment of crab chitin was deacetylated with the mixture of 40% aqueous solution of NaOH with isopropyl alcohol (1 : 16 v/v) under heating for 7 h at 100°C. The pellet was filtered and dissolved in water acidified with hydrochloric acid (pH 3.5), dialyzed against water, and lyophilized. As a result, chitosan was obtained (30.5 g) with molecular mass of 80 kD determined by viscosimetry [12] and acetylation degree of 1.7% determined by ^{1}H-NMR spectroscopy [13].

Preparation of low molecular weight chitosan (LC). Commercially available chitosan with acetylation degree of 4% was depolymerized with hydrogen peroxide, and chitosan with molecular mass of 15 kD was isolated by the method described earlier [12]. Oligochitosan (COL) with average molecular mass of 5.5 kD (6.2 g) was prepared by depolymerization of HC (8 g) with hydrogen peroxide (1.7%) for 48 h at 37°C, precipitation with isopropyl alcohol, and ultrafiltration on a membrane with exclusion limit of 3 kD. Molecular mass of COL was determined by the amount of reducing terminal monosaccharides according with a published method [14]. Di-, tri-, and tetrachitooligosaccharides were produced by acidic hydrolysis of HC and subsequent ion-exchange chromatography of the oligosaccharide mixture as described in [15].

Preparation of N-acetylated chitosans. HC samples (0.5 g) were dissolved in a mixture of water (40 ml) with methanol (48 ml), and various amounts (0.028, 0.05, 0.12, 0.25, and 0.5 ml) of acetic acid anhydride were added to the solutions. The solutions were incubated for 2 h at room temperature and then alkalified with ammonium solution to pH 9.0. The resulting pellet was separated by centrifugation, washed with methanol, dissolved in dilute hydrochloric acid (pH 3.5), dialyzed against water, and lyophilized. Acetylation degree for the obtained chitosans determined by ^{1}H-NMR spectroscopy [13] was 2.8, 3.7, 8.1, 9.3, and 14.6%.

Preparation of N-acylated chitosans. Chitooligosaccharides (COS) were acylated with 3-hydroxytetradecanoic acid N-hydroxysuccinimide ester as described in [15]. Oligochitosans (COL) (0.8 g) were acylated with 3-hydroxytetradecanoic acid N-hydroxysuccinimide ester (0.4 g) in the solvent mixture of water with N,N′-dimethylformamide (200 ml, 1 : 2 v/v) for 48 h at 37°C. The mixture was acidified with hydrochloric acid to pH 3.0, and free fatty acid was extracted three times with *n*-butanol, low molecular weight admixtures were removed by ultrafiltration of aqueous solution, and the final solution was lyophilized. The raw product yield was 0.65 g. For further purification, the acylated COS (0.15 g) was dissolved in 1.5 ml of acidified water, and the solution was applied on a column with 5 ml of the reverse-phase sorbent Octadecyl Si100-Polyol (Serva, Germany); chitosan not bound to the sorbent was eluted with water, and bound chitosan was eluted with increasing gradient of acetonitrile in water (5-100%). Chitosan in fractions was determined from the reaction of amino groups with trinitrobenzenesulfonic acid [16]. By the data of gas–liquid chromatography and ^{1}H-NMR spectroscopy, the fraction eluted with 20-40% acetonitrile contained, on average, one fatty acid residue per molecule of COL. The yield of acylated COL was 0.062 g.

Biotinylation of chitosan. LC (6 mg) was dissolved in 0.1 ml of 0.1 M acetic acid, the solution was diluted to 1 ml with water, and the pH was adjusted 7.5 with 0.1 M NaOH. Then 1 mg of biotinamidohexanoic acid N-hydroxysuccinimide ester dissolved in 0.1 ml of dimethylsulfoxide was added, and the mixture was stirred for 16 h at 20°C. Biotinylated chitosan (B-LC) was isolated by gel chromatography on Sephadex G-50 in buffer A. The biotinylation degree of labeled chitosan determined by fluorometry, as described in [17], was 4 moles of biotin per mole of chitosan.

Determination of LPS-binding activity of chitosans by ligand–enzyme solid-phase assay (LESPA). The solution of LPS from *E. coli* 055 (0.1 ml, 10 μg/ml) in carbonate buffer, pH 9.5, was applied on a Polysorp polystyrene plate (NUNC, USA) and incubated overnight at 37°C. Nonspecific sites of binding were blocked with buffer B. The plates were washed with buffer A, and 0.1 ml of B-LC solution in buffer A at various concentrations (7.7-250 μM) was applied into each well (the molar concentrations of chitosan were counting based on the glucosamine residue) and incubated for 4 h at 37°C. In additional experiments, the amount of bound B-LC was found to achieve a constant level during this time range (data not shown). After the incubation, plates were washed with buffer A, and streptavidin–horseradish peroxidase conjugate at the dilution of 1 : 1000 was applied to the wells and incubated for 1 h at 37°C. *o*-Phenylenediamine was used as a chromogenic substrate in the substrate mixture. Absorption was determined on a μQuant Bio-TEK Instruments, Inc. spectrophotometer (USA) at 492 nm in three parallel samples. The value of $\Delta D = D_{ex} - D_0$ was calculated, where D_{ex} is optical density after the addition of B-LC and D_0 is optical density without B-LC. Dissociation constant values (K_d) were calculated graphically from the Scatchard plot, where ΔD is abscissa and $\Delta D/C_{B\text{-}LC}$ is ordinate.

Competitive LESPA was used to determine LPS-binding activity of acetylated and acylated derivatives of chitosans. Ligands at various concentrations (12.5-350 μM, three parallel samples for each concentration) were applied into the wells simultaneously with B-LC (30.8 μM), and the binding of B-LC with LPS was determined as described above. The inhibition of B-LC binding with components of LPS from *E. coli* was carried out according to a method similar to that described for chitosans. Inhibition constant (K_i) was determined from the equation:

$$K_i = [I]_{50}/(1 + [L]/K_d),$$

where $[L]$ is molar concentration of B-LC, K_d is equilibrium dissociation constant, and $[I]_{50}$ is concentration of unlabeled ligand inducing 50% inhibition of specific binding of B-LC with LPS. The $[I]_{50}$ value was determined graphically on the basis of inhibition curve (plot of inhibition dependence (%) on molar concentration of inhibitor). The K_d value was previously determined as described above.

Modeling of low molecular weight chitosans, N-acetylated and N-acylated derivatives of chitosans, and their complexes with LPS. Theoretical models of chitosan spatial structure (number of repeated links n = 2, 3, 4, 8, and 30) were built using the carbohydrate construction module of the MOE software package (Molecular Operating Environment; http://www.chemcomp.com/). Structures of oligochitosan (n = 30) with various number of acetate groups and acyl derivatives of chitooligosaccharides were built using the molecular construction module of the MOE software. Crystal structure of *E. coli* K12 LPS primarily stated in work [18] (code PDB 2FCP) was obtained from the PDB database [19]. An acyl moiety (absent in the initial file) was built-in using the molecular construction module of the MOE software. Energy minimization was carried out for LPS and chitooligosaccharide structures in vacuum and in water. Energy minimization for molecules was performed by the method of steepest descent using the energy minimization module of the MOE software with parameters of force potential MMFF94x.

Molecular docking of chitosans and their acetyl and acyl derivatives was performed using the docking module (FlexX) of the MOE software. A lipopolysaccharide molecule was taken for a receptor, and chitosan molecules were taken for conformationally flexible ligands when docking was performed. The contacts in complexes were analyzed using the MOE software.

RESULTS

Synthesis and characterization of chitosan derivatives. Chitosan specimens differing in degree of N-acetylation were synthesized by stepped acetylation of preliminarily deacetylated chitosan. This approach allows preparation of chitosan specimens with equal molecular masses and random distribution of N-acetates along the polysaccharide chain. This is necessary for elimination of effects of chemical structure and chain length on the LPS-binding activity of chitosan. Elimination of N-acetyl groups of chitin commonly occurs under stringent alkaline conditions [12]. We have applied the method of chitin deacetylation under mild conditions using a mixture of aqueous alkali and isopropyl alcohol as the reagent. This resulted in high yield (75%) of HC with molecular mass of 80 kD determined by viscosimetry [12] and acetylation degree of 1.6-2% by the data of ^{1}H-NMR spectroscopy [13]. Then HC was N-acetylated by acetic anhydride in water–methanol solution with increase in anhydride/glucosamine weight ratio. As a result, we obtained five HC specimens containing from 2.8 to 15.0% acetate determined by ^{1}H-NMR spectroscopy.

To prepare specimens of chitooligosaccharides and low molecular weight chitosan derivatives, we used two different methods of carbohydrate chain splitting. Di-, tri-, and tetra-COS were prepared by acidic hydrolysis of HC followed by ion-exchange chromatography of the hydrolyzate [15]. Depolymerization of HC with hydrogen peroxide resulted in COL (average molecular mass of 5.5 kD) and LC (15 kD). COS were N-acylated with 3-hydroxytetradecanoic acid hydroxysuccinimide ester and isolated by reverse-phase chromatography on octadecyl silica gel. By the data of chemical analysis and ^{1}H- and ^{13}C-NMR spectroscopy, molecules of acylated di-, tri-, and tetra-chitooligosaccharides contain one residue of 3-hydroxytetradecanoic acid bound with the amino group of the glucosamine residue localized on the reducing terminus of the molecule [15]. Acylated COL derivatives also contain, on average, one residue of 3-hydroxytetradecanoic acid, which presumably acylates the amino group of glucosamine on the reducing terminus of the polysaccharide. Acylated COS are known to aggregate in aqueous solutions to form micelles of various size [20]. The critical micelle concentrations determined using a fluorescence probe for di- and tetrachitooligosaccharides N-acylated with 3-hydroxytetradecanoic acid are 490 and 1024 μg/ml, respectively [21], which is higher than concentrations of chitosan derivatives (5-200 μg/ml) used in this study, so one can suppose that LPS interact with monomolecular forms of acylated chitosans.

Characterization of the LESPA test-system. To determine the equilibrium dissociation constant (K_d) for chitosan–LPS complex formation, we used a method based on interaction of biotinylated chitosan with LPS immobilized on a solid carrier. It was shown previously that effective binding of LPS with chitosan occurs after preliminary incubation of the components in a solution at 37°C followed by holding of their mixture at the same temperature [22]. So, before immobilization, the LPS solution was held at 37°C for 24 h for partial dissociation of LPS aggregates. Both binding of LPS with B-LC and inhibition of the binding were also carried out at 37°C, and the reaction mixtures were held for 4 h to achieve equilibrium. The K_d of reaction between LPS and LC determined by LESPA was $5.1 \cdot 10^{-6}$ M. This is near the K_d determined earlier for this complex by other methods [11] and may evidence reliability of data obtained using the LESPA test-system.

Activity of chitosans and their derivatives in interaction with LPS was determined by competitive binding using LESPA. The following system was used for inhibition: *E. coli* LPS immobilized on the surface of solid phase and LC labeled with biotin (B-LC). The amount of B-LC bound with LPS was determined using streptavidin–horseradish peroxidase conjugate.

The IC_{50} values (the inhibitor concentration required for reduction of ligand binding by half) were determined for all chitosan specimens, and K_i were calculated from the determined K_d value. As it is known, the inverse relationship is observed between K_i and affinity of binding, that is, K_i increase evidences decrease in affinity of chitosan derivative binding with LPS [23].

Effect of N-acetylation degree of chitosan on its LPS-binding activity. Figure 2 shows the plot of K_i of chitosan specimens with different contents of N-acetates (1.6, 2.8, 3.7, 7.2, 9.3, and 15.0%) versus their acetylation degree. Analysis of the data indicates that the affinity of chitosan binding with LPS decreases with increase in chitosan acetylation degree from 1.6 to 9% and on the whole decreases about eightfold (Fig. 2). Further increase in amount of acetate groups from 9 to 15% does not lead to

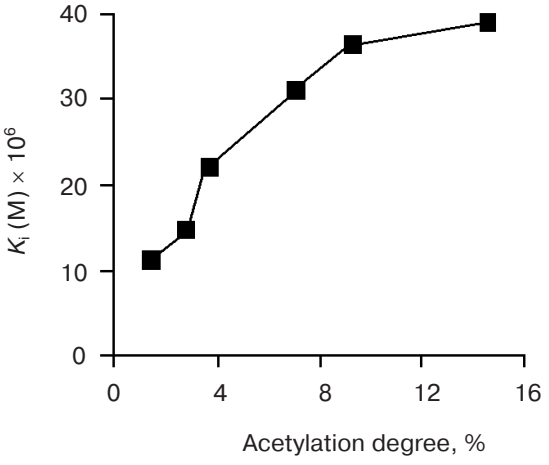

Fig. 2. Effect of N-acetylation degree of chitosans on binding with LPS.

change in LPS-binding activity of chitosans. Thus, the N-acetylation degree of chitosan influences affinity of its binding with LPS.

Binding of N-acyl derivatives of chitosan with LPS. We studied the binding affinity of a series of chitosan derivatives N-acylated with 3-hydroxymyristic acid: water-soluble N-3-hydroxymyristoyl chitooligosaccharides and N-3-hydroxymyristoyl oligochitosan in comparison with corresponding non-acylated compounds. Comparison of 50% inhibition concentration and inhibition constant values given in the table showed that the affinity of the studied chitosan derivative specimen binding with LPS falls in the order: N-acyl-COL > N-acyl-chitotetraose > N-acyl-chitotriose > N-acyl-chitobiose > LC > chitotetraose. Analysis of the data shows that introduction of acyl substituent into low molecular weight chitosan derivatives significantly increases their LPS-binding activity (compare I–V, II–VI). Elongation of the carbohydrate chain of the studied chitosans (n = 2-30) also leads to increase in LPS-binding activity—both of substituted and non-substituted chitosan derivatives.

Interaction of LPS fragments with chitosan. It is known that in the LPS molecule both lipid A and inner area of core oligosaccharide carry negatively charged groups which may be involved in interaction of LPS with chitosan [4]. We studied the ability of these fragments of the LPS molecule to inhibit binding in the B-LC–LPS system. As is evident from Fig. 3, both structural fragments of the molecule rather effectively (by 60-80%) inhibit the binding. However, the inhibitory activity of lipid A is somewhat higher than that of core. One can see by comparison that O-specific polysaccharide, the uncharged LPS fragment, virtually does not inhibit B-LC binding with LPS.

Modeling of chitosans with different contents of acetate groups and their complexes with R-LPS. We built the structures of low molecular weight chitosans (n = 30) containing different amounts (3.3, 6.6, 9.9, 13.2, and 16.6%) of acetate groups with the following positions on monosaccharide residues of chitosan: two groups on residues 1 and 15; three groups on residues 1, 15, and 25; four groups on residues 1, 7, 15, and 25; and five groups on residues 1, 7, 15, 17, and 25. The structure of the molecule exhibits an elongate stalk consisting of the glucosamine residues, forming a spiral. We conducted molecular docking of these chitosans with R-LPS using parameters allowing calculation of the energy of molecule binding into a complex and predicted the structures of the most energetically advantageous complexes. The energies of chitosan binding with LPS are −4.2, −3.9, −3.5, and −3.7 kcal/mol for chitosans without acetate groups and containing two, four, and five acetate groups, respectively. Increase in acetate group number leads to increase in free binding energy and, hence, to decrease in affinity of LPS interaction with chitosan. Analysis of contacts between the components in complexes showed that the structure of the complex is stabilized by several hydrogen and ionic bonds. The ionic bonds are formed between the nitrogen atoms of chitosans with the oxygen atoms GP4901 O7A, O8A, O9A, EA21008 O2P, and KDO1002 O1A, O1B of lipopolysaccharide. The hydrogen bonds are formed by nitrogen atoms and oxygen atoms O3 and O6 of chitosans and GP4901 O7A, O8A, O9A, EA21008 O2P, KDO1002 O1A, O1B, GAL1007 O2, PO41010 O2, KDO1003 O1A, KDO1002 O7, and GMH1004 O7 of lipopolysaccharide. Increase in amount of acetic groups in chitooligosaccharides leads to change in the most advantageous binding site and decrease in bonds stabilizing the complex.

Modeling of di-, tri-, tetra-, and octachitooligosaccharides, their acyl derivatives, and complexes of chitooligosaccharides with R-LPS. The structures of di-, tri-, tetra-, and octachitooligosaccharides and their deriv-

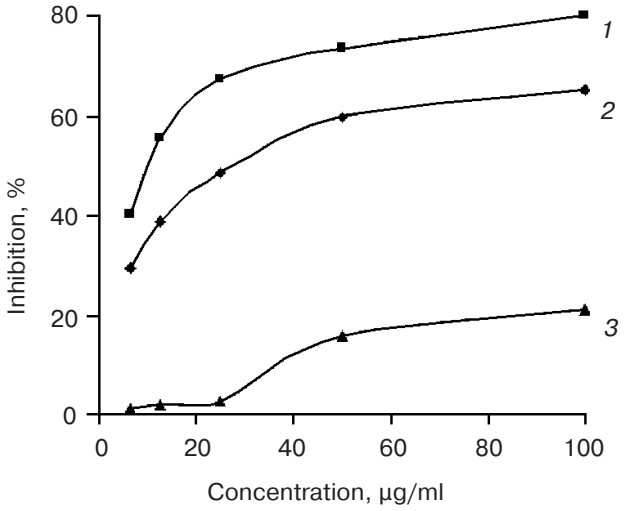

Fig. 3. Inhibition of B-LC binding with LPS. Inhibitors: *1*) lipid A; *2*) core; *3*) O-specific polysaccharide.

Inhibition of B-LC binding with LPS by acylated COLs

Derivative	50% inhibition concentration, μM	K_i (M) × 10^6
Chitotetraose	333.3	81.7
COL	125.0	30.8
C14-OH chitobiose	166.7	40.8
C14-OH chitotriose	88.9	21.8
C14-OH chitotetraose	44.4	10.9
C14-OH COL	13.9	3.4

atives N-acylated with 3-hydroxytetradecanoic acid on the reducing terminus were reconstructed using the carbohydrate construction module of the MOE software. The structures after energy minimization in water were used in experiments on molecular docking of chitooligosaccharides and lipopolysaccharide (Figs. 4 and 5). The distances were determined between amino groups in the chitosan molecule and O-atoms of phosphate groups in the lipid A molecule. It has been shown that with elongation of the carbohydrate chain of chitosan the conformational sites appear with the distance 1.23 nm between amino groups, which is close to the distance 1.26 nm between the atoms of lipid A phosphate groups the chitosan amino groups can interact with. The elongation of the chitosan carbohydrate chain leads to multipoint binding on several anionic sites localized on both core and lipid A of the LPS molecule. The fatty acid residue of N-acylated COS is parallel to the fatty acid residues of lipid A that enforces their hydrophobic interaction and increases affinity of the complex between acylated chitosan and LPS. The energies of binding of di-, tri-, tetra-, and octachitooligosaccharides with R-LPS calculated using the MOE software are –4.47, –4.84, –4.61, and –4.30 kcal/mol, respectively. These energies for their acylated derivatives are –4.49, –3.95, –5.43, and –4.38 kcal/mol, respectively.

DISCUSSION

We have shown earlier that chitosan, the natural cationic polyelectrolyte, forms stable complexes with LPS [11]. It is generally admitted that LPS binding with polycationic compounds occurs basically via electrostatic interactions between oppositely charged groups of the two components and includes carboxylic and phosphate groups of core and lipid A in the LPS molecule [4, 5]. The main contribution to the negative LPS charge belongs to phosphate substituents localized in lipid A, which is a hydrophobic fragment and the main LPS site for binding of polycations capable of neutralizing the endotoxin toxicity.

The fact that LPS displaces the anionic dye Tropaeolin 000-2 from its complex with chitosan [11] suggests for involvement of ionized amino groups of chitosan in LPS binding. The results obtained in the present work on inhibition of LPS binding to B-LC by the lipid A and core is an indirect evidence for the role of charged residues in the interaction between LPS and chitosan. The significant role of electrostatic forces in formation of LPS–chitosan complexes is confirmed by their marked dissociation, sometimes to total decomposition, when incubated in 0.6 and 1.0 M sodium chloride solutions. However, some LPS–chitosan complexes are revealed maintaining their stability in solutions of high ionic strength [22]. This suggests that other interaction types, besides electrostatic forces, are involved in the complex formation. Hydrogen bonds with participation of chitosan hydroxyl groups as proton donors and hydrophobic forces may be involved in stabilization of the complexes. Increase in contribution of hydrophobic bonds into the formation of LPS–chitosan complex can be regarded as one of possible ways to increasing its stability.

The chitosan molecule has hydrophobic substituents, namely acetate groups, which partially substitute amino groups in the polymer. At present, there is no data on direct involvement of N-acetates in chitosan binding with LPS; there exists a possibility of interaction between these groups and acyl domain of lipid A. Moreover, N-acetate groups can indirectly influence the binding via their effect on both the conformation and physical properties of chitosan.

We have studied the influence of N-acetylation degree of chitosans on the formation of its complexes with LPS for chitosans with relatively low content of acetates (<15%). Chitosan specimens with various amounts of acetates but of equal molecular masses and random character of distribution of N-acetates along the polysaccharide chain were taken for the comparative study. This allowed elimination of the effect of chemical structure and chain length on the LPS-binding activity of chitosans. The increase in the content of acetates in the chitosan molecule (from 1.5 to 9.0%) was found to result in decrease in efficacy of its binding to LPS in spite of the increase in general hydrophobicity of the polymer molecule. When the amount of acetate groups further increases (up to 15%), a disturbance of this regularity occurs: chitosans with different acetate contents demonstrate equal inhibitory activities. This may be due to rather high hydrophobicity of chitosan specimen with 15% of acetates, which can enhance its affinity to hydrophobic sites of LPS molecule and thereby compensate the decrease in contribution to interaction of electrostatic component resulting from the increase in amount of N-acetates.

The mechanism of decrease in affinity of interaction between LPS and chitosan when N-acetylation degree of the latter increases is probably complex. One cause of the observed dependence can be the decrease in linear density of charges and flexibility of chitosan polymer chain resulting from the increase in the content of N-acetate groups in the molecule [24]. In fact, the force of electrostatic interaction between polyelectrolytes (including chitosan) and oppositely charged amphiphilic compounds decreases with the increase in rigidity of polymer molecule and decrease in the charge density. It may be that the higher linear charge density in chitosan with acetylation degree of 1% compare to that in chitosan with 12% N-acetates is the cause of stronger binding with negatively charged detergent micelles [25]. The linear charge density in the chitosan molecule and the extent of its flexibility can probably influence the size and localization of chitosan-binding sites on the LPS molecule, as it has been observed earlier when studying the interactions between polyelectrolytes and proteins [26]. A comparative analysis

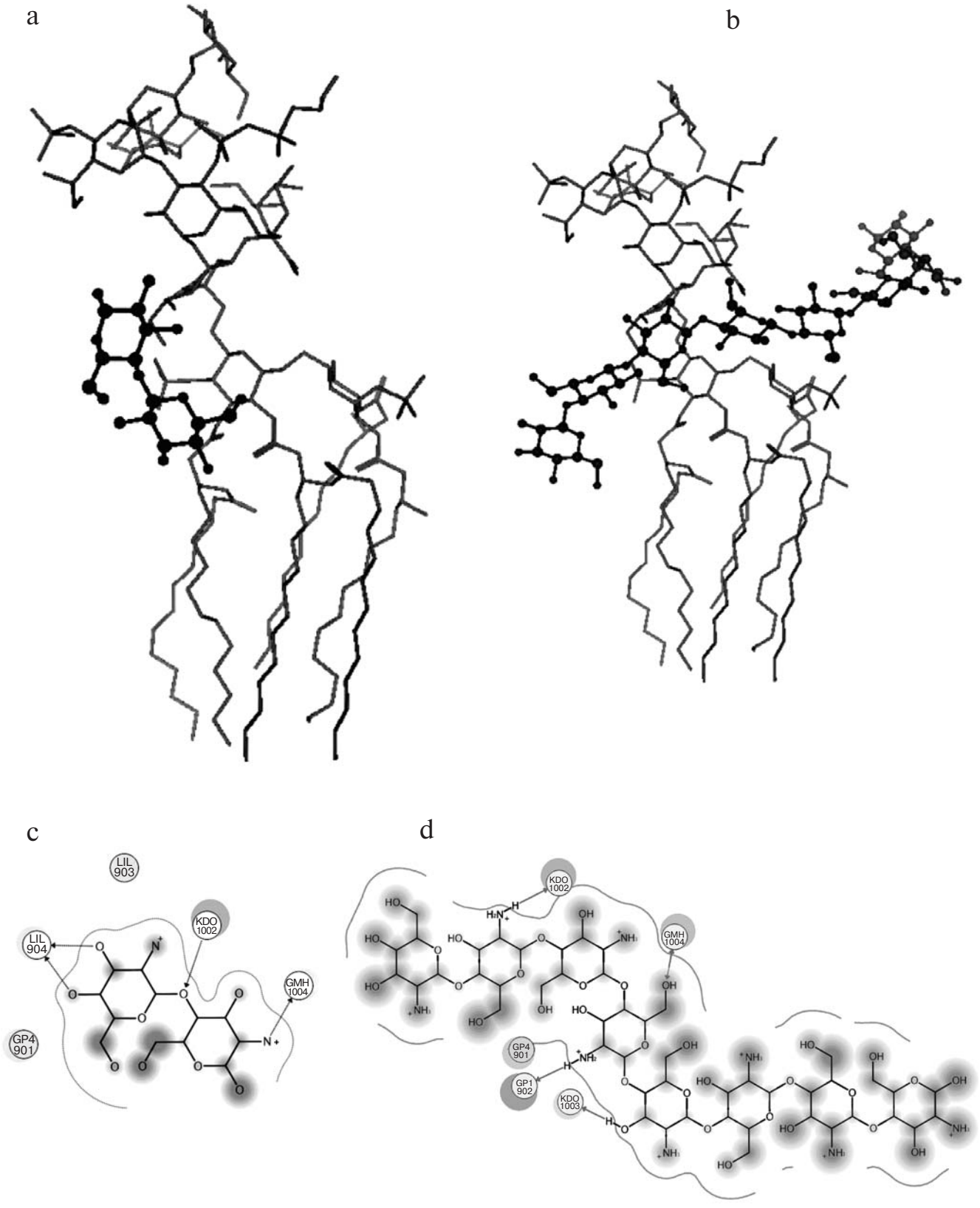

Fig. 4. Structures of the lowest energy complexes of lipopolysaccharide with chitobiose (a) and chitooctaose (b) calculated using the docking module of the MOE software and analysis of contacts in these complexes (c, d). The LPS molecule is represented in stick form and chitooligosaccharides in ball-and-stick form. The denotations for the lipopolysaccharide molecule sites are analogous to those given in the Fig. 1.

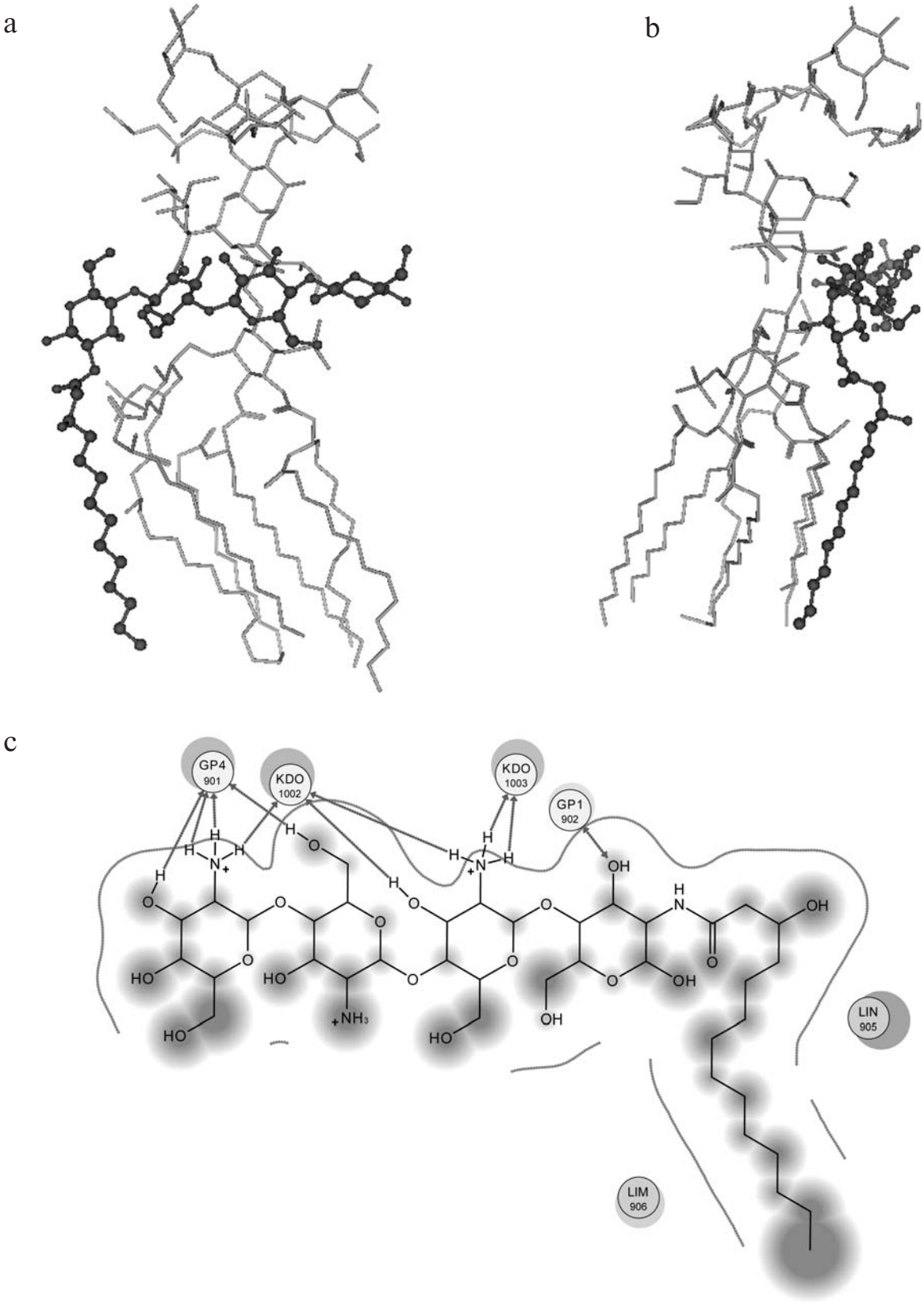

Fig. 5. Structure of complex between the N-acylated derivative of chitotetraose with lipopolysaccharide (a), side-view of their complex (b), and analysis of contacts in the model complex (c) drawn using the MOE software. The LPS molecule is represented in stick form and N-acylated chitotetraose in ball-and-stick form. The denotations for the lipopolysaccharide molecule sites are analogous to those given in the Fig. 1.

of LPS complexes with chitosans of various N-acetylation degrees constructed by molecular modeling suggests such supposition. Hence, the content of N-acetates, the above-mentioned polymer properties depend on, must influence the features of the binding sites. This can contribute to the dependence of the LPS–chitosan binding affinity on the content of N-acetates in the chitosan.

As has been revealed earlier, the increase in the content of N-acetate groups in chitosan results in the increase in association between molecules in aqueous media [27]. Although we conduct our experiments in dilute solutions, we cannot completely exclude the contribution of this process to alteration of binding affinity that is observed under alteration of acetylation degree.

Thus, the elevation of hydrophobicity of the chitosan molecule due to the increase in N-acetylation degree cannot serve as an effective way to increase its LPS-binding activity.

It has been demonstrated earlier that long-chain aliphatic lipophilic groups, when presented in a polycation, stabilize its complexes with LPS through hydrophobic interactions of hydrophobic substituents with acyl lipid A domains [4, 6]. A chemical modification of chitosan was performed to increase the affinity of LPS–chitosan binding. Hydrophobic derivatives of chitosan were synthesized substituted at the amino group by 3-hydroxytetradecanoic acid. The solubility of these compounds in aqueous media was achieved via utilization of low molecular weight chitosan derivatives and low degree of their acylation. Mono-acyl-chitooligosaccharides (di–tetra) and oligochitosan (5.5 kD) were produced as a result, with the position of acyl substituent at the reducing terminus of the molecule. As mentioned earlier [6], amphiphilic compounds of such type with distantly localized hydrophobic and hydrophilic regions inside the same molecule interact preferentially with the lipid A fragment, which is important for neutralization of LPS. The study of LPS-binding activity of acylated chitosan derivatives in comparison with non-substituted derivatives has demonstrated that the introduction of long acyl substituents into the chitosan molecule substantially increases its affinity to LPS.

A computer simulation of LPS–chitosan complexes has been carried out by the method of molecular docking to obtain information on their structure. The complexes were selected for each chitosan specimen with the strongest binding between the components (minimum free energy of the interaction). Generally, the models for LPS complexes with three types of chitosan derivatives were obtained: for chitosans with various contents of acetamide groups, COS, and N-acyl-COS. When the constructed and experimental complexes of LPS with chitosan of various N-acetylation degrees were compared, we observed that the character of change in theoretical free energy values for complex binding coincided generally with that in the experimental K_i values (table) depending on the content of N-acetates in the chitosan. The analysis of the complexes has demonstrated involvement of ionic, hydroxyl, and carbonyl groups of both components in the binding with the formation of a net of hydrogen bonds, and involvement in the interaction of simple sugar and fatty acid residues of core and lipid A in LPS. The putative sites of chitosan binding on the LPS molecule are thus not the same for the chitosan specimens with different content of N-acetates.

The analysis of models of LPS–chitooligosaccharide complexes has demonstrated that the free energy of the binding decreases with the increase in polymerization degree of the oligosaccharide, which coincides with the experimental data suggesting that K_i values for the studied chitosan derivatives increase (the affinity decreases) in the order: N-acyl-COL < N-acyl-chitotetraose < N-acyl-chitotriose < N-acyl-chitobiose < COL < chitotetraose. It follows from the models that the increase in length of chitosan carbohydrate chain results in multipoint binding on several anionic sites which are localized on core and lipid A of LPS molecule, which apparently determines the increase in the binding affinity.

Thus, our results show that water-soluble derivatives of chitosan with hydrophobic substituents and low substitution extent of amino groups are promising compounds for binding endotoxin. In connection with this, the synthesis and study of LPS-binding activity of new chitosans modified with hydrophobic substituents are of interest.

The study on the *in silico* interaction between LPS and chitosan is a difficult task. The method of molecular docking is used generally for prediction of the structure of a complex between protein (receptor, enzyme) and ligand. In this case, a binding site localized in a spatially limited inner space of the protein molecule is only regarded. In our case, the molecular surface of receptor (LPS) is exposed to the solvent to greater extent, and the ligand (chitosan) possesses substantially greater rotary and conformational freedom due to the absence of strict steric limitation. Our work represents the initial step in the investigation on computer simulation of LPS–chitosan complexes and will be continued.

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