Use of Colorimetric Method for Evaluation of LPS of Different Structure

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We studied the possibility of using the colorimetric method with carbocyanin dye for quantitative measurements of LPS of different structure in aqueous solutions. The absorption spectra (intensity and position of absorption peaks) of LPS—carbocyanin complexes depend not only on the presence of polysaccharide fragment in LPS structure, but also on the core structure.

Key Words: *lipopolysaccharides; carbocyanin; bacterial chemotypes*

LPS are components of the cell wall in gram-negative bacteria [5]. The interest to LPS is constantly increasing due to their role in vital activity of microorganism and because of the involvement of endotoxins (LPS by chemical structure) in the development of endotoxin shock leading to severe consequences and lethal outcome [6]. Biological, serological, and physicochemical methods are used for LPS measurements in drugs and biological fluids. However, biological methods are laborious. LAL test widely used in recent years is sufficiently simple and highly sensitive, but its availability is limited by high price of reagents.

Among the physicochemical methods for LPS measurements, the colorimetric method is worthy of note [8]. LPS contain no chromophore groups and do not absorb light in visible spectrum. The proposed method is based on the capacity of cation carbocyanin dye to form colored complexes with LPS, the spectral peaks of these complexes are shifted towards shorter wavelengths (λ=468-478 nm) compared to dye maximum (λ=510 nm). Later detailed evaluation of the method potentialities showed that it was difficult to attain reproducible results with LPS isolated from different microorganisms. Several modifications of the original method were developed for more effective evaluation of LPS, for example, modifications rendering it higher sensitivity and better reproducibility [1]. Other solvents for carbocyanin were proposed in order to improve color stability.

It is known that LPS consists of hydrophobic lipid A, oligosaccharide core, and O-polysaccharide. However, no relationship was detected between changes in the spectra of LPS—carbocyanin complex and composition of LPS molecule.

We studied the possibility of using the colorimetric method for measurements of LPS differing by their core structure.

MATERIALS AND METHODS

Commercial LPS preparations were used: *E. coli* O55:B5 (S-chemotype) *E. coli* EH100 (Ra mutant), *E. coli* J5 (Rc mutant), and *Salmonella typhimurium* SL1181 (Re mutant; Sigma). Carbocyanin dye (1-ethyl-2-(3-(1-ethylnaphtho(1,2d)-thiasolin-2-ilidene)-2-methylpropenyl)naphtho(1,2d)-thiasolium bromide; Aldrich) was used. LPS from *Rhodobacter capsulatus* was isolated by phenol extraction [4].

LPS were evaluated by the classical method and its modifications were studied [1,8]. The absorption spectra were recorded on a Hitachi 557 spectrophotometer. The final concentration of LPS

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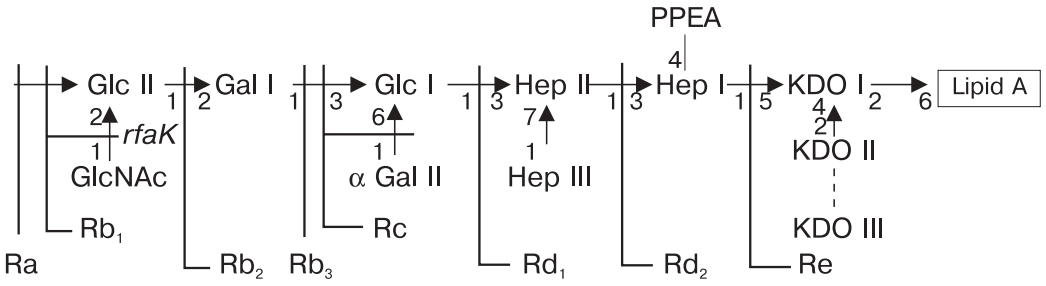

Fig. 1. *E. coli* K-12 LPS core structures (from Ra to Re; adapted after [9]).

in samples was 100 μg/ml, of carbocyanin stain 0.003%.

RESULTS

Numerous bacterial strains form colonies of different shape determined by the structure of cell wall LPS. Bacteria forming smooth colonies (S-chemotype) contain complete LPS molecules in their cell wall consisting of hydrophobic lipid A, oligosaccharide core, and polysaccharide O-chain [3]. Microorganisms forming rough colonies produce LPS lacking polysaccharide chain (R-chemotype) or containing its rudimentary form.

The R-chemotype LPS can possess core structures differing by the quantity and composition of sugars (from maximally shortened Re-structure to complete Ra core; Fig. 1). We studied the dependence of LPS—carbocyanin complex absorption spectra on S, Ra, Rc, and Re structure of LPS.

In previous studies using carbocyanin dye for LPS measurements different positions of the absorption peaks were noted not only for LPS from different bacteria, but even for LPS from the same strain [1]. This can be due to different methods of extraction, purity of preparations, specific features of the strains, and culturing conditions essential for the structure of the appropriate LPS.

Our previous studies on *Rb. capsulatus* culture showed that modification of growth conditions was essential for the structure of LPS released into the medium. This can be seen in the position of absorption peaks of the LPS—carbocyanin complexes [2].

Analysis of absorption spectra after LPS hydrolysis showed that the absorption peaks of the lipid A—carbocyanin and polysaccharide—carbocyanin complexes are shifted towards short-wave and long-wave regions, respectively, from the absorption peak of free dye [1]. The contribution of the core to spectrum modification and positions of the absorption peaks was never determined.

Solutions of carbocyanin with LPS preparations free from polysaccharide fragment (R-chemotype) turned different shades of yellow, while solutions of S-chemotype LPS were always blue. Only S-LPS exhibited additional absorption at 500-600 nm (Fig. 2). This can mean that blue color indicates the presence of O-polysaccharide in LPS preparations. This is in line with the data on the spectrum of polysaccharide—carbocyanin complex absorption for the polysaccharide fragment obtained by LPS hydrolysis [1]. Our results suggest that the color of LPS solution with carbocyanin indicates the chemotype of the bacterium.

Analysis of absorption spectra of LPS—carbocyanin complexes (Fig. 2) indicates that the spectra of LPS of different chemotypes differ by shape, absorption, and position of absorption peaks. The most long-wave absorption maximum was recorded for S-LPS (at λ=465 nm). With shortening core structure the peaks shifted towards the short-wave area: λ=462 nm for Ra-core, λ=461.5 nm for Rc-core, and λ=458 nm for Re-core (Fig. 2). The shorter the LPS structure, the lower absorption of the complex (LPS concentration in all samples 100 μg/ml). It is noteworthy that the weight percent of lipid A differs in different LPS samples, decreasing from Re- to S-structure. This can affect absorption of the LPS—dye complex.

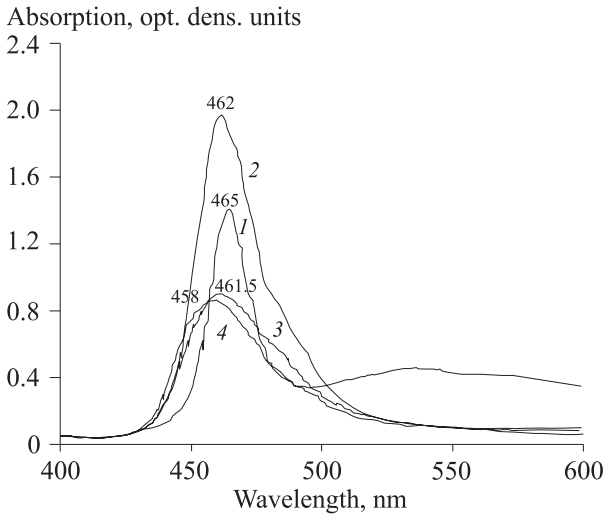

Fig. 2. Absorption spectra of LPS—carbocyanin complexes for LPS of different structure (LPS concentration 100 μg/ml; carbocyanin concentration 0.003%). *1*) S-LPS; *2*) Ra-LPS; *3*) Rc-LPS; *4*) Re-LPS.

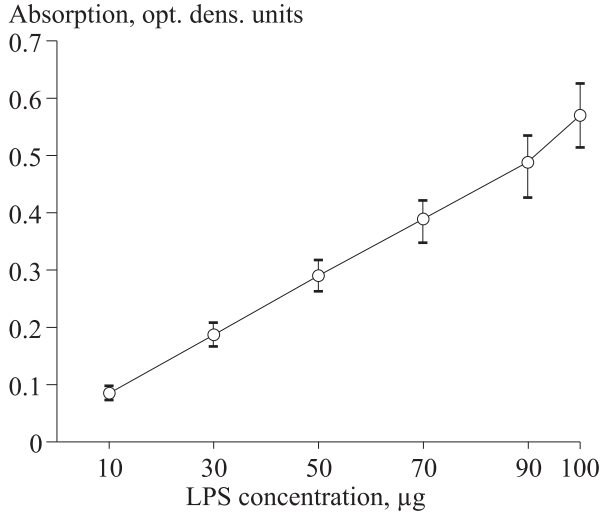

Fig. 3. Relationship between carbocyanin—$LPS_{Rb.\ caps.}$ complex absorption and LPS concentration.

As was noted above, a principal difference between S- and R-LPS is the presence of O-antigen. Molecules shortened by this fragment are more hydrophobic and liable to aggregation. LPS hydrophobia also increases with shortening the core, which leads to the formation of micelles in solutions. This reduced the availability of individual molecules for dye, which was clearly reflected by the absorption spectra intensity (Fig. 2). This assumption is confirmed by the data on proportional reduction of dye-LPS interaction after addition of the aggregating agent (PEG-6000) [1]. With increasing LPS hydrophobia from S- to Re-chemotype the absorption intensity decreases, the spectra are extended, and the peaks are shifted towards the short-wave region. This means that quantitative evaluation of LPS in samples requires plotting the absorption-concentration curves for each LPS.

In order to extent the interval of linear relationship between LPS content and absorption, increasing dye concentration to 0.02% was proposed [1]. This method proved to be unjustified in experiments with *Rb. capsulatus* LPS. We obtained a linear relationship between the complex absorption intensity and the concentration of this LPS at 10-100 μg/ml in the presence of 0.003% dye in the sample (Fig. 3).

In order to prevent absorption of LPS and carbocyanin dye, it is convenient to use borosilicate glass tubes.

Summing up our findings, we conclude that the absorption spectra of LPS—carbocyanin complexes provide information about the composition of the studied LPS, for example, the presence of the polysaccharide fragment and core structure.

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