Biotechnology Reports 24 (2019) e00381

Contents lists available at ScienceDirect

Biotechnology Reports

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

Covalently immobilized chemically modified lysozyme as a sorbent for bacterial endotoxins (lipopolysaccharides)

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ARTICLE INFO

Article history:
Received 1 August 2019
Received in revised form 23 August 2019
Accepted 25 September 2019

Keywords:
Immobilized lysozyme
Chemically modified lysozyme
Anisaldehyde
Benzaldehyde
Bacteriolytic activity
Endotoxin adsorption
Immunoglobulin G adsorption
Sepsis

ABSTRACT

Chemical modification of lysozyme was carried out using benzaldehyde and anisaldehyde. It was shown that chemical modification affects only 1–2 amino groups of the protein molecule which does not prevent further covalent immobilization of lysozyme using the remaining free amino groups. The bacteriolytic activity of lysozyme is preserved after chemical modification and after subsequent covalent immobilization. As a result of chemical modification immobilized lysozyme more effectively adsorbs bacterial lipopolysaccharides (endotoxins). Adsorption of immunoglobulin G does not increase after modification. The sorbents obtained in this work can be used for the future development of new medical material for the extracorporeal treatment of sepsis. The proposed scheme for the modification and immobilization of lysozyme can be used with various aldehydes for the preparation of sorbents with different properties.

1. Introduction

Bacteriolytic enzymes are more and more often regarded as an alternative to antibiotics due to the increasing number of resistant bacteria [1,2]. These enzymes are widely distributed in nature: they are isolated from preparations of animals, plants, fungi, bacteria and bacteriophages. Bacteriolytic enzymes have many different applications in biotechnology and medicine. Chicken egg lysozyme is the most well-known and the most used bacteriolytic enzyme in modern medicine and biotechnology. In our recent work we have shown that immobilized lysozyme can not only destroy bacteria but also effectively adsorbs the endotoxins (bacteria lipopolysaccharides) [3]. Endotoxin adsorbtion is required for the extracorporeal treatment of sepsis [4,5]. In our recent work it was also shown that the new sorbent is blood-compatible so it can potentially be used in extracorporeal therapy technologies to remove pathogenic components from the patient's bloodstream [3,6]. The practice of using extracorporeal therapy in the treatment of severe diseases including sepsis has significantly expanded in recent years [4,5,7]. In addition we have shown that immobilized lysozyme absorbs not only endotoxins but also a certain amount of immunoglobulin G (IgG) [3]. Binding to IgG may explain the possible additional physiological role of lysozyme as opsonin [3,8], but this binding is not required in the treatment of sepsis. We set ourselves a goal through chemical modification to further improve the properties of the new sorbent based on lysozyme.

Different chemical modification of lysozyme in order to improve its antimicrobial properties is described in the literature [9]. There is a good example of "improving lysozyme properties" — a modification of amino groups by cinnamaldehyde [10]. Cinnamaldehyde with ε-amino groups of lysine residues forms a Schiff base which can be reduced with sodium borohydride to obtain a chemically stable modification. It was shown that such modification significantly increases the antibacterial activity of lysozyme in relation to a number of bacterial species [10]. A similar modification to increase antibacterial activity also is possible with caffeic and cinnamic acids in which unlike the previous example no secondary amine is formed

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https://doi.org/10.1016/j.btre.2019.e00381

but carbodiimide activation is used to form an amide bond [11]. Perillic aldehyde and palmitic acid can also be used to modify lysozyme to enhance lysozyme efficacy against different bacteria [12,13].

In our work we decided to use the idea of modifying lysozyme with aromatic aldehydes. We chose benzaldehyde and anisaldehyde because of their relatively better solubility in water which simplifies the technology of lysozyme modification and therefore is more convenient for future production scaling. In addition these aldehydes do not have an alkene double bond which can be reduced during the modification procedure which can make product composition more uncertain.

In this work we set a goal to determine the resulting degree of modification (the number of modified amino groups per enzyme molecule) at various modifier/protein molar ratios in the reaction mixture, compare bacteriolytic properties of native and modified lysozyme, then obtain covalently immobilized lysozyme with different degrees of chemical modification and study bacteriolytic activity and sorption characteristics of new materials. As a polymer agarose matrix for lysozyme immobilization we chose WorkBeads 200SEC with a granule diameter of 200 μm for which its hemocompatibility and suitability for use in medicine in extracorporeal blood purification procedures was previously shown [3,6,14]. To determine the bacteriolytic activity we chose gram-negative *Escherichia coli* and gram-positive *Micrococcus luteus* cells as model bacteria substrates. To determine the adsorption of bacterial endotoxins we selected *Escherichia coli* and *Pseudomonas aerugenosa* lipopolysaccharide preparations.

2. Materials and methods

2.1. Reagents and equipment

The following reagents were used: Lysozyme from chicken egg white, *Micrococcus luteus* (lyophilized cells), $NaIO_4$, 1,6-diaminohexane, sodium acetate, Tris, $NaBH_4$, EDTA, MES, 2,4,6-trinitrobenzenesulfonic acid (5% water solution), benzaldehyde, 4-anisaldehyde, ε-aminocaproic acid, Coomassie Brilliant Blue G-250 (Sigma, USA); KOH, NaOH, H_3BO_3, $NaHCO_3$, Na_2CO_3, KH_2PO_4, K_2HPO_4, HCl, yeast extract (Helicon, Russia); ethanol (Ferrein, Russia); agar (Ferak, Germany); WorkBeads 200SEC polymer matrix (Bio-Works, Sweden); phosphoric acid, NaOH, glutaraldehyde (25% water solution) (Panreac, Spain); citric acid, acetic acid, $CuSO_4$ (Reakhim, Russia); Sepadex G-25 Superfine (GE Healthcare, Sweden); human immunoglobulin (IgG) "Sandoglobulin" (Sandoz, Germany); *Escherichia coli* endotoxin standard (Charles River Laboratories, USA); *Pseudomonas aeruginosa* endotoxin standard "Pyrogenal" (Medgamal, Russia); LAL (Limulus amebocyte lysate) chromogenic endpoint assay for endotoxin determination (Hycult Biotech, Netherland), water for LAL-Assay (Pirotest, Russia); Museum strain *E. coli* JM109 was kindly provided by Dr. J. Messing (Waksman Institute, New Jersey, USA). All solutions were prepared in bidistilled water. The following equipment was used in the studies: UV-1800 spectrophotometer (Shimadzu, Japan), TV-80-1 electrical thermostatic air dry oven (MedLife, Russia), LT-105 thermostatic circulating water bath (LOIP, Russia), analytical scales OH- PA64 (Ohaus, USA), Thermo Orion pH-meter model-420 (Thermo Scientific, USA), Multi Bio RS-24 rotator (BioSan, Latvia), MiniSpin centrifuge (Eppendorf, Germany).

2.2. Modification of lysozyme

Lysozyme (2 mg/mL) and aldehyde solutions (1.5 mg/mL) were prepared in $NaHCO_3$-Na_2CO_3 buffer (50 mM, pH 8.0). Lysozyme/aldehyde molar ratios in a reaction medium at 1/0 (control), 1/3, 1/7 and 1/15. The reaction mixture was incubated at 25 °C for 3 h on a rotator (10 rpm). A freshly prepared 2% $NaBH_4$ aqueous solution was added to the obtained preparation (to a final concentration of 0.1%) and incubated at 25 °C for 30 min while stirring, this procedure was repeated twice. To separate the lysozyme from low molecular weight components a chromatography was carried out on Sephadex G-25 column (7cm•5cm^2) equilibrated with K_2HPO_4–KOH buffer (20 mM, pH 8.0) at a flow rate of 2 mL/min.

2.3. Determination of modification degree of lysozyme

The decrease in the number of free amino groups of lysozyme was determined using trinitrobenzenesulfonic acid in accordance with the previously described procedures [15,16] with some modifications. Measurements were carried out at 37 °C in H_3BO_3-NaOH buffer (0.1 M, pH 9.5). A solution of TNBS was added to the cuvette with the protein solution to a concentration of 0.04%, the growth of absorption was recorded at 420 nm before reaching the plateau (~50 min). The absorbance value at the plateau (D_{420}) was used to plot calibration curves using data for unmodified lysozyme (0.7–4.0 μM) and ε-aminocaproic acid (2–16 μM). The modification degree of lysozyme was calculated from the ratio D_{420} values for the modified and native enzyme solutions with the equal protein concentrations.

2.4. Protein quantitation

Protein concentration was determined via the Bradford method with Coomassie G250 [17] and by microbiuret method [18] with a modified Benedict reagent [19].

2.5. Lysozyme immobilization

Covalent immobilization of lysozyme was performed by attaching a protein to an aminated matrix using the approaches in our previous works [6,20]. For amination the matrix was first activated (oxidized) with $NaIO_4$ [21]. First the matrix was washed (using Buchner funnel with sintered disc) with a 20-fold volume (relative to the matrix volume) of distilled water, and then a two-fold volume of 2% $NaIO_4$ solution was added. Then the mixture was incubated at 20 °C for 2 h on a rotary shaker (5 rpm). After incubation the matrix was washed with 20-fold volume of distilled water. A single volume of 2 M 1,6-diaminohexane solution was added to the activated matrix followed by incubation of the mixture at 20 °C for 2 h on a rotary shaker (5 rpm). A double volume of freshly prepared 0.5% $NaBH_4$ aqueous solution was added to the obtained preparation and incubated for 30 min while stirring then another similar portion of freshly prepared $NaBH_4$ solution was added and the mixture was incubated for an additional 30 min. Then aminated matrix was washed with a 5-fold volume of 1 M NaCl solution and a 10-fold volume of a KH_2PO_4-K_2HPO_4 buffer (10 mM, pH 7.0, 130 mM NaCl) (buffer A).

0.56 mL of 25% glutaraldehyde solution was added to 10 mL of 50% aminated matrix suspension (containing 50% sediment by volume) in a $NaHCO_3$-NaOH buffer (30 mM, pH 10.0). This mixture wad stirred at 25 °C for 30 min on a rotary shaker (5 rpm). Then the gel was washed with 50 mL of a $NaHCO_3$-NaOH buffer (30 mM, pH 10.0) and transferred to a separate container. 10 mL of 7.5 mg/mL lysozyme solution in the same buffer was added to the gel. The mixture was incubated at 25 °C for 3 h on a rotary shaker (5 rpm). A double volume of freshly prepared 0.5% $NaBH_4$ aqueous solution was added to the obtained preparation and incubated for 20 min while stirring, then another similar portion of freshly prepared $NaBH_4$ solution was added and the mixture was incubated for an additional 20 min. Finally the sorbent was washed with 200 mL of distilled water and then was washed with a 150 mL of buffer A.

2.6. Determination of the amount of immobilized lysozyme

The amount of immobilized lysozyme was determined by the difference between the amount added and the amount in the supernatant after the immobilization procedure (after incubation of lysozyme solution with gel before $NaBH_4$ adding). The concentration of lysozyme was determined spectrophotometrically by optical absorption of the solution at 280 nm.

2.7. Immobilized lysozyme storage

The preparations were stored at 5 °C as a 50% suspension (containing 50% sediment by volume) in buffer A with 2 mM EDTA. Before the experiments the preparation was washed with a 10-fold volume of the same buffer. The activity and sorption characteristics of immobilized lysozyme did not change in the range of experimental error during 2 weeks of storage.

2.8. Bacteria substrate preparation

The growth of *E. coli* cells was carried out in accordance with the standard method [22]. The cell suspension (approximately 10^9 cells (colony-forming unit, CFU) per mL) in 0.15 M NaCl was frozen by immersing the tubes in liquid nitrogen and stored at −70 °C for not more than 3 weeks. Samples of *E. coli* cells were defreezed immediately prior to the experiment. The suspension of *M. luteus* cells was prepared by adding 5 mg of dried cells in 10 mL of of Tris-MES-acetate buffer (0.01 M, pH 8.8) at 20 °C. Before using, suspensions of *E. coli* and *M. luteus* cells were centrifuged for 5 min at 1360 *g* at 5 °C and resuspended in a Tris-MES-acetate-carbonate buffer (0.01 M, pH 8.8).

2.9. Assay of soluble lysozyme activity

Bacteriolytic activity of lysozyme was determined by turbidimetric method as decreasing of optical density (OD) of cell suspension at a wavelength of 650 nm. The rate of change in optical density ($-dOD_{650}/dt$) is directly proportional to the rate of cell lysis ($-dCFU/dt$) [23,24]. The measurements were carried out at 37 °C in a Tris-MES-acetate-carbonate buffer (0.01 M, pH 8.8). The amount of bacterial cells added to the reaction mixture was selected in such a way that the initial optical density was 0.5-0.55. The measurements were carried out in 0.5 mL cuvettes with teflon stopper. Lysozyme concentration was 0.1 μg/mL of solution. After the enzyme was added to the cuvette, the kinetics of optical density change was recorded for 5–7 minutes and the initial velocities were determined in the first 2–3 minute interval. For the correction of the rate of lysis on the background change in the optical density, control experiments were performed without the addition of an enzyme. Enzyme activity was expressed as $-dOD_{650}/dt$ (OD units/min).

Km (Michaelis constant) values were expressed in OD_{650} units. For the Km determination the concentration of substrate (bacteria cells) in cuvett was changed from 0.1 to 0.9 OD_{650} units. In our condition OD_{650} units value is directly proportional to cell concentration [23,24].

For determination of the pH-optimum of enzyme activity the pH value of the mixture was changed from 6 to 10.

2.10. Assay of immobilized lysozyme activity

Similarly as in the case of soluble lysozyme optical absorbance decrease of the cell suspension at 650 nm was measured. The measurements were carried out in the same buffer mixture and at the same temperature. The amount of bacterial cells added to the reaction mixture was selected in such a way that the initial optical density was 0.5-0.55. The amount of immobilized lysozyme added was 35 μL per 1 mL of mixture. The reaction mixture was incubated in test tubes (10 mL each) in thermostatic air dry oven on a rotary shaker at 10 rpm (at speeds of more than 14 rpm, the effect of cell destruction without the action of the enzyme appears, probably due to mechanical action on the cells). Samples (1 mL) were taken from the mixture every 2 min, leaving the rest mixture for further incubation. Selected samples of 1 mL were placed in tubes, the particles of the immobilized enzyme were allowed to settle (0.5 min) and then the optical density of the supernatant (suspension of cells without immobilized enzyme) was measured. The dependence of the change in supernatant optical density over time was plotted for 16 min and the rate of change of optical density with time (activity) was determined from the slope of the dependence. For the correction of the lysis rate on the background change in the optical density, control experiments were performed by adding to the mixture a matrix without lysozyme. Enzyme activity was expressed as in the previous section.

2.11. Study of endotoxin adsorption on immobilized lysozyme

Adsorption was investigated by "batch chromatography method". 100 μL of sorbents were placed in plastic tubes and washed with 20 volumes of water for the LAL-test. Concentrated solutions of endotoxins were diluted by buffer A to a final concentration of 75 ng/mL. Then the 10 volume of endotoxin solution was added to sorbents and incubated for 30 min at 37 °C on a shaker (5 rpm). After the incubation and centrifugation (3 min at 6708 *g*), samples of supernatant were taken and diluted 200 times with water for the LAL-test. The endotoxin concentration was determined from its activity in the samples by the end point LAL method [25]. As a control, endotoxin sorption was tested on the raw WorkBeads 200SEC matrix. The efficiency of sorption was calculated as the ratio of endotoxin quantity after and before incubation with sorbent.

2.12. Study of IgG adsorption on immobilized lysozyme

Adsorption was investigated by "batch chromatography method". Before experiment IgG solution was additionally purified by exclusion chromatography on Sephadex G-25 column (10 cm•1 cm^2) equilibrated with buffer A at a flow rate of 1 mL/min. Immobilized lysozyme was washed with 10 volumes of buffer A and 50% suspension of sorbent was prepared. A 0.9 mL portion of IgG solution (10 mg/mL in the same buffer) was added to 200 mL of suspension. The resulting mixture was incubated for 30 min at 37 °C on a shaker (5 rpm) and then centrifuged (3 min at 6708 *g*). The adsorbed IgG amount was calculated by the difference between the quantity after and before incubation with the sorbent. The concentration of IgG in the supernatant was determined spectrophotometrically via optical absorption of the solution at 280 nm.

3. Results and discussion

The strategy of our work was to modify chemically and subsequently covalently immobilize the enzyme on the matrix. The reverse scheme (immobilization of the enzyme with its subsequent modification) is more complex from a technological point of view. The fact is that the pores of used by us matrix are rather large, therefore, lysozyme during immobilization is distributed inside the particles [3,6,20]. Modification of such a preparation with aldehydes in this case will be complicated by the diffusion of reagents inside the particles. Both the first and the second step of the proposed process (modification and immobilization) are carried out using free amine groups of lysine. As only

three lysine residues in lysozyme are available for reaction without disturbing the tertiary structure [26] it was first necessary to choose the conditions under which the modification of the lysozyme with aldehydes is affected between one to two amino groups.

The results of modification were controlled by titration of free amino groups in lysozyme preparations using TNBS. When comparing the calibration dependences of titration of native lysozyme and ε-aminocaproic acid (example is given on Fig. 1) it was found that the analytical signal of the enzyme is about 7 times larger. Indeed lysozyme contains 6 lysine residues and one free amino group of terminal amino acid [27]. Therefore the calculation of modification degree of enzyme was carried out under the assumption of the presence of 7 titratable amino groups. For all modified lysozyme preparations dependence of the analytical signal on protein concentration was linear and passes through zero.

Table 1 shows modified lysozyme properties. Increasing the aldehyde concentration in the reaction mixture leads to a higher degree of protein modification. As a modifier benzaldehyde looks slightly less active than anisaldehyde. In all cases, the modification affects from 0.7 to 1.7 amino groups per enzyme molecule. The other amino groups are either less reactive or sterically unavailable for interaction with the aldehyde in the case of the native enzyme. A further increase in the concentration of the modifier was considered inappropriate. First already at enzyme/modifier molar ratio of 1/15, the concentration of aldehydes used is close to their solubility in water. A further increase in aldehyde concentration would require the use of organic solvents, which would adversely affect the lysozyme. Secondly the obtaining of an enzyme with one-two modified amino groups fully corresponded to our tasks.

It is found that protein concentrations in preparations determined by the Bradford and microbiuret methods are close within the experimental error. The ratio of the optical absorption of the protein solution at 280 nm and at 260 nm also does not change after modification within the experimental error. Thus chemical modification within the experimental error does not affect the binding of Coomassie G-250 with the protein and the formation of the biuret complex by peptide bonds, practically does not change the UV spectrum.

A control experiment showed that lysozyme treated in the modification procedure without the addition of aldehydes did not change its bacteriolytic properties. The activity of the enzyme after modification varies slightly. Modified lysozyme demonstrates the same pH-optimum of activity against *M. luteus* cells and a little shift to alkaline in pH-optimum in activity against *E. coli* cells. After modifying the lysozyme with both benzaldehyde and anisaldehyde the Km values for the substrate *M. luteus* are reduced to one and a half times. After modification of lysozyme by benzaldehyde the Km value for the substrate *E. coli* reduces twice. After modification of the lysozyme with anisaldehyde the Km for the substrate *E. coli* almost does not change. The decrease in the Km after modification can be explained by the improved binding of bacterial cells on the lysozyme.

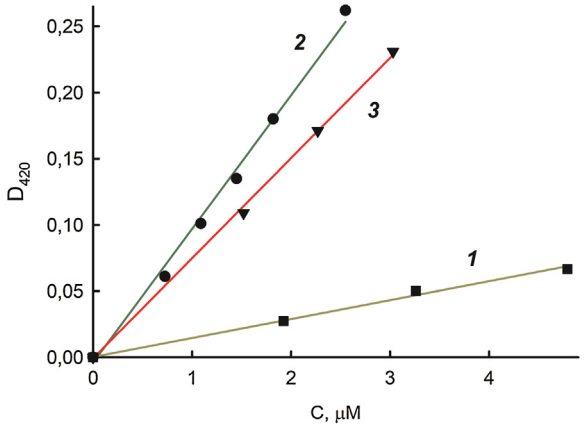

Fig. 1. The dependence of optical density at 420 nm on the concentration: ε-aminocaproic acid (***1***, ■), native lysozyme (***2***, ●) and lysozyme modified with anisaldehyde (enzyme/aldehyde molar ratio 1:15) (***3***, ▼).

Subsequently the obtained preparations of modified lysozyme were covalently immobilized on the WorkBeads 200SEC matrix. Table 2 shows a summary of the immobilized modified lysozyme properties in comparison with the immobilized unmodified lysozyme properties: immobilization yield, bacteriolytic activity, endotoxin adsorption and IgG adsorption. Control experiments showed that the matrix without immobilized lysozyme do not affect bacterial lysis and don't absorb endotoxin or IgG. It is very important that the developed modification procedure did not affect the efficiency of protein immobilization on the carrier - in all cases the yield of immobilization ranges from 92 to 96%. This fact confirms the validity of the chosen strategy for obtaining immobilized lysozyme preparations - the presence of even one available free amino group in a protein molecule is quite enough for its complete binding to the carrier.

As we see both modified lysozyme and unmodified lysozyme after immobilization retains activity against bacterial cells. Relative bacteriolytic activity of immobilized modified lysozyme in comparison with immobilized unmodified lysozyme is higher against *M. luteus* cells (approximately one and a half times) and almost the same in the case of *E. coli* cells. As noted in our previous work in the case of immobilized lysozyme only a small part of the enzyme is sterically available to the bacterial cells substrate [3,6,20]. In general it can be said that the lysozyme amino groups involved in the modification and subsequent immobilization are not necessary to maintain bacteriolytic activity with respect to living bacterial cells.

To determine the sorption characteristics we used a tenfold solution volume in relation to the sorbent volume. Such a volume ratio corresponds volume ratio value order in real extracorporeal therapy procedures [28]. To test the effectiveness of endotoxin sorption we chose its concentration of 75 ng per mL which is 600–2000 times higher than blood plasma concentration of real patients with sepsis of different severity [29]. As can be seen from Table 2, after modification the ability of immobilized lysozyme to sorb *E. coli* and *P. aeruginosa* endotoxins improves. In the case of benzaldehyde modification the improvement in endotoxin sorption is more significant up to one and a half times higher. In view of such high sorption capacity in the future for medical applications it will be possible to use a lower amount of sorbent with a large volume of fluid (blood or blood plasma). As already mentioned, immobilized lysozyme is able to adsorb IgG [3] which is not a positive feature if the sorbent is used in the treatment of sepsis. To test the sorption, a solution of IgG with a concentration of 10 mg mL was used, which is close to the average values of its concentration in human blood plasma [30]. As can be seen from Table 2, the removal of IgG is less than 10% and this value remains almost unchanged after chemical modification of the lysozyme. Thus the sorption of IgG is relatively low and does not pose a threat to the patient if the sorbent is used in medical procedures. The sorption of IgG will be even less significant in the case of a decrease in the amount of sorbent compared with the volume of the treated fluid. The fact that there is no change in the level of sorption of IgG adsorption after chemical modification may indicate that the interaction between the lysozyme and IgG is possible not accidentally but there is a specific binding site that lacks exposed lysine residues. This fact can confirm that opsonin

Table 1
Characteristics of native and modified lysozyme.

Characteristic		Native lysozyme	Control[a]	Enzyme/aldehyde molar ratio					
				benzaldehyde			anisaldehyde		
				1:3	1:7	1:15	1:3	1:7	1:15
Amount of modified amino groups		–	–	68 ± 0.08	1.19 ± 0.09	1.52 ± 0.12	0.86 ± 0.07	1.28 ± 0.07	1.70 ± 0.09
[Protein] (microbiuret method)/[protein] (Bradford method)		1.01 ± 0.05	1.03 ± 0.05	0.97 ± 0.10	0.99 ± 0.07	1.13 ± 0.08	1.09 ± 0.08	1.03 ± 0.09	1.18 ± 0.13
Absorbans at 280 nm / Absorbans at 260nm		1.69 ± 0.04	1.69 ± 0.04	1.66 ± 0.05	1.67 ± 0.05	1.70 ± 0.04	1.65 ± 0.04	1.66 ± 0.05	1.64 ± 0.05
M. luteus	Activity 10^{-3} OD/min	8.3 ± 0.5	8.1 ± 0.5	9.9 ± 0.6	9.7 ± 0.6	9.6 ± 0.6	9.9 ± 0.5	9.8 ± 0.5	9.8 ± 0.5
	K_m, optical units	0.14 ± 0.02	0.15 ± 0.03	0.12 ± 0.03	0.11 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	0.13 ± 0.03
	pH-optimum	8.7 ± 0.2	8.7 ± 0.2	8.8 ± 0.2	8.8 ± 0.2	8.8 ± 0.2	8.9 ± 0.2	8.9 ± 0.2	9.0 ± 0.2
E. coli	Activity 10^{-3} OD/min	12.2 ± 0.8	11.9 ± 0.9	12.0 ± 0.9	12.2 ± 0.9	12.7 ± 0.9	12.0 ± 0.6	12.1 ± 0.7	11.9 ± 0.7
	K_m, optical units	0.17 ± 0.02	0.18 ± 0.03	0.14 ± 0.03	0.10 ± 0.02	0.08 ± 0.02	0.16 ± 0.02	0.18 ± 0.03	0.18 ± 0.03
	pH-optimum	8.8 ± 0.2	8.8 ± 0.2	8.9 ± 0.2	9.2 ± 0.2	9.4 ± 0.2	9.0 ± 0.2	9.1 ± 0.2	9.3 ± 0.2

Experimental data are presented as mean and 95% confidence interval calculated by Student's t-test.
[a] Lysozyme treated without the addition of aldehydes (enzyme/aldehyde molar ratio 1:0).

Table 2
Characteristics of composite materials based on immobilized native and modified lysozyme.

Characteristic		Native lysozyme	Enzyme/aldehyde molar ratio					
			benzaldehyde			anisaldehyde		
			1:3	1:7	1:15	1:3	1:7	1:15
Yield of immobilization, %		95 ± 3	92 ± 3	93 ± 3	96 ± 3	92 ± 3	92 ± 3	95 ± 3
Activity (*M. luteus* cells), 10^{-3} OD/min		10.5 ± 1.7	16.2 ± 1.9	16.1 ± 1.7	16.5 ± 2.0	14.6 ± 1.6	14.9 ± 1.5	15.3 ± 1.7
Activity (*E. coli* cells), 10^{-3} OD/min		11.1 ± 2.0	11.3 ± 1.8	11.2 ± 1.8	11.0 ± 2.0	11.4 ± 1.8	11.3 ± 2.0	11.1 ± 1.9
E. coli endotoxin adsorption[b]	Capacity, ng per mL of sorbent	466 ± 39	598 ± 43	670 ± 48	682 ± 47	581 ± 45	596 ± 47	598 ± 49
	Removal efficiency, %	62	80	89	91	78	80	80
P. aerugenosa endotoxin adsorption[b]	Capacity, ng per mL of sorbent	342 ± 37	548 ± 42	572 ± 46	587 ± 52	433 ± 48	443 ± 52	449 ± 51
	Removal efficiency, %	46	73	76	78	58	59	60
IgG adsorption[c]	Capacity, mg per mL of sorbent	8.7 ± 0.8	8.2 ± 0.8	7.9 ± 0.9	7.4 ± 0.8	7.8 ± 0.9	7.3 ± 0.8	7.1 ± 0.9
	Removal efficiency, %	8.7	8.2	7.9	7.4	7.8	7.3	7.1

Experimental data are presented as mean and 95% confidence interval calculated by Student's t-test.
[b] 75 ng/mL endotoxin solution, 10:1 solution/sorbent volume rate.
[c] 10 mg/mL IgG solution, 10:1 solution/sorbent volume rate.

function of lysozyme is the result of its ability to specifically bind to IgG [3] and not simply be a result of a surface charge change in an antigen when it binds randomly with the lysozyme [8].

We tested competitive sorption from a solution containing both *P. aeruginosa* endotoxin and IgG. There were three variants of immobilized lysozyme: unmodified, modified with benzaldehyde (1.19 of modified amino groups), modified with anisialdehyde (1.28 of modified amino groups). Within the experimental error, the sorption of endotoxin and IgG did not change in the case of the simultaneous presence of both components in the solution. This fact also confirms the assumption that IgG and endotoxin bind independently at different sites of immobilized lysozyme.

4. Conclusion

Composite material based on modified covalently immobilized lysozyme shows excellent endotoxin adsorption. The adsorption of IgG on immobilized lysozyme did not increase after chemical modification which in combination with increased sorption of endotoxin improves the specificity of the sorbent. Thus the developed material has broad prospects for future medical sorbent development for the extracorporeal therapy in the treatment of sepsis. The proposed scheme for obtaining immobilized modified lysozyme can be used with various aldehydes for the preparation of sorbents with different properties for various purposes of biotechnology and medicine.

Author contribution

PAL, NLE, DAM, IYA and AVL conceived and designed the project. PAL, DAM, EDO, and OAD acquired the data. PAL, DAM, VAN, IYA, NVK, NLE and AVL analyzed and interpreted the data. PAL and NLE wrote the paper. All the authors have read and approved the manuscript.

Funding

Funding was received for this work.

The work was supported by the Federal Target Program "Research and Development in Priority Areas for the Development of the Russian Science and Technology Complex for 2014-2020", section "Development of new medical sorbents for extracorporeal treatment of sepsis with antimicrobial activity and ability to adsorb bacterial toxins" (application code 2017-14-576-0053-142; unique project ID RFMEFI57417X0181; agreement no. 14.574.21.0181).

Intellectual Property

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual properly. In so doing we

confirm that we have followed the regulations of our institutions concerning intellectual property.

Declaration of Competing Interest

All authors declare no conflicts of interest in this paper.

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