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LIPOPOLYSACCHARIDE CONTAMINATION OF RECOMBINANT PROTEINS AND ITS SIGNIFICANCE FOR IMMUNOLOGICAL STUDIES

Bachelor thesis

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ANNOTATION

The aim of this work was to purify recombinant proteins from lipopolysaccharides by extraction with detergent Triton X-114. Followingly the level of endotoxin left in the protein solution was determined by Lymulus Amoebocyte Lysate assay. Next an attempt to find the lowest level of LPS that is recognized by suspension of splenocytes was performed.

Tato studie se zabývá jedním z možných způsobů odstranění lipopolysacharidu z roztoku rekombinantního proteinu. Pro tento účel byla použita extrakce detergentem Triton X-114, vysoce účinná eliminační metoda LPS. Koncentrace zbylého LPS ve vzorku proteinu byla zjištěna pomocí Limulus Amoebocyte Lysate testu. Za účelem zjištění reakce organizmu na LPS byla suspenze splenocytů stimulována škálou různých koncentrací LPS s následnou detekcí cytokinů TNF-α a INF-γ pomocí ELISA.

AFFIRMATION

I declare that the work summarized in the thesis was done on my own or in collaboration with the co-author presented in the manuscript. The literature used was properly cited or quoted.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my dissertation thesis, in full / in shortened form resulting from deletion of indicated parts to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

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České Budějovice, 20th of May 2010 Barbora Svobodová

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LIST OF ABBREVIATIONS

ELISA	Enzyme-Linked ImmunoSorbent assay
EU	Endotoxin unit; 1 EU = 0,1 ng
HDL	High density lipoprotein
IFN-γ	Interferon γ
LPS	Lipopolysaccharide
LTA	Lipotechoic acid
LBP	LPS binding protein
LAL	Lymulus amoebocyte lysate
LAL RW	Lymulus amoebocyte lysate reagent water
pNA	*p*-nitroaniline
PMN	Polymorphonuclear leukocytes
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor

CONTENT

1. ABSTRACT

Because of the pathophysiological effects of endotoxin on organisms this study discusses one way of its removal. The technique of endotoxin removal from recombinant protein solutions that seems to be most effective nowadays - the extraction with detergent Triton X-114 was used. To evaluate the effectivity of LPS removal the concentration of remaining LPS in the protein solution was quantified by Limulus Amoebocyte Lysate assay. Efficiency of 94,1% removal was achieved. Making a connection between the level of endotoxin left in the protein solution and its effect on organisms the attempt to find the lowest level of LPS that is recognized by suspension of splenocytes is described. Because of imperfect data the boarder concentration is approximated to range of 1,56 – 0,39 ng/ml endotoxin concentration.

2. INTRODUCTION

Endotoxin which is a different name for lipopolysaccharide, is a major component of the outer membrane of Gram-negative bacteria. In pharmaceutical industries it is possible to find endotoxins during production processes. As a result they are present in commercially available products such as recombinant proteins of biologically active substances and often complicate study of their biological effects [2].

The release of endotoxins from Gram-negative bacteria into the environment is continuous regardless to the stage of bacteria development (cell death, division, etc.) [1]. Since bacteria exhibit ability to colonize almost any environment being an undemanding species the endotoxins liberated from them are everywhere contaminating everything.

In immunological studies endotoxins are undesirable contamination because of their pathophysiological effects. The presence of even small amounts of endotoxin from preparations of recombinant proteins can cause adverse reactions when the proteins are used as antigens for immunizing experimental animals [3, 4]. These effects include shock, tissue injury or even death [5-7].

From the variety of techniques of endotoxin removal from recombinant protein the one found most effective - extraction with detergent Triton X-114 is described in this article. To assess the effectivity of LPS removal the concentration of remaining LPS in the

protein is quantified by Limulus Amoebocyte Lysate assay. Finally the attempt to find the lowest level of LPS that is recognized by suspension of splenocytes is described.

3. THEORETICAL BACKGROUND

3.1. LPS structure and properties

Endotoxins are very stable molecules, resisting extreme temperatuers and pH values in comparison to proteins [8, 9]. That is why they can be found on the outer membrane of Gram-negative bacteria (*Figure 1*).

Cell membrane of Gram-negative bacteria

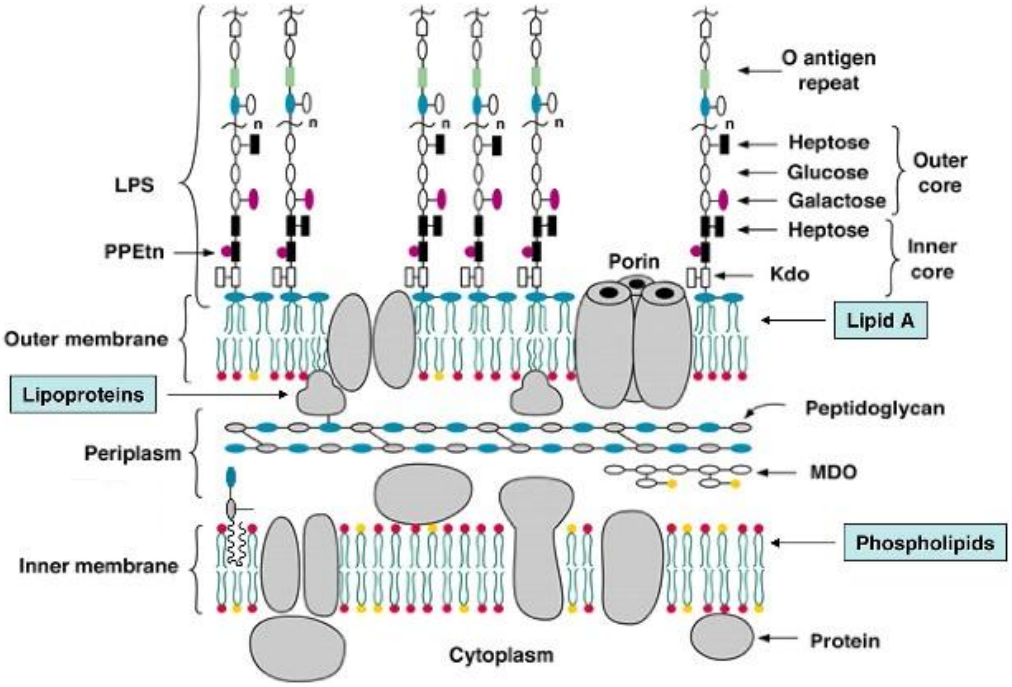


Figure 1: Molecular model of the cell wall of Gram-negative bacteria according to Raetz and Whitfield 2002 [10]. Main parts of LPS are denoted in the picture. Ovals and rectagles stand for sugar residues, circles represent polar head groups of various lipids. PPEtn (ethanolamine pryrophostphate); Kdo (2-keto-3deoxyoctonic acid).

Lipopolysacchride molecule is composed of 3 major regions: core oligosaccharide - a hydrophilic polysaccharide moiety , O antigen - also formed by saccharides, and lipid A which is hydrophobic (*Figure 2*). Lipid A is the part that is responsible for most of biological activities of endotoxin, in particular its toxicity [1].

Schematic picture of LPS molecule

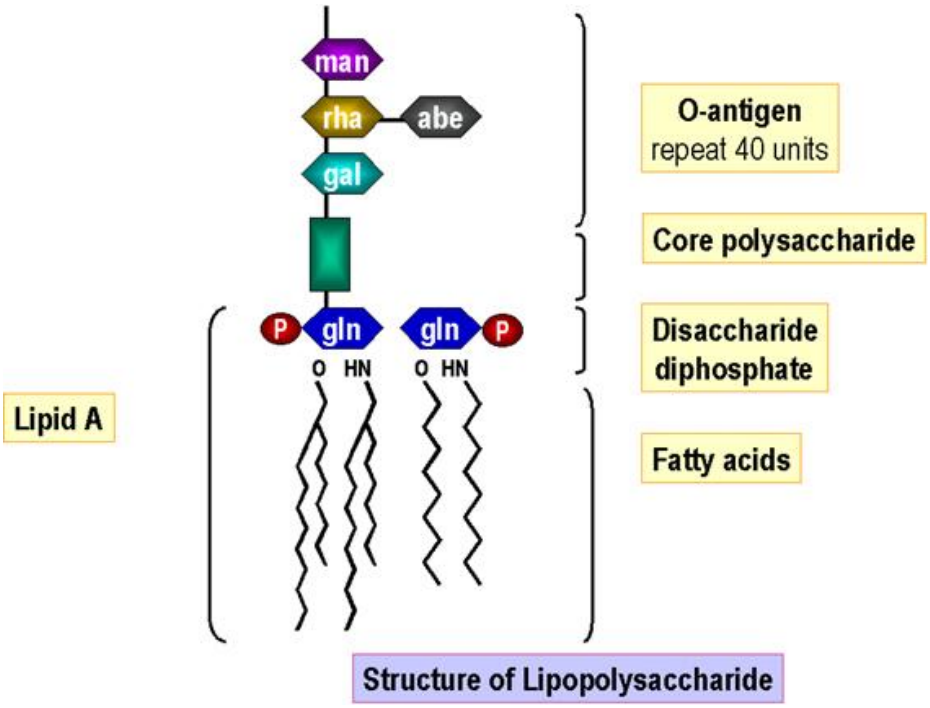


Figure 2: More detailed picture showing the parts of lipopolysaccharide molecule. The hexagonal structures represent the saccharide units.

In the structure of LPS there are regions close to lipid A that are phosphorylated. Therefore in a solution the LPS molecules exhibit a net negative charge. Since LPS molecule possesses amphiphilic character in solution it creates aggregates resulting from non-polar interactions between lipid chains and bridges generated among phosphate groups by divalent cations [1].

3.2. Activating the immune system

Upon a foreign substance entering the body two kinds of immune defense are activated: humoral and cellular line. The humoral part mediates the activation of immunity through factors - complement, antibodies, and acute phase proteins. The latter one involves cell structures as monocytes, macrophages, and neutrophils which are able to recognize bacterial cell wall constituents [26]. Eventhough the different mode of action of both lines their mechanisms are closely interconnected.

As Magalhães et al. (2007) suggest, endotoxins do not influence cells and organs directly, instead they act through the activation of immune system, affecting mostly monocytes and macrophages. Consequently the immune response is enhanced. The immune response signalizing pathway works through messengers called mediators [11, 12]. These messengers are responsible for spreading of the immune response. The activation of immunity include increased body temperature, alterations in the structure and function of organs and cells, activation of the coagulation cascade, modification of hemodynamics and induction of shock.

3. 2. 1. Cellular defense

Complement and antibodies are able to recognize components of bacterial cell wall such as LPS and its parts (lipid A, O-antigen) or LTA. Binding them they mediate a mechanism of their elimination. An antigen labeled in this way is predestined to destruction. Now the phagocytosing cells (monocytes, macrophages, and PMN) are able to identify these objects by complement and Fc receptors [27]. For instance activated PMN express CD14 and thus are able to recognize and phagocytose LPS and other bacterial fragments [26].

By identification of foreign substances in the body a cascade of release of inflammatory mediators, vascular and physiological changes is initiated. LPS-induced activation of macrophages results in production of microbicidal agents (oxygen free radicals, lysozyme, cationic proteins, acid hydrolases, and lactoferrin) and inflammatory mediators [28, 29, 30], especially TNF-α which is one of the first cytokines to be secreted after exposure to LPS. The site of rapid TNF-α manufacture after an inflammatory challenge

is in Kupffer cells - macrophages in liver. The endothelial cells react to the circulating cytokines by release of other meadiators which in turn attract and activate B and T lymphocytes [31]. T lymphocytes, among other mediators, secrete INF-γ which enhances the effects of LPS on mononuclear cells [32, 33].

3. 2. 2. Humoral defense

As stated before the humoral defense works through bunch of chemicals where antibodies predominate. B cells are responsible for producing the antibodies. When an antigen is recognized, specific B cells are stimulated to proliferate and differentiate into plasma cells [37]. This activation of B cell is usually stimulated by helper T cells. As soon as T cell encounters an antigen it starts to release lymphokines which in turn stimulate B cells to produce antibodies.

Antibodies serve as a tool in both, viral and bacterial infection. They are able to block the the entry of a virus into the host cell by binding its entire surface [38]. In a similar way antibodies can coat the surface of a bacterium to prevent its attachment to the different body linings such as gut wall. Antibody signaling can finally result in enhancing the phagocytosis by macrophages and neutrophils.

The stimulation of parenchymal cells in liver by TNF-α during infection results in production of acute-phase proteins such as LBP which also activates phagocytes and antigen-presenting cells [34, 35]. The level of LBP currently present in serum binds to LPS making it more accessible for immune cells [36, 38, 39]. LBP also posseses anti-inflammatory properties catalysing the transfer of LPS and LTA to HDL or other lipoproteins [40, 41, 42]. The LPS monomer is carried by LBP to the cell where it is transfered to CD14 molecule.

CD14 is a membrane protein situated on the outer membrane of monocytes, macrophages and neutrophils [38]. It lacks the transmembrane domain therefore another accessory molecule for signal transduction is required [43, 44]. The molecule fullfilling this function is TLR4. As da Silva (2001) [45] states, recent studies showed that microbial components interact primarily with CD14 and then with the TLRs. It was found out that LPS creates cross-linkages to TLR4 and MD-2 only if both, TLR4 and MD-2 are coexpressed with

CD14. MD-2 is an extracellular protein that is closely associated with TLR4, making the binding of LPS to this receptor possible [46].

3. 3. Techniques of LPS quantification

The two still commonly used methods for endotoxin detection are rabbit pyrogen test and LAL assay [13, 14]. The rabbit pyrogen test was developed in the 1920's and is based on measuring the rise in body temperature of rabbits after intravenous injection of a test solution [1]. Because of relatively high time and financial requirements the rabbit pyrogen is nowadays being replaced by LAL test. The test is based on the coagulation reaction of the lysate from the blood cells of *Limulus polyphemus* (horseshoe crab – *Figure 3*) in the presence of endotoxin.

Limulus polyphemus

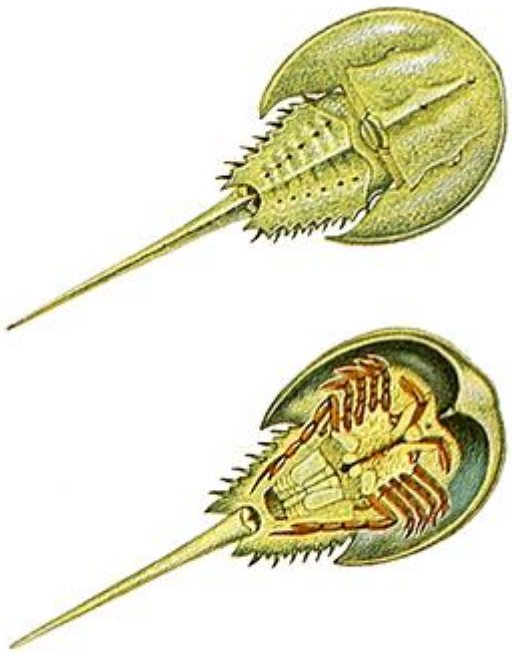


Figure 3: Illustrative picture of horseshoe crab.The animal from which the LAL lysate is extracted.

The use of LAL for the detection of endotoxin evolved from the observation that a Gram-negative infection of Limulus polyphemus resulted in fatal intra-vascular coagulation. This report came in 1956 from Fred Bang. It was discovered that the animal's blood cells, mobile cells called amoebocytes, contain granules with a clotting factor (coagulogen). This substance is released outside the cell when bacterial endotoxin is encountered [15].

Limulus amoebocyte lysate is an aqueous extract of blood cells from the horseshoe crab. LAL reacts with bacterial endotoxin, which is a membrane component of Gram-negative bacteria. This reaction is the basis of the LAL test, which was used for the detection and quantification of endotoxins.

There are several modes of LAL assay, gel-clot, turbidimetric LAL and chromogenic LAL technigque. The first is coupled with sequential dilution of the sample containing endotoxin until a no more observable clot is formed. The other two are based on the kinetics of the reaction with endotoxin. In turbidimetric LAL assay there is only as much of coagulogen to form turbidity but not enough to form a clot [16]. The optical densities of various test-sample dilutions are measured and correlated to values from calibration curve. In the chromogenic substrate assay the coagulotgen is partially or completely repaced by a chromogenic substrate [17]. When hydrolyzed by the pre-clotting enzyme, the chromogenic substrate releases a yellow substance known as *p*-nitroaniline. The intensity of the colouring is dependent on the endotoxin concentration [18].

3. 4. Possible ways of endotoxin removal

According to the properties of the protein solution one would need to clean it from endotoxins. The appropriate isolation technique is chosen from the following ones: Anionic-exchange chromatography suitable for solution of positively charged proteins [19], ultrafiltration suitable for solutions of small protein molecules contaminated with large endotoxin aggregates, ion-exchange chromatography, affinity adsorbents, gel filtration chromatography, sucrose gradient centrifugation [20], extraction with detergent Triton X-114 [21, 22, 1].

In our research we used Triton X-114 phase separation to eliminate the portion of endotoxin in the protein solution. This technique benefits from the properties of the detergent. Triton X-114 in aqueous solution is able to create two immiscible aqueous

phases by arranging itself in conical-shaped micelles. Consequently there is a micelle-rich phase and micelle-poor phase[23]. This immiscibility of certain surfactants is observed above a critical temperature of the solution, which is called cloud point. The cloud point of Triton X-114 is at 22°C. Thanks to the nonionic structure of the surfactant the endotoxin migrates to the micelle-rich phase. It is attracted there because of the non-polar interactions between alkyl chains of lipid A and the surfactant tail groups. Increasing the temperature of a protein solution above the cloud point brings the endotoxins into the micelles. After centrifugation at that temperature the surfactant-rich phase containing most of the endotoxin is the bottom phase [21, 24, 25] and can be separated from the rest of the protein solution.

4. MATERIALS AND METHODS

4.1. LPS REMOVAL FROM PROTEIN SOLUTIONS

4.1.1. Removal of endotoxin by phase separation with Triton X-114

To the protein solution (IRS protein of c = 0,7mg/ml) 1% of Triton X-114 was added. The solution was incubated 5 min on ice and afterwards vortexed thoroughly. To achieve the micelle-rich phase formation the mixture was incubated 5-10 min at 37°C. Centrifugation at 37°C (the rotor was prewarmed to 37°C) for 1 min at 13000 rpm followed. The upper phase of the purified protein was transfered to a new clean pyrogen free test tube and the procedure was repeated once more. The stress was put on working in pyrogen free environment to reduce the risk of recontamination with LPS. For this reason most of the procedure was performed in a flow-box. LPS free equipment was required (test tubes, pipette tips, etc.).

4.1.2. Bio-Beads preparation

To get rid of the residual Triton X-114 from the protein solution the Bio-Beads SM2 (BioRad, cat. No. 152-8920) were used. Not to contaminate the protein with LPS again the beads were cleaned from endotoxin as well. 1 g of beads was mixed with 10 ml of

methanol in 15 ml tube. All was well mixed by inverting and vortexing. After 3 min the beads settled at the bottom of the tube and methanol was pipetted away. 10 ml of 2% Triton X-114 solution in 20 mM Tris-Cl (pH = 8) were added to the beads and the mixture was stirred for 2 hours at 4°C. Next the triton buffer was removed and the beads were washed once with methanol and 2-3 times with LPS free buffer (the same buffer used in the protein solution).

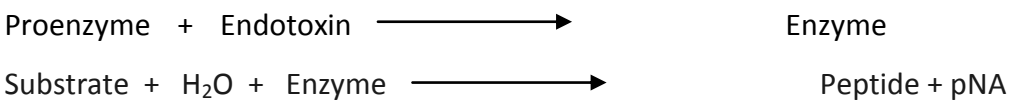
4.1.3. Removal of residual Triton X-114

The washing buffer was removed from the beads and the protein solution was added instead. The mixture was stirred for 1 hour at 4°C. The beads were removed with the help of LPS free column, catching the protein solution.

4.2. LAL ASSAY

Introduction

The effectivity of LPS removal from protein solution by Triton X-114 was determined by LAL test. The principle of this test is based on an enzymatic reaction where the endotoxin activates a proenzyme which in turn makes the substrate to release *p*-nitroaniline (pNA). *P*-nitroaniline possessing a yellow colour is then photometrically measured at 405 – 410 nm.



The substrate is a colourless synthetically produced peptide Ac-Ile-Glu-Ala-Arg-pNA. The reaction is stopped with a stop reagent. Since the absorbance is in direct proportion to the amount of endotoxin present, the concentration of endotoxin can be calculated from a standard curve.

In each LAL assay kit following chemicals can be found: Limulus amoebocyte lysate (containing the proenzyme), chromogenic substrate, endotoxin and LAL reagent water (LAL

RW) for dilution of the other substances. As a stop reagent 25 % acetic acid or 10 % SDS solution may be used. The detection range of endotoxin which is valid for the kit is 1 – 0,1 EU/ml.

Procedure

All the reagents were prepared by dissolving them in LAL RW. Calibration solutions of endotoxin of concentrations 1; 0,5; 0,25; 0,1 EU/ml (endotoxin unit/ml = 1 ng/ml) were diluted. Sample solutions were 10x diluted not to exceed the range of detection of LAL test. 50 µl of standard endotoxin solutions, negative control (LAL RW), sample solutions were transfered into Ependorf tubes and thermostated at 37°C. The reagents were prewarmed to 37°C as well. To each ependorf tube 45 µl of LAL were added and the solutions were incubated for 10 min at 37°C. Next 90 µl of the chromogenic substrate were added to all ependorfs in the same order as LAL and were again icubated 6 min at 37°C. After approximatelly 6 min the reaction was stopped with 50 µl of 25 % acetic acid. All solutions were pipetted to 96-well microtiter plate and the absorbance was measured at 405 nm on a spectrophotometer.

Results

The absorbances obtained from the spectrophotometer (see *Table 1* and *2*) were plotted against the endotoxin concentration. Using method of regression analysis calibration line was constructed (see *Figure 4*). Thereafter the endotoxin content of the samples was calculated from the equation given by the calibration line:

$$A = 1{,}312 * c + 0{,}006$$

$$c = (A - 0{,}006)/1{,}312.$$

The concentration of endotoxin in the solutions are shown both in EU/ml and ng/ml.

Endotoxin concentration of standards [EU/ml]	Absorbances of the standards at 405 nm
1	1,348
0,5	0,631
0,25	0,292
0,1	0,116
0	0,075

Table 1: Data obtained from the spectrophotometer for standard solutions.

Sample	IRS after treatment with Triton X-114 including removal of residual Triton X-114	IRS before treatment with Triton X-114	IRS after 1st extraction with Triton X-114	Negative control after treatment with Triton X-114 including removal of residual Triton X-114	Negative control after 1st extraction with Triton X-114
Absorbance	0,112	1,787	0,107	0,091	0,092
Concetration of LPS [EU/ml] in 10x diluted soltutions	0,080094	1,356577	0,076284	0,064091	0,064853
Concetration of LPS [EU/ml] in concentrated soltutions	0,800945	13,56577	0,762841	0,640908	0,648529
Concetration of LPS [ng/ml] in concentrated soltutions	**0,080094**	**1,356577**	0,076284	0,064091	0,064853

Table 2: This table shows which sample solutions were investigated and the values for them.

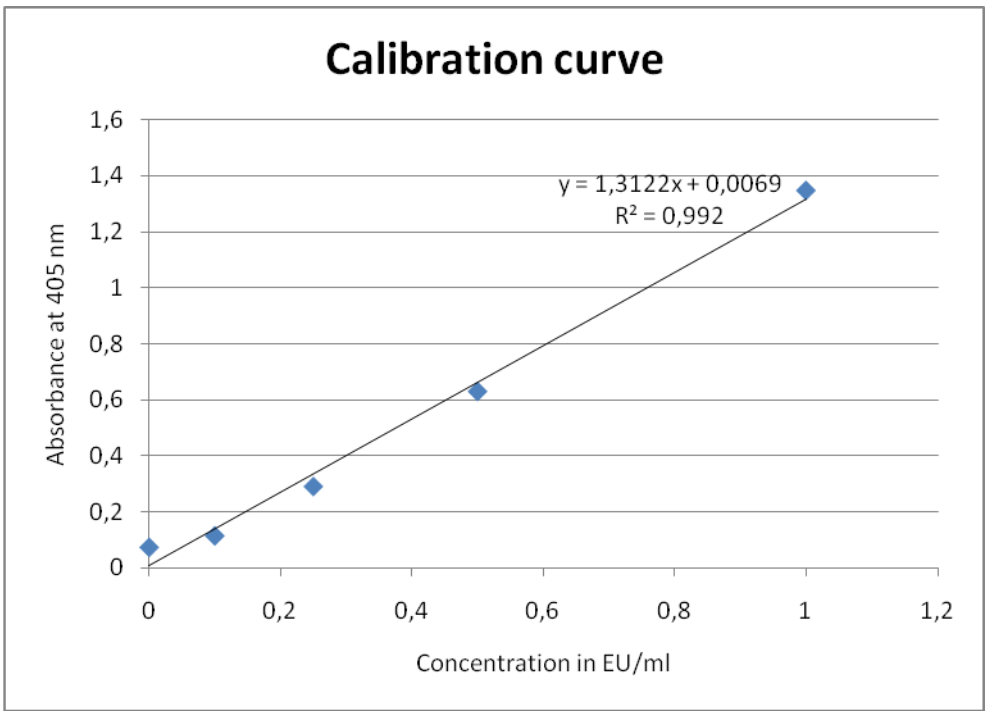


Figure 4: Calibration curve constructed from values of standard solutions using regression analysis.

Before treatment of the protein solution (IRS) with Triton X-114 the amount of endotoxin present was determined to be 1,356577 ng/ml. After 2-serial phase separation with Triton X-114 the remaining amount of endotoxin in the protein solution was 0,080094 ng/ml. From this we can see that the protein solution was decontaminated by 94,1%.

Discussion

As was expected, the level of endotoxin in the protein solution after 2-serial extraction was reduced by the significant amount 94,1%. Nevertheless, as found in the literature we did not reach the highest level of reduction of LPS contamination possible. As Aida and Pabst (1990, [21]) states 1000-fold reduction in LPS concentration already in the first cycle of phase separation is possible. According to Liu et al. (1997, [22]) it is feasible to show 99 % decontamination after 3 cycles of extraction with Triton X-114. Eventhough, this lower reduction in LPS level is enough for farther utilization of the protein for immunological studies (this is the objective of the other part of the study).

The control measurements prove that there was no major misshandling the samples during the laboratory procedure. The laboratory conditions were pyrogen free enough not to violate the validity of the investigation. The LPS concentration does not change distinctly after individual extractions.

The drawback of this LPS removal method is the protein loss. Certain portion of the protein stays in the detergent phase. The exact amount was not measured but according to Aida and Pabst (1990) it might approach 2 %. In our case the protein loss resulted mostly from the laboratory procedure of the phase separation with Triton X-114 and its following removal from protein solution. The protein loss approached 25% of the initial volume of the protein solution.

4.3. INDUCTION OF CYTOKINES IN CULTURES OF MOUSE SPLENOCYTES BY LPS AND FOLLOWING DETERMINATION OF A MINIMUM EFFECTIVE CONCENTRATION OF LPS

Introduction

As stated before endotoxins might interfere in the immunological studies, for instance when using recombinant proteins as antigens in experimental animals. In order to find out the minimum effective concentration of endotoxin we performed investigation stimulating splenocyte cells with range of LPS concentrations. Splenocytes take part in the immune defense by producing several substances. Doing so they activate the immune system and enhance its response. The suspension of splenocytes was prepared from a mouseb spleen. The production of cytokines TNF-α and IFN-γ were monitored and detected by ELISA.

Procedure

Under restricted LPS environment the suspension of splenocytes was prepared. The spleen was freshly obtained from a mouse and kept in a medium (RPMI-1640 base) on ice under sterile conditions. Next it was homogenized using a piston of a syringe. The mixture was washed in a sieve with the medium. The leaking solution containing the

splenocyte cells was collected into a Petri dish. Then, it was transferred to a test tube and centrifuged for 5-6 min at 1000 RPM. The supernatant was discarded and the splenocytes were washed once more with the medium.

Mixing the suspension 1:1 with 0,5% trypan blue the dead cells were stained. The concentration of living cells in the resulting solution was determined under the light microscope and adjusted to final concentration of approximatelly 10^7 cells/ml. This suspension was transferred to 96-well microtiter plate and LPS solutions of different concentrations were added. Considering the dilution by cell suspension, the final concentrations of LPS standards were 500; 100, 25; 6,25; 1,56; 0,39; 0,098 ng/ml. The positive (c_{LPS} = 5 µg/ml) and negative controls were included. The well microtiter plate with these mixtures was kept in an incubator at 37°C and 5% CO_2 atmosphere.

After 24 hours samples for determination of TNF-α content were collected. For IFN-γ determination samples were collected after 48 hours incubation. These samples were submitted to ELISA. As a reference, calibration solutions of TNF-α and IFN-γ of known concentrations underwent ELISA simultaneously. The absorbances of the solutions were measured on spectrophotometer, TNF-α at 450 nm and IFN-γ at 490 nm.

Results

The LPS stimulated cells showed no regular behaviour corresponding to the increasing concentration of LPS. Values for content of TNF-α after stimulation of the spelnocyte cell can be found in *Table 4*. *Table 3* represents ELISA calibration curve.

TNF α concentration of standards [ng/ml]	Absorbances of the standards at 450 nm
2	0,688
1	0,711
0,5	0,681
0,25	0,503
0,125	0,358
0,063	0,228
0,031	0,131
0	0,041

Table 3: Data after ELISA assay from the spectrophotometer. The absorbances for calibration solutions of TNF-α

Concentration of LPS stimulating the splenocytes [ng/ml]	Absorbance		
	Measurement 1	Measurement 2	Measurement 3
Possitive control - 5000	0,156	0,232	0,299
Negative control - 0	0,071	0,065	0,068
500	0,378	0,376	0,517
100	0,442	0,440	0,446
25	0,322	0,464	0,419
6,25	0,403	0,410	0,543
1,56	0,339	0,410	0,366
0,39	0,144	0,171	0,205
0,098	0,067	0,094	0,098

Table 4: Absorbance data after ELISA for all the other solutions.

Discussion

After several repetitions of this experiment no reliable response of production of IFN-γ by splenocytes after simulation with LPS could be recorded. A few of these trials failed because of ELISA did not work properly. In the other measurements the values fluctuated so much that a regular behaviour could not be assumed.

The production of TNF α by splenocytes showed more promising behaviour though a sharp point of the LPS concentration could not be stated. Plotting the absorbance against the concentrations of TNF-α calibration solutions does not show linear behaviour. Nevertheless there is a remarkable difference in TNF-α produced after stimulation of spenocytes with LPS solutions of concentrations 1,56 and 0,39 ng/ml. Therefore the minimum effective concentration of LPS to stimulate the activity of splenocytes must be in range 1,56 – 0,39 ng/ml.

5. CONCLUSION

Eventhough the study wan not successful in determination of the exact minimum effective concentration of LPS we showed that splenocytes – cells of immune system respond to the presence of endotoxin. Therefore when using recombinant proteins for immunological studies one needs to be careful about the level of LPS contamination. Phase separation with Triton X-114 turned out to be a very effective technique of endotoxin removal from protein solutions despite the protein loss, which might be reduced through optimization of the laboratory procedure.

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