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Studies on endotoxin removal mechanism of adsorbents with amino acid ligands

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Abstract

In this paper, a series of adsorbents with different amino acid ligands for endotoxin removal were prepared and endotoxin adsorption capacities (EAC) in aqueous solution were studied using an affinity column. The results showed that the property and structure of amino acid ligands have great influence on EAC. As the increasing of isoelectric point and polarity of amino acids ligands, EACs of the adsorbents increased. In addition, computer simulation method was employed to a further investigation on the interaction between endotoxins and ligands. Based on the results, some adsorbents were applied to remove endotoxin from endotoxemia rabbit's serum. Similar adsorption results were observed and the removal efficiency of adsorbents with Arg, Ser ligands is up to 78%.

Keywords: Endotoxin; Amino acids; Affinity adsorbents; Computer simulation

1. Introduction

Endotoxins are lipopolysaccharides (LPSs) of the outer cell wall of Gram-negative bacteria [1] and show strong biological effects on human beings and animals even at very low concentration when entering the blood stream. The biological effects include affecting structure and function of organs and cells, changing metabolic functions, raising body temperature, triggering the coagulation cascade, modifying haemodynamics and causing shock [2]. Serious endotoxin intoxication can cause endotoxermia. Because of its toxicity, removal of endotoxin duly and effectually from patients' blood is very important in clinic.

The general structure of endotoxins is a polar heteropolysaccharide chain covalently linked to a non-polar lipid moiety, lipid A, which anchors endotoxin in the outer bacterium membrane [3]. Lipid A is the most conserved part of endotoxin and is responsible for most of the biological activities of endotoxin [4]. Due to the complicated structure of endotoxin, there is not a generally applicable method for the removal of different endotoxins. Although many methods were developed to decontaminate endotoxin, such as ultrafiltration, two-phase extraction, HPLC, etc. [5,6], the facts that affinity adsorption takes place in mild conditions similar to the environment within living beings and has a very large combined constant make it suitable for the removal of trace endotoxin from varied samples to a relatively low level [7]. Affinity techniques, especially tailor-made endotoxin-selective affinity adsorbents exhibit a favorable foreground and were studied extensively in the recent years. It is interesting to investigate common rules in process of endotoxin removal and find out efficient affinity ligand for selective removal of endotoxin.

In this study, a series of adsorbents based on a non-specific matrix agarose and different amino acids ligands were prepared. An affinity chromatography column was packed and employed to investigate the adsorption capacity for endotoxin in aqueous solution. Computer simulation method was also used to study the interaction between endotoxin and ligand. Besides, some adsorbents were applied to removal endotoxin from endotoxemia rabbit's serum.

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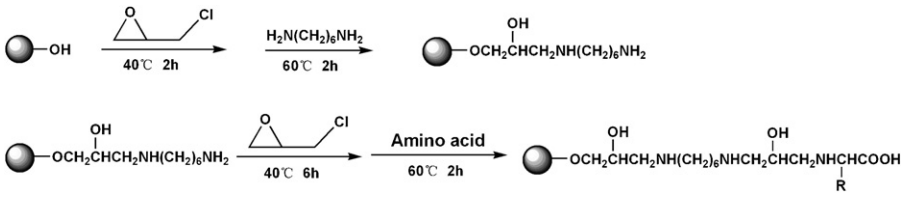

Fig. 1. Preparation of agarose adsorbents: R is lateral group of amino acid.

2. Experimental

2.1. Materials

Endotoxin standards, Limulus amoebocyte lysate (LAL) for endotoxin detection and endotoxin-free water were purchased from BoKang Marine Organisms Products Company (Zhanjiang, PRC) and National Institute of Pharmaceutical and Biological Products Control (Beijing, PRC). Amino acids were BR grade and purchased from Zhujiang Biomaterials Factory (Tianjin, PRC). Agar powder was BR grade and purchased from Reagents Company of National Pharmaceutical Group Corporation (Shanghai, PRC). All other chemicals were of analytical grade and purchased from local chemical reagent Companies.

BET-32 endotoxin test apparatus was from Electronic Instrument Factory of Tianjin University (Tianjin, PRC). The minimum detection limit of the Limulus Amebocyte Lysate was 0.03 EU/mL. The term EU describes the biological activity of endotoxin and 100 pg the standard endotoxin corresponds to 1 EU.

Endotoxin-free equipment was used throughout. All glassware was treated with detergent in an ultrasonic oscillator device followed by heat treatment at 220 °C overnight. All solution transfers were performed with endotoxin free devices [8].

2.2. Preparation of agarose adsorbents with amino acids ligands

Two grams tween-80 (as a dispersion agent) was dissolved in a solution of toluene and carbon tetrachloride at 75 °C. Four grams agarose powder and 70 mL distilled water were mixed and heated to reflux, and then was poured into the dispersed phase under stirring and the mixture was stirred at 75 °C for 1 h. After cooling to room temperature, the resultant agarose beads were sieved (20–40 meshes) and washed with distilled water.

One gram agarose beads was activated with a solution containing 2 mL epichlorohydrin and 3 mL 1.25 mol/L NaOH, shook at 40 °C for 2 h and washed with distilled water. The activated beads were suspended in 4 mL 0.4 mol/L hexamethylendiamine solution and were shaken at 60 °C for 2 h, washed with distilled water to obtain the aminoalkylagarose beads.

For the modification, 1 g aminoalkylagarose beads was incubated in a mixture of 1 mL epichlorohydrin and 5 mL 0.02 mol/L NaOH aqueous solution, and the reaction was performed at 40 °C for 6 h. After washing with distilled water, the beads were suspended in 15 mL solution, which contained 0.1 g amino acids and were adjusted to pH 12 with 2 mol/L NaOH. The beads were shaken at 60 °C for 2 h and were washed with distilled water. Ten different amino acids such as L-serine (Ser), L-arginine (Arg), L-phenylalanine (Phe), L-asparagic acid (Asp), glycine (Gly), L-histidine (His), L-glutamic acid (Glu), L-glutamine (Gln), L-asparagine (Asn) and L-tryptophane (Trp) were employed to modify the beads.

The synthetic scheme was showed in Fig. 1 and the contents of modified amino acids ligands were directly calculated from elemental analysis results.

2.3. Column

The experiments were conducted on a tailor-made column with dimensions of 50 mm × 5 mm which can autocontrol an approximate flow-rate of 0.4 mL/min. The column was packed with the newly synthesized adsorbents. Each time before it was used the column was cleaned by perfusion with 0.1 mol/L NaOH containing 20% ethanol, 1.5 mol/L NaCl, normal saline and endotoxin-free water to pH neutrality in turn [9].

2.4. Endotoxin (LAL) assay in aqueous solution

Endotoxin was assayed by the Limulus test, which involves a turbidimetric time assay at 450 nm with a BET-32 endotoxin test apparatus. Limulus amoebocyte lysate was used as the reagent for the reaction [10]. All samples were measured in duplicate. If the deviation exceeded 10%, the measurement was repeated.

The endotoxin removal ability of the adsorbent was quantitatively evaluated using endotoxin adsorption capacity (EAC), which represented the adsorption ability of per μmol ligand and was calculated by the following formula:

$$\mathrm{EAC} = \frac{(m_{\mathrm{feed}} - m_{\mathrm{filtr}})}{M_{\mathrm{ligand}}}$$

where m_{feed} is the amount of endotoxin (in endotoxin unit, EU) in the feed loaded on the column, m_{filtr} is the amount of endotoxin (EU) in filtrates and M_{ligand} (μmol) is the content of ligands immobilized on the adsorbents.

2.5. Dynamic adsorption of endotoxin from aqueous solution

Three hundred EU endotoxin standards were dissolved in 2 mL endotoxin-free water (pH 7.36) and the solution was shaken for 15 min at room temperature, and then the prepared 150 EU/mL endotoxin feed was perfused through the treated column at a flow-rate of 0.4 mL/min. The residual solution in the column was also extruded and the filtrate was collected for the LAL assay.

2.6. Computer simulation

The Sillicon Graphics SGI INDIGO 2 workstation was used for the stimulation. Molecular building, geometry optimization, conformational search were done using the molecular modeling package Sybyl version 6.91. In calculation, the molecular mechanics method and molecular dynamics method were used [11].

The results of computer simulation were expressed using binding energy (E_{bind}, kJ/mol), which was determined as following:

$$E_{bind} = E_{complex} - E_{endo} - E_{ligand}$$

where $E_{complex}$ is the energy after binding of endotoxin and ligand fragment, E_{endo} and E_{ligand} represent the energy of endotoxin fragment and ligand fragment, respectively.

2.7. Static adsorption of endotoxin from endotoxemia rabbit's serum

Specific pathogen-free male Beijing rabbits were kept in the Nankai Hospital of Tianjin. The rabbits fasted for 24 h before use. The acute model of endotoxemia used in our experiments was a sublethal, single-dose injection of *Escherichia coli* endotoxin (1.5 mg/kg) in a solution of 0.9% normal saline. After 4 h exposure, the blood of the rabbits was extracted and centrifuged for assay.

0.13 g adsorbents were incubated with 0.6 mL 1.01 EU/mL serum of endotoxemia rabbit and shaken for 0.5 h at 37 °C. The content of endotoxin was measured in Nankai Hospital of Tianjin according to the literature [12].

3. Results and discussion

3.1. Endotoxin adsorption experiments in aqueous solution

In this part, 10 amino acids were immobilized on agarose beads via a diaminohexane (DAH) spacer to investigate the influence of ligand's property and structure on endotoxin adsorption capacity in aqueous solution. The contents of amino acid ligand were directly calculated from elemental analysis results which are approximately between 46 and 119 μmol/g wet adsorbent, as summarized in Table 1. The experiments were performed with endotoxin-free water and the pH is about 7.4.

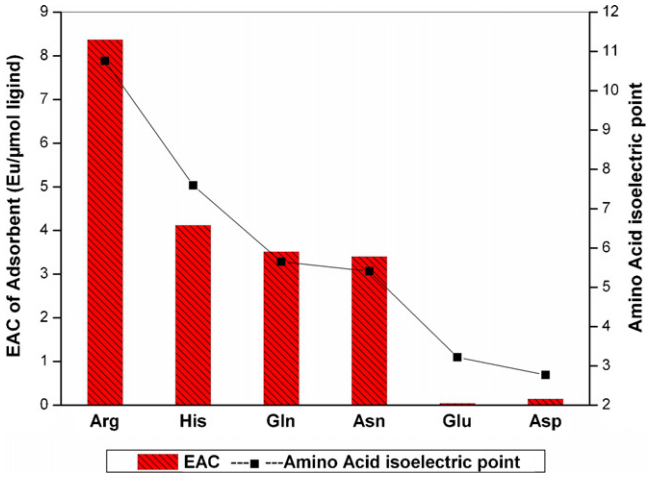

Fig. 2. Influence of amino acid ligand isoelectric point on EAC.

Considering the distinction of amino acids ligands contents immobilized on adsorbents, we used EAC (adsorption capacity of per μmol ligand) to express the adsorption contribution of amino acids ligands. As can be discerned from Figs. 2 and 3, EAC of Arg and Ser adsorbents are much higher than that of the others, which are 8.37 and 7.07 EU/μmol ligand, respectively (Table 1).

3.2. Influence of charges bearing on amino acids ligand on EAC

In this part, six amino acids were employed as ligands to investigate the influence of electrostatic interaction on EAC. The isoelectric point of amino acid ligand was used to indicate the amount of charge bearing on the adsorbent. The results were showed in Fig. 2.

It can be seen from Fig. 2 that with the decrease of isoelectric point amino acid, the EAC of adsorbent decreased as well. As we

Table 1
Adsorption and computer simulation of amino acids ligands adsorbents

Ligand	The content of ligand (μmol/g wet adsorbent)	Endotoxin adsorption capacity (EAC, EU/μmol ligand)	Isoelectric point	Binding energy (E_{bind}, kJ/mol)
Arg[a]	46	8.37	10.76	−308.7
His[a]	81	3.61	7.59	−188.6
Glu[a]	80	0.04	3.22	−82.8
Asp[a]	86	0.14	2.77	−13.2
Ser[b]	50	7.07	5.68	−228.1
Gln[b]	72	3.51	5.65	−177.8
Asn[b]	75	3.4	5.41	−141.4
Gly[b]	119	0.25	5.97	−100.2
Trp[c]	94	2.58	5.89	−130.7
Phe[c]	99	0.58	5.48	−109.8

[a] Amino acids with charges.
[b] Polar amino acids with no charges.
[c] Apolar amino acids.

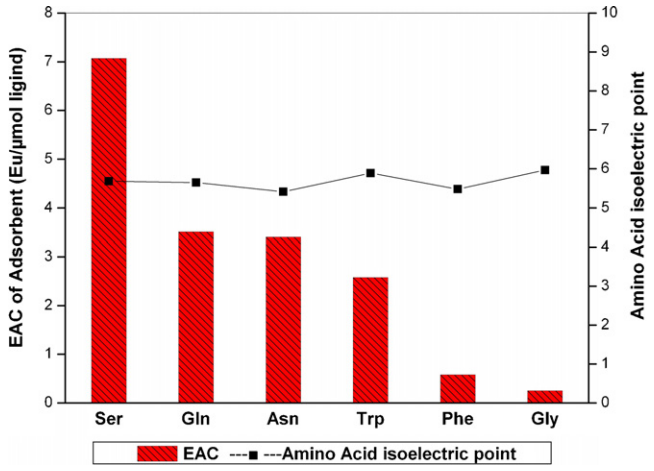

Fig. 3. Influence of amino acid ligand polarity on EAC.

know, endotoxin molecule carries large amounts of phosphate groups and exhibits a net negative charge in aqueous solution ($pK_a = 1.3$ [13]) at neutrality and the electrostatic force plays an important role in the process of endotoxin adsorption. The charges bearing on amino acids ligands could contribute much to EAC of adsorbent.

For the acidic amino acids such as Glu and Asp, which have the isoelectric values of 3.22 and 2.77, respectively, have strong negative charges due to their second carboxy groups at neutrality. When they were immobilized on the adsorbent as ligands, the repulsion between endotoxin and ligands make them difficult to approach each other. As a result, the EACs of adsorbents are quite low. On the other hand, Arg ligand exhibits strong positive charges due to its basic (pK_a around 12.0) guanidine group, and the EAC of Arg adsorbent is the highest among the others.

3.3. Influence of amino acid ligand polarity on EAC

Six amino acids were chosen to study the influence of ligand polarity on EAC. The immobilized amino acids have similar isoelectric value (5–6) but different polarity. The results are summarized in Fig. 3.

Among the six amino acids, Trp and Phe are apolar amino acids and the others display polar properties owing to their hydrophilic R groups. The EACs of polar amino acids adsorbents were generally higher than those of apolar ones. The only exception is Gly, which is polar amino acid but the Gly adsorbent showed the lowest EAC value among the six samples. This is because there is no hydrogen bond and hydrophobic interaction existing between Gly and endotoxin.

With the assist of hydroxyl group on the lateral chain of Ser, Ser adsorbent could form hydrogen bond with endotoxin, and the minor steric interference and flexible structure make it easy to form a stable structure and EAC of Ser adsorbent is higher than the others.

Trp and Phe have similar property but big distinction on EACs of adsorbents. It could be their different structures lead to this. The large phenyl group on the lateral group of Phe interrupted the interaction between endotoxin and ligand, while the high hydrophobicity of lateral group on Trp was tempered by the presence of the indole NH group, which could serve as a hydrogen bond donor and favor contacting with polar groups.

The results above indicate that polarity of amino acid ligand greatly influence EAC of adsorbent. The hydrophilic R groups of amino acids ligand make them possible form hydrogen bond with endotoxin and enhance the interaction between ligand and endotoxin. At the same time hydrophobic interaction, steric effect and possibility of structure matching could also exist.

3.4. Computer simulation

For a further understanding of the interaction between amino acid ligand and endotoxin, computer simulation method was employed to investigate possible interaction pattern in three-dimensional space. We chose the conserved phosphorylated residues of Lipid A as our object and acted it with amino acids ligands of adsorbents. The binding energies were summarized in Table 1.

It can be seen from the results that these binding energies showed a similar trend with the EACs of amino acids adsorbents. Fig. 4 displayed some photos of simulation.

With the assistance of hydroxyl group on the ligand and space, Ser adsorbent formed a cage structure with phosphoric residue of endotoxin via three couple of hydrogen bonds (Fig. 4a). The minor steric interference makes it easy to form the stable structure and Ser adsorbent shows an excellent adsorption performance.

In Fig. 4b, the ligand Trp formed hydrogen bonds with endotoxin for the existing of indole NH group. On the other hand, the large phenyl group of Phe made it difficult to approach to endotoxin molecule. The phenyl group was pushed away from the interaction center. The EAC of Trp adsorbent is higher than that of Phe although they exhibited similar properties on charge and polarity.

From the simulation models of Arg and Try adsorbent with endotoxin (Fig. 4), some proofs indicate that a cooperative hydrophobic interaction exist and help them to achieve better performance.

Computer simulation method offered some references to our work. The photos obtained by simulation displayed an imaginable interaction pattern between endotoxin and amino acids ligands. We hope that the method could assist to design endotoxin removal adsorbent to a certain extent.

3.5. Endotoxin adsorption from endotoxemia rabbit's serum

It had been proved in our previous work that the agarose gel matrix had good blood compatibility [14,15]. Based on the adsorption results in aqueous solution, some adsorbents were applied to remove endotoxin from endotoxemia rabbit's serum. The results were showed in Table 2.

From the results we can see that adsorption results obtained were similar to those in aqueous solution. Arg and Ser adsorbents displayed much higher EAC than the others and the removal efficiencies are up to 78.3%. They could effectively remove endotoxin from endotoxemia rabbit's serum.

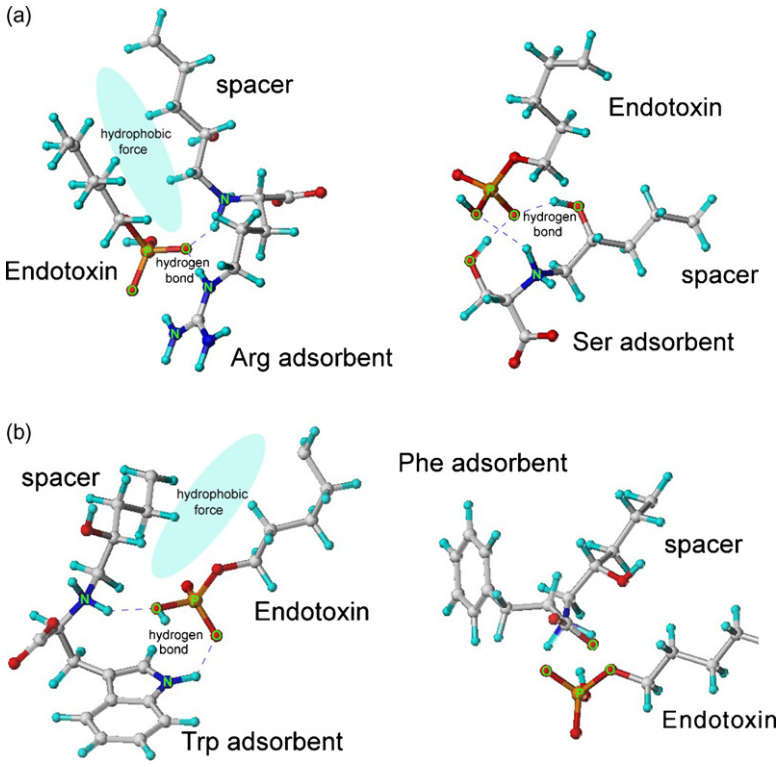

Fig. 4. Computer simulation of interaction models between endotoxin and ligand: the broken lines are hydrogen bonds formed between endotoxins and ligands, the light blue elliptical regions represent hydrophobic interaction.

Table 2
Endotoxin removal from endotoxemia rabbit's serum

Ligand	Before adsorption (EU/mL)	After adsorption (EU/mL)	EAC (10^{-3} EU/μmol ligand)	Endotoxin removal efficiency (%)
Arg	1.011	0.219	78.14	78.3
Ser	1.011	0.219	68.03	78.3
His	1.011	0.636	21.20	37.1
Gln	1.011	0.745	16.41	26.3

4. Conclusions

In this work a series of affinity adsorbents with amino acids ligands were prepared to investigate the influence of ligand structure on endotoxin adsorption. It was found that the charge and polarity of amino acid ligand have great influence on the adsorption of endotoxin. The electrostatic force plays a dominant role in the adsorption process and the R group of polar amino acid ligand makes it possible to form hydrogen bond with endotoxin, which enhances the adsorption of endotoxin significantly. We found that Arg and Ser are better ligands for the adsorption of endotoxin. They could effectively remove endotoxin from endotoxemia rabbit's serum. The removal efficiency of adsorbents is up to 78%. Besides, computer simulation method could be used to investigate imaginable interaction pattern between endotoxin and adsorbent.

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References

[1] M. Vaara, H. Nikaido, Handbook of Endotoxin, vol.1, Elsevier, Amsterdam, 1984, p. 1.
[2] G.D. Martich, A.J. Boujoukos, A.F. Suffredini, Immunobiology 187 (1993) 403.
[3] C.R.H. Raetz, Annu. Rev. Biochem. 59 (1990) 129.
[4] C. Hirayama, M. Sakata, J. Chromatogr. B 781 (2002) 419.
[5] D. Petsch, F.B. Anspach, J. Biotechnol. 76 (2000) 97.
[6] Y. Li, R. Lander, W. Manger, A. Lee, J. Chromatogr. B 804 (2004) 353.
[7] S. Minobe, T. Watanabe, T. Sato, T. Tosa, J. Chromatogr. 248 (1982) 401.
[8] D. Petsch, E. Rantze, F.B. Anspach, J. Mol. Recognit. 11 (1998) 222.
[9] S.H. Lee, J.S. Kim, C.W. Kim, Process Biochem. 38 (2003) 1091.
[10] J.C. Hurley, Clin. Microbiol. Rev. 8 (1995) 268.
[11] J. Vaidyanathan, T.K. Vaidyanathan, P. Yadav, C.E. Linaras, Biomaterials 22 (2001) 2911.
[12] R.S. Lin, F.Y. Lee, S.D. Lee, Y.T. Tsai, H.C. Lin, R.H. Lu, W.C. Hsu, C.C. Huang, S.S. Wang, K.J. Lo, J. Hepatol. 22 (1995) 165.
[13] K.C. Hou, R. Zaniewski, J. Parenter. Sci. Technol. 44 (1990) 204.
[14] G.H. Hou, H.Y. Wang, T. Liu, Z. Yuan, J. Fang, D.H. Li, Q. Qiu, S.D. Guo, Chem. J. Chinese U. 26 (2005) 1277.
[15] G.H. Hou, T. Liu, H.Y. Wang, Artif, Cell Blood Sub. 33 (2005) 227.