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Quantitative Determination of Ca^{2+} Effects on Endotoxin Removal and Protein Yield in a Two-Stage Ultrafiltration Process

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ABSTRACT

Endotoxin subunits normally aggregate into vesicles with a molecular mass around 1,000,000. Some proteins can bind and disaggregate endotoxin to form protein–endotoxin complexes, which makes endotoxin removal from protein solution more complicated. It is also known that divalent cations such as Ca^{2+} can act as "bridges" between lipopolysaccharide subunits, causing large vesicles to form. Such a property has potential utility in endotoxin removal from biological solutions. A two-stage ultrafiltration was used in this study during which Ca^{2+} reaggregated lipopolysaccharide subunits in protein solutions into large vesicles that hence were retained by a 300,000 nominal molecular weight cutoff ultrafiltration membrane. After the reaggregation of lipopolysaccharide subunits, the endotoxin removal efficiency was in the range of 54.78 to 73.10%, meanwhile protein yield was in the range of 71.57 to 89.54% at various protein/endotoxin concentration ratios.

Key Words. Endotoxin removal; Protein purification; Membrane; Ultrafiltration; Lipopolysaccharide; Protein–LPS complex

INTRODUCTION

Bacterial endotoxins are lipopolysaccharides (LPS) derived from the outer membranes of gram-negative bacteria (1). Endotoxin contamination during manufacture of water for injection (WFI) and parenteral solutions is of great

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concern to the pharmaceutical industry. Endotoxins are responsible for most pyrogenic reactions if they occur in sufficient amounts in large volume parenterals (infusion fluids) (2, 3). Endotoxins in distilled water cause serious complications as they can be present in kidney dialysis machines and intravenously infused nutrients, pharmaceutical agents, and various other products. Endotoxins were also shown to cause disease in animals (4). The physiopathological effects shown by affected humans or animals include pyrogenicity, endotoxemia, leucopenia, leucocytosis, shock, allergic reactions, and lowered blood pressure (5).

Gram-negative bacteria are widely used in the biotechnology industry to produce recombinant DNA products such as peptides and proteins. Bacterial endotoxins have been recognized by the industry as a major cause of the pyrogenic reactions that can be encountered during the administration of biotherapeutics. The removal of these physiologically active agents from final bioproducts has always been a challenge. Because proteins are biologically active and sensitive to pH or temperature changes, endotoxin removal from protein solutions is more complicated than that from water. Moreover, it is known that some proteins can bind endotoxins and form protein–LPS complexes in biological solutions (6–10). It was reported that hemoglobin not only can bind but also disaggregate LPS, and enhance LPS activation of *Limulus* amebocyte lysate (LAL) in a concentration-dependent manner (11, 12). We reported previously the concentration effects of hemoglobin and albumin on protein–LPS binding and endotoxin removal (13). It was found that the proteins interacted with LPS and formed protein–LPS complexes, resulting in the disaggregation of LPS vesicles.

Endotoxin subunits have molecular weights of 10,000 or less, depending on the composition of the solution in which they are present (4). Aggregated forms of endotoxin range from 300,000 to 1,000,000 daltons (14–17). The aggregation is believed to be facilitated by cations. Because LPS has negatively charged phosphate groups, cations, especially divalent cations such as Ca^{2+} and Mg^{2+}, can act as "bridges" between LPS subunits (18), resulting in LPS bilayer sheets or vesicles with a diameter of the order of 0.1 μm in water (19, 20). In a previous study, researchers in our research group investigated the cation effects on endotoxin removal efficiency and protein recovery in an affinity chromatographic process (21). In another study, we investigated the effects of Ca^{2+} on LPS reaggregation in hemoglobin solutions and the removal of endotoxin. When LPS subunits in complexes were reaggregated and formed large vesicles again in the solution, they did not pass the membrane, and endotoxin was not found in the filtrate (22).

In the work presented in this article, the method of two-stage ultrafiltration was also used to study the effect of Ca^{2+} on LPS reaggregation. Since the chromogenic LAL assay was employed to detect endotoxin, it was possible to

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determine endotoxin concentration quantitatively. The endotoxin removal efficiency after reaggregation in the presence of Ca^{2+} was studied. The yield of hemoglobin was also investigated.

EXPERIMENTAL

Materials and Methods

Glassware

All glassware used was autoclaved in an autoclave (Series 300, Harvard/LTE) for 1 hour and followed with heating in an oven (Thelco Model 16, Precision Scientific Co.) at 175°C for 3 hours. All solution transfers were performed by endotoxin-free devices. Sterile, disposable plasticware was used to prevent endotoxin contamination.

Reagents

Endotoxin-free water from BioWhittaker (BioWhittaker Inc., Walkersville, MD) was used for endotoxin dilution and all solution preparations. Endotoxin from *Escherichia coli* 026:B6, human hemoglobin A_0 (HbA_0, ferrous), 0.1 M endotoxin-free HCl, 0.1 M endotoxin-free NaOH, NaCl (molecular biology reagent), and $CaCl_2 \cdot 2H_2O$ (molecular biology reagent) were from Sigma (Sigma Chemical Co., St. Louis, MO). Hemoglobin and endotoxin solutions were prepared with endotoxin-free 0.15 M NaCl solution. Ultrafree-CL polysulfone 300,000 NMWCO (Nominal Molecular Weight Cutoff) ultrafiltration membrane filters were from Millipore (Millipore Corporation, Bedford, MA).

Endotoxin Assay

To determine endotoxin concentration, a chromogenic *Limulus* Amebocyte Lysate (LAL) test kit QCL-1000 from BioWhittaker (BioWhittaker Inc., Walkersville, MD) was used. The chromogenic substrate is a short synthetic polypeptide with an amino acid sequence that mimics a natural cleavage site in a clotting protein present in the lysate. The chromogenic *p*-nitro aniline (*p*NA) moiety is attached to the end of the peptide as Ac-Ile-Glu-Ala-Arg-*p*NA. In the chromogenic LAL test the proenzyme which exists in the *Limulus* amebocyte lysate (LAL) was activated by the endotoxin in a water bath at 37°C for 10 minutes. Chromogenic substrate was then added, and the active enzyme caused the release of *p*NA from the substrate, producing a yellow color. After 20 minutes the reaction was stopped with 25% acetic acid. The intensity of the color change produced by the substrate cleavage was measured with a UV/VIS spectrophotometer (Hitachi U-2000, Hitachi Instruments Inc., Danbury, CT) at 405 nm. The results were compared to a calibration curve to obtain endotoxin concentration. However HbA_0 also has a strong absorbance at 405 nm, and it should not be counted in the endotoxin absorbance. The cor-

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rection was made by deducting the HbA_0 absorbance from the total absorbance of the reaction mixture.

Endotoxin removal efficiency, *ERE*, of the ultrafiltration was determined by

$$ERE = \frac{C_{LPS}^{load} - C_{LPS}^{filt.}}{C_{LPS}^{load}} \quad (1)$$

where C_{LPS}^{load} is the endotoxin concentration in the solution loaded on the ultrafiltration membrane filter (EU/mL) and $C_{LPS}^{filt.}$ is the endotoxin concentration in the filtrate of the ultrafiltration (EU/mL).

Protein Assay

The concentration of HbA_0 in the solution was measured at 410 nm by a UV/VIS spectrophotometer (Hitachi U-2000, Hitachi Instruments Inc., Danbury, CT). Protein yield, *Y*, of the ultrafiltration was determined by

$$Y = C_{HbA_0}^{filt.}/C_{HbA_0}^{load} \quad (2)$$

where $C_{HbA0}^{filt.}$ is the HbA_0 concentration in the filtrate (μg/mL) and C_{HbA0}^{load} is the HbA_0 concentration in the solution loaded on the ultrafiltration membrane filter (μg/mL).

The First-Stage Ultrafiltration

3.6 mL HbA_0 solutions at various concentrations (Table 1) were incubated with 0.4 mL 50.00 EU/mL endotoxin solution at 37°C in a water bath for 30 minutes; the concentration ratios of HbA_0 to endotoxin were 0.90, 1.80, 2.70, and 3.60 μg/EU. Sterile, endotoxin-free culture tubes were used for the incubation. After incubation, the mixture solutions were placed on 300,000 NMWCO polysulfone ultrafiltration membrane filters and ultrafiltration was performed by centrifuging the mixture samples at 1800*g* for 5 minutes. This is the first-stage ultrafiltration, as shown in Fig. 1. 0.1 mL filtrate of each so-

TABLE 1
Protein and Endotoxin Concentrations in Feeds Loaded to the First-Stage Ultrafiltration

HbA_0 concentration in feed (μg/mL)	Endotoxin concentration in feed (EU/mL)	$[HbA_0]/[LPS]^a$ (μg/EU)
45.00	50.00	0.90
90.00	50.00	1.80
135.00	50.00	2.70
180.00	50.00	3.60

[a] The concentration ratio of HbA_0 and LPS.

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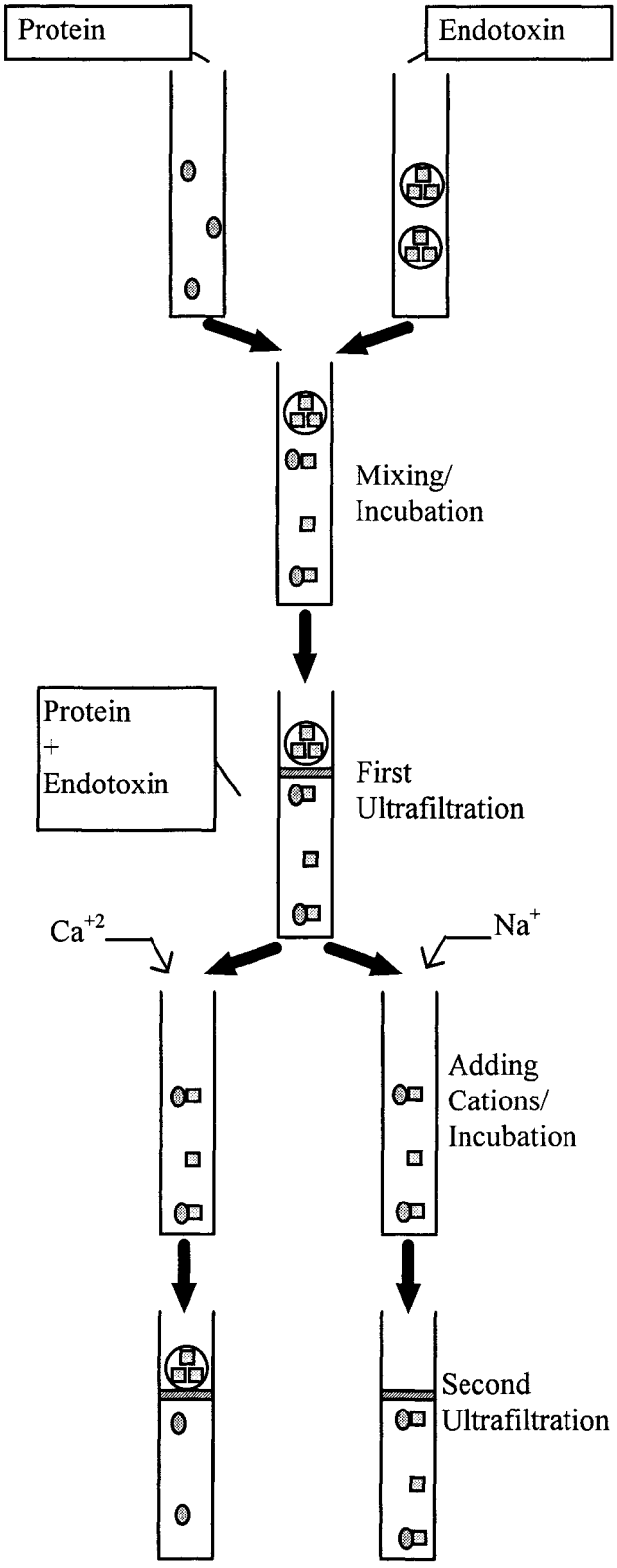

FIG. 1 The experimental procedure.

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lution was set aside for the LAL test, and the remaining filtrate was divided into two parts. One part was used for the second-stage ultrafiltration to investigate Ca^{2+} effect on endotoxin removal and protein purification with $CaCl_2$; the other was used for a control experiment with NaCl.

The Second-Stage Ultrafiltration

Four 1 mL filtrates at various hemoglobin concentrations from the first-stage ultrafiltration were put into sterile endotoxin-free culture tubes. Then 1 mL 0.05 M endotoxin-free $CaCl_2$ solution was added to each tube. The ionic strength of the $CaCl_2$ solution was 0.15 M. The mixtures were incubated in a water bath at 37°C for 30 minutes. After incubation, the solutions were applied to 300,000 NMWCO polysulfone ultrafiltration membranes and the ultrafiltration was performed by centrifuging at 1800*g* for 5 minutes. This is the second-stage ultrafiltration as shown in Fig. 1. Filtrate (0.1 mL) of each solution was subjected to the LAL test. In order to conduct a control experiment that was used as a comparison to the endotoxin reaggregation study in $CaCl_2$ solution, 0.15 M NaCl solution was used to study the aggregation state of LPS in the mixtures. The ionic strength of NaCl solution was 0.15 M, as same as that of $CaCl_2$ solution. The experimental procedure was similar to that of the study with $CaCl_2$ solution.

RESULTS AND DISCUSSION

Endotoxin Removal of the First-Stage Ultrafiltration

The endotoxin removal efficiency of the first-stage ultrafiltration is shown in Table 2. As the HbA_0/LPS concentration ratio increased from 0.90 to 3.60 μg/EU, the endotoxin removal efficiency, *ERE*, decreased from 98.84 to 88.36%. This phenomena can be explained as follows: Pure endotoxin in water cannot pass through the ultrafiltration membranes rated at 300,000 nominal molecular weight cutoff (NMWCO). When endotoxin aggregates were

TABLE 2
Endotoxin Removal Efficiency, *ERE*, and HbA_0 Yield, *Y*, of the First-Stage Ultrafiltration

$[HbA_0]/[LPS]$ (μg/EU)	*ERE* (%)	*Y* (%)
0.90	98.84	79.91
1.80	94.96	90.67
2.70	92.64	95.39
3.60	88.36	95.19

broken down by protein molecules and smaller protein–LPS complexes were formed, the complexes passed the membrane and endotoxin was found in the filtrate. As observed in our previous study (13), the protein capacity of breaking down endotoxin is protein concentration dependent. The higher the protein concentration, the more the endotoxin is dissociated. Therefore, at high HbA_0/LPS concentration ratios, more endotoxin was broken down to form smaller protein–LPS complexes, which passed through the membrane. This resulted in less endotoxin retained by the membrane and lower *ERE*.

Protein Yield of the First-Stage Ultrafiltration

The protein yield of the first-stage ultrafiltration is shown in Table 2. As the HbA_0/LPS concentration ratio increased from 0.90 to 3.60 μg/EU, the protein yield, *Y*, increased from 79.91 to 95.19%. Binding between the HbA_0 and the membrane may be the cause of protein loss during filtration.

Endotoxin Removal of the Second-Stage Ultrafiltration—The Effect of Ca^{2+} on Endotoxin Removal

Figure 2 shows endotoxin concentrations in feeds loaded on the second-stage ultrafilters and endotoxin concentrations in membrane filtrates of the second-stage ultrafiltration in situations when Ca^{2+} or Na^+ was added. From Fig. 2 we can see that at various HbA_0/LPS concentration ratios, the endotoxin concentrations in the filtrates with Ca^{2+} were much lower than those in the control samples (with Na^+). Figure 3 shows the amount of endotoxin removed from the solution during the second-stage ultrafiltration. The amount of endotoxin removed from the solution with Ca^{2+} were much higher than those in the solution with Na^+. The effect of Ca^{2+} on the amount of endotoxin removed is clearly shown in Table 3, The *ERE* with Ca^{2+} was two to three times of the *ERE* with Na^+.

TABLE 3
Effect of Ca^{2+} on Endotoxin Removal Efficiency, *ERE*, of the Second-Stage Ultrafiltration

[HbA_0/LPS (μg/EU)	*ERE* with Ca^{2+} (%)	*ERE* with Na^+ (%)
0.90	59.09	19.60
1.80	72.73	34.17
2.70	73.10	28.15
3.60	54.78	24.98

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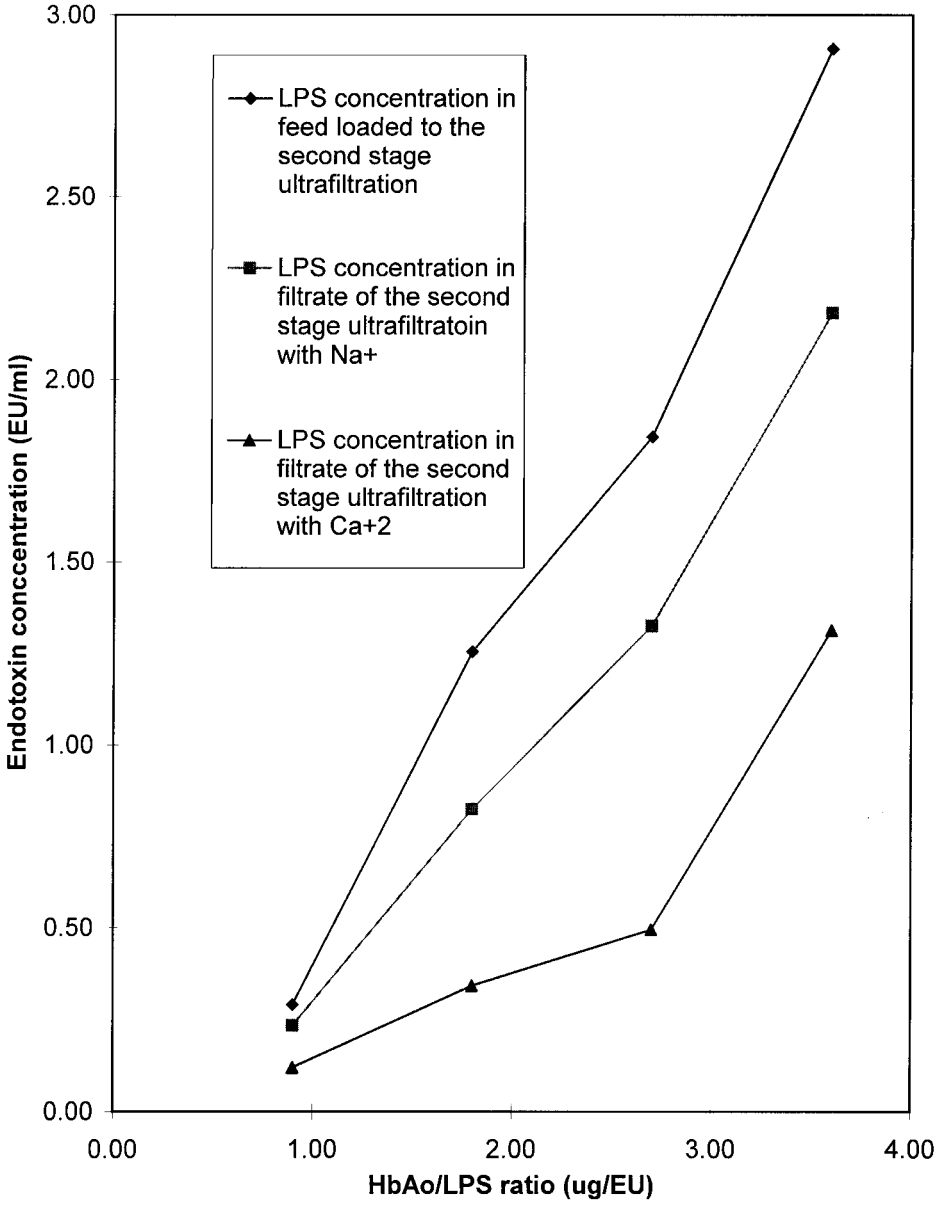

FIG. 2 Endotoxin concentration in the second-stage ultrafiltration.

Protein Yield of the Second-Stage Ultrafiltration—The Effect of Ca^{2+} on Protein Yield

Figure 4 shows HbA_0 concentrations in feeds loaded on the second-stage ultrafilters and HbA_0 concentrations in filtrates of the second-stage ultrafiltration. From Fig. 4 we can see that at various HbA_0/LPS concentration ratios, the curves representing HbA_0 concentrations with Ca^{2+} and with Na^+ almost overlap, which indicates that the HbA_0 concentrations in the filtrates with Ca^{2+} were very close to those in the control experiments (with Na^+).

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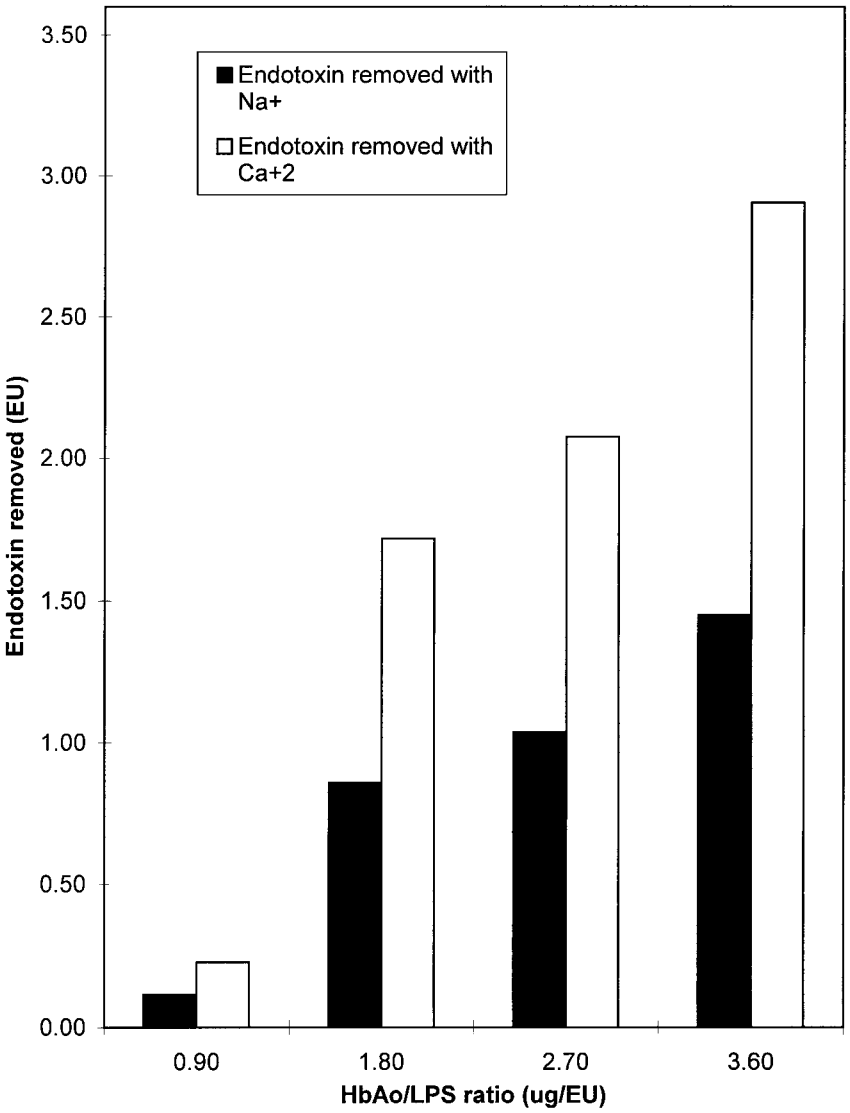

FIG. 3 Endotoxin removed during the second-stage ultrafiltration.

Figure 5 illustrates the protein loss during the second-stage ultrafiltration. The amount of HbA_0 lost from solutions with Ca^{2+} and Na^+ are almost the same, which indicates that the introduction of Ca^{2+} has almost no effect on protein yield. Table 4 shows that the HbA_0 yields with Ca^{2+} are very close to the HbA_0 yields with Na^+ at various HbA_0/LPS concentration ratios.

The physical meanings of above results may be explained as follows. Pure endotoxin is usually in the aggregate state with a molecular weight around 1,000,000 daltons and is retained by a 300,000 NMWCO membrane. In this study HbA_0 and endotoxin mixtures at four different concentration ratios were incubated before the first ultrafiltration. Hemoglobin molecules broke the en-

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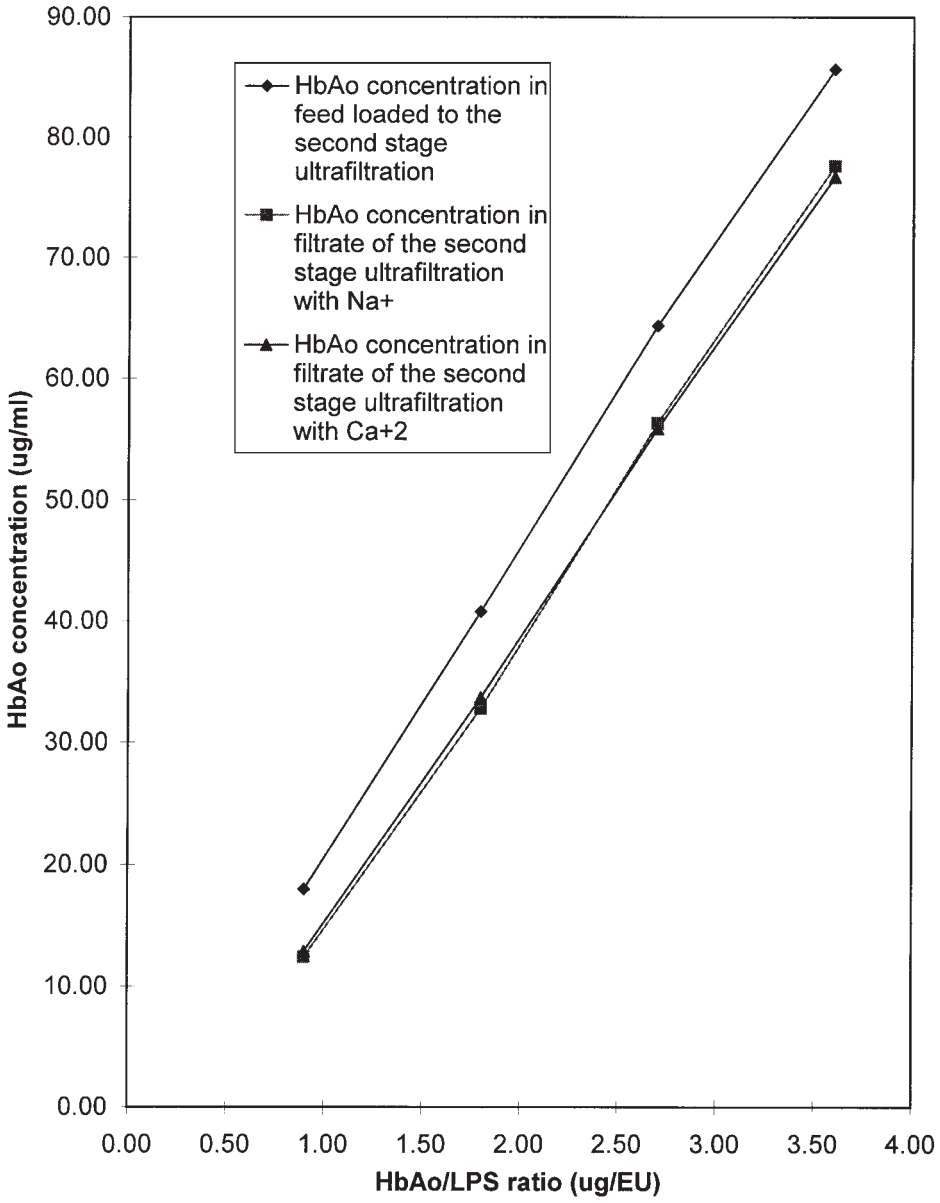

FIG. 4 Protein concentration in the second-stage ultrafiltration.

dotoxin aggregates and formed protein-LPS complexes with LPS subunits. As we reported in our previous study (13), protein concentration has a significant effect on protein–LPS binding and the amount of endotoxin disaggregated. In other words, the following equilibrium moved toward the RIGHT-HAND side as the protein concentration was increased:

$$\text{LPS aggregate} + \text{protein} \rightleftharpoons \text{LPS subunits} + \text{protein–LPS} \qquad (3)$$

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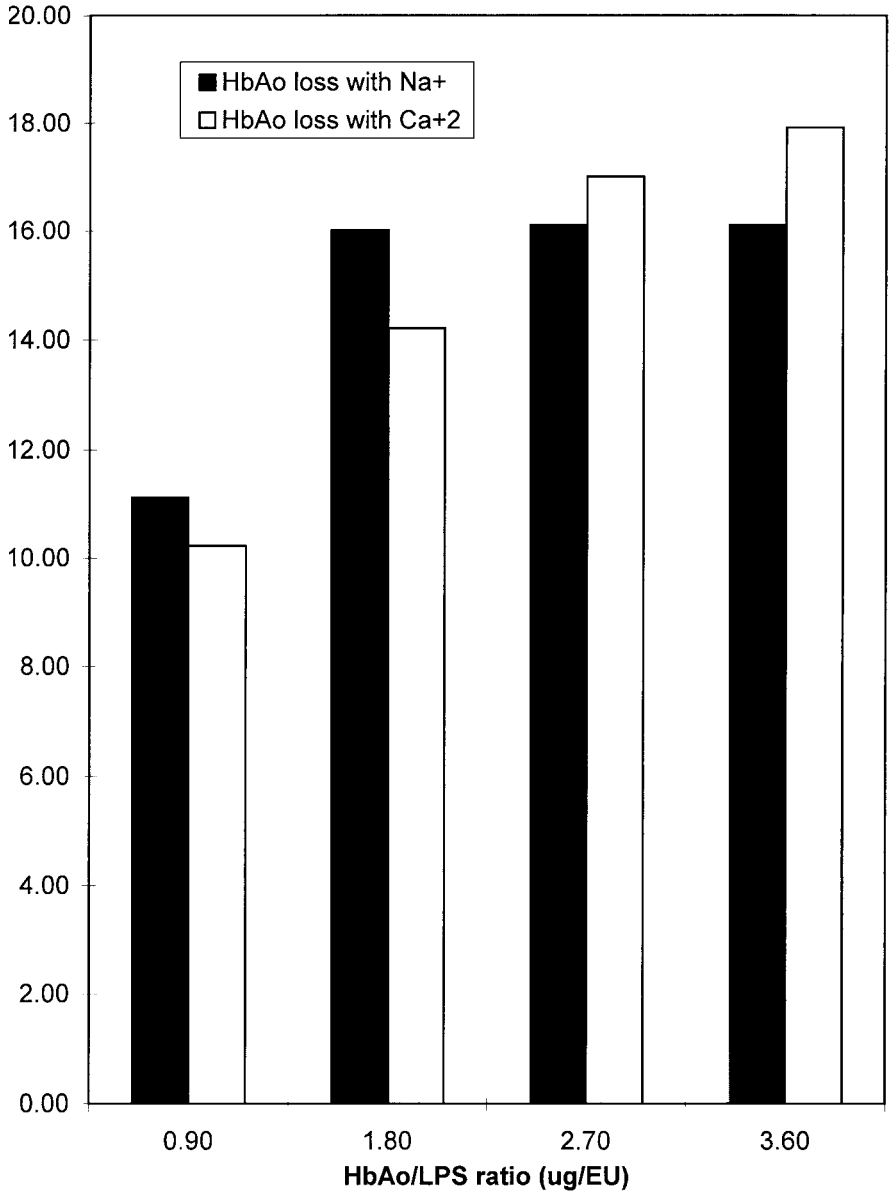

FIG. 5 Protein loss during the second-stage ultrafiltration.

TABLE 4
Effect of Ca^{2+} on Protein Yield, *Y*, of the Second-Stage Ultrafiltration

$[HbA_0]/[LPS]$ (μg/EU)	*Y* with Ca^{2+} (%)	*Y* with Na^{+} (%)
0.90	71.57	69.05
1.80	82.54	80.32
2.70	86.79	87.49
3.60	89.54	90.60

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As a result, *ERE* decreased from 98.84 to 88.36%. However, when Ca^{2+} was added to all samples after the first ultrafiltration, the cation began to move the above equilibrium toward the LEFT-HAND side due to the "bridging effect," which resulted in the reaggregation of LPS subunits. Therefore, less endotoxin was detected in the filtrates of the second-stage ultrafiltration with Ca^{2+} (Figure 2), and more endotoxin was removed (Fig. 3) compared with the control experiments (with Na^{+}).

CONCLUSIONS

The results of this study showed that Ca^{2+} can affect endotoxin aggregate states not only in water, as reported before, but also in protein solutions where protein–endotoxin complexes exist. The addition of Ca^{2+} to protein–endotoxin mixture can result in the reaggregation of endotoxin subunits, which makes it easier to remove endotoxin from protein solutions through ultrafiltration. The effects of Ca^{2+} on endotoxin removal efficiency in second-stage ultrafiltration can be clearly seen by comparing the *ERE* with Ca^{2+} to the *ERE* with Na^{+} in the control experiments.

The results also showed that the protein yield during ultrafiltration with Ca^{2+} or with Na^{+} is almost the same. This indicates that the protein yield was not sacrificed in the process of LPS reaggregation in the presence of Ca^{2+}. This phenomenon has potential utility in ultrafiltration processes to increase endotoxin removal efficiency and in the meantime maintain protein yield.

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