Journal of Immunological Methods, 132 (1990) 191–195
Elsevier

JIM 05674

Removal of endotoxin from protein solutions by phase separation using Triton X-114

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(Received 10 April 1990, accepted 11 May 1990)

Endotoxin contamination of protein solutions was reduced by a phase separation technique using the detergent, Triton X-114. Protein solutions containing endotoxin were treated with Triton X-114 on ice. The solution was then warmed to 37°C, whereupon two phases formed. The Triton X-114 phase, containing the endotoxin, was precipitated by centrifugation. The first cycle of phase separation produced a 1000-fold reduction of endotoxin from contaminated preparations of cytochrome *c*, catalase and albumin. Complete removal of endotoxin could be achieved by further cycles of phase separation. Each cycle of phase separation resulted in only a 2% loss of protein, and could be completed within 15 min. The small amount of detergent (0.018%) that persisted in protein solution could be removed by gel filtration or absorption. Proteins treated by this procedure retained normal functions. This phase separation technique provides a rapid and gentle method for removing endotoxin from protein solutions.

Key words: Endotoxin; Lipopolysaccharide; Phase separation; Triton X-114

Introduction

Endotoxins are major contaminants in commercially available proteins or biologically active substances, and often complicate study of their biological effects (Pabst et al., 1989). Polymyxin B affinity chromatography is effective in reducing endotoxin in solutions (Issekutz, 1983). Recently, Karplus et al. (1987) reported an improved method of polymyxin B affinity chromatography in which endotoxin could be absorbed effectively after dissociation of the endotoxin from the proteins by a nonionic detergent, octyl-β-D-glucopyranoside. We report here a method to reduce endotoxin in protein solutions using Triton X-114, in which the detergent aids in dissociation of endotoxin from the protein, while also providing a convenient phase separation capability for removing the dissociated endotoxin.

Materials and methods

Removal of endotoxin by phase separation

As test samples, bovine liver catalase (40 mg/ml, Sigma, St. Louis, MO), horse heart cytochrome *c* (13 mg/ml, Sigma, type III), bovine serum albumin (BSA, 30 mg/ml, Sigma) and radiolabelled lipopolysaccharide (^{3}H and ^{14}C, 200 ng/ml, from *Salmonella typhimurium*, provided by

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Abbreviations: BSA, bovine serum albumin; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline, O_2^-; superoxide anion.

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Dr. R.S. Munford, University of Texas, Dallas) (Hass et al., 1983) were dissolved in phosphate-buffered saline (PBS). Phase separation was carried out as described except that pre-condensation of Triton X-114 was not performed (Bordier, 1981). Test solutions (0.5 ml) were mixed with Triton X-114 (Sigma) at 1% (5 μl) by vigorous vortexing in 1.5 ml conical microfuge tubes. Samples were placed in an ice bath for 5 min to ensure a homogenous solution. After vortexing the chilled samples, the tubes were warmed at 37°C for 5 min to allow two phases to form. Samples were then centrifuged for 7 s with a microcentrifuge, equilibrated at 37°C in an incubator. In some experiments, tubes were placed in a 56°C water bath for 1 min before centrifugation. After centrifugation, the detergent phase was found as an oily droplet at the bottom of the tube. The upper aqueous phase was removed with care so as not to aspirate the detergent phase.

Removal of Triton X-114

Residual detergent in the aqueous phase was removed by gel filtration on BioGel P-300 (Bio-Rad Laboratories, Richmond, CA) or by treatment with Bio-Beads SM-4 (Bio-Rad). The P-300 column was prewashed with 0.1 column volume of 1% sodium dodecyl sulfate and then with three column volumes of PBS containing 0.01% sodium azide. This washing was performed after each use. Residual Triton X-114 in the aqueous phase eluted *after* cytochrome *c* (MW 12,800) on the P-300 column. Alternatively, residual detergent was removed by absorption with Bio-Beads SM-4. The beads were made endotoxin-free as follows: SM-4 beads (1 g wet weight) were suspended in 10 ml of 2% Triton X-114 and stirred for 2 h at 4°C. Then the beads were transferred to a column and washed with 20 ml of methanol and then with 20 ml of PBS. To remove Triton X-114 from protein solutions, the aqueous phase (1 ml) was stirred with SM-4 beads (0.5 g) at 4°C for 1 h.

Detection of endotoxin

The *Limulus* amoebocyte lysate gelation test was employed to detect endotoxin. Ten-fold serial dilutions of test samples were incubated with *Limulus* lysate (E-Toxate, Sigma) at 37°C for 60 min in glass tubes. Results are reported as the highest dilution of the sample that formed a firm gel. The test detected 10 pg/ml of our standard endotoxin, purified from *E. coli* K235 (McIntire et al., 1969).

Radioactivity measurement

Samples (500 μl) were mixed with 4 ml of scintillant (Opti-Fluor, Packard Instrument Co., Downers Grove, IL). Scintillation counting was performed with a Packard Tri-Carb 4640 with quench correction and external standard to calculate dpm. Results are reported as total dpm in the ^{3}H region, due to both ^{3}H and ^{14}C.

Measurement of superoxide anion release by neutrophils

Human neutrophils were isolated from venous blood by dextran sedimentation followed by Ficoll-Hypaque gradient centrifugation (Haslett et al., 1985). Neutrophils were suspended in Krebs-Ringer phosphate buffer with 0.2% glucose. For measurement of superoxide anion (O_2^-) release, neutrophils were suspended at 1×10^6 cells/ml in 40 μM cytochrome *c*, then stimulated with 0.1 μM phorbol myristate acetate (Sigma) for 15 min at 37°C. After the incubation, the supernatants were scanned from 560 to 540 nm, and the height of the peak at 550 nm was determined with reference to the isosbestic points at 542 and 556 nm. The extinction coefficient of 0.021 μM^{-1} was used to calculate the total amount of O_2^- released.

Assay of catalase activity

The activity of catalase was determined by the method of Beers and Sizer (1952), with disappearance of peroxide followed spectrophotometrically at 240 nm. Specific activity (U/mg) = ΔA_{240}/min ÷ 0.0436 absorbance/μmol of H_2O_2 ÷ mg of catalase/ml, where ΔA_{240}/min is the initial rate of decrease in absorbance measured in the first 45 s.

Results

Removal of endotoxin from protein solutions by Triton X-114 phase separation

Phase separation using Triton X-114 was effective in reducing endotoxin from solutions of three

TABLE I

REDUCTION OF ENDOTOXIN IN PROTEIN SOLUTIONS BY PHASE SEPARATION

Results are expressed as the highest dilution of protein forming a solid gel when mixed with *Limulus* lysate. The number of independent samples tested is shown in parentheses.

Cycles of phase separation	Phase tested	*Limulus* gelation		
		Catalase (40 mg/ml)	Cytochrome *c* (13 mg/ml)	BSA (30 mg/ml)
0	Detergent + aqueous	1:100,000 (3)	1:10,000 (3)	1:100 (2)
1	Aqueous	1:100 (3)	1:10 (4)	– [b] (2)
1	Detergent [a]	1:100,000 (2)	1:10,000 (2)	ND [c]
2	Aqueous	1:1 (2)	– [b] (6)	ND
3	Aqueous	– [b] (2)	ND	ND

[a] PBS was added to restore original volume.
[b] Negative in the gelation test at 1:1.
[c] Not determined.

different proteins. The first cycle of phase separation reduced endotoxin contamination by 1000-fold (Table I). Further cycles of phase separation resulted in complete removal of endotoxin. The endotoxin was found in the detergent phase. After treatment of cytochrome *c* with Triton X-114, the protein content of the detergent phase was 2% and of the aqueous phase was 98%. Thus, detergent treatment caused little loss of protein. No dilution of protein occurred during treatment with Triton X-114.

Distribution of radiolabelled LPS during phase separation

Radiolabelled LPS was treated with Triton X-114, and the radioactivity was determined in the aqueous phase and the detergent phase. As shown in Table II, the first cycle of phase separation removed 99% of radioactivity from the aqueous phase, and repeated treatment caused complete removal of radioactivity as well as *Limulus* reactivity.

Removal of residual detergent from protein solutions

When PBS was treated with Triton X-114, and the Triton X-114 content of the aqueous phase was determined by measuring the absorbance at 275 nm, the amount of Triton X-114 remaining in the aqueous phase was 0.018% (0.016–0.024%, $n = 4$). This residual detergent in the cytochrome *c* solution could be removed by gel filtration on Bio-Gel P-300. Triton X-114 was eluted after cytochrome *c*. Alternatively, residual detergent could be removed by incubation with Bio-Beads SM-4. When the aqueous phase (1 ml) was treated with SM-4 (0.5 g) for 1 h, detergent (A_{275} nm) was completely absorbed. However, some protein (15% of a 1 mg/ml solution of BSA) was also absorbed by treatment with SM-4 (0.5 g). Cytochrome *c* solution treated by either method was negative in the *Limulus* assay (< 10 pg/ml). Thus, endotoxin-free protein solutions could be easily and rapidly obtained by combining removal of endotoxin by phase separation using Triton X-114, followed by removal of residual detergent by gel filtration or absorption with SM-4.

TABLE II

PHASE SEPARATION OF RADIOLABELLED ENDOTOXIN

Radiolabelled endotoxin (0.5 ml of 200 ng/ml of ^{3}H and ^{14}C-labelled *S. typhimurium* LPS in saline) was treated twice with 5 μl of Triton X-114. The radioactivity of each fraction was then counted in the ^{3}H region. Background counts (5–8 dpm) were subtracted. Results are means ± SE from three experiments.

Cycles of phase separation	Phase tested	Radioactivity (dpm)
0	Detergent + aqueous	2853 ± 11
1	Detergent	2819 ± 54
2	Aqueous [b]	−1 ± 1
2	Detergent	12 ± 2

[b] *Limulus* assay was negative.

TABLE III

EFFECT OF TRITON X-114 PHASE SEPARATION ON THE ACTIVITY OF CATALASE

Treatment of catalase	Specific activity (U/mg)
Untreated catalase	3776 ± 125 ($n = 4$)
Aqueous phase from third cycle of phase separation	3748 ± 66 ($n = 8$)
250 kDa fraction from P-300 column	4649 ± 122 ($n = 3$)

Stability of proteins during Triton X-114 phase separation

We tested the effect of detergent treatment on the enzyme activity of catalase. We compared the enzyme activities of untreated catalase, the aqueous phase of the third cycle of phase separation, and the 250 kDa fraction from the P-300 column. As shown in Table III, enzyme activity was not affected by three cycles of treatment with Triton X-114. (The gel filtration actually increased the specific activity of the catalase. We observed that gel filtration increased the clarity and specific activity of solutions of commercial catalase, irrespective of detergent treatment.)

To test the efficiency of removal of detergent, we examined the function of cytochrome *c* in the assay for O_2^- release from PMA-stimulated neutrophils. As shown in Table IV, a small amount of Triton X-114 was harmful to the cellular activity of neutrophils. However, after gel filtration on P-300 or treatment with SM-4, cytochrome *c* could be used in the O_2^- assay. These results indicated that these two methods were effective in removing residual detergent from the aqueous phase.

TABLE IV

REMOVAL OF TRITON X-114 FROM THE AQUEOUS PHASE OF CYTOCHROME *c* SOLUTION

Neutrophils were stimulated with 100 nM PMA for 15 min at 37 °C in the presence of cytochrome *c* treated as indicated.

Treatment of cytochrome *c*	PMA-stimulated O_2^- release [a] (nmol $O_2^-/10^6$ neutrophils)
Untreated cytochrome *c*	14.95 ± 0.10
Triton X-114 added to cytochrome at 0.002%	11.24 ± 0
Triton X-114 added to cytochrome at 0.02%	0.95 ± 0.19
Phase separation, then gel filtration on P-300	14.73 ± 0.14
Phase separation, then absorption with SM-4 beads	13.62 ± 0.29

Discussion

We describe a procedure for reduction of endotoxin in protein solutions. Phase separation using Triton X-114 was developed to separate integral membrane proteins from hydrophilic proteins (Bordier, 1981). We adapted this method to reduction of endotoxin from protein solutions. The effectiveness of Triton X-114 in reducing endotoxin results from two characteristics of the detergent. (1) Triton X-114 allows endotoxin to dissociate from proteins in the homogenous solution that forms below the clouding point temperature (20 °C), as described for octyl-β-D-glucopyranoside (Karplus et al., 1987). Our unpublished result that polymyxin B affinity chromatography was effective in reducing endotoxin in Percoll solutions, but not in cytochrome *c* solutions suggested that endotoxin *bound to proteins* could not be removed by polymyxin B affinity chromatography. (2) Triton X-114 is easily precipitated together with hydrophobic molecules like endotoxin at temperatures above 20 °C (Bordier, 1981). We found that the endotoxin was indeed concentrated in the detergent phase (Table I). These characteristics made the procedure reported here simpler than another procedure in which endotoxin is dissociated from proteins by octyl-β-D-glucopyranoside and then subsequently absorbed by polymyxin B-Sepharose 4B, followed by removal of detergent by dialysis (Karplus et al., 1987). With the procedure reported here, > 95% of protein could be recovered without dilution. One cycle of phase separation could be completed within 15 min. Thus, the phase separation could be repeated easily until endotoxin in the aqueous phase became undetectable by *Limulus* assay.

The major disadvantage of the procedure reported here is that a small amount of detergent (0.018%) persisted in the aqueous phase. Because of this residual detergent, the aqueous phase could be harmful to living cells. For example, we found

that residual detergent in cytochrome *c* solutions damaged neutrophils. Residual detergent could be removed by absorption on Bio-Beads SM-4 or by gel filtration on Bio-Gel P-300. However, a substantial amount of protein might be absorbed on the SM-4 beads, and dilution of proteins unavoidably occurred in the gel filtration method.

A major advantage of the procedure was that it appeared not to harm the proteins. Catalase and cytochrome *c* that had been depleted of endotoxin and residual detergent retained normal functions.

In addition to depletion of endotoxin from proteins like recombinant products or monoclonal antibodies, phase separation using Triton X-114 should be useful for depletion of lipids from albumin or lipoproteins, or for concentration of endotoxin.

Acknowledgments

We thank Dr. Robert S. Munford for providing radiolabelled LPS.

This work was supported by NIH Grants DE 05494 and RR 05994.

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