

Single step protocol to purify recombinant proteins with low endotoxin contents

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Abstract

Endotoxin is an unwanted by product of recombinant proteins purified from *Escherichia coli*. The inherent toxicity of endotoxins makes their removal an important step for the proteins' application in several biological assays and for safe parenteral administration. The method described in this paper is a one-step protocol which is effective at removing tightly bound endotoxin from over-expressed tagged proteins in *E. coli*. We combined affinity chromatography with a non-ionic detergent washing step, to remove most of the endotoxin contaminants from the end product. An endotoxin reduction of less than 4 to 0.2 EU mg⁻¹ was achieved with protein recovery close to a yield 100%. As this new protocol requires only one step to simultaneously purify tagged proteins and eliminate endotoxins, it represents a substantial advantage in time, effort, and expense.

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Different fusion tag systems have been exploited to rapidly separate foreign recombinant proteins from the bulk of other *Escherichia coli* proteins via affinity chromatography. The most commonly used epitope tags are glutathione *S*-transferase [1], maltose-binding protein [2], and histidine-tag [3–5]. Proteins with up to 95% purity however still contain a significant amount of endotoxins. Endotoxins are lipopolysaccharides associated with the outer membrane of gram-negative bacteria. The inherent toxicity of these lipopolysaccharides has a profound effect on biochemical events of certain cells and can interfere with in vitro experiments [6]. In addition, the presence of even minute amounts of endotoxin from therapeutic protein preparations can cause inflammation and septic shock in humans and experimental animals [7]. To avoid these adverse effects, near complete elimination of endotoxins from final recombinant proteins is necessary prior to the proteins' parenteral administration and biological cell-based application. So far

various techniques have been established to decontaminate protein solutions with varying degrees of success [8].

Ultra filtration, although effective in removing endotoxins from water, is an inefficient method in the presence of proteins, which can be damaged by physical forces [9]. Anion exchangers, which take advantage of the negative net charge of endotoxins, have been extensively used for endotoxin adsorption. However, when acidic proteins need to be decontaminated, proteins may co-adsorb onto the matrix and cause a significant loss of biological material. Also, net-positively charged proteins form complexes with endotoxins, causing the proteins to drag endotoxin along the column and consequently minimizing the endotoxin removal efficiency [10].

Endotoxin-selective affinity sorbents, such as histidine, histamine, polymyxin B, and poly-L-lysine sorbents facilitate the adsorption of endotoxins onto the matrix through electrostatic and hydrophobic interactions [11–14]. Disadvantages are several fold dilution of proteins and multiple rounds of binding to eliminate substantial amounts of endotoxins, which often lead to the loss of biological material [10].

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Phase separation based on non-ionic detergent, like Triton X-114, has been described as the most effective method to date for removing endotoxins from protein solution [13–15]. Below the temperature of its cloud point, Triton X-114 efficiently dissociate endotoxins from proteins. Above the clouding point temperature of Triton X-114, endotoxins get trapped in the detergent phase, due to their hydrophobic properties, whereas the hydrophilic proteins remain in the aqueous phase. However, this method is time consuming, as several rounds of extractions are required to reduce the endotoxin content by 98–99%. Moreover, temperature shifts can lead to protein denaturation and traces of triton can remain in protein solutions. Finally, the one-step purification under denaturing conditions using 60% isopropanol cannot be used for purification systems under native conditions, such as required for glutathione *S*-transferase and maltose-binding fusion proteins [16]. As shown by these different methodologies, endotoxins can be considered to be temperature and pH stable molecules, rendering their removal as one of the most difficult tasks in downstream processes during protein purification. As of present literature, no economical large scale method for endotoxin removal has been established.

The following study introduces a one-step method for the use of affinity chromatography for recombinant protein purification by which the simultaneous removal of endotoxins is made possible. To test the feasibility of our protocol, we examined six recombinant proteins of various physico-chemical properties.

The six proteins were expressed as either glutathione *S*-transferase or as histidine-tagged proteins: (a) the human protein TAR RNA binding protein (TRBP)² which has previously been shown to bind the TAR response element of human immunodeficiency virus type I [17]; (b) the transcription activator Tax protein from the human T-cell leukemia virus type 1, a potent oncogenic deregulator of cellular gene expression [18,19]; (c) the cAMP receptor protein SyCRP, a regulator of gene expression [20]; (d) the LysR-type transcription regulator NdhR, which is implicated in the inorganic carbon pathway (Ci) from Cyanobacterium *Synechocystis* PCC 6803 [21]; and finally, (e) Oxa1 (of both *Saccharomyces cerevisiae* and *Neurospora crassa*), one of a central component in the insertion process of mitochondrially encoded proteins [22,23].

An advantage of the present protocol is that tightly bound endotoxins are being dissociated from recombinant proteins regardless of the affinity chromatography method used. With protein recovery up to 99%, this method permits the reduction of endotoxins to levels as low as 4 to 0.2 EU mg⁻¹, without compromising the structural and functional integrity of proteins.

Materials and methods

Strains and plasmids

Escherichia coli BL21 DE3 (Stratagene) and TG1 strains were used to express proteins from pET and pGEX constructs, respectively. pGEX-Tax, pET11d-TRPB, and GST-Oxa1 ‘tail-domain’ from *S. cerevisiae* and *N. crassa* expression vectors have been described elsewhere [22–24]. SyCRP and NdhR genes were amplified by PCR from *Synechocystis* sp. PCC6803 genomic DNA using the following oligonucleotides; 5'-CATATGGGCACTAGTCCC AAA-3', 5'-CTCGAGGGAAATTAGATCTTCTAAAT CC-3' and 5'-CATATGCAAGCAACCTTACACCA-3', 5'-CTCGAGAAGAACTAAGTTCACAGGGGT-3'. The PCR products were digested with *NdeI/XhoI* and cloned into the corresponding restriction sites of pET22b (Novagen).

Expression

Four milliliter of overnight culture was used to inoculate 400 ml of LB containing 100 µg/ml ampicillin. Cultures were agitated at 250 rpm until the *A*_{600nm} reached 0.5 and expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Cells were harvested by centrifugation at 5000 rpm for 20 min and stored frozen. TRBP and NdhR were induced overnight at 22 °C.

Purification and endotoxin removal

Cell pellet containing His-tagged SyCRP protein was resuspended in 20 ml buffer A (100 mM KCl, 200 mM NaCl, 10 mM imidazole, 20 mM Tris-HCl, pH 8) and 10 U/ml benzonase. For His-tagged TRBP and NdhR proteins expressing cells, cell pellets were resuspended in denaturation buffer B (8 M urea, 100 mM KCl, 200 mM NaCl, 10 mM imidazole, 20 mM Tris-HCl, pH 8). Pellets of cells expressing GST fusion proteins were resuspended in 20 ml phosphate buffer, PBS (3 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄, 173 mM NaCl, pH 7.3). Cells were disrupted with the French press and lysates were centrifuged at 15,000 rpm, 4 °C for 30 min.

A suspension of 1 ml Ni-NTA agarose (Qiagen, Germany) or GST sepharose (Pharmacia, Germany) beads was poured into two columns. Cell extract was divided into two aliquots of 10 ml and each aliquot was loaded onto one column that was previously equilibrated with fifteen column volumes of either buffer A, B or PBS. The first column was washed with 50 column volumes of buffer A, B or PBS containing 0.1% Triton X-114 (Sigma-Aldrich, Germany) and then washed with 20 column volumes of the same buffer without detergent at 4 °C. The second column was washed with 70 column volumes of the same buffer but lacking detergent (Table 1). Elution was achieved with 150 mM imidazole (Sigma-Aldrich, Germany) or 20 mM reduced glutathione (Sigma-Aldrich, Germany). TRBP purified under denaturing

² Abbreviations used: TRBP, TAR RNA binding protein; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

Table 1
Purification conditions

Fusion tag	Buffer used	Wash conditions				Elution conditions
		Standard protocol		Triton X-114		
		CV ^a	Triton in % (v/v)	CV ^a	Triton in % (v/v)	
Glutathione <i>S</i> -transferase	PBS	70	0	50 20	0.1 0	20 mM free reduced glutathione
Histidine tagged	A or B	70	0	50 20	0.1 0	150 mM imidazole

^a Column volumes.

conditions was refolded onto the column applying step gradients from denaturation to renaturation in buffer B with a decreasing amount of urea. NdhR was renaturated using column exchange buffer. To eliminate the imidazole or reduced glutathione from samples, the eluted fractions were dialyzed against binding buffer containing 100 mM KCl, 20 mM Tris-HCl, pH 7.3 or buffer A for 4 h at 4 °C.

In vitro binding experiments

Purified His-TRBP was incubated either with GST-Tax or GST bound to the glutathione–Sephadex beads (Pharmacia) in a binding buffer containing 20% glycerol, 5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and was incubated for 2 h at 4 °C. The beads were washed with 10 column volumes of the incubation buffer. The bound proteins were then released by boiling in sodium dodecyl sulfate (SDS) and analyzed on Western blots with both, an anti-His antibody and an anti-GST antibody (Sigma-Aldrich, Germany).

Electrophoresis mobility shift

SyCRP-DNA-binding assay was performed as previously described [20]. A double-stranded ICAP DNA fragment, which contains the 22-bp consensus sequence for *E. coli* CRP was prepared by annealing the oligonucleotides 5'-GCAACGAATAAATGTGATCTAGATCACATTTT AGGCACCC-3' and 5'-GGGGTGCCTAAAATGTGATCTAGATCACATTTATTCGTTG-3'. Binding reactions were set up with 125 pmol ICAP DNA and various amounts of SyCRP protein in 30 µl binding buffer (50 mM Tris-HCl, pH 7.5, 60 mM NaCl, 1 mM EDTA, 8.3% glycerol, 0.1 mg/ml bovine serum albumin) with a final concentration of 40 µM cAMP. After 20 min incubation at room temperature, the samples were loaded onto a 10% non-denaturing polyacrylamide gel. Electrophoresis was performed at 100 V in 0.25 mM TBE buffer. Gels were stained by SYBR Safe fluorescence dye (Molecular Probes, Germany) and detected using UV illumination [25].

Endotoxin and protein concentration

The endotoxin content of different fractions was determined by the Limulus amoebocyte lysate chromogenic

assay according to the manufacturer's instructions, (Bio Whittaker). To determine whether components of the different fractions interfered with the assay, defined amounts of endotoxin were spiked into the samples. Protein concentrations were determined using the BCA protocol (Pierce). Triton X-114 content of the aqueous phase was determined by measuring the absorbance at 280 nm.

Results and discussion

Endotoxin clearance in the course of protein purification

Two phase extraction using a non-ionic detergent, such as Triton X-114, is known to be a very effective method for removing endotoxins from purified proteins [15]. We tested whether the removal of endotoxin could be achieved during chromatography purification with the use of Triton X-114 in the washing steps. To this end, three His-tagged proteins (SyCRP, NdhR, and TRBP) and three GST fusion proteins (Tax and Oxa1 from *S. cerevisiae* and *N. crassa*) were over-expressed in *E. coli*, and purified according to the method described above.

The application of 0.1% Triton X-114 in the washing steps was successful at reducing endotoxins during histidine and during GST fusion protein purification, whereas washing steps lacking detergent were ineffective in eliminating endotoxins. In contrast to purified materials employing the standard protocol which contained from 2500 to 34,000 EU mg⁻¹, purified recombinant proteins treated with Triton X-114 contained concentrations as low as 4 to 0.2 EU mg⁻¹ (less than 99% of initial endotoxin content) (see Fig. 1 and Table 2). Furthermore, recovery of protein was up to 99% as compared to the standard protocol. It is important to note that the elimination of endotoxins using Triton X-114 was only successful by performing the complete procedure at 4 °C. At room temperature, the same protocol was ineffective at removing endotoxin (data not shown). Triton X-114 is thought to dissociate the endotoxin molecule from the protein in a clear micellar homogenous solution at a temperature beyond its cloud point [8]. As the temperature is raised, micelles become larger and ultimately aggregate at a temperature above the cloud point of Triton X-114 [8]. Thus, we supposed that at 4 °C, Triton X-114 drag endotoxin along the column after releasing it from the protein whereas at room temperature, the dissociation

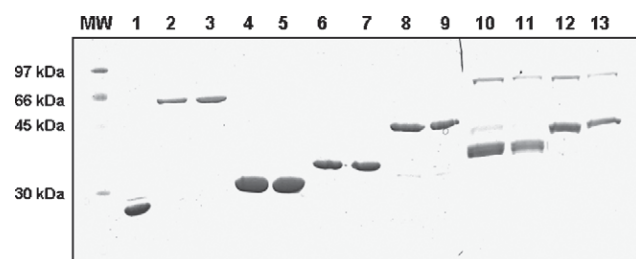


Fig. 1. SDS-PAGE analysis of various recombinant proteins; 2–5 μ g of each recombinant protein were loaded onto the 15% polyacrylamide gel and visualized by Coomassie blue staining. Lane 1, GST; lanes 2 and 3, GST-Tax; lanes 4 and 5, SyCRP; lanes 6 and 7, NdhR; lanes 8 and 9, TRBP, lanes 10 and 11, Oxa1 from *S. cerevisiae*; lanes 12 and 13, Oxa1 from *N. crassa*. Lanes 1, 3, 5, 7, 9, 11, and 13, purified proteins with Triton X-114; lanes 2, 4, 6, 8, 10, and 12, proteins purified using standard protocol.

Table 2
Endotoxin levels of purified recombinant proteins

Samples	pI	Triton	Purification conditions	Protein recovery	EU ml ⁻¹	EU mg ⁻¹
Tax	6.55	–	N	100%	>8000	>16,000
		+	N	>99%	1.9 $\pm$ 0.3	3.5 $\pm$ 0.5
Oxa1 N.c	8.50	–	N	100%	>30,000	>15,000
		+	N	>99%	5 $\pm$ 0.5	2.5 $\pm$ 0.5
Oxa1 S.C	9.66	–	N	100%	>32,000	>20,000
		+	N	>99%	6 $\pm$ 1	4 $\pm$ 0.5
GST	6.19	+	N	100%	0.9 $\pm$ 0.5	1.8 $\pm$ 0.5
SyCRP	5.76	–	N	100%	>22,000	>34,000
		+	N	>99%	3 $\pm$ 0.5	1 $\pm$ 0.5
TRBP	7.19	–	D	100%	>5000	>3000
		+	D	>99%	0.1 $\pm$ 0.05	0.2 $\pm$ 0.05
NdhR	6.49	–	D	100%	>4000	>2500
		+	D	>99%	2 $\pm$ 0.07	0.36 $\pm$ 0.03

Endotoxin contents of various recombinant proteins. Each recombinant protein was purified either on Ni-NTA or glutathione-Sepharose columns under native (N) or denaturing (D) conditions in the presence (+) or absence (–) of 0.1% Triton X-114 in washing steps. Three different preparations of each recombinant protein were subjected to endotoxin removal using Triton X-114.

process appears not to take place and endotoxin remains associated to the protein.

Residual endotoxins in solubilized inclusion bodies can reach levels of 8×10^6 EU ml⁻¹ despite the fact that endotoxin levels were found to be higher in recombinant proteins which are isolated from soluble fractions [26]. We demonstrate that the histidine-tagged proteins TRBP and NdhR, purified under denaturing conditions, were almost free of endotoxins. The residual amount of endotoxins found in the elution fraction was as low as 0.4 to 0.2 EU mg⁻¹ as compared to more than 2500 EU mg⁻¹ using wash steps without Triton X-114 (Table 2).

Endotoxins have been shown to form complexes with proteins of different isoelectric points [8] where electrostatic interactions are thought to be the main driving force. As a result, the removal of endotoxins from basic proteins should prove to be more difficult than from acidic proteins [27]. We tested whether Triton X-114 in washing steps

could eliminate endotoxins from proteins with a pI above 8.5. We found that Triton X-114 in connection with affinity chromatography was as effective at removing endotoxins from the acidic proteins (SyCRP and NdhR) as it was from the positively charged proteins Oxa1 (Table 2).

Following of the endotoxin content during purification

Start concentrations of endotoxin in *E. coli* total extracts were ranging from 500,000 to 1,000,000 EU ml⁻¹. Consequently, differences of 1800 to 20,000 EU ml⁻¹ between Triton X-114 treatment and standard protocol found in wash fractions could not be distinguished. To overcome this problem, we employed a methodology previously described in the literature; removal of endotoxin from purified preparation of recombinant protein [13]. Thus, by using a purified preparation of both His-tagged SyCRP and GST-Tax proteins spiked with a defined amount of endotoxin, we could easily chase the endotoxin content in the different fractions and appreciate the efficiency of Triton X-114 wash to remove tightly bound endotoxin in comparison to the standard protocol wash. An equal amount of purified His-tagged SyCRP and GST-Tax protein containing up to 20,000 EU mg⁻¹ was loaded onto either two Ni-NTA columns or two GST columns, respectively. As shown in Fig. 2, washing steps without detergent were ineffective at dissociating tightly bound endotoxins from the recombinant proteins. Most endotoxins were recovered in the eluted product.

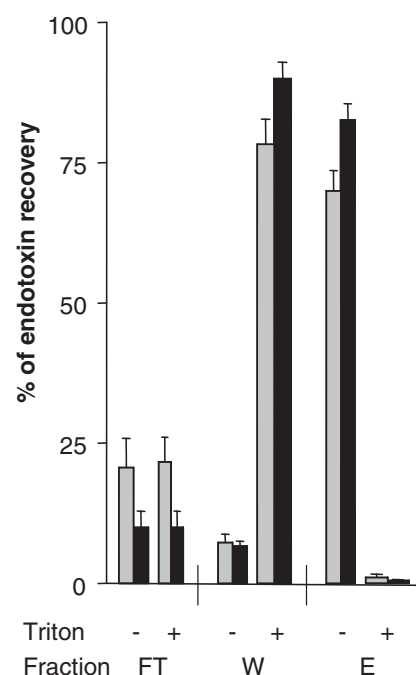


Fig. 2. Comparison of endotoxin contents in the different fractions. Purified SyCRP (grey) and GST-Tax (black) spiked with 20,000 EU mg⁻¹ were bound to either Ni-NTA or GST beads and washed in the presence or in the absence of 0.1% Triton X-114 as indicated. Endotoxin content was determined in flow through (FT), washing steps (W), and bound materials (E). For each protein, three experiments were performed independently.

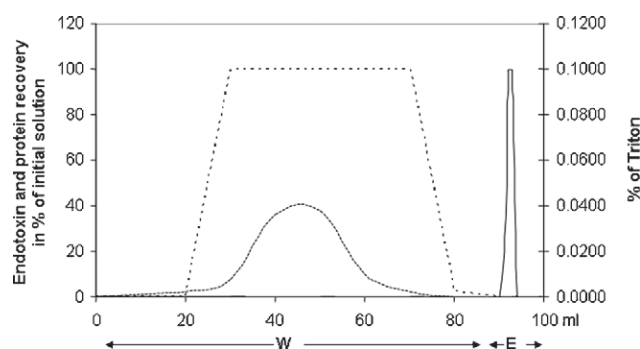


Fig. 3. Chromatography run of purified preparation of His-tagged SyCRP spiked with 20,000 EU mg⁻¹. Washing steps (W) and elution fractions (E) were tested for endotoxin contents (dash line) and protein recovery (continuous line) using LAL-test and BCA protein assay, respectively. The presence of Triton X-114 was monitored at 280 nm in the washing steps (dot line).

In the presence of the detergent, more than 99% of the initial endotoxin content was detected in the washing fractions and a final concentration of 1 ± 0.5 EU mg⁻¹ was found in the elution fraction (Figs. 2 and 3). The use of Triton X-114 was successful at dissociating tightly bound endotoxins from recombinant proteins. Additionally, the detergent could be reduced to a concentration of less than 0.001%, within the detection limit of Triton X-114 by 280 nm absorption (Fig. 3) in the last wash fraction. We assumed therefore, that the elution fractions do not contain more than 0.001%, thereby overcoming the disadvantage of residual traces of Triton in the end product.

Protein integrity

To test whether protein integrity was affected by the Triton X-114 treatment, we analyzed *E. coli*-expressed proteins in various in vitro assays.

TRBP has previously been reported to specifically bind to Tax [24]. We analyzed the interactions between His-TRBP and GST-Tax fusion proteins, which were either purified in the presence of or lack of Triton X-114, in vitro. TRBP specifically bound to GST-Tax (Fig. 4) but not to GST alone. No noticeable difference in activity was observed between the different recombinant proteins.

Functionality of SyCRP was assessed by a mobility shift assay using purified His-SyCRP, treated or not with Triton

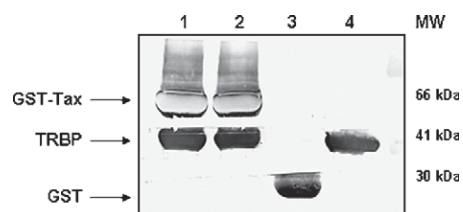


Fig. 4. TRBP directly interacts with Tax. Purified endotoxin free TRBP (lane 4, input) is retained on GST-Tax purified using Triton X-114 (lane 1) as well as on GST-Tax purified using the standard protocol (lane 2) but not on GST (lane 3) columns, as demonstrated by Western blot analysis of bound elute fractions.

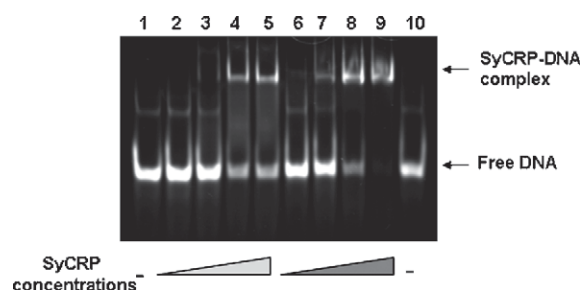


Fig. 5. Electrophoretic mobility shift assay. Purified SyCRP recombinant proteins using the standard protocol (lanes 2–5) and the Triton X-114 treatment (lanes 6–9) have been compared for their ability to bind to ICAP sequence. Final concentrations of recombinant protein are 0 M (lanes 1 and 10), 10 nM (lanes 2 and 6), 50 nM (lanes 3 and 7), 125 nM (lanes 4 and 8), and 250 nM (lanes 5 and 9).

X-114, and the *E. coli* ICAP consensus DNA sequence as previously described [20]. As shown in Fig. 5, incubation of SyCRP with the double-stranded DNA resulted in a shifted band. The intensity of the SyCRP-DNA complex was amplified as the concentration of the added SyCRP increased. Again, no obvious dissimilarity in binding activity was observed between the two SyCRP proteins. Therefore, treatment with Triton X-114 did not compromise protein functional integrity.

Conclusion

The procedure described in this study enables the removal of endotoxins in the course of chromatography purification of tagged fusion proteins. The addition of a non-ionic detergent, such as Triton X-114, in wash buffer successfully removes more than 99% of the endotoxin contaminants. The minimal endotoxin concentration achieved was lower than 0.2 EU mg⁻¹; protein recovery and yield were close to 100%. Ni-NTA agarose and glutathione-Sepharose matrix could be employed for the six proteins used in this study. We predict that the procedure described here can easily be adapted to other affinity chromatography methods, such as maltose-binding fusion proteins and Strep-II-tagged proteins. This paper describes a rapid, cost-effective, and large scale applicable alternative method for the efficient removal of endotoxins from recombinant proteins using affinity chromatography together with a non-ionic detergent.

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