Published in final edited form as:

Anal Biochem. 2009 December 15; 395(2): 268–270. doi:10.1016/j.ab.2009.08.042.

Removal of Bound Triton X-100 from Purified Bovine Heart Cytochrome *bc*₁

Rastislav Varhač, Neal C. Robinson, and Andrej Musatov*

Department of Biochemistry, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900

Abstract

Cytochrome *bc*₁ isolated from Triton X-100 solubilized mitochondrial membranes contains up to 120 nmol of Triton X-100 bound per nmol of the enzyme. Purified cytochrome *bc*₁ is fully active; however, protein bound Triton X-100 significantly interferes with structural studies of the enzyme. Removal of Triton X-100 bound to bovine cytochrome *bc*₁ was accomplished by incubation with Bio-Beads SM-2 in presence of sodium cholate. Sodium cholate is critical since it does not interfere with the adsorption of protein on the hydrophobic surface of the beads. The resulting Triton X-100 free cytochrome *bc*₁ retained nearly full activity, absorption spectra, subunit and phospholipid composition.

Keywords

Bio-Beads; cytochrome *bc*₁; Triton X-100; sodium cholate

Detergents are an indispensable tool in experimental membrane protein biochemistry. They are used during extraction, isolation and purification of membrane proteins, and also during structural and functional studies. The initial extraction and isolation of protein often requires different detergents from later structural and functional studies. Removal or exchange of unbound detergent is typically achieved by ion-exchange, hydrophobic or gel chromatography [1-3]. Alternatively, the hydrophobic surface of Bio-Beads¹ (styrene-divinylbenzene copolymers) can be used for removal of the unbound detergent and has been described for both batch and column procedures [4-8]. A detergent with small micelle size, such as cholate or deoxycholate, can be easily removed by dialysis allowing it to be replaced by a different detergent [9]. However, removal of unbound detergent in many cases does not completely solve the problem, since commercially available detergents also bind to the protein and consequently affect structural and/or functional studies. Based on our studies with cytochrome *c* oxidase, Cyt *bc*₁, and also on data published by others it is clear that the detergent in the initial stage of isolation remains bound to the final purified membrane proteins and potentially may interfere with further analysis [1,9,10]. The extent of interference depends on the nature of the bound detergent, its amount and, most likely, on the location within the protein where the detergent is bound. The removal of such protein bound detergent without structural and functional alteration of the proteins is one of the difficulties encountered in the study of isolated and detergent-solubilized mitochondrial electron-transfer complexes. In this work, Bio-Beads

*Corresponding author. FAX: (210) 567-6595; Phone: (210) 567-3779; musatov@uthscsa.edu

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were successfully used for removal of Cyt *bc*₁ bound nonionic detergent TX-100. TX-100 (octylphenolpoly(ethyleneglycolether)) is one of the commonly used detergents in membrane protein biochemistry. Its a nonionic detergent with a hydrophilic polyethylene oxide group and a hydrocarbon hydrophobic group. TX-100 has an average of 9-10 ethylene oxide groups per molecule.

The model system used in this work is purified from bovine heart cytochrome *bc*₁ complex. Cyt *bc*₁ was isolated from TX-100 (Roche) solubilized bovine heart mitochondrial membranes [11]. Although further purification required ammonium acetate and ammonium sulfate precipitation from the deoxycholate and cholate solubilized membrane protein-phospholipid mixture, purified Cyt *bc*₁ still contained up to 120 nmol of TX-100 bound per nmol of Cyt *bc*₁. After the final ammonium acetate precipitation, the purified enzyme was dissolved in the 20 mM TrisHCl pH 8.0 buffer, containing 0.66 M sucrose and 0.001 M histidine. Purified Cyt *bc*₁ with bound TX-100 was fully active when the enzymatic activity was analyzed in 50 mM TrisHCl buffer pH 7.9 containing 0.1 mg/mL dodecyl maltoside; however, protein bound TX-100 significantly interfered with structural studies on the enzyme, such as phospholipid analysis. A normal phase silicic acid HPLC with a gradient elution according to Nissen and Kreysel [12] and minor modifications [13] was used to determine phospholipid composition. Phospholipids were extracted from the 2.2 - 3.7 nmol purified enzyme using chloroform/methanol/water extraction [14]. Essentially all of the TX-100 was co-extracted with the phospholipids, i.e. extracted phospholipid also contained up to 480 nmol detergent. The extracted phospholipid-detergent mixture was dried under nitrogen, dissolved in a chloroform:methanol mixture (2:1, v/v), and subjected to HPLC analysis. Strong absorbance of the TX-100 separated polyoxyethylene chains in the UV area made phospholipid determination unfeasible (Figure 1, upper panel).

Removal of protein bound TX-100 was achieved by incubation of Cyt *bc*₁ with Bio-Beads (20-50 mesh, Bio-Rad Lab), that had been washed with methanol and water [14]. To remove TX-100, 3.0 μM Cyt *bc*₁ was solubilized in 10 mM TrisHCl buffer pH 8.0 containing 23 mM sodium cholate. This was placed into a 1.5 mL plastic tube with 200 mg of moist Bio-Beads. The presence of sodium cholate was critical for successful TX-100 removal without precipitation of Cyt *bc*₁. In contrast, nonionic dodecyl maltoside at a concentration up to 20 mM could not be substituted for sodium cholate because of significant Cyt *bc*₁ precipitation. We believe this result from adsorption of dodecyl maltoside to the Bio-Beads [15], although it has been demonstrated that Bio-Beads can remove cholate as well [16]. The Cyt *bc*₁-cholate

¹ABBREVIATION

Cyt <i>bc</i>₁	bovine heart cytochrome <i>bc</i> ₁ complex, ubiquinol-cytochrome <i>c</i> oxidoreductase, Complex III
Bio-Beads	Bio-Beads SM-2, styrene-divinylbenzene copolymers
TX-100	Triton X-100, octylphenolpoly(ethyleneglycolether)
RP-HPLC	reversed phase high-performance liquid chromatography
CL	cardiolipin
PE	phosphatidylethanolamine
PC	phosphatidylcholine

mixture was stirred at 4 °C. The removal of TX-100 by Bio-Beads was time-dependent. More than 50% of TX-100 was removed during the first 2 min, but complete TX-100 removal required 60 min. After incubation, the Bio-Beads were separated from Cyt *bc*₁ by centrifugation at 12,800 x g for 6 min (Brinkmann Eppendorf centrifuge). The recovery of the protein was 90-100%. Phospholipid composition of Cyt *bc*₁ after incubation with Bio-Beads was determined by silicic acid HPLC (Figure 1, lower panel). Quantification of phospholipid was done using a standard phospholipid mixture (cardiolipin, phosphatidylethanolamine, and phosphatidylcholine purchased from Avanti Polar Lipids, Inc.). By this analysis 1 nmol of Cyt *bc*₁ contained 10 ± 2 , 88 ± 10 , 62 ± 7 nmol of cardiolipin, phosphatidylethanolamine, and phosphatidylcholine, respectively. The presence of phospholipids provides evidence that the Bio-Beads treatment does not lead to delipidation. In fact the cardiolipin content determined by phosphate analysis of the TLC spot of phospholipids extracted from Cyt *bc*₁, isolated by the same method, is 9.2 ± 0.7 nmol of cardiolipin bound to nmol of Cyt *bc*₁ [17]. The phosphorus content of purified Cyt *bc*₁ is 120-140 P/Cyt *bc*₁ suggests that the phospholipid composition is unaffected by Bio-Beads [2].

Treatment of Cyt *bc*₁ with Bio-Beads did not measurably alter either the oxidized or reduced visible spectra suggesting that TX-100 removal did not perturb the heme *b*'s or heme *c*₁. Subunit composition of Cyt *bc*₁ was also unaffected by incubation with Bio-Beads (Figure 2), but the RP-HPLC elution profile shows almost complete absence of a TX-100 elution peak at 44 min. Most importantly, the resulting TX-100 free Cyt *bc*₁ retained nearly full activity. The enzymatic activity of Cyt *bc*₁ before and after incubation with Bio-Beads was measured spectrophotometrically at 550 nm as a rate of cytochrome *c* reduction with decylubiquinol (Sigma) as the substrate [11] and was found to be 200 ± 10 s⁻¹ and 185 ± 14 s⁻¹, respectively. The electron-transfer activities of some preparation of Cyt *bc*₁ are susceptible to the Bio-Beads treatment; e.g. sometimes resulting in 5-30% of activity loss. The reason of such variation is not clear, since no structural alterations were observed.

Summary

In the present work, removal of Triton X-100 from Cyt *bc*₁ without interfering with its structural and functional properties was achieved by incubation of enzyme with Bio-Beads in the presence of sodium cholate. The method is simple, rapid, inexpensive, and characterized by almost 100% recovery of enzyme. The resulting Cyt *bc*₁ maintains functional and structural integrity. We believe that the described method has general use and can be applied to most detergent-solubilized membrane proteins.

ACKNOWLEDGMENT

This work was supported by grants from UTHSCSA (URC grant 128240 for AM), National Institute of Health (NIH GM024795 for NCR) and the Robert A. Welch Foundation (AQ1481 for NCR). The authors thank Dr. LeAnn K. Robinson for editorial help in preparing the manuscript.

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Figure 1. Silicic acid HPLC of phospholipids extracted from purified bovine heart cytochrome *bc*₁

Upper main panel: Separation of phospholipid-TX-100 mixture extracted from 1 nmol of cytochrome *bc*₁ before treatment with Bio-Beads. Inset: Separation of TX-100 (230 nmol) polyoxyethylene chains by silicic acid HPLC column. Lower panel: HPLC analysis of phospholipids extracted from Bio-Beads treated cytochrome *bc*₁. Phospholipids were extracted from 1 nmol of Bio-Beads treated cytochrome *bc*₁. Inset: Separation of mixed phospholipid standards (5 nmol of cardiolipin (CL), 5 nmol of phosphatidylethanolamine (PE), and 5 nmol of phosphatidylcholine (PC)). Detection of phospholipids elution was monitored at 203 nm with a Waters 2487 Dual λ Absorbance Detector.

Figure 2. C₁₈ reversed-phase HPLC purification of bovine heart cytochrome *bc*₁ subunits
Upper chromatogram: RP-HPLC purification of cytochrome *bc*₁ subunits before treatment with Bio-Beads. Subunits of cytochrome *bc*₁ (0.5 nmol) were separated on 10- μ m, 300- $\text{\AA}$ pore size Vydac C₁₈ column (0.46 $\times$ 25 cm). The sample preparation and water/acetonitrile gradient were as described previously [9] with minor modifications. Quantification of cytochrome *bc*₁ bound TX-100 was based upon integration of C₁₈ RP-HPLC peak area since TX-100 elutes as a well-defined single peak at ~44 min during C₁₈ RP-HPLC. Lower chromatogram: RP-HPLC purification of cytochrome *bc*₁ subunits after treatment with Bio-Beads. Subunits of Cyt *bc*₁ (0.5 nmol) were purified by RP-HPLC after cytochrome *bc*₁ was incubated with Bio-Beads as described in the text.