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Research Article

# Interference of phenol during quantification of a bacterial lipoprotein

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**ABSTRACT:** **Accurate protein estimation is an essential requirement for any biochemical investigation. The bacterial Braun liporotein (BLP) from *E. coli* (a Toll-2 receptor ligand) is purified via phenol extraction on the basis of selective extraction of the lipoprotein. The procedure leaves behind the major endotoxin lipopolysaccharide (LPS) that acts through the related Toll-4 receptor. However, as low as 0.00001% of phenol carried over during lipoprotein isolation interferes in the Lowry's method of protein estimation. A simple gel filtration on sephadex G-50 efficiently separates lipoproteins from phenol thereby avoiding inaccurate protein estimation of the lipoprotein content and making it suitable ligand for Toll-2 receptor.**

**KEYWORDS:** **Lipoproteins; Lipopolysaccharide (LPS); Lowry's method; Phenol interference.**

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## INTRODUCTION

Proteins are abundant macromolecules that carry out diverse array of biological functions and their accurate estimation is a routine procedure in biochemical experiments. Although, many simple and rapid colorimetric methods of protein estimation are currently available, none are ideal (Okutucu *et al.*, 2007). Each method has both advantages and disadvantages with respect to sensitivity, rapidity, relative ease of performance, reproducibility and level of interference by non-compatible substances. For example, thiols, lipids and reducing sugars interfere in the bicinchoninic acid (BCA) method (Brown *et al.*, 1989; Morton & Evans, 1992; Kessler & Fanestil, 1986), while detergents interfere in Bradford method of protein estimation (Compton & Jones, 1985). Levels of interference are least in the case of Biuret method, but the assay is less sensitive compared to other methods and is not suitable when the amount of protein under study is available in small quantities with the linear range covering just 1 to 5 mg/ml (Sapan *et al.*, 1999). Among the several methods used to quantitate proteins, Lowry's method ranks as the most cited method (Lowry *et al.*, 1951; Kresge *et al.*, 2005) because of its simplicity and wide acceptance.

While isolating and quantitating a bacterial lipoprotein, Braun lipoprotein (BLP) by the methods described by Inouye *et al.* (1976) and Neilsen *et al.* (2001), isolated lipoprotein failed to show any significant band even when 2 µg of protein was loaded and visualized using silver stain. In parallel, equivalent amounts of BSA appeared as an intense band. Loading higher amounts of lipoprotein enabled visualization of the band of correct molecular size.

We suspected possible interference of chemicals/reagents used during protein purification and hence screened all the reagents/chemicals for interference. Among all the reagents tested, we found only phenol to interfere in the popular Lowry's method and also in BCA method of protein estimation.

Although, phenol does not interfere in Bradford and Biuret methods, they are not feasible for the estimation of the purified BLP since, the yield of the total lipoprotein isolated per batch is less (approx. 1mg/10g bacterial cells) and the minimum detectable amount of protein in Biuret method is 1 mg/ml. Moreover, purified BLP is dissolved in 1% SDS (since detergent is necessary to solubilise lipoproteins) which is known to interfere in the Bradford method (Compton *et al.*, 1985). In addition, monitoring absorbance at 280 nm is also not suitable since phenol has a significant absorbance at 280 nm. The demerits of the other methods of protein estimation left us to estimate the isolated BLP by BCA and Lowry's method. Hence, removal of phenol is an absolute requirement to estimate BLP by Lowry's method.

Here, we describe a simple gel filtration procedure using sephadex G-50 to efficiently remove traces of phenol from lipoprotein preparation.

## MATERIALS AND METHODS

Reagents and chemicals were obtained from the following sources: *E. coli* DH5α was obtained from Microbial Type Culture Collection, Chandigarh, India. Gelatin was obtained from porcine skin, ammonium persulphate, sephadex G-50 and acrylamide 3X crystals were purchased from Sigma Aldrich, Chemicals, St. Louis, Missouri, USA; bovine transferrin holoform was from GIBCO life technologies, Scotland; bovine serum albumin (BSA), Folin and Ciocalteu's phenol reagent and sodium dodecyl sulphate were purchased from SISCO Research Laboratories, Mumbai, India; sodium potassium tartarate, copper sulphate, sodium carbonate, acetone, disodium monohydrogen phosphate, monosodium dihydrogen phosphate, sodium hydroxide and sodium chloride were obtained from RANKEM, RFCL limited, Gujarat, India; trypsin and orcinol were from Loba Chemie, Mumbai, India; orthophosphoric acid and coomassie brilliant blue R 250 were purchased from S.D.Fine Chemical LTD (SDFCL), Mumbai, India; 2-naphthol and resorcinol were obtained from Indian drugs and pharmaceuticals LTD, Hyderabad, India and 1-naphthol was from Merck specialties private limited, Mumbai, India; bicinchoninic acid (BCA) protein assay kit was purchased from Boster Immunoleader, California, USA and spin concentrator was from Agilent Technologies, California, USA.

### Isolation and purification of BLP

BLP was isolated from E. coli DH5α as described by Neilsen *et al.* (2001). Briefly, cells grown in Luria broth were pelleted and resuspended in 10mM sodium phosphate buffer (pH 7.5) containing 5 mM EDTA (S-buffer) and 1 mM PMSF per gramme of wet bacteria and lysed on ice by sonication. Cell debris were removed by centrifuging at 2000g for 5min and the membrane was collected by centrifuging at 40,000 g for 40 minutes and solubilized using 4% SDS & 0.5% β-mercaptoethanol. Contaminating proteins were precipitated using 4N sodium acetate buffer (pH 5.2) and 5% acetone. Lipoprotein was pelleted with 30% acetone and treated with saturated phenol to remove LPS (repeated 5-6 times). Lipoprotein was then recovered from the phenol phase using 30% acetone and the pellet was dissolved in S-buffer containing 1% SDS.

### Protein estimation

Protein estimation was done by Lowry's method according to Lowry *et al.* (1951) with a linear range of 15-75 µg/mL, Biuret's method as described by Gornall *et al.* (1949) with a linear range of 1-5 mg/mL, by Bradford method according to Bradford *et al.* (1976) with a linear range of 1-10 µg/mL and using BCA kit with a linear range of 20-2000 µg/mL as per the manufacturer's instructions.

### SDS-Polyacrylamide gel electrophoresis

Samples of 1 and 2 µg of BSA and apparently purified BLP were subjected to 15% reducing SDS-PAGE after heating the samples for 1 min at 90° C and silver stained according to

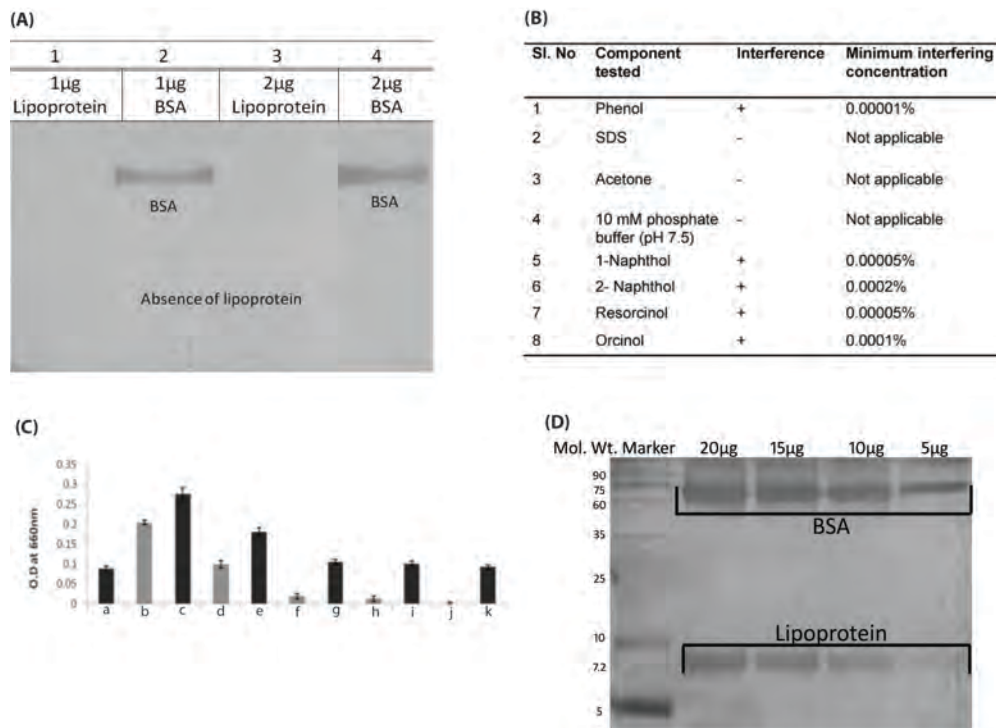
Mortz *et al*. (2001). In a separate experiment equal concentrations of BSA and purified BLP (5 and 10 μg) were mixed and resolved on a 15% reducing SDS-PAGE and silver stained as before. The molecular masses of BSA and BLP were compared with the mobility of standard molecular weight markers.

**Concentration-dependent interference of phenol during Lowry's method**

To determine the minimum concentration of phenol interfering during protein estimation by BCA and Lowry's method, increasing concentration of phenol (0.00001% to 0.001%) was used in the presence and absence of 45 μg of BSA or other proteins.

**Sephadex G-50 chromatography**

For gel filtration chromatography, 10 mg of apparently purified BLP in a total volume of 1mL, was loaded on a 30 cm x 1 cm column packed with 25 ml of swollen sephadex G-50 gel beads, equilibrated with 10mM phosphate buffer (pH 7.5) containing 1% SDS (necessary to solubilize this membrane lipoprotein). Fractions were collected at a flow rate of 0.2 ml/minute and monitored for absorbance at 280 nm and alternate fractions were subjected to SDS-PAGE and visualized after silver staining.




| Sl. No | Component tested | Interference | Minimum interfering concentration |
|---|---|---|---|
| 1 | Phenol | + | 0.00001% |
| 2 | SDS | - | Not applicable |
| 3 | Acetone | - | Not applicable |
| 4 | 10 mM phosphate buffer (pH 7.5) | - | Not applicable |
| 5 | 1-Naphthol | + | 0.00005% |
| 6 | 2- Naphthol | + | 0.0002% |
| 7 | Resorcinol | + | 0.00005% |
| 8 | Orcinol | + | 0.0001% |

**Figure 1: Interference of phenol during the quantification of lipoprotein purified from *E. coli.*** (A) Inability of silver nitrate to stain the indicated amounts of bacterial lipoprotein: 1 μg and 2 μg of purified bacterial lipoprotein (Lanes 1 & 3 respectively) and 1 μg and 2 μg of BSA (Lanes 2 & 4 respectively) were subjected to SDS-PAGE analysis and silver stained. Note the absence of bands in lanes 1 & 3 with intense bands of BSA in lanes 2 & 4. Data represents typical banding pattern obtained in more than 3 experiments. (B) All the chemicals and reagents used during the purification of lipoprotein were screened for their ability to interfere in Lowry's method of protein estimation. As low as 0.00001% of phenol interfered in the assay. This was true for other structural analogues of phenols. Data represents the mean of 6 independent experiments carried out in triplicates. (C) Increasing concentrations of phenol in a total volume of 1mLwithout or with fixed amount of BSA (45 μg/ml) was subjected to Lowry's method of protein estimation. (D) SDS-PAGE analysis of purified lipoprotein (5 μg and 10 μg) respectively mixed with equal amount of BSA. Notice the difference in relative band intensities of BSA and lipoprotein. Data represents typical banding pattern obtained in more than 3 experiments. (a) BSA, 45 μg; (b) Phenol 0.001%; (c) BSA, 45 μg + Phenol 0.001%; (d) Phenol 0.005%; (e) BSA, 45 μg + Phenol 0.005%;(f) Phenol 0.0001%; (g) BSA, 45 μg + Phenol 0.0001%; (h) Phenol 0.0005%; (i) BSA, 45 μg + Phenol 0.0005%; (j) Phenol 0.00001%; (k) BSA, 45 μg + Phenol 0.00001%.

### Statistical analysis

All the experiments were carried out in triplicates and the data presented are representative of six individual trial. Values are presented as mean ± SD.

## RESULTS

### Failure of the apparently purified BLP to be visualized on SDS-PAGE in comparison with BSA

Lowry's method is ideal when low concentration of proteins (15-75 µg/ml) needs to be quantitated. Hence, we quantified the amount of lipoprotein in our BLP preparation by Lowry's method. When we subjected 2 µg of purified BLP to 15% SDS-PAGE analysis and silver stained (silver nitrate can detect proteins at concentrations as low as 100 ng), we failed to see any band (Figure 1A). However, 2 µg BSA showed intense band after silver staining. In parallel experiments, when we mixed equal amounts (5-20 µg) of BSA with BLP and resolved the mixture on a 15% SDS-PAGE, we observed change in band intensities of BLP in comparison with equivalent BSA bands (Figure 1D). This clearly suggested over estimation of the purified BLP by Lowry's method. Inability to visualize the BLP band made us to screen all the chemicals/reagents for a possible substance interfering in the Lowry's method.

### Phenol interferes in the Lowry's method of protein estimation

Screening of all the reagents/chemicals used in BLP purification showed only phenol interfering in Lowry's method (Figure 1B). To determine if interference of phenol in Lowry's method is specific to lipoprotein, or can occur with other proteins as well, since numerous chemicals and reagents can interfere in standard protein assays (Morton & Evans, 1992; Kessler & Fanestil, 1986), we tested BSA, ovalbumin, transferrin, human serum albumin and gelatin. We found, irrespective of the protein being assayed phenol interfered with determination of all these proteins (data not shown). We see, concentration of phenol as low as 0.00001% interfered in Lowry's method of protein estimation (Figure 1C). In the next series of experiments, we examined whether or not types of phenols make a difference. Almost all the phenols we used (1-naphthol, 2-naphthol, resorcinol and orcinol) interfered in the Lowry's method of protein estimation in a fashion similar to phenol (Figure 1B). Following this, we checked if phenol interfered in other methods of protein estimations and found interference specific to Lowry's and BCA method. Phenol concentration as low as 0.001% interfered with the BCA method, while concentrations as high as 0.01 % of phenol did not interfere in Biuret and Bradford method (data not shown).

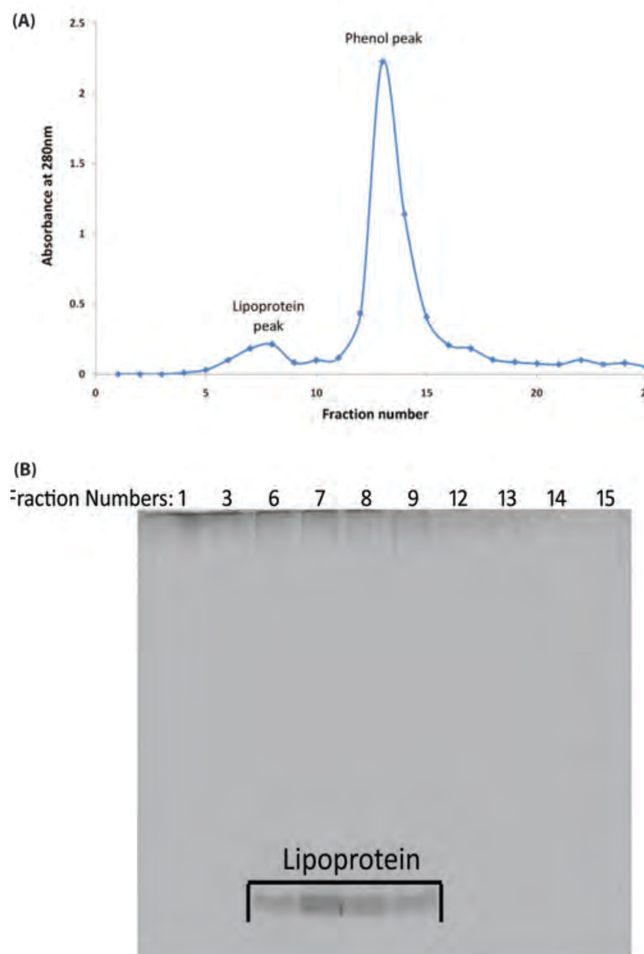



**Figure 2: Separation of bacterial lipoprotein from phenol using sephadex G-50.** (A)10 mg of apparently purified lipoprotein was subjected to gel filtration using sephadex G-50 and the fractions were monitored for their absorption at 280nm. The small peak of material eluting in fractions 6-9 was lipoprotein, while the larger peak corresponding to fractions 11-16 is due to elution of phenol. Data represents typical elution profile in more than 2 experiments. (B) SDS-PAGE analysis of fractions eluting from sephadex G-50. Fractions numbering 1, 3 ,6, 7, 8, 9, 12, 13, 14 and 15 were subjected for SDS-PAGE analysis followed by silver staining. Notice the absence of bands corresponding to phenol peak and presence of bands corresponding to lipoprotein peak. Data represents typical banding pattern obtained in more than 2 experiments.

### Sephadex G-50 gel filtration separates phenol from lipoprotein

Once we realized phenol carried over was responsible for incorrect quantification of proteins by Lowry's method, we attempted to remove the phenol using centrifugal spin concentrator with a 5 kDa cut-off limit. Unfortunately, this approach failed to remove sufficient amounts of phenol to abolish interference in the protein assay. We therefore resolved the lipoproteins by molecular sieving using sephadex G-50 gel beads. A typical elution profile by

monitoring absorption at 280 nm was obtained as shown in Figure 2A. Sephadex G-50 effectively separated the lipoprotein from phenol as evident from the elution profile and the corresponding SDS-PAGE analysis (Figure 2B). Lipoprotein eluted in fractions 6-9 (as visualized on a 15% SDS-PAGE), while, phenol eluted in fractions 11-16 (Figure 2B). Since, phenol also absorbs at 280nm like proteins, separating the two on gel beads and subjecting the fractions to SDS-PAGE analysis allowed visualization of the proteins on the gel. For example, in a representative experiment of the loaded 10mg of lipoprotein only 3.5 mg was lipoprotein while, the bulk of the material absorbing at 280nm was actually phenol.

## DISCUSSION

Although protein estimation is simple, it is essential to define the amount of protein present in the given sample with multiple methods to obtain accurate protein estimations. Failure to perform accurate protein estimation may result in a disaster as happened in the case of melamine adulteration in milk in China, where protein estimation was solely done by Kjeldahl method which is based on determination of total nitrogen. High nitrogen content of non-proteinaceous melamine encouraged people to include it in the food to increase its apparent protein content resulting in devastating health effects (Yang *et al.* 2009). Therefore, accurate protein estimation should also be a routine exercise in any biochemistry laboratory or food industry to normalize the values especially when different proteins are under scrutiny.

As in our case of purifying the isolated bacterial lipoprotein, phenol typically extracts lipoprotein but not bacterial endotoxin LPS (Neilsen *et al*., 2001), thus use of phenol becomes inevitable. Removal of bacterial endotoxin LPS in lipoprotein preparation is a prerequisite as LPS is also a ligand for related Toll-4 receptor and in fact many commercially available LPS preparations were known to have lipoproteins as contaminants and these lipoproteins may have accounted for responses incorrectly ascribed to LPS (Lee *et al*., 2002; Watanabe *et al*., 2003). Lipoprotein contamination can be effectively removed by phenol extraction as reported by Hirschfeld *et al.* (2000). This is also critical when one examines the specific effects mediated by Toll-4 in comparison with Toll-2 receptors.

### Conclusion

In summary, we find unavoidable contamination of phenol occurring during lipoprotein isolation from *E.coli* and this variable contamination disrupts accurate protein determination. As phenols readily undergo oxidation under Lowry's reaction conditions, they interfere during protein estimation by Lowry's method (Singleton *et al*., 1999) hence separating the lipoproteins from phenol that was carried over during the isolation of the lipoprotein is essential and was easily accomplished by molecular sieving. Phenol interference in Lowry's protocol for protein determination is not new (Lowry *et al*., 1951; Niamke *et al*., 2005), but, the presence of phenol carried over during lipoprotein purification is. Since, interest in Toll receptors is rapidly increasing, identifying the precise ligand free of other contaminants is a prerequisite while studying the functions of receptors for bacterial lipidated material. In this report, we have carried out a simple molecular sieving experiment where phenol carried over during protein purification can be effectively removed from lipoprotein preparation.

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