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Extraction and Purification of Lipopolysaccharide of *Klebsiella pneumoniae* Isolates

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A B S T R A C T

The genus *Klebsiella* contains a number of opportunistic pathogens which cause several serious infections, including pneumonia, bacteremia, and urinary tract infections. Characteristically, *Klebsiella spp.* produce large mucoid colonies because of the synthesis of large amounts of capsular polysaccharide (CPS). The LPS molecule is a virulence determinant in *Klebsiella pneumoniae*. In the present work from a pathogenic urine sample *Klebsiella pneumoniae* was obtained, the lipopolysaccharide extracted and completely purified by gel filtration chromatography. The purity was confirmed through determination of protein concentration and carbohydrate.

Introduction

Lipopolysaccharide

The lipopolysaccharide (LPS) molecule is composed of three distinct sections; lipid A, a core polysaccharide and a side chain O-antigen (O-Ag) polysaccharide. Nine O antigen types are distinguished in *K. pneumoniae*, O1 being the most frequent. The most important role of the O-antigen is to protect *K. pneumoniae* from complement mediated killing (Podschun and Ullmann 1998; Hansen, Mestre *et al.*, 1999; Brisse, Grimont *et al.*, 2006).

Lipopolysaccharides are made up of a hydrophobic lipid (lipid A, which is responsible for the toxic properties of the molecule), a hydrophilic core polysaccharide chain, and a hydrophilic O-antigenic polysaccharide side chain. In most cases, O-specific chains are built of repeating units of oligosaccharides which exhibit a strain-specific structural diversity. The sugar constituents, their sequence, and their mode of linkage determine the serological O specificity of respective strains. They are the main determinants of the classifications of the serotypes of Salmonella species. The CPS of *K. pneumoniae* is complex acidic polysaccharide consisting of repeating units of 3–6 sugars. In the past two decades a number of *K. pneumoniae* strains have been found to cause primary pyogenic liver abscess (PLA) (Wang *et al.*, 1998; Yang *et al.*, 2004;

Wacharotayankun *et al.,* 1993; Fung *et al.,* 2002), with the capsular serotype K_1 being the most virulent (Mizuta *et al.,* 1983). The K_1 structure has been reported previously (lacking the acetyl-decoration on fucose) to possess two unique features- a fucose subunit (also found only in K54 and K63), and a unique cyclic 2,3-(S)-pyruvate appendix differing from a commonly seen 4,6-(R)-pyruvate in CPS repeat units (Arakawa *et al.,* 1995).

K. pneumoniae contains a capsule around its cell, known as K antigen. The reason for its pathogenicity is the thick capsule layer surrounding the bacterium, it is 160 nm thick of fine fibers that protrudes out from the outer membrane at right angles (Lawlor *et al.,* 2005).

Function and application

The lipid A moiety has been identified as critical to the endotoxin activity of lipopolysaccharide. This was demonstrated by finding identical bioactive results, including endotoxic activity, between synthetic and natural-sourced *E. coli* lipid A preparations (Galanos *et al.,* 1985). The active receptor for lipopolysaccharide has been identified as the CD14/TLR4/MD2 receptor complex, which promotes the secretion of proinflammatory cytokines including tumor necrosis factor-α and interleukin-1 (Palsson-McDermott and O'Neill, 2004). While the lipid A component is primarily responsible for immune response activation, the polysaccharide component of *Salmonella enterica* LPS is also necessary for NF-κB activation (Muroi and Tanamoto, 2002).

An important characteristic of *K. pneumoniae* growth is the production of capsular polysaccharide (CPS). A thick CPS layer, referred to as a capsule, envelops these bacteria and thwarts protective host defences (Simoons-Smit *et al.,* 1986; Williams *et al.,* 1983; Williams and Tomas, 1990). Encapsulated *K. pneumoniae* also produces large quantities of cell-free CPS which is aggregated with toxic lipopolysaccharide (LPS) and contributes to pathogenicity (Batshon *et al.,* 1963; Straus, 1987). Circulating cell-free CPS may also bind and neutralize antibodies that would otherwise attach to and opsonize bacteria (Cryz *et al.,* 1986).

Bacterial pathogenicity has been shown to be due to different causes, including the structures of capsular polysaccharides (CPS; the K antigen), lipopolysaccharide (LPS; the O antigen), secreted toxins, drug resistance, and genetics (Abbott, 2003; Podschun and Ullman, 1998; Chang *et al.,* 2000; Cheng *et al.,* 1991). *Klebsiella pneumoniae* is an opportunistic pathogen of the Enterobacteriaceae and usually causes pneumonia or urinary tract infections (Chiu *et al.,* 1988). In addition, the hospital outbreak of multidrug resistant *Klebsiella* spp., especially the so-called extended-spectrum beta lactamase (ESBL) and *Klebsiella pneumoniae* carbapenemase (KPC) subtypes has drawn much attention in recent years (Chiu *et al.,* 1988; Fang *et al.,* 2004; Ko *et al.,* 2002).

The CPS of *K. pneumoniae* is complex acidic polysaccharide consisting of repeating units of 3–6 sugars. The type of sugars seems to correlate with the virulence, and 78 capsule types have been identified (Lin *et al.,* 2004) In the past two decades a number of *K. pneumoniae* strains have been found to cause primary pyogenic liver abscess (PLA) (Wang *et al.,* 1998; Yang *et al.,* 2004; Wacharotayankun *et al.,* 1993; Fung *et al., et al.,* 2002), with the capsular serotype K_1 being the most virulent (Mizuta *et al.,* 1983). The K_1 structure has been reported previously (lacking the acetyl-decoration on fucose) to possess two unique features- a fucose subunit (also found only in K54 and K63), and a

unique cyclic 2,3-(S)-pyruvate appendix differing from a commonly seen 4,6-(R)-pyruvate in CPS repeat units (Arakawa *et al.*, 1995).

Materials and Methods

Solutions for determination of protein concentration (Bradford, 1976)

Coomassie brilliant blue G-250 stain

It was prepared by dissolving 0.1g of Coomassie brilliant blue G-250 in 50ml of 95% ethanol, then 100ml of 85% phosphoric acid was added with agitation and the volume was completed to one litre with distilled water, then it was filtered through Whattman filter paper (No.1) and kept in a dark container.

Bovine Serum Albumin (BSA)

It was prepared by dissolving 0.1g of BSA in Tris-HCl buffer, and then the volume was completed to 100ml with the same buffer.

Sodium hydroxide (1M)

It was prepared by dissolving (40g) of sodium hydroxide in (950 ml) of distilled water. Volume was completed to one liter.

Solutions and chemicals for purification of LPS

Ion exchange

Sodium chloride (NaCl), 0.25 M

It was prepared by dissolving 3.65g of sodium chloride in 250 ml distilled water.

DEAE-cellulose

It was prepared by suspending 20g of DEAE-cellulose in 1leter distilled water.

PBS buffer (0.1M pH 7.1)

It was prepared by dissolving 8.5 gm of PBS into 1000ml of distilled water and the pH was adjusted to 7.1.

Gel filteration chromatography

PBS buffer (0.1M pH 7.1)

It was prepared by dissolving 8.5 gm of PBS into 1000ml of distilled water and the pH was adjusted to 7.1.

Blue dextran 2000

It was prepared by dissolving 0.06 g of blue dextran 2000 in 2 ml of distilled water.

Sepharose CL-6B

It was prepared as recommended by the manufacturing company (Pharmacia).

Chemical analysis of lipopolysaccharide

Solutions for determination of protein concentration

Phosphoric acid (85%)

It was prepared by the addition of 15 ml of distilled water to 85 ml of phosphoric acid.

Sodium hydroxide (1M)

It was prepared by dissolving (40g) of sodium hydroxide in (950 ml) of distilled water. Volume was completed to one liter.

Coomassie brilliant blue G-250 (Bradford, 1976)

It was prepared by dissolving 0.1g of Coommassie-brilliant blue G-250 in a solution composed of 100 ml of phosphoric acid 85% and 50 ml of absolute ethanol. Then

the volume was completed to 1litter by distilled water. The solution was filtrated through filter paper Whatman No.1.

Bovine serum albumin (BSA) (100 µg/ml)

It was prepared by dissolving 0.025g of BSA in 25 ml of distilled water. From this stock solution the concentrations 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/ml were prepared by diluting the stock in distilled water.

Cell preparation

Bacterial cells (*K. Pneumoniae* isolate) for lipopolysaccharide (LPS) extraction were cultured on 60 plates of nutrient agar, incubated at a temperature 37^0C for a period 18-24 hr. Then harvested by spreader and kept in a funnel containing 200 ml broth.

The inoculated flasks were incubated at 37ºC for 24hours with shaking at 150 rpm. After incubation, cultures were centrifuged (3000 rpm for 15 minutes), and the pellet was washed twice with phosphate buffer.

Cells were suspended in phosphate buffer containing 0.5% formalin (pH= 7.2) and were kept at 4ºC for 18 hours. After that, the cells were centrifuged (3000 rpm for 15 minutes) and washed with phosphate buffer.

Finally, cells were dried using cold acetone by ten times the sample's volume (Silipo *et al.,* 2002).

Destruction of bacterial cells

The *Klebsiella* LPS was extracted by a method given by (Chandan and Fraser, 1994), which is summarized in the following steps:

The dried cells (20 g) were suspended in 50 ml phosphate buffer, and then 0.5 ml of 0.5M EDTA solution was added. The suspension was then homogenized with magnetic stirrer for 2 minutes. The homogenized suspension was autoclaved and then left to cool.

After cooling, DNase and RNase solutions were added to at a final concentration of 1µg/ml for each solution and incubated at 37ºC for 10 minutes.

Proteinase K solution at a final concentration of 0.1mg/ml was added and incubated at 56ºC for 10 minutes and then the temperature was increased to 60ºC for further 10 minutes. Finally the mixture was left to cool.

The extraction mixture was centrifuged at 10000 rpm for 15 minutes. Two phases were formed; the aqueous phase (upper phase) was aspirated off with sterilized Pasteur's pipette and dialyzed for 4-6 days against distilled water at 4ºC with changing the water every day. The dialyzed sample that contained endotoxin was lyophilized to obtain crude endotoxin.

Determination of protein concentration

Protein concentration was carried out using the Bradford method (1976) as follows: Add 20µl of sample (crude or purified) was mixed with 50 µl of 1M NaOH with continuous shaking for (2-3minutes).then (1ml) of Coomasie Brilliant Blue G-250 was added with shaking. Absorbance was measured at 595 nm by a spectrophotometer. A standard curve of bovine serum albumin was carried out using different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µg / ml). Each was pipetted in duplicate in sterilized test tubes, then absorbency was plotted against the corresponding concentration of bovine serum albumin (Figure 1).

Carbohydrate determination

According to Dubois *et al.,* (1956), the phenol-sulphuric acid method was used to determine carbohydrate concentration in LPS,

in which the following steps were adopted: Standard solutions (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml) of glucose were prepared from the glucose stock solution (100 µg/ml), in order to plot the standard curve.

One ml of 5% phenol was added to each tube and shaken well, followed by addition of 5 ml of H2SO4 was added to each tube. After well-shaking, the tubes were cooled in ice bath.

Absorbance was read at 490 nm for each tube, and then the standard curve was plotted.

The total carbohydrates concentration in LPS sample was determined by transferring 0.5 ml of each gel-filtration fraction to a test tube, and then 0.5 ml of 5% phenol and 2.5 ml of H2SO4were added. The tubes were shaken well, and then cooled in ice bath. The absorbance was read at 490 nm, and the total carbohydrate concentration was estimated from the standard curve using curve fitting equation (Fig. 2).

Purification of LPS from *K. pneumoniae* (Jonson and perry, 1975)

Ion exchange chromatography using diethyl aminoethyl- cellulose (DEAE-Cellulose)

Preparation of ion exchange column (DEAE-cellulose)

The DEAE-Cellulose was prepared according to the method suggested by (Whitaker and Bernhard, 1972). A 20 gram of ion exchange resin was suspended in 1 liter distilled water, left in graduated cylinder to stagnate, after that the supernatant was removed, this step was repeated many times, until the supernatant becomes clear, the ion exchange resin was filtered by using Bukhner's funnel under vacuum (without drying the ion exchange resin), then the resin was activated in 250 ml from buffer which contains 0.25M sodium hydroxide and 0.25M sodium chloride for 30 minutes, the resin was re-filtered and washed under vacuum using distilled water, then the resin was suspended in 250ml hydrochloride acid 0.25M with agitation for 30 minutes. After that, the resin was washed with distilled water under vacuum, the resin was suspended in PBS buffer (0.1M, pH 7.1) and the ion exchange resin was degassed by using vacuum. The resin was packaged gently in glass column (3.5×7cm), the equilibration was achieved by the same PBS buffer (Fig. 4).

Separation through ion exchange resin (DEAE-cellulose) (Schutte *et al.*, (23)

DEAE-cellulose was packed in a column with dimensions of (1.5 × 25) cm. Ten ml solution (resultant extraction step) was loaded in ion exchange column carefully, the separated fractions were collected at flow rate 30 ml/hour (approximately, 5 ml for each fraction), the wash was obtained by 0.05M phosphate buffer, pH 7.5 (the same buffer used in equilibration).

The elution was achieved by the same buffer (100ml) of 0.05M phosphate buffer (pH 7.5) with gradual increase in concentration of 0.5M sodium chloride (100ml), the flow rate was 30ml/ hour too, the protein concentration of the fractions was measured at wavelength 280nm by UV-Visual Beam Spectrometer (Labomed) to the washed and eluted fractions, protein concentration then was calculated.

The presence of the LPS was estimated from each fraction of the major peaks. LPS was then measured the absorbance to every cool fractions at 280nm to assessment the amount of whole protein in the fragement (Bruck *et al.*, 1982) whereas at 490nm to calculation the carbohydrate amount according to (Dubios *et al.*, 1956).

Gel filtration chromatography using Sepharose CL-6B

Column preparation

One hundred milliliters of Sepharose CL-6B gel was washed with 0.05M phosphate buffer (pH 7.5) degassed under vacuum for 10 minutes for the removal of air bubbles that found in the gel, subsequently the gel suspension was poured into a glass column (1.5× 80cm) and allowed the matrix to be settled down.

This gel is supplied swollen and ready to use as a suspension containing 20% ethanol as a preservative. The suspension is highly thick to be poured directly into a chromatography column therefore it must first be diluted with eluent to the required consistency (as described by company instruction Pharmecia).

Column equilibration

The gel was equilibrated with 0.05M phosphate buffer (pH 7.5) with flow rate 48ml/h (4ml/5min).

Purification of LPS through gel filtration chromatography using sepharose CL-6B (Chhibber and Vadehra, 1986):

A (5ml) sample of each concentrated partially purified LPS was added to the column (1.5 x87 cm) carefully using pasture pipette, Elution of 0.05M Phosphate buffer (pH 7.5), (5ml) fraction was collected with flow rate of 60ml/hr.

The protein contents were estimated by measuring the absorbance at 280nm by UV-Visual Beam Spectrometer (Labomed) (Bruck *et al.,* 1982), whereas at 490nm to calculation the carbohydrate amount according to (Dubios *et al.,* 1956).

Determination of molecular weight of LPS by gel filtration chromatography

Determination of the void volume of the column

Sepharose CL-6B column was prepared and packed as described in A 2 ml blue dextran 2000 solution was passed through the column, and of 0.05M phosphate buffer (pH 7.5) was added. Fractions of 5 ml were collected.

The absorbency at 600 nm for each fraction was measured. The column void volume (Vo) was determined, by the estimation of the total volume of fractions as characterized with start point movement of the blue dextran to that of climax of absorbency of the blue dextran.

Determination of LPS elution volume (Ve)

Sepharose-6B column (80× 1.5 cm) was prepared, packed and Equilibrated for a second time. 5 ml of purified LPS sample was passed through the column carefully, and equilibrated with 0.05M phosphate buffer (pH 7.5), with a flow rate of 50 ml/hour.

Fractions of 5 ml were collected. The elution volume (Ve) was estimated for the separated fractions of purified exotoxin, by following the absorbance at 280 nm.

Measurement of standard protein elution volume (Ve)

Different standard proteins were applied through Sepharose CL - 6B column, and then eluted with 0.05M phosphate buffer, with a flow rate of 50 ml/hour, as shown in (Table 1).

The elution volume was estimated for each standard protein by following the absorbance for the separated fractions at wave length 280

nm. The (Ve/Vo) ratio was calculated for each standard protein and for the separated fractions of purified LPS. Then standardization was done by plotting the elution volume (Ve) of each standard proteins to the void volume (Vo) of the blue dextran 2000 (Ve/Vo) versus the log value of molecular weight (Stellwagen, 1990). The LPS molecular weight was accordingly calculated.

Results and Discussion

Purification of LPS

The collected fractions were first subjected for the determination of protein through reading the absorbance of each fraction at 280nm as submitted by (Bruck *et al.*, 1982). Afterward, each fraction was handled according to Dubois *et al.*, (1956) to calculate carbohydrate content, then the absorbance was read at a wave length of 490nm.The correlation between absorbency and fractions number of both concentration (protein and carbohydrate) was drawn in figure 3.

Determination of molecular weight

The method of filtrating gel on a column Sepharose 6B was followed to estimate the molecular weight of the LPS, using a standard protein; by drawing the relationship between the logarithm of a standard protein molecular weight and the size of recovery/ size of Void (Ve/Vo) (Fig. 5).

Fig.1 A standard curve of bovine serum albumin

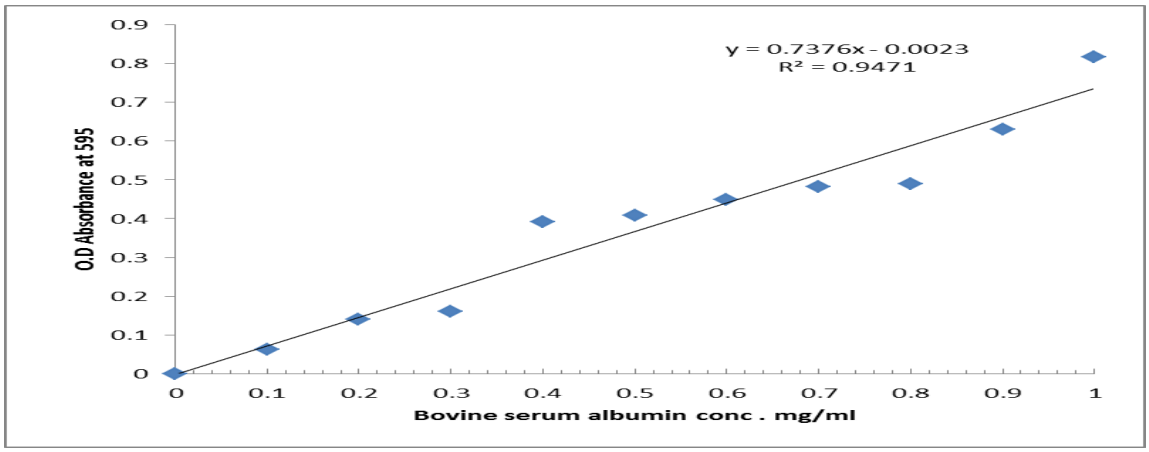
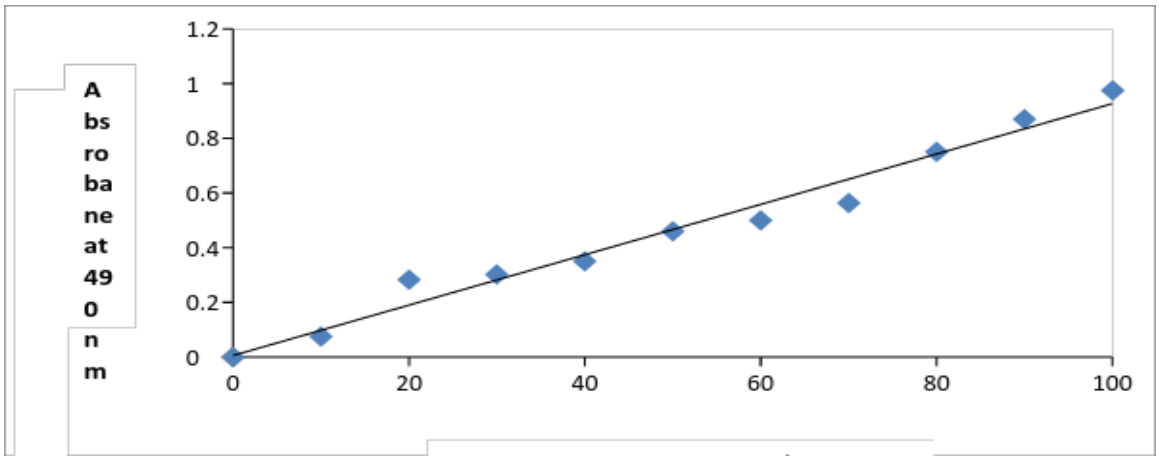


Fig.2 Standard curve of glucose



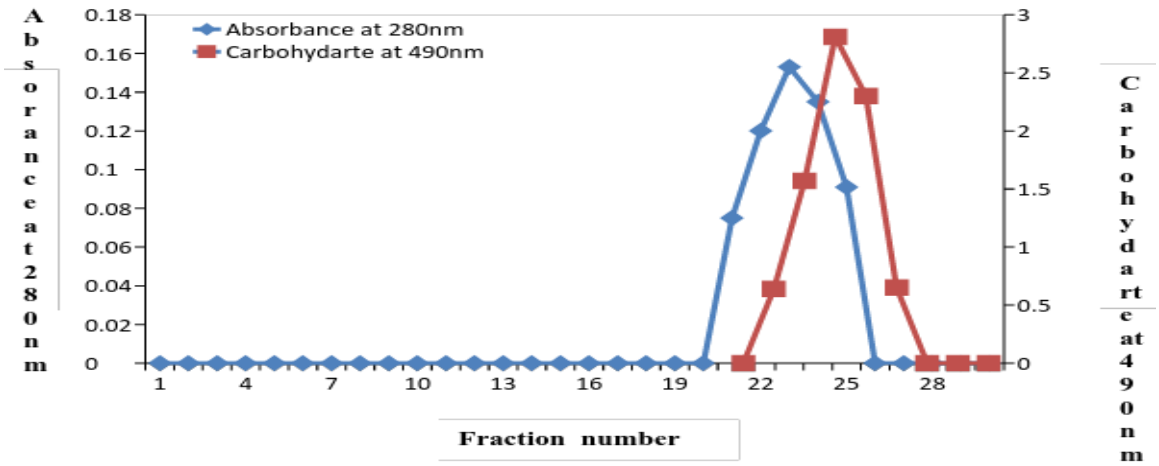


Figure (3): Gel filtration chromatography for purification LPS by using Sepharose 6B column 1.5 X 87 cm . The collumn calibrated with phosphate buffer pH 7.5, flow rate 60ml/ hrs and 5 ml/fraction in the second time.

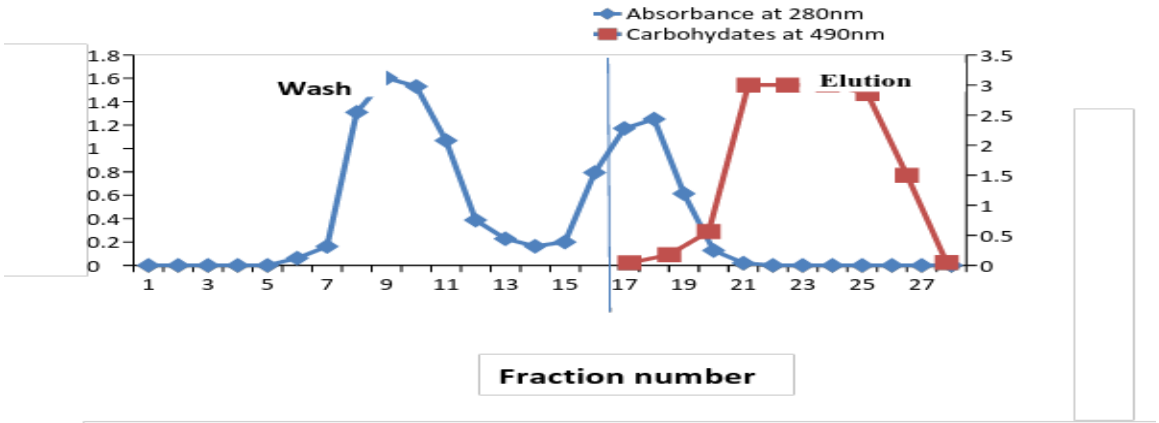


Figure (4): Gel filteration Chromatography for LPS of K.pneumonia throught Sepl 6B , column 1.5 X 80cm , elution wasdone with PBS PH 7.2 at flow rate 60ml/h , 5ml f fraction in the first time

Fig.5 Standardization of LPS from *Klebsiella pneumoniae* according to the ratio of Void volume and elution volume (Ve/Vo) ratio

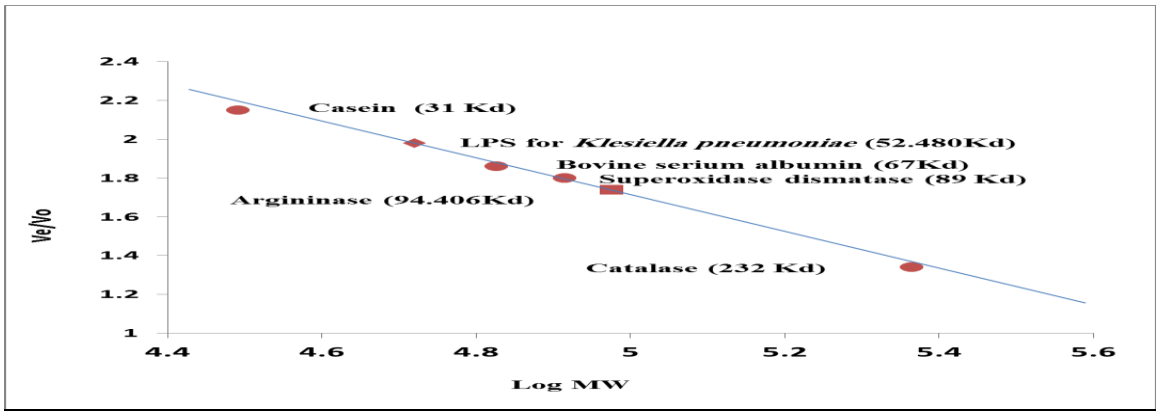


Table.1 Standard proteins used in determination of the molecular weight of lipopolysaccharide from *K. Pneumoniae*

Molecular weight (KDaltons)	Standard proteins
31	Casein
67	Bovine Serum Albumin
89	Superoxidase dismutase
94.40	Argininase
232	Catalase

Table.2 Standardization of LPS from *Klebsiella pneumoniae* according to the ratio of Void volume and elution volume (Ve/Vo) ratio

Ve/Vo	Standard protein and purified LPS
2.15	Casein
1.86	Bovine Serum Albumin
1.8	Superoxidase dismutase
1.74	Argininase
1.341	Catalase
1.98	LPS

Determination elution volume for standard protein

Gel filtration was carried out for four of standard proteins (Table 2); absorption was measured at 280nm in separated volumes to determine elution volume (V0) for each standard protein.

The relationship between elution volume percentages was blotted for each standard protein to the elution volume of blue dextrin (Ve/Vo) against molecular weight logarithm. This way helped measure enzymatic molecular weight.

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