Preparation of antibodies type IgY against *Salmonella typhi* lipopolysaccharide in chicken eggs

تحضير أضداد نوع IgY ضد متعدد السكريد الشحمي لبكتريا *Salmonella typhi* في بيض الدجاج

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Abstract

Lipopolysaccharide was extracted from local isolate *Sallmonella typhi* (previously isolated and characterized) by hot EDTA method, and the extract was partially purified by gel filtration chromatography on sepharose Cl-6B gel. The results showed that the percentage of the carbohydrates amount in the partially purified LPS extract was 43.7%, while the percentage of binding proteins in the same extract was 0.7% with no nucleic acids was found. The molecular weight for the LPS was measured by the gel filtration chromatography method using Sepharose Cl-6B gel and was equivalent to 263000 Dalton. The LD50 of LPS was determined by injection of chicken embryos type Ice Brown in the charioallantoic membrane, and was 14.66 µg/Kg. In order to obtain anti *S. typhi* IgY antibodies, chickens were immunized with the partially purified *S. typhi* subcutaneously. The IgY antibodies were extracted from eggs yolk by water dilution method and the extract was partially purified by ammonium sulphate precipitation at ratio 60% saturation, and gel filtration chromatography on sepharose Cl-6B gel. The results showed that the protein amount was equivalent to 23.5 mg/ml; specific activity was 0.268, and an overall yield of 70%. The molecular weight for the IgY antibodies was measured by the gel filtration chromatography method using Sepharose Cl-6B gel and was found to be 178000 dalton. The concentration of anti *S.typhi* LPS IgY antibodies in chicken eggs were investigated by ELISA and was found to be 6.3 mg/ml, and there is a significant differences (P<0.01).

المستخلص

استخلص متعدد السكريد الشحمي (LPS) من بكتريا *Sal. typhi* التي سبق ان تم عزلها وتوصيفها ، بطريقة الـ EDTA الساخن ، ونقي المستخلص جزئيا بطريقة كروموتوغرافيا الترشيح الهلامي باستعمال هلام الـ Sepharose Cl-6B . بينت النتائج ان كمية الكاربوهيدرات في المستخلص كانت 43.7 % ونسبة البروتين المرتبط 0.7% وانه خالي من الاحماض النووية . قدر الوزن الجزيئي لل -LPS بوساطة كروموتوغرافيا الترشيح الهلامي كان مساويا الى 263000 دالتون ، وتم تحديد الجرعة المميتة النصفية LD50 لعديد السكريد الشحمي المنقى جزئيا عن طريق حقن اجنة الدجاج نوع Ice Brown في غشاء الالنتويس وكانت مساوية الى 14.66µg / كغم . ولغرض الحصول على اضداد نوع IgY متخصصة لعديد السكريد الشحمي ، تم تمنيع الدجاج تحت الجلد ، بعد ذلك استخلصت الاضداد من صفار البيض بطريقة التخفيف المائي ، ونقي المستخلص جزئيا بخطوتين الاملى بوساطة الترسيب باستعمال كبريتات الا مونيوم بنسبة تشبع 60% ، والثانية بوساطة الترشيح الهلامي ، اظهرت النتائج ان كمية البروتينات في مستخلص اضداد IgY المنقى جزئيا كانت مساوية الى 23.5 ملغم/مل مع فعالية نوعية 0.268 ملغم IgY/ ملغم بروتين وحصيلة 70% ، وقدر الوزن الجزيئي للأضداد بالترشيح الهلامي باستعمال هلام Sepharose Cl-6B

وكان مساويا الى 178000 دالتون . تم التحري عن تركيز اضداد IgY في بيض الدجاج بوساطة اختبار الـ ELISA ، وكان مساويا الى 6.3 ملغم / مل ، وظهر ان هناك فروقات معنوية على مستوى احتمالية 0.01 .

Introduction

Typhoid fever is enteric disease caused by *Sal. typhi*, it is one of the major health problems. Eradication therapy for this disease employs two or more antibiotics; this therapy has undesirable side effects, including increasing the prevalence of antibiotic-resistant strains and medical coast. Vaccine development is the preferred approach for *S. typhi* treatment, but an effective vaccine that offers complete protection has not yet been developed. The vaccine can offer between (33, 78) % protection and could not be used in large scale public prevention programmes [1]. Non antibiotic substances are easily obtained from edible sources that inhibit proliferation of pathogenic bacteria and prevent adherence of pathogenic bacteria to epithelial cells. Thus anti-adhesion and mucosal protective agents could represent potential targets for pathogenic bacteria treatment [2,3]. The protective effect of egg yolk immunoglobulin (IgY antibody) obtained from chickens immunized with bacterial whole –cell lysate is able to inhibit bacterial growth [4]. These facts suggest that IgY could be used as a novel modality against *S. typhi* associated typhoid. Howevever, IgY produced by whole-cell lysates may cross react with other bacteria, and this could decrease the efficiency of IgY. Therefore, immunization using a selective antigen is required to produce a more effective *S. typhi* specific IgY. *S. typhi* has a variety of surface components which are virulence related, among them LPS play an important role in pathogenicity, and required to elicit an effective immune response and antibodies which presumably recognized epitopes present in LPS were protective against endotoxemia and typhoid [5].So as a result of increasing antibiotic – resistant strains problem and in attempt to find alternatives to decrease the pathogenicity of *S. typhi* by producing more efficient immunotherapeutic agent.

Materials and Methods

Extraction and partial purification of LPS from *S. typhi*: the bacteria was cultivated on nutrient agar medium, (150 Petri dishes), were incubated at 37˚C under aerobic condition for 18 hr, then the colonies were harvested by scraping process using spreader with phosphate buffer saline pH 7.2(NaCl:8g, K2HPO4:1.12g, KH2PO4 :0.34g, in 1000ml distilled water), the cells were washed three times with PBS, the cells were precipitated by cooling centrifuge at 3000 rpm/min for 15 min. at 4˚C the cells were dried by cooled acetone at 4˚C with ratio of 1:10 after that 16g of dried cells were obtained, stored at -16˚C the dried cells were resuspend with ratio 1:1 (wt:v) with PBS, 0.1 ml of EDTA solution(0.5M) was added for each 10 ml of cells suspension. The mixture was autoclaved at 121˚C for 10 min then the mixture was left to cool. Then RNase and DNase were added at final conc. 1µg/ml, and the mixture was incubated at 37˚C for 10 min., after that proteinase K enzyme was added at final conc. of 1µg/ml and the mixture was incubated in water bath at 56˚C for 10 min, then the temp. Was increased to 60˚C for 10 min, the mixture was left to cool at room temp. Two phases were separated, the supernatant aqueous upper phase at the top and the sediment phase at the bottom. The supernatant which supposed to contain LPS was dialysed against dist. Water for 4 days, and then lyophilized [6].

Partial purification by gel filtration using sepharose Cl-6B

Amount of sepharose Cl-6B gel was prepared as suspension, and then poured in to a glass column (2*71cm). Nine mg of crude lyophilized LPS was dissolved in 3ml of phosphate buffer saline [7]. Then the sample was added carefully, and washed by phosphate buffer saline twicelly, 3mL was collected for each fraction at the flow rate (40 ml/ hr.), the optical density of the collected fraction were measured at the wave length 280nm (absorbance) to estimate the quantity of total protein in the fractions, and also measured at the wave length 260 nm to estimate the total quantity of nucleic acid in the fractions[8], and at 490nm to estimate the total quantity of carbohydrate[9].

Determination of LPS molecular weight: The molecular weight of the partially purified LPS was determined by gel filtration chromatography method, using sepharose Cl-6B, standard proteins, Catalase (232 KD), Alkaline phosphatase (80KD), Bovine serum albumin (67KD), Endonuclease (28KD), were used as molecular weight markers.

Determination of LD50 for *S.typhi* LPS: four equal groups of fertilized ice brown eggs were used; each group consisted of 6 fertilized eggs, incubated routinely in a humidified, self turning incubator maintained at 37˚C. Different concentration (5, 10, 20,40)µg/Kg were used to treat chick embryos age of 8 days, and 1 ml of the test solution was gently directly injected in the chorioallantoic mem., control embryos were injected with 1 ml of PBS. The embryos lethality was followed up within 24 hrs. and the LD50 was calculated according to the logarithmic method [10] as follows :

Log LD50=Log Da+ d(f+1)

Da= Log of the lowest dosage levels used.

d= Log of the constant ratio between dosage levels.

f= factor was taken from the standard table.

Preparation of antigen-adjuvant emulsion: Aspirate desired volume of antigen (14.66 µg of LPS was dissolved in 0.5 ml of PBS) mixed with an equal volume of adjuvant (0.5ml). Mixing was need to be continued until the emulsion becomes milky and then pasty.

The schedule of immunization: Immunization was carried out according to the modified [11]. One ml of the solution was injected in to the chest region of the chickens (14 weeks old) subcutaneously. Two booster injections for the same dose were given after (3, 6) weeks with incomplete Freund's adjuvant. The eggs were collected after a week of the last injection.

Extraction and partial purification of IgY antibodies

Extraction of IgY antibodies: The crude antibodies from yolk were extracted by water dilution method [12]. The yolks of 8 eggs were separated from the white and washed with deionized water, then diluted 6 times with cold 3mM HCl adjusted with 10% acetic acid to give the suspension final pH 5, incubated at 4˚C for 6 hr. followed by centrifugation at 10000 rpm for 15 min. The water soluble fraction (WSF) was carefully collected and further purified by ammonium sulphate at 60% saturation. The precipitate was collected by centrifugation, dialyzed for 4 days at 4˚C against distilled water and lyophilized.

Partial purification of IgY antibodies by gel filtration using Sepharose Cl-6B: Glass column was loaded with Sepharose Cl-6B, the matrix size was (2x 67) cm.

Partial purification of IgY antibodies extract: Ten mg of precipitant was dissolved in PBS buffer containing 0.5M Na2SO4 and loaded to column, and eluted with PBS containing 0.5 M Na2SO4, 3ml fraction were collected in test tubes. The absorbance of the collected fractions was measured at 280 nm.

Determination of protein content of IgY antibodies: Lowery method [13] was used to determine the amount of protein that combined with crude and partial purified IgY antibodies.

Determination of IgY antibodies molecular weight: The molecular weight was determined by gel filtration chromatography method, using sepharose Cl-6B, standard proteins were used as molecular markers (Endoglucnase, Bovine serum albumin, Alkaline phosphatase, Catalase).

Determination of specific activity of IgY antibodies: The specific activity was estimated according to [14].

Sp.act. = Elisa value mg / total protein mg.

Determination of IgY antibodies recovery: The recovery percentage was calculated according to [14] as follows:

Rec.= OD 492nm(A) / OD492nm (B) X 100

A= is OD 492 of crude extract (WSF).

B= OD 492 nm in the egg yolk.

Enzyme immunoassay for quantitative measurement of IgY antibodies: The specific antibodies is estimated according to [15].

Statistical analysis: The results were statistically analyzed by t-test and $p<0.01$ was considered statistically significant difference [14].

Results and discussion: Extraction of LPS: The percentage of carbohydrates and protein in the crude extract were (34.3, 1.4) % respectively Table (1).

Table (1): Amount of carbohydrates, protein, nucleic acid in the crude extract and partial purified LPS

LPS	Carbohydrates (%)	Protein (%)	Nucleic acid (%)
Crude	34.3	1.4	0
Partial purified	43.7	0.7	0

Moreover, the amount of nucleic acids contamination the extract were measured according to [16] method, the results showed that there was no nucleic acids in the sample and that due to the destruction effect of nuclease enzymes on nucleic acids.

Partial purification of LPS: the results in Figure (1) show that two peaks of carbohydrates were separated; one of them was small and the other was big; the two peaks contain protein components linked with LPS which was difficult to separate, activity of LPS in chickens was measured by following LD50 of the lyophilized fractions (21, 22, 23, 24, 25, 26, 27) and (44, 45, 46, 47, 48, 49, 50) by injection in chick embryos, the results showed that the first peak was contained active LPS while there is no activity in the second one so the small peak was taken and the big one was omitted.

The chemical analysis to partial purified LPS was done, and the amount of carbohydrates, protein and nucleic acids were estimated in the collected fractions.

The results showed that the percentage ratio of carbohydrates was 43.7% Table (1). On the other hand, the results record the protein percentage as 0.7% Table (2), while the results of Nucleic acids content in the partial purified LPS extract were shown to be nill as long as an efficient nucleases were used in removal of nucleic acid from the extract.

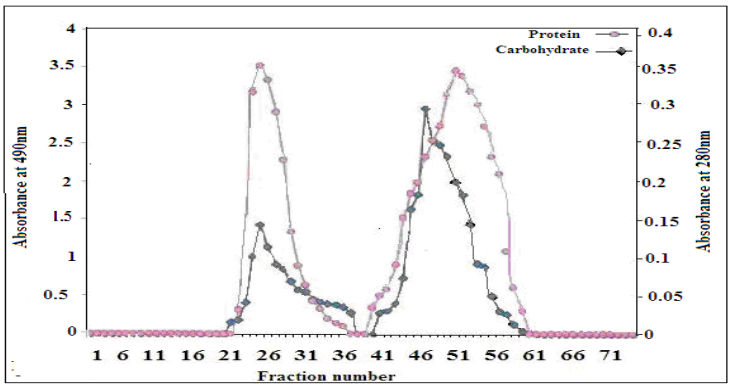

Figure (1): Gel filtration chromatography of *Sal.typhi* LPS by using SepharoseCl-6B, column dimension was (2x71) cm., elution was done with phosphate buffer saline pH7.2 at flow rate 40 ml/h., 3ml for each fraction

Determination of LPS molecular weight: The molecular weight of LPS was estimated as equivalent to 263000 Dalton Figure (2), the reason for high molecular weight value of the isolated LPS may be related to the type of bacterial strain.

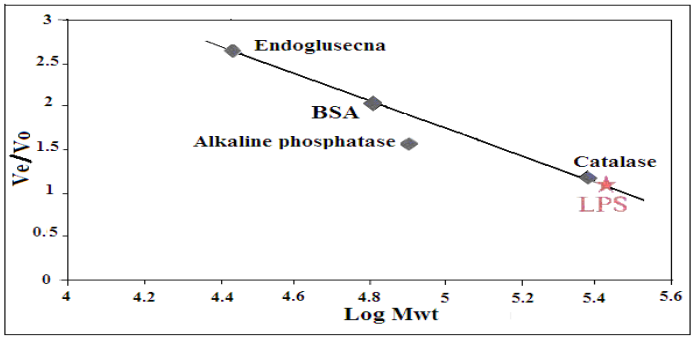

Figure (2): Molecular weight determination by gel filtration chromatography using Sepharose Cl-6B.

Determination of LD50 for *S.typhi* LPS: The results with peak one LPS showed that doses less than 10ug/Kg of LPS didn't have toxic effect on the chick embryos while the embryos death reached 2-5 dose with 14.66 ug/egg of LPS has median lethal effect .
The results with peak two LPS showed no toxic effects on the chick embryos to all LPS concentrations doses. The differences in LD50 values may be due to type of antigen which used in immunization, degree of its purity, sensitivity of the embryos, period which used and age.

Extraction and partial purification of lgY antibodies: Extraction and partial purification of lgY antibodies using ammonium sulfate: the extracted proteins were measured according to [13] depending on standard curve of bovine serum albumin and the results showed that the concentration of protein was 37.6 mg/ml with specific activity of 0.1718 mglgY/mg protein and an over yield of 74% Table (3).

Table (3): Protein content and IgY concentration.

Purification steps	Volume (%)	Protein conc. (%)	IgY Conce. (/mg/ml)	Specific (mg IgY/mg Protein)
Soluble yolk extract (WSF)	200	48.6	N.D*	N.D*
Pellet after 60% amm. Sulphate	5	37.6	6.46	0.1718
Gel filtration with Sepharose cl-6B	15	23.5	6.30	0.2680

*** N.D :Not Determinant**

Partial purification of lgY antibodies by gel filtration chromatography using **Sepharose CI-6B:** The eluted solution in the transmitted fractions was investigated by measuring the absorbance at 280nm and the relationship between the absorbance and transmitted fractions were plotted Figure (3). The results showed that two peaks of protein were separated. Estimation of lgY antibodies specific activity for the two peaks was tested by ELISA. The fractions of the first peak (25,26,27,28,29) and the second peak (37,38,39,40,41) were collected and lyophilized separately. The test results showed that the first peak contains active lgY antibodies, while no activity was found in the second one. So the first peak was taken and the second peak was neglected. In order to confirm the efficiency of purification process, the chemical analysis was made to determine the protein concentration in partially purified lgY antibodies Figure (2). The results showed that the concentration of protein in the partial purified lgY antibodies by gel filtration was 23.5 mg/ml with specific activity of 0.2680 mg lgY/mg protein and overall yield of 70% Table (3).

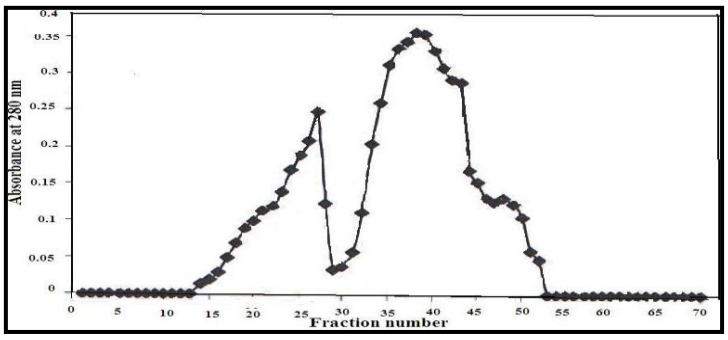

Figure (3): Gel filtration chromatography of anti *Sal. typhi* LPS-IgY antibodies by using Sepharose cl-6B, column dimension (2x67)cm, elution was done with PBS containing 0.5 M sod. Sulphate pH7.2 at flow rate 40ml/hr. , 3ml for each fraction

Determination of the molecular weight of lgY antibodies: The molecular weight value of lgY antibodies was determined and it was equivalent to 178000Dalton Figure (4). Many studies referred that molecular weight of lgY antibodies reached to 180000 Dalton [17, 18, 19]

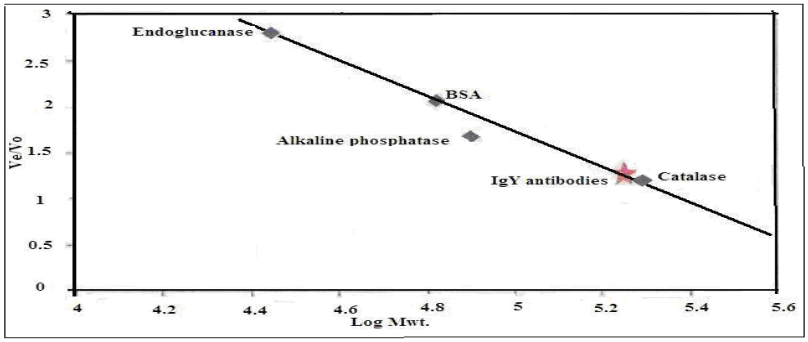

Figure (4): Determination of molecular weight of IgY antibodies by gel filtration chromatography using Sepharose Cl-6B (2x67)cm.

Specific activity of IgY antibodies: Specific activity of anti *S.typhi* LPS antibodies using ammonium sulphate and partially purified anti *S.typhi* LPS- IgY antibodies using gel filtration (peak one and peak two) were determined by using ELISA [20]. In order to determine the fraction which contained higher specific activity of IgY antibodies against conc., the comparison were done between graduated conc. of peak 1, peak 2 and the precipitant which resulted from ammonium sulphate with control. The results showed that the peak one contain higher specific activity of anti *S.typhi* LPS- IgY antibodies (0.2680 mg IgY/ mg protein).When compared with peak two and partially purified IgY antibodies using ammonium sulphate were (0, 0.1718) mg/mg protein respectively .

Enzyme immunoassay for quantitative determination of IgY antibodies: The results showed that slightly gradual decrease were occurred in IgY antibodies conc., a higher conc. was reported at the first well for anti *S.typhi* LPS- IgY antibodies in compared with control Figure (5) with statistically significant differences ($P<0.01$). However 1:1024 IgY dilution was considered optimal conc. (6.3 mg/ ml) because this dilution rate gave an optimal absorbance (change in absorbance of 1.0 at 450 nm after 30 min. of substrate conversion at 25˚C).

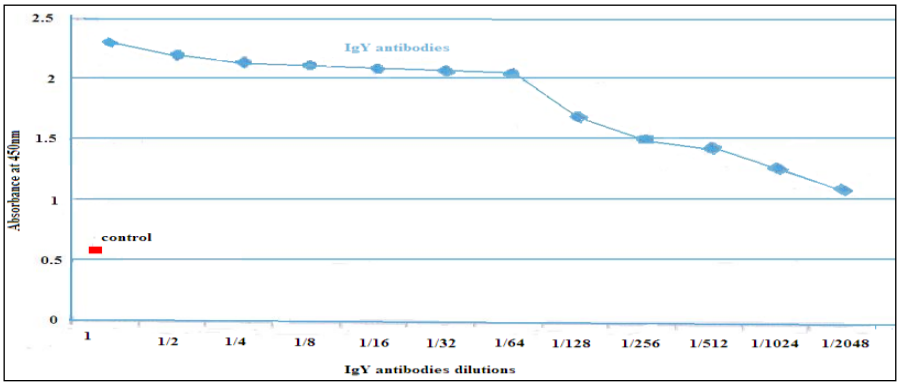

Figure (5): Determination of anti *S. typhi* LPS-IgY antibodies by ELISA.

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