Separation of *Salmonella enteritidis* from Experimentally Contaminated Liquid Eggs Using a Hen IgY Immobilized Immunomagnetic Separation System

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Immunomagnetic separation with immunomagnetic beads was used to separate *Salmonella enteritidis* from experimentally contaminated liquid eggs. Immunoglobulin (IgY) from egg yolk was prepared of hens immunized with *S. enteritidis* PT4 and its lipopolysaccharide (LPS). The specific antibodies against *S. enteritidis* and its LPS were produced and deposited into hen egg yolk. These antibodies were immobilized to immunomagnetic beads and used to separate *S. enteritidis* from liquid eggs. The antimicrobial activity of the IgY was higher when the LPS was used as an antigen rather than whole cells. The amounts of coating immunoglobulin and incubation time influenced the isolation ratio of the bacteria in the samples. Using anti-LPS antibody immobilized magnetic beads, a maximum of 93.2% of *S. enteritidis* was separated from the contaminated liquid eggs (10^2–10^3 colony forming units/mL).

Keywords: *Immunomagnetic separation; Salmonella enteritidis; egg yolk antibody; eggs; lipopolysaccharide*

INTRODUCTION

As with any food of animal origin, eggs may be contaminated with organisms that are potentially pathogenic for humans. In the past decade, the incidence of *Salmonella enteritidis* as a cause of human salmonellosis has increased dramatically worldwide (Rodrique *et al*., 1990). Data from an *S. enteritidis* trace back program indicate that in the 1990s, 32% of all food-borne illness was caused by *S. enteritidis* in eggs (APHIS Veterinary Services, 1994). It has been demonstrated that intact shell eggs may be contaminated through vertical transmission from the hen (Gast and Beard, 1990, 1992). It is generally recognized that *S. enteritidis* PT4 is the most important strain which is closely associated with poultry and eggs in North America (Stevens *et al*., 1989). Although lysozyme, ovotransferrin, and the alkaline pH in egg white are known to kill or inhibit the growth of a wide variety of microorganisms, Salmonellae are known to secrete their own chelators during extended storage, which enables them to compete with ovotransferrin and to survive and grow in eggs (Garibaldi, 1970).

The Egg Products Inspection Act of 1970 (FDA, 1971) requires that all egg products should be rendered free of *Salmonella* by appppropriate pasteurization process. It required that all liquid egg products be heated to a temperature of not less than 64.4 °C for 2.5 min. *S. enteritidis* PT4 also appears to be more heat resistant than some other *Salmonella* species (Humphrey *et al*., 1990). Removal of bacteria without heat treatment from liquid egg was studied by means of centrifugal process (Kosikowski and Moreno, 1970). Continuous bacterial centrifugation of liquid egg white reduced total bacterial count 50−99.7% depending on the processing temperature. However, a large number of organisms containing *Salmonella* remained in the samples. Immunochemical separation has been shown to be an effective tool for the separation and isolation of specific materials from heterogeneous solution (Hammock *et al*., 1995). Recently, hen egg yolk has been attracting considerable attention as a source of antibody (Larsson and Sjoquist, 1990; Nakai *et al*., 1994). Antigen-specific immunoglobulin G (IgG) has been widely used for immunological analysis in the field of diagnosis, food safety, and affinity chromatography. Higher antimicrobial IgG production in serum caused by proper immunization of hens with bacteria should induce higher specific IgG deposition in eggs.

A new immunomagnetic separation technique was developed (Skjerve *et al*., 1990; Skjerve and Olsvik, 1991). This technique has been shown to be a very effective tool for the separation and isolation of specific cells from heterogeneous cell suspensions (Skjerve *et al*., 1990; Sonti and Bose, 1995). The immunomagnetic separation has been used to extract *Staphylococcus aureus* from milk (Johne *et al*., 1989), and specific *Escherichia coli* strains have been isolated from bacterial suspensions and feces (Lund *et al*., 1988). However, the antibodies used in these methods were mainly of mammalian origin from mouse or rabbit. Hen immunoglobulin in egg yolk (IgY) could be expected to be a potential source of antibody for immobilizing to the magnetic beads. In this study, specific IgY against *S. enteritidis* were produced in hen egg yolk for the selective separation of the bacteria from experimentally contaminated liquid eggs.

MATERIALS AND METHODS

Immunization of Hens. *S. enteritidis* PT4 was incubated in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37 °C for 20 h. Cells were harvested and treated with 0.5% formalin for 3 h. Lipopolysaccharide (LPS) of *S. enteritidis* was isolated according to the hot phenol extraction method (50% phenol, 70 °C) of Westphal *et al*. (1952). The antigen suspensions [2×10^7 cells/mL and 2 mg of LPS/mL in phosphate buffered saline (PBS)] were emulsified with an equal volume of Freund's complete adjuvant (FCA) (Sigma, St. Louis, MO). One milliliter of this emulsion was injected intramuscularly per hen at two sites (at 0.50 mL each). Three hens (White Leghorn, 20 weeks old) were used per antigen.

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Three booster injections of antigens were given 2, 4, and 6 weeks after the first injection. Eggs were collected every day during the immunization period. The antibody from egg yolk was purified from the water soluble fraction of egg yolk by precipitating the lipoprotein according to the method of Akita and Nakai (1992).

Enzyme-Linked Immunosorbent Assay (ELISA). Each well of the 96 well polystyrene plates (Corning Costar, Cambridge, MA) was coated with 100 μL of *S. enteritidis* whole cell (10^7/well) suspension or LPS solution (10 μg/well) in 0.05 M carbonate buffer (pH 9.6) for overnight at 4 °C. After the wells were washed with PBS containing 0.05% Tween 20 (PBS-Tween), they were blocked by incubation for 1 h at 37 °C with 150 μL per well of 2.0% (w/v) bovine serum albumin (Sigma, grade V) in carbonate buffer. After three washings with PBS-Tween, appropriately diluted IgY was added and the plates were incubated for 1 h at 37 °C. After four washings with PBS-Tween, 100 μL of alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma, St. Louis. MO) was added and incubated at 37 °C for 1 h. Plates were washed with PBS-Tween, and 100 μL of the substrate solution (*p*-nitrophenyl phosphate, 1 mg/mL, pH 9.8, Sigma) was added. After 30 min of incubation at 37 °C, 25 μL of 2 N NaOH was added to stop the reaction and the color was read on an ELISA plate reader (Model 550, Bio-Rad, Mississauga, ON) with a 405 nm filter. Competitive ELISA was carried out as follows: Polystyrene ELISA plates were coated with 100 μL of LPS solution (10 μg/well) in 0.05 M carbonate buffer, pH 9.6. The plates were blocked by the same method described above. After the plates were washed with PBS-Tween, 50 μL of serially diluted anti-whole cell or anti-LPS antibodies (3^0−3^{-4} dilution) and 50 μL of alkaline phosphatase conjugate anti-whole cell IgY (1:1000 dilution with PBS) were incubated in the same well at 37 °C for 2 h. The plates were washed four times with PBS-Tween and developed by adding 100 μL of the substrate of *p*-nitrophenyl phosphate (1 mg/mL) and incubated 30 min at 37 °C. The reaction was stopped by adding 25 μL of 2 N NaOH, and the plates were read at 405 nm. The conjugate of alkaline phosphatase IgY was prepared according to the method described by Kemeny (1991).

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out as described by Laemmli (1970) using a 5−15% gradient gel (Bio-Rad, Mississauga, ON). The protein was stained with a Coomassie brilliant blue R-250 (Sigma) in 10% acetic acid/30% methanol. Gels were scanned on a Sharp JX-330 scanner (Sharp Electronics, Tokyo, Japan) and the raw volume of each band determined using the Pharmacia Imagemaster.

Coating Procedure of Egg Yolk IgY Antibodies. Tosyl-activated magnetic beads were purchased from Dynal (Dynabeads M-280, Dynal AS, Oslo, Norway). The coupling of anti-whole *S. enteritidis* cell and anti-LPS antibodies to tosyl-activated beads was carried out according to the method of Nilsson and Mosbach (1984) at room temperature. The antibody was dissolved in 3 mL of 0.25 M $NaHCO_3$ at pH 9.5 and mixed with 1 mL of the activated beads, followed by overnight agitation at 4 °C. Remaining interfering tosyl groups were removed by adding 5 mL of 2% bovine serum albumin in 0.8 M Tris-HCl buffer, pH 9.5. The amount of antibodies bound to the beads was determined by analyzing the unadsorbed protein in serum using the Lowry method (Lowry *et al*., 1951). The final coating of antibodies onto immunomagnetic particles was adjusted to achieve a final concentration of 5−40 mg of IgY/mg of particles by incubation with various amounts of antibody solution (7−50 mg of IgY/mg of particles) (Funderud *et al*., 1987). The binding of the antibodies onto the particles was measured by ELISA using a alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma) according to the method of Holt *et al*. (1995).

Immunomagnetic Separation of *Salmonella* in Eggs. The homogenized experimentally contaminated whole liquid eggs containing about 10 cells/10 mL of sample were incubated at 37 °C for 4 h. Sterile disposable 15 mL conical tubes were used. Ten milliliters of each sample was mixed with 100 μL of coated immunomagnetic particles (10 mg of particle/mL) and incubated for 3, 5, 10, 20, and 30 min at room temperature on a rotating device. Gentle rotation during incubation prevents particles from settling and improves the binding kinetics. At the end of incubation, particles were sedimented using the magnetic particle concentrator (Dynal MPC-1). After removal of the magnetic beads, the number of cells remaining in the liquid eggs was determined. After vigorous mixing, a 0.1 g aliquot of the sample was plated onto duplicate xylose lysine desoxycholate (XLD) agar (Taylor, 1965). Colony forming units (cfu) were enumerated after incubation at 37 °C for 48 h.

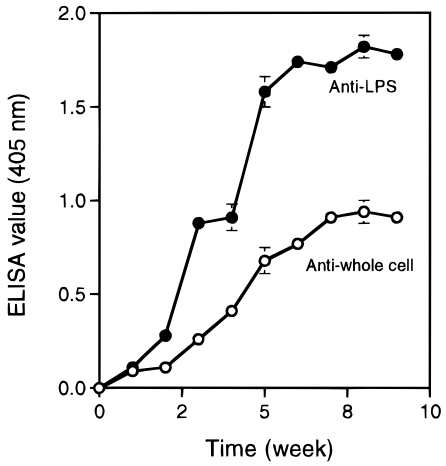

Figure 1. Changes of antibody levels in hen egg yolk during the immunization period. Levels of anti-whole cell and anti-LPS antibodies in egg yolk are expressed as ELISA absorbances (OD 405 nm) at 1:500 dilution using the whole cell and LPS as antigens, respectively. Booster injections were given at 2, 4, and 6 weeks after the first injection. Vertical bars show the standard deviation.

Antimicrobial Activity of Egg Yolk Antibody. The IgY solution was serially diluted with PBS and mixed with an equal volume of *S. enteritidis* suspension (10^7 cells/mL of PBS). Agglutination of the bacterial cells was observed after 1 h of incubation at 37 °C. Two volumes of Trypticase soy broth medium was added to the above mixture of IgY, and the mixture was then incubated at 37 °C for 2 h. The turbidity of the supernatant reflecting bacterial growth was measured at 600 nm, and values relative to that of the control medium (without IgY) were calculated (Shimizu *et al*., 1989). The data are expressed as the average at triplicate measurement.

RESULTS AND DISCUSSION

Production of Anti-*S. enteritidis* Antibodies in Hen Egg Yolk. Traditionally, the species chosen for antibody production have been of mammalian origin, most frequently rabbits, but recently there has been an increasing use of hens. There are several advantages of using hen instead of mammalian antibodies. The obvious advantage is the ease of collecting eggs from laying hens rather than collecting blood from animals. As hen IgY can be purified from yolk, there is no need to sacrifice the animal. Another advantage of chicken IgY is that hen eggs seem to be a suitable material for a large scale and low-cost production of IgG (Larsson and Sjoquist, 1990). Hens were immunized with *S. enteritidis* cells and its LPS, and the antibodies that were deposited into the egg yolk were analyzed by ELISA (Figure 1). The LPS gave higher sensitivity than whole cells. Anti-whole cell antibody activity appeared in the yolk 2 weeks later than that of anti-LPS antibody. The higher antibody activity of the yolk persisted longer than that of the serum antibody and was maintained at high levels by repeated injection at 1 month intervals (data not shown). Production of antibodies recognizing *S. enteritidis* and LPS was enhanced by FCA, and the antibodies were efficiently transferred to egg yolk,

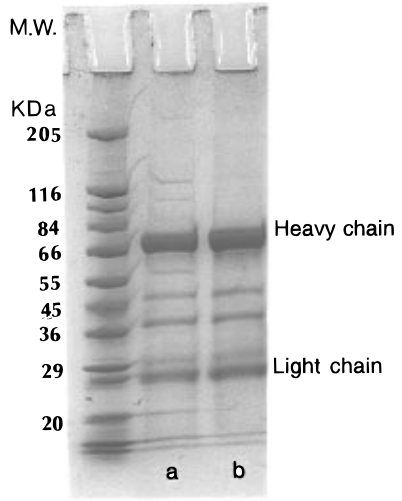

Figure 2. SDS−PAGE patterns of egg yolk antibodies: (a) anti-whole cell antibody; (b) anti-LPS antibody.

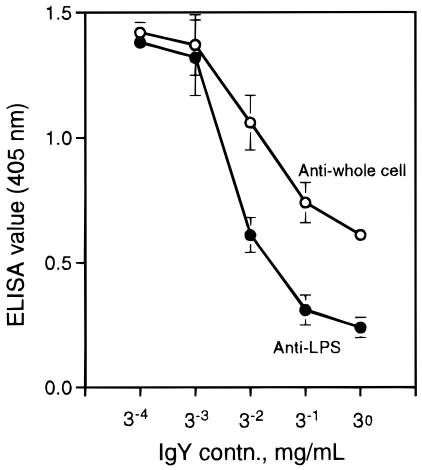

Figure 3. Antigen (LPS)-binding activity measured by competitive ELISA of anti-whole cell and anti-LPS antibodies. Data are the average of triplicate measurements, and error bars represent standard deviation.

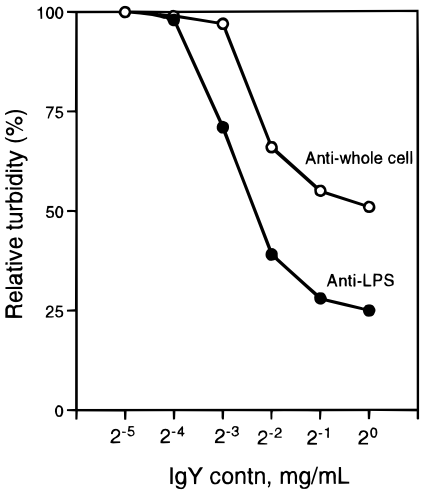

Figure 4. Inhibition of *S. enteritidis* growth by the antibodies against the whole cell and LPS. Data are the average of triplicate measurements.

giving higher antibody activity. In general, bacteria are usually injected into animals as antigen without adjuvant. However, the production of antibodies against *E. coli* cells and their LPS was enhanced by FCA (Shimizu *et al.*, 1989). In this study, I did not compare the effect of adjuvants on immunoresponse of hens. FCA must be useful to enhance the production of antibodies recognizing *S. enteritidis* whole cells and its LPS as well as *E. coli*. The crude IgY from egg yolk, which showed high anti-LPS and whole cell activities (e.g. eggs obtained in week 8 of the immunization period shown in Figure 1), was further purified by ammonium sulfate precipitation and cold ethanol precipitation (Akita and Nakai, 1992). The IgY obtained by using this method was subjected to electrophoresis, and its purity was about 80% as determined by the scanner (Figure 2). Some contaminating proteins (especially the 53 and 39 kDa proteins) from the water soluble fraction remained in the IgY fraction. The purity and specificity of antibody are required for the desired application of immunochemical techniques. The purity of the IgY purified by the above method could be enough for coating onto the magnetic beads. The activity was detected by ELISA with sensitivities of 0.002−0.8 mg of IgY/mL. The antigen binding activities of the anti-whole cell and LPS antibodies were compared by competitive ELISA (Figure 3). The anti-LPS antibody showed higher binding activity than anti-whole cell antibody. Anti-whole cell antibody might include antibodies against other cell surface proteins in addition to LPS in *S. enteritidis*.

Antimicrobial Activity of Anti-Whole Cell and Anti-LPS Antibodies. Anti-*S. enteritidis* whole cell and anti-LPS antibodies were diluted with PBS and mixed with the bacteria suspension (Figure 4). The cell agglutinating activities (the grade of sample dilution at which agglutination of the cells was observed) of anti-*S. enteritidis* whole cell and anti-LPS antibodies were 2^4 and 2^3, respectively. The anti-LPS antibody also showed higher bacterial agglutinating activities and more strongly inhibited the cell growth of *S. enteritidis* than did anti-*S. enteritidis* whole cell. These results agree with the ELISA data shown in Figure 3. The antibody against *E. coli* also aggregated the bacterial cells and inhibited their growth *in vitro* (Shimizu *et al.*, 1989). The inhibition of cell growth was probably caused by intense agglutination of bacterial cell in the presence of IgY, preventing cell division and growth. It is suggested that LPS in *S. enteritidis* may be an important immunogenetic site in the cell surface.

Separation of *S. enteritidis* in Eggs by Immunomagnetic Beads. Immunochemical methods based on the specific affinity between the antigen and antibody have been shown to be very effective for the separation of specific targets from a heterogeneous suspension. A close correlation between competitive ELISA and agglutination test was found (Figures 3 and 4). For optimizing the coating of the antibody to the magnetic beads, the effect of varying the amount of antibody to the immunomagnetic beads for the separation of *S. enteritidis* was studied (Figure 5). A marked reduction in the number of cfu of *S. enteritidis* was found when the binding IgY (anti-LPS antibody) was increased to over 20 mg of IgY/mg of beads. Microscopic examination also showed that the immunomagnetic beads with more linked IgY formed more aggregates. In subsequent experiments, a ratio of 20 mg of IgY/mg of the beads was used, giving nearly maximum binding capacities without using too much antibody.

The hen egg yolk IgY immobilized on immunomagnetic beads was successfully used for isolation of the target bacteria from experimentally contaminated liquid egg (Figure 6). An increase of the incubation time from 3 to 10 min increased the maximum bacteria reduction

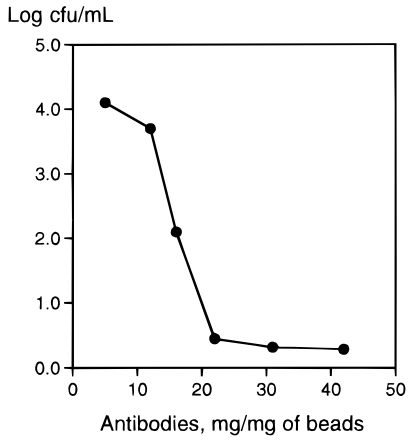

Figure 5. Effect of the amount of immobilized antibody relative to immunomagnetic separation. The residual number of *S. enteritidis* colonies after separation by using the antibody immobilized beads is shown. Data are the average of triplicate measurements.

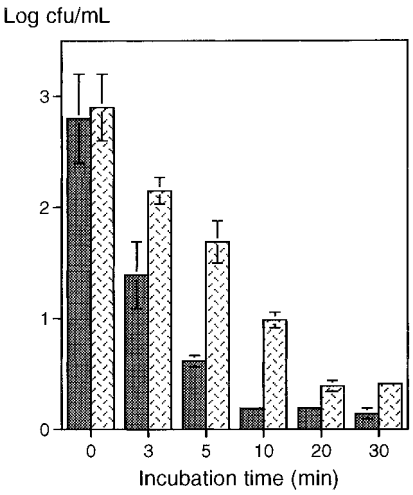

Figure 6. Changes of *S. enteritidis* cfu after treatment with immunomagnetic beads as a function of incubation time: (dark bar) anti-LPS antibody; (patterned bar) anti-whole cell antibody. Data are the average of triplicate measurements, and error bars represent standard deviation.

level from 50.4 to 93.2% by anti-LPS antibody. The adsorbed level of bacteria reached a plateau with an incubation time of 30 min. On the other hand, the adsorption rate of the anti-whole cell antibody coated beads was slower than that of anti-LPS antibody. These results are well correlated with the antimicrobial activity of the antibodies (Figure 4). The reason for the more effective separation of the bacteria using anti-LPS IgY than anti-whole cell antibody is not clear from the present work. It may be closely related to the specificity of the antibody and surface structure of *S. enteritidis*. Outer membrane LPS are an important bacterial cell wall of *S. enteritidis*. LPS are composed of three components: lipid A, inner core, and O-side chain oilgosaccharides (Lindberg, 1980). The O-side chain is composed of repeating oligosaccharide units, which vary between *Salmonella* species. Differences in length and composition significantly alter the virulence (Finlay and Falkow, 1988). However, their immunogenecity is not clear. The present work revealed that anti-LPS IgY is more effective in separating *S. enteritidis* from liquid eggs, indicating its suitability for use in the isolation of live *S. enteritidis* from food. The immunocapture step employs antibodies immobilized on magnetic beads for the detection of bacteria by eliminating a selective enrichment procedure. Further investigation on antibody specificity of anti-LPS IgY including main epitope mapping recognized by hens could be useful for further application of hen IgY for the immunoassay and separation of *S. enteritidis* in eggs.

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