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Anti-Bacterial Activities of Chicken Egg Yolk Antibody (IgY) against Enteric Pathogens

By

Eun-Na Lee 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall 2000

University of Alberta

Library Release Form

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Degree: Master of Science

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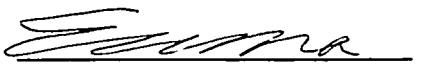

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Anti-Bacterial Activities of Chicken Egg Yolk Antibody (IgY) against Enteric Pathogens** submitted by **Eun-Na Lee** in partial fulfillment of the requirements for the degree of **Master of Science** in **Food Science and Technology.**

J. S. Sim, Supervisor

R. O. Ball

M. R. Suresh

Date: Sept 28 /2000

Abstract

Hyperimmunized laying chickens against antigenic proteins, viruses or bacterial origin produce antibodies (IgY) specific against the immunized antigens both in the egg yolk and serum. This system exploits a wide range of potential applications in food production. In this research, the anti-bacterial properties of IgY were investigated. A flock of Single Comb White Leghorn (SCWL) chickens were hyperimmunized with four enteric pathogens, including *Escherichia coli* (*E. coli*) O157:H7, *E. coli* 987P, *Salmonella enteritidis* or *S. typhimurium*, which are today's most threatening enteric microorganisms associated with food poisoning pathogens in humans and animals.

Polyclonal IgY loaded eggs were produced and then the water-soluble fraction (WSF) containing IgY (average 28.8% of the total protein) was prepared. Specific binding activity and concentration (average 9.5% of total IgY content) of the specific antibody were determined by the ELISA method using bacterial whole cells. A high degree of cross-reactivity of anti-*Salmonella* IgY between *S. enteritidis* and *S. typhimurium* was demonstrated. Specific IgY inhibited bacterial growth *in vitro* to the maximum of 2.3 log CFU/ml reduction in bacterial counts. The specific binding activity of IgY was further demonstrated in the immunofluorescence and immunoelectron micrographs, which revealed the structural alterations of bacterial surface bound by specific IgY molecules.

In conclusion, this research work shows potential applications of egg yolk antibodies as anti-bacterial prophylactic uses for infectious diseases and suggests an antibody farming concept as an alternative to conventional egg farming.

To

God

And

My family

Who are always with me

I dedicate this thesis

Acknowledgments

I would like to thank my respectful supervisor, Dr. J. S. Sim, with all my heart for his guidance and inspiration throughout the course of my studies. I am also sincerely grateful to my committee members, Dr. M. R. Suresh and Dr. R. O. Ball for their academic direction. Special thanks are extended to Dr. J. S. Sim and Dr. M. R. Suresh, who have given me the valuable opportunity to further my studies.

Sincere thanks are expressed to Dr. H. H. Sunwoo for his invaluable advice and consideration, which aided me to complete my studies. I am indebted to Mr. K. Manninen for placing the facilities at my disposal and making revisions to my thesis. To my colleagues, Ms. L. Chui and Mr. Y. Wang, I am grateful for their assistance and encouragement. The instruction and help of Dr. M. Chen for electron microscopy and other technical staff throughout various experiments are deeply acknowledged as well.

I wish to thank my dear friends for their companionship which provided me with comfort and encouragement. Special thanks are also expressed to my uncle, aunt and cousin for their support and concern. To many other people, I am grateful for the enriching spare time that I have had while studying.

I would like to express my deep appreciation to my family: Parents, sister, brother-in-law, brother, and niece. Without their prayer, love, concern, and inspiration, I would not have accomplished my studies.

Most of all, gratitude and honor be to God with all my heart for his blessings upon me, and the opportunity to be thankful for all the above.

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List of Abbreviations

Abbreviation	Meaning
BSA	Bovine serum albumin
C	Constant domain
CDRs	Complementarity-determining regions
CFU	Colony-forming unit
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
H	Heavy chain
h	Hour
Ig	Immunoglobulin
L	Light chain
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
min	Minute
OD	Optical density
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PBS-Tw	Phosphate buffered saline containing 0.05% Tween 20
rpm	Revolution per minute
SCWL	Single comb white leghorn
TSB	Tryptic soy broth
V	Variable domain
vol	Volume
wk	Week
WSF	Water-soluble fraction
wt	Weight

Chapter 1. General Introduction

1.1. Chicken Egg Yolk Antibody (IgY)

Chickens can produce antibodies in blood against foreign substances in host defense where antibodies can specifically bind their antigens to neutralize their effects. There are three kinds of immunoglobulins (Igs), IgG, IgA, and IgM in blood serum, which are distinguishable in concentration, structure, and immunological function. The major immunoglobulin is IgG, which makes up about 75% of the total immunoglobulin pool. The concentrations have been reported to be 5.0, 1.25, and 0.61 mg/ml serum for IgG, IgA, and IgM, respectively (Lesile & Martin, 1973).

Antibodies are transferred from hen to chick *via* the latent stage of the egg, and play an important role in immunological function for the relatively immuno-incompetent chick to resist various infectious diseases (Schaaf, 1959; Parry & Porter, 1981). Immunoglobulin, especially IgG, is selectively secreted from the hen's circulatory system across the oolemma into the maturing oocyte in the ovarian follicle (Rose & Orlans, 1981). This transfer occurs via a receptor-mediated mechanism and an ovarian IgG receptor allows the selective transport of all IgG subpopulations presented by the maternal blood (Brambell, 1970; Locken & Roth, 1983).

Maternal IgA and IgM are incorporated into the egg white in the oviduct along with the egg albumen secretion. IgA and IgM in egg white are subsequently transferred to the embryonic gut via swallowed amniotic fluid (Rose et al., 1974) and IgG in egg yolk circulates in the blood of the chick *via* the endoderm of the yolk sac (Patterson et al.,

1962). The concentrations of IgA (~0.7 mg/ml) and IgM (~0.15 mg/ml) in egg white are relatively low while that of IgG (8~25 mg/ml) in egg yolk is considerably high (Rose et al., 1974).

The antibody response in the chicken serum is reflected in the corresponding egg yolk with a delay of approximately a week, which suggests that the level of serum antibody may have to reach a certain level before the serum antibody transfers to the yolk. The correlation of specific antibody levels between the serum and the egg yolk exhibits a significant linear pattern (Losch et al., 1986; Bollen & Hau, 1999). The concentrations of egg yolk antibodies have a constant relationship with those of serum antibodies and are on average 1.23 ± 0.07 times greater than their serum counterparts (Wolley & Landon, 1995).

The major antibody, IgG, in both blood serum and egg yolk has been termed IgY (Leslie & Clem, 1969) because it is distinct from mammalian IgG in structural and functional properties.

1.2. Characteristics of IgY

1.2.1. Structure

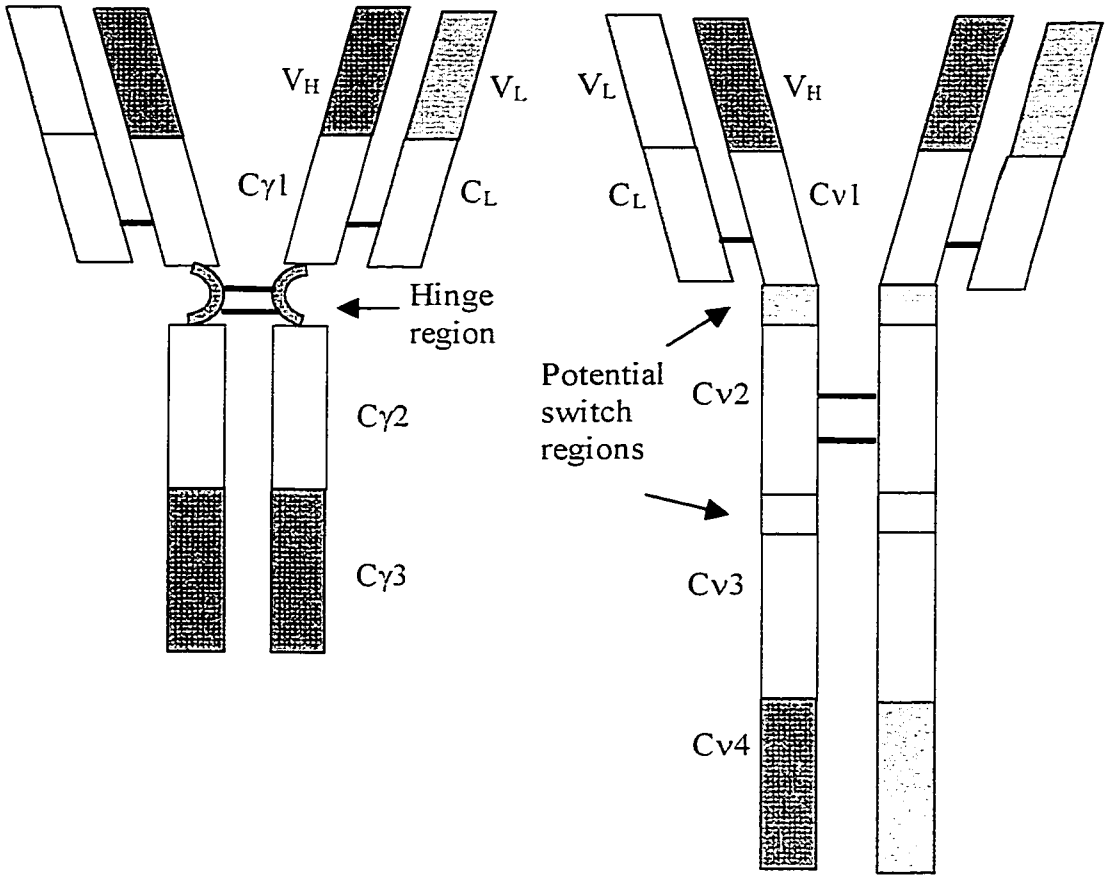
IgY consists of two identical heavy (H) chains and two identical light (L) chains, which are linked by disulfide bridge. IgY has a molecular mass of ~180 kDa which is heavier than that (~150 kDa) of mammalian IgG. The H chains of IgY possess one variable domain (V), four constant domains (C) and no genetic hinge, unlike mammalian IgG which has three C domains and a hinge region. The molecular structure of IgY,

therefore, is similar to mammalian IgM or IgE, which consist of four C domains. Comparisons of C-region sequences in IgG and IgY show that the Cγ2 and Cγ3 domains of IgG are most closely related to the Cv3 and Cv4 domains of IgY, respectively, and that the equivalent of the Cv2 domain is absent in γ chains of IgG (Figure 1-1). The Cv2 domain is probably 'condensed' to form the structure similar to the IgG hinge region (Burton, 1987; Parvari et al., 1988; Fellah et al., 1993; Magor et al., 1994).

In IgY, there are regions near the boundaries of the Cv1- Cv2 and Cv2- Cv3 domains that contain proline and glycine residues. These regions have the potential to confer limited flexibility on the molecule, in a manner analogous to the 'switch' regions in some mammalian Igs (Helm et al., 1991). The content of β-sheet structure in C domains of IgY is lower than that of mammalian IgG; therefore, the conformation of IgY domains is more disordered in comparison to mammalian IgG. Intramolecular force as well as non-covalent interaction between H and L chains are important factors to support the protein conformation. Intrachain disulfide linkage between the V region and C region of L-chain to stabilize the structure of the mammalian IgG L-chain is not present in the IgY L-chain and thus intramolecular forces of IgY are weaker than those of mammalian IgG (Shimizu et al., 1992).

The structural properties of IgY (e.g. molecular size, lower flexibility, conformation of domains, intramolecular bonding) are considered to influence the overall properties of IgY molecule. They also may be structural factors that have bearing on the lower molecular stability of IgY compared to mammalian IgG (Pilz et al., 1977; Shimizu et al., 1992).

Figure 1-1. Comparison of structures of IgY and mammalian IgG



Mammalian IgG **Chicken IgY**

1.2.2. Stability

The conformation stability of IgY is lower than that of mammalian IgG in any type of treatment such as acid, heat, and proteolytic enzyme, which suggests that the overall stability of IgY molecule is lower than that of mammalian IgG molecule (Otani et al., 1991; Shimizu et al., 1992; Hatta et al., 1993).

The stability of IgY to acid and alkali has been studied under various conditions. It was found that the activity of IgY was decreased at pH 3.5 or lower and almost completely lost with irreversible change at pH 3.0. Rapid decrease of the IgY activity at low pHs indicated conformational changes and damage in the Fab portion including the antigen-binding site. Under alkaline conditions, the activity of IgY did not change until the pH increased to 11; however, it was markedly diminished at pH 12 or higher (Shimizu et al., 1988, 1992).

IgY has been thermally treated at various temperatures for different periods of time. The binding activity of IgY with antigen decreased with increasing temperature and heating time. The activity of IgY decreased by heating for 15 min at 70°C or higher (Shimizu et al., 1988, 1992) and IgY denatured seriously when thermally treated at temperatures higher than 75°C (Chang et al., 1999). IgY is relatively stable to pressure as reported with no detectable inactivation of IgY by pressure up to 4,000 kg/cm^2 (Shimizu et al., 1994).

Freezing and freeze-drying are low temperature processes that are usually considered to be less destructive. However, proteins may suffer loss of activity as a result of conformational changes, aggregation or adsorption (Skrabanja et al., 1994). There have been some reports on the stability of IgY in regards to these methods. Freezing and

freeze-drying did not affect the activity of IgY unless repeated several times (Shimizu et al., 1988). Chansarkar (1998), however, showed that frozen or freeze-dried IgY resulted in some loss of antigen-binding activity and a significant drop in the solubility under the conditions of high salt and protein concentrations.

IgY is relatively resistant to trypsin or chymotrypsin digestion, but is fairly sensitive to pepsin digestion. The tryptic digestion of IgY retained its antigen-binding and cell-agglutinating activities in spite of a definite breakdown of the polypeptides. Unlike the trypsin digestion, no definite cleavage of the IgY chains was observed for chymotryptic digestion and the activities of IgY remained high for these digests (Shimizu et al., 1988; Otani et al., 1991). Hatta et al. (1993) also demonstrated the behavior of IgY against trypsin and chymotrypsin, showing that 39% and 41% of the activity by neutralization titer remained for the mixture with trypsin and chymotrypsin, respectively, after 8 h of incubation. The stability of IgY against pepsin appears to be highly dependent on pH and the enzyme/substrate ratio. At pH 5 or higher, IgY was fairly resistant to pepsin and retained its antigen–binding and cell-agglutinating activities. However, at pH 4.5 or below, both activities were lost (Shimizu et al., 1988). The neutralization titer of IgY with pepsin under the different incubation time and pH was observed by Hatta et al. (1993). The results confirmed the susceptibility of IgY to pepsin at low pH, which showed the loss of IgY activity at pH 2 and 91% and 63% of activity at pH 4 after 1 h and 4 h incubation time, respectively.

1.2.3. Immunological Properties

The structural characteristics of IgY is relevant to the immunological properties,

which are somewhat different from those of mammalian IgG. IgY has a valency of ~2.0, reflecting large antigen-binding sites, and binds an antigen strongly. However, IgY displays precipitating or agglutinating properties only at raised salt concentrations (Kubo et al., 1973). The two Fab arms may be so closely aligned that steric hindrance precludes the cross-linking of epitopes on two large antigens. The effect of conditions that permit those properties (e.g., salt or low pH) would be to release the Fab arms, providing functional independence of the binding sites (Gallagher & Voss, 1974).

The differences of Fc regions between IgY and mammalian IgG, which include number and nature of carbohydrate chains, flexibility of switch region and the number of constant regions, lead to the different interaction of IgY with molecules as an antigen in comparison to that of mammalian IgG (Table 1-1).

Antibody diversity is achieved differently in chickens than in mammals (Reynaud et al., 1985; Weill & Reynaud, 1987). The IgY light chain locus consists of a single J-C unit to which the same V gene becomes rearranged in nearly all bursal cells. The chicken light chain repertoire thus appears to be of an extremely somatic type (Reynaud et al., 1985). Consequently, the overall antibody diversity of IgY is lower than that of mammalian IgG, which is indicative of the restricted diversity of IgY specificities.

1.3. Production of IgY

1.3.1. Influencing Factors

Immunization of laying hens with an antigen is required to obtain high specific antibodies against a target antigen. Immunization protocols should be practical and effec-

Table 1-1. Comparison of immunological properties of IgY and mammalian IgG

Interaction	IgY	Mammalian IgG
Interference with mammalian IgG	No	Yes
Interference with rheumatoid factor	No	Yes
Interference with human anti-mouse IgG antibody	No	Yes
Activation of mammalian complement	No	Yes
Protein A/G binding	No	Yes
Mammalian Fc receptor binding	No	Yes

Based on Larsson et al. (1993)

-tive to ensure the large production of high specific antibodies. There are several critical factors that influence antibody production dependent on egg-laying capacity and immune response of chickens.

The egg-laying capacity of chickens can be a primary factor related to the large production of antibodies. The strain of chickens, the immunization time and the nature of antigens or adjuvant have an influence on the egg production. Li et al. (1998) showed that the Single Comb White Leghorn (SCWL) chickens produce twice eggs as many as the Rhode Island Red (RIR) hens. The laying performance of chickens can be affected by the stress derived from the immunization. Therefore, the immunization of chickens prior to starting laying can decrease the adverse effect of the stress (Schade et al., 1996). The immunization of chickens with Freund's complete adjuvant (FCA) significantly reduces the egg-laying frequency in comparison to the use of Freund's incomplete adjuvant (FIA) (Bollen & Hau, 1999). The egg-laying capacity of chickens is also influenced by the kinds and origin of antigens used for the immunization (Schade et al., 1994).

The immune response of chickens to antigens is an important determinant to raise high specific antibodies. In addition to the strain of chickens, the properties of an antigen and adjuvant and the route, frequency, and interval of immunization are of critical importance in inducing the strong immune response of chickens. Chicken strains used for breeding purpose show higher antibody responses than those used commercially for egg production due to the healthier status of inbred chickens (Schade et al., 1996). The type and dose of antigens also should be considered since the extent of an antibody response is directly related to the foreign nature of the immunogen for the host to which an optimal antigen dose should be used. Hatta et al. (1997a) reported that the percentage of specific

antibody in total IgY ranged from 5 (anti-insulin antibody) to 28% (anti-mouse IgG antibody). Using bovine IgG and lactoferrin as antigens, approximately 10-15% of total IgY were specific antibodies (Akita & Li-Chan, 1998; Li-Chan et al., 1998). The immunopotentiating actions of various adjuvants have been compared, demonstrating that FCA resulted in a higher immune response of chickens than FIA and Hunter's TiterMax adjuvant (Bollen et al., 1996). However, the adverse effects of FCA, including the reduction in egg production and long-term tissue damage of chickens, were observed in some studies (Erhard et al., 1997; Bollen & Hau, 1999). There are different immunization methods of chickens; intramuscularly (Akita & Nakai, 1993), subcutaneously (Svendsen et al., 1995), intrabursally and intracoelomicly (Elfaki et al., 1992). Intramuscular immunization in the breast muscle and subcutaneous immunization in the neck are recommended for young chickens and older chickens, respectively. The total number of immunizations required depend on the type and dose of the antigen as well as the particular adjuvant employed. Two to four booster injections are usually required to maintain production of high levels of specific antibodies, which appear at 5-6 weeks after the initial immunization and continue for up to a year (Schade et al., 1996; Li-Chan, 2000).

1.3.2. Comparison to Mammalian IgG Production

Mammals such as rabbits, mice, sheep and goats are traditionally the most common sources of antibody production that require an adequate amount of antibodies with high specificity and high avidity (Warr, 1982). Chickens are recognized as a

potential alternative for antibody production since they meet the above requirements and moreover have advantages over mammals.

A laying hen produces an average of 240 eggs in a year with a yolk volume of approximately 15 ml/egg. The amount of serum collected from an immunized rabbit per year is about 40 ml. One gram of egg yolk contains about 10 mg of IgY whereas 1 ml of rabbit serum yields about 35 mg of IgG. Consequently, an immunized hen and rabbit produce 36 g and 1.4 g of antibodies/year, respectively. This is indicative of the higher productivity of chickens compared to mammals of similar size. Only large mammals such as cows or horses can produce more antibodies than a laying hen (Larsson et al., 1993; Hatta et al., 1997a).

In general, the development of a chicken immune response closely resembles the curves observed in mammals. This classical response to immunization comprises the primary response showing low titer and low affinity antibodies followed by an enhanced secondary response (Kokko & Karenkampi, 1992; Wolley & Landon, 1995). In contrast, there have been reports on the primary response characterized by high titer and high affinity antibodies and little change in response to re-immunization (Steinberg et al., 1970; Losch et al., 1986). Discrepancies in the responses of chickens to immunization may originate from differences in B cell population sizes between individuals (Wolley & Landon, 1995).

There have been some studies that show the higher production of specific antibodies in mammals than in chickens. Specific antibodies produced by sheep were six to ten times greater than those produced by chickens (Erhart et al., 1991; Woolley & Landon, 1995). Bollen et al.(1996) showed that the response elicited by rabbits was 1.5-

2.0 times higher compared to chicken response. However, it can not be ruled out that these differences may be derived from the shorter half-life of chicken IgY and less sensitivity to adjuvant in comparison to mammalian IgG (Patterson et al., 1962; Watson, 1992; Steinberg et al., 1970; Erhart et al., 1991).

Antibodies obtained from mammals and chickens are comparable in terms of specificity and avidity. Mammalian antibodies were found to possess slightly higher specificity and similar or lower avidity for their antigens as compared to chicken antibodies (Ikemori et al., 1993; Woolley & Landon, 1995).

1.4. Isolation of IgY

Lipids and proteins are the major constituents of egg yolk. The lipid fraction, including triglycerides, phospholipids, and cholesterol, constitutes approximately one-third of the yolk and mainly egg yolk solids (Powrie, 1976). Proteins consist of 15-17% of the yolk, which can be separated by centrifugation into particles, 'the granules' and a clear fluid supernatant, 'the plasma' (Stadeklman & Cotterill, 1977). Granules are composed of 70% α- and β- lipovitellins, 16% phosvitin, and 12% low-density lipoproteins (LDL) (Burley & Cook, 1961). Plasma is about 78% of the total yolk proteins and composed of a lipid-free globular protein, livetin (α-, β-, and γ-) and low-density lipoproteins (MaCully et al., 1962). IgY is known as γ- livetin and exists in egg yolk plasma proteins together with two other water-soluble proteins, α- and β-livetin, and lipoprotein; therefore, separation of IgY or γ-livetin requires extraction of the water-

soluble fraction (WSF) from yolk lipoprotein followed by purification from other livetins (Polson & Von Wechmar, 1980).

The WSF can be obtained by using the water dilution method based on the aggregation of yolk lipoproteins at low ionic strengths as reported by Jensenius et al. (1981). Centrifugation or filtration is subsequently used to fractionate the WSF from water-insoluble components of egg yolk (Kwan et al., 1991; Akita & Nakai, 1992). For extraction of the WSF from egg yolk with water, two factors are of critical importance: the pH and the extent of egg yolk dilution (Akita & Nakai, 1992).

The pH has an influence on the amount of lipids in the WSF. Acidic conditions change the integrity of the egg yolk granules and lead to increases in the lipid binding ability. Therefore, lowering pH results in not only increasing the recovery of IgY but also decreasing the amount of LDL in the supernatant. The WSF was almost devoid of lipids at mild acidic conditions (pH 4.6 to 5.2) and the highest yield of IgY was obtained between pH 5.0-5.2 (Akita & Nakai, 1992).

The amount of protein recovered in the WSF decreases with increasing dilution factor. In contrast, the purity of IgY recovered in the WSF is higher with increasing dilution factor, probably due to greater removal of other protein components into the water-insoluble fraction. Eight to ten-fold dilution is preferred for more efficient separation of lipid (90-93%) from the WSF, with moderate (60%) recovery of IgY (Kwan et al., 1991).

In addition to the water dilution method, many IgY isolation methods have been reported; separation of lipoprotein by ultracentrifugation (McBee & Cotterill, 1979), delipidation by organic solvents (Bade & Stegemann, 1984), and precipitation of

lipoproteins by polyethyleneglycol (Polson et al., 1985), sodium dextran sulfate (Jensenius et al., 1981), polyacryl acid resins (Hamada et al, 1991), carrageenan and xanthan gum (Hatta et al., 1990). Akita & Nakai (1993) compared the water dilution method to other methods such as polyethylene glycol, sodium dextran sulfate, and xanthan methods in terms of the yield, purity and activity of IgY. The water dilution method yielded IgY in the highest level (91%), purity (31%) and with similar activity to that obtained by using other methods.

For the wide utilization of IgY, a large-scale production of IgY with high recovery and purity from egg yolk is necessary. Such a separation process should be simple and economical requiring few chemicals for food applications. In view of these requirements, the water dilution method appears the most appropriate technique, offering the following advantages over other isolation methods.

1. Simple and rapid procedure;

2. Production of high yields of functionally active IgY with varying levels of purity;

3. Easily scaled-up for large-scale production of IgY;

4. Enabling the utilization of the rest of the egg yolk as a food product or further fractionation of other biologically active components (Akita & Nakai, 1993).

The isolation of the WSF containing livetins from the yolk granule is followed by the additional step to separate IgY from the other water-soluble proteins, α- and β-livetins, and remaining LDL. Various methods used for the purification of IgY have been reported, including ultrafiltration (Akita & Nakai, 1992), ion exchange chromatography

(McCannel & Nakai, 1990) and metal chelate interaction chromatography (Cam & Peter, 1997).

1.5. Advantages of IgY

Chickens are potent antibody producers that can serve as a successful substitution of mammals. Chickens offer several advantages in terms of economical and practical aspects. Maintenance of a large flock of laying hens is economical because of large-scale feeding of hens (Hamada et al., 1991). Vaccination of laying hens to control various avian infectious diseases is already a common practice, which is well accepted by both poultry farmers and the consuming public (Hatta et al., 1997a). The number of chickens used for antibody production can be reduced due to their capabilities of producing larger amounts of antibodies as compared to the number and capability of mammals (Schade et al., 1996).

Polyclonal antibody production in mammals involves three procedures: immunizing with antigens, blood sampling, and purification of antibodies. In chickens, egg yolk serves as an antibody source, replacing blood sampling. The collection of egg yolk is less labor intensive and more hygienic than blood sampling (Hatta et al., 1997a). In addition, chickens are not distressed by the non-invasive sampling method, which is compatible with modern animal protection regulations (Gottstein & Hemmeler, 1985).

Egg yolk can be separated automatically by using a machine, which enables IgY production to be largely scaled-up. Egg yolk contains only IgY while mammalian blood contains IgG together with other Ig classes (Hatta et al., 1997a). Therefore, the isolation

of IgY from egg yolk can be more simply carried out than that of mammalian IgG. This, combined with the all above advantages, characterize IgY production as less expensive and more convenient (Polson & Von Wechmar, 1980).

Chickens display strong and stable immune responses for a long laying period, which is indicative of long-term use of chickens for antibody production (Tsunemitsu et al., 1989; Kuroki et al., 1993). Chickens can also produce more specific antibodies against mammalian antigens because of the phylogenetic distance between avian and mammals (Jensenius et al., 1981). That is, IgY as a polyclonal antibody can frequently recognize more sites on a mammalian antigen as foreign. The restricted diversity of IgY, combined with this attribute, can make it more possible to produce specific antibodies against antigens that are well-conserved and rarely immunogenic in mammals (Reynaud et al., 1985; Song et al., 1985). There have been numerous studies that succeeded in producing IgY specific to low immunogenic antigens against mammals (Table 1-2).

1.6. Applications of IgY

1.6.1. Existing Applications

1.6.1.1. Passive Immunization

Pathogens, including bacteria, viruses, parasites, and toxins, invade the body either through a number of natural entry routes (e.g., respiratory tract, gastrointestinal tract, and genitourinary tract) or through unnatural routes opened up by breaks in mucous membranes or skin (Kuby, 1997c). The gastrointestinal (GI) tract, which is a major route of invasion, possesses a protective characteristic of immunological tolerance to ingested

Table 1-2. Production of IgY specific to low immunogenic antigens against mammals

Antigen	Reference
Proliferating cell nuclear antigen of calf thymus	Gassmann et al., 1990
Heat-shock protein (Hsp 70)	Gutierrez & Guerriero, 1991
Human insulin	Lee et al., 1991
Rat glutathion peroxide	Yoshimura, 1991
Peptidylglycine α-amidating enzyme	Sturmer et al., 1992
Von Willebrand factor	Toti et al., 1992
Platelet glycoprotein Iib-IIIa	Toti et al., 1992
Parathyroid hormone related protein	Rosol et al., 1993
Mouse erythroprotein receptor	Morishita et al., 1996

Based on Sim et al. (2000)

foreign materials (Tomasi & McNabb, 1987). Secretory IgA is the predominant immunoglobulin class in external secretions and plays an important role in the immune protection of the GI tract. IgA produced by GI tract-associated lymphoid tissue is transported into the lumen of the GI tract and assists in protection against infections by inhibiting binding, preventing colonization, or neutralizing toxins (Williams & Gibbons, 1972). However, the secretory IgA system is absent or inadequate in certain circumstances, where pathogens cause infectious diseases. A particularly vulnerable period is in the weeks and months after birth, when the immune system is still immature (Stiehm& Fudnberg, 1966). Numerous studies have documented the role of colostrum and breast milk in protecting the newborn against GI tract infections (Welsh & May, 1979; Glass et al., 1983; Jason et al., 1984). Furthermore, non-maternal antibodies have been used for passive immunization to protect young humans or animals from infectious diseases (Hilpert et al., 1987; Davidson et al., 1989; Schaller et al., 1992).

Passive immunization differs from active immunization in the provision of an antibody obtained from other animals. Pathogen-specific antibodies administered orally or systemically neutralize infectious activity or toxicity of the antigens. For effective passive immunization, the administration of antibody requires large amounts of antibody and thus a practical method is needed to prepare antibodies. Antigen-specific IgY can be produced on a large scale from eggs laid by chickens immunized with selected antigens (Hatta et al., 1997a). Therefore, the use of IgY for passive immunization has been studied extensively, demonstrating its effectiveness in preventing or treating infectious diseases caused by various pathogens such as viruses and bacteria (Table 1-3).

Table 1-3. Effect of passive immunization by pathogen-specific IgY

Pathogen	Effect	Reference
Salmonella	• Preventing gut colonization and organ invasion in chicks infected with *S. enteritidis* • Protecting mice challenged with *S. enteritidis* or *S. typhimurium* from experimental salmonellosis • Preventing fatal salmonellosis in neonatal calves exposed with *S. typhimurium* or *S. dublin*	Opitz et al., 1993 Yokoyama et al., 1998a Yokoyama et al., 1998b
Escherichia coli	• Curing diarrhea affected piglets in a field study • Protecting neonatal calves from fatal enteric colibacillosis by K99-pilated enterotoxigenic *E. coli* (ETEC) • Preventing diarrhea in rabbits challenged with ETEC • Protecting pigs challenged with K88+ ETEC from *E. coli*-induced enterotoxemia • Reducing diarrhea incidences of neonatal calves in the field trial	Wiedemann et al., 1991 Ikemori et al., 1992 O'Farrelly et al., 1992 Yokoyama et al., 1993 Ozpinar et al., 1996
Streptococcus	• Reducing caries formation in rats infected with *S. mutans* • Preventing the establishment of *S. mutans* in dental plaque of humans	Hamada et al., 1991 Hatta et al., 1997b
Edwardsiella	• Preventing edwardsiellosis of Japanese eels infected with *Edwardsiella tarda*	Hatta et al., 1994
Rotavirus	• Preventing bovine rotavirus (BRV) induced diarrhea in murine model • Protecting calves from BRV disease • Protecting neonatal calves from BRV diarrhea under field conditions. • Preventing human rotavirus induced gastroenteritis in mice	Kuroki et al., 1993 Kuroki et al., 1994 Kuroki et al., 1997 Ebina, 1996
Coronavirus	• Protecting neonatal calves from bovine coronavirus-induced diarrhea	Ikemori et al., 1997
Infectious bursal disease (IBD) virus	• Protecting chicks from IBD	Eterradossi et al., 1997

Based on Sim et al. (2000)

IgY was found to be effective in passive immunization for therapeutic or prophylactic purpose. Wiedemann et al. (1991) reported that IgY is as successful as a common antibiotic therapy in curing piglets with diarrhea. IgY raised against fimbrial antigens of *Escherichia coli* was protective in newborn piglets against a challenge with homologous strains (Yokoyama et al., 1992; Kim et al., 1996).

1.6.1.2. Immunological Tool

A frequently used approach for the detection of antigens involves an immobilized capture antibody, an antigen, and a labeled detection antibody. The antibodies in this assay are usually derived from mammals and the samples to be tested are often serum and plasma (Larsson et al., 1993). If anti-mammalian IgG antibodies or complement are present in the samples, they may block the antigen binding sites of the capture antibody and cause false positive reactions (Boscato & Stuart, 1986, 1988; Larsson & Sjoquist, 1989). In a bacterial or viral specimen test, mammalian capture antibody may also result in erroneous reactions due to the binding activity to protein A/G expressed by bacteria probably existing in samples (Katz et al., 1985; Guss et al., 1986).

In contrast, IgY does not possess such immunological properties and thus can be used to avoid these interference problems (Zrein et al., 1988; Larsson et al., 1991, 1992; Larsson & Mellstedt, 1992). Many studies, therefore, have been conducted to show the feasibility of IgY application in diagnostic assays. Larsson et al. (1991, 1992) and Larsson & Mellstedt (1992) proved the potential of IgY for an efficient alternative tool to avoid interference by rheumatoid factors, human anti-mouse antibodies, or complement

in enzyme immunoassays. The use of IgY was also effective in detecting viruses by a solid phase protein A radioimmunoassay (Katz et al., 1985).

The high specificity of IgY against mammalian antigens is an additional advantage to IgY application for a diagnostic tool. The phylogenetic distance between chickens and mammals enables chickens to produce IgY specific to mammalian antigen, which makes it possible to detect a wider range of antigens, especially, those well-conserved in mammals. In addition, various biological compounds that occur at low concentrations have been proven to be detectable by using antigen-specific IgY. A variety of antigens against which IgY has been raised include: hematoside (NeuGc) (Hirabayashi et al., 1983), plasma kallikrein (Burger et al., 1985), 1.25-dihydroxyvitamin D (Bauwens et al., 1988), human dimeric IgY (Polson et al., 1989), human transferrin (Ntakarutimana et al., 1992), ochratoxin A (Clarke et al., 1994) and high-molecular weight mucin-like glycoprotein-A (HAGP-A) (Shimizu, 1995).

Moreover, IgY has been successfully used as an immunological tool for other immunoassays such as western blotting (Gutmann et al. 1995; Murata et al. 1996), rocket immunoelectrophoresis (Schade et al. 1997b), immunoprecipitation (Gutmann et al. 1995; Camenisch et al. 1999), immunogold labelling (Fortagens et al. 1997), and immunohistochemistry (Rosol et al. 1993; Schmidt et al. 1993).

Immunoaffinity chromatography is a process for the isolation and purification of target molecules, using immobilized specific antibodies directed against the target molecule. This technique is considered a simple and mild processe which can isolate materials with high purity, activity and stability. However, a more widespread use has been limited by high cost of the technique requiring large amounts of antibodies which

should fall within parameters such as the efficiency of immobilization, antigen-binding capacity, useful life and re-usability of immunoadsorbents (Yarmush et al., 1992; Škrabanja et al., 1994). IgY, which can be simply produced in large quantities and high titers, may reduce such limitations and replace other sources of polyclonal antibodies or monoclonal antibodies conventionally used in immunoaffinity chromatography (Kim & Li-Chan, 1998; Kim et al., 1999; Li-Chan, 2000). Therefore, immobilized IgY has been used successfully for the purpose of immunoaffinity isolation of lactoferrin (Li-Chan et al., 1998) and immunoglobulins from colostrum, milk or chesse whey (Akita & Li-Chan, 1998; Kim & Li-Chan, 1998).

1.6.2. Future applications

Advantageous properties as well as existing applications of IgY have spurred extensive studies to explore the potential of IgY for possible applications. In a recent study, the inhibitory effect of IgY on pig-to-human xenograft rejection was demonstrated, showing the possibility of IgY application in pig-to-human xenotransplantation (Fryer et al., 1999). The success of monoclonal antibody-based immunotherapy in colorectal cancer as reported by Holz et al. (1996) may suggest an additional future application of IgY. Accordingly, the range of areas in which IgY can be applied may be remarkably extended as studies in progress provide more results.

Above all, the promising role that IgY plays as a food supplement has received much attention based on adequate evidence. There are many considerations of various aspects to implement the use of an antibody as a food supplement, which are as follows (Facon et al., 1993).

1. Economical and practical immunization of antibody in donor animal;
2. Determination of most immunogenic antigen to raise most effective antibody;
3. Large-scale production of antibodies in large quantities;
4. Sterile, safe, and stable preparation without loss of antibody activity;
5. Available measurement of effective amount of specific antibody;
6. Synergistic effect of antibody;
7. Adverse effect of antibody;
8. Long-term and stable storage.

The immunization of chickens and the production of IgY have been well recognized to be simple, practical and economical as described in the above sections. A number of studies have also provided sufficient evidence of the suitability of IgY preparation for food supplementation: a safe and stable preparation of IgY by the water dilution method requiring no chemicals and resulting in no significant loss of IgY activity (60-90% of recovery) (Kwan et al., 1991; Akita & Nakai, 1993); the stability of IgY to pasteurization at 60°C for 3.5 min (Hatta et al., 1993); GRAS (generally regarded as safe) status of IgY given from both the USDA and FDA (Coleman, 2000). However, the susceptibility of IgY to heat (> 75°C) and acid (< pH 3.0) may be a hindrance to the application of IgY as a food supplement. Some investigations have solved this problem by developing effective means, which is addition of sugars, glycerol, or glycine to IgY solution to improve the stability of IgY under processing conditions such as heat, acid, and high-pressure treatment (Shimizu et al., 1994; Chang et al., 1999).

Synergistic or adverse effects that IgY obtains or gives may enhance or limit the applicability of IgY. Akita et al. (1998) suggested that a cocktail of IgY against various

antigens could possibly enhance the immunological effect of antibodies. Other protein components together with IgY in egg yolk can provide IgY with more resistance to heat or acid if IgY is used directly in the form of the whole egg yolk (Schmidt et al., 1989), which is indicative of an additional synergistic effect. On the other hand, an adverse effect may be encountered with the utilization of IgY. Allergenicity of IgY is the potential problem derived from the difference of IgY structure from that of mammalian IgG (Bernhisel-Broadbent et al., 1991). IgY was found to be less allergenic than egg white proteins, recognized as major egg protein allergens. The cross-reactivity between IgY and egg white proteins is also low, which indicates a low possibility of an adverse effect (Akita et al., 1999).

The preparation of IgY having appropriate storage properties is another essential consideration in its application as a food supplement. This may include storage of liquid products in the frozen state or at 2-4°C with added preservatives to retard microbial growth, or storage of dried products (Li-Chan, 2000). IgY preparations could be stored for 5 to 10 years at 4°C without significant loss in antibody activity and also retain their activities after 6 months at room temperature or 1 month at 37°C (Larsson et al., 1993). Freeze-drying for a dried product is a low temperature process, which is considered to minimize risk of bacterial growth and to be less destructive than spray-drying (Rousell & McCue, 1991). However, careful attention should be paid to this process which may lead to freezing and drying stresses (Pikal, 1994). In Chansarkar's study (1998) on the effects of freeze-drying, IgY which was freeze-dried at high protein (30 mg/ml) and low salt (0.14 M NaCl) concentrations showed no significant loss of solubility and antigen-binding activity.

To date there has been ample information to elucidate the potential application of IgY as a food supplement. Therefore, more studies are encouraged to explore immunological properties of IgY, especially the beneficial effects that are the key consideration for prospective IgY application.

1.7. Plan of Studies

1.7.1. Hypothesis

Monoclonal antibodies which are raised against one particular epitope of antigen have been utilized for a wide range of studies. Their characteristic of recognizing only one target epitope can be advantageous to studies on characterizing specific molecules of an antigen in comparison to polyclonal antibodies. There have been some reports on bacteriostatic or bactericidal effects of monoclonal antibodies against particular molecules possibly associated with bacterial growth (Sadziene et al., 1993, 1994; Yamaguchi et al., 1997; Lin et al., 1998). In other words, the reaction of antibodies with bacteria could result in the inhibition of bacterial growth *in vitro* without complement or phagocytosis.

The success of IgY application in passive immunization has been reported in many studies, demonstrating anti-microbial activities of IgY (Table 1-3). The effect of IgY on preventing infectious diseases caused by pathogenic bacteria suggests that IgY may suppress bacterial activity leading to infections by some mechanisms. Bacterial proliferation, following adhesion to host intestinal epithelium may be inhibited by bacteria-specific IgY.

On the basis of above findings, it was postulated that IgY directed against bacteria also might possess anti-bacterial properties resulting in bacteriostatic or bactericidal effects as shown in studies using monoclonal antibodies. Therefore, the hypothesis that bacteria-specific IgY may inhibit bacterial growth *in vitro* in the absence of complement or phagocytes could be suggested.

1.7.2. Objective of Studies

To substantiate the suggested hypothesis, the studies were aimed at investigating the growth inhibitory effect of IgY on enteric pathogens, including *Escherichia coli* O157:H7, *E. coli* 987P, *Salmonella enteritidis* and *S. typhimurium*, *in vitro*. Demonstration of an anti-bacterial effect of IgY may support the application of IgY as food or feed supplements that can possibly serve to prevent bacterial contamination of foods or feeds considered as major vehicles of bacterial infection in humans or animals.

The studies were performed by using simple and practical protocols in an effort to increase the suitability for food or feed production. Therefore, this characteristic may provide additional evidence to facilitate the implementation of IgY technology.

1.7.3. Outline of Studies

Figure 1-2. outlines the sequence of studies conducted with an ultimate goal to demonstrate the anti-bacterial effect of IgY. The details of studies, focused on simple and practical protocols, are as follows:

1. Chickens were immunized with bacterial whole cells which had not been purified for the provision of specific antigens.

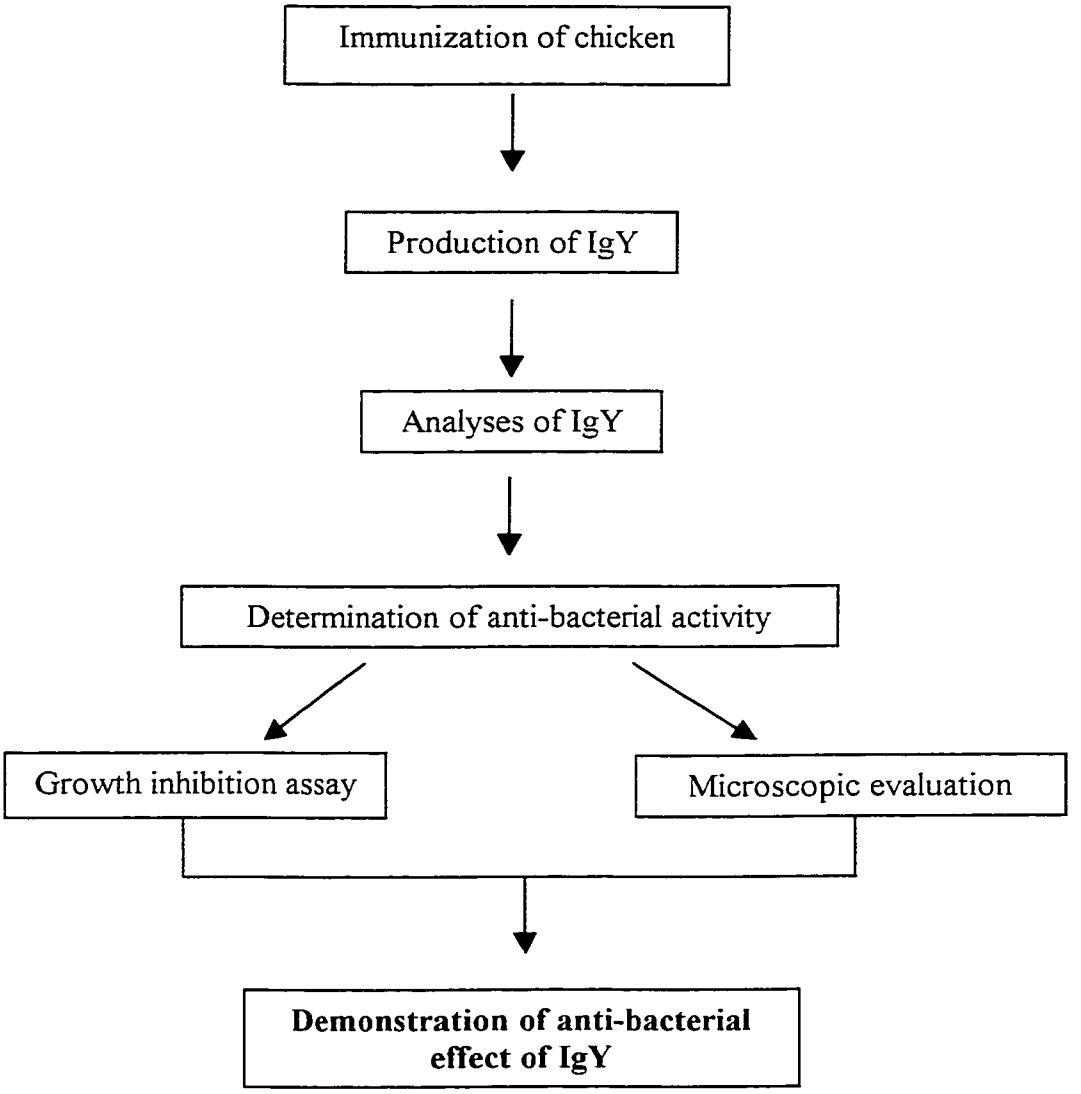
2. IgY was isolated in the form of water-soluble fraction from egg yolk by using the water-dilution method.

3. The water-soluble fraction containing high levels of specific IgY was concentrated to IgY powder as a dried product by using freeze-drying.

4. IgY powder was analyzed for specific IgY concentration by the quantitative ELISA method.

5. IgY powder was used for determining the anti-bacterial effect of IgY in growth inhibition assays and microscopic evaluation studies.

Figure 1-2. Outline of studies



Chapter 2. Growth Inhibitory Effect of IgY on *Escherichia coli* O157:H7

2.1. Introduction

Escherichia coli (E. coli) O157:H7 has been the major cause of increasing outbreaks of foodborne diseases. *E. coli* O157:H7 is classified in the enterohaemorrhagic *E. coli* (EHEC) group of pathogenic *E. coli*, which has major virulence properties, including adherence to the intestinal epithelium and production of verocytotoxin (Shiga-like toxin). *E. coli* O157:H7 causes a spectrum of diseases ranging from a mild diarrhea to haemorrhagic colitis, haemolytic uraemic syndrome (HUS) and, in some cases, death (Griffin et al., 1988; Padyhe & Doyle, 1991, 1992). Dietary risk factors associated with *E. coli* O157:H7 infection have found sources from apple cider, vegetables as well as foods of bovine origin such as undercooked ground beef and raw milk (Bell et al, 1994; Besser et al., 1993; Keene et al., 1997; Watanabe & Ozasa, 1997; Ackers et al., 1998).

In view of the magnitude and severity of outbreaks of foodborne diseases caused by *E. coli* O157:H7, there is an urgent need to reduce or eliminate related risk factors. However, it is difficult to take preventive measures due to the ability of organisms to colonize in the intestines of healthy animals and their resistance to acid, dehydration, and high salt concentrations (Glass et al., 1992; Benjamin & Datta, 1995; Tilden et al., 1996). Although technologies such as chlorination and irradiation have been developed to prevent food contamination from the pathogens, potential problems related to adverse effects of their use in food safety must be elucidated (Brotman et al., 1995). Therefore,

alternative methods satisfying food safety remain to be explored for the prevention of foodborne bacterial infection.

As a food grade for the antibody production, chicken egg yolk antibody (IgY) has been extensively studied to substantiate the effective use for passive immunization in a form of feed additive (Leslie & Clem, 1969; Shimizu et al., 1988; Wiedemann et al., 1991; Ozpinar et al., 1996; Yokoyama et al., 1993,1998). There are several advantages of utilizing IgY as a food additive to prevent bacterial infection. IgY can simply be isolated from egg yolk by the water-dilution method on a large-scale without using any chemicals or organic solvents (Kwan et al., 1991; Akita & Nakai, 1993). It is relatively stable under various conditions, including heat, pressure, acid, alkali, and proteolytic enzyme as well (Shimizu et al., 1988, 1992, 1994; Otani et al., 1991). Therefore, large and simple production of IgY with relatively high stability is considered to be practically applicable for food supplementation.

In this study, the anti-bacterial activity of chicken egg yolk antibody (IgY) prepared in a powder form was examined *in vitro* to determine the growth inhibitory effect of IgY on *E. coli* O157:H7. The present study may result in the prospective future application of IgY for an effective measure to prevent the contamination of food with *E. coli* O157:H7.

2.2. Materials and Methods

2.2.1. Bacteria and Culture Conditions

A strain of *E. coli* O157:H7 (V241) was obtained from Animal Health Laboratories Branch, Alberta Agriculture, Food and Rural Development, Edmonton, Alberta, Canada. Bacteria were cultured in tryptic soy broth (TSB) at 37°C for 24 h with shaking. After incubation, cells were harvested by centrifugation at 8,000 rpm for 15 min and were treated with 3.7% formalin overnight. The inactivated cells were washed three times, suspended in sterile saline and then freeze-dried. Lyophilized whole-cell cultures were stored at – 20°C until used.

2.2.2. Immunization of Chickens

All chickens were cared for in accordance with the Canadian Council on Animal Care guidelines of animal welfare. Immunization of hens was carried out as described (Sunwoo et al., 1996). Lyophilized *E. coli* O157:H7 whole cells were suspended in sterilized phosphate-buffered saline (PBS, pH 7.2) (500 μg of cell/ml; 16 μg of protein/ml) and emulsified with an equal volume of Freund's incomplete adjuvant. Eight 23-wk-old Single Comb White Leghorn (SCWL) chickens were immunized intra-muscularly at four different sites (0.25 ml per site) of breast muscles (two sites per left or right breast muscle) with cells or without cells as a control. Booster immunizations were given at 2 wk and 4 wk after the initial immunization in the same manner. Eggs were collected daily and stored at 4°C until used.

2.2.3. Isolation of Water-Soluble Fraction (WSF) Containing IgY from Egg Yolk

The WSF containing IgY was prepared from egg yolk using the water dilution method developed by Akita & Nakai (1992). The water dilution method was used to

retain most of the lipids in the egg yolk pellet while recovering IgY in the WSF (supernatant) after centrifugation. The plasma proteins were also separated from the granular proteins since the egg yolk granules were found to aggregate with dilution. The livetins, including γ-livetin (IgY), in the supernatant were then recovered by centrifugation. The egg yolk was physically separated from egg white and rolled on paper towels to remove adhering egg white. The membrane was punctured and the yolk was allowed to flow into a graduated cylinder without the membrane. The egg yolk was first mixed gently with 8 volumes of cold distilled water (acidified with 0.1 M HCl to give pH 4.0) to avoid possible disruptions of egg yolk granules due to presence of high concentrations of acid. Cold acidified distilled water (pH 2.0) was then added to make the final dilution of 1:10. After mixing well, the mixture was adjusted to pH 5.0~5.2 and incubated at 4°C for 12 h. The WSF was obtained by centrifugation at 3,000 rpm and 4°C for 20 min and then stored at –20°C until analyzed.

2.2.4. Preparation of IgY Powder

The WSF that contained specific IgY with high levels of activities or non-specific IgY was neutralized with 0.1 N NaOH to ensure that the results would not be confounded by the acidity and lyophilized to obtain IgY powder.

2.2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

The prepared WSF and IgY powder were assayed by an ELISA procedure as described by Sunwoo et al. (1996, 2000).

2.2.5.1. Specific Activity of IgY

A microtiter plate was coated with 150 µl of lyophilized *E. coli* O157:H7 whole cells in carbonate-bicarbonate buffer (0.05 M, pH 9.6) (0.31 mg of cell/ml;10 µg of protein/ml) and was incubated at 37°C for 90 min. The plate was washed four times with PBS containing 0.05% Tween 20 (PBS-Tw). After washing, 200 µl of a 1% (wt/vol) solution of bovine serum albumin (BSA) in carbonate-bicarbonate buffer were added to each well and incubated at 37°C for 45 min. The BSA solution was then discarded and each well was washed four times with PBS-Tw. The WSF (diluted 1:1,000 in PBS) containing specific egg yolk antibodies (IgY) or non-specific IgY as a control were added to wells (150 µl per well) and incubated at 37°C for 90 min. After the plate was washed four times with PBS-Tw, 150 µl of rabbit anti-chicken IgG conjugated with horseradish peroxidase (diluted 1:1,000 in PBS) was added to each well and incubated at 37°C for 90 min. The plate was washed four times with PBS-Tw, followed by addition of 150 µl of freshly prepared substrate solution, 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) in 0.05 M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide. The reaction was continued for 30 min. Absorbance of the mixture was read at 405 nm using a Bio-Tek EL 309 microplate reader. The ELISA value of antibody activity was determined by subtracting the value of control antibody from that of specific antibody.

2.2.5.2. Cross-Reactivity of IgY

The cross-reactivity of IgY was determined by using the above ELISA method and the following bacterial cells were used: *E. coli* O157:H7, *E. coli* 987P, *Salmonella enteritidis*, and *S. typhimurium*. Wells of the microtiter plate were coated with 150 µl of

lyophilized whole cells in carbonate-bicarbonate buffer (*E. coli* O157:H7 (0.31 mg/ml), *E. coli* 987P (0.5 mg/ml), *S. enteritidis* (1.67 mg/ml), and *S. typhimurium* (1.67 mg/ml)). Specific IgY powder was reconstituted and serially diluted with PBS (25 to 6.25 μg/ml). Reconstituted IgY powder (150 μl per well) was added to react with coated antigens. The cross-reactivity of IgY against other bacteria was determined by comparing activities against those bacteria with activity against *E. coli* O157:H7.

2.2.5.3. Total IgY Concentration

The ELISA was performed as described above, except the plate was coated with 150 μl of rabbit anti-chicken IgG at a final concentration of 3.75 μg/ml. Samples of the WSF were diluted 1:90,000 with PBS. Specific or non-specific IgY powder was reconstituted and serially diluted with PBS (2 to 0.125 μg/ml). Two-fold serial dilutions of purified chicken IgG (1 mg/ml) in PBS (0.5 to 0.031 μg/ml) were used as the reference antibody to prepare a standard curve. The standard curve (appendix 1) was then compared to provide a relative measurement of total IgY concentration.

2.2.5.4. Specific IgY Concentration

The concentration of *E. coli* O157:H7-specific IgY was measured using the ELISA method as described by Sunwoo et al. (2000). Wells of a microtiter plate were coated with 150 μl of rabbit anti-chicken IgG and lyophilized *E. coli* O157:H7 whole cells at a concentration of 3.75 μg/ml and 0.31 mg/ml in carbonate-bicarbonate buffer, respectively. After incubation at 37°C for 90 min, the plate was washed four times with PBS-Tw. Two hundred microliters of a 1% (wt/vol) solution of BSA in carbonate-

bicarbonate buffer was then added and incubated at 37°C for 45 min. After washing, three dilutions of reconstituted specific (6.25, 3.13, and 1.56 μg/ml) and non-specific (4.5, 2.25, and 1.13 mg/ml) IgY powder in PBS were added to wells (150 μl per well) coated with *E. coli* O157:H7 whole cells. Wells coated with rabbit anti-chicken IgG were filled with two-fold serial dilutions of purified chicken IgG (1 mg/ml) in PBS (0.5 to 0.008 μg/ml). The plate was subsequently incubated at 37°C for 90 min and washed. Secondary antibody, substrate and the measurement of absorbance were identical as described in section 2.2.5.1. The optical density at 405 nm was converted to μg of specific IgY/mg of IgY powder by using a quantitative standard curve (appendix 2(a)) determined by the titration between rabbit anti-chicken IgG and purified chicken IgG.

2.2.6. Protein Assay

The Bio-Rad protein assay (Microtiter Plate Protocol), based on the method of Bradford, was performed using purified chicken IgG (1 mg of protein/ml) as the reference protein. The WSF (diluted 1:100 in PBS) and two-fold serial dilutions of the reference protein in PBS (0.5 to 0.0625 mg/ml) were assayed on the microtiter plate. Absorbance at 595 nm after 5 min reaction was measured by a Bio-Tek EL 309 micro-plate reader.

The protein concentrations of specific or non-specific IgY powder were also determined by the same procedures. The reconstituted specific or control IgY powder to be tested were serially diluted 1:2 with PBS (0.5 to 0.0625 mg/ml).

2.2.7. Growth Inhibition Assay

2.2.7.1. Preparation of Bacteria

The same strain of *E. coli* O157:H7 used as an antigen for immunizing chickens was subcultured on a blood agar plate at 37°C overnight and then suspended in TSB. The suspension was adjusted to an optical density of 0.05 at 600 nm, corresponding to a cell density of about 2.7×10^7 CFU/ml. The same volume of 20% (vol/vol) of glycerol in TSB was added and stored at – 70°C until used.

2.2.7.2. Bacterial Growth Curve

Two milliliters of prepared bacterial culture were mixed with 2 ml of TSB and incubated at 37°C with shaking. The turbidity of culture (OD at 600 nm) was measured by spectrophotometer (Spectronic 20) at 1 h intervals. The growth curve was plotted until reaching stationary phase.

2.2.7.3. Preparation of IgY Solution

Specific or non-specific IgY powder was reconstituted to 90, 180, and 360 mg/ml with TSB and then centrifuged at 3,500 rpm and 4°C for 20 min. The supernatant was sterilized by using a 0.22 μm-pore-size membrane filter.

2.2.7.4. Growth of E. coli O157:H7 with IgY

Two milliliters of specific or non-specific IgY solution were added to the same volume of prepared *E. coli* O157:H7 culture. The bacteria and IgY mixtures were incubated at 37°C with shaking. Aliquots of samples (100 μl) were taken at 0, 2, 4, and 6

h of incubation time. Plate counts were performed by the spread plate method on TSB agar plates in duplicate. The inoculated plates were incubated at 37°C overnight. The number of colony-forming units (CFU) per plate was counted to determine the total number of bacterial CFU per ml of sample.

2.2.8. Microscopic Analyses

2.2.8.1. Immunofluorescence Microscopy

One hundred microliters of *E. coli* O157:H7 (62.5 μg/ml) cells suspended in PBS were incubated with the same volume of specific IgY or non-specific IgY (100 μg of IgY powder/ml PBS) or without IgY at 37°C for 1 h. After washing with PBS two times, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken IgG diluted 1:250 in PBS was added and then incubated at 37°C for 1 h. The samples were washed as before and resuspended in 50 μl of PBS. Cell suspension (10 μl) was smeared on the microscope slide, which was then air dried and coverslip was mounted by using a drop of mounting buffer (Glycerol-PBS, pH 7.2). Immunofluorescent staining of specimens was detected by a 2001 confocal laser scanning microscope (Molecular Dynamic).

2.2.8.2. Immunoelectron Microscopy

One milliliter of *E. coli* O157:H7 cells suspended in PBS (62.5 μg/ml) was centrifuged at 12,000 rpm for 10 min. To the cell pellets was added 1 ml of specific IgY or non-specific IgY (500 μg of IgY powder/ml of 1% BSA in PBS). After incubation at 37°C for 1 h, samples were washed with 1% BSA in PBS two times and then mixed with

100 μl of rabbit anti-chicken IgG as the bridge (diluted 1:14 with 1% BSA in PBS), followed by incubation at 37°C for 1 h. After washing, samples were incubated with 300 μl of goat anti-rabbit IgG gold conjugate (diluted 1:25 with 1% BSA in PBS). The suspended cells were used for negative staining and ultrathin sectioning.

For negative staining, bacterial cells were washed with distilled water two times and subsequently mounted on a 300 mesh copper grid. Grid-mounted samples were stained with 2% uranyl acetate. After washing and drying, specimens were observed with a transmission electron microscope (Hitachi H-7,000 TEM, Tokyo, Japan).

To perform ultrathin section method, bacteria treated with antibodies were washed with 1% BSA in PBS two times, fixed with 2.5% glutaraldehyde for 1 h, and postfixed with 1% osmium tetroxide for 1 h. The fixed samples were dehydrated in a graded series of ethanol and embedded in Spurr's medium. After infiltration with Spurr's medium, polymerization was accomplished at 70°C for 12 h. The specimens were then thin sectioned with an ultramicrotome (Ultracut E model, Reichert-Jung, Austria). Ultrathin sections were mounted on the 200 mesh copper grid and stained with 2% uranyl acetate and then with lead citrate. The specimens were examined with a transmission electron microscope (Hitachi H-7,000 TEM, Tokyo, Japan).

2.3. Results and Discussion

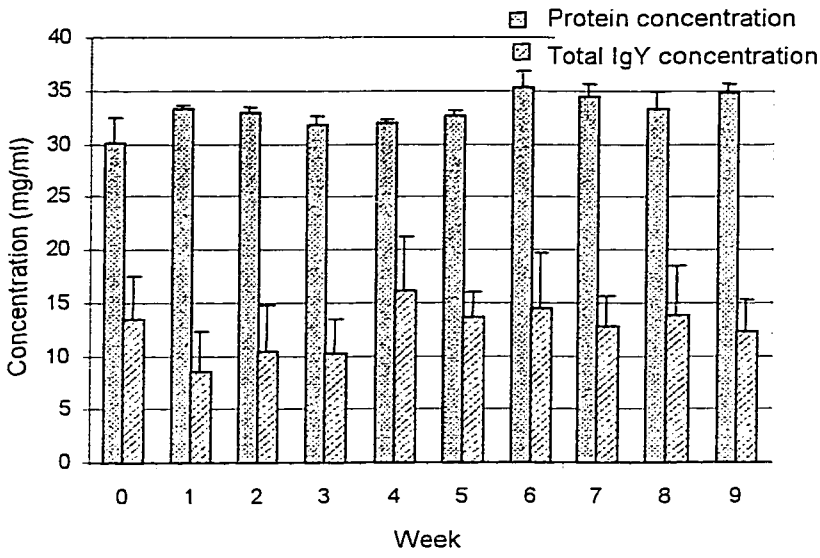
2.3.1. Concentrations of Protein and Total IgY in the WSF

The concentrations of protein and total IgY in the WSF obtained from egg yolk by using the water dilution method under the conditions of 10-fold dilution and pH of 5.0-

5.2 were determined as shown in Figure 2-1. Both protein and total IgY concentrations in the WSF were relatively constant over the immunization period as reported previously (Sunwoo et al., 1996; Li et al., 1998). The average (±standard deviation) concentrations of protein and total IgY in the WSF obtained during the immunization period were 33.04 ± 1.56 and 12.58 ± 2.24 mg/ml, respectively. It has been reported that total IgY concentration in egg yolk from the immunized chicken was not affected by the difference of a strain of chicken (Li et al., 1998), of antigen (Sunwoo et al., 1996), and of adjuvant (Erhard et al., 1997) that were used for immunization. However, the different results concerning total IgY concentration in egg yolk have been shown ranging from 8 to 25 mg/ml (Rose et al., 1974; Shimizu et al., 1989; Sunwoo et al., 1996; Erhard et al., 1997; Li et al., 1998). This variation may be attributed to the various methods of separating IgY from egg yolk and of measuring IgY concentration.

Therefore, the purity of IgY is an important consideration to analyze IgY-related properties and utilize IgY for the further study. The purity of IgY (total IgY in protein) in the WSF with these values was obtained by using the water dilution method characterized as simple and practical, showing an appropriate purity of IgY. For the wide utilization of IgY, a large-scale production of IgY with high recovery and purity from egg yolk is necessary. Such a separation process should be simple and economical requiring few chemicals for food applications. The water dilution method was likely to meet those needs and was carried out to obtain the WSF containing antigen-specific IgY without a further purification step.

Figure 2-1. The concentrations of protein and total IgY in the WSF obtained from chickens immunized with *E. coli* O157:H7 whole cells during the immunization period. Values are the mean of quadruple samples. Vertical bars indicate the standard deviation.

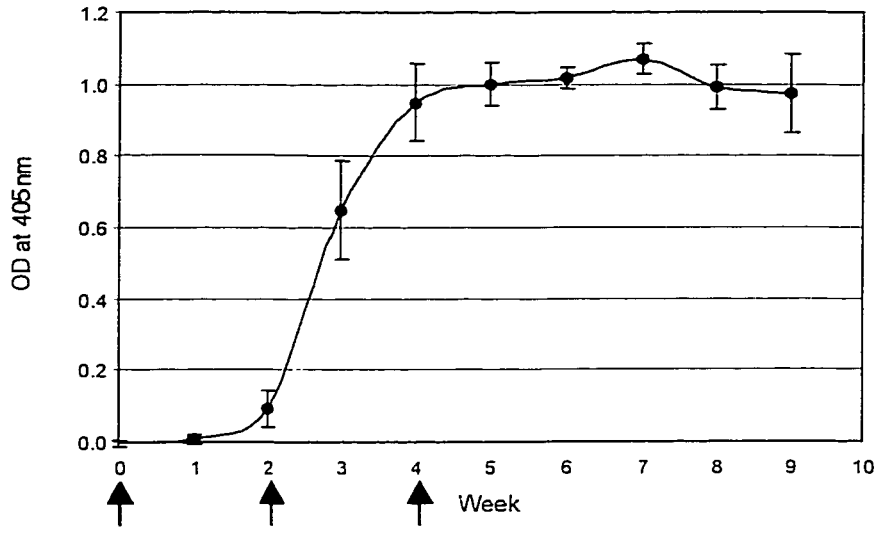


2.3.2. Specific Activity of IgY against *E. coli* O157:H7

Specific activities of IgY in the WSF from chickens immunized with *E. coli* O157:H7 whole cells was monitored by the ELISA using *E. coli* O157:H7 whole cells during the immunization period (Figure 2-2). SCWL chickens showed a relatively strong immune response against *E. coli* O157:H7 whole cells. *E. coli* O157:H7 whole cells (250 μg of cells; 8 μg of protein) were used to immunize a chicken one time, which were sufficiently immunogenic to induce an immune response of a chicken. Immunogenicity of the antigen is influenced by several factors, including the species or strain being immunized, antigen properties and dosage, the route of administration and adjuvant (Kuby, 1997a). This result indicates that the production of antigen-specific antibody can be efficiently elicited in SCWL chickens immunized intramuscularly with *E. coli* O157:H7 whole cell emulsified with Freund's incomplete adjuvant.

The initial immunization of chickens generated the primary response characterized by the lag phase during which a slight increase of *E. coli* O157:H7-specific IgY activity was detected in egg yolk. After the first booster immunization, the secondary response occurred rapidly and reached a greater magnitude. As shown in the result, the OD value of 0.949 was attained after exponential rise. This pattern of antibody activity can be explained that memory B cells formed during a primary response are more easily activated by the booster immunization than naive B cells by the first contact of antigen (Kuby, 1997b). The change of antibody activity was not remarkable after the second booster immunization (slight rise up to 1.07). Thereafter, the level of antibody activity

Figure 2-2. The change of specific activity of IgY in the egg yolk from chickens immunized with *E. coli* O157:H7 whole cells. The level of IgY activity in a 1000-fold dilution of the WSF was measured by the ELISA using *E. coli* O157:H7 whole cells and expressed as ELISA value (OD at 405 nm). Values are the mean of quadruple samples. Vertical bars indicate the standard deviation. Arrows indicate the week of immunization.



remained relatively high, showing no considerable decline during the experimental period (up to 9 wk). It seems that the second booster immunization was not necessary to develop high levels of antibody activity.

There are two types of curves with respect to the development of antibody activity (Behn et al., 1996), which are a 'mammalian-like' curve and a 'saltatory' curve. The one shows that the activity increases after the first booster immunization and remains relatively stable for several weeks or months. The latter is a different pattern that the activity increases during a period of 10 days after the first booster immunization, maintains a plateau level during another 10 days, and then decreases. This result shows the activity development which closely resembles the 'mammalian-like' curve as generally seen in chickens (Schade et al., 1997a).

As a result, antibodies (IgY) specific for *E. coli* O157:H7 whole cells, that is, antibodies able to react with a number of antigenic determinants (epitopes) on the bacterial whole cell, could be obtained from the egg yolk of eggs laid by chickens immunized with *E. coli* O157:H7 whole cells. Anti- *E. coli* O157:H7 IgY in the WSF that was produced during the immunization period of 5 to 9 wk showed high antigen-binding specificity and could be used for the further study to investigate the effect of IgY on the bacterial growth (See below).

2.3.3. Properties of IgY Powder

The WSF was isolated from pooled egg yolks containing high levels of IgY specific for *E. coli* O157:H7 (eggs collected during the immunization period of 5-9 wk). IgY powder was then obtained from freeze-drying the prepared WSF. IgY powder

containing non-specific IgY was also obtained as a control. For the future food application of IgY, it is necessary to provide IgY product in concentrated form which is stable for long-term storage. IgY powder, as a concentrated and dried product, may be suitable form for these considerations. Therefore, IgY prepared in powder form was utilized to investigate immunological properties of IgY in further studies.

To provide the basic information on IgY powder, the properties of IgY powder were characterized by analyzing the concentrations of protein, total IgY, and specific IgY as shown in Table 2-1. The purity of IgY powder (total IgY in protein) was 27.6%, which was decreased by approximately 10% in comparison to that of the WSF from the result of foregoing study. When reconstituted for the assay, IgY powder was found to be not completely dissolved. It is, therefore, speculated that the decrease in the purity of IgY powder resulted from freeze-drying effects that reduced the solubility and ELISA value of IgY. However, the freeze-drying method appears to be more suitable for the preparation of IgY powder than other methods such as spray-drying from the result (Yokoyama et al., 1992) that higher yield and lower moisture content of powder and higher antibody titer were observed in the powder prepared by freeze-drying than by spray-drying.

The concentration of *E. coli* O157:H7-specific IgY in IgY powder was also assessed by the ELISA as described. Specific IgY concentration in control IgY powder was significantly lower than that in specific IgY powder as expected. The proportion of *E. coli* O157:H7-specific IgY in the total egg yolk IgY averaged 9.2%, which was close to the result (9.0%) obtained by using the affinity chromatography (Li et al.,1998).

Table 2-1. The concentrations of protein, total IgY and specific IgY in IgY powder prepared from the WSF containing *E. coli* O157:H7-specific or non-specific IgY. Values are the mean ± standard deviation.

IgY powder	Concentration (mg/g)		
	Protein	Total IgY	Specific IgY
E. coli O157:H7-specific	510 ± 38	141 ± 24	13 ± 4
Non-specific	468 ± 62	93 ± 21	< 0.1

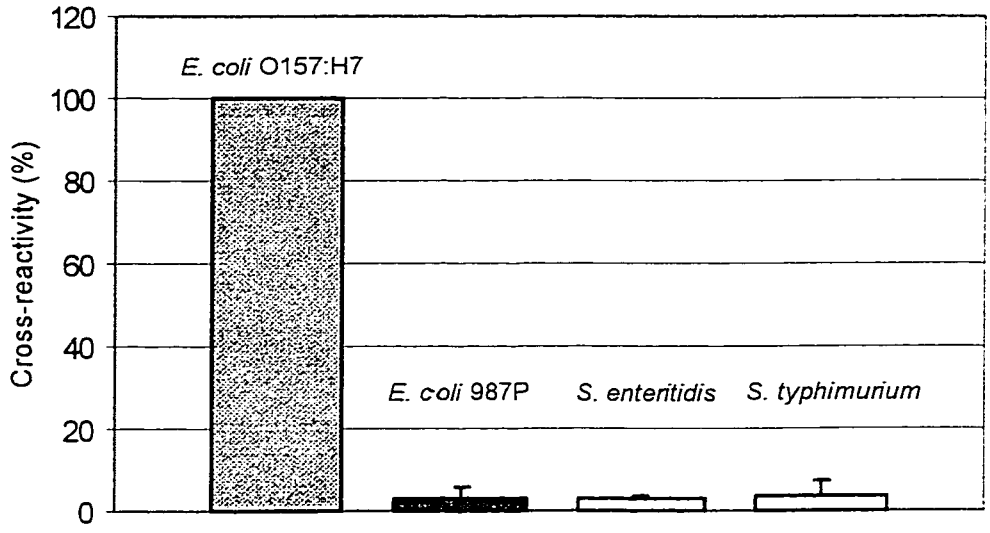
Thus far, there has been limited information available on determining the concentration of antigen-specific antibodies. It is, therefore, necessary to develop a practical assay in this matter since the quantity of specific antibodies used for food application is a crucial factor influencing the effective reactivity of antibodies with antigens. The quantitative ELISA performed in the current study appears to be an appropriate technique to measure the amount of specific antibodies. By this method, specific IgY powder was demonstrated to contain certain amounts of specific antibodies with antigen-binding activity and was used to further evaluate its anti-bacterial properties.

2.3.4. Cross-Reactivity of IgY

Activities of anti-*E. coli* O157:H7 IgY against other members of the family Enterobacteriaceae, including *E. coli* 987P, *S. enteritidis*, and *S. typhimurium*, were investigated to determine the cross-reactivity of anti-*E. coli* O157:H7 IgY. The cross-reactivity of IgY, if any, may provide an additional advantage for food application of IgY due to its anti-bacterial effects on other pathogenic bacteria as well as target bacteria. In this regard, the above enteric pathogens were chosen to assess the cross-reactivity of IgY.

There might be considerable possibilities of IgY to cross-react with antigenic determinants (epitopes) on selected bacteria since IgY was raised against *E. coli* O157:H7 whole cells with a large number of epitopes that might be shared in part by other bacteria belonging to the same family. However, the cross-reactivity of anti-*E. coli* O157:H7 IgY was very low as shown in Figure 2-3. This property of IgY, in contrast to the assumption, indicates that there are a small number of identical epitopes or unrelated

Figure 2-3. The cross-reactivity of anti-*E. coli* O157:H7 IgY with other members of Enterobacteriaceae, including *E. coli* 987P, *S. enteritidis*, and *S. typhimurium*. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation.



epitopes possessing similar chemical properties between *E. coli* O157:H7 and selected bacteria. As a result, anti-*E. coli* O157:H7 IgY was found to be highly specific for *E. coli* O157:H7, which implies that anti-*E. coli* O157:H7 IgY can implement its anti-bacterial function specifically against *E. coli* O157:H7.

2.3.5. Growth Inhibitory Effect of *E. coli* O157:H7-Specific IgY

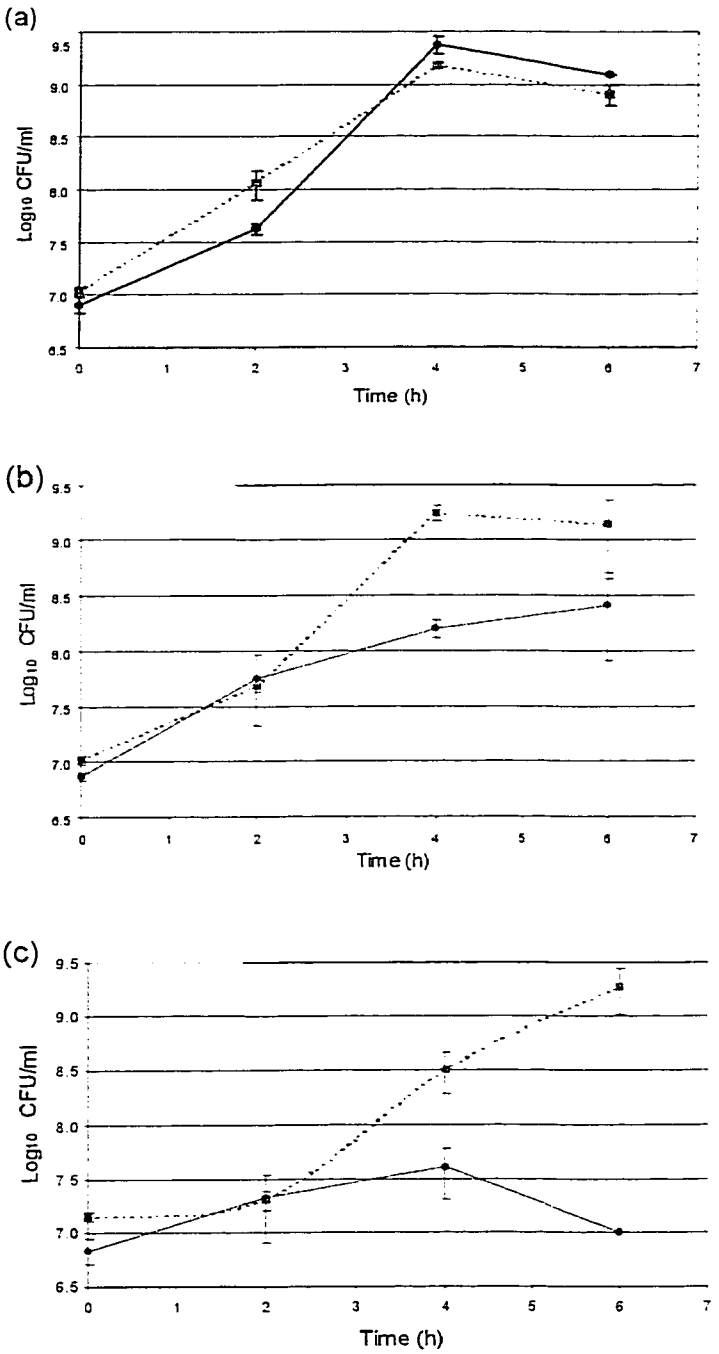
It is not surprising that many reports have shown the bactericidal effects of specific antibodies in the presence of phagocytes or complement, which resulted from antibody ability to opsonize bacteria for phagocytosis or activate complement for lysis. More notable are subsequent studies that antibodies by themselves were inhibiting bacterial growth or killing bacteria. Specific monoclonal antibodies directed against particular molecules on the bacterial surface have bacteriostatic or bactericidal effects *in vitro* (Avakian et al., 1993; Sadziene et al., 1993; Yamaguchi et al., 1997; Lin et al., 1998). These results could be a rationale for using IgY to evaluate its ability to exhibit the same effect although IgY is a polyclonal antibody.

There was other encouragement to explore the immunological function of IgY. There were several reports that animals challenged with enterotoxigenic *E. coli* were protected from diarrhea by oral administration of IgY (O'Farrelly et al., 1992; Yokoyama et al., 1992; Ikemori et al., 1992; Imberechts, et al., 1997; Marquardt et al., 1999) and also showed reduction in the bacterial level of small intestine colonization (Zuniga et al., 1997). It was evident from these results that IgY suitable for food application can react with pathogenic microorganisms and can neutralize or inhibit pathogen activities related to infectious diseases.

To substantiate the hypothesis on the anti-bacterial effect of IgY, the growth inhibition assay was performed to prove whether IgY can inhibit *E. coli* O157:H7 growth in a liquid medium. The growth curve of *E. coli* O157:H7 under the same condition that was used in the growth inhibition assay was first plotted, which reached the stationary phase at approximately 6 h of incubation time (Data not shown). Accordingly, the time of sampling to perform growth inhibition assay was determined. The different concentrations of specific or non-specific IgY powder (0.18, 0.09, and 0.045 g/ml) were used for the test, considering the effect of antibody amount on the interaction with antigen.

There was no significant difference between the specific and non-specific (control) group in the test using IgY at a concentration of 0.045 g/ml (Figure 2-4(a)). In contrast, the bacterial growth in the presence of more concentrated IgY was different between the two groups. As shown in Figure 2-4(b), the number of *E. coli* O157:H7 incubated with specific IgY at a concentration of 0.09 g/ml decreased in growth rate compared to that of the control group after 2 h incubation. Cell counts of the specific group and control group rose by 0.5 logCFU/ml and 1.5 logCFU/ml, respectively, during 2-4 h of incubation time, which indicated three times more growth of bacteria in the control group. At 6 h of incubation time, the difference of the bacterial growth between the two groups was 0.7 logCFU/ml. A two-fold greater concentration of IgY (0.18 g/ml) had an almost similar effect on the pattern of *E. coli* O157:H7 growth as shown in the assay using 0.09 g/ml of IgY during 4 h incubation (Figure 2-4(c)). However, there was a 0.8 logCFU/ml increase and 0.6 logCFU/ml decrease in the number of bacteria in the control and the specific group, respectively, after 4 h incubation, which resulted in the

Figure 2-4. The effect of IgY on the growth of *E. coli* O157:H7 in a liquid medium. Bacteria (approximately 1×10^7 CFU/ml) were grown in TSB mixed with different concentrations of *E. coli* O157:H7-specific or non-specific IgY powder at 37°C with shaking: (a) 0.045g/ml; (b) 0.09g/ml; (c) 0.18g/ml. The viable cells were counted by the plate count method. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation. The solid line shows the growth pattern of *E. coli* O157:H7 incubated with specific IgY. The dotted line shows that of *E. coli* O157:H7 incubated with non-specific IgY as a control.



significant reduction by 2.3 logCFU/ml in the amount of bacterial growth of the specific group compared to the control group.

The concentration of *E. coli* O157:H7-specific IgY, which was effective in the inhibition of bacterial growth, was more than 1.17 mg/ml without considering losses of specific IgY during the preparation of the IgY solution. The growth inhibitory effect of IgY was dose-dependent and could be enhanced by higher concentrations of IgY. As a result, the growth of *E. coli* O157:H7 in a liquid medium was inhibited in the presence of specific IgY raised against homologous strain.

The mechanism of growth inhibition by antibodies is not precisely understood. Agglutination, which is the interaction between antibodies and particulate antigens resulting in visible clumping, may be one mediator of growth inhibition; bacteria competing with each other in large aggregates conceivably grow more slowly than their free-swimming, single counterparts. However, agglutination may not be an important mediator of growth inhibition since the steric hindrance of two Fab arms of IgY precludes the cross-linking of bacteria (Kubo et al., 1973; Gallagher & Voss, 1974).

Binding of antibodies to certain specific components on the bacterial surface may be a crucial mediator. Outer membrane protein (OMP), lipopolysaccharide (LPS), flagella, and fimbriae (or pili) could be categorized into these components. It is hypothesized that cell surface components can easily be recognized and bound by antibodies. This binding may lead to the impairment of biological functions of those components which play an essential role in the bacterial growth (Sim et al., 2000). Further study on the mechanism for the inhibitory effect of IgY on the growth of *E. coli* O157:H7 is necessary to understand immunological properties of IgY. The growth inhibitory property of IgY

may encourage IgY to be applied in food preparation to confer novel protection from *E. coli* O157:H7 and reduce the risk of outbreak caused by contaminated foods with those pathogens.

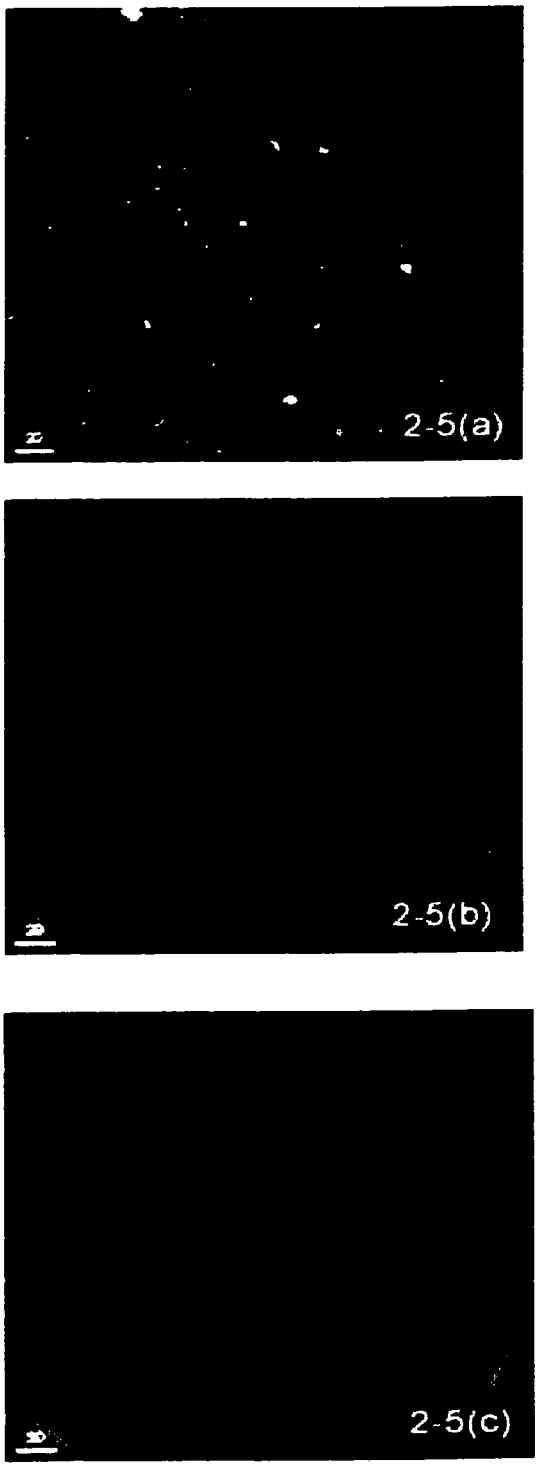
2.3.6. Microscopic Analyses of Specific IgY Binding to *E. coli* O157:H7

The specific-binding activity of IgY against *E. coli* O157:H7, which resulted in inhibiting bacterial growth, was proven by the methods such as ELISA and growth inhibition assay as presented above. To visualize *E. coli* O157:H7 with bound specific IgY, further evaluation of IgY binding activity was conducted by using immuno-fluorescence and immunoelectron microscopy.

Firstly, immunofluorescence microscopy was employed for a preliminary test to provide the positive result of binding reaction between IgY and *E. coli* O157:H7. *E. coli* O157:H7 was incubated with specific or non-specific IgY, followed by the incubation of bacterial cells with FITC-conjugated rabbit anti-chicken IgG. Bacteria were also incubated without IgY to examine the presence of the autofluorescence from bacteria. FITC used as a fluorescent dye or fluorochrome in this study absorbs blue light at one wavelength (490 nm) and emits an intense yellow-green fluorescence at longer wavelength (517 nm) (Herman, 1998). Specific IgY binding to cells was visualized by tagging antibodies with FITC, i.e., FITC conjugated anti-IgY.

E. coli O157:H7 reacted with specific IgY and fluoresced as shown in Figure 2-5(a). On the contrary, fluorescence was not observed in bacteria incubated with non-specific IgY as a positive control (Figure 2-5(b)) or without IgY as a negative control (Figure 2- 5(c)). As a result, the observation of fluorescence from bacteria reacted with

Figure 2-5. Immunofluorescence micrographs of *E. coli* O157:H7 incubated with (a) specific IgY; (b) non-specific IgY; or (c) without IgY (magnification 400×, Bar = 20 μm)



specific IgY demonstrated the binding of specific IgY to *E. coli* O157:H7. The antigen-antibody interaction is a biomolecular association that does not lead to an irreversible chemical alteration in either the antibody or antigen. The interaction between an antibody and an antigen involves various noncovalent interactions between the antigenic-binding site, or epitope, of the antigen and the variable-region (V_H /V_L) domain of the antibody molecule, particularly the hypervariable regions, or complementarity-determining regions (CDR_S) (Kuby, 1997b). Accordingly, the binding of IgY to bacteria as shown here results from the reaction between CDR_S of IgY and epitopes of bacteria.

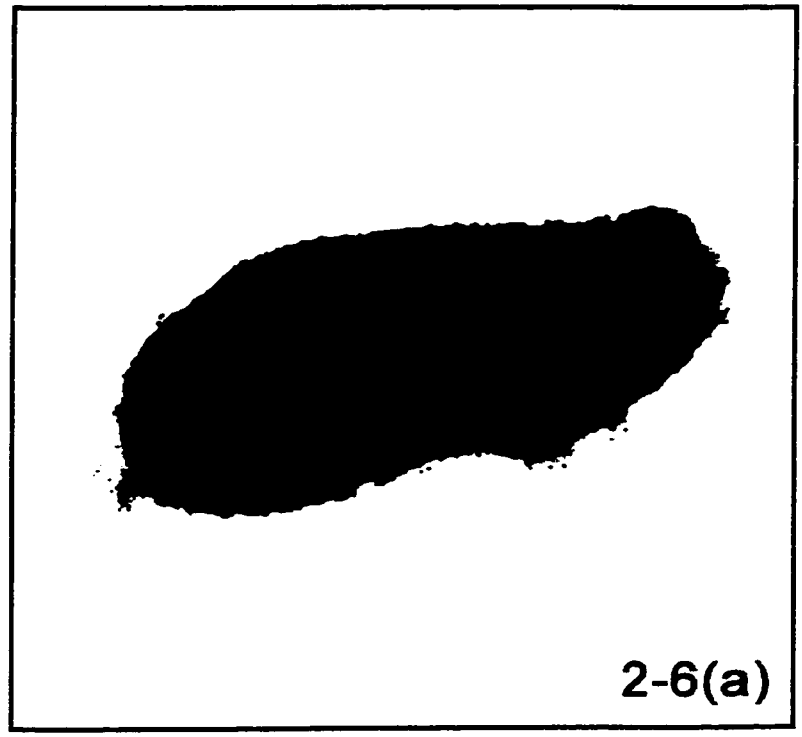
The interaction between IgY and bacteria was further investigated by immunoelectron microscopy after the positive results were confirmed in immuno-flourescence microscopic analyses. Immunoelectron microscopy is a technique that uses colloidal gold as a marker to stain antigens observed. The binding of gold labeled antibody to antigen or antibody and the observation of scattered gold particles around antigen make it possible to demonstrate the specific-binding activity of antibody against antigen.

E. coli O157:H7 was incubated with specific or non-specific IgY and then was mixed with rabbit anti-chicken IgG. The mixture of bacteria with antibodies was subsequently incubated, followed by reacting with goat anti-rabbit IgG conjugated with gold. Negatively stained or ultrathin sectioned specimens were observed by transmission electron microscopy. The results showed the distribution of gold particles around bacteria reacted with specific IgY (Figure 2-6(a), 2-7(a)) while there were no gold particles observed around bacteria incubated with non-specific IgY as a control (Figure 2-6(b), 2-

7(b)). The presence of gold particles demonstrated that specific IgY reacts with bacteria, specifically epitopes of bacteria.

In particular, gold particles were observed around the surface of bacteria, which is indicative of the epitopes derived from the surface components of bacteria such as LPS, OMP, fimbriae (or pili) and flagella. It was also noted that the morphological change of bacteria incubated with specific IgY was observed in a sectioned specimen as shown in Figure 2-7(a). IgY binding to the surface components expressed on bacteria may cause structural alterations of the bacterial surface. These observations may support the hypothesis suggested in the study of growth inhibition assay. Specific IgY binding to *E. coli* O157:H7 may immobilize bacteria and disturb cellular processes, resulting in the impairment of bacterial growth. The findings from this study suggest further examination related to the role of specific-binding activity of IgY against bacterial surface components playing in inhibiting bacterial growth.

Figure 2-6. Immunoelectron micrographs of negatively stained *E. coli* O157:H7 incubated with (a) specific IgY; (b) non-specific IgY (magnification 9,000 ×)



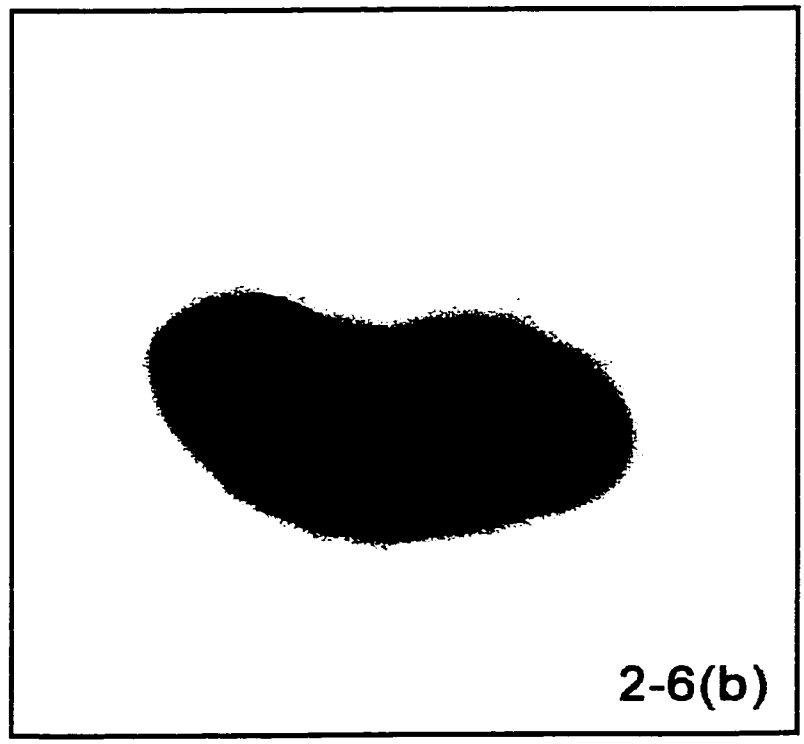
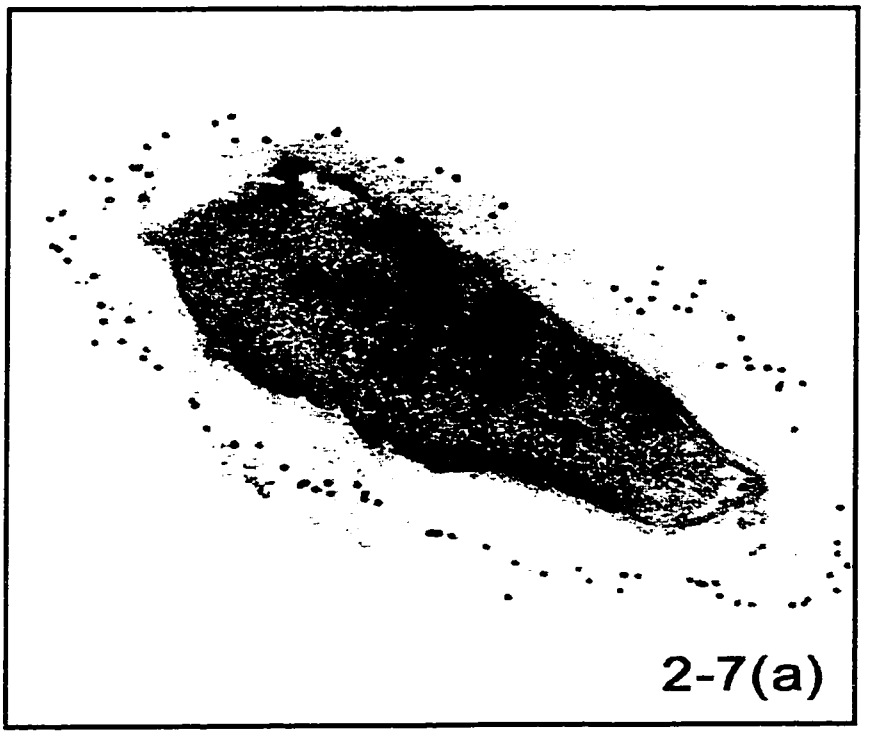
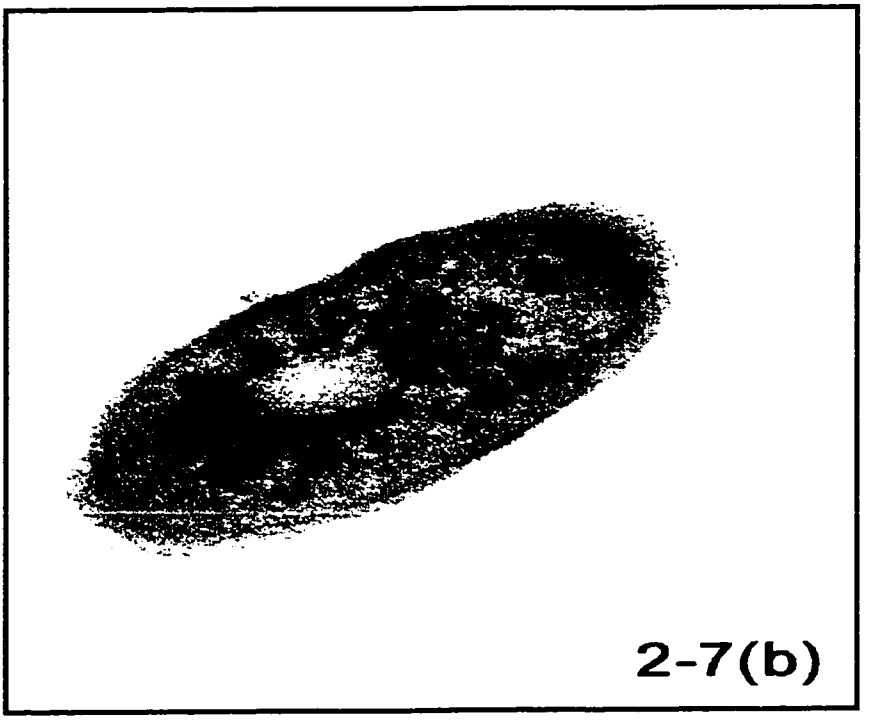


Figure 2-7. Immunoelectron micrographs of ultrathin sectioned *E. coli* O157:H7 incubated with (a) specific IgY (magnification 12,000×); (b) non-specific IgY (magnification 6,000 ×)





Chapter 3. Anti-Bacterial Activities of IgY against Enterotoxigenic *Escherichia coli* 987P

3.1. Introduction

Diseases due to *Esherichia coli* (*E. coli*) are a primary cause of morbidity and mortality in young animals. Enterotoxigenic *E. coli* (ETEC) is most frequently implicated in the most common type of disease, diarrhea, during the first week of life and again around the time of weaning. Because of the incidence and severity of the disease and the potential for large economic losses, ETEC have been the subject of intensive study. The pathogenesis of ETEC-causing diarrhea has two main steps: intestinal colonization mediated by fimbriae and hypersecretion of water and electrolytes caused by heat-stable (ST) or heat-labile (LT) enterotoxins, or both (Moon et al., 1978; Saeed et al., 1983; Parry & Rooke, 1985; Holmgren, 1985). The strains of ETEC associated with intestinal colonization are those that express the K88, K99, and 987P fimbrial adhesins (Moon et al., 1977; Isaacson & Richter, 1981; Morris et al., 1982).

ETEC strains carrying 987P are commonly responsible for enteric enterotoxic colibacillosis in newborn or postweaning pigs (Nagy et al., 1977; Isaacson & Richter, 1981). Postweaning diarrhea is usually the most constant disease problem of large-scale farms, which is mainly caused by the weaning itself (Nagy et al., 1999). While feeds primarily serve as sources of nutrients for animals, they can also be important vehicles for transmission of infectious diseases to animals. Therefore, the prevention of feed

contamination may be the first step to be taken into action where control could be effective in reducing the risk of ETEC infection in piglets.

In view of productivity and stability, IgY can be considered practically applicable for food or feed supplementation. There have been several reports on the use of IgY in the prevention and control of ETEC infection. Specific IgY against K88, K99, and 987P fimbrial adhesins of ETEC prevented neonatal piglets from fatal enteric colibacillosis (Yokoyama et al., 1992). Weaned pigs challenged with F18+ ETEC were fully protected by oral administration of IgY against the same fimbriae (Zuniga et al., 1997). *In vitro* studies showed that the inhibition of ETEC adhesion to piglet intestinal cells or mucosa by IgY reduced the virulence of this pathogen (Jungling et al., 1991; Yokoyama et al., 1992; Jin et al., 1998).

IgY, which possesses anti-ETEC activity and the suitability for feed supplementation, suggests the potential application in preventing feed contamination. In-feed IgY specific for ETEC may reduce the risk of feed contamination with ETEC through inhibiting bacterial proliferation. Therefore, the present study is aimed at investigating the growth inhibitory effect of egg yolk antibody (IgY) on ETEC 987P *in vitro* to support the possibility of future application.

3.2. Materials and Methods

3.2.1. Bacteria and Culture Conditions

A local strain of enterotoxigenic *Escherichia coli* (ETEC) 987P (Em 88-1604) was obtained from Animal Health Laboratories Branch, Alberta Agriculture, Food and

Rural Development, Edmonton, Alberta, Canada. Bacteria were cultured in E-media at 37°C for 48 h with shaking. After incubation, cells were harvested by centrifugation at 8,000 rpm for 15 min and were treated with 3.7% formalin overnight. The inactivated cells were washed three times, suspended in sterile saline and then freeze-dried. Lyophilized whole-cell cultures were stored at –20°C until used.

3.2.2. Immunization of Chickens

All chickens were cared for in accordance with the Canadian Council on Animal Care guidelines of animal welfare. Immunization of hens was carried out as described (Sunwoo et al., 1996). Lyophilized ETEC 987P whole cells were suspended in sterilized phosphate-buffered saline (PBS, pH 7.2) (500 μg of cell/ml; 15.6 μg of protein/ml) and emulsified with an equal volume of Freund's incomplete adjuvant. Eight 23-wk-old Single Comb White Leghorn (SCWL) chickens were immunized intramuscularly at four different sites (0.25 ml per site) of breast muscles (two sites per left or right breast muscle) with cells or without cells as a control. Two booster immunizations were given at 2 wk and 4 wk after the initial immunization in the same manner. Eggs were collected daily and stored at 4°C until used.

3.2.3. Isolation of Water-Soluble Fraction (WSF) Containing IgY from Egg Yolk

The WSF containing IgY was prepared from egg yolk using the water dilution method developed by Akita & Nakai (1992). The water dilution method was used to retain most of the lipids in the egg yolk pellet while recovering IgY in the WSF (supernatant) after centrifugation. The plasma proteins were also separated from the

granular proteins since the egg yolk granules were found to aggregate with dilution. The livetins, including γ-livetin (IgY), in the supernatant was then recovered by centrifugation. The egg yolk was physically separated from egg white and rolled on paper towels to remove adhering egg white. The membrane was punctured and the yolk was allowed to flow into a graduated cylinder without the membrane. The egg yolk was first mixed gently with 8 volumes of cold distilled water (acidified with 0.1 M HCl to give pH 4.0) to avoid possible disruptions of egg yolk granules due to presence of high concentrations of acid. Cold acidified distilled water (pH 2.0) was then added to make the final dilution of 1:10. After mixing well, the mixture was adjusted to pH 5.0~5.2 and incubated at 4°C for 12 h. The WSF was obtained by centrifugation at 3,000 rpm and 4°C for 20 min and then stored at -20°C until analyzed.

3.2.4. Preparation of IgY Powder

The WSF that contained specific IgY with high levels of activities or non-specific IgY was neutralized with 0.1 N NaOH to ensure that the results would not be confounded by the acidity and lyophilized to obtain IgY powder.

3.2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

The prepared WSF and IgY powder were assayed by an ELISA procedure as described by Sunwoo et al. (1996, 2000).

3.2.5.1. Specific Activity of IgY

A microtiter plate was coated with 150 µl of lyophilized ETEC 987P whole cells in carbonate-bicarbonate buffer (0.05 M, pH 9.6) (0.5 mg of cell/ml; 15.6 µg of protein/ml) and was incubated at 37°C for 90 min. The plate was washed four times with PBS containing 0.05% Tween 20 (PBS-Tw). After washing, 200 µl of a 1% (wt/vol) solution of bovine serum albumin (BSA) in carbonate-bicarbonate buffer were added to each well and incubated at 37°C for 45 min. The BSA solution was then discarded and each well was washed four times with PBS-Tw. The WSF (diluted 1:1,000 in PBS) containing specific IgY or non-specific IgY as a control were added to wells (150 µl per well) and incubated at 37°C for 90 min. After the plate was washed four times with PBS-Tween, 150 µl of rabbit anti-chicken IgG conjugated with horseradish peroxidase (1:1,000 in PBS) was added to each well and incubated at 37°C for 90 min. The plate was washed four times with PBS-Tw, followed by addition of 150 µl of freshly prepared substrate solution, 2-2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) in 0.05 M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide. The reaction was continued for 30 min. Absorbance of the mixture was read at 405 nm using a Bio-Tek EL 309 microplate reader. The ELISA value of antibody activity was determined by subtracting the value of control antibody from that of specific antibody.

3.2.5.2. Cross-Reactivity of IgY

The cross-reactivity of IgY was determined by using the above ELISA method and following bacterial cells were used: ETEC 987P, *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *S. typhimurium*. Wells of the microtiter plate were coated with 150 µl of lyophilized whole cells in carbonate-bicarbonate buffer (ETEC 987P (0.5

mg/ml), *E. coli* O157:H7 (0.31 mg/ml), *Salmonella enteritidis* (1.67 mg/ml), and *S. typhimurium* (1.67 mg/ml)). Specific IgY powder was reconstituted and serially diluted with PBS (0.16 to 0.04 mg/ml). Reconstituted IgY powder (150 µl per well) was added to react with coated antigens. The cross-reactivity of IgY against selected bacteria was determined by comparing activities against those bacteria with activity against ETEC 987P.

3.2.5.3. Total IgY Concentration

The ELISA was performed as described above, except the plate was coated with 150 µl of rabbit anti-chicken IgG at a final concentration of 3.75 µg/ml. Samples of the WSF were diluted 1:90,000 with PBS. Specific or non-specific IgY powder was reconstituted and serially diluted with PBS (2 to 0.125 µg/ml). Two-fold serial dilutions of purified chicken IgG (1 mg/ml) in PBS (0.5 to 0.031 µg/ml) were used as the reference antibody to prepare standard curve. The standard curve (appendix 1) was then compared to provide a relative measurement of total IgY concentration.

3.2.5.4. Specific IgY Concentration

The concentration of ETEC 987P-specific IgY was measured using the ELISA method as described by Sunwoo et al. (2000). Wells of a microtiter plate were coated with 150 µl of rabbit anti-chicken IgG and lyophilized ETEC 987P whole cells at a concentration of 3.75 µg/ml and 0.5 mg/ml in carbonate-bicarbonate buffer, respectively. After incubation at 37°C for 90 min, the plate was washed four times with PBS-Tw. Two hundred microliters of a 1% (wt/vol) solution of BSA in carbonate-bicarbonate buffer

was then added and incubated at 37°C for 45 min. After washing, three dilutions of reconstituted specific (25, 12.5 and 6.25 μg/ml) and non-specific (4.5, 2.25, and 1.13 mg/ml) IgY powder in PBS were added to wells (150 μl per well) coated with ETEC 987P whole cells. Wells coated with rabbit anti-chicken IgG were filled with two-fold serial dilutions of purified chicken IgG (1 mg/ml) in PBS (0.5 to 0.008 μg/ml). The plate was subsequently incubated at 37°C for 90 min and washed. Secondary antibody, substrate and the measurement of absorbance were identical as described in section 3.2.5.1. The optical density at 405 nm was converted to μg of specific IgY/mg of IgY powder by using a quantitative standard curve (appendix 2(b)) determined by the titration between rabbit anti-chicken IgG and purified chicken IgG.

3.2.6. Protein Assay

The Bio-Rad protein assay (Microtiter Plate Protocol), based on the method of Bradford, was performed using purified chicken IgG (1 mg of protein/ml) as the reference protein. The WSF (diluted 1:100 in PBS) and two-fold serial dilutions of the reference protein in PBS (0.5 to 0.0625 mg/ml) were assayed on the microtiter plate. Absorbance at 595 nm after 5 min reaction was measured by a Bio-Tek EL 309 micro-plate reader.

The protein concentrations of specific or non-specific IgY powder were also determined by the same procedures. The reconstituted specific or control IgY powder to be tested were serially diluted 1:2 with PBS (0.5 to 0.0625 mg/ml).

3.2.7. Growth Inhibition Assay

3.2.7.1. Preparation of Bacteria

The same strain of ETEC 987P used as an antigen for immunizing chickens was subcultured on a blood agar plate at 37°C overnight and then suspended in E-media. The suspension was adjusted to an optical density of 0.05 at 600 nm, corresponding to a cell density of about 2.7×10^7 CFU/ml. The same volume of 20% (vol/vol) of glycerol in E-media was added and stored at – 70°C until used.

3.2.7.2. Bacterial Growth Curve

Two milliliters of prepared bacterial culture was mixed with 2 ml of E-media and incubated at 37°C with shaking. The turbidity of culture (OD at 600 nm) was measured by spectrophotometer (Spectronic 20) at 1 h intervals. The growth curve was plotted until reaching stationary phase.

3.2.7.3. Preparation of IgY Solution

Specific or non-specific IgY powder was reconstituted to 90, 180, and 360 mg/ml with E-media and then centrifuged at 3,500 rpm and 4°C for 20 min. The supernatant was sterilized by using a 0.22 μm-pore-size membrane filter.

3.2.7.4. Growth of ETEC 987P with IgY

Two milliliters of specific or non-specific IgY solution was added to the same volume of prepared ETEC 987P culture. The bacteria and IgY mixtures were incubated at 37°C with shaking. Aliquots of samples (100 μl) were taken at 0, 2, 4, and 6 h of

incubation time. Plate counts were performed by the spread plate method on TSB agar plates in duplicate. The inoculated plates were incubated at 37°C overnight. The number of colony-forming units (CFU) per plate was counted to determine the total number of bacterial CFU per ml of sample.

3.2.8. Microscopic Analyses

3.2.8.1. Immunofluorescence Microscopy

One hundred microliters of ETEC 987P (100 μg/ml) cells suspended in PBS were incubated with the same volume of specific IgY or non-specific IgY (100 μg of IgY powder/ml PBS) or without IgY at 37°C for 1 h. After washing with PBS two times, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken IgG diluted 1:250 in PBS was added and then incubated at 37°C for 1 h. The samples were washed as before and resuspended in 50 μl of PBS. Cell suspension (10 μl) was smeared on the microscope slide, which was then air dried and coverslips was mounted by using a drop of mounting buffer (Glycerol-PBS, pH 7.2). Immunofluorescent staining of specimens was detected by a 2001 confocal laser scanning microscope (Molecular Dynamic).

3.2.8.2. Immunoelectron Microscopy

One milliliter of ETEC 987P cells suspended in PBS (100 μg/ml) was centrifuged at 12,000 rpm for 10 min. To the cell pellets was added 1 ml of specific IgY or non-specific IgY (500 μg of IgY powder/ml of 1% BSA in PBS). After incubation at 37°C for 1 h, samples were washed with 1% BSA in PBS two times and then mixed with 100 μl of

rabbit anti-chicken IgG as the bridge (diluted 1:14 in 1% BSA in PBS), followed by incubation at 37°C for 1 h. After washing, samples were incubated with 300 μl of goat anti-rabbit IgG gold conjugate (diluted 1:25 with 1% BSA in PBS). The suspended cells were used for negative staining and ultrathin sectioning.

For negative staining, bacterial cells were washed with distilled water two times and subsequently mounted on a 300 mesh copper grid. Grid-mounted samples were stained with 2% uranyl acetate. After washing and drying, specimens were observed with a transmission electron microscope (Hitachi H-7,000 TEM, Tokyo, Japan).

To perform ultathin section method, bacteria treated with antibodies were washed with 1% BSA in PBS two times, fixed with 2.5% glutaraldehyde for 1 h, and postfixed with 1% osmium tetroxide for 1 h. The fixed samples were dehydrated in a graded series of ethanol and embedded in Spurr's medium. After infiltration with Spurr's medium, polymerization was accomplished at 70°C for 12 h. The specimens were then thin sectioned with an ultramicrotome (Ultracut E model, Reichert-Jung, Austria). Ultrathin sections were mounted on a 200 mesh copper grid and stained with 2% uranyl acetate and then with lead citrate. The specimens were examined with a transmission electron microscope (Hitachi H-7,000 TEM, Tokyo, Japan).

3.3. Results and Discussion

3.3.1. Concentrations of Protein and Total IgY in the WSF

The WSF containing IgY was isolated from egg yolk of chickens immunized with ETEC 987P by using the water dilution method under conditions of 10-fold dilution and pH of

5.0-5.2. The concentrations of protein and total IgY in the WSF were subsequently analyzed by protein assay and the ELISA as described. As shown in Figure 3-1, both protein and total IgY concentrations in the WSF were relatively constant over the immunization period. This pattern was similar to those presented by Sunwoo et al. (1996) and Li et al. (1998). The average (±standard deviation) concentrations of protein and total IgY in the WSF obtained during the immunization period were 32.90 ± 1.84 and 11.16 ± 1.66 mg/ml, respectively. The concentration of total IgY was similar to that (12.58 ± 2.24 mg/ml) obtained from the WSF containing anti-*E. coli* O157:H7 as shown in the previous chapter 2. This result demonstrated that the total IgY concentration in egg yolk is independent of the nature of immunogens as reported by Sunwoo et al. (1996).

The purity of IgY (the total IgY in protein) in the WSF was 33.9%, which should be considered as an important factor for further test and was relatively high in respect to the water dilution method used as an isolation process. Therefore, the water dilution method is practically suitable for food or feed application of IgY since it can produce IgY simply in large quantities requiring no chemicals.

3.3.2. Specific Activity of IgY against ETEC 987P

The change of IgY specific activity in the WSF was monitored by the ELISA using ETEC 987P whole cells during the immunization period (Figure 3-2). The level of ETEC 987P-specific IgY activity increased a week after the first immunization. After the first booster immunization, the level of antibody activity showed a slight fall, followed by an exponential rise. The level increased continuously to a maximum of 0.855 three wks

Figure 3-1. The concentrations of protein and total IgY in the WSF obtained from chickens immunized with ETEC 987P whole cells during the immunization period. Values are the mean of quadruple samples. Vertical bars indicate the standard deviation.

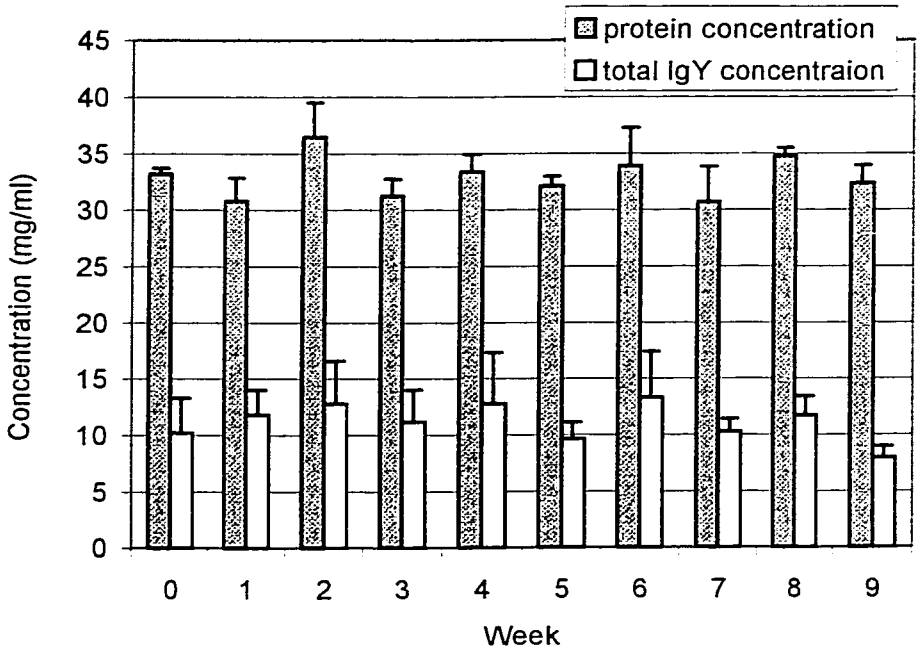
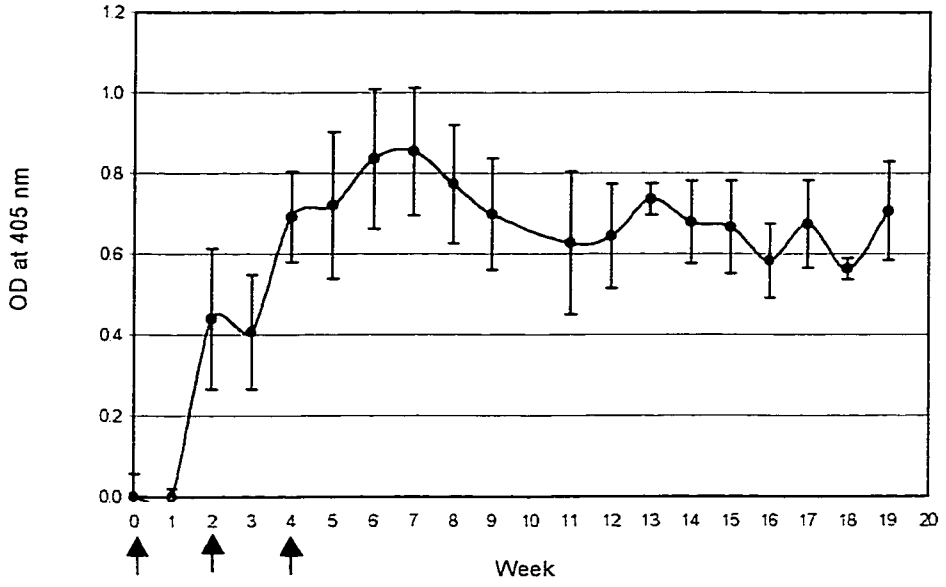


Figure 3-2. The change of specific activity of IgY in the egg yolk from chickens immunized with ETEC 987P whole cells. The level of specific activity in a 1000-fold dilution of the WSF was measured by the ELISA using bacterial whole cells as an antigen and expressed as ELISA value (OD at 405 nm). Values are the mean of quadruple samples. Vertical bars indicate the standard deviation. Arrows indicate weeks of immunization.



after the second booster immunization. IgY activity, thereafter, fell to a minimum of 0.564, which was a decrease of 34% in comparison to the maximum value. The level of IgY activity remained relatively high showing no considerable decline throughout the period that the eggs were monitored (up to 19 wk).

SCWL chickens showed a relatively strong immune response against ETEC 987P whole cells by the immunization that was performed simply since there was no need to purify particular portions of bacteria for more immunogenicity. ETEC 987P whole cells (250 μg of cell; 7.8 μg of protein) were used to immunize a chicken once, which was sufficiently immunogenic to induce an immune response of a chicken. This result indicates that the production of antigen-specific IgY can be efficiently elicited in SCWL chickens immunized intramuscularly with ETEC 987P whole cells emulsified with Freund's incomplete adjuvant.

To implement application of IgY for feed supplementation, antibody production should be characterized as simple and practical. IgY possessing long duration of specific activities could be obtained from hyperimmunized chickens by using simple and practical immunization protocols as presented above. Therefore, these characteristics of IgY production may encourage IgY to be utilized in future application.

3.3.3. Properties of IgY Powder

Specific IgY powder was obtained from the WSF containing IgY with high levels of specific activity against ETEC 987P. IgY powder containing non-specific IgY was also prepared as a control (eggs collected during the immunization period of 5-9 wk). The

concentrations of protein, total and specific IgY were subsequently determined as shown in Table 3-1.

The ratio of total IgY to protein concentration was not significantly different between specific (16.7%) and non-specific (19.9%) IgY powder, which is indicative of both powders having similar IgY purity. The purity of IgY powder was decreased by approximately 17% in comparison to that of the WSF in the foregoing study. This may result from freeze-drying effect, which reduces solubility and ELISA value of IgY (Chansarkar, 1998). The concentration of ETEC 987P-specific IgY in IgY powder was assessed by the quantitative ELISA as described. Specific IgY concentration in control IgY powder was significantly lower than that in specific IgY powder as expected. The proportion of ETEC 987P-specific IgY in total IgY averaged 6.7%, which was lower than that (9.2%) of *E. coli* O157:H7-specific IgY as determined previously (Chapter 2). ETEC 987P may be less immunogenic than *E. coli* O157:H7 and induce immune responses of SCWL chickens to a lesser extent. Consequtently, specific IgY powder contained certain amounts of specific antibodies with antigen-binding activity and was used to further evaluate its anti-bacterial properties.

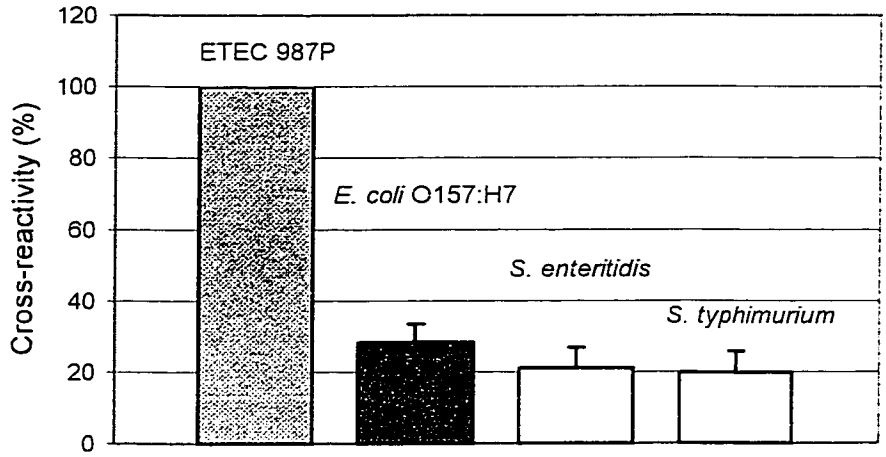
3.3.4. Cross-Reactivity of IgY

The cross-reactivity of anti-ETEC 987P IgY against other members of the family Enterobacteriaceae (*E. coli* O157:H7, *S. enteritidis*, and *S. typhimurium*) was measured by using the ELISA as described above. Anti-ETEC 987P IgY cross-reacted slightly with *E. coli* O157:H7, *S. enteritidis*, and *S. typhimurium* by 28.26%, 21.1% and 19.69%, respectively (Figure 3-3). It is speculated that those bacteria share some antigenic binding

Table 3-1. The concentrations of protein, total IgY and specific IgY in IgY powder prepared from the WSF containing ETEC 987P-specific or non-specific IgY. Values are the mean ± standard deviation.

IgY powder	Concentration (mg/g)		
	Protein	Total IgY	Specific IgY
ETEC 987P-specific	540 ± 40	90 ± 26	6 ± 0.7
Non-specific	468 ± 62	93 ± 21	<0.0001

Figure 3-3. The cross-reactivity of anti-ETEC987P IgY with other members of Enterobacteriaceae, including *E. coli* O157:H7, *S. enteritidis*, and *S. typhimurium*. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation.



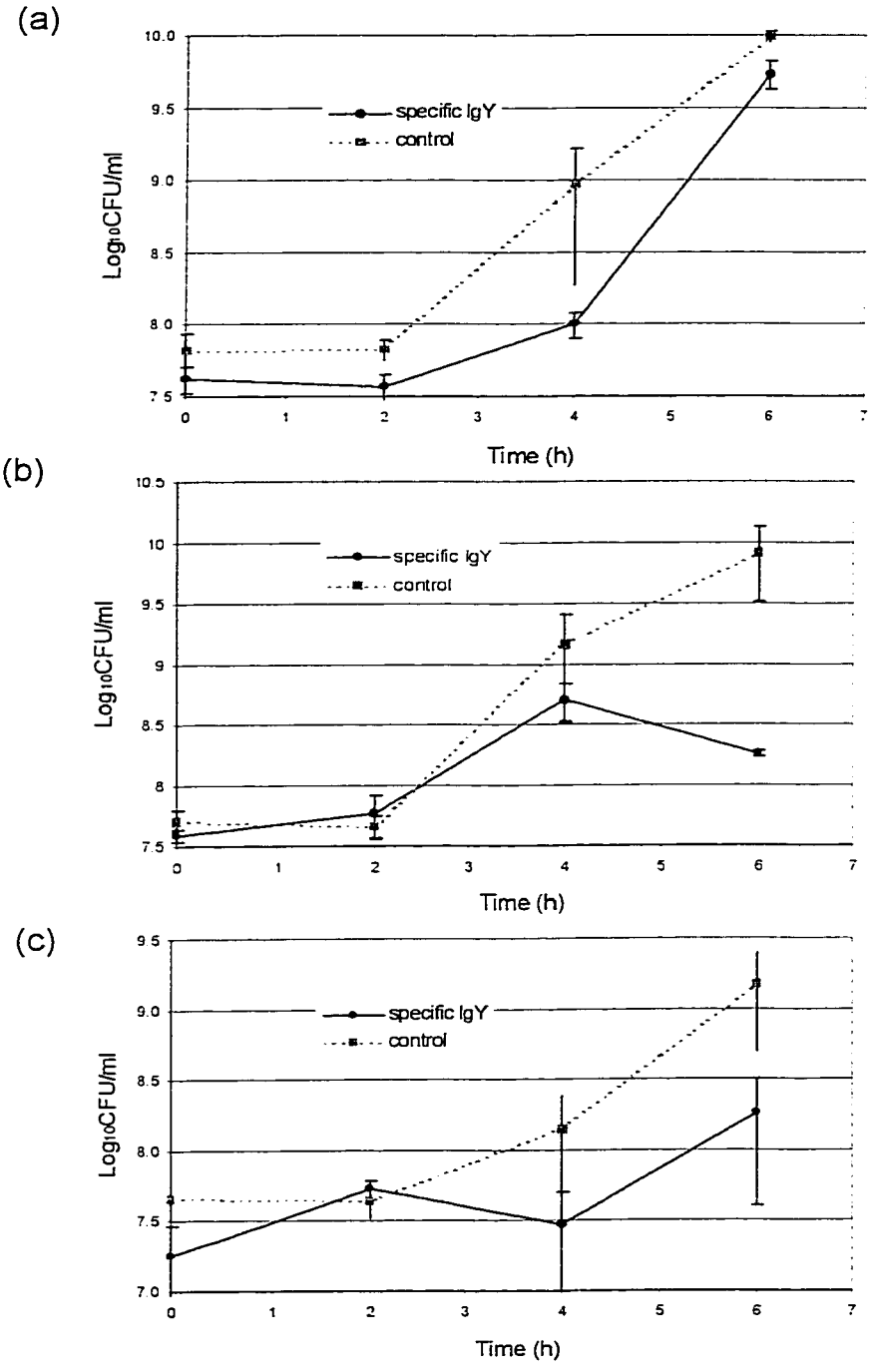
sites, epitopes, with ETEC 987P. Common lipopolysaccharide (LPS) of Gram-negative bacteria is likely to be one of the factors leading to cross-reactivtiy (Braun et al., 1976). IgY is a polyclonal antibody derived from large numbers of epitopes on an antigen and thus may share components that react with other pathogenic bacteria. This cross-reactivity may add more advantages to the use of IgY for preventing bacterial contamination of feeds.

3.3.5. Growth Inhibitory Effect of ETEC 987P-Specific IgY

The effect of IgY on ETEC 987P growth *in vitro* was investigated to prove whether IgY can inhibit the bacterial growth in a liquid medium. The growth curve of ETEC 987P generated under the same conditions as were applied in the growth inhibition assay was first plotted. It showed that 0 to 2 h of incubation time was lag phase, followed by exponential phase and then stationary phase reached at approximately 6 h of incubation time (Data not shown). Accordingly, the time of sampling to perform the growth inhibition assay was determined. The different concentrations of ETEC 987P-specific or non-specific IgY powder (0.045, 0.09, and 0.18 g/ml) were used for the test considering the effect of antibody amount on the interaction with antigen.

There was no significant difference between specific and non-specific (control) groups in the test using 0.045 g/ml of IgY (Figure 3-4(a)). On the other hand, the bacterial growth in the presence of more concentrated IgY was different between the two groups. As shown in Figure 3-4(b), bacteria were in the lag phase during the first 2 h incubation, which showed no growth of bacteria. This was followed by exponential phase after that time. This growth pattern was like the growth curve determined above. The

Figure 3-4. The effect of IgY on the growth of ETEC 987P in a liquid medium. Bacteria (approximately 1×10^7 CFU/ml) were grown in E-media mixed with different concentrations of ETEC 987P-specific or non-specific IgY powder at 37°C with shaking: (a) 0.045g/ml; (b) 0.09g/ml; (c) 0.18g/ml. The viable cells were counted by the plate count method. Values are the mean of duplicate samples. Vertical bars indicate the standard deviation. The solid line shows the growth pattern of ETEC 987P with specific IgY. The dotted line shows that of ETEC 987P with non-specific IgY as a control.



ncrease in number of ETEC 987P incubated with specific IgY was less compared to that of the control group during the exponential phase. Cell counts of the control group rose by 1.5 $\log_{10}$CFU/ml during 2-4 h of incubation time while those of specific group rose by 1.0 logCFU/ml. After 4 h incubation, there was a 0.7 logCFU/ml increase and 0.4 logCFU/ml decrease in the number of control and specific group, respectively. The difference of the bacterial growth between the two groups was 1.5 logCFU/ml at 6 h of incubation time. The concentration of ETEC 987P-specific IgY, which was effective in the inhibition of bacterial growth, was 0.54 mg/ml without considering losses of specific IgY during preparation of IgY solution. Two-fold greater concentration of IgY (1.08 mg/ml) also had an inhibitory effect on the growth of bacteria. The number of bacteria grown with specific IgY was reduced by 0.8 logCFU/ml when compared to that of the control group after 6 h incubation (Figure 3-4(c)). As such, the growth of ETEC 987P in liquid media was inhibited in the presence of IgY raised against homologous strain.

The mechanism of the inhibitory effect of IgY on bacterial growth is not clearly understood. The binding of antibody to bacteria may be the main step necessary to cause inhibition of bacterial growth. Antibodies bound to particular components on the bacterial surface possibly blocks the function of components that may be crucial factors for motility and growth of bacteria (Sim et al., 2000). IgY used in this study possesses binding activities against various epitopes derived from the bacterial surface due to the characteristic of a polyclonal antibody raised against bacterial whole cells. The binding activities of IgY against bacterial surface components, including fimbriae (pili), outer membrane protein and LPS, may facilitate the growth inhibition of bacteria. Further studies are necessary to provide an understanding of immunological properties of IgY.

3.3.6. Microscopic Observation of IgY Binding to ETEC 987P

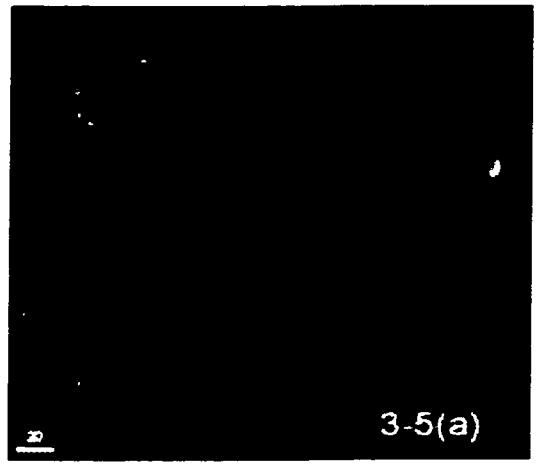
The binding of IgY to ETEC 987P was observed by using immunofluorescence microscopy. Bacteria were incubated with specific IgY or non-specific IgY as a positive control and without IgY as a negative control. Bacteria with bound IgY were then visualized by tagging IgY with rabbit anti-chicken IgG conjugated with FITC. Bacteria reacting with specific IgY fluoresced (Figure 3-5(a)) while bacteria in both control groups showed no fluorescence (Figure 3-5(b),(c)). The presence of fluorescence is indicative of the binding of specific IgY to bacteria. The positive result obtained from this immunofluorescence microscopy could be a preliminary test for further study on immunoelectron microscopy.

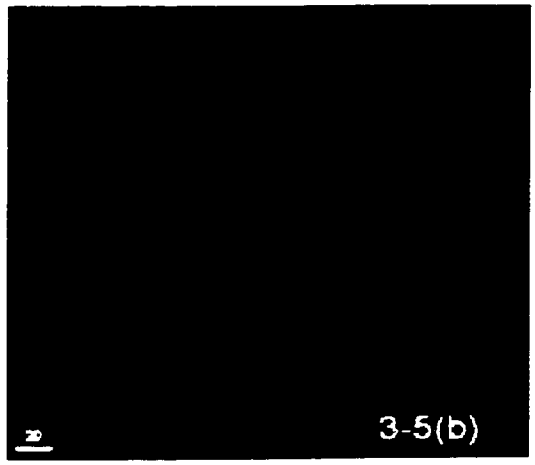
Bacteria incubated with antibodies, including IgY, rabbit anti-chicken IgG, and goat anti-rabbit IgG conjugated with immunogold, in this order, were observed by using transmission electron microscopy. The distribution of immunogold particles was observed around bacteria that reacted with specific IgY (Figure 3-6,7(a)). In contrast, there was no immunogold in the observation of bacteria incubated with non-specific IgY (Figure 3-6,7(b)). As a result, specific IgY was demonstrated to have the binding activity to bacteria.

It was noted that there were gold particles specifically on the surface of bacteria. Furthermore, structural alteration was observed in sectioned bacteria with bound specific IgY as displayed in Figure 3-7(a). These findings indicate that IgY binding to the surface components of bacteria may cause structural alterations. As speculated in the growth inhibition assay, particular components of the bacterial surface may be blocked by the binding of IgY, showing morphological changes and resulting in the impairment of

bacterial growth. The characterization of these components, which may be crucial for bacterial growth, remains to be studied further. The question of mechanism that leads to the inhibition of bacterial growth following the binding of IgY to bacteria needs to be elucidated as well.

Figure 3-5. Immunofluorescence micrographs of ETEC 987P incubated with (a) specific IgY; (b) non-specific IgY;and (c) without IgY (magnification 400 ×, Bar = 20 μm)





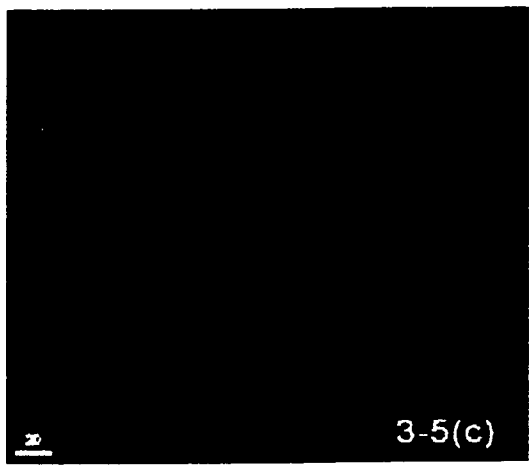
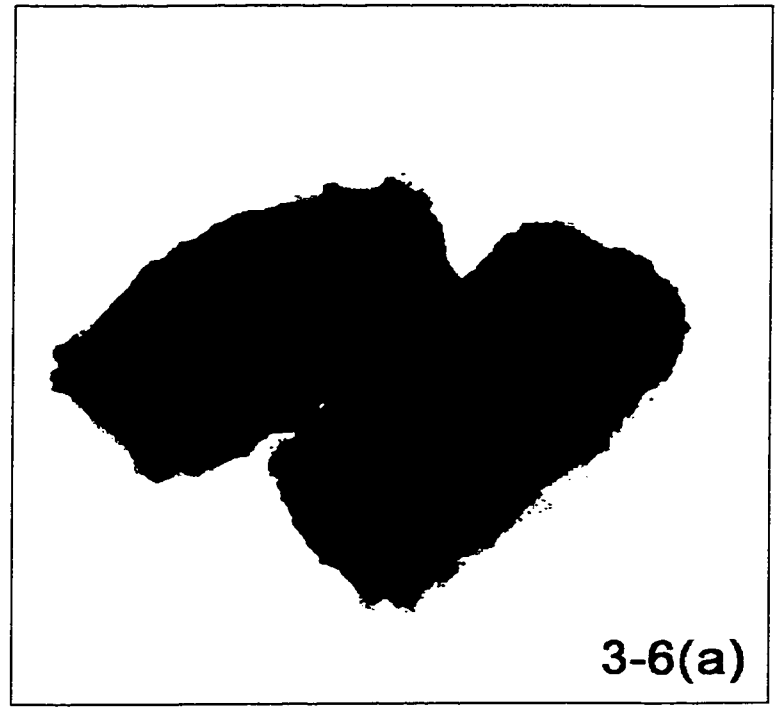


Figure 3-6. Immunoelectron micrographs of negatively stained ETEC 987P incubated with (a) specific IgY (magnification 6000×); (b) non-specific IgY (magnification 9000×)



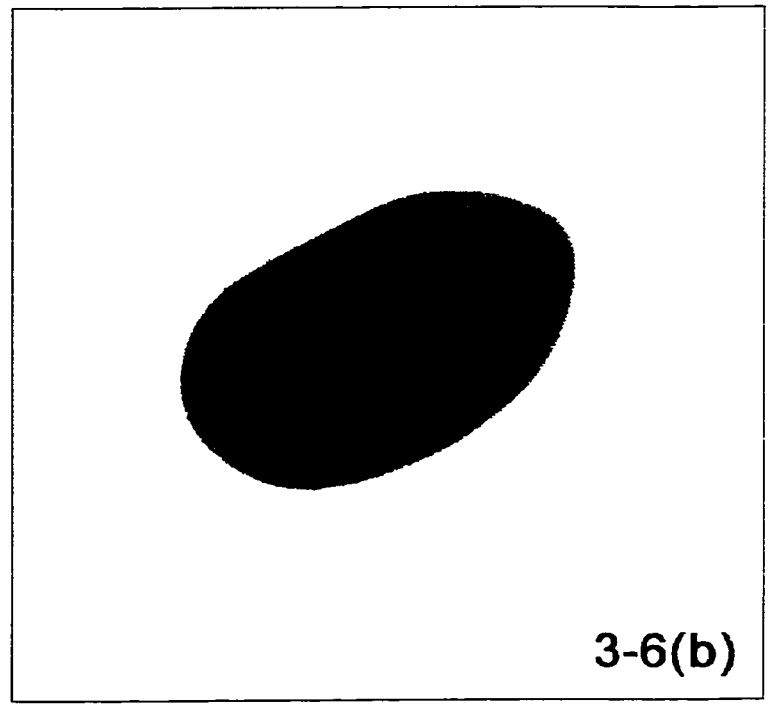
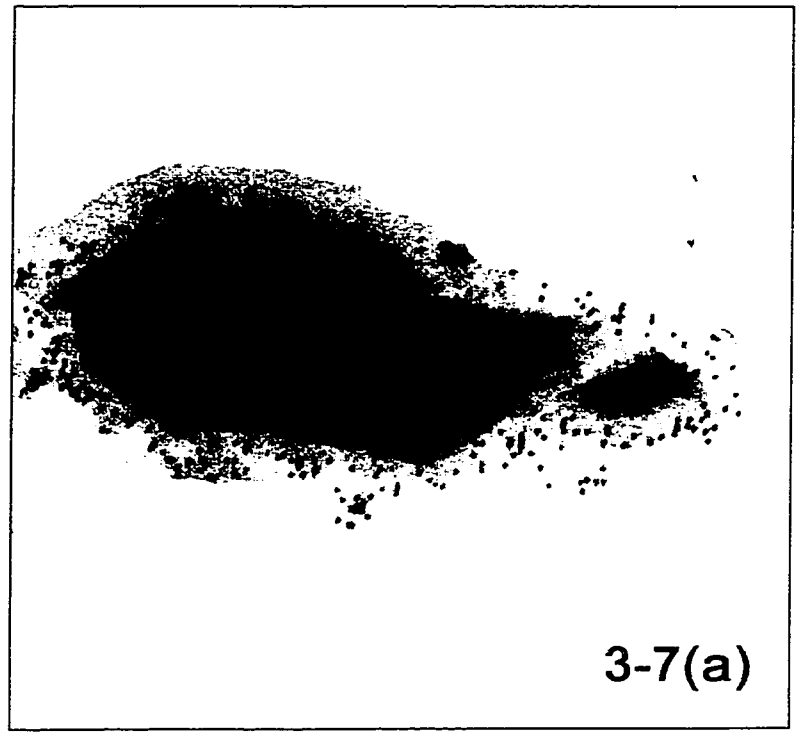
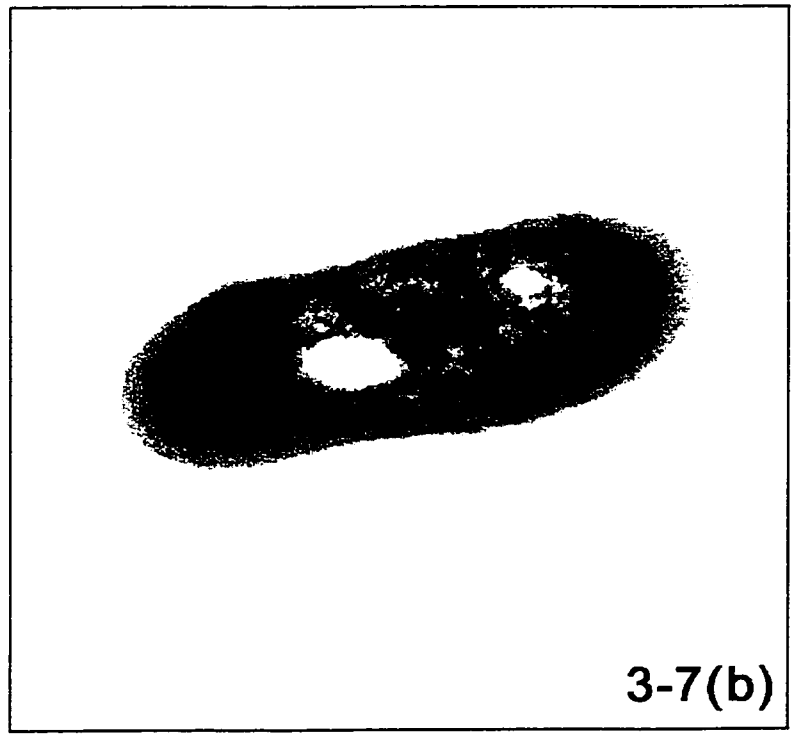


Figure 3-7. Immunoelectron micrographs of ultrathin sectioned ETEC 987P incubated with (a) specific IgY; (b) non-specific IgY (magnification 9,000×)





Chapter 4. Anti-Bacterial Properties of IgY against *Salmonella enteritidis* and *S. typhimurium*

4.1. Introduction

The high incidence of foodborne salmonellosis outbreaks has posed a threat to public health and has remained an important issue to be solved. *Salmonella* can cause a spectrum of pathological conditions such as gastroenteritis and bacteraemia in humans by the mechanisms of invasion, multiplication in, and penetration of the intestinal epithelium (Roberts et al., 1996). Two serovars, *Salmonella enteritidis* and *S. typhimurium*, account for most salmonellosis associated with foods of animal origin, including milk, poultry meat, and eggs (Houston, 1987; O'Brien, 1988; St Louis et al., 1988; CDC, 1990; Khakhria et al., 1997). Improper pasteurization has led to contaminated milk associated with salmonellosis outbreaks (Ryan et al., 1987). *Salmonella* infection of broiler or laying chickens mainly implicates poultry products in food contamination. The contamination of poultry meat products originates primarily from chickens infected with *Salmonella* during processing (MaGarr et al., 1980). *Salmonella*-infected laying hens produce eggs contaminated with *Salmonella* by two possible routes, transovarian and trans-shell (St Louis et al., 1988; Barnhart et al., 1991; Tauxe, 1991). Therefore, an effective strategy to prevent poultry products from contamination can be focused on the control of chickens infected with *Salmonella*.

There have been a number of reports showing different aspects of chicken infection: the greatest susceptibility of chickens to *Salmonella* infection during the first

few days of life (Nurmi et al., 1973; Pivnick, 1981) and the identification of contaminated feed and drinking water as important sources of *Salmonella* infection in chickens (Williams, 1981; Cox et al., 1983). On the basis of these findings, control measures such as the use of probiotics in chicks and organic acids in feeds or water have been studied and found effective in controlling *Salmonella* infection (Hinton et al., 1985; Rouse et al., 1988; Weirup et al., 1988; Fox, 1989). In recent years, increasing egg-associated outbreaks have resulted in need of preventive actions to be taken for reducing the risk of egg contamination with *Salmonella*, specifically, *S. enteritidis*. The more hazardous aspect of egg contamination is that intact shell eggs can be contaminated with these pathogenic bacteria that have heat-resistance and grow rapidly in the egg yolk (Humphrey, et al., 1989). The use of irradiation as a control measure has been recently approved in the U.S. to protect consumers from egg-associated bacterial infection. However, irradiation may have adverse effects such as the depletion of vitamins and unappealing visual and textural characteristics and should be taken into account when considering its efficacy (Federal Register, 2,000).

Chicken egg yolk antibody (IgY), which is practical for large-scale food or feed production, may serve as a potent salmonellosis-control measure, suggesting multi-faceted applications. Some studies reported that *Salmonella*-specific IgY can prevent fatal salmonellosis in mice or calves by oral administration (Peralta et al., 1994; Yokoyama et al., 1998 a, b). In addition, Promsopone et al. (1998) demonstrated that the mixture of probiotics and *Salmonella*-specific IgY have the beneficial effect of reducing the colonization of *S. typhimurium* in broiler chicks. These support a possible role for IgY in providing the passive immunization for broiler or laying chicks to protect them from

Salmonella infection. Furthermore, IgY may play other roles in controlling *Salmonella* contamination if the specific activity of IgY against *Salmonella* results in the growth inhibition of those bacteria. Laying hens immunized with *Salmonella* may produce egg yolks loaded with *Salmonella*-specific IgY. The egg yolk is the most susceptible part to *Salmonella* contamination and may be protected from *Salmonella* proliferation. The supplementation of IgY in chicken feed or drinking water or in milk could be considered as a control tool for preventing *Salmonella* contamination as well.

This study was, therefore, attempted to evaluate the anti-bacterial properties of IgY against *S. enteritidis* and *S. typhimurium* for possible future IgY application.

4.2. Materials and Methods

4.2.1. Bacteria and Culture Conditions

S. enteritidis (ESO 9325-92) and *S. typhimurium* (ATCC 14028) were obtained from Animal Health Laboratories Branch, Alberta Agriculture, Food and Rural Development, Edmonton, Alberta, Canada. Bacteria were cultured in tryptic soy broth (TSB) at 37°C for 24 h with shaking. After incubation, cells were harvested by centrifugation at 8,000 rpm for 15 min and were treated with 3.7% formalin overnight. The inactivated cells were washed three times, suspended in sterile saline and then freeze-dried. Lyophilized whole-cell cultures were stored at – 20°C until used.

4.2.2. Immunization of Chickens

All chickens were cared for in accordance with the Canadian Council on Animal Care guidelines of animal welfare. Immunization of hens was carried out as described (Sunwoo et al., 1996). Lyophilized *S. enteritidis* (500 μg of cell/ml; 3 μg of protein/ml) and *S. typhimurium* (500 μg of cell/ml; 4.5 μg of protein/ml) whole cells were suspended in sterilized PBS (pH 7.2) and were emulsified with an equal volume of Freund's incomplete adjuvant. Eight 23-wk-old Single Comb White Leghorn (SCWL) chickens were immunized intramuscularly at four different sites (0.25 ml per site) of breast muscles (two sites per left or right breast muscle) with cells or without cells as a control. Booster immunizations were given at 2 wk and 4 wk after the initial immunization in the same manner. Eggs were collected daily and stored at 4°C until used.

4.2.3. Isolation of Water-Soluble Fraction (WSF) Containing IgY from Egg Yolk

The WSF containing IgY was prepared from egg yolk using the water dilution method developed by Akita and Nakai (1992). The egg yolk was physically separated from egg white and rolled on paper towels to remove adhering egg white. The membrane was punctured and the yolk was allowed to flow into a graduated cylinder without the membrane. The egg yolk was first mixed gently with 8 volumes of cold distilled water (acidified with 0.1 M HCl to give pH 4.0) to avoid possible disruptions of egg yolk granules due to presence of high concentrations of acid and mixed gently. Cold acidified distilled water (pH 2.0) was then added to make the final dilution of 1:10. After mixing well, the mixture was adjusted to pH 5.0~5.2 and incubated at 4°C for 12 h. The WSF was obtained by centrifugation at 3,000 rpm and 4°C for 20 min and then stored at −20°C until analyzed.

4.2.4. Preparation of IgY Powder

The WSF that contained specific IgY with high levels of activities or non-specific IgY was neutralized with 0.1N NaOH to ensure that the results would not be confounded by the acidity and lyophilized to obtain IgY powder.

4.2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

The prepared WSF and IgY powder were assayed by an ELISA procedure as described by Sunwoo et al. (1996, 2,000).

4.2.5.1. Specific Activity of IgY

A microtiter plate was coated with 150 µl of lyophilized *S. enteritidis* (1.67 mg of cell/ml; 10 µg of protein/ml) or *S. typhimurium* (1.11 mg of cell/ml; 10 µg of protein/ml) whole cells in carbonate-bicarbonate buffer (0.05 M, pH 9.6) and was incubated at 37°C for 90 min. The plate was washed four times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tw). After washing, 200 µl of a 1% (wt/vol) solution of bovine serum albumin (BSA) in carbonate-bicarbonate buffer were added to each well and incubated at 37°C for 45 min. The BSA solution was then discarded and each well was washed four times with PBS-Tw. The WSF (diluted 1:1,000 in PBS) containing specific IgY or non-specific IgY as a control were added to wells (150 µl per well) and incubated at 37°C for 90 min. After the plate was washed four times with PBS-Tw, 150 µl of rabbit anti-chicken IgG conjugated with horseradish peroxidase (1:1,000 in PBS) was added to each well and incubated at 37°C for 90 min. The plate was washed four times with PBS-

Tw, followed by addition of 150 µl of freshly prepared substrate solution, 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) in 0.05 M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide. The reaction was continued for 30 min. Absorbance of the mixture was read at 405 nm using a Bio-Tek EL 309 microplate reader. The ELISA value of antibody activity was determined by subtracting the value of control antibody from that of specific antibody.

4.2.5.2. Cross-Reactivity of IgY

The cross-reactivity of IgY was determined by using the above ELISA method and the following bacterial cells were used: *S. enteritidis*, or *S. typhimurium, Escherichia coli* O157:H7 and *E. coli* 987P. Wells of the microtiter plate were coated with 150 µl of lyophilized whole cells in carbonate-bicarbonate buffer (*S. enteritidis* (1.67 mg/ml), *S. typhimurium* (1.67 mg/ml), *E. coli* O157:H7 (0.31 mg/ml), and *E. coli* 987P (0.5 mg/ml)). IgY powder specific for *S. enteritidis* (40 to 10 µg/ml) or *S. typhimurium* (64 to 16 µg/ml) was reconstituted and serially diluted with PBS. Reconstituted IgY powder (150 µl per well) was added to react with coated antigens. The cross-reactivity of anti- *S. enteritidis* or anti- *S. typhimurium* IgY against selected bacteria was determined by comparing activities against those bacteria with activities against *S. enteritidis* or *S. typhimurium*, respectively.

4.2.5.3. Total IgY Concentration

The ELISA was performed as described above, except the plate was coated with 150 µl of rabbit anti-chicken IgG at a final concentration of 3.75 µg/ml. Samples of the

WSF were diluted 1:90,000 with PBS. Specific or non-specific IgY powder was reconstituted and serially diluted with PBS (2 to 0.125 μg/ml). Two-fold serial dilutions of purified chicken IgG (1 mg/ml) in PBS (0.5 to 0.031 μg/ml) were used as the reference antibodies to prepare a standard curve. The standard curve (appendix 1) was then compared to provide a relative measurement of total IgY concentration.

4.2.5.4. Specific IgY Concentration

The concentrations of *S. enteritidis*- or *S. typhimurium*-specific IgY was measured by the ELISA as described by Sunwoo et al. (2,000). Wells of a microtiter plate were coated with 150 μl of rabbit anti-chicken IgG (3.75 μg/ml) and lyophilized *S. enteritidis* (1.67 mg/ml) or *S. typhimurium* (1.67 mg/ml) whole cells in carbonate-bicarbonate buffer, respectively. After incubation at 37°C for 90 min, the plate was washed four times with PBS-Tw. Two hundred microliters of a 1% (wt/vol) solution of BSA in carbonate-bicarbonate buffer was then added and incubated at 37°C for 45 min. After washing, two-fold serial dilutions of reconstituted specific (12.5 to 1.56 μg/ml) and non-specific (4.5 to 0.28 mg/ml) IgY powder in PBS were added to wells (150 μl per well) coated with *S. enteritidis* or *S. typhimurium* whole cells. Wells coated with rabbit anti-chicken IgG were filled with two-fold serial dilutions of purified chicken IgG (1 mg/ml) in PBS (0.5 to 0.008 μg/ml). The plate was subsequently incubated at 37°C for 90 min and washed. Secondary antibody, substrate and the measurement of absorbance were identical as described in section 4.2.5.1. The optical density at 405 nm was converted to μg of specific IgY/mg of IgY powder by using a quantitative standard curve (appendix 3) determined by the titration between rabbit anti-chicken IgG and purified chicken IgG.

4.2.6. Protein assay

The Bio-Rad protein assay (Microtiter Plate Protocol), based on the method of Bradford, was performed using purified chicken IgG (1 mg of protein/ml) as the reference protein. The WSF (diluted 1:100 in PBS) and two-fold serial dilutions of the reference protein in PBS (0.5 to 0.0625 mg/ml) were assayed on the microtiter plate. Absorbance at 595 nm after 5 min reaction was measured by a Bio-Tek EL 309 microplate reader.

The protein concentrations of specific or non-specific IgY powder were also measured by the same procedure. The reconstituted specific or control IgY powder to be tested were serially diluted 1:2 with PBS (0.5 to 0.0625 mg/ml).

4.2.7. Growth Inhibition Assay

4.2.7.1. Preparation of Bacteria

The same strain of *S. enteritidis* or *S. typhimurium* used as an antigen for immunizing chickens was subcultured on a blood agar plate at 37°C overnight and then suspended in TSB. The suspension was adjusted to an optical density of 0.05 at 600 nm, corresponding to a cell density of about 2.7×10^7 CFU/ml. The same volume of 20% (vol/vol) of glycerol in TSB was added for storage at – 70°C until used.

4.2.7.2. Bacterial Growth Curve

Two milliliters of prepared bacterial culture were mixed with 2 ml of TSB and incubated at 37°C with shaking. The turbidity of culture (OD at 600 nm) was measured by spectrophotometer (Spectronic 20) at 1 h intervals. The growth curve was plotted until reaching stationary phase.

4.2.7.3. Preparation of IgY Solution

Specific or non-specific IgY powder was reconstituted to 90, 180, and 360 mg/ml with TSB and then centrifuged at 3,500 rpm at 4°C for 20 min. The supernatant was sterilized by using a 0.22 μm-pore-size membrane filter.

4.2.7.4. Growth of S. enteritidis or S. typhimurium with IgY

Two milliliters of specific or non-specfic IgY solution were added to the same volume of prepared *S. enteritidis* or *S. typhimurium* culture. The bacteria and IgY mixtures were incubated at 37°C with shaking. Aliquots of samples (100 μl) were taken at 0, 2, 4, and 6 h of incubation time. Plate counts were performed by the spread plate method on TSB agar plates in duplicate. The inoculated plates were incubated at 37°C overnight. The number of colony-forming units (CFU) per plate was counted to determine the total number of bacterial CFU per ml of sample.

4.2.8. Microscopic Analyses

4.2.8.1. Immunofluorescence Microscopy

One hundred microliters of *S. enteritidis* (33.3 μg/ml) or *S. typhimurium* (33.3 μg/ml) cells suspended in PBS were incubated with the same volume of specific IgY or non-specific IgY (100 μg of IgY powder/ml PBS) or without IgY at 37°C for 1 h. After washing with PBS two times, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken IgG diluted 1:250 in PBS was added and then incubated at 37°C for 1 h. The samples were washed as before and resuspended in 50 μl of PBS. Cell suspension (10 μl) was smeared on the microscope slide, which was then air dried and coverslip was mounted by using a drop of mounting buffer (Glycerol-PBS, pH 7.2). Immunofluorescent staining of specimens was detected using a 2001 confocal laser scanning microscope (Molecular Dynamic).

4.2.8.2. Immunoelectron Microscopy

One milliliter of *S. enteritidis* (33.3 μg/ml) or *S. typhimurium* (33.3 μg/ml) cells suspended in PBS was centrifuged at 12,000 rpm for 10 min. To the cell pellets was added 1 ml of specific IgY or non-specific IgY (100 μg of IgY powder/ml of 1% BSA in PBS). After incubation at 37°C for 1 h, samples were washed with 1% BSA in PBS two times before adding 100 μl of rabbit anti-IgY IgG as the bridge (diluted 1:14 in 1% BSA in PBS), followed by incubation at 37°C for 1 h. After washing, samples were incubation with 300 μl of goat anti-rabbit IgG gold conjugate (diluted 1:25 in 1% BSA in PBS). The suspended cells were used for negative staining and ultrathin sectioning.

For negative staining, bacterial cells were washed with distilled water two times and subsequently mounted on a 300 mesh copper grid. Grid-mounted samples were

stained with 2% uranyl acetate. After washing and drying, specimens were observed with a transmission electron microscope (Hitachi H-7,000 TEM, Tokyo, Japan).

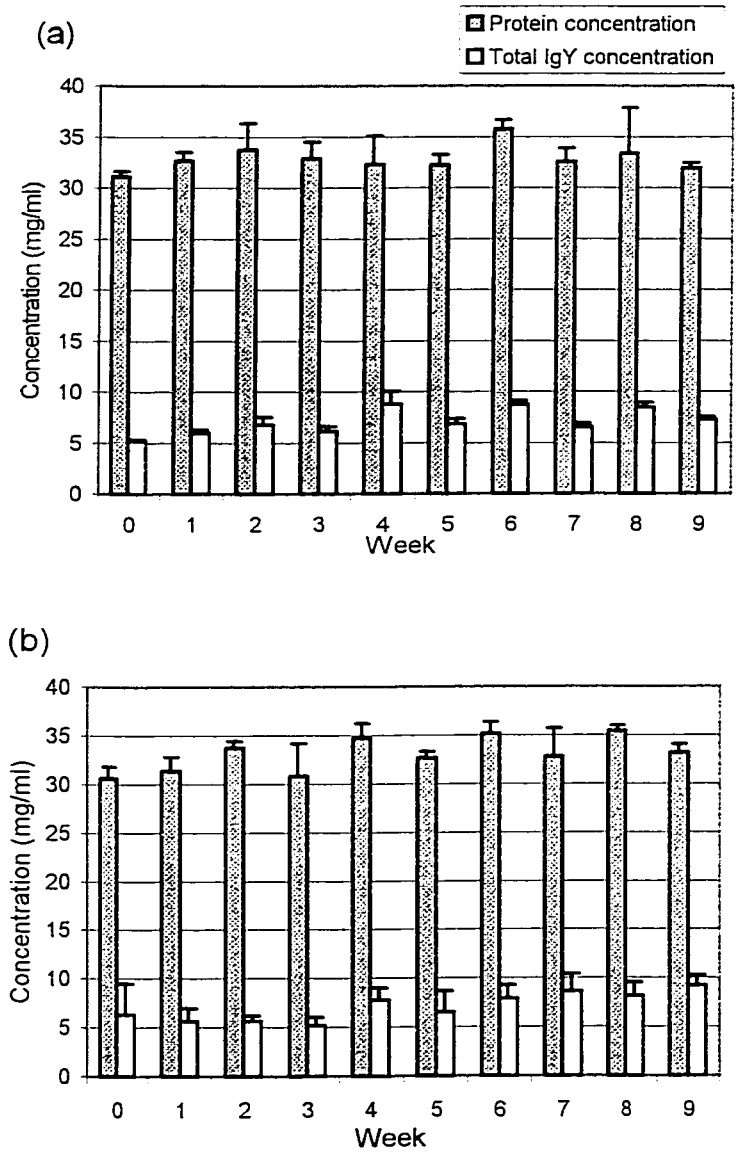
To perform ultathin section method, bacteria treated with antibodies were washed with 1% BSA in PBS two times, fixed with 2.5% glutaraldehyde for 1 h, and postfixed with 1% osmium tetroxide for 1 h. The fixed samples were dehydrated in a graded series of ethanol and embedded in Spurr's medium. After infiltration with Spurr's medium, polymerization was accomplished at 70°C for 12 h. The specimens were then thin sectioned using an ultramicrotome (Ultracut E model, Reichert-Jung, Austria). Ultrathin sections were mounted on a 200 mesh copper grid and stained with 2% uranyl acetate and then with lead citrate. The specimens were examined with a transmission electron microscope (Hitachi H-7,000 TEM, Tokyo, Japan).

4.3. Results and Discussion

4.3.1. Concentrations of Protein and Total IgY in the Egg Yolk WSF

The WSF was obtained weekly from egg yolks of immunized chickens by the water dilution method and analyzed to determine protein and total IgY concentrations. As shown in Figure 4-1(a)(b), the total IgY concentration of the WSF during the immunization period was relatively constant. Similar results were presented in other reports (Sunwoo et al., 1996; Li et al., 1998) and the previous chapter 2 and 3. The antibody content in the egg yolk of chickens remains constant regardless of immunization.

Figure 4-1. The concentrations of protein and total IgY in the WSF obtained from chickens immunized with (a) *S. enteritidis*; (b) *S. typhimurium* whole cells during the immunization period. Values are the mean of quadruple samples. Vertical bars indicate the standard deviation.



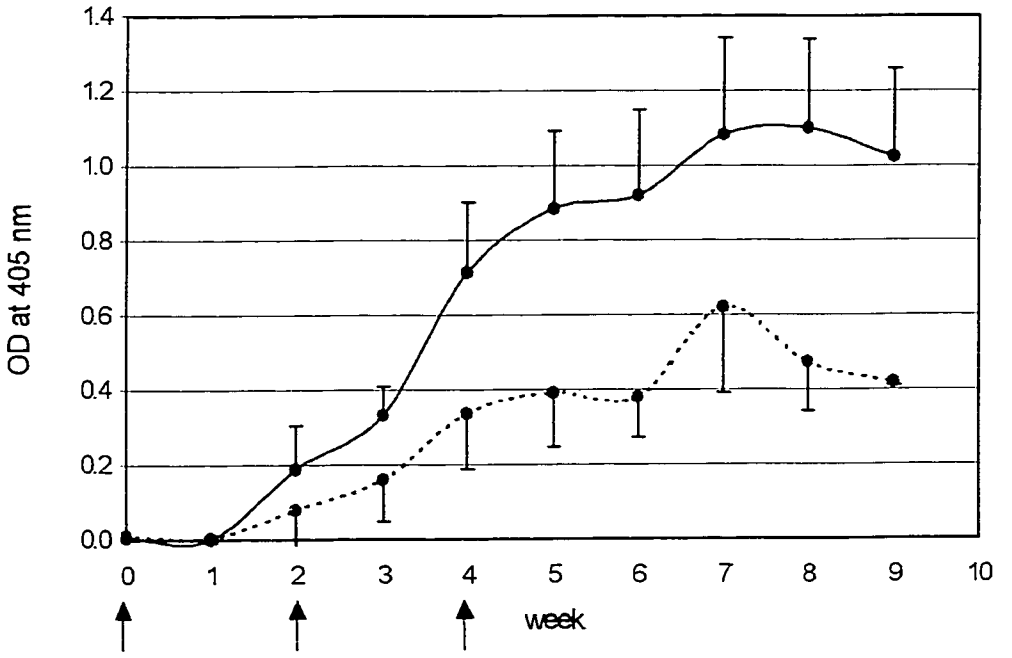
The average total anti-*S. enteritidis* IgY concentration (± standard deviation) in the WSF was 7.08± 1.24 mg/ml and that of anti-*S. typhimurium* was 7.09± 1.40 mg/ml. There was no difference between those two values, which indicates that the total IgY concentration in the egg yolk is independent of the kinds of antigens to raise antibodies. This result is consistent with reports that the differences in chicken strain (Li et al., 1998), bacterial antigen (Sunwoo et al., 1996), and adjuvant (Erhard et al., 1997) that were used for immunization did not affect the total IgY concentration in the egg yolk of immunized chickens.

In this study, IgY was extracted from egg yolk by using the water dilution method, where the extent of dilution and the pH are critical factors to be considered for the recovery, purity and activity of IgY in the WSF (Akita & Nakai, 1992). Thus, egg yolk was diluted 10-fold with acidified water to a final pH of 5.0-5.2, which is considered as a more efficient separation of lipid from the WSF with moderate recovery of IgY. The WSF containing anti-*S. enteritidis* IgY or anti-*S. typhimurium* IgY was obtained under these conditions with a purity (total IgY in protein) of 21.5% or 21.4%, respectively. The recovery of IgY might exist in the range of 92 to 96% on the basis of Akita and Nakai (1993) using the same conditions to obtain the WSF. Consequently, the water dilution method appears to be highly suitable for food application of IgY in that IgY present in the WSF can be produced in a large quantity by using a simple and economical method.

4.3.2. Specific Activities of IgY against *Salmonella* Antigens

Specific activities of anti-*Salmonella* IgY against *Salmonella* whole cells were monitored weekly by using the ELISA method. Figure 4-2 shows the change of specific

Figure 4-2. The change of specific activity of IgY in the egg yolk from chickens immunized with *S. enteritidis* whole cells (the solid line) or *S. typhimurium* whole cells (the dotted line) during the immunization period. The level of specific activity in a 1000-fold dilution of the WSF was measured by the ELISA using bacterial whole cells as an antigen and expressed as an ELISA value (OD at 405 nm). Values are the mean of quadruple samples. Vertical bars indicate the standard deviation. Arrows indicate the week of immunization.



activities of IgY in the WSF against antigens during the immunization period (up to 9 wk). The levels of activities increased one week after the initial immunization and then rose constantly. The lag time of one week can be explained in that it takes approximately one week for specific antibodies produced in chicken's serum to be transferred and accumulated in egg yolk. A one-week gap of specific-antibody occurrence between serum and egg yolk was also shown by Li et al. (1998), explaining the reason for the lag time.

The activity of anti-*S. enteritidis* IgY against *S. enteritidis* reached a peak (OD value of 1.10) at 8 wk and declined thereafter. Anti-*S. typhimurium* IgY activity against *S. typhimurium* also showed almost the same pattern as that of anti-*S. enteritidis* IgY. The level of activity increased to the maximum OD of 0.62 at 7 wk and then decreased. The overall activity of anti-*S. typhimurium* IgY was lower than that of anti-*S. enteritidis* IgY. This may be attributed to the prior immune state of chickens or inactivated *S. typhimurium* as a weak immunogen. Chickens immunized in this study were not tested to confirm they had not been previously exposed to *S. typhimurium* or to bacteria carrying cross-reacting antigens with *S. typhimurium*. This assumption can be made on the basis of which chickens are the major reservoir of other strains of *Salmonella* as well as *S. typhimurium*. The other reason for comparatively low activity of anti- *S. typhimurium* IgY could possibly be suggested from findings that inactivated *S. typhimurium* are less immunogenic than live *S. typhimurium* and thus inactivated *S. typhimurium* immunogens do not stimulate enough immune response to eliminate those bacteria in chickens (Germanier, 1972; Barrow et al., 1990; Hassan et al., 1991). However, it can not be concluded that the value of anti- *S. typhimurium* IgY activity is low compared to another

study which was performed under the same conditions as this study except for using different antigens derived from the same bacteria. Sunwoo et al. (1996) found that IgY raised against purified lipopolysaccharide (LPS) from *S. typhimurium*, which is known as a major immunogenic component, showed a lower value of activity than the value obtained in this study using *S. typhimurium* whole cells as crude antigens without further purification.

It is thus likely that both *Salmonella*-antigens used in the form of inactivated bacterial whole cell are immunogenic enough to induce an immune response and produce *Salmonella*-specific antibodies in chickens. The immunogenicity of an antigen is influenced by several factors, including the species or strain being immunized, antigen properties and dosage, the route of administration and the adjuvant (Kuby, 1997a). In this study, the production of *Salmonella*-specific antibody could be efficiently elicited in SCWL chickens immunized intramuscularly with *S. enteritidis* or *S. typhimurium* whole cells emulsified with Freund's incomplete adjuvant. As a consequence, IgY in the WSF was assessed to possess specific activity against *Salmonella* whole cells and could be used to determine anti-bacterial properties for further study.

4.3.3. Properties of IgY Powder

The WSF was obtained from egg yolk containing IgY with high levels of specific activity based on results shown in Figure 4-2 (eggs collected during the immunization period of 4 to 9 wk). IgY powder containing specific or non-specific IgY was then prepared by freeze-drying the WSF and was analyzed for protein, total IgY and specific IgY concentrations as presented in Table 4-1.

Table 4-1. The concentrations of protein, total IgY and specific IgY in IgY powder prepared from the WSF containing *Salmonella*-specific or non-specific IgY. Values are the mean ± standard deviation.

IgY powder	Concentration (mg/g)		
	Protein	Total IgY	Specific IgY
Anti-*S. enteritidis*			
Specific IgY	590 ± 38	129 ± 10	20 ± 7
Non-specific IgY	468 ± 62	93 ± 21	0.14 ± 0.036
Anti-*S. typhimurium*			
Specific IgY	605 ± 41	140 ± 35	14 ± 6
Non-specific IgY	468 ± 62	93 ± 21	0.14 ± 0.037

The purity (total IgY in protein) of *S. enteritidis*-specific IgY powder and *S. typhimurium*-specific IgY powder was 21.9% and 23.1%, respectively. These values were similar to those of the WSF as presented previously, which indicates that freeze-drying to prepare IgY powder from the WSF may be an appropriate method for stable preparation of IgY product in a concentrated and dried form.

The specific IgY concentration in IgY powder was 20 ± 7 and 14 ± 6 mg/g for *S. enteritidis*-specific IgY powder and *S. typhimurium*-specific IgY powder, respectively, which was 16% and 10% of total IgY. *S. typhimurium*-specific IgY powder contained lower amounts of specific IgY than *S. enteritidis*-specific IgY powder, which corresponds to previous results that the specific activity of anti-*S. typhimurium* IgY was lower than that of anti-*S. enteritidis* IgY. The specific IgY concentration in non-specific IgY powder, which was prepared as a control from non-immunized chicken egg yolk WSF, was significantly lower than that of specific-IgY powder as expected. Both control IgY powders contained 0.14 mg/g of specific IgY.

The specific IgY concentration in IgY powder is one of the major factors needed to be considered for the application of IgY in food production since the determination of the optimum amount of IgY to accomplish its immunological function can lead to the maximum effectiveness with the minimum cost. IgY powder properties characterized by containing certain amounts of specific IgY prompted the further evaluation to unveil the outcome of antibody-antigen binding activity.

4.3.4. Cross-Reactivity of IgY

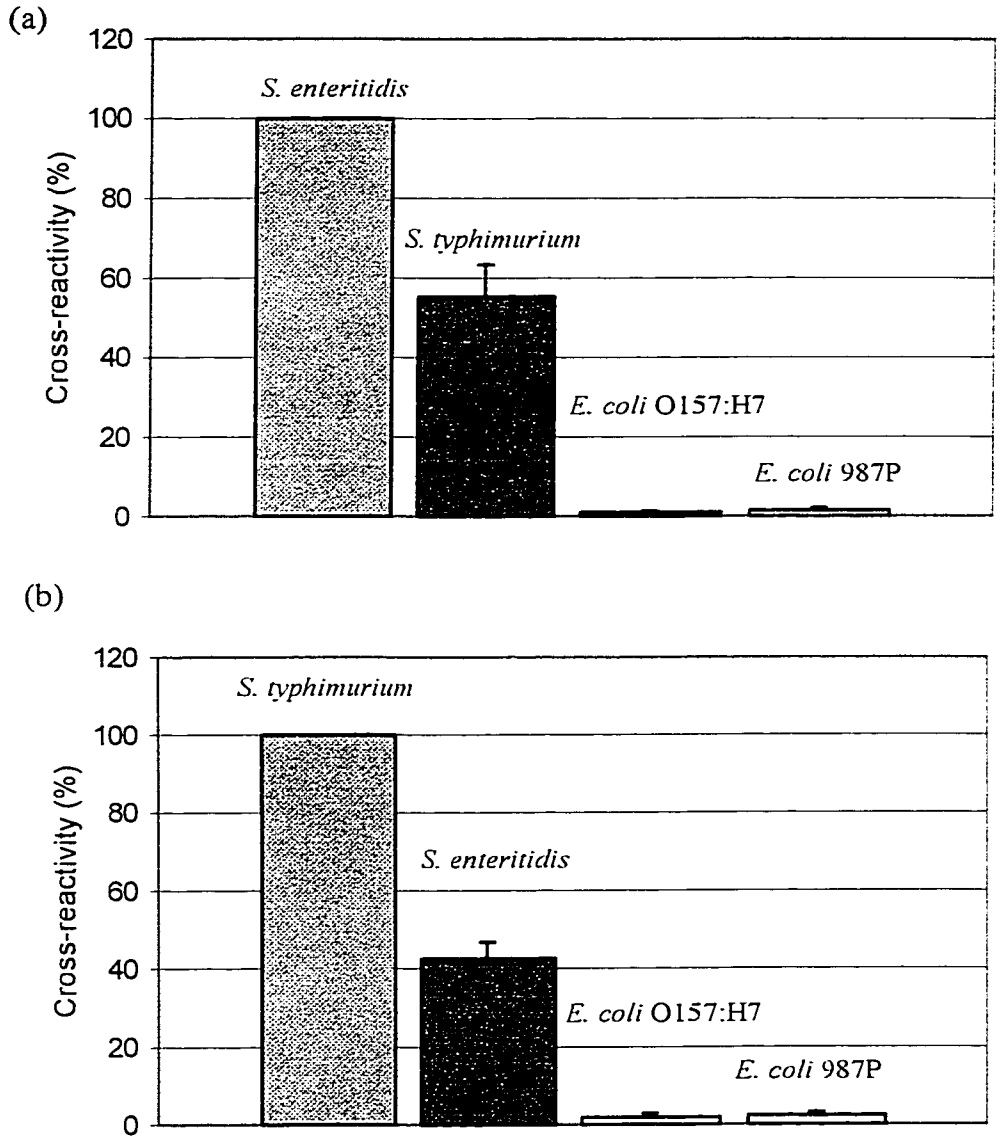
The specificity of anti-*Salmonella* IgY was investigated by measuring the cross-reactivity of IgY with other members of the family Enterobacteriaceae, including *Escherichia coli* O157:H7 and *E. coli* 987P. The cross-reactivity of anti-*Salmonella* IgY between *S. enteritidis* and *S. typhimurium* was also determined by using the ELISA method. The cross-reactivity of anti-*S. enteritidis* IgY with *E. coli* O157:H7 or *E. coli* 987P was significantly low as indicated by Figure 4-3(a). In contrast, anti-*S. enteritidis* IgY cross-reacted highly with *S. typhimurium* by 55.3%. Anti-*S. typhimurium* IgY also showed quite low cross-reactivity with both *E. coli* stains, while it did demonstrate high cross-reactivity with *S. enteritidis* by 42.4% (Figure 4-3(b)).

The high cross-reactivity of anti-*Salmonella* IgY between *S. enteritidis* and *S. typhimurium* can be explained by the fact that both *Salmonella spp.* share somatic antigens (O:1 and O:12) and common epitopes on the flagellin (Le Minor, 1984; van Zijderveld et al., 1992). Anti-*Salmonella* IgY used in this study was a polyclonal antibody against bacterial whole cells and thus there may be more possibility to raise antibodies against cross-reacting antigens. The cross-reactivity of IgY can add more values to anti-bacterial properties of IgY in that IgY may have an anti-bacterial effect on bacteria with cross-reacting antigen as well as target bacteria.

4.3.5. Growth Inhibitory Effect of *Salmonella*-Specific IgY

The specific binding activity of anti-*Salmonella* IgY against *Salmonella* was demonstrated by using the ELISA method as presented above. This result could prompt further study on growth inhibitory effect of IgY to be conducted. Growth curves of *S. enteritidis* and *S. typhimurium* were first determined under the same conditions as were

Figure 4-3. The cross-reactivity of (a) anti-*S. enteritidis* IgY; (b) anti-*S. typhimurium* IgY with other members of Enterobacteriaceae, including *Escherichia coli* O157:H7 and *E. coli* 987P. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation.



used in the growth inhibition assay. They were plotted until reaching stationary phase, which could provide information on incubation time and sampling time for the growth inhibition assay. Both growth curves showed similar patterns that comprised lag (0-2 h of incubation time), exponential (2-6 h of incubation time), and stationary phase (Data not shown). Accordingly, *S. enteritidis* or *S. typhimurium* was incubated with IgY for 6 h during which samples were taken at 2 h intervals to perform the growth inhibition assay. In this assay, different concentrations of specific or non-specific IgY were used considering the importance of antibody quantities to effectively interact with antigens.

Figure 4-4(a) shows the growth of *S. enteritidis* incubated with *S. enteritidis*-specific or non-specific IgY (control) in a liquid medium during 6 h incubation. Control IgY had no effect on bacterial growth, which maintained a lag phase and exponential phase during 0-2 and 2-6 h of incubation time, respectively. However, the growth of *S. enteritidis* incubated with specific IgY was different showing a reduction in bacterial growth after 4 h incubation. Cell counts of the specific and control groups increased by 0.3 logCFU/ml and 1.2 logCFU/ml, respectively, during 4-6 of incubation time, indicating that bacteria in the specific treatment group proliferated four times less than the control group. The difference in bacterial growth between the two groups implied that specific IgY has an inhibitory effect on the growth of *S. enteritidis*. The concentration of specific IgY that was effective in inhibiting bacterial growth was 0.45 mg/ml, assuming that there was no loss of IgY content in IgY solution in the process of filtration for the preparation of IgY solution.

The growth of *S. typhimurium* incubated with *S. typhimurium*-specific or non-specific IgY in a liquid medium was also monitored during 6 h incubation as shown in

Figure 4-4. The effect of IgY on the growth of *Salmonella* in a liquid medium. Bacteria (approximately 1×10^7 CFU/ml) were grown in TSB mixed with *Salmonella*-specific or non-specific IgY powder at 37 °C with shaking: (a) the growth of *S. enteritidis* incubated with 0.045 g of IgY powder/ml; (b) the growth of *S. typhimurium* incubated with 0.045 g of IgY powder/ml. The viable cells were counted by the plate count method. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation. The solid line shows the growth pattern of *Salmonella* with specific IgY. The dotted line shows that of *Salmonella* with non-specific IgY as a control.

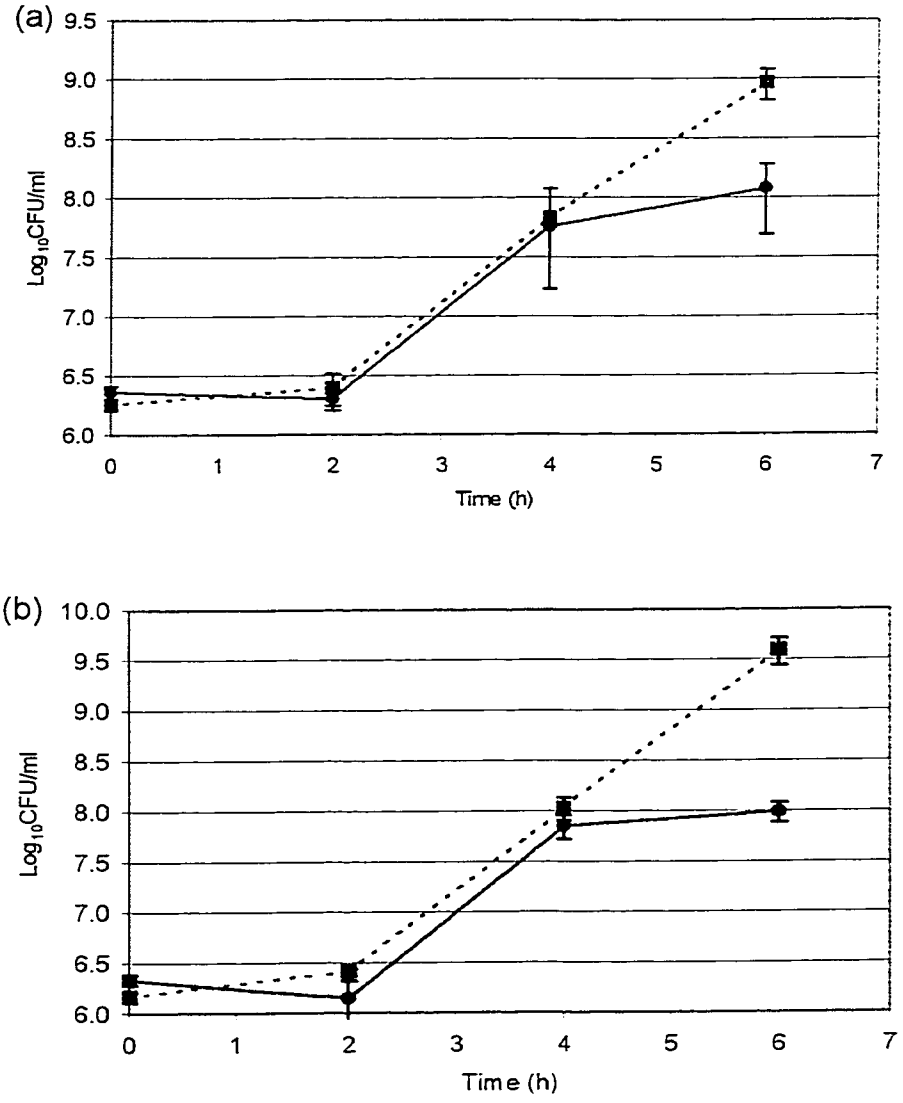


Figure 4-4(b). The growth patterns of the specific and control groups were similar to those observed in the experiment of *S. enteritidis* with IgY. There was no difference in bacterial growth between the two groups during 4 h incubation. However, the degree of bacterial growth when incubated with specific IgY decreased 16-fold less than that of the control group. Consequently, cell counts of the specific group were reduced by 1.6 log CFU/ml in comparison to those of the control group at 6 h of incubation time. The concentration of specific IgY that showed the growth inhibitory effect was 0.63 mg/ml.

As a result, both *Salmonella*-specific IgY were found to inhibit the growth of homologous *Salmonella* in a liquid medium. The mechanism by which antibodies can suppress bacterial growth is not clearly understood. The agglutination of bacterial cells cross-linked by antibodies may be one of mediators to cause the growth inhibition of bacteria. Bacterial cells clumping together may have less motility and opportunity to take nutrients and proliferate than free-motile single bacterial cell. However, there have been reports to show that bacterial growth is inhibited by antibodies in solid medium where bacteria cannot aggregate, demonstrating that the reduction of bacterial colony counts mediated by antibodies is not attributed to the agglutination reaction (Sadziene et al., 1992; Feldmann et al., 1992). In addition, Kubo et al.(1973) reported that the agglutinating property of IgY can be displayed only at raised salt concentrations or low pH conditions due to the steric hindrance caused by so closely aligned Fab arms of IgY.

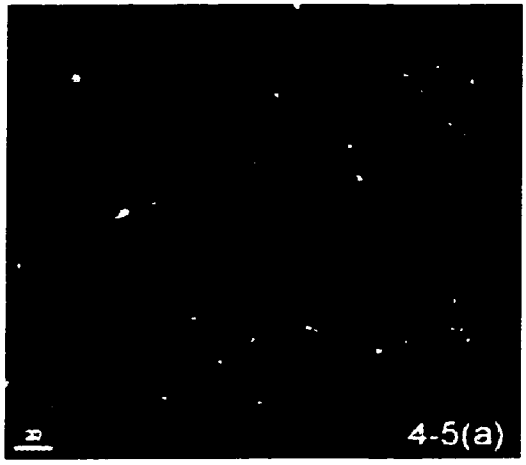
Therefore, there may be other reactions between IgY and bacteria to cause the growth inhibition of bacteria than the agglutination reaction. The specific binding of IgY to bacteria appears to be involved in bacterial growth inhibition. Particular components expressed on the bacterial surface, which are crucial factors for the bacterial growth, may

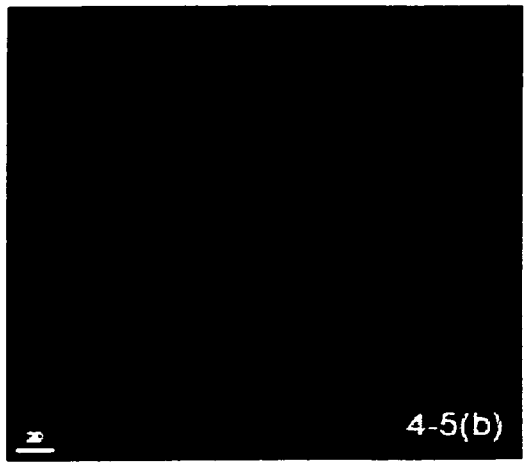
be recognized and bound by related polyclonal antibody, IgY. This binding may block or impair the function of growth-related bacterial components and lead to bacterial growth inhibition. Outer membrane protein, lipopolysaccharide, fimbriae (or pili), and flagella may be included in these bacterial surface components (Sim et al., 2,000). Therefore, the specific binding activity of IgY, as a potential candidate for the major anti-bacterial property, requires more intensive studies uncover the mechanism of inhibition.

4.3.6. Microscopic Observation of IgY Binding to *Salmonella*

The specific binding activity of *Salmonella*-specific IgY against *Salmonella* was further evaluated by microscopic observation. Immunofluorescence and immunoelectron microscopy were carried out to visualize *Salmonella* bound by IgY. Figure 4-5 shows micrographs of *S. enteritidis* incubated with IgY observed using immunofluorescence microscopy. Bacteria were incubated with specific IgY or non-specific IgY as a positive control and without IgY as a negative control. IgY bound to bacteria could be then tagged by rabbit anti-chicken IgG conjugated with FITC, which is a fluorescent dye. As presented in Figure 4-5, fluorescence was observed in *S. enteritidis* incubated with *S. enteritidis*-specific IgY while there was no fluorescence in the control groups. The presence of fluorescence implies that IgY is specifically bound to bacteria, demonstrating the specific binding activity of IgY against the bacteria. A positive result was also obtained from the experiment of *S. typhimurium* with IgY. *S. typhimurium*-specific IgY was proven to possess the specific binding activity against *S. typhimurium* as indicated by fluorescence in Figure 4-6(a). The immunofluorescence microscopy performed could be

Figure 4-5. Immunofluorescence micrographs of *S. enteritidis* incubated with (a) specific IgY; (b) non-specific IgY; (c) without IgY (magnification 400 ×, Bar = 20 μm)





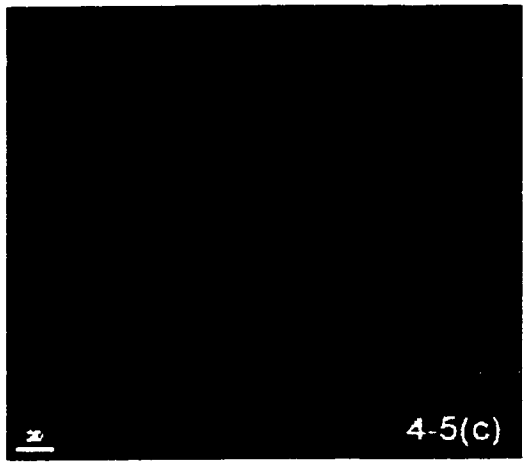
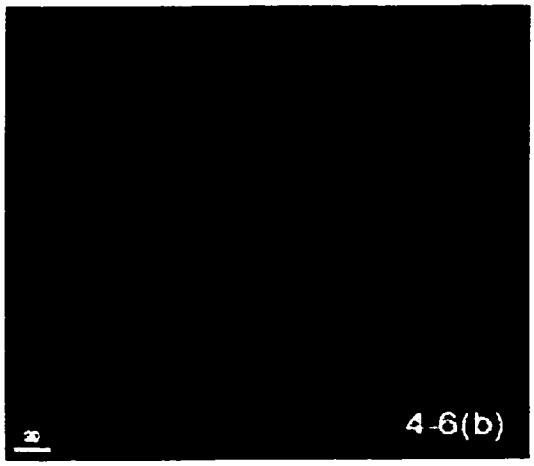
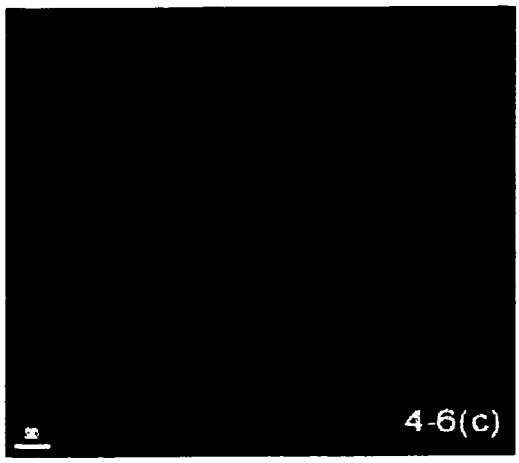


Figure 4-6. Immunofluorescence micrographs of *S. typhimurium* incubated with (a) specific IgY; (b) non-specific IgY; (c) without IgY (magnification 400 ×, Bar = 20 μm)





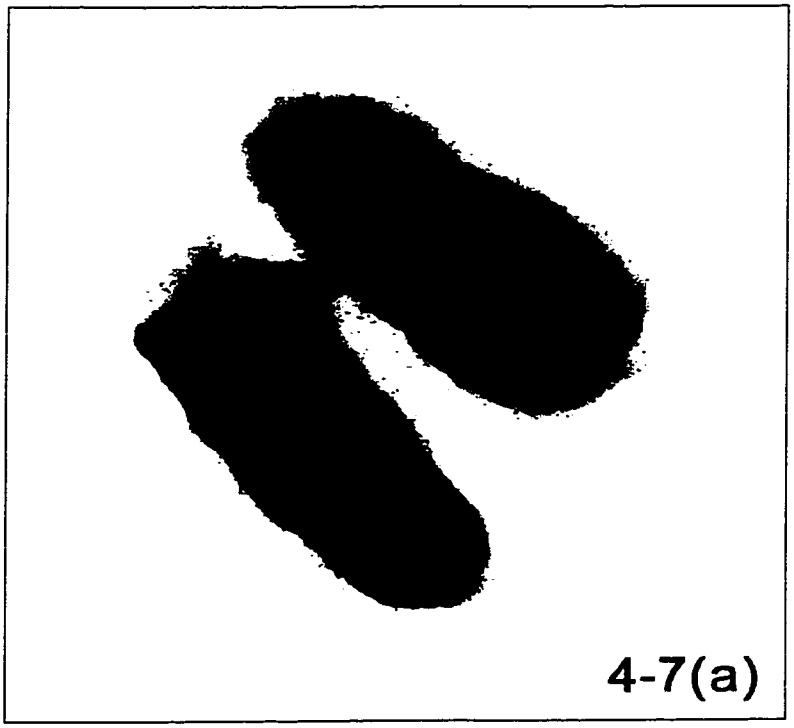
a preliminary test to confirm the specific binding activity of IgY and to further evaluate its activity through immunoelectron microscopy.

Immunoelectron microscopy was conducted by procedures described previously, which were a series of incubation of *Salmonella* with IgY, rabbit anti-chicken IgG, and goat anti-rabbit IgG conjugated with immunogold, in this order. Bacteria were then negatively stained or sectioned to be observed with a transmission electron microscope. Figure 4-7,8 show micrographs of *S. enteritidis* incubated with *S. enteritidis*-specific IgY or non-specific IgY. Immunogold particles were observed around bacteria incubated with specific IgY (Figure 4-7,8(a)), in contrast to the observation of bacteria incubated with non-specific IgY (Figure 4-7,8(b)). Bacteria labeled with immunogold indicated that bacteria were bound by specific IgY, which substantiates the specific binding property of IgY against bacteria. The experiment of *S. typhimurium* with IgY also resulted in the presence of immunogold particles around bacteria incubated with *S. typhimurium*-specific IgY as shown in Figure 4-9,10(a). *S. typhimurium*-specific IgY was verified to have the specific binding activity against *S. typhimurium* as well.

Furthermore, immunoelectron microscopic observation revealed the distribution of immunogold particles around the bacterial surface. It was also found that the bacterial surface was structurally altered as shown in Figure 4-8,10(a). These findings can be indicative that specific IgY is attached to components exposed on the bacterial surface, resulting in the structural alterations of the bacterial surface. One of the possible causes leading to the inhibition of bacterial growth is the reaction between bacterial surface components and IgY raised against those components as explained previously. This assumption may be corroborated on the basis of this morphological change of bacteria

with bound IgY. Further studies remain to be carried out to attest which bacterial surface components are bound by specific IgY and how those binding activities of IgY result in the growth inhibition of bacteria.

Figure 4-7. Immunoelectron Micrographs of negatively stained *S. enteritidis* incubated with (a) specific IgY (magnification 6300×); (b) non-specific IgY (magnification 6000×)



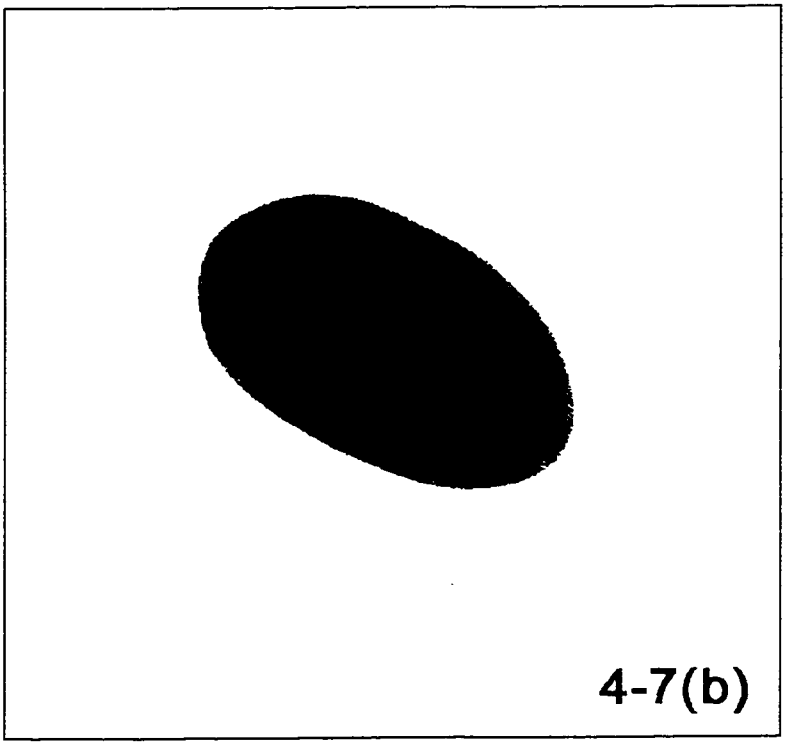
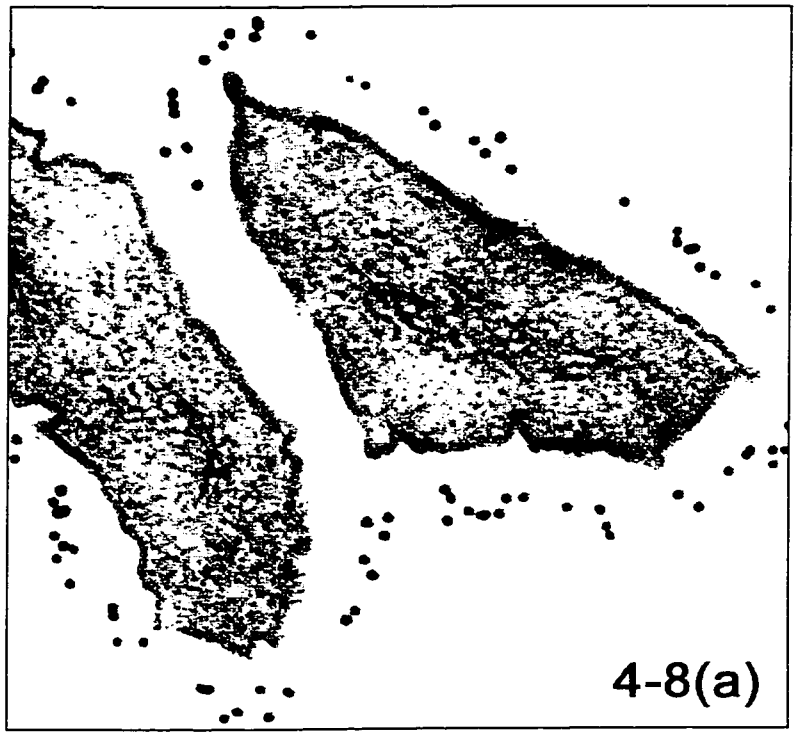


Figure 4-8. Immunoelectron micrographs of ultrathin sectioned *S. enteritidis* incubated with (a) specific IgY (magnification 9000×); (b) non-specific IgY (magnification 5100×)



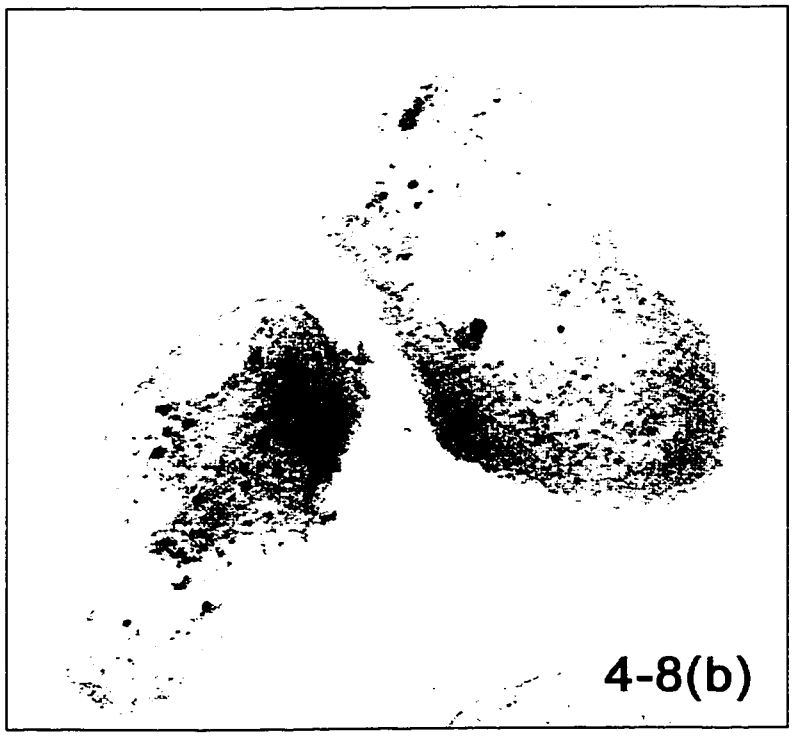


Figure 4-9. Immunoelectron micrographs of negatively stained *S. typhimurium* incubated with (a) specific IgY; (b) non-specific IgY (magnification 9,000×)

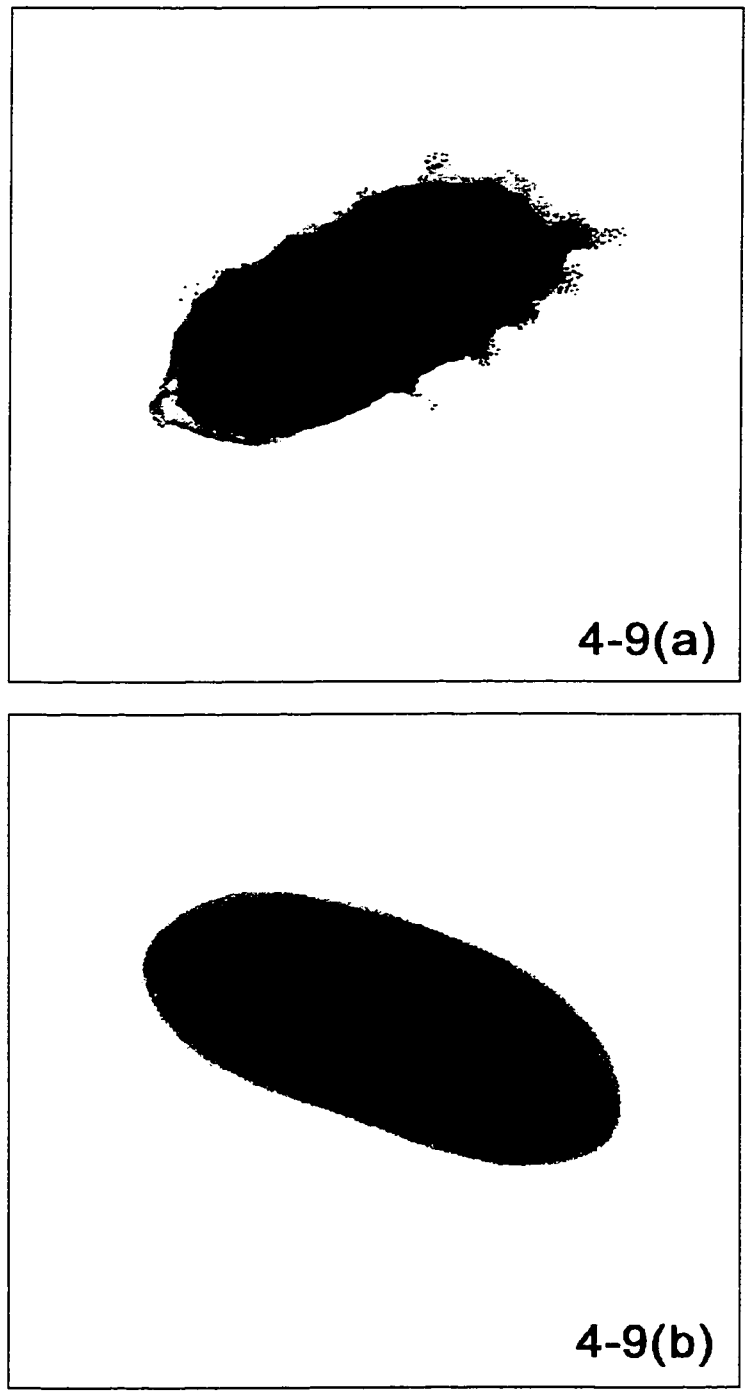
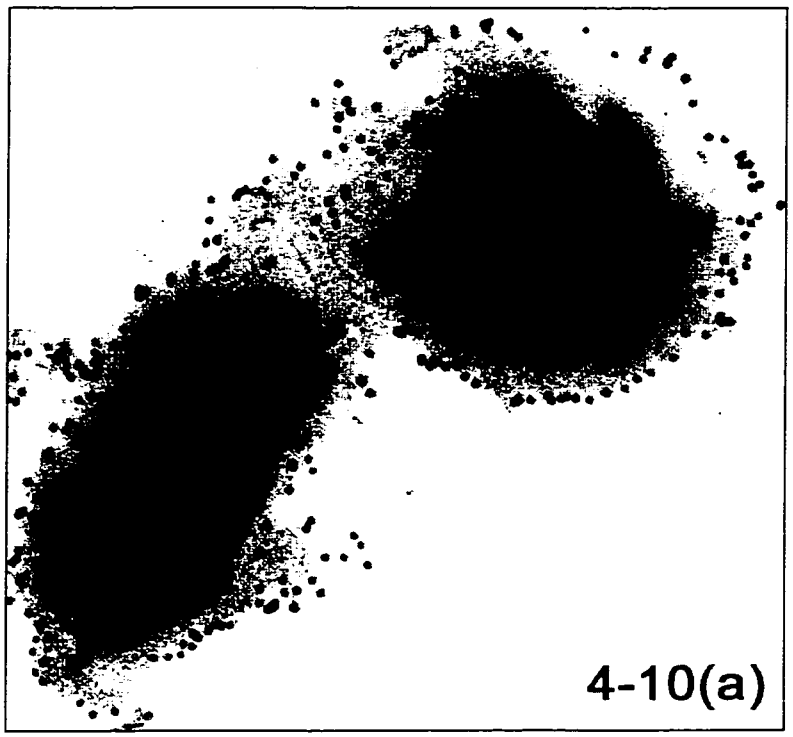
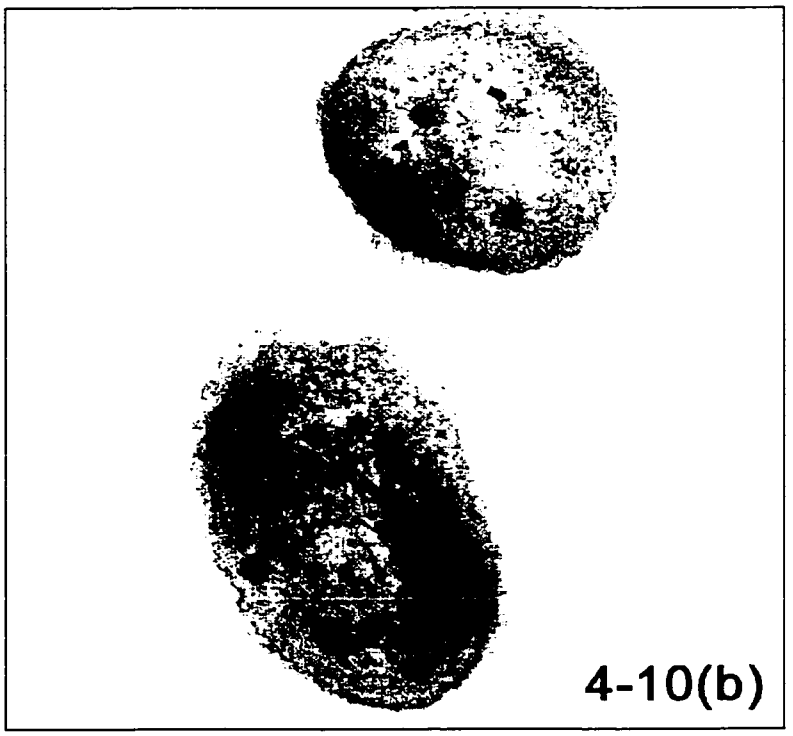


Figure 4-10. Immunoelectron micrographs of ultrathin sectioned *S. typhimurium* incubated with (a) specific IgY; (b) non-specific IgY (magnification 6,000×)





Chapter 5. Conclusions

The existence of antibody in humans and animals is one of the marvelous phenomena in the biological world. The function of antibody as a defence mechanism is so complicated that incessant studies have been long carried out to unveil antibody-related facts. On the basis of knowledge about the reaction of antibody with antigen, antibody has been used for a wide range of applications. In other words, the binding activity of antibody against antigen, which is the first step to process antigen, has made it possible to develop antibody technology in many areas such as biological and medical science. Various antibody sources, including colostrum of cows, milk and blood of animals, thus, have been explored to obtain antibody suitable for different applications.

Chicken egg yolk is also a valuable source of antibody, possessing advantageous characteristics for broad application. Food science and product technology is a potential area in which chicken egg yolk antibody (IgY) may be practically applied due to increasing interest in value-added foods and the applicability of food-based IgY. Therefore, the immunological property of IgY, which can serve as 'value' in foods, needs to be investigated to provide more evidence for IgY application.

The binding activity of antibody is commonly known to aid in processing of antigen by immunolgical effectors such as phagocytes and complement. However, there may be another possible function of antibody, which may encourge IgY to be applied in foods. In this regard, the hypothesis based on the previous findings of anti-bacterial activity of IgY and growth inhibitory effect of monoclonal antibody was suggested: the binding activity of IgY against bacteria may result in their growth inhibition.

Accordingly, the studies were designed to substantiate this hypothesis, considering future IgY application for food product, that is, food supplements. Antigens against which IgY may have an effect were chosen as major enteric pathogens associated with foodborne diseases in humans or animals. In addition, simple and practical methods were used for IgY production to support the suitability of IgY for food production.

IgY specific for pathogenic bacteria, such as *Escherichia coli* O157:H7, enterotoxigenic *E. coli* 987P (ETEC 987P), *Salmonella enteritidis*, or *S. typhimurium*, was produced in egg yolks of Single Comb white leghorn (SCWL) chickens immunized with whole cells. Simple protocols for immunizing chickens, which consist of bacterial whole cells as an antigen (250 μg of cell/ml) and two booster immunizations, were sufficiently applicable to elicit immune responses in chickens. This immune response could be characterized as long-lasting and strong, resulting in the production of high levels of antigen-specific IgY during the long period (up to 19 wk for anti-ETEC 987P IgY).

IgY was isolated in the form of the water-soluble fraction (WSF) from egg yolk by using the water dilution method. The WSF could be obtained with an appropriate purity of IgY (average 28.8% total IgY in protein) by only adjusting pH to 5.0-5.2 and diluting ten times with water. The WSF containing high levels of specific IgY against bacteria was concentrated to IgY powder as a dried product by using freeze-drying. IgY powder possessed an average 22.3% of IgY purity (based on protein) and 10.5% specific IgY in total IgY. The large amount of specific IgY determined by the simple quantitative ELISA technique could demonstrate the advantageous utilization of IgY for food application. Furthermore, the analysis of IgY powder showed an IgY property of cross-

reactivity. Anti-*E. coli* IgY had low cross-reactivity with other members of the family Enterobacteriacae. In contrast, anti-*Salmonella* IgY showed high cross-reactivity between *S. enteritidis* and *S. typhimurium*. The cross-reactivity of IgY may increase the efficiency of the anti-bacterial property of IgY against various bacteria sharing epitopes.

In addition to the specific binding activity of IgY against bacteria, the anti-bacterial property of IgY was evaluated further to substantiate the hypothesis of the inhibitory effect of IgY on bacterial growth *in vitro*. Growth inhibition assays, using *E. coli* O157:H7, *E. coli* 987P, *S. enteritidis*, or *S. typhimurium*, demonstrated that bacterial growth was inhibited by respective bacteria-specific IgY. Bacterial counts of the specific treatment group were reduced by a maximum of 2.3 logCFU/ml after 6 h incubation, in comparison to that of the control group. The amount of specific IgY effective in inhibiting bacterial growth varied in a range between 0.54 to 2.34 mg/ml. As a result, the specific binding activity of IgY was found to have an inhibitory effect on the bacterial growth.

The specific binding activity of IgY leading to the inhibition of bacterial growth was further explored by using immunofluorescence and immunoelectron microscopy. These studies could visualize the interaction of bacteria with IgY in more detail than the ELISA technique and growth inhibition assay. Immunofluorescence microscopic analyses showed the presence of fluorescence in bacteria reacted with specific IgY, which was indicative of the binding of specific IgY to bacteria. This study could be used as a preliminary test for immunoelectron microscopy, including negative staining and ultrathin sectioning methods. The specific binding of IgY to bacteria was also observed by immunoelectron microscopic analyses. The observation of immunogold particles

labeling bacteria, furthermore, revealed the distribution of gold particles on and structural alterations of the bacterial surface. These findings may support the possibility that specific IgY binds to various components, including lipopolysaccharide, outer membrane protein, fimbriae (or pili) and flagella, of bacterial surface structure, which are crucial for bacterial growth. Bound IgY may cause structural alterations of bacteria, resulting in the impairment of bacterial growth.

In conclusion, anti-bacterial properties of IgY demonstrated in these studies may be of value for food or feed supplementation. Bacteria-specific IgY may play a protective role in foods or feeds, preventing contamination by pathogenic bacteria and consequently reducing the risk of pathogens-causing infection in humans or animals. To date there have been efforts to develop effective means for controlling or preventing foodborne diseases, which are mainly caused by pathogenic bacteria contaminating foods. IgY, as a food-based deterrent, may serve as a novel protective measure characterized by being economical, efficacious and safe. Evidence based on these studies may verify the applicability of IgY as follows:

1. Simple and practical immunization of chickens;
2. Use of bacterial whole cells for antigens to raise high levels of specific IgY;
3. Water-dilution method for isolating IgY in relatively large amount and high purity;
4. Preparation of IgY powder as a food or feed product;
5. Available measurement of the amount of specific IgY;
6. Cross-reactivity of IgY;
7. Growth inhibitory effect of IgY on enteric pathogens.

Furthermore, future studies can be proposed to provide an understanding and efficiency of the anti-bacterial property of IgY for the development of IgY technology.

1. Study on the mechanism of growth inhibitory effect of IgY;
2. Study on the determination of bacterial surface components bound by IgY;
3. Study on the efficiency of IgY cocktails obtained from chickens immunized with various pathogenic bacteria;
4. Study on the effectiveness of the anti-bacterial property of IgY in foods or feeds.

Chapter 6. References

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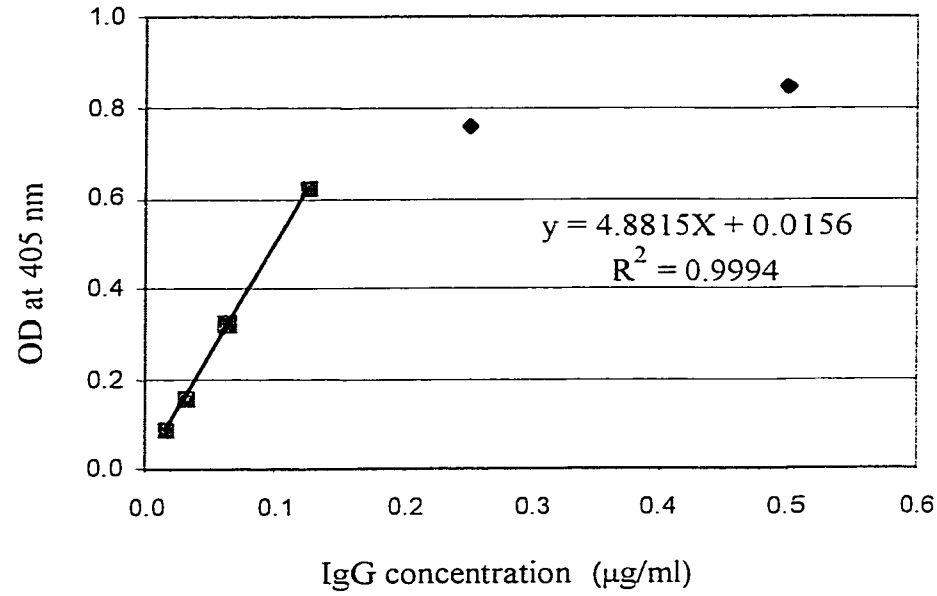
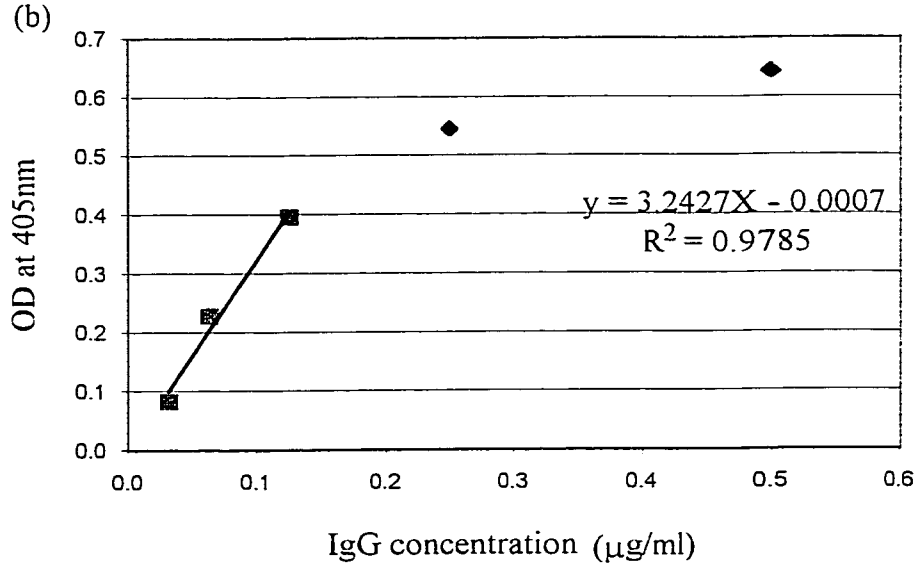
Appendix 1. The standard curves for determination of total IgY concentrations (a) in the WSF; (b) in IgY powder

(a)

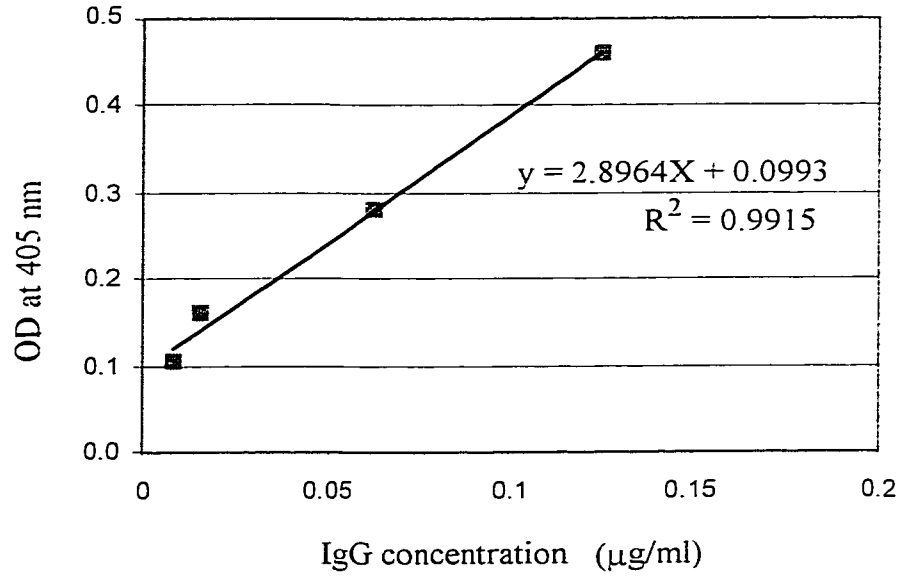


(b)

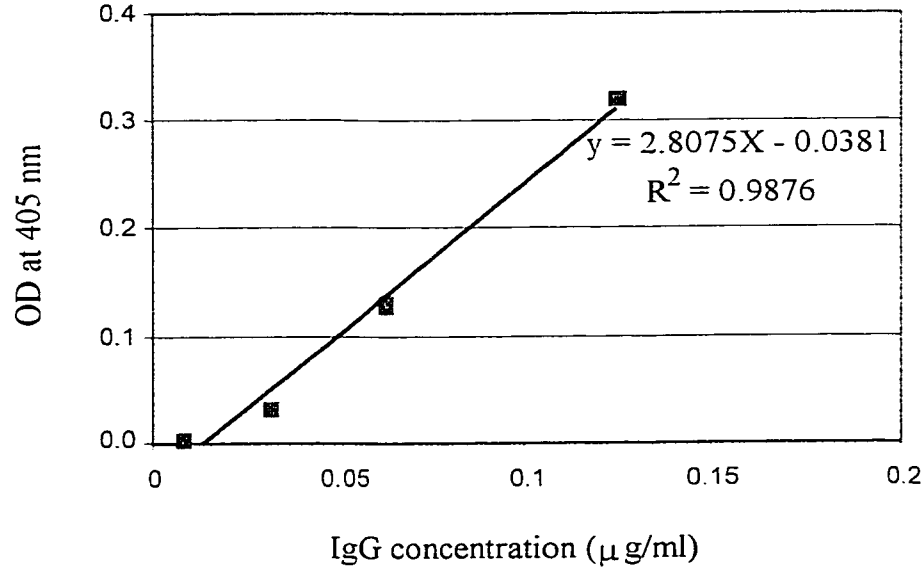


Appendix 2. The standard curves for determination of concentrations of (a) *E. coli* O157:H7-specific IgY; (b) ETEC 987P-specific IgY

(a)



(b)



Appendix 3. The standard curve for determination of *Salmonella*-specific IgY concentrations