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PRODUCTION, APPLICATION, AND STABILITY OF CHICKEN EGG YOLK ANTIBODY (IGY)

BY

XIUJIE LI 

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **DOCTOR OF PHILOSOPHY**

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TO MY PARENTS, MY HUSBAND AND MY DAUGHTER FOR THEIR LOVE AND ENCOURAGEMENT

ABSTRACT

Single Comb White Leghorn (SCWL) and Rhode Island Red (RIR) hens were used to examine the effects of egg and yolk weights on egg antibody (IgY) production in the two strains of chickens immunized with bovine serum albumin (BSA). The SCWL chickens had a greater percent hen-day production and greater egg and yolk weights than did the RIR chickens. However, the anti-BSA antibody activities determinated by ELISA in the serum and the egg yolk were similar between the SCWL and RIR chickens. Similarities between the two strains of hens were also observed in protein and total IgY contents and the percents of BSA-specific antibody in the total IgY. It was concluded that both the SCWL and RIR chickens immunized with BSA can produce IgY containing similar proportions of BSA-specific antibodies. Therefore, the egg yolk weight and the percent hen-day production, both of which are greater in SCWL hens, are considered to be important factors for the efficient production of IgY.

Experiments were also conducted to examine immune response of chickens to bovine decorin, chondroitin sulfate proteoglycan (CSPG) of high molecular weight extracted from the porcine stifle meniscus, and chondroitin sulfate peptide of bovine aggrecan. 35 wks old SCWL hens were immunized, respectively, with the above antigens, and egg produced by each bird were examined by ELISA for antibody activity in the yolk. High antibody activities were found in those eggs, which suggested that chickens are efficient host birds that can produce specific antibodies against mammalian proteoglycan which is thought to be a relatively weak antigen in mammals.

The polyclonal antibodies obtained from proteoglycans immunized chickens were also examined by ELISA inhibition assay with samples of proteoglycans treated with and without enzymes. Digestion of proteoglycans with papain caused complete loss in antigenicity, while chondroitinase-ABC treatment showed no appreciable effect. This indicated that specific antibodies recognize the protein core but not glycosaminoglycan chain.

IgY stability study was conducted using *E.coli.* 987p as antigen. The results indicated that pH 4 is the ideal condition at both 4°C and –20°C. –20°C is the better storage temperature in terms of long time storage in Water-soluble fraction.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CS	Chondroitin sulfate
CSPG	Chondroitin sulfate Proteoglycan
E. coli	*Escherichia coli*
ELISA	Enzyme linked-immunosorbent assay
GAG	Glycosaminoglycan
IC50	50% Inhibition
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Egg yolk immunoglobulin
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.05% Tween
RID	Radial immunodiffusion
RIR	Rhode Island Red
SCWL	Single Comb White Leghorn
WSF	Water-soluble fraction

LIST OF UNITS USED IN THE TEXT

UNIT	DEFINITION
°C	Degree Celsius
d	Day
g	Gram
h	Hour
kg	Kilogram
mg	Milligram
µg	Microgram
L	Liter
mL	Milliliter
nm	nanameter
µL	Microliter

INTRODUCTION

The rapidly declining per capita consumption of eggs over the past three decades is a major challenge faced by the egg industry. To meet this challenge, researchers around the world look at the egg beyond its traditional food value, and to explore many economically viable biomedicals, nutraceuticals and ovo-biotechnologies. As a part of the Poultry-Product Technology Program at the University of Alberta, a series of experiments has been conducted to understand the unique immune physiology of commercially bred laying chickens, produce egg antibodies by chickens and to explore the feasibility to turn the conventional commercial egg production system to a value-added antibody farming system.

It has been long recognized that the hen, like her mammalian counterparts, provides her young with antibodies as protection against hostile invaders. This system exploits the transfer by immunized hens of specific antibodies from serum to egg yolk, and provides a supply of antibodies called immunoglobulin Y (IgY) to the developing embryo and the hatched chicken. The protection against pathogens that the relatively immuno-incompetent newly hatched chick has, is through transmission of antibodies from the mother via the egg. Egg yolk, therefore, can be loaded with large amount of IgY against pathogens which can immobilize the existing or invading pathogens during the embryo development and day-old chicks.

A commercially bred-laying chicken lays an average of 250 eggs a year. Egg yolk contains 8 - 20 mg of immunoglobulins (IgY) per ml or 136 - 340 mg per yolk, suggesting that more than 34 g of immunoglobulins can be produced from one

immunized hen in a year. By immunizing the hens with antigens and collecting IgY from the egg yolk, low cost antibodies at less than $10 per g (compared to more than $20,000 per g of mammalian IgG) can be obtained. This calculation offers a potential for antibody farming as an alternative to conventional egg farming. The immunization of laying hens to various pathogens makes it possible to readily available antigen specific IgY, which would open a new IgY market for medical uses as preventive medicine, biological or diagnostic tool, and nutraceuticals or functional food uses as well as oral immunosupplementation for prophylaxis.

Immunoglobulin in avian blood is transferred to the yolk of eggs to give acquired immunity to the offspring. In the egg, the white contains IgA and IgM at the relatively low concentrations of 0.7 and 0.15 mg/ml, respectively, while yolk contains a considerably higher concentrations of 25 mg IgG/ml. IgY is an efficient source of specific antibodies when used as a food ingredient for a passive local immunization of the gastrointestinal tract, or as a reagent for detection, estimation and isolation of different molecules in foods and biological fluids.

Despite the exciting prospects offered by this system, no research information is available concerning antibody farming using the commercial egg production system. Therefore, the objective of the research herein reported were to investigate 1), biological and environmental factors influencing the immune responses and antibody production of laying chickens, 2), IgY production against bovine biomolecules (non-avian) and pathogens, 3), physico-chemical characteristics of IgY and stability, and 4), lastly potential applications of specific antibody for food and research purposes.

CHAPTER 1. LITERATURE REVIEW

1.1 Egg Antibody (IgY)

Immunoglobulin in avian blood is transferred to the yolk of eggs to give acquired immunity to the offspring (Rose and Orlans, 1981). The protection against pathogens is provided for the relatively immuno-incompetent newly hatched chick. In the egg, the white contains IgA and IgM at the relatively low concentrations of 0.7 and 0.15 mg/ml, respectively, while yolk contain a considerably higher concentrations of 25 mg IgG/ml (Rose et al., 1974). The antibody in egg yolk has been referred to as IgY (Leslie and Clem, 1969) because it is somewhat different from mammalian IgG in structure and immunological properties. IgY is a larger molecule (Kobayashi and Hirai, 1980) slightly more acidic with lower molecular rigidity (Higgins, 1975) than that of mammalian IgG. Unlike mammalian IgG, IgY does not fix mammalian complement. It neither binds to protein A, G nor F_C receptors (Jensenius et al., 1981; Akerstrom et al., 1985). Since IgY is mainly composed of γ-livetin, which is a larger molecule than any other α-, β-livetin in egg yolk, it is relatively easy to separate from other proteins in the water-soluble fraction of egg yolk. Several methods have been used for the isolation and purification and even large-scale purification of IgY from egg yolk (Polson et al., 1980, 1985; Jensenius et al., 1981; Bade and Stegemann, 1984; Hassl and Aspock, 1988; McCannel and Nakai, 1989, 1990; Hatta et al., 1990; Akita and Nakai, 1992).

A hen lays an average of 240 eggs a year (Canadian Egg Marketing Agency, 1986). The annual production of IgY by a hen may be 24 g, which is high productivity compared to antibody production by mammals such as rabbits, mice and goats. Chicken egg yolk attracted much attention as a good source of antibodies (Leslie and Clem, 1969).

At present, a tremendous number of hens are being immunized with several antigens (vaccination) to protect them from infectious diseases and are being managed to lay eggs for commercial production. Hen eggs, therefore, are now considered to be a potential source of large-scale production of antibody (IgY). Chickens are potent antibody producers, and the immunological responsiveness of their antibodies is similar to that of mammals. Therefore, IgY can be substituted for mammalian antibodies. The use of chickens for antibody production, as opposed to mammals, represents both a refinement of and a reduction in animal use. Antibody production in mammals involves two steps which cause physical and psychological stress. The first step is the immunization itself, while the second step consists of the bleeding of the animal as a prerequisite for antibody preparation. It is a refinement in that the second painful step is replaced by antibody extraction from egg yolk. It entails a reduction in the number of animals used because chickens produce larger amounts of antibodies than mammals (Schade et al., 1996).

IgY is an efficient source of specific antibodies when used as a food ingredient for a passive local immunization of the gastrointestinal tract, or as a reagent for detection, estimation and isolation of different molecules in foods and biological fluids (Lösch et al., 1986; Shimizu et al., 1988; Bar-Joseph et al., 1980; Vieira et al., 1984; Yolken et al., 1988). Large-scale isolation of IgY does not require any chemicals or organic solvents, therefore, purified IgY is considered to be practically applicable for passive immunization by oral administration. The effectiveness of passive immunization by oral administration of IgY to prevent infection has been reported for rotavirus diarrhea in man and animals (Kuroki et al., 1993, 1994, 1997; Hatta, et al., 1993), dental caries (Otake et

al., 1991; Hatta et al., 1997a), enteric colibacillosis (Yokoyama et al., 1992; Imberechts et al., 1997; Zuniga et al., 1997), and salmonellosis (Yokoyama et al., 1998).

Passive immunization of IgY could be achieved by antimicrobial activity against essential virulence determinants of pathogens. Antibodies interfere with the adhesion of pathogens to the intestinal wall and neutralize partially, or completely, their colonization potential. In the case of porcine enterotoxigenicic *Escherichia coli* (ETEC), antibodies against the adhesive fimbriae were shown to protect against intestinal colonization and disease (Rutter and Jones, 1973; Moon, 1981). The activity of IgY may change with pH, temperature, digestive enzymes in the intestinal tract and dose of antibodies as well. These variables enhance the effectiveness of antibody application. Shimizu et al.(1988) reported that the activity of IgY specific to *E. coli* was quite stable on incubation with trypsin or chymotrypsin, but sensitive to pepsin especially at pH lower than 4.5. Dose-dependent effects of specific IgY in controlling diarrhea of newborn calves has been demonstrated (Ozpinar et al., 1996).

1.2. Biosynthesis of Immunoglobulins

Avian circulatory system consists of at least three kinds of immunoglobulins, IgG, IgA, and IgM, 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 0.61mg for IgG, IgA, and IgM/ml serum, respectively (Lesile and Martin, 1973). Chickens produce immunoglobulins against almost all kinds of antigens including bacteria, virus,

and foreign substances in host defense. The immunoglobulins thus produced specifically bind their antigens to neutralize their effects.

In mammals, transfer of maternal antibody occurs after birth via the mammary secretions and the neonatal gut, whereas in birds, all the maternal antibody needed to protect the newly hatched chick must be present in the egg. Specific antibody, especially IgG, is transferred from hen serum to yolk and to the circulation of the chick via the endoderm of the yolk sac. Antibody is secreted into the ripening egg follicle (Patterson et al., 1962) and is incorporated into the egg white in the oviduct along with the egg albumen secretion. The concentrations of IgM and IgA in egg white and IgG in egg yolk are about 0.15 mg, 0.7 mg, and 25 mg per ml, respectively (Rose et al., 1974). Compared to other plasma proteins, IgG, is selectively secreted into egg follicle and the IgG secretion from the hen's circulatory system into the ovarian follicle is increased at a specific stage in its development. This transfer to the ovarian follicle is receptor dependent and the ovarian IgG receptor allows the selective transport of all IgG subpopulations presented by the maternal blood (Locken and Roth, 1983). Maternal IgA and IgM, present in oviduct secretions, are acquired by the egg as it passes down the oviduct where the egg white is laid down. At this time the yolk, which is fully formed on leaving the ovary, is surrounded by the vitelline membrane. The subsequent transfer of these immunoglobulins to the embryonic gut via swallowed amniotic fluid, at a time when neither is present in the serum, resembles the transfer of certain of the colostral or milk immunoglobulin to the newborn mammal.

Immunoglobulins in eggs provide passive immunity in that the antibodies in eggs originated from the mother hen are used to protect the newly hatched chick from a variety

of infectious diseases. In fact, IgY in egg yolk circulates in the blood and IgA and IgM in the digestive tract of the chick. Therefore, antibodies transferred from hen to chick via the latent stage of the egg play an important role in immunological function for the newly developed chicks to resist various infectious diseases.

1.3. IgY Production

Several papers compared the antibody production in hen egg and rabbit serum. Jensenius and co-workers estimated the total antibody activity of the eggs laid by a hen in a month is equivalent to that produced in a half liter of serum from an immunized rabbit (Jensenius et al., 1981). Gottstein and Hemmeler (1985) compared the antibody production efficiency for *Echinococcus granulosus* as an antigen. They reported that the quantity of IgY obtained from eggs laid by an immunized hen was 18 times greater than that of IgG isolated from the serum of an immunized rabbit. The authors also compared the productivity of IgY from the eggs laid by a hen over a year with that of IgG from the whole serum of a rabbit in which both animals were immunized with the same several antigens. The results are summarized in Table 1.1.

Hens usually lay about 250 eggs (about 4,000 g of egg yolk) in a year. On the other hand, the serum collected from a rabbit is about 40 ml. One gram of egg yolk laid by the immunized hen contains about 10 mg of IgY whereas 1 ml of rabbit serum yields about 35 mg of IgG. Therefore, an immunized hen yields 40 g of IgY compared to 1.4 g of IgG produced by an immunized rabbit. Thus, the antibody production in hens is nearly thirty times greater than of rabbits based on the weight of antibody produced per head per year.

1.4. Isolation and Purification

Egg yolk is a complex food which can be separated by centrifugation into particles, 'the granules' and a clear fluid supernatant, 'the plasma' (Stadelman and Cotterill, 1977). Granules are composed of 70% α- and β- lipovitellins, 16% phosvitin, and 12% low-density lipoproteins (LDL and HDL) (Burley and Cook, 1961). Plasma is about 78% of the total yolk and composed of a lipid-free globular protein, livetin (α- , β- , and γ-) which represent about 10.6% of the total yolk solids and low-density lipoproteins (McCully et al., 1962). IgY is known as γ- livetin and exists in egg yolk together with other two water-soluble proteins, α-, and β- livetin, and lipoprotein; therefore, separation of IgY or γ- livetin requires extraction of water-soluble fraction (WSF) from yolk lipoprotein followed by purification from other livetins (Polson et al., 1980).

Based on the aggregation of yolk lipoproteins at low ionic strengths as reported by Jensenius et al. (1981), several researchers used water dilution followed by centrifugation or filtration to fractionate WSF from water-insoluble components of egg yolk (Kwan et al., 1991; Akita and Nakai, 1992). For extraction of WSF from egg yolk with water, two factors are critical: the pH and the extent of egg yolk dilution (Akita and Nakai, 1992). It was found that pH was extremely important to obtain the highest recovery of IgY. Fichtali et al.(1993) had maximum recovery (54%) of IgY in WSF by 10-fold dilution and pH 5.5. IgY recovery was increased to 93-96% under the condition of 6 times dilution and pH 5.0 after incubation at 4C for 6hr followed by centrifugation or filtration (Akita and Nakai, 1992).

After separation of WSF containing livetins from the egg granule, the next step is to isolate IgY from the other water-soluble proteins, α-, and β- livetin, and low-density lipoproteins. A variety of methods have been used for purification of IgY: ultracentrifugation (McBee and Coteterill, 1979), organic solvents (Bade and Stegemann, 1984; Polson et al., 1980), precipitation using sodium dextran sulphate (Jensenius et al., 1981), or natural gums (Hatta et al., 1988, 1990), ultrafiltration (Akita and Nakai, 1992), and chromatography such as ion exchange chromatography (McCannel and Nakai, 1990) and metal chelate interaction chromatography (McCannel and Nakai, 1989).

Many purification methods of IgY have been reported so far: separation of lipoprotein by ultracentrifugation (McBee and Cotterill, 1979), delipidation by organic solvents (Bade and 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 were found to be highly effective as a precipitation of yolk lipoproteins (Hatta et al., 1990). etc. These methods, however, were questionable to apply practically for isolation of IgY on a large scale. The above methods seem costly and not necessarily effective for isolation of IgY. Also, the IgY prepared by using organic solvents or chemicals for delipidation seemed to have some problems in safety. The effective gums selected above have been used as food ingredients, and thus their use for purification of IgY will present no problem, as far as the IgY thus purified is used for oral administration. Egg yolk antibodies (IgY) have been isolated, purified, and then successfully applied in a wide variety of areas such as immunoassay and immunotherapy.

However, to produce food grade IgY, water dilution may be considered as the most appropriate technique and its efficiency for lipoprotein precipitation may be further improved by adding natural gums such as carrageenan (Hatta et al., 1990). In addition, water dilution is less expensive and allows the use of the remaining sedimented fraction in food applications of for the separation of other biologically active components (Kwan et al., 1991). The effective methods for large-scale isolation and purification of IgY have been also developed, which might be automated and easily scaled-up using industrially separated yolk.

1.5. Quantitative Assays

1.5.1. IgY ELISA

Egg yolk antibodies raised against antigens can be assayed by an enzyme-linked immunosorbent assay. This assay uses the principle that specific antibody can bind to antigen, especially antigenic binding site. Antibodies bound to antigen can be quantified by the reaction of substrate and enzyme conjugated with secondary antibody which can bind to specific antibody bound to antigen. The procedures are as follows. Microtiter plates are used as a solid support and are coated with antigens. A 10 μg/ml concentration of antigen in carbonate-bicarbonate buffer (0.05 M, pH 9.6) is added to each well and incubated for 24 h at room temperature. The plates are washed three times with deionized water. After washing, 150 μl of 1 % (wt/vol) solution of bovine serum albumin (BSA) in carbonate-bicarbonate buffer (0.05 M, pH 9.6) is added to each well, and incubated at 37 °C for 30 min. The BSA solution is then discarded and each well is washed three times with phosphate buffered saline-Tween 20 (0.05%) (PBS-Tween). Diluted sample

containing egg yolk antibodies (IgY) are added to the plate and incubated at 37 °C for 1 h. The plates are washed three times with PBS-Tween and 150 µl of rabbit anti-chicken IgG conjugated with horseradish peroxidase (1:1,000 in PBS-Tween) is added to each well. After incubation at 37 °C for 1 h, plates are washed three times with PBS-Tween, followed by addition of 100 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 0.03 % sodium perborate. The reaction is continued for 30 min. Absorbance of chromophore produced in the reaction mixture is read at 405 nm using a microplate reader (Sunwoo et al., 1996).

1.5.2. Antimicrobial assay

The inhibitory effect of IgY antibody on the growth of antigen such as bacteria and virus can be measured by turbidity (OD at 550 nm) and plate counting methods. Sugita-konishi et al. (1996) showed the effects of specific IgY on the growth of three bacterial strains by turbidity assay. Each kind of bacteria (10^4 cfu/ml) in TSB was incubated with different concentrations of specific and control IgY at 37 °C. The turbidity of the culture was measured at OD 550 nm at 2 hr intervals after culture. Plate counting was used as another method for the measurement of antimicrobial activity by Shimizu and colleagues (Shimizu et al., 1988). IgY (1-10 mg/ml) was added to a test culture (10^6 cfu *E.coli*/ml in TSB medium) and samples of the culture were taken after 1, 3, and 5 hr. A viable cell count was made on TSB agar plates after appropriate dilution of the bacteria-IgY mixtures. The inoculated plates were incubated at 37 °C overnight.

1.6. Physico-chemistry and Stability

The structure of IgY is identical to the major immunoglobulin found in serum, but can be distinguished from that of mammalian IgG. IgY consists of two heavy chains (H) and two light chains (L) and has a molecular mass of ~180 kDa. The H chains of IgY possess one variable (V) region domain, four constant (C) region domains and no genetic hinge, unlike mammalian IgG which has three constant region domains and a hinge region (Parvari et al., 1988). The molecular structure of IgY, therefore, is similar to mammalian IgM or IgE, which consist of four 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. The Cv2 domain was probably 'condensed' to form the IgG hinge region (Burton, 1987; Parvari et al., 1988; Fellah et al., 1993; Magor et al., 1994).

Shimizu et al. (1992) reported that the content of β-sheet structure in the constant domains of IgY was presumed to be lower than that of rabbit IgG, and the flexibility of the boundary region between Cv1 and Cv2 domains corresponding to the hinge region of IgG was less than that of rabbit IgG. Ohta and co-workers (1991) have made clear the whole structure of the various sugar chains of IgY, indicating that 27.1% of asparagine-linked carbohydrate chains of IgY have glucose as the nonreducing end residue of the glycolic chains.

The structural properties of IgY (e.g. molecular size, conformation of domains, intramolecular bonding, lack of disulfide linkage in the IgY L-chain, and lower flexibility of the hinge region) were considered to influence the overall properties of IgY molecule

and are structural factors that might have bearing on the lower molecular stability of IgY, compared with mammalian IgG (Pilz et al., 1977; Shimizu et al., 1992).

IgY, is different from the IgG of mammals in molecular weight, isoelectric point, binding behavior with complements, etc. as summarized in Table 1.2.

The heat and pH stability of IgY and rabbit IgG specific to human rotavirus were compared by measuring the antibody activity by ELISA (Hatta et al., 1993). IgY is more sensitive than that of IgG at temperatures higher than 70°C. The study also showed that the temperature corresponding to the maximum of the denaturation endotherm (T_{max}) of IgY was 73.9 °C while that of IgG was 77.0 °C according to differential scanning calorimetry. The IgY activity under various acidic conditions (pH 2 and 3) was more sensitive than that of rabbit IgG. These observations were supported by Otani and co-workers (1991) in studies comparing anti-α_{S1} casein IgY and rabbit IgG specific to mouse IgG. These differences in heat and acid sensitivity between IgY and IgG may be attributed to the variations in their protein structures.

Shimizu et al.(1988, 1992, 1993) reported on the molecular stability of IgY antibodies in comparison with that of mammalian IgG antibodies and found that heat (>75 °C) or acid (<pH 3.0) treatment reduced the antibody activity of IgY. The activity of IgY was decreased by incubating at pH 3.5 or lower and almost completely lost at pH 3.0 The activity of IgY was decreased by heating for 15 min at 65 °C or higher. Under alkaline conditions, changes in the activity of IgY did not occur until the pH increased to 11 but was markedly diminished by incubation at pH 12 or higher.

IgY antibody is relatively resistant to trypsin or chymotrypsin digestion, but is fairly sensitive to pepsin digestion. However, the IgY is more susceptible to pepsin,

trypsin or chymotrypsin digestion than the rabbit IgG antibody. Otani et al. (1991) showed the digestion profiles of egg yolk IgY and rabbit serum IgG antibodies specific to -α_{S1} casein by determination of a percentage decrease in undigested heavy chain over a time period by pepsin, trypsin or chymotrypsin digestion.

Susceptibility of IgY against *E.coli* (Simizu et al., 1993), and human rotavirus (Hatta et al., 1993) was also examined and the results were also demonstrated. SDS-PAGE profiles of IgY after incubation with pepsin revealed that IgY at pH 2.0 was hydrolyzed into small peptides, and no bands corresponding to IgY were detected in linear-gradient polyacrylamide gel electrophoresis (Hatta et al., 1993). On the contrary, incubation with pepsin at pH 4.0, heavy (H)-and light (L)-chain were clearly observed after 4 h, although certain new bands appeared between H- and L-chains.

The behavior of IgY with trypsin and chymotrypsin was also examined. Changes in the neutralization titer of IgY were almost the same for the incubation with trypsin and with chymotrypsin. After 8h incubation, the activity of IgY in neutralization titer remained 39% and 41% for the mixtures with trypsin and chymotrypsin, respectively. On incubation with trypsin, the IgY H-chain disappeared, and several bands between H- and L- chain appeared on SDS-PAGE. In the case with chymotrypsin, both H- and L-chains of IgY remained unchanged, although a small band below H-chain was observed.

1.7. Advantage of Chicken IgY

The antibody titers of concentrated IgY and the initial yolk-water soluble protein fraction are stable over a half year period; a long duration of a high immune response in the hen against BRV antigen was noted in Kuroki et al. (1993). The maintenance of a

large flock of layers is more economical than keeping large numbers of cows for antibody production. This makes IgY more feasible for large scale-production (Hamada et al., 1991). Another advantage of IgY over colostrum antibody is that a high neutralizing antibody titer remains stable for a long period as compared to that of colostrum which decreases a few days after initial harvest (Tsunemitsu et al., 1989).

The recovery of pigs that received egg powders with F18ab was comparable with that of pigs treated with medicated feed containing 1000 ppm colistin sulfate and 250 ppm ampicilin. Since at present no product is commercially available to prevent edema disease or postweaning diarrhea, egg powders with specific antibodies may be an alternative therapy.

It has been reported by several workers that the activity of IgY is not adversely affected by pasteurization conditions (Yolken et al., 1988; Otani et al., 1991; Hatta et al, 1993). The neutralization activities of both IgY and its Fab fragments were not reduced by heating at 65°C for 15min. The implication of this finding is that possible concerns with transmission of *salmonella* or other bacterial contamination associated with egg products may not be a major concern. Consequently, pasteurization could be used to eliminate pathogenic contaminants, with the exception of heat-resistant spores, without adverse effect on antibody activity (Akita et al., 1998).

It was demonstrated that cooking eggs did not denature or functionally inactivate IgY. Yolken et al. (1988) showed that antibodies to rotaviruses persisted in commercially pooled egg preparations that had been pasteurized.

Components of chicken egg such as lysozyme (Pellegrini et al., 1993) and ovotransferrin (Valenti et al., 1983) display anti-bacterial effects. The latter may become

more marked if higher doses are used. Therefore, dried egg can provide anti-microbial activity more efficiently.

The activity of IgY was found not to be affected by pasteurization at 60°C for 3.5min. This fact together with the higher productivity and the mass production at industrial scale strongly suggests the possibility for IgY as a practical reality to be applied by oral administration to prevent infectious disease (Hatta et al., 1993).

An antigen-specific IgG has been conventionally isolated from sera of animals, such as rabbits, which have been superimmunized with an aimed antigen. IgY is also to be isolated from the egg yolk laid by the hen superimmunized previously. Several advantages in the preparation of antibody using hens over using animals are summarized as follows (Hatta et al., 1997b).

The conventional method inevitably sacrifices animals which have produced the specific IgG in their circulating blood. On the other hand, the method of using hens is sufficient only to collect the eggs laid by superimmunized hens. For separation IgY, a large scale method is now applicable by automatic separation of the egg yolk with a machine.

As egg yolk contains only IgY, the isolation of IgY from the yolk is much easier than that of IgG from animal blood sera. Large-scale feeding of hens for egg production now being carried out is also a merit for collecting the source of a specific antibody. Also, immunization of hens (vaccination) has long been applied to prevent hens from infectious diseases, indicating that immunization of hens is much more systematized to be effective than doing it for animals. Egg yolk as the source of IgY is much more hygienic than mammal's sera from which IgG is separated. Because of a taxonomical

difference, the hen has the possibility to produce the antibodies whose formation is difficult or impossible in mammals.

Chickens are potent antibody producers, and their immunological responsiveness is similar to that of mammal. Therefore, IgY can be used as successful substitution of mammalian antibodies. The use of chickens for antibody production, as opposed to mammals, represents both a refinement and a reduction in animal use. It is a refinement in that the second painful step, the collection of blood, is replaced by antibody extraction from egg yolk. It entails a reduction in the number of animals used because chickens produce larger amounts of antibodies than mammals (Schade et al., 1996).

1.8. Possible Applications

The observation that the protective effect of IgY was not destroyed by removal of the Fc fragment, and that the Fab fragment was stable against further peptic digestion at pH above 4.2 (Akita and Nakai, 1993), suggests that IgY could be effective in young infants under 6 months of age. According to Nakai (1962) the stomach pH of infants under 6 months is normally in the range of 4-5 even 2-3h after intake of milk. Consequently, peptic digestion of IgY in the stomach of such infants will lead to a stable functional Fab fragment.

IgG is often digested and inactivated by gastric juice. Hydroxypropyl methylcellulose phthalate (HPMCP) used to separate yolk lipids has been used as an enteric coating substance for some drugs (Dressman and Amidon, 1984; Takada et al., 1989). Apparently, HPMCP-coated drugs are resistant to gastric juice, and dissolution in intestinal fluid is pH – dependent. It is likely that antibody powder coated with HPMCP,

although not perfectly, may have been conferred enteric resistance properties against low pH, thereby allowing safe passage through the stomach and ensuring the ultimate release of functioning antibodies in the small intestine.

In studies by O'Farrelly et al. (1992), carbonate-bicarbonate buffer was used to suspend the egg yolks during oral inoculation to protect IgY in its passage through the stomach so that it could reach the small intestine.

1.8.1. Passive immunization

An important application of IgY is for passive immunization therapy in which the specific binding ability to the antigens (pathogens, venoms, etc) serves to neutralize the biological activities of those antigens. Passive immunization seems to be one of the most valuable applications of antibodies in which pathogen-specific IgG is administered to individuals to result in prevention of infectious diseases. Passive immunization differ from active immunization (vaccination) in that the former employs an antibody obtained from other animals. The administration of this antibody specific to certain antigens (bacteria, virus, toxin, etc.) to individuals orally or systemically works to neutralize infectious activity or toxicity of the antigens. For practical application of passive immunization, an effective method of preparation of the antibody will be necessary, because large amounts of antibody may be required to administer the antibody for the passive immunization. The antigen-specific IgY can now be prepared on an industrial scale from eggs laid by hens immunized with selected antigens. Passive immunization using IgY will be widely practiced in the near future (Hatta et al., 1997b).

Bacteria enter the body either through a number of natural entry routes (e.g., the respiratory tract, the gastrointestinal tract, and the genitourinary tract) or through unnatural routes opened up by breaks in mucous membranes or skin. The gastrointestinal (GI) tract processes large quantities of foreign material daily (Tomasi and McNabb, 1987). It is important that the gut immunologically tolerates ingested dietary antigens. There is a general consensus that IgA, which is produced by GI tract-associated lymphoid tissue and transported into the lumen of the gut, assists in protection against bacterial, viral, and parasitic infections by inhibiting binding, preventing colonization, and neutralizing toxins (Williams and Gibbons, 1972). In certain circumstances, the secretory IgA system is absent or inadequate; increased colonization of the GI tract by bacterial or viral pathogens may be one of the sequels of this condition. A particularly vulnerable period is in the weeks and months after birth, when the immune system is still immature (Stiehm and Fudnberg, 1966). Numerous studies have documented the role of colostrum and breast milk in protecting the newborn against GI tract infections (Glass et al., 1983; Jason et al., 1984; Welsh and May, 1979). More recent studies have examined the possibility of using nonmaternal antibodies to passively immunize the GI tract.

Because *E. coli* is a major cause of infectious diseases in domestic animals, strategies to reduce the incidence and severity of disease have been considered important priorities to reduce the large economic losses resulting from these diseases (Moon and Bunn, 1993). The principle of vaccination, discovered in 1796 by Jenner, has provided the means to effectively reduce diseases caused by pathogenic *E. coli*. In particular, there are numerous commercial vaccines for the prevention of neonatal diarrhea in pigs, calves, and lambs. While vaccines have had a major impact on disease, other management

strategies that employ improved sanitation, the use of antibiotics, and the assurance that neonates received colostrum early in life also are important in the reduction of disease and will remain an integral part of disease prevention. Colonization of the intestine with *E. coli* leads to strong anti-colonization immunity associated with the appearance in the serum of anti-*E. coli* antibody of the IgA class. However, so far no effective vaccination protocol has been developed (Bianchi et al., 1996). Passive immunization may therefore be an attractive alternative. It may be achieved by the ingestion of antibody against essential virulence determinants. In the case of porcine ETEC, colostral antibodies against the adhesive fimbriae were shown to protect against intestinal colonization and disease (Rutter and Jones, 1973; Moon, 1981).

IgY can be used therapeutically as well as prophylactically. Wiedemann et al. (1991) found that IgY was as successful as a common antibiotic therapy in curing piglets with diarrhea. Yokoyama et al. (1992) showed that antibodies prepared from the yolk of eggs from hens immunized with fimbrial antigens of *E. coli* were protective in newborn piglets against a challenge with homologous ETEC strains. Kim et al. (1996) also reported that the 21-day old piglets that received IgY against ETEC were protected against the deleterious effects of this organism.

1.8.2. Immunological tool

Antigen-specific IgG isolated from sera of superimmunized animals, such as rabbits, cows, and goats has been widely applied as an immunological tool in the field of diagnosis as well as pure research. The antigen-specific IgY is useful in its binding specificity as well as mammalian IgG is specific to given antigens. They both serve to

detect antigens for which specificity will never be achieved by any other method (Hatta et al., 1997b).

1.8.3. Diagnosis

Altscuch et al. (1984) reported that IgY against human antibody (IgG and IgM) was applicable to determining their concentration in biological fluid by the method of rocket-immunoeletrophoresis. In the case of using rabbit IgG in this method, chemical modification, such as carbamylation, of the IgG is generally needed to change its isoelectric point from that of the human antibody. However, in application of IgY by this method, the carbamylation was not necessary since its isoelectric point is different from that of the human antibody. Fertel et al. (1981) demonstrated the application of IgY in determining prostaglandin in serum using radioimmunoassay in which they used prostaglandin conjugated with hemocyanin (hapten) as an antigen for immunization of hens. Gardner and Kaye (1982) prepared IgY specific to rotavirus, adenovirus, and influenza virus, demonstrated immunological detection of these viruses using the IgY as the first antibody, and fluorescein isothiocyanate (FITC)-conjugated rabbit IgG specific to IgY as the second antibody. The preparation of IgY to these viruses was achieved with much more convenience over the conventional rabbit IgG, because of no necessity of purification of the virus as antigen. Since these viruses can be cultivated using fertilized eggs, the contaminants in the virus culture that are components of egg must not show any immunogenicity to hens as far as hens are immunized with the virus culture as an antigen. It was suggested that IgY is a suitable antibody for detecting pathogens in stool samples, because it does not bind protein A derived from Staphylococcus aureus usually found in

stool. Owing to this property of IgY, the fault of the detection method of pathogens in stool can be avoided. Many researchers have also demonstrated the application of IgY for determination of various important, but very minor biological substances, such as plasma kallikrein (Burger et al., 1985), 1.25-dihydroxyvitamin D (Bauwens et al., 1988), hematoside (NeuGc) (Hirabayashi et al., 1983), human transferrin (Ntakarutimana et al., 1992), ochratoxin A (Clarke et al., 1994), human dimeric IgY (Polson et al., 1989), and high-molecular weight mucin-like glycoprotein-A (HAGP-A) (Shimizu et al., 1995).

Another advantage of IgY as an immunological tool over using rabbit IgG is the sensitivity of hens to antigens originated from mammals. A number of proteins exist whose amino acid sequence are well preserved among mammals, and many of these proteins have no or little antigenicity toward mammals. Therefore, for preparing antibodies against those proteinous antigens, the hen is highly promising as an alternative animal, because of the immunological distance of hen from mammals. In fact, Carroll and Stollar (1983) succeeded in preparing IgY against RNA polymerase II which has not generated its specific antibody in mammals. Many researchers have succeeded in producing IgY specific to less immunogenic antigen against mammals (Table 1.4).

1.8.4 Ligand of an immunoadsorbent

Immuno-affinity chromatography has been applied as a useful method for purification of proteins (antigens). Rabbit IgG has been conventionally used as a ligand to be immobilized to the absorbent, such as cellulose or agarose. However, several disadvantages of this affinity chromatography have been pointed out when rabbit IgG was used as a ligand, because acidic pH values less than pH 2 are necessary for

dissociation of the protein immunologically bound to the rabbit IgG on the immunoadsorbent. Therefore, the dissociated protein is often denatured depending on its nature. Moreover, production of rabbit IgG in large amount is generally expensive. The immuno-affinity chromatography using rabbit IgG as a ligand has thus been applied for isolation of only certain proteins.

It was demonstrated that IgY is an effective alternative antibody as a ligand for an immunoadsorbent. In the experiment, IgY and rabbit IgG specific to mouse IgG were immobilized on Sepharose 4B, respectively, in order to compare its dissociation efficiency for the purification of mouse IgG. Mouse serum was applied on immunoadsorbents, and the adsorbent was eluted with the buffer solution of pH4.0 and 2.0 stepwise. The mouse IgG dissociated at pH4.0 was only half of that applied, and the remaining IgG was eluted with pH2.0 buffer solution in an immunoadsorbent using rabbit IgG as a ligand. On the other hand, 97% of the mouse IgG was dissociated even at pH4.0 on the immunoadsorbent using IgY as a ligand (Hatta et al., 1997b).

Although use of specific IgY against pathogenic microorganism was intended as a prophylactic agent, the IgY was most probably also partially therapeutic, because its continued oral use after challenge exposure may have lessened the numbers of proliferating bacterial cells adhering to intestinal colonization sites. In fact, the antibody product when used in the field, is intended as an adjunct to other methods of prevention and treatment. In instances where antimicrobial resistance has emerged, which has been the trend for recent *Salmonella* field isolates, IgY can be the next best alternative to dam vaccination while taking advantage of the unlikely emergence of resistance to the antibody. In the field, use of antibody in combination with other preventive measures is

expected to greatly improve survivability of calves during *Salmonella* epizootics. The value of oral antibody administration when used in a combination treatment lies in the fact that it acts as a first line of defense in mucosal protection involving fast elimination of invading bacteria, thereby preventing or lessening the severity of morbidity arising from infection. It is recommended that oral passive immunization, using IgY, be considered as an adjunct to vaccination or antimicrobial treatment, or both, when there is real danger of exposure to *salmonellae* (Yokoyama et al., 1998b).

Hatta et al. (1997a) indicated that the immune IgY could be a novel ingredient for foods and mouth rinses to prevent the colonization of *S. mutans*, especially in the presence of sugar.

The protective effect of IgY may be enhanced by producing a cocktail of antibodies against several important antigens including the various CFAs, enterotoxins and the more important O antigens endemic to a particular region. Although anti-adherence antibodies may offer better protection because of serotype specificity, anti-enterotoxin antibodies may also confer cross-protection against heterologous strains producing the enterotoxins (Akita et al., 1998).

1.9. Limitations

Antigen was detected despite oral dosing with protective IgY concentrations indicating that protection did not necessarily mean prevention of infection but rather reduction in the amount of infective virus (Kuroki et al., 1993).

The most vulnerable population group is older children, especially in developing countries, since pepsin production increases and gastric pH decreases with age. In vitro

studies on pepsin digestion of IgY showed both rapid and complete digestion at low pH (Shimizu et al., 1988; Schmidt et al., 1989). In addition, the fraction of IgY absorbed into the circulation from the same dose of IgY administered to pigs decreased with increasing age of pigs (Yokoyama et al., 1993). There is, therefore, a need to find ways to protect IgY against peptic action and acidity of the stomach if IgY is to be administered to older children or adults.

Although IgY inhibited adhesion of *E. coli* $K88^+$ MB to piglet mucus, prolonged incubation of IgY and *E. coli* did not further reduce the degree of adhesion of *E. coli* to mucus. It was also shown that antibodies were not able to displace *E. coli* $K88^+$ MB once they are bound to the mucus of the small intestine. This indicates that the antibodies have a lower affinity for the receptor than that of $K88^+$ fimbriae. Since IgY did not remove previously bound *E. coli* $K88^+$ MB from the mucus, prophylactic use of IgY is suggested (Jin et al., 1998). However, therapeutic effects of IgY in diarrhea of piglets caused by *E. coli* K88 have been reported (Yokoyama et al., 1992; Kim et al., 1996; Wiedemann et al., 1991).

While both IgY and whole egg yolk appear to have the same protective effect, various theoretical and practical concerns may alter the usefulness of each preparation. While IgY precipitate may be more stable for long periods of storage, it is more time-consuming and expensive to prepare. However, egg yolk precipitations carry the concern of transmission of *salmonellae* or other bacterial contamination and, in the developed world, the concern of cholesterol content (O'Farrelly et al., 1992).

IgY has a serum half-life of 1.85 days in newborn pigs. This is considerably shorter than the reported serum half-life of 12 to 14 days for homologous IgG (colostrum antibodies) (Curtis and Bourne, 1971, 1973).

IgY is less rigid and stable under acidic conditions, and passage of the molecule in the stomach at low pH could weaken its overall conformational stability more readily than that of mammalian IgG, which could influence its faster rate of decay in the circulation (Yokoyama et al., 1993).

In the Zuniga et al.(1997), the intake of antibody was most probably too low. Comparability of experimental results as well as of prospective commercial products suffers from a lack of standardization of antibody content.

Table 1.1. Productivity of IgY and IgG

Immunized animal	Rabbit	Hen
Source of antibody	Blood serum	Egg yolk
Kind of antibody	Polyclonal	Polyclonal
Quantity of antibody	1,400 mg/rabbit	40,000 mg/hen
(Quantity of specific antibody)		
Anti-HRV (MO) antibody	5.6×10^6 NT	600×10^6NT
Anti-HRV (Wa) antibody	37.8×10^6 NT	520×10^6 NT
Anti-Mouse IgG antibody	700 mg	11,200 mg
Anti-Insulin antibody	0 mg	2,000 mg

NT: Neutralization titer

HRV: Human rotavirus

Table 1.2. Comparison of avian IgY and mammalian IgG

Character	Avian IgY	Mammalian IgG
Molecular weight	180 kDa	150 kDa
Isoelectric point	>acidic	<acidic
Heat stability	>sensitive	<sensitive
pH stability	>sensitive	<sensitive
F_c receptor binding activity	Low	High
Protein A/ protein G binding	no	yes
Interference with mammalian IgG	no	yes
Interference with rheumatoid factor	no	yes
Complement activation	no	yes

Table 1.3. Production of IgY specific to less immunogenic antigen against mammals

Antigen	Reference
proliferating cell nuclear antigen of calf thymus	Gassmann et al., 1990
heat-shock protein (Hsp 70)	Gutierrez and Guerriero, 1991
human insulin	Lee et al., 1991
rat glutathion peroxide	Yoshimura et al., 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

1.10. REFERENCES

Akerstrom, G., Brodin, T., Reis, K., and Borg, L. 1985. Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. *J. Immunol.* 135:2589-2592.

Akita, E.M., and Nakai, S. 1992. Immunoglobulins from egg yolk: Isolation and purification. *J. Food Sci.* 57:629-634.

Akita, E.M., and Nakai, S. 1993. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E.coli* strain. *J. Immunol. Methods.* 160:207-214.

Akita, E.M., Li-Chan, E.C.Y., and Nakai, S. 1998. Neutralization of enterotoxigenic *Escherichia coli* heat-labile toxin by chicken egg yolk immunoglobulin Y and its antigen-binding fragments. *Food Agri. Immunol.* 10:161-172.

Altschuh, D., Hennache, G., and Regenmortel, M.H.V. 1984. Determination of IgG and IgM levels in serum by rocket immunoelectrophoresis using yolk antibodies from immunized chickens. *J. Immunol. Methods.* 69:1-7.

Bade, H., and Stegemann, H. 1984. Rapid method of extraction of antibodies from hen egg yolk. *J. Immunol. Methods.* 72:421-426.

Bar-Joseph, M.,and Malkinson, M. 1980. Hen egg yolk as a source of antiviral antibodies in the enzyme linked immunosorbent assay (ELISA): a comparison of two plant viruses. *J. Virol. Methods.* 1:179-183.

Bauwens, R.M., Devos, M.P., Kint, J.A., and Leenheer, A.P. 1988. Chicken egg yolk and rabbit serum compared as sources of antibody for radioimmunoassay of 1,25-dihydroxyvitamin D in serum or plasma. *Clin. Chem.* 34:2153-2154.

Bianchi, A.T.J., Scholten, J.W., van Zijderveld, A.M., van Zijderveld F.G., and Bokhout, B.A. 1996. Parenteral vaccination of mice and piglets with $F4^+$ *Eshcerichia coli* suppresses the enteric anti-F4 response upon oral infection. *Vaccine.* 14:199-206.

Burger, D., Ramus, M.A., and Schapira, M. 1985. Antibodies to human plasma kallikrein from egg yolks of an immunized hen: Preparation and characterization, *Thromb. Res.* 40:283-288.

Burley, R.W., and Cook, W.H. 1961. Isolation and composition of avian yolk granules and their constituents alpha- and beta-lipovitellins. *Can. J. Biochem. Physiol.* 39:1295-1307.

Burton, D.R. 1987. Molecular Genetics of Immunoglobulins (Calabi, F., and Neuberger, M.S., eds.), pp. 1-50 Elsevier Science Publishers.

Canadian Egg Marketing Agency. 1986. The Amazing Egg. Canadian Egg Marketing Agency, Ottawa, Ontaio.

Carroll, S.B., and Strollar, B.D. 1983. Antibodies to calf thymus RNA polymerase II from egg yolks of immunized hens. *J. Biol. Chem.* 258:24-26.

Clarke, J.R., Marquardt, R.R., Frohlich, A.A., Oosterveld, A., and Madrid, F.J. 1994. Isolation, characterization, and application of hen egg yolk polyclonal antibodies. In *Egg uses and processing technologies-New developments.* ed. Sim, J.S., and Nakai, S. pp207. CAB International, Oxon.

Curtis, J., and Bourne, F.J. 1971. Immunoglobulin quantitation in sow serum, colostrum, and milk and in the serum of young pigs. *Biochem. Biophys. Acta.* 236:319-332.

Curtis, J., and Bourne, F.J. 1973. Half-lives of immunoglobulins IgG, IgA, and IgM in the serum of new-born pigs. *Immunology.* 24:147-155.

Dressman, J.B., and Amidon, G.L. 1984. Radiotelemetric method for evaluating enteric coatings in vivo. *J. Pharm. Sci.* 73:935-938.

Fellah, J.S., Kerfourn, F., Wiles, M.V., Schwager, J.,and Charlemagne, J. 1993. Phylogeny of immunoglobulin heavy chain isotypes: structure of the constant region of Ambystoma mexicanum upsilon chain deduced from cDNA sequence. *Immunogenetics.* 38:311-317.

Fertel, R., Yetiv, J. Z., Coleman, M. A., Schwarz, R. D., Greenwald, J.E., and Bianchine, J. R. 1981. Formation of antibodies to prostaglandins in the yolk of chicken eggs. *Biochem. Biophys. Res. Commun.* 102:1028-1033.

Fichtali, J., Charter, E.A., Lo, K.V., and Nakai, S. 1993. Purification of antibodies from industrially separated egg yolk . *J. Food Sci.* 58:1282-1285.

Gardner, P.S., and Kaye, S. 1982. Egg globulins in rapid virus diagnosis. *J. Virol. Methods.* 4:257-262.

Gassmann, M., Töhmmes, P., Weister, T., and Hubsgher, U. 1990. Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. *FASEB J.* 4:2528-2532.

Glass, R.I., Svennerholm, A.M., and Stoll, B.J. 1983. Protection against cholera in breast-fed children by antibodies in breast-milk. *N. Engl. J. Med.* 308:1389-1392.

Gottstein, B., and Hemmeler, E. 1985. Egg yolk immunoglobulin Y as an alternative antibody in the serology of echinococcosis. *Z. Parasitenkid.* 71:273-278.

Gutierrez, J.A., and Guerriero, V. 1991. Quantitation of Hsp70 in tissues using a competitive enzyme-linked immunosorbent assay. *J. Immunol. Methods.* 143:81-88.

Hamada, S., Horikoshi, T., Minami, T., Kawabata, S., Hiraoka, J., Fujiwara, T., and Ooshima, T. 1991. Oral passive immunization against dental caries in rats by use of hen egg yolk antibodies specific for cell-associated glucosyltransferase of Streptococcus mutans. *Infect. Immun.* 59:4161-4167.

Hassl, A., and Aspock, H. 1988. Purification of egg yolk immunoglobulins: a two-step procedure using hydrophobic interaction chromatography and gel filtration. *J. Immunol. Methods.* 110:225-228.

Hatta, H., Kim, M., and Yamamoto, T. 1990. A novel isolation method for hen egg yolk antibody "IgY". *Agri. Biol. Chem.* 54:2531-2535.

Hatta, H., Ozeki, M., and Tsuda, K. 1997b. Egg yolk antibody IgY and its application. In *Hen eggs: Their basic and applied science*, ed. Yamamoto, T., Juneja, L.R., Hatta, H., and Kim, M. pp151-178. CRC press.

Hatta, H., Tsuda, K., Akachi, S., Kim, M., Yamamoto, T., and Ebina, T. 1993. Oral passive immunization effect of anti-human rotavirus IgY and its behavior against proteolytic enzymes. *Biosci. Biotech. Biochem.* 57:1077-1081.

Hatta, H., Tsuda, K., Ozeki, M., Kim, M., Yamamoto, T., Otake, S., Hirasawa, M., Katz, J., Katz, J., Childers, N.K., and Michalek, S.M. 1997a. Passive immunization against dental plaque formation in humans: Effect of a mouth rinse containing egg yolk antibodies (IgY) specific to Streptococcus mutans. *Caries Res.* 31:268-274.

Higgins, D.A. 1975. Physical and chemical properties of fowl immunoglobulins. *Vet.Bull.* 45:139-154.

Hirabayashi, Y., Suzuki, T., Suzuki, Y., Taki, T., Matumoto, M., Higashi, H., and Kato, S. 1983. A new method for purification of antiglycospingolipid antibody. Avian antihematoside (NeuGc) antibody. *J. Biochem.* 94:327-330.

Imberechts, H., Deprez, P., Van Driessche, E., and Pohl, P. 1997. Chicken egg yolk antibodies against F18ab fimbriae of Escherichia coli inhibit shedding of F18 positive E.coli by experimentally infected pigs. *Vet. Microbiol.* 54:329-341.

Jason, J.M., Nieburg, P., and Marks, J.S. 1984. Mortality and infectious disease associated with infant-feeding practices in developing countries. *Pediatrics.* 74:702-727.

Jensenius, J.C., Andersen, I., Hau, J., Crone, M., and Koch, C. 1981. Eggs: conveniently packaged antibodies. Methods for purification of yolk IgG. *J. Immunol. Methods.* 46:63-68.

Jin, L.Z., Baidoo, S.K., Marquardt, R.R., and Frohlich, A.A. 1998. In vitro inhibition of adhesion of enterotoxigenic *Esherichia coli* K88 to piglet intestinal mucus by egg-yolk antibodies. *FEMS Immunol. Med. Microbiol.* 21:313-321.

Kim, J.W., Marquardt, R.R., Baidoo, S.K., and Frohlich, A.A. 1996. The use of egg antibodies to counteract diarrheal diseases in piglets. *J. Anim. Sci.* 74:195.

Kobayashi, K., and Hirai, H. 1980. Studies on subunit components of chicken polymeric immunoglobulins. *J. Immunol.* 124:1695-1704.

Kuroki, M. Ohta, M., Ikemori, Y., Peralta, R.C., Yokoyama, and H., Kodama, Y. 1994. Passive protection against bovine rotavirus in calves by specific immunoglobulins from chicken egg yolk. *Arch. Virol.* 138:143-148.

Kuroki, M., Ikemori, Y., Yokoyama, H., Peralta, R.C., Icatlo, F.C., and Kodama, Y., 1993. Passive protection against bovine rotavirus-induced diarrhea in murine model by specific immunoglobulins from chicken egg yolk. *Vet. Microbiol.* 37:135-146.

Kuroki, M., Ohta, M., Ikemori, Y., Icatlo, F.C., Kobayashi, C., Yokoyama, H., and Kodama, Y. 1997. Field evaluation of chicken egg yolk immunoglobulins specific for bovine rotavirus in neonatal calves. *Arch. Virol.* 142:843-851.

Kwan, L., Li-Chan, E., Helbig, N., and Nakai, S. 1991. Fractionation of water-soluble and –insoluble components from egg yolk with minimum use of organic solvents. *J. Food Sci.* 56:1537-1541.

Lee, K., Ametani, A., Shimizu, M., Hatta, H., Yamamoto, T., and Kaminogawa, S. 1991. Production and characterization o antihuman insulin antibodies in the hen's egg. *Agric. Biol. Chem.* 55:2141-2143.

Lesile, G.A., and Clem, L.W. 1969. Phylogeny of immunoglobulin structure and function III. Immunoglobulins of the chicken. *J. Exp. Med.* 130:1337-1352.

Leslie, G., and Martin, L.N. 1973. Studies on the secretory immunologic system of fowl. III. Serum and secretory IgA of the chicken. *J. Immunol.* 110: 1-9.

Locken, M.R., and Roth, R.E. 1983. Analysis of maternal IgG subpopulations which are transported into the chicken oocyte. *Immunol.* 49: 21-28.

Lösch, Y., Schranner, I., Wanke, R., and Jurgens, L. 1986. The chicken egg, an antibody source. *J.Vet. Med.* B33: 609-619.

Magor, K.E., Higgins, D.A., Middleton, D., and Warr, G.W. 1994. One gene encodes the heavy chains for three different forms of IgY in the duck. *J. Immunol.* 153:5549-5555.

McBee, L.E., and Cotterill, O.J. 1979. Ion-exchange chromatography and electrophoresis of egg yolk proteins. *J. Food Sci.* 44:656-660.

McCannel, A.A., and Nakai, S. 1989. Isolation of egg yolk immunoglobulin-rich fractions using copper-loaded metal chelate interaction chromatography. *Can. Inst. Food Sci. Techonol. J.* 22:487-490.

McCannel, A.A., and Nakai, S. 1990. Separation of egg yolk immunoglobulins into subpopulations using DEAE-ion exchange chromatography. *Can. Inst. Food Sci. Techonol. J.* 23:42-46.

McCully, K.A., Mok, C.C., and Common, R.H. 1962. Paper electrophotetic characterization of proteins and lipoproteins of hen's egg yolk. *Can. J. Biochem. Physiol.* 40:937-952.

Moon, H.W. 1981. Protection against enteric colibacillosis in pigs suckling orally vaccinated dams: Evidence for pili as protective antigens. *Am. J. Vet. Res.* 42:173-177.

Moon, H.W., and Bunn, T.O. 1993. Vaccines for preventing enterotoxigenic *Eshcerichia coli* infections in farm animals. Vaccine. 11:213-220.

Moon, H.W., Whipp, S.C., and Skartvedt, S.M. 1976. Etiologic diagnosis of diarrheal diseases of calves: frequency and methods for detecting enterotoxin and K99 antigen production by *Escherichia coli. Am. J. Vet. Res.* 37:1025-1029.

Morishita, E., Narita, H., Nishida, M., Kawashima, N., Yamagishi, K., Masuda, S., Nagao, M., Hatta, H., and Sasaki, R. 1996. Anti-Erythoroprotein receptor monoclonal antibody: Epitope mapping, quantification of the soluble receptor, and detection of the solublized transmembrane receptor and the receptor-expressing cells. *Blood.* 88:465-471.

Nakai, S. 1962. Study on digestion of milk protein and its improvement, PhD thesis, University of Tokyo.

Ntakarutimana, V., Demedts, P., Sande, M.V., and Scharpe, S. 1992. A simple and economical strategy for downstream processing of specific antibodies to human transferrin from egg yolk. *J. Immunol. Methods.* 153:133-140.

O'Farrelly, C., Branton, D., and Wanke, C.A. 1992. Oral ingestion of egg yolk immunoglobulin from hens immunized with an enterotoxigenic *Escherichia coli* strain prevents diarrhea in rabbits challenged with the same strain. *Infect. Immun.* 60:2593-2597.

Ohta, M., Hamako, J., Yamamoto, S., Hatta, H., Kim, M., Yamamoto, T., Oka, S., Mizuochi, T., and Matuura, F. 1991. Structures of asparagine-linked oligosaccharides from hen egg-yolk antibody (IgY). Occurrence of unusual glucosylated oligomannose type oligosaccharides in a mature glycoprotein. *Glycoconjugate J.* 8: 400-413.

Otake, S., Nishihara, Y., Makimura, M., Hatta, H., Kim, M., Yamamoto, T.,and Hirasawa, M. 1991. Protection of rats against caries by passive immunization with hen-egg-yolk antibody (IgY). J. *Dent. Res.* 70:162-166.

Otani, H., Matsumoto, K., Saeki, A., and Hosono, A. 1991. Comparative studies on properties of hen egg yolk IgY and rabbit serum IgG antibodies. *Lebensm Wiss. u. Techonol.* 24:152-158.

Ozpinar, H., Erhard, M.H., Aytug, N., Ozpinar, A., Baklaci, C., Karamuptuoglu, S., Hofmann, A., and Losch, U. 1996. Dose-dependent effects of specific egg-yolk antibodies on diarrhea of newborn calves. *Preventive Vet. Med.* 27: 67-73.

Parvari, R., Avivi, A., Lentner, F., Ziv, E., Tel-Or, S., Burstein, Y., and Schechter, I. 1988. Chicken immunoglobulin γ-heavy chains: Limited V_H gene repertoire, combinatorial diversification by D gene segments and evolution of the heavy chain locus. *EMBO J.* 7:739-744.

Patterson, R., Younger, J.S., Weigle, W.O., and Dixon, F.J. 1962. The metabolism of serum proteins in the hen and chick and secretions of serum proteins by the ovary of the hen. *J. Physiol.* 45:501-513.

Pellegrini, A., Thomas,U., Von Fellenberg, R., and Wild, P. 1993. Bactericidal activities of lysozyme and aprotinin against Gram-negative and Gram-positive bacteria related to their basic character. *J. Appl. Bacteriol.* 72:180-187.

Pilz, I., Schwarz, E., and Palm, W. 1977. Small-angle X-ray studies of the human immunoglobulin molecule kol.

Polson, A., and Von Wechmar, M.B. 1980. Isolation of viral IgY antibodies from yolks of immunized hens. *Immunol. Comm.* 9:476-493.

Polson, A., Coetzer, T., Kruger, J., Von Maltzahn, E., and Vac der Merwe, K. J. 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol. Invest.* 14:323-327.

Polson, A., Maass, R., and Van Der Merwe, K.J. 1989. Eliciting antibodies in chickens to human dimeric IgA removal of factors from human colostrum depressing anti IgA antibody production. *Immunol. Invest.* 17:465-489.

Rose, M.E., and Orlans, E. 1981. Immunoglobulins in the egg, embryo and young chick. *Devel. and Comp. Immunol.* 5:15-20, 371-375.

Rose, M.E., Orlans, E., and Buttress, N. 1974. Immunoglobulin classes in the hen's egg: their segregation in yolk and white. *Europ. J.Immunol.* 4:521-523.

Rosol, T.J., Steinmeyer, C.L., McCauley, L.K., Merryman, J.I. Werkmeister, J.R., Grone, A., Weekmann, M.T., Swayne, D.E., and Capen, C.C. 1993. Studies on chicken polyclonal anti-peptide antibodies specific for parathyroid hormone related protein (1-36). *Vet. Immunol. Immunopathol.* 35:321-337.

Rutter, I.W., and Jones, G.W. 1973. Protection against enteric disease caused by Escherichia coli- a model for vaccination with a virulence determinant. *Nature.* 242:531-532.

Schade, R., Staak, C., Hendriksen, C., Erhard, M., Hugl, H., Koch.G., Larsson, A., Pollmann, W., Regenmortel, M. Rike, E., Spielmann, H., Steinbusch, H., and Straughan, D. 1996. The production of avian (egg yolk) antibodies : IgY. *ALTA* 24:925-934.

Shimizu, M., Fitzsimmons, R.C., and Nakai, S. 1988. Anti-E.coli immunoglobulin Y isolated from egg yolk of immunized chickens as a potential food ingredient. *J. Food Sci.* 53:1360-1366.

Shimizu, M., Nagashima, H., Sano, K., Hashimoto, K., Ozeki, M., Tsuda, K., and Hatta, H. 1992. Molecular stability of chicken and rabbit immunoglobulin G. *Biosci. Biotech. Biochem.* 56:270-274.

Shimizu, M., Watanabe, A., and Tanaka, A. 1995. Detection of high-molecular weight mucin-like glycoprotein-A (HMGP-A) of human milk by chicken egg yolk antibody. *Biosci. Biotech. Biochem.* 59:138-139.

Shmidt, P., Wanke, R., Linckh, E., Wiedemann, V., Kuhlmann, R., and Losch, U. 1989. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. II. In vitro studies on gastric and enteric digestion of egg yolk antibodies specific against pathogenic *Escherichia coli* strains. *J. Vet. Med.* B36:619-629.

Stadeklman, W. J., and Cotterill, O.J. 1977. Egg science and Technology. 2nd ed. AVI Pub. Co. Westport, CT, pp. 65-91.

Stiehm, E.R., and Fudenberg, H.H. 1966. Serum levels of immune globulins in health and disease: a survey. *Pediatrics.* 37:715-720.

Sturmer, A.M., Driscoll, D.P., and Jackson-Matthews, D.E. 1992. A quantitative immunoassay using chicken antibodies for detection of native and recombinant a-amidating enzyme. *J. Immunol. Methods.* 146:105-110.

Sugita-konishi, Y., Shibata, K., Yun, S.S., Hara-kudo, Y., Yamaguchi, K., and Kumagai, S. 1996. Immune functions of immunoglobulin Y isolated from egg yolk of hens immunized with various infectious bacteria. *Biosci. Biotech. Biochem.* 60:886-888.

Sunwoo, H.H., Nakano, T., Dixon, W.T., and Sim, J. S. 1996. Immune responses in chickens against lipopolysaccharide of Escherichia coli and Salmonella typhimurium. *Poultry Sci.* 75:342-345.

Takada, K., Ohhashi, M., Furuya, Y., Yoshikawa, H., and Muranishi, S. 1989. Enteric solid dispersion of ciclosporin A (CiA) having potential to deliver CiA into lymphatics. *Chem. Pharm. Bull.* 37:471-474.

Tomasi, T.B., and McNabb, P.C. 1987. The secretory immune system. In Basic and clinical immunology, ed. Stobo, J.D., and Wells, J.V. Appleton & Lange, Norwalk, Conn.

Toti, F., Gachet, C., Ohlmann, P., Stierle, A., Grunebaum, L., Wiesel, M.L., and Cazenave, J.P. 1992. Electrophoretic studies on molecular defects of von Willebrand factor and platelet glycoprotein Iib-IIIa with antibodies produced in egg yolk from laying hens. Haemostasis. 22:32-40.

Tsunemitsu, H., Shimizu, M., Hirai, T., Yonemichi, H., Kudo, T., Mori, K., and Onoe, S. 1989. Protection against bovine rotaviruses in newborn calves by continuous feeding of immune colostrum. *Jap. J. Vet. Sci.* 51:300-308.

Valenti, P., Antonini, G., Von Hunolstein, C., Orsi, N., and Antonini, E. 1983. Studies on the antimicrobial activity of ovotransferrin. *Int. J. Tissue React.* 1:97-105.

Vieira, J.G.H., Oliveira, M.A.D., Russo, E.M.K., Maciel, R.M.B., and Pereira, A.B. 1984. Egg yolk as a source of antibodies for human parathyroid hormone (hPTH) radioimmunoassay. *J. Immunoassay.* 5:121-129.

Welsh, J.K., and May, J.T. 1979. Anti-infective properties of breast-milk. *J. Pediatr.* 94:1-9.

Wiedemann, V., Linckh, E., Kuhlmann, R., Schmidt, P., and Losch, U. 1991. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. *J. Vet. Med.* B38:283-291.

Williams, R.C., and Gibbons, R.J. 1972. Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. *Science.* 177:697-699.

Yokoyama, H., Peralta, R.C., Diaz, R., Sendo, S., Ikemori, Y., and Kodama, Y. 1992. Passive protective effect of chicken egg yolk immunoglobulins against experimental enterotoxigenic *Escherichia coli. Infet. Immun.* 60: 998-1007.

Yokoyama, H., Peralta., R.C., Sendo, S., Ikemori, Y., and Kodama, Y. 1993. Detection of passage and absorption of chicken egg yolk immunoglobulins in the gastrointestinal tract of pigs by use of enzyme-linked immunosorbent assay and fluorescent antibody testing. *Am. J. Vet. Res.* 54:867-872.

Yokoyama, H., Umeda, K., Peralta, R.C., Hashi, T., Icatlo, F.C., Kuroki, M., Ikemori, Y., and Kodama, Y. 1998a. Oral passive immunization against experimental salmonellosis in mice using chicken egg yolk antibodies specific for Salmonella enteritidis and S. typhimurium. *Vaccine.*16: 388-393.

Yokoyama,H., Peralta R.C., Umeda, K., Hashi, T., Icatlo, F.C., Kuroki, M., Ikemori, Y., Kodama, Y. 1998b. Prevention of fatal salmonellosis in neonatal calves, using orally administered chicken egg yolk Salmonella-specific antibodies. *Am. J.Vet.Res.* 59: 4, 416-420.

Yolken, R.H., Leister, F., Wee, S.B., Miskuff, R.,and Vonderfecht, S. 1988. Antibodies to rotaviruses in chickens'eggs: A potential source of antiviral immunoglobulins suitable for human consumption. *Pediatrics.* 81:291-295.

Zuniga, A., Yokiayama, H., Albicker-Rippinger, P., Eggenberger, E., and Bertschinger, H.U. 1997. Reduced intestinal colonization with F18-positive enterotoxigenic *Escherichia coli* in weaned pigs fed chicken egg antibody against the fimbriae. *FEMS Immunol. Med. Microbiol.* 18:153-161.

CHAPTER 2. EFFECTS OF EGG AND YOLK WEIGHTS ON YOLK ANTIBODY (IGY) PRODUCTION IN LAYING CHICKENS

2.1. INTRODUCTION

Immune responses by producing antibodies (immunoglobulins) against foreign materials (antigen) are important for chickens to protect themselves from infection. In laying hens, the immunoglobulin, IgG in the blood is efficiently transferred across the follicular epithelium of the ovary and accumulated in the yolk during oogenesis (Rose and Orlans, 1981). This is important to protect their progeny. The concentration of IgG in the yolk, which is more commonly called IgY (Leslie and Clem, 1969), is higher than that in the serum (Rose et al., 1974; Larsson et al., 1993). The IgY is continuously absorbed by the embryo during embryogenesis until the second day after hatching providing the evidence of passive immunization protection acquired from the hen.

Chickens tore large amounts of IgY in the yolk and are considered efficient antibody producers (Gottstein and Hemmeler, 1985). In a period of six weeks, one immunized hen produces 298 g of IgY, which is much higher than the serum antibody (16.6 mg) obtained from one rabbit (Sim and Nakai, 1994). Moreover, due to the phylogenic distance between birds and mammals, chickens produce more specific antibodies against mammalian antigens than do mammals (Jensenius et al., 1981). For example, bovine serum albumin (BSA) (Ermeling et al., 1992) and human serum (Lösch et al., 1986) are more antigenic in avian species than in mammals. The IgY is superior to

[1]A version of this chapter has been published. Li, X., Nakano, T., Sunwoo, H. H., Paek, B. H., Chae, H. S., and Sim, J. S., 1998. Poultry Sci. 77: 266 – 270.

serum antibody due to higher levels of specific antibodies (Orlans, 1967; Rose et al., 1974) and relative ease of purification (Akita and Nakai, 1992) with low cost (Polson and Von Wechmar, 1980). Egg yolk antibody has also an advantage over serum antibody because of its compatibility with modern animal welfare (Gottstein and Hemmeler., 1985).

Although chickens have been used as antibody producers for more than ten years and some antibodies have been obtained from egg yolk, there is still limited information available concerning the IgY production in relation to egg traits and hen productivity. The present study was undertaken 1) to examine the immune response against BSA as the antigen in two breeds (Shaver vs. Rhode Island Red, RIR) of chickens, and 2) to examine the effect of laying performance (e.g. rate of egg production, and egg and yolk size) on the efficiency of IgY production.

2.2. MATERIALS AND METHODS

Experimental Birds

Twenty 35 wk old chickens, including 10 Shaver and 10 RIR hens, averaging in body weights 1.53 ± 0.20 (SD) and 2.06 ± 0.06 kg, respectively, were kept in the University of Alberta Poultry Unit. The Shaver hens were used as those with high laying performance, while the RIR hens as those with low laying performance. All chickens were cared for in accordance with the Canadian Council on Animal Care guidelines of animal welfare.

Immunization of Chickens with Bovine Serum Albumin

Procedures of chicken immunization followed those described by Sunwoo *et al.* (1996). For the first injection, 2 mg BSA[2] was dissolved in phosphate buffered saline (PBS, 0.14 *M* NaCl, 0.0015 *M* KH_2PO_4, 0.0081 *M* Na_2HPO_4 and 0.0027 *M* KCl, pH 7.2), and emulsified with an equal volume of complete Freund's adjuvant to obtain the final concentration of 1 mg BSA/mL. Each hen was intramuscularly injected with 1 mg BSA (Shimizu *et al.*,1992) at four different sites (0.25 mg per site) of breast muscles (2 sites per left or right breast muscle). Booster injection was given intramuscularly 2 wk after the first injection with the same dose emulsified with Freund's incomplete adjuvant. Blood samples were collected from the wing vein on 0,14, 35, 56, 63 and 70 days after the initial injection. Eggs were collected daily and stored at 4°C until analyzed.

Isolation of Water Soluble Fraction

Isolation of water-soluble fraction (WSF) was carried out by the method described by Akita and Nakai (1992) with minor modifications. Egg yolk was separated from the white using an egg yolk separator and then rolled on paper towels to remove adhering egg white. The yolk membrane was punched and the yolk without membrane was transferred to a graduated cylinder. The yolk samples were mixed with six volumes of cold acidified water, pH 2.5 adjusted with 0.1 *N* HCl. The mixture, which had a pH of 5.6, was kept at 4°C for 6 h, and then centrifuged at 12,100 x *g* and 4°C for 15 min.

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[2]Sigma Chemical Co., St. Louis, MO 63178-9916

After centrifugation, supernatant was collected as WSF. The WSF samples obtained were used for determination of the anti-BSA antibody activity by ELISA, the total IgY content by the radial immunodiffusion (RID) technique, and the content of IgY specific to BSA by BSA-agarose affinity column chromatography.

ELISA

Antibody activities in sera and WSF were determined using microtiter plates with 96 wells[3], which were coated by adding 150 mL of a BSA solution (25 mg/mL 0.05 *M* carbonate buffer, pH 9.6) to each well and incubating at 22°C for 2 h. Plates were washed with PBS containing 0.05% Tween (PBST). Samples (150 mL) of sera or WSF were then prepared for ELISA. Sera were diluted 1000 times, while the WSF, obtained by six times dilution of yolk (see above) was diluted 167 times to obtain 1000 times dilution. Diluted sample were added to the well, and incubated at 22°C for 3 h. Subsequent to incubation, plates were washed with PBST, and incubated with the second antibody (150 mL of a 1:1000 dilution of peroxidase conjugated rabbit anti-chicken IgG[2]) at 37°C for 1 h. Plates were washed with PBST, and 100 mL of substrate solution, 2, 2' - azino - bis (3 - ethylbenzthiazoline-6-sulfonic acid)[2] in 0.05 *M* phosphate-citrate buffer, pH 5.0 containing 0.03% sodium perborate[2], was added to the well. After 30 min, the absorbance of reaction mixture was read at 405 nm using an ELISA reader[4] against the reaction mixture prepared with PBS.

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[3]Costar Corp., Cambridge, MA 02139

[4]Bio-Tek Instruments, Inc. Winooski, VT 05404

ELISA was also carried out to examine the reactivity of chicken anti-BSA antibody to sera from elk and rabbit, which were chosen as species representing ruminant and non-ruminant species, respectively. If a positive reaction is observed, the anti-BSA IgY produced may be useful to purify serum albumin from those species by preparing an anti-BSA IgY conjugated affinity column. Elk serum was a gift from Mr. S. L. Kurylo[5], and rabbit serum was obtained from Gibco BRL[6]. Bovine serum used as control was obtained from a local abattoir. Chicken sera containing anti-BSA antibody diluted at 1 to 1000 were added to the plates coated with diluted serum (0.5 mL /mL 0.05 *M* carbonate buffer, pH 9.6) from different species, and ELISA was performed as above.

Radial Immunodiffusion

Radial immunodiffusion of WSF was performed by the method previously described (Sunwoo *et al.*, 1996). The solution A was prepared by mixing 0.3 mL rabbit anti-chicken IgG[4] with 1.7 mL barbital buffer[2] (50 m*M* sodium barbital and 10 m*M* barbital, pH 8.6) and incubated in a 56°C water bath. Solution B was prepared by mixing 70 mg of agarose[2] with 4.6 mL barbital buffer and 0.4 mL of 0.35% (wt/vol) sodium azide, and holding the mixture in a boiling water bath until the agarose dissolved. Solutions A and B were mixed well, equilibrated at 56°C, and poured into RID plate. Serum and WSF (both 6mL) and standard IgG from chicken[2] (0 to 1.0 mg in 6mL of PBS) were them added to 2.5 mm diameter wells, and incubated at room temperature for

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[5]Inner Sense International Inc., Sherwood Park, AB Canada, T8A 2A6.

[6]Burlington, ON Canada, L7P 1A1.

3 days. The diameter of precipitation ring formed was measured, and the concentration of IgG was calculated by referring to the standard curve prepared by using known amounts of IgG (see above).

Bovine serum albumin-agarose affinity chromatography

Affinity column of BSA-agarose (15 mg BSA immobilized per mL of 4% beaded agarose) was used to isolate specific anti-BSA IgY from WSF. The WSF sample containing approximately 18 mg of total IgY was applied to a 1 x 1.3 cm column of BSA-agarose equilibrated with 0.01 *M* sodium phosphate buffer pH 7.2 containing 0.5 *M* NaCl and 0.02% sodium azide. After loading the sample, the column was washed with 3 mL of this buffer to remove unbound materials. The column was then eluted with 0.5 *M* acetic acid containing 0.5 *M* NaCl, pH 2.4. Fractions (1 mL) were collected into tubes containing 0.81 mL of 0.5 *M* Tris to neutralize acidic elutes. All fractions collected were determined for protein content by measuring absorbance at 280 nm, antibody activities by ELISA, and the content of IgY by the RID method.

Analytical Methods

The content of moisture in egg yolk was measured by weighing before and after heating at 110°C overnight. The content of crude fat was determined by measuring the content of petroleum ether (boiling range 37.8-56.9°C) extractable material using a Goldfish extraction apparatus[7]. The content of nitrogen in yolk was determinated by the

[7]Perstorp Analytical / Tecator Inc. Herndon, VA 22071

Kjeldahl method (A.O.A.C. 1980), and the content of protein was calculated using a factor of 6.25 to convert from nitrogen content. All data were calculated as the mean ± SD. A *t*-test was used to evaluate the difference between means (Yu et al., 1985).

2.3. RESULTS AND DISCUSSION

Percent hen-day production in chickens and their egg and yolk weights during the experimental periods are given in Table2.1.The percent hen-day production was approximately two times higher ($P < 0.01$) in the Shaver than in the RIR hens, and both the egg and yolk weights were 1.3 times greater ($P < 0.01$) in the Shaver than in the RIR hens as expected. The ratio of yolk weight to egg weight was similar (average 0.3, $P > 0.05$) between the two strains of chickens. Moisture, fat and protein contents in the yolk (Table 2.1) were similar ($P > 0.05$) between the strains of chickens averaging 49.9, 31.5, and 14.3% of wet weight, respectively. These are close to those reported previously (Stadelmen and Cotteril, 1986; Burley and Vadehra, 1989). The total protein content per yolk was accordingly lower in the RIR hens.

The changes of anti-BSA antibody activity, determined by ELISA in the serum and egg yolk from either Shaver or RIR birds are shown in Figure 2.1. In both strains, the antibody activity in serum rapidly increased and reached a plateau on day 14. In contrast, the antibody activity in the egg yolk continued to increase after day 14, and reached the peak on day 56. Similar changes in antibody activities in the serum and egg yolk have previously been observed in SCWL hens immunized with E. *coli* (Nakai et al., 1988) and lipopolysaccharides (Sunwoo et al., 1996). There was no significant ($P > 0.05$) difference in the antibody activity between the two strains of chickens in both the serum and egg

yolk throughout the experimental period. These results indicated that BSA is highly antigenic to both breeds of chickens. The chicken anti-BSA antibody from either strain was found to react with sera from cattle, elk and rabbit. Absorbances at 405 nm in ELISA were 0.261, 0.180, and 0.088 for bovine, elk, and rabbit sera, respectively (Figure. 2.2).

The concentration of IgY in the egg yolk was relatively constant (average 0.6%, w/w Table 2.2) among the chickens regardless of the breeds, egg weight, egg production or days of the experimental period. A similar trend has been reported previously in the concentration of egg yolk IgY during immunization of chickens (Shimizu et al., 1988; Sunwoo et al., 1996). However, the total content of IgY in the yolk was approximately 1.3 times greater, and the total IgY produced during the 18wk experimental period was three times greater in the Shaver than in the RIR hens (Table 2.2). This is expected since both the rate of egg production and yolk weight were less in the latter (Table 2.1).

The foregoing results showed that both the antibody activity and total IgY concentration in the egg yolk, were similar between the two strains of chickens, suggesting that the relative concentration of IgY which is specific to BSA is similar between the two strains of hens. However, the absolute concentration of specific IgY is unknown. To examine the content of BSA-specific IgY, we used a BSA-agarose affinity column. The elution pattern of WSF on BSA-agarose was similar between the two strains of hens. A representative chromatography is shown in Figure 2.3. Majority (94 %) of anti-BSA antibody activity was retained in the column, and eluted with 0.5 *M* acetic acid in fractions 7 to 11. The ratio of anti-BSA titre to protein was 50 times greater in the BSA-specific IgY than in the WSF. The proportion of BSA-specific IgY in the total egg

yolk IgY was similar between the two strains of hens as suggested above, and averaged 9.0%.

There is limited information available on the content of antigen specific immunoglobulin. Affinity chromatography with antigen as a ligand appears to be an appropriate technique. Gassmann et al. (1990) raised IgY against proliferating cell nuclear antigen (PCNA), and purified specific antibodies by using affinity chromatography on PCNA-agarose. These authors reported that 3.2% of IgY was specific to the antigen. Available information from a chemical supplier[8] showed that loading the BSA-cellulofine affinity column with 19 mL of immune rabbit serum resulted in elution of 26.85 mg of anti-BSA antibodies from the column. Assuming that the concentration of immunoglobulin in rabbit serum is 13 mg/mL, the proportion of BSA-specific antibody in total immunoglobulin, which is 10.9% by calculation, is apparently close to the proportion of BSA-specific antibody found in this study (see above).

The purified of anti-BSA IgY may be useful for preparing anti-BSA IgY coupled affinity column for small or large scale purification of BSA. The anti-BSA IgY affinity column may also be useful for purifying serum albumin from non-bovine species (e.g. elk) to which the anti-BSA IgY can bind.

In conclusion, the present results suggest that both the Shaver and RIR hens immunized with BSA can produce egg yolk IgY with similar proportions of BSA-specific antibodies. Thus, the egg yolk weight and the percent hen-day production, both of which

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[8]Cellulofine affinity chromatography (in Japanese). Seikagaku Kogyo Co., Ltd. Tokyo Yakugyo Bldg., 1-5, Nihonbashi-honocho 2-chome, Chuo-ku, Tokyo, 103 Japan.

were greater in the Shaver hens, appear to be important factors for the efficient production of anti-BSA IgY.

Table 2.1. Laying performance and egg yolk composition during the experimental period

Variables	Shaver	Rhode Island Red	p
Percentage hen-day production	85.1± 0.0	46.6 ± 11.8	< 0.01
Egg weight g[1]	50.9 ± 2.4	38.7 ± 2.3	< 0.01
Yolk weight g[1]	17.0 ± 1.2	13.2 ± 0.9	< 0.01
Yolk weight / egg weight	0.33 ± 0.0	0.34 ± 0.0	> 0.05
Moisture,[2] % of wet weight	50.5 ± 0.2	49.3 ± 0.6	> 0.05
Fat,[2] % of wet weight	31.1 ± 0.1	31.9 ± 0.4	> 0.05
Protein in yolk,[2] % of wet weight	14.5 ± 0.1	14.1 ± 0.0	> 0.05
Protein per yolk, g	2.46 ± 0.1	1.85 ± 0.1	< 0.01

[1]Both egg and yolk weights were derived from 200 eggs of Shaver (Single Comb White Leghorn) hens and 150 eggs of Rhode Island Red hens.

[2]Six eggs from each breed were analyzed.

Table 2.2. IgY content in the yolk and the total IgY produced during the experimental period

Variable	Shaver	RIR	P
IgY in yolk[1] (% of wet weight)	0.62 ± 0.5	0.63 ± 0.5	> 0.05
IgY per yolk (mg)[2]	105.4 ± 0.7	83.2 ± 0.5	< 0.01
Total IgY production (g)[3]	61.1 ± 0.6	23.4 ± 0.5	< 0.01

[1]Thirty eggs collected from each strain on day 0, 14, 21, 28, 35, 42, 49, 56, 63, and 70 were analysed by RID.

[2] Calculated from the concentration of IgY in the yolk and the yolk weight (Table 2.1).

[3]Calculated based on the content of IgY produced during the 18 wk experimental period.

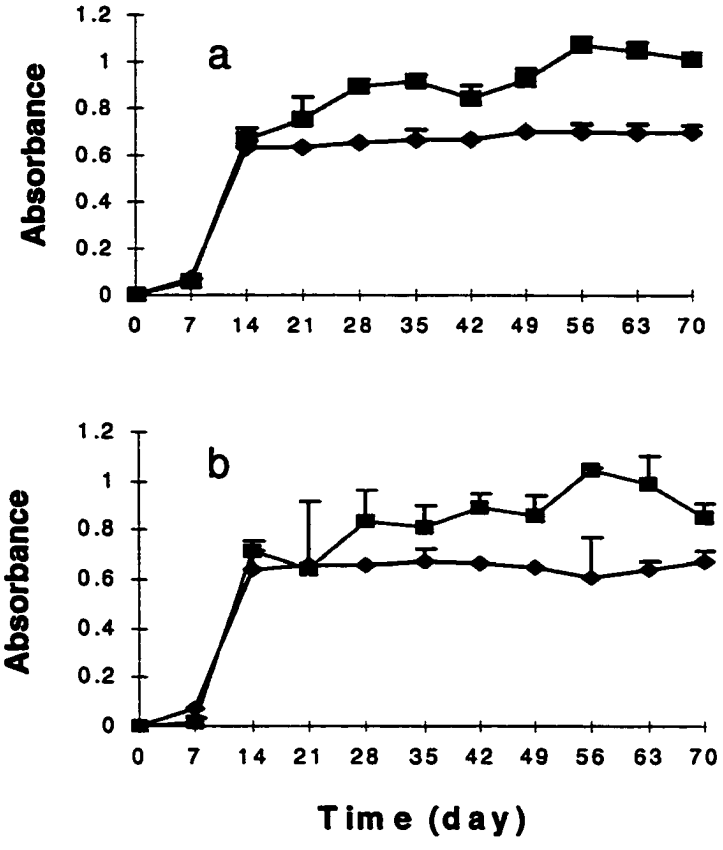

Figure 2.1. Changes of antibody activities in serum and egg yolk during the immunization period. Antibody activities in sera (♦) and egg yolk (■) (both at 1:1000 dilution) from SCWL (a) and RIR (b) hens were measured by ELISA, and were expressed as ELISA absorbance at 405 nm. Vertical bars indicate standard deviation.

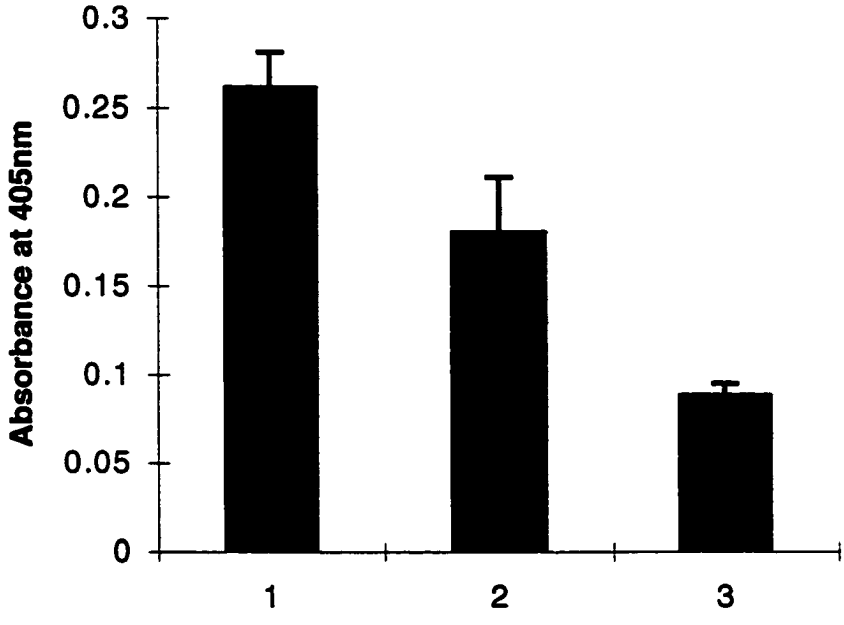

Figure 2.2. Reactivity of chicken anti-BSA antibody to cattle, elk, and rabbit sera 1) bovine serum, 2) elk serum, 3) rabbit serum, Vertical bars indicate SD based on six observations from the same serum sample.

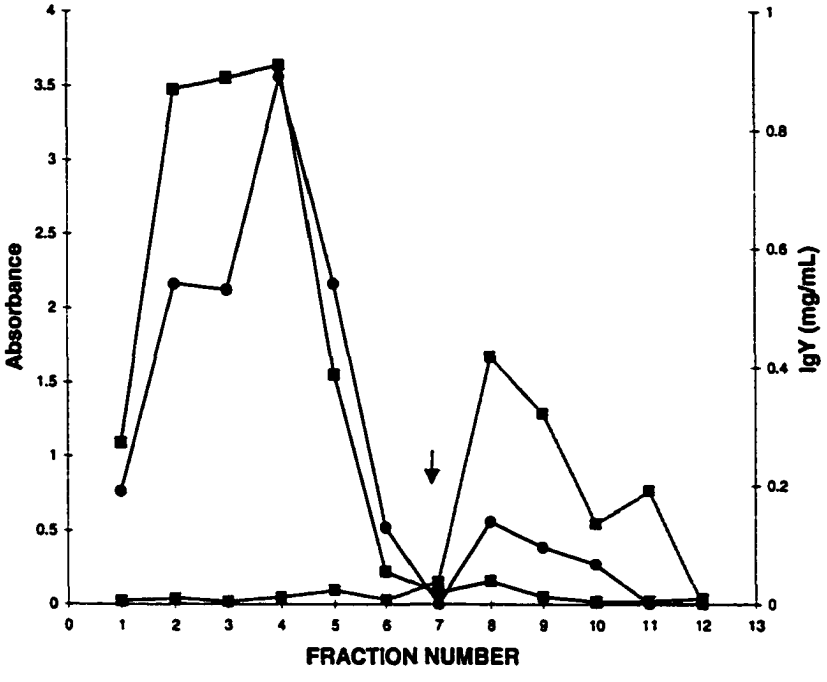

Figure 2.3. Purification of anti-BSA antibody by affinity chromatography. Fractions (1 mL) collected were monitored for anti-BSA antibody activity (absorbance at 405 nm, ▲), and contents of IgY (●) and protein (absorbance at 280 nm, ■). Arrow indicates the application of 0.5 M acetic acid containing 0.5 M NaCl, pH 2.4. See text for other details.

2.4. REFERENCES

Akita, E. M., and Nakai, S. 1992. Immunoglobulins from egg yolk: Isolation and purification. J. Food Sci. 57:629-634.

Association of Official Analytical Chemists. 1980. Official Methods of Analysis. 13th ed. Washington, DC.

Burley, R. W. and Vadehra, D. V. 1989. The Avian Egg: Chemistry and Biology, John Wiley & Sons, Inc. New York.

Ermeling, B. L., Steffen, E. K., Fish, R. E., and Hook, R. R. 1992. Evaluation of subcutaneous chambers as an alternative to conventional methods of antibody production in chickens. Lab. Anim. Sci. 42:402-407.

Gassmann, M., Töhmmes, P., Weiser, T., and Hübscher, U. 1990. Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. FASEB J. 4:2528-2532.

Gottstein, B., and Hemmeler, E. 1985. Egg yolk immunoglobulin Y as an alternative antibody in the serology of echinococcosis. Z. Parasitenkunde 71:273-278.

Jensenius, J.C., Andersen, I., Hau, J., Crone, M., and Koch, C. 1981. Eggs: Conveniently packaged antibodies. Methods for purification of yolk IgG. J Immunol. Meth. 46:63-68.

Larsson, A., Balow, R. M., Lindahl, T. L., and Forsberg, P. O. 1993. Chicken antibodies: taking advantage of evolution. A review. Poultry Sci. 72:1807-1812.

Leslie, G. A., and Clem, W. L. 1969 Phylogeny of immunoglobulin structure and function. III. Immunoglobulin of the chicken. J. Exp. Med. 130:1337-1352.

Lösch, Y., Schranner, I., Wanke, R., and Jurgens, L. 1986. The chicken egg, an antibody source. J. Am. Vet. Med. B33:609-619.

Nakai, S., Shimizu, M., and Fitzsimmons, R. C. 1988. Anti-E. coli immunoglobulin Y isolated from egg yolk of immunized chickens as a potential food ingredient. J. Food Sci. 5:1360-1366.

Orlans, E. 1967. Fowl antibody VIII. A comparison of nature, primary and secondary antibodies to erythrocytes in hen sera; their transmission to yolk and chick. Immunology 12:27-37.

Polson, A., and Von Wechmar, M. B. 1980. Isolation of viral IgY antibodies from yolk of immunized hens. Immunol. Comm. 9:475-493.

Rose, M. E., and Orlans, E. 1981. Immunoglobulins in the egg, embryo and young chick. Dev. Comp. Immun. 5:15-20.

Rose, M. E., Orlans, E., and Buttress, N. 1974. Immunogloblin classes on the hen's egg: Their segregation in yolk and white. Eur. J. Immunol. 4:521-523.

Sim, J. S., and Nakai, S. 1994. Egg usage and processing technologies: New development. Guildford UK pp.94-103.

Shimizu, M., Fitzsimmons, R. C., and Nakai, S. 1988. Anti-E. Coli immunoglobulin Y isolated from egg yolk of immunized chickens as a potential food ingredient. J. Food Sci. 53:1360-1366.

Stadelmen, W. J., and Cotteril, O. J. 1986. Egg Science and Technology 3rd ed. Avi Co., Westport, CT.

Sunwoo, H. H., Nakano, T., Dixon, W. T., and Sim, J. S. 1996. Immune responses in chickens against lipopolysaccharide of *Escherichia coli* and *Salmonella typhimurium*. Poultry Sci. 75:342-345.

Yu, W., Cao, S., Wang, Z., Du, R., Xie, W., Ming, D., and Yu, H. 1985.Bio-statistics and experimental design. 5th ed. Agriculture Beijing. pp 43-63.

CHAPTER 3. PRODUCTION OF CHICKEN EGG YOLK ANTIBODY (IGY) AGAINST BOVINE PROTEOGLYCAN

3.1. INTRODUCTION

Chickens, like mammals, protect their offsprings by transferring maternal immunoglobulin G (IgG) antibodies from serum to egg yolk (Patterson et al., 1962; Rose et al., 1974). Chicken IgG is often called IgY because its physicochemical properties differ from those of mammalian IgG (Leslie and Clem, 1969). Antibodies are highly specific tools to identify proteins. Most antibodies are produced by immunizing animals. However, mammalian proteins are often more immunogenic in phylogenetically distant chickens than in mammals. Carroll and Stollar (1983) and Gassmann et al. (1990) reported efficient production of IgY against poorly immunogenic proteins. Another advantage of raising IgY is its large quantity. Gottstein and Hemmeler (1985) reported that the amount of purified IgY produced in one month is 18 times higher than that of IgG produced in a rabbit.

Proteoglycans are the major non-collagenous proteins in the extracellular matrix of connective tissues such as skin and cartilage, and have important physiological functions to maintain the integrity of tissues (Hardingham et al., 1992). It is interesting to know immune responses of chickens against mammalian proteoglycans. Anti-proteoglycan IgY, if available, will be useful to study roles of proteoglycans. A search of

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the literature indicated no report of immunizing chickens with proteoglycans. This study was therefore undertaken to examine whether we could raise IgY antibodies against bovine skin proteoglycan decorin. Decorin is a low molecular weight proteoglycan having ~37 KDa core protein to which a single dermatan sulphate chain and three N-linked oligosaccharides are covalently attached, and is believed to have a role in collagen fibrillogenesis (Kresse et al., 1994).

3.2. MATERIALS AND METHODS

Immunization of Hens with Decorin

Three 35 wk old Single Comb White Leghorn hens, averaging 1.74 ± 0.24 (SD) kg in body weight were kept at the University of Alberta Poultry Unit. All chickens were cared for in accordance with the Canadian Council on Animal Care guideline for animal welfare. For production of antibody, chickens were injected intramuscularly into breast muscle (Li et al., 1998) on day 0 with 1 mg decorin emulsified in 1 mL of 50% (v/v) Freund's complete adjuvant in phosphate buffered saline (PBS). Decorin was extracted from bovine skin and purified as described by Pearson et al. (1983). On day 14, chickens were injected with the same amount of antigen in Freund's incomplete adjuvant. Eggs were collected daily and stored at 4 °C until analyzed.

Isolation of Water Soluble Fraction

Isolation of the water soluble fraction (WSF) was carried out by the method described by Li et al. (1998). Egg yolk was separated from white and transferred into a

graduated cylinder. Egg yolk samples were diluted with 6 times cold acidified water to make the final pH 5.0, and held at 4°C over night. These samples were then centrifuged at 12,100 x g and 4°C for 25 min, Each supernatant was collected as WSF, and was determined for the anti-decorin antibody activity by ELISA. The anti-decorin IgY used for the ELISA inhibition assay and immunohistochemical stainings was purified using decorin-conjugated cellufine affinity column. Conjugation of decorin agarose was carried out according to the manufacturer's (Chisso Corp. Japan) instruction.

ELISA

Antibody activity in WSF was determined according to Li et al. (1998). Microtitre plates with 96 wells were coated by adding 150 μL of decorin solution (5 μg/mL in 0.05 M , carbonate buffer, pH 9.6) to each well and incubated at 22°C for 2 h. After washing with PBS containing 0.05% Tween-20 (PBST), 150 μL of WSF, which was diluted 1000 times, was added to each well, and incubated at 37°C for 1 h. Subsequent to incubation, plates were washed with PBST. The secondary antibody (150 μL of a 1 : 1000 dilution of peroxidase conjugated rabbit anti-chicken IgG) was then added and incubation was continued at 37°C for 1 h. Plates were washed again with PBST, and 100 μL of substrate solution, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfate acid) in 0.05 M phosphate-citrate buffer, pH 5.0 containing 0.03% sodium perborate, was added to each well. After 30 min of incubation at 22°C, the absorbance of the reaction mixture was measured at 405 nm using ELISA reader.

ELISA inhibition assay was carried out by incubating 50 μL of increasing concentration of competing antigen (0 to 100 μg/ mL) in the decorin coated plates

prepared as above with an equal volume of 5 times diluted anti-decorin IgY purified by the affinity column (see above). Incubation was at 4°C over night. ELISA was then performed as above. The percentage of inhibition of antibody binding was calculated using the formula: percentage inhibition = 100 - (100 x absorbance in the presence of inhibitor / absorbance in the absence of inhibitor).

Enzymatic Digestion of Decorin for ELISA

Papain digests of decorin were produced by incubation of 1mg of decorin in 1mL of a 0.1 M sodium acetate buffer, pH 5.5 containing 5 mM EDTA, 5 mM cysteine-HCl, 0.02% sodium azide and 6.2 units of twice crystallized papain (Sigma Chemical Co., St. Louis, MO) at 65°C for 4 h. Digestion with chondroitinase-ABC was carried out by incubating 1 mg of decorin in 1 mL of 0.1 M Tris -HCl buffer, pH 8.0 containing 0.1 M sodium acetate, 0.02% sodium azide and 0.05 unit of chondroitinase-ABC (Sigma Chemical Co.) at 37°C for 1 h. Both papain and chondroitinase-ABC were inactivated after digestion by heating the incubation mixtures in boiling water for 15 min.

Radial Immunodiffusion

Radial immunodiffusion (RID) of WSF was performed by the method previously described by Sunwoo et al. (1996). The solution A was prepared by mixing 1.2 mL rabbit anti-chicken IgG with 6.8 mL barbital buffer (50 mM sodium barbital and 10 mM barbital, pH 8.6) and incubating in a 56°C water bath. Solution B was prepared by mixing 280 mg of agarose with 18.4 mL barbital buffer and 1.6 mL of 0.35% (wt /vol) sodium azide, and holding the mixture in a boiling water bath until the agarose was dissolved.

Solutions A and B were mixed well, equilibrated at 56°C, and poured into RID plate. WSF (6 μL) and an equal volume of standard IgG (0 to 1 mg / mL in PBS) were then added to 2.5 mm diameter wells, and incubated at room temperature for 3 days. The diameter of precipitation rings formed was measured to estimate the content of IgG.

Immunohistochemical Staining of Tissues

Fresh tissues from different species including bovine longissimus dorsi, porcine temporomandibular joint disc and chicken comb were fixed in 4% buffered formalin containing 0.5% cetylpyridinium chloride, and embedded in paraffin (Drury and Wallington,. 1967). Five micron thick sections were cut, deparaffinized and treated with 2% of H_2O_2 in methanol for 30 min to eliminate endogenous peroxidase activity. Sections were then washed in PBS, soaked in normal rabbit serum (diluted 1 in 20 in PBS) for 30 min and incubated at 37°C for 1 h with 50 times diluted anti-decorin IgY purified by the affinity column chromatography (see above). Control sections were incubated with WSF from non-immune eggs. After washing in PBS, sections were incubated for 45 min with peroxidase conjugated rabbit anti-chicken IgG (Sigma) diluted 1 : 250 in PBS. Sections were washed again and incubated for 3 min in a solution containing 0.05% diaminobenzidine tetrahydrochloride (Sigma) and 0.01% H_2O_2 to develop the color.

Statistical Analysis

A t-test was used to detect significant ($P < 0.05$) differences between means of ELISA absorbance and total IgY concentration (Yu et al., 1985).

3.3. RESULTS AND DISCUSSION

The anti-decorin antibody activity measured as ELISA absorbance in WSF was undetectable on day 0, rapidly increased ($P < 0.05$) from day 7 to day 35, and was relatively constant ($P > 0.05$) thereafter (Figure. 3.1). These results indicated that bovine decorin is highly antigenic to chickens. The total IgY concentrations monitored in the egg yolk from the three chickens during the experimental period were relatively constant (9.8 ± 1.5 mg/mL, $P > 0.05$) as reported previously (Sunwoo et al., 1996).

ELISA inhibition assays (Figure. 3.2) showed a positive binding of IgY to bovine decorin. The concentration of antigen required for 50% inhibition was 1.3 μg / mL. There was no inhibition observed with dermatan sulphate, a constituent polysaccharide of decorin, suggesting that the protein core of decorin only is antigenic (see below). There was also no inhibition with many other components found in connective tissues including chondroitin sulphates A and C, aggrecan, keratan sulphate, fibronectin and collagen type I.

To further confirm the above suggestion of the antigenicity of protein core, samples of decorin were digested separately with either papain or chondroitinase-ABC, and the resulting fragments were examined using the ELISA inhibition assay. Digestion with papain caused a complete loss in antigenicity, while chondroitinase-ABC had no appreciable effect (Figure. 3.2). These results indicated that the protein core but not the dermatan sulphate chain is recognized by the anti-decorin IgY.

Immunohistochemical stainings of tissues from different species showed that control stainings with WSF from non-immune eggs were negative (results not shown). In contrast positive immunostainings with anti-decorin IgY were seen in the bovine

longissimus dorsi muscle connective tissues (Figure. 3.3a), porcine temporomandibular joint disc (Figure. 3.3b), and chicken comb (Figure. 3.3c).

The present results demonstrate for the first time the production of anti-bovine decorin IgY. Chickens appear to be highly efficient producers of anti-bovine decorin IgY. This IgY can recognize bovine, porcine, and chicken decorins. The anti-decorin IgY may be a useful tool to monitor decorin, which is an interesting protein to animal and meat scientists. For example, a recent study suggested importance of collagen binding proteoglycan (probably decorin) in the post-mortem aging of meat (Nishimura et al., 1996). Decorin is also suggested to have roles in regulating collagen fibrillogenesis and neutralizing the effect of transforming growth factor-β, which stimulates collagen and proteoglycan synthesis (Kresse et al., 1994).

In conclusion, chickens are efficient host birds that produce specific IgY to the protein core of bovine decorin.

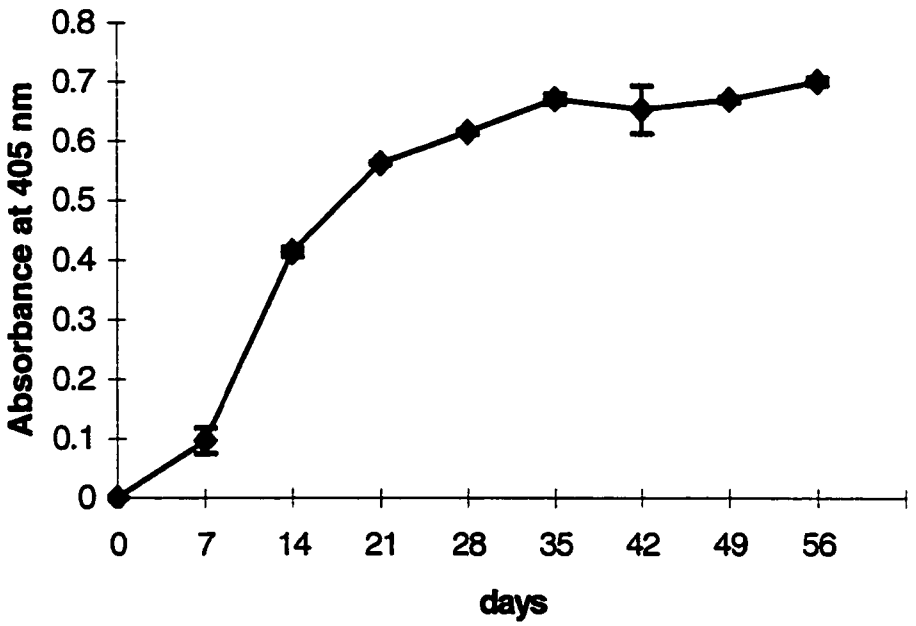


Figure 3.1. Changes in the anti-decorin antibody activity in the egg yolk during the immunization period. Antibody activities in the WSF of egg yolk (at 1 : 1000 dilution) were measured by ELISA and were expressed as ELISA absorbance at 405 nm. Vertical bars indicate standard deviation.

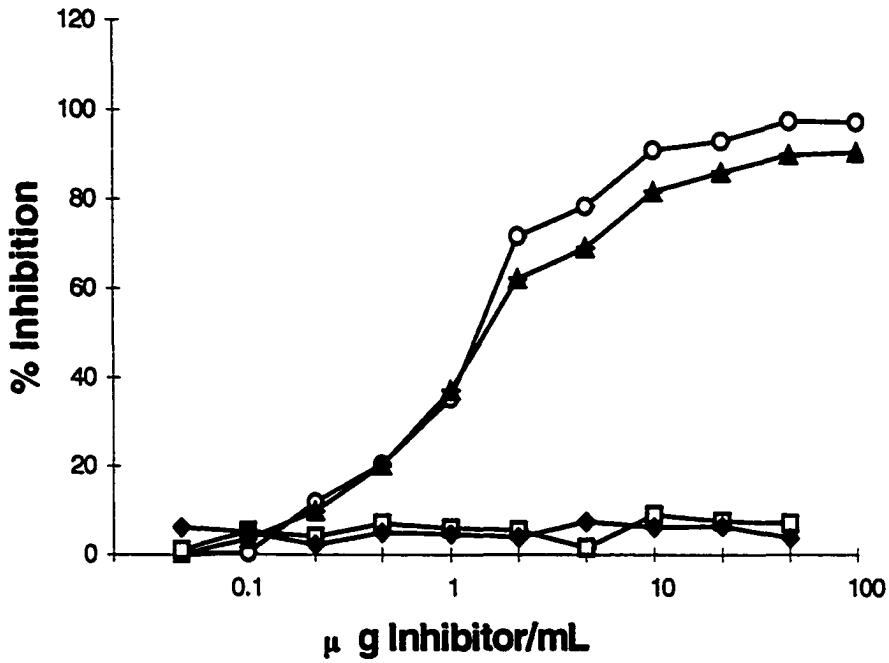


Figure 3.2. ELISA inhibition assay with enzymatically degraded decorin and dermatan sulphate. The antigenicity of each of decorin digested with papain (♦), decorin digested with chondroitinase-ABC (▲), and dermatan sulphate (□) was compared with that of enzyme untreated decorin (○).

a

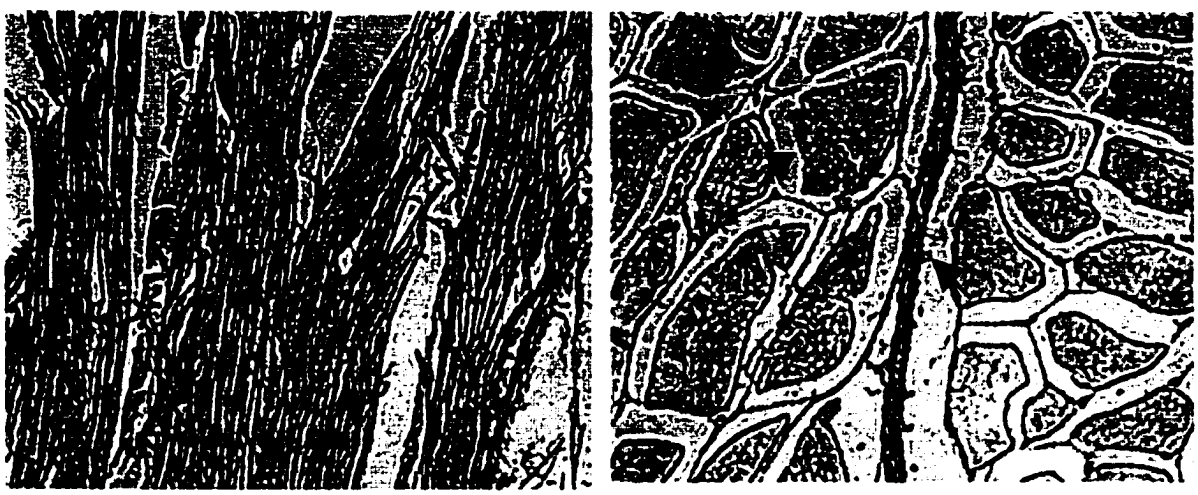
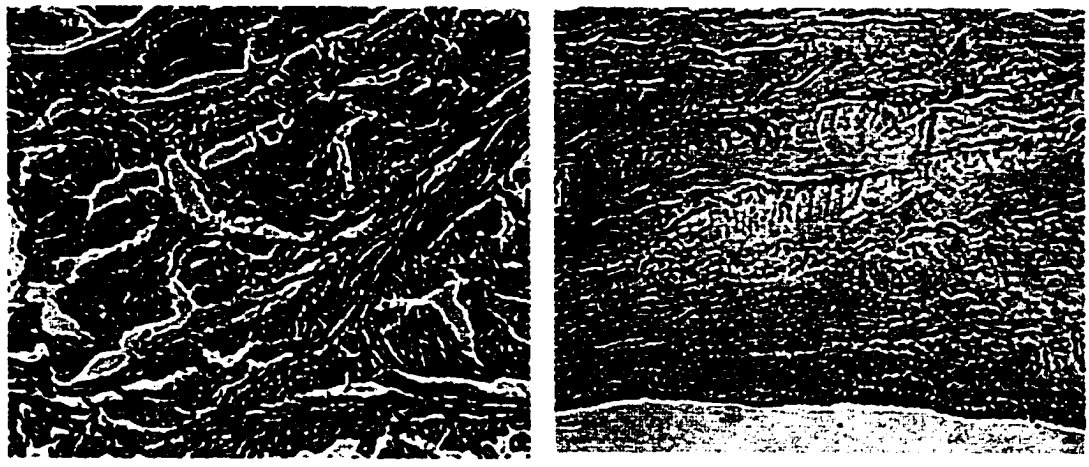


Figure 3.3. Immunohistochemical staining of tissues from different species with IgY raised against bovine skin decorin. a) Bovine longissimus dorsi showing positive immunostainings in the epimysium (left micrograph), and intramuscular connective tissues (right micrograph). Arrow and arrow head point perimysium and endomysium, respectively. x 150.

b



c

b). Porcine temporomandibular joint disc. Positive immunostainings were observed throughout the oval shaped disc with biconcave structure. However, the staining intensity tended to be lower in the central thinner part (left micrograph) than in the remaining thicker part (right micrograph) of the disc. x 150.

c). chicken comb showing positive stained collagen fibres in the surface layer. Magnificantion was x 150.

3.4. REFERENCES

Carroll, S. B., and Stollar, B. D.1983. Antibodies to calf thymus RNA polymerase II from egg yolks of immunized hens. J. Bio. Chem. 258:24-26.

Drury, R. A. B., and Wallington, E. A. 1967. Carleton's Histological Technique. Oxford University Press, New York, N.Y.

Gassmann, M., Töhmmes, P., Weiser, T., and Hübscher, U. 1990. Efficent production of chicken egg yolk antibodies against a conserved mammalian protein. FASEB J. 4:2528-2532.

Gottstein, B., and Hemmeler, E. 1985. Egg yolk immunoglobulin Y as an alternative antibody in the serology of echinococcosis. Z. Parasitenkunde. 71:273-278.

Hardingham, T. E., and Fosang, A. J. 1992. Proteoglycans: many forms and many functions. FASEB J. 6:861-870.

Kresse, H., Hausser, H., and Schönherr, E. 1994. Small proteoglycans. Page 73-100 *in* P. Jollès, ed. Proteoglycans. Birkhäuser Verlag, Basel. Switzerland.

Leslie, G. A., and Clem, W. L. 1969. Phylogeny of immunoglobulin structure and function. III. Immunoglobulin of the chicken. J. Exp. Med. 130:1337-1352.

Li, X., Nakano, T., Sunwoo, H. H., Sim, J. S., Paek, B. H., and Chae, H. S.1998. Effects of egg yolk weights on yolk antibody (IgY) production in laying chickens. Poultry Sci. 77:266-270.

Nishimura, T., Hattori, A., and Takahashi, K. 1996. Relationship between degradation of proteoglycans and weakening of the intramuscular connective tissue during post-mortem aging of beef. Meat Sci. 42:251-260.

Patterson, R., Younger, J. S., Weigle, W. O., and Dixson, F. J. 1962. The metabolism of serum proteins in the hen and chick and secretions of serum proteins by the ovary of the hen. J. Gen. Physiol. 45:501-513.

Pearson, C. H., Winterbottom, N., Fackre, J. S., Scott, P. G., and Carpenter, M. R. 1983. The NH_2-terminal amino acid sequence of bovine skin proteodermatan sulfate. J. Biol. Chem. 258:15101-15104.

Rose, M. E., Orlans, E., and Buttress, N. 1974. Immunoglobulin classes on the hen's eggs: Their segregation in yolk and white. Eur. J. Immunol. 4:521-523.

Sunwoo, H. H., Nakano, T., Dixon, W. T., and Sim, J. S. 1996. Immune responses in chickens against lipopolysaccharide of *Escherichia coli* and *Salmonella typhimurium*. Poultry Sci. 75:342-345.

Yu, W., Cao, S., Wang, Z., Du, R., Xie, W., Ming, D., and Yu, H. 1985. Bio-statistics and experiment design. 5th ed. Agriculture Beijing. pp. 43-63.

CHAPTER 4. ISOLATION OF HIGH MOLECULAR WEIGHT PROTEOGLYCAN FROM PORCINE STIFLE MENISCUS AND PRODUCTION OF ANTI-PROTEOGLYCAN ANTIBODIES IN CHICKENS

4.1. INTRODUCTION

Stifle (or knee) meniscus is a fibrocartilaginous weight bearing structure located between the femoral and tibial condyles. The mechanical functions of the meniscus depend on the proper composition and organization of its constituent macromolecules. Since in athletes the incidence of knee meniscus injury is considerably high, an understanding of the chemical composition of meniscus is of interest by analyzing tissues from pigs. The meniscus contains collagen fibres and a high molecular weight (MW) proteoglycan (a protein substituted with glycosaminoglycans, GAGs) as major extracellular matrix constituents (Fithian et al., 1990; McDevitt and Webber, 1990; Nakano et al., 1997). Collagen fibres provide the load bearing tissues with tensile strength, while the proteoglycan is thought to be responsible in large part for the resilience or compressive stiffness of the tissue (Fithian et al. 1990). Previous studies of human and canine menisci (Adams et al., 1986; McNicol and Roughley, 1980; Roughley et al., 1981) have shown that the structure of high MW proteoglycan resembles that of aggrecan, a well characterized chondroitin sulfate proteoglycan (CSPG) from hyaline cartilage (Hardingham et al., 1994, Heinegård and Oldberg, 1989). A high MW

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proteoglycan has recently been reported in the pig stifle meniscus, and characterized by gel electrophoresis and immunoblotting (Nakano et al., 1996). However, there is limited information available concerning a quantitative analytical study of high MW proteoglycan isolated from the porcine meniscus.

Immunochemical analysis using specific antibodies is one of the useful methods to identify protein molecules. It is, however, thought that production of antibodies against its core protein by immunizing mammals with intact proteoglycan may be difficult if its protein core is heavily substituted with GAGs as seen in cartilage proteoglycan, aggrecan. Little is known about the immune response of chickens against porcine proteoglycans. Because of the phylogenetic distance between avian and mammalian species (Jensenius et al., 1981), chickens may be superior to mammals in the production of antibodies against mammalian antigens. Moreover, chickens as antibody producers have several other advantages over mammals (Akita and Nakai, 1992; Gottstain and Hemmeler, 1985; Orlans, 1967; Polson and Wechmar, 1980; Rose et al., 1974). For example, antibodies produced in the chicken are stored in the egg yolk, and thus the amount of egg yolk antibody (IgY) collected from one bird is much higher than the amount of serum antibodies collected by sacrificing the host animal (Sim and Nakai, 1994). It is, therefore, our interest to determine whether chickens can produce IgY against the pig proteoglycan. This report describes the isolation and characterization of high MW proteoglycan from the porcine stifle meniscus and the production of IgY against its core protein.

4.2. MATERIALS AND METHODS

Materials

Medial and lateral menisci were obtained from the left and right stifles of three market weight pigs at a local abattoir. Carcasses were kept at 4°C overnight before menisci were removed. All menisci were visually normal. The meniscus body was then dissected free of adherent synovial membranes and fibrous attachments, rinsed with cold distilled water to remove synovial fluid, and stored at -20°C until extracted.

Chondroitin sulfate from pig rib cartilage, dermatan sulfate from hog skin and hyaluronic acid from human umbilical cord were obtained from Sigma Chemical Co. Mississauga, Ontario, Canada. Chondroitin sulfate from whale cartilage was from Miles Laboratories, Elkhart, IN. U.S.A., and keratan sulfate from bovine cornea was from Seikagaku America Inc. Rockville, MD., U.S.A.

Isolation of proteoglycan

The tissues were cut into small pieces and stirred at 4°C for 44 h with 10 volumes of 4 M guanidine-HCl / 0.05 M Tris-HCl / 0.15 M NaCl / 0.02% (wt / vol) sodium azide, pH 6.6 containing proteinase inhibitors (0.01 M EDTA, 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine-HCl, 0.0005 M N-ethylmaleimide, 0.001 M phenylmethanesulphonyl fluoride, 5 µg / ml pepstatin) (Pearson et. al., 1983). The tissues were extracted a second time for 24 h with a fresh aliquot of the same solution. The extracts were filtrated through glass wool and combined. To determine the content of uronic acid in the extracts, three volumes of 95% ethanol were added to an aliquot and the mixture was left overnight at 4°C. The precipitate that formed was washed three times with 75% ethanol, dissolved in 0.5 M urea, and used to estimate uronic acid content. The standard curve was prepared from varying concentrations of glucuronolactone in 0.5 M

urea. The amount of uronic acid remaining in the tissue was estimated after the residues were washed with water and subsequently digested with papain, as described previously (Nakano et al., 1997).

The guanidine-HCl in the extracts was then exchanged by dialysis for 7 M urea, and the extracts were chromatographed on a column of DEAE-Sephacel (Pharmacia Biotech Inc. Baie d'Urfé, Quebec, Canada) eluted with a linear gradient of 0.15 - 1 M NaCl. Fractions containing proteoglycans, as detected by the dimethylmethylene blue (DMB) dye binding method (Farndale et al., 1992), were pooled, concentrated, and applied to a column of Sepharose CL-4B (Pharmacia) in 7 M urea / 0.05 M sodium acetate / 0.05 M Tris- HCl / 0.02% (wt / vol) sodium azide, pH 6.6. Proteoglycans were separated into high, intermediate and low MW fractions referred to as fraction I, II and III, respectively, and the effluents within each fraction were pooled, dialyzed in water and freeze-dried for subsequent analyses.

Enzymatic digestion of proteoglycans and GAGs

Digestions with chondroitinase-ABC (Sigma) and Chondroitinase-ACI (ICN Pharmaceuticals Canada Ltd., Montreal, Quebec, Canada) were carried out separately with 0.005 unit of each enzyme per μg uronic acid in 0.01 M sodium acetate buffer containing 0.02% sodium azide at 37°C for 1 h. The pH of the buffer was 8.0 for chondroitinase-ABC and 7.3 for chondroitinase-ACI. Digestion with chondroitinase-B (Sigma, 0.05 unit per μg uronic acid) was in 0.01 M sodium acetate buffer containing 0.02% sodium azide, pH 7.5 at 25°C for 1 h. Digestion with endo-ß-galactosidase (Seikagaku America, 0.05 unit per mg of proteoglycan) was in 0.01 M sodium acetate

buffer containing 0.02% sodium azide, pH 5.8 at 50°C for 1 h. Chondroitinase-ABC digests both chondroitin sulfate and dermatan sulfate, while condroitinase-ACI and chondroitinase-B digest chondroitin sulfate and dermatan sulfate (with chondritinase-B (Sigma, 0.05 unit per μg uronic acid) was in 0.01 M sodium acetate buffer containing 0.02% sodium azide, pH 7.5 at 25°C for 1 h. Digestion with endo-ß-galactosidase (Seikagaku America, 0.05 unit per mg of proteoglycan) was in 0.01 M sodium acetate buffer containing 0.02% sodium azide, pH 5.8 at 50°C for 1 h. Chondroitinase-ABC digests both chondroitin sulfate and dermatan sulfate, while condroitinase-ACI and chondroitinase-B digest chondroitin sulfate and dermatan sulfate (with idoronosyl residues), respectively (Jandik, 1994). Endo—galactosidase is a keratan sulfate degrading enzyme (Nakazawa, 1989).

The enzymes used were checked for activity against standard samples of the appropriate GAGs : chondroitin sulfate from whale cartilage, dermatan sulfate from hog skin and keratan sulfate from bovine cornea.

Analytical methods

Uronic acid content was determined by the carbazole reaction (Kosakai and Yoshizawa, 1979) using glucuronolactone as a standard, and protein content by the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumin (Sigma) as a standard. Amino acid composition of fraction I proteoglycan was determined using a Beckman 6300 amino acid analyzer (Beckmen Center B065 SUMC Palo Alto CA., U.S.A.).

Cellulose acetate electrophoresis

Electrophoresis of proteoglycans on cellulose acetate strips (Gelman Sciences, Ann Arbor, MI., U.S.A.) was carried out in 0.1 M pyridine and 1.2 M acetic acid (Habuchi et al., 1973; Nakano et al., 1996). The strips were stained in 0.1% (wt / vol) Alcian Blue 8GX in 0.1% acetic acid.

Immunodiffusion

Undiluted ascites fluid (8 µl) containing anti-keratan sulfate monoclonal antibody AH12 (Nakano et al., 1993) and chondroitinase-ACI treated fraction I proteoglycan (40 µg in 8 µl) were applied to wells in gels, which were made from 1% (wt / vol) agar in saline containing 0.05% sodium azide (Koga et al., 1990).

Interaction of proteoglycan fraction with hyaluronic acid

Hyaluronic acid from human umbilical cord was dissolved in 0.5 M sodium acetate buffer, pH 6.8 and added to the test solution of proteoglycan in the same buffer at a weight ratio of hyaluronic acid to proteoglycan of 1 : 100. After allowing the solution to stand at 4 °C for 12 h, the extent of aggregation was examined by chromatography on Sepharose CL-2B (Sigma) by monitoring fractions with the DMB dye binding method (Farndale et al., 1992).

Preparation of chicken egg yolk antibodies (IgY) against high MW CSPG from pig meniscus

Antibody production was carried out following the procedures described by Li et al. (1998). Three Single Comb White Leghorn hens were used for immunization. These chickens were kept at the University of Alberta Poultry Unit and cared for in accordance with the Canadian Council on Animal Care guideline of animal welfare. Each chicken was injected intramuscularly on day 0 with 1 mg of high MW CSPG from pig meniscus emulsified in 1 ml of 50% (vol / vol) Freund's complete adjuvant in phosphate buffered saline (PBS, 0.14 M NaCl, 0.0015 M KH_2PO_4, 0.0081 M Na_2HPO_4 and 0.0027 M KCl, pH 7.2). On day 14, chickens were injected with the same amount of antigen emulsified in Freund's incomplete adjuvant. Eggs were collected daily, and stored at 4°C until used for isolation of the water soluble fraction (WSF) from egg yolk.

The method of preparation of WSF was described in our previous report (Li et al., 1998). Samples of WSF were determined for the anti-CSPG IgY activities by ELISA (see below) and for total IgY concentrations by the radial immunodiffusion assay (Li et al., 1998).

ELISA

The procedures of ELISA followed those described previously (Li et al., 1998). Antibody activities were determined by incubating 150 µl of 1000 times diluted WSF in microtiter plates precoated with 5 µg / ml concentration of pig meniscus CSPG (fraction I, see above).

ELISA inhibition assay was carried out by incubating 50 µl of increasing concentration of competing antigen (0 - 100 µg / ml) in the plates prepared as described above with an equal volume of 1000 times diluted WSF prepared from day 42 eggs.

Other methods

Gel chromatography on Sephacryl S-300 was carried out as described (Nakano et al., 1996). A t-test was used to detect significant ($P < 0.05$) differences between means of ELISA absorbance and total IgY concentration (Yu et al., 1985).

4.3.RESULTS AND DISCUSSION

Isolation and characterization of proteoglycans

Approximately 50% of the total uronic acid recovered was extracted from the pig meniscus tissue with 4 M guanidine-HCl. Chromatography of the extract on DEAE-Sephacel gave a proteoglycan peak eluting at 0.4-0.7 M NaCl (Figure. 4.1). The peak fractions were separated by Sepharose CL-4B chromatography into three fractions (I, II and III) containing high, intermediate and low MW proteoglycans, respectively (Figure. 4.2). The relative proportions and concentrations of uronic acid and protein for each fraction are shown in Table 4.1. The ratio of uronic acid to protein was highest in fraction I (1.60), and greater in fraction II (0.74) than in fraction III (0.45). This indicated that the extent of GAG substitution in the protein core was highest in fraction I. Only this fraction was studied further.

Fraction I was found to be highly susceptible to either chondroitinase-ABC (data not shown) or chondroitinase-ACI (Figure.4.3, lane 1) as evidenced by a complete disappearance of its band on cellulose acetate. There was, however, no appreciable digestion observed in this fraction with either chondroitinase-B (Figure. 4.3, lane 3) or

endo—glactosidase (keratanase) (Figure. 4.3, lane 5). Therefore, fraction I contained CSPG.

On immunodiffusion analysis, a clear precipitin line was seen with the chondroitinase-ACI digest of fraction I and anti-keratan sulfate monoclonal antibody AH 12 (data not shown). Therefore, the fraction I CSPG contained keratan sulfate of which concentration was too low to be detected in its keratanase digest examined by cellulose acetate electrophoresis (Figure. 4.3, lane 5). The positive results of immunodiffusion with monoclonal antibody is consistent with the previous report of immunoblotting of high MW CSPG from pig meniscus (Nakano et al., 1997), and further suggested the evidence for the presence of multiple epitopes on each CSPG molecule.

To examine the molecular size of chondroitin sulfate, fraction I CSPG was digested with papain and chromatographed on Sephacryl S-300. Fraction I chondroitin sulfate had a broad peak (Figure. 4.4a) with K_{av} 0.36, which was close to that (0.41) of pig rib cartilage chondroitin sulfate but greater than that (0.25) of whale cartilage chondroitin sulfate (data not shown). Aliquots of fraction I chondroitin sulfate were then digested with chondroitinase-ACI (as positive control) and chondroitinase-B, and each digest was chromatographed on the same column to examine whether there are any iduronosyl residues present in the chondroitin sulfate chains to form copolymeric GAG chains. Chondroitinase-ACI digestion resulted in fragmentation of all chondroitin sulfate chains (Figure. 4.4b), while the effect of chondroitinase-B was undetectable (Figure. 4.4c). These results were consistent with those obtained with electrophoresis (Figure. 4.3).

In order to determine whether the pig meniscus CSPG was able to form high MW aggregates with hyaluronic acid, aliquots of fraction I were chromatographed on Sepharose CL-4B with and without prior incubation with hyaluronic acid. Approximately 40% of fraction I CSPG aggregated and was excluded from the column (Figure. 4.5). Amino acid composition in fraction I CSPG showed high content of glutamic acid, serine and glycine (Table 4.2).

The porcine meniscus contains CSPG with high MW (probably > 1000,000 Dalton) as do menisci of other species (Adams et al., 1986, McNicol and Roughley, 1980, Roughley et al., 1981). The meniscus CSPGs appear to share with aggrecan (a well characterized CSPG from cartilage) various characteristics including high ratio of uronic acid to protein, presence of keratan sulfate, ability to aggregate with hyaluronic acid and the amino acid composition with high contents of glutamic acid, serine and glycine. Chondroitin sulfate chains with their charged hydrophilic nature (Fithian et al., 1990, Mow et al., 1992, Muir, 1997) and consequent high swelling pressure likely provide compressive stiffness to the stifle meniscus, a weight bearing structure. Thus, the abundance of chondroitin sulfate chains within this proteoglycan may be important for the normal mechanical function of the structure.

Production of IgY against the fraction I CSPG

The IgY activity against the fraction I CSPG was undetectable on day 0, rapidly increased from day 7 to day 35, and remained relatively constant thereafter (Figure 4.6). This indicated that CSPG is highly antigenic to chickens. The total IgY concentrations

determined in the egg yolk during the experimental period were constant (7.6 ± SD 0.3 mg / ml, $P > 0.05$) as reported previously (Li et al., 1998, Sunwoo et al., 1996).

ELISA inhibition assays using fraction I CSPG coated plates (Figure 4.7) showed that the IgY recognized epitopes on the CSPG. The concentration of CSPG required for 50% inhibition (IC_{50}) was 13 μg / ml. The IC_{50} decreased to 3 μg / ml when the CSPG was treated with chondroitinase-ABC suggesting that the protein core without chondroitin sulfate chain is a more efficient inhibitor (approximately 4 fold) than the intact CSPG. This is probably due to increased accessibility of the antigen to the antibody after the removal of chondroitin sulfate chains. In contrast, no inhibition was observed with either the papain digest of CSPG or the commercial chondroitin sulfate ($IC_{50} > 1000$ μg / ml). These results indicated that the protein core but not the chondroitin sulfate chain is recognized by the anti-CSPG IgY. No inhibition of IgY binding was observed with 1000 μg / ml of dermatan sulfate, hyaluronic acid and collagen type I (data not shown).

Production of mammalian antibodies against the core protein of proteoglycan with abundant GAG chains (e.g. aggrecan) is thought to be relatively difficult. For example, Christner et al. (Christner et al., 1980) immunized mice with chondroitinase-ABC treated aggrecan, and found no antibodies against its protein core. Instead they reported production of antibodies against unsaturated disaccharide generated after enzymatic cleavage of chondroitin sulfate chains. The present results, however, suggested that chickens are efficient producers of IgY against the protein core of chondroitin sulfate rich proteoglycan from pig meniscus. We have also been able to raise IgY specific for the protein core of bovine aggrecan (T. Nakano and X. Li, unpublished data). The IgY obtained in the present experiment is a useful tool to monitor high MW CSPG in the

meniscus of the pig, which can be used as an experimental model to study knee meniscus injury, a common problem among athletes. Immunohistochemical localization of CSPG in the pig meniscus is under progress in our laboratory.

Table 4.1. Relative proportion, and uronic acid and protein concentrations in the proteoglycan fractions from porcine stifle meniscus

Fraction	% of Total proteoglycan[a]	Uronic acid (mg/g of dry weight)	Protein (mg/g of dry weight)	Ratio Uronic acid/protein
I	35.2	21.3	13.3	1.6
II	29.2	22.0	29.8	0.74
III	35.6	13.7	30.6	0.45

[a]Proteoglycan was determined as uronic acid.

Table 4.2. Amino acid composition of fraction I CSPG

Amino acid	Residues / 1000 total
Aspartic acid	95
Threonine	62
Serine	106
Glutamic acid	143
Glycine	134
Alanine	76
Valine	81
Isoleucine	44
Leucine	86
Tyrosine	26
Phenylalanine	37
Histidine	26
Lysine	38
Arginine	46

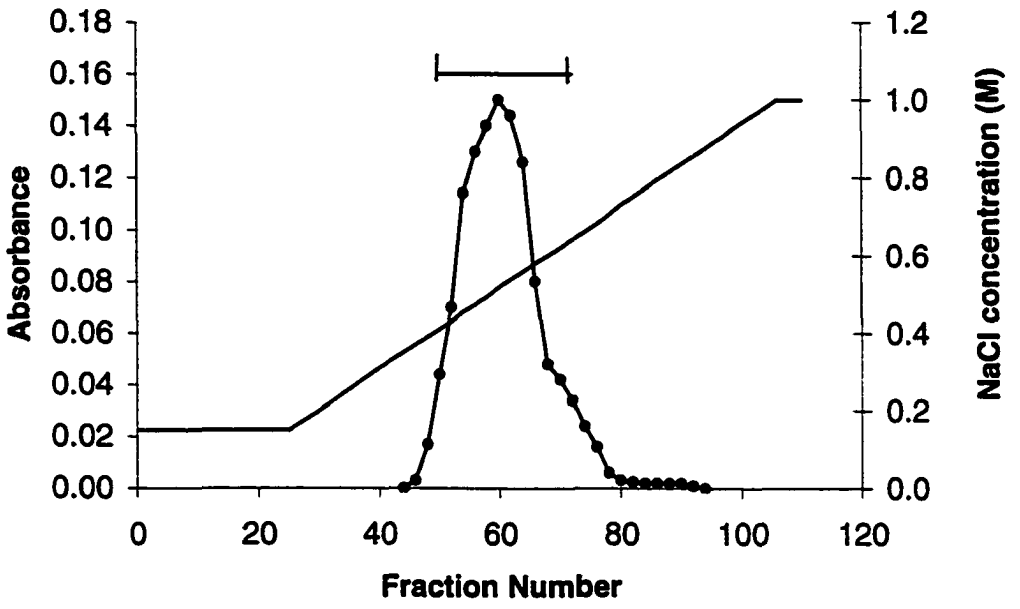

Figure 4.1. Chromatography of extract of pig stifle meniscus on a 2.5 x 14 cm column of DEAE-Sephacel. Fractions of 11 ml were collected at a flow rate of 35 ml / h and determined for proteoglycan by the DMB dye reaction (absorbance at 595 nm). Bar denotes fraction pooled for further study.

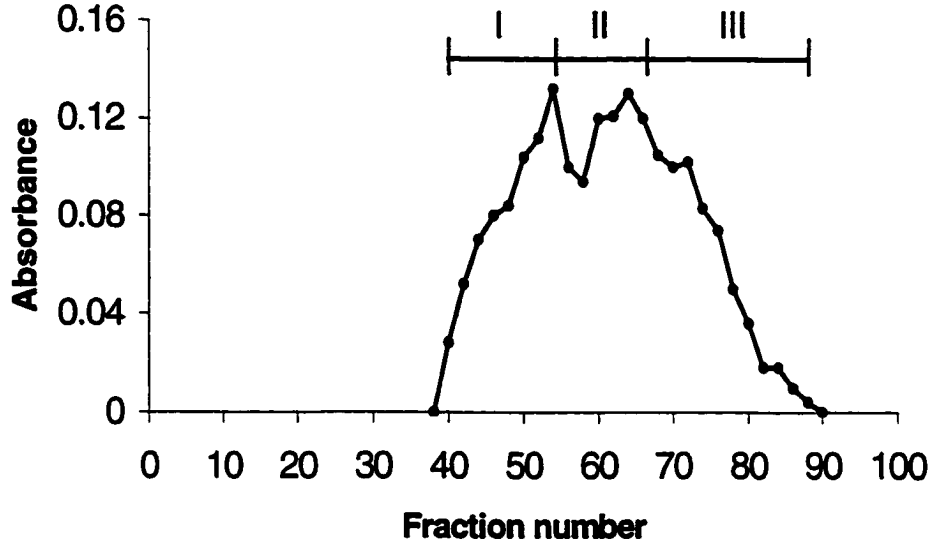

Figure 4.2. Chromatography of proteoglycan containing fraction from the DEAE-Sephacel column (Fig. 1) on a 1 x110 cm column of Sepharose CL-4B. Fractions of 1ml were collected at a flow rate of 2 ml / h and determined for proteoglycan by the DMB dye reaction (absorbance at 595 nm). Bar denotes fractions (I, II and III) pooled for further study.

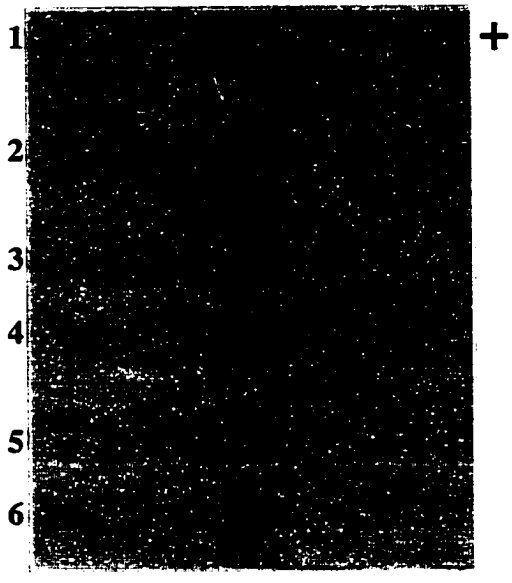

Figure 4.3. Cellulose acetate electrophoresis of fraction I CSPG. Lines 1, 3 and 5 respresent samples treated with chondroitinase-ABC, chondroitinase-B and endo-β-galactosidise, respectively. The remaining lanes 2, 4 and 6 represent samples not treated with enzymes. Samples treated with and without each enzyme were electrophoresed on a same strip.

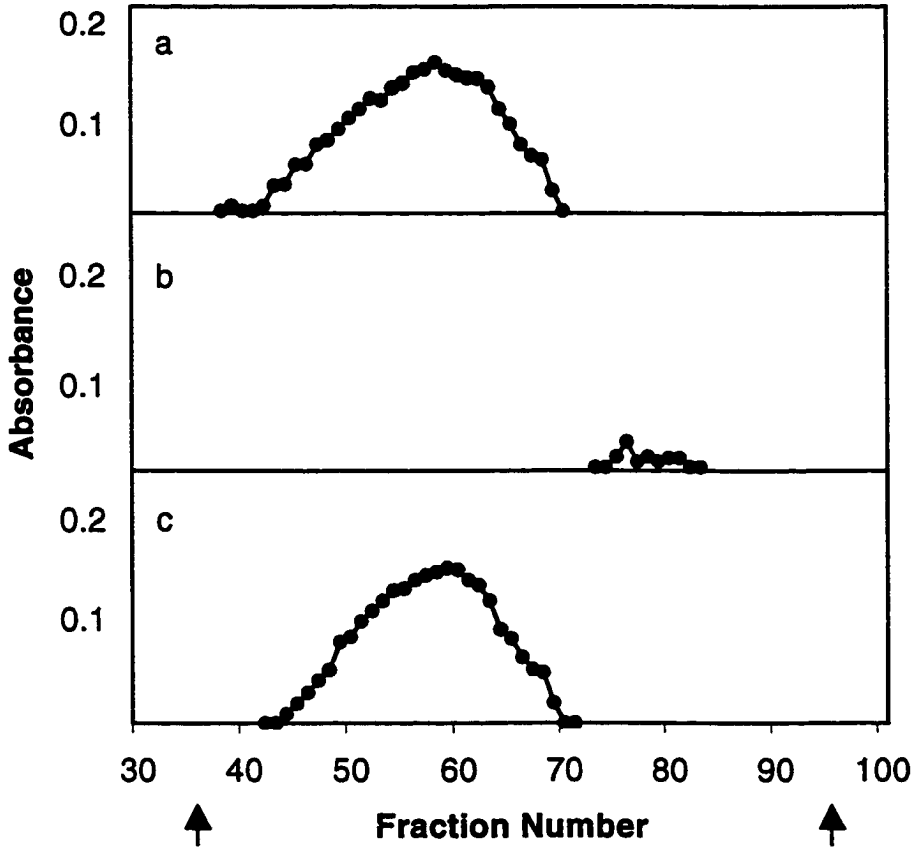

Figure 4.4. Gel chromatography of fraction I chondroitin sulfate on a 1 x110 cm column of Sephacryl S-300. Fractions (1 ml) collected were determined for sulfated GAG by the DMB reaction (absorbance at 595 nm). a) Not treated with enzyme. b) Treated with chondroitinase-ACI. c) Treated with chondroitinase-B. The left and right arrows show the void volume and total column volume, respectively.

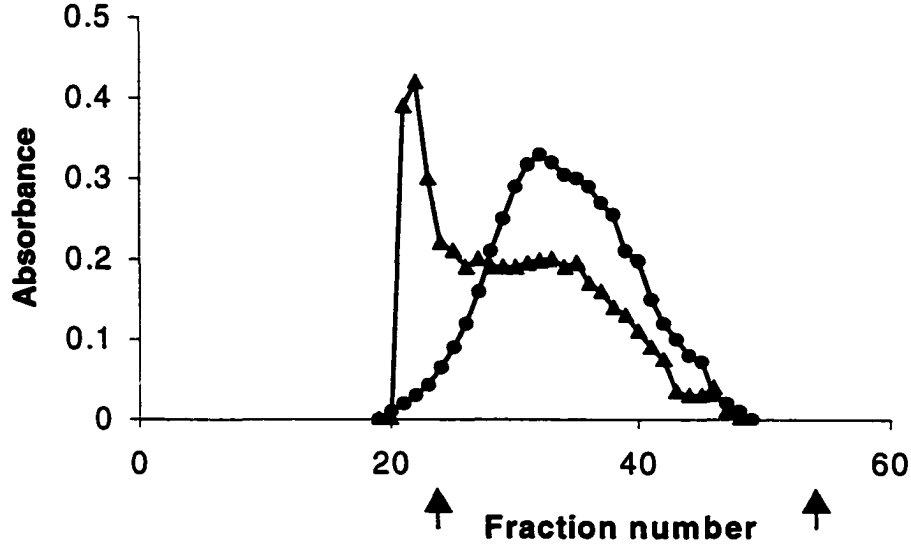

Figure 4.5. Chromatography of Sepharose CL-2B of fraction I CSPG with (▲) and without (●) prior incubation with hyaluronic acid. Each sample was applied to a 0.7 x 70 cm column equilibrated and eluted with 0.5 M sodium acetate, pH 5.8 containing 0.02% sodium azide, and fractions (0.55 ml) collected at a flow rate of 0.60 ml / h were monitored for proteoglycan by the DMB reaction (absorbance at 595 nm). The left and right arrows show the void volume and total column volume, respectively.

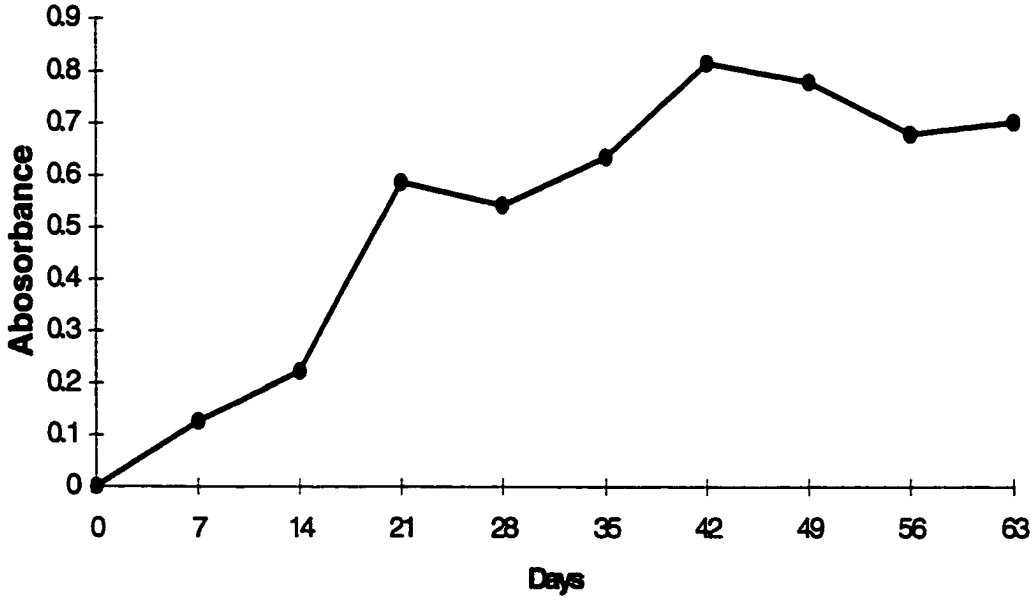

Figure 4.6. Changes in the IgY activity against fraction I CSPG during the immunization period. IgY activities were determined by ELISA and expressed as absorbance at 405 nm. Average values of absorbance from three chickens were plotted.

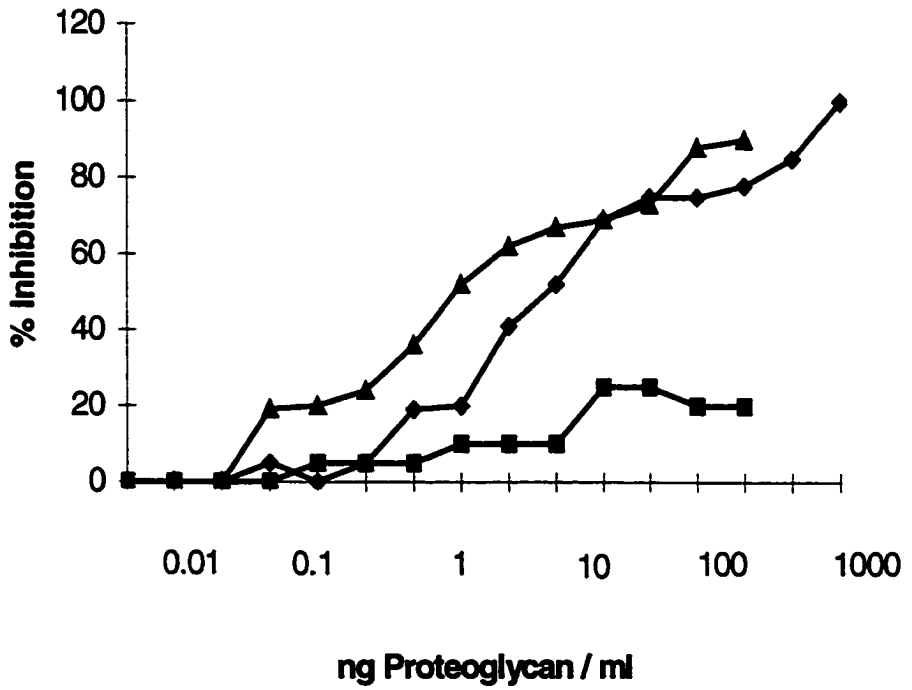

Figure 4.7. Effects of enzyme digestion on antigenicity of fraction I CSPG determined by ELISA inhibition assay. (●): Not treated with enzyme (control). (▲): Treated with chondroitinase-ABC. (■): Treated with papain.

4.4. REFERENCES

Adams, M. E., Mcdevitt, C, A., Ho, A., and Muir, H, 1986. Isolation and characterization of high-buoyant-density proteoglycans from semilunar menisci. J. Bone Joint Surg. 68A:55-64.

Akita, E. M., and Nakai, S, 1992. Immunoglobulins from egg yolk: isolation and purification. J. Food Sci. 57:629-634.

Christner, J. E., Caterson, B., and Baker, J. R, 1980. Immunological determinants of proteoglycans. Antibodies against the unsaturated oligosaccharide products of chondroitinase-ABC-digested cartilage proteoglycans. J. Biol. Chem. 255:7102-7105.

Farndale, R.W., Sayers, C. A., and Barett, A. J, 1992. A direct spectrophotometric assay for sulfated glycosaminoglycans in cartilage culture. Connect. Tissue Res. 9:247-248.

Fithian, D. C., Kelly, M. A., and Mow, V. C, 1990. Material properties and structure-function relationship in the menisci. Clin. Orthop. Relat. Res. 252:19-31.

Gottstein, B, and Hemmeler, E, 1985. Egg yolk immunoglobulin Y as an alternative antibody in the serology of echinococcosis. Z. Parasitenkunde 71:273-278.

Habuchi, H., Yamagata, T, Iwata, H., and Suzuki, S. 1973. The occurrence of a wide variety of dermatan sulfate-chondroitin sulfate copolymers in fibrous cartilage. J. Biol. Chem. 248:6019-6028.

Hardingham, T. E., Fosang, A. J., and Dudhia, J. 1994. The structure, function and turnover of aggrecan, the large aggregating proteoglycan from cartilage. Eur. J. Clin. Chem. Clin. Biochem. 32:249-257.

Heinegård, K., and Oldberg. Å. 1989. Structure and biology of cartilage and bone matrix noncollagenous macromolecules. FASEB J. 3:2042-2051.

Jandik, K. A., Gu, K.,and Linhardt, R. J. 1994. Action pattern of polysaccharide lyases on glycosaminoglycans. Glycobiology 4:289-296.

Jensenius, J. C., Andersen, I., Hau, J., Crone, M., Koch, C. 1981. Eggs: Conveniently packaged antibodies. Methods for purification of yolk IgG. J. Immunol. Meth. 46:63-68.

Koga, T., Okahashi, N., Takahashi, I., Kanamoto, T., Asakawa, H., and Iwaki, M. 1990. Surface hydrophobicity, adherence and aggregation of cell surface protein antigen mutants of Streptococcus mutans serotype C. Infect. Immun. 58:289-296.

Kosakai,, M., and Yoshizawa, Z. 1979. A partial modification of the carbazole method of Bitter and Muir for quantitation of hexuronic acid. Anal. Biochem. 93:295-298.

Li, X,; Nakano, T, Sunwoo, H, H., Paek, B. H., Chae H. S., and Sim, J. S. 1998. Effects of egg and yolk weight on yolk antibody (IgY) production in laying chickens. Poultry Sci. 77:266-270.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall. R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

McDevitt, C. A., and Webber, R. J. 1990. The ultrastructure and biochemistry of meniscal cartilage. Clin. Orthop. Relat. Res. 252:8-18.

McNicol, D., and Roughley, P. J. 1980. Extraction and characterization of proteoglycan from human meniscus. Biochem. J. 185:705-713.

Mow, V. C., Ratcliffe, A., and Poole, A. R. 1992. Cartilage and diarthrodial joint as paradigms for hierarchical materials and structures. Biomaterials 13:67-97.

Muir, H. 1997. Molecular approach to the understanding of osteoarthrosis. Ann Rheum. Dis. 36:199-208.

Nakano, T., Imai, S., Koga, T., Dodd, C. M., and Scott, P. G. 1993. Monoclonal antibodies to the large chondroitin sulphate proteoglycan from bovine temporomandibular joint disc. Matrix 13:243-254.

Nakano, T., Sunwoo, H. H., Li. X., Price, M. A., and Sim, J. S. 1996. Study of sulfated glycosaminoglycans from porcine skeletal muscle epimysium including analysis of iduronosyl and glucuronosyl residues in galactosaminoglycan fractions. J. Agric. Food Chem. 44:1421-1434.

Nakano, T., Dodd, C. M., and Scott, P. G. 1997. Glycosaminoglycans and proteoglycans from different zones of the porcine knee meniscus. J. Orthop. Res. 15:213-220.

Nakazawa, K., Ito, M., Yamagata, T., and Suzuki, S. 1989. Substrate specificity of keratan sulphate – degrading enzymes (endo -β- galactosidase, keratanase and keratanase II) from microorganisms. In : Greiling, H. and Scott, J. E., eds. Keratan sulfate, chemistry biology, chemical pathology, London: The Biochemical Society.

Orlans, E. 1967. Fowl antibody VIII. A comparison of nature, primary and secondary antibodies to erythrocytes in hen sera; their transmission to yolk and chick. Immunology 12:27-37.

Pearson, C. H., Winterbottom, N., Fackre, J. S., Scott, P. G., and Carpenter, M. R. 1983. The NH_2-terminal amino acid sequence of bovine skin proteodermatan sulfate. J. Biol. Chem. 258:15101-15104.

Polson, A., and Von Wechmar, M. B. 1980. Isolation of viral IgY antibodies from yolk of immunized hens. Immunol. Comm. 9:475-493.

Rose, M. E., Orlans, E., and Buttress, N. 1974. Immunoglobulin class on the hen's eggs: their segregation in yolk and white. Eur. J. Immunol. 4:521-523.

Roughley, P. J., McNicol, D., Santer, V., and Buckwalter, J. 1981. The presence of a cartilage-like proteoglycan in the adult human meniscus, Biochem. J. 197: 77-83.

Sim, J. S., and Nakai, S. 1994. Egg usage and processing technologies: new development. Guildford UK pp. 94-103.

Sunwoo, H. H., Nakano, T., Dixon, W. T., and Sim, J. S. 1996. Immune response in chickens against lipopolysacchride of *Escherichia coli.* and *Salmonella typhimurium.* Poultry Sci. 75:342-345.

Yu, W., Cao, S., Wang, Z., Du, R., Xie, W., Ming, D., and Yu, H. 1985. Bio-statistics and experiment design. 5th ed. Agriculture Beijing. pp. 43-63.

CHAPTER 5. IMMUNE RESPONSE OF CHICKENS AGAINST BOVINE AGGRECAN CHONDROITIN SULFATE-PEPTIDE

5.1. INTRODUCTION

Chondroitin sulfate is the major acidic polysaccharide found in the extracellular matrix of cartilage. It is covalently attached to the core protein to form proteoglycan, aggrecan (Hardingham et al., 1994) which has functions to maintain the resilience of cartilage (Muir, 1977), and regulates endochondral ossification (Hunter, 1991) and differentiation and proliferation of chondrocyte (Goetinck, 1982).

Mammalian aggrecan is a relatively weak immunogen in mammalian species (Doege et al., 1990). There are, however, few reports on the immunization of chickens with mammalian aggrecan. Antibodies, if so available, are sensitive tools to detect antigens in biological samples. They can also be used to purify antigens. Because of phylogenetic distance between avian and mammalian species (Jensenius et al., 1981), chickens may be superior to mammals in the production of antibodies against mammalian antigens. Moreover, chickens as antibody producers have several other advantages over mammals (Gottstein and Hemmeler, 1985; Akita and Nakai, 1992). For example, antibodies produced in the chicken are stored in the egg yolk, and thus the amount of egg yolk antibody (IgY) collected from one bird is much higher than the amount of serum antibodies collected by sacrificing the host animal (Sim and Nakai, 1994). This study was

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[1]A version of this chapter has been submitted for publication. Li, X., Nakano, T., Sim, J. S. Can. J. Anim. Sci.

therefore, undertaken to examine immune response of chickens against chondroitin sulfate peptide of bovine aggrecan.

5.2. MATERIALS AND METHODS

Three 35 wk old Single Comb White Leghorn hens were used for immunization. These chickens were kept at the University of Alberta Poultry Unit and cared for in accordance with the Canadian Council on Animal Care guideline of animal welfare. Each chicken was injected intramuscularly on day 0 with 1 mg of CS-peptide emulsified in 1 mL of 50% (vol / vol) Freund's complete adjuvant in phosphate buffered saline (0.14 M NaCl, 0.0015 M KH_2PO4, 0.0081 M Na_2HPO4 and 0.0027 M KCl, pH 7.2). The chondroitin sulfate peptide was prepared from bovine nasal cartilage as described previously (Nakano et al., 1998). On day 14, chickens were injected with the same amount of antigen emulsified in Freund's incomplete adjuvant. Eggs were collected daily, and stored at 4°C until used for isolation of water soluble fraction (WSF) from egg yolk.

The method of preparation of WSF was described in our previous report (Li et al., 1998). Samples of WSF were tested for IgY activities against bovine chondroitin sulfate peptide by ELISA (see below) and for total IgY concentrations by the radial immunodiffussion assay (Li et al., 1998). The procedures of ELISA followed those described previously (Li et al., 1998). Antibody activities were determined by incubating 150 μL of 1000 times diluted WSF in microtiter plates precoated with 5 μg / mL concentration of bovine chondroitin sulfate peptide. ELISA inhibition assay was carried out by incubating 50 μL of increasing concentration of competing antigen (0 to 100 μg of

bovine chondroitin sulfate peptide / mL) in the plates prepared as described above with an equal volume of 1000 times diluted WSF. Immunohistochemical staining was carried out as described previously (Li et al., 1998).

5.3. RESULTS AND DISCUSSION

The IgY activities against bovine chondroitin sulfate peptide measured in three chickens were undetectable on day 0, rapidly increase in 2 wk, and remained relatively constant thereafter (Figure. 5.1). This suggested that the CS-peptide of bovine aggrecan is highly antigenic to chickens. The total IgY concentrations determined during the experimental period were relatively constant and averaged 5.9 mg / mL. This is consistent with our previous report (Li et al., 1998).

To determine whether the IgY produced is specific to the peptide CS chains, the CS-peptide was digested with enzymes and the antigenicities of the resulting fragments were tested using the ELISA inhibition assay. Digestion with papain caused a loss in antigenicity, while digestion with chondroitinase-ABC showed no appreciable effect (Figure. 5.2). The concentration of competing antigen required for 50% inhibition of antibody binding was > 100 μg / mL for papain digest, and 20 and 19 μg / mL for chondroitinase-ABC digest and enzyme untreated control, respectively. No inhibition of antibody binding was observed with 100 μg / mL of CS, keratan sulfate, dermatan sulfate and collagen type II (data not shown). These results indicated that the epitopes recognized by the IgY are probably located in the peptide but not in the chondroitin sulfate chain of aggrecan.

Immunohistochemical staining of bovine nasal cartilage showed an intense staining reaction with the IgY raised against the chondroitin sulfate peptide (Figure. 5.3a). Control staining with WSF from non-immune eggs was negative (Figure. 5.3b) as expected.

On conclusion, chickens are efficient producers of IgY against the peptide (but not chondroitin sulfate chains) of bovine aggrecan.

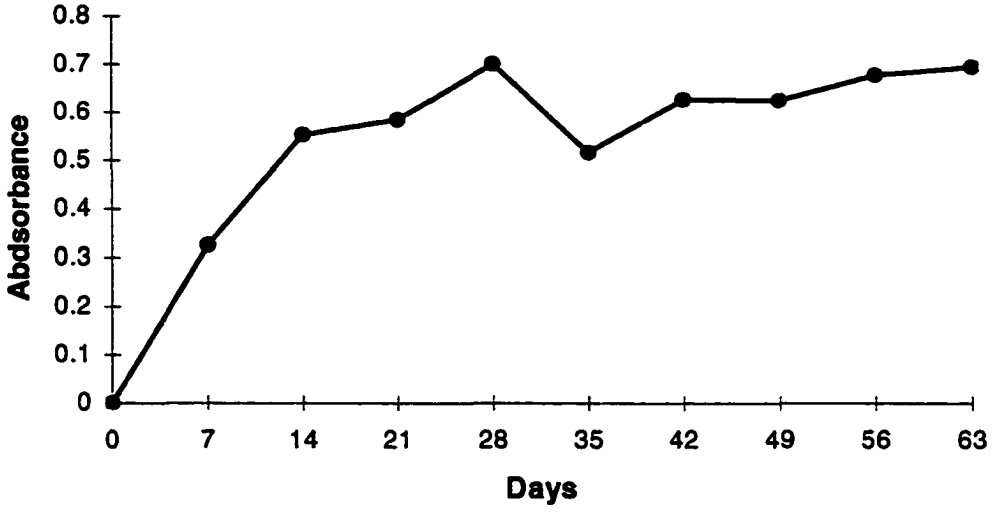

Figure. 5.1. Changers in the IgY activity against CS-peptide of bovine aggrecan during the immunization period. The IgY activity was measured by ELISA and expressed as ELISA absorbances from three chickens were plotted.

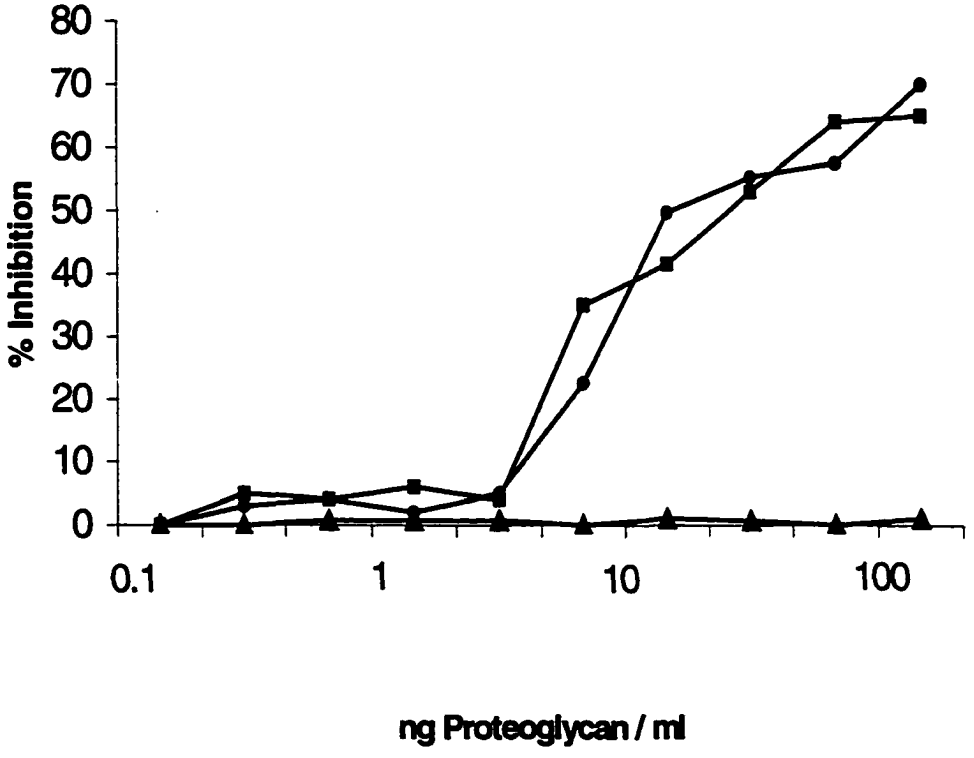

Figure. 5.2. Inhibition of binding of the IgY to CS-peptide of bovine aggrecan. Inhibitors used were CS-peptides treated with papain (▲) and chondroitinase-ABC (■), and enzyme untreated control (●).

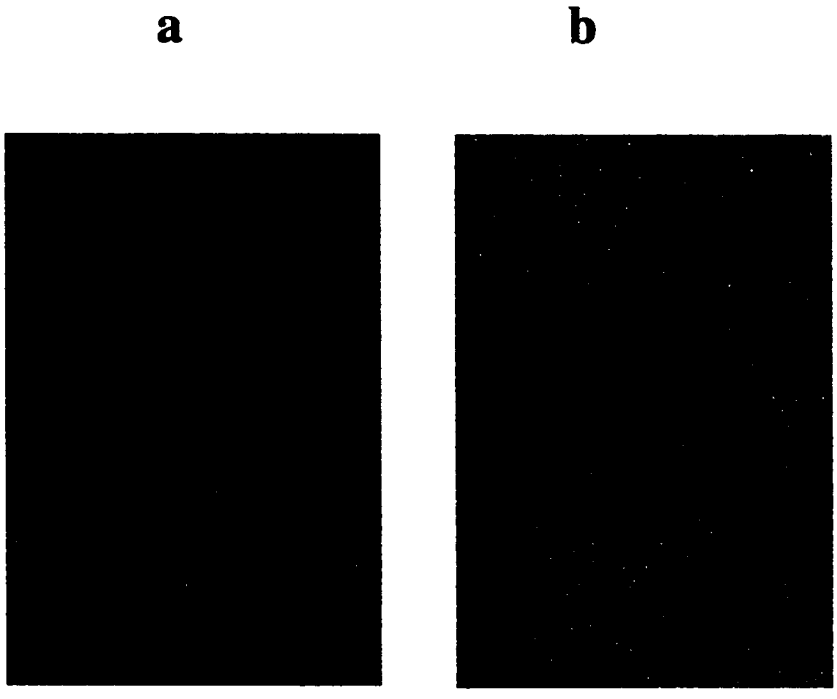

Figure. 5.3. Immunohistochemical staining of bovine nasal cartilage stained with (a) and without (b) the IgY raised against CS-peptide of bovine aggrecan. Magnification: x 150.

5.4. REFERENCES

Akita, E. M., and Nakai, S. 1992. Immunoglobulins from egg yolk: Isolation and purification. J. Food Sci. 57:629-634.

Doege, K., Rhodes, C., Sasaki,M., Hassel, J. R., and Yamada, Y.1990 Molecular biology of cartilage proteoglycan (aggrecan) and link protein, in: Extracellular Matrix Gene (L. Sandel and C. Boyd, eds), Academic Press, New York. Pp. 137-155.

Goetinck, P, F. 1982. Proteoglycan in developing embryonic cartilage. In The glycoconjugates. Glycoproteins, glycolipids and proteoglycans. Edited by M. I. Horowitz. Part A 3:197-229.

Gottstein, B., and Hemmeler, E. 1985. Egg yolk immunoglubulin Y as an alternative antibody in the serology of echinococcosis. Z. Parasitenkunde 71:273-278.

Hardingham, T. E., Fosang, A. J., and Dudhia, J. 1994. The structure, function and turnover of aggrecan, the large aggregating proteoglycan from cartilage. Eur, J. Clin. Chem. Clin. Biochem. 32:249-257.

Hunter, G. K. 1991. Role of proteoglycan in the provisional calcification of cartilage, A review and reinterpretation. Clin. Orthop. Rel. Res. 262:256-280.

Jensenius, J. C., Andersen, I., Hau, J., Crone, M., and Koch, C. 1981. Eggs: Conveniently packaged antibodies. Methods for purification of yolk IgG. J. Immunol. Meth. 46:63-68.

Li, X. Nakano, T., Sunwoo, H.H., Paek, B.H., Chae, H. S., and Sim, J.S. 1998. Effect of egg and yolk weights on yolk antibody (IgY) production in laying chickens. Poultry Sci.77:266-270.

Muir, H. 1977. Molecular approach to the understanding of osteoarthrosis. Ann. Rheum. Dis. 36: 199-208.

Nakano, T., Nakano, K., and Sim, J. S. 1998. Extraction of glycosaminoglycan peptide from bovine nasal cartilage with 0.1 M sodium acetate. J. Agric. Food Chem. 46: 772-778.

Sim, J. S., and Nakai, S. 1994. Egg usage and processing technologies: New development. Guildford UK pp. 94-103.

Yu, W., Cao, S., Wang, Z., Du, R., Xie, W., Ming, D., and Yu, H. 1985. Bio-statistics and experiment design. 5th ed. Agriculture Beijing. pp. 43-63.

CHAPTER 6. STUDY OF IMMUNE RESPONSE OF CHICKEN TO *E. COLI* AND ANTI-*E.COLI* ANTIBODY STABILITY DURING STORAGE

6.1 INTRODUCTION

As early as 1893, Klemperer found that a simple alternative to conventional antiserum production is the production of antibodies in chicken eggs. More antibody is exist in egg yolk than in serum. Since then, antibodies against many different antigens have been produced based on the advantage of using chicken as low cost (Polson and Von Wechmar, 1980), easy to handle animal model as well as antibody in egg yolk easy to purify (Akita and Nakai, 1992). However, the purpose of producing antibodies is the application of them. Many egg yolk antibodies have been used as diagnostic reagents (Gross and Speck, 1996). In addition, IgY also has been used to detect (Lösch et al., 1986) and treat (Kuhlmann et al., 1988; Yolken and Wechmar, 1980) intestinal virus, avian viral arthritis (Tang et al., 1995), predicts contamination of flock by *salmonella enteritidis* (Gast et al., 1997), and to prevent fish disease (Miyazaki, 1993). Egg yolk antibody against *Escherichia coli* K88 has also been used in preventing diarrhea caused by *E. coli* K88 in weaning piglets (Erhard et al., 1996). The successful application of chicken antibodies depends largely on their stability. Knowing the molecular stability of IgY is particularly important when IgY is used as a reagent under various conditions. The antibody activity under pH 2-7 and 11-13 and a short incubating period has been investigated (Shimizu et al., 1992). However, the therapeutic application of chicken egg yolk antibodies requires that the antibody is to be stored for some time during the feeding period. There is limited information concerning the stability of IgY under different pHs in

the storage condition. The objective of this chapter are1) to examine the immune response of chickens to *E. coli* and 2) to examine the stability of the water – soluble fraction of IgY under different pHs and different storage times.

6.2. MATERIALS AND METHODS

Immunization of Chickens with *E. coli*

Procedures of chicken immunization followed those described by Sunwoo et al. (1996). For the first injection, 1 x 10^9 *E. coli* cells were dissolved in phosphate buffered saline (PBS, 0.14 *M* NaCl, 0.0015 *M* KH_2PO_4, 0.0081 *M* Na_2HPO_4 and 0.0027 *M* KCl, pH 7.2), and emulsified with an equal volume of Freund's complete adjuvant to obtain the final concentration of 1 x 10^9 cells /mL. Each hen was intramuscularly injected with 1 x 10^9 cells (Shimizu et al., 1988) at four different sites of breast muscles (2 sites per left or right breast muscle). A booster injection was given intramuscularly 2 wk after the first injection with the same dose emulsified with Freund's incomplete adjuvant. Blood samples were collected from the wing vein on 0,14, 35, 56, 63 and 70 days after the initial injection. Eggs were collected daily and stored at 4°C until analyzed.

Isolation of Water Soluble Fraction

Isolation of water soluble fraction (WSF) was carried out by the method described by Akita and Nakai (1992) with small modification. Egg yolk was separated from the white using an egg yolk separator and then rolled on paper towels to remove adhering egg white. The yolk membrane was punctured and the yolk without membrane was

transferred into a graduated cylinder. The yolk samples were mixed with six volumes of cold acidified water, pH 2.5 adjusted with 0.1 *N* HCl. The mixture, which had a pH of 5.0, was kept at 4°C for 6 h, and then centrifuged at 12,100 x *g* and 4°C for 15 min. After centrifugation, the supernatant was collected as WSF. The WSF samples obtained were used for determination of the anti-BSA antibody activity by ELISA and radial immunodiffusion.

Acid and Alkali Treatment

The pH of WSF was adjusted with HCl or NaOH to make the final pH 2 – 12. The samples were then stored at 4°C or –20°C for 30 and 60 days.

ELISA

Antibody activities in WSF samples were determined using microtiter plates with 96 wells, which were coated by adding 150 μL of a solution (1 x 10^9cells/mL 0.05 *M* carbonate buffer, pH 9.6) to each well and incubating at 22°C for 2 h. Plates were washed with PBS containing 0.05% Tween (PBST). Samples (150 μL) of WSF were then prepared for ELISA. The WSF, obtained by six times dilution of yolk (see above) was further diluted 167 times to result in a 1000 times diluted sample. Diluted samples were added to the well, and incubated at 22°C for 3 h. Subsequent to incubation, plates were washed with PBST, and incubated with the second antibody (150 μL of a 1:1000 dilution of peroxidase conjugated rabbit anti-chicken IgG) at 37°C for 1 h. Plates were washed with PBST, and 100 μL of substrate solution, 2, 2' - azino - bis (3 - ethylbenzthiazoline-6-sulfonic acid) in 0.05 *M* phosphate-citrate buffer, pH 5.0 containing 0.03% sodium

perborate, was added to the well. After 30 min, the absorbance of the reaction mixture was read at 405 nm using an ELISA reader against the reaction mixture prepared with PBS.

Statistical Analysis

An F-test was used to detect significant difference among means of pH, temperature and storage time (Yu et al., 1985).

6.3. RESULTS AND DISCUSSION

Immune Response

The changes of antibody activity, determined by ELISA are shown in Figure 6.1. The antibody activity in egg yolk was very weak on day 7, rapidly increased by day 14, and gradually increased to reach the peak on day 42, and remained relatively constant thereafter. This indicates that *E. coli* is highly antigenic to chickens.

Stability

The stability of IgY was measured as antibody activity and expressed as absorbance at 405 nm. Results show that in our experiment, temperature is the major effect only under an alkaline condition. Under pH 12 at 4°C antibody activity was almost completely lost, but little loss of activity was found at –20°C at both 30 days and 60 days storage. These results are similar to that found by Shimizu et al. (1992), who used

chicken anti-mouse IgG antibody. They reported that the activity of IgY did not change until pH increased to 11, and was markedly diminished by incubating at pH 12 or higher.

Figures 6.2, and 6.3 show the changes of anti - E. coli chicken egg yolk antibody activity between 30 days and 60 days storage under pH 1 – 3 at 4°C (c) and -20°C (d). The IgY activity was decreased with the decrease in pH form 3 to 1, indicating that the lower the pH, the more unstable ($p < 0.001$) the antibody under the condition when pH is 3 or lower than 3. Figures 6.2, 6.3 also show that all the IgY activity under pH 1 – 3 of storage are significantly higher ($p < 0.001$) than that during 60 days of storage, indicating that short storage time favours the antibody stability.

The changes of anti – E. coli antibody activity of IgY WSF under pH 1 – 12 during 30 days and 60 days are shown in Figures 6.4 and 6.5. In both 4°C and –20°C storage, antibody activity was the highest value under pH 4 (Figure 6.6) ($p < 0.001$). The results indicate that pH 4 is the best storage pH at both 4°C and –20°C 30 or 60 days storage. Although no changes of activity were found under pH 5 – 11 from 30 to 60 days of storage, the activity of IgY are relatively low ($p < 0.001$) under these pHs. The above results show that there may be a balance that exists in the IgY molecule to keep the IgY activity constant.

In conclusion, chickens can produce antibody against *E. coli*. pH 4 is the best storage condition at both 4°C and –20°C. –20°C is the better storage temperature in terms of long time storage of IgY in the water -soluble fraction.

IgY against *E. coli*. in water soluble fraction should be able to be introduced to infants and piglets because it is acid resistant. Also, it can be applied in vitro to prevent hamburger disease because it is stable under 30 or 60 days of storage at –20°C.

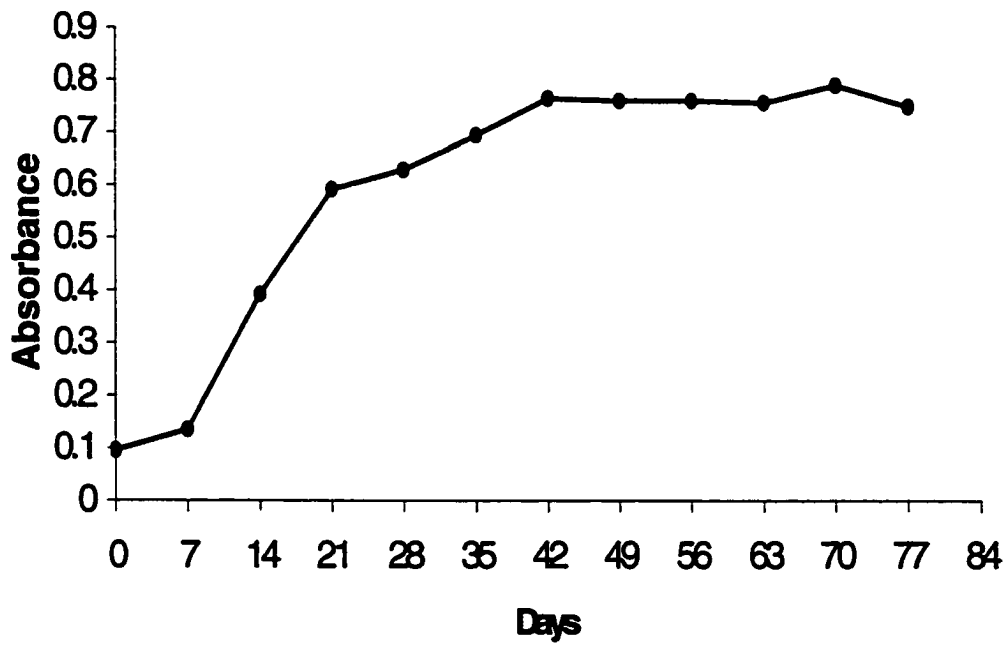

Figure. 6.1. Changes in the IgY activity against E. coli during the immunization period. The IgY activity was measured by ELISA and expressed as ELISA absorbance at 405 nm.

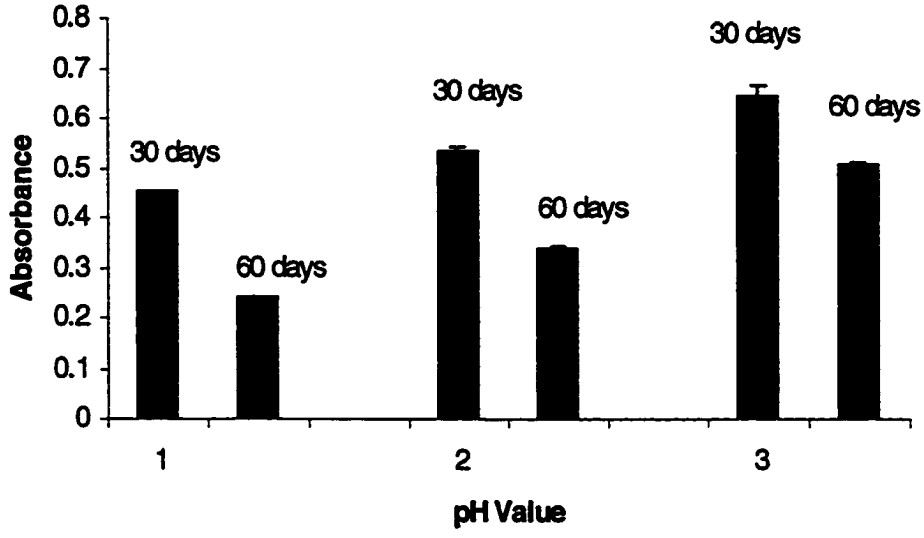

Figure. 6.2. The differences of IgY activity against E. coli between 30 days and 60 days of storage under pH 1 - 3, at 4°C. The IgY activity was measured by ELISA and expressed as ELISA absorbance at 405 nm.

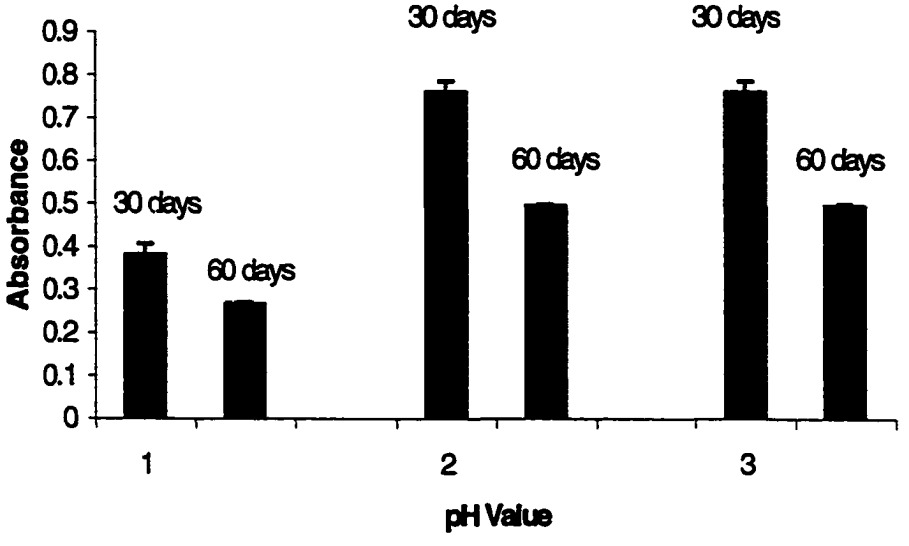

Figure. 6.3. The differences of IgY activity against E. coli between 30 days and 60 days of storage under pH 1 – 3, at – 20°C. The IgY activity was measured by ELISA and expressed as ELISA absorbance at 405 nm.

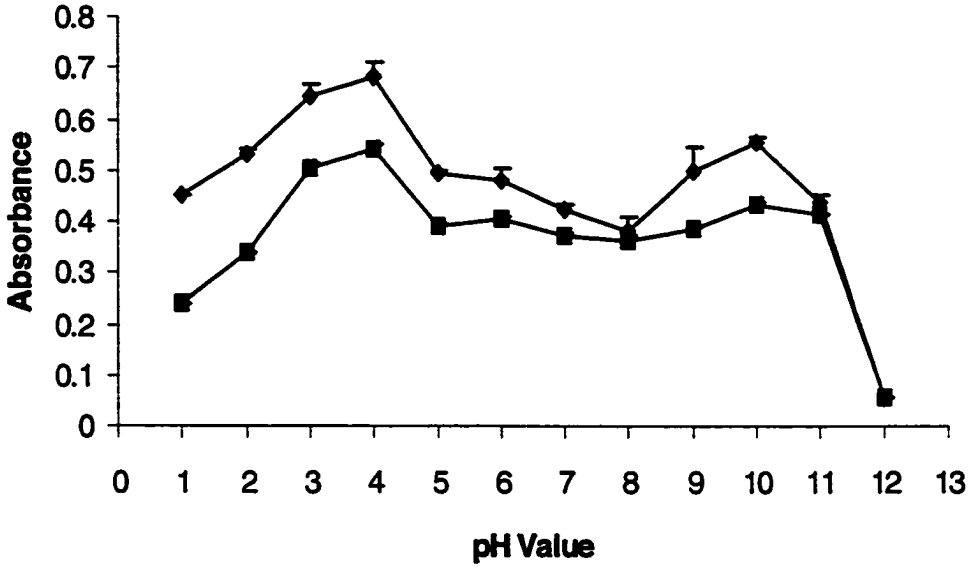

Figure. 6.4. Changes in the IgY activity against E. coli under pH 1 – 12 at 4°C during 30 days (●) and 60 days (■) of storage. The IgY activity was measured by ELISA and expressed as ELISA absorbance at 405 nm.

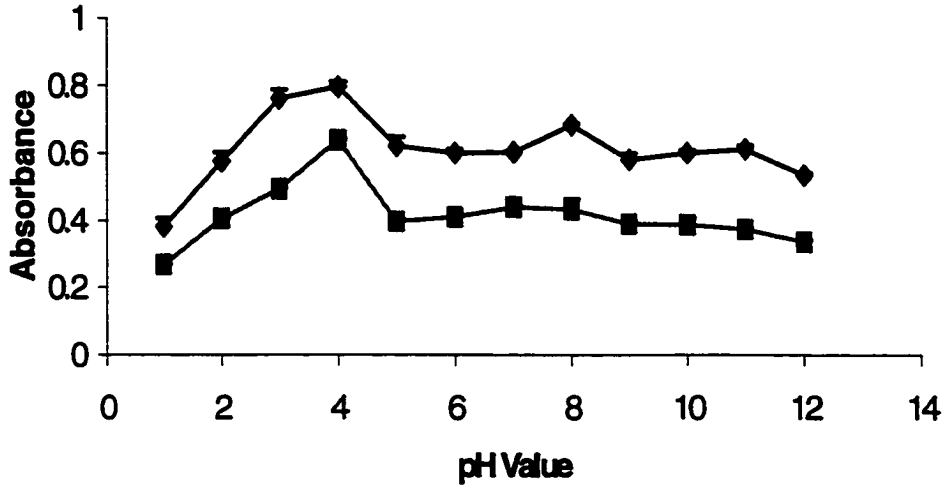

Figure. 6.5. Changes in the IgY activity against E. coli under pH 1 – 12 at – 20°C during 30 (●) days and 60 days (■) of storage. The IgY activity was measured by ELISA and expressed as ELISA absorbance at 405 nm.

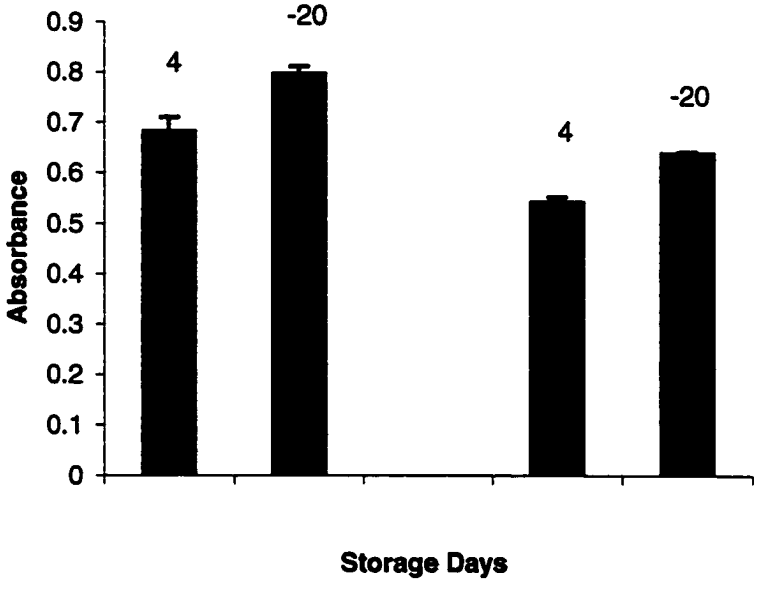

Figure. 6.6. The differences of IgY activity against E. coli under pH 4, at 4°C and – 20°C during 30 days (left) and 60 days (right) of storage. The IgY activity was measured by ELISA and expressed as ELISA absorbance at 405 nm.

6.4. REFERENCES

Akita, E. M., and Nakai, S. 1992. Immunoglobulins from egg yolk: isolation and purification. J. Food Sci. 57:629-634.

Erhard, M. H., Bergmann, J., Renner, M., Hofmann, A., and Heinritzi, K. 1996. Prophylactic effect of specific egg yolk antibodies in diarrhea caused by *Escherichia coli* K88 (F4) in weaned piglets. Zentralblatt Fur Veterinarmedizin – Reihe A. 43:217-223.

Gast, R. K., Porter R. E. Jr., and Holt, P. S. 1997. Applying tests for specific yolk antibodies to predict contamination by *Salmonella enteritidis* in eggs from experimentally infected laying hens. Avian Diseases. 41:195-202.

Gross, M., and Speck, J. 1996. Avian yolk antibodies in diagnosis and research. DTW – Deutsche Tierarztliche Wochenschrift. 103:417-422.

Klemperer, F. 1893. Über natürliche Immunität und ihre Verwerthung für die Immunisierungs therapie. Arch. Exptl. Pathol. Und Pharmakol. 3:356-382.

Kuhlmann, R. H., Wiedemann, V., and Schmidt, P. 1988. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. J. Am. Vet. Med. Assoc. 35:610-616.

Lösch, U., Schranner, I., and Wanke, R. 1986. The chicken egg: An antibody source. J. Am. Vet. Med. Assoc. 33:609-619.

Miyazaki, T. 1993. Application of egg yolk antibody to prevention of fish disease. Nippon Nogeikagaku Kaishi 67:1440-1443.

Polson, A., and M. B. Von Wechmar, 1980. Isolation of viral IgY antibodies from yolk of immunized hens. Immunol. Comm. 9:475-493.

Shimizu, M., R. C. Fitzsimmons, and Nakai, S. 1988. Anti-E. Coli immunoglovulin Y isolated from egg yolk of immunized chickens as a potential food ingredient. J. Food Sci. 53:1360-1366.

Shimizu, M., Nagashima, H., Sano, K., Hashimoto, K., Ozeki, M., Tsuda, K., and Hatta, H. 1992. Molecular stability of chicken and rabbit immunoglobulin G. Biosci. Biotech. Biochem. 56:270-274.

Sunwoo, H. H., T. Nakano, W. T. Dixon, and J. S. Sim, 1996. Immune responses in chickens against lipopolysaccharide of *Escherichia coli* and *Salmonella typhimurium*. Poultry Sci. 75:342-345.

Tang, Y. D., Z. A. Zhou, C. S. Zhai, Y. L. Wang, and Z. X. Gu, 1995. Development, detection and application of egg yolk antibodies produced against avian viral arthritis (AVA) by hyperimmunization. China Journal of Veterinary Sci. 15:66-69.

Yolken, R. H., and M. B. Wechmar. 1980. Isolation of viral IgY antibodies from yolks of immunized hens. Immunol. Commun. 9:475-493.

Yu, W., Cao, S., Wang, Z., Du, R., Xie, W., Ming, D., and Yu, H. 1985. Bio-statistics and experiment design. 5th ed. Agriculture Beijing. pp. 64-86.

SUMMARY AND CONCLUSION

Chickens immunized against antigenic molecules such as protein, lipopolysaccharides, lipid, glycoprotein, carbohydrate and pathogens produce specific antibodies both in serum and egg yolk. The physicochemical characterization of egg yolk antibody, called IgY, differs from mammalian IgG in terms of molecular size, isoelectric point, susceptibility to proteolysis and attachment to mammalian complement. Production of IgY in egg yolk is very efficient and economical, in comparison with mammalian IgG in serum, because of its relatively easy preparation and superior specificity. Therefore, IgY can be applied in the scientific area as a biological or diagnostic agent and in the food area as a functional food supplement. The major purpose of this thesis work is to determine what are biological factors, to develop the research reagent for biochemistry or immunohistochemistry and to examine the stability of IgY for potential food uses. Thus, findings obtained from this research can be further applied to avian biology, bioassay, protein purification, and preventive medicine.

Biological Factors Influencing IgY Production

Chickens have been used to produce antibody, but no information is available regarding the efficiency of IgY production and biological factors inherent to commercial laying performance. In the present research, the effects of egg production and egg weight on antibody yield were investigated.

Two groups of laying chickens showing low and high egg production were hyperimmunized with bovine serum albumin (BSA) as an antigen. IgY production

(mg/hen/time) was compared and statistically analyzed in relation to egg production rate and egg size. The study clearly showed that antibody concentration (percent or mg/g) in the egg yolk did not change with egg size or the number of eggs laid. Total antibody production (mg/hen/time) was directly proportional to egg sizes and egg numbers. This suggests that modern commercial laying chickens may be the optimum IgY producer for commercial antibody farming, since they are intensively selected for egg production and egg weight.

IgY as Research Tool

Avian egg yolk IgY has been used as a biological tool in cancer therapy and as a biochemical tool in protein characterization. The purified IgY can recognize a specified protein from cancer cells and then be an important agent for cancer treatment. IgY is useful for characterizing the high molecular weight protein like mucin-like glycoprotein-A of human milk and insulin of pig islet cells, because of the phylogenetic distance between birds and mammals.

The present study of specific IgY against a high molecule protein of proteoglycan (> 1,000,000 daltons) extracted from porcine stifle meniscus and bovine cartilage tissues was undertaken to be used as a research reagent in biochemistry or immunohistochemistry. Decorin, a member of the proteoglycan family consisting of a dermatan sulphate chain and a protein core, is a relatively weak immunogen in mammalian species. The molecule maintains the integrity of tissues.

Three 35-week-old SCWL chickens were immunized by intramuscular injection with 1 mg of decorin antigens. The activity of specific IgY was determined by ELISA

showing that the decorin was highly antigenic to chickens. Thus, the purified IgY was turned out be useful in the study of the biochemical and immunohistochemical roles of proteoglycans.

Anti E.coli IgY and Stability

Passive immunization of IgY against infectious pathogens could be achieved by antimicrobial activity against pathogens. Antibodies interfere with the adhesion of pathogens to the intestinal wall and neutralize partially, or completely their colonization potential. However, the stability and specific antigenicity of IgY at the gut level have been questioned. In this study, we chose porcine enterotoxigenic *Escherichia coli* (ETEC) to study the stability of IgY activity at various pH, temperature, and time periods. The results showed that the antigenicity of IgY was sustained at the gastric acid barrier suitable for passive immune protection. The effectiveness of antibody application for therapeutic and prophylactic use of IgY against infectious *E. coli* in baby pigs is currently under investigation.

IgY immunized with a porcine ETEC 987P was isolated. The activity of IgY against ETEC was determined by ELISA absorbance at 405 nm. The result indicated that the IgY reacted specifically with the ETEC. The further study on the stability for long time storage was undertaken to examine the activity of IgY under the different condition of temperature, pH and time periods. Therefore, IgY mixed with other feeds can maintain its activity and effectively neutralize or reduce the proliferation of bacteria in feed and gut of piglet for the prevention of diarrhea.

Conclusion

The present study of this thesis shows the potential for IgY application to open up a new exciting research area and potential market of IgY for medical and food uses, biological or diagnostic tool, and animal feed industry. Furthermore, these findings may substantiate this possibility of IgY research as below: 1). Laying chickens immunized with various antigens (BSA protein, proteoglycan, bacteria) produced highly specific polyclonal antibodies (IgY). 2). IgY concentration (mg/g) in the egg yolk is relatively constant irrespectively with egg size and productivity. 3). Total IgY production (mg/hen/time) is directly related to the egg size and the number of eggs laid. 4). A known amount of specific IgY is calculated by quantitative ELISA technique and can be formulated with other feeds. 5). IgY against the highly conserved molecule of mammalian proteoglycan is used as the useful reagent of immunohistochemical staining technique. 6). The isolated IgY from egg yolk shows the most stable at pH 4 and below 4°C. Therefore, the stable IgY mixed with other feeds can be used for the prevention of infectious diseases such as diarrhea in pig industry. The antibacterial IgY will attract considerable attention as a natural antimicrobial functional food, which justifies the need of antibody farming using the modern commercial egg production system. In this respect, further research requires to harvest anti-bacterial IgY loaded eggs, to isolate and purify high titre IgY from egg yolk and to examine the bacteriostatic activity of IgY in vivo and in vitro.