大韓獸醫學會誌(1996) 第36卷 第4號
Korean J Vet Res(1996) 36(4) : 895~902

Production of chicken egg yolk antibody to *Canine parvovirus*

Tae-ho Oh, Hong-ryul Han*

Veterinary Referral Center of Colorado, Denver, Colorado, USA
*College of Veterinary Medicine Seoul National University**
(Received May 12, 1996)

개의 파보바이러스에 대한 난황 항체 생산

오 태 호 · 한 홍 율*

미국 콜로라도 수의 위탁 진료센터
서울대학교 수의과대학*
(1996년 5월 12일 접수)

초 록 : 산란계에 불활화 개 파보바이러스 백신을 근육내로 1주 간격으로 4회 접종하여 면역화시키고 최종 접종 2주후에 채란하여 4℃에 보관하여 사용하였다.

난황항체는 5% HPMCP를 이용하여 분리하였고 0.5% HPMCP 용액은 lipid 침전에 매우 효과적이었으며 희석배수 10배에서 투명한 상층액을 나타내었다. 1차분리한 상층액의 단백질 농도는 2.5mg/ml이었고 최종 단백질 용액의 경우는 26.53mg/ml이었다. SDS-PAGE 전기영동상에서 분자량 60~70 KD 및 30~40 KD의 2 band가 나타났으며 non-reducing 전기영동에서는 닭 혈청 IgG와 같은 120~160 KD의 분자량을 보인 band가 각각의 분리용액에서 나타났다.

난황 항체의 개 파보바이러스에 대한 혈구응집억제반응 항체역가는 혈청의 역가에 비해 1주의 차이를 주며 증가했으며 난황 항체는 1:640에서 1:2560, 혈청은 1:640에서 1:5120을 나타내었다.

Key words : *canine parvovirus*, egg yolk antibodies, HPMCP.

Introduction

The yolk of hen eggs is a rich source of immunoglobulin[1]. Also egg yolks can be easily incorporated into animals or human diets. After immunization of laying hens, the specific humoral response is seen in the serum if hens are mirrored in the egg yolks some days later and remains elevated for several weeks, and the IgG-antibodies produced are transferred into the egg follicle[2,3]. In that way large amounts of specific antibodies can be produced with little effort. Use of chickens for production of antibodies on large scale is associated with obvious advantages. When eggs are used as the antibody source, productivity is much higher than in mammals, and no technical assistance is necessary for collection and marking of eggs. There has been no report so far on the use of chicken egg yolk antibodies for the control of canine parvovirus infection in neonatal pups.

Address reprint requests to Dr. Hong R. Han. College of Veterinary Medicine, Seoul National University, Suwon 441-744, Republic of Korea.

Antigen-specific IgG has been widely used for immunological analysis in the fields of diagnosis as well as pure research[4]. Another important application of IgG is passive immunization therapy in which antigen-specific IgG is administered to unimmunized individual.

Hens egg yolk contains IgG, derived from the hen serum and transmitted to the chick. IgM and IgA absent in yolk[5]. For production of heterologous, polyclonal antibody after immunization of laying hens with antigen the IgG-antibodies produced are transferred into the egg follicle and persist a long period after the second boost[2]. Antibody from the circulating blood plasma is secreted into the ripening egg follicle and in incorporated into the egg white in the oviduct along with the egg albumin[5]. At present, a tremendous number of hens, immunized to protect them from several diseases, are managed to lay eggs systematically, and the eggs containing IgG are consumed as food.

The immunoglobulin of egg yolk which is now called IgY, differs from mammalian IgG in the molecular size, isoelectric point, and binding ability with mammalian complement and protein A[2]. IgY is also known as -livetin and exists in egg yolk together with other two water-soluble proteins, -livetin(chicken serum albumin) and -livetin[7], and various lipoproteins(LDL and HDL) which are the major components of egg yolk. Thus, a simple alternative to conventional antiserum production is the production of antibodies in chicken eggs[2,8].

Materials and Methods

Experimental Animals : Adult egg-laying Hyline hens were obtained from the commercial poultry farm. They were housed in double-layer cages individually for laying throughout the experiment. Commercial laying ration(Sun Jin laying formula, Sun Jin Co) and water were provided *ad libitum*.

Immunizations : Chickens were immunized with the commercial inactivated canine parvovirus vaccine intramuscularly in the breast muscle 4 times at intervals of one week.

Blood and egg samples : Blood samples were taken from each hen before immunization and at 1, 2, 3, 4 and 6 weeks by wing veinpuncture. The sera were separated after overnight incubation at 4℃. Eggs for the passive immunization of animal were harvested 2 weeks later after completion of immunizations and stored at 4℃ until processed. The period of storage was not exceed 7 days. Control eggs were collected from each chicken non-immunized.

Isolation of antibody from egg yolk for HI test : A method described by Piela *et al*[8] was used with minor modification. Individual egg yolks from immunized or control hens were separated from egg whites and egg yolk membranes. One ml of egg yolk was mixed 1:1(vol/vol) with phosphate buffered saline(pH 7.4) and 1:2(vol/vol) with chloroform. After vortex, the tube was incubated for 1 hour at 4℃ and centrifuged at 2,000 rpm for 20 min. The clear supernatant was used for testing of antibody titer to canine parvovirus.

Isolation of antibody from egg yolk for passive immunization : The egg yolks were separated from the egg white and the yolk membranes carefully using sterile gauze to remove as much of the albumin as possible, and rolled on filter paper to remove adhering egg white. The membrane was punctured and yolk allowed to flow into a graduated cylinder without membrane. Thirteen to fifteen yolks were used for one lipid-precipitation procedure as follows. Total 200 ml of yolk was diluted with 10 volume of distilled water which was precooled at 4℃. The diluted egg yolks were mixed and an aqueous dispersion of 5%(vol/vol) HPMCP in 80% ethyl alcohol was slowly added to the diluted egg yolk mixture to make a 0.5%(vol/vol) mixture for lipid precipitation. After the mixture were kept at 4℃ for 24 hours, clear liquid supernatant(water-soluble protein fraction) in the upper layer of the mixture was obtained and was passed through a 0.45nm membrane filter into a sterile beaker to remove solid lipid materials and bacteria, and was stirred gently. While agitating gently on a magnetic stirrer, ammonium sulfate(60%) was added slowly, and kept at 4℃ for overnight. After the precipitate was obtained by centrifugation at 3,000g for 30 minutes, the supernatant was discarded and precipitate was resuspended in 1-2 pellet volumes of PBS(pH 7.4). The final antibody solution was transferred to dialysis tube and dialyzed against three changes of PBS for overnight. Aliquots as the source of IgY were stored at -20℃ until use.

Protein measurement : The MicroBCA protein assay kit (Pharmacia Co) was used with bovine serum albumin as the reference protein.

SDS-polyacrylamide gel electrophoresis : SDS-PAGE was done under nonreducing condition using a pre-made excel gel 15% gradient acrylamide gel(Pharmacia Biotech Inc. USA). For the running buffer anode SDS buffer stripe(pH 6. 6) and cathode SDS buffer(pH 7.1) was used. Protein samples were incubated with sample buffer(SDS 1.0g, EDTA 3mg, BPB 10mg, pH 6.8) without reducing agent as the molecular weight markers. SDS-PAGE molecular weight standards proteins and purified chicken serum IgG(Promega Co) were used, and stained with a mixture of Coomassie Brilliant Blue R-250. SDS-PAGE was also done under the reducing conditions with 2-mercaptoethanol.

Hemagglutination Inhibition Test : Serum and IgY samples were tested using a method by Carmichael *et al*[5]. Each test series had appropriate RBC and virus controls.

Results

Immune response of chicken given inactivated CPV vaccine : All chickens immunized had a significant levels of HI antibody to canine parvovirus except non-immunized control chickens(Fig 1). The antibody responses of chicken were usually detected 1 weeks after initial immunization. The highest serum antibody titer was reached 1 week after the third infection of inactivated canine parvovirus vaccine, and HI antibody titers to canine parvovirus were ranged from 1 : 640 to 1 : 5120 in serum from immunized chickens at 2 weeks after last injection. While antibody titers of the control chickens were remained negative during the experiment.

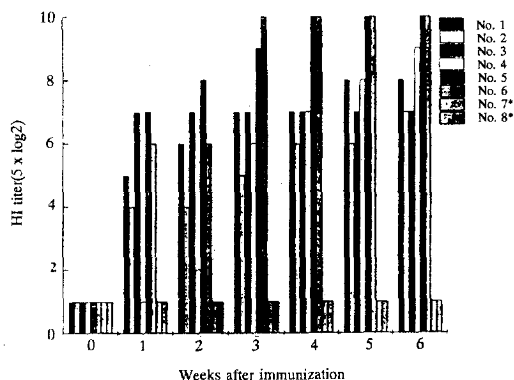

Fig 1. Serum antibody responses of chickens immunized with inactivated canine parvovirus vaccine. * Non-vaccinated control chicken.

The hemagglutination inhibition titer of egg yolk from nonimmunized hens was less than 1 : 10. The antibody response in yolk was increased at 1 weeks after the second immunization(Fig 2). The serum antibody titer was reflected in the egg yolk antibody concentration of immunized hens. With a delay of approximately a week, yolk antibody responses were similar fashion to that of serum antibody. Nevertheless the levels of yolk antibody were generally lower than those serum antibody(Fig 2). The data showed that the antibody titers to canine parvovirus were ranged from 1 : 640 to 1 : 2560 in egg yolk from immunized chickens at 2 weeks after the last infection. Thereafter the antibody

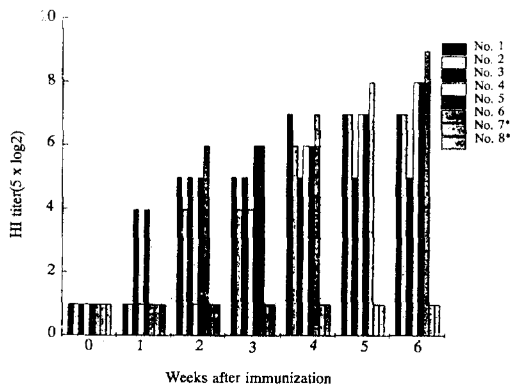

Fig 2. Yolk antibody responses of chicken immunized with inactivated canine parvovirus vaccine. * Non-vaccinated control chicken.

response persisted in the egg yolk throughout the experimental period of 20 weeks.

Isolation of antibody from egg yolk : Using a 5% aqueous HPMCP followed by ammonium sulfate precipitation and dialysis was effective method for the precipitation of isolation of egg yolk antibodies to canine parvovirus. Egg yolk was diluted 4, 6, 8, 10 and 12 times with distilled water for simple

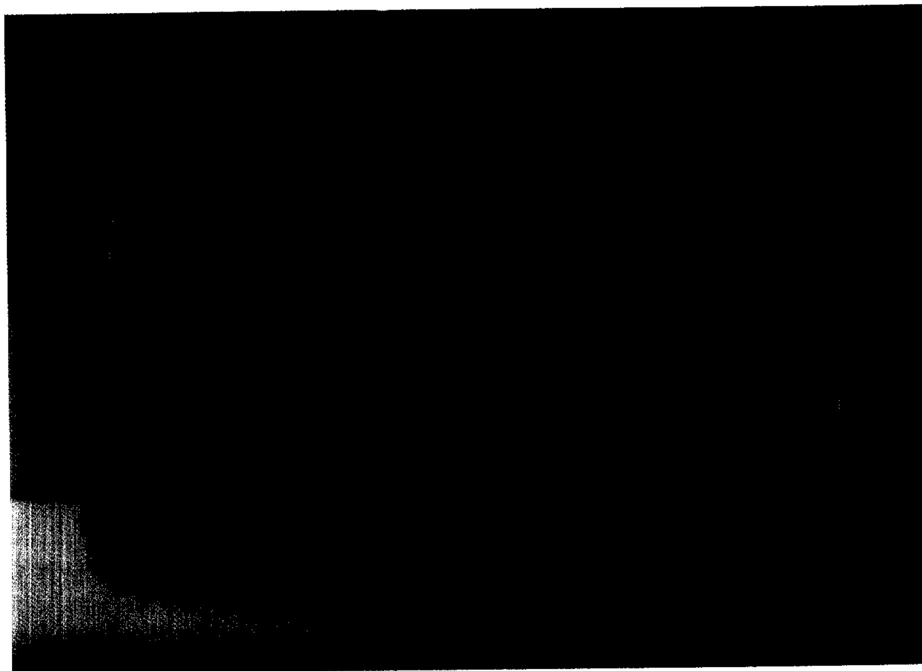

Fig 3.Effect of HPMCP treatment for precipitation of egg yolk lipoprotein. Mixture diluted egg yolk solution(9 parts) and 5% HPMCP solution(1 part) was incubated at 4℃ for 24 hours.

dilution. The pH of the diluted egg yolk was from 6.3 to 6.6. After overnight incubation, samples diluted 10 times gave relatively clear supernatants with only slight lipid contamination. The supernatant containing lipid appeared yellowish due to lipid-soluble pigments(Fig 3). Further experiments were done using 1/10 diluted yolk solution.

The dilution of egg yolk mixture with 0.5% of HPMCP precipitated the lipid faster than the mixture with 0.25% or 1% of HPMCP. The clear supernatant(water-soluble protein) in the upper layer of the mixture began to be formed at 2-3 hours after adding the solution of 5% HPMCP, and was found to remain almost constant after 12 hours. Settling of egg yolk granules was continued after 24 hours but amount of water-soluble fraction was small. The precipitated egg yolk granules appeared fluffy, forming a loose aggregate and settling to more than 90% of total volume giving a very clear colorless solution(Fig 3). The nature of precipitates formed was light-yellowish and emulsive.

The amount of HPMCP treated supernatant was approximately 1,200ml(60% of egg yolk mixture) after incubation at 4℃ overnight and final solution after dialysis was usually 42ml(21% of original egg yolk volume). Total proteins in the egg yolk mixture, HPMCP treated supernatant, salting-out solution and dialyzed solution was 2.4, 2.2, 32.78 and 26.53 mg/ml, respectively(Table 1). The dilution of egg yolks with 10 volume of distilled water and mixture with 0.5% of HPMCP gave a supernatant containing 55% of total egg yolk protein. About 203mg of total protein in supernatant was produced from on egg. The recovery rate of total proteins was 23.2% of the yolk protein after ammonium sulfate precipitation and dialysis of supernatant.

SDS-PAGE of water-soluble fraction, ammonium sulfate precipitated solution and dialyzed solution has a heavy-chain with high molecular weight, estimated MW; 60-70KD and a light-chain with low molecular weight, estimated MW;

Table 1. Total protein of various solutions for isolation of antibody from egg yolk

	Egg yolk mixture[a]	HPMCP treated Supernatant[b]	Salting-out solution[c]	Dialyzed solution[d]
Total volume (ml)	2,000	1,200	36	42
Total protein (mg/ml)	2.4	2.2	32.78	26.53

a : 200g of egg yolk was used
b : After lipid precipitation by 5% HPMCP HP-50
c : ammonium sulfate precipitation(60%)
d : used dialysis membrane

30-40KD(Fig 4). These bands were considered to be IgY H and L chains, respectively. In non-reducing gel(1% SDS) SDS-PAGE of IgY at various stages of isolation, each isolated solution gave a protein band estimated 120-160KD of molecular weight and was considered to be egg yolk immunoglobulin. Chicken serum IgG gave a protein band of the same molecular mass 120-160KD of molecular weight (Fig 5). Several proteins were obtained in the supernatant including IgY after treating the egg yolk with HPMCP. Other proteins were seems to be and livetin.

Fig 4.SDS-PAGE of various stages of solution in reducing 15% pre-made SDS gel. Protein samples were prepared in the presence of 2-mercaptoethanol. Lane 1. ammonium sulfate precipitation; Lane 2. water-soluble fraction; Lane 3. ammonium sulfate precipitation and dialysis solution.

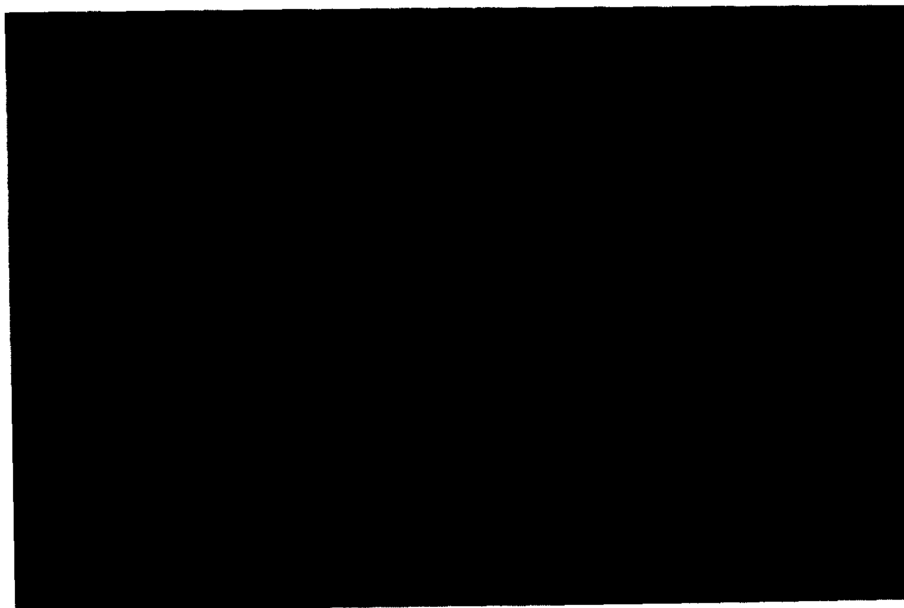

Fig 5. SDS-PAGE of various stages of solution in nonreducing pre-made 15% SDS gel. Protein samples were prepared in the 1% SDS without 2-mercaptoethanol. Lane 1. Chicken serum IgG; Lane 2. Ammonium sulfate precipitation; Lane 3. Water-soluble fraction; Lane 4. Ammonium sulfate precipitation and dialysis solution; Lane 5. Whole egg yolk.

Discussion

Egg yolk is an fluid emulsion with continuos phase of protein(livetin) and a dispersed phase of lipoprotein particles and yolk lipid[7]. IgY is one of the livetins(-livetin). Therefore, removal of lipoprotein or lipid is an important step for the purification of IgY[7]. Partial lipoprotein precipitation could be performed from simple dilution of egg yolks with distilled water alone[10]. The separation of the supernatant from the precipitate could be obtained without requiring centrifugation. Improving the procedure with a lipid precipitating agent facilitates delipidation and recovery of large water-soluble fraction.

Beside mere precipitation, many purification methods for IgY have been reported using lipoprotein separation by ultracentrifugation[11], delipidation by organic solvent[12], and lipoprotein precipitation by polyethyleneglycol[13], sodium dextran sulfate[1] or carageenan[7]. However, these methods seem to be impractical, because of their scale and cost. Moreover, IgY obtained from these methods using organic solvent or chemicals may not be suitable for pharmaceutical purpose[7]. In present study 5% aqueous hydrosypropylmethylcellulosephthalate(HPMCP) dispersion effective for precipitating lipid. Because HPMCP have been used as an enteric coating substances, IgY prepared by this reagent should by suitable for oral administration. Furthermore the isolation of IgY using HPMCP is quite practical, because the conjugation of HPMCP and lipids was easily precipitated by standing overnight and a clear supernatant was obtained.

The mechanism of precipitation by HPMCP is unknown[14]. It is presumed to be similar to that with carrageenan, natural gum[7], wherein the main force causing precipitation with lipoprotein is the ionic binding elaborated between the negative charge of the lipid acting substance and the net position charge of the lipoprotein. IgG is often digested and inactivated by gastric juice. The HPMCP used to separated yolk lipids in this study has been used as an enteric coating substances for several drugs[15]. Apparently, HPMCP-coated drugs are resistant to gastric juice, and dissolution in intestinal fluid is pH-dependent. In this study it is likely that the concentrated antibody solution coated with HPMCP, may have enteric resistance properties against low pH, thereby allowing stable passage through the stomach and ensuring the absorption of intact antibodies in the small intestine.

The major proteins present in the water soluble fraction include - and - livetins with molecular weight of 70KD and 42KD, respectively[16], and low-density lipoproteins. Rose *et al*[5] reported that molecular weight estimation of IgG from serum and yolk gave identical results and molecular weight of 170KD. Non-reducing SDS-PAGE electrophoresis in this study showed a same m.w. band with chicken serum IgG at various stages. Salt precipitation and dialysis caused selective removal of some contaminating proteins especially the 38KD and 53KD fraction with reduction of the 70KD proteins.

A number of factors may influence the purification process, including the quality of eggs used, the ambient temperature, and optimal ratio of egg yolk and HPMCP. It was observed that separation of the water-soluble fraction and solid phase of the egg yolk and HPMCP mixture after standing at 4℃ overnight was more distinct, and a clearer supernatant could be obtained when fresh eggs were used for isolation. Long storaged stale eggs usually have fragile yolks that easily break upon handling and incidence of egg white contamination is high, leading to poor delipidation upon treatment with HPMCP. The poor protein composition of these eggs may also inhibit an effective binding with HPMCP due to changes in molecular stability of proteins. To avoid the problem freshly harvested eggs were used in the present study.

Although water-soluble proteins constitute 42.4% of the total proteins in egg yolk[17]. Seventeen point four percent of the total protein was recovered in the supernatant and that results was concurred with previous finding by Kwan *et al*[18].

Chicken eggs contain as much as 200mg of IgG which is found almost exclusively in the yolk[5,19]. The immunoglobulin, which is not only readily produced but also easily purified[20]. Egg yolk IgG is described as being like mammalian IgG, although recently, it was suggested that this avian im-

munoglobulin, called either IgG and IgY is antigenically similar to mammalian IgG, IgA and IgE[21,22]. In present study, the previous finding that the specific humoral response to injected antigens in hens is mirrored in the egg yolk[2,23] have been confirmed. Increased levels of serum antibodies to immunizing CPV vaccine could be detected 1 week after immunization and reached a peak at 1 week after 4th injection. As expected, since the antibody in the yolks persisted for 16 to 20 weeks, the protective effect persisted when the egg yolks were collected from eggs laid by hens up to 16 to 20 weeks post-immunization.

Conclusions

The semipurified antibody solutions were obtained by concentration of the water soluble protein fraction of egg yolks using hydroxypropylmethylcellulose phthalate and ammonium sulfate precipitation from immunized hens with commercial inactivated canine parvovirus vaccine.

A 5% aqueous HPMCP treatment and ammonium sulfate precipitation was effective to remove yolk lipid. The anti-canine parvovirus antibody preparations showed clear reactions in HI test. Yolk antibody titers reached peak levels at 5 to 6 weeks postinoculation. Antibody response of immunized chickens was significantly greater than that of non-immunized chicken($p<0.01$).

References

1. Jensenius J, Anderson I, Han J, *et al.* Eggs:conveniently packaged antibodies methods for purification of yolk IgG. *J Immunol Methods,* 46:63-68, 1981.
2. Losch U, Schranner I, Wanke R, *et al.* The chicken egg, an antibody source. *J Vet Med* B 33:609-616, 1986.
3. Kuhlman R, Wiedemann V, Schmidt P, *et al.* Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. I. Immunization and antibody determination. *J Vet Med B,* 35:610-616, 1988.
4. Svendsen L, Crowley A, Ostergaard LH, *et al.* Development and comparison of purification strategies for chicken antibodies from egg yolk. *Lab Anim Sci,* 45;89-93, 1995.
5. Rose ME, Orlans E, Buttress N. Immunoglobulin classes in the hens egg : their segregation in yolk and white. *Europ J Immunol,* 4:521-523, 1974.
6. Kramer TT, Cho HC. Transfer of immunoglobulins and antibodies in the hens egg. *Immunology,* 19:157-167, 1970.
7. Hatta H, Kim M, Yamamoto T. A novel isolation method for hen egg yolk antibody, IgY. *Agri Biol Chem,* 54:2531-2535, 1990.
8. Piela TH, Hulka CM, Yates VJ, *et al.* Use of egg yolk in serological tests (ELISA and HI) to detect antibody to Newcastle disease, infectious bronchitis and Mycoplasma gallisepticum. *Avian Dis,* 28:877-883, 1984.
9. Carmichael LE, Joubert JC, Pollock RVH. Response of puppies to canine - origin parvovirus vaccines. *Mod Vet Prat,* 65:99-101, 1984.
10. Akita EM, Nakai S. Immunoglobulins from egg yolk : isolation and purification. *J Food Sci,* 57:629-634, 1992.
11. McBee LF, Cotterill OJ. Ion-exchange chromatography and electrophoresis of egg yolk proteins. *J Food Sci,* 44:656-667, 1979.
12. Bade H, Stegemann H. Rapid method of extraction of antibodies from hen egg yolk. *J Immunol Methods,* 72: 421-426, 1984.
13. Hatta H, Sim JS, Nakai S. Separation of phospholipids from egg yolk and recovery of water-soluble proteins. *J Food Sci,* 53:425-431, 1988.
14. Yokoyama H, Peralta RC, Sendo S, *et al.* 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, 1993.
15. Dressman JB, Amidon GL. Radiotelemetric method for evaluating enteric coatings *in vivo. J Pharm Sci,* 73: 935-938, 1984.
16. Burley RW, Vadehra DV. Chromatographic separation

of the soluble proteins of hens egg yolk : an analytical and preparative study. *Anal Biochem,* 94:53-56, 1979.

17. Osuga DT, Feeney RE. Egg proteins, In Whitaker JR and Tannenbaum SR, ed *Food Proteins,* AVI Pub, Westport, CT, 1977.
18. Kwan L, Li-Chan E, Helbig N, *et al.* Fractionation of water-soluble and -insoluble components from egg yolk with minimum use of organic solvents. *J Food Sci,* 56:1537-1541, 1991.
19. Leslie GA, Clem LW. Phylogeny of immunoglobulin structure and function: Immunoglobulins of the chicken. *J Exp Med,* 130:1337-1352, 1962.
20. Polson A, Coetzer TH, Kruger J, *et al.* Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol Investigations,* 14:323-327, 1985.
21. Hadge D, Ambrosius H. Evolution of low molecular weight immunoglobulins. IV. IgY-like immunoglobulins of birds, reptiles and amphibians, precursors of mammalian IgA. *Mol Immunol,* 21:699-707, 1991.
22. Warr GW, Magor KE, Higgins DA. IgY:clues to the origin of modern antibodies. *Immunol Today,* 16;392-398, 1995.
23. Ofarrely C, Branton D, Wanke CA. 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, 1992.