Generation and Characterization of Polyclonal Antibody Against Part of Immunoglobulin Constant Heavy ν Chain of Goose

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Immunoglobulin Y (abbreviated as IgY) is a type of immunoglobulin that is the major antibody in bird, reptile, and lungfish blood. IgY consists of two light (λ) and two heavy (ν) chains. In the present study, polyclonal antibody against IgYFc was generated and evaluated. rIgYC ν 3/C ν 4 was expressed in *Escherichia coli*, purified and utilized to raise polyclonal antibody in rabbit. High affinity antisera were obtained, which successfully detected the antigen at a dilution of 1:204,800 for ELISA assay. The antibody can specifically recognize both rIgYC ν 3/C ν 4 and native IgY by Western blot analysis. Furthermore, the serum of *Grus japonensis* or immunoglobulin of chicken, duck, turkey, and silkie samples and dynamic changes of serum GoIgY after immunogenicity with GPV-VP3-virus-like particles (GPV-VP3-VLPs) can be detected with the anti-GoIgYFc polyclonal antibody. These results suggested that the antibody is valuable for the investigation of biochemical properties and biological functions of GoIgY.

Introduction

AVIAN SPECIES EXPRESS THREE IMMUNOGLOBULIN classes, IgY, IgA, and IgM, that consist of an λ light chain and one of ν , α , or μ heavy chains.^(1–4) IgY is the major antibody in blood that is often mislabeled as immunoglobulin G (IgG) in older literature, and sometimes even in commercial product catalogues, due to its functional similarity to mammalian IgG. However, this older nomenclature is obsolete, since IgY differs both structurally and functionally from mammalian IgG⁽⁵⁾ and does not cross-react with antibodies raised against mammalian IgG.⁽⁶⁾

Like IgG, IgY is composed of two light and two heavy chains. Domains at the amino-terminus are highly variable (V) and the VH and VL domain pairings create the antigen (Ag)-binding site, which confers the antibody specificity. On the contrary, very little genetic variability is found in the other domains and these are referred to as the constant regions. The IgY heavy chain consists of four constant domains; the Fc fragment mainly contains two constant domains on the C-terminus, the C ν 3 domain and the C ν 4 domain,⁽⁷⁾ which relate directly to biological properties such as half-life, initiating secondary effector functions, the content of which also could reveal the immune status of geese.⁽⁸⁾

In this study, a polyclonal antibody against immunoglobulin Y Fc fragment of goose was generated and evaluated. Furthermore, IgY can be detected with the antibody by Western blotting and ELISA assay. These results suggested

that the antibody is useful in biochemical and functional studies on the humoral immune response of goose.

Materials and Methods

Plasmid construction

The total RNA of goose spleen was extracted using TRIzol (Invitrogen, Carlsbad, CA) from spleen tissue, according to the manufacturer's instructions; the GoIgYC ν 3/C ν 4 was amplified using RT-PCR by forward primer 5'-CAGAGCTGCAGCCCCATCCAG-3' and reverse primer 5-AACCTATTTACCGGGGTCT-3'. *Nde*I and *Xho*I sites are on the 5' and 3' termini, which is for cloning into pET-30a(+) (Novagen, Darmstadt, Germany). The plasmid was confirmed by DNA sequencing (BGI, Beijing, China).

Protein expression and purification

We transformed the expression plasmid into *Escherichia coli* Rossetta (DE3) for expression of the recombinant proteins. An aliquot of 100 μ L of overnight *E. coli* culture was diluted into 5 mL of Luria-Bertani medium. When the bacteria had reached a density (OD₆₀₀) of 0.6 at 37°C, isopropyl- β -D-thiogalactoside (IPTG) was added to a final concentration of 1 mM and the cells were grown for 4 h to induce recombinant proteins. For purification of recombinant proteins, SDS-PAGE gels were stained with 1 M KCl; the area of the gels corresponding to the bands representing proteins were cut comminuted and added to an appropriate volume of phosphate-buffered saline (PBS).

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Antibody production and purification

Polyclonal antibody was raised against purified recombinant GoIgYCv3/Cv4 (rGoIgYCv3/Cv4) protein in two rabbits (6 weeks old). For the first injection, 2 mg purified protein was emulsified with an equal volume of complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) and injected subcutaneously. Two booster injections of 2 mg protein emulsified with incomplete Freund's adjuvant were performed at 2-week intervals. Ten days after the last immunization, the antiserum was collected and stored at -20°C . For purification of polyclonal antibody, the protein G-Sepharose (GenScript, Nanjing, China) was used. After washing the column with $5\times$ volume of binding buffer (20 mM NaH_2PO_4 , 0.15 mM NaCl [pH 7.0]), the diluted serum was added onto the column with an equal volume of binding buffer. The column was then washed 5 to 10 times with the binding buffer after binding for 30 min at ambient temperature; then the IgG was collected by elution buffer (0.1 M citric acid [pH 2.0]) and stored at -20°C .

SDS-PAGE and Western blot analysis

For electrophoresis and Western blot analysis, the serum of goose, *Grus japonensis*, or immunoglobulin of chicken, duck, turkey, or silkie samples were subjected to 12% SDS-PAGE followed by transferring to polyvinylidene fluoride (PVDF) membranes⁽⁹⁾ and a cell extract from *E. coli* with empty pET-30a was used as negative control. The PVDF membrane was blocked with blocking buffer (1 $\times$ PBS, 0.5% skim milk) and probed with antibodies diluted in blocking buffer. Detection was employed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Invitrogen) and the strips were developed with EasySee Western blot kit (Transgen, Beijing, China).

Indirect ELISA

ELISA was conducted according to Engvall's method⁽¹⁰⁾ with minor modifications. The 96-well microplates were coated with 200 ng of serum or immunoglobulin samples in carbonate coating buffer (pH 9.6) overnight at 4°C . A cell extract from *E. coli* with empty pET-30a and rGoIgYCv3/Cv4 protein were used as controls. The plates were incubated for 2 h at 37°C with blocking buffer. 100 μL of four individual goose serum samples against GPV-VP3-VLPs diluted in blocking buffer were added to each well and incubated for 2 h at 37°C . Then a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG in PBS was added to each well and incubated for 1 h at 37°C . 3,3',5,5'-tetramethylbenzidine peroxidase substrate was added, and the plates were incubated for 10 min at room temperature. The reaction was stopped by adding 100 μL of 1 M H_2SO_4 . The absorbance values were measured immediately at 450 nm.

Results

Expression and purification of rGoIgYCv3/Cv4

The 0.65 kb DNA fragment encoding the Cv3 and Cv4 regions was amplified and cloned into a pET30a(+) expression vector, which was expressed in *E. coli* and purified (Fig. 1). SDS-PAGE analysis revealed that the rGoIgYCv3/Cv4 was about 30 kDa. The IPTG-induced rGoIgYCv3/Cv4 was detected in the inclusion body status almost exclusively.

FIG. 1. Expression and purification of rGoIgYCv3/Cv4; the plasmid pET30a-rGoIgYFc was transformed in *E. coli*. Lane M, protein molecular weight marker broad (TaKaRa Biotech, Dalian, Japan); lane 1, total protein from uninduced *E. coli* harboring pET30a-rGoIgYCv3/Cv4; lanes 2–4, total, inclusion body, and soluble proteins from *E. coli* harboring pET30a-rGoIgYCv3/Cv4, induced with IPTG; lane 4, purified rGoIgYCv3/Cv4.

Finally 1.059 mg/mL purified rGoIgYCv3/Cv4 could be obtained from 200 mL bacterial culture by cutting gels.

Purification and characterization of anti-GoIgY polyclonal antibody

Polyclonal antibodies were generated in rabbit by using the purified rGoIgYCv3/Cv4. The rGoIgYCv3/Cv4 antigen was detected with the antisera at the dilution of 1:204,800 (Fig. 2) by indirect ELISA. Then the polyclonal antibodies were purified by using protein G-Sepharose. SDS-PAGE analysis revealed that both the heavy (about 50 kDa) and light (about 20 kDa) chains of IgG were purified (Fig. 3). Western blot analyses were conducted with purified rGoIgYCv3/Cv4 and native GoIgY from serum and bile. A single band at about 30 kDa or 70 kDa can be detected (Fig. 4). These results demonstrated that the antibody can recognize the rGoIgYCv3/Cv4 and the native GoIgY specifically.

FIG. 2. Serum titers were tested by indirect ELISA; dilution ranged from 1:100 to 1:16,038,400. Rabbit pre-immune serum was used as negative control.

FIG. 3. Purification of anti-GoIgY polyclonal antibody; the antibody was purified by protein G-Sepharose. Lane M, protein molecular weight marker broad; lane 1, total protein from unpurified serum harboring anti-GoIgY polyclonal antibody; lane 2, unbound proteins from serum harboring anti-GoIgY polyclonal antibody; lanes 3–5, protein complex washed by binding buffer; lanes 6–9, protein eluted by elution buffer.

Cross-reactivity analysis of IgY Fc fragment of six avians and purified anti-GolY polyclonal antibody

The extraction IgY of chicken, duck, turkey, silkie, the serum of goose, and *Grus japonensis* were transferred to PVDF membrane. A single band at about 70 kDa could be detected in every sample (Fig. 5). To further verify the result of the Western blot described above, six kinds of IgY, purified rGoIgYCv3/Cv4 (positive control), and negative control were coated on a 96-well plate. Eventually, the ELISA results showed that the IgY or serum of six avians are recognized specifically by anti-GoIgY polyclonal antibody in different degrees (Fig. 6).

Dynamic change of GolY after immunogenicity with GPV-VP3-VLPs

The dynamic change of GoIgY of four individual goose serum samples against GPV-VP3-VLPs^(11–13) which possessed the satisfied immunogenicity, was evaluated by indirect ELISA assay. Each serum at 1:100,000 dilution was coated in 96-well plates and detected with purified antibody at dilutions of 1:20 (Fig. 7).

FIG. 4. Evaluation of anti-GoIgY polyclonal antibody by Western blot. Lane M, EasySee protein weight marker (Transgen); lanes 1,3,4, purified rGoIgYCv3/Cv4 (lane 1) and native GoIgY from serum (lane 3) and bile (lane 4) were analyzed by Western blot with purified antibody at dilutions of 1:400; lane 2, negative control.

FIG. 5. Cross-reactivity analysis of IgY from six avians by Western blot. Lane M, EasySee protein weight marker; lane 1, purified rGoIgYCv3/Cv4 was used as positive control; lane 2, negative control; lanes 3–8, IgY of goose (lane 3), turkey (lane 4), duck (lane 5), *Grus japonensis* (lane 6), chicken (lane 7), and silkie (lane 8) were analyzed by Western blot with purified antibody at dilutions of 1:400.

Discussion

In this study, we report on the generation and evaluation of a polyclonal antibody against GoIgY by using an Fc fragment of GoIgY. High avidity antisera were obtained, which are functional in two different assays, ELISA and Western blot. The crude antisera that were purified by protein G-Sepharose showed that the antibody we raised is highly specific to activation with either rGoIgYCv3/Cv4 or native GoIgY.

In chicken, duck, and goose, the cDNAs encoding the heavy chain constant regions of IgY, IgA, and IgM have been cloned or partially cloned.^(2,4,8,14) The deduced peptide sequences suggest that they have four structurally similar domains with cysteine residues, the potential disulphide bond donor, in appropriate positions, indicate that avian species may share structural features and evolutionary precursors of immunoglobulins. At the genomic level, there is only limited information available in other avians. Therefore, based on the results in this report, the degree of reactivity by Western blot and ELISA from high to low are goose, duck, chicken,

FIG. 6. Further study of the IgY of six avians by indirect ELISA. Three repeated tests were analyzed with purified antibody at dilutions of 1:50.

FIG. 7. Data are expressed as mean $\pm$ SEM of each group of sera ($n=4$ per group) at indicated time points from three separate experiments.

turkey, silkie, and *Grus japonensis*. It can be concluded from this study that ducks have the greatest cross-reactivity with GoIgYCv3/Cv4 and *Grus japonensis* have the lowest.

GoIgY, the major antibody in serum,^(16,17) concerning immunity and infectious status, plays an important role in the humoral immunity.⁽¹⁸⁾ In the present study, dynamic changes of serums of GPV-VP3-VLP IgY were detected by indirect ELISA. The study showed that titers of total IgY were lowest before immunization with GPV-VP3-VLPs. Following immunization, concentrations of total IgY increased for 2 weeks; after reaching a peak at week 2, they remained at a high level until week 5, after which the concentrations of IgY decreased. Based on these results, it can be concluded that IgY plays a later but major role in humoral immunity.

In conclusion, a sensitive and specific polyclonal antibody against GoIgY was generated and applied to analyze the immunity cross-reaction between six avians and dynamic changes of serum IgY. Since Cv3/Cv4 is a key domain of immunoglobulin and has different recruitment capabilities for a variety of immune effector functions, the antibody generated in the present study will provide a powerful tool for the detection of GoIgYCv3/Cv4 under various conditions for the investigation of biochemical properties and biological functions of GoIgYCv3/Cv4.

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