RESEARCH NOTE

Research Note: Development of a sandwich ELISA for determining plasma growth hormone concentrations in goose

R. Chen, R. H. Guo, M. M. Lei, H. X. Zhu, L. Y. Yan, and Z. D. Shi [1]

Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base of Ministry of Science and Technology, Institute of Animal Science, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China

ABSTRACT Growth hormone (**GH**) is required for normal postnatal development in poultry; however, no immunoassay exists to assess its levels in geese plasma, hindering the study of endocrine regulation in this species. We developed a sandwich ELISA to determine the GH concentrations in the plasma of geese. Recombinant goose GH was produced using a eukaryotic expression system and purified for use as the reference standard in ELISA and the antigen for producing the polyclonal antibodies in rabbits. Rabbit anti-goose GH polyclonal antibody was used to coat the wells of the ELISA plate, and its biotinylated form served as the detection antibody. An avidin-conjugated horseradish peroxidase was used to bind the detection antibody and catalyze the chromogenic reaction of 3,3,5,5-tetramethylbenzidine and H_2O_2. A sigmoidal curve was fitted to the optical density and the log of the standard GH concentration using the four-parameter logistic model. The sensitivity of the assay was less than 0.156 ng/mL. The intra- and interassay coefficients of variation were less than 9 and 13%, respectively. The response curve of the serially diluted plasma samples from geese exhibited a good parallel relationship with that observed for the reference standards. The assay effectively detected differences in GH concentrations in plasma samples from geese at various physiological stages; thus, it will be useful for future study of their growth and metabolism.

Key words: growth hormone, concentration, sandwich, ELISA, goose

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INTRODUCTION

Growth hormone (**GH**), a polypeptide hormone secreted by the anterior pituitary gland, is essential for postnatal growth in mammals and birds. The importance of pituitary GH in avian growth is demonstrated by growth suppression following surgical removal of the pituitary gland (Scanes et al., 1986; Proudman et al., 1994), and reduced growth that occurs in dwarf chickens that lack the GH receptor (Agarwal, et al., 1994). Pituitary GH stimulates the hepatic production of insulin-like growth factor-I, which in turn stimulates growth through the regulation of lipid, protein, and carbohydrate metabolism (Scanes, 2009; Jiang and Ge, 2014; Carter-Su et al., 2016). It is of prime importance to accurately determine the concentration of GH in the blood circulation to study the endocrine regulation of avian growth.

Existing assays for the determination of GH in fowl blood samples are mainly based on homologous and heterologous radioimmunoassay (**RIA**) using native GH protein extracted from a large number of pituitary glands (Harvey and Scanes, 1977; Proudman and Wentworth, 1978; Leung et al., 1984; Zheng et al., 2007) or recombinant avian GH (Proudman, 1984; Picaper et al., 1986). However, RIA requires the use of hazardous radioisotopes that restrict assay use to specialized laboratories. Therefore, an enzyme linked immunosorbent assay (**ELISA**), which does not involve the use of these substances, has been developed to determine the concentration of GH in chicken plasma samples (Houston et al., 1991).

Currently, there are some commercially available chicken GH ELISA kits that have been adopted for the quantitative determination of chicken GH concentrations in plasma, tissue homogenates and other biological fluids (Jawad et al., 2016; Morita et al., 2016; Ibitoye et al., 2019). However, the feasibility of these kits for measuring GH in goose plasma has not been evaluated. No immunoassay has thus far been developed for goose plasma GH, which has hindered the study of goose growth endocrinology. We have previously reported a simple and sensitive sandwich ELISA that

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[1]Corresponding author: zdshi@jaas.ac.cn

was validated to measure prolactin (**PRL**) concentrations in goose plasma (Chen et al., 2019). In this study, a sandwich ELISA was developed to determine the concentration of GH in the plasma of geese based on a rabbit anti-goose GH polyclonal antibody with a recombinant goose GH used as the antigen.

MATERIALS AND METHODS

Ethics Approval

The experimental procedures were approved by the Jiangsu Academy of Agricultural Sciences Experimental Animal Ethics Committee and conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Decree No. 63 of the Jiangsu Academy of Agricultural Science on July 8, 2014).

Animals and Sample Collection

A total of 30 healthy, one-day-old Yangzhou male geese were obtained from a commercial farm and kept in floor pens during an experimental period of 70 d. A commercial diet and water were provided ad libitum. The geese were given 24 h of light from d 1 to 7, and adjusted to 16 h of light and 8 h of darkness (16L: 8D) per day from d 8 onward. Blood samples (n = 8) were collected every 10 d by brachial venipuncture into heparinized tubes. The plasma was separated by centrifugation at 2,500 × *g* for 10 min at 4°C and stored at −20°C until analysis. Two 70-day-old male geese were killed by cervical dislocation and pituitary gland was collected. They were homogenized in 10 volumes of radio immunoprecipitation assay (**RIPA**) buffer containing 1mM phenylmethylsulfonyl fluoride (#P0013B, Beyotime, Shanghai, China) at 4°C, and centrifuged for 5 min at 12,000 × *g* to obtain the supernatants. The protein concentration was determined using a Bicinchoninic Acid (**BCA**) protein assay kit (#P0012S, Beyotime, and the supernatant was stored at −20°C until analysis.

To evaluate the physiological suitability of the assay, 3-mo-old Yangzhou ganders (n = 8) were selected from a goose farm and provided food and water ad libitum under a natural photoperiod. The chicken growth hormone-releasing hormone (**GHRH**) peptide HADAIFTDNYRKFLGQISARKFLQTIIGK, identified in a previous study (Harvey et al., 2014), was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). Peptides were freshly dissolved in 0.9% NaCl containing 0.01% (w/v) ascorbic acid (to prevent oxidation) and administered by intravenous injection at a dose of 10 μg/kg body weight. Blood samples were collected from the contralateral brachial vein 10, 20, 30, and 60 min after intravenous injection. Samples were collected in heparinized tubes and kept on ice until centrifugation. The plasma was separated via centrifugation at 2,500 × *g* for 10 min at 4°C and stored at −20°C until analysis.

Preparation of Recombinant Goose GH Protein

The DNA coding sequence of the 191-amino acid mature peptide of goose GH (GenBank accession number: AY149895.2) was cloned into a commercially available eukaryotic expression vector, pCZN4b (Zoonbio, Nanjing, China). This expression vector contained the DNA coding sequence for a hexa-His tag at the C-terminus of the target protein. The recombinant plasmid was then transfected into HEK293 cells using the method described by Longo et al. (2013). The transfection system comprised of 27 mL cell culture and 3 mL Opti-MEM medium (#31985070, Invitrogen, Carlsbad, CA) containing 30 μg plasmid DNA and 90 μg polyethylenimine (#23966, Polyscience, Warrington, PA). Once the cell vitality was lower than 50%, the culture medium was collected and centrifuged; the supernatant obtained and loaded onto a column packed with Ni-NTA resin (#R90110, Thermo Fisher Scientific, San Jose, CA) for separation of the recombinant goose GH, according to the manufacturer's instructions. The eluted fractions were dialyzed overnight in 10 mM phosphate-buffered saline (**PBS**, pH 7.4) at 4°C. The recombinant goose GH was analyzed by 12% SDS-PAGE and western blotting using an anti-His tag antibody (#HT501-01, Transgen, Beijing, China), and the protein concentration was determined using BCA protein assay kit. The purified protein was aliquoted and stored at −20°C until use.

Preparation of Polyclonal Rabbit Anti-GH Antibody

Recombinant goose GH was used as an antigen for the generation of polyclonal antibody in 2 New Zealand white rabbits weighing 2 to 2.5 kg. For primary immunization, each rabbit was subcutaneously injected with 400 μg of the recombinant protein, emulsified with Freund's complete adjuvant (1:1, v/v, #F5881, Sigma-Aldrich, St. Louis, MO). Two booster immunization doses, each containing 400 μg of the recombinant protein emulsified with Freund's incomplete adjuvant (1:1, v/v, #F5506, Sigma-Aldrich), were administered at 3-wk intervals. Blood samples were collected approximately 7 d after each booster immunization to determine the antibody titers. The final blood sampling was performed 2 weeks after the second booster immunization. Antisera were isolated for the purification of the antibodies through antigen-affinity chromatography. In brief, recombinant GH was conjugated to cyanogen bromide-activated Sepharose 4B (#17-0430-02, GE Healthcare Bio-Sciences AB, Uppsala, Sweden), according to the manufacturer's instructions. The antisera were loaded onto the GH-Sepharose 4B column, and the anti-GH antibody was eluted with 100 mM glycine buffer (pH 2.7). The purified antibody was dialyzed overnight in 10 mM PBS buffer (pH 7.4) at 4°C. The concentration of the purified antibodies was determined using BCA

protein assay kit, and the antibody was aliquoted and stored at −20°C until use.

Biotin Labeling of the Antibody

The purified antibody was reconstituted to 2 mg/mL in 10 mM PBS buffer (pH 7.4). To 1 mL of this antibody, 27 μL biotin N-hydroxysuccinimide ester (#H1759, Sigma-Aldrich), dissolved in dimethylsulfoxide (DMSO) (10 mM), was added. The solution was then mixed gently by inversion for 90 min at 20°C in the dark. Any unconjugated biotin N-hydroxysuccinimide ester was removed by dialyzing against 10 mM PBS buffer (pH 7.4) for 48 h at 4°C. These biotin labeled antibodies were fractionated and stored at −20°C until use.

Procedure for ELISA

Polystyrene microtiter plates (#3590, Corning Inc., Corning, NY) were coated with affinity purified anti-GH polyclonal antibody (1 μg/mL in 10 mM PBS, pH 7.4; 100 μL/well). The plates were then incubated overnight at 4°C. The antibody solution was removed by inverting the plates, and the wells were washed three times with PBST (10 mM PBS, pH 7.4, containing 0.05% Tween-20). The remaining reactive sites on the wells were saturated by incubation with 200 μL/well of 5% bovine serum albumin (dissolved in PBST) for 1 h at 37°C. After 3 washes, the plates were immediately used for the following ELISA tests.

The recombinant goose GH protein was used as the reference standard with 2-fold serial dilution in PBST starting from an initial concentration of 10 ng/mL. One hundred microliters of the standard protein solution or plasma sample was transferred into each well. After incubation at 37°C for 1 h and 3 washes with PBST, the biotinylated anti-GH antibody (0.4 μg/mL in PBST) was added (100 μL/well) and the plate was incubated for 1 h at 37°C. After 3 washes with PBST, 100 μL of a solution of avidin-conjugated horseradish peroxidase (1:5,000 diluted in PBST; #E030100-01, EarthOX, Millbrae, CA) was added to each reaction well, and the plate was incubated at 37°C for 30 min. After 5 further washes, 100 μL of freshly prepared horseradish peroxidase substrate solution (0.1 mg/mL 3,3,5,5-tetramethylbenzidine (Sigma-Aldrich) and 0.02% H_2O_2 in 0.1 M Na_2HPO_4, 0.05 M citric acid, pH 4.5−5.5) was added to each well. The plate was incubated for 15 min at 37°C in the dark, and the reaction was stopped by the addition of 100 μL/well of 1 M HCl. The optical density (**OD**) of each sample was read at 450 nm using a spectrophotometer (Eon, BioTek Instruments Inc., Winooski, VT). All samples and standards were assayed in duplicates.

Validation of the Assay

To validate the assay, the dose response curve of the reference standards was used to demonstrate a parallel relationship to the response curves of 2-fold serially diluted pooled plasma samples (n = 5) of Yangzhou geese. The sensitivity of the assay was determined as the minimal concentration difference from the zero standard and was calculated by the addition of 2 standard deviations to the mean OD value of 10 zero standard replicates. Three plasma samples of Yangzhou geese were tested 6 times on one plate to assess the intra-assay precision and in three separate assays to assess the interassay precision. A coefficient of variation <15% was deemed acceptable. Cross-reactivity was assessed by substituting the standards with serial dilutions of purified chicken pituitary follicle-stimulating hormone (FSH, USDA-cLH-I-1, procured from Dr. J.A. Proudman, USDA), luteinizing hormone (LH, USDA-cLH-I-3, procured from Dr. J.A. Proudman, USDA), and PRL (AFP-10328B, procured from Dr. A.F. Parlow, NIPP, UCLA). Recovery was evaluated by spiking a high and a low concentration of recombinant goose GH protein to the pooled goose plasma (n = 6) using the following formula: (measured spike concentration/expected spike concentration) times 100. The expected spike concentration was determined by adding the concentration of the pooled geese plasma to the concentration of the added recombinant protein. The acceptable range for recovery was between 80 and 120%.

Statistical Analysis

The mean values of duplicate OD readings for each standard and sample were calculated and subtracted from the average reading value of the zero standard. An equation of the standard curve was constructed by regression analysis of the mean OD readings and the log of standard concentrations using the four-parameter logistic model (GraphPad Prism 8, GraphPad Software, San Diego, CA). Additionally, linear regression was fitted to the data points and a Student's t test was performed to compare slopes, thereby assessing parallelism between plasma and recombinant GH (Bouquet et al., 2020). The GH concentration in each sample was calculated using this fitted equation based on the mean OD reading of each sample and presented as means ± SEM. Differences between means was analyzed using one-way ANOVA with least significant difference test for multiple comparisons using SPSS 20 software (IBM Corp., Armonk, NY). Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Production of Recombinant Goose GH Protein and its Polyclonal Antibody

The recombinant goose GH protein contained 218 amino acid residues with a predicted molecular mass of ∼25 kDa, including a 21-residue leading sequence derived from the expression plasmid and the 191-residue sequence of the goose GH with a hexa-His tag at the C-terminus. A band with a molecular mass of ∼25 kDa, which is similar to the predicted mass of the recombinant goose GH

protein, was detected via SDS-PAGE (Figure 1A). The same band was also detected via Western blot analysis using an anti-His-tag antibody (Figure 1B). In western blot analysis, the purified anti-GH polyclonal antibody specifically detected four distinct bands with molecular masses below 60 kDa in the homogenates of goose pituitary glands (Figure 1C). Different bands correspond to the GH isoforms that are derived from alternative splicing, glycosylation, and phosphorylation of the immature GH molecule. This shows the high affinity and detectability of the rabbit anti-GH polyclonal antibody generated in this study.

Development and Validation of a Sandwich ELISA for Detection of Goose GH

A sandwich ELISA was developed in which the affinity-purified anti-GH polyclonal antibody was used as the capture antibody and the recombinant GH protein was bound to it, which was further bound by biotin-labeled detection antibody. A standard curve fitted for the OD values and the corresponding log of the reference standard concentrations is shown in Figure 2A ($R^2 = 0.998$). The detection range of the curve was from 0.078 to 10 ng/mL. The sensitivity of the assay was less than 0.156 ng/mL. Both the intra- and interassay coefficients of variation were respectively less than 9 and 13% (Table 1), suggesting that the assay has a higher accuracy. No cross-reactivity of the anti-GH antibody or assay system was detected when high concentrations of FSH, LH, and PRL from chicken were added (data not shown). Recovery was assessed based on the ability to retrieve known amounts of recombinant GH protein. The acceptable recovery rate of 94 to 105% suggested that the sample matrix did not affect the response signal for GH (data not shown).

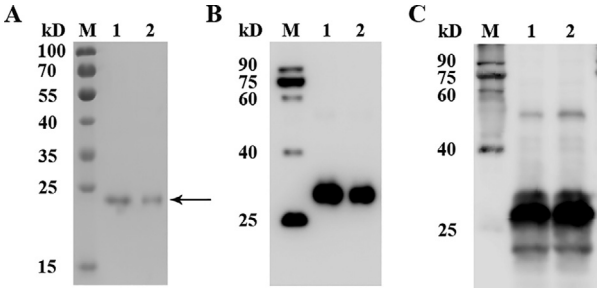

Figure 1. Identification of recombinant goose GH protein and specificity analysis of the anti-GH polyclonal antibody. (A) Purified recombinant goose GH protein was separated by 12% SDS-PAGE and the gel was stained with Coomassie blue. The arrow indicates the target protein. (B) Western blot analysis of the purified recombinant goose GH protein, recognized by an anti-His tag antibody (1:1,000 dilution). The band was visualized with ECL reagent after washing and incubation with HRP-conjugated secondary antibody. Lane M, molecular weight standards; Lane 1and 2, purified recombinant goose GH protein. (C) Western blot analysis of pituitary gland homogenates from geese using the anti-GH polyclonal antibody (1:10,000 dilution) revealed four specific bands with molecular mass below 60 kDa. Lane M, molecular weight standards; Lane 1and 2, pituitary gland homogenates from two 70-day-old male Yangzhou goose.

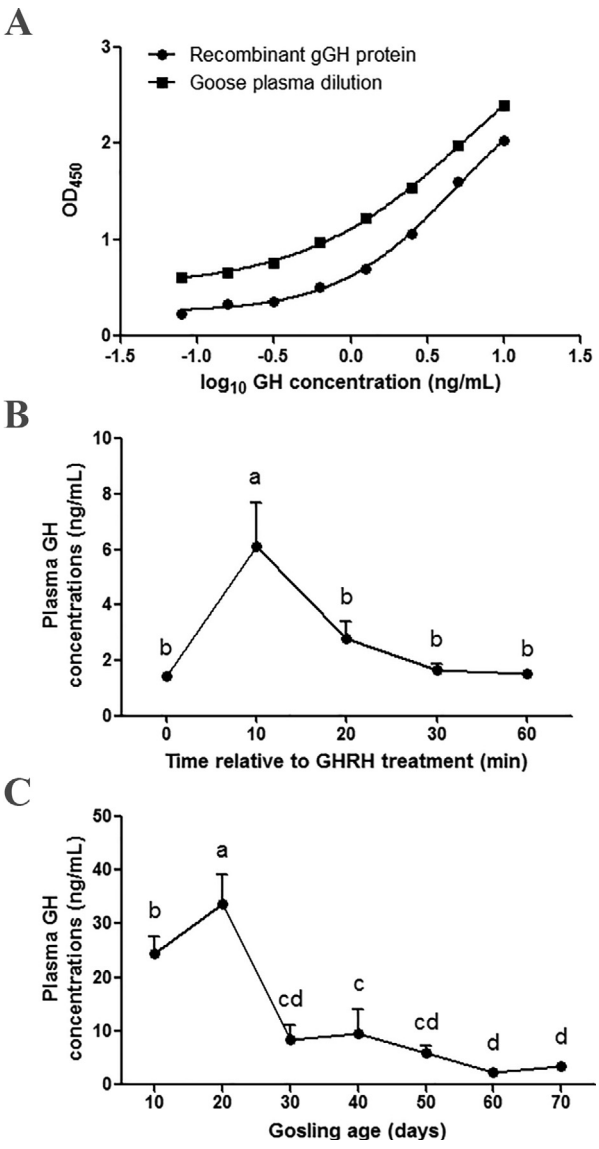

Figure 2. Development and Validation of a Sandwich ELISA for Detection of Goose GH. (A) The response curve of 2-fold serially diluted pooled plasma samples (n = 5) from Yangzhou geese (□) was parallel to the standard curve obtained using the recombinant goose GH standards (•). (B) Plasma GH concentrations in Yangzhou ganders after chicken GHRH treatment. Zero represented the time point when intravenous injection of chicken GHRH peptide (10 μg/kg body weight) was done. (C) Plasma GH concentrations in Yangzhou geese at different days of age. Data are shown as means ± SEM. Different lowercase letters indicate significant differences ($P < 0.05$).

Parallelism was observed between the serial dilution curve of the pooled goose plasma samples ($R^2 = 0.999$) and the recombinant standard (Figure 2A). A Student's

Table 1. Precision of the assay.

	Intraassay precision			Interassay precision		
Sample	1	2	3	1	2	3
n	6	6	6	3	3	3
Mean (ng/mL)	30.0	15.7	3.3	28.3	13.3	4
Standard deviation	0.9	1.4	0.2	1.5	1.2	0.5
CV (%)	2.9	8.8	5.3	5.4	9.6	12.2

Three plasma samples of Yangzhou geese were tested six times on one plate to assess the intra-assay precision and in three separate assays to assess the interassay precision.

Abbreviation: CV, coefficient of variation.

t test revealed that the slopes did not differ significantly from each other ($P > 0.05$). The plasma GH concentrations of chicken GHRH-treated Yangzhou ganders, determined using the developed assay, are presented in Figure 2B. Following chicken GHRH challenge, the plasm GH concentration increased from a baseline of 1.45 ng/mL to 6.1 ng/mL, a 4.2-fold increase. The trend in plasma GH response to chicken GHRH challenge in geese confirmed the biological validation of the present assay for the determination of GH in goose plasma.

GH Concentrations in the Plasma of Geese at Different Days of Age

Plasma GH concentrations of geese were assayed at 10-d intervals over a period of 70 d (Figure 2C). At d 10, the GH concentration was at a moderately high level of approximately 25 ng/mL. It reached a significantly ($P < 0.05$) higher peak level of approximately 33 ng/mL at d 20. Subsequently, the GH concentrations in the plasma decreased rapidly to below 10 ng/mL on d 30 and remained at a lower level from d 30 to 70. The concentration of plasma GH was high in the early stage of rapid growth and low in the latter stages. This pattern of plasma GH concentration with age has been observed in domestic fowl (Proudman et al., 1995; Reiprich et al., 1995).

In this study, a sandwich ELISA was developed to determine the plasma GH concentration in geese, and the validity of the assay was demonstrated. Using the assay, the GH concentrations in the plasma samples from geese of different ages were found to be different. In addition, the assay reliably quantified the increase in GH concentrations occurring in response to GHRH administration to the ganders. These results demonstrate that the developed ELISA is suitable for the measurement of plasma GH concentrations and can serve as a useful tool for studying the regulation of GH secretion and growth in geese.

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DISCLOSURES

The authors declare no conflicts of interest.

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