—Research Note—

Specific Anti-peptide Antibody to β Subunit of Chicken Thyrotropin: Production and Characterization

Atsushi IWASAWA¹⁾, Hirofumi NAKAYAMA¹⁾, Yoshio YAMAMOTO²⁾,
Osamu DOI¹⁾, Takao NAKAMURA¹⁾# and Yukio KATO³⁾

Departments of ¹⁾Animal Science and Technology and ²⁾Veterinary Anatomy, Faculty of Agriculture, Gifu University, Gifu 501-1193 and ³⁾Department of Life Science, Faculty of Agriculture, Meiji University, Kawasaki 314-8571, Japan

Present: #Gifu Prefectural College of Agriculture, Kani 509-0241, Japan

Abstract. The unavailability of homologous antibodies to avian thyrotropin (TSH) has been a major hindrance in the study of avian reproduction and metabolism. Here we report the production and characterization of an anti-peptide antibody specific to chicken TSH β subunit. According to the cDNA sequence of chicken TSH β subunit, a synthetic peptide corresponding to the amino acid residues 35-50 (Tb3550; Asp-Ser-Asn-Gly-Lys-Lys-Leu-Leu-Leu-Lys-Ser-Ala-Leu-Ser-Gln-Asn) was prepared to produce polyclonal antibody in rabbits. This peptide sequence is highly specific to avian TSH β and was designed for the antibody to show no cross-reaction to pituitary and other chicken proteins with known amino acid sequences. Anti-Tb3550 recognized a 18.2-kDa band on immunoblot of the cephalic lobe homogenate, but not of the caudal lobe homogenate, of chicken anterior pituitary gland. Positive immunohistochemical staining with anti-Tb3550 was restricted to the cephalic lobe, whereas the cells positive to anti-chicken luteinizing hormone were scattered throughout the anterior pituitary. Anti-Tb3550 did not bind either to purified chicken luteinizing hormone or to follicle stimulating hormone preparation. These results indicate that this antibody specifically recognizes chicken TSH β subunit. This antibody should be useful for immunochemical study of avian TSH and for tracing TSH activity during the purification of the hormone.

Key words: Chicken, TSH β , Anti-peptide antibody, Immunoblotting, Immunohistochemistry
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Avian thyroid hormones have been reported to affect not only heat production, metabolism and growth as in mammals, but also gonadal development, molting and migration (see [1-3] for review). The involvement of thyroid hormones in induced molt of the hen, where hens are forced into molting to improve egg production and egg quality, in particular has attracted the attention of investigators due to its biological interest as well as economic importance to the poultry industry [4-6]. Whether the natural seasonal molt is brought about

by increased thyroid activity seems less certain, but there have been reports suggesting the involvement of the thyroid in wild avian species (see [7] for review). Although thyrotropin (TSH), as a tropic hormone of the pituitary gland, is supposed to regulate circulating thyroid hormone levels under various physiological and artificial conditions such as molting, the exact roles of TSH are not well understood, since purification with thorough characterization of avian TSH has not been successful and hence no homologous immunoassays specific for avian TSH are yet available.

Because the cDNA sequences of TSH β subunits of avian species have been recently determined (chicken: [8, 9]; Japanese quail: [10]), an understanding of avian TSH is now possible from molecular aspects. The aim of this study was to prepare a sequence-specific antibody directed against a peptide that was synthesized according to the amino acid sequence deduced from the cDNA sequence. We obtained results showing that this antibody specifically recognizes the β subunit of chicken TSH (but not the α subunit). Our study confirmed the applicability of this antibody to immunological methods such as immunohistochemistry and immunoblot for specific determination of the chicken TSH β subunit, representing the first report on a homologous antibody to avian TSH.

Materials and Methods

Peptide synthesis

A custom peptide with the sequence Asp-Ser-Asn-Gly-Lys-Lys-Leu-Leu-Leu-Lys-Ser-Ala-Leu-Ser-Gln-Asn (Tb3550), corresponding to residues 35-50 of the β subunit of chicken TSH, was obtained from a commercial source (Sawady Technology Inc., Tokyo).

Production of antiserum

The synthetic peptide Tb3550 was coupled to a carrier protein, keyhole limpet hemocyanin (Sigma Chemical Co., St. Louis, MO, USA), at a peptide/protein ratio of 500:1 (mol:mol) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturers' instructions. Female New Zealand White rabbits were immunized with the peptide-protein conjugate into the popliteal lymph nodes [11] for the first immunization and intradermally into numerous sites on the back for succeeding immunizations every 2 weeks for 5 months as described previously [12, 13]. The whole procedure was conducted in accordance with the Guidelines for the Care and Use of Animals of our institutions.

Western blot

Chicken pituitary homogenate and highly purified chicken luteinizing hormone (LH) and follicle stimulating hormone (FSH) preparations (USDA-cLH-I-3 and cFSH-I-1; supplied through the

USDA Animal Hormone Program, Beltsville, MD, USA) were each separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described [14].

To localize TSH immunoreactivity, the anterior pituitary glands of White Leghorn laying hens were dissected into cephalic, middle and caudal portions with a scalpel blade as described [15]. The cephalic and caudal homogenates were electrophoresed as for the whole anterior pituitary.

For electrophoresis, a 1-mm-thick slab gel with an acrylamide concentration of 12.5% or of 5–20% gradient (Bio Craft, Tokyo) was used. After electrophoresis, the separated proteins were transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA, USA) using a semi-dry electroblotting apparatus (Bio Craft).

After protein transfer was completed, a part of the membrane was stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA) and the rest was used for immunoblotting. For immunoblotting, the membrane was incubated sequentially with the following reagents: a blocking solution consisting of 3% skim milk and 0.1% Tween 20 in Tris-buffered saline (TBS), pH 7.4, overnight at 4 °C; anti-Tb3550 (diluted to 1:6,000 with the blocking solution), anti-chicken LH IRC-2 *Gunma* [16] (1:6,000; kindly provided by the Biosignal Research Center, Gumna University) or non-immune rabbit serum (1:6,000) overnight at 4 °C; biotinylated goat anti-rabbit γ -globulin (Gibco BRL, Gaithersburg, MD, USA) for 1 h at room temperature; and finally peroxidase-labeled streptavidin (Gibco BRL) for 20 min at room temperature, with rinsing with 0.1% Tween 20 in TBS for 15 min between the incubations. A final rinse was followed by a detection of peroxidase activity with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Gibco BRL). The membrane was finally rinsed with distilled water, dried and photographed. Molecular weights of the bands appearing on the immunoblots were calculated using a public software package (NIH Image; National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

The anterior pituitary glands of White Leghorn laying hens were fixed by immersion in Bouin-Hollande fixative for 24 h, dehydrated with an

ethanol series, and embedded in paraffin. Two- μ m-thick serial sections were mounted on gelatin-coated slides, deparaffinized with xylene, and incubated in a solution of 0.3% H_2O_2 in methanol for 20 min to inhibit endogenous peroxidase activity. After rinsing with phosphate-buffered saline (PBS), the sections were incubated sequentially with the following reagents at room temperature unless otherwise mentioned: normal goat serum (diluted 1:50 with PBS containing 0.5% Triton X-100) for 30 min; anti-Tb3550 (1:4,000) or anti-chicken LH IRC-2 *Gunma* (1:500,000) sera overnight at 4 C; biotinylated goat anti-rabbit γ -globulin (1:200; Vector Laboratories, Burlingame, CA, USA) for 30 min; and finally peroxidase-labeled avidin (Vector) for 30 min with rinsing with PBS for 15 min between the incubations. A final rinse with PBS was followed by immersion of the sections for 5 min in 0.02% 3, 3'-diaminobenzidine (Dojindo, Kumamoto) and 0.005% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.4, to visualize peroxidase activity. The sections were counter-stained with hematoxylin, dehydrated in an ethanol series, cleared with xylene and finally sealed with coverslips.

Absorption test

The anti-Tb3550 antiserum (10 μ l) was incubated in a micro centrifuge tube with the Tb3550 peptide

(unconjugated, 20 μ g) dissolved in PBS overnight at 4 C. After the incubation, the mixture was used as the primary antisera for immunoblotting and immunohistochemistry at the same final dilution as for the standard procedures described above.

Results

Amino acid comparison

Figure 1 compares amino acid sequences of TSH β , LH β and FSH β subunits of avian and mammalian species. The residues 35-50 of chicken TSH β subunit (sequence of the peptide Tb3550) are identical to those of Japanese quail, but this region shows some amino acid substitution in rat TSH β subunit. The sequence of Tb3550 is highly specific for chicken (and Japanese quail) TSH. Residues 33-34 (Thr and Arg), which are among hydrophilic amino acids and highly antigenic, were not included in the synthetic peptide since they are also borne by chicken LH β subunit.

Immunoblotting

Immunoblot of the homogenate of whole anterior pituitary using anti-Tb3550 revealed a typical broad band of glycoprotein at 18.2 kDa (Fig. 2). This 18.2-kDa band disappeared when the anti-Tb3550 antibody pre-incubated with an excess

	1.....10.....20.....30.....40.....50.....60
chicken TSH	VCAPSEYTIHVEKRECAyclAINTTICAGFCMT DSNGKKLLLSALSQN VCTYKEMFYQ
quail TSH	-----L-----
rat TSH	F-I-T--MMY-DR-----T-----Y-----I--LF-P-Y---D---RDFT-R
chicken LH	LGGGGRPP-R-INV-VA---DG-PQ-M-VT--A-G-Y-R--EPVYR SPLGPPP-SA---GALR-E
rat LH	SRGPLRPL-R-VNA-LAA-NEF-PV-ITFT-S---Y-PSMVRVLP AALPPVP-P---R-LRFA
quail FSH	-ELTNI--A--RE--EM-ITV-A-W-S-Y-F---PVY- YPPVSSV-QI--F--VV-E
rat FSH	HS-ELTNI--S---E--RF-IS---W-E-Y-Y---LVY- DPAPRNT-K---F--LV-E
	70.....80.....90.....100.....110....
chicken TSH	TALIPGCPHHTIPYYSYPVAISCKCGKCNTDYSDCVHEKVRTNYCTKPQKLCNM
quail TSH	-----
rat TSH	-VE-----VA--F-----T--A-K-----TFYLGGSFG
chicken LH	RWALW---IGSD-RVLL---L--R-AR-PMAT---TVQGLGPAF-GA-GGFGGE
rat LH	SVRL---PGVD-IV-F---L--R--P-RLSS---GGPRTQPM-T-DL-HLPGLLLF
quail FSH	-VK---GD-PESF-----TG-H-ET-D--ST--TVRGLGPS--SFSHNGS-Q
rat FSH	-IRL---AR-SDSL-T---TE-H---DS-ST--TVRGLGPS--SFGEMKE

Fig. 1. Amino acid sequences of the mature peptides of the β subunits of TSH, LH and FSH from avian and mammalian species. Amino acids are numbered with the N-terminal residue of chicken TSH β as No. 1. Amino acids identical to those of chicken TSH β subunit are indicated by a hyphen (-). Gaps have been inserted to maximize homology. Reversed letters indicate the sequence of the synthetic peptide Tb3550. See [25] for rat TSH β , [26] for chicken LH β , [27] for rat LH β , [28] for quail FSH β and [29] for rat FSH β subunit sequences.

Fig. 2. Immunoblots of chicken anterior pituitary homogenate. Samples: M, molecular weight marker; lanes 1–4, whole anterior pituitary homogenate; lane 5, cephalic lobe homogenate; lane 6, caudal lobe homogenate. Stainings: lanes M and 1, Coomassie brilliant blue; lanes 2, 5 and 6, anti-Tb3550; lane 3, anti-Tb3550 pre-incubated with Tb3550 peptide; lane 4, non-immune rabbit serum.

amount of peptide Tb3550 was used as the antibody. The staining pattern with the absorbed antibody was quite similar to that with non-immune rabbit serum used as a control. For localization of the 18.2-kDa band in the pituitary, the band was detected in the cephalic lobe homogenate, but not in the caudal lobe homogenate.

Hormone specificity of anti-Tb3550 was examined by immunoblotting of highly purified chicken LH (USDA-cLH-I-3) and FSH (cFSH-I-1). As shown in Fig. 3, chicken LH was stained with anti-chicken LH serum but not with anti-Tb3550. Anti-Tb3550 did not stain chicken FSH either. We could not use anti-chicken FSH sera because of the limited supply from the distributor.

Immunohistochemistry

Immunoreactive cells stained with anti-Tb3550 were distributed in the ventral region of the cephalic lobe of the anterior pituitary (Fig. 4a). On the other hand, cells positive for anti-chicken LH were observed throughout the anterior pituitary gland (Fig. 4b).

Adjacent sagittal sections were each reacted with anti-Tb3550 (Fig. 4c) and anti-Tb3550 absorbed with the peptide Tb3550 (Fig. 4d). The cells

Fig. 3. Immunoblots of chicken pituitary glycoprotein hormone preparations. M: prestained molecular weight marker. Lane 1: USDA-cLH-I-3, stained with anti-chicken LH IRC-2 *Gunma*. Lane 2: USDA-cLH-I-3, stained with anti-Tb3550. Lane 3: USDA-cFSH-I-1, stained with anti-Tb3550. For lanes 1–3, 0.5 μ g of the each hormone preparation was used for electrophoresis.

positive for anti-Tb3550 were polygonal or ovoid in shape and their cell bodies were of various sizes. The positive staining disappeared when the

Fig. 4. Immunohistochemical staining of chicken anterior pituitary gland. Plates a and b, and c and d are each adjacent sections. Immunostained with anti-Tb3550 (a, c), anti-chicken LH IRC-2 *Gunma* (b) and anti-Tb3550 pre-incubated with Tb3550 peptide (d). a, b: $\times 40$; c, d: $\times 1,000$.

absorbed antibody was used instead of anti-Tb3550.

Discussion

The present study demonstrated that anti-Tb3550 specifically recognized chicken TSH β subunit. The disappearance of the 18.2-kDa band on the immunoblot with anti-Tb3550 antibody that had been incubated with peptide Tb3550 indicates that the peptide and the 18.2-kDa glycoprotein bind competitively to anti-Tb3550. Therefore, it is suggested that the 18.2-kDa protein contains the same amino acid sequence (or epitope) as the peptide, a sequence which is highly specific for chicken TSH β subunit. The disappearance of immuno-positive cells on the use of absorbed antibody supports the result of the immunoblot.

The antigenic peptide Tb3550 was designed for the antibody to show no cross-reaction to any chicken proteins with known amino acid sequences. Our results in immunohistochemistry and immunoblot provided evidence that this antibody does not recognize LH β , FSH β or the common α subunit, since they are present both in the cephalic and caudal lobes [17]. This conclusion is supported by the result that anti-Tb3550 did not bind either to purified chicken LH or FSH preparation on immunoblot.

Gonadotrophs have been found located both in the cephalic and caudal lobes of the avian anterior pituitary gland, whereas thyrotrophs are almost exclusively distributed in the cephalic lobe, as revealed by immunohistochemistry [17–20]. Nevertheless, these findings on thyrotrophs have been based on the use of anti-human, anti-bovine and anti-rat TSH antisera owing to the lack of anti-avian TSH antiserum. The use of heterologous antibodies has a disadvantage in that absorption tests are impossible without purified antigens, and hence one cannot confirm the specificity of the antibody to the antigen of the heterologous animal species. Production of a homologous antibody is therefore necessary for reliable results. Our immunohistochemical findings and immunoblot data obtained with the homologous antibody to chicken TSH β revealed localization of immuno-positive staining restricted to the cephalic lobe, thereby confirming the previous findings with heterologous antibodies on TSH localization in the

pituitary.

Amino acids of gonadotropin and TSH β subunits corresponding to the region 32–51 between Cys-31 and -52 are considered to form a loop structure and be involved in receptor binding in various animal species [21]. Some amino acids (Thr-33, Arg-34, Tyr-38 and Pro-43) in this region of chicken LH β subunit are common in rat FSH β but not in rat LH β subunit. This partial homology of chicken LH β and rat FSH β has been considered to explain experiment results showing that chicken LH acts as FSH in mammalian gonads *in vitro* [22, 23], which is of comparative endocrinological interest [24]. The corresponding region of chicken TSH β subunit also contains some amino acids (Thr-33, Arg-34, Asp-35, Lys-39) in common with rat FSH β subunit, and thus chicken TSH might exert FSH-like effects if it were administered to mammalian gonads *in vitro*. Since Anti-Tb3550 was produced against this receptor-binding region, it might be useful as a tool in endocrine studies for an epitope mapping of this specific region.

In addition, this antibody should be helpful for a specific immunoassay of chicken TSH based on a competitive binding principle with iodine-labeled peptide Tb3550 or a non-competitive binding principle in combination with an antibody to common α subunit. Work is proceeding in our laboratories to establish a highly specific immunoassay to distinguish TSH, LH and FSH in the chicken serum with the use of specific, anti-peptide antibodies to common α subunit and respective β subunits.

In conclusion, we raised an antibody that specifically recognizes chicken TSH β subunit. This antibody proved to be useful for immunohistochemistry and immunoblotting. It should be an important tool in the study of avian TSH, and should also be profitable for tracing immunological TSH activity during the purification of TSH, the only avian pituitary hormone that has yet to be purified and characterized.

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