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Thyroid-stimulating hormone (TSH): Measurement of intracellular, secreted, and circulating hormone in *Xenopus laevis* and *Xenopus tropicalis*

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ABSTRACT

Thyroid-stimulating hormone (TSH) is an important regulator of the hypothalamic–pituitary–thyroid (HPT) axis in *Xenopus laevis*. To evaluate the role of this hormone on developing tadpoles, immunologically-based Western blots and sandwich ELISAs were developed for measuring intracellular (within pituitaries), secreted (*ex vivo* pituitary culture), and circulating (serum) amounts. Despite the small size of the tadpoles, these methods were able to easily measure intracellular and secreted TSH, and circulating TSH was measurable in situations where high levels were induced. The method was validated after obtaining a highly purified and enriched TSH sample using anti-TSH-β antibodies conjugated to magnetic beads. Subsequent mass-spectrometric analysis of the bands from SDS–PAGE and Western procedures identified the presence of amino acid sequences corresponding to TSH subunits. The purified sample was also used to prepare standard curves for quantitative analysis. The Western and ELISA methods had limits of detection in the low nanogram range. While the majority of the developmental work for these methods was done with *X. laevis*, the methods also detected TSH in *Xenopus tropicalis*. To our knowledge this is the first report of a specific detection method for TSH in these species, and the first to measure circulating TSH in amphibians. Examples of the utility of the methods include measuring a gradual increase in pituitary TSH at key stages of development, peaking at stages 58–62; the suppression of TSH secretion from cultured pituitaries in the presence of thyroid hormone (T4); and increases in serum TSH following thyroidectomy.

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1. Introduction

Xenopus laevis is widely used as a model species for studying the hypothalamic–pituitary–thyroid (HPT) axis due to the ease of observation of thyroid hormone (TH) regulated metamorphic events [3,7,8,12]. The traditional model for the HPT axis in mature organisms suggests that as TH levels rise or fall, TSH levels decrease or increase, respectively. However, the model appears to be more complex during the TH-dependent stages of metamorphosis when circulating TSH and TH levels seem to rise concurrently. Methods for measuring the circulating thyroid hormones, thyroxine and triiodothyronine (T4 and T3, respectively), in this species have been developed [23]. Another major regulator of the HPT axis is TSH. Although measurements in *X. laevis* have not directly confirmed this simultaneous rise in TSH, it is hypothesized to be true based on measurements of pituitary TSH mRNA expression [3,12,18] and somewhat indirect bioassay methods [7]. Additional TSH measurements in *X. laevis* have come from immunologically-based assays developed in other species. For example; Boorse and Denver [1] used antibodies directed against human TSH in a Western blotting procedure; Ogawa et al. [16] were able to identify TSH producing cells in pituitaries with anti-bovine TSH antisera; Bray and Sicard [2] measured TSH in crude fractions and whole tadpoles using an unspecified commercially available radioimmunoassay.

Prior to this study, a robust procedure designed specifically for measuring TSH in this species did not exist. The inability to measure TSH within the pituitary and its rate of secretion, as well as the circulating levels in the serum, limited the ability to more fully elucidate the feedback controls and compensatory mechanisms of the HPT axis. Therefore, to gain a greater understanding of the role of TSH, we set out to develop sensitive immunoassays for TSH in *X. laevis*. We also tested these assays in *Xenopus tropicalis*, a closely related species that is gaining importance in studies of amphibian development.

As in other species, *X. laevis* TSH is made up of alpha and beta subunits. The alpha subunit is common to other pituitary glycoproteins, but the beta subunit is unique to TSH [5,9]. Typical antibody

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production methods using purified TSH-β protein are somewhat impractical for this species due to the small size of the organism. However, Buckbinder and Brown [3] have reported the mRNA sequence of *X. laevis* TSH-β (Accession No. L07618 [three other sequences exist for *X. laevis* TSH-β, but all have the same amino acid sequence: another mRNA BC094208.1, and two ESTs: CD302274 and CF547864.1]). Thus knowing the amino acid sequence, we based our immunoassay development on the approach of generating antibodies to likely peptide candidates. This approach was used successfully by Okada et al. [17] to develop an RIA for *Rana catesbeiana* TSH using a peptide from the C-terminal of the beta subunit. We began the development of these methods with the assumption that a peptide from the C-terminal sequence of TSH-β would be a candidate for the production of functional antibodies. Additionally, since we wanted to have the potential to develop a sensitive sandwich ELISA, we selected another peptide sequence that had the potential to be an exposed immunogenic site. We hypothesized that having two independent antibody recognition sites would increase the specificity and sensitivity of the assay.

2. Methods

2.1. Animals

Animals were obtained from in-house culture and are described in more detail by Degitz et al. [4]. Staging information was determined according to Nieuwkoop and Faber (NF) [14]. For thyroidectomy, tadpoles at NF stage 58 were anesthetized and the thyroid gland was removed using a forceps. The animals were allowed to recover in clean water and then returned to the culture unit. The serum was collected 72 h after thyroidectomy and compared to control animals that were not thyroidectomized.

2.2. Anti-TSH-β antibody production

Peptide synthesis, immunization, preliminary testing, and purification were performed by Chemicon International (Temecula, CA, USA). Antibodies to the C-terminal site of *X. laevis* TSH-β were made using the amino acid sequence: CTKPFEPQYLGFSNYIQ. Additionally, an internal peptide sequence expected to be a potential immunogenic site was selected: DPNLKEGLPKMLMSQKAC. Both peptides were conjugated to ovalbumin and used to immunize rabbits. Preliminary ELISAs with the antisera confirmed positive interactions with both the free and conjugated peptide for both sites. Affinity chromatography was then used to purify the immunoglobulins interacting with the peptides. Purified antibody preparations (0.3 and 0.65 mg protein/mL for the C-terminal and internal sites, respectively) were mixed with equal volumes of glycerol, divided into aliquots, and stored at −20 °C. A subsequent polyclonal antibody to the same C-terminal peptide conjugated to KLH was produced by Genscript Corp. (Piscataway, NJ, USA). Both C-terminal antibody preparations functioned similarly.

2.3. SDS–PAGE and Western blotting

Samples were prepared for SDS–PAGE according to the manufacturer's instructions for the type of gel being run. We used either precast Criterion 15% Tris–HCl gels or Criterion XT 12% Bis–Tris gels (Bio-Rad, Hercules, CA, USA), with 18–26 lanes per gel. The treatment buffers for these two gel systems are different, but both included a reducing agent and a 5 min heating step at 95 °C. The two systems produced essentially the same results. Media from whole pituitary explant cultures were generally collected at 24 or 48 h intervals for up to 144 h in culture and stored at −80 °C. Typically, after sample preparation in the treatment buffer, the equivalent of 5 μL of media was loaded per lane. Pituitaries were homogenized at the time of dissection by vortexing for 1 min in a small volume (15–30 μL) of the appropriate treatment buffer. These were stored at −80 °C until further processing. At the time of analysis, these samples were heated for 5 min at 95 °C, followed by dilution with additional 1× treatment buffer such that the equivalent of 0.05–0.005 pituitary was typically loaded per lane. The dilution was adjusted up or down based on the strength of the expected response. Serum samples were frozen at −80 °C after collection and then prepared with treatment buffer similar to above at the time of analysis. Because of the high protein content, the maximum amount of serum that could be loaded before the bands became distorted was the equivalent of 3 μL of serum. When processing a large number of samples, up to four gels were used at a time. In addition to the samples, pre-stained molecular weight markers (Bio-Rad, Precision Plus All Blue) were loaded onto the gels to aid in determining location, size, and blotting efficiency. If there was a desire to quantify the amount of TSH from the membranes, a "standard curve" was also included. This consisted of a dilution series from an aliquot of a pooled sample of media collected after culturing high TSH-producing pituitaries (i.e., collected from NF stage 58/59 tadpoles). The concentration of TSH in this pool was calibrated using a purified sample (see below).

After electrophoresis, the proteins were transferred onto 8 × 12 cm, 0.2 μm nitrocellulose membranes (Pierce, Rockford, IL, USA) using the HEP-1 semi-dry blotting system (Owl Separation Systems, Portsmouth, NH, USA). When processing a large number of samples, two gels were trimmed to include only the bottom half of the gel, which contained the area of interest around the TSH band. Transferring two gels onto one membrane allowed a greater number of samples to be analyzed at one time. Additionally, two membranes could be processed at a time in the transfer apparatus. Thus, up to 104 samples, minus standards and controls, could be analyzed at a time when using the 26-well gels. The transfer was performed at 300 mA/membrane for 1–2 h in 25 mM Tris/192 mM glycine (pH 8.3) and 15% methanol. After transfer, the membranes were treated with Qentix Signal Enhancement Reagent (Pierce) according to the manufacturer's directions.

2.4. TSH detection

Following the enhancement step, the membranes were blocked for 15 min in 30 mL of Starting Block T20 blocking buffer (Pierce). All membrane incubation steps were done on a rocking platform at room temperature, except the primary antibody incubation step which was done at 4 °C. Incubation with the primary antibody was done overnight with 30 mL of a 1:250 dilution of the purified antibody to the C-terminal TSH-β peptide in blocking buffer. The membranes were washed for 10 min in 50 mL wash buffer (25 mM Tris–Cl [pH 7.2], 150 mM NaCl, and 0.05% Tween-20). The wash step was repeated four more times. Next, the membranes were incubated for 1 h with 30 mL of a 1500-fold dilution of HRP-conjugated goat anti-rabbit secondary antibody (Pierce) in blocking buffer. The membranes were again washed five times, as described above. Finally, the membranes were incubated with 8 mL of Super Signal West Dura chemiluminescent substrate (Pierce) for 5 min. The membranes were briefly blotted on filter paper and enclosed in plastic wrap. Chemiluminescent images were captured with the Fluor-S Multi-Imager (Bio-Rad). The imager was equipped with a model 166d Tamron lens and was used in high resolution mode with a 40 mm zoom and maximum aperture. Exposure times were adjusted to capture sufficient light without saturating pixels, typically 15 min. The volume/area counts from an area representing the TSH band of each lane was quantified using Quantity One Software (Bio-Rad).