Specific Antibodies against Synthetic Oligopeptides of Eel GTH/TSH Subunits

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Specific antibodies were raised against Japanese eel gonadotropic hormone I/β (GTH I/β), thyroid-stimulating hormone β (TSHβ), and common α (GTH/TSHα) subunits using synthetic oligopeptides as antigens in order to examine GTH and TSH synthesis. The specificity of the antibodies was evaluated by Western blot and immunohistochemistry. By Western blot of eel pituitary, each antibody detected one specific band. The immunohistochemical results also indicate that GTH I and TSH can be reliably identified by these antibodies in eel. As details on the identity of eel GTH I have not been published, we attempted to identify these cells by subtraction of GTH I/β- and TSHβ cells from the entire population of GTH/TSHα positive cells. In chum salmon, the anti-GTH/TSHα positive cells which reacted with neither anti-salmon GTH I/β nor anti-human TSHβ (β−α+ cells) proved to be anti-salmon GTH I/β positive in an adjacent section. In early vitellogenic Japanese eels and mid-vitellogenic New Zealand longfinned eels from the wild, many β−α+ cells were observed. These results suggest that the β−α+ cells in Japanese eels are probably GTH I cells. The development and validation of these antibodies will be significant for future studies on the role of GTHs and TSH in Japanese eels.

Key words: gonadotropin, thyrotropin, synthetic peptide, immunohistochemistry, Anguilla japonica, Anguilla dieffenbachii

The Japanese eel Anguilla japonica is commercially important in the aquaculture industry of Japan. For stocking of farms, juveniles are caught from the wild, as artificial propagation of eels has not yet been established. Indeed, it is difficult to obtain mature eels for production of larvae, because adult eels have immature gonads when held in captivity. Moreover, gonadal development ceases when maturing eels are taken from the wild. As a result, hormone treatments are required to promote gonadal development. It has been reported that multiple injections of chum salmon pituitary homogenate (sPH), which contains much gonadotropic hormone (GTH), can induce oogenesis.1) In many cases, however, sPH-administration is not successful in inducing final oocyte maturation and spontaneous ovulation following vitellogenesis. This may conceivably be alleviated by using endogenous, rather than exogenous, heterologous, GTHs for that purpose. It is necessary to measure GTH plasma levels and cell contents. At present, it is not possible to quantify endogenous GTH levels after correcting for 'noise' due to cross-reaction of antibodies with salmon GTHs, contained in salmon pituitary homogenates.

In teleosts, two distinct GTHs, designated GTH I and GTH II have been isolated and characterized from several species. GTH I and GTH II, together with thyroid-stimulating hormone (TSH), belong to the GTH/TSH family, whose members consist of a common α subunit (GTH/TSHα) and a hormone-specific β subunit. The amino acid sequences for GTH/TSHα and GTH I/β are 70% and 67% identical in Japanese eel and chum salmon, respectively. Accordingly, as stated above it is highly likely that an antibody which is raised against purified eel GTH I/β will cross-react with salmon GTH I/β. Besides, the purification of eel GTH is particularly difficult because it is hard to obtain mature eels whose pituitaries contain much GTH. It was reported, however, that, by using synthetic oligopeptides for salmon-specific sequences as antigens, specific antibodies which did not show cross-reaction with eel pituitary could be generated against salmon GTH Iβ and GTH Iβ.2) Therefore, when employing synthetic oligopeptides for eel-specific sequences as antigens, specific antibodies, which distinguish between endogenous GTHs and exogenous, heterogeneous GTHs, can probably be obtained.

The cDNAs encoding Japanese eel GTH/TSHα and GTH I/β subunits were cloned and their nucleotide sequences determined.3) Furthermore, the nucleotide sequences of the cDNAs encoding TSHβ have been determined in both European4) and Japanese eels.5) Accordingly, the amino acid sequences of GTH/TSHα, GTH

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5 Nagae et al., in preparation.
IIβ, and TSHβ could be deduced. Recently, GTH IIβ cDNA was isolated from the Japanese eel pituitary, but its nucleotide and deduced amino acid sequences were not published. Therefore, we attempted to identify this hormone-producing cell-type by subtraction of GTH IIβ- and TSHβ-positive cells from the total GTH/TSHα-positive cell population using immunohistochemical techniques.

As stated above, the Japanese eels are relatively immature at the onset of the spawning migration, with gonadosomatic indices (GSI) ranging from about 2-4% in females. The New Zealand longfinned eels A. dieffenbachii are much more sexually developed at the beginning of the downstream spawning migration, with GSIs ranging from about 5-10% in females. Relatively advanced gonadal development, together with the ease of capture of migratory individuals directly from the wild, suggests that studies on A. dieffenbachii should add to our understanding of eel reproductive physiology.

The present paper reports on the generation and validation of specific antibodies which recognize eel GTH IIβ, TSHβ or GTH/TSHα using synthetic oligopeptides as antigens in order to examine changes in GTHs and TSH in the eel pituitary.

**Materials and Methods**

**Animals**

Cultivated female Japanese eels A. japonica (GSI 0.6-1.3%) were purchased from a commercial eel supplier, while females at the silver stage (GSI 1.4-2.8%) were captured in Takase River, Aomori prefecture.

Vitellogenic female New Zealand longfinned eels A. dieffenbachii (GSI 7.2-7.7%) were caught in Lake Ellesmere, New Zealand, as described in Lokman et al. 5.

Chum salmon Oncorhynchus keta, serving as positive controls in this study, were collected from the Yurappu River in September 1993.

Following terminal anesthesia in ethyl aminobenzoate, pituitaries were collected and frozen for Western blot analysis or fixed with Bouin-Hollande sublimate for immunohistochemistry (4-5 pituitaries per group).

**Peptide Synthesis and Immunization**

A computer analysis of the deduced amino acid sequences of Japanese eel GTH IIβ (eGTH IIβ), GTH/TSHα (eGTH/TSHα), and TSHβ (eTSHβ) subunits (Nagae et al.; in preparation) was employed for prediction of the secondary structure and the areas of hydrophilicity. One possible antigenic and specific site was chosen within each protein, corresponding to 16-20 amino acid residues in eGTH IIβ-(97-116), eTSHβ-(88-103), and eGTH/TSHα-(36-51), respectively (Table 1).

Oligopeptides were then synthesized, conjugated with bovine serum albumin (BSA), and used to immunize male New Zealand white rabbits as described in detail previously. 5 Sera were absorbed with an equal volume of 1% BSA in phosphate buffered saline (PBS; 10 mm sodium phosphate, 0.15 M sodium chloride, pH 7.5). Sera against eGTH IIβ and eTSHβ were re-absorbed with an equal volume of 10μg/ml salmon pituitary glycoprotein fraction (sPG). The absorbed antisera were used as the antipeptide antibodies.

### Table 1. Sequence of oligopeptides used for generation of antisera against eel glycoprotein hormones

<table>
<thead>
<tr>
<th>Hormone*</th>
<th>Synthetic region</th>
<th>Oligopeptide sequence**</th>
</tr>
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<tbody>
<tr>
<td>GTH IIβ</td>
<td>97–116</td>
<td>CAAQLRLPDFCMVRSALPA</td>
</tr>
<tr>
<td>TSHβ</td>
<td>88–103</td>
<td>CDPARDECTHSAADG</td>
</tr>
<tr>
<td>GTH/TSHα</td>
<td>36–51</td>
<td>CFSAAYTPLRSLKTM</td>
</tr>
</tbody>
</table>

* GTH: gonadotropin; TSH: thyrotropin.
** Amino acid sequence deduced from reference (Nagae, 3). Salmons et al. 4.
*** Amino acid sequences are given in the one-letter code.

**Immunostaining**

The specificity of the resulting antipeptide antibodies was tested by Western blot and immunohistochemical analyses, as described previously. For Western blotting, Japanese eel pituitary homogenate at the silver stage (ePH), sPH, and hCG (Sankyo-Zoki Co., Tokyo, Japan) were used. All electrophoreses were performed under reducing conditions with 10% 2-mercaptoethanol.

To distinguish anti-eGTH/TSHα positive cells (α cells) from anti-eGTH IIβ positive cells (GTH IIβ cells) or anti-eTSHβ positive cells (TSHβ cells) more definitively, double immunostaining was performed on pituitaries from each animal group. After color development to blue with a mixture of anti-eGTH IIβ and anti-eTSHβ, the steps from incubation with anti-eGTH/TSHα as the primary antibody onwards were performed again and stained to red. Alkaline phosphatase (AP; Funakosi Co., Tokyo, Japan) was used as the enzyme conjugated streptavidin. As the substrate for AP, Vector® Red and Vector® Blue (VECTOR Lab., CA, USA) were used to stain GTH/TSHα cells and both GTH IIβ and TSHβ cells, respectively.

To confirm the specificity of the immunostaining, anti-BSA was substituted for the primary antibody, or anti-eGTH IIβ-(97-116), anti-eTSHβ-(88-103), and anti-eGTH/TSHα-(36-51) were absorbed with corresponding oligopeptides (5 μg/0.1 ml antibody at working dilution). Adherent sections were stained with alcan blue (AB)-periodic acid Schiff (PAS)-orange G or with Gomori's aldehyde fuchsin (AF). For immunostaining chum salmon pituitary cell-types, anti-salmon GTH Iβ (sGTH Iβ), anti-salmon GTH IIβ (sGTH IIβ), and anti-human TSHβ (hTSHβ) (UCB Bioproducts) were used. Specificity of anti-sGTH Iβ and anti-sGTH IIβ were previously confirmed.

The identification of cell types in the pars distalis was based on histophysiological and immunohistochemical criteria described previously.

**Results**

**Western Blot Analysis**

Electrophoretic and Western blot analysis using anti-eGTH IIβ-(97-116), anti-eTSHβ-(88-103), and anti-eGTH/TSHα-(36-51) are shown in Fig. 1. By anti-eGTH IIβ, one specific band presumed GTH IIβ (17 kDa) was detected in ePH, but no specific bands were detected in sPH and hCG. By anti-eTSHβ, one specific band presumed TSHβ (17 kDa) was detected in ePH, but no specific bands were detected in sPH and hCG. By anti-eGTH/TSHα, one specific band presumed GTH/TSHα (19 kDa) was detected in ePH, one specific band presumed GTH/TSHα (19 kDa) was detected in ePH, one specific band presumed GTH/TSHα...
Fig. 1. SDS-PAGE gel stained with Coomassie brilliant blue (a) and Western blotting analysis (b-h) of Japanese eel pituitary homogenate (1), chum salmon pituitary homogenate (2), and human chorionic gonadotropin (3). Membranes were immunostained with anti-eGTH (b), anti-eGTH, absorbed with eGTH-(97-116) (c), anti-eTSH (d), anti-eTSH absorbed with eTSH-(88-103) (e), anti-eGTH/TSH (f), anti-eGTH/?TSH absorbed with eGTH/TSH-(36-51) (g), or anti-BSA (h), respectively. Positions of molecular weight markers, expressed in kilodaltons, are indicated to the left of the figure.

Fig. 2. Four consecutive sections from a pituitary gland of an untreated Japanese eel stained with anti-eGTH IIβ (a), anti-eTSHβ (b), anti-eGTH/TSHα (c), or aldehyde fuchsin (d). Right photographs (e–h) show their left-hand counterparts (a–d) at high magnification. Arrows indicate β negative α positive cells. Bar 25 μm.

Immunohistochemistry (Single Staining)

In cultivated eels, few GTH IIβ cells, only situated in the proximal pars distalis (PPD) were observed (Fig. 2a). These cells were mostly oval in shape, and stained intensely with AF (Fig. 2a, d, e, h) or faintly with both AB and PAS. In some eels, however, GTH IIβ cells appeared to be completely absent.

Subunit (around 22 kDa) was detected in sPH and one specific band presumed hCG (22 kDa) was detected in hCG. After absorption by oligopeptides, no specific bands were reacted. No specific bands were detected by anti-BSA.

Immunohistochemistry (Double Staining)

In maturing chum salmon, β − α+ cells stained red and corresponded to sGTH Iβ positive cells in adjacent sections (Fig. 3a, b).

In immature cultivated eels, β − α+ cells were mostly seen in the PPD (Fig. 4a), but only few violet-stained GTH IIβ cells were noted (Fig. 4a). A similar staining pattern

TSHβ cells, located in the rostral pars distalis (RPD), were mostly elongated in shape and they stained faintly with AF (Fig. 2b, d, f, h) or intensely with AB.

Anti-eGTH/TSHα positive cells were observed in both the PPD and RPD (Fig. 2c). These cells stained with AF and could be categorized into one of three cell-types. The first and second cell-types corresponded to anti-eGTH IIβ immunoreactive (Fig. 2a, c, e, g) and anti-eTSHβ positive cells (Fig. 2b, c, f, g) respectively. The third type comprised the cells which neither reacted with anti-eGTH IIβ nor with anti-eTSHβ (Fig. 2a, b, c, e, f, g). These cells were designated β negative α positive cells (β − α+ cells). The β − α+ cells were round or elongated in shape, and situated in the periphery of the glandular cords of the PPD (Fig. 2g). They stained slightly with anti-eGTH/TSHα and stained intensely with AF (Fig. 2c, d, g, h) or with AB and faintly with PAS.
was seen in pituitaries from wild, early vitellogenic silver eels (Fig. 4b).

In New Zealand eels (mid vitellogenesis), many GTH II β cells were situated in the central parts of the glandular cords of the PPD and at least an equivalent number of β−α+ cells were noted in the periphery of the same area (Fig. 4c). As opposed to the Japanese eel, GTH II β cells in A. dieffenbachii reacted with both AB and PAS, while β−α+ cells stained intensely with AB (Fig. 5a, b).

In control sections, using anti-BSA or primary antibodies absorbed by corresponding oligopeptides, positively staining cells were not observed (data not shown). Moreover, no red cells appeared after second staining, when NRS were used instead of anti-GTH/TSHα.

**Discussion**

The specificity of antibodies raised against synthetic oligopeptides of Japanese eel GTH II β, TSH β, and GTH/TSHα was evaluated by Western blot analysis and immunohistochemistry. There are two main arguments in favor of regarding these antibodies as specific for their respective subunits. Firstly, the molecular weights of the bands that immunoreacted with these antibodies fall within the range of sizes reported for other teleostean GTH II β,9−13 TSH β,14−17 and GTH/TSHα,9,18 respectively. Indeed, we detected one specific band in chum salmon PH with anti-eGTH/TSHα, whose molecular weight was the same as that of the α subunit (22 kDa) in chum salmon,9 previously determined by electrophoretic technique.

Secondly, these antibodies stained only their corresponding hormone-producing cells. To identify GTH cells, AF staining was performed because GTH/TSH-producing cells react with this stain.11 In eel, although GTH cells and TSH cells have similar staining properties,
it is often possible to distinguish these cell-types by light microscopy on the basis of their topographical separation. Thus, GTH cells are localized in PPD basophils, TSH cells in RPD basophils. In this study, anti-eGTH IIβ and anti-eTSHβ specifically reacted with PPD and RPD basophils, respectively. However, few GTH IIβ cells were observed in the RPD also. Anti-eGTH/TSHα is also immunohistochemically specific for GTH and TSH subunits on the following two grounds. Firstly, α cells were AF positive, and vice versa, in the Japanese eel. Secondly, anti-sGTH Iβ positive cells corresponded with β−α+ cells in chum salmon pituitary. It can be concluded that these antibodies have high specificity for their corresponding subunits, since our findings concerning some characteristics of the antigens, such as molecular weight, histochemical staining property, and localization, agree with previous reports.

Evaluation of the oligopeptide designs used in the present study deserves some consideration. The synthetic peptides for eGTH IIβ were intentionally chosen based on the lack of homology with chum salmon GTH IIβ. Overall, the β subunits of chum salmon GTH II and Japanese eel GTH II have 70% amino acid sequence identity. The antibody subsequently obtained, anti-eGTH IIβ, did not react with salmon GTH IIβ. Thus, it is confirmed that this antibody can distinguish between endogenous GTH II and exogenous salmonid GTHs.

From only three teleosts, the European eel, the Japanese eel, and the rainbow trout, the amino acid sequences of the TSHβ subunit have been determined. TSHβ sequences in Japanese and European eels are nearly identical and do not differ in the synthesized region. In contrast, the β subunit of eTSH and that of rainbow trout share only 53% of their amino acid sequence, and eTSHβ (88-103) and the corresponding region in rainbow trout just 47%. Accordingly, anti-eTSHβ did not react with salmonid TSH.

TSHs of teleosts have been substantially less investigated than those of mammals and GTHs of teleosts. This may be attributed to the relatively small amounts of TSH present in the pituitary glands in fish, and to the extensive structural similarities that exist between TSH and GTH in teleosts. These limitations have made it difficult to purify TSH and, hence, to generate antibodies against TSH. The present study revealed that anti-eTSHβ labeled specifically the RPD basophils, previously identified as TSH cells in the Japanese eel. Thus, anti-eTSHβ (88-103) will prove useful for studies on biological and chemical properties of eel TSH.

The region corresponding to the third synthetic oligopeptide evaluated here, eGTH/TSHα-(36-51), has 100% amino acid sequence identity with European eel, pike eel, common carp, mouse, rat, pig and human GTH/TSHα subunits. The amino acid sequence Gly44 to Lys49 within GTH/TSHα is part of a highly conserved region, although chum salmon α1 is different at positions 46 and 50, with substitutions of a glutamine for an arginine and an alanine for a threonine, respectively. Chum salmon α2 is also marginally different, namely at position 49, where a glutamine is substituted for a lysine. Nevertheless, anti-eGTH/TSHα reacted with anti-sGTH Iβ positive cells and anti-sGTH IIβ positive cells in chum salmon pituitary, suggesting that it is likely that this antibody can be used in many species.

In the present study, immunohistochemical techniques were adopted to identify GTH I cells in Japanese eels. Previously, Kawauchi et al. reported that GTH I and GTH II were contained in different cells in salmon. If assuming this also applies for the Japanese eel, then it may be possible to detect eel GTH I cells by the subtraction of GTH IIβ cells and TSH β cells from the total GTH/TSHα cell population. In fact, a similar strategy was performed in African catfish to observe GTH I cells. In our study, α cells, which reacted neither with anti-eGTH IIβ nor with anti-eTSHβ, were observed in the PPD.

Keeping in mind the antibody specificities, two possible interpretations concerning the identity of these β−α+ cells can be proposed. Firstly, like salmon, the β−α+ cells are GTH I cells. In salmonids, GTH I cells are present prior to gametogenesis and GTH II cells appear coincident with the onset of spermatogenesis and vitellogenesis and they
are distributed in the periphery and in the central parts, respectively, of the glandular cords of the PPD.20 In non-treated eels, which have previtellogenic gonads, many β-α" cells were found as opposed to only a few GTH II cells and they were localized in the periphery of the glandular cords in the PPD of New Zealand eel. These facts suggest that the β-α" cells are GTH I cells in eels also. Alternatively, the β-α" cells are GTH II cells that either contain α subunits only or fewer β- than α subunits. It has been reported that free α subunits exist in the pituitary of bullfrog27,28 and in the pituitary tumor of humans.29,30 Given that the β-α" cells were observed in wild silver eels, it appears that free α subunits are not reflecting aberrant hormone production.

In summary, we have generated three antibodies, that specifically recognize eel GTH II β, eel TSH β or eel GTH/ TSHα. These antibodies will be useful for development of quantitative assays, which, in combination with histochimical techniques, can be applied for detailed studies into the roles of GTHs in the Japanese eel. The analysis of one such study, employing artificially-induced maturation, is currently in progress.

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