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Abstract

A method for the purification of chinook salmon (*Oncorhynchus tshawytscha*) GH, which retains its biological activity, is described. The biological activity was investigated with an established radioreceptor assay using liver membranes from pregnant rabbits and bovine GH as standard and labelled hormone. The enrichment of the preparation was checked with electrophoresis (SDS-PAGE). Extraction and further steps were carried out using low molarity alkaline buffer (pH 8-10, M = 100 mM). Three chromatography steps were performed (Concanavalin-A sepharose, Bio-gel P60, DEAE). Ion exchange chromatography was performed under isocratic conditions (using a 50 cm column). Two isoforms (sGH1 and sGH2) were isolated. The purification yield is 0.7% compared to lyophilized pituitaries. The molecule is homogeneous in SDS-PAGE. Contamination by prolactin, gonadotrophin and corticotrophin is negligible (< 0.5%). It could be demonstrated that the biological activity of the preparation is maintained since this preparation stimulates the growth of juvenile trout (*Salmo gairdneri*) and binds specifically (35%) to trout liver membranes.

Introduction

To date, there has been little study of the endocrine mechanisms of growth in teleost fish. The development of radioimmunoassays for fish growth hormones during the last few years (Cook *et al.* 1983; Wagner and McKeown 1986; Bolton *et al.* 1986) should enable a better understanding of this process. Knowledge of both the circulating hormone levels and the receptors of these hormones are equally important. However, among the different GH's purified (Cook *et al.* 1983; Famer *et al.* 1976; Wagner *et al.* 1985; Specker *et al.* 1985; Kawauchi *et al.* 1986; Kishida *et al.* 1987) only that from the tilapia has been used in a homologous system to demonstrate the presence of receptors (Fryer 1979). Evidence that such receptors exist in other species of fish has necessitated the use of GH heterologues such as that used for tilapia (Fryer 1979; Fryer and Bern 1979) or bovine GH (Tarpey and Nicoll 1985). The absence of homologous studies on salmonid GH may be a reflection of the fact that the isolated peptides are weakly hydrophilic; Bolton *et al.* (1986) found it necessary to add Triton × 100 to dissolve chum salmon (*Oncorhynchus keta*) GH. It is possible that this hydrophobic property was acquired during their purification which involved 'harsh' techniques such as precipitation. Thus, our aim was to purify the growth hormone of the chinook salmon (*O. tsawytscha*) using relatively 'gentle' techniques to conserve the hydrophilic properties of the resulting GH, properties necessary for it to bind to its receptor and to demonstrate its biological activity.

Materials and methods

Pituitaries

Chinook salmon (*O. tshawytscha*) pituitary glands were collected (from adult females) at the Spring Creek hatcheries (Colombia River) of Washington State (U.S.A.) during the 1976 spawning season. They were frozen immediately in liquid nitrogen and maintained thus until lyophilisation.

Isolation

The purification protocol used was based on the method of Komourdjian and Idler (1979) with modifications. Purification took 5 days at 4°C, 1g of lyophilised pituitary powder was homogenized with a Dounce homogenizer (Kontes glass C.O.) in 70ml of ammonium bicarbonate buffer (100mM, pH 10, PMSF 0.1%) for 10 minutes. The homogenate was centrifuged at 40,000 × g for 45 minutes and the supernatant was retained; this step was repeated. The combined supernatants were applied to a column (3 × 18 cm) of Concanavalin A-Sepharose (Con-A) (Pharmacia) and eluted with Tris-HCl buffer (50mM NaCl, 150mM, $MgCl_2$ 1mM, $CaCl_2$ 1mM, $MnCl_2$ 1mM, pH 7.8, 20ml/hour). The Con-A eluent containing unbound material was concentrated to 15ml in an ultrafiltration cell (Amicon Diaflo, D = 76 mm, PM 10). The sample was applied directly to a column of Bio-gel P 60 (Biorad, 5 × 100 cm, 70ml/h) and eluted with ammonium bicarbonate buffer (100mM, pH 10). The 10,000 to 30,000 MW fraction was diluted with distilled water to 5mM and concentrated to 20ml with an ultrafiltration cell. The sample was applied to a column of DEAE Trisacryl M (IBF, 1.6 × 50 cm, 40ml/h) equilibrated with 500ml 5mM ammonium bicarbonate and eluted with 20mM buffer. The GH eluted as two peaks. Each peak was concentrated and dialyzed in 10mM ammonium bicarbonate with a Micro-Prodicon system (Bio-Molecular Dynamics, Prodimem PA-10), GH, at final concentration of 0.5 mg/ml, was lyophilized.

Characterisation

sGH was localized by comparison with bGH (NIH bGH B1) in SDS-PAGE electrophoresis (6.5–16% gradient) after denaturation with 2-mercaptoethanol (Eastman Kodak Co.) for 5 minutes at 100°C (Laemmli 1970). Homogeneity was studied on HPLC TSK 2000 analytical column (Toyo Soda M Co., 0.75 × 60 cm) with phosphate buffer (200mM, pH 7.5, 0.5 ml/min). Immunological properties of sGH_1 and sGH_2 were compared by Ouchterlony's (1967) gel diffusion technique. Antibodies anti sGH_2 (7302F) were obtained from New Zealand white rabbits injected intradermally with 100μg of sGH_2 every two weeks for three months; sGH was dissolved in 20μl of 10mM NaOH before mixing with 1ml of NaCl 0.9% – Freunds adjuvent (50/50) solution. Radioimmunoassay of a solution containing 3μg/ml of purified sGH were performed to estimate any contamination by sGTH (Breton *et al.* 1978), sPRL (Hirano *et al.* 1985) and ACTH (Sumpter and Donaldson 1986). For an immunocytochemical study, mature rainbow trout pituitaries were fixed in a Bouin Holland solution and serial sagittal sections (5μm) stained using the Cleveland Ricker Wolfe method (Gabe 1968). Adjacent sections were used to react with sGH antibody (dilution 1/100). The peroxidase-conjugated swine immunoglobulin anti-rabbit serum (Dako) was revealed with 4-chloro-1-naphthol (0.2 mg/ml in phosphate buffer 50mM).

Biological activity

To determine the best conditions of its extraction from pituitaries, sGH activity was tested in a mammalian radioreceptor assay (Gerasimo *et al.* 1979). At the end of purification, sGH binding activity was tested on trout liver membranes in excess (330μg of protein; Le Bail unpublished data) prepared using the Fryer method (1979), bGH and sGH were iodinated with ^{125}I using the modified chloramine T method (Kelly *et al.* 1979). ^{125}I labelled protein was separated from unreacted iodine by gel filtration on a ACA-54 column (0.5 × 20 cm) using phosphate buffer (100mM, pH 7.5) with

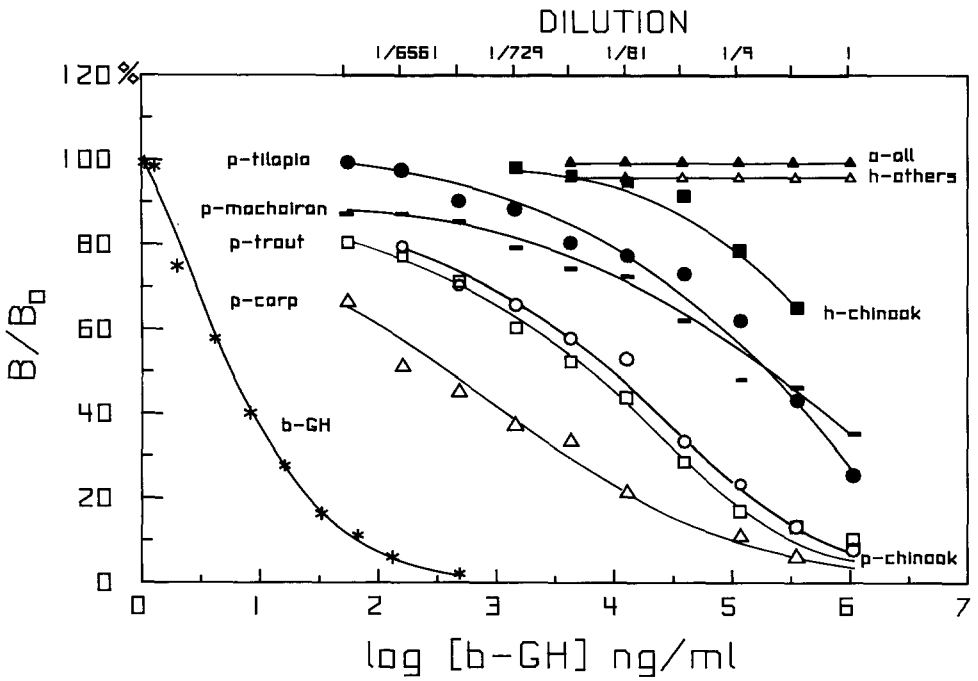

Fig. 1. Representative mammalian radioreceptorassay (RRA): dose-response inhibition curves for bovine growth hormone (bGH) and serial dilutions of crude pituitary (p), hypothalamus (h) and optic lobe (o) extracts from carp (*Cyprinus carpio*), rainbow trout (*Salmo gairdneri*), chinook salmon (*Oncorhynchus tshawytscha*), machoiran (*Chrysichthys nigrodigitatus*) and tilapia (*Oreochromis niloticus*). Dilution 1 corresponds to the part of brain diluted in one milliliter of buffer.

BSA (0.5%) and NaCl (9%). The specific activity was estimated (about 60 μci/μg) from the rate of iodine incorporation in GH. Liver membranes were incubated with 20,000 cpm of GH for 16h at 4°C in the same buffer. One μg of GH was added to determine the nonspecific binding. Bound and free hormone were separated by the addition of 3 ml of assay buffer (Tris-HCL 20 mM, $MgCl_2$ 10mM, BSA 0.5%, pH 7.5) and centrifugation at 3,000 × g for 10 minutes. The supernatant was decanted and the ^{125}I labelled GH, bound to the membrane pellet, was counted in an automatic gamma counter (Packard Multi-Prias 2). Different parts of brain (optic lobes, hypothalamus and pituitary) from a range of species were extracted in 1ml of assay buffer. Serial dilutions of these initial extracts were tested with the mammalian radioreceptor assay. The species used were carp (*Cyprinus carpio*, 5 kg), rainbow trout (*Salmo gairdneri*, 2 kg), chinook salmon (*O. tshawytscha*), machoiran (*Chrysichthys nigrodigitatus*, 100 g), and tilapia (*Oreochromis niloticus*, 150 g).

A trout bioassay was performed on intact juvenile 1+ rainbow trout (21.48 ± 11.00 g, N = 27). The 3 groups of 9 fish, distinguished with different Alcian Blue spots (Societé chimique Pointed Girard S.A.), were maintained in the same tank (300 l) at 13°C for the duration of the experiment. Fish were fed *ad libitum* with pellets (Aqualim). Two days before injection and weighing, the fish were starved. Each week for 8 weeks fish either received an injection of NaCl 9% or NaCl 9% + sGH (0.1μg/g).

Results

Detection of the fish growth hormone (Fig. 1)

Optic lobe and hypothalamic extracts of all species studied did not cross react in the mammalian radioreceptor assay, the only exception being the hypothalamus of chinook salmon. Conversely, all pituitary extracts displaced bovine GH from its

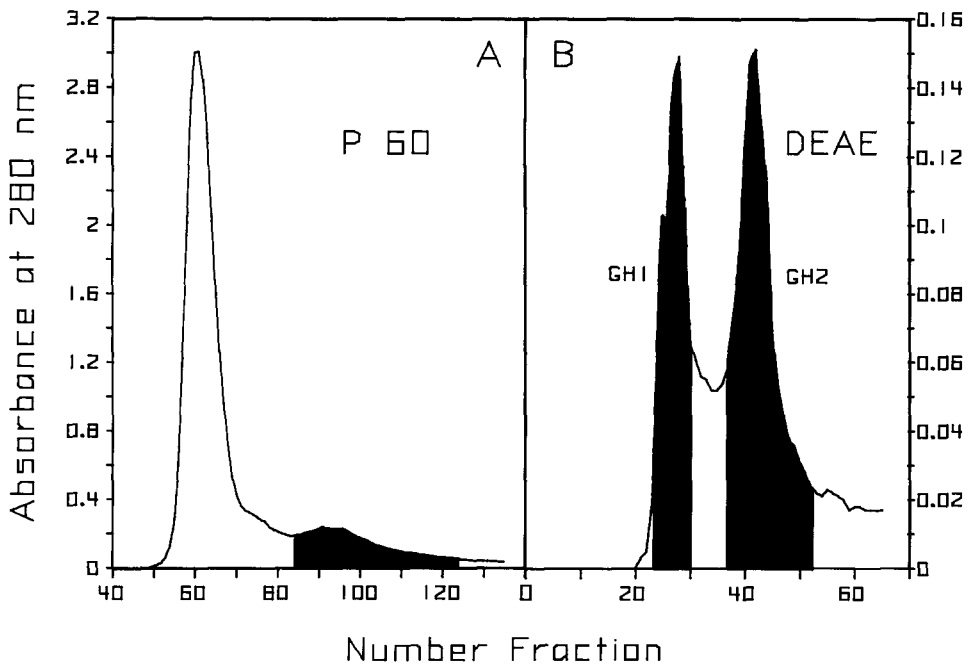

Fig. 2. Chromatography patterns for the purification of sGH. (A) Gel filtration Bio-gel P60 (5 × 100 cm) in 100mM ammonium bicarbonate buffer (pH 10). The second peak (in black) was diluted 5mM, concentrated to 20 ml with an ultrafiltration cell (Amicon Diaflo), then applied to the DEAE column. (B) Ion exchange chromatography of the concentrate on DEAE Trisacryl M (1,6 × 50 cm) with 5mM ammonium bicarbonate buffer (pH 10) and eluted with 20mM of the same buffer.

receptor. The gradient of the displacement curve was not as steep as that of standard curve of bGH. There was no difference between the gradients of the different fish species.

Using this radioreceptorassay and constant protein concentration, chinook pituitary extract obtained at pH 10 induced the best displacement (range of pH tested: 2–11; results not shown). An electrophoresis (SDS-PAGE) of this extract, has permitted to estimate at about 8% (Vernon photometer) the band comigrating with the heaviest bGH band. For this two reasons, this pH was used for sGH purification.

Table 1. Recovery yield at the different steps of sGH purification.

Steps	% recovery	Technique used
Lyophilized pituitaries	100	Lyophilized weight
Alkaline extract	48	Lowry *et al.* (1951)
Non glycoproteins	24	Lowry *et al.* (1951)
Proteins < 30,000 dalton	3.1	Lowry *et al.* (1951)
Purified sGH sGH_1	0.36	Lyophilized weight
sGH_2	0.34	Lyophilized weight

Purification

During the different steps of purification, sGH was identified by electrophoresis (SDS-PAGE).

When the non-glycoprotein fraction from Con-A was analysed chromatographically, using P60 gel, the void volume showed as a very large first peak (Fig. 2A). The second peak contained proteins having a MW between 30,000 and 10,000 daltons. The second peak corresponded to about 3% of the original lyophilized pituitary weight and displayed two distinct peaks using isocratic chromatography on DEAE (Fig. 2B) corresponding to sGH_1 and sGH_2, respectively. The yield of purification is 0.7% (Table 1).

Characterisation

With electrophoresis and after denaturation by

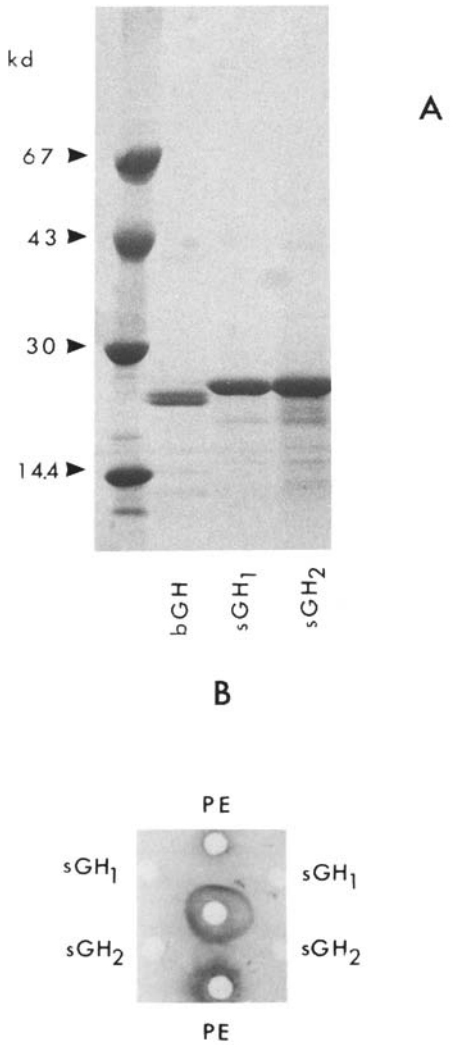

Fig. 3. (A) – Gel electrophoresis (SDS-PAGE) patterns of s-GH. (B) – Immunodiffusion of sGH_1, sGH_2 and pituitary extract (PE) against an antiserum raised in rabbit to sGH_2.

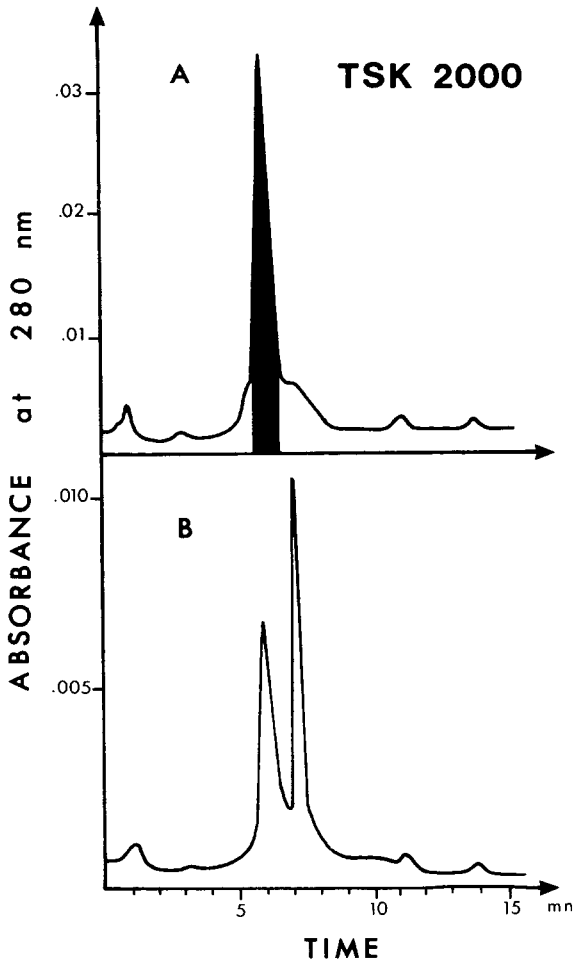

Fig. 4. Homogeneity of sGH. (A) Analytical chromatography was done on HPLC TSK 2000 column (0.75 × 60 cm) with 200 mM phosphate buffer pH 7.5 with a flow rate of 0.5 ml/min. After concentration, the major peak (in black) was applied for a second time on the same column under the same elution conditions (B).

mercaptoethanol, sGH_1 and sGH_2 had the same profile (Fig. 3A). The major band migrated with the heaviest bGH band and corresponded to the monomeric sGH. The minor bands had a MW between 24,000 and 14,000 daltons.

To test whether the minor bands were contaminants, sGH_2 was chromatography on a HPLC TSK 2000 column (Fig. 4A). The largest peak, when concentrated and rechromatographed in the same conditions, showed eluent peaks at the same level as those obtained after the first chromatography (Fig. 4B). Two of them were dominant, the heaviest form corresponding to the major peak of the first chromatography.

Immunodiffusion studies (Fig. 3B) showed that the sGH_1 and sGH_2 were immunologically identical, the precipitation arcs being perfectly continuous.

Purity

By using radioimmunoassays for different pituitary

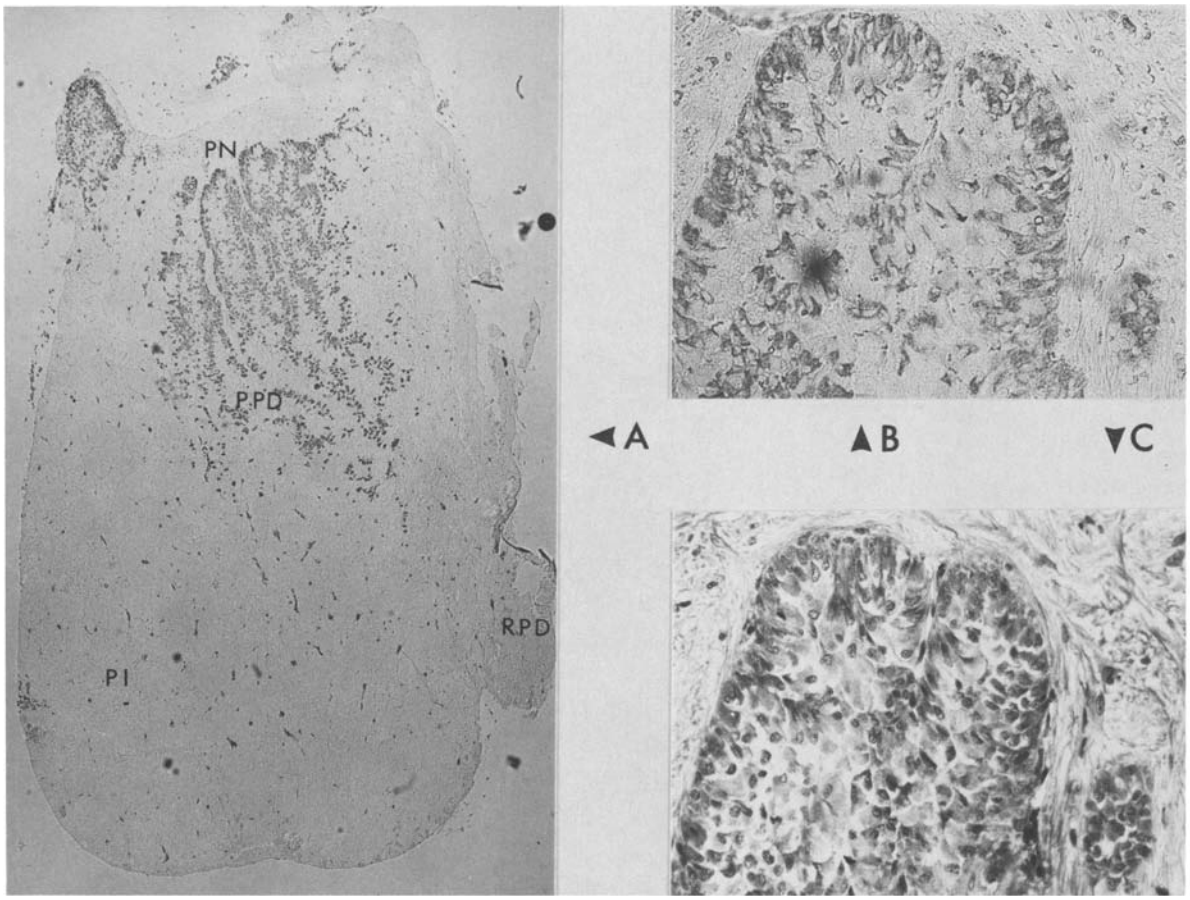

Fig. 5. Sagittal section of rainbow trout pituitary labelled with anti-chinook GH_2 rabbit serum (7302 F) at a dilution of 1/100. (A) RPD: rostral pars distalis, PPD: proximal pars distalis, PI: pars intermedia, PN: pars nervosa, (×30).

Adjacent sections of PPD were labelled with anti sGH_2 (7302F) (B) or stained using Cleveland Wolf method (somatotroph cells appear in black) (C).

hormones which were available for salmonids, it was estimated that contamination of the sGH_1 and sGH_2 respectively were : ≤ 0.2% and ≤ 0.06% for prolactin, ≤ 0.4% and ≤ 0.3% for gonadotrophin. ACTH contamination was only estimated for sGH_2 and this was undetectable, that is to say < 0.01%.

In immunocytochemical studies, a low dilution (1/100) of anti sGH_2 was used to show up any cross reactivity. The antibody did not bind to either the prolactin cells in the rostral pars distalis, or cells in the pars intermedia (Fig. 5A). In the proximal pars distalis, the anti sGH_2 binds only to the cell types (Fig. 5B) which showed up as orange (dark grey in the photography) using the Cleveland Wolf technique (Fig. 5C). These were found very specifically along the pars nervosa ramifications.

Biological activity

The effect of sGH on the growth of intact juvenile trout is shown in Fig. 6A. Weekly injections of sGH (0.1μg/g of live weight) induced an increased growth rate, which after 8 weeks was 61% greater compared to controls.

Lyophilised sGH_2 was soluble in phosphate buffer and 70% of the iodine linked with the hormone. After incubation for 24h at room temperature, the labelled sGH_2 showed a total binding of

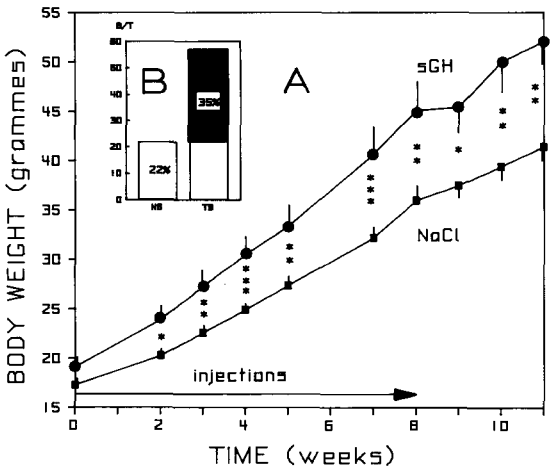

Fig. 6. Biological activity of sGH. (A) Effects of sGH on the growth of juvenile rainbow trout. Fish received one intraperitoneal injection each week at a dose of 0.1 μg/g of body weight during 2 months. Vertical lines represent the standard error of mean. Significant differences from controls (Student's t-test) are as follows: *(0.05), **(0.01), ***(0.005). (B) Binding of ^{125}I labelled sGH to trout liver membrane in excess expressed as a percentage of the total radioactivity. Specific binding (black bar) was determined by subtracting the nonspecific binding (NS = ^{125}I bound in the presence of 1μg of unlabelled sGH) from the total binding (TB).

57% with excess trout liver membranes (Fig. 6B). Non-specific binding appears relatively elevated (22%) but specific binding is much greater (35%).

Discussion

Fryer (1979) was the first to use a tilapia hepatic radioreceptor assay to evaluate the biological activity of teleostean GH; such an assay requires a source of purified tilapia GH. In this study we applied a method developed for mammals using bovine GH (bGH) which is readily available. The pituitary extracts from all the species tested (salmoniformes, cypriformes, siluriformes and perciformes) specifically displaced bGH from its receptor which suggests that this bioassay is applicable to all teleosts. Contrary to the other species for which the extracts were taken from fresh tissue, the hypothalamic extracts from chinook salmon came from lyophilized tissue in which pituitary material may have been present. This could explain the displacement obtained. The affinity of fish GH for rabbit receptors is weaker than that of bGH. This explains the loss of sensitivity of this technique and the impossibility to follow the increase in biological activity at the different purification steps. However, this technique allows to determine the best conditions of extraction of biologically active GH from pituitaries of different teleost species.

Before using the purification procedure presented in this article, preliminary investigations using other techniques such as precipitation, hydrophobic chromatography and coloured gel were studied, during which the GH became insoluble. Ionic strengths used in these experiments were high (> 500mM). As a results of these findings, subsequent experiments were carried out systematically at an alkaline pH, with buffer concentrations not exceeding 100mM. This enabled a totally soluble GH to be obtained with a purification yield (approximately 7mg/10g fresh pituitaries) greatly higher than that found by Wagner *et al.* (1986) and equivalent to that obtained by Kawauchi *et al.* (1986).

In opposition to the findings of Kawauchi *et al.* (1986), we were unable to establish any hydrophobic properties of the sGH. This may be due to the fact that there are differences in the primary structures of the GH of chum and chinook salmon. However the aminoacid sequence of the GHs of 3 salmonid species (Kawauchi *et al.* 1986; Agellon and Chen 1986; Nicoll *et al.* 1987), show that there is no difference between rainbow trout GH and chum salmon GH and the degree of homogeneity with the coho salmon is 97%. Thus, it seems that the hydrophobic or hydrophilic properties depend on the conditions of extraction and purification. It is evident however that the sGH is unstable as demonstrated by the effect of a simple HPLC rechromatography after concentration of the fractions. Similarly after electrophoresis (SDS-PAGE) of heavily loaded gels (*ca* 100μg sGH) several minor bands are seen in addition to the principal one. These are not due to contamination as the latter is negligible for gonadotrophin, prolactin and for molecules from the corticotrophin family. Using the less sensitive technique of immunoblotting (sensitive at 50ng) no TSH contamination was detected (< 5%) (Swanson, personal communication).

These forms could be due to a modification of the tertiary molecular structure (HPLC) or to a break in the polypeptidic chain (SDS-PAGE). Further, a low concentration of anti sGH_2 applied on histological sections of the trout pituitaries binds to only one cell type localized in the proximal pars distalis. The acidophil cells (stained by orange G) in proximal pars distalis are considered to be GH cells in mammals (Gabe 1968; Dubois and Herlant 1968) and also in fish (Ball 1965; Olivereau 1970; Wagner and McKeown 1983; Farbridge *et al.* 1985). Electron microscopy demonstrates that this antibody only binds to GH cell secretory granules (Dubourg, Kah and Le Bail, unpublished data). These results confirm that the chinook GH that was isolated is extremely pure.

With ion exchange chromatography, chinook salmon GH appeared as 2 forms, called sGH_1 and sGH_2 with respect to their elution speed. Their electrical charges are thus different. However, the MW estimated with electrophoresis is the same (23,000 daltons) and it was not possible to distinguish them immunologically. These two forms with a different electrical charge were also observed in the chum salmon (Wagner *et al.* 1985; Kawauchi *et al.* 1986). Equal amounts of sGH_1 and sGH_2 were purified from the same pituitaries. These two forms synthesized in the pituitary could be two genetic variations resulting from the duplication of the salmonid genome (Kawauchi *et al.* 1986).

Two techniques have been used to characterize the biological activity of the salmonid GH. In this study intraperitoneal injection of sGH in juvenile trout resulted in an appreciable weight gain (+61%), greater than that obtained by Wagner *et al.* (1985), but less than that obtained by Kawauchi *et al.* (1986). However such a comparison is not strictly valid since there are differences in the frequency of injection, quantities administered as well as factors involved in rearing of the fish (exteral factors, strains, etc.) which varied between one experiment and another. Thus it was considered that the percentage of hormone binding to the receptors when they were present in excess is a better indication of biological activity. The best percentage of specific binding (36% presented in our results) was obtained using labelled sGH just after iodination, it is 3 times more than that obtained with tilapia GH by Fryer (1979). This percentage of specific binding decreases with time, and after two weeks it is about 20% (result not shown). This is the first time that such binding has been established in a homologous system in salmonids, preceding results coming from a tilapia system (Fryer 1979; Fryer and Bern 1979).

The properties of our iodinated sGH and the specificity of our anti sGH suggest that further research could produce a radioimmunoassay system for measurement of circulating levels in salmonids as well as a better understanding of the properties their GH receptors.

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