Pergamon
0300-9629(94)00178-2

Comp. Biochem. Physiol. Vol. 110A, No. 4, pp. 335–340, 1995

0300-9629/95 $9.50+0.00

The use of recombinant gilthead sea bream (*Sparus aurata*) growth hormone for radioiodination and standard preparation in radioimmunoassay

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A gilthead sea bream growth hormone (sbGH) obtained by cloning and expression of sbGH cDNA was used to develop a sensitive and specific radioimmunoassay (RIA). Iodination of recombinant sbGH (rsbGH) was performed by the classical Chloramine-T method. Specific antiserum, raised in rabbits, was added in a final dilution of 1/36,000. The minimum detectable dose was 30 pg, and the midrange of the assay (ED_{50}) was 275 pg. Intra- and inter-assay coefficients of variation (CV) were 3.3 and 5.8% at ED_{50} levels. Human GH (hGH), ovine GH (oGH), carp gonadotropin (cGtH), chinook salmon gonadotropin (sGtH), ovine prolactin (oPRL) and recombinant tilapia prolactin (rtiPRL) did not show cross-reactivity. Serial dilutions of chinook salmon GH (sGH) and recombinant rainbow trout GH (rtGH) showed a low but significant cross-reactivity. A good parallelism between rsbGH standard and serial dilutions of native sbGH, plasma and pituitary extracts was observed. In addition, when plasma and pituitary samples were analyzed for GH quantification, non-significant differences were observed within this and previous RIA for native sbGH. Therefore, it appears conclusive that our rsbGH can be used successfully as a standard and radioiodinated hormone in GH assays for gilthead sea bream, which is extensively cultured in the Mediterranean area.

Key words: Radioimmunoassay; Rabbit antiserum; Recombinant growth hormone; Gilthead sea bream; Teleost; Fish.

Comp. Biochem. Physiol. 110A, 335–340, 1995.

Introduction

Growth hormone (GH), produced and secreted by the pituitary gland, is the main regulator of somatic growth (Isaksson *et al.*, 1987). GH excess results in gigantism, whereas GH deficiency leads to dwarfism; in either condition, normalization of GH secretion restores normal growth rate (Jansson *et al.*, 1986; Lamberts, 1988). Detectable amounts of GH and GH-binding sites have been demonstrated in gilthead sea bream (*Sparus aurata*) larvae a few days after hatching, which suggests a physiological role of GH during the early growth and development of fish species (Martí-Palanca and Pérez-Sánchez, 1994). Seasonal (Pérez-Sánchez *et al.*, 1994c) and nutritional (Pérez-Sánchez *et al.*, 1994a) changes in circulating GH and hepatic GH-binding have

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Received 31 May 1994; revised 22 September 1994; accepted 28 September 1994.

also been studied in older fish. Thus far, we have used as standard and tracer a gilthead sea bream GH (sbGH) purified from pituitary extracts (Le Bail *et al.*, 1993; Pérez-Sánchez *et al.*, 1994b). However, to facilitate further research into the action of GH and to avoid the long and tedious purification of pituitary hormones, we have cloned and expressed the sbGH cDNA. The resulting recombinant GH migrated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with an apparent molecular weight of 22 kDa, which is identical to that of the native sbGH and matches well with the full length of sbGH cDNA (Martínez-Barberá *et al.*, 1994). In this paper, we report the development of a sensitive and specific radioimmunoassay (RIA) for recombinant sbGH (rsbGH), which is on hand in a practically unlimited amount due to the availability of an expression system, together with a standardized purification process.

Materials and Methods

Hormones

sbGH cDNA was expressed in *E. coli* by using the pET-3a expression system. Induction was done by adding 0.1 M IPTG (isopropylthio-β-D-galactoside) to transformed cells. rsbGH stored as inclusion bodies was isolated after a brief sonication by solubilization in 6 M guanidinium HCl and further dialysis against 50 mM $(NH_4)HCO_3$ (pH = 7.8). Human GH (hGH), ovine GH (oGH), and ovine prolactin (oPRL) were obtained from the National Hormone and Pituitary Distribution Program (Baltimore, MD). Chinook salmon GH (sGH) was generously provided by Dr P-Y. Le Bail (Lab. Physiologie des Poissons, INRA, Rennes, France). Recombinant rainbow trout GH (rtGH), and recombinant tilapia prolactin (rtiPRL) were a gift of Dr J. Smal (Eurogentec, Liège, Belgium). Chinook salmon gonadotropin (sGtH) and carp gonadotropin were generously provided by Dr B. Breton (Lab. Physiologie des Poissons, INRA, Rennes, France). Recombinant human insulin-like growth factor-I (rhIGF-I) was generously provided by Dr K. Müller (Ciba-Geigy, Basel, Switzerland).

Hormone-specific antiserum

Female rabbits were immunized against rsbGH as follows: 200 μg rsbGH in 1 ml phosphate-buffer saline (PBS), emulsified in 1 ml complete Freund's adjuvant, were injected subcutaneously. One hundred micrograms of rsbGH in PBS and incomplete Freund's adjuvant were administered at 5, 8 and 11 weeks after the first injection. Seven days after the last booster, blood was collected from the ear vein weekly for a period of 3 weeks. After coagulation at 37°C for 2–3 hr, blood was centrifuged for 20 min at 3000 ***g*** to remove clotted cells. The clarified serum, whose titre did not change along the bleedings, was stored at −30°C until further use.

Radioiodination

The ^{125}I-labelling of rsbGH was carried out by the Chloramine-T method (Greenwood *et al.*, 1963) employing 0.6–0.7 mCi (3.7 GBq/ml) of radioisotope, 10 μg of hormone in 10 μl 0.3 M sodium phosphate buffer (1% NaCl, pH = 7.2), and 2.8 μg chloramine-T in 7 μl 0.06 M sodium phosphate buffer (0.2% NaCl, pH = 7.2). The reaction proceeded at room temperature for 3 min, then adding 2.8 μg sodium metabisulfite in 7 μl of 0.06 M phosphate buffer. Unreacted iodide was separated from labelled proteins by gel filtration in a Sephadex G-25 column (PD10, Pharmacia). The tracer was repurified through a Sephacryl S-200 column (1 × 100 cm), calibrated before use with Dextran Blue (2000 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), iodinated rhIGF-I (7.5 kDa), and vitamin B-12 (1.35 kDa). The column was eluted with 20 mM Tris–HCl (pH = 7.5), containing 0.15 M NaCl, 0.02% sodium azide and 0.3% bovine serum albumin (BSA), at a flow rate of 10 ml/hr. Iodinated rsbGH was stable for about 2 months when stored in glycerol (1:1) at −20°C.

RIA procedure

The assay was performed using a double-antibody method under disequilibrium conditions as previously described for native sbGH RIA (Le Bail *et al.*, 1993). Rabbit antiserum against rsbGH was added in a ratio of 1/24,000 to 200 μl of standard and unknown samples supplemented with non-immunized rabbit serum (1/800) in 50 mM Tris–HCl buffer (pH = 7.5), containing 10 mM $MgCl_2$ ($6 \cdot H_2O$), 0.05% sodium azide, and 1% BSA. After overnight incubation at 15°C, 100 μl of tracer (12,000 cpm) in 50 mM Tris–HCl buffer were added to each tube in a final volume of 300 μl, and incubation continued for 24 hr at 15°C. Precipitation of the antibody-bound hormone was made by adding 100 μl of sheep anti-rabbit gamma-globulin, diluted 1/5 in 50 mM Tris–HCl buffer (pH = 7.5) containing 10 mM $MgCl_2$ ($6 \cdot H_2O$), and 0.05% sodium azide. The incubation was prolonged overnight, and the assay was terminated by adding 3 ml of the last 50 mM Tris–HCl buffer. The tubes were then centrifuged for 45 min at 3000 ***g***. The supernatant was decanted and the radioactivity in the bound fraction was determined in a gamma counter.

The cross-reactivity of native sbGH, sGtH, cGtH, rtiPRL, oPRL, hGH, oGH, sGH and

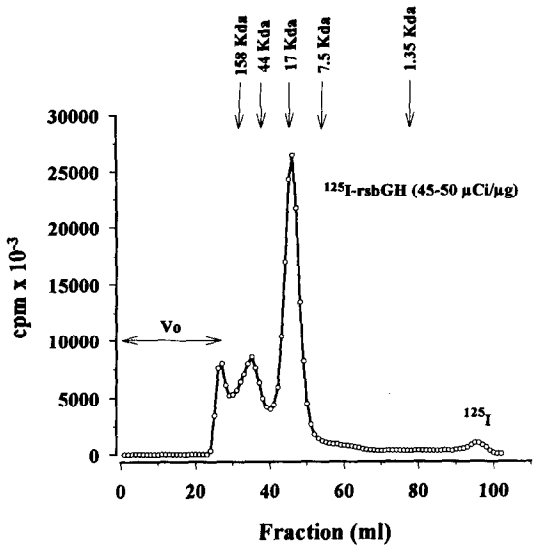

Fig. 1. Chromatographic profile of iodinated recombinant sbGH on Sephacryl S-200 column (1 × 100 cm); flow rate 10 ml/hr. Arrows indicate molecular weight markers, including bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), iodinated rhIGF-1 (7.5 kDa), and vitamin B-12 (1.35 kDa). Vo; void volume (volume of elution of Dextran Blue, 2000 kDa).

rtGH was examined. Paralellism between rsbGH and serial dilutions of plasma and pituitary extracts was also surveyed. To further validate the assay, GH concentrations in unknown plasma samples were determined by this and previous RIA for native sbGH. From January to December, 70 fish were sampled and anaesthetized with MS-222 (3-aminobenzoic acid ethyl ester; Sigma) (100 ppm). Blood was taken from caudal vessels, and plasma was removed by centrifugation at 3000 *g* for 30 min and kept frozen at −30°C until assayed.

Statistics

B/Bo values derived from serial dilutions of hormones, plasma and pituitary homogenates were converted to logits; the slopes were calculated for each set of points and then compared with the rsbGH standard curve by analysis of covariance. The slopes were considered to be significantly different if $P<0.05$. Linear regression was used to correlate plasma GH concentrations measured by native and recombinant sbGH RIAs. The data were analyzed by analysis of variance and the slope and the coefficient of regression did not differ significantly from 1 ($P<0.05$).

Results and Discussion

Figure 1 shows the chromatographic profile of recombinant sbGH following radioiodination and separation over a Sephacryl S-200 column. Reacted iodide was eluted as a large peak with an apparent molecular weight of 17–18 kDa, which corresponds well to the monomeric form of sbGH. Two minor peaks which could correspond to GH oligomers or other contaminant proteins were also detected (see Howanitz, 1993). The rsbGH antiserum precipitated up to 90–95% of the monomeric form (Fig. 2A), and only

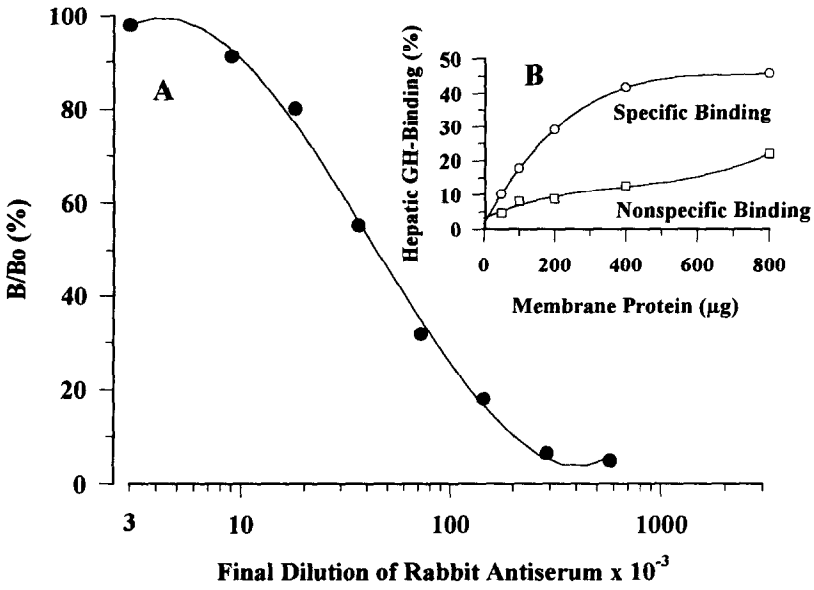

Fig. 2. (A) Precipitation of the monomeric recombinant sbGH (12,000 cpm) specifically bound to rabbit antiserum, diluted 1/36,000 in a final volume of 300 µl. (B) Binding of the monomeric recombinant sbGH (24,000 cpm) to increasing amounts of liver membrane preparations.

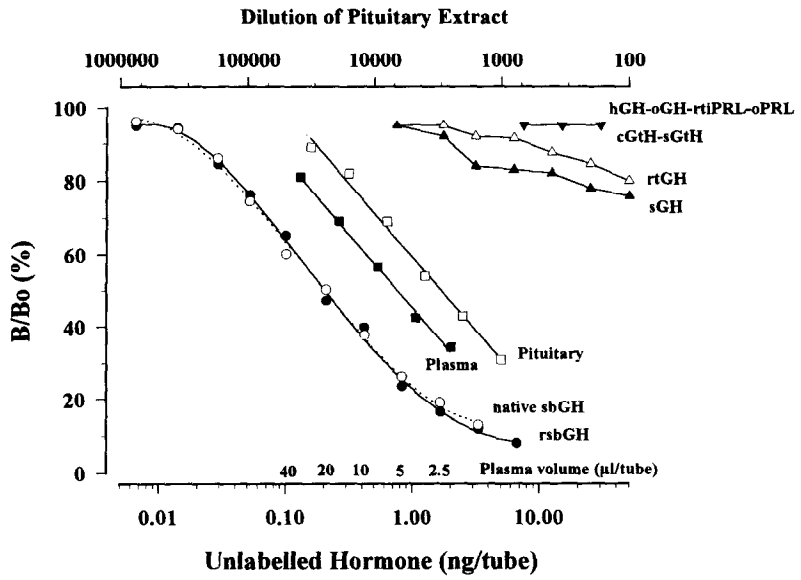

Fig. 3. Competitive displacement of labelled rsbGH bound to rabbit antiserum by pituitary hormones, and serial dilutions of plasma and pituitary extracts. Chinook salmon GH (sGH), recombinant trout GH (rtGH), human GH (hGH), ovine GH (oGH), ovine prolactin (oPRL), recombinant tilapia prolactin (rtiPRL), chinook salmon gonadotropin (sGtH), carp gonadotropin (cGtH). Initial dilution of pituitary extract = 1 pituitary/ml RIA buffer/100 g body weight.

8–10% of labelled proteins in the minor peaks (data not shown). To further determine the suitability of our iodinated GH fraction, we incubated a fixed amount of tracer with increasing amounts of liver membrane preparations, as it has been described for native sbGH (Pérez-Sánchez *et al.*, 1994b). In our experiments, up to 45% of iodinated hormone can be specifically bound to liver membranes (Fig. 2B), considered as the most important target tissue for the direct action of GH (Mendelshon, 1988). This value is similar to that established for sGH (Yao *et al.*, 1991), and higher than that found for GH preparations of coho salmon (Gray *et al.*, 1990), Japanese eel (Hirano, 1991), and tilapia (Ng *et al.*, 1992), in which the maximum specific binding ranged between 35 and 10% of total counts added.

Under our assay conditions, the radioactivity specifically bound by the antibody in the absence of unlabelled hormone (Bo) ranged between 30 and 35% of total counts added. Non-specific binding was up to 0.5% of total radioactivity added to each tube. The sensitivity of the assay, defined as the smallest amount of antigen distinguished with a 99% probability from the zero dose, was 30 pg. The mid-range of the assay (ED_{50}), calculated as the amount of rsbGH that displaces the 50% of labelled hormone bound to rabbit antiserum, was 275 ± 0.12 pg (mean $\pm$SEM, $N = 5$). This value is of the same order of magnitude than that established for tilapia (Farmer *et al.*, 1976), Japanese eel (Kishida and Hirano, 1988), chinook salmon (Le Bail *et al.*, 1991), and carp (Fine *et al.*, 1993) GH RIAs, and markedly lower than the value reported for chum salmon (Bolton *et al.*, 1986; Wagner and Mckeown, 1986) assays. A good performance was also shown when comparisons were made with human (Ribela *et al.*, 1993) and bovine GH assays (Hennies and Holtz, 1993). In addition, the precision of the assay was reasonably consistent when intra- and inter-assay coefficients of variation (CV) were considered. The intra-assay CVs for three plasma samples (1.3, 2.8, 5.6 ng/ml), assayed four times in a single assay, were 2.1, 3.3 and 3.5%, respectively. The corresponding inter-assay CVs, determined in three separate assays were 3.5, 5.8, and 8.5%, respectively.

Figure 3 illustrates the competitive displacement of rsbGH bound to rabbit antiserum. hGH, oGH, rtiPRL, oPRL, cGtH and sGtH did not cross-react in our rsbGH RIA. A significant displacement was observed with serial dilutions of sGH and rtGH, but only at concentrations 80–160-fold those of rsbGH. By contrast, when recombinant and native sbGH were used as reference preparations, a good superimposition between the two standard curves was observed. In agreement with these results, plasma and pituitary homogenates gave inhibition slopes that were not significantly different from the sbGH standard. Moreover, the regression curve obtained with the use of recombinant and native sbGH RIAs for the determination of GH levels

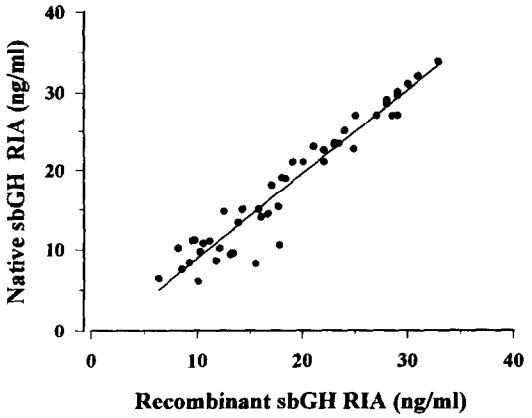

Fig. 4. Linear regression showing the comparison between recombinant and native sbGH RIA systems for the determination of GH concentration in 60 unknown plasma samples of gilthead sea bream. Coefficient of regression ($r = 0.95$); slope ($s = 1.06$).

in unknown plasma samples provides suitable evidence for the equivalence of measures in both RIA systems (Fig. 4). In fact, the coefficient of regression ($r = 0.95$) and the slope ($s = 1.06$) of linear regression did not differ significantly from 1 ($P < 0.05$). All this is quite relevant, especially considering that one of the main problems in immunoassay reagent preparation and standardization is the frequent existence of GH isoforms (Ekins, 1992). As has been indicated above, GH oligomers can be present in either the recombinant product or the pituitary extract, and may have quite different immunological activities.

In conclusion, from our results, it appears conclusive that rsbGH can be used for immunoassay reagent preparations. The availability of rsbGH in a practically unlimited amount facilitates further research into the action of GH, and avoids further extraction and purification from pituitary glands. In this regard, it is interesting to denote that up to date recombinant products have not been used to develop a specific GH immunoassay for a typical marine fish, such as gilthead sea bream which is successfully cultured in the Mediterranean area.

Acknowledgements—We thank Ciba Geigy and National Hormone and Pituitary distribution Program for the supply of rhIGF-I and mammalian pituitary hormones. We are grateful to Drs J. Smal, P.-Y. Le Bail, and B. Breton for the supply of fish pituitary hormones. This work is supported in part by a grant of Ministerio de Educacion y Ciencia, Spain (Mar 91-1020).

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