Recombinant somatolactin as a stable and bioactive protein in a cell culture bioassay: development and validation of a sensitive and reproducible radioimmunoassay

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Abstract

A recombinant somatolactin (SL) obtained by cloning and expression of sole SL cDNA was analyzed and used to develop a sensitive and specific RIA. In contrast to native proteins, which tend to dimerize and aggregate immediately after pituitary isolation, the majority of recombinant sole SL (rsSL) remained as a monomeric protein after long-term storage, as shown by size exclusion chromatography and Western blot. Using rsSL as a tracer and standard in the RIA, the minimum detectable dose and the midrange (ED_{50}) of the assay were 0·15 and 1·8–2·1 ng/ml respectively. Intra- and interassay coefficients of variation were 4·3% and 6·5% at ED_{50} levels. Recombinant gilthead sea bream GH and recombinant trout GH did not show cross-reactivity, whereas a good parallelism between rsSL standard and serial dilutions of plasma and sole pituitary extracts was observed. In order to demonstrate some biological activity of rsSL, the ability of this recombinant product to prime gilthead sea bream phagocytes for *in vitro* enhancement of mitochondrial activity was examined by a chromogenic assay. A bell-shape dose–response curve was obtained with a maximum at 50 nM (1·2 μg/ml), similar to that reported previously for GH. Therefore, taking together all these data, it appears conclusive that rsSL is a long-term stable protein which retains, at least in part, biological activity, providing a useful tool to clarify the physiological role of fish SL.

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Introduction

Somatolactin (SL), a new member of the growth hormone (GH) and prolactin (PRL) family, was initially isolated from Atlantic cod (*Gadus morhua*) pituitaries as a 26 kDa glycoprotein (Rand-Weaver *et al.* 1991*a*). The corresponding protein was subsequently isolated from the pituitaries of Japanese flounder (*Paralichthys olivaceus*) (Ono *et al.* 1990), coho salmon (*Oncorhynchus kisutch*) (Planas *et al.* 1992, Rand-Weaver *et al.* 1992, Lu *et al.* 1995), chum salmon (*Oncorhynchus keta*) (Kakizawa *et al.* 1993), gilthead sea bream (*Sparus aurata*) (Cavari *et al.* 1995), red drum (*Sciaenops ocellatus*) (Zhu & Thomas 1995), and halibut (*Hippoglossus hippoglossus*) (Johnson *et al.* 1997). In addition, SL clones have been isolated and characterized from cDNA libraries of Japanese flounder (Ono *et al.* 1990), Atlantic cod (Takayama *et al.* 1991*a*), chum salmon (Takayama *et al.* 1991*a*), lumpfish (*Cyclopterus lumpus*) (Iraqui *et al.* 1993), halibut (Iraqui *et al.* 1993), sole (*Solea senegalensis*) (Pendón *et al.* 1994) and gilthead sea bream (Astola *et al.* 1996), which indicates a high degree of homology (70–80%), with seven cysteine residues in the consensus sequence, six of which are involved in disulfide bonding.

Immunocytochemical studies indicate that SL-immunoreactive cells are located in the pars intermedia of teleostean and holostean fish (Rand-Weaver *et al.* 1991*b*, Kaneko *et al.* 1993, Parhar & Iwata 1994, Dores *et al.* 1996). These cells are periodic acid–Schiff positive in most species, but chromophobic in salmonids in which cloning and sequencing of cDNA SL shows a lack of N-glycosylation sites in the mature protein (Takayama *et al.* 1991*b*). The physicochemical and biochemical properties of SL are becoming, therefore, increasingly clear. Nevertheless, the formation of undesirable high molecular weight aggregates, due to aberrant interchain disulfide bonding and non-specific hydrophobic interactions, is the major problem limiting the final yield of monomeric SL. Recently, we have cloned and expressed the cDNA coding for sole SL (Pendón *et al.* 1996), and the aim of this work was to assess the suitability of the folding-purification procedure to obtain a bioactive and long-term stable

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protein preparation. The recombinant protein was analyzed by Western blot and size exclusion chromatography. In the absence of a well recognized bioassay, we examined the capacity of our recombinant sole SL (rsSL) for *in vitro* priming of gilthead sea bream phagocytes. Additionally, the use of this recombinant product as standard and radioligand in a sensitive and reproducible RIA is reported.

Materials and Methods

Animals

Blood and pituitary glands were taken in November from 2-year-old soles reared in running sea water under natural conditions of temperature and photoperiod at the fish farm of Cupimar (Cádiz, Spain). The animals were anesthetized with MS-222 (Sigma Chemical Company, St Louis, MO, USA), before killing by cervical section following CIOMS (Council for International Organizations of Medical Sciences) guidelines.

Hormones

Recombinant rainbow trout (*Oncorhynchus mykiss*) GH (rtGH) (lot no. BP19) was a gift of J Smal (Eurogentec, Liège, Belgium). Ovine GH (oGH) (lot no. AFP-9220A) was a gift of the National Institute of Health (NIH) and the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK) (Bethesda, MD, USA). Recombinant gilthead sea bream GH (rsbGH) was obtained as described elsewhere (Martínez-Barberá *et al.* 1994). rsSL, expressed in *E. coli* BL21 cells, was first solubilized in guanidinium hydrochloride (6 M) and 2-β-mercaptoethanol (1%). Renaturalization of the solubilized protein was performed by dialysis against 50 mM $(NH_4)HC0_3$ (pH 7·8). The resulting recombinant product was lyophilized, solubilized with 20 mM Tris–HCl (pH 8·0), and purified to homogeneity by gel filtration on a Superose 6 HR column (Pharmacia Fine Chemicals, Piscataway, NJ, USA) (Pendón *et al.* 1996). Fractions containing the monomeric form were pooled, and stored as a lyophilized powder for more than 1 year at − 30 °C.

Purity and stability

rsSL was dissolved in NaOH (10 mM), and 50 mM Tris–HCl buffer (pH 7·5) was rapidly added to reach a final protein concentration of 1–2 mg/ml. The resulting protein suspension was chromatographed through a Protein Pak 300 sw column (30 × 0·75 cm) (Waters Corporation, Milford, MA, USA), calibrated before use with molecular weight markers (Bio-Rad, Hercules, CA, USA). The column was eluted with 50 mM Tris–HCl buffer (pH 7·5), containing 0·1 M NaCl and 0·02% sodium azide, at a flow rate of 30 ml/h. oGH and rtGH were chromatographed for comparative purposes, and absorbance was monitored at 280 nm in an ultraviolet monitor (Pharmacia). rsSL was further characterized by SDS-PAGE and Western blot, using a rabbit antiserum (1/5000) raised in female rabbits. Immunostaining was carried out with a peroxidase-conjugate and 4-chloro-1-naphthol as chromogenic substrate (Sigma). Pituitary culture medium containing the native sole SL was used as a reference. For this purpose, isolated pituitary glands were incubated at 22 °C for 48 h in 96-well plates with 200 µl of complete medium: RPMI 1640 (Sigma), supplemented with 10% fetal calf serum (Serva, Heidelberg, Germany).

Hormone-specific antiserum

Female rabbits were immunized against rsSL as follows: 200 µg rsSL in 0·5 ml PBS, emulsified in 0·5 ml complete Freund's adjuvant, were injected subcutaneously. Two hundred micrograms of rsSL in PBS and incomplete Freund's adjuvant were administered at 3, 6, 9 and 12 weeks after the first injection. Seven days after the last booster, blood was collected from the ear vein. After coagulation at 37 °C for 2–3 h, blood was centrifuged for 20 min at 3000 ***g*** to remove clotted cells. The clarified serum was stored at − 30 °C until further use.

Radioiodination

rsSL was iodinated by the chloramine T method as described elsewhere (Pérez-Sánchez *et al.* 1994). Unreacted iodine was separated by gel filtration on a Sephadex G-25 column (PD10, Pharmacia). The tracer was repurified through a Sephacryl S-200 (Pharmacia) column (50 × 0·7 cm). The column was eluted with 20 mM Tris–HCl (pH 7·5), containing 0·15 M NaCl, 0·02% sodium azide and 0·3% BSA (RIA grade, Sigma), at a flow rate of 30 ml/h. Specific activity ranged between 50 and 70 µCi/µg. Iodinated rsSL was stored in 50% glycerol at − 20 °C, and was stable for more than 2 months.

RIA procedure

The assay was performed using a double-antibody method under disequilibrium conditions as previously described for native and recombinant gilthead sea bream GH (Le Bail *et al.* 1993, Martínez-Barberá *et al.* 1995). Rabbit antiserum against rsSL was added at an initial ratio of 1/125 000 to 200 µl of standard and unknown samples supplemented with non-immunized serum (1/500) in 50 mM Tris–HCl buffer (pH 7·5), containing 10 mM $MgCl_2$, 0·1% Triton X-100, 0·05% sodium azide and 1% BSA. After overnight incubation at 15 °C, 9000–10 000 c.p.m. of tracer in assay buffer were added to each tube, and incubation continued for 24 h at 15 °C. The antibody-bound hormone was precipitated by adding 100 µl of sheep anti-rabbit gamma globulin, diluted 1/5 in 50 mM Tris–HCl (pH 7·5) containing 10 mM $MgCl_2$ and

0·05% sodium azide. The incubation was continued 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 Packard gamma counter with an efficiency of 75%.

Bioactivity

To test the bioactivity of rsSL, an assay involving the cleavage of XTT (sodium 3′-[phenyl-amino-carbonyl-3,4-tetrazolium]-bis-(4-methyl-6-nitro)-benzene sulfonic acid hydrate) by gilthead sea bream phagocytes was used (Calduch-Giner *et al.* 1997). Briefly, head kidney was homogenized in PBS, and the resulting cell suspension allowed to settle for 30 min. The upper fraction was decanted from the undissociated tissue, centrifuged at 400 ***g*** for 15 min and resuspended in culture medium. The resulting cell suspension was further allowed to sediment for 15 min on ice. The cell concentration in the upper fraction was determined in a hemocytometer, and adjusted to 7–8 × 10^6 leukocytes/ml in the culture medium. The culture medium, isosmotic to gilthead sea bream serum (350 mOsm/kg, pH 7·2), was endotoxin free and consisted of Leibovitz medium (L-15, Sigma), supplemented with L-glutamine (0·3 g/l, Sigma), 0·1% fetal calf serum, 0·25 μg fungizone/ml, 100 IU potassium penicillin/ml and 100 IU streptomycin sulfate/ml (Serva).

The enriched leukocyte fraction was seeded in 96-well plates (100 μl/well), and incubated overnight at 24 °C in a humidified chamber. Non-adherent cells were discarded by several washings with PBS. The remaining cells (macrophages, 80%; neutrophils, 20%) were incubated in the culture medium (24 °C) with/without rsSL. After 5–6 h of priming culture, phagocytes were pulsed with an XTT solution (final concentration 0·3 mg/ml), which is cleaved by mitochondrial dehydrogenases to form an orange formazan dye. The yield color was measured 4–6 h later in an ELISA plate reader (Bio-Rad) at 495 nm with a reference wavelength of 650 nm. For comparative purposes, the effect of bacterial lipopolysaccharide (LPS, *Salmonella thyphimurium*, Sigma) on XTT cleavage was monitored.

Statistical analysis

The data were examined for significance by one-way ANOVA, followed by a Student–Newman–Keuls test. In the RIA procedure, curve parallelism between rsSL and serial dilutions of hormones, plasma and pituitary homogenates was tested by covariance analysis ($P<0·05$ was considered statistically significant).

Results and Discussion

The target organ(s) and physiological significance of fish SL remain uncertain, although a role in reproduction (Planas *et al.* 1992, Rand-Weaver *et al.* 1992, Olivereau & Rand-Weaver 1994), stress (Rand-Weaver *et al.* 1993), smoltification (Rand-Weaver & Swanson 1993), calcium regulation (Kakizawa *et al.* 1993, Kaneko & Hirano 1993), metabolic activity (Rand-Weaver *et al.* 1995) and background adaptation (Zhu & Thomas 1995) has been suggested, based on the factors which evoke changes in pituitary and circulating SL levels. At the present time, coho salmon (Rand-Weaver *et al.* 1992), chum salmon (Kakizawa *et al.* 1993), red drum (Zhu & Thomas 1995) and halibut (Johnson *et al.* 1997) SLs have been used to develop fish SL RIAs. However, in both these and other assays, a major problem is the tendency of purified SL to dimerize and aggregate rapidly following isolation.

The monomeric form of coho salmon SL increases transepithelial phosphate reabsorption by winter flounder (*Pleuronectes americanus*) renal tubules (Lu *et al.* 1995). A weak effect has been reported on coho salmon steroidogenesis, but this effect was only found within 24 h of SL purification from pituitary extracts (Planas *et al.* 1992). In fact, as has been indicated by Rand-Weaver and coworkers (1992), coho salmon SL can dimerize and aggregate even in the lyophilized state. In a similar way, Zhu & Thomas (1995) showed that red drum SL aggregates immediately after chromatographic separation, decreasing the recovery of the monomeric form with the storage of gel filtration fractions at 4 °C. Therefore, as indicated by those authors, the use of red drum and coho salmon SL in the RIA procedure is limited to a few days after purification. In contrast, as determined by size exclusion chromatography, up to 95% of rsSL is conserved after 1 year of storage as a monomer with an apparent molecular weight of 22–23 kDa (Fig. 1). Using an rsSL antiserum, a single immunoreactive band was found by Western blot following SDS-PAGE under non-reducing conditions. Moreover, as indicated previously (Pendón *et al.* 1996, 1998), the electrophoretic mobility of rsSL was equal to that reported for the native protein in its non-glycosylated form (Fig. 2). Nevertheless, different degrees of aggregation were observed when the expressed protein was solubilized in 10 mM NaOH before the addition of 20 mM Tris–HCl (pH 8·0) in the chromatographic purification procedure (data not shown). This undesirable effect was not found when the folding product was directly solubilized in Tris–HCl buffer, probably due to the low solubility of reactive folding intermediates at pH 8·0. However, when the purified rsSL (stored without reactive folding intermediates) was used for routine assays, it was first dissolved with 10 mM NaOH to ensure a total solubilization of the lyophilized protein.

rsSL remained stable after iodination, with a percentage of deiodination near to 10% after 1 month of storage. In agreement with this, using a 15-day-old tracer, up to 90% of ^{125}I-rsSL could be precipitated after overnight incubation with a high concentration of rsSL antiserum. To evaluate further the binding affinity of this antiserum, a

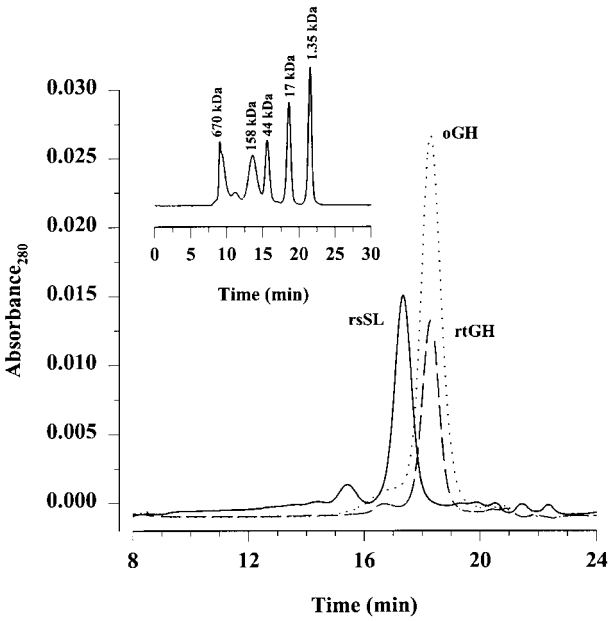

Figure 1 Chromatographic profile of oGH (2 mg/ml), rtGH (1 mg/ml), and rsSL (1·3 mg/ml) on a Pak 300 sw column (30 × 0·75 cm); loop 20 µl, flow rate 30 ml/h. The inset shows the chromatographic profile of molecular weight markers, including bovine thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B-12 (1·35 kDa).

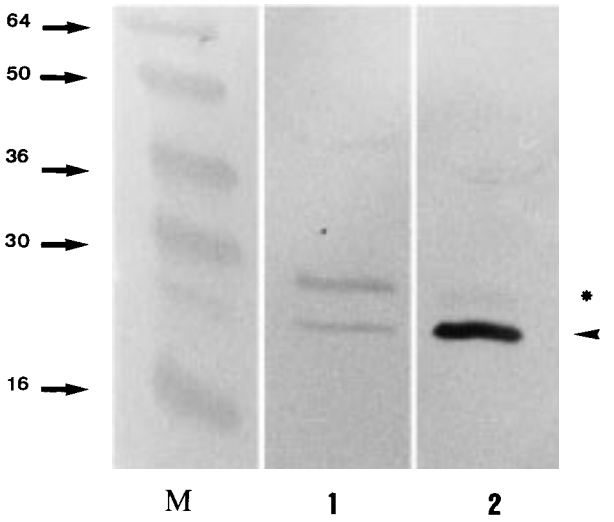

Figure 2 Immunoreactivity of native SL and rsSL with a polyclonal serum against rsSL. Lane M, protein markers (kDa). Note the presence of two different forms of native SL in the pituitary culture medium (lane 1) (the putative glycosylated form is shown by an asterisk). rsSL (lane 2) runs identically to the non-glycosylated form.

fixed amount of radioligand (9000 c.p.m.) was incubated overnight with serial dilutions of the antiserum (1/2000–1/4 000 000). The transformation Scatchard plot of data showed two major populations of SL-binding sites, with affinity constants of 7·5 and $1·3 \times 10^{10}$ l/mol respectively (Fig. 3). This high binding affinity shows that 50% of the tracer can be bound with a relatively high dilution of the antibody (1/125 000). Thus, in the RIA procedure, the antiserum was used at a final dilution of 1/500 000, which precipitates up to 40% of total counts added (9000–10 000). The non-specific binding was 1·5%, and the midrange of the assay (ED_{50}) was 1·8–2·1 ng/ml, which is significantly lower than that found in previous assays, in which the ED_{50} value was 3·1 ng/ml (Zhu & Thomas 1995), 6·4 ng/ml (Rand-Weaver *et al.* 1992), 11·6 ng/ml (Kakizawa *et al.* 1993) and 36·0 ng/ml (Johnson *et al.* 1997) for red drum, coho salmon, chum salmon and halibut SL RIAs respectively.

In our assay system, repeated determinations of the SL concentration in a plasma pool (2·4 ng/ml) gave intra- and interassay coefficients of variation of 4·3% (n=8) and 6·5% (n=5) respectively. The recovery of rsSL, calculated as the slope of the linear regression of the amount of SL added to plasma and that recovered, was near 100% over the entire range assayed (Fig. 4). Furthermore, as shown in Fig. 5, displacement curves resulting from serial dilutions of plasma and pituitary extracts were parallel to the standard curve. rtGH and rsbGH did not cross-react in the assay, while pituitary extracts from gilthead sea bream and sea bass (*Dicentrarchus labrax*) were recognized at relatively low concentrations. However, the resulting slopes did not parallel the standard curve (data not shown), probably due to differences in SL sequence between the different orders of bony fish. Therefore, taking into account all these results, it appears conclusive that rsSL provides a useful tool to develop a sensitive and specific assay to measure plasma and pituitary SL levels, avoiding the problems of dimerization and aggregation of the tracer and/or unlabeled hormone.

The demonstration of purity, identity and stability of rsSL monomer is not sufficient to prove biological activity because all of the chemical and physical aspects contributing to SL bioactivity are not known. An SL bioassay is needed, therefore, as an adjunct to liquid chromatography and SDS-PAGE. Some success has been reported with *in vitro* culture of red drum scales (Zhu & Thomas 1997), and primary monolayer cultures of flounder renal tubules (Lu *et al.* 1995). Nevertheless, the large dose (500 nM) required to elicit melanosome aggregation in the former, and the long and tedious procedure to quantify phosphate reabsorption in the latter (15 days) did not allow the development of a routine assay for biopotency measurement. In this context, it is of interest that both GH and PRL exert a direct effect upon mammalian leukocytes (Gala & Shevach 1993, Murphy *et al.* 1995), and we cannot exclude the possibility that a new member of the GH/PRL family would also exert some immunoregulatory actions. In this regard, it must be noted that the

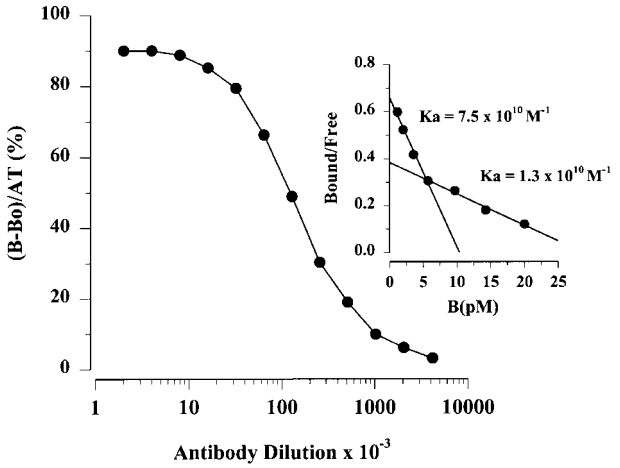

Figure 3 Binding of ^{125}I-rsSL (9000 c.p.m.) to serial dilutions of rsSL antiserum. Inset is a transformation Scatchard plot of data (Ka; affinity constant).

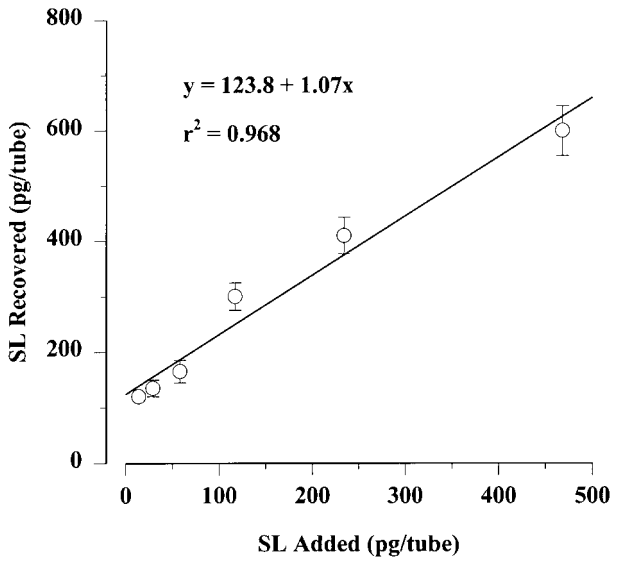

Figure 4 Recovery of rsSL added to 0·1 ml of a plasma pool. The level of SL in this plasma pool is around 1·1 ng/ml. Each value is the mean ± S.E.M. of two or three separate determinations.

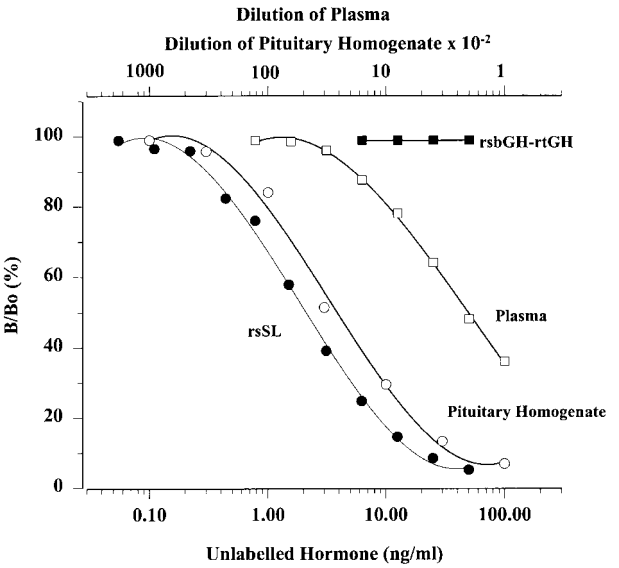

Figure 5 Competitive displacement of ^{125}I-rsSL bound to rabbit antiserum by pituitary hormones, and serial dilutions of plasma and sole pituitary homogenates. Initial dilution of pituitary homogenate: 1 ml RIA buffer/100 g body weight.

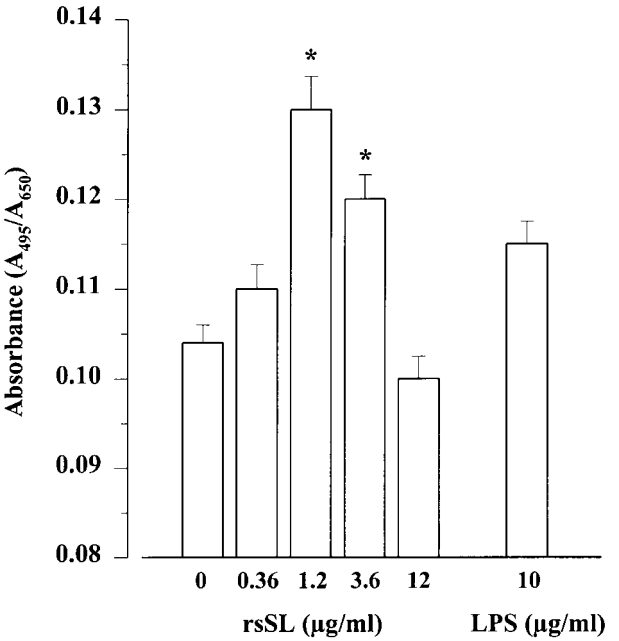

Figure 6 Representative graph of the effect of rsSL and LPS on *in vitro* XTT cleavage by gilthead sea bream phagocytes. Each value is the mean ± S.E.M. of four to six replicates (*P<0·05).

presence of GH receptors has been demonstrated in erythroid, lymphoid and myeloid cells of gilthead sea bream (Calduch-Giner *et al.* 1995). Furthermore, both GH and PRL are able to enhance the proliferative response of chum salmon leukocytes (Sakai *et al.* 1996*a*), as well as to prime trout phagocytes for *in vitro* superoxide anion release (Sakai *et al.* 1996*b*). In this last work, Sakai and coworkers failed to demonstrate any effect with chum salmon SL (10–100 ng/ml). However, with the increase of the dose range assayed (360–12 000 ng/ml), we found that rsSL is able to enhance the mitochondrial activity of gilthead sea bream phagocytes (Fig. 6). A bell-shape dose–response curve was obtained with a maximum at 1200 ng/ml (50 nM), similar to that established with rtGH (Calduch-Giner *et al.* 1997). Compared with rtGH, the minimum effective dose of rsSL is 5–10 fold higher, but the diminished response following LPS priming suggests

that the SL effect cannot be attributed to bacterial contaminant of our recombinant product. At the present time, it remains to be established whether this SL action is mediated by SL and/or GH/PRL receptors. In any case, this XTT assay seems to be suitable for monitoring SL bioactivity, in a manner similar to that proposed for GH. In fact, in comparison with a traditional weight gain bioassay, a less time-consuming and more accurate measure of GH biopotency is accomplished with cell proliferative assays, using stable clones of mouse myeloid cells transfected with a full length cDNA coding for GH receptors (Roswall *et al.* 1996).

In summary, in contrast to previously isolated pituitary SLs, rsSL is a protein with long-term stability which can be used to develop sensitive and easily reproducible assays. The capacity of rsSL to activate gilthead sea bream macrophages indicates that this protein retains, at least in part, biological activity. Therefore, both this and other recombinant products can provide a useful tool to further clarify the physiological role of fish SL.

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