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Cloning and Expression of Somatolactin, a Pituitary Hormone Related to Growth Hormone and Prolactin from Gilthead Seabream, *Sparus aurata*

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A pituitary hormone, somatolactin (SL), belonging to the GH/PRL family, is produced in the intermediate lobe of the teleost pituitary. The function of this protein is uncertain. Clones coding for SL were isolated and sequenced from a gilthead seabream pituitary cDNA expression library. The nucleotide sequence of the larger cDNA isolated was 1.5 kb containing a 0.8-kb 3′-untranslated region and two potential polyadenylation signals (AATAAA). The mature polypeptide is composed of 207 amino acids, and a signal peptide of 24 residues was also found in the SL precursor. A potential N-glycosylation site Asn-Lys-Thr was identified in gilthead seabream SL. A comparison of the SL amino acid sequences of several fishes indicated that seven cysteine residues are characteristically present in all the SLs so far isolated. Six of those residues are present in homologous positions in SL and GH *Sparus aurata* proteins. SL and GH from *S. aurata* showed a 43% homology at the nucleotide level and 22% identity at the amino acid level. Expression of recombinant SL (rSL) in *Escherichia coli* and isolation from inclusion bodies led to a monomeric form of SL identical in electrophoretic mobility to one of the two forms of the native SL secreted from gilthead seabream pituitaries cultured *in vitro.* Further, a native glycosylated modified SL secreted *in vitro* as shown by N-glycosidase treatment was identified. Specific anti-SL antibodies that discriminate well against gilthead seabream GH and PRL in immunoblotting were also raised against rSL.

Somatolactin is a recently discovered polypeptide produced from the pars intermedia of several teleosts. Its characterization by direct protein sequencing and complementary DNA (cDNA)[2] cloning reveals that it is a single-chain polypeptide structurally related to growth hormone (GH) and prolactin (PRL). Currently, the cDNA clones coding for the SLs of several fishes are known (Ono *et al.*, 1990; Takayama *et al.*, 1991a; Iraqi *et al.*, 1993; Pendón *et al.*, 1994). All these genes code for a single presomatolactin polypeptide of approximately 229 to 235 amino acids with 68 to 83% homology among the mature SLs. The function of SL is unknown, although involvements in environmental salinity adaptation (Wendelaar *et al.*, 1986), low osmolarity (Olivereau *et al.*, 1980), black background (Ball and Batten 1981), calcium regulation (Kaneko and Hirano, 1993), stress (Rand-Weaver *et al.*, 1993), and reproduction (Rand-Weaver *et al.*, 1992; Kawauchi *et al.*, 1991) have been suggested.

Sequence data from this article have been deposited with the GenBank / EMBL Data Libraries under Accession No. L49205.

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[2] Abbreviations used: SL, somatolactin; SDS, sodium dodecyl sulfate; kb, kilobases; cDNA, complementary DNA; sa, *Sparus aurata;* PAGE, polyacrylamide gel electrophoresis; kDa, kilodaltons; GH, growth hormone; PRL, prolactin; rSL, recombinant somatolactin.

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SL has been purified from the pituitaries of several species of fish, although in amounts too low for extensive biochemical characterization (Rand-Weaver *et al.*, 1991b; 1992). *Sparus aurata* is a well-studied fish from the Mediterranean sea. As a first step to study the function of this new pituitary hormone in the development of the gilthead seabream, a SL cDNA from *S. aurata* pituitary has been cloned and expressed.

The present report concerns the cDNA nucleotide sequence of SL from the gilthead seabream *S. aurata* (saSL) and the expression and purification to homogeneity of this hormone in *Escherichia coli.* It also shows that gilthead seabream native SL is secreted *in vitro* both in glycosylated and nonglycosylated forms.

MATERIALS AND METHODS

E. coli strain Y1090 was used as the bacterial host for cloning. Strain XL1Blue was used for subcloning, and BL21 (DE3) was used for SL cDNA expression in the pET-3a system. The enzymes used in this study were purchased from Pharmacia (Uppsala, Sweden) and Boehringer Mannheim Biochemicals (Germany). Yeast extract, bactotrypton, and bactoagar were purchased from Difco (Grand Island, NY); isopropyl-β-D-thiogalactopyranoside (IPTG) and restriction enzymes were purchased from Pharmacia. All other reagents were commercial preparations of analytical grade or of the highest purity available.

Cloning and Sequencing of Gilthead Seabream SL

Phage (10^5) from a λgt11 gilthead seabream pituitary cDNA expression library (Martínez-Barberá *et al.*, 1994) was screened with a *Solea senegalensis* SL cDNA probe (Pendón *et al.*, 1994) using published protocols (Sambrook *et al.*, 1989). Positive plaques were eluted and rescreened with the same probe until single plaques were identified. cDNAs were isolated from the recombinant phage DNA by *Pst*I–*Hin*dIII digestion and subcloned into the same sites of pBluescript SK(−) vector.

The complete cDNA was determined from the larger clone from both strands by sequencing overlapping restriction fragments. DNA sequencing was performed with T7 DNA polymerase (Pharmacia). Nucleic acid comparison with other SL genes applied the program BLASTN to the GenBank and EMBL databases.

Expression and Renaturation of Recombinant SL

Oligonucleotides designed to the 5′ and 3′ ends of the cDNA isolated were used by PCR to produce a full-length mature gilthead seabream SL protein. The primers contained specific sequences to facilitate the insertion of the PCR product in the *Nde*I–*Bam*HI sites of the pET-3a expression vector. First, *E. coli* strain DH5α was used to propagate the culture and then for expression the BL21(DE3) strain was used. Protein expression was induced with 0.5 m*M* IPTG when cultures reached an OD_{600} of 0.6. After 2–5 hr at 37°, bacteria were harvested, washed, and sonicated. After centrifugation at 12000 rpm for 20 min, the insoluble fraction was found by SDS–PAGE analysis to contain the expressed protein (Laemmli, 1970). Inclusion bodies were then isolated, solubilized in 6 *M* guanidinium hydrochloride containing 1% β-mercaptoethanol, and dialyzed for renaturation of rSL in 50 m*M* ammonium biocarbonate dialysis buffer, pH 7.8 (Puri *et al.*, 1991, 1992; Martínez-Barberá *et al.*, 1994).

SL Antibody Production and Western Blotting Analysis

Approximately 200 μg of renaturated rSL was injected into rabbits. Each animal was boosted twice at 3-week intervals before the serum was tested by immunoblotting. For immunodetection, extracts from uninduced *E. coli* culture cells, IPTG-induced cells, and inclusion body-isolated SL were separated by SDS–PAGE. The gels were transferred onto nitrocellulose membranes (Millipore, Bedford, MA) (Towbin *et al.*, 1979), blocked with 5% nonfat dry milk in TBS, and incubated with anti-SL serum (1:10,000 dilution in TBS). Bound antibodies were detected with goat anti-rabbit peroxidase-conjugated IgG (Boehringer Mannheim) and developed with α-chloronaphthol. Rabbit preimmune serum and gilthead seabream recombinant GH were used as controls in the immunoblotting analysis.

In Vitro Culture of Gilthead Seabream Pituitaries

Sparus aurata weighing 100–150 g were decapitated and individual pituitaries were removed. Cultures

were in 200 µl of complete medium (RPMI 1640 supplemented with 10% fetal calf serum) in the bottom of 96-well multiple plates. The pituitaries were cultured at 23° for several days. SL release to cultured pituitary medium was determined at 24-hr intervals by Western analysis using the anti-rSL serum. To identify a glycosylated form of native SL secreted from *in vitro* cultures of gilthead seabream pituitaries, culture medium containing SL was incubated at 37° for 16 hr with N-glycosidase (Sigma Chemicals, St. Louis, MO) at 10 units / ml. Proteins were further analyzed by SDS–PAGE and Western blotting.

RESULTS AND DISCUSSION

Cloning and Sequencing of SL from Gilthead Seabream

Sparus aurata SL cDNA was cloned and sequenced from a gilthead seabream pituitary expression library (Figs. 1 and 2). The probe used for the library screening was a PCR product obtained from a previously described sole cDNA clone (Pendón *et al.*, 1994). At the nucleotide level, the cDNA isolated showed a highly conserved sequence compared with other published fish SLs. The amino acid sequence of gilthead seabream SL is compared with other SLs deduced from different fish species in Fig. 3. Amino acid identities of *S. aurata* SL with other fish species are as follows: 83% with flounder, 79% with sole, 77% with lumpfish, 73% with chum salmon, and 68% with atlantic cod. This remarkable conservation of the SL sequence is higher than that observed for other pituitary hormones such as growth hormone (Takayama *et al.*, 1991b). The degree of homology seen in SLs is much greater than in GHs, where only 45% of the residues are invariant between fish species. On the other hand the sequence data show that SL is larger than GH in *S. aurata*, the most distinctive feature being the putative presence of three disulfide loops as described for other fish SLs. SL and GH in *S. aurata* share 42 strictly conserved amino acid residues and it seems likely that these are involved in maintaining the conformation of the proteins, assuming that they share a similar tertiary structure (Rand-Weaver and Kawauchi, 1993).

Expression and Isolation of a Recombinant SL

The SL protein was expressed in *E. coli* and, isolated from inclusion bodies, yielded approximately 15 mg per liter of culture. This rSL was used to generate an anti-SL serum that reacts with the native hormone (Fig. 4, lane 1). The 24-kDa rSL-expressed protein migrates in SDS–PAGE gels at a position identical to a nonglycosylated variant of native SL gilthead seabream as shown by immunoblotting (Fig. 4). As indicate in Fig. 5, *in vitro* cultures of pituitaries from *S. aurata* secreted both glycosylated and nonglycosylated SL forms. The results of the Western blot analyses suggest that the

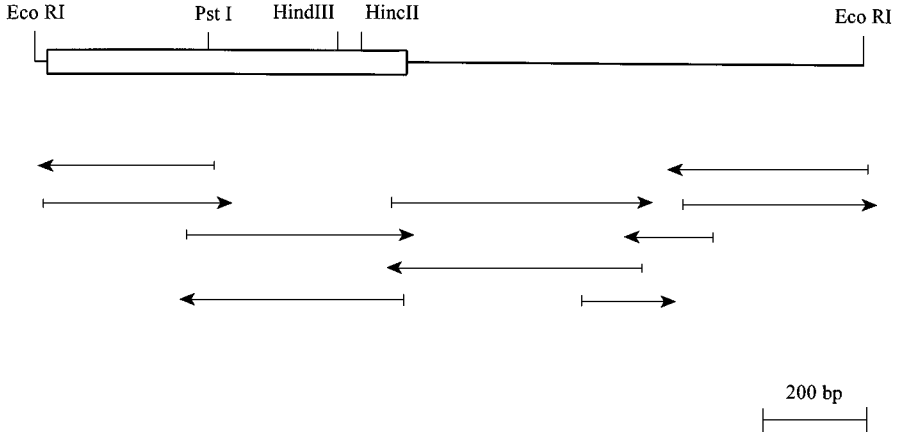

FIG. 1. Restriction map and sequencing strategy of a gilthead seabream Somatolactin cDNA clone. Restriction enzymes sites used for subcloning and sequencing of gene fragments are illustrated.

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ACAATGCGCATGATAAGAGTAATAAAGCAGGGTCAATGGGCTGTACTGCTCTGGCCCTAT    60
   MetArgMetIleArgValIleLysGlnGlyGlnTrpAlaValLeuLeuTrpProTyr    -19

CTGCTCACTGCGAGCATCCCACTGGACTGCAGGGACGAGCAGGGCGGCCTCTCCCACTGT   120
LeuLeuThrAlaSerIleProLeuAspCysArgAspGluGlnGlyGlyLeuSerHisCys    15

CCCTCTATCTCCCAGGAGAAACTTCTAGACCGAGTCATCCAGCATGCTGAGCTCATCTAC   180
ProSerIleSerGlnGluLysLeuLeuAspArgValIleGlnHisAlaGluLeuIleTyr    35

CGTGTCTCTGAAGAATCGTGCTCTTTGTTTGAGGAGATGTTCATCCCATTCCCACTGCAG   240
ArgValSerGluGluSerCysSerLeuPheGluGluMetPheIleProPheProLeuGln    55

CTTCAGAGGAACCAAGCTGGCTATCCCTGCATCACCAAAGCCTTACCCATCCCCAGCTCC   300
LeuGlnArgAsnGlnAlaGlyTyrProCysIleThrLysAlaLeuProIleProSerSer    75

AAAAGTGAAATCCAACAGATATCTGACAAATGGCTGCTTCATTCTGTGCTGATGCTGGTC   360
LysSerGluIleGlnGlnIleSerAspLysTrpLeuLeuHisSerValLeuMetLeuVal    95

CAGTCTTGGATCGAGCCTTTGGTCTACCTGCAGACCACACTGAACCGCTACGATGGAGTT   420
GlnSerTrpIleGluProLeuValTyrLeuGlnThrThrLeuAsnArgTyrAspGlyVal   115

CCTGACATGCTGCTCAACAAGACCAAGTGGGTGTCTGAGAAACTGATGAGTCTGGAGCAA   480
ProAspMetLeuLeuAsnLysThrLysTrpValSerGluLysLeuMetSerLeuGluGln   135

GGTGTGGTGGTCCTCATCAAGAAGATGCTGGACGAGGAAATGATGACCACAACGTACAGC   540
GlyValValValLeuIleLysLysMetLeuAspGluGluMetMetThrThrThrTyrSer   155

GAACAAGGCCTCTTCCAAGACGACGGGCAGCCCGAGATGCTGGAATACGTTATGAGAGAC   600
GluGlnGlyLeuPheGlnAspAspGlyGlnProGluMetLeuGluTyrValMetArgAsp   175

TACACCTTGCTCAGCTGCTTCAAGAAAGATGCCCACAAGATGGAGATTTTGCTCAAGCTT   660
TyrThrLeuLeuSerCysPheLysLysAspAlaHisLysMetGluIleLeuLeuLysLeu   195

CTCAAGTGTCGACAAAATGACATGCACAGCTGTCGATAAAACATCGAGTCGGGCTTTTGA   720
LeuLysCysArgGlnAsnAspMetHisSerCysArg *                         207

ATACGTGTTGTTTGGCTTTAAATAAATTCCTGGTAGCCGTCCACTTACAGATATGACCAT   780

GCCTCAGGCGGTTCAGCCTCGCTCGCAAAGCAGTACATTCTTTATTGATTGTTTTGGAAC   840

ACCTTCACACAGAAATAACTTTATATTTCCCTGCACAGTTCTTATTTTAACCTGGCAAAG   900

GCAACAGAGGGCAAAGTGGAAAAGATTATTTGTGTGTCGAGCTGTCAAAAAAATCTACAT   960

ATCTGCCATTGATTTCCATTTCCTTTGTTCTTAACTGGAGTTTGTATTCCTCGCTGGCTC  1020

TTGCAGTGTTTTGATTATTCCCGCGACCCCAGTGAGACCCCTCTTTCAAATGGAGCCGGT  1080

TTCACTTCTGCATTATTGAAATGAAACACTTTCACTGGAGACGGGAGTCAAACAGAGACT  1140

CACTACTTAACTTTGATTGGTGAAGATGAGTGTGCGAGAGACGGCGAGCGGAACAAATGG  1200

CAGTTAAAATTAATTATGTGGCGTTTTTGGTTCCACCCAGGTTTGGATTTGTTGAGTGCT  1260

CATGAAAGAGTCGTGAATTTTGCAGTGGCTTAACTCGCCTAATTAAACCTGAATGTAATA  1320

ACATGCGTTTATTATTTACTTTAAATGATACACAATTTGGATTTTTATGGCAAGATGATA  1380

TGAATATGAAATGCATAATGCAAACTTTCCTTTTCTGTACTCTCCTGTGTAAGTAGAATA  1440

GGCCCAGTGTGCTGTGAAAATCTTTTTGTCATCAATCACGAATGATTTTAGCCGCTAATA  1500

AAGCATGTTGTAGAAAAAAAAAA                                        1523
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FIG. 2. Complete amino acid sequence of Somatolactin from *Sparus aurata* deduced from a cDNA clone. Nucleotide sequence was obtained from overlapping clones covering both strands of the cDNA using T_3 and T_7 universal primers. Nucleotides (nt) are arranged with the Met codon ATG at nt 4 and the stop codon TAA (asterisk) at nt 697. The aa sequence is numbered beginning with the putative signal peptide of 24 aa and the coding sequence for mature saSL with Ile 1. A potential gycosylation site is shaded, seven cysteine residues are underlined, and two potential polyadenylation sites (AATAAA) are in boldface.

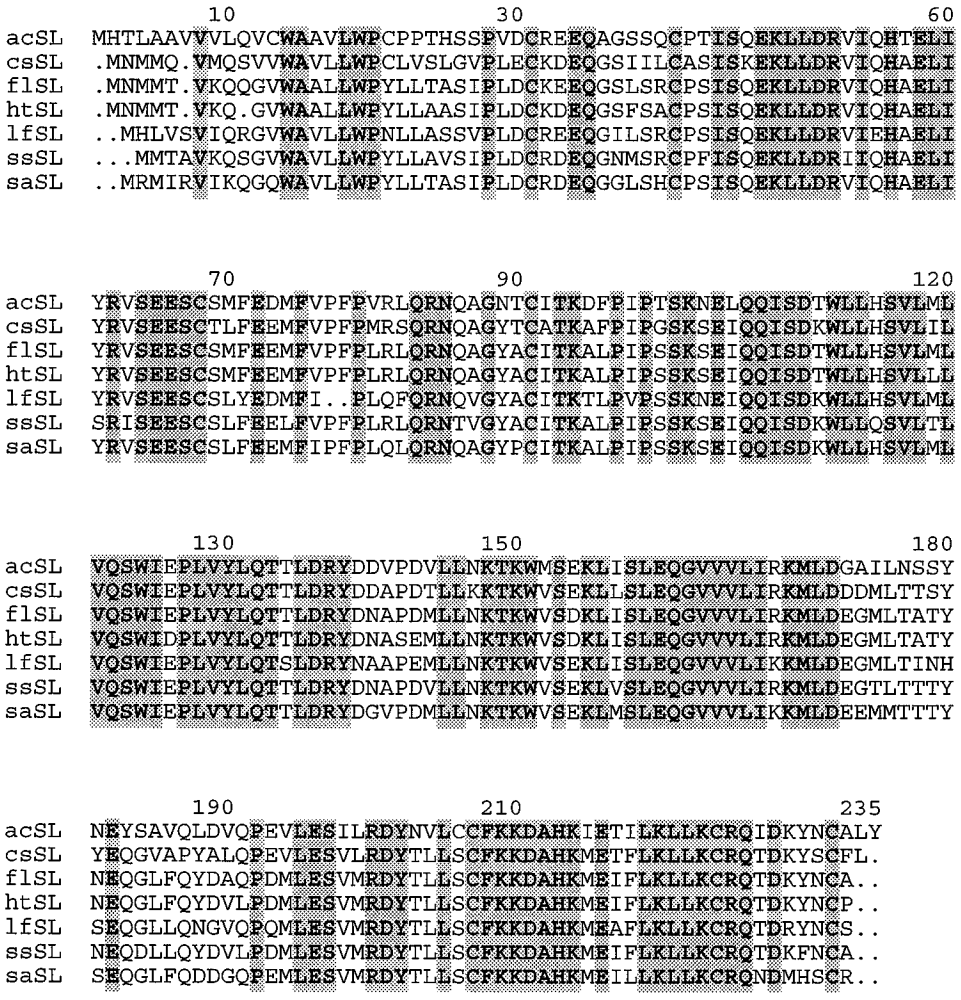

FIG. 3. Comparison of amino acid sequences of SL from different fish species. Residues are numbered beginning with the putative start Met for acSL. To allow for maximal alignment, gaps have been introduced (dots). Consensus amino acid residues are shaded. Note that seven Cys residues are conserved in all the sequences. saSL shows a 68, 73, 83, 75, 77, and 79% aa sequence identity to Atlantic cod (acSL), chum salmon (csSL), flounder (flSL), halibut (htSL), lumpfish (lfSL), and sole (ssSL), respectively.

native SL polypeptides secreted from pituitaries of this fish genuinely represent different processed forms of the hormone as was suggested by Cavari *et al.* (1995). Both SL forms are recognized by the anti-rSL antiserum (Fig. 5), raising questions about the distinct functional role(s) of the *S. aurata* SLs.

Although *S. aurata* native SL have been recently isolated from pituitaries (Cavari *et al.*, 1995), the yield from the biochemical procedures was low and required large quantities of biological material. In addition, the procedure requires alkaline extraction and solvent treatment which may alter the biological actions of the hormone. To date no assay is available for this pituitary hormone and further investigation is necessary. The availability of large amounts of recombinant SL from *S. aurata* aids the elucidation of the biological action(s) of this protein in *in vitro* and *in vivo* assays.

The function(s) of SL is unknown. Based on biochemical, physiological, and histocytological studies, the pars intermedia cells which secrete SL (Rand-Weaver *et al.*, 1991a) have been implicated in diverse areas including calcium regulation (Olivereau *et al.*, 1981), ion phosphate transport (Lu *et al.*, 1995), acid–base balance (Wendelaar *et al.*, 1986), background adaptation (Ball and Batten, 1981; Zhu and Thomas, 1995),

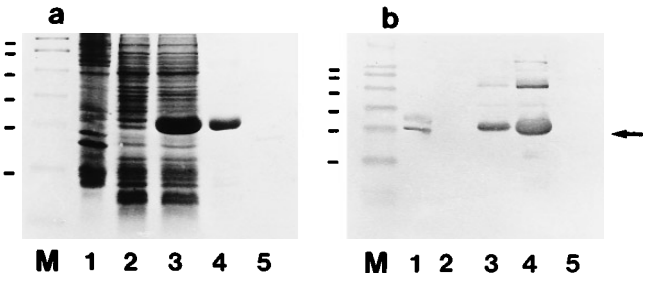

FIG. 4. Overexpression of gilthead seabream SL in *E. coli.* (a) Coomassie blue-stained SDS–PAGE of *Sparus aurata* pituitary cell extract (lane 1), solubilized proteins from *E. coli* overexpressing somatolactin (lane 3), *E. coli* expressed rSL isolated from inclusion bodies (lane 4), protein extracted from an induced *E. coli* culture (lane 2), and recombinant gilthead seabream growth hormone (lane 5). (b) Western blot analysis of the protein pattern shown in (a) with anti-rSL serum. Note that rSL shows the same electrophoretic mobility as a native SL from *Sparus aurata* pituitary (arrow) (compare lanes 3 and 4 with lane 1). The upper bands in lanes 3 and 4 in (b) represent aggregates of rSL. Putative glycosylated and nonglycosylated forms of native SL are detected in pituitary culture extracts (lane 1 in (b)). Lane M, molecular mass markers (top to bottom: 116, 97, 66, 45, 21, 14).

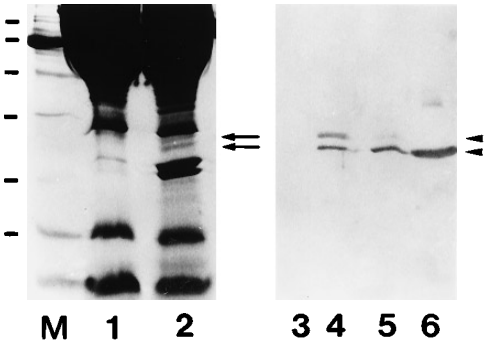

FIG. 5. Identification of two forms of native gilthead seabream SL. Coomassie blue-stained SDS–PAGE (lanes 1–2) and Western blot analysis (lanes 3–6) of primary pituitary culture medium and rSL with anti-rSL serum. Overloaded samples of pituitary-free culture medium (lane 1) and pituitary cultured *in vitro* (lane 2) are used on stained gels to show the secreted forms of native SLs (arrows). The two major bands running ahead of SL represent glycosylated and nonglycosylated forms of GH. The overrepresented protein at the top of the stained gel (lanes 1 and 2) is albumin from serum culture medium. Native gilthead seabream SL secreted *in vitro* (lane 2) was identified by an anti-SL serum in the immunoblot (lane 4). Proteins from pituitary-free culture medium are used as a control on the immunoblot (lane 3). N-glycosidase treatment assayed as described under Materials and Methods indicates that the upper native SL band in lane 2 (upper arrow) is a glycosylated SL form. The glycosylated upper band in lane 4 is removed after the N-glycosidase treatment (lane 5), as shown by immunoblotting. Recombinant gilthead seabream SL migrates as the native nonglycosylated SL (lane 6). Lane M represents molecular markers as in Fig. 4.

and reproduction (Planas *et al.*, 1992). Although these studies are indicative, the precise function(s) of SL is uncertain. It is also unclear whether an SL-like protein is expressed in other vertebrate groups.

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