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Bacterial Production and Purification of the Fish Pituitary Hormone Somatolactin

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Somatolactin, a pituitary hormone belonging to the growth hormone/prolactin family, is produced in the intermediate lobe of teleost pituitary. To date, the functions of this new hormone and the target tissues are unknown. A *Solea senegalensis* somatolactin (ssSL) cDNA has previously been cloned and isolated. Here we have inserted this cDNA into a pET-3a plasmid in order to produce recombinant ssSL in *E. coli* BL21 (DE3) cells. The protein induced was isolated from inclusion bodies by a solubilization–renaturation procedure originally developed to generate native disulfide bonds, to get putative active proteins. The recombinant somatolactin was further purified to homogeneity by gel filtration on FPLC. The estimated molecular weight of 26 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis agrees well with the molecular mass calculated from the translated cDNA sequence and with native somatolactin (SL). The recombinant protein showed electrophoretic mobility identical to that of one of the native forms of SL secreted *in vitro* by cultured pituitaries from sole. Another native SL expressed in *S. senegalensis* represented a glycosylated modified hormone as shown by *N*-glycosidase treatment. Further, recombinant SL was recognized by an anti-native SL antibody and used to generate polyclonal sera reactive with the native pituitary hormone. To date, this represents the first recombinant SL protein isolated in sufficient quantities for biophysical and biochemical investigation and for studies on its physiological actions.

Somatolactin is a new pituitary hormone, structurally related to both growth hormone and prolactin. SL-producing cells[2] are present in the intermediate lobe of the fish pituitary (1). SL was found and purified from pituitaries of Atlantic cod (*Gadus morhua*) as a 26-kDa glycoprotein by Rand-Weaver *et al.* (2). However, the yield of SL from this natural source is usually low and hence has limited the number of detailed studies of this hormone. The complete structure was subsequently elucidated by the cDNA sequence, and in view of its structural similarity to GH and PRL, it was named somatolactin (3). Recently several SL cDNAs sequences were reported for flounder (*Paralichthys olivaceus*) (3), chum salmon (*Oncorhynchus keta*) (4), Atlantic cod (*G. morhua*) (5), sole (*Solea senegalensis*) (6), halibut (*Hippoglossus hippoglossus*) (7), and lumpfish (*Cyclopterus lumpus*) (7). The protein has been found in glycosylated and nonglycosylated forms depending on the fish species used for isolation (1,4).

Although the biochemical properties of SL are becoming increasingly clear, its physiological significance still remains unknown. Preliminary evidences indicate that SL stimulates *in vitro* gonadal steroidogenesis (8), and plasma SL level revealed a gradual increase during sexual maturation (9). Nonspecific environmental stress has also been described to induce a rapid activation of SL-secreting cells in the pars intermedia of the fish pituitary (10). Diverse functions for somatolactin in acid–base regulation and calcium regulation have also been reported (11,12). On the other hand, the putative expression of SL in pituitaries from organisms other than fish is a matter of active investigation. To obtain a better understanding of the function of fish SL and allow studies of factors affecting regulation of its synthesis and secretion, we have previously cloned and sequenced the cDNA coding for this protein in *S.*

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[2] Abbreviations used: SL, somatolactin; rSL, recombinant somatolactin; ssSL, *Solea senegalensis* somatolactin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside.

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senegalensis (6). We report herein the expression and purification to homogeneity for the first time of a fish recombinant SL from *S. senegalensis*. Further, immunological evidence of reactivity of the recombinant SL with an anti-cod native SL serum and generation of a polyclonal serum to recombinant SL is also documented.

MATERIALS AND METHODS

Reagents, enzymes, and strains. *Escherichia coli* strains DH5α and BL21 (DE3) were used as the bacterial host for cloning and expression of ssSL, respectively. Restriction enzymes and DNA modification enzymes were purchased from Pharmacia and Boehringer Mannheim and used according to the manufacturers' recomendations. Synthetic oligonucleotides were obtained from Eurogentec S.A. (Belgium). Yeast extract, bactotryptone, and agarose were from Hispanalab (Madrid, Spain). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Promega. *N*-glycosidase from *Flavobacterium meningosepticum* was obtained from Boehringer Mannheim. An anti-native SL serum was a gift of Dr. M. Rand-Weaver (Sheffield, UK). Superose 6 HR 10/30 and FPLC were from Pharmacia. All other reagents were commercial preparations of analytical grade or of the highest purity available. Animals were obtained from a local fishery plant (Cupimar, San Fernando, Cádiz, Spain).

Isolation of ssSL cDNA sequences and construction of pET-SL. Somatolactin protein coding sequence was amplified from a ssSL cDNA clone (6) using two oligonucleotides, SL1 (5′-GGGGGGCATATGATCCCACTAGACTGTAGGG-3′), which contained nucleotides encoding amino acids 1 to 7 and created a *Nde*I site at the initiation codon, and SL2 (5′-GGGGGGGGATCCTTATGCACAGTTGAATTTGTC-3′), which contained bases complementary to nucleotides coding for the C-terminal 6 amino acids (202–207) plus the stop codon and created a *Bam*HI site. This construction results in a protein with 23 amino acids residues removed from the original cDNA isolated, corresponding to those of the putative signal peptide (5–7). Thirty cycles of PCR were performed (93°C for 1 min, 50°C for 30 s, 72°C for 2 min) in 100 μl of 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM Mg Cl_2, 0.2 mM dNTPs, 1 mg/ml gelatin, 50 ng SL1, 50 ng SL2, and 1 unit *Taq* polymerase. The amplified ssSL fragment was gel purified, digested with *Bam*HI and *Nde*I, and subcloned into the *Nde*I–*Bam*HI sites of the expression vector pET-3a to construct pET-SL.

Expression, solubilization, and refolding of somatolactin. *E. coli* strain DH5α was transformed with pET-SL using standard procedure (13). Transformants were selected on LB ampicillin/agar plates and single recombinant plasmids were sequenced by the dideoxynucleotide method (14) to ensure that no changes had occurred during amplification. Then an *E. coli* BL21 (DE3) strain was transformed with isolated pET-SL for expression of ssSL. Transformants were selected on LB plates with 100 μg/ml ampicillin. A small inoculum of *E. coli* BL21 (DE3) containing pET-SL was grown at 37°C in 3.5 ml of L-broth supplemented with ampicillin. The overnight culture was diluted into 250 ml of L-broth supplemented with ampicillin and grown at 37°C. Expression of rSL protein was induced at OD_{600} of 0.6 by addition of IPTG to 0.5 mM and cells were harvested 4 h later by centrifugation at 7500*g* for 20 min at 4°C. The production of recombinant SL was monitored by SDS–PAGE analysis (15) of *E. coli*-soluble and -insoluble protein extracts after disruption of cells by sonication. The protein analysis was performed using a 4% stacking gel and a 15% separation gel. When needed, gels were run under nonreducing conditions, omitting 2-β-mercaptoethanol. Gels were stained with Coomassie brilliant blue R-200. rSL was found associated with the insoluble fraction.

Solubilization and formation of disulfide bonds of rSL from inclusion bodies were performed basically following a method originally developed for the folding of proteins successfully reactivated from *E. coli* (16). By using this procedure we have renaturated and obtained a physiological active growth hormone from fish (17). Inclusion bodies were so, washed briefly with 20 mM Tris–HCl, pH 8.0, 5 mM EDTA, containing 4% Triton to eliminate adhering impurities, followed by four washes with 20 mM Tris–HCl, pH 8.0, to remove most of the detergent. Then, they were dissolved in 20 mM Tris–HCl, pH 8.0, containing 6 M guanidinium hydrochloride and 1% 2-β-mercaptoethanol at a ratio of 10 mg of rSL to 70 ml of buffer, approximately. The reducer agent was included to break existing nonnative intramolecular and intermolecular disulfide bonds (16,18). The solution was incubated at room temperature for 2 h and then centrifuged at 10,000 rpm for 30 min at 4°C. Renaturation of the solubilized rSL was performed by dialysis against 25 vol of 50 mM NH_4HCO_3 at 4°C for 3 h with sequential changes of buffer. The precipitate formed during dialysis was removed by centrifugation at 30,000 rpm for 30 min at 4°C. The supernatant was lyophilized in 30-ml aliquots for several days. For FPLC purification, a small amount of the lyophilized refolded rSL was dissolved in 400 μl of 1.0 mM NaOH and 20 mM Tris–HCl, pH 8.0, was rapidly added to reach a final protein concentration of 3–4 mg/ml. Two hundred microliters of the dissolved protein was purified by gel filtration chromatography on FPLC on a Superose 6 HR 10/30 column (25 ml) eluted in a flow rate of 0.25 ml/min and monitored with a UV-M monitor (Pharmacia). Fractions of 325 μl were collected and analyzed by SDS–PAGE, and those containing rSL as

a monomer form were pooled, lyophilized, and stored at −80°C.

Immunological characterization of recombinant somatolactin. Purified proteins were further characterized by the Western blot procedure using anti-cod SL serum (1). Briefly, proteins were transferred to nitrocellulose membrane essentially as described (19), and membranes were blocked with 5% nonfat dry milk in TBS buffer for 1 h at room temperature. Immunostaining was carried out using a polyclonal antibody made against cod native SL (1) at 1:5000 dilution made in TBS−milk for 14 h at room temperature. Then, after brief 3× washes with TBS containing 0.5% Tween 20, the membranes were proved with peroxidase-conjugated anti-rabbit IgG for 2 h at room temperature and finally after several washes in TBS−Tween 20 they were developed using 4-chloro-1-naphthol as chromogenic substrate.

Antibody production. Antiserum was produced against rSL in two rabbits. rSL isolated from inclusion bodies was dissolved as indicated above and PBS was added to a protein concentration of 2 mg/ml. The antigen (200 μg) was emulsified with an equal volume of complete Freund's adjuvant (first injection) or incomplete Freund's adjuvant (subsequent injections). Intradermal injections were given at intervals of 3 weeks for a period of 3 months. These polyclonal sera were assayed by immunoblots as indicated above for anti-native cod SL.

In vitro culture of sole pituitary glands. *S. senegalensis* animals weighing 150−200 g were decapitated and pituitaries were removed. Culture of individual glands were in 200 μl of complete medium (RPMI 1600 supplemented with 10% fetal calf serum) in a 96-well multiple plate. Pituitaries were incubated at 22°C for 2−3 days. SL release to the medium from sole cultured pituitaries was determined by Western analysis using an anti-SL serum. To identify a glycosylated form of native SL, culture medium was incubated with *N*-glycosidase (0.2 units/μl) at 37°C for 16 h (20). Proteins were further analyzed by SDS−PAGE and Western blot with SL antibody.

RESULTS AND DISCUSSION

Although SL have been isolated from fish pituitaries (2), those isolation methods produce only limited quantities of the protein and hence have limited the number of detailed studies of this hormone. The availability of sufficient amount of SL will help to elucidate the physiological function of this protein and to study the regulation of its metabolism in fish. Consequently, we decided on a molecular approach. The strategy for introducing a previously isolated ssSL cDNA (6) into pET-3a is described above. Upon induction with IPTG, BL21 (DE3) cells harboring the pET-SL plasmid expressed SL at a level approaching approximately 20% of the total cellular protein (see Fig. 1, lane 2). The expressed protein was almost exclusively found in inclusion bodies and was recovered mostly in the pellet fraction after low-speed centrifugation of broken cells (Fig. 1, lanes 3 and 5). Several growth conditions were tested by changing both the temperature of the culture and the level of induction of the T7 promoter. Results showed that the recombinant protein remains in the pellet fraction when either the temperature of induction was lowered or when a 20-fold reduction of the IPTG concentration was included in the growth conditions of bacteria (data not shown). The results in Fig. 1 also indicate yields of up to 90% monomeric ssSL against low levels of SL aggregates (Fig. 1, lanes 5 and 6). When the recombinant protein was analyzed by SDS−PAGE under nonreducing conditions, inclusion bodies were composed mostly of partially reduced monomers, with the electrophoretic mobility of rSL being that of the reduced form of rSL after refolding (Fig. 1, lanes 5 and 6). Disulfide-bonded oligomers were also found in these conditions (Fig. 1, lane 5). The presence of reduced rSL monomers in the inclusion bodies can be explained by the reducing environment of the *E. coli* cytoplasm. This has also been observed for other proteins with disulfide bridges in their native state such as growth hormone (21) and human prolactin (22). The oligomers are probably due to intermolecular disulfide bonds since they are disaggregated in the presence of 2-β-mercaptoethanol, a re-

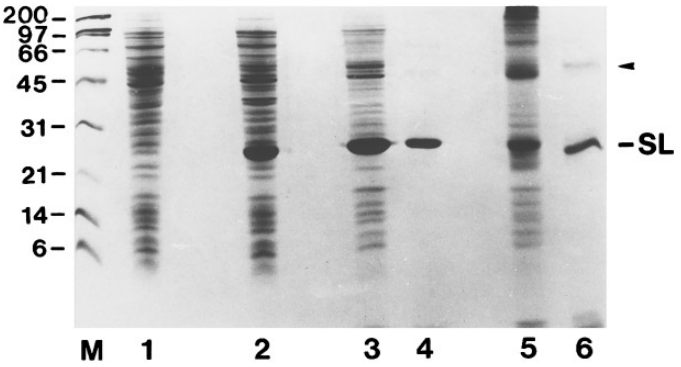

FIG. 1. SDS−PAGE of protein expression and refolded SL. Expression of recombinant SL was induced in BL21 (DE3) cells as described under Materials and Methods. Whole proteins of bacteria from noninduced (lane 1) and induced (lane 2) culture, isolated inclusion bodies (lane 3), and solubilized and refolded rSL (lane 4) were boiled in the presence of 1% SDS and 2-β-mercaptoethanol before electrophoresis on a 15% polyacrylamide gel. The sizes (kDa) of the molecular weight markers (lane M) are indicated. The same amounts of proteins from inclusion bodies and refolded rSL as in lanes 3 and 4 were analyzed, omitting 2-β-mercaptoethanol (lanes 5, 6). Recombinant SL was obtained as monomer and aggregate forms after renaturation (lane 6). Partially reduced forms of SL are present in bacterial inclusion bodies (lane 5) which migrate on the gel as the reduced form of refolded rSL (lane 6). Some aggregates of rSL on inclusion bodies (lane 5) and refolded rSL (lane 6) are indicated by an arrow.

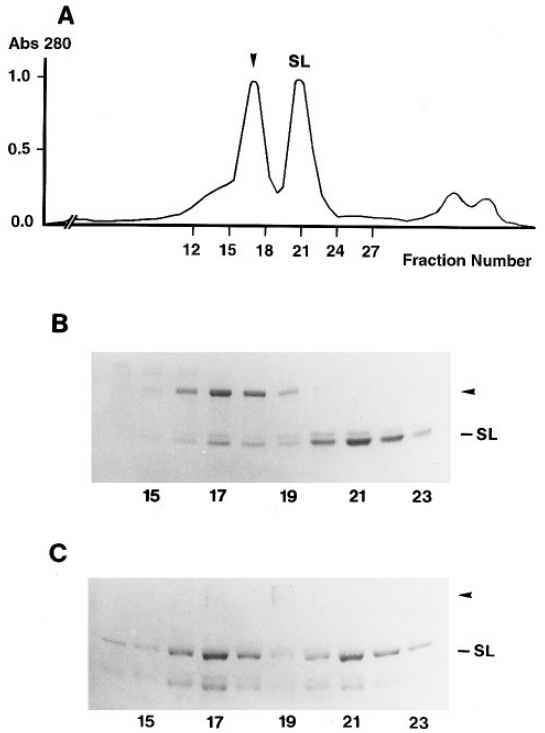

FIG. 2. Purification of recombinant SL by FPLC. Gel filtration analysis was done using a Pharmacia Superose 6 HR column. One milligram of renaturated rSL from inclusion bodies in 200 μl of 20 mM Tris, pH 8.0, 2 mM EDTA (column buffer) was injected into the gel filtration column. Two major peaks corresponding to SL aggregates (arrow) and SL monomers (SL) were eluted (A). SDS–PAGE (15% polyacrylamide) of the peak fractions indicated in A was performed in the absence (B) and in the presence of 2-β-mercaptoethanol (C). Most of the SL aggregates and the oxidized forms of SL in B are reduced in C. Some breakdown products of SL are also observed.

ducing agent which maintains cysteine residues in a reduced form (Fig. 1, lane 3). Solubilization of inclusion bodies and refolding during the renaturation procedure were checked by SDS–PAGE under reducing or nonreducing conditions. In this case the presence of oxidized and reduced forms of SL was more evident. By the analysis shown in Fig. 1 emerges the fact that most of the refolded rSL from inclusion bodies was on the monomeric oxidized form with a low yield of monomeric reduced SL (see Fig. 1, lanes 4 and 6).

The refolded recombinant ssSL was further purified by gel filtration chromatography on FPLC and was eluted in two peaks: a first one with large amounts of aggregates (Fig. 2B, fractions 15–19) and a second peak which contained the highly purified monomeric form of recombinant ssSL (Fig. 2B, fractions 20–23). In the presence of 2-β-mercaptoethanol, the expressed SL migrated as a single band, corresponding to the fully reduced monomer free of oligomers (Fig. 2C). This isolated rSL comigrated with a native form of ssSL on SDS–PAGE under reducing conditions (see Figs. 4 and 5) and reacts with an anti-cod SL serum generated against native SL (Fig. 3). Furthermore, amino acid peptide sequencing analysis demonstrated the homogeneity of our SL purification (data not shown). By using this purification procedure we estimate that 40–50 mg of FPLC-purified SL can be obtained from 1 liter of induced cells (10^8 cells/ml), of which more than 95% is in the oxidized monomeric form (see Fig. 2B, lanes 20–23). The recovery of the total protein expressed represents approximately 15–20%.

The scientific literature reports on the use of inclusion bodies as a method of production of useful proteins. Most of the procedures depend on the availability of simple, rapid, and economical solubilization and renaturation schemes. A major problem used to be a low recovery or potential irreversible modification to the protein structure. Although some expressed rSL was not recovered from inclusion bodies or after dialysis in a solubilized form, that seems not to be a major problem to purify a high amount of fish rSL. First, the amount of insoluble material during the dialysis method does not represent a major drawback to the yield we obtained with rSL. Second, aggregate material (SL) is also not a major contaminant of the expressed SL.

Because of the great interest in this hormone for studies on pituitary function in fish, we decided to generate an additional probe such as a polyclonal serum. This result is shown in Fig. 4. Reactivity is maintained with recombinant SL at very high dilutions (up to 1:30,000 in immunoblots). Results shown on Fig. 4 suggested that SL in pituitaries from *S. senegalensis*, as it occurs in others fishes, is probably present in glycosylated and nonglycosylated forms (see reactivity of anti-SL with pituitary extracts in Fig. 4, lane 1). However, it seems that in sole pituitaries the nonglycosyl-

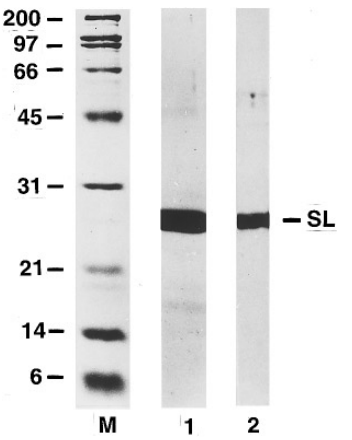

FIG. 3. Protein blot analysis of FPLC isolated rSL with anti-native cod SL serum. Lane M, protein markers. Lane 1, purified recombinant ssSL from FPLC chromatography. Lane 2, immunoreactivity of rSL with anti-native SL serum generated against a native SL protein (1).

ated SL form is quite abundant in contrast with what has been described for Atlantic cod (2). The expressed sole rSL shows electrophoretic mobility identical to that of a native form of ssSL (compare, in Fig. 4, lanes 2–4 with lane 1). To further investigate this physiological aspect of ssSL, we decided to analyze if both forms of SL are not only synthetized but also secreted as well. *In vitro* culture media of sole pituitary glands were tested by immunoblot with SL antibody. Our results demonstrate that *in vitro*, sole pituitaries secrete both forms of SL (see lanes 2 and 6 in Fig. 5). Further, *N*-glycosidase treatment demonstrated that the slower migrating form of native SL represents a glycosylated polypeptide (compare, in Fig. 5, lanes 2 and 6 with lanes 3 and 5). Pituitary-free culture media served as negative controls (Fig. 5, lanes 1 and 7). More significantly, the recombinant SL comigrated exactly with the nonglycosylated secreted form of the native sole SL. The significance of both forms of native SL is presently unknown but the fact that both are secreted opens new questions on the role of glycosylated and nonglycosylated SLs in fishes. Our rSL hormone could serve for studies in that direction. These results together with preliminary evidence of membrane binding reactivity of recombinant SL (radioreceptor analysis) induce us to think that rSL should show some biological activity and it may be of use for elucidating the target tissues of this hormone. This remains to be demonstrated and is currently under investigation.

On the other hand, anti-rSL serum and rSL protein are useful tools for looking up somatolactin-like proteins in expression libraries in other organisms, including mammals. So far, all pituitary hormones present in mammals have been identified in lower vertebrates and, thus, a somatolactin-like protein should eventually be present in all vertebrate classes.

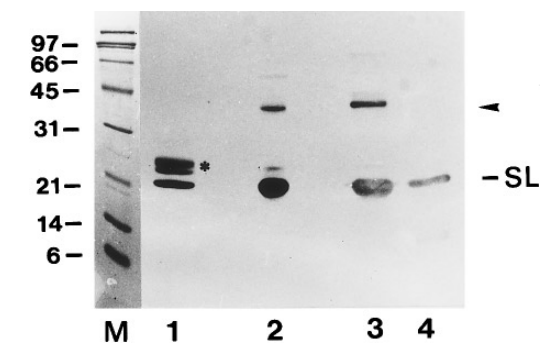

FIG. 4. Immunoreactivity of native and recombinant sole SL with polyclonal serum generated against rSL. Note the presence of different forms of native SL in whole pituitary extracts of *Solea senegalensis* (lane 1) (putative glycosylated form is shown by asterisk). IPTG-induced BL21 (DE3) cell extracts harboring pET-SL were analyzed by immunoblot with anti-rSL serum as shown: whole bacteria protein extract (lane 2), isolated inclusion bodies (lane 3), and FPLC purified rSL (lane 4). rSL (lanes 2, 3, 4) runs identically to a native ssSL form (lane 1). Some aggregates of rSL are detected in lanes 2 and 3 (arrowhead).

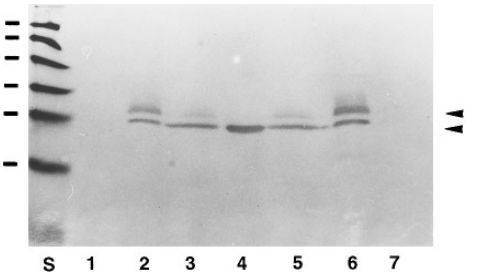

FIG. 5. Identification of glycosylated and nonglycosylated forms of sole native SLs. *Solea senegalensis* pituitary culture medium and recombinant SL were tested by immunoblot with anti-rSL serum. Lanes 2 and 6 represent duplicate individual pituitary cultures. *N*-glycosidase-treated culture pituitary medium containing native SL shown in lanes 3 and 5 indicates that the top SL band represents a glycosylated form of the hormone. Recombinant SL (lane 4) runs identically to the nonglycosylated form of the native SL secreted *in vitro* (lanes 2, 3, 5, 6). Pituitary-free culture medium is used as a control in lanes 1 and 7.

In conclusion, this paper represents the first scientific report of a large-scale production of recombinant SL in *E. coli*. Its physiological role, still unknown, could be studied now among other approaches by identifying target receptors in fish membrane tissue preparations.

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