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Production and purification of biologically active recombinant tilapia (*Oreochromis niloticus*) prolactins

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REvised Manuscript received 17 June 1991

ABSTRACT

Recombinant expression vectors carrying tilapia prolactin-I or -II (tiPRL-I or tiPRL-II) cDNA were constructed and the tiPRL-I and II proteins were produced in *E. coli* as inclusion bodies. These inclusion bodies were dissolved in 6 mol urea/1. Refolding of the proteins was followed by SDS-PAGE under non-reducing conditions so as to visualize the oxidized state of the molecules. Proteins tiPRL-I and tiPRL-II were purified by gel filtration and ion-exchange chromatography. The N-terminal sequence and bioactivities of both purified proteins were then analysed. Recombinant tiPRL-I and tiPRL-II induced a significant rise in plasma calcium levels as well as in mucocyte density in the abdominal skin epithelium. When tested on kidney membrane, both proteins exhibited potency in competing with $^{125}$I-labelled tiPRL-I for binding sites, but tiPRL-I seemed to be more potent than tiPRL-II in competing for these sites. The results obtained for the biological activities tested suggest that both recombinant prolactins were correctly refolded and had retained the full biological activity previously observed with the natural hormone preparations extracted from the animals.


INTRODUCTION

In teleost fish, prolactin is involved in several important physiological processes such as growth, reproduction, metabolism and mucus production; the hormone’s primary role being regulation of water and electrolyte homeostasis (Clarke & Bern, 1980; Loretz & Bern, 1982; Hirano, 1986; Wendelaar Bonga & Pang, 1989; Prunet, Avella, Fostier et al. 1990).

Tilapia has two prolactins (tiPRL-I and tiPRL-II) which have been well characterized (Specker, King, Nishioka et al. 1985a; Specker, King, Rivas & Young, 1985b; Flik, Fenwick, Kolar et al. 1986; Yamagushi, Specker, King et al. 1988; Young, McCormick, Demarest et al. 1988; Rentier-Delrue, Swennen, Prunet et al. 1989; Specker, Brown & Brown, 1989). Because of their ability to promote sodium retention, both prolactins are considered to be pivotal regulators in freshwater fish (Specker et al. 1985b). These proteins have been implicated in improved survival of tilapia acclimated to prolonged acid stress (Flik, Van Der Velden, Seegers et al. 1989b). Moreover, both prolactins restore the plasma osmolality and electrolyte concentration to normal levels, as well as the integumental transepithelial potential difference of hypophysectomized tilapia (Young et al. 1988). Both prolactins behave similarly with respect to these aspects of osmoregulation. However, Specker et al. (1989) have shown that salamandrid integumental prolactin receptors may discriminate between tiPRL-I and -II. Until now, there have been no data indicating the different actions of the two prolactins in a homologous bioassay except the preliminary results of Specker et al. (1985b) showing that the large prolactin promotes growth whereas the small prolactin does not.

One important prerequisite for investigating possible differences in the roles of the two prolactins is the availability of large amounts of active proteins. In this
study, we describe the production of both tilapia prolactins in *Escherichia coli* and their purification. We also demonstrate that both recombinant prolactins synthesized in *E. coli* are able to raise blood calcium levels, stimulate mucus cells and bind tilapia kidney membranes, as do natural tilapia prolactins (Fryer, 1979; Wendelaar Bonga & Meis, 1981; Wendelaar Bonga & Flik, 1982; Edery, Young, Bern & Steiny, 1984; Flik, Wendelaar Bonga & Fenwick, 1984; Flik et al. 1986; Flik, Fenwick & Wendelaar Bonga, 1989a; Dauder, Young, Hass & Bern, 1990).

**MATERIALS AND METHODS**

**Molecular genetics**

**Plasmids**

Plasmid ptiPRL-II contains the cDNA coding for tiPRL-II in plasmid pUC13 (Amersham laboratories, Brussels, Belgium). Plasmid pARAE is a derivative of pAR3040 (Rosenberg, Lade, Chui et al. 1987) constructed in one of our laboratories. Plasmid pT7tiPRL-I is the plasmid expressing tiPRL-I. All three plasmids have been described previously (Rentier-Delrue et al. 1989). Plasmid pSP73 was purchased from Promega (Leiden, The Netherlands).

**Bacterial strain**

*E. coli* strain HB101 and *E. coli* strain BL21 (DE3) provided by F. W. Studier (Studier & Moffat, 1986; Biology Department, Brookhaven National Laboratory, Upton, NY, U.S.A.) were used for construction and protein production respectively.

**Production of tiPRL-II proteins**

*E. coli* BL21 (DE3) harbouring plasmid pT7tiPRL-II was grown at 37 °C in 5 litres Luria–Bertani medium (Sambrook, Fritsch & Maniatis, 1989) containing 200 µg ampicillin/ml. When the culture has reached an OD$_{600}$ of 0.9, synthesis was induced with isopropyl-β-thiogalactosidase (IPTG; Eurogentec S.A., Liège, Belgium) and the culture was grown for 4 h at 37 °C.

**Purification of recombinant tiPRL-I and tiPRL-II proteins**

**Chemicals**

Urea solutions were deionized on mixed-bed resin (Bio-Rad, AG 501-X8; Eke, Belgium), filtered and stored at 4 °C. Cells were broken in a French cell press (American Instrument Company; Silver Spring, MD, U.S.A.) at 10$^8$ Pa. Dialysis was performed in Molecularporous Membrane (Spectra/Por, Polylab; Antwerp, Belgium) with a molecular weight cut-off of 6–8 kDa.

**Chromatography**

Proteins were filtered through Millex (Millipore, Brussels, Belgium) and applied to a gel filtration column (Sephadex G-100; Pharmacia, Brussels, Belgium). This was followed by an anion-exchange chromatography on a fast-performance liquid chromatography (FPLC) system (MonoQ HR 10/10; Pharmacia). All purification procedures were performed at 4 °C. Purified proteins were concentrated by lyophilization.

**Biochemical and chemical characterization**

Proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Weber & Osborn, 1969) under reducing or non-reducing conditions, with Coomassie brilliant blue R-250 staining. Protein concentration was determined by a simplified Lowry assay (Peterson, 1977). Western blot analysis of tiPRL-II protein was performed after electrotransfer of the proteins from SDS-polyacrylamide gel to nitrocellulose paper (Towbin, Staehelin & Gordon, 1979). TiPRL-II was detected using a salmon prolactin antiserum (Prunet, Boeuf & Houdebine, 1985), the immune complexes being revealed by peroxidase-conjugated antibodies. The N-terminal sequence of both prolactins was determined by the Edman degradation method (Wittman-Liebold & Lehmann, 1980).

**Analysis of biological activity**

**Animals**

Sexually mature male tilapia (*Oreochromis mossambicus*) were obtained from laboratory stock. They were kept in running tapwater Ca$^{2+}$ (0.7 mmol/l), Mg$^{2+}$ (0.2 mmol/l), Na$^+$ (0.5 mmol/l) and K$^+$ (0.06 mmol/l) at 27 °C; lights were on for 12 h per day. The fish were fed fixed rations of food (commercial trout pellet; Trouvit, Putten, The Netherlands) amounting to 1.5% of the body weight of the fish per day. The experiments were carried out in December and May.

**Analysis**

Total plasma calcium was determined with a commercial colorimetric calcium kit (Sigma, St Louis, MO, U.S.A.; catalogue no. 586). Combined calcium phosphate standards (Sigma catalogue no. 360-11) were used as references. Plasma osmolalities were determined with a Roebling osmometer on fresh 50 µl plasma samples.

**Hormone treatments and plasma sampling**

TiPRL-I and tiPRL-II were dissolved in Cortland saline NaCl (126 mmol/l), KCl (3.5 mmol/l), MgSO$_4$·7H$_2$O (3 mmol/l), Na$_2$HPO$_4$·2H$_2$O (4.6 mmol/l), K$_2$PO$_4$...
RESULTS

Construction of tiPRL-II expression plasmid

The expression system based on T7 RNA polymerase was used in this study (Studier, Rosenberg, Dunn & Dubendorff, 1990). Text-figure 1 outlines the construction scheme of the E. coli vector used to direct the expression of tiPRL-II. Plasmid pSP73 was used to subclone the cDNA corresponding to the first 128 codons of the mature tiPRL-II protein. The TaqI–PvuII fragment of ptiPRL-II was inserted with the PstI–TaqI oligonucleotide into the plasmid pSP73 cut with PstI and PvuII. The oligonucleotide contains the NdeI restriction site which includes the ATG (coding for the initiator methionine) followed by the first eight codons of tiPRL-II. Plasmid pARAE was used in this study (Studier, Rosenberg, Dunn & Dubendorff, 1990). Text-figure 1 outlines the construction scheme of the intermediate plasmid pSPtiPRL-IIA and the PvuII–BamHI fragment of ptiPRL-II were cloned between the NdeI and BamHI sites of the pARAE vector to yield the pT7tiPRL-II.

The vector similarly constructed to express tiPRL-I has been described previously (Rentier-Delrue et al. 1989).

Expression of tiPRL-II cDNA

Production of tiPRL-II was carried on in the BL21 (DE3) E. coli strain. After induction with IPTG, a major protein was produced in cells containing plasmid pT7tiPRL-II (Pl. 1, fig. 1). On reducing SDS-PAGE, this protein had an apparent molecular weight of 23 kDa. To establish that the protein produced was in fact tiPRL-II, a Western immunoblotting experiment was performed on total extract from non-induced and induced cells using salmon antiserum (Pl. 1, fig. 2). The newly synthesized protein was shown to react with the antiserum and was identified as tiPRL-II protein. Some synthesis of this product was observed even in non-induced conditions, probably due to the basal level of the T7 RNA polymerase production (Studier et al. 1990).
TEXT-Figure 1. Schematic representation of the construction of plasmid pT7tiPRL-II. Plasmid pSPtiPRL-IIΔ was constructed from pSP73 and ptiPRL-II using synthetic oligonucleotides. Plasmid pT7tiPRL-II was constructed from pARAE, pSPtiPRL-IIΔ and ptiPRL-II. Both constructions are described in the Results section. The hatched and stippled boxes represent the Φ10 promoter and TΦ terminator for T7 RNA polymerase respectively. The arrow under the hatched box indicates the orientation of the Φ10 promoter. The shaded box in ptiPRL-II represents the entire tilapia prolactin-II cDNA. Ap®, gene coding for the ampicillin resistance. T4 kinase and T4 DNA ligase, isolated from phage T4, were provided by BRL laboratories.
Recombinant tilapia prolactins

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TEXT-Figure 2. Ion-exchange purification of refolded (a,c) tilapia prolactin-I (tiPRL-I) and (b,d) tiPRL-II. (a,b). Ion-exchange chromatography was performed on a MonoQ column equilibrated with ethanolamine (20 mmol/l) at pH 9.5 for tiPRL-I and pH 9 for tiPRL-II. The proteins were eluted by a 0-0-5 mol NaCl/l gradient. Fractions (1 ml) were collected at a flow rate of 2 ml/min and were monitored by U.V. absorbance at 280 nm. (c,d). Coomassie blue-stained SDS-PAGE, under non-reducing conditions, of column fractions 27 to 34 for tiPRL-I and fractions 21 to 29 for tiPRL-II. The sample loaded into each well contained 10 μl of the corresponding fraction.

TABLE 1. Effects of tilapia prolactin-I (tiPRL-I) and tiPRL-II injections on total plasma calcium and osmotic value and on the number of mucocytes per unit length of cross-sections of abdominal skin epithelium. Values are means ± s.d. with the number of samples shown in parentheses

<table>
<thead>
<tr>
<th></th>
<th>Plasma Ca (mmol/l)</th>
<th>Plasma osmotic value (mOsmol/kg)</th>
<th>Mucocytes (no./mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tiPRL-I Control</td>
<td>2.99 ± 0.22*(7)</td>
<td>305 ± 2 (7)</td>
<td>18.8 ± 3.5*(5)</td>
</tr>
<tr>
<td>tiPRL-II Control</td>
<td>2.48 ± 0.48 (5)</td>
<td>305 ± 4 (5)</td>
<td>9.1 ± 3.6 (5)</td>
</tr>
<tr>
<td>tiPRL-I Control</td>
<td>2.90 ± 0.25 (5)</td>
<td>308 ± 2 (5)</td>
<td>25.6 ± 4.4*(5)</td>
</tr>
<tr>
<td>tiPRL-II Control</td>
<td>2.57 ± 0.12 (6)</td>
<td>308 ± 4 (6)</td>
<td>13.8 ± 3.0 (5)</td>
</tr>
</tbody>
</table>

*P < 0.025 compared with respective control (Mann-Whitney U test).

Production and purification of recombinant tiPRL-I and tiPRL-II proteins

TiPRL-II as well as tiPRL-I (Rentier-Delrue et al. 1989) proteins were produced by growing the transformed cells in rich medium with 2 μg ampicillin/ml at 37°C. Both prolactins were overexpressed as inclusion bodies (Marston, 1986). The respective yields for tiPRL-I and tiPRL-II were approximately 70 and 45 mg/l culture, as estimated by SDS-PAGE. To purify the produced proteins, cells were collected by centrifugation at 4500 g for 15 min, resuspended in 100 ml of Tris–HCl (10 mmol/l, pH 8), EDTA (1 mmol/l), PMSF (0.1 mmol/l) and kept at −20°C. They were disrupted as described in Materials and Methods. The inclusion bodies were harvested by centrifugation at 10 000 g for 15 min while most of the cellular debris remained in the supernatant.

Journal of Endocrinology (1991) 131, 219-227
The N-terminal sequence of both proteins is NH$_2$-Met-Val-Pro. This sequence corresponds to the sequence of the mature proteins assuming that the initiating methionine added for allowing translation initiation is not cleaved.

**Biological activity**

**Plasma analysis**

Both prolactins induced significant hypercalcaemia in tilapia ($P<0.009$ for tiPRL-I and $P<0.015$ for tiPRL-II). No effect of either hormone preparation on the plasma osmolality was observed (Table 1).

**Mucocytes**

Both tiPRL-I ($P<0.004$) and tiPRL-II ($P<0.001$) doubled the epidermal mucocyte density of tilapia. No significant difference ($P>0.15$) was observed between the control groups (Table 1).

**Binding assay**

Both tiPRL-I and -II preparations were able to displace labelled tiPRL-I from its binding sites on a kidney membrane preparation. Specific binding for tiPRL-I and tiPRL-II were 19.0% and 17.8% respectively at 0.15 g wet initial tissue weight/ml. The ratio specific binding tiPRL-I/specific binding tiPRL-II was 1.1±0.01 (mean±s.e.m., n=4) with different tissue concentrations. As indicated in Text-fig. 3, curves for displacement of tiPRL-I by increasing amount of tiPRL-I or tiPRL-II indicated that these two hormone preparations have different ED$_{50}$ values (18 and 310 ng/ml for a membrane preparation of 0.15 g initial tissue weight/ml respectively for tiPRL-I and -II). The ratio ED$_{50}$ tiPRL-I/ED$_{50}$ tiPRL-II was 12.9±2.6 (mean±s.e.m., n=3) with different tissue concentrations. However, tiPRL-curves appeared to be parallel to displacement curves obtained with a pituitary extract collected from freshwater adapted Oreochromis niloticus (Text-fig. 3).

**DISCUSSION**

We have described the production of significant amounts of tiPRL-I and -II in E. coli by means of a T7-promotor expression system. The two prolactins are produced as insoluble inclusion bodies which can be easily isolated. This approach, which allows recovery of correctly refolded proteins, has become classical and involves a denaturing step under reducing conditions followed by a refolding step. FPLC purification, leading to a single major band of tiPRL-I or tiPRL-II in its oxidized form, indicates that the protein preparations are quite homogeneous. The yield for pure recombinant...
proteins estimated by the Lowry method (Peterson, 1977) was between 10 and 15 mg/l of E. coli culture. Our purification procedure allows the recovery of approximately 25% of the recombinant tiPRLs present in the cell lysate.

Analysis of the N-terminal sequence of both proteins indicates that the initiator methionine is not cleaved off during synthesis. The absence of cleavage can be explained by the apparent difficulty for aminopeptidase to cleave off a methionine when it is followed by a valine residue (Tsunasawa, Stewart & Sherman, 1985) and by the presence of a proline in position which may create a local conformation that prevents binding of aminopeptidase (Liang, Allet, Rose et al. 1985).

We used three different biological assays to test refolding of both proteins. Two well-established effects of prolactin administration to tilapia are the induction of hypercalcaemia and an increased numerical density of skin mucocytes: this was shown for both ovine and tilapia prolactins (Wendelaar Bonga & Meis, 1981; Wendelaar Bonga & Flik, 1982; Flik et al. 1984). In the absence of sufficient homologous prolactin, the latter was administered by implantation of prolactin cells in the intraperitoneal cavity of the recipient fish. The hypercalcaemic effect is mainly due to stimulation of calcium uptake via the gills (Flik et al. 1984, 1989a). As shown in these previous studies, we can see that both tiPRL-I and tiPRL-II induced a significant rise in plasma calcium levels as well as in mucocyte density in the abdominal skin epithelium. Plasma osmolality was unchanged, similar to the natural prolactins tested in tilapia (Wendelaar Bonga & Meis, 1981) indicating that the increase in plasma calcium is not caused by a general increase in plasma electrolytes. Thus, the two prolactin preparations exhibited biological activities similar to natural prolactins for all the parameters tested. These results agree with a previous study indicating that the two forms of tilapia prolactin isolated from pituitaries are indistinguishable with regard to their effects on plasma ion regulation in freshwater tilapia (Young et al. 1988).

The third test was a binding assay. Several binding studies with ovine prolactin have confirmed the presence of prolactin-binding sites in osmoregulatory organs such as gill or kidney (Fryer, 1979; Edery et al. 1984; Dauder et al. 1990) and binding appeared to be higher in freshwater tilapia than in seawater-acclimated fish. Thus, the ability to bind specifically to kidney membranes, collected from freshwater fish, appeared to be another way to characterize bioactivity of both tiPRL preparations. When tested on kidney membrane the two forms of recombinant ti-PRL exhibit potency in competing with 125I-labelled tiPRL-I for binding sites. However, tiPRL-I appeared to be more potent than tiPRL-II in competition for the tiPRL-I-binding sites. One possible explanation is that recombinant tiPRL-I preparation has regained more biological activity, after the refolding step, than tiPRL-II. However, this explanation is not supported by the other biological assays where the two prolactins appeared equipotent in stimulating mucocyte density and plasma calcium level. Another explanation is that natural tiPRL-II has lower affinity than natural tiPRL-I for tiPRL-I-binding sites. Dauder et al. (1990) have shown that the two tiPRLs are equipotent in displacing ovine prolactin from its binding sites on kidney membranes. However, these results obtained using mammalian prolactin do not allow definitive conclusions with respect to relative affinity of the two tiPRLs for their receptors. Moreover, the presence of one or two prolactin-binding sites is still questionable (Edery et al. 1984; Dauder et al. 1990). Complete characterization of both tiPRL-I and -II binding sites in kidney tissue is needed before any conclusion can be drawn about this problem. Finally, our results (Text-fig. 3) show a parallelism between displacement curves obtained with pituitary extracts (containing both natural tiPRLs) and with recombinant tiPRL-I or -II. This suggests that our recombinant prolactins recognize the same binding sites on kidney membranes as natural tiPRLs. Thus, qualitatively, these hormonal preparations have the same activity in this bioassay as natural pituitary products.

In conclusion, the results of the three biological assays suggest that the two forms of recombinant prolactin obtained in this study have the biological activity previously observed with natural tilapia preparations. The T7 expression system used in this study thus provides a simple way to obtain large amounts of active prolactin of both types for further biological studies.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Mr F. A. T. Spanning’s excellent organization of fish husbandry. We also thank V. Goffin (Laboratoire de Biologie Moléculaire et de Génie Génétique, Université de Liège, Belgium) for helpful discussions. This work was supported, in part, by a grant from Eurogentec S.A. (Liège, Belgium).

REFERENCES


DESCRIPTION OF PLATES

Plate 1
SDS-PAGE and Western blot analysis of tilapia prolactin-II (tiPRL-II) synthesis. Total cell lysate was prepared by centrifuging 1 ml cells. The pellet was dissolved in 200 µl Laemmli buffer (Laemmli, 1970) and 20 µl were heated at 90 °C for 5 min and loaded onto a 15% polyacrylamide gel. Figure 1. Total cellular protein of uninduced (lane 1) and induced (lane 2) cells.

Figure 2. Western blot analysis. Total protein was transferred to nitrocellulose and tiPRL-II was detected using a salmon prolactin antiserum. M, molecular weight standards (kDa).

Plate 2
SDS-PAGE analysis of the refolding of proteins (Fig. 3) tiPRL-I and (Fig. 4) tiPRL-II. Samples of inclusion bodies were dissolved in Laemmli buffer with (lane 1) or without (lane 3) reducing agent (β-mercaptoethanol). Faster-migrating oxidized molecules and dimeric forms can be found under non-reducing conditions. Samples of proteins after denaturation-renaturation steps were dissolved in Laemmli buffer with (lane 2) or without (lane 4) reducing agent. Both recombinant proteins migrated faster under non-reducing than under reducing conditions.