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Journal of Chromatography A, 922 (2001) 165–175

JOURNAL OF CHROMATOGRAPHY A

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Ni(II)-based immobilized metal ion affinity chromatography of recombinant human prolactin from periplasmic *Escherichia coli* extracts

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Received 26 January 2001; received in revised form 10 April 2001; accepted 10 April 2001

Abstract

A novel, two-step preparative technique is described for the purification of authentic recombinant human prolactin (rhPRL) secreted into the periplasm of transformed *Escherichia coli* cells. The first step is based on immobilized metal ion affinity chromatography of periplasmic extract, using Ni(II) as a relatively specific ligand for hPRL in this system. It gives superior resolution and yield than established ion-exchange chromatography. Size-exclusion chromatography is used for further purification to >99.5% purity. The methodology is reproducible, leading to 77% recovery. Identity and purity of the rhPRL were demonstrated using sodium dodecylsulphate–polyacrylamide electrophoresis, isoelectric focusing, mass spectrometry (matrix-assisted laser desorption ionization time-of-flight), radioimmunoassay, RP-HPLC and high-performance size-exclusion chromatography. In the Nb2 bioassay, the hormone showed a bioactivity of 40.9 IU/mg. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Immobilized metal affinity chromatography; *Escherichia coli*; Prolactin; Polypeptides; Peptides; Hormones; Proteins

1. Introduction

Human prolactin (hPRL) is a polypeptide hormone produced by the anterior pituitary gland and, to a lesser extent, by some other tissues [1]. Several structural variants of the hormone exist as a result of pre- and post-translational modifications (e.g., phosphorylation, glycosylation). The basic form of hPRL is a single-chain protein consisting of 199 amino acids and three disulphide bridges. While PRL is best known for its stimulation of lactation, it displays a wide range of diverse biological properties, including regulatory roles in breast development, reproduction and the immune response. Its various functions can be attributed, in part, to its molecular heterogeneity [1,2].

Purified PRL was first obtained from pituitary extracts. However, the methodology used proved to be harsh to its molecular structure and also cumbersome due to the various purification steps involved [3,4]. Whereas expression of PRL in transformed cells, whether prokaryotic or eukaryotic, circumvented some of the problems associated with PRL

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PII: S0021-9673(01)00875-5

extraction from glands, the recombinant technology created different dilemmas. Low yield refolding [5], flawed processing of a fusion protein [6,7], poor cleavage of polyhistidine tails [8] and low production [9] have been cited in the generation of PRL by transformed *Escherichia coli* bacteria. In eukaryotic cells, the presence of low potency glycosylated hPRL forms [10,11] and cleaved isoforms [11,12] hampered the purification process due to physicochemical similarities between the variants and the hormone. PRL heterogeneity may also be produced by protein handling and the presence of such artificial variants cannot only impair the stability of the final product, but also its biological potency [2]. It therefore appears that there is a compelling need for an improved methodology aimed at producing PRL and its variants in highly purified forms which can be used to delineate their functional differences.

PRL and the closely related hormone, human growth hormone (hGH), are known to be zinc-binding proteins [13–15]. The affinity of hGH for zinc forms the basis of its purification by Zn (II)-based, immobilized metal affinity chromatography (IMAC) [16]. In the present study, the metal ion affinity of PRL was utilized for purification of authentic, recombinant hPRL secreted into the periplasmic fluid of transformed *E. coli* cells. It was found that the yield and purity level of the hormone, as obtained with procedures involving an initial well-established, cationic exchange resin chromatography step [10], could be substantially improved by substituting this step with IMAC based on Ni^{2+} acting as a highly specific ligand for PRL.

2. Materials and methods

2.1. Materials

A pituitary hPRL preparation (NIDDK-hPRL-RP-2 with a stated potency of 35.0 IU/mg) and rabbit anti-hPRL antiserum (NIDDK-anti-hPRL-3) from the National Institute of Diabetes and Digestive and Kidney Diseases were kindly donated from Dr. A.F. Parlow, National Hormone and Pituitary Program, Torrance, CA, USA. The first International Standard for hPRL, coded 84-500, with a formally assigned specific activity of 21.1 IU/mg was kindly provided by the National Institute for Biological Standards and Control (South Mimms, UK). Highly purified pituitary hPRL (hPRL-NOR) was obtained from Aker University Hospital, Oslo, Norway. Chromatographic resins, ampholytes, molecular mass markers, Coomassie Blue R-250, protein A and β-D-thiogalactopyranoside (IPTG) were purchased from Amersham Pharmacia Biotech (São Paulo, Brazil). Antifoam A, cytochrome *c*, myoglobin and proteins used for isoelectric point (p*I*) standards were obtained from Sigma (St. Louis, MO, USA). Culture media and antibiotics were obtained from Gibco-BRL (Gaithersburg, MD, USA).

2.2. Methods

2.2.1. Expression of recombinant hPRL (rhPRL)

The starting material for rhPRL purification was prepared from *E. coli* RB791 bacteria harboring an expression vector in which the hPRL complementary DNA (cDNA) was inserted downstream of the tac promoter and a signal sequence similar to the one described for the *E. coli* maltose binding protein [17]. The vector construction was carried out in collaboration with Sanofi Recherche (Toulouse, France). Following transformation, cryotubes containing the bacterial culture and 15% glycerol were stored at −80°C; for each fermentation procedure a different tube was used to plate the recombinant bacteria. One or two isolated colonies were used to inoculate 100 ml of Luria-Bertani medium (containing 100 μg/ml ampicillin) and the culture was incubated in a shaker for 14 h at 37°C. After a (1:50) dilution culturing was continued in a bioreactor, maintaining the pH (7.2), temperature (37°C) and dissolved oxygen tension (35%). The foam level was controlled by addition of antifoam A. Expression of rhPRL by the cultured *E. coli* cells was induced after 2 h by adding IPTG up to 1 m*M* and fermentation was allowed to continue for a further 3 h.

2.2.2. Preparation of periplasmic extract

The fermentation product was centrifuged at 16 000 *g* for 30 min and the ice-cooled pellet resuspended in ice-cold 10 m*M* Tris–HCl, 16 m*M* EDTA, pH 7.5, containing 23% (w/v) sucrose (volume=$A_{600\ nm}$·culture volume/100) and incubated for 15 min on ice. Following centrifugation,

the pellet was resuspended in the same volume of cold water and the suspension incubated for 30 min on ice and then centrifuged again for 10 min. The supernatant (osmotic shock fluid) was saved as the periplasmic fraction [18].

2.2.3. Purification of rhPRL

2.2.3.1. Ion-exchange chromatography on Sp-Sepharose fast flow

Procedures employed were a modification of the methodology previously described by Price et al. [10]. Briefly, the pH of the periplasmic extract was adjusted to 5.0 using 3 *M* acetic acid and the extract centrifuged (2000 *g*, 10 min, 4°C) to remove proteins that had precipitated at this pH. The supernatant was then applied onto a SP-Sepharose Fast Flow column (Pharmacia Biotech, Upsala, Sweden, XK16/40; 14 cm×16 mm I.D.), equilibrated in 50 m*M* sodium acetate, pH 5.0 (buffer A) and the effluent absorbance monitored at 280 nm. After washing to baseline absorbance, the column was washed with buffer A containing 200 m*M* sodium chloride. rhPRL was then eluted with 50 m*M* *N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid) (HEPES) (pH 9.0). Fractions were analysed using sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and radioimmunoassay.

2.2.3.2. IMAC on Ni(II)-chelating Sepharose Fast Flow

The periplasmic extract was dialysed against 50 m*M* ammonium hydrogencarbonate (pH 7.9) to remove traces of EDTA utilised in the osmotic shock procedure. The extract, containing up to 7 mg PRL, was then applied onto a column pre-packed with chelating Sepharose Fast Flow gel (Pharmacia Biotech, Upsala, Sweden, XK16/40; 25 cm×16 mm I.D.) and precharged with Ni^{2+} following recommendations by the manufacturer. Equilibration was carried out with 50 m*M* sodium phosphate (pH 7.2), 0.8 *M* NaCl, 0.1% Tween 20 (buffer B). After washing to baseline absorbance, the column was washed with buffer B containing 30 m*M* imidazole. The rhPRL was then eluted with buffer B containing 60 m*M* imidazole. Contaminants with high affinity for the resin were eluted with buffer B containing 100 m*M* imidazole. Fractions were analysed using SDS–PAGE and radioimmunoassay.

2.2.3.3. Size-exclusion chromatography

Pooled rhPRL activity obtained by either the SP-Sepharose or Ni(II)-chelating Sepharose chromatography was applied (maximum volume, 15 ml) onto a 300 ml Sephacryl S-100 HR column (Pharmacia Biotech, Upsala, Sweden, XK26/70; 57 cm×26 mm I.D.) pre-equilibrated with 0.05 *M* ammonium hydrogencarbonate (pH 7.9). Fractions were collected, analyzed for purity by SDS–PAGE and the identity confirmed by radioimmunoassay. Fractions were pooled for maximal recovery. Mock chromatography with buffer utilized in the first chromatographic step was executed as a control for reagent-induced artefacts. Fractions corresponding to the hPRL peak were also collected as controls of buffer-induced artefacts in RP-HPLC analysis.

2.2.4. Analytical procedures

2.2.4.1. hPRL and total protein determination

Human PRL immunoactivity was assayed using a double-antibody liquid-phase radioimmunoassay (RIA), as previously described [8]. Total protein concentration was determined using the Bradford reaction [19] with bovine serum albumin as a standard.

2.2.4.2. Electrophoresis and immunoblotting

Analysis of hPRL-containing fractions was performed by electrophoresis on 12% SDS–polyacrylamide gels under non-reducing conditions [20]. The gels were stained with Coomassie Blue R-250 or the proteins transferred by electrophoresis to a nitrocellulose membrane for immunoblotting using rabbit polyclonal anti-hPRL antibody. Bound antibody was detected with protein A labelled with ^{125}I, followed by autoradiography [9].

2.2.4.3. Mass spectrometry

The purity and molecular mass of purified rhPRL samples were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Analysis was performed on a Voyager DE (PE Biosystems, Foster City, USA) in positive ion mode using 40 kV

acceleration voltage. Calibration of the machine was carried out with a mixture of cytochrome *c* (M_r 12 362) and myoglobin (M_r 16 952). The matrix consisted of 100 m*M* sinapinic acid in a mixture of acetonitrile–methanol–water (1:1:1).

2.2.4.4. HPLC

Quantitative analysis of periplasmic extracts and its fractions was performed using isocratic RP-HPLC for hPRL, methodology described elsewhere [21]. Sham chromatography was performed to establish buffer-induced artefacts. High-performance size-exclusion chromatography (HPSEC) was used to identify the presence of hPRL and its oligomers and to quantify the purified hormone. Procedures were used as reported for hGH [22].

2.2.4.5. Isoelectric focusing (IEF)

IEF of purified rhPRL samples and of a pituitary hPRL preparation (hPRL-NOR) were performed on 8×8 cm, 5% acrylamide gels with pH 3.5–10 ampholytes. The gels were stained with Coomassie Blue R-250. Bovine β-lactoglobulin A, bovine carbonic anhydrase type II (A and B) and human carbonic anhydrase type I were used as IEF standards.

2.2.4.6. Amino acid sequencing and composition

The N-terminal amino acid sequence of purified rhPRL was determined following its electroelution from polyacrylamide gel and blotting onto a poly(vinylidene diflouride) (PVDF) membrane. Automated Edman degradation [23] on an Applied Biosystems Model 494 sequencer was used. The amino acid composition analysis of the rhPRL preparation was carried out using acid hydrolysis in a Perkin-Elmer Hydrolyzer/Derivatizer ABI 420 A [2]. Since protein quantification through this methodology is considered to be the most accurate [24], it was performed to validate the PRL determinations by RIA and RP-HPLC demonstrating good agreement among the methodologies.

2.2.4.7. PRL bioactivity

PRL bioactivity was determined using the Nb2 lymphoma cell proliferation assay [25]. The ability of purified rhPRL to stimulate ^{3}H-thymidine incorporation in quiescent, lactogen-dependent Nb2 lymphoma cell cultures was compared with that of pituitary hPRL preparations. Incubations were performed in 98-well plates in a final volume of 200 μl/well, with initial cell concentrations of 50×10^3 cells/ml. [^{3}H]-Thymidine (0.5 μCi/10 μl/well) was added at 44 h and the incubation continued for another 4 h, followed by harvesting of the cells and determination of the radioactivity incorporated into DNA in a Betaplate counter (LKB Wallac, St. Quentin en Yvelines, France) [26].

3. Results

3.1. Purification of rhPRL

Two procedures for purification of rhPRL from periplasmic *E. coli* extract were compared. They consisted of (i) an ion-exchange and (ii) an IMAC purification step, both followed by Sephacryl S-100 size-exclusion chromatography as a refinement procedure. Quantification of hPRL in crude extracts, homogeneous or semi-purified preparations was carried out by RP-HPLC coupled to radioimmunoassay. The RP-HPLC, based on high hydrophobicity of the PRL molecule, allows separation of the hormone from the bulk of bacterial proteins, hence enabling highly accurate and sensitive quantification of hPRL [21].

The first procedure (Fig. 1) involved an SP-Sepharose chromatography step used by Price et al. for purifying hPRL secreted by transformed murine C127 cell cultures [10]. This step was optimized for purification of rhPRL from periplasmic *E. coli* fluid. Following adjustment of the latter to pH 5.0, precipitation of bacterial proteins occurred, probably due to the fact that many *E. coli* proteins have a p*I* of about 5.0 [27]. The precipitate was eliminated, without PRL loss, by a centrifugation step prior to chromatography, instead of a clarification by filtration utilized by Price et al. [10]. Increasing the NaCl concentration in the washing fluid to 200 m*M*, while omitting Tween 80, led to enhanced elution of contaminants without significant loss of the hormone when compared with the lower molarity washing buffer (90 m*M*) previously described [10]. As shown in Fig. 1A, rhPRL was eluted with 50 m*M* HEPES (pH 9.0) as one sharp peak. The HEPES concen-

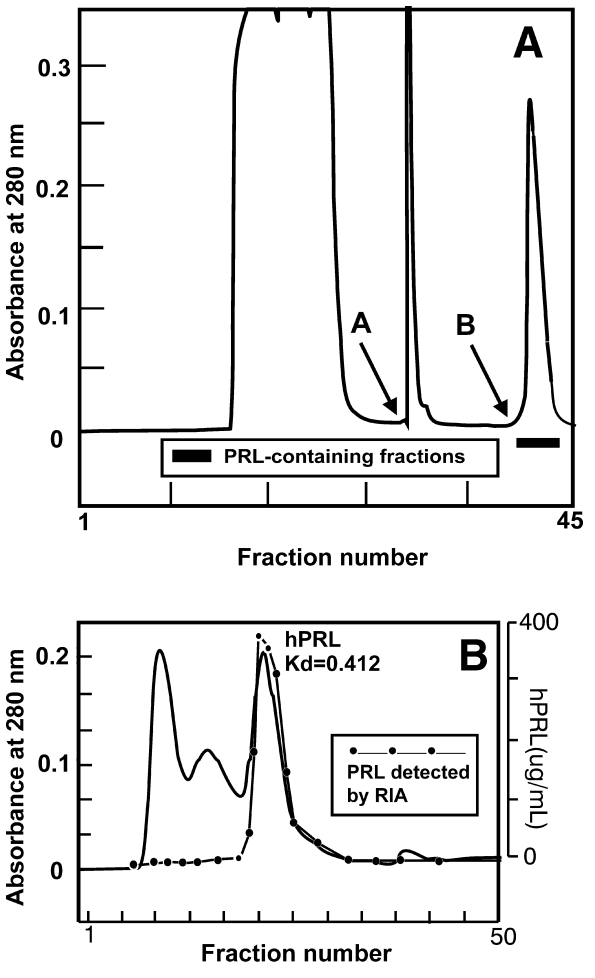

Fig. 1. Typical results obtained with cation-exchange and size-exclusion chromatography of periplasmic rhPRL. (A) SP-Sepharose Fast Flow chromatography. After application of the periplasmic extract (100 mL containing 7.5 mg of rhPRL), washings were performed with 50 m*M* sodium acetate (pH 5.0) and with the same buffer containing 200 m*M* NaCl (arrow A); hPRL was eluted with 50 m*M* HEPES (pH 9.0) (arrow B). The flow-rate was maintained at 110 mL/h. (B) Size-exclusion chromatography on Sephacryl S100 of pooled fractions. A flow-rate of 60 mL/h (50 m*M* ammonium hydrogencarbonate buffer, pH 7.9) was used throughout.

tration and pH were increased to avoid contaminant precipitation observed with the unmodified methodology. Results of the subsequent purification using a Sephacryl S-100 column are shown in Fig. 1B. In this column, the distribution coefficient (K_d) for hPRL was compatible with a 23 000 protein (K_d = 0.412). The hormone was resolved from higher molecular mass protein contaminants (peak I, Fig. 1B), but not from the adjacent overlapping peak of impurities (peak II, Fig. 1B). This led to losses during collection of the PRL-containing fractions.

In the second procedure, a novel method for purification of hPRL was used. It is based on affinity of PRL for divalent metal ions [13–15,28,29], a feature which is not so common for bacterial proteins [30]. IMAC was carried out using Ni(II) ions as the ligand. Ni(II) was selected since it had already been used in IMAC for partial purification of hGH, which is a PRL analogue [16]. Initial optimal elution conditions were first established with a linear imidazole gradient from 0 to 100 m*M* (data not shown). In order to reduce separation times and obtain more reproducible results, we designed a step elution based on the method optimisation. It was found that 30 m*M* imidazole efficiently removed contaminants with low affinity for the resin (peak I, Fig. 2A). For elution of PRL, 60 m*M* imidazole was selected out of 45, 55, 60 and 100 m*M* imidazole, since molarities lower than 60 m*M* led to incomplete PRL elution resulting in broad peaks and diluted fractions. Although with a higher molarity (100 m*M*) we obtained a sharper peak and a faster PRL elution, co-elution of tightly bound contaminants occurred (data not shown). PRL elution with 60 m*M* of imidazole yielded a sharp peak (peak II, Fig. 2A) free of strongly adsorbed impurities (peak III, Fig. 2A). Subsequent size-exclusion chromatography on Sephacryl S-100 led to a single peak of rhPRL (K_d = 0.421); the smaller peaks were due to buffer-induced artefacts (Fig. 2B).

The results in Fig. 3 clearly show that use of Ni(II)-based IMAC led to a much purer initial rhPRL fraction than SP-Sepharose ion-exchange chromatography (lanes 3 and 5). This probably reflects a higher selective affinity of the IMAC column for the hormone. Following the second purification step, single bands of an M_r of 23 000, consistent with the M_r of hPRL, were obtained in both methodologies when the rhPRL was analysed on SDS–PAGE (Fig. 3, lanes 4 and 6). Table 1 shows a comparison between the recovery and purity levels achieved by the two methodologies employed. In the IMAC methodology better recoveries were obtained for both steps (84 and 91%) than in the

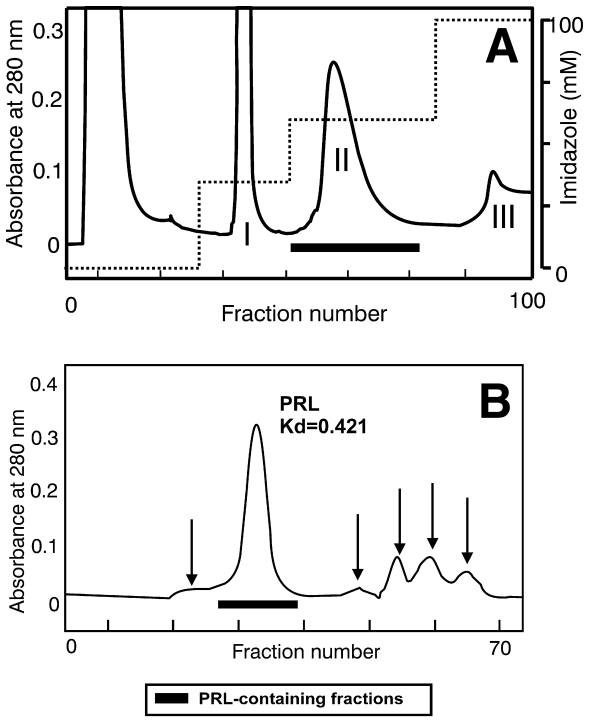

Fig. 2. Typical results obtained with purification of periplasmic rhPRL using IMAC as the first step. (A) Ni (II)-based IMAC. After application of the dialyzed periplasmic extract (100 mL containing 10 mg of rhPRL), washings were performed with 50 m*M* sodium phosphate buffer, 0.8 *M* NaCl, pH 7.2 and with the same buffer containing 30 m*M* imidazole to remove impurities with low affinity for the matrix (peak I). The hormone (peak II) was eluted with buffer containing 60 m*M* imidazole and a final wash with 100 m*M* imidazole was used to elute contaminants (peak III). A flow-rate of 100 mL/h was maintained throughout. The dotted line refers to the imidazole concentration (m*M*) employed. (B) Size-exclusion chromatography on Sephacryl S100 of pooled fractions. A flow-rate of 60 mL/h of ammonium hydrogencarbonate buffer, pH 7.9, was used. The arrows indicate buffer-induced artefacts. For further experimental details, see text.

ion-exchange chromatography methodology (61 and 84%). It can be inferred from these results that the major factor that contributed to the final PRL recovery observed in the IMAC procedure (ii) (77 vs. 46%) was the high PRL-adsorptive IMAC step, since the final chromatography was common for both methods and presented similar recovery yields. Final purity levels accomplished through the IMAC procedure were also higher than that attained by the cation-exchange method (99.5 vs. 97%).

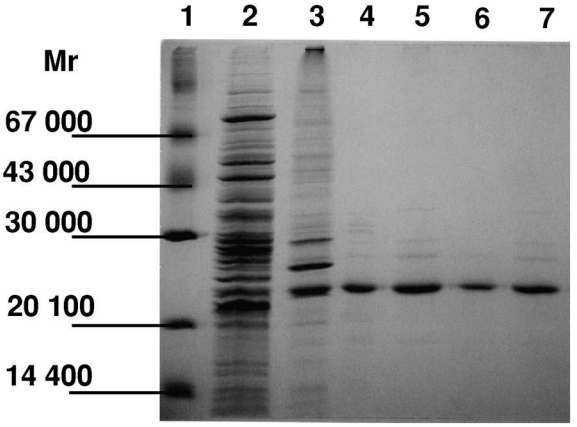

Fig. 3. Coomassie Blue stained SDS–PAGE (12.5%) analysis of samples obtained during periplasmic rhPRL isolation and purification steps. Lanes: 1, molecular-mass markers; 2, periplasmic extract; 3, SP-FF eluted hPRL; 4, size-exclusion chromatography of hPRL eluted from SP-FF; 5, CS-FF-Ni^{2+} eluted hPRL; 6, size-exclusion chromatography of hPRL eluted from CS-FF-Ni^{2+}; 7, pituitary hPRL-NOR.

3.2. Characterization of IMAC-purified rhPRL

MALDI-TOF-MS of IMAC-purified rhPRL proved its identity and showed a high degree of purity (Fig. 4). The M_r obtained was 22 888, a value consistent with the M_r of hPRL described in the literature, i.e. 22 892 [10], and its theoretical value, 22 899.

Analysis of the purified rhPRL by analytical HPSEC showed a main symmetrical peak (Fig. 5B), with a retention time (t_R) consistent with a 23 000 protein and smaller peaks (total of 6%) that are attributed to PRL oligomers.

It has previously been reported that deamidation of hGH leads to alterations in its hydrophobicity, allowing separation of the variant from the unaltered form by RP-HPLC [31]. Analysis of IMAC-purified rhPRL by RP-HPLC (Fig. 5A) generated two peaks (in addition to several reagent-induced peaks). The major one (24 756 t_R) is hPRL and the other peak (22 284 t_R) is probably due to a deamidated rhPRL derivative corresponding to 4.0% of total protein. A similar distribution was found for rhPRL purified by the ion-exchange methodology (data not shown). Many peaks were observed when a purified pituitary

Table 1
Recovery of PRL after each purification method used for its purification from the periplasmic fluid. The values obtained are averages of five independent experiments performed on 100 mL of periplasmic fluid. The recovery and purification factors are calculated relative to the total amount (mg) of PRL in 100 mL of periplasmic fluid. Details of the pooled fractions referred to here are shown in Figs. 1 and 2. The numbers in parentheses are ranges of values obtained for the recovery

	Periplasmic fluid		Step I		Step II	
	Method (i)	Method (ii)	(i) Cation exchange	(ii) IMAC	(i) Size exclusion	(ii) Size exclusion
hPRL (mg)[a]	7.5	10.0	4.5	8.4	3.4	7.6
Total protein (mg)[b]	171.5	120.0	8.1	7.9	3.1	6.2
Mass fraction[c]	0.04	0.08	0.56	1.06	1.10	1.23
Purity (%)[d]				97.7	97.0	99.5
Purification factor	1	1	13.0	12.6	25.5	14.6
Recovery (%)	100	100	61±21 (45–67) $n=5$	84±9 (76–86) $n=5$	46±9 (40–52) $n=5$	77±9 (67–79) $n=5$

[a] Estimated by RIA and RP-HPLC.
[b] Estimated by the Bradford method.
[c] Mass fraction is defined as PRL content divided by total protein mass.
[d] The purity level was estimated by laser densitometry of the PRL staining-bands. Method (i) consists of a cation-exchange chromatography followed by a size-exclusion chromatography; method (ii) consists of a IMAC followed by a size-exclusion chromatography.

hPRL preparation (hPRL-NOR) was analysed by this method, probably a result of contaminants present in this preparation (data not shown).

Isoelectric focusing analysis of purified rhPRL and of the purified pituitary hPRL-NOR preparation (Fig. 6A), shows that both preparations contain two bands corresponding to p*I* values of 6.16 and 5.95. The band with a p*I* of 5.95, which corresponds to an acid form and probably represents the deamidated form, is present to a lesser extent in the rhPRL than in the pituitary preparation. This finding is consistent with the data obtained by RP-HPLC (Fig. 5A). The higher concentration of deamidated PRL in the pituitary preparation probably reflects a higher degree of manipulation.

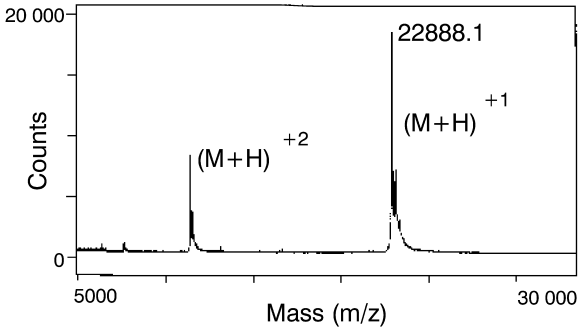

Fig. 4. M_r determination of hPRL by MALDI-TOFI-MS performed as described in Materials and methods. The monocharged 22 888.1 M_r is indicated above the corresponding peak.

In addition to the SDS–PAGE analysis (see above), immunoblot analysis showed an identical profile for the purified rhPRL and the pituitary hPRL standard (Fig. 6B). N-Terminal amino acid analysis confirmed that the rhPRL had the same N-terminal sequences and signal peptide cleavage as the natural hormone [32] (Table 2). The amino acid composition of the rhPRL was also in agreement with that reported for the natural hormone [33] (Table 3).

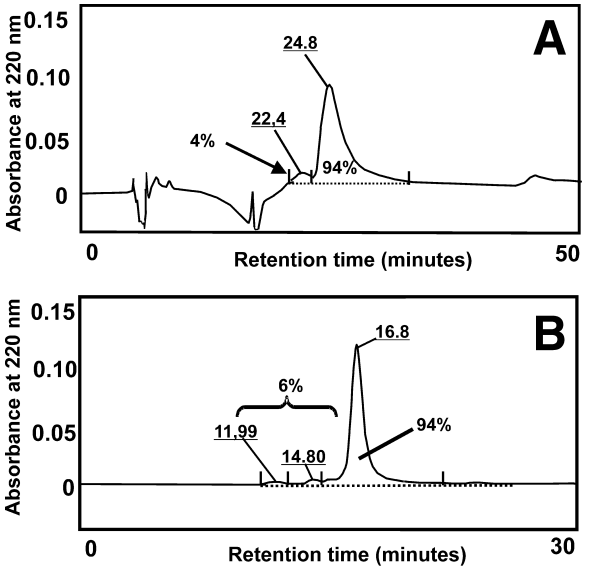

Fig. 5. HPLC analysis of rhPRL purified by IMAC and size-exclusion chromatography. (A) Isocratic RP-HPLC on a C_4 Vydac 214 TP 54 column (25 cm×4.6 mm I.D.; pore diameter 300 Å and particle diameter 5 μm), connected to a Vydac 214 FSK 54 guard column. The mobile phase consisted of 71% Tris–HCl buffer (50 m*M*, pH 7.5) and 29% *n*-propanol, with a flow-rate of 0.5 mL/min and column temperature maintained at 45°C. (B) Isocratic HPSEC on a G2000SW column (60 cm×7.5 mm I.D.; particle diameter 10 μm and pore size 125 Å) connected to a 7.5 cm×7.5 mm I.D. SW guard column. The mobile phase was 0.025 *M* ammonium hydrogencarbonate, pH 7.0, with a flow-rate of 1.0 mL/min. The arrow and bracket represent respectively the deamidated and oligomeric PRL isoforms. The retention times are displayed above the correspondent peaks. The amount of rhPRL injected was 25 μg in a 250 μL volume.

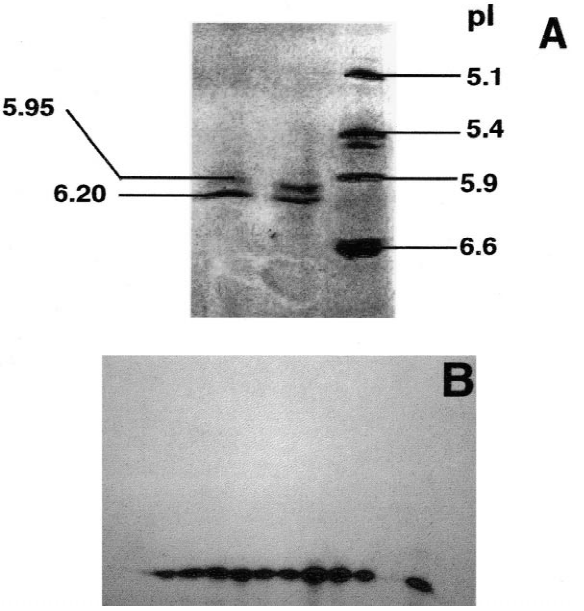

Fig. 6. (A) Analysis of rhPRL in IEF in 5% acrylamide gels with pH 3.5–10 ampholytes. Lanes: 1, purified rhPRL; 2, pituitary hPRL-NOR; 3, IEF standards (β-lactoglobulin A, p*I* 5.1; bovine carbonic anhydrase, type II A, p*I* 5.4; bovine carbonic anhydrase, type II B, p*I* 5.9 and human carbonic anhydrase type I, p*I* 5.6). (B) Assessment of immunological activity of hPRL by Western blotting. hPRL was subjected to SDS–PAGE and analysed by immunoblot using anti-hPRL antisera.

3.3. Bioactivity of rhPRL

The Nb2 cell proliferation assay was used to determine the mitogenic activities of various hPRL preparations (Fig. 7). The activities obtained were: IMAC-purified rhPRL, 41.0 IU/mg; crude rhPRL-containing periplasmic fluid, 38.5 IU/mg; hPRL-NIDDK, 35.0 IU/mg; and WHO-hPRL, 21.1 IU/mg. The slightly higher activities of the rhPRL preparations would be consistent with higher sample purity, less harsh purification conditions and absence of low-potency PRL isoforms currently observed in pituitary extracts such as glycosylated and phosphorylated variants.

4. Discussion

Bacterial production of periplasmic rhPRL in a secreted, properly folded, biologically potent, monomeric form has obvious advantages when compared to the production of the hormone in a reduced, insoluble form as cytoplasmic inclusion bodies. The main problem associated with the production of the secreted rhPRL is its low expression, with consequent difficulties with regard to its separation from bacterial proteins [9]. To counteract this difficulty, we developed a more efficient procedure for the purification of rhPRL from periplasmic extracts.

The hypothesis that bacterially-produced rhPRL could be purified on the basis of its affinity for divalent cations [13–15,28] was confirmed. In fact, the binding of rhPRL to immobilized Ni^{2+} was sufficiently tight and selective to obtain a highly purified homogeneous preparation of hPRL in one

Table 2
Amino-terminal sequence of residues 1-11 of purified PRL. The sequence is generated from data obtained after analysis of a purified sample of PRL on an Applied Biosystems Model 494 A automatic micro-sequencing apparatus according to standardized procedures

		1				5				10		--------199
Expected N-terminal sequence hPRL [32]	H_2N	Leu	Pro	ile	Cys	Pro	Gly	Gly	Ala	Arg	Cys	--------COOH
Observed sequence	H_2N	Leu	Pro	ile	Cys	Pro	Gly	Gly	Ala	Arg	Cys	--------COOH

single step. The imidazole concentration used for elution of the hormone was higher than for some His-tagged fusion proteins described in the literature [34], probably due to a tighter binding of the hormone to the immobilized Ni^{2+} ions. Furthermore, the Ni(II)-based IMAC allows purification of the rhPRL in the absence of poly-histidine tails which, even in cases where an enzymatic cleavage site is present, are sometimes difficult to remove [8].

A comparison of the SP-Sepharose-based purification of rhPRL in the present study with the SP-Sepharose-based purification of rhPRL produced by transformed murine cell cultures [10] shows that, in the latter study, better values were obtained for the recovery (100.0 vs. 61%) and mass fraction (1.0 vs. 0.6). These differences can be attributed to differences in the quantity and nature of the contaminating proteins. Whereas the mass fraction for hPRL in the conditioned culture medium was 0.318 [10], it was as low as 0.043 in the periplasmic extract (Table 1), indicating that the latter contained relatively more contaminants. It is likely that the higher concentration of protein contaminants in the periplasmic fluid led to lower adsorption of hPRL by the matrix (via competitive inhibition) and hence to lower recoveries of the hormone despite modifications and adjustments made in the sample preparation pro-

Table 3
Aminoacid composition of purified PRL

Amino acid	Expected composition [33]	Observed composition
Asp	9.05	9.75
Thr	4.02	4.45
Ser	8.54	8.68
Glu	13.57	14.36
Pro	4.02	4.44
Gly	3.52	4.3
Ala	5.53	6.00
Cys	X	X
Val	5.03	4.22
Met	2.51	2.36
Ile	6.53	5.27
Leu	12.06	12.32
Tyr	3.52	3.02
Phe	2.51	2.83
Lys	5.03	5.7
His	4.52	4.24
Trp	X	X
Arg	6.03	6.46
Total	100.0	100.0

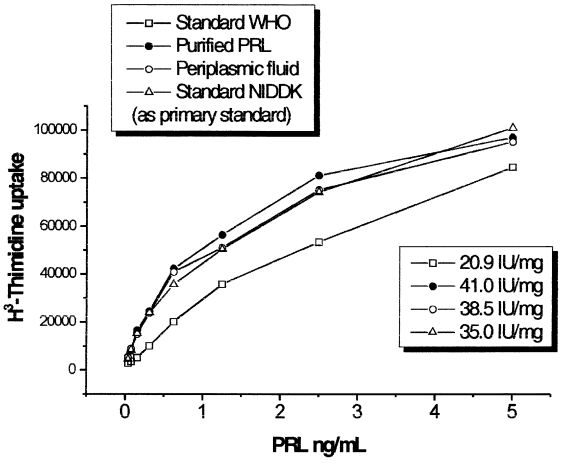

Fig. 7. Assessment of in vitro bioactivity of rhPRL in the rat Nb2 lymphoma cell proliferation assay. The mitogenic activities of the recombinant hPRLs were compared against two pituitary standards. Symbols: (□) International Standard of Pituitary hPRL-WHO 84/500; (△) NIDDK-hPRL-RP-2-Standard NIDDK as primary standard; (●) purified PRL; (○) periplasmic fluid.

cedure, washing and eluting conditions of the original methodology developed by Price et al. [10]. This also emphasizes the need for a highly efficient purification step for the separation of rhPRL from the bacterial proteins in the periplasmic extract, as appears to be provided by the Ni(II)-based IMAC (Fig. 3).

A comparison of the SP-Sepharose Fast Flow and the Ni(II)-based IMAC purifications of periplasmic rhPRL clearly shows that the novel, metal ion affinity-based purification of the hormone has better recovery values with much lower protein contamination, minimizing the chance of hormone proteolysis (Figs 1–3; Table 1). It is notable that subsequent size-exclusion chromatography was not only useful as a refinement step in the purification, but also for desalting and minimizing the presence of aggregated forms of the protein.

It has been reported that post-translational modifications of recombinant proteins can occur in *E. coli* bacteria following their expression (by proteolytic cleavage) or during purification (by deamidation, oligomerization and proteolysis) [2,35,36]. In view of this, characterization of the recombinant product is essential for verification of its identity, quality and biological activity. The immunologic activity of the IMAC-purified rhPRL was confirmed by Western blotting and by radioimmunoassay. Analysis by SDS–PAGE, mass spectrometry, HPSEC and Sephacryl S-100 chromatography confirmed that the recombinant product consisted of a protein with a molecular mass of 23 000, with only minor contamination by hPRL oligomers (6.0%). RP-HPLC and isoelectric focusing showed the presence of a protein with the characteristic t_R of hPRL and the presence of 4.0% of a less hydrophobic, more acidic form which is probably due to deamidated hPRL. Much higher percentages of this isoform were present in a pituitary hPRL preparation used as a control, underscoring the higher quality of our developed PRL preparation.

The results obtained in the present study indicate that the novel, Ni(II)-based IMAC protocol for purifying rhPRL from periplasmic *E. coli* fluid is most useful for the generation of highly purified hPRL. It is likely that this method will also be valuable for the purification of a number of hPRL variants as confirmed by initial promising data gathered in our laboratory (data not shown) where glycosylated isoforms showed a peculiar affinity towards the Ni(II) charged matrix, paving the way for a feasible separation methodology to resolve the non-modified PRL from variants with carbohydrate residues. The availability of highly purified hPRL and its variants is essential for investigations into their differential actions.

Acknowledgements

This work was supported by FAPESP, São Paulo, Brazil (project 98/05518-6).

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