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Short Communication

Purification to homogeneity of bovine prolactin by high-performance ion-exchange chromatography

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ABSTRACT

Homogeneous bovine prolactin (bPRL) has been obtained using a procedure based on high-performance anion-exchange chromatography. The procedure enables up to 6 mg of 99.4% pure bPRL to be obtained per hour, with a recovery of 32.4%. The purity of the protein was checked by N-terminal sequencing and sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The highly purified bPRL obtained with this method is suitable for complete structural and immunochemical studies.

INTRODUCTION

Prolactin (PRL) is a polypeptide hormone of molecular weight 23 000 which is secreted by the acydophil cells of the anterior pituitary. The single chain of 199 residues has three disulphide-bonded loops, two of which are located near the two termini of the molecule. It is secreted in the form of a prohormone with a sequence of 229 amino acids. An enzymatic cleavage occurs at position 30 giving the native form of PRL [1]. Belonging to the same hormonal family as growth hormone, it shares a number of biological, immunological and structural features with this molecule [2], as well as with mammalian placental lactogen and proliferin [3].

Pituitary PRL preparations always contain a certain degree of heterogeneity because of the presence of various post-translational modified forms of the molecule [4–6]. Using traditional chromatographic systems, it is difficult and time consuming to remove the heterogeneous forms from the native PRL preparation.

This paper describes the application of a high-performance liquid chromatography (HPLC) system with an anion-exchange column for the purification to homogeneity of pituitary bovine PRL (bPRL).

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EXPERIMENTAL

Purification of bPRL

The starting material was prepared by following the first steps of the procedure described by Reichert [7]. The ethanolic precipitate obtained (bPRL 1) was chromatographed (100 mg per 10 ml per run) on a Sephacryl S-200 (Pharmacia LKB, Uppsala, Sweden) column (70 × 2.6 cm) in a 25 m*M* phosphate buffer solution, pH 7.5. Eluate fractions (10 ml) were collected and aliquots were assayed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The bPRL peak (bPRL S-200) was concentrated and further purified by anion-exchange HPLC.

The HPLC apparatus used was a Waters 625 LC system equipped with a Waters tunable absorbance detector (Waters Millipore, Milan, Italy). A TSK-DEAE 5PW column (10 μm; 75 × 7.5 mm) (Toyo Soda, Japan) was used, which was operated at a flow-rate of 1 ml/min in phosphate buffer solution (25 m*M*, pH 7.5). The eluate was recorded at 280 nm. The sample (2–10 mg per injection) was eluted with a 16 min linear gradient to 0.9 *M* NaCl in the phosphate buffer. The fraction containing bPRL was identified by SDS-PAGE, assayed for protein content [8] and stored in a lyophilized form at −20°C (bPRL-DEAE).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

The SDS-PAGE was performed in polyacrylamide gradient gels (7–20%) overlain with 5% stacking gel [9], using a Minigel apparatus (Biometra, Göttingen, Germany). The electrophoresis was performed at a constant current of 10 mA for 15 min and successively at constant voltage (200 V) for about 1 h. The gels were stained with Coomassie Brilliant Blue R250 and with silver nitrate [9]. The stained bands were quantified by scanning the electrophoretic patterns at 595 nm using a Quick Scan R & D densitometer (Helena Labs, Beaumont, TX, USA).

Amino acid sequence

A sample of 2.7 nmol of bPRL-DEAE was submitted to 10 cycles of sequence analysis using an automatic amino acid sequencer (Applied Biosystems, Milan, Italy).

RESULTS AND DISCUSSION

The yields and purification rate at each stage of the purification procedure are given in Table I. The total protein concentration was determined using the method of Lowry *et al.* [8]. The amount of bPRL in the fractions was determined by SDS-PAGE, comparing the integrated intensity of the hormone band with the integrated intensity of known amounts of the purified hormone migrated on the same gel [10]. The total yield of homogeneous native bPRL was about 20 mg per 100 g wet weight of pituitaries. The recovery was 32.4% and the purification was 99.4%.

Fig. 1 shows the elution profile obtained after the application of 2 mg of bPRL S-200 to the anion-exchange column. Homogeneous bPRL eluted in the first peak (6.36 min after the injection) at a sodium chloride concentration of 0.21 *M*. Using HPLC it was possible to purify large amounts of bPRL in a short time. To isolate less than 20 mg/h, an analytical column (75 × 7.5 mm) was chosen, but it is possible to scale-up the method for preparative separations.

TABLE I

SEQUENCE OF PURIFICATION OF bPRL

Stage	Total protein (mg)	bPRL (mg)	Yield (%)	Purification (%)
bPRL 1	185.25	59.09	100	31.9
bPRL S-200	62.06	42.09	71.2	67.8
bPRL-DEAE	19.25	19.13	32.4	99.4

The purification rate during the bPRL preparation was monitored by SDS-PAGE and the Coomassie-stained bands were quantified by densitometry. The electrophoretic patterns of bPRL after each stage of the purification are shown in Fig. 2. The bPRL-DEAE formed (samples 3 and 4) was homogeneous with a purity of more than 95%, also when the gel was stained with silver nitrate.

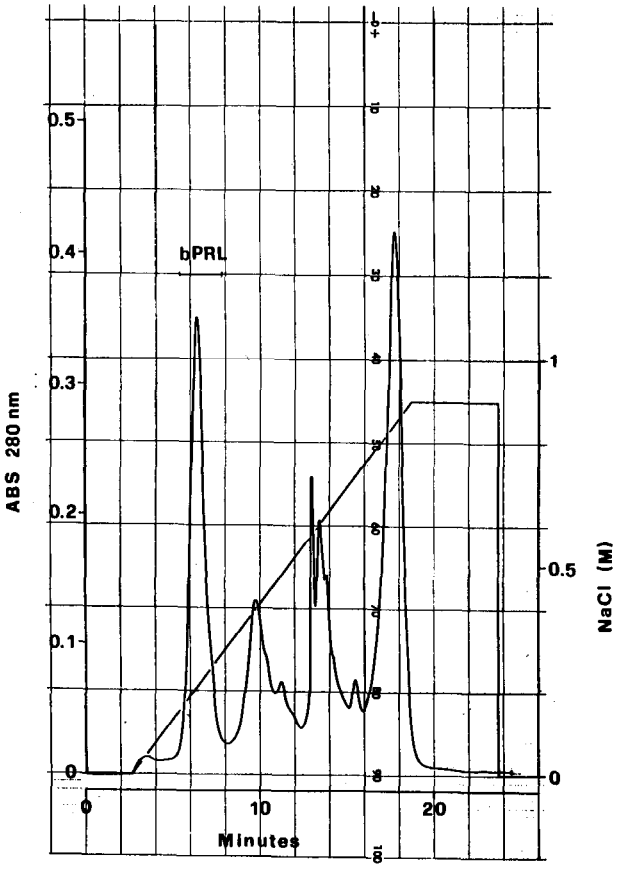

Fig. 1. High-performance liquid chromatogram obtained after the application of 2 mg of bPRL S-200 to the anion-exchange column. The sodium chloride concentration gradient used for the elution is shown on the graph.

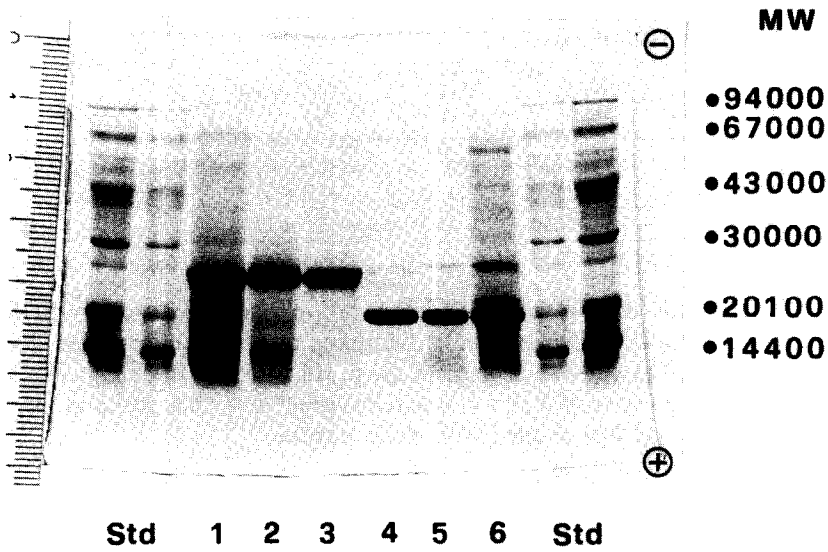

Fig. 2. Gradient SDS-PAGE of bPRL during the purification procedure. Std = standard proteins for molecular weight (MW); 1, 6 = bPRL 1; 2, 5 = bPRL S-200; 3, 4 = bPRL-DEAE. Samples 1–3 were dissolved in the presence of the reducing agent 2-mercaptoethanol (5%). Samples 4–6 were in the oxidized form.

Samples were analysed under reducing (samples 1–3) and non-reducing (samples 4–6) conditions. As the figure shows, bPRL has a mobility which is related to the reduced or oxidized state of its disulphide bridges. This phenomenon has already been observed for rat [5] and human PRL [11]. It has been suggested [11] that these proteins in their S–S bonded form have a more compact structure which results in lower apparent molecular weight.

The first ten amino acid residues of purified bPRL obtained from sequence analysis were: H_2N–Thr–Pro–Val–Cys–Pro–Asn–Gly–Pro–Gly–Asn. This sequence was obtained free of contamination by other amino acids and corresponds exactly to the bPRL sequence [1].

Using the procedure described here, bPRL can be purified from a crude extract to homogeneity with only two chromatographic steps, one of which exploits the resolving power of HPLC. The bPRL purification procedures described previously involve the use of preparative electrophoresis, which is useful in minimizing microheterogeneity in the protein preparation [10]. This kind of methodology offers the advantage of a high resolution separation, but it is not very reproducible and requires a very long time to obtain the separation and elution of the protein bands [9]. In comparison, the method reported in this paper gives a fast and reproducible recovery of highly homogeneous bPRL, which is readily available for structural and immunochemical characterization.

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