Membrane-bound prolactin receptors from the mammary gland of 6–7-day postpartum lactating rabbits were solubilized using the zwitterionic detergent, Zwittergent 3-12 (3-dodecyl(dimethylammonio)-1-propanesulfonate). The solubilized receptor from one rabbit was bound to an ovine prolactin-agarose affinity gel and eluted at pH 4.2. The receptor appeared as a single band upon staining sodium dodecyl sulfate-polyacrylamide gels with Coomassie blue. The protein yield from one rabbit was about 8 µg and the overall yield of receptor was over 50%. The apparent molecular weight was 42,000 on sodium dodecyl sulfate gels but varied on molecular weight columns due to the type of detergent. Receptor inactivated by iodoniation had an apparent molecular weight of 21,000 on sodium dodecyl sulfate gels. The purified receptor did not bind to concanavalin A-agarose or Lens culinaris-agarose.

Protein hormone receptors are present in low numbers in the plasma membrane of cells, making purification difficult. Only the receptors for insulin (1), epidermal growth factor (2), luteinizing hormone-chorionic gonadotropin (3), and insulin-like growth factor II (4) have been purified to homogeneity, and, recently, the receptor for nerve growth factor (5) was purified to near homogeneity.

Prolactin receptors are located primarily in the plasma membrane of mammary tissue and liver from lactating or pregnant animals. The difficulty in purifying the prolactin receptor (6–9) has been ineffective dissociation of the receptor-hormone complex from hormone affinity columns. When Triton X-100 was used to purify the receptor from the rabbit mammary gland membranes, prolactin aggregated and it was necessary to use hGH1 as the binding ligand (6). The receptor was purified 189-fold with a 12.4% recovery of binding activity and was heterogeneous on SDS-PAGE. Recently, the receptor from lactating mouse liver was purified using CHAPS as the solubilizing detergent and a prolactin affinity column (8). Although the reported purification was only 118-fold with a 3.6% recovery of binding activity, the receptor appeared to be nearly pure on SDS-PAGE. In another recent report, Triton X-100 and a streptavidin-affinity column were used to purify prolactin receptors from the rabbit mammary gland and liver that had first been allowed to interact with biotinylated human growth hormone (9). No yields were reported, though a 10% recovery of binding activity was obtained from the mammary gland.

Previous attempts at purification (6-8) used 5 mM MgCl2 to elute the receptor from the immobilized lactogenic hormone affinity column, although 5 mM MgCl2 has been reported to irreversibly denature the PRL receptor (8, 10) and this may account for the low recovery of prolactin-binding activity.

We recently reported that a low pH ammonium acetate buffer can dissociate membrane-bound receptor from prolactin without denaturation of the receptor (11) despite some reports to the contrary (6, 12). The zwitterionic detergent, Zwittergent 3-12, was effective in solubilizing the receptor from the membranes and did not interfere in the radioreceptor assay with 125I-oPRL when used under carefully controlled conditions (13). Accordingly, a purification procedure was developed which used Zwittergent 3-12 for solubilization, prolactin-agarose for binding the receptor and a pH 4.2 buffer for eluting the thoroughly washed receptor from the affinity column in an effective and non-denaturing manner. This procedure resulted in about a 37,500-fold purification and at least 54% recovery of binding activity.

MATERIALS AND METHODS

A summary of the purification of receptor from about 100 g of mammary tissue from a single rabbit is presented in Table I. Solubilization of the membranes with 2% Zwittergent 3-12 resulted in a doubling of the binding activity and a halving of the amount of protein, a result previously observed (8). The overall recovery based on the crude membranes was 54%; the recovery from the solubilized receptor was 24%. Under the conditions used, over 90% of the total prolactin-binding activity bound to the prolactin affinity gel overnight. Rigorous washing of the receptor-hormone complex was used to maximize purity. The receptor was eluted from the affinity columns with a pH 4.2 buffer in a sharp peak and was collected in a neutralizing buffer (Fig. 1). The use of Extracti-Gel D allowed for effective removal of detergent, and concentration was

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1 The abbreviations used are: hGH, human growth hormone; Zwittergent 3-12, 3-(dodecyl(dimethylammonio))-1-propanesulfonate; SDS, sodium dodecyl sulfate; oPRL, ovine prolactin; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PRL, prolactin; PMSF, phenylmethylsulfonyl fluoride; TLCK, 1-chloro-3-tosylamido-7-aminomethyl-2-heptanone; EC050, concentration of unlabeled prolactin required to displace 50% of the 125I-prolactin from the receptor; PEG, polyethylene glycol.

2 Portions of this paper (including "Materials and Methods" and Figs. 3–6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-2975, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Receptor

Particulate 560  1.1  616  100 1.0

Solubilized acetate, 0.5% Zwittergent 3-12, pH 4.2. Two-ml fractions were collected and binding to prolactin was assayed (see "Materials and Methods").

The purified receptor appeared as a single protein upon electrophoresis on SDS gels under reducing conditions and staining with Coomassie blue and the molecular weight estimated from the standards used was 42,000 (Fig. 2). Gels containing known amounts of the six standard marker proteins as well as the purified receptor from one rabbit were scanned and the amount of receptor was approximated to be about 8 μg of protein (Table I). In another attempt to determine the amount of protein recovered from the affinity column, the solubilized membrane proteins were labeled lightly with '*'I-(specific activity, 0.028 mCi/mg of protein).

The entire purification procedure, starting with homogenization of the mammary tissue, was also done in the presence of the protease inhibitors; 1 mM TLCK or 3 mM PMSF; or 3 mM PMSF and 5000 Kallikrein units of Trasylol. The results were identical to those obtained in the absence of protease inhibitors. The receptor was also purified using 0.5% CHAPS as the detergent, and the results obtained were similar to those with Zwittergent 3-12.

The purified receptor had a binding affinity for prolactin, EC50 of (3.71 ± 0.66) × 10^-10 M (Fig. 3), indicating that the binding activity of the purified receptor was the same as the native receptor.

![Image](image-url)

**FIG. 1.** Elution of prolactin receptor from a prolactin-agarose column. The receptor was eluted with 0.05 M ammonium acetate, 0.5% Zwittergent 3-12, pH 4.2. Two-ml fractions were collected and binding to prolactin was assayed (see "Materials and Methods").

![Image](image-url)

**FIG. 2.** Plot of log molecular weight versus Rf of standard proteins. Bovine serum albumin (BSA), ovalbumin, pepsin, trypsinogen A, β-lactoglobulin, lysozyme, and the total prolactin receptor (arrow) from one rabbit. The inset is the SDS-PAGE electrophoresis of the prolactin receptor stained with Coomassie blue. The apparent molecular weight (arrow) of the prolactin receptor was Mr = 42,000.

Earlier studies demonstrated that the crude prolactin receptor solubilized from lactating mouse mammary glands or liver (14) was retained on a concanavalin A-agarose column. However, the purified rabbit mammary gland receptor was not retained on a concanavalin A-agarose column in the present studies. Similar results were obtained using a Lens culinaris-agarose column, which has the same carbohydrate specificity as concanavalin A. Furthermore, neither of these lectins significantly affected the binding of '*'I-prolactin to purified receptor in the radioreceptor assay at concentrations up to 500 μg of lectin/assay tube.

A number of other methods were used in an attempt to assess the purity of the receptor, and these included chromatography of the purified and iodinated purified receptor. Chromatography on Bio-Gel P-100 of the purified receptor using 0.5% CHAPS as the detergent and assaying for prolactin-binding activity resulted in a single peak of activity which had an apparent molecular weight of 55,000 (Fig. 4). Receptor, purified in Zwittergent 3-12, iodinated with Iodobeads and chromatographed on Bio-Gel P-150, resulted in an apparent molecular weight of 37,000 (Fig. 5), but when this material was chromatographed on SDS gels under reducing conditions the apparent molecular weight was 21,000 (Fig. 6). In all cases, there was only a single iodinated peak on the columns and gels, indicating a highly purified protein. However, it is also clear that there is a variance in the apparent molecular weight of the prolactin receptor, and this appears to be a function of the detergent present and the extent of iodination of the receptor.

The apparent molecular weights of the purified and iodinated receptor on a calibrated CL-6B column (1.4 × 100 cm) in 0.1% v/v Triton X-100 was 180,000; in Zwittergent 3-12, 37,000 (Fig. 5); and in 1% CHAPS on a P-100 column it was 17,000. With unpurified receptor (prior to placing on the affinity column) the apparent molecular weight in CHAPS was 55,000, whereas in Triton X-100 the apparent molecular

![Image](image-url)

**TABLE I**

| Receptor form         | Total protein | oPRL bound | Total oPRL binding capacity recovered | -Fold purification |
|-----------------------|---------------|------------|---------------------------------------|--------------------|
| Particulate membrane  | 560           | 1.1        | 616                                   | 100                | 1.0                |
| Solubilized membrane  | 280           | 5.0        | 1400                                  | 227                | 4.5                |
| Affinity gel purified | ~8 μg         | 41,230     | 330                                   | 54                 | 37,500             |
weight was 350,000. It appears that the type of detergent has an influence on the apparent molecular weight of the receptor, and it appears to be related to the micelle size of the detergent.

**DISCUSSION**

The prolactin receptor from the lactating rabbit mammary gland was purified to homogeneity with over 50% recovery of binding activity. The procedure allows for the purification of receptor from a single rabbit, which corresponds to about 100 g of tissue in a single step on a prolactin affinity column with a yield of about 8 µg of pure receptor. The method utilizes Zwittergent 3-12, a zwitterionic detergent, for solubilization of the receptor from the membranes and was particularly useful at a concentration just above the critical micelle concentration. It provides comparable results to CHAPS as used previously to purify prolactin receptor from mouse livers (8, 15) but at a much lower cost. The extensive washing of the bound receptor-prolactin-agarose complex resulted in a pure receptor upon elution from the column. The most useful innovation in the purification procedure was the use of a pH 4.2 elution buffer which rapidly dissociated the receptor from the immobilized prolactin in high yields. Concentrating the small amounts of receptor was effected by use of Extracti-Gel D to remove essentially all the detergent and vacuum dialysis and concentration in a Micro-ProDiCon apparatus. In this manner, 30 ml could be concentrated to 25 µl if desired. The concentrated prolactin receptor in the presence of high amounts of detergent gave anomalous migrations on SDS gels. The use of Extracti-Gel D alleviated this problem, as evidenced by the single sharp protein staining band observed in Fig. 2. The protein isolated has the same binding affinity for prolactin as the impure solubilized material and does not appear to be the product of proteolysis since a variety of proteolytic inhibitors added to the original mammary homogenate did not affect the size or quantity of the receptor purified. Whether the entire intact receptor has been isolated is not known at this time, but Haeuptle et al. (9) reported a \( M_r \approx 35,000 \) for receptor purified from rabbit mammary gland and Triton X-100 solubilized receptor cross-linked to \(^{125}\text{I}}\-hGH, suggesting that this is the size of the binding unit.

The molecular weight of the prolactin receptor isolated in this study was \( M_r = 42,000 \) on SDS gels (Fig. 2) done under reducing conditions, and this \( M_r \) was also comparable to the mouse liver prolactin receptor (8) which had a reported \( M_r = 37,000 \). However, the molecular weight of the purified receptor in 0.5% CHAPS was \( M_r = 55,000 \) (Fig. 4). Lactogenic receptors from rat liver plasma membranes solubilized in Triton X-100 and bound to \(^{125}\text{I}}\-hGH had molecular weights of \( M_r = 77,300 \) (16) and \( M_r = 73,000 \) (17), as determined by hydrodynamic measurements. However, the molecular weight of the lactogenic receptor in livers of estrogen-treated male rats in CHAPS utilizing \(^{125}\text{I}}\-oPRL and chromatography of Sepharose 6B was \( M_r = 320,000 \) (18), comparable to previous findings (13). It is not clear whether these higher forms represent the true molecular weight of the receptor, aggregated forms, or some unique interaction of the receptor with detergent, in comparison to the standard proteins used as markers.

Iodination of the purified receptor with Iodobeads, which contain a strong oxidant, rapidly inactivates receptor binding to prolactin. This material was useful for assessing purity and searching for contaminant proteins because of the incorporation of a large amount of \(^{125}\text{I}}\. As shown in Fig. 6, there was only a single iodinated protein providing further evidence for the purity of the receptor. However, the apparent molecular weight of the iodinated receptor on SDS gels was \( M_r = 21,000 \) and, in gel filtration in CHAPS, the \( M_r = 17,000 \). The reason for the apparent reduction in molecular weight of the iodinated receptor is under current investigation.

The purified rabbit mammary receptor did not bind to a concanavalin A or \( L.\) culinaris lectin-agarose column, which are specific for glucose and/or mannose residues. The lectins did not inhibit the binding of oPRL to receptor. The results suggest that the purified receptor is devoid of the carbohydrate units binding these lectins. The binding of crude mouse liver prolactin receptors to concanavalin A columns observed in earlier studies (14) may be due to interactions between the lectin and other proteins associated with the receptor in the crude state.

The procedure described in this paper allows for the routine purification of the prolactin receptor from rabbit mammary gland as a single homogeneous protein in a single step and in high yields. The availability of purified receptor should contribute significantly to studies concerned with the properties of the receptor, prolactin-receptor interaction, and the mechanism of action of prolactin.

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SUPPLEMENTAL MATERIAL TO PURIFICATION OF RABBIT MAMMARY PROLACTIN RECEPTOR
BY ACIDIC ELUTION FROM A PROLACTIN AFFINITY COLUMN

P. Callejas-Torres, P. A. Hruby, P. B. Mahler and K. E. Einar

MATERIALS AND METHODS

Materials: Purified mouse prolactin (Sigma M-3381) was provided by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases through Dr. A. P. Fox of the Pituitary Hormones and Antigens Center, Tucson, Arizona. Stock solutions (1 mg/ml) were made in pH 7.4 saline plus 0.1 M PMSF, and 0.01 M PMA. Culture supernatants were provided by Dr. Richard P. Levy of the San Diego Cancer Institute, San Diego, California. A 30% solution of IgG was prepared in phosphate-buffered saline (PBS), pH 7.4.

Methods: BLOT cells were grown in either 100-mm dishes at 37°C in 5% CO2. 24 h after inoculation, the cultures were washed with PBS and incubated in DMEM containing 10% FBS. At the time of harvesting, the cells were rinsed twice with PBS and harvested in RIPA buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% SDS; 0.5% NP-40). After centrifugation at 1000 g for 5 min, the supernatants were used for immunoprecipitation experiments.

Immunoprecipitation: Rabbit anti-mouse prolactin receptor IgG was generated in rabbits by immunization with the purified mouse prolactin receptor expressed in BLOT cells. Immunoprecipitation was performed using 100 ml of culture supernatant in a total volume of 1 ml of PBS buffer (pH 7.4). The culture supernatant was mixed with 1 ml of 1 gl of protein A-Sepharose for 2 h at 4°C. The reaction was then centrifuged at 14,000 g for 30 min at 4°C. The pellet was washed twice with PBS and resuspended in 1 ml of 2X SDS sample buffer. The sample was then boiled for 5 min and the supernatant was removed for electrophoresis. The fraction was then resolved on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with a 1:1000 dilution of anti-MICE anti-prolactin receptor IgG and washed three times with TBST (Tris-buffered saline containing 0.05% Tween 20) before being probed with anti-rabbit IgG-HRP. The reaction was visualized using an ECL detection kit.

Immunoblotting: Immunoblotting was performed using a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with a 1:1000 dilution of anti-MICE anti-prolactin receptor IgG and washed three times with TBST (Tris-buffered saline containing 0.05% Tween 20) before being probed with anti-rabbit IgG-HRP. The reaction was visualized using an ECL detection kit.

Immunofluorescence: Immunofluorescence was performed using a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with a 1:1000 dilution of anti-MICE anti-prolactin receptor IgG and washed three times with TBST (Tris-buffered saline containing 0.05% Tween 20) before being probed with anti-rabbit IgG-HRP. The reaction was visualized using an ECL detection kit.

Crude receptors were purified by incubation for 1 h at 4°C with 1 ml of culture supernatant in a total volume of 1 ml of PBS buffer (pH 7.4). The culture supernatant was then added to 10 ml of protein A-Sepharose for 2 h at 4°C. The reaction was then centrifuged at 14,000 g for 30 min at 4°C. The pellet was washed twice with PBS and resuspended in 1 ml of 2X SDS sample buffer. The sample was then boiled for 5 min and the supernatant was removed for electrophoresis. The fraction was then resolved on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with a 1:1000 dilution of anti-MICE anti-prolactin receptor IgG and washed three times with TBST (Tris-buffered saline containing 0.05% Tween 20) before being probed with anti-rabbit IgG-HRP. The reaction was visualized using an ECL detection kit.

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Rabbit Mammary Prolactin Receptor Purification by Acidic Elution

Fig. 4. Elution profile of purified prolactin receptor on a P-100 column (1.3 x 107 cm) equilibrated and eluted with 0.1 M phosphate, 0.02% NaCl, 0.1% CHAPS (pH 7.5). Five hundred ml from the peak tube of the affinity column were used. Fractions, 1.8 ml, were assayed for prolactin binding (Methods). The proteins used to calibrate the column were bovine serum albumin, 66K; ovalbumin, 44K; chymotrypsinogen A, 25K; and lysozyme, 14K. Maximum binding occurred at 1:10.

Fig. 5. Chromatography of purified and iodinated prolactin receptor on Bio-Gel P-150. Fractions were 1 ml (Methods).