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Note

Separation of porcine zona pellucida components by high-performance liquid chromatography on styro-gel protein columns

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The zona pellucida is an acellular glycoprotein-like structure surrounding the mammalian oocyte. The chemical and physical properties of the zona have been the subject of intensive research during the last ten years. Because of the immunological similarities between human and pig zona, research has focused on this particular topic [1—4]. Despite this intensive effort little is known about the molecular structure of the zona because of the difficulties involved in obtaining adequate separation of the individual components.

Since it has been shown that some of these components might play a role in immunological fertility [5—7], it is imperative that a method is developed to separate the solubilized zonae into individual fractions in order to characterize the biochemical properties of each component.

Using purification methods such as gel filtration or sodium dodecyl sulphate—polyacrylamide gel electrophoresis (PAGE-SDS), only one to four fractions could be obtained by various investigators [2, 8, 9]. In this paper, we report the solubilization of porcine zona pellucida glycoproteins by lithium-3,5-diiodosalicylate and subsequent purification of the components on two styro-gel protein columns by high-performance liquid chromatography (HPLC). The solubilization procedure is similar to the one described by Dietl et al. [10], who showed that four glycoproteins react with specific anti-porcine zona serum. By this procedure it was possible to separate up to eleven individual glycoprotein fractions, as opposed to the conventional techniques that have previously been used.

EXPERIMENTAL

Isolation of zonae pellucidae

Porcine zonae, approx. 50,000, were isolated according to the modified

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method of Gwatkin et al. [8] and Dunbar et al. [2]. The procedure was briefly as follows. Fresh pig ovaries were obtained from the local slaughterhouse, chopped up and the suspension was filtered through a 210-μm nylon screen followed by a 75-μm nylon screen. The cell debris was digested with collagenase (Sigma, St. Louis, MO, U.S.A.) at a concentration of 1 mg/ml for 30 min at 37°C. Following incubation with the collagenase the suspension was filtered as described above.

The isolated zonae remaining on the nylon screen were checked under a microscope for purity and only batches with a contamination of less than 1% oocytes were used for the solubilization process.

Solubilization of zonae

The solubilization procedure was basically the same as described by Dietl et al. [10]. Approx. 50,000 zonae were suspended in 1 ml of 0.3 *M* lithium-3,5-diiodosalicylate in 0.05 *M* Tris—HCl buffer, pH 7.5. The suspension was stirred for 15 min at room temperature; the volume was doubled with bi-distilled water and the mixture was reincubated for 10 min.

The mixture was then centrifuged at 45,000 *g* for 90 min at 4°C. The sediment was discarded and the supernatant mixed with a 50% phenol—water solution (1:1, v/v) to eliminate possible contamination by unspecific proteins in the glycoprotein mixture. The suspension was then stirred for another 50 min at 4°C, centrifuged at 4000 *g* for 60 min (4°C) and the aqueous phase separated. The latter was then dialysed for 48 h against several changes of bidistilled water at 4°C. The dialysate was lyophilized and the glycoproteins were precipitated in absolute ethanol. The precipitate was then collected by centrifugation at 4000 *g* for 60 min at 4°C, and dissolved in 1 ml of phosphate-buffered saline (0.05 *M*, pH 7.3).

High-performance liquid chromatography

The high performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) was equipped with a M 6000 pump (420 bar, 0.1—9.9 ml), a septumless sample injector U6K (injector loop capacity 1 μl to 2 ml), and an integrator. The absorption was monitored by a constant-wavelength detector (M 440) at 254 nm. Styro-gel protein columns (Waters) of I-125 and I-60 were used with dimensions of 30 × 0.78 cm and with pore sizes of 125 Å and 60 Å, respectively. The molecular weight separation range for the I-60 column is given by the manufacturers as 1000—20,000 for globular proteins and 600—8000 for proteins of random coil configuration. For the I-125 column the molecular weight ranges were indicated as 2000—80,000 for native globular configurations and 1000—30,000 for random coils. For mobile phases either 0.06 *M* phosphate buffer, pH 7.4, or 0.2 *M* formic acid in 6 *M* urea was used at a flow-rate of 2.5 ml/min. Chromatography was carried out either with the I-125 column alone or with the I-60 used as a precolumn.

The following molecular-weight markers were included: ribonuclease M_r = 13,700, chymotrypsinogen A M_r = 25,000, ovalbumin M_r = 43,000, and bovine serum albumin M_r = 67,000 (Pharmacia, Freiburg, G.F.R.).

For qualitative analyses approx. 100 μg of protein were injected at a time, for preparative HPLC up to 1 mg of protein, and the various fractions

collected. For purification purposes, repeated injections of the same fractions were made until homogeneity was obtained.

Determination of the N-terminal amino acids

The N-terminal amino acids were detected according to the Dns chloride method. The purified HPLC fractions were lyophilized and dissolved in 10 μl of 0.2 *M* $NaHCO_3$. This mixture was then incubated with 10 μl of a 9.27 μM Dns chloride solution in acetone. This was followed by a 60-min incubation at 37°C. The mixture was then dried over sodium hydroxide and hydrolysed in 50 μl of 6 *M* hydrochloric acid for 24 h at 110°C. After hydrolysis the reaction mixture was dried again, dissolved in 50% pyridine and the individual Dns amino acids were identified by thin-layer chromatography (TLC) on polyamide sheets (Nano-plates F 1700, Schleicher und Schüll, Dassel, G.F.R.), in three different systems: 1.5% (v/v) formic acid, benzene—acetic acid (9:1, v/v), and ethyl acetate—methanol—acetic acid (20:1:1, v/v), where the first solvent was used in one direction and the last two successively in the second direction. Dns derivatives of the 21 amino acids (Sigma) served as standards.

RESULTS AND DISCUSSION

A typical HPLC elution profile of the solubilized zonae is shown in Fig. 1. As can be seen, a clear-cut separation of at least nine individual components with molecular weights between > 100,000 and 8200 could be obtained compared to molecular weight standards of ribonuclease (M_r = 13,700), chymotrypsinogen A (M_r = 25,000), ovalbumin (M_r = 43,000), and albumin (M_r = 67,000). These were found to be: M_r > 100,000 (1), = 78,000 (2), = 40,000 (3), = 30,000 (4), = 27,000 (5), = 25,000 (6), = 18,000 (7), = 16,000 (8), = 8200 (9). For calibration of the molecular weights the data module was set to calculate the linear least-square fit for each of the standards, according to the

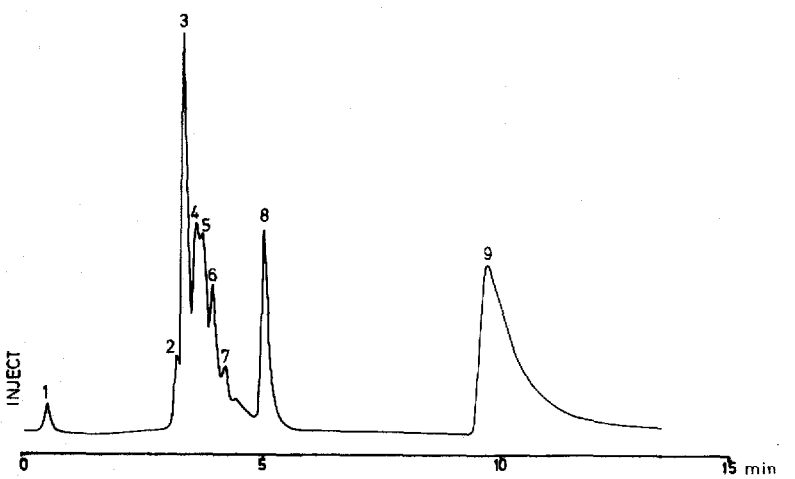

Fig. 1. Elution profile of 50,000 solubilized zonae pellucidae by HPLC using the I-125 protein column. Molecular weights are: 1, > 100,000; 2, 78,000; 3, 40,000; 4, 30,000; 5, 27,000; 6, 25,000; 7, 18,000; 8, 16,000; 9, 8200 (mobile phase 0.06 *M* phosphate buffer, pH 7.4; flow-rate 2.5 ml/min; wavelength 254 nm).

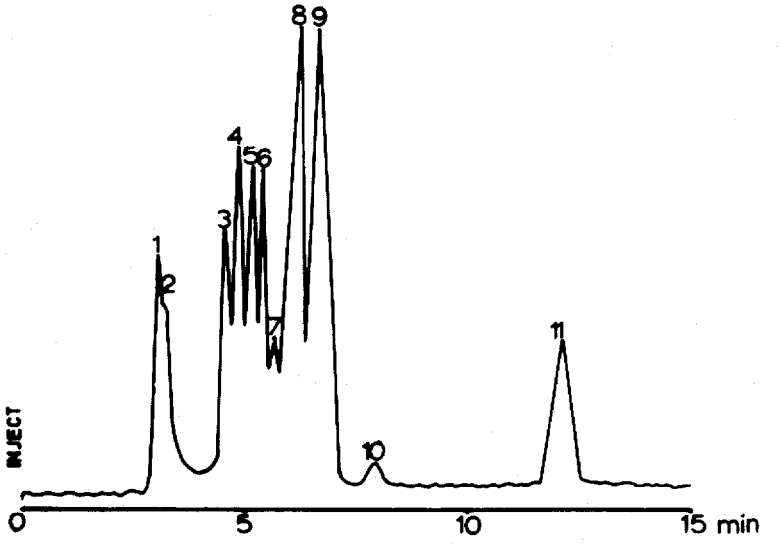

Fig. 2. Elution profile of the solubilized glycoproteins from 50,000 zonae pellucidae by HPLC, using the I-125 column and the I-60 precolumn (mobile phase 0.2 *M* formic acid in 6 *M* urea; flow-rate 2.5 ml/min; wavelength 254 nm).

equation $M_r = D_0 + D_1 (t_R)$, where the calibration coefficients D_0, and D_1 were computed for each experiment and t_R is the retention time of the various standards. Standard errors of estimation ranged from 0.072 to 0.076 and correlation coefficients equalled 0.99.

Fractions 2, 3, 4 and 8 isolated here showed molecular weights similar to those found earlier using immunoaffinity chromatography [8]. In addition, one component with a molecular weight of 40,000 and N-terminal residue of alanine (Ala) has been found to possess boar spermatozoal receptor properties [11].

As can be seen from Fig. 2, it is possible to isolate up to eleven components using the I-60 precolumn and 6 *M* urea in the mobile phase, thus obtaining an even better separation of the individual components (additional peaks 9 and 10). By repeated injections of the four fractions mentioned above, each of these fractions could be purified to homogeneity using the I-125 column alone. This was further confirmed by the fact that only one N-terminal amino acid was found in each of these fractions. The N-terminal amino acids were Arg for M_r = 78,000, Ala for M_r = 40,000, Ala for M_r = 30,000, and H is for M_r = 16,000. This indicates that with this purification procedure it is possible to obtain the various components in a pure enough form that biochemical analysis of the molecules can be carried out.

This seems a much better separation procedure than the various other methods that have been used to date. Gwatkin et al. [8] and Dunbar et al. [2] could only demonstrate ill-defined fractions by PAGE-SDS. Dependent on the solubilization procedure the most homogeneous fractions that have been found up to now were reported by Menino and Wright [9]. They isolated four individual fractions by PAGE-SDS.

Depending on the pH and/or ionic strength of the various mobile phases that we have tried besides 0.2 *M* formic acid and 6 *M* urea, we observed slight varia-

tions in retention times for the same compounds. However, retention times of the same components relative to the molecular-weight markers remained unchanged in a particular mobile phase. Therefore we assume that factors other than merely gel exclusion, i.e. pH, hydrophobic interactions with the solid phase and ionic strength, affect partition coefficients for a given compound.

Purification of zona components is time-consuming and difficult because of the possibility of denaturation of these molecules during prolonged purification procedures. In addition, common methods like gel-filtration chromatography and PAGE-SDS do not give satisfactory separation of the various zona components. Separation of the zona components by HPLC as described here requires 15 min, and collection of the various fractions makes it possible to carry out biochemical analyses in a relatively short time.

Since it was shown previously that porcine zona pellucida antigens cross-react with anti-human zona sera [1], the method presented here could enable the development of an immunological test as a diagnostic aid in immunological sterility cases.

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