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STUDIES OF FISH ZONA PELLUCIDA BY HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY ON AGAROSE COLUMNS AND FREE ZONE ELECTROPHORESIS

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SUMMARY

By either free zone electrophoresis or high-performance ion-exchange chromatography on DEAE agarose, zona pellucida from Baltic small herring (*Clupea harengus* L.) was separated into several fractions. These fractions had very similar protein compositions, since on polyacrylamide gel electrophoresis in sodium dodecyl sulphate they all gave the same pattern: chiefly one major and three minor bands corresponding to proteins with the following estimated molecular weights: 78 000, 96 000 (the major component), 115 000, and 125 000. It is likely that these proteins constitute the so-called supramolecular complexes of zona pellucida from Baltic small herring. Only one electrophoretic and one chromatographic fraction gave precipitin arcs on immunodiffusion with rabbit antiserum against zona pellucida from the fish *Aristichthys nobilis* (Richardson).

INTRODUCTION

Zona pellucida, the acellular coat surrounding the mammalian oocyte to which the sperm binds in the initial step of fertilization, is of great interest in reproductive biology research, especially in connection with attempts to devise an immunological contraceptive method. Antibodies against zona pellucida prevent the sperm from binding, and female rabbits immunized with antigens from zona pellucida become temporarily infertile [1]. Some cases of female sterility may be due to autoantibodies against antigens from zona pellucida [2, 3].

For studies of the fertilization process at the molecular level and for the development of a contraceptive vaccine, it is of primary importance to have

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access to highly purified zona proteins and the complexes in which they exist in zona pellucida (called supramolecular complexes by Dunbar et al. [4]. Since zona pellucida can often be prepared in only minute amounts and is relatively labile, methods such as high-performance liquid chromatography (HPLC) and free zone electrophoresis with their high resolution, short run times, and applicability with very small amounts of material, are attractive for the separation of the proteins and the supramolecular complexes from zona pellucida. In this paper we describe the application of these methods to zona pellucida from Baltic small herring (*Clupea harengus* L.). The main reason for the choice of fish eggs as starting material is that these are available in the relatively large amounts that might be required during the development of a purification procedure, which ought to be applicable with zona pellucida from other animal species.

MATERIALS, METHODS AND EQUIPMENT

Rabbit antiserum against zona pellucida from the fish *Aristichthys nobilis* (Richardson) was used for analyses of chromatographic and electrophoretic fractions by the Ouchterlony double-immunodiffusion test.

The following standard proteins from Bio-Rad (Richmond, CA, U.S.A.) were used for estimation of molecular weights of zona pellucida proteins by polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulphate (SDS): ovalbumin (mol. wt. 43 000), bovine serum albumin (67 000), phosphorylase b (94 000), and β-galactosidase (116 000). For estimation of protein molecular weights by pore gradient electrophoresis in the absence of SDS we used the following proteins, available from Pharmacia, Uppsala, Sweden: ovalbumin (43 000), bovine serum albumin (67 000), catalase (232 000), ferritin (440 000), thyroglobulin (669 000). Sephadex G-25 was also obtained from Pharmacia.

Diethylaminoethanol was purchased from Serva Feinbiochemica (Heidelberg, F.R.G.).

G3707, a neutral detergent, was a gift from Dr. D. De Coster (Atlas Chemie, Everberg, Belgium).

Free zone electrophoresis was performed, as previously described, in a quartz tube (390 × 3 mm I.D.) slowly rotating around its long axis to suppress disturbing convection [5, 6].

Preparation of heat-soluble zona pellucida

The method was similar to that described by Wu and co-workers [7, 8]. The fish eggs (ca. 2 g) were suspended in 6 ml of 0.4 M Tris—acetic acid (pH 8.7) containing 0.58% (w/v) sodium chloride, 0.29% (w/v) EDTA and 0.2% (w/v) sucrose. Following incubation at 72°C for 20 min (with stirring) the suspension was cooled to 4°C. Most of the heavy material was removed by centrifugation at 1500 g for 10 min. The supernatant obtained on centrifugation at 23 000 g for 30 min contained the zona pellucida and some contaminants, which were removed by further centrifugation under the same experimental conditions. This zona pellucida preparation was used directly in the free zone electrophoresis experiments. However, prior to application onto the HPLC columns, the preparation was passed through a Sephadex G-25 bed equilibrated with 0.05 M sodium chloride to remove low-molecular-weight compounds.

Coupling of DEAE groups to agarose

The 15% agarose beads (15 g; 5—15 μm) were prepared as previously described [9] and cross-linked with divinylsulphone [10, 11]; the deactivation step with mannitol was omitted. The beads were washed with water until the supernatant had a pH of 7. These and other washings were made by centrifugation at 600 g for 3 min. Diethylaminoethanol (6 ml) was mixed with 14 ml of 2 M potassium hydroxide, and the mixture was added dropwise and with stirring to the centrifuged agarose beads. The stirring was continued for 12 h at room temperature to let the alcohol react with vinyl groups in the agarose. The DEAE—agarose beads obtained were then washed five times with 20-ml portions of water, the pH of which had been adjusted to 12 with potassium hydroxide. Finally, the gel was washed with water until pH 7 was reached.

The HPLC equipment (UV-monitor 2158 SD, pump 2150, controller 2152, recorder 2210) was from LKB Produkter (Bromma, Sweden).

RESULTS

Estimation of the molecular weights of the zona pellucida proteins

Electrophoresis of fresh zona pellucida from Baltic small herring in a polyacrylamide gel in the presence of SDS according to the method of Neville [12], as slightly modified by Jergil and Ohlsson [13], gave the pattern shown in Fig. 1a. The electropherogram shows four main protein bands, denoted ZP1, ZP2, ZP3 and ZP4. By means of a calibration curve derived with the standard proteins listed earlier, the molecular weights were estimated at 125 000 (ZP1), 115 000 (ZP2), 96 000 (ZP3), and 78 000 (ZP4).

Zona pellucida material that had been kept at room temperature at pH 8 for 4—5 h gave a more complex pattern (Fig. 1b), probably owing to proteolysis. The proteins were found to be more stable at pH 5.

In an attempt to estimate the molecular weights in the absence of SDS, we employed electrophoresis in a gel gradient of polyacrylamide from $T = 24\%$ $C = 4\%$ to $T = 4\%$ $C = 4\%$ [T is the total concentration (w/v) of acrylamide and N,N-methylenebisacrylamide; C is the cross-linking concentration (w/w), see ref. 14]. The gel had the following dimensions: 6.5 × 6.5 × 0.3 cm. The run was conducted at 250 V (4.8 mA) for 11 h at pH 8.4 in a buffer consisting of 0.09 M Tris, 0.08 M boric acid and 0.0025 M EDTA [15]. Only one major protein band with pronounced tailing was obtained, corresponding to a molecular weight of 220 000 as estimated roughly from a comparison with the standard proteins (Fig. 2a). However, a heavy protein precipitate was also seen at the top of the gel. A sample that had been stored at room temperature (pH 8) for one day gave the pattern shown in Fig. 2b (cf. Fig. 1b). It should be emphasized that all of the molecular weights given above are very approximate, since zona pellucida proteins contain carbohydrate [16, 17] and it is well known that there are large uncertainties in the estimation of molecular weights of glycoproteins by the electrophoresis methods used in this study [18].

Anion-exchange chromatography of zona pellucida

After ultrasonic treatment for 10 min, the DEAE—agarose beads were packed in water to a height of 33 cm in a Plexiglas column (0.6 cm I.D.). Water, adjusted to pH 4 with hydrochloric acid, was passed through the column overnight at a
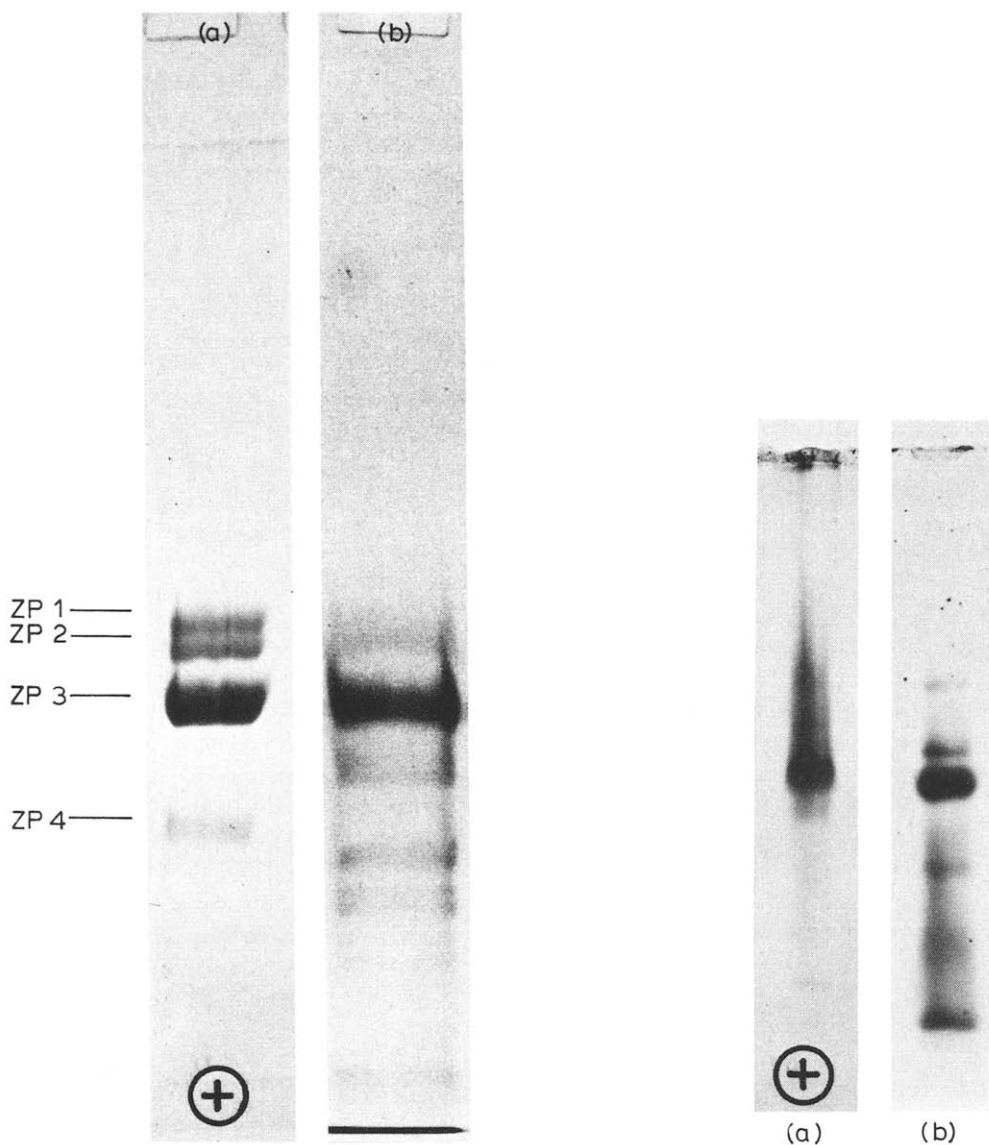
Fig. 1. Polyacrylamide gel electrophoresis in SDS of zona pellucida from Baltic small herring (*Clupea harengus* L.). (a) Fresh material; (b) material stored at room temperature (pH 8) for 4—5 h. The molecular weights of the proteins ZP1, ZP2, ZP3, and ZP4 were estimated roughly at 125 000, 115 000, 96 000, and 78 000, respectively. A comparison between the two protein patterns shows that zona pellucida is easily degraded.

Fig. 2. Pore gradient gel electrophoresis of zona pellucida from Baltic small herring. The run was made in the absence of SDS. (a) Fresh material; (b) material stored at room temperature (pH 8) for one day. The molecular weight of the main protein in fresh material was estimated at 220 000. Much material was retained at the top of the gel.

flow-rate of 0.1 ml/min, followed by 0.05 M Tris—acetic acid (pH 7.0) for equilibration of the bed. About 40 μl of a sample of heat-solubilized zona pellucida was applied. A linear gradient from 0.05 M Tris—acetic acid (pH 7.0)

to 0.25 M Tris—acetic acid (pH 7.0) during 30 min was used for elution at a flow-rate of 1 ml/min and a pressure of 2 bars (Fig. 3a). The experiment was repeated with other compositions of the buffer (see Fig. 3b and c). Upon immunodiffusion, only fractions corresponding to the highest peak in each of the three chromatograms gave a precipitin arc (e.g. Fig. 4), which was visible even when these fractions were strongly diluted (sometimes twenty-fold). No arcs were observed when the chromatographic experiment was performed in buffers of alkaline pH (Tris—acetic acid, pH 8.8; sodium phosphate, pH 8.2; sodium borate, pH 8.0). However, when the Tris—acetic acid buffer (pH 8.8) contained 20% (v/v) ethylene glycol, an immunoprecipitate was again obtained with the material from the highest peak in the chromatogram (ethylene glycol is known to stabilize proteins [19]).

The immunologically active fractions corresponding to the highest peak in Fig. 3a, were rechromatographed on the same column and under the same experimental conditions as the run in Fig. 3a. The chromatogram showed only one peak in the same position as the highest peak in Fig. 3a. The immunologically active material was thus chromatographically homogeneous in this system.

When analyzed by PAGE in the presence of SDS, all of the chromatographic fractions in Fig. 3 (except those corresponding to the first small peaks, which did not contain protein) gave the same electrophoretic pattern as the applied sample, i.e. that shown in Fig. 1a.
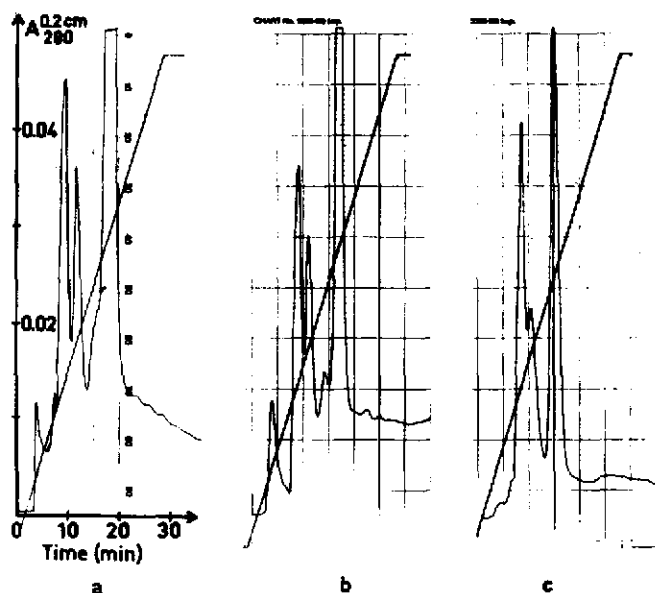

Fig. 3. High-performance ion-exchange chromatogram of zona pellucida from Baltic small herring on DEAE—agarose. (a) Gradient from 0.05 M Tris—acetic acid (pH 7.0) to 0.25 M Tris—acetic acid (pH 7.0) for 30 min. (b) The same as (a) except that both buffers also contained 1% (w/v) G3707, a neutral detergent [20]. (c) The same as (a) except that both buffers also contained 20% (v/v) ethylene glycol. In all experiments only material corresponding to the highest peak gave a precipitin line on immunodiffusion (Fig. 4). When analyzed by polyacrylamide gel electrophoresis in SDS all peaks gave the same electrophoretic pattern, similar to that shown in Fig. 1a.
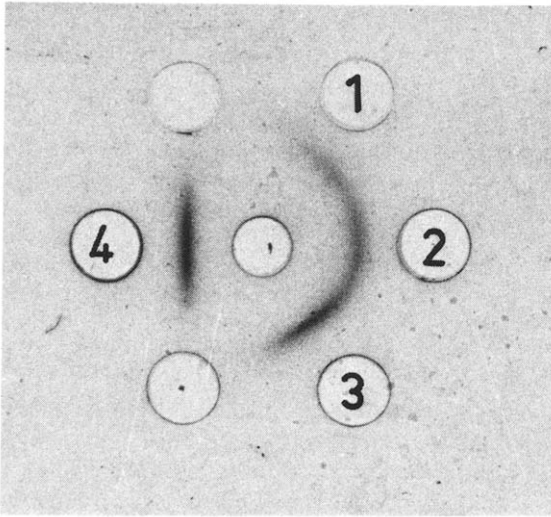
Fig. 4. Immunodiffusion of zona pellucida material. Well 1: heat-solubilized zona pellucida, prepared as described under Materials, methods and equipment, and diluted 1:1 with the buffer used in the extraction of zona pellucida (the same buffer was used in the immuno-diffusion experiment). Well 2: the same as well 1, but no dilution of the sample. Well 3: the same as well 1 after "desalting" on Sephadex G-25. Well 4: material corresponding to the highest peak in Fig. 3b.

Free zone electrophoresis of zona pellucida

About $5 \mu l$ of heat-solubilized zona pellucida were placed in the rotating electrophoresis tube filled with $0.05 M$ Tris—acetic acid buffer (pH 7.0). The run was conducted at 1580 V (4 mA). Fig. 5a shows the scans obtained after electrophoresis for 0, 10 and 20 min. After the last scan, material corresponding to each of the peaks was withdrawn for analysis by SDS electrophoresis: all peaks gave the same electrophoretic pattern, namely that of the applied sample, i.e. the pattern shown in Fig. 1a. In immunodiffusion, only material corresponding to the highest peak gave a precipitin line.

When the experiment was repeated in a buffer containing 1% (w/v) of the detergent G3707 [20], a similar electropherogram was obtained (Fig. 5b). The analyses by SDS electrophoresis and immunodiffusion gave the same result as the run done without detergent.

DISCUSSION

As the term "free zone electrophoresis" implies, this technique permits separations in a carrier-free medium (in buffer alone) and is therefore suitable for the fractionation of particles that are too large to penetrate the pores in a polyacrylamide gel. The supramolecular complexes of zona pellucida from porcine oocytes seem to be as large as that, to judge from a paper by Dunbar et al. [4]: "Attempts to separate the zona components by electrophoresis in the absence of SDS were totally unsuccessful, as supramolecular aggregates are present which do not enter the gel". Similarly, the corresponding protein complexes in zona pellucida from Baltic small herring were retained at the top
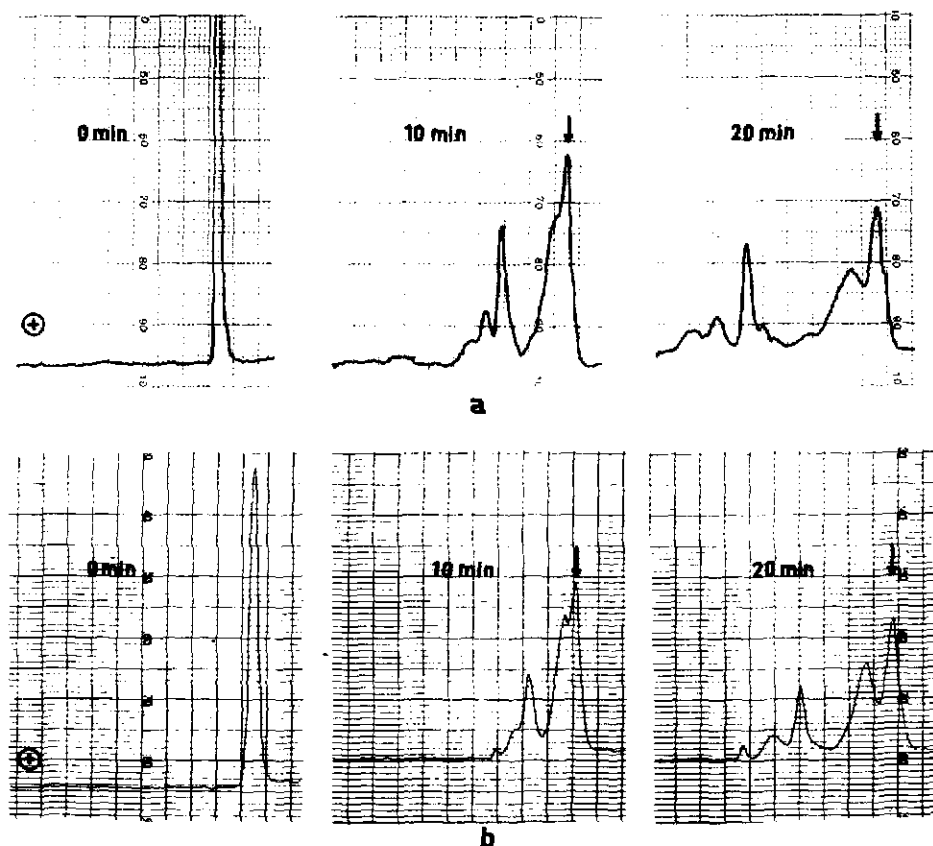
Fig. 5. Free zone electrophoresis of zona pellucida from Baltic small herring. The experiments were performed in (a) 0.05 M Tris—acetic acid (pH 7.0) and (b) the same buffer containing 1% (w/v) G3707, a neutral detergent [20]. The arrow indicates the position of the starting zone. When analyzed by immunodiffusion, only fractions corresponding to the highest (stationary) peak gave precipitin arcs. When these fractions were analyzed by polyacrylamide gel electrophoresis in SDS they showed the same pattern as that in Fig. 1a, i.e. that of the applied sample.

of a pore-gradient gel in the absence of SDS: only a complex of a molecular weight ca. 220 000 migrated into the gel (Fig. 2a). It should be emphasized that any supporting medium can induce the formation of protein aggregates and precipitates (see below and ref. 19). Free zone electrophoresis is accordingly a valuable method for analysis and separation of the large supramolecular complexes of zona pellucida.

Both in free zone electrophoresis (Fig. 5) and high-performance ion-exchange chromatography (Fig. 3), zona pellucida from Baltic small herring showed a complex pattern with several peaks. Only one electrophoretic and one chromatographic fraction gave a precipitin arc in the Ouchterlony double-immunodiffusion test, indicating that the peaks do not correspond to identical material. Furthermore, the rechromatography experiment showed that the immunologically active fractions were chromatographically homogeneous. However, when analyzed by PAGE in SDS, all of the electrophoretic and

chromatographic protein-containing peaks in Figs. 3 and 5 gave the same pattern: that of the original, unfractionated material shown in Fig. 1a. This finding indicates that the proteins of zona pellucida, under the conditions used in the free zone electrophoresis and HPLC experiments, are not in free form but are associated into complexes of very similar protein composition. These complexes certainly exist also in the intact zona pellucida, since the chance is very small that complexes formed artificially during the solubilization and the purification of zona pellucida would have the same protein composition as the unfractionated material. However, all experiments on zona pellucida proteins should be interpreted with great caution, since it has been shown recently that membrane proteins have a tendency to form artifactual aggregates on contact with chromatographic and electrophoretic supporting media [19], and zona pellucida proteins resemble membrane proteins in the sense that they have a strong tendency to aggregate [21]. It is therefore an obvious advantage to use separation methods (such as free zone electrophoresis) that do not require the presence of a supporting medium and therefore should not induce the formation of artificial aggregates.

Dunbar et al. [4] state in a study of the zona pellucida from porcine oocytes: "We conclude that the zona pellucida is composed of several glycoprotein macromolecules; interaction of these macromolecules to form supramolecular complexes and the integral zona pellucida is dependent on noncovalent forces". In the above discussion we have put forward several indications that supramolecular complexes also constitute the zona pellucida from Baltic small herring and that these complexes (mainly built up of the protein bands ZP1, ZP2, ZP3, and ZP4 in Fig. 1) have small differences in charge and can thus be (partially) separated by high-performance ion-exchange chromatography (Fig. 3) and free zone electrophoresis (Fig. 5). We have not investigated how these differences in charge among the supramolecular complexes can be explained in molecular terms. It is not unlikely, however, that the proteins in the different complexes have somewhat different structures since it has been stated that the relatively large width of the electrophoretic bands of zona pellucida is caused by microheterogeneity in the carbohydrate moiety [16, 17]. It has been reported that these broad bands can be split into a series of narrow protein zones by two-dimensional electrophoresis [22].

It is interesting to note that the detergent G3707 [20] cannot split the bonds between the proteins in the supramolecular complexes, to judge from analysis of all the chromatographic and electrophoretic fractions in Figs. 3 and 5 by PAGE in SDS, since all of these fractions gave the same gel electrophoresis pattern as unfractionated zona pellucida, independent of whether detergent was used or not in the fractionations. This inability of G3707 to dissociate the zona pellucida proteins is also evident from the observation that addition of G3707 to the buffer did not affect the general appearance of the chromatograms (cf. Fig 3a and b) or the electropherograms (cf. Fig. 5a and b). However, from the PAGE experiments in SDS (Fig. 1a), it is obvious that SDS has the desired bond-splitting property. It has in fact been used already for the isolation of zona proteins [21]. The drawback of SDS is its strongly denaturing power. However, we have recently put forward the hypothesis that hydrophobic proteins should be easier to renature after an SDS treatment than are water-

soluble proteins [19]. Since zona pellucida proteins evidently are hydrophobic there is a good chance that they can be renatured following PAGE in SDS. If this appears to be the case (at least their immunological properties can be restored after removal of SDS [21, 24]), all zona proteins separable by this electrophoresis technique can be purified and recovered in a native state by the methods we have introduced [25—27]. These methods are based on excision of the gel slice containing the protein of interest and recovery of the protein from the gel slice in high yield and without significant dilution of the protein.

The precipitin arcs observed in the immunodiffusion experiments were more distinct and clearly visible following "desalting" (Fig. 4, well 3), chromatography (Fig. 4, well 4) and electrophoresis (not shown) than they were in the original sample (Fig. 4, wells 1 and 2), in spite of the fact the protein concentration was higher in the latter sample. This observation supports the suggestion of Dunbar and Raynor [24] that "there may be some component interfering with the antigen—antibody complexes and that this component can be removed by electrophoretic separation".

A comparison of the electrophoretic patterns presented in Fig. 1a and b (and Fig. 2a and b) show that zona pellucida proteins are degraded rapidly, probably by proteolysis. It is therefore essential to use fresh material and fast separation methods, for instance the high-performance electrophoresis and chromatographic techniques described herein, particularly when the experiments are performed at alkaline pH (ethylene glycol seems to have some stabilizing power). These methods permit fractionation within 20 min. If shorter run times are required one can increase the field strength (voltage) in the electrophoresis experiment and the flow-rate in the HPLC run. An increase in flow-rate will, of course, decrease the resolution, but not very much for flow-rates up to 1.5 ml/ min as indicated by experiments not described here. An HPLC separation (molecular sieving) of porcine zona pellucida has recently been published [28]. Unfortunately, few data were given regarding the homogeneity of the fractions obtained.

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