Assembly of the Sea Urchin Fertilization Membrane: Isolation of Proteoliaisin, a Calcium-dependent Ovoperoxidase Binding Protein

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ABSTRACT Fertilization of the sea urchin egg is accompanied by the assembly of an extracellular glycoprotein coat, the fertilization membrane. Assembly of the fertilization membrane involves exocytosis of egg cortical granules, divalent cation-mediated association of exudate proteins with the egg glycocalyx (the vitelline layer), and cross-linking of the assembled structure by ovoperoxidase, a fertilization membrane component derived from the cortical granules. We have identified and isolated a new protein, which we call proteoliaisin, that appears to be responsible for inserting ovoperoxidase into the fertilization membrane. Proteoliaisin is a 250,000-Mr protein that binds ovoperoxidase in a Ca²⁺-dependent manner, with half-maximal binding at 50 μM Ca²⁺. Other divalent cations are less effective (Ba²⁺, Mn²⁺, and Sr²⁺) or ineffective (Mg²⁺ and Cd²⁺) in mediating the binding interaction. Binding is optimal over the physiological pH range of fertilization membrane assembly (pH 5.5–7.5). Both proteoliaisin and ovoperoxidase are found in isolated, uncross-linked fertilization membranes. We have identified several macromolecular aggregates that are released from uncross-linked fertilization membranes after dilution into divalent cation-free buffer. One of these is an ovoperoxidase-proteoliaisin complex that is further disrupted only upon the addition of EGTA. These results suggest that a Ca²⁺-stabilized complex of ovoperoxidase and proteoliaisin forms one structural subunit of the fertilization membrane.

Fertilization initiates a complex series of extracellular and intracellular reactions that transform the sea urchin egg into an embryo (reviewed in reference 1). The earliest visible change after fertilization is the elevation and assembly of the fertilization membrane (2), a glycoprotein coat that surrounds and protects the embryo. In the unfertilized egg, the components that give rise to the fertilization membrane are segregated into two domains. Some reside in the cortical granules, secretory vesicles located in the egg cortex, and some are in the vitelline layer, a glycoprotein coat attached to the external surface of the plasma membrane. Fertilization induces cortical granule exocytosis, and the mixing of components from these two cell compartments results in the assembly of a soft fertilization membrane (3). After a few minutes, this soft fertilization membrane is hardened by the introduction of dityrosine cross-links between adjacent polypeptide components (4, 5). Cross-linking is catalyzed by an enzyme called ovoperoxidase (6) which originates in the cortical granules and is assembled into the soft fertilization membrane (7). Once cross-linked, the hardened fertilization membrane is resistant to mechanical, chemical, and enzymatic degradation. At the blastula stage, the embryo secretes a specific hatching enzyme in order to remove this protective envelope (8).

The comparative ease with which fertilization membrane assembly can be manipulated and the relative simplicity of the system provide a unique opportunity to analyze the biochemical mechanisms by which proteins assemble into specific structures in the absence of intracellular controls. Several observations suggest that fertilization membrane assembly is a carefully regulated process. The surface of the fertilization membrane is paracrystalline (9) and arises from the divalent cation-mediated (10, 11) insertion of discrete structural units onto a modified vitelline layer, which acts as a scaffold (12). Only a subset of the assembled proteins is cross-linked by dityrosine residues during hardening (13), which indicates that cross-linking is also regulated by the substrate specificity of ovoperoxidase and/or the location of ovoperoxidase within the fertilization membrane.
The fertilization product (14), which is released upon fertilization of eggs with disrupted vitelline layers, forms a paracrystalline precipitate with divalent cations which is analogous to the paracrystalline surface material of the fertilization membrane (15, 16). This suggests that certain portions of the assembly system can be isolated and studied in partial reactions that do not lead to the formation of a fertilization membrane. One component of the assembly system, ovoperoxidase, has been purified and characterized (6). In this report, we present the isolation, identification, and preliminary characterization of a second fertilization product protein, proteoliaisin, that is implicated in fertilization membrane assembly. Proteoliaisin is a Ca2+-dependent ovoperoxidase binding protein that appears to mediate the insertion of ovoperoxidase into the fertilization membrane. Some of these results have been previously published in abstract form (17).

MATERIALS AND METHODS

Sea urchins of the species Strongylocentrotus purpuratus were collected, stored, and handled as previously described (4). Before further treatment, eggs were washed twice by suspension in Millipore-filtered sea water (0.45-μm filter; Millipore Corp., Bedford, MA), which was followed by settling and decantation. Subsequent steps were performed at 10°C.

Ovoperoxidase was purified as previously described (6). We prepared soft fertilization membranes (13) from aliquots of the same egg batch used to prepare fertilization product. For subsequent analyses, soft fertilization membranes were disaggregated (converted to "wraiths") by suspension in divalent cation-free buffer (13). Fertilization product was prepared as previously described (6) with the following modifications. After treatment with acetic acid, pH 8.5, and benzamidine and glycine ethyl ester were added to final concentrations of 10 and 30 mM, respectively.

We determined protein concentrations by the method of Bradford (18), using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard. All spectrophotometric assays were performed on a Gilford model 240 spectrophotometer (Gilford Instruments Laboratories, Inc., Oberlin, OH). STROMION chloride was purchased from Aldrich Chemical Co. (Milwaukee, WI). Other divalent cations were purchased from J. T. Baker Chemical Co., (Phillipsburg, NJ). These and all other reagents used were of the highest purity available.

Assay for Proteoliaisin: Ovoperoxidase-binding Activity.

Samples of proteoliaisin (20–100 μg), diluted to 200 μl in 20 mM HEPES, pH 7.5, 30 mM CaCl2 (buffer A), were mixed with 5–15 μl of pure ovoperoxidase (3–4 U, see below) in buffer A and incubated at room temperature for 1 h. The entire sample was applied to a minicolumn (Evergreen Scientific, Los Angeles, CA) containing 0.5 ml packed DEAE-Sepharose (Sigma Chemical Co.) equilibrated with buffer A. The protein that did not bind to the DEAE was removed by washing with 1 ml of buffer A. Ovoperoxidase that bound to the DEAE but was not complexed with proteoliaisin was eluted with 5 ml of 600 mM NaCl in buffer A. Ovoperoxidase-proteoliaisin complexes were then eluted with 1.5 ml of 600 mM NaCl in buffer A. Ovoperoxidase activity was determined by adding solid ammonium sulfate to 10% saturation. The precipitated protein was collected by centrifugation at 17,900 g for 15 min.

Before DEAE-Sepharose chromatography, the proteoliaisin-enriched fertilization product extract was precipitated with solid ammonium sulfate at 30% saturation to remove benzamidine. The precipitate was collected by centrifugation at 17,900 g for 15 min and resuspended in 20 mM 2(N-morpholino)ethanesulfonic acid, pH 6.0, 50 mM glycine ethyl ester, 1 mM EGTA, 100 mM NaCl. The protein was dissolved in a 1:10 dilution of 1% Triton X-100 and was stored at −20°C when necessary.

SDS Electrophoresis.

SDS electrophoresis was performed at 60 mA per gel, constant current in a water-cooled apparatus (Bio-Rad Protein Cell, Bio-Rad Laboratories). Gels and blots were photographed with Polaroid type 55 positive/negative film, and appropriate filters were used when necessary.

Electrophoresis Procedures.

All electrophoresis procedures were performed at 60 mA per gel, constant current in a water-cooled apparatus (Bio-Rad Protein Cell, Bio-Rad Laboratories). Gels and blots were photographed with Polaroid type 55 positive/negative film, and appropriate filters were used when necessary.
Nondenaturing (Native) Gel Electrophoresis: The gel system consisted of a 5% polyacrylamide separating gel containing 400 mM imidazole, pH 7.5, and a 4% stacking gel containing 200 mM imidazole, pH 6.5. The running buffer contained 50 mM HEPES and 13 mM imidazole, pH 6.8. Samples were prepared for electrophoresis by dialysis against 20 mM HEPES, pH 7.5, containing either 10 mM CaCl₂ or 10 mM EGTA, followed by the addition of glycerol and bromophenol blue to final concentrations of 20 and 0.1%, respectively. The dialysis buffer concentration was sufficient to prevent any pH changes resulting from chelation of divalent cations by EGTA. Gels were stained for protein with Coomassie Blue R as described for SDS gels. Identical lanes were stained for endogenous ovoperoxidase activity by the use of modified guaiacol assay (6). Proteoliaisin activity was detected using a blotting technique. In this procedure, the protein was transferred electrophoretically (transfer apparatus, Hoefer Scientific Instruments, San Francisco, CA) from the gel to a nitrocellulose sheet. We blocked the remaining protein binding sites by incubation for a minimum of 30 min in 1% bovine serum albumin, 1% gelatin in buffer A containing 1 mM H₂O₂ to inactivate endogenous ovoperoxidase (6). After extensive washing was done, a solution of pure ovoperoxidase (5 U/ml) in buffer A was added. 60 min later, the ovoperoxidase solution was removed, and the blot was washed several times with buffer A and then stained for bound ovoperoxidase by use of 4-chloro-naphthol as substrate (21). We prepared the reaction mixture by mixing 50 ml of buffer A with 5 ml of methanol containing 30 mg of 4-chloro-naphthol (Sigma Chemical Co.). The reaction was initiated by the addition of 2 ml of 15 mM H₂O₂. Control experiments were performed to ensure that efficient protein transfer was obtained under these conditions and that no ovoperoxidase activity was detected on the blot when either ovoperoxidase or calcium was omitted from the incubation mixture.

RESULTS
Evidence for the Existence of an Ovoperoxidase Binding Protein (Proteoliaisin)

Calcium-mediated binding of ovoperoxidase to a fertilization product component was suggested during the development of a purification scheme for ovoperoxidase (6). When fertilization product was fractionated by DEAE ion-exchange chromatography, the elution characteristics of ovoperoxidase were dramatically altered by the presence of Ca²⁺ in the elution buffers. In buffers containing EGTA, a single peak of ovoperoxidase activity was eluted by 80 mM NaCl. In the presence of Ca²⁺, we obtained two peaks of activity, one eluted by 80 mM NaCl and one by 310 mM NaCl. The elution characteristics of purified ovoperoxidase, however, were not affected by the presence of Ca²⁺. When purified ovoperoxidase and Ca²⁺ were added back to protein fractions from an EGTA column and rechromatographed in the presence of calcium, specific fractions exhibited the capacity to alter the ovoperoxidase elution profile. Based on these observations, we developed a quantitative assay for proteoliaisin, as described in Materials and Methods. The proteoliaisin assay was linear with the concentration of proteoliaisin when up to 50% of the added ovoperoxidase was bound (Fig. 1). To conserve material, we used 3–5 U ovoperoxidase per assay and adjusted the amount of proteoliaisin assayed to fall within the linear range. Each sample was assayed in triplicate, and the values reported are averages. The reproducibility of the assay was ±20%. Although the range of conditions under which binding could be determined was somewhat limited by the nature of the assay, the assay was useful in monitoring the purification of proteoliaisin and for a preliminary analysis of the ovoperoxidase-binding interaction.

Purification of Proteoliaisin

Proteoliaisin was purified from the fertilization product released from eggs with disrupted vitelline layers (14). We...
have modified the method of obtaining fertilization product (6) by including benzamidine and glycine ethyl ester during exocytosis to stabilize proteoliaisin activity. In the presence of these substances, the Ca\textsuperscript{2+}-dependent precipitation of fertilization membrane structural proteins (15, 16) was inhibited. Hyalin, an egg surface glycoprotein also released at fertilization (15), precipitated normally and was removed by low speed centrifugation. The soluble fertilization product contained most of the major protein species found in vivo assembled soft fertilization membranes (Fig. 2, lanes 1 and 2), including ovoperoxidase, as identified by its apparent $M_r$ of 70,000 (Fig. 2, lane 4). The amounts of protein and proteoliaisin obtained in a typical preparation of fertilization product are illustrated in Table I.

Preliminary fractionation and concentration of the fertilization product was effected by ammonium sulfate precipitation followed by dialysis against buffer containing benzamidine, glycine ethyl ester, and Ca\textsubscript{Cl2} (see Materials and Methods). The soluble extract thus obtained was enriched in both ovoperoxidase and proteoliaisin (Table I and Fig. 2, lane 3). Ovoperoxidase was separated from proteoliaisin by DEAE-Sepharose chromatography in the presence of EGTA (see Materials and Methods) and was recovered in the column load and preliminary wash fractions (Fig. 3A). This ovoperoxidase fraction could then be purified by chromatography on carboxymethyl-Sepharose (data not shown) as in the standard ovoperoxidase purification (6). Proteoliaisin activity eluted with the major protein peak (Fig. 3A), which contained three major protein components of $M_r > 200,000$ (Fig. 2, lane 5). The results of a typical purification obtained with material that had never been frozen are illustrated in Table I.

One of two techniques has been used to further purify proteoliaisin: ovoperoxidase affinity purification or a second DEAE-Sepharose fractionation step. The purification obtained by either technique was quantitatively and qualitatively similar (Table II and Fig. 2, lanes 6 and 7). In the affinity purification step, nitrocellulose was used as an affinity support (see Materials and Methods). This procedure was useful only for the purification of small quantities of proteoliaisin (Table II). The protein eluted from the affinity support with EGTA contained one major protein with an $M_r$ of 250,000 and trace contaminants of other high molecular weight components and ovoperoxidase (Fig. 2, lane 6). Under nonreducing conditions, an $M_r$ of 200,000 was observed for the major protein species (Fig. 2, lane 8), which suggests the presence of intra-

![Figure 3](image_url)

**Figure 3** DEAE-Sepharose chromatography. All chromatography buffers contained 1 mM EGTA (see Materials and Methods). (A) Fractionation of the proteoliaisin-enriched supernatant obtained after dialysis against Ca\textsuperscript{2+}, benzamidine, and glycine ethyl ester. (B) DEAE-purified proteoliaisin rechromatographed with a shallow NaCl gradient (see Results). O, protein by $A_{280}$; ., proteoliaisin activity in units per milliliter; ■, ovoperoxidase activity in units per milliliter; X, NaCl gradient. Conditions were as described in Materials and Methods.

### Table I

| Purification step                  | Total protein mg | Specific activity U/mg | Total activity U | Purification over FP |
|------------------------------------|------------------|------------------------|-----------------|---------------------|
| Fertilization product (FP)         | 212              | 12                     | 2,544           | —                   |
| 10% Ammonium sulfate precipitation | 156              | 16                     | 2,496           | 1.3                 |
| Dialysis against glycine ethyl ester, benzamidine, Ca\textsuperscript{2+} | 100              | 23                     | 2,300           | 1.9                 |
| 30% ammonium sulfate precipitation | 82               | 25                     | 2,050           | 2.1                 |
| DEAE-Sepharose chromatography      | 19               | 53                     | 1,007           | 4.4                 |
| Recovery                           | 9%               | 40%                    |                 |                     |

Fertilization product was prepared from 100 ml of packed, dejellied eggs as described in the text, and was not frozen before the purification was begun.

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molecular disulfide linkages. The results of a typical affinity purification step are illustrated in Table II.

The second DEAE-Sepharose fractionation step was performed under conditions similar to the first; however, a much broader NaCl gradient was used to maximize resolution (see Materials and Methods). Under these conditions, two protein peaks were obtained (Fig. 3B), only one of which contained proteoliaisin activity. The proteoliaisin fractions contained one major protein with a Mₙ of 250,000, as determined by SDS electrophoresis (Fig. 2, lane 7). The results obtained from a typical second DEAE-Sepharose fractionation step are illustrated in Table II. The material used for this fractionation had been frozen before purification. The lower absolute proteoliaisin activity reflects the loss of activity that occurred after freezing, as discussed below.

Proteoliaisin obtained by these procedures was purified eightfold from fertilization product (Table II) and was ~75% pure (see Fig. 2, lane 8). Thus, it comprises ~9% of the protein in fertilization product. Proteoliaisin was concentrated (Amicon pressure cell, PM 10 filters, Amicon Corp., Scientific Systems Div., Danvers, MA), dialyzed against 20 mM HEPES, pH 7.5, 50 mM glycine ethyl ester, 100 mM NaCl, 10% glycerol, and then filtered through 0.45-μm Millipore filters. This purified proteoliaisin was used to analyze the ovoperoxidase-binding interaction in vitro as described below.

Stability of Proteoliaisin

The proteoliaisin activity in fertilization product prepared by our original method (6) was unstable. Loss of binding activity was accompanied by a decrease in trichloroacetic acid-precipitable protein, and several high molecular weight components normally found on SDS gels of fertilization product were absent or greatly diminished. We tested several inhibitors of cortical granule proteases (14, 22) for their ability to prevent the loss of proteoliaisin activity. Of these, benzamidine was the most effective, increasing the half-life of proteoliaisin activity from 15 to 35 days at 20°C.

Several other compounds were also tested, in combination with benzamidine, for the ability to further stabilize proteoliaisin activity. These included: l-threitol, an inhibitor of the glucanase activity found in cortical granule exudate (23); aminotriazole, an inhibitor of ovoperoxidase activity (4); ascorbate, an antioxidant that reduces peroxide substrates of ovoperoxidase; and glycine ethyl ester, an inhibitor of the morphological change in fertilization membrane assembly known as the I-to-T transition (3). In combination with benzamidine, glycine ethyl ester was the only compound that significantly increased the half-life of proteoliaisin activity in fertilization product. The half-life when it was stored at 20°C increased from 35 d for 10 mM benzamidine alone to 90 d for the combination of 10 mM benzamidine and 50 mM glycine ethyl ester.

Benzamidine had other effects on our preparations: It inhibited precipitation of the paracrystalline fertilization product proteins (data not shown) and prevented the binding of fertilization product proteins to DEAE-Sepharose, necessitating its removal during the purification of proteoliaisin (see Materials and Methods). The proteoliaisin-enriched supernatant fraction obtained after dialysis of fertilization product extracts against benzamidine and Ca²⁺ has been stored at ~60°C for up to 8 mo without significant loss of activity. After thawing, however, the proteoliaisin activity in these supernatants declined over ~7 d until a plateau of ~50% of the original activity was reached. We do not know the significance of this loss of activity, but it occurred even in the presence of benzamidine and glycine ethyl ester. It was not prevented by protease inhibitors (soybean trypsin inhibitor, pepstatin A or leupeptin) and was not associated with any change in polypeptide size as detected by SDS electrophoresis. Proteoliaisin activity was stable for 1–2 wk when stored on ice. For characterization of the binding activity, all experiments were performed within 3 wk of purification.

Divalent Cation Dependence of Proteoliaisin Activity

Both precipitation of the fertilization membrane structural proteins from fertilization product (15, 16) and the maintenance of soft fertilization membrane structural integrity (13) depend upon divalent cations in the surrounding medium. To determine the relationship of proteoliaisin activity to these phenomena, we investigated the divalent cation-dependence of proteoliaisin activity. The results of this analysis are shown in Table III. The interaction of ovoperoxidase and proteoliaisin in the assay was very specific for Ca²⁺, which was half-maximally effective at 50 μM. Ba²⁺ and Mn²⁺ were only partially effective in replacing Ca²⁺, at 16- and 40-fold higher concentrations, respectively. At the highest concentration that could be tested (20 mM), saturable binding was not achieved with Sr²⁺ and no binding was detected with either Mg²⁺ or Cd²⁺ (data not shown).

| TABLE II |
| Two Alternate Proteoliaisin Final Purification Steps |
| --- |
| Purification step | Total protein mg | Purification compared with |
| --- | --- | --- | --- |
| Protelolaisin activity ug | Starting material | FP |
| Ovoperoxidase affinity chromatography | 2.0 | 93 | 4.4 |
| Protelolaisin | 0.8 | 98 | 1.9 | 8.2 |
| Repeat DEAE-Sepharose chromatography | 44 | 26 | — | 2.6 |
| Protelolaisin | 16 | 44 | 1.8 | 4.4 |

For ovoperoxidase affinity chromatography, DEAE-purified proteoliaisin from the preparation described in Table I was used as starting material. In the repeat DEAE-Sepharose fractionation step, the starting material was DEAE-purified proteoliaisin prepared from fertilization product that had been stored frozen prior to purification and had lost 46% of its activity (see Results).

| TABLE III |
| Divalent Cation Dependence of Proteoliaisin Activity |
| --- |
| Divalent cation | KD μM | % Maximum activity |
| Ca²⁺ | 0.05 | 100 |
| Sr²⁺ | 19.0 | ND |
| Ba²⁺ | 0.80 | 43 |
| Mn²⁺ | 2.0 | 19 |

Divalent cations (up to 20 mM) were tested as described in the text. Results are expressed as the concentration of divalent cation that gave half-maximal activity for that cation (KD). The maximal activity obtained with each cation is shown as the percentage of maximal activity obtained with Ca²⁺. The maximal activity of Sr²⁺ was not determined and the KD for Sr²⁺ is given as the concentration that gave half the maximal binding obtained with Ca²⁺.
The pH Dependence of Proteoliasin Activity

During assembly of the fertilization membrane, the local pH around the egg may change, since proton efflux accompanies egg metabolic activation (24, 25). The equilibrium pH of the sea water is 7.5-8.2. The effects of pH on various components of the fertilization membrane and their interactions are, therefore, of interest in the analysis of the assembly process. Proteoliasin activity was assayed over the pH range 5.5–9.0 (see Materials and Methods). In the pH range expected during fertilization membrane assembly (pH 5.5–7.5), the binding activity was relatively constant (Fig. 4). Above pH 7.5, the activity began to decline and decreased to ~50% of maximal by pH 9.0, the highest pH that could be tested with this assay.

Analysis of Proteoliasin Activity in In Vivo Assembled Soft Fertilization Membranes

Ovoperoxidase is a component of the assembled fertilization membrane (7). To confirm that proteoliasin is also a fertilization membrane component and to characterize its interaction with ovoperoxidase in the membrane, the native gel procedure described in Materials and Methods was employed. After we disaggregated soft fertilization membranes by diluting them into divalent cation-free buffer (13), we treated the soluble protein and residual membrane structures (wraiths) with either Ca\(^{2+}\) or EGTA. The electrophoretic properties of these proteins were then directly compared with those of purified proteoliasin and purified ovoperoxidase treated with either Ca\(^{2+}\) or EGTA. After electrophoresis, the locations of protein, endogenous ovoperoxidase activity, and proteoliasin activity in the gels were determined by use of specific stains. The results of these studies are presented in Figs. 5 and 6.

The location of purified ovoperoxidase in these gels, as determined by protein and activity stains, was for the most part not affected by the preincubation conditions (Figs. 5A and 6A, lanes 1 and 2). As previously reported (6), purified ovoperoxidase is electrophoretically heterogeneous, and, in this system, migrated to a position overlapping the stacking and separating gel interface. Purified proteoliasin migrated as a single protein band and the mobility of this band was increased after preincubation with EGTA (R\(_{f}\) 0.39, Fig. 6B, lanes 1 and 3), as compared with preincubation with Ca\(^{2+}\) (R\(_{f}\) 0.36, Fig. 5B, lanes 1 and 3). The pI of ovoperoxidase is in the range of 6.3-6.7 (P. Weidman, unpublished observation) whereas that of proteoliasin is much lower. So, although ovoperoxidase is much smaller than proteoliasin, it migrated more slowly in this native gel system, presumably because of its lower net charge at pH 6.5-7.5.

The electrophoretic profile of disaggregated (13) soft fertilization membrane proteins, both with and without (data not shown) preincubation with Ca\(^{2+}\), was distinct from that of the purified components. The disaggregated soft fertilization membrane proteins fractionated into approximately three protein complexes, seen as bands in Fig. 5C, lane 1. One of these bands contained endogenous ovoperoxidase activity (R\(_{f}\) 0.32, Fig. 5C, lane 2). This band and the band immediately below it stained for proteoliasin activity (R\(_{f}\) 0.32 and 0.34, Fig. 5C, lane 3). Preincubation with EGTA generated a distinctly different profile of protein complexes (Fig. 6C, lane 3). There was an increase in the number and the mobility of the protein bands. Ovoperoxidase was now found in the region of the stacking gel interface (Fig. 6C, lane 2) and comigrated with purified ovoperoxidase (Fig. 6A, lane 2). We observed a single band of proteoliasin activity (R\(_{f}\) 0.39, Fig. 6C, lane 3), which comigrated with purified proteoliasin (R\(_{f}\) 0.39, Fig. 6B, lane 3). We did not observe dissociation of ovoperoxidase and proteoliasin when soft fertilization membranes were

![Figure 4](image-url) The pH activity profile of proteoliasin. The buffer and modified assay conditions are described in Materials and Methods.

![Figure 5](image-url) Native gel electrophoresis after preincubation in 10 mM CaCl\(_2\). Samples were dialyzed in 20 mM HEPES, pH 7.5, 10 mM CaCl\(_2\) for 20 h and electrophoresis was run as described in Materials and Methods. (A) Purified ovoperoxidase; (B) purified proteoliasin; (C) disaggregated soft fertilization membranes; (lane 1) protein stain; (lane 2) ovoperoxidase activity stain; (lane 3) proteoliasin activity stain. Numbers on the right are R\(_{f}\) values of the respective bands.
diluted into Ca\textsuperscript{2+}-free buffers without chelators such as EGTA, and Methods. Lanes are the same as for Fig. 5.

The composition of the protein bands obtained after native gel electrophoresis of disaggregated soft fertilization membranes was further analyzed by SDS electrophoresis in a second dimension (Fig. 7). The location of proteoliaisin activity in the native gels coincides with the presence of a 230,000 \( M_r \) protein. The \( M_r \) of purified proteoliaisin is 250,000 \( \pm \) 15,000. The location of endogenous ovoperoxidase activity in the native gel coincided with a protein of \( M_r 70,000 \) on the SDS gel, which is the \( M_r \) of ovoperoxidase. In disaggregated soft fertilization membranes preincubated in Ca\textsuperscript{2+}, the proteins of 230,000 and 70,000 \( M_r \) were associated with the same native gel protein band (Fig. 7A). Preincubation of disaggregated soft fertilization membranes in EGTA resulted in a separation of these components in the native gel (Fig. 7B). The soft fertilization membrane complex that contained proteoliaisin activity but not ovoperoxidase activity (Fig. 7A) was also disrupted after treatment with EGTA (Fig. 7B). In particular, two proteins of \(~57,000 \) and \( 60,000 \ M_r \), exhibited a dramatic increase in mobility after this treatment (Fig. 7, X), which suggests that they are members of a larger complex that was stabilized by Ca\textsuperscript{2+}. The relationship of proteoliaisin to this complex remains to be defined.

DISCUSSION

The requirement for divalent cations in the assembly of the fertilization membrane led us to investigate divalent cation-mediated interactions of ovoperoxidase with other components of fertilization product that were potential precursors of the fertilization membrane. This report presents the identification and partial purification of proteoliaisin, a calcium-dependent ovoperoxidase binding protein. Identification of this 250,000 \( M_r \) protein was based on the enrichment of this component during purification of the ovoperoxidase binding activity from fertilization product (Fig. 2) and upon direct evidence obtained through the use of a specific activity stain in conjunction with nondenaturing polyacrylamide gel electrophoresis (Figs 5 and 6).

Purification of proteoliaisin was aided by use of the compounds benzamidine and glycine ethyl ester not only to stabilize proteoliaisin activity during purification but also to assist in the preliminary fractionation of fertilization product. The addition of benzamidine and glycine ethyl ester to fertilization product specifically inhibited the formation of the paracrystalline precipitate but had no effect on the precipitation of hyalin. Baginski and co-workers (26) recently reported a similar inhibition of paracrystalline precipitation in cortical granule exudate prepared in the presence of 5 mM benzamidine and 1 mM aminotriazole, an effect attributed to the inhibition of normal posterior fusion process. We have found that 10 mM benzamidine alone was sufficient to prevent paracrystalline precipitation and that the inhibition may be due, in part, to the binding of benzamidine to fertilization product proteins. Glycine ethyl ester appeared to prevent proteolytic degradation of proteoliaisin and prevented the precipitation of ovoperoxidase and proteoliaisin from fertilization product extracts treated with benzamidine and Ca\textsuperscript{2+}. This effect may be similar to that of glycine and lysine, compounds known to interfere in the assembly of the fertilization membrane (27, 5). These results emphasize the need for a more thorough analysis of the effects of such inhibitors on individual steps in fertilization membrane assembly.

The ovoperoxidase-proteoliaisin interaction is specific for Ca\textsuperscript{2+}, with optimal concentrations in the micromolar range (Table III) and is relatively independent of pH (Fig. 4). These physical characteristics are distinct from the previously studied divalent cation-dependent associations in fertilization membrane assembly. For example, divalent cations are required for maintenance of soft fertilization membrane structural integrity (13) and for precipitation of fertilization membrane structural proteins from fertilization product (16). In both systems, the divalent cation requirement is relatively nonspecific: Mg\textsuperscript{2+}, Sr\textsuperscript{2+}, Ba\textsuperscript{2+}, and Mn\textsuperscript{2+} are as effective as Ca\textsuperscript{2+}. Optimum cation concentrations are in the low millimolar range. In the case of the formation of the paracrystalline precipitate, a distinct pH optimum in the range 7.5-8.0 has been shown. The differences between these phenomena and the binding of ovoperoxidase by proteoliaisin are probably not an artifact of the in vitro assay system, since the native gel electrophoresis studies (Fig. 5) demonstrate that conditions that lead to disaggregation of soft fertilization membranes do not disrupt the ovoperoxidase-proteoliaisin interaction. These data suggest that ovoperoxidase may be incorporated into the fertilization membrane as a preformed ovoperoxidase-proteoliaisin complex. The interaction of this complex with other fertilization membrane components probably occurs via a different divalent cation-dependent mechanism. We do not yet know whether the ovoperoxidase-binding protein complex exists within the cortical granules (and is therefore assembled during oogenesis) or is assembled after exocytosis.

The association of ovoperoxidase and proteoliaisin in dis-
aggregated soft fertilization membranes pretreated with Ca\(^{2+}\) (Fig. 5), as opposed to their dissociation after pretreatment with EGTA (Fig. 6), is consistent with a Ca\(^{2+}\)-mediated interaction of these components in the soft fertilization membrane. Dilution of soft fertilization membranes into divalent cation-free buffer did not disrupt this interaction; the disruption occurred only after treatment with chelators. Hence, the divalent cation-dependence of the ovoperoxidase-proteoliasin interaction in vivo may be similar to that found in the in vitro assay system, with Ca\(^{2+}\) being specifically employed at relatively low concentrations to mediate the interaction. In addition to binding ovoperoxidase, proteoliasin appears to interact with other soft fertilization membrane components, e.g. the 57,000 and 60,000 \(M_r\) proteins (Fig. 7A). This suggests that proteoliasin may be a critical component in other interactions that lead to fertilization membrane assembly.

There is also evidence that proteoliasin is important in fertilization membrane hardening itself. When soft fertilization membranes are hardened in vitro (13), the fertilization membrane components of >100,000 \(M_r\) are excluded from SDS polyacrylamide gels, suggesting that these components have been cross-linked by ovoperoxidase. The same proteins are excluded from SDS-polyacrylamide gels of in vivo hardened fertilization membranes (13). These results indicate that once the soft fertilization membrane is assembled, proteoliasin may function as a substrate for its ligand, ovoperoxidase, in the cross-linking of membrane components.

The findings reported here are consistent with a crucial role for proteoliasin in the assembly and hardening of the fertilization membrane. Proteoliasin could mediate the insertion of ovoperoxidase into the fertilization membrane either by acting as a cortical granule vector to the assembling membrane or as a vitelline layer component for intercepting this enzyme. The association of proteoliasin with other fertilization membrane proteins may also be important in determining the orientation of ovoperoxidase with respect to its substrates. We are now attempting to identify the prefertilization location of proteoliasin, and the egg surface or cortical granule component(s) that interact with the ovoperoxidase-proteoliasin complex to effect assembly. Such an approach should indicate at which steps vitelline layer components enter into the overall process and whether other covalent modifications (such as proteolysis) attend the assembly reaction. Ultimately, it should be possible to formulate a pathway that describes all of the biochemical interactions involved in fertilization membrane assembly.

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