Sea Urchin Fertilization Envelope: Isolation, Extraction, and Characterization of a Major Protein Fraction from *Strongylocentrotus purpuratus* Embryos†

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ABSTRACT: The sea urchin fertilization envelope (FE) is an extracellular embryonic coat formed from an extraoval layer, the vitelline envelope (VE), and components derived from egg exocytotic vesicles, the cortical granules. The FE passes through several stages of development post-insemination, each characterized by discrete changes in morphology, permeability, and solubility; the definitive "hardened" FE which results is, according to the present paradigm, resistant to proteolytic digestion and insoluble in a variety of reducing and denaturing solvents. We now report a gentle isolation procedure for hardened FEs which results in highly purified envelopes essentially free of cytoplasmic contamination with the morphology and ultrastructure of in vivo FEs. Isolated FEs from *Strongylocentrotus purpuratus* embryos were repetitively extracted with 6.0 M urea–1.5 M mercaptoethanol, pH 10 at 100 °C for 10 min, and examined ultrastructurally. Dramatic changes in ultrastructure of the extracted FEs were observed when compared with controls and a minimum of 71% of the total protein content of the FEs was removed as determined by quantitative ninhydrin analysis. The temperature dependence of the rate of protein extraction from isolated FEs was studied in 0.010 M ethanolamine at pH 10.0; the maximal rate of extraction was achieved at 80 °C with one-half maximum rate observed at approximately 60 °C. The effect of pH on the rate of protein extraction from isolated FEs was examined in 0.01 M ethanolamine–0.1 M NaCl and in 0.15 M mercaptoethanol–0.1 M NaCl. The extraction rate increased with increasing pH but the mercaptoethanol buffer curve had a much greater slope than the ethanolamine one with the former curve having an inflection point at pH 9.8 which is quite close to the pK_a of mercaptoethanol. The urea–mercaptoethanol soluble protein fraction of hardened FEs was composed of three glycoproteins (mol wt 91 600, 71 200, and 53 000) and two proteins (mol wt 32 600 and 18 200) as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. It was deduced from in vivo ^{125}I-labeling experiments that these proteins/glycoproteins are on the outer aspect of the FE surface.

The sea urchin embryo fertilization envelope (FE)[1] is an extracellular structure formed from a surface VE template and components derived from the cortical granules. The cortical granules in the unfertilized egg lie just beneath the egg plasma membrane and undergo a wave of exocytosis beginning at the site of contact with the fertilizing sperm (reviewed by Czihak, 1975; Giudice, 1973; Runnström, 1966). As exocytosis proceeds, the VE elevates away from the egg surface and some of the cortical granule components become associated with the VE to form the unhardened FE. Shortly after elevation, the VE undergoes morphological and biochemical changes (Veron et al., 1977; also reviewed by Czihak, 1975; Giudice, 1973) and is transformed into a hardened FE capable of preventing excess sperm entry into the egg (Hagström & Runnström, 1959; Tyler et al., 1956; Hagström & Hagström, 1959; Sugiyama, 1951). The transition from an unhardened to a hardened FE is accompanied by a dramatic solubility change, i.e., from facile dissolution in dilute alkaline mercaptan solutions to extreme resistance to solubilization in various denaturing and reducing solutions (Foerder & Shapiro, 1977; Veron et al., 1977; Shapiro, 1975; Runnström, 1969).

Although electron microscopic analysis has demonstrated many morphological features of the hardened FE and the VE to FE transition, biochemical information about FE macromolecular structure is minimal because of its extreme resistance to solubilization. The present report describes conditions which result in extraction of a major protein fraction of the FE and details a characterization of these soluble proteins. The results now open the possibility of isolation and further characterization of macromolecules from hardened FEs.

Materials and Methods

Procurement and Preparation of Gametes. Shedding of eggs and sperm from *Strongylocentrotus purpuratus* and *Lytechinus pictus* was induced by intracoelomic injection of 0.5 M KCl. Sperm were collected "dry" and stored at 0 °C until needed; females were inverted over beakers and eggs were shed into filtered seawater obtained from aquaria facilities at Scripps Institution of Oceanography. Egg jelly was routinely removed by titrating egg suspensions to pH 5.0–5.4 with 0.1 N to 1.0 N HCl for 3 min; the egg suspension was then readjusted to pH 8.0 with unneutralized 1.0 M Tris base. The eggs were washed several times with normal seawater by repeated resuspension and decantation of supernatant seawater. Shedding and manipulations of eggs prior to the preparation of FEs were done at 16–18 °C in a water bath. Quantitation of egg suspensions was according to the method of Vacquier & Payne (1973).

Preparation of FEs. A 1–2% egg suspension (v/v) buffered with 5 mM Tris-HCl (pH 8.0) was gently stirred while approximately 1 mL of a diluted sperm suspension was added (1 drop "dry" sperm into 1 mL of seawater). The embryos were allowed to settle by gravity for approximately 25 min at which time the supernatant seawater was removed by aspiration. The embryos were further packed by sedimentation in the hand

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[1] Abbreviations used: FE, fertilization envelope; Tris, tris(hydroxymethyl)aminomethane; VE, vitelline envelope.

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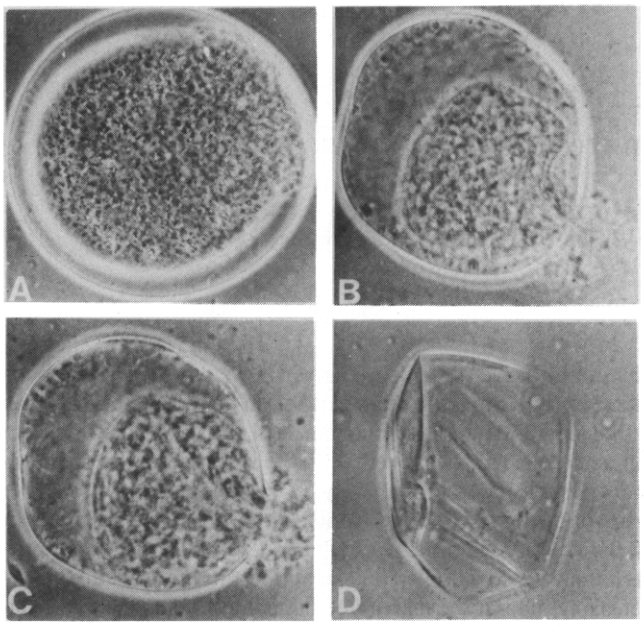

FIGURE 1: Time course of embryo lysis and release of cytoplasmic contents through ruptured hardened FEs. *S. purpuratus* embryos at 30 min post-insemination were suspended in glass distilled water (A), a nick was produced in the FE with the Dounce homogenizer and the cytoplasmic contents oozed out of the hole (B, C) to produce a FE "ghost" (D); 370×.

centrifuge. At 30 min post-insemination, the egg pellets were placed in an ice bath and rapidly chilled to 0–4 °C. Ten volumes of ice-cold glass distilled water was added to each volume of pellet and the suspenson was vigorously stirred at 4 °C for 10 min. The suspension was homogenized in a Dounce apparatus with the tight fitting pestle using ten strokes. The homogenate was centrifuged at top speed in a clinical centrifuge for 10 min. The supernatant fluid was removed by aspiration and the pellet was homogenized again using another 10 volumes of ice-cold glass distilled water. The homogenate was recentrifuged as above. A minimum of ten cycles of homogenization, centrifugation, and resuspension was performed with each FE preparation. Final purification of FEs involved centrifugation of FE suspensions on a discontinuous sucrose gradient. A bottom pad of 5 mL of 75% sucrose was placed in a 30-mL centrifuge tube; then 5 mL each of 60%, 50%, 40%, 30%, 20%, and 10% sucrose were carefully layered over the pad. A 1-mL sample of FE suspension was centrifuged for approximately 10 min at top speed in a clinical centrifuge. Typically three fractions of envelopes collected at the 75/60, 60/50, and 50/40 interfaces; fractions of greatest purity as assessed by phase contrast microscopic examination were pooled.

Light and Electron Microscopy. Light microscopy was performed with a Zeiss phase contrast microscope equipped with a Nikon AFM microflex camera attachment. Scanning electron microscopic procedures used were essentially those of Tegner & Epel (1973) except that isolated envelopes were examined without fixation. We used the transmission electron microscopic procedures of Gould-Somero & Holland (1975).

Protein Determination. Protein concentrations were estimated using the procedure of Lowry et al. (1951).

Amino Acid Analysis. FEs or extracted fractions were hydrolyzed in 6.0 N HCl at 110 °C for 12, 24, and 72 h, and the amino acid content was determined on a Spinco Model 120 amino acid analyzer. Alternatively, total amino acid content was determined by the quantitative ninhydrin procedure of Lee & Takahashi (1966) with glycine as the standard.

Sodium Dodecyl Sulfate/Urea–Polyacrylamide Gel Electrophoresis. The procedures of Weber & Osborn (1969) were used except that gels were adjusted to 6.0 M urea, a final acrylamide concentration of 6.6% and a monomer/bis ratio of 37/1 (w/w). Electrophoresis grade acrylamide and methylenebisacrylamide from Bio-Rad Laboratories (Richmond, Calif.) were used in all experiments. Extracts of FE preparations were dialyzed against 6.0 M urea–0.015 M mercaptoethanol (pH 7.0) for 18 h with two changes. The gels were stained for protein or carbohydrate according to the procedures of Fairbanks et al. (1971). Gels were scanned at a rate of 1 cm/min, slit width of 0.05 mm at 600 nm (protein) or 530 nm (carbohydrate) using a Gilford spectrophotometer equipped with a linear transport device. The recorder speed was usually 1 cm/min.

Labeling of Eggs, Embryos, FEs, or Egg Homogenate with $Na^{125}I$. A 10% (v/v) suspension of unfertilized eggs, embryos, or FEs was used; lactoperoxidase (150 μg/mL) and $Na^{125}I$ (50 μCi/mL) were added in 0.01 M Tris-HCl buffered seawater (or 0.01 M Tris-HCl) at pH 8.0. The reactions were started with an addition of H_2O_2 (0.0006% final); additional aliquots of H_2O_2 were added every 2 min for a total of 8 min. The eggs, embryos, or FEs were washed several times in either normal seawater (eggs or embryos) or glass distilled water (FEs) by centrifugation in a hand (eggs or embryos) or clinical centrifuge (FEs) and resuspension in fresh medium. To label an unfertilized egg homogenate, ca. 1 mL packed cell volume of *S. purpuratus* eggs were treated with dithiothreitol (Epel et al., 1970) to partially remove the VE (Carroll et al., 1977) and homogenized in 10-mL glass distilled water with a Dounce homogenizer. The homogenate was diluted to 50 mL and 1.0 M Tris-HCl was added to a final concentration of 0.010 M and a pH of 8.0; 600 μCi of $Na^{125}I$ and 1.0 mg of lactoperoxidase were added. The reaction was initiated with 200 μL of 0.06% H_2O_2 (0.00024% final); additional aliquots were added every 2 min for a total of 8 min. The preparation was dialyzed against four changes of a 20-fold excess of glass distilled water at 4 °C and the small amount of precipitate was removed by centrifugation at 20 000*g* for 30 min.

Determination of Radioactivity. ^{125}I radioactivity was determined by either liquid scintillation counting (Carroll et al., 1977) or by crystal scintillation counting.

Results

Isolation, Structure, and Purity of Hardened FEs. When embryos (at 30 min post-insemination) were suspended in glass distilled water they immediately lysed (Figure 1A). After production of a nick in the FE with the Dounce homogenizer, the cytoplasmic contents rapidly escaped the FE (Figures 1B and 1C) to yield an intact FE "ghost" (Figure 1D). Several cycles of homogenization, centrifugation, and washing were required to lyse all of the embryos and remove contaminating particles. Any remaining particles were removed following centrifugation of FE suspensions on sucrose gradients. Approximately 2.7 mL packed FE volume was obtained from 100 mL of packed unfertilized *S. purpuratus* eggs and about 1.8 mL of packed FEs was recovered after the sucrose gradient step. Isolated FEs retained the surface "T" projections (Figure 2A) described by Tegner & Epel (1973) and Veron et al. (1977) and had an ultrastructure (Figure 2B) which was typical of FEs on normal, fertilized eggs (Anderson, 1968).

We estimated the extent to which our FE preparations were contaminated with cytoplasmic proteins by performing a mixing experiment. An unfertilized egg homogenate was prepared from dithiothreitol-treated cells to remove most of

TABLE I: Cytoplasmic Contamination of Isolated FEs.[a]

Fraction	Total cpm of ^{125}I	Total μmol of Gly equiv[b]	Spec radioact. (cpm/μmol of Gly equiv)	% contamination of FE by cytoplasmic proteins[c]
Crude homogenate	9.34×10^6	2.00×10^3	4.67×10^3	
FE before sucrose gradient	4.51×10^4	1.12×10^2	4.03×10^2	8.6
FE after sucrose gradient[d]	1.15×10^3	4.06×10^0	2.84×10^2	6.1

[a] Three milliliters packed cell volume of unfertilized *S. purpuratus* eggs were inseminated with a minimal amount of sperm in a total volume of 14 mL of 0.010 M Tris-HCl seawater (pH 8.0). Within 5 min post-insemination, the inseminated eggs were diluted to 240 mL with normal seawater and allowed to settle by gravity. At 30 min post-insemination, the supernatant seawater was aspirated, and 9.6 mL of radioiodinated protein and glass distilled water to 30 mL total volume were added. The suspension was rapidly chilled to 4 °C and homogenized with a Dounce apparatus and FE were isolated as described in Methods. [b] A quantitative amino acid analysis after hydrolysis in 6.0 N HCl at 100 °C for 72 h was as described by Lee & Takahashi (1966). [c] Percent contamination = (cpm in isolated FE/specific radioactivity of cytoplasmic homogenate)/μmol of glycine equivalents in isolated FE. [d] A fraction of the total FE preparation containing 3900 cpm was loaded on a discontinuous sucrose gradient and centrifuged as described in Methods. FEs were collected at interfaces above the 75% sucrose cushion (1750 cpm), washed by centrifugation and resuspension in glass distilled water, hydrolyzed, and analyzed for amino acid content according to the procedure of Lee & Takahashi (1966).

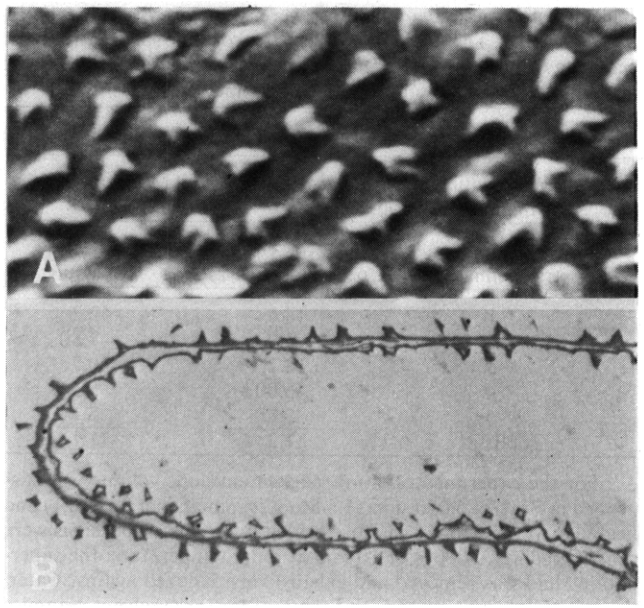

FIGURE 2: Electron micrographs of isolated hardened *S. purpuratus* FEs. (A) Scanning electron micrograph; 23 000×; (B) transmission electron micrograph; 8500×.

TABLE II: Measurements of *S. purpuratus* Packed FE Volume during and after Extraction with 6.0 M Urea–1.5 M Mercaptoethanol (pH 10.1).[a]

Step no.	Procedure	Packed FE vol (mL)
1	Suspension in water	0.4
2	Centrifugation immediately after suspension in solvent	1.5
3	1st extraction	0.9
4	2nd extraction	0.5
5	3rd extraction	0.4
6	4th extraction	0.35
7	5th extraction	0.25
8	Suspension, unsolubilized fraction in water after washing in water	1.1

[a] Initial FE packed volume was determined by centrifugation for 10 min in a graduated conical test tube at top speed in a clinical centrifuge. The FE were mixed with 5 mL of 6.0 M urea–1.5 M mercaptoethanol (pH 10.1) and immediately centrifuged as in step 1 to determine the packed FE volume. The FE were resuspended and heated in a sealed tube at 100 °C for 15 min. The packed FE volume was again determined as in step 1 and the FE were resuspended and centrifuged at 20 000*g* for 15 min at room temperature. The supernatant fluid was removed, a fresh 5-mL aliquot of 6.0 M urea–1.5 M mercaptoethanol (pH 10.1) was added, and a second extraction was started. In step 8, the washing procedure was performed several times as described in Methods except the preparation was not resuspended with the Dounce apparatus, but mixed on a vortex device.

the VE, radioiodinated, mixed with an inseminated egg suspension at 30 min post-insemination and the FEs isolated. Most of the contaminating cytoplasmic proteins (ca. 93%) were removed from the FE preparation by the initial washing procedures (Table I). Further purification of the FEs by discontinuous sucrose gradient centrifugation further reduced cytoplasmic contamination to approximately 6%.

Physical and Morphological Evidence for Disruption of FE Structure by Urea–Mercaptoethanol Extraction. FE solubility was qualitatively assessed in initial experiments by microscopic examination at 200–400× and by measurements of packed FE volume. Although suspension of FEs in 6.0 M urea–1.5 M mercaptoethanol (pH 10–10.5) at 90–100 °C resulted in a rapid clearing of the opaque FE suspension, microscopic examination demonstrated that large numbers of FEs were still present. The packed FE volume decreased in the urea–mercaptoethanol solvent following an initial swelling process (Table II). After five extractions, approximately 17% of the original packed FE volume in the urea–mercaptoethanol solvent remained; this "insoluble fraction" swelled threefold relative to the original packed FE volume in water when resuspended and washed in water. Additonally, the extracted FEs appeared very transparent and generally appeared to have a less rigid structure compared with control unextracted FEs when examined with phase contrast microscopy.

We examined the effects of the urea–mercaptoethanol solvent on FE ultrastructure using scanning and transmission electron microscopy. For scanning electron microscopic analysis, an FE suspension was adjusted to 10% (v/v) envelope concentration with 6.0 M urea–1.5 M mercaptoethanol (pH 10.5) and treated at 100 °C for 10 min. The preparation was cooled to 23 °C and centrifuged at 20 000*g* for 10 min at 23 °C; a second extraction was also performed. The extracted FEs were dialyzed against glass distilled water overnight and prepared for scanning electron microscopy. The FEs which were extracted in urea–mercaptoethanol did not contain the surface "T" projections, but rather had donut-shaped structures with the same periodicity (Figure 3A,B). FEs were also extracted in an identical manner (except five extractions were per-

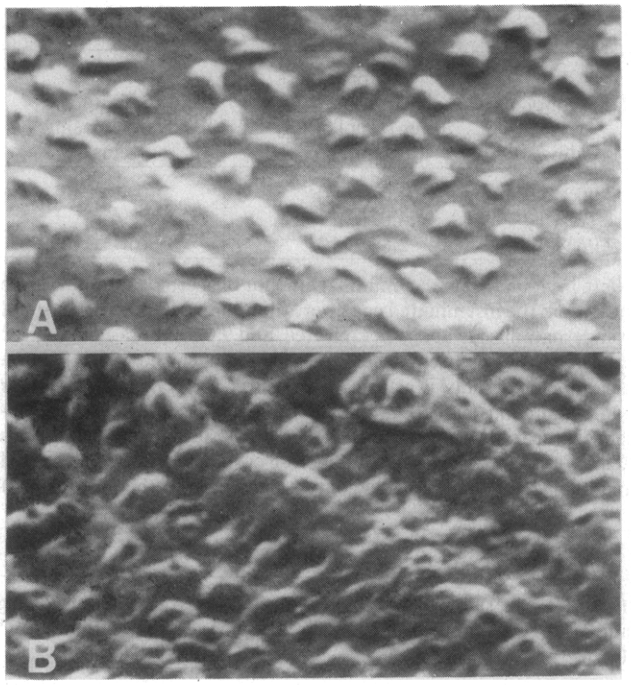

FIGURE 3: Scanning electron micrographs of normal hardened *S. purpuratus* FEs (A) and urea–mercaptoethanol extracted FEs (B); 20 000×. The FE suspension for extraction was adjusted to 10% (v/v) envelope concentration with 6.0 M urea–1.5 M mercaptoethanol (pH 10.5) and heated at 100 °C for 10 min. The preparation was cooled to 23 °C and centrifuged at 20 000*g* for 10 min at 23 °C; a second extraction was also performed. The extracted FEs were dialyzed against glass distilled water overnight and prepared for scanning electron microscopy as described in Methods.

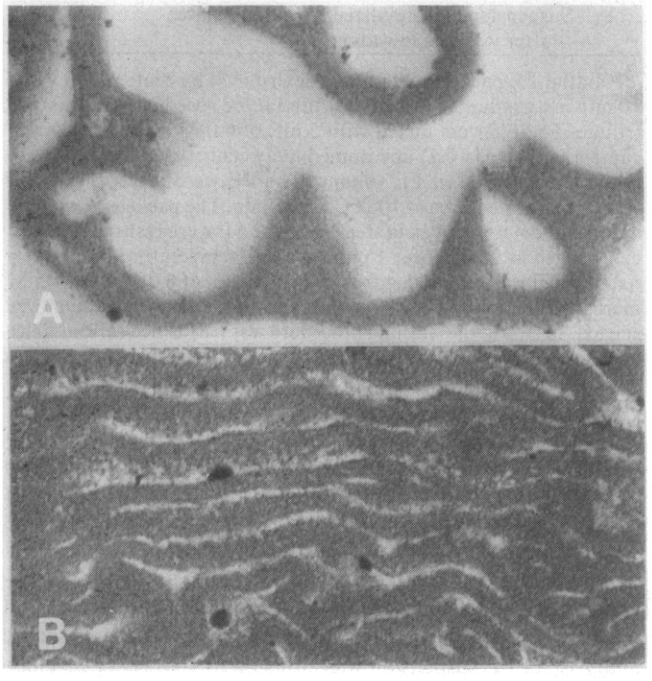

FIGURE 4: Transmission electron micrographs of normal hardened *S. purpuratus* (A) and urea–mercaptoethanol extracted FEs (B); 101 000×. The FEs were extracted in an identical manner as described in the legend to Figure 3 except five extractions were performed.

formed) and prepared for transmission electron microscopy. Extracted FEs were packed more densely during the embedding process to give a high concentration of extracted FEs; extracted FEs showed no evidence of the "T" structures seen in the control preparation (Figure 4A,B).

TABLE III: Extraction of *L. pictus* ^{125}I-Labeled FE in Various Solvents.[a]

Solvent	% ^{125}I-label extracted (mean ± SEM, *N* in parentheses)
6.0 M urea–1.0 M mercaptoethanol, pH 10.5	103.1 ± 4.7 (9)
6.0 M urea–0.1 M mercaptoethanol, pH 10.5	87.3 ± 8.0 (5)
6.0 M urea–0.01 M mercaptoethanol, pH 10.5	58.9 ± 4.8 (5)
6.0 M urea–0.001 M mercaptoethanol, pH 10.5	37.8 ± 8.7 (5)
0.01 M Tris-HCl, pH 8.0	16.5 ± 1.2 (9)

[a] Unfertilized eggs (1 mL packed cell volume) were labeled with ^{125}I and fertilized, and [^{125}I]FE was isolated as described in Methods. The final FE preparation was suspended in ca. 30 mL of water and 200-μL aliquots of this suspension were added to conical centrifuge tubes on ice. Each suspension (10–20 μL) was counted to determined the total number of counts in each tube. Two milliliters of each of the solvents listed was added; the tubes were then capped and incubated at 83 °C for 1 h. The samples were centrifuged at top speed in a clinical centrifuge for 10 min and 1 mL of the supernatant fluid was removed and counted by liquid scintillation spectrometry.

TABLE IV: Extraction of *S. purpuratus* FE in 6.0 M Urea–1.5 M Mercaptoethanol (pH 10.1).[a]

Extraction no.	Av Gly equiv per μmol (*n* = 2)	% of total Gly equiv
1	48.3	42.2
2	8.93	7.80
3	12.2	10.7
4	6.00	5.24
5	5.88	5.14
Totals	81.3	71.1

[a] For the experiment, 0.4 mL packed envelope volume was suspended in 5 mL of 6.0 M urea–1.5 M mercaptoethanol (pH 10.1) and heated at 100 °C for 10 min in a sealed tube. The suspensions were centrifuged at 20 000*g* for 10 min at room temperature; the supernatant fluid was removed and exhaustively dialyzed against water; the pellet was reextracted with 5.0 mL of the same solvent. Samples of untreated FE, the insoluble fraction, and the extracts were hydrolyzed in 6.0 N HCl at 105 °C for 72 h and analyzed by the quantitative ninhydrin procedure of Lee & Takahashi (1966). An average recovery of ninhydrin positive material (soluble extracts plus insoluble fraction compared to direct hydrolysis of FE) of 91% was obtained.

Quantitation of FE Extraction. Two experimental designs were utilized to chemically determine the extent of FE solubility in various solvents quantitatively. One experiment utilized FEs which had been labeled by the [^{125}I]lactoperoxidase procedure as the VE precursor (*L. pictus*). Solubility was also quantitatively measured by determining the fraction of total ninhydrin reactive material released after dialysis and acid hydrolysis following five extractions with a urea-mercaptoethanol solvent.

Table III shows the percent of extraction of ^{125}I counts from *L. pictus* FEs after exposure to various solvents at 83 °C for 1 h. All solvents tested extracted a significant fraction of the ^{125}I label with 6.0 M urea–1.0 M mercaptoethanol giving complete extraction. In addition, there was a direct relationship between the percent of label extracted and the mercaptoethanol concentration used.

The solubility of *S. purpuratus* FEs in 6.0 M urea–1.5 M mercaptoethanol (pH 10.1) was determined in a quantitative ninhydrin experiment (Table IV); after five extractions ap-

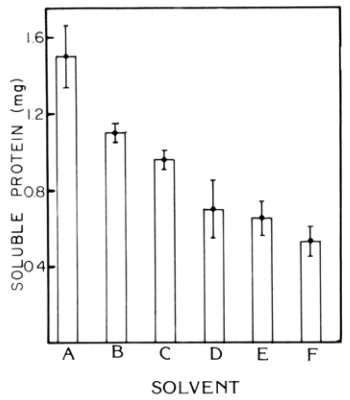

FIGURE 5: Extraction of hardened *S. purpuratus* FEs in various solvents. FEs (3.72 mg, 100 μL packed volume/tube) were heated at 100 °C in sealed tubes with 10 mL of solvent for 10 min; the different suspensions were centrifuged at 20 000*g* for 10 min at 25 °C and the soluble fractions were dialyzed against glass distilled water. The retentates were adjusted to 0.1 N NaOH and the amount of protein was determined as described in Methods. The bars represent the mean and SEM ($n = 4$) for each solvent: (A) 6.0 M urea–1.5 M mercaptoethanol (pH 10.0); (B) 6.0 M urea–0.1 M Tris-HCl (pH 10.0); (C) 1.5 M mercaptoethanol (pH 10.0); (D) 1.0 M urea–0.1 M Tris-HCl (pH 10.0); (E) 0.015 M mercaptoethanol (pH 10.0); (F) 0.1 M Tris-HCl (pH 10.0).

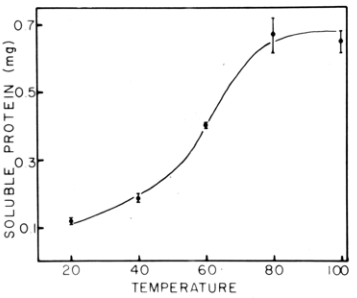

FIGURE 6: Effect of temperature on protein extraction rate from hardened *S. purpuratus* FEs. FEs (3.72 mg, 100 μL packed volume/tube) were incubated for 10 min in sealed tubes with 1.0 mL of 10 mM ethanolamine (pH 10.0); the suspension were then analyzed for soluble protein content as described in the legend to Figure 5. The bars represent the range of duplicate analysis.

proximately 70% of the total ninhydrin reactive material (primarily amino acids as amino sugars undergo destruction under the conditions of acid hydrolysis prior to ninhydrin analysis) was removed from the FE preparation.

The efficiency of FE extraction by several solvents was determined. The effect of temperature and pH on extraction rate was also examined.

Six different solvents were used to extract *S. purpuratus* FEs (Figure 5); in the experiment only a single extraction for 10 min was used, so 6.0 M urea–1.5 M mercaptoethanol (pH 10.0) only gave approximately 40% extraction of total FE protein. The mercaptoethanol component of the solvent was not absolutely required for extraction of FE proteins as 6.0 M urea alone extracted approximately 30% of the total protein. Likewise, there was little difference between 6.0 M urea–0.01 M Tris (pH 10.0), 1.5 M mercaptoethanol (pH 10.0), 1.0 M urea–0.01 M Tris (pH 10.0), 0.015 M mercaptoethanol (pH 10.0), or 0.01 M Tris (pH 10.0). The molecular state(s) of the species extracted with each of the solvents used in this experiment was unknown, but after reduction and denaturation in preparation for electrophoresis on sodium dodecyl sulfate–urea polyacrylamide gels, the same electrophoretic profiles were obtained for each extract (see Results, Characterization).

The effect of temperature on the rate of protein extraction

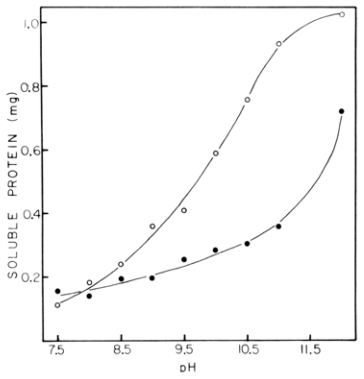

FIGURE 7: Effect of pH on the rate of protein extraction from hardened *S. purpuratus* FEs in 0.01 M ethanolamine–0.1 M NaCl (●) or 0.15 M mercaptoethanol–0.1 M NaCl (○). FEs (3.72 mg, 100 μL packed volume/tube) were incubated in 1.0 mL of solvent for 10 min at 40 °C. The suspensions were then analyzed for protein content as described in the legend to Figure 5.

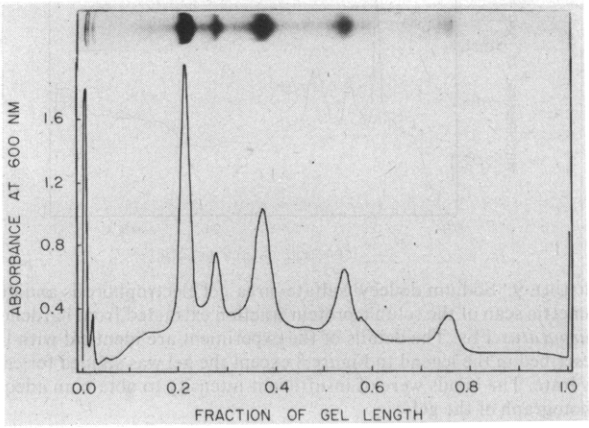

FIGURE 8: Sodium dodecyl sulfate–urea gel and densitometric scan of the soluble protein fraction extracted from hardened *S. purpuratus* FEs. FEs (3.72 mg, 100 μL packed volume) were mixed with 0.9 mL of 6.6 M urea–1.65 M mercaptoethanol (pH 10.0), heated at 100 °C for 10 min, and centrifuged 20 000*g* for 10 min at 25 °C. The supernatant solution was dialyzed against 100 volumes of 6.0 M urea overnight; the solution was changed to 6.0 M urea–0.015 M mercaptoethanol (pH 7.0) the next morning and dialysis continued for several hours. Gels were prepared as described in Methods and 100 μL of the soluble protein fraction of the FE was applied; at the end of the electrophoresis, the gel was stained for protein.

from *S. purpuratus* FEs was studied in an ethanolamine buffer (Figure 6). The rate of protein extraction dramatically increased with increasing temperature up to 80 °C. Temperatures greater than 80 °C gave no further increase in the rate of protein extraction.

The effect of pH on the rate of protein extraction from FEs was determined in mercaptoethanol and ethanolamine buffers. Sodium chloride was included in the buffers to minimize ionic strength effects. The extraction rate (between pH 8.5 and 12.0) was greater with the mercaptoethanol buffer compared with the ethanolamine buffer (Figure 7) with an inflection point in the former curve at a pH of approximately 9.8 which is close to the pK_a for the dissociation of the sulfhydryl group of mercaptoethanol (Edsall & Wyman, 1958). This contrasts markedly with the pH dependency of protein extraction from FEs observed with the ethanolamine buffer.

Characterization of the Extractable Protein Fraction of the FE. We determined some of the macromolecular characteristics of the urea–mercaptoethanol extractable protein fraction of *S. purpuratus* FEs using sodium dodecyl sulfate–urea polyacrylamide gel electrophoresis. Figure 8 shows a typical

TABLE V: Sodium Dodecyl Sulfate–Urea Polyacrylamide Gel Electrophoresis of the Soluble FE Macromolecules from *S. purpuratus* Embryos.[a]

Rel mobility ± SEM	Mol wt 10^{-3} ± SEM	% composition ± SEM[b]
0.219 (0.006)	91.6 (1.7)	21.0 (2.4)
0.292 (0.009)	71.2 (2.1)	15.0 (1.0)
0.382 (0.006)	53.0 (1.1)	26.0 (1.1)
0.542 (0.005)	32.6 (2.4)	22.0 (1.7)
0.749 (0.007)	18.2 (2.5)	7.0 (0.4)
Total		91.0

[a] Five different FE preparations were used in the analysis; duplicate or triplicate gels of each preparation were run and stained for protein. [b] The peaks from tracings of electrophoretograms were cut and weighed to estimate the percentage composition.

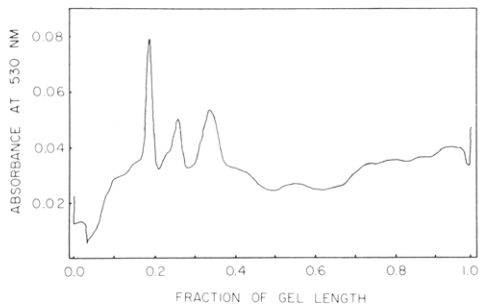

FIGURE 9: Sodium dodecyl sulfate–urea gel electrophoresis and densitometric scan of the soluble protein fraction extracted from hardened *S. purpuratus* FEs. The details of the experiment are identical with those described in the legend to Figure 8 except the gel was stained for carbohydrate. The bands were of insufficient intensity to obtain an adequate photograph of the gel.

Coomassie blue stained gel and its spectrophotometric scan. A small amount of material typically does not enter the gel but we estimate that approximately 91% of the total protein in the gel is contained in five major polypeptides (Table V).

Table V also shows the relative mobilities, molecular weights, and percentage composition estimates for five different FE preparations. Molecular weight estimates were standardized using eight different proteins ranging in molecular weight from 15 000 to 220 000. Parallel gels stained for carbohydrate using the periodic acid–Schiff base reaction showed that the 91 600, 71 200, 53 000 and possibly the 32 600 molecular weight components contained small amounts of carbohydrate (Figure 9) and thus are probably glycoproteins. The gel patterns (stained with Coomassie blue) were quantitatively and qualitatively similar (within experimental error) after FE suspensions were extracted with each of the solvents described in the legend to Figure 5 (data not shown).

We used the polyacrylamide gel electrophoresis procedure as an assay to determine if polypeptide bond hydrolysis had occurred during our extraction. Polyacrylamide gels of aliquots of an FE preparation exposed to 6.0 M urea–1.5 M mercaptoethanol (pH 10.1) and 100 °C for varying periods of time are shown in Figure 10. A "zero" time point is quite difficult to obtain and probably represents 10–20 s of exposure. Decomposition of the electrophoretic profile was evident by 15–30 min with a total loss of discrete polypeptides by 240 min. Bovine serum albumin at 1.0 mg/mL in the same urea–mercaptoethanol solvent was also hydrolyzed at a similar rate.

Polyacrylamide gel electrophoresis was also used to examine the possibility that some of the five polypeptides might be

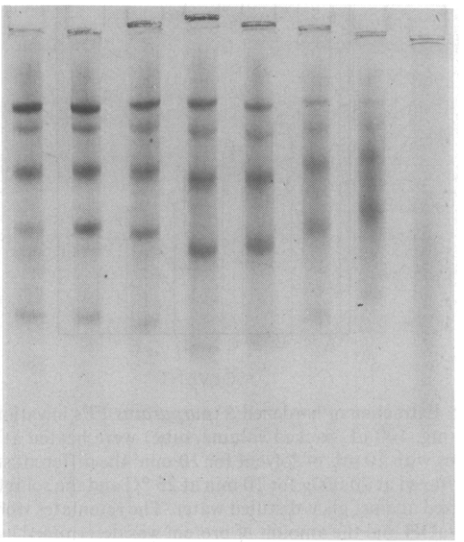

FIGURE 10: Time course of protein hydrolysis of soluble FE proteins during the extraction. The details of the experiment are identical with those given in the legend to Figure 8 except extraction was carried out for 0, 1, 5, 10, 15, 30, 60, and 240 min (left to right).

TABLE VI: Amino Acid Composition of Normal FE and FE Extracted in 6.0 M Urea–1.5 M Mercaptoethanol.[a]

Amino acid	Normal FE mol %	Extracted FE mol %	Normal − extracted FE (% change)
Ala	5.19	4.44	+14.5
Arg	5.83	4.36	+25.2
Asx	13.8	16.2	−17.4
½-Cystine[b]	8.43	10.3	−22.2
Glx	10.7	11.3	−9.9
Gly	10.4	11.8	−13.5
His	1.46	0.84	+42.5
Ile	4.05	4.02	+0.7
Leu	4.70	2.93	+37.7
Lys	3.73	1.68	+55.0
Met[b]	0.49	0.25	+49.0
Phe	3.24	2.34	+27.8
Pro	6.65	7.88	−18.5
Ser	7.13	7.21	−1.1
Thr	5.35	5.70	−6.5
Trp[c]			
Tyr	2.59	2.01	+22.4
Val	6.32	6.71	−6.2

[a] Amino acid analysis was accomplished following acid hydrolysis as described in Methods. FEs were extracted five times in 6.0 M urea–1.5 M mercaptoethanol (pH 10.1) for 10 min at 100 °C. [b] No special precautions were taken to protect these residues. [c] Not determined.

modified by proteolysis during FE isolation. For this experiment an egg suspension was inseminated, split into equal portions in each of two beakers and FEs were isolated using embryos from one beaker as described in Methods and from the other batch of embryos in the same manner except that 0.001 M phenylmethanesulfonyl fluoride–0.01 M Tris-HCl (pH 8.0) was used as the isolation medium. Gel electrophoretic analysis showed no significant differences in the urea–mercaptoethanol extracts of FEs isolated in the presence or absence of phenylmethanesulfonyl fluoride (data not shown).

The amino acid composition of FEs isolated from embryos at 30 min post-insemination was compared with FE extracted in the urea–mercaptoethanol solvent. The results presented

in Table VI show significant changes in several amino acids; e.g., histidine, leucine, and lysine as a result of the extraction procedure.

Localization of the Extractable Protein fraction of the FE. The electron microscopic, amino acid and gel electrophoretic analyses of urea-mercaptoethanol extracted FEs presented in this paper suggest that the extraction procedure removes proteins from the FE surface. We tested this hypothesis using in vivo lactoperoxidase catalyzed radioiodination of embryos. Recent work of Veron et al. (1977) and Veron & Shapiro (1977) has shown that, within 7 min post-insemination, the FE of *S. purpuratus* embryos become essentially impermeable to either ^{125}I-labeled or fluorescein-conjugated concanavalin A but will bind radioiodinated concanavalin A with high affinity. We have taken advantage of the FE permeability change to macromolecules to determine if the FE macromolecules that we can extract in urea-mercaptoethanol are on the outer aspect of the FE surface.

An unfertilized *S. purpuratus* egg suspension (20 mL of a 10% (v/v) suspension) was inseminated and washed with normal seawater a few minutes later. At 30 min post-insemination the embryos were iodinated and the iodinated FEs were isolated. The preparation was adjusted to a 10% (v/v) FE suspension and was incubated in 6.0 M urea–1.5 M mercaptoethanol (pH 10.5) at 100 °C for 15 min. The preparation was then cooled and centrifuged at 20 000*g* for 10 min at room temperature. The supernatant solution was dialyzed against two changes (50 mL each) of 6.0 M urea–0.015 M mercaptoethanol (pH 7.0) over a 24-h period. A portion of the retentate fraction was layered on a polyacrylamide gel. The gels were stained for protein, scanned sliced into ca. 1-mm sections, and counted. The densitometric scan of the gel and radioactivity in each slice (after background correction) were normalized on the same scale as shown in Figure 11. Polypeptides of relative mobility = 0.210 and 0.364 had relatively high specific radioactivities compared with the low, but significant specific radioactivity of the polypeptides of relative mobility = 0.545 and 0.750. Resolution on the gel was diminished in order to increase the number of counts; thus, it was difficult to determine if coincident label and Coomassie blue staining band were present for the polypeptide of relative mobility = 0.292. When the protein load applied was decreased by two- to fourfold to improve resolution, the level of counts in some of the bands was below detection (data not shown).

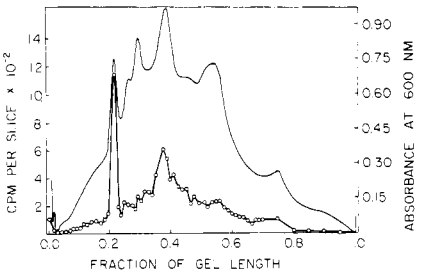

FIGURE 11: Sodium dodecyl sulfate gel electrophoresis of the soluble protein fraction of in vivo surface ^{125}I-labeled hardened *S. purpuratus* FEs. Twenty milliliters of a 10% (v/v) unfertilized *S. purpuratus* egg suspension was inseminated and washed with normal seawater a few minutes later. At 30-min post-insemination the embryos were iodinated and subsequently the iodinated FEs were isolated as described in Methods. The preparation was adjusted to a 10% (v/v) FE suspension and was incubated in 6.0 M urea–1.5 M mercaptoethanol (pH 10.5) at 100 °C for 15 min. The preparation was cooled and centrifuged at 20 000*g* for 10 min at room temperature. The supernatant solution was dialyzed against two changes (50 mL each) of 6.0 M urea–0.015 M mercaptoethanol (pH 7.0) over a 24-h period. A portion of the retentate fraction was layered on polyacrylamide gels as described in Methods. The gels were stained for protein, scanned, sliced into ca. 1-mm sections, and counted. The densitometric scan and radioactivity data (after background correction) were normalized and plotted on the same scale. Solid line, absorbance at 600 nm; (○) cpm/slice × 10^{-2}.

Discussion

In this paper we report an isolation procedure, extraction, and characterization of a major protein/glycoprotein fraction from hardened sea urchin egg FEs. Our isolation procedure is gentle and results in FE preparations which are essentially free of contaminating cytoplasmic protein. Analysis of our isolated FEs by scanning and transmission electron microscopy shows that the center-to-center spacing of the "T" projections, which is a good marker of the hardened envelope, is 0.35 ± 0.06 μm (n = 20). This compares favorably the spacing of FE "T" projections fixed in vivo (0.43 ± 0.04 μm (n = 20) Tegner & Epel (1973), 0.40 ± 0.10 μm (n = 20) Veron et al. (1977)). Thus, we are confident that our FE preparations represent highly purified, intact FEs with an ultrastructure which has been observed for FEs in vivo.

We have extracted isolated FEs in several solvents using a variety of conditions. Ultrastructural analysis of urea-mercaptoethanol extracted FEs clearly shows that material(s) is being removed from the envelope. At the scanning electron microscopic level of examination the "T" projections were dramatically altered in their morphology and appeared to be converted into donut-shaped structures with the same periodicity as the original "T" projections; e.g., from 0.361 ± 0.06 μm (n = 20) to 0.32 ± 0.05 μm (n = 20) after extraction, respectively. Examination of urea-mercaptoethanol extracted FEs by transmission electron microscopy also showed evidence of "T" projection modification or removal. While it is tempting to speculate that the extraction procedure only removes "T" projection protein(s) on the basis of thickness measurements, it is clear that an additional dramatic physical change in FE structure, i.e., swelling, takes place following extraction. Thus material(s) from other regions of the FE surface may also be removed by urea-mercaptoethanol extraction. Inoue & Hardy (1971) have also observed dissociation and solubilization of fully developed FEs with urea-mercaptoethanol using a surface replica-transmission electron microscopic procedure.

Direct biochemical analysis of various extracts of isolated, hardened FEs also demonstrated that considerable material(s) was removed from the structure. Radioiodinated FEs (labeled as the VE precursor in vivo) released a significant soluble fraction of the total radioactivity in response to extraction by the urea-mercaptoethanol solvent. The ^{125}I-label extracted was complete but this number represents an upper limit since not all portions of the VE precursor are likely to be labeled and because none of the cortical granule contents are labeled by this procedure. The best estimate of the total urea-mercaptoethanol extractable protein of the FE is probably given by the quantitative ninhydrin experiments. In these experiments FEs were repetitively extracted with urea-mercaptoethanol for only 10 min at 100 °C, conditions which minimize hydrolysis of the soluble protein fraction (Figure 10). Analysis of the urea-mercaptoethanol extracts showed 71% of the total amino acid content of the FEs was removed by extraction.

Temperature and mercaptan are extremely important parameters in extracting proteins from the hardened FE. The "T_m" of approximately 60 °C for extraction of FEs in ethanolamine (pH 10.0) shown in Figure 6 closely resembles that observed for fully developed FE of the amphibian *Xenopus laevis* (Wyrick, 1974; Wolf et al., 1976). The increased rate of protein extraction from FEs in mercaptoethanol buffers was shown in Figure 7 and has also been noted in sea urchin egg VE

solubilization (Glabe & Vacquier, 1977; Carroll et al., 1977; Epel et al., 1970; reviewed by Berg, 1967). The mercaptan effect on increased rate of egg coat dissolution has also been noted in the horned beetle *Xylotrupes dichotomus* (Kawaski et al., 1976), the crinoid *Comanthus japonica* (Holland, 1976), the keyhole limpet (Heller & Raftery, 1976a,b), *Xenopus laevis* (Yurewicz et al., 1975; Wolf et al., 1976), and mouse (Inoue & Wolf, 1974a,b, 1975a,b). It should be noted, however, that in the present work, the presence of mercaptoethanol in the extraction medium only enhances the rate of protein extraction from FEs, the qualitative and quantitative appearance of the extracted proteins determined by gel electrophoresis is identical regardless of extraction medium.

Our characterization of the urea–mercaptoethanol extractable protein fraction from hardened sea urchin FEs indicates that the fraction is composed of five proteins, at least three of which are glycoproteins ranging in molecular weight from 18 200 to 91 600. The low degree of contamination of our FE preparation by cytoplasmic proteins (ca. 6% w/w) makes it seem unlikely that any of these five proteins are due to cytoplasmic contamination. These results are in basic agreement with the macromolecular characteristics of isolated sea urchin VEs (Glabe & Vacquier, 1977) and *Xenopus laevis* isolated VEs and FEs (Wolf et al., 1976). Since the in vivo lactoperoxidase catalyzed ^{125}I-labeling experiments indicate these five urea-mercaptoethanol extractable FE proteins are perhaps on the surface of the envelope, some of the proteins may represent the paracrystalline proteins of the FE described by Bryan (1970a,b).

The transition of the VE to the FE and the hardening of the FE involves release of cortical granule contents (for reviews, see Giudice, 1973; Runnström, 1966) and perhaps the formation of dityrosine cross-links catalyzed by an egg peroxidase (Foerder & Shapiro, 1977). This simple procedure for isolation and extraction of a major protein fraction of the hardened FE should facilitate further studies of the VE to FE transition.

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