The Organic Matrix of the Skeletal Spicule of Sea Urchin Embryos

Stephen C. Benson,* Nancy Crise Benson,* and Fred Wilt‡

*Department of Biological Sciences, California State University, Hayward, California 94542; and ‡Department of Zoology, University of California, Berkeley, California 94720. Address reprint requests to Dr. Wilt.

Abstract. The micromeres that arise at the fourth cell division in developing sea urchin embryos give rise to primary mesenchyme, which in turn differentiates and produces calcareous endoskeletal spicules. These spicules have been isolated and purified from pluteus larvae by washing in combinations of ionic and nonionic detergents followed by brief exposure to sodium hypochlorite. The spicules may be demineralized and the integral matrix dissolves. The matrix is composed of a limited number of glycoproteins rich in aspx, glux, gly, ser, and ala, a composition not unlike that found in matrix proteins of biomineralized tissues of molluscs, sponges, and arthropods. There is no evidence for collagen as a component of the matrix. The matrix contains N-linked glycoproteins of the complex type. The matrix arises primarily from proteins synthesized from late gastrulation onward, during the time that spicule deposition occurs. The mixture of proteins binds calcium and is an effective immunogen. Electrophoresis of the glycoproteins on SDS-containing acrylamide gels, followed by blotting and immunocytochemical detection, reveals major components of ~47, 50, 57, and 64 kD, and several minor components. These same components may be detected with silver staining or fluorography of amino acid–labeled proteins. In addition to providing convenient molecular marker for the study of the development of the micromere lineage, the spicule matrix glycoproteins provide an interesting system for investigations in biomineralization.

We report here our initial results on purification and characterization of the organic matrix of the endoskeletal spicule of the sea urchin embryo. The pluteus larva has calcareous rods, which are intracellular deposits of calcite (mainly $CaCO_3$ and some $MgCO_3$ on an organic ground substance; Benson et al., 1983). The matrix of the skeletal element is of interest because of its probable role in the biomineralization process and because of the obvious parallel between biomineralization of hydroxylapatite in vertebrates and calcite in marine invertebrates. An equally important consideration is the opportunity the spicule presents to study the determination and differentiation of the micromere lineage, a group of cells whose developmental history is very well known. At the fourth cell division in the sea urchin embryo four micromeres arise at the vegetal pole. These micromeres give rise to primary mesenchyme cells, which in turn differentiate into the skeletal spicules (reviewed by Wilt et al., 1985). Micromeres may be isolated from the embryo and will differentiate autonomously in culture to form spicules (Okazaki, 1975). Hence, information on the matrix of the spicules may be useful in the study of tissue-specific differentiation in sea urchin embryos. We have devised ways to purify the matrix of the spicule, and the data support the conclusion that the matrix is composed of a small number of soluble N-linked glycoproteins that have a strong biochemical similarity to acidic proteins present in calcareous structures of other invertebrates.

Materials and Methods

Embryo Culture

Individuals of *Strongylocentrotus purpuratus* were collected locally. Gametes were obtained and fertilized, and embryos were cultured by standard techniques (Hinegardner, 1967) using Millipore (0.45 μm)-filtered seawater that contained 10 μg/ml of gentamycin sulfate. When cultures were raised to pre-gastrula stages, 1×10^4 embryos/ml or less was the usual concentration. For later stages, cultures were diluted to contain 2×10^3 embryos/ml.

Isolation of Spicules

Embryos were collected by centrifugation and washed once in calcium- and magnesium-free seawater (CMFSW)[1] and twice in cold 1.5 M glucose. The pellet of embryos was resuspended by homogenizing in a loose fitting Dounce homogenizer in 10 vol of ice cold 10 mM Tris buffer, pH 7.4, that contained a mixture of protease inhibitors. The final concentration of inhibitors was: 5 mM benzamidine, 50 mM ε-aminocaproic acid, 5 mM *N*-ethylmaleimide, and 0.5 mM phenylmethylsulfonyl fluoride (added just before use). The homogenate was allowed to sit on ice for 10 min, then centrifuged at 800 *g* for 3 min, the supernatant discarded, and the entire procedure repeated.

The pellet of lysed embryos was resuspended in 10 vol of 2% Triton X-100, 4% sodium deoxycholate (Trito-DOC), 20 mM Tris buffer, pH 7.4, and homogenized in a loose fitting Dounce homogenizer. The suspension was centrifuged 800 *g* for 3 min and the supernatant discarded. This procedure was repeated 4–5 times until most of the cellular debris was absent and the pellet appeared light brown or off-white. This preparation, which is designated "bags",

[1] *Abbreviations used in this paper:* CMFSW, calcium- and magnesium-free seawater; FCS, fetal calf serum; Trito-DOC, a solution of 2% Triton X-100, 4% sodium deoxycholate.

shows large numbers of spicules, a few cells, and the blastocoel space surrounded by the basal lamina.

The preparation of spicules and basal laminae was resuspended in a homogenizer that contained 5 vol of the same ice cold detergent solution used above, and while moving the pestle, 5 embryo volumes of cold 5% sodium hypochlorite, 10 mM Tris buffer, pH 8, were added. Homogenization was continued on ice for 15–60 s while the preparation turned clear white, then quickly centrifuged at 800 *g* for 1 min, and the spicules subsequently washed by resuspending the pellet with a pasteur pipette in 1 embryo volume of cold 2% hypochlorite, 10 mM Tris. After another centrifugation, the spicules were washed 2–3 times in cold distilled water.

Spicule preparations were demineralized by resuspending the water washed pellet in 50 mM EDTA, pH 8, or 0.1 N acetic acid. There is often a minute residue present after demineralization, composed of dust and possibly some insoluble component of the spicule matrix. This is removed by centrifugation at 1,000 *g* for 2 min. The soluble spicule matrix is dialyzed against four changes of 1 liter of cold distilled water, lyophilized, and stored frozen. The lyophilized powder easily redissolves in aqueous solutions. Poor recoveries have been obtained when glass rather than plastic containers were used.

Labeling Procedure

Embryos were labeled with radioactive amino acids or monosaccharides by washing ~10^7 embryos twice in artificial seawater (Hinegardner, 1967) and then resuspending them in 50 ml of artificial seawater that contained 10 mg/ml of gentamycin. [^{35}S]Methionine (500 Ci/mM), ^{3}H-L-amino acid mixture (196 mCi/mg), L-4,5[^{3}H]leucine (130 Ci/mM), or L-5[^{3}H]proline (31.5 Ci/mM), were added to a final concentration of 5 mCi/ml and the culture incubated 6–10 h at 15°C with gentle (80 rpm) rotation in a flask that allowed the seawater layer to be ~1-cm deep. D-1,6[^{3}H]Glucosamine (47.6 Ci/mM) or D-2[^{3}H] mannose (22 Ci/mM) was used for labeling in the same way. After the labeling, embryos were diluted to 2 liters with Millipore-filtered seawater (final concentration = 2×10^3/ml) and cultured an additional 48–72 h. Aliquots of labeled preparations were removed during the spicule preparation and precipitated with trichloroacetic acid that contained 1 mM quantities of the unlabeled congener of the tracer used.

A pulse-chase labeling procedure slightly different from the above was used to determine the approximate time of synthesis of spicule matrix proteins. Embryos were fertilized and cultured continuously at 15°C with 2×10^3 embryos/ml in seawater that contained 0.1 mCi/ml of $^{45}CaCl_2$. At various times during the culture period, [^{3}H]leucine (130 Ci/mM) was added to a final concentration of 0.5 mCi/ml. After exposure to the radioactive leucine for 20–24 h, the embryos were washed three times by centrifugation and resuspended in seawater that contained 10^{-5} M unlabeled leucine, then diluted to the 2×10^3 embryos/ml, and development continued in seawater at 15°C that contained 1 mM leucine. This concentration of leucines effectively stops further detectable incorporation of label because the pools are expanded.

Samples from embryos labeled with radioactive amino acids or carbohydrates were treated with hot (90°C) trichloroacetic acid, collected on Whatman glass fiber filters (Whatman Inc., Clifton, NJ), and washed with 10% trichloroacetic acid, water, and methanol. Samples that contained ^{45}Ca were counted by adjusting aqueous aliquots to contain 0.5 N NaOH, heating 2 min at 80°C, and collecting samples on a glass fiber filter, which was then rinsed with 0.5 N NaOH, water, and methanol. All samples were counted by liquid scintillation counting.

Immunological Procedures

Antisera were raised to soluble spicule matrix proteins after subcutaneous immunization of rabbits using Freund's complete adjuvant. The immunoglobulin fraction was isolated from preimmune and immune sera by ammonium sulfate precipitation and DEAE cellulose chromatography (Fahey, 1969).

Light Microscopy

Plutei were fixed in freshly prepared 2% paraformaldehyde, pH 7.4 made in CFSW. After the embryos were rinsed in CFSW, they were dehydrated through a graded series of ethanol to 95% and were embedded in JB-4 (Polysciences, Inc., Warrington, PA) glycol methacrylate and polymerized overnight. Sections were cut at 2–3 mm with a Reichert OM-2 ultramicrotome (Reichert Scientific Instruments, Buffalo, NY) with glass knives, and the sections picked up on slides coated with L-polylysine.

The Vectastain (R) ABC system using avidin–biotin horseradish peroxidase complex was used to localize the spicule matrix antigens. The primary antibody was used at a dilution of 1:100–1:200. Subsequent steps were done according to manufacturer's instructions.

Colloidal Gold Immunoelectron Microscopy

Embryos were fixed in 0.75% glutaraldehyde in CFSW for 1 h at room temperature, and then washed with cold CFSW and postfixed with 1% OsO_4 in 0.2 N sodium phosphate buffer, pH 7.0 for 45 min at 21°C. Embryos were dehydrated through a graded series of ethanol and embedded in Spurr's resin. Silver sections were picked up on stainless steel grids.

A technique described by Wessel and McClay (1985) for the indirect application of colloidal gold was applied to sections as follows: the grids were incubated in 10% goat serum/20% fetal calf (FCS) serum in phosphate-buffered saline (PBS), followed by 10% FCS in PBS. Grids were then incubated for 1 h at 21°C in the anti-spicule matrix antibody diluted 1:100–1:200 dissolved in 10% FCS-PBS. After thorough washing in 10% FCS in PBS, 15-nm gold particles conjugated to goat anti-rabbit IgG (Janssen) diluted 1:30 in 10% FCS-PBS was applied for 1 h, then washed extensively with PBS. 2% glutaraldehyde in PBS was used to fix the gold particles to the sections. After thorough washing with distilled water, the sections were poststained with uranyl acetate and lead citrate and viewed with a Hitachi HS-8 electron microscope.

Immunoblotting

Electrophoresis was in 12.5% acrylamide gels that contained SDS by the method of Laemmli (1970) as modified by Dreyfus et al. (1984). Immunoblotting was done essentially as described by Towbin et al. (1979). The main modification was to use 5% (wt/vol) non-fat dry milk as the blocking agent, as described by Johnson et al. (1984). The binding of swine anti-rabbit IgG complexed with horseradish peroxidase (Accurate Chemical & Scientific Corp., Westbury, NY) was visualized using diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as the substrate.

Spicule Matrix Characterization

Electrophoresis. Electrophoresis was done in 12.5% acrylamide gels as described above. Visualization of radioactive protein was achieved by fluorography as described by Laskey and Mills (1975). Gels were silver stained, essentially by the technique of Morrisey (1981) after overnight fixation in 0.037% formaldehyde in 50% methanol.

Carbohydrate Analysis. Total carbohydrate was estimated by the phenol–sulfuric acid method described by Ashwell (1966) using galactose or mannose as a standard. Hexosamine content was estimated by the procedure of Swann and Balazs (1966) using galactosamine as a standard. Uronic acid was determined by the procedure of Blumenkrantz and Asboe-Hansen (1973) and sialic acid by the resorcinol reaction as described by Spiro (1966).

Endoglycosidase F digestion of radioactive spicule matrix proteins was done by following the manufacturer's instructions. Samples were adjusted to contain 0.1 M sodium phosphate buffer, pH 6.1, 50 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 1% 2 mercaptoethanol. 6 U of enzyme (New England Nuclear, Boston, MA) were added and the reaction incubated at 37°C for either 1 or 18 h.

Endoglycosidase H digestions were done in 50 mM sodium citrate, pH 5.5, 0.02% SDS, 0.4 mM phenylmethylsulfonyl fluoride as detailed by the manufacturer. 0.3 U of enzyme (Miles Laboratories, Inc., Naperville, TN) was added and the reaction incubated at 37°C for 18 h. After digestion with either glycosidase, the samples were diluted with an equal volume of doubly concentrated Laemmli sample buffer and subjected to electrophoresis.

Calcium Binding. Calcium binding activity was determined for the acid-demineralized, water-soluble spicule matrix by equilibrium dialysis as described by Potter et al. (1983). The solution used in these experiments was one-fifth the concentration of calcium-free seawater (Hinegardner, 1967).

Amino Acid Analysis. The spicule matrix proteins were hydrolyzed in 6 N HCl at 110°C for 20 h, and were separated and quantitated on a Beckman model 120 amino acid analyzer (Beckman Instruments, Inc., Palo Alto, CA). Recoveries of total amino acid were monitored by recovery of L-norleucine added before hydrolysis. Reproducibility of analyses varied <5%. Quantitation of proline and hydroxyproline in spicule matrix isolated from embryos cultured in seawater that contained [^{3}H]proline was examined after acid hydrolysis of the protein. The samples were chromatographed on a Dowex 50-X8 column as described by Cutroneo et al. (1972). The column was calibrated with [^{14}C]-proline and [^{3}H]hydroxyproline. Protein concentrations were estimated according to the colorimetric methods of Lowry et al. (1951) using bovine serum albumin as a standard.

Results

Purification of Spicule Matrix

The endoskeletal spicule is a relatively large and prominent structure in prism and pluteus stage embryos. Previous attempts to purify it have relied primarily on lysis and repeated washing in detergent (Pucci-Minafra et al., 1972). If the entire embryo is repeatedly washed in distilled water or in various detergents, the cellular portions of the embryo are gradually lysed and removed, leaving the insoluble calcite rods of the spicule behind. Fig. 1 shows larvae after a brief exposure to Triton X-100 and sodium deoxycholate. Many cells have lysed, though many still remain, and the outlines of the bag-like basal lamina may be seen in relief. The basal lamina and the fibrous blastocoel contents (Solursh and Katow, 1982) are very difficult to solubilize. Fig. 2*B* shows a whole mount of a spicule viewed by electron microscopy after repeated washings in detergents; the remaining laminae and fibrous contaminants are very evident, even though they were barely perceptible by phase-contrast microscopy.

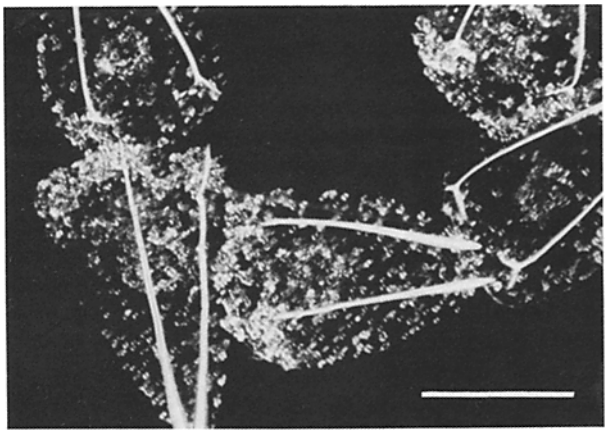

Figure 1. Partially purified spicules. Pluteus larvae were washed twice with Triton-DOC and viewed by dark field microscopy. The basal lamina, still adorned with some cells, encloses the long skeletal spicules and some gut and mesenchyme cells. Bar, 10 μm.

The partially contaminated spicules that result from detergent washing may be further purified by exposure to urea and SDS, a method described by us previously (Benson et al., 1983). Another efficacious and simple way to further purify the spicule is brief exposure to alkaline sodium hypochlorite. Fig. 2*A* shows the outlines of a spicule prepared by this method. It lacks the obvious contamination present in spicules that are not exposed to hypochlorite. The method is very rapid and simple, and exposure to bleach is very brief. The possibility that this oxidizing agent might modify organic components of the matrix has not been borne out (vide infra). It is important in this procedure to partially purify the spicule with detergent before exposure to bleach or considerable impurities may contaminate the preparation. In the next section we shall present evidence that the chemical composition of the spicule matrix is sufficiently different from the basal lamina and blastocoel contents that one may conclude that the spicules are, indeed, extensively purified by this method.

Composition and Properties of the Spicule Matrix

When the mineral component of the fixed spicule is dissolved with chelating agents or dilute acids, a lamellar reticulum with the overall outlines of the spicule are visible (Benson et al., 1983). If the spicule is not exposed to fixative for many hours before demineralization, all structure visible in the light microscope disappears, which indicates that at least some of the matrix is water soluble.

When isolated spicules are washed exhaustively with water, then dried to constant weight over P_2O_5, their dry weight is equivalent to 5% of the protein mass present in the egg (Table I). Presumably most of the mass of the spicule is calcium carbonate. Demineralization of the calcified spicule by dilute acid, dialysis, and subsequent determination of the protein of the soluble material indicates that ~0.1% of the total weight of the calcified spicule is protein. Hence, ~0.005% of the total

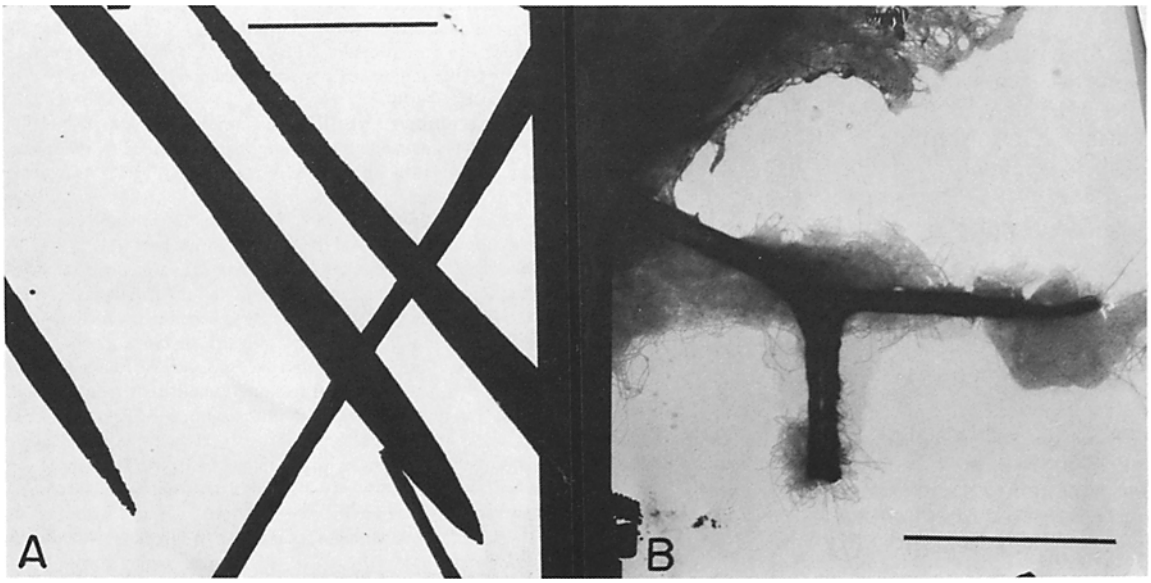

Figure 2. Transmission electron micrographs of spicules. Spicules were prepared by Triton-DOC washing with (*A*) and without (*B*) subsequent treatment with alkaline hypochlorite. The spicules were prepared as whole mounts and examined by electron microscopy. Bars, 1 μm.

Table I. Recovery of Protein and Label in Isolated Spicules

Substance or label	% Starting material in "bags"*	% Starting material in isolated spicules‡	% Starting material in soluble matrix§
Total protein‖	14.2 (2)	4.9¶ (3)	0.006** (3)
			0.005‡‡ (22)
^{45}Ca	51 (9)	44.4 (10)	nd
[^{35}S]Methionine	8.3 (2)	0.013 (2)	0.007

nd, not detectable. Numbers in parentheses indicate the number of preparations examined to obtain the average values shown.

* This is the stage of purification after extensive washing in deoxycholate-Triton X-100 when the cells are removed but the basal lamina and other material is still present.

‡ Spicules were prepared from the bags of basal lamina by alkaline hypochlorite.

§ Spicules were demineralized and centrifuged at 10,000 *g* for 10 min. 85% of the radioactivity was nonsedimentable, and was then dialyzed.

‖ The total protein per embryo is 40 ng, and there are 1.8×10^6 embryos/ml of packed eggs.

¶ Material recovered is the dry weight of the spicule, including both mineral and organic material.

** Demineralized by EDTA. Protein determined by ninhydrin reactions on hydrolyzed protein.

‡‡ Demineralized by acetic acid. Protein determined by the Lowry reaction.

embryo protein is soluble spicule matrix protein. If EDTA is used to demineralize the spicule, spurious values for protein recovery may be obtained, probably because the spicule matrix protein contains considerable EDTA that is not dialyzable, a behavior noted by others (Weiner, 1984).

The efficiency of obtaining spicules may be conveniently followed by recovery of ^{45}Ca. Nakano et al. (1963) and Mintz et al. (1981) showed that ^{45}Ca uptake into an insoluble form is a reliable indication of spicule formation. We find about one-half of the insoluble ^{45}Ca of the pluteus is recovered in the final spicule preparation, and most of the loss of calcium occurs during detergent washing. We cannot distinguish slow demineralization in detergent from physical loss of small pieces of the spicule during homogenization and centrifugation. Since the calcium recovery indicates that spicule recovery is ~40–50%, we conclude from this data that the mineralized spicule is ~10% of the dry weight of the pluteus, and ~0.01% of the protein of the pluteus is soluble spicule matrix protein.

When the embryo is labeled with radioactive methionine, ~8% of the acid-insoluble label is found associated with the bag preparation after washing with Triton-DOC. A small proportion of this label is eventually found in the thoroughly purified spicule. At least 90–95% of the label at the "bag" stage is removed by exposure to alkaline hypochlorite. Usually half, or sometimes more, of the radioactivity in the final purified spicule preparation is recovered after demineralization, dialysis, and centrifugation to clarify the solution. Some 10–15% of the demineralized spicule component sediments at 10,000 *g* in 10 min, and presumably represents a water-insoluble element of the spicule matrix. Recovery of [^{35}S]-methionine is similar to that found for total protein (Table I). Similar results are obtained when [^{3}H]leucine is used as a label. The morphological and biochemical data on the extent of contamination after simple Triton-DOC washing and the removal of contaminants with hypochlorite are reasonably concordant. The spicule preparation may also be labeled after exposure of embryos to radioactive glucosamine or mannose (data not shown), but the amount of radioactivity that could be obtained was too low to allow further analysis.

The soluble spicule matrix proteins have an unusual amino acid composition (Table II). No substantial differences have been found in the composition when the spicules are demineralized with either EDTA or acetic acid. The soluble spicule matrix proteins are very rich in asparate + asparagine, glutamine + glutamate, glycine, serine, and alanine. These five amino acid groups constitute ~64% of the amino acid residues present. This unusual composition is often found with proteins associated with calcite skeletal elements in various phyla (Weiner, 1984; Weiner et al., 1983). The analysis represents an average of different components in the mixture. The amino acid composition is very different from the collagens, which had been reported by others to be a component of the spicule matrix (Pucci-Minafra et al., 1972). The amino acid analyses showed no detectable hydroxyproline, even though the characteristic high absorbance at 440 nm of the hydroxyproline–ninhydrin reaction product allows easy identification of hydroxyproline in a conventional amino acid analysis. We have also labeled embryos on several occasions with [^{3}H]proline and subsequently subjected the hydrolysate of the soluble spicule matrix protein to chromatographic techniques that resolve hydroxyproline from proline. Hydroxyproline has not been detectable by these procedures, even though the presence of 1% of the proline label as hydroxyproline could have been detected.

The amino acid analysis did not reveal any peaks that correspond to chlorotyrosine, an amino acid derivative that might be expected if the brief exposure of the spicules to sodium hypochlorite resulted in a reaction of the spicule matrix protein with hypochlorite. The amino acid analysis did indicate the presence of amino sugars that may correspond to glucosamine, galactosamine, and mannosamine.

*Table II. Amino Acid Composition of Soluble Spicule Matrix Proteins**

Amino acid	Mole % spicule matrix‡	Mole % hypochlorite-solubilized material§
Lysine	3.3	2.4
Histidine	3.7	0.3
Arginine	2.3	0.3
Aspartic acid + asparagine	11.0	16.0
Glutamic acid + glutamine	12.1	14.0
Glycine	21.3	7.1
Serine	12.5	1.5
Threonine	4.4	2.2
Tyrosine	1.3	tr
½ Cysteine	2.9	5.5
Alanine	8.3	6.6
Leucine	4.2	9.2
Isoleucine	2.5	6.3
Valine	3.4	9.1
Proline	4.5	16.4
Phenylalanine	1.8	2.6
Methionine	0.5	nd
Hydroxyproline	nd	0.6

nd, not detectable.

* Uncorrected for losses of individual amino acids, including cysteine, threonine, and methionine.

‡ Average of six determinations of separate preparations. Variability for any given amino acid did not exceed 10%, and replicates varied by <5%.

§ Average of two determinations of separate preparations.

‖ Traces of putative chlorotyrosine were noted.

The material that is solubilized by the hypochlorite washing of the "bag" preparation was also hydrolyzed and its amino acid composition determined; this is also shown in Table II. Of course, this analysis will be the result of hydrolysis of many different solubilized proteins, but the composition is strikingly different from the soluble spicule matrix. Though rich in glx and asx, basic amino acids are much less prevalent, glycine is low, and proline is high. The presence of hydroxyproline almost certainly represents blastocoel and basal lamina collagens (Crise-Benson and Benson, 1979; Wessel et al., 1984). A considerable amount of amino sugar, which has not been characterized, is also present in hydrolysates of this material. There is an indication that hypochlorite does convert some tyrosine to putative chlorotyrosine.

The soluble spicule matrix proteins have also been analyzed for the presence of carbohydrate. Total reducing sugar is ~4% of the amount of protein present, based on determination of protein by the Lowry colorimetric method. About one-fourth of the carbohydrate present is amino sugar, probably the amino forms of glucose, galactose, and mannose, based on the elution positions of peaks from columns used for amino acid analyses. Conventional chemical assays were done for the detection of uronic acid and sialic acid, and we could not detect the presence of these compounds. The sensitivity of the procedures were such that uronic acid and sialic acid would have had to be present at 1% or more of the amount of protein present. The electrophoretic mobility of spicule matrix proteins is not affected by digestion with neuraminidase (data not shown).

Soluble spicule matrix proteins were assayed for calcium binding activity by equilibrium dialysis using $^{45}CaCl_2$. Typical binding data are shown in Fig. 3. If one assumes that the average molecular weight of protein species binding calcium is 51,000 D, one may calculate that there are ~2.4 binding sites for calcium per mole of protein, and that the sites have

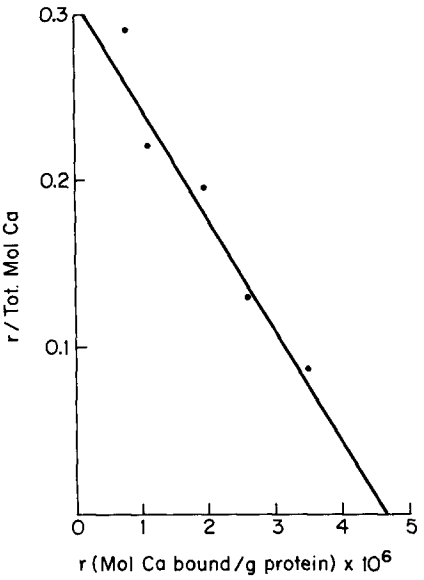

Figure 3. The binding of ^{45}Ca to spicule matrix protein. The Scatchard plot of Ca^{+2} binding to spicule matrix protein after equilibrium dialysis is shown. The protein concentration was 43 mg/ml. Calcium concentrations ranged from 4×10^{-4} to 2×10^{-5} M. The best fit line was drawn using linear regression analysis.

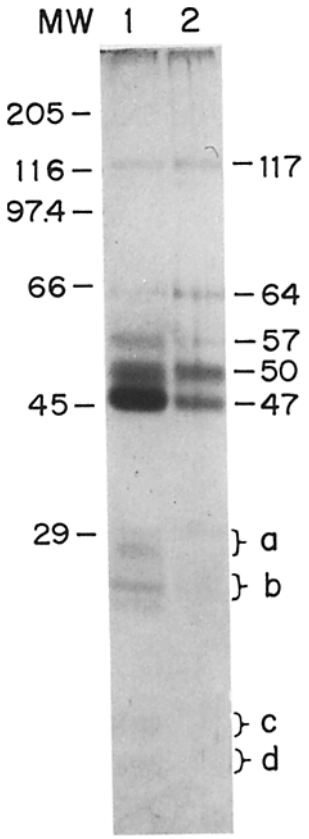

Figure 4. Silver staining of spicule matrix after electrophoresis. Two independent preparations (*1* and *2*) of spicule matrix protein were electrophoresed in 12.5% acrylamide gels that contained SDS. The gel was silver stained. Numbers and letters on the right margin are designations assigned to the different spicule matrix proteins. Numbers on the left margin indicate the positions of molecular weight markers.

an approximate binding constant (K_d) of 1.5×10^{-4} mol. We shall discuss below data that indicate there are several proteins present in the matrix, and which ones and how many of them bind calcium is not yet known.

The Components of the Matrix

Early attempts to visualize the number of components in the soluble matrix proteins were disappointing, primarily because of the difficulty in visualizing any bands after SDS polyacrylamide gel electrophoresis. Conventional staining methods do not stain acidic proteins well, and Coomassie Blue does not show any bands in a gel. We have found the silver staining method of Morrissey (1981) particularly well suited for visualizing these proteins. Fig. 4 shows the fractionation of two different spicule matrix preparations on SDS-containing acrylamide gels. This gel was somewhat overstained to help visualize minor components. The principle bands are labeled by designation with the apparent molecular weights. Four of the components shown here, labeled 64, 57, 50 and 47, comprise stainable bands that have been observed in dozens of preparations. A distinct component at 117 kD, while clear, is sometimes missing from some preparations. Components labeled 29, 25, and low molecular weight components labeled *a* and *b*, are only visualized after extensive overstaining and are present in very low amounts, estimated at <2% of the total based on densitometry. These latter minor components often show multiple bands or are visualized as a smear. Densitometric scans of gels shown in Fig. 4 indicate that the four predominant components (47, 50, 57, and 64) constitute from 71 to 82% of the total silver staining material. The relative proportions of the different bands differ somewhat among different preparations, as seen in the figure, and we

do not yet understand the reason for this. All bands are eliminated by pronase treatment before electrophoresis. None of the bands described above stain with the usual acidic dyes like Coomassie Blue, and all are presumably acidic.

Embryos have been labeled with ^{3}H-algal hydrolysate or [^{35}S]methionine, the spicule matrix proteins isolated and fractionated on gels, and then the radioactivity visualized by autoradiography or fluorography. If silver staining and fluorography are done on the same preparation, the visualized bands are congruent, except the 64-kD component is very poorly labeled with methionine and the low molecular weight components are more evident in fluorograms. Components 47, 50, and 57 represent 70% of the total label on the gel. Fig. 5*A* shows an example of such a fluorograph. Dots show bands of radioactivity that also showed bands at the identical location on the silver stained gel of this preparation.

The components of the spicule matrix may also be visualized by immunoblotting procedures. A polyspecific, polyclonal antibody was raised in rabbits against soluble spicule matrix proteins; immunoblotting of the proteins separated on SDS acrylamide gels followed by visualization using horseradish peroxidase–porcine anti–rabbit IgG is shown in Fig. 5*B*. This preparation is identical to the one used for fluorography shown in Fig. 5*A*, and it is apparent that the same bands that label with methionine are stained by the antibody, with two exceptions. The 64-kD band that labels poorly with methionine is visualized by the antibody. Also, there is a component at ~114 kD demonstrated by the antibody that is seen neither with fluorography nor silver staining, and about which we have no further information. It has only been observed in a few preparations. Application of these same immunochemical procedures to blotted proteins using preimmune IgG were completely negative. With the exception of the methionine-deficient band at 64 kD, the major (57, 50, and 47 kD) and minor (117, 29, 25, *a* and *b*) components of the spicule matrix are demonstrable with silver staining, radioactivity, and antigenicity. Several of the identified minor components are certainly composed of multiple components, and the component 57 and component 47 sometimes appear as doublets. Thus, the four major components and numerous minor components represent a minimum estimate of the complexity of the soluble spicule matrix.

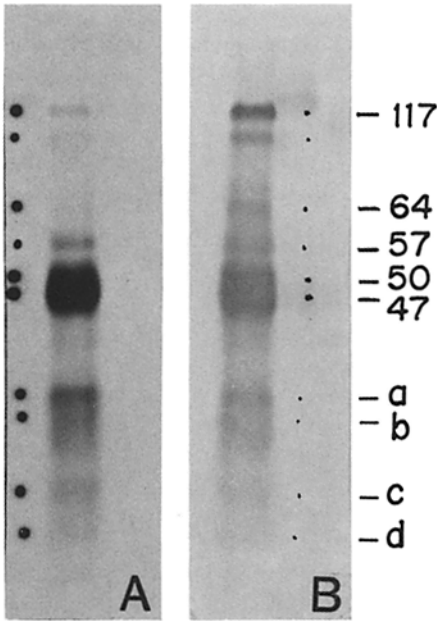

Figure 5. Immunochemical and radiochemical visualization of spicule matrix. A preparation of spicule matrix protein labeled in vivo with [^{35}S]methionine was fractionated by SDS polyacrylamide gel electrophoresis, electroblotted to nitrocellulose, and visualized with antibody against spicule matrix proteins as described in Materials and Methods. The immunostained nitrocellulose strip was then autoradiographed. *A* shows the autoradiogram of the radioactive protein, and *B* shows the staining from the horseradish peroxidase–swine anti–rabbit IgG. Dots of radioactive ink were used to align and compare bands of the two images, and the visible bands are congruent.

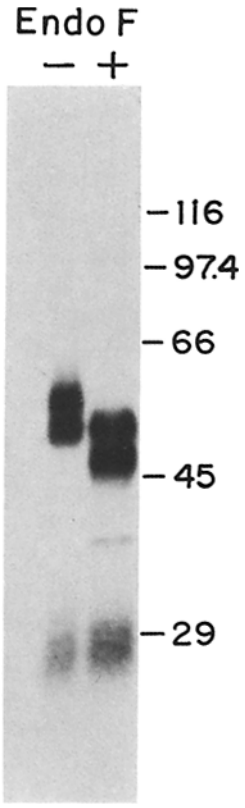

Figure 6. Endoglycosidase treatment of spicule matrix protein. A preparation of [^{35}S]methionine-labeled spicule matrix protein was digested with endoglycosidase F, as described in Materials and Methods, and then subjected to electrophoresis on SDS that contained acrylamide gels and fluorographed. Molecular weight markers are indicated on the right.

Specificity of the polyclonal IgG preparation was determined by light and electron immunochemical techniques. Spicule matrix components react specifically when observed by biotin–avidin immunoperoxidase technique. With this procedure intense staining is observed in the spicule matrix, which was partially demineralized during sample preparation. The blastocoel cavity and wall show a faint reaction, which is also seen with preimmune IgG and presumably represents nonspecific binding of IgG molecules. The antigens seem localized to the products of primary mesenchyme cells. To further confirm the specificity of the antibody preparation and to gain some insight into the subcellular localization of the matrix protein, some specimens were prepared for electron microscopy and stained with rabbit antibody IgG and goat anti–rabbit IgG coupled to colloidal gold. There is a significant deposition of colloidal gold over the demineralized spicule matrix and this is not seen in other cells of the embryo. Only background levels of labeling are seen with preimmune IgG. Photographs of these cytological results are available upon request.

Treatment of the soluble matrix proteins with endoglycosidase F, a wide spectrum enzyme that can hydrolyze N-glycosidic bonds, including those that contain high mannose and complex oligosaccharides, induces a pronounced electrophoretic shift in several of the components. Fig. 6 shows an example of this treatment on a ^{35}S-labeled spicule matrix preparation. It is clear in this example that components designated 117, 57, 50, and 47 migrate more rapidly after endo F treatment, and in each case there is an approximate reduction of ~4 kD in apparent molecular weight. Similar experiments on five different preparations indicate that components 117, 57, 50, 47, and 29 contain complex carbohydrate. Component 64 does not. We have not yet determined the status of components 25, *a* and *b*. Similar results were obtained when the components were visualized with immunoblotting. Mild alkaline hydrolysis of spicule matrix protein under conditions where O-linked oligosaccharides are sensitive to hydrolysis fails to alter the mobility of the proteins on SDS-containing acrylamide gels (data not shown). Treatment of the same spicule matrix preparation with endoglycosidase H, an enzyme that only hydrolyzes N-glycosidic bonds of the high mannose type, fails to alter the mobility of the components affected by endo F (data not shown). This indicates that several of the soluble spicule matrix proteins, including three of the four major ones, are probably N-glycosylated with oligosaccharides of the complex type.

The Time and Place of Spicule Matrix Accumulation

Two approaches were used to detect the time of synthesis and accumulation of the matrix proteins. The first approach takes advantage of the fact that ^{45}Ca, insoluble in neutral or mildly alkaline aqueous conditions, is solely associated with the spicule (Nakano et al., 1963; Mintz et al., 1981). Hence continuous labeling of embryos with ^{45}Ca may be used to normalize the recovery of amino acid in the spicule. Embryos were therefore continuously labeled with ^{45}Ca and pulsed with [^{3}H]leucine at various times during development. After the labeling, the embryos were washed and cultured in seawater that contained nonradioactive leucine under conditions known to be an effective amino acid chase (Allen and Wilt, 1975). All experiments were terminated after 92 h when well-developed spicules were present. The spicules were then prepared and the ratio of ^{3}H to ^{45}Ca determined. This experimental design allows one to determine the time of synthesis of matrix even though it may be assembled later. Individual matrix components are, of course, not distinguished and we can refer only to an average behavior of the main bulk of the matrix. The experiment was repeated four times with different ^{3}H and ^{45}Ca inputs and the results always showed that exposure to leucine before the mid-late gastrula stage resulted in little labeling of the spicule matrix (Table III). On the other hand, labeling during prism and pluteus stages produced extensive labeling. This experiment indicates that the greater part of the matrix is probably synthesized concomitantly with the actual magnesian calcite deposition.

A second approach is to use the polyclonal antibody against the soluble spicule matrix proteins as an immunocytochemical tool to determine the time and place of antigen accumulation. The immunoglobulins react specifically with the spicule matrix of 72-h larval mesenchyme cells, as mentioned earlier. We do not observe localized immunoperoxidase or fluorescent antibody signals when rudimentary spicules are not present. Once again, the localization and accumulation of the predominant spicule antigens seem to coincide approximately with the actual deposition of the skeletal element.

Table III. The Timing of Spicule Matrix Synthesis

Stage of ^{3}H-amino acid labeling	Normalized $^{3}H/^{45}Ca$ ratios	
	Exp. 1	Exp. 2
Fertilization to hatching (1–20 h)	0.175	0.037
Hatching to gastrula (20–44 h)	0.11	0.05
Prism (44–68 h)	1.0	1.0
Pluteus (68–92 h)	0.31	0.40

Embryos were continuously cultured in seawater at 15°C containing $^{45}CaCl_2$, and exposed transiently at the indicated times to [^{3}H]leucine, after which the embryos were cultured without radioactive leucine until they reached the mature pluteus stage. Thus, embryos were labeled with amino acid at different times, but were all allowed to develop to the same stage. The spicules were then isolated and purified, and aliquots counted to determine the isotope ratio. The absolute level of counts obtained depend on the input of isotope, the recovery of spicules, and their purity. The two experiments here had very different absolute levels of incorporation ($^{3}H/^{45}Ca$ = 0.04 in exp. 1 and 3.74 in exp. 2 in the original homogenate), and so the ratios obtained were normalized by setting the ratio obtained at the prism stage 1.0. Two other experiments of similar design gave equivalent results.

Discussion

The formation of calcareous spicules by primary mesenchyme cell of sea urchin embryos has been studied for many decades by embryologists because it is the end product of an interesting lineage of cells that derive from the micromeres. The spicules have been used as an index of differentiation, determination, and pattern formation. We have presented here novel information on their constitution and composition that opens the way for a molecular study of the modes of determination and differentiation in this lineage of cells of the echinoderm embryo.

The purification scheme, which has antecedents in the pioneering work of Okazaki (1960), has now been used many times with embryos of both *S. purpuratus* and *L. pictus*, and it is rapid and efficacious. Based on morphological criteria, there is little if any contamination of the product with basal lamina, cells, or other debris. If the spicules are demineralized, a mixture of several proteins of unusual composition is obtained. Our estimate of the amount of matrix, based on recovery of protein, suggests that at least 0.01% of the total embryo protein is present in the matrix. The recovery of amino acid, as shown in Table I, also suggests that ~0.01% of the total amino acid label in protein is associated with the spicule matrix. Since the cells that elaborate the spicule are enclosed within a continuous epithelium formed by the blastocoel wall, one might expect that the primary mesenchyme cells would label poorly, and hence, recovery of radioactive amino acid would only provide a minimal estimate of the amount of new protein (cf. Harkey and Whitely, 1983). The spicule itself is a prominent object in the embryo, but most of it is mineral, and only ~0.1% of it is composed of the organic matrix. However, since mesenchyme cells probably contain only 1–2% of the total protein in the embryo (there are ~32 mesenchyme cells in the 600-cell gastrula, and the cells are all of similar size), a group of proteins that is 0.01% of the total embryo protein may be rather abundant if restricted to the mesenchyme cells (constituting >1% of the

protein synthesized by primary mesenchyme). The results of the immunocytochemistry, which show these antigens localized to the spicule matrix within mesenchyme cells, is consistent with the idea that these proteins may represent a significant biosynthetic commitment by these cells.

The properties of the soluble matrix proteins are unlike those previously described by others. Pucci-Minafra et al. (1972) have described the organic component of the spicule as a collagen-containing matrix. The basis for this was the presence of [^{3}H]hydroxyproline in spicules isolated from embryos isolated by pulse label with [^{3}H]proline. We have consistently failed to detect hydroxyproline in amino acid analysis of these proteins prepared from clean, purified spicules. In addition, Mintz et al. (1981) failed to detect the presence of purified collagenase sensitive peptides within spicule-producing mesenchyme cells. We feel the presence of collagen in spicule preparations of Pucci-Minafra derives from the impure condition of the spicules produced by their method. Electron microscopy of spicules (Fig. 2) prepared by their method reveals amorphous and fibrillar material, no doubt derived from basal lamina and blastocoelic collagen shown to be present at the time spicules are prominent (Crise-Benson and Benson, 1979; Wessel et al., 1984; Solursh and Katow, 1982). The matrix is composed of acidic glycoproteins with amino acid composition similar to the proteins found in marine invertebrate mineralized structures (Weiner et al., 1983). It should be noted that the properties reported here are averages. There seem to be four predominant proteins with apparent molecular weights, when glycosylated, of 49, 50, 57, 64 kD, and some of these may be composed of more than one protein. Conditions for a two-dimensional gel resolution of this mixture are now being sought. The sensitivity of these proteins to digestion with endoglycosidase F and insensitivity to endoglycosidase H or mild alkaline hydrolysis allow the conclusion that the carbohydrate moiety of the spicule proteins if of the N-linked complex type. While the significance of the glycosylation is unknown, it is consistent with an observation by Schneider et al. (1978) that treatment of gastrulating embryos with tunicamycin, an inhibitor of N-linked glycosylation, prevents the formation of spicules by primary mesenchyme cells. The presence of carbohydrate has also been detected associated with the matrix protein(s) from other invertebrate calcified tissue (Kingsley and Watabe, 1983).

The calcium binding activity of the spicule matrix proteins is similar to that observed for soluble matrix extracted with EDTA from invertebrate calcium carbonate–containing structures (e.g., Sikes and Wheeler, 1983). In various studies the dissociation constants have ranged from 10^{-3} to 10^{-5} M. While some authors have termed 10^{-5} M a "high affinity" constant, it is a much lower affinity than dissociation constants for vertebrate intracellular calcium binding proteins, which have values in the range of 10^{-6}–10^{-7} M (Williams, 1980). We do not know which of the several proteins of the spicule matrix is involved in calcium binding. Several authors have attributed calcium binding activity to the acidic amino acid residues of the calcified organic matrix (Weiner, 1979; 1983), whereas others have proposed other potential anionic sites, such as phosphate or sulfate groups (Lee et al., 1977; Sikes and Wheeler, 1983). It is interesting to note that the low capacity of binding by the matrix suggests that the binding may involve multiple coordination complexes between matrix ligands and calcium ions (Williams, 1980). The role of such low affinity, low capacity calcium binding by matrix proteins is unclear since it is somewhat inconsistent with matrices acting as crystal nucleators. High capacity and low affinity binding is implicit in expitaxial binding mechanism in which calcium binding occurs on matrix with spacing that approximates the calcium–calcium distances in the calcium carbonate lattice (Weiner and Traub, 1980; Weiner and Hood, 1975). A resolution of this question may require the purification of the individual matrix components.

The available evidence indicates that much of the glycoprotein matrix is synthesized and assembled as the calcareous spicule is being formed in the intracellular vacuole of the syncytial mesenchyme cells. The evidence does not rule out the possibility that small amounts of some components are formed much earlier, even during oogenesis, nor is the precise timing of synthesis of the several different matrix proteins yet known.

We expect a continued exploration of these questions will be of interest for a molecular study of the determination and differentiation of a particular lineage during sea urchin development. The polyclonal antibody described here has been used to isolate a cDNA that encodes one of the principal matrix proteins, and characterization of this cDNA, its cognate gene, mRNA, and product, is now underway (Sucov, H., S. Benson, J. Robinson, F. Wilt, R. Britten, and E. Davidson, manuscript in preparation). Furthermore, continued characterization of spicule matrix glycoproteins may offer opportunities for the study of the molecular basis of biomineralization in developing embryos.

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