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Isolation and Purification of Three Egg-Membrane Lysins from Sperm of the Marine Invertebrate *Megathura crenulata* (Giant Keyhole Limpet)†

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ABSTRACT: Three enzymes which lyse the egg-membrane of the marine invertebrate *Megathura crenulata* were obtained from the sperm of a single individual as well as from pooled samples of sperm from several males of the same species. The three enzymes ("lysins") were purified to homogeneity by a combination of gel filtration and ion-exchange chromatography. The three egg-membrane lysins were different as judged by chromatographic behavior, gel electrophoresis in the presence of sodium dodecyl sulfate, amino acid composition, isoelectric point, and circular dichroism spectra. The three egg-membrane lysins had molecular weights of 57,250 (±572), 53,688 (±1387), and 58,000 (±580) as determined by sedimentation equilibrium in the presence of 6 M guanidine–HCl. Similar experiments carried out in the absence of guanidine–HCl yielded similar molecular weight values, showing that all three enzymes exist as monomeric species.

Egg-membrane lysins are enzymes, found in the sperm extracts of marine invertebrates and other animals, that are capable of dissolving the egg vitelline membrane[1] (Dan, 1967). Tyler (1939) was able to show the existence of an egg-membrane lysin in the sperm extract of the giant keyhole limpet *Megathura crenulata* and suggested that such an enzyme might facilitate the penetration of the spermatozoon through the vitelline membrane into the egg. Further studies on the *Megathura crenulata* egg-membrane lysin were done by Krauss (1950). Recently, an egg-membrane lysin having a molecular weight of 8800 has been isolated from a sperm extract of *Tegula pfeifferi* (Haino, 1971). A similar enzyme, reported to be located in the acrosomes of mammalian spermatozoa, has been isolated (Stambaugh and Buckley, 1972; Zaneveld *et al.*, 1972) and it is thought that this enzyme dissolves a small tunnel in the zona pellucida through which the spermatozoon can penetrate the egg (Stambaugh and Buckley, 1972).

Further knowledge of such lysins could be helpful in understanding the fundamentals of fertilization. Egg-membrane lysins may serve as tools in elucidating the structure and function of the vitelline membrane, in understanding species specificity, enhancing fertilization, or in developing fertilization inhibitors. This study describes the isolation, purification, and some properties of three egg-membrane lysins from sperm extracts of *Megathura crenulata*.

Experimental Section

Materials. Giant keyhole limpets (*Megathura crenulata*) were purchased from Pacific Bio-Marine, Venice, Calif. Sepharose 6-B, Sephadex G-75, and Sephadex QAE-50 were purchased from Pharmacia; cellulose phosphate was obtained from Sigma; hydroxylapatite HTP and Bio-Gel P-150 were purchased from Bio-Rad Laboratories.

Methods. Isolation of Sperm. Testes of dissected limpets were suspended in 0.01 M Tris-HCl (pH 8.3) containing 0.5 M NaCl allowing the sperm to shed into the buffer solution. The mixture was passed through cheesecloth and the sperm suspension that passed through was centrifuged at 5000 rpm in a Sorvell-SS 34 rotor for 10 min. The supernatant was discarded and the pellet containing the sperm was washed twice with the Tris buffer.

Isolation of Egg-Membranes. Ovaries of dissected limpets were suspended in 0.005 M glycine–NaOH (pH 8.55) containing 0.5 M NaCl and stirred gently with a magnetic stirrer at 4° overnight. The suspension was then passed through cheesecloth and centrifuged at 9000 rpm in a Sorvall GS-3 rotor for 40 min. The pellet, containing the jellyless eggs, was resuspended in the glycine–NaOH buffer. The eggs were then homogenized in a Teflon homogenizer (Thomas, Phil.) causing the egg cytoplasm to escape from the sack-like membrane. The homogenization was followed by inspecting samples under the light microscope. The suspension was then passed through a nylon screen cloth, Nitex 30 (Kressilk Products, Inc.); all the cell debris except the egg-membrane passed through. After thorough washing with 0.005 M glycine–NaOH containing 0.5 M NaCl, the membranes were collected from the screen and stored in the glycine–NaOH buffer at 4°. The membranes seemed homogeneous as judged by light

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[1] The term egg-membrane does not imply any definite known structure such as a lipid bilayer. Other names which were used to describe the same structure are vitelline membrane, vitelline coat, or vitelline envelope. Since the actual molecular structure of this morphological entity is not known at present, we prefer to use the name which has been in longest use, namely egg-membrane.

microscopy. A few drops of toluene were added as an antibacterial agent.

Enzyme Assay. The assay is based on the dissolution of the egg-membrane by the lysins, *i.e.*, the decrease in turbidity at 640 nm of an egg-membrane suspension following the addition of the lysin. Egg-membranes suspended in 0.005 M glycine–NaOH (pH 8.55) (17°) containing 0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M $CaCl_2$, and 0.01 M KCl were sonicated in a Branson Model S75 sonifier setting 6 for 45 sec and adjusted to an absorbance between 0.6 and 0.7 at 640 nm. The assay was performed in a Gilford 240 spectrophotometer whose measuring chamber was maintained at 17 ± 1°. A 10-mm light path cuvette was used. The sonicated egg-membrane suspension, 0.5 ml, was equilibrated at 17° and transferred to the cooled cuvette in the spectrophotometer. The reaction was started by adding 20 μl of a suitably diluted enzyme solution. Readings were made every 15 sec for 3–4 min at 640 nm using a digital absorbance meter and data lister (Gilford Instruments). One unit of enzyme activity is defined as that amount of enzyme which will cause a decrease of 0.01 absorbance units in an egg-membrane suspension in 1 min at pH 8.55 at 17° in a 0.5-ml reaction mixture. The specific activity is expressed in units per milligram of protein.

Protein was determined according to Lowry *et al.* (1951) with crystalline bovine serum albumin as a standard.

Amino Acid Analysis. Samples (approximately 0.5 mg) were lyophilized and hydrolyzed under reduced pressure at 110° for 24, 48, and 72 hr in 3 N *p*-toluenesulfonic acid containing 0.2% indole (Liu and Chang, 1971). The hydrolysate was then analyzed according to Spackman *et al.* (1958) on a Beckman Model 120B amino acid analyzer. Cysteine and methionine were determined after performic acid oxidation.

Conductivity measurements were done in a Radiometer CDM 3 conductivity meter equipped with a CDA 100 manual temperature compensator. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Fairbanks *et al.* (1971). Electrofocusing was performed in a 110-ml LKB 8101 column. The procedure was that described in the LKB manual. The 3-10 ampholine solution was also supplied by LKB Produkter, Stockholm.

Circular dichroism (CD) spectra were recorded on a modified Beckman CD spectrophotometer coupled to a Hewlett-Packard 5480 A signal analyzer. Low noise CD spectra were obtained by averaging 64 scans. The CD intensity was calibrated with an aqueous solution of camphorsulfonic-*d-10* acid, $\Delta\epsilon = 2.2\ M^{-1}\ cm^{-1}$ at 290 nm where $\Delta\epsilon$ is the molar absorptivity for left circulary polarized light minus that for right circulary polarized light (Cassim and Yang, 1969).

Sedimentation Equilibrium. Weight average molecular weights and homogeneity were determined in the analytical ultracentrifuge by the meniscus depletion method of Yphantis (1964) with the use of a multichannel, double-sector interference cell. Centrifugation was carried out at 10° in a Spinco Model E centrifuge. Each lysin was dialyzed against 0.01 M phosphate buffer containing 0.4 M NaCl at pH 7.0, and molecular weight evaluations were performed at three enzyme concentrations (0.4, 0.6, and 0.8 mg/ml) in the three double-channeled centerpiece at 20,000 rpm. The same enzyme samples were dialyzed for 48 hr against 6 M guanidine–HCl containing 0.5% β-mercaptoethanol. The material was again run at the same three concentrations at 30,000 rpm. The interference patterns were recorded on Kodak metallographic plates with an exposure of 35 sec and the plates were read with the aid of a Gaertner microcomparator. Plates were analyzed at 24 and 48 hr to ensure that equilibrium was complete. After correction for the density of the solvent, refractive index was used as a measure of protein concentrations. The apparent molecular weights of the proteins at different concentrations at equilibrium were calculated from the log C *vs.* X^2 plots by the method of Yphantis (1964). The partial specific volume $\bar{V}$ was calculated from the amino acid composition (Cohn and Edsall, 1943).

TABLE I: Purification of Egg-Membrane Lysins A, B, and C.

Purification Step	Volume (ml)	Total protein (mg)	Total Activity (units × 10^{-5})	Sp Act. (units/mg × 10^{-3})
I Cell-free extract	1700	1595	158.6	9.9
II Ammonium sulfate precipitate 0–80% saturation	208	1040	114.0	11.0
III Sepharose 6-B	445	610	100.0	16.4
Egg-Membrane Lysin A				
(I–III same as above)				
IV Hydroxylapatite fraction I	196	174	8.2	4.7
V QAE Sephadex egg-membrane lysin A	110	82	6.3	7.7
Egg-Membrane Lysin B				
(I–III same as above)				
IV Hydroxylapatite fraction II	265	234	57.5	24.5
V First cellulose phosphate fraction I	305	101	47.0	46.5
VI Second cellulose phosphate egg-membrane lysin B	226	75	32.0	42.6
Egg-Membrane Lysin C				
(I–III same as above, IV as for lysin B)				
V First cellulose phosphate fraction II	115	29	5.4	18.6
VI Second cellulose phosphate egg-membrane lysin C	142	11	2.1	19.1

Results

Purification of Egg-Membrane Lysins A, B, and C. The purification steps are summarized in Figure 1 and Table I. It was found that the same three enzymes can be obtained from either isolated sperm (see Methods) or from whole testes.

Dissected testes were stored at −20° until use. All subsequent operations were carried out at 4°. Frozen testes (31.6 g) from one individual were thawed and suspended in 700 ml of 0.01 M Tris-HCl (pH 8.3) containing 0.5 M NaCl and left stirring for 12 hr. The suspension was then centrifuged using a GS-3 rotor in a Sorvall RC2-B at 9000 rpm. The pellet was resuspended in 1000 ml of 0.01 M Tris-HCl buffer containing

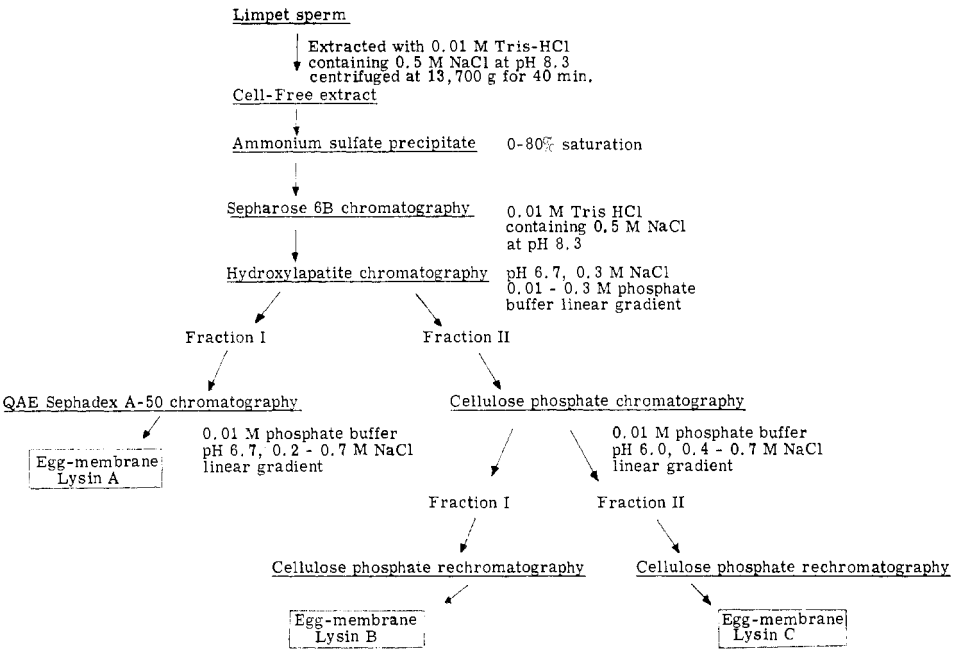

FIGURE 1: Schematic presentation of the purification steps of egg-membrane lysins A, B, and C.

0.5 M NaCl and stirred for another 12 hr. This method of extraction was applied also when isolated sperm was the starting material. After centrifugation the extracts were combined and 950 g of solid ammonium sulfate was added to 80% saturation. The ammonium sulfate solution was stirred for 24 hr and the precipitate collected by centrifugation in the GS-3 rotor at 9000 rpm for 40 min. The pellet was suspended in 170 ml of 0.01 M Tris-HCl (pH 8.3) containing 0.5 M NaCl and dialyzed against the same buffer with three buffer changes.

Sepharose 6-B Column Chromatography. The dialyzed enzyme solution (208 ml) was concentrated by ultrafiltration on a UM-2 Amicon membrane to 68 ml and applied to a 5 × 80 cm column of Sepharose 6-B equilibrated with 0.01 M Tris-HCl (pH 8.3) containing 0.5 M NaCl. Elution was carried out with the starting buffer. The elution profile (Figure 2) shows one lytic activity peak.

Hydroxylapatite Column Chromatography. Egg-membrane lysin fractions were pooled (fractions 50–75, Figure 1), dialyzed against 0.01 M potassium phosphate (pH 6.7) containing 0.3 M NaCl, and loaded on a 2.5 × 40 cm hydroxylapatite column equilibrated with 0.01 M potassium phosphate (pH 6.7) containing 0.3 M NaCl. A linear gradient of 0.01 M potassium phosphate (pH 6.7) containing 0.3 M NaCl to 0.3 M potassium phosphate (pH 6.7), 0.3 M NaCl in a total volume of 1400 ml was applied to elute the proteins. Two lytic activity peaks (I, II) emerged, differing in their specific activity (Figure 3).

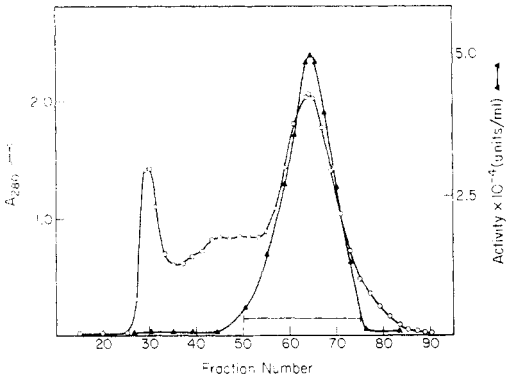

FIGURE 2: Chromatography of egg-membrane lysin A, B, and C on Sepharose 6-B at pH 8.3. The column (5 × 79 cm) was equilibrated with 0.01 M Tris-HCl buffer containing 0.5 M NaCl. After applying the protein sample (68 ml) the column was developed with the starting buffer. The fraction volume was 20 ml and the flow rate was 45 ml/hr. Solid bar denotes pooled fractions for further purification.

QAE Sephadex A-50 Column Chromatography, Egg-Membrane Lysin A. Fraction I of the hydroxylapatite column (fractions 47–73, Figure 3) was pooled, dialyzed against 0.01 M potassium phosphate (pH 6.7) containing 0.2 M NaCl, and loaded on a 1.5 × 25 cm Sephadex QAE A-50 column equilibrated with the same buffer. The column was washed with the starting buffer until all unadsorbed protein was removed and developed with a linear gradient of 0.01 M potassium phosphate (pH 6.7) containing 0.2 M NaCl to 0.01 M potassium phosphate (pH 6.7) containing 0.7 M NaCl in a total volume of 800 ml. The lytic activity emerged as a single peak (Figure 4). This peak is designated as egg-membrane lysin A.

Cellulose Phosphate Column Chromatography, Egg-Membrane Lysins B and C. Fraction II of the hydroxylapatite step (fractions 74–110, Figure 3) was pooled and dialyzed against 0.01 M potassium phosphate (pH 6.0) containing 0.4 M NaCl. The protein was loaded on a 1.5 × 26 cm cellulose phosphate column which was recycled twice with 0.5 M NaOH and 0.5 M HCl and equilibrated with 0.01 M potassium phosphate (pH 6.0) containing 0.4 M NaCl. A linear gradient of 0.01 M potassium phsophate (pH 6.0) containing 0.4 M NaCl to 0.01 M potassium phosphate (pH 6.0) containing 0.7 M NaCl in a total volume of 1000 ml was applied. Two lytic activity peaks emerged from the column (Figure 5a). Peak I is designated as egg-membrane lysine B and peak II as egg-membrane lysin C.

Second Cellulose Phosphate Column Chromatography, Egg-Membrane Lysin B. Fraction I (fractions 40–77, Figure 5a) was dialyzed against 0.01 M potassium phosphate (pH 6.0) containing 0.4 M NaCl and loaded on a 1.5 × 25 cm cellulose phosphate column equilibrated with the same buffer. The same elution conditions as described for the first cellulose phosphate

TABLE II: Summary of Molecular Weight Estimations.

Method	Egg-Membrane Lysin A	Egg-Membrane Lysin B	Egg-Membrane Lysin C
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	42,000	53,000	49,000
Sedimentation equilibrium	59,743 ± 2,787[a]	61,330 ± 1,863[a]	61,325 ± 826[a]
Sedimentation equilibrium in the presence of 6 M guanidine–HCl containing 0.5% β-mercaptoethanol	57,250 ± 572[a]	53,688 ± 1,387[a]	58,000 ± 580[a]
Sephadex-G-75 chromatography	23,000	18,000	30,000
Bio-Gel P-150 chromatography	42,000	30,500	29,500

[a] Average of six determinations at different protein concentrations and different times of centrifugation. Other details are given in Methods.

chromatography were applied. A single lytic activity peak emerged (Figure 5b).

Second Cellulose Phosphate Column Chromatography, Egg-Membrane Lysin C. Fraction II (fractions 83–96, Figure 5a) was dialyzed against 0.01 M potassium phosphate (pH 6.0) containing 0.4 M NaCl and loaded on a 1.5 × 26 cm cellulose phosphate column equilibrated with the same buffer. The same elution procedures as described for the first cellulose phosphate chromatography were applied. A single activity peak emerged from the column (Figure 5c).

The isolation procedure described for the purification of the three egg-membrane lysins from sperm extracts of *Megathura crenulata* was reproducible with regard to ratio, yield, and purity. The same results were achieved whether the sperm of only one individual animal was used (as described in this paper) or a pool from a number of animals. The same results were also achieved whether isolated sperm or whole testes were extracted.

Physicochemical Characterization of the Egg-Membrane Lysins. Sodium Dodecyl Sulfate Gel Electrophoresis. Egg-membrane lysins A, B, and C were subjected to polyacrylamide electrophoresis in the presence of 1% sodium dodecyl sulfate as further evidence for their purity and for the determination of the molecular weights. A single protein band was seen for each of the egg-membrane lysins (Figure 6) corresponding to molecular weights of 42,000, 53,000, and 49,000, respectively.

Sedimentation Equilibrium. The molecular weights of egg-membrane lysins A, B, and C as determined by sedimentation equilibrium in the absence and presence of 6 M guanidine–HCl are summarized in Table II. These results suggest that all three enzymes exist as monomeric species.

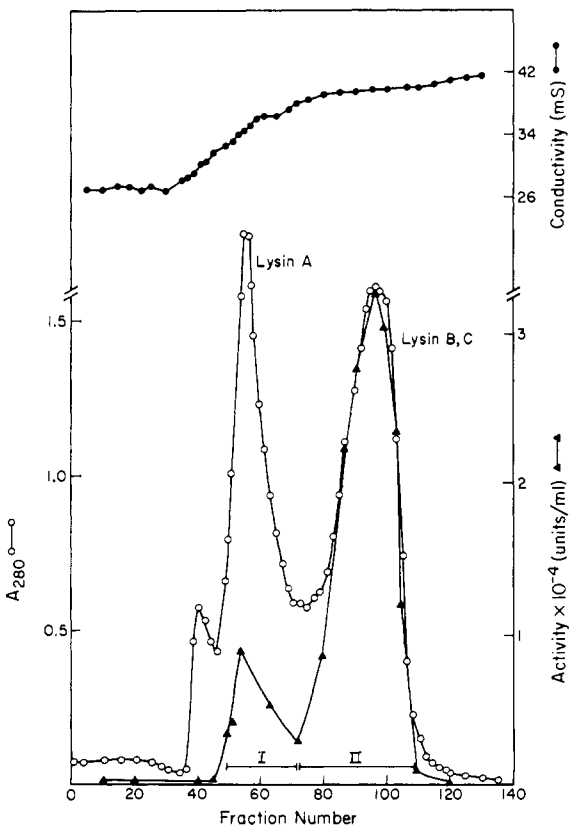

FIGURE 3: Chromatography of egg-membrane lysin A, B, and C on hydroxylapatite at pH 6.7. The column (2.5 × 40 cm) was equilibrated with 0.01 M potassium phosphate buffer containing 0.3 M NaCl. The fraction volume was 7.5 ml and the flow rate rate 31 ml/hr. Solid bar denotes pooled fractions for further purification: fraction I contains lysin A activity; fraction II contains lysins B and C activities.

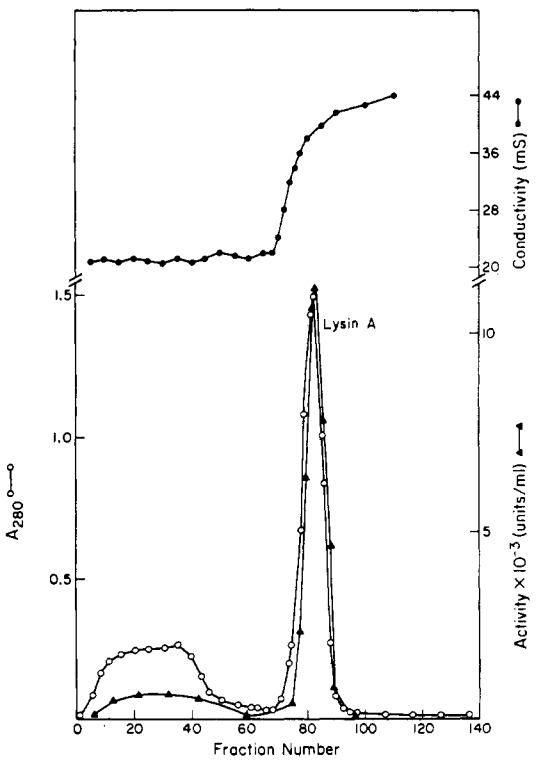

FIGURE 4: Chromatography of egg-membrane lysin A on Sephadex QAE A-50 at pH 6.7. The column (1.5 × 25 cm) was equilibrated with 0.01 M potassium phosphate buffer containing 0.2 M NaCl. The fraction volume was 8.5 ml and the flow rate 25 ml/hr.

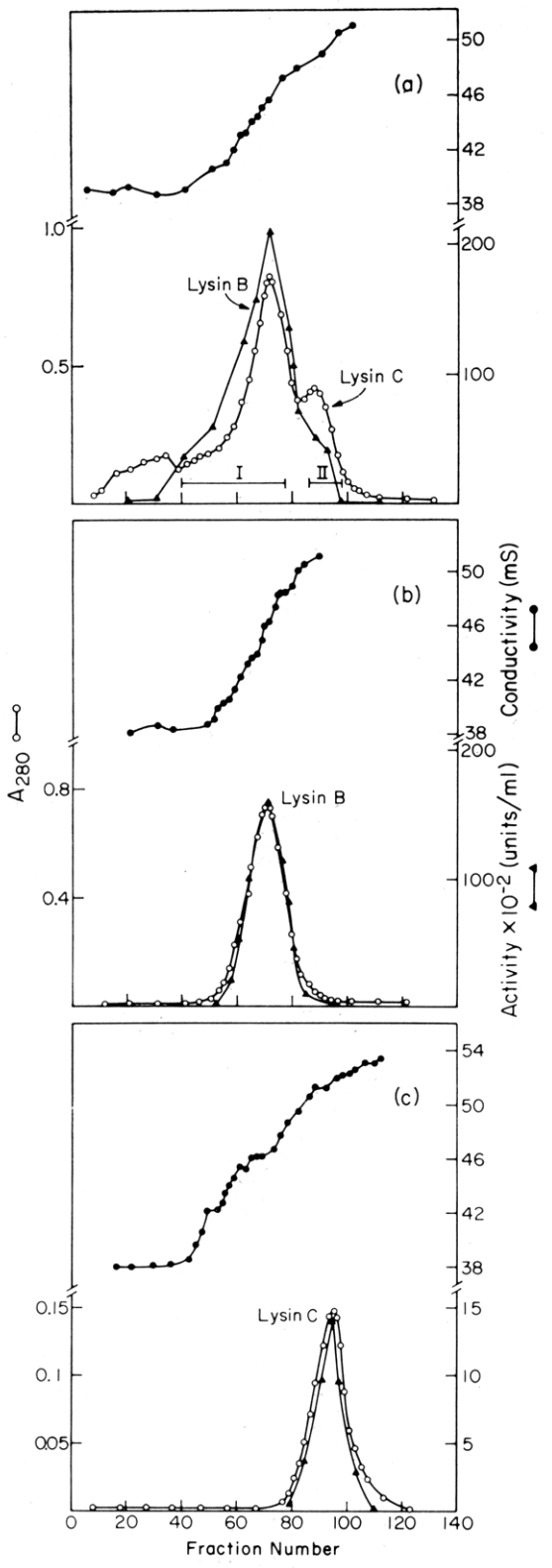

FIGURE 5: (a) Chromatography of egg-membrane lysin B and C on cellulose phosphate at pH 6.0. The column (1.5 × 26 cm) was equilibrated with 0.01 M potassium phosphate buffer containing 0.4 M NaCl. The fraction volume was 8.5 ml and the flow rate 35 ml/hr. Solid bar denotes pooled fraction for further purification. Fraction I contains lysin B activity and fraction II contains lysin C activity. (b) Rechromatography of egg-membrane lysin B on cellulose phosphate at pH 6.0. The column (1.5 × 25 cm) was equilibrated with 0.01 M potassium phosphate containing 0.4 M NaCl. The fraction volume was 8.5 ml and the flow rate was 35 ml/hr. (c) Rechromatography of egg-membrane lysin C on cellulose phosphate at pH 6.0. The column (1.5 × 27 cm) was equilibrated with 0.01 M potassium phosphate containing 0.4 M NaCl. The fraction volume was 8.5 ml and the flow rate 30 ml/hr.

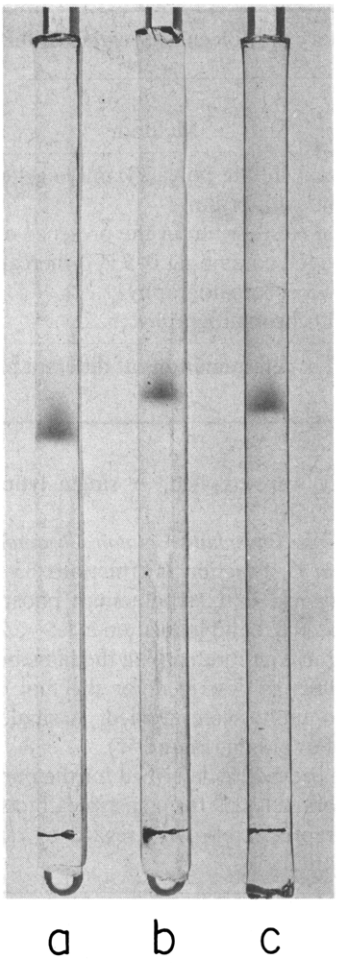

FIGURE 6: Sodium dodecyl sulfate gel electrophoresis of 40 μg of egg-membrane lysin A (a), 30 μg of egg-membrane lysin B (b), and 25 μg of egg-membrane lysin C (c).

Figure 7 represents three typical plots derived from these determinations. The results indicated homogeneity of the preparations.

Molecular Weight Estimation on Sephadex G-75. A (2.5 × 86 cm) column equilibrated with 0.01 M potassium phosphate buffer (pH 6.7) containing 0.3 M NaCl was calibrated with standard proteins. Calculation of the apparent molecular weight from the elution volumes gave 23,000, 18,000, and 30,000 for egg-membrane lysins A, B, and C, respectively.

Molecular Weight Estimation on Bio-Gel P-150. A (1.5 × 95 cm) column equilibrated with 0.01 M potassium phosphate buffer (pH 6.7) containing 0.3 M NaCl was calibrated with standard proteins. The apparent molecular weights are 42,000, 30,500, and 29,500 for egg-membrane lysins A, B, and C, respectively. The molecular weight estimation of the three lysins are summarized in Table II.

Circular dichroism spectra of egg-membrane lysins A, B, and C in the region of 200–300 nm are given in Figure 8. When the different concentrations of egg-membrane lysin A, B, and C which were used in the CD measurements (Figure 8) were taken into account, the ratio of the magnitudes of the rotatory strength ($\Delta\epsilon$) of the lysins was 1:1.2:6.2, respectively. In addition, the shape and peak position of the CD bands were considerably different. The results suggest that the three egg-membrane lysins differ to some extent in their secondary structures. This is taken as further evidence that the three egg-membrane lysins are actually three different enzymes.

Electrofocusing. The pI values obtained for the three egg-

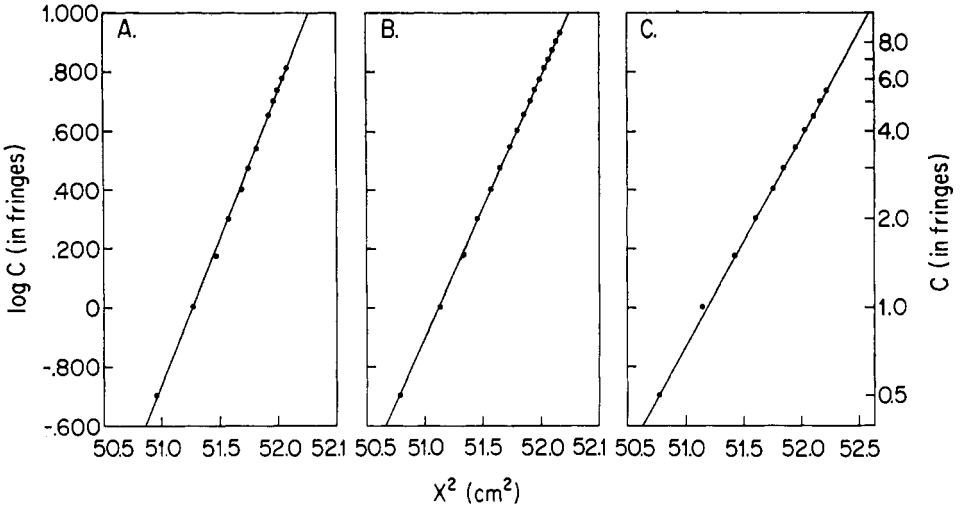

FIGURE 7: Sedimentation equilibrium of egg-membrane lysins in 6 M guanidine-HCl containing 0.5% mercaptoethanol (pH 7.1) following a 48-hr dialysis at 4° against the same solvent. The three representative plots are: (A) lysin A, 0.8 mg/ml at 44 hr, 34,000 rpm; (B) lysin B, 0.8 mg/ml at 48 hr, 30,000 rpm; (C) lysin C, 0.6 mg/ml at 47 hr, 30,000 rpm, centrifugation was carried out at 10°.

TABLE III: Amino Acid Composition of Egg-Membrane Lysin A, B, and C.

	Egg-Membrane Lysin A[a] Residues/Molecule			Egg-Membrane Lysin B[b] Residues/Molecule			Egg-Membrane Lysin C[c] Residues/Molecule		
	Hydrolysis		Nearest	Hydrolysis		Nearest	Hydrolysis		Nearest
Amino Acid	24 hr	72 hr	Integer	24 hr	72 hr	Integer	24 hr	72 hr	Integer
Lysine	29.8	30.1	30	28.3	28.1	28	36.2	32.9	33–36
Histidine	14.6	15.9	16	15.1	15.1	15	20.8	21.8	22
Arginine	28.0	28.7	28–29	36.1	35.1	35–36	33.7	33.5	34
Tryptophan	6.9	7.1	7	4.1	5.0	5	5.0	6.5	6–7
Aspartic acid	68.0	66.1	66–68	63.7	63.4	63–64	71.9	64.3	72
Threonine	22.8	21.2	23	21.5	21.6	22	31.6	29.5	32
Serine	29.7	26.7	30	39.1	32.9	40	41.3	35.8	42
Glutamic acid	54.8	52.3	52–55	46.7	45.2	45–47	51.3	49.4	50–51
Proline	16.9	24.7	25	11.3	19.9	20	10.9	22.3	23
Glycine	36.2	35.5	36	34.1	32.4	34	33.8	31.9	34
Alanine	32.0	31.5	32	27.3	25.8	27	41.9	38.6	42
Half-cystine[d]	29.4	29.0	29	26.8	26.6	27	28.2	28.4	28
Valine	21.4	23.4	24	18.0	21.3	22	18.9	26.1	26
Methionine[d]	9.2	8.9	9	8.8	8.8	9	7.2	7.2	7
Isoleucine	18.4	18.1	18	13.4	15.0	15	14.3	15.8	16
Leucine	30.9	29.9	31	24.3	23.0	24	23.0	21.4	23
Tyrosine	30.1	28.8	30	28.3	27.2	28	22.9	23.7	24
Phenylalanine	19.1	18.8	20	17.0	16.6	17	17.0	19.0	20
Total			506–512			476–480			534–539

[a] Based on molecular weight of 57,250. [b] Based on molecular weight of 53,688. [c] Based on molecular weight of 58,000. [d] Determined as cysteic acid and methionine sulfone following oxidation with performic acid.

membrane lysins are: lysin A, 5.9; lysin B, 9.9; and lysin C, 9.8.

Amino Acid Composition. This is given in Table III. The amino acid compositions of the three egg-membrane lysins are fairly similar although it is clear that significant differences exist. This is especially obvious when comparing the amino acid residues arginine, threonine, serine, alanine, leucine, and tyrosine of the three enzymes. No amino sugars were found in any of the egg-membrane lysins.

pH Optimum. Under the conditions of the assay the lytic reaction was linear with amounts of up to 5 μg/ml for each of the enzymes. The optimal pH for the lytic reaction was 8.5 at 17° for the three egg-membrane lysins (Figure 9), as determined in 0.005 M Tris-maleate containing 0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M $CaCl_2$, and 0.01 M KCl.

Discussion

This paper describes the isolation of three lytic enzymes from the sperm of the giant keyhole limpet *Megathura crenulata*. The three enzymes were found in sperm extracts of a single limpet as well as in pooled samples from several animals.

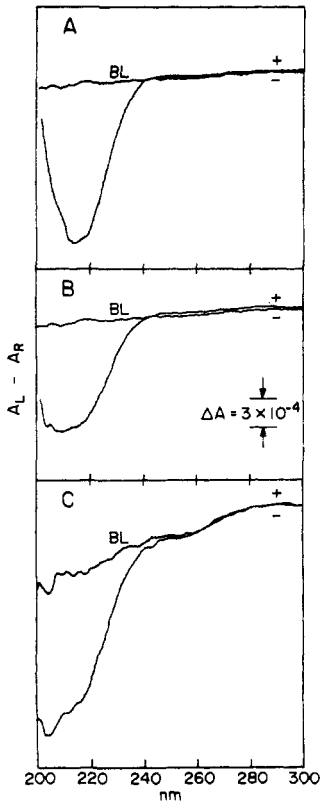

FIGURE 8: Circular dichroism spectra of egg-membrane lysin A, B, and C. The CD spectra are instrument tracing of the average of 64 scans. Lysin A had an A_{280} of 0.133, lysin B had an A_{280} of 0.074, and lysin C had an A_{280} of 0.019. Light path was 1 mm. All lysins were in 0.01 M phosphate buffer (pH 7.0) containing 0.3 M NaCl. Peak to peak noise at 210 nm was less than 6×10^{-5} ΔA. BL instrument base line. A, B, and C stand for egg-membrane A, B, and C, respectively.

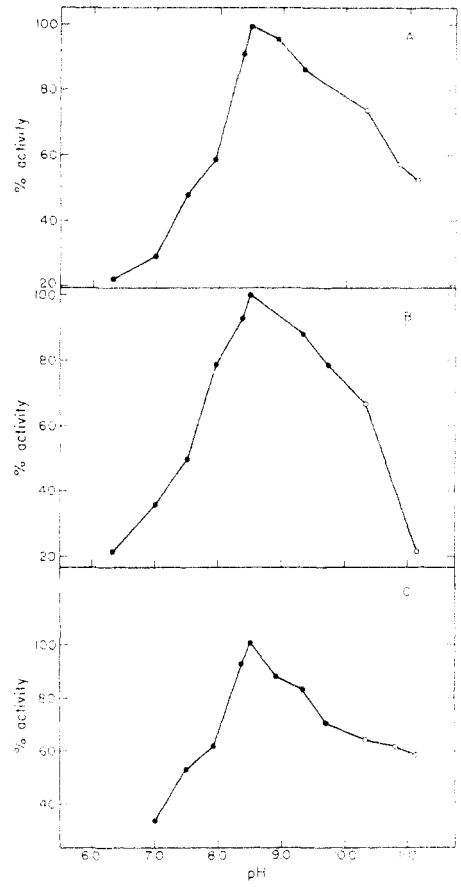

FIGURE 9: pH dependence of the lytic activity: (○) 0.005 M Tris-maleate containing 0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M $CaCl_2$, and 0.01 M KCl; (●) 0.005 M glycine–NaOH containing 0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M $CaCl_2$, and 0.01 M KCl. Other details are given in Methods. A, egg-membrane lysin A; B, egg-membrane lysin B; C, egg-membrane lysin C.

The different chromatographic behavior, amino acid composition, different specific activity, isoelectric point, CD spectra, and molecular weights lead us to believe that these egg-membrane lysins are indeed different enzymes.

The purification procedure for the three egg-membrane lysins was complicated due to the fact that it was necessary to use high ionic strength solutions in order to avoid precipitation of the proteins and loss of activity, therefore excluding use of ion exchangers such as DEAE- or CM-cellulose.

Since the assay for the enzymic activity measures decrease in turbidity and thus does not distinguish between the three egg-membrane lysins on a chemical basis (reaction substrate and products), it is quite possible that the three egg-membrane lysins have different functions and all are necessary for fertilization. They may be analogous to enzymes such as trypsin and chymotrypsin, both having similar molecular weights and amino acid compositions, yet differing in their chemical specificity.

A comparison with the egg-membrane lysin of *Tegula pfeifferi* (Haino, 1971) shows that egg-membrane lysins A, B, and C of *Megathura crenulata* are different in some respects, such as molecular weight, pI, and amino acid composition. This species difference might support the view that the egg-membrane lysins are species specific.

The molecular weight obtained from sodium dodecyl sulfate polyarcylamide gel electrophoresis and sedimentation equilibrium are higher than those obtained from Sephadex G-75 chromatography (Table II). This might suggest binding of the egg-membrane lysins to Sephadex G-75, as has been reported for the galactose-binding protein from *E. coli* (Boos and Gordon, 1971; Boos *et al.*, 1972). This is supported by the fact that the natural substrate for the three lysins, the egg-membrane of *Megathura crenulata*, contains a glycoprotein (E. Heller and M. A. Raftery, unpublished results) as does the egg membrane of *Tegula pfeifferi* (Haino and Kigawa, 1966). In this way the egg-membrane lysins would tend to bind more strongly to a carbohydrate resin like Sephadex G-75. The values that are obtained from the Bio-Gel P-150 chromatography (Table II) suggest residual binding of egg-membrane lysins A, B, and C to this resin also, though this binding must be of a different nature than that observed with Sephadex.

On the other hand, the molecular weight values obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis are fairly close to the sedimentation equilibrium values; therefore we tend to accept these values as the true ones. In addition, the molecular weight values obtained by centrifugation in the presence and absence of guanidine–HCl are close to the values determined by sodium dodecyl sulfate gel electrophoresis suggesting that egg-membrane lysins A, B, and C are each composed if a single polypeptide chain.

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Evolutionary Stability of the Histone Genes of Sea Urchins†

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ABSTRACT: Hybridization of sea urchin histone mRNA and filter-bound DNA has been used to estimate the base-sequence divergence of histone genes in evolution. Saturation hybridization experiments indicate that approximately 1000 copies of each histone gene are present in DNA isolated from sea urchin sperm. Thermal stabilities of the hybrids show that there is little if any heterogeneity in these repeated genes, *i.e.*, genes for a particular histone appear to be identical or very nearly so. Divergence among histone genes from sea urchins and other species was studied by hybridization of ^{3}H-labeled 9–12S sea urchin RNA with filter-bound DNA from various organisms. Thermal stability measurements and RNase susceptibility were used to estimate nucleotide substitutions. Some of the histone genes, like their protein gene products, are extremely conserved in evolution. Sea urchin 9–12S mRNA forms stable hybrids with numerous invertebrate DNAs as well as DNA from vertebrates and a higher plant. Comparisons of nucleotide substitutions with amino acid replacements in the histone proteins suggest that low levels of third position changes leading to synonymous codons have occurred. However, these substitutions have not accumulated at the rate predicted if these substitutions were strictly neutral. This suggests that selection pressure operates at the level of nucleic acids as well as on the proteins for which they code.

Early embryogenesis in echinoderms is characterized by a period of rapid DNA synthesis and cell division (see Hinegardner, 1967). The rate of protein synthesis increases rapidly upon fertilization (Hultin, 1961) as revealed by amino acid incorporation associated with polyribosomes (Rinaldi and Monroy, 1969). Small polysomes predominate during cleavage stages, although several size classes are present by blastulation (Nemer, 1972). As might be expected in rapidly dividing cells, a large fraction of the small polysomes are involved in the synthesis of nuclear and chromosomal proteins. Kedes *et al.* (1969) have estimated that approximately 50% of the proteins synthesized during cleavage accumulate in the nucleus and about half of these appear to be histones. Labeling patterns of the nascent polypeptide chains of small polysomes showed incorporation of large amounts of lysine or arginine and trace amounts of tryptophan, typical of the amino acid composition of histones (Nemer and Lindsay, 1969; Kedes *et al.*, 1969). More recent experiments have shown that during *in vitro* incubation of small polysomes, amino acid chain elongation occurs and identifiable histones are synthesized (Moav and Nemer, 1971).

Pulse-labeled RNA from these small polysomes contains a predominant 9S species which has many of the properties predicted for histone mRNA (Kedes and Gross, 1969). Recent reports which described 9S RNA-dependent *in vitro* translation of histones have unquestionably identified histone mRNA as a component of the 9S fraction (Gross *et al.*, 1973). Similar experiments have also been reported for 9S HeLa cell RNA (Jacobs-Lorena *et al.*, 1972). The *in vitro* translation products include proteins which correspond to HeLa cell histones in electrophoretic mobility and tryptic peptide patterns (Breindl and Gallwitz, 1973).

The 9–12S fraction of sea urchin RNA also displays characteristic hybridization properties (Kedes and Birnstiel, 1971; Weinberg *et al.*, 1972; McCarthy and Farquhar, 1972). A major fraction hybridizes with reiterated DNA (Kedes and Birnstiel, 1971) and cross-reacts with heterologous DNA (Weinberg *et al.*, 1972; McCarthy and Farquhar, 1972). Each

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