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STUDIES ON THE EGG-MEMBRANE LYSIN OF *TEGULA PFEIFFERI* THE REACTION MECHANISM OF THE EGG-MEMBRANE LYSIN

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SUMMARY

The mechanism of lysis of egg membrane was studied using pure egg-membrane lysin and the isolated egg membrane of a sea snail, *Tegula pfeifferi*. Kinetic analysis of the reaction and chromatographic fractionation of the reaction mixture demonstrated that the lysis can be divided into three phases in terms of lysin concentration and product release. At low concentrations of lysin (Phase I), all the lysin added was precipitated with a part of the egg membrane and nothing appeared in the supernatant. At medial concentrations of lysin (Phase II), all the lysin added precipitated and a substance(s) from egg membrane was released into the supernatant. At high concentrations of lysin (Phase III), excess lysin remained in the supernatant along with the soluble product(s) released from the egg membrane at the medial concentrations.

Either when or after the lysin reacted with egg membrane, it adsorbed to an insoluble part of egg membrane and lost its activity. The maximal amount of lysin adsorbed is proportional to the amount of egg membrane. The strong bond between lysin and an insoluble part of egg membrane cannot be dissociated by 8 M urea, 0.1 M HCl, 0.1 M KOH or 10^{-3} M dithiothreitol.

The plot of lysin concentration versus product release showed a sigmoidal curve. In the presence of excess lysin, the amount of the product released is proportional to the amount of egg membrane. The product(s) from egg membrane is a mucopolysaccharide–protein complex(es) with a molecular weight of $5 \cdot 10^6$ or more. Stoichiometric analysis showed that about 2000 (or more) molecules of lysin are required to liberate one molecule of the soluble product.

These results strongly indicate that the lytic action of egg-membrane lysin is a stoichiometric rather than an enzymatic reaction.

INTRODUCTION

The existence of species specificity in fertilization suggests the presence of a precise mechanism that elicits the action. This subject commands the interest of many biologists. Since Tyler [1] revealed that the sperm extract of the giant keyhole limpet,

Megathura crenulata, lyses the egg membrane of the same species, several workers have demonstrated the presence of "egg-membrane lysin" in sperm extracts of a number of marine animals [2–4]. It is believed that, in these species, the egg-membrane lysin bears the species specificity for fertilization.

Knowledge of the protein nature and the strict specificity exhibited by the egg-membrane lysin has prompted some workers to assume that these lysins are enzymatic in nature [2, 5–7].

In a previous investigation of the mechanism through which egg-membrane lysin reacts, we used isolated egg membrane and partially purified lysin of the sea snail (*Tegula pfeifferi*) [8]. Our observations seemed to discount the assumption that lysin is an enzyme.

The present paper deals with further studies on the mechanism of lysis, wherein pure lysin was used. These studies indicated that the action of the egg-membrane lysin was not enzymatic.

MATERIALS AND METHODS

Materials

Egg membrane from live *T. pfeifferi* (from local fisheries around the Izu peninsula of Japan) was isolated [4] and suspended in 0.5 M KCl containing 0.03 M phosphate buffer (pH 7.8) and 1 mM EDTA [8]. Egg-membrane lysin was purified by the method described previously. The concentration of this lysin was determined by using the ultraviolet extinction coefficient at 280 nm ($E^{1\%}_{1\,cm}$ (280 nm) = 23.8) [9].

Determination of the lytic activity

To start the reaction, 0.2 ml of egg-membrane lysin solution was mixed with 3 ml of 0.1% egg-membrane suspension that had been incubated at 17 °C. The reaction mixture was incubated for a specified time and stirred at proper intervals. Lytic activity was measured by two methods. The turbidimetric method is based on the change induced by lysin in the turbidity of the egg-membrane suspension [8, 9]. This change in turbidity was measured for the time required for the incubation of the reaction mixture and the lytic unit was determined by the method described previously [9]. A second method is based on the release, through lytic action, of a soluble substance(s) from egg membrane [8]. Each reaction mixture was incubated for 60 min either to complete the reaction or to reach an equilibrium for the reaction; then, the mixture was centrifuged at 2600 rev./min for 10 min in a tapered glass tube placed in a swinging-bucket rotor. The amount of neutral sugar which had been shown to be proportional to the lysis [8] was determined as a measure of the soluble substance(s) released from egg membrane.

Because the quantity of arginine found in lysin (15%) exceeds that found in egg membrane (0.5%) [4], the arginine content of the supernatant fluid was determined in order to measure the amount of egg-membrane lysin present. The ultraviolet absorption in the fluid at 280 nm was also determined. This determination can be the sum of the soluble substance(s) from egg membrane plus the lysin content.

Chemical analysis

Neutral sugar content was determined by the Anthrone method [10] with glucose used as the standard.

Amino sugar was quantitated according to the Strange [11] modification of the method of Rondle and Morgan [12]; the buffer was that of Immers and Vasseur [13] and glucosamine served as the standard.

After hydrolysis with 6 M HCl at 110 °C for 20 h, protein was estimated by the ninhydrin method [14] with leucine used as the standard. Ultraviolet absorbance (at 280 nm) was also measured. After hydrolysis with 6 M HCl at 110 °C for 20 h, the arginine content was determined by the Sakaguchi reaction [15]. Amino acids were analyzed with a Beckman–Spinco Model MS amino acid analyzer by the method of Moore and Stein [16].

Column chromatography

Column chromatography was performed in the cold room with Sephadex G-25, Sephadex G-100, Sepharose 4B and Sepharose 2B.

RESULTS

Kinetic of the lytic action

Results in a previous report [8] showed that no lytic activity was detected (measured by neutral sugar release or turbidity change) when the amount of lysin was small. The amount of lysin required for the lysis was restricted to a narrow range (about 1–10% of the dry weight of egg membrane even with the use of pure lysin). These results suggested the possibility that the reaction might not be enzymatic. Hence, we performed more detailed experiments with prolonged incubation (from 1 to 60 min).

Several reactions were made in a fixed egg-membrane concentration (0.1 %) with various concentrations of lysin. Neutral sugar and arginine content as well as

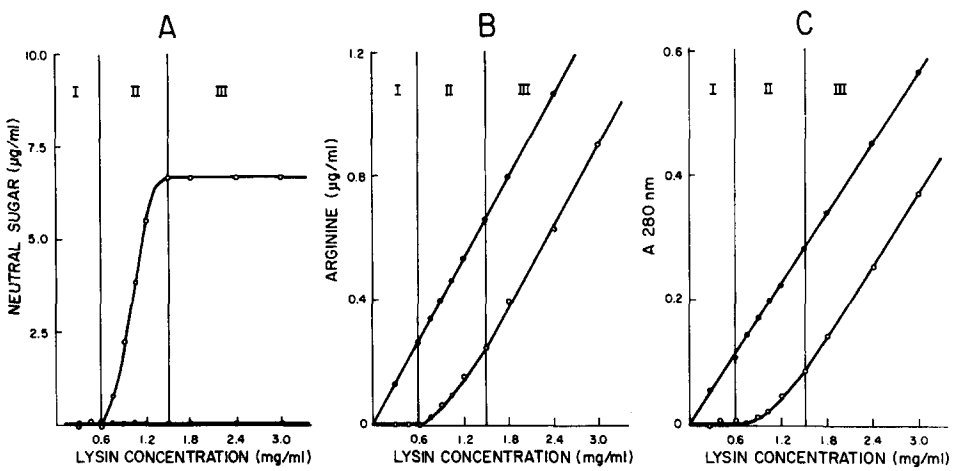

Fig. 1. The effect of lysin concentration on the release of various substances. (A) Neutral sugar. (B) Arginine. (C) $A_{280\ nm}$. As is shown in Figs 5A and 5B, the supernatant from egg-membrane suspension contains P_3 and P_4. P_3 is an Anthrone-positive substance and is light absorptive at 280 nm. The amounts shown in these figures have been corrected by the use of the supernatant of an egg-membrane suspension. ○–○, egg-membrane suspension plus lysin; ●–●, KCl–EDTA–phosphate buffer (pH 7.8) plus lysin.

ultraviolet absorption (280 nm) of the supernatant fluids were determined. The results are presented in Figs 1A–1C. Parameters were plotted against the lysin concentration in each group determined (open circle). KCl–EDTA-phosphate lysin buffer (pH 7.8) without egg-membrane suspension was used as the control in all cases (solid circle).

Results derived from determining the sugar content (Fig. 1A) showed that, in terms of lysin concentration, the reactions can be divided into three phases; with the first ranging between 0 and 0.6 mg/ml, the second between 0.6 and 1.5 mg/ml, and the third from 1.5 mg/ml or higher. In Phase I, no sugar was released in the supernatant. In Phase II, sugar release began at the lowest concentration and reached a maximum at the highest concentration. In Phase III, sugar content in the supernatant remained constant. In Phase II, the plot exhibited a sigmoidal curve rather than a straight line. This might be a reflection of the multiple hits by lysin that caused the release of substance(s) that contained sugar.

Fig. 1B shows the relation between the arginine contents of the supernatant and the lysin concentration. As in Fig. 1A, the reactions in Figs 1B and 1C are divided into three phases. In Phase I, arginine was detected in the control but not in the test solution. In higher concentrations of lysin (0.6–1.5 mg/ml), arginine appears in the supernatant and forms a concave curve (Phase II), and in even higher concentrations a straight line is formed parallel to the control (Phase III). In each test case, however, the amount of arginine present is smaller than that in its control. This data is contrary to the hypothesis that lysin is an enzyme and that it remains in the solution and acts catalytically.

Ultraviolet absorbance at 280 nm (Fig. 1C) shows a similar pattern to that described in Fig. 1B; no release of substances was noted in any concentration of less than 0.6 mg/ml (Phase I), a gradual increase was evident in 0.6–1.5 mg/ml (Phase II), and an increase proportional to that of the lysin concentration was present in over 1.5 mg/ml (Phase III).

In order to determine the amount of lysin remaining in the supernatant fluids, a two-stage experiment was performed. The first stage was described in Fig. 1, and the second stage, the assay of the supernatant fluids from the first stage for residual lysin activity. The final concentration of the egg membrane in the second mixture is the same as that used for the first stage. The lytic activity in the supernatant fluid was measured by the turbidimetric method. Fig. 2 shows that when the lysin concentration was below 1.2 mg/ml, no lytic activity was detected in the supernatant from the first mixture. However, lytic action was noted when the concentration exceeded 1.8 mg/ml. Therefore, the arginine which appears in the supernatant under the conditions of Phase II (Figs 1B and 1C) apparently stems from a source other than the added, active lysin. The disparity between the activity line of the supernatant in Phase III (dotted line) and the control (solid line) may be attributed to the inhibition by the product(s) of lysis or to the nature of the turbidimetric method in which a lower lysin concentration tends to give a higher specific activity [9].

For further analyses, reactions were tested in various concentrations of lysin and of egg-membrane suspensions. Fig. 3A shows the determination of neutral sugar in the supernatant. In each concentration of egg membrane, the neutral sugar released was limited to a certain level when excess lysin was added. For each egg-membrane concentration, the maximum amounts of neutral sugar were plotted

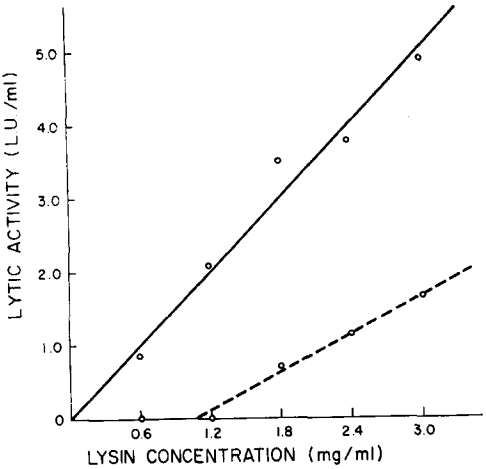

Fig. 2. Lytic activity remaining in the supernatant after lysis shown in Fig. 1. Conditions are described in the text. O–O, lytic activity in the initial mixing; O- - -O, remaining lytic activity in the supernatant of reaction mixture.

(Fig. 3B). This plotting shows that the neutral sugar released was proportional to the amount of egg membrane when optimal or excess lysin was present. 1 mg egg membrane can yield 5.3 μg neutral sugar.

It is obvious that the added lysin is in the precipitate when the lysin concentration is low in the reaction mixture (Phase I) (Figs 1B and 1C). Therefore, we investigated the quantitative relationship among lysin concentration, the amount of arginine in the supernatant, and the amount of egg membrane. Reaction mixtures similar to those described in Fig. 3, produced the results shown in Fig. 4A. When the egg-membrane concentration is high, the maximal lysin concentration that disappears from the supernatant is high. The maximal concentration of lysin when arginine was not detected in the supernatant (intersection between Phase I and II) was plotted against the egg-membrane concentration (Fig. 4B (solid line)). Intersecting points between Phase II and III in each group were also plotted in this figure

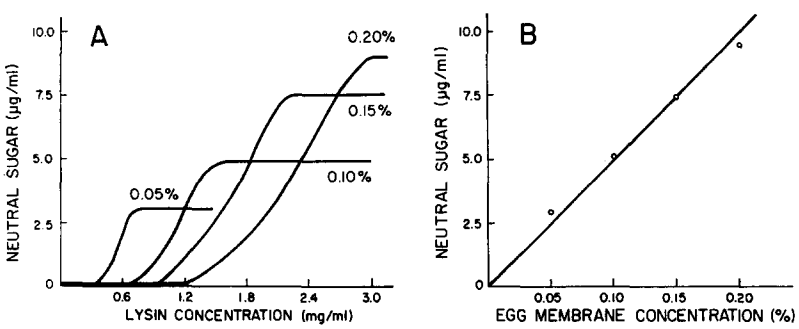

Fig. 3. Effect of lysin and of egg-membrane concentrations on the release of neutral sugar. (A) Concentrations of egg-membrane suspensions used were as follows: 0.05, 0.10, 0.15 and 0.20 %. (B) The maximal amounts of neutral sugar (in each concentration of egg membrane) were plotted against the egg-membrane concentration.

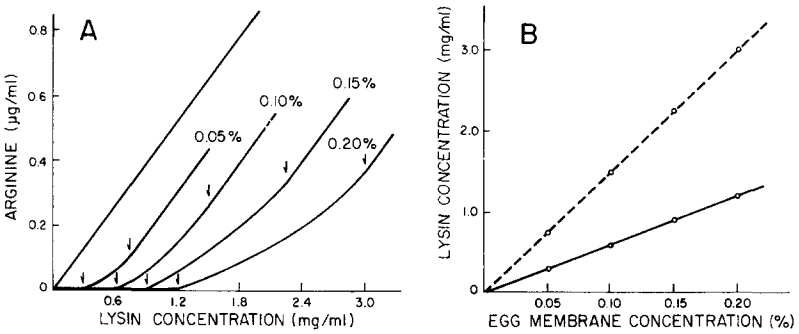

Fig. 4. (A) Effect of lysin on the amount of arginine present in the reaction supernatant. The following concentrations of egg-membrane suspensions were used: 0.05, 0.10, 0.15 and 0.20 %. The straight line extrapolated to the origin shows the amount of arginine in lysin that was added to KCl–EDTA–phosphate buffer (pH 7.8). (B) Effect of egg membrane on amount of lysin contained in the precipitate from the reaction mixture. The lysin concentrations were estimated from Fig. 4A. ○–○, the maximal lysin concentrations in which no arginine appears in the supernatant of the reaction mixture; ○- - -○, the minimal lysin concentrations for which the amount of arginine released is parallel to the control.

(dotted line). Both lines are straight and can be extrapolated to the origin. The solid line shows that the maximal lysin concentration, when no arginine is released in the supernatant, is proportional to the concentration of the egg membrane. Since little lytic activity was detected in the reaction supernatant (Fig. 2) in the range of lysin concentration of Phase II, the dotted line also indicates the proportionality of egg membrane and adsorbed, precipitated lysin; therefore, this line can be used to calculate that 1 mg of egg membrane can precipitate a maximum of 0.1 mg of lysin. The lysin in solution seems to be precipitated by adsorption to the insoluble part of the egg membrane (Table III). In Phase II, soluble substance from the egg membrane contributes the arginine present in the supernatant.

Fractionation of the reaction supernatant

The results described above showed that the soluble substance(s) from egg membrane was released by the action of lysin; therefore, we attempted to fractionate the soluble substance(s). To prevent the appearance of lysin in the reaction supernatant (see Figs 1 and 2), a proper ratio of egg membrane and lysin was determined for use in the preparation of soluble substance(s). 8 ml of solution, containing 0.9 mg/ml lysin was added to 120 ml of 0.1 % egg-membrane suspension; this mixture was incubated and then centrifuged. The supernatant fluid, concentrated 3-fold by lyophilization, was fractionated by a Sephadex G-25 column, using a phosphate buffer (0.03 M, pH 7.8). The results are shown in Fig. 5A. The elution pattern obtained by measuring the absorbance at 280 and at 215 nm showed four peaks (P_1, P_2, P_3 and P_4). P_2 is due to the ethylenediamine tetraacetate contained in the reaction mixture. Egg-membrane suspension without lysin served as a control and was treated similarly and chromatographed with the same system. The results (Fig. 5B) show three peaks that correspond to P_2–P_4 in the experimental group (Fig. 5A). Therefore, substances from egg membrane that are released in P_3 and in P_4 are not

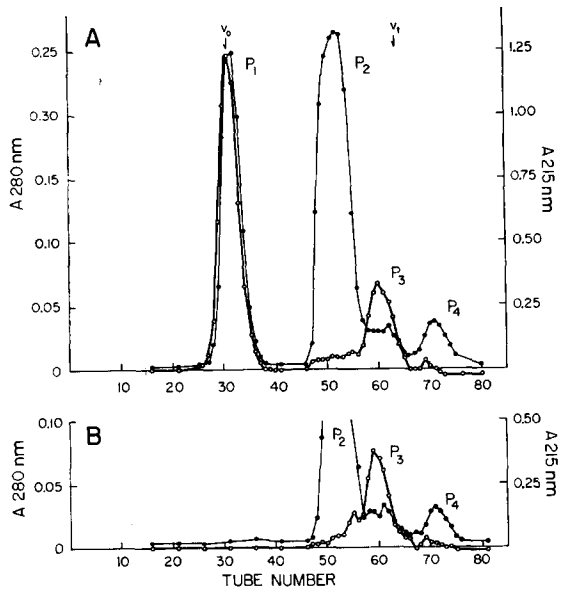

Fig. 5. Column chromatography of the reaction supernatant on Sephadex G-25. (A) The reaction supernatant. (B) The supernatant of egg-membrane suspension. The column (3.5 cm × 105 cm) was developed with 0.03 M phosphate buffer (pH 7.8) and 11-ml fractions were collected. ○–○, $A_{280\ nm}$; ●–●, $A_{215\ nm}$.

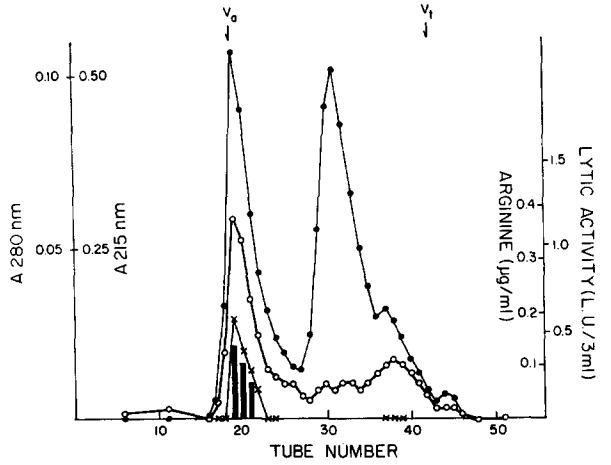

Fig. 6. Column chromatography of the supernatant from lysate prepared by addition of excess lysin on Sephadex G-25. A mixture of 0.8 ml of 1.8 mg/ml lysin and 6 ml of 0.1 % egg-membrane suspension was incubated at 17 °C for 40 min. The supernatant from the reaction mixture was charged on the column (2.0 cm × 36.7 cm) and fractionated (3 ml per tube). ○–○, $A_{280\ nm}$; ●–●, $A_{215\ nm}$; ×–×, arginine; solid bar, lytic activity.

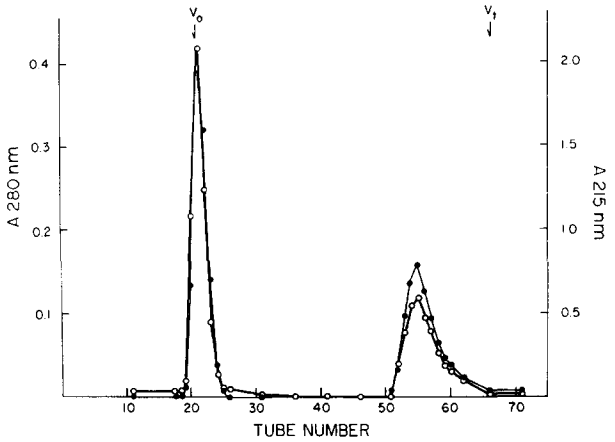

Fig. 7. Column chromatography of P_1 fraction from Sephadex G-25 on Sephadex G-100. P_1 fraction was prepared by the same column chromatographic system (but with excess lysin) as that for fractions described in Fig. 5A. Fractions 28–35, corresponding to those in Fig. 5A, were combined and concentrated to 9.4 ml by lyophilization. The resulting concentrate was charged on Sephadex G-100 (2.5 cm × 102 cm) and fractionated (7.6 ml per tube) using the buffer of 0.03 M phosphate (pH 7.8). ○–○, $A_{280\ nm}$; ●–●, $A_{215\ nm}$.

influenced by addition of lysin. The substance in P_3 is Anthrone positive which indicates the presence of neutral sugar.

Because P_1 was eluted at $K_d = 0$ from a column with Sephadex G-25, the fractions of P_1 were combined, concentrated and charged on a Sephadex G-100 column. On elution with the buffer (0.03 M phosphate, pH 7.8), a single peak appeared at the point of $K_d = 0$. Since the lysin is eluted around $K_d = 1$ from the G-100 column, this result shows that lysin is absent from P_1, i.e. the substance in P_1 is the product(s) formed by the lytic action on the egg membrane.

This finding was confirmed by the use of excess lysin in the reaction. In this case, P_1 from the Sephadex G-25 column exhibited lytic activity (Fig. 6) and was eluted in two peaks when it had been fractionated by the Sephadex G-100 column (Fig. 7). The reaction product caused one peak and excess lysin caused a second. No other peaks were eluted.

Characteristics of the reaction product(s)

Reaction product(s) obtained by column chromatography through Sephadex G-25 and G-100, was analyzed with Sepharose 4B and Sepharose 2B. Columns were developed with a pH 7.8 phosphate buffer (0.03 M) containing 0.2 M KCl. The substance(s) was eluted as a single peak around the point of $K_d = 0$ from each column. These results show that the product(s) released from egg membrane by lytic action may consist of one or more substances having molecular weight of $5 \cdot 10^6$ or more. This substance(s) is labile and, when stored at 4 °C for 1 or 2 days, additional peaks at $K_d = 0.17$ and 1.0 were observed by Sepharose 4B column chromatography. The substance(s) can be adsorbed on a DEAE-cellulose column. However, elution from the column caused degradation of the substance(s), thereby prohibiting the use of this method at the purification stage of the substance.

The reaction product(s) which had been obtained by column chromatography (Sephadex G-25 and G-100) was desalted through a Sephadex G-25 column. Analysis of an aliquot of the desalted product(s) (Table I) showed this substance(s) to be a mucopolysaccharide–protein complex, which contains 77% amino acids and 17.8% neutral sugar. The presence of 2.5% arginine in the product(s) explains the appearance of arginine in Phase II (Fig. 1B). This finding is compatible with the interpretation

TABLE I

COMPOSITION OF THE SOLUBLE PRODUCT(S) FROM EGG MEMBRANE

	Dry weight of egg membrane (%)	Dry weight of soluble product(s) (%)
Amino acids*	2.30	77.0
Arginine	0.08	2.5
Neutral sugar	0.53	17.8
Amino sugar	0.16	5.2
Total	2.99	(100.0)

* The amount of amino acids was estimated as the amount measured by the ninhydrin method minus the amount of amino sugar.

TABLE II

AMINO ACID COMPOSITIONS OF THE SOLUBLE PRODUCT(S) AND THE EGG MEMBRANE

	Molar ratio of soluble product(s)	Molar ratio of egg membrane **
Lysine	7	7
Histidine	3	4
Ammonia	74	71
Arginine	10	5–6
Tryptophan	–	7
Hydroxyproline	12	10
Aspartic acid	19	19
Threonine	83	97
Serine	46	56
Glutamic acid	15–16	11–12
Proline	15–16	6
Glycine	15	17
Alanine	14	13
Half-cystine	1	1
Valine	11	14–15
Methionine	1	1
Isoleucine	6	8–9
Leucine	11	11
Tyrosine*	2	2
Phenylalanine*	2	6

* Because of the presence of hexosamines in large quantity, these amino acids could not be determined accurately.

** These data are cited from Exp. Cell Res. 42 (1966) 631.

TABLE III

EXTRACTION OF LYSIN FROM THE PRECIPITATE OF REACTION MIXTURE

The precipitate obtained from a mixture of 30 ml of 0.1 % egg-membrane suspension and 2 ml of 0.9 mg/ml lysin solution was suspended in 15 ml distilled water. This 3-ml suspension was centrifuged and the precipitate was extracted by 3 ml of each solvent. After standing for 60 min, the extraction was completed by centrifugation. A 0.2 % suspension of untreated egg membrane in distilled water was extracted by the same solvents. All the lysin in the solution used under conditions described was adsorbed to insoluble egg membrane. When all lysin is extracted, the expected $A_{280\ nm}$ of the 3-ml extract is 0.272 and the amount of arginine is 82.5 μg.

Solvent of extraction	Insoluble egg membrane after lysis		Untreated egg membrane	
	$A_{280\ nm}$ of extract	Amount of arginine extracted (mg)	$A_{280\ nm}$ of extract	Amount of arginine extracted (mg)
KCl–EDTA–phosphate buffer (pH 7.8)	0	–	0	–
1 M KCl	0.010	–	0	–
8 M urea, 1st	0.114	–	0.140	–
8 M urea, 2nd	0.154	–	0.222	–
0.1 M KOH	0.654	91.5	0.472	22.8
0.2 M HCl	0.020	0	0.038	0
$2.3 \cdot 10^{-3}$ M dithiothreitol	0.172	0	0.128	0

that the amount of lysin added adsorbed to the precipitated residual egg membrane (dotted line in Fig. 4B). Table II shows that the amino acid composition of the soluble product resembles egg membrane.

Fate of the reacted lysin

It has been shown that the lysin is precipitated with the insoluble portion of the egg membrane after the lysis; therefore, we used six different solvent systems in an attempt to extract lysin from the precipitate (Table III). Some of the reaction precipitate was mixed with each solvent, and allowed to stand 60 min at room temperature. The mixture was then centrifuged and the supernatant fluid was analyzed. As a control, unreacted egg membrane was treated similarly. Only 0.1 M KOH could dissolve most of the precipitate (Table III). The large amount of arginine in the KOH extract indicates the presence of lysin in a soluble state.

After neutralizing, the KOH extract was applied to a Sephadex G-100 column; the elution profile is given in Fig. 8A. Since no peak was detected in fractions around $K_d = 0.74$ where lysin would appear, the lysin was apparently eluted in complex with a part of the egg membrane. A similar elution pattern to that in Fig. 8A was obtained when unneutralized KOH extract was chromatographed in a 0.01 M KOH solution containing 0.2 M KCl.

Untreated egg membrane, extracted by 0.1 M KOH, was neutralized and then chromatographed on the Sephadex G-100 column (Fig. 8B). This extract contained much less arginine than that described in Fig. 8A. Fractions 20–26 were combined, as were Fractions 29–36, and each combination was then mixed with active lysin, in-

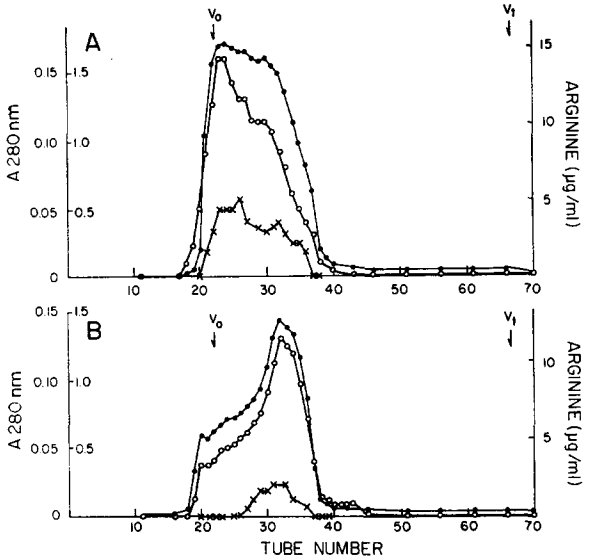

Fig. 8. Column chromatography of alkaline extracts of egg membrane on Sephadex G-100. (A) Alkaline extract from insoluble part of reaction mixture after lysis. The reaction precipitate obtained from 120 ml of 0.1 % egg-membrane suspension and 8 ml of 0.9 mg/ml lysin solution was mixed with 60 ml of 0.1 M KOH for 60 min at room temperature. After centrifugation, the supernatant was neutralized, concentrated to 7.6 ml by lyophilization, and analyzed. The column (2.5 cm × 100 cm) was developed by 0.03 M phosphate buffer (pH 7.8) and fractionated (7.6 ml per tube). (B) Alkaline extract of untreated egg membrane. 100 and 20 ml of 0.1 % egg-membrane suspension was centrifuged and 60 ml of 0.1 M KOH was mixed with the resulting precipitate. Other conditions were the same as described previously. ○–○, $A_{280\ nm}$; ●–●, $A_{215\ nm}$; ×–×, arginine.

cubated for 60 min at 17 °C, and chromatographed on the Sephadex G-100 column. In both instances a single peak appeared at $K_d = 0$ but there were no peaks around $K_d = 0.74$. These data also show the adsorption of lysin to a part of egg membrane. The neutralized KOH extract from reacted egg membrane was treated with 0.1 M HCl and 10^{-3} M dithiothreitol. These treatments caused subdivisions of some components in the extract, but failed to free the lysin from a part of the egg membrane.

DISCUSSION

Judging from the results described, the lytic reaction of the egg-membrane lysin exhibits the following characteristics: (a) a soluble product originating from egg membrane is released by multiple hits by the egg-membrane lysin; (b) no product is derived from the egg-membrane lysin in the supernatant of the reaction mixture; (c) the egg-membrane lysin is adsorbed to the unlysed part of the egg membrane in the reaction. The following equation is based on the reaction of the lysin with the egg membrane:

$$[M]+n[L] \rightarrow [P_{sup}]+[P_{ppt}-nL]$$

where M is the amount of the egg membrane; n, an integral number; L, the amount of the egg-membrane lysin; P_{sup}, the amount of the product that originated in the

supernatant from the egg membrane; and P_{ppt}, the amount of the product that originated in the precipitate from the egg membrane.

According to the results depicted in Figs 1A and 3A, 1 mg of egg membrane reacted with a maximum of 0.1 mg of egg-membrane lysin and produced 5.3 μg of neutral sugar. With a molecular weight of 8800, 0.1 mg of lysin corresponds to $1.13 \cdot 10^{-2}$ μmoles. Since the ratio of neutral sugar in the soluble product is 17.8 % and the molecular weight of this product is postulated to be $5 \cdot 10^6$ or more as is described above, 5.3 μg of neutral sugar corresponds to $5.92 \cdot 10^{-6}$ μmoles or less of the product. Consequently, $1.91 \cdot 10^3$ or more of the lysin molecules are required to release one molecule of the soluble product from the egg membrane. In the above equation, n is $1.91 \cdot 10^3$ or more. Together with the other characteristics of lysin, this stoichiometry indicates that the lysin acts neither enzymatically nor catalytically in the reaction.

These considerations might still be interpreted as supporting the possibility that the lysin is an enzyme, as follows. (A) The egg membrane has two types of sites for the lysin; one type of site only adsorbs lysin with a high affinity and the other is attacked by the lysin enzymatically. When lysin molecules are added to the egg membrane, they are first adsorbed by the former type of site until saturation, after which the remaining lysin acts catalytically on the latter type of site and releases a product(s). The amount of lysin to cause saturation can be calculated as 0.6 mg/ml at the concentration of 0.1% egg membrane. Doubling the amount (1.2 mg/ml) caused half as much product to be released into the supernatant. For the release of the total product, however, only one-half of the amount for saturation need be further added (Fig. 1A). In addition, prolonged incubation with a half-maximal concentration of lysin (0.9 mg/ml) did not increase the product release [8]. These results support the conclusion that lysin acts stoichiometrically rather than enzymatically. (B) It could alternatively be assumed that a lysin preparation contains two types of proteins: one which adsorbs to egg membrane, and another which acts enzymatically. However, physicochemical analysis of the lysin preparation did not show the presence of two types of proteins.

The mechanism of the lytic reaction of the egg membrane of *T. pfeifferi* can be described as follows. The release of one molecule of egg-membrane product results only when egg membrane reacts with about 2000 molecules or more of lysin (which corresponds to 1/10 of the weight of the egg membrane). The adsorption of egg-membrane lysin to egg membrane causes an initial increase in the turbidity of the egg-membrane suspension and the subsequent release of the product from egg membrane in concurrance with a decreasing turbidity [8, 9]. The lysin becomes inactive after the reaction and combines with an insoluble part of the egg membrane. The strong bond between lysin and this part of the egg membrane cannot be dissociated by 8 M urea, 0.1 M HCl, 0.1 M KOH or 10^{-3} M dithiothreitol.

The non-enzymatic action of the egg-membrane lysin described above could be rationalized as follows: to achieve fertilization, a spermatozoon must quickly perforate the egg membrane and create a hole just large enough so that it can enter the egg. The stoichiometric nature is, therefore, preferred to the enzymatic one for the lysin, since the former case offers a mechanism to limit the size of the hole.

Only because of the protein nature of lysin, several workers [2, 5–7] believed that its action with egg membrane was enzymatic. Inoue et al. [17] described the egg-

membrane lysin of sea urchin, but made no comment on the nature of its activity, enzymatic or otherwise. Further studies on this factor would be of interest.

The dissolution of cell walls [18–20] by many enzymes, such as lysozyme, has prompted a widespread assumption that an enzymatic reaction occurs when similar types of membrane are lysed by a protein. However, it has been indicated that the lysin from *T. pfeifferi* is not an enzyme. Hence, it seems desirable to re-investigate, from this point of view, the reaction mechanisms of the hatching enzyme [21, 22] the jelly dispersing enzyme [23], and the zona lysin [24, 25].

There is a distinct species specificity among four species of *Tegula* for the lytic reaction of the egg membrane by the lysin. It would seem worthwhile to investigate the types of physicochemical properties of the egg membrane and the lysin that determine the species specificity. Further research along this line will be pursued.

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