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Biochimica et Biophysica Acta 1564 (2002) 375–383

BIOCHIMICA ET BIOPHYSICA ACTA
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Behavior of silkworm yolk protein on phospholipid membranes

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Received 16 November 2001; received in revised form 26 April 2002; accepted 4 June 2002

Abstract

During early development, the plasma membrane of silkworm (*Bombyx mori*) eggs undergoes a superficial cleavage that separates the blastodermal protoplasm and the yolk. To test whether the blastoderm absorbs yolk through the plasma membrane in *B. mori*, we studied the interaction of phospholipid membranes and yolk using a phospholipid planar bilayer membrane (PBM) and liposomes. In addition, egg-specific protein (ESP; 225 kDa), a yolk protein that is specific to *B. mori* eggs, was collected by fractionating the eggs. Liposomes were mixed with either *B. mori* yolk or ESP, and observed under an electron microscope. This showed that the phospholipid membrane was spanned by fine particles 10–20 nm in diameter. Both yolk and ESP caused the PBM to become extraordinarily leaky, with a membrane potential of −70 mV for yolk and −198 mV for ESP. These results suggest that although it is a water-soluble protein, ESP permeates the phospholipid membrane without the help of enzymes.

Keywords: Silkworm yolk protein; Liposome; Membrane structure; Planar bilayer membrane; Transmembrane-soluble protein

1. Introduction

Mature silkworm (*Bombyx mori*) eggs consist mostly of yolk in the center, and also contain mitochondria and endoplasmic reticula in the thin cortical layer (the peripheral protoplasm) between the yolk and the eggshell. Within 10 h after fertilization, the blastoderm develops in the peripheral protoplasm via superficial cleavage. This type of cleavage results in the plasma membrane separating the yolk and the blastodermal protoplasm. The yolk is generally regarded as a source of nutrients for embryogenesis, which implies that the blastoderm ingests the yolk, although this remains to be determined. We speculated that the blastoderm absorbs the yolk, especially the yolk protein, through the plasma membrane. The stiff, opaque eggshell prevents microscopic observation of these processes, however, and even slight injury to the eggshell is enough to stop embryogenesis, making it almost impossible to continuously observe the course of embryogenesis in vivo. Therefore, the transport of yolk protein through the plasma membrane must be studied in vitro.

In this study, we investigated the interaction of phospholipid membrane and yolk by electrophysiologic analysis of the phospholipid planar bilayer membrane (PBM), and by observing liposome membranes under an electron microscope.

2. Materials and methods

2.1. Materials

Partially purified soybean phospholipid [1] was provided by Dr. H. Hirata, Laboratory of Biological Signaling, Department of Life Science, Himeji Institute of Technology, Japan. Cholesterol was purchased from Wako Pure Chemicals (Osaka, Japan). Bovine serum albumin (BSA; initial fractionation by cold alcohol precipitation), Hepes, and Tris were purchased from Sigma (St. Louis, MO, USA). All other chemicals were commercial, analytic-grade products. Unless specified, purified water from a Millipore Milli-Q SP TOC system was used.

2.2. Preparation of yolk proteins

Unfertilized, mature eggs and oviducts were removed from female *B. mori* adults (Shi 146 strain) reared on fresh

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PII: S0005-2736(02)00473-X

mulberry leaves. The oviducts were removed from the eggs by hand under tap water. The following procedures were conducted at 4 °C or on ice, unless otherwise specified. Eggs were homogenized with a mortar and then mixed with buffer A (50 mM phosphate buffer, pH 7.2; 60 ml/10 g eggs). The eggshells were removed with a tea strainer, and then the homogenate was centrifuged at 10,000×*g* for 20 min. The supernatant was collected as a yolk fraction (designated Yf), which was applied to chromatography columns, as follows.

We used two types of chromatography columns to fractionate the protein: Bio-Gel A-15m (1 cm (i.d.)×80 cm, Bio-Rad), and DEAE-cellulose (1.5 cm (i.d.)×10 cm, Sigma). The Bio-Gel A-15 m column was equilibrated with buffer B (150 mM KCl, 0.5 mM EDTA, 50 mM Tris–HCl, pH 7.4), and Yf was eluted in 1 ml aliquots at a flow rate of 0.1 ml/min at room temperature with buffer B. The DEAE-cellulose column was preconditioned with buffer A and used to elute 2 ml aliquots of protein at a flow rate of 1 ml/min at 4 °C with buffer A plus stepwise additions of KCl, as described in Zhu et al. [2]. Proteins were evaluated on each column by optical absorbance at 280 nm and were identified via electrophoresis on a 10% SDS-PAGE gel at room temperature as described by Zhu et al. [2]. We checked the purity of the protein from the DEAE-cellulose column using an HPLC instrument (LC-6A, Shimadzu, Kyoto, Japan) equipped with a molecular sieving column (TSK gel G3000SW, 7.5 mm (i.d.)×600 mm, Tosoh Co., Tokyo, Japan). The protein was eluted with buffer C (100 mM sodium sulfate, 50 mM phosphate buffer, pH 7.2) at a constant 1 ml/min flow rate at room temperature. The elution profile was monitored at 280 nm.

2.3. Observation of liposomes by electron microscopy

A soybean phospholipid film was dried under a vacuum overnight, hydrated with buffer A plus 0.3 M KCl, and sonicated under nitrogen to obtain small unilamellar liposomes. These liposomes were frozen and thawed three times and then sieved through 0.4-μm double-stacked Nuclepore filters to obtain large unilamellar liposomes for observation under an electron microscope. Yolk protein was dispersed in buffer A plus 0.3 M KCl and then mixed with liposomes. Liposomes with a final phospholipid concentration of 10 mg/ml were frozen quickly using the technique of Heuser [3]. The frozen liposomes were fractured at −110 °C and etched at −90 °C for 2 min

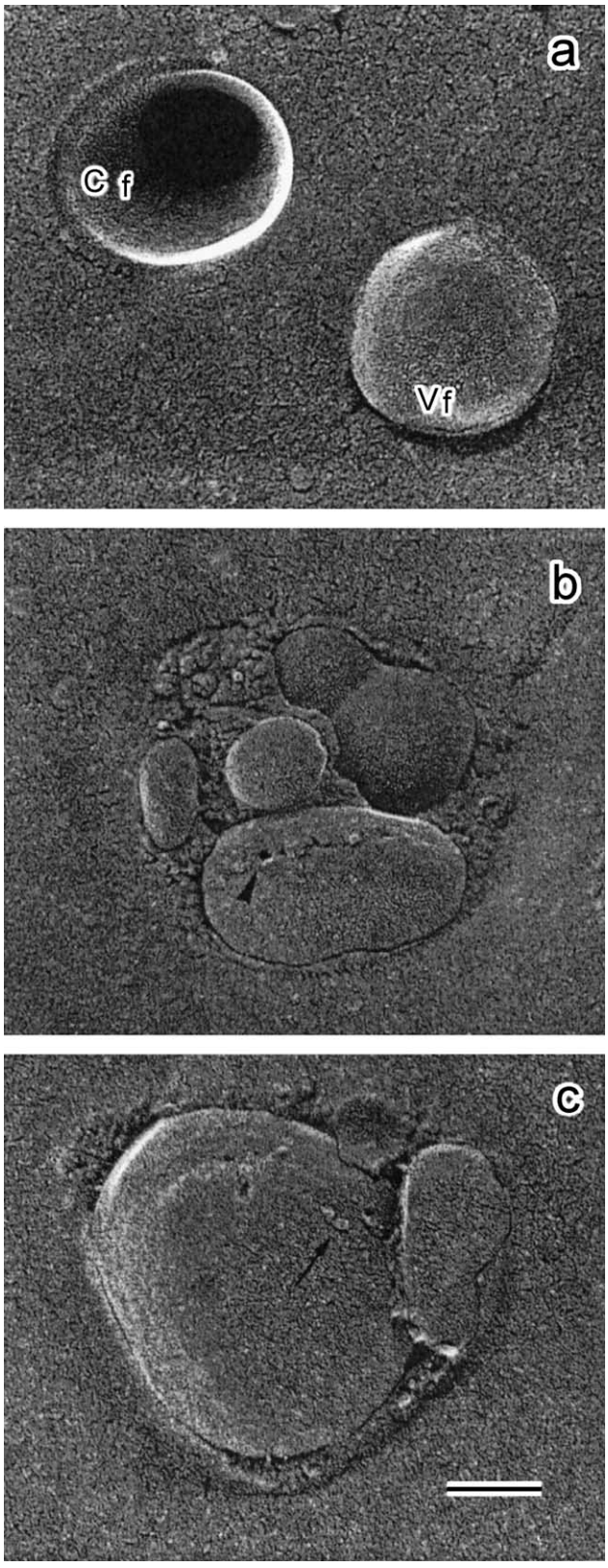

Fig. 1. Freeze–fracture electron micrograph of liposomes alone or mixed with *B. mori* yolk and incubated at room temperature. The fracturing method splits the liposome membrane along its hydrophobic interior, resulting in a convex face (Vf; stained light) and a concave face (Cf; stained dark). Scale bar=100 nm. (a) Liposomes alone; Vf and Cf are exposed. (b) Liposomes plus yolk after a 30-min incubation. (c) Liposomes plus yolk after a 2-h incubation. (b and c) Fine particles aggregate around liposomes and result in the assembly of several liposomes. Several fine particles (arrow) and pores (arrowhead) (10–20 nm in diameter) are on the Vf of the liposome membrane. The pores on the fractured face show that fine particles once spanned the membrane, because when the liposome membrane is split, some of the fine particles spanning the membrane fall away from the fractured face, leaving pores on it.

in a freeze–fracture apparatus (BAF400D, Balzers, Liechtenstein). Liposome replicas were obtained by rotary shadowing at 25° with ca. 6 nm Pt/C and at 90° with ca. 20 nm C, and were examined under an electron microscope (H-7000, Hitachi) operating at 100 kV.

2.4. Experiments with PBM

PBM was prepared according to Montal and Mueller [4] using a lipid mixture composed of soybean phospholipid and cholesterol (40:1 by weight). Monolayers were made from a 10 mg/ml solution of the lipid mixture in *n*-hexane on the surface of two buffered salt solutions (250 mM KCl, 10 mM HEPES–Tris, pH 7.4) separated by Teflon film (25 μm thick) in a Teflon cell. After the solvent had evaporated, the water level was raised, causing the two monolayers to become joined through a hole (0.1 mm in diameter) that was pretreated with a 0.2% solution of *n*-hexadecane in *n*-hexane. The capacitance of the PBM was 45–55 pF when triangular-wave voltage (±12.5 mV, 20 Hz) was applied. The two 1.5-ml chambers flanking the PBM were designated as *cis*, the chamber connected to the virtual ground, and *trans*, the chamber to which the command voltage was applied. Since *cis* and *trans* represented the extracellular and intracellular sides of the plasma membrane, respectively, the PBM potential (command voltage) corresponded to the intracellular potential.

Experiments were conducted at room temperature (24–26 °C) using a voltage clamp. The current across the PBM was measured via salt bridges (1% agarose, 1 M KCl) with Ag/AgCl electrodes connected to a voltage source and a current-to-voltage converter. The low-impedance electrodes were very stable, and the junction potentials were compensated for electrically. The amplifier (CEZ-2300, Nihon Kohden, Tokyo, Japan) signal was monitored using a low-pass filter (E-3201A, NF Co., Yokohama, Japan) with an oscilloscope (VC-6045, Hitachi) and a strip chart recorder (R-61, Rika Denki, Tokyo, Japan), and was simultaneously recorded on a videocassette recorder using a pulse code modulation processor (PCM-501ES, Sony). Just after for-

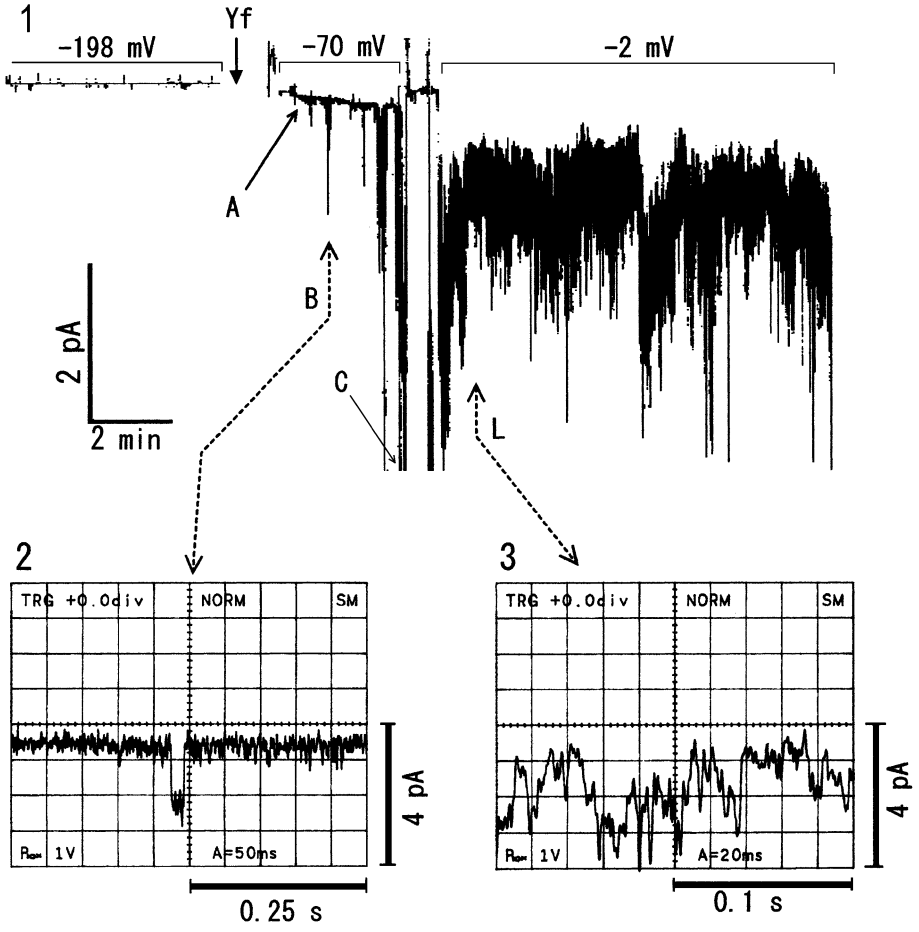

Fig. 2. Effect of yolk on the electric current across PBM. Horizontal axis=time, vertical axis=electric current. (1) Currents during one measurement. Holding voltages are indicated above the trace. After adding 40 μg protein/ml yolk (Yf) and setting the voltage at −70 mV, the current gradually increased and exhibited light fluctuation (A) frequently, channel-like fluctuation (B) occasionally, and an explosive spike (C) every time. The type C fluctuation was quickly followed by PBM rupture during most measurements. PBM that did not rupture became leaky (4 of 23 membranes) and exhibited the chaotically fluctuating current of the L state (L). (2) Type B wave shape enlarged from (1). (3) L state wave shape from (1). The L state fluctuations were quite random compared to those of type B.

mation, PBM alone was tested at ±198 mV (the maximum command voltage of the amplifier) for 1 h. This normal PBM had an electrical resistance of ca. 2000 GΩ; current fluctuation was trivial and was attributed to background noise. Sample aliquots (2.7–60 μg protein) were then added to the *cis* chamber and stirred gently for 5 min with a magnetic stirrer. The PBM potential was maintained at the initial voltages, −70 mV for Yf and −198 mV for isolated proteins, until the PBM threatened to rupture. When large spikes in current occurred, the potential was quickly switched to 0 V and then carefully adjusted to negative values at which current fluctuations continued moderately.

3. Results and discussion

3.1. Crude yolk

Liposomes and Yf were mixed (for a final total protein content of 2.5 mg/ml), incubated at room temperature for 0.5 or 2 h, and quickly frozen. The electron micrographs of these samples show that most of the fine particles in Yf tend to aggregate on the phospholipid membrane, and some can even span the membrane (Fig. 1). After both the 0.5- and 2-h incubations, fine particles were adsorbed by and accumulated around liposomes, resulting in liposome assembly, and several fine particles (10–20 nm in diameter) spanned the liposome membranes (Fig. 1).

PBM experiments for Yf were performed using 23 membranes (Fig. 2). Early in the measurement period, three types of current fluctuation were identified and designated types A, B, and C. Type-A fluctuation gradually increased during measurement and was observed in most membranes. Type B, which occasionally appeared within type A, was similar to the current produced by gating activity of ion channels. The maximum conductance for types A and B was 10 pS and 20–30 pS, respectively. Type C was observed in every membrane, and reflected an abrupt, drastic increase in conductance. As mentioned in Materials and methods, the PBM potential was reduced as soon as type C occurred, to avoid membrane rupture. However, of the 23 membranes tested, only four survived type-C fluctuations, and those then exhibited chaotic fluctuations even at lower voltages; we call this the L state (Fig. 2). The random wave shape of the L state, characterized by a mean conductance of at least 100 pS and sometimes in excess of 1 nS, is far different than any produced by ion channels known to date. We suspect that type-C fluctuation indicates the beginning of the L state.

Since a PBM can control membrane potential and thus is more useful than liposomes as a model, we can induce the same fine Yf particles as those spanning the liposome membranes to enter and span the PBM at the appropriate voltage more readily than they would enter liposomes. A PBM that contains fine particles nearly 10 nm in diameter must be leaky and in danger of rupture. In preliminary PBM experiments, Yf caused current fluctuations, usually at negative voltages (−50 to −70 mV) but seldom at positive voltages (+50 to +70 mV); thus, we chose to use an initial voltage of −70 mV in subsequent experiments. We predicted that −70 mV is the voltage at which fine particles can pass into the PBM. The PBM tends to rupture at the onset of the L state, during which current fluctuations are chaotic. Therefore, we suggest that the L state is induced by fine particles entering the PBM, which then gains about 100 pS conductance per particle.

Since an initial voltage of −70 mV is physiologically reasonable for a plasma membrane potential in the laboratory, the event depicted in Fig. 2 probably also occurs in the plasma membrane of the *B. mori* blastoderm; fine particles

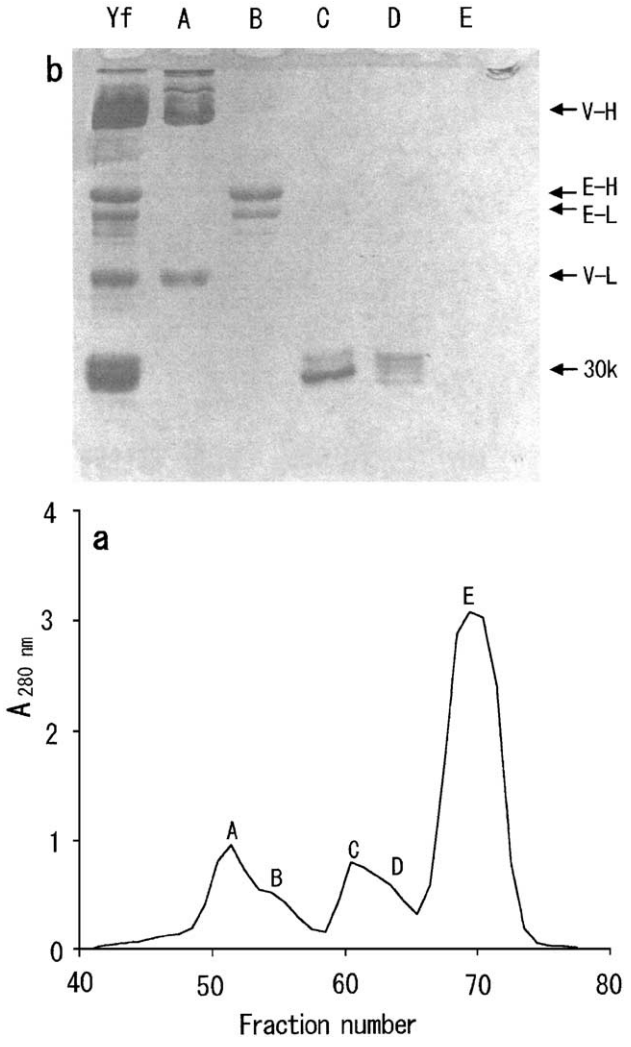

Fig. 3. Fractionation of *B. mori* yolk. (a) Elution profile of each fraction on a Bio-Gel A-15m column chromatography monitored at 280 nm absorbance. Buffer (150 mM KCl, 0.5 mM EDTA, 50 mM Tris–HCl, pH 7.4) was used for equilibration and elution at room temperature. Five peaks (A, B, C, D, and E) were recovered from Yf (from 0.25 g eggs). (b) SDS-PAGE was used to analyze Yf, A, B, C, D, and E from the column on a 10% gel stained with Coomassie blue. Vtn is represented by two bands (V–H, V–L), ESP by two bands (E–H, E–L), and 30k by four adjacent bands (30k). Faint Vtn bands appear in lane B. No bands appear in lane E.

in *B. mori* yolk slowly move into the blastodermal plasma membrane during early embryogenesis, even if there are no enzymes on the membrane.

3.2. Isolated proteins

Yf from 0.25 g of *B. mori* eggs was applied to a Bio-Gel A-15 m column. The Yf elution profile is characterized by five peaks (corresponding to fractions A, B, C, D, E) (Fig. 3). SDS-PAGE analysis showed that fraction A contained vitellin (Vtn), fraction B contained egg-specific protein (ESP), and fractions C and D contained 30-kDa proteins (30k). These four fractions are known as the major *B. mori* yolk proteins; 30k is a mixture of four proteins with molecular weights of approximately 30 kDa, whereas the molecular weights of Vtn and ESP are 420 and 225 kDa, respectively. Vtn is heterotetramer, consisting of two 178 kDa polypeptides and two 42 kDa polypeptides. ESP

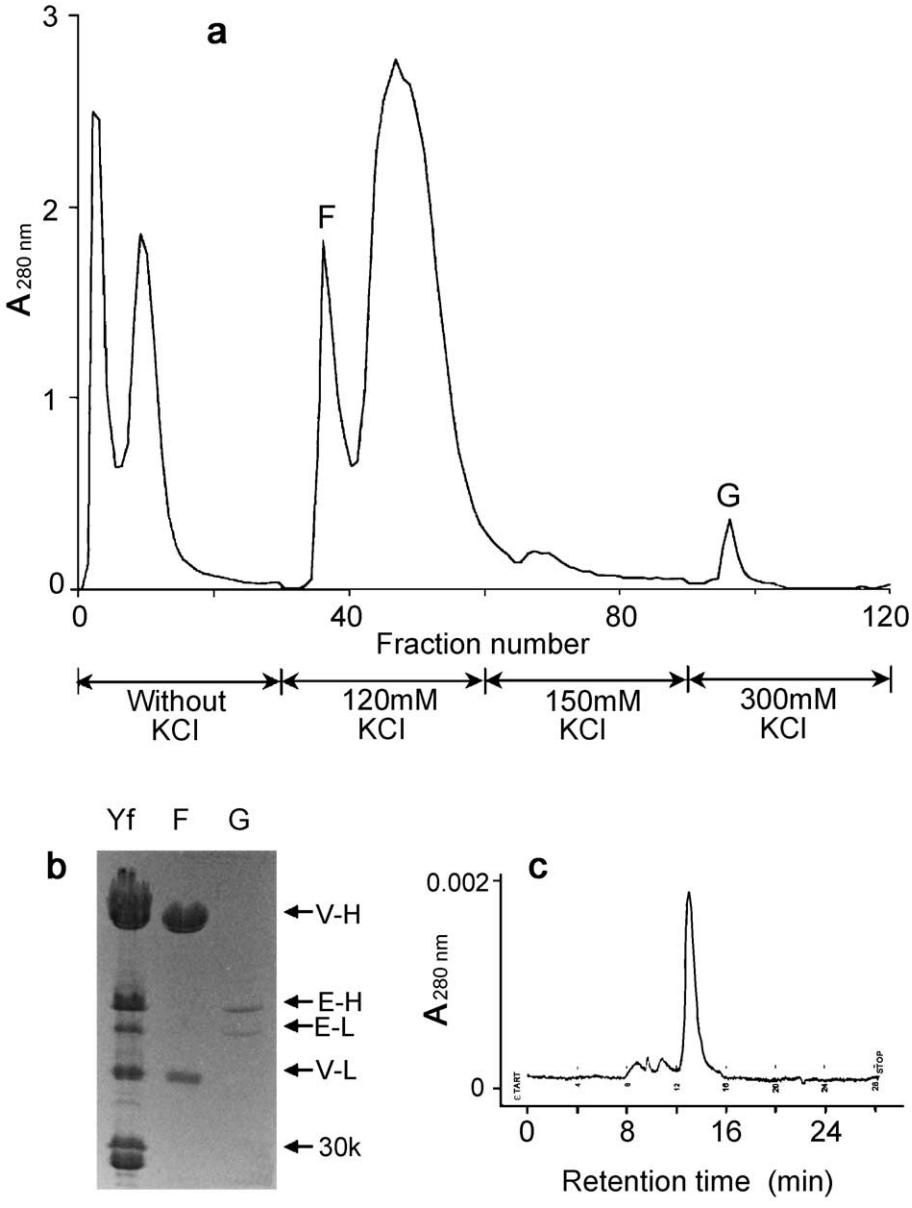

Fig. 4. Separation of ESP from *B. mori* yolk. (a) Elution profile of ESP on a DEAE-cellulose column monitored at 280 nm absorbance. Buffer (50 mM phosphate, pH 7.2) was used for preconditioning and initial elution; KCl was added stepwise for further elution. The column was maintained at 4 °C. Peaks F and G were recovered from Yf (from 0.5 g of eggs). (b) SDS-PAGE was used to analyze Yf, F, and G from the column as described in Fig. 3b. Each band is labeled as in Fig. 3b. (c) Fraction G from DEAE-cellulose column was injected into a TSK gel G3000SW column, eluted using HPLC and buffer (100 mM sodium sulfate, 50 mM phosphate buffer, pH 7.2) at room temperature, and monitored at 280 nm absorbance for testing the purity of recovered ESP. The fraction yielded a single major peak.

is heterotrimer consisting of two 72 kDa polypeptides and one 64 kDa polypeptide [2,5–8]. Fraction E was slightly yellow and yielded no band on the SDS-PAGE gel, so this fraction likely contained pigment, not protein, and is responsible for the yellow color of *B. mori* yolk. The A and B peaks overlapped on the elution profile, and SDS-PAGE analysis of fraction B yielded pale Vtn bands. We applied each of the five fractions to the PBM and measured their activity within 100 mV. Although fractions C, D, and E induced no fluctuation in the electric current, fractions A and B sometimes induced fluctuations at −70 to −100 mV (data not shown), suggesting that Vtn and ESP are responsible for the current fluctuations observed in the PBM.

To efficiently isolate ESP, we applied Yf from 0.5 g of *B. mori* eggs to the DEAE-cellulose column, and eluted proteins stepwise, using 0.12, 0.15, and 0.3 M KCl plus buffer A (Fig. 4). Vtn and ESP were recovered in fractions F and G, respectively. However, fraction F was slightly yellow, and was assumed to be contaminated with the same pigment found in fraction E (Fig. 3). The HPLC elution profile of fraction G had a single peak (Fig. 4c). PBM experiments with ESP and Vtn were repeated using fractions G (iESP) and A (gVtn), respectively (gVtn was used instead of fraction F to avoid contamination by the yellow pigment). For reference, we used BSA, a soluble protein well-known for its interaction with phospholipid membranes [9].

Figs. 5 and 6 show the results of PBM experiments with iESP, gVtn, and BSA. Fifty membranes were tested with iESP, and whereas none exhibited type-A or -B fluctuations, each exhibited a drastic fluctuation in transmembrane current identical to type C (Fig. 2). Those PBMs that survived type-C fluctuations then exhibited the L state (Fig. 5), which was maintained at lower voltages (−2 to −20 mV), as in Fig. 2. The survival rate of PBM treated with iESP was 40% (20 of 50 membranes). All PBMs treated with gVtn and BSA experienced type-C fluctuations, and the surviving PBMs—only 11–13%—showed the L state at lower voltages. Since we chose −198 mV as the initial voltage specifically to induce type-C fluctuation within 1 h, it was not surprising that type-C fluctuation was found with the iESP, gVtn, and BSA samples. However, the survival rate of PBM was higher if treated with iESP than with gVtn or BSA, suggesting that iESP can easily induce the L state in PBM. Since the movement of fine particles into PBM likely accounts for the L state, as was shown using Yf, the ESP molecule can probably also pass

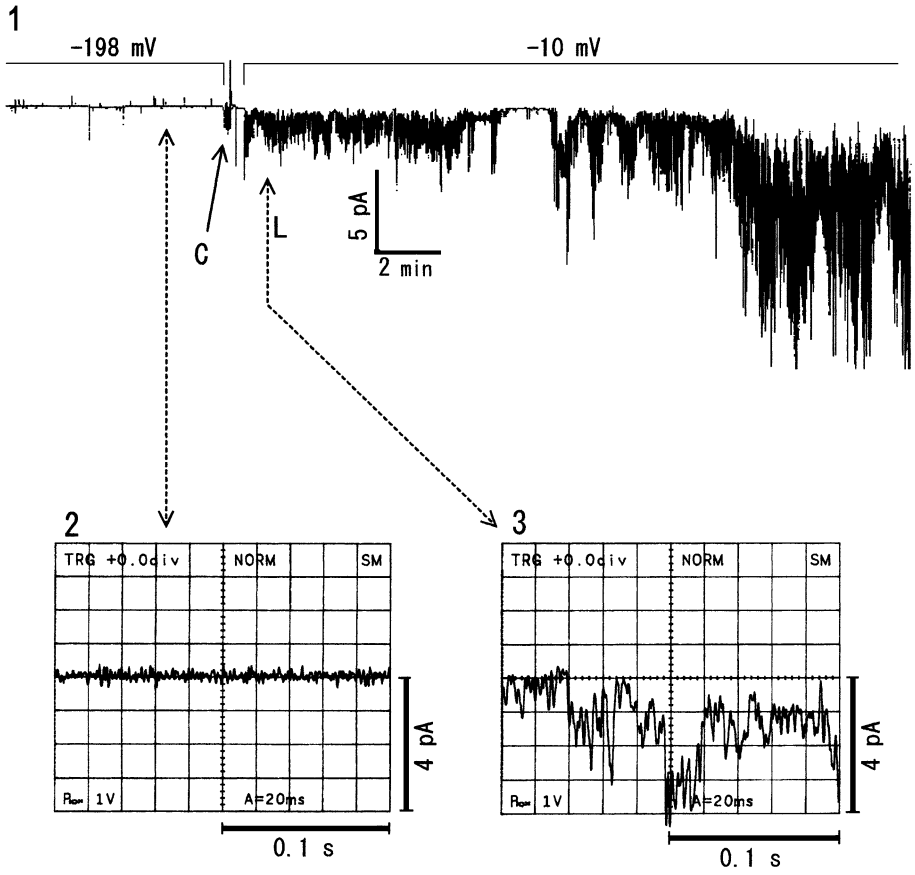

Fig. 5. Effect of ESP on electric currents across the PBM. Horizontal axis=time, vertical axis=electric current. (1) Currents during one measurement, before which ESP (4 μg/ml) was added to the *cis* side. The current was almost zero at the beginning of the −198 mV application. A spike occurred suddenly (C), and 20 of 50 membranes survived, although these became leaky and experienced L states (L). These C and L fluctuations are similar to type C fluctuations and the L state (Fig. 2). (2) Enlargement of the wave shape before the spike and (3) in the L state.

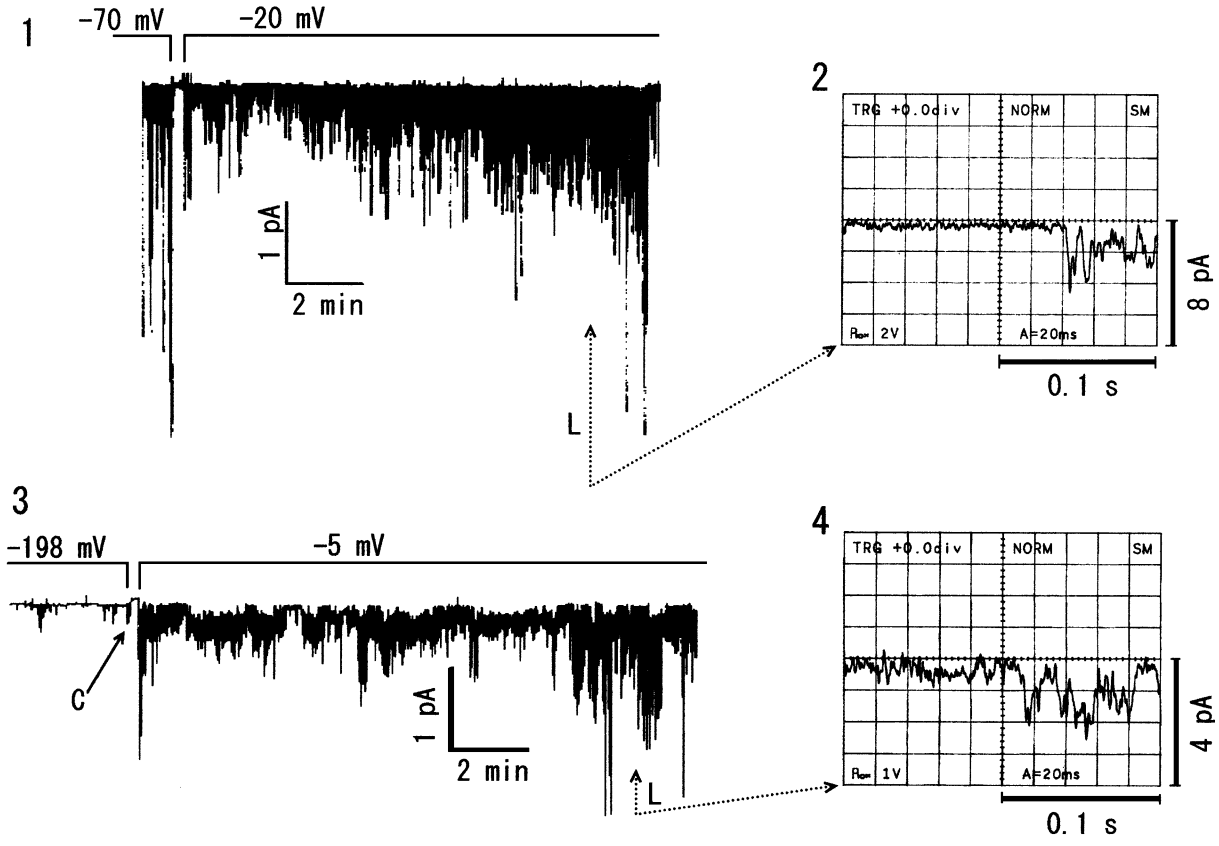

Fig. 6. Effect of Vtn and BSA on electric currents across PBM. Horizontal axis=time, vertical axis=electric current. (1) Currents with Vtn during one measurement. Before this trace, the current spiked at −198 mV in response to the addition of Vtn (5 μg/ml) to the *cis* side, then voltage was reduced to −70 mV. Only 2 of 16 membranes survived the spike and these exhibited the L state (L). (2) Enlargement of the L state wave shape in panel 1. (3) Currents with BSA during one measurement, before which BSA (2.7 μg/ml) was added to the *cis* side. Slight current fluctuations sometimes occurred at −198 mV, and an explosive spike occurred (C). Only 2 of 18 membranes survived the spike and these exhibited the L state (L). (4) Enlargement of the L state wave shape in panel 3.

into the phospholipid membrane without rupturing it. To test this prediction, we incubated a mixture of liposomes and iESP (0.1 mg/ml protein) for 24 h at room temperature. Quick-freeze fracturing of the mixture showed a fine particle (oval in shape; 10×20 nm) spanning the liposome membrane (Fig. 7). Despite the long incubation period (24 h), fewer fine particles were found spanning the membrane or aggregating on the liposome than with the Yf particles, possibly because the ESP concentration was low or the membrane potential was not optimal for particle movement.

3.3. Discussion of ESP

ESP and Vtn are complex proteins. The amino acid sequences of ESP and vitellogenin (a precursor of Vtn) contain putative asparagine-linked glycosylation sites and serine-rich domains that tend to be phosphorylated during posttranslational processing. Furthermore, in ESP, the sequences of a putative lipid binding site and a metal binding site were also found [10–13]. SDS-PAGE gels stained with the appropriate dyes indicated that both ESP and Vtn contain sugar, phosphate, and lipids [14], and a radioactive labeling method proved that phosphorylation

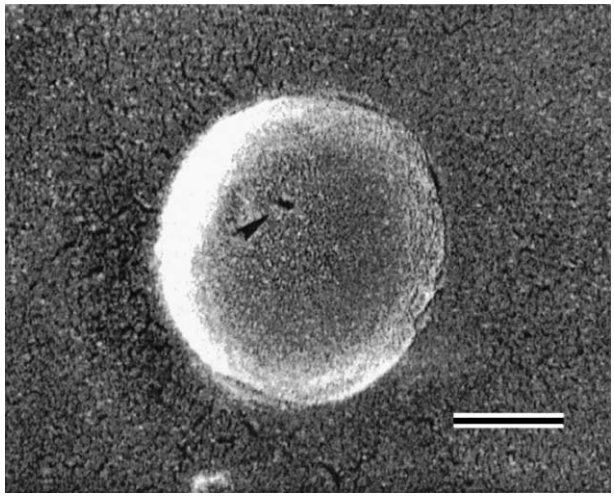

Fig. 7. Freeze–fracture electron micrograph of liposomes mixed with ESP and incubated at room temperature for 24 h. Refer to Fig. 1 for the methodologic details and legend. The oval pore (10×20 nm) on the Vf (arrowhead) indicates that a fine particle spans the membrane. Scale bar=100 nm.

occurred on the serine residues of both proteins [15,16]. Moreover, atomic absorption/flame spectrometry indicated that these proteins contain Zn, Ca, and Mg, and that ESP also contains Cu [8]. Incidentally, although the isoelectric points of these proteins have never been reported, the elution profile of the DEAE-cellulose column (Fig. 4) suggests that at pH 7.2, Vtn is negatively charged and that ESP is more negative. As for the secondary structure, ESP was estimated to be 26% α-helix, 18% β-sheet, 24% β-turn, and 32% random coil [17].

Several reports have introduced circumstantial evidence for the peculiarity of ESP. In newly laid *B. mori* eggs, Vtn, ESP, and 30k account for approximately 40%, 25%, and 35% (w/w) of the total soluble proteins, respectively. In the eggs of many other insects or oviparous animals, however, the yolk has a simpler protein composition, wherein Vtn accounts for more than 80% of the total soluble protein [2,18–21]. Yolk proteins similar to ESP have been found only in eggs of some lepidopteran insects [2]. Vtn is not essential for embryogenesis in *B. mori*; Vtn-deficient eggs can complete embryonic development [22]. *B. mori* Vtn and 30k are produced in the fat body and are transported to oocytes via the hemolymph, whereas ESP is produced by the ovary in *B. mori* [5–7]. ESP stores are exhausted completely during embryogenesis in *B. mori*, but Vtn and 30k are present at larval hatching [2]. Although they do not describe the role or function of ESP, these reports suggest that ESP, compared with other yolk proteins, is significant to *B. mori* embryogenesis.

Our results indicate that ESP has a specific function; however, it is worth adding a statistical elucidation. Therefore, using the null hypothesis that the PBM survival rates of ESP and the other protein should be identical and setting 0.05 as the level of significance, the differences in the survival rates of these proteins were tested by comparison with the estimated value of the standard error (z-test). When treated with ESP, Vtn, and BSA the numbers of PBMs that survived were 20 of 50, 2 of 16, and 2 of 18, respectively. For ESP versus Vtn, the difference in the survival rates is given by 20/50−2/16=0.275, whereas the standard error is estimated to be 0.135. Consequently, z is 0.275/0.135=2.03, which is large enough (>1.96) to reject the null hypothesis. For ESP versus BSA, z is 0.289/0.129=2.24, resulting in rejection of the null hypothesis. Therefore, the main role of ESP in determining the PBM survival rate is demonstrated statistically.

The Yf results show that *B. mori* yolk contains fine particles that can permeate phospholipid membrane, and suggest that such permeation induces the L state in PBM and is promoted by negative membrane potential. The iESP results demonstrate that ESP can easily induce the L state in PBM at −198 mV and can pass into the membrane unaided. However, which components of Yf can permeate the phospholipid membrane? The diameter of the ESP molecule, approximately 20 nm when determined by electron micrograph using rotary shadowing (data not shown), is consistent with that of the fine particles that spanned the membrane (Fig. 1). Given that ESP is similar in size to Yf particles (spanning the membrane) and in its tendency to induce the L state, we conclude that ESP is the most probable candidate for the Yf particles, and that ESP likely permeates the blastodermal plasma membrane during early embryogenesis in *B. mori*. Why, then, does Yf induce the L state at lower negative potentials than iESP? Yf alone causes a gradual increase in PBM conductance (Type A) that precedes the L state. ESP, a soluble protein 20 nm in diameter, can permeate the phospholipid membrane (about 5 nm thick) without bursting it. Permeation of the phospholipid membrane by ESP probably aids development of the *B. mori* blastoderm, although the intrusion of proteins or peptides generally results in cytotoxicity, e.g., defensin [23], magainin [24], cecropin [25], sarcotoxin [26], and islet amyloid polypeptide [27]. These issues should be studied in the future.

4. Conclusions

We studied *B. mori* yolk proteins by observing liposome membrane structure and conducting experiments with PBM, and came to the following conclusions:

1. We could induce soluble protein to permeate PBM and observe protein behavior on a molecular scale.
2. ESP can permeate PBM unaided and without causing the membrane to burst.
3. When contained in the yolk, ESP permeates PBM at a physiologically normal membrane potential much more quickly than isolated ESP.
4. ESP likely permeates the blastodermal plasma membrane without the help of other enzymes during embryogenesis in *B. mori*.

Acknowledgements

We thank Dr. Hajime Hirata for his invaluable advice on PBM techniques and for providing the phospholipid. We also thank Dr. Yasushi Tamada for his instruction on protein fractionation, and Ms. Takako Seki for technical assistance.

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