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Rapid report

Unusual protein behavior illustrated with silk fibroin

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Abstract

We investigated the interaction between phospholipid membranes and silk fibroin recovered from the posterior silk gland of the silkworm. Observations of the planar lipid bilayer membrane and electron microscopic observations of liposomes showed that newly constructed silk fibroin, existing in the form of filaments, quickly penetrates phospholipid membranes without bursting them.

Keywords: Silk fibroin; Bilayer membrane; Transmembrane fibril; Silk gland

Silk fibroin is a representative fibril-forming protein that is synthesized in cells located on the surface of the posterior part of the silk gland in the silkworm *Bombyx mori*; it is subsequently secreted into the lumen and transported to the lumen of the middle part of the silk gland for storage until required for spinning [1,2]. Silk fibroin, which will fibrillate on spinning, is in a weak gel state in the posterior silk gland [3]. Amyloidogenic proteins behave in several ways, leading to fibril formation or membrane disruption [4–6]. State changeability and fibril formation are characteristics common to amyloidogenic proteins and silk fibroin, which led us to suspect that they share other common characteristics, including their interaction with membranes. However, the molecular mass of silk fibroin is far greater than that of amyloidogenic proteins. Silk fibroin exists as a 2300-kDa molecular complex, which consists of heavy (350 kDa) and light (26 kDa) chains, and P25 (30 kDa) [1]. Silk fibroin may thus be too large to interact with membranes. Previously [7], we showed that *Bombyx mori* yolk protein (225 kDa), which is an almost spherical molecule 20 nm in diameter, was incorporated smoothly into a phospholipid bilayer membrane (about 5 nm thick [8]), suggesting a possible interaction between silk fibroin and membranes. Furthermore, such an interaction may play a physiologically important role in secretion via cell membranes in the posterior silk gland. Although two reports suggest that silk fibroin is secreted by exocytosis [9,10], the secretion mechanism remains unclear, as those reports depended on circumstantial evidence. It is possible that the secretion involves an interaction between the cell membrane and silk fibroin.

Therefore, we examined the interaction between phospholipid bilayer membranes and silk fibroin of the posterior silk gland using both planar lipid bilayer membranes (PBM) and electron micrographic observations using liposomes and quick-freeze fracturing. To collect intact silk fibroin, which aggregates readily even with a slight stimulus, the posterior silk glands were excised from *Bombyx mori* (N137 × C146) on the 7th day of the fifth larval instar and were carefully homogenized in buffer (150 mM KCl, 5 mM Tris–HCl, pH 7.4) by pressing slowly. After keeping the homogenate on ice for 1 h, the supernatant was recovered as silk fibroin from the posterior silk gland (PSGF) and stored at −85 °C until used for experiments. The experiments with PBM and observations of liposomes used essentially the same methods as reported previously [7], except for slight modification as follows. The buffered salt solution (150 mM KCl, 5 mM Tris–HCl, pH 7.4) used to homogenize the posterior silk gland was also used to prepare the PBM and liposomes in order to unify the composition of

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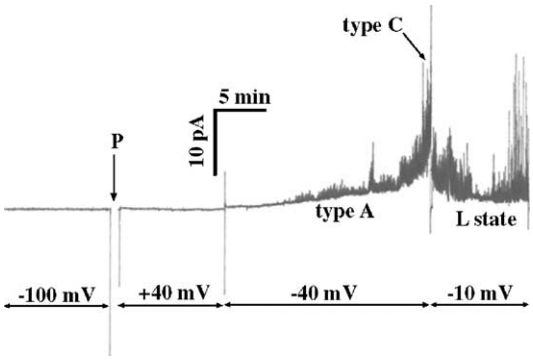

Fig. 1. Electric current across the PBM during one measurement. Horizontal axis: time, vertical axis: electric current. The holding voltages are indicated below the trace. Despite the addition of PSGF (arrow P), the current remained near zero at the membrane potential of +40 mV, as well as in the control at −100 mV. Upon switching to −40 mV, the current increased gradually with small fluctuations (type A) and finally exhibited explosive spikes (type C). A PBM that survived the type C fluctuation exhibited a continuously fluctuating current (L state), even at a lower potential, e.g., −10 mV.

the aqueous phase. The lipid used for the PBM and liposomes was soybean phospholipid with no cholesterol added. As in the previous report, the two 1.5-ml chambers flanking the PBM were designated *cis*, the chamber connected to the virtual ground, and *trans*, the chamber to which the command voltage was applied. Just after formation, the PBM was tested alone at ±100 mV for 1 h. PSGF aliquots (60 μg protein) were then added to the *cis* chamber and stirred for 20 s with a magnetic stirrer.

PBM experiments with PSGF were performed using 46 membranes, of which 28 showed the typical transition of electric currents across the membrane after adding PSGF to PBM (Fig. 1). Unexpectedly, these results were similar to those of PBM measurements using crude *Bombyx mori* yolk protein [7]. On giving the membrane potential a negative value, two types of current fluctuation were usually induced, one after the other, and were designated types A and C. The type-A fluctuation was a gradual, long-term increase. In most cases, type A started just after the switchover to −40 mV and lasted for several minutes, along with increasing tremor. Type C, which was characterized by an abrupt, drastic increase in membrane conductance, usually appeared after the current was increased moderately with type A and often threatened to disrupt the PBM. Therefore, the PBM potential was reduced when type C occurred to avoid membrane rupture; consequently, the PBMs exhibited chaotic fluctuations at even lower voltages, we termed this the L state (Fig. 1). The random wave shape of the L state was characterized by minimal conductance of 100 pS and sometimes indicated a conductance of several nS. Note that the occurrence of current fluctuations was dependent on the direction of the membrane potential. Type-A fluctuation occurred only at a negative potential (−40 mV), while a current fluctuation other than type A occasionally appeared at +40 mV. We chose −40 mV because current fluctuations seldom occurred at −30 mV, but type A nearly always occurred at −40 mV.

Liposomes and PSGF were mixed (for a final total protein content of 2 mg/ml), incubated at room temperature for 30 min, and frozen quickly. For reference, PSGF alone was frozen quickly. Replicates of fractured samples showed that PSGF contained filaments nearly 15 nm in diameter and that those filaments adhered to liposomes (Fig. 2). Furthermore, it was evident that the filaments skewered the liposome, i.e., the filament penetrated the liposome and traversed the liposome lumen. One filament even appeared to skewer two liposomes.

The membrane potential of a PBM can be controlled, making it a more useful model than liposomes. Thus, at the appropriate voltage, we could induce the penetration of PBM by filaments like those skewering the liposomes more readily than in the case of liposomes. That is, the time required for a filament to penetrate a PBM was much shorter

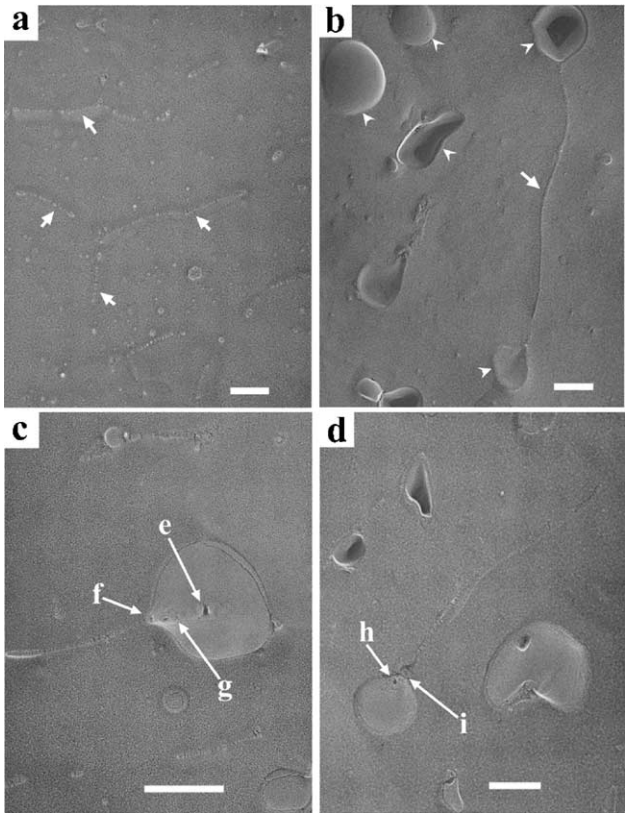

Fig. 2. Freeze-fracture electron micrographs of liposomes plus PSGF. The fracturing method splits the liposome membrane along its hydrophobic interior, resulting in a convex (stained light) or concave (stained dark) face. Scale bar=200 nm in each panel. (a) PSGF alone as a reference, featuring a filament similar to a string of beads (arrows), nearly 15 nm in width. (b) Liposomes plus PSGF, showing a filament (arrow) and fractured liposomes (arrowheads). Two separated liposomes adhere to a filament. (c) Liposome skewered by a filament. The pore in the liposome (e) indicates that something pierced the membrane, leading to the conclusion that a filament pierced the membrane on the left side of the liposome (f), traversed the lumen while raising the membrane (g), and escaped from the lumen (e). (d) A liposome similarly skewered by a filament that pierced the membrane twice (h, i).

than 30 min. A PBM that is penetrated by filaments, which are nearly 15 nm in diameter, will be leaky; PSGF usually produced a leaky state in the PBM at negative voltages (−40 mV) but seldom at positive voltages (+40 mV; Fig. 1). Therefore, we postulated that a membrane potential of −40 mV causes filaments to penetrate the PBM and that the L state of the PBM is induced by a filament perforating the PBM, which then develops a conductance of at least 100 pS. As one study elucidated that silk fibroin in the posterior silk gland exists in the form of filaments [11], we postulated that the filament present in PSGF (Fig. 2) is silk fibroin and that the method used to prepare PSGF was sufficient to maintain native silk fibroin.

Our results include four unique features. (1) Huge protein molecules dispersed in the aqueous phase were incorporated into the phospholipid membrane unaided. (2) The phospholipid membrane did not burst on incorporation of the protein molecules, although the protein was in the form of filaments with diameters far in excess of the membrane thickness. (3) After being incorporated into the membrane, the protein molecules escaped to the opposite side of the aqueous phase unaided. (4) These unique behaviors are unknown in a fibril-forming protein, although this protein has been utilized since antiquity. Some proteins or peptides can spontaneously enter a bilayer membrane without disrupting it; however, such molecules are small compared with the membrane thickness and are toxic to the host cell [12,13]. In an exceptional case, egg-specific protein (ESP; 225 kDa), which is one of the *Bombyx mori* yolk proteins, was found in a previous study to enter a membrane, using the same methods as used here [7]. The rate at which PBMs converted to the L state was postulated to correlate with the ability of the protein to be incorporated into the membrane without disruption. The L state production rates were 40% for ESP [7] and 61% for silk fibroin, which clearly differed. Silk fibroin was incorporated into phospholipid membranes in the form of a filament (Fig. 2), which is a super-molecular complex that is much larger than an ESP molecule. Despite its large size, silk fibroin is more easily and more safely incorporated into membranes than is ESP, indicating that silk fibroin has unique characteristics with respect to membranes. The primary element of the filament of silk fibroin is thought to be the heavy chain, which is estimated to be 60 nm long and 15 nm wide [5], closely matching the diameter of the filament. We found that filaments could traverse the liposome lumen through a 200 nm range (Fig. 2) and that these filaments contained several heavy chain molecules. The heavy chains slipped into the membrane from the outer aqueous phase and escaped from the membrane to the opposite aqueous phase (liposome lumen). With ESP, incorporation into the membrane was observed, but escape to the opposite side was never detected, which is the most crucial difference between ESP and silk fibroin. Therefore, silk fibroin, which is a very familiar material, has unprecedented and unique characteristics that are distinct from those of ESP.

When skewering the liposome, the direction of filament transfer may be axial or perpendicular to the filament axis. In the latter case, the filament must swing perpendicular to its axis to cut the liposome. The former case, in which the motion of the filament relative to the liposome is similar to that of a train passing through a tunnel, is unlikely, as this would require a long-distance transfer (200 nm) within 30 min, necessitating a driving force from an electrical field or motor protein. Therefore, the motion perpendicular to the axis is probable. This is supported by the finding that the filament traversed the liposome lumen through a peripheral zone close to the membrane (Fig. 2), indicating that the putative transfer against the liposome involves a reasonably short distance.

Our results strongly suggest that silk fibroin, newly biosynthesized in the posterior silk gland cell, has the ability to pass through cell membranes unaided, but we cannot elucidate whether its secretion into the silk gland lumen is owing to this ability, to exocytosis, or to both. We plan to develop a technique for anchoring cells to the surface of a material, with one side of the filament skewering the cell and the other side adhering to the material coated with silk fibroin. To do this, we will take advantage of two features of silk fibroin: its newly discovered ability to be incorporated into phospholipid membranes and its well-known property of assembly for solidification. However, we will need to assess whether the filament skewering damages cells.

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