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Silk production in a spider involves acid bath treatment

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We studied physiological conditions during the spinning of dragline silk by the garden cross spider, *Araneus diadematus*. Silk is converted from the liquid feedstock in the gland into a solid thread via a tapering tubular duct and exits at a spigot. The distal part of the tubule appears specialized for ion transport and the management of the pH inside the lumen. Thus, it appears that spider silk *in vivo*, like some industrial polymers *in vitro*, is spun through an acid bath.

Keywords: biopolymer; extrusion; fibre; proton pump; spinning

1. INTRODUCTION

The largest silk gland of the garden cross spider *Araneus diadematus* is called the major ampullate and produces the safety- or drag-line (Wilson 1962*a*; Vollrath 1992). As in other spiders, the linear pathway for the production of this important silk thread has the following components: a progressively widening secretory region; a storage ampulla; a progressively narrowing spinning tubule; a valve; a distal duct; and a spigot where the formed silk thread passes to the exterior (Kovoor & Zylberberg 1972; Townley *et al.* 1991).

It is unclear how the thread is actually formed, although it is commonly assumed that shear forces somewhere along the production line will transform the aqueous liquid silk into insoluble solid thread through a liquid crystalline transition (Kerkam *et al.* 1991). It has further been suggested that a muscle-actuated valve acts as a clamp on the thread and as an extrusion die to further reduce the thread's diameter (Wilson 1962*b*).

We studied the duct leading out of the glandular sac to describe the extrusion process up to the valve, as processes along the duct seem rather important for the formation of the silk. Our observations of the epithelium of the duct suggest that water is extracted from the feedstock while the pH of the lumen is lowered to facilitate the gelling of the silk.

2. MATERIAL AND METHODS

Mature specimens of the spider *Araneus diadematus* were obtained from the wild. For ultrastructural examination, material was fixed in a solution containing 2% glutaraldehyde, 0.1 M sodium cacodylate–HCl buffer (pH 7.4) and 1% sodium chloride. After embedding in Epon, sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. The localization of a proton pump was investigated in whole mounts of fresh major ampullate glands by vital staining with 0.001% neutral red. The uncharged dye is free to diffuse through vacuolar membranes, but becomes trapped when it acquires a positive charge in the acidic (proton-rich) environment (Bulychev *et al.* 1978; del' Antone 1979; Sit *et al.* 1996), i.e. at pH 7.4. Controls were incubated (1 h; 20 °C) in spider Ringer's solution (Schartau & Leiderscher 1983) containing either sodium vanadate (1 mM) or bafilomycin Al (2 mM) or 10 mM ouabain before vital staining; sodium vanadate is a specific inhibitor of v-ATPase (Lubansky & Arruda 1985). Magnesium-dependent p-phenylphosphatase activity was demonstrated at the ultrastructural level using the method of Mayahara *et al.* (1980). The material was first fixed (4 °C; 30 min) in a solution 0.1 M phosphate buffer (pH 7.4) 2% formaldehyde; 0.25% glutaraldehyde and 1% sodium chloride. The control incubation medium contained 10 mM ouabain. To examine the effects of pH on the viscosity of the protein feedstock, fresh major ampullate glands were dissected in Ringer's solution at pH 7.4 and samples of the contents of the ampulla were transferred to solutions at 20 °C of different pH. The solutions had been prepared by adjusting Trizma-buffered Ringer's solution with dilute hydrochloric acid or sodium hydroxide solutions.

3. RESULTS

In *Araneus diadematus* the spinning tubule is surprisingly long and looped back on itself twice and bound tightly together within a connective tissue sheath to form an elongated 'S' (see figure 1*a*). The cuticle lining of the lumen is positively birefringent, appearing blue or yellow depending on orientation (figure 1*b*).

Examination by transmission electron microscopy of ultrathin sections of the distal part of the spinning tubule and the distal duct revealed a single layer of tall columnar epithelial cells underlying the cuticle of the duct (see figure 2*a*). The epithelium shows a range of morphological characteristics which suggest specialization for ion and water transport (figure 2*b*): (i) the apical surfaces of the epithelial cells show numerous fine and decorated microvilli (60 nm in diameter) closely packed together; (ii) the high density of the material between the apical surface of the

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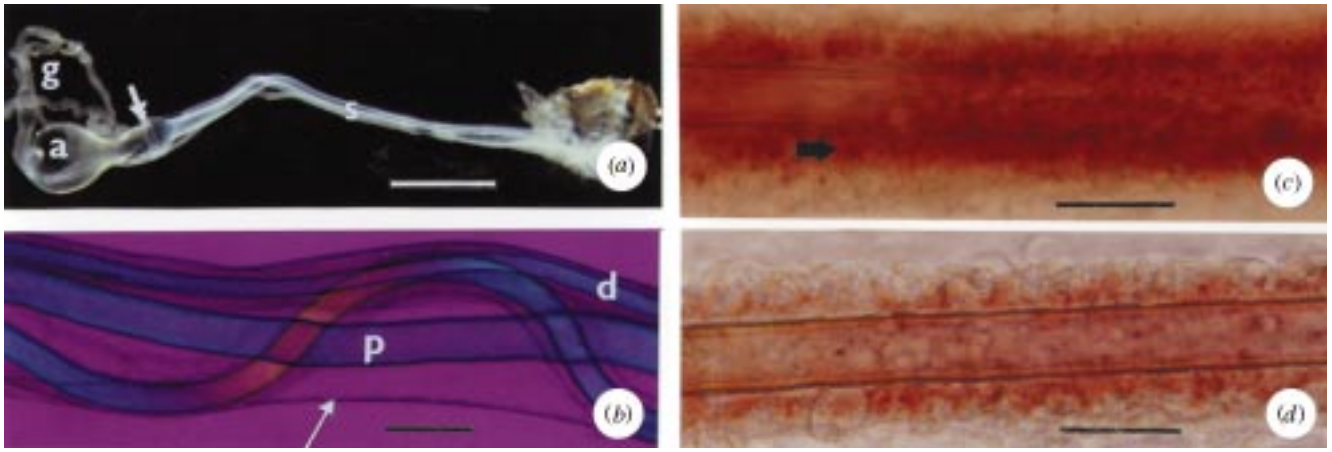

Figure 1. Unfixed major ampullate gland and duct of the spider *Araneus diadematus* mounted in spider Ringer's solution. (*a*) Dark field illumination. The secretory region (g) passes secretion to the ampulla (a) which opens (arrow) into the spinning tubule. The three limbs of the spinneret (s) are bound together. Scale bar, 2 mm. (*b*) Crossed polars with a first-order red plate showing positive birefringence. The three limbs of the spinneret, decreasing in diameter from proximal (p) to distal (d), are bound together in an S-shaped arrangement within a common connective tissue sheath (arrow). Epithelium appears to be absent from the proximal part of the tubule. Scale bar, 200 mm. Distal duct of the major ampullate gland. (*c*) The duct was vitally stained in 0.001% neutral red in spider Ringer's solution at pH 7.4 and mounted whole. Numerous vesicles containing neutral red can be seen in the apical cytoplasm (arrow) of the columnar epithelial cells. Scale bar, 50 mm. (*d*) The distal duct shows a marked reduction in vital staining after pretreatment with 1 mM sodium vanadate in spider Ringer's solution. Scale bar, 50 mm.

cell and the cuticle lining the lumen supports the implication that water is transported out of the lumen; (iii) the lateral surfaces of the cells are sealed apically by elongated septate desmosomes and show extensive interdigitations (figure 2); (iv) the basal membrane of the cells show numerous deep infoldings; and (v) the cells contain numerous mitochondria located above and below the centrally positioned nucleus and, in the apical cytoplasm, large numbers of vesicles, some with decorated membranes. In contrast, the epithelium surrounding the proximal parts of the spinning tubule is greatly reduced and is partly absent in the initial segment.

Ultrathin sections of the distal duct show small quantities of low-density, flocular material surrounding the silk thread in the lumen of the duct. Examination of freshly dissected tubules showed this to be an aqueous solution. We studied the location of the draw-down process by examining 1 μm-thick plastic sections with the light microscope. Drawdown of the thread—indicated by its reduction in diameter and pulling away from the cuticle lining the lumen takes place in the distal third of the spinning tubule and is almost complete before the thread reaches the valve. We used vital staining with neutral red to investigate whether a proton pump was present in this material (figure 1*c*,*d*). The epithelial cells of the distal part of the spinning tubule and the distal duct rapidly took-up neutral red into vesicles concentrated in the apical cytoplasm in large amounts (figure 1*c*). A gradient of neutral red in the epithelial cells started at low levels in the proximal part of the spinning tubule and increased progressively towards the spigot. Both contents and cuticle lining of the lumen rapidly stain red, possibly indicating intraluminal transport of protons. Almost no neutral red was taken up by the epithelial cells of the secretory part of the ampullate gland or those lining the ampulla itself. Staining of the spinning tubule and distal part of the duct appeared to be (i) slightly inhibited by pre-incubation for 1 h with 10 mM ouabain (not shown); and (ii) markedly inhibited by 1 mM sodium vanadate (figure 1*d*), and by 2 mM bafilomycin A, specific inhibitors of v-ATPase (Lubansky & Arruda 1985).

The ultrastructural localization of a transport ATPase in the epithelial cells of the distal part of the duct was investigated using p-phenylphosphate as a substrate (see figure 3). An intense reaction was seen on the lateral and basal surfaces. The reaction was largely inhibited by 10 mM ouabain. Thus, two ATPases could be present: (i) an ouabain-sensitive ATPase at the basal and lateral surfaces of the cell; and (ii) a vanadium-sensitive v-ATPase pumping protons into vesicles within the cytoplasm.

The viscosity of the secreted silk precursor within the lumen of the ampulla was assessed subjectively by microscopic examination in spider Ringer's solution at pH 5.5 and pH 7.4. The secretion behaved as a viscous liquid at pH 7.4, but set to a gel at pH 5.5. Transferring the gelled material to Ringer's solution at pH 7.4 restored it to a viscous solution.

4. DISCUSSION

Our observations of the epithelium lining the distal part of the spinning tubule of the major ampullate gland in the spider *Araneus diadematus* demonstrated that it strongly resembles comparable epithelium in insect Malpighian tubules which is implicated in water and ion transport (Berridge & Oschman 1969, 1972). There is direct evidence for the progressive removal of water as the silk feedstock flows through the distal part of the secretory pathway in the spider *Argiope aurantia* (Tillinghast *et al*. 1984). In our *Araneus* micrographs, the numerous microvilli, and some of the apical vesicles of the epithelial cells, were decorated in a way

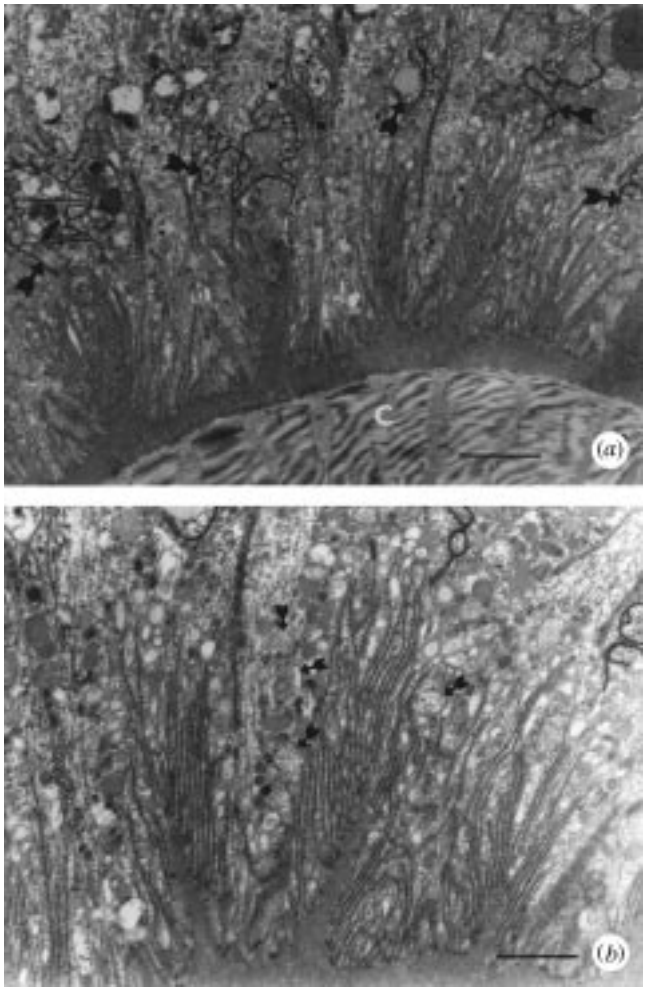

Figure 2. Transmission electron micrograph of an ultrathin section of the apical part of the transporting epithelium of the distal duct of the major ampullate gland of *Araneus diadematus*. (*a*) The epithelium showing extensive septate desmosomes (arrows), numerous apical microvilli and the cuticle (c) which lines the lumen. Scale bar, 2 mm. (*b*) A higher magnification of the apical surface of the epithelial cells showing numerous vesicles (arrows) and microvilli. Scale bar, 1 mm.

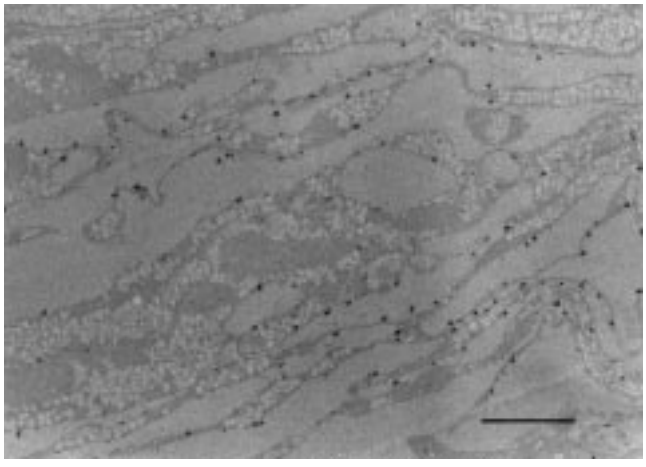

Figure 3. Cytochemical demonstration of a transport ATPase in the distal duct using magnesium p-phenylphosphate (Mayahara *et al*. 1980; Knight & Lewis 1992). Reaction product is seen on the lateral cell surfaces close to the base of the epithelial cells. Scale bar, 1 mm.

that resembles the proton-transporting membranes in the contractile vacuole of *Paramecium caudatum* (Fok *et al.* 1995).

Our observations on neutral red uptake suggest that the distal part of the spider's spinning duct used a v-ATPase to transport hydrogen ions into apical vesicles. If these are then secreted by exocytosis, it may increase the pH of the lumen and consequently the viscosity of the feedstock, enabling a thread to be drawn and/or processed after drawing. It is possible that the bundling of the three limbs of the S-shaped tubule in connective tissue allows hydrogen ions pumped into the two distal limbs to be withdrawn from the proximal limb which lacks a transporting epithelium. This would maintain a relatively high pH in the proximal limb helping to keep the viscosity of the protein feedstock low before the pH is lowered distally. These findings are of interest in view of evidence that there is a progressive acidification in the silkworm *Bombyx mori* as silk feedstock flows through the distal part of the secretory pathway. This may favour the β-configuration of the fibroin (Magoshi *et al.* 1994).

The exceptional toughness and high tensile strength of some spider silks (Kaplan *et al.* 1994) has generated considerable interest in the commercial use of these biopolymers. In view of this, several spider-silk genes have been isolated, cloned and expressed in prokaryotes (see, for example, Lewis 1992; Kaplan *et al*. 1994). Whereas this represents an important step, little progress has so far been made towards elucidating the conditions involved in the spinning of silk by the spider. Our study suggests that the spider's processing of her silken life-line may resemble the processing of industrial polymers, such as rayon, which are spun or extruded into an acid bath. However, Nature's method of drawing-down a filament directly from protein feedstock is no doubt more sophisticated, as well as more economical, than industry's methods for extruding fibres through orifices or extrusion dies.

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