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Electron microscopy of fibril–matrix interactions in a natural composite, insect cuticle

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The extent and stability of the interactions between the fibrils and the matrix of insect cuticle are shown. Effectively the fibrous phase is a polysaccharide with a very closely attached protein coat. The matrix consists of more loosely bound protein.

1. Introduction

Insect cuticle is a composite material made of the polysaccharide chitin (poly N-acetyl glucosamine) and a mixture of proteins. Cuticle differs from most composites found in animals in that the polysaccharide is the fibrous phase of cuticle and it occurs as a much larger volume fraction (10–50%). The fibres are bundles of polymer molecules with a fibril diameter of 2.8 nm [1]. The length of the fibres is imprecisely determined but probably of the order of several micrometers. The fibres are arranged in sheets with all the fibres in each sheet parallel. The arrangement of sheets varies resulting in structures varying from purely helicoidal material (in which the orientation of the fibres is rotated a small amount from sheet to sheet) to purely preferred orientation. How the proteins are attached to the fibres is not known. It has been suggested [2] that they may be covalently linked through glucosides but confirmation is still required. All of the protein is not in contact with the fibres and the interactions between matrix proteins is a more vexed question [3, 4].

Cuticle behaves, mechanically, as a composite [5] and has been subjected to its most rigorous analysis to date on the assumption that it is a two-phase composite [6, 7]. This is a first approximation since the matrix is heterogeneous both chemically and physically. For an understanding of the dynamic mechanical properties of cuticle it is important that something is known about the extent and type of interactions between the components. Some aspects of the chitin–protein interactions are reported here.

2. Materials and methods

The abdominal cuticle of fifth instar *Rhodnius prolixus* Stål, was used. *Rhodnius* is a blood-sucking bug; larvae can take a meal 10 times their unfed body weight. To accommodate this the abdominal cuticle increases in surface area four-fold. The abdominal cuticle is completely helicoidal. The chitin content is approximately 12% dry weight and 80% of the rest of the dry weight (protein) can be easily removed [8].

All cuticles were prepared by scraping off the attached tissue with a blade, soaking the cuticle in distilled water for 1 min then cleaning the cuticle with a pad of damp filter paper until all the epidermis was removed. Some cuticles were then extracted with 2:1 (v/v) methanol:chloroform for 24 h, or 0.1 M phosphate buffer (pH 7) or 8 M urea for 24 h or incubated in pronase in pH 8 buffer for 24 h, all at room temperature, or digested in 1 M NaOH at 70° C for 24 h.

For electron microscopy (EM) treated and untreated cuticles were homogenized in 0.1 M ammonium acetate (pH 7) in a blender then sonicated for 5 min. Large lumps of material were removed by a short spin in a bench centrifuge. The supernatant was examined. The sample was sprayed onto carbon-coated EM grids which were then sprayed with 1% uranyl acetate and the grids dried at room temperature. Coated samples were prepared by drying a drop of sample on to a carbon coated grid than shadowing the grids at 45° with tungsten in an Edwards coating unit. All samples were examined in a Phillips EM 300.

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3. Results

Fibres were found to be similar to those reported from other insects and isolated by other methods (Fig. 1), they have a diameter of approximately 3 nm. Negatively stained fibres from whole cuticle show no apparent structure. If cuticle has been extracted with urea the fibres clump making determination of any structure difficult with negative staining. Shadowed preparations are more successful.

It is necessary to sonicate macerated cuticle to obtain a dispersed enough suspension to allow a sufficiently thin layer of material to be produced for examination by electron microscopy. Preparations made by this method from whole cuticle show individual fibrils which taper at the ends up to 1 μm long with irregularly spaced lumps of material attached (Fig. 2). When cuticle has been extracted with an aqueous solvent prior to maceration these large lumps do not persist although tapered fibrils are present (Fig. 3). Extraction with an aqueous solvent prior to, or during, preparation of the material can remove much of the protein, especially after maceration [8]. In order to preserve as much protein in the preparation as possible, cuticles were extracted and prepared with an organic solvent. Because of hydrophobic effects this will render much of the protein globular but it also shows that the protein is fairly regularly spaced along the fibrils (Fig. 4). These proteins

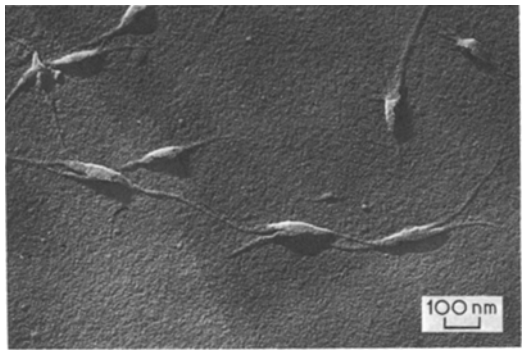

Figure 2 Electron micrograph of an individual fibril with large lumps of protein attached, prepared from whole, untreated *Rhodnius* cuticle, shadowed with tungsten.

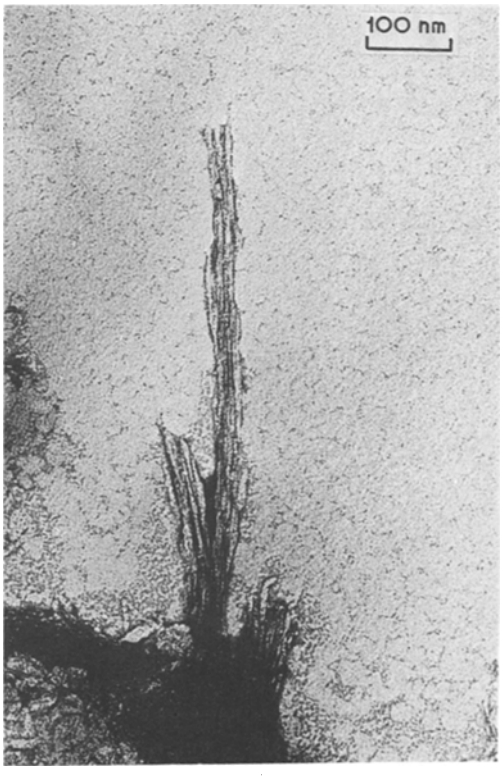

Figure 1 Electron micrograph of chitin microfibrils prepared from *Rhodnius* cuticle after digestion in M NaOH. Negatively stained with uranyl acetate.

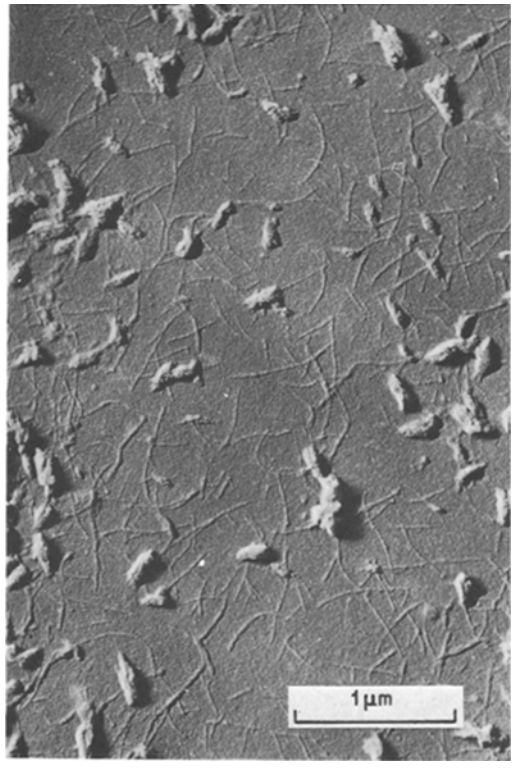

Figure 3 Electron micrograph of individual fibrils from *Rhodnius* cuticle after extraction with pH 7 buffer, no large amounts of protein appear. The preparation has been shadowed with tungsten.

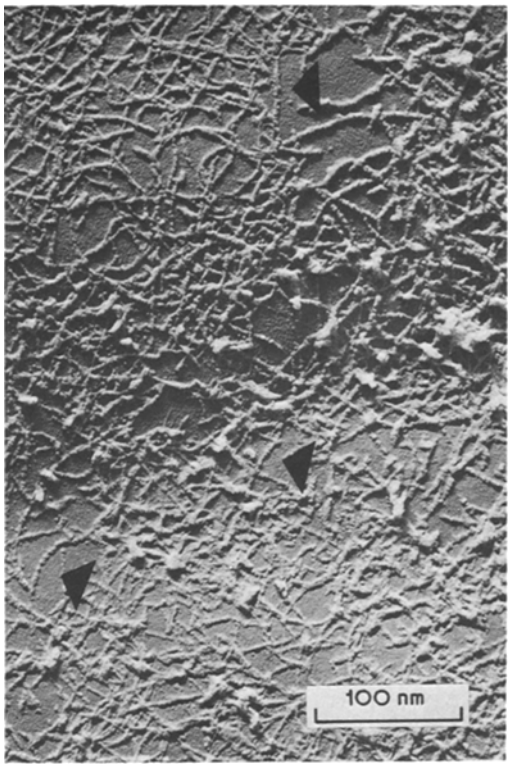

Figure 4 Electron micrograph of individual fibrils prepared from *Rhodnius* cuticle after extraction with methanol:chloroform. The proteins have formed globular beads due to the organic solvent. The preparation has been shadowed with tungsten.

must have a labile attachment to the fibrils as they are removed by aqueous solvents (Fig. 3).

After pronase treatment high magnification of shadowed preparations show a regular structure with a periodicity of less than 10 nm (Fig. 5).

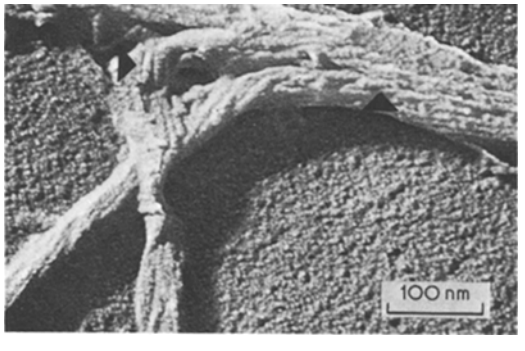

Figure 5 Electron micrography of fibrils prepared from *Rhodnius* cuticle after incubation in pronase showing a very fine regular structure. The preparation has been shadowed with tungsten.

4. Discussion

The interactions between the components of insect cuticle are not well understood. It has been suggested [3, 4] that quite stable secondary bonds are formed between matrix proteins and that these are sufficient to explain the mechanical properties of cuticle. Other views suggest covalent bonds may be present but there is little opinion regarding the bonding of protein to chitin.

The results presented here suggest a strong affinity of protein for chitin in cuticle; other work suggests the involvement of covalent bonds [2]. Chitin has been used as the substrate in affinity chromatography of soluble cuticular proteins and it has been shown that close secondary interactions can exist between the two [9]. This, however, is an artificial system as the chitin has been extracted with alkali before use. The regular repeat structure and tapered fibres shown in Fig. 5 even after digestion in pronase suggest that there is a very stable layer of protein attached to the chitin so that it is fairly resistant to enzymic digestion. The complex should be considered a true glycoprotein. Superimposed upon the glycoprotein are layers of more loosely bound proteins, the lumps seen in most figures. These are probably not covalently bound in *Rhodnius* cuticle as they can be removed by denaturants but they are sufficiently well attached to survive treatment with an organic solvent.

Extractable matrix proteins from insect cuticle have been partly characterized and have been shown for *Rhodnius* to be different, at least in amino acid composition, to those which are inextractable [10]. Cuticle therefore consists of two phases. There is the chitin microfibril which is covered with a well attached protein sheath, the coated fibril is then in contact, in a more or less regular way, with the true matrix consisting of several different proteins. The matrix then, because of the affinity of protein for protein, interacts with the fibrils to produce an efficient composite.

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