SOME ASPECTS OF THE BIOCHEMISTRY OF CHITIN-PROTEIN

COMPLEX IN THE INSECT PROCUTICLE

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Introduction

The epicuticle--notwithstanding its thinness--is probably the most efficacious and important obstacle to the penetration of pathogenic fungi in the insect body. But the bulk of the insect cuticle is made of the procuticle, which is almost exclusively made of chitin and protein, and which always contains chitin everywhere. The procuticle is much thicker than the epicuticle (several hundred times thicker) and obviously represents a further obstacle to the parasitic fungi.

As fungi rely on chitin and thus on a chitin-synthesizing system for the elaboration of their cell walls, it is obvious that interactions are likely to occur between the development of pathogenic fungi and the metabolic events in the insect integument, in which chitin biosynthesis and chitin biodegradation processes play a prominent part.

Because lipids, waxes, and epicuticle are treated elsewhere in this publication (see articles by M. Locke and C. J. Blomquist), we will focus this short review on some fundamental aspects of chitin and chitin-protein biochemistry in the insect cuticle. Recent, more thorough, reviews can be found in Andersen (1979), Hepburn (1967), Locke (1974), and Richards (1978).

The Procuticle: Main Characteristics

The procuticle is entirely built up by the epidermal cells, which form a continuous and monolayered epithelium in direct contact with the

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innermost layers of the cuticle. The procuticle lies beneath the epicuticle, which includes, from inside, a protein epicuticle layer (a cross-linked network of lipids and proteins), a cuticulin envelope, and wax layers, covered by a superficial cement layer.

When the cuticle is stained, for instance with the Mallory's triple stain procedure or with the "Azan" (azocarmin-alinin blue-Orange C) of Haidenhain, three or four principal layers appear conspicuously: exocuticle, mesocuticle, endocuticle, and an innermost "deposition layer."

With the light microscope, the procuticle is seen as being formed of a succession of lamellae. With the electron microscope, these lamellae appear as beds of fibers, which sometimes appear to be parabolically arranged, passing from one lamella to the next one. According to the original interpretation of Bouligand (1965, 1971), this image of parabolically arranged fibers is only the result of optical illusion. The fibers are thought, on the contrary, to run parallel to the surface, but with a slight and progressive change in the fiber orientation, from one bed to another, so that there is a change of 180° between two successive laminae. This feature would give rise to the optical image of parabolically arranged filaments.

The lamellate aspect of the procuticle thus is due to the arrangement of the chitin-protein fibers. According to Blackwell and Weih (1980), the basic structural unit is a microfibril made up of a central crystalline chitin, about 2.8 nm thick, containing 18 to 21 chitin molecules running antiparallel (which gives the typical X-ray diffraction pattern of the crystallographic form α-chitin, according to Rudall, 1955). The crystalline chitin is surrounded by a protein sheath, so that the microfibril diameter is 7.25 nm. The protein subunits are arranged around the chitin core, as shown in Figure 1. Of course, the association between chitin and protein in such a microfibril is supposed to be maintained by some bonds between the molecules. We will come back to this point later.

A. Chitin

Chitin is a high linear polymer of β-1,4 linked N-acetyl-D-glucosamine units. Some residues in the chitin chain may be non-acetylated and are perhaps involved in covalent linkages with proteins (Hackman, 1960). Chitin itself mainly exists in the form of a glyco-protein or chitin-protein complex, as already pointed out in 1951 by Richards.
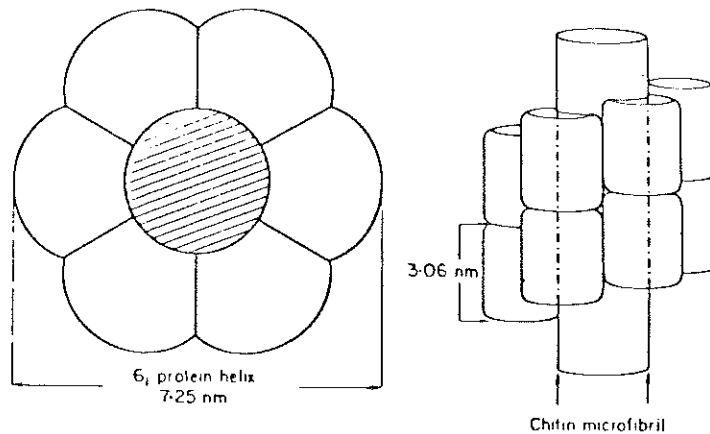

Figure 1. Arrangement of the protein subunits around the chitin core in the microfibril viewed (left) perpendicular to the fiber axis, and (right) along the fiber axis (Blackwell and Weih, 1980).

In insects, the amount of chitin is usually comprised of between 20% and 45% of the cuticle dry weight. Chitin offers a high resistance to usual chemical reagents: it is insoluble in dilute acids or alkalies, and in all the organic solvents. After removal of the other components, the isolated chitin retains all the macroscopic morphological features of the cuticle, but none of its physical properties (hardness, impermeability, color, elasticity), which are given by the other cuticular organic compounds.

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The nature of the proteins associated with chitin and the type of links between proteins give a cuticle layer its hardness (= exocuticle) or its flexibility and softness (= endocuticle). Hard parts (sclerites) correspond to cuticular regions where exocuticle takes up a prominent part of the cuticle thickness, while soft or flexible parts are almost entirely built up of endocuticle.

B. Proteins

There are several different kinds of proteins, with probably different amino-acid compositions, not only from species to species, but also with respect to age, function, and localization, a situation complicated by the possibility of artifacts due to extraction procedures. As a matter of fact, the purification of one single cuticular protein species has never been achieved. A clear picture of protein composition could thus hardly be proposed (Richards, 1978).

Following Trim (1941), these proteins are named "arthropodins" when soluble, and "sclerotins" when insoluble in water or buffers. The protein fractions generally have a relatively high percentage of glycine and alanine (10 to 30%) as well as of proline (7 to 13%) and valine (5 to 12%). Tyrosine is always present (4 to 12%), but sulfur-containing amino acids are almost lacking.

Different protein fractions can be extracted using a series of solvents. These fractions do not correspond to different protein species and composition, but merely to the nature of the binding forces (Hackman, 1960, 1974; Hackman and Golberg, 1978). The cuticular protein pool can thus be fractionated into soluble free protein molecules, proteins linked by Van der Waals forces, hydrogen-bonded proteins, electrovalent bonded proteins, and proteins linked by strong covalent bonds, the latter being only extractable by hot N NaOH.

In the hard exocuticles, the main part of the protein fraction is stabilized in the form of sclerotins. The sclerotization processes involve cross-links between adjacent protein chains. Tyrosine derivatives, especially N-acetyldopamine, seem to be involved in all cases.

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Among the different types of linkages which have been postulated, we will mention:

1. The attachment of β-carbon of N-acetyldopamine to the protein chain through the second amine of diamino acids (what yields keto-catechols by acid hydrolysis) (Andersen, 1976, 1977).

2. The quinone tanning through ortho-diphenols formed from tyrosine through dopa, dopamine, protocatechuic acid, a.s.o., linked in different possible ways to free amino groups of diamino acids in the protein molecule.

However, it must be pointed out that Vincent and Hillerton (1979) believe that there is no obvious chemical evidence for the existence of covalent cross-links, and that this type of linkage, if it does occur, cannot explain the degree of stiffening of the cuticle at scleroti-zation. In the authors' opinion, and as suggested earlier by Fraenkel and Rudall (1940), the strong dehydration which occurs when procuticle turns up to exocuticle (from 70% water down to 12% in some cases) would deeply affect the stiffness of the cuticle and induce secondary bonds (hydrogen bonds) insuring the protein insolubility without covalent cross linkings. Dehydration itself would be controlled by quinones.

A careful reexamination of this mechanism by Andersen (1981) in the locust led him to conclude that dehydration may contribute to cuticle stabilization, but that the observed properties are nevertheless better explained by the existence of covalent cross-links.

In some cuticular portions exposed to mechanical stretching, a rubberlike protein, resilin, may be found. Its properties give the integument a much higher elasticity.

C. Chitin-protein linkage

After careful purification by extraction of all other components, isolated chitin samples always contain small amounts of amino acids, mainly aspartic acid and histidine (Hackman, 1960; Strout and Lipke, 1974; Kimura et al., 1976; Brine and Austin, 1981). It thus appears that strong covalent linkages are involved between chitin and protein

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chains. Hackman (1960, 1976) proposed N-acyl-glucosamine covalent links from the carboxyl group of aspartyl- or histidyl-residues in the protein chain to the free NH$_2$-group of some glucosamine unit in the chitin molecule.

According to Lipke, in highly sclerotized cuticles such as Sarcophaga puparium, chitin may be bound to protein via some neutral sugar (Lipke et al., 1965, 1971a,b). But they also admit that physical interactions may contribute to the stability of the cuticle, in addition to covalent bonds.

Quite recently, Brine (1982) suggested that, at least in Crustacean cuticle, the amide type bonding could be the predominant covalent linkage, mainly in the form of a N-glycosidic linkage between the terminal N-acetylglucosamine of the chitin chain and aspartic acid (or asparagine) in the protein molecule (Figure 2).
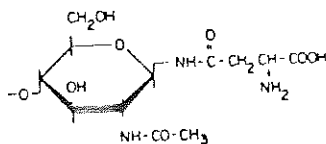

Figure 2. See Brine, 1982.

Whatever the chemical nature of the bonds between chitin and proteins, it is obvious that when associated in some way to protein, chitin is protected against direct hydrolysis by pure chitinases (Jeuniaux, 1963, 1965; Lipke et al., 1971a). Using a purified chitinase as a tool, it is thus possible to estimate the amount of "free" chitin (which is directly accessible to chitinase) and the amount of "bound" chitin, which is only susceptible to chitinase hydrolysis after protein removal, either by NaOH or by proteolytic enzymes (Jeuniaux, 1963, 1964).

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The proportion of free and bound chitin varies considerably according to the type of structure and the zoological origin. For instance, the proportion of free chitin is extremely low in polychaete worms setae, while it has generally been found higher than 60% of the total chitin in mollusk shells.

In insect cuticles, the proportion of free chitin is low (generally less than 5% of the total chitin), even in soft larval cuticles. There is, however, a remarkable exception in the case of the peritrophic membrane, a chitino-proteic muff which is secreted in the middle intestine and which protects the mesenteron cells against injury by food particles. In the peritrophic membranes, the proportion of free chitin is generally much higher than that found in the cuticles (Jeuniaux, 1963).

These observations imply that the enzymatic hydrolysis of chitin in a cuticle is impossible without concomitant hydrolysis of proteins.

Biochemical Alterations During Molting: The Role of the Hydrolases of the Exuvial Fluid

The physiology of the epidermal cells during the molt/intermolt sequence varies according to a precise schedule. The apolysis, or detachment of the apical epithelial membrane, is due to turnover of the apical membrane plaques under hormonal command by ecdysone (Locke and Huie, 1979). In the exuvial lumen thus created, the cells begin to secrete the exuvial fluid containing the hydrolases able to digest the organic material of endocuticle: proteolytic enzymes, chitinase, and β-N-acetylglucosaminidase (or chitobiase)(Passonneau and Williams, 1953; Jeuniaux and Amanieu, 1955; Jeuniaux, 1963). The simultaneous action of proteolytic and chitinolytic enzymes is required to hydrolyze chitin in the chitin-protein complex. The digestion products are chitobiose (= di-N-acetylchitobiose) and free N-acetyl-D-glucosamine (Jeuniaux, 1963; Kimura, 1976), peptides, and free amino acids, which are rapidly absorbed by the epithelial cells.

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The exocuticle generally resists enzymatic hydrolysis, but is able to adsorb and retain the enzymes of the exuvial fluid. The external layers of the old cuticle thus contain high amounts of chitinolytic enzymes, even in the exuviae after ecdysis (Jeuniaux, 1955a, b; David et al., 1971). This fact may have some implications in the control of penetration of parasitic fungi through the cuticle during the premolt period, as the insect is surrounded by a film of chitinolytic and proteolytic enzymes fixed to the old cuticle.

Chitinosynthesis and the Edification of the New Cuticle

After apolysis and the secretion of the molting fluid, the edification of the new cuticle takes place. The first step is the formation of the new apical membrane plaques and of the cuticulin envelope (Locke, 1974) followed by the secretion of the protein epicuticle and of the fibrous chitinoprotein procuticle. The latter is laid down at the level of the apical membrane, at the tip of the microvilli, due to the activity of the same membrane plaques, which are responsible for the orientation of the chitin microfibers (Locke, 1976).

Chitin is synthesized from glucose, mainly liberated from the hemolymph trehalose just before apolysis thanks to the activation of a hemolymph trehalase (Duchateau-Bosson et al., 1963). Other chitin-precursors are fructose, glucosamine, or N-acetylglucosamine (AGA) (Mayer et al., 1980). These compounds are turned into uridine diphospho-N-acetylglucosamine (UDPAGA), which is the direct precursor of chitin biosynthesis. The transfer of one AGA to the growing chitin chain is catalyzed, as in fungi, by a UDPAGA-chitin transferase (Candy and Kilby, 1962; Jaworski et al., 1963, 1965; Surholt and Zebe, 1972).

Our knowledge of chitin synthesis biochemistry in insects has been far behind that of fungi, but recent development of chitin biosynthesis studies in cultivated imaginal discs or cell-free systems will allow a better comparison between these mechanisms. Chitin biosynthesis has been shown to increase with increasing β-ecdyson concentration

(Oberlander et al., 1978), but only after a 24-hour lag period after exposure to the hormone (Ferkovich et al., 1981). According to Cohen (1982), chitin microfibrils in Tribolium tissue culture are elaborated by spheroidal particles which are structurally similar to chitosomes observed in fungi. The relation with apical membrane plaques remains to be examined.

Chitin biosynthesis is probably going to receive better attention, due to the interest of insecticides derived from benzolurea (difluben-zuron, dimilin), which inhibit the achievement of the molt, and are thought to block the formation of chitin.

The edification of the new cuticle does not stop after ecdysis and cuticle expansion. While the deposition of postecdysial procuticle is going on, the pore canals are routes through which the organic material for epicuticle achievement (lipids, waxes) and exocuticle sclerotization and melanization (quinone precursors, phenolases, polyphenoloxidases) are driven.

Conclusion

Both chitin degradation by chitinases and the hormonal control of chitin biosynthesis can interfere with the development of fungi in the invaded insect cuticle. As far as prospective research in applied biology is concerned, it may be suggested that a better knowledge of the biochemistry of chitin biosynthesis in insects would allow a fruitful comparison with that of fungi. The influence of insect molting hormones on the chitin synthesizing system of fungi could also open interesting developments.

Finally, a better knowledge of the mechanisms allowing the penetration of fungal filaments through the cuticle is of course needed: the exceptional resistance of the chitin-protein complex of the exocuticle to enzymatic breakdown by the molting fluid hydrolasas suggests a careful examination of the adaptation of parasitic fungi at the level of their exocellular enzymatic equipment.

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