Sclerotization of Insect Cuticle in a Cell-Free System

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Incubation of deproteinized larval cuticle (chitin flakes) with purified arylphorin (calliphorin) or larval haemolymph of *Calliphora vicina* resulted in the formation of a chitin-protein complex. Enzymatic oxidation of N-β-alanyldopamine (NDAB) in the presence of chitin flakes or the chitin-protein complex, resulted in various degrees of cross-linking of NBAD-quinone formed with chitin. This study confirms the involvement of arylphorin in the process of quinone tanning of insect cuticle.

Introduction

Sclerotization of the exoskeleton is an essential process for insect metamorphosis in which 1,2-diphenolic (N-acetyl-catecholamines) substrates are oxidized by tyrosinase to form nascent quinones in the exocuticle. The diphenolic substrates are ultimately derived from the precursor N-acetyl-dopamine (NADA). Amino groups are added and/or condensed to *o*-quinone derivatives resulting in the covalent linking of structural proteins. The sclerotized cuticle is more or less insoluble in aqueous solutions. Therefore, only few experimental evidences about the nature of cuticle proteins are available. The arylphorins, the major haemolymph proteins of holometabolous insect larvae, which serve as storage of aromatic amino acids [1] and carrier for ecdysteroid hormones [2], are also shown to be involved in cuticle sclerotization [3–10]. Arylphorin appears to be a highly suitable protein to study the mechanism of sclerotization. In order to provide experimental support to the proposed scheme of quinone tanning, we have investigated, using an *in vitro* system, whether chitin flakes could be tanned in the presence of necessary factors.

Materials and Methods

Larvae of the blowfly, *Calliphora vicina,* were reared on goat meat at 25 °C and relative humidity of 65%.

Integuments from 3rd instar larvae were successively extracted in (1) water, 1 h, 20 °C, (2) 7 M urea, 12 h, 2 °C, (3) 0.01 M NaOH, 24 h, 2 °C, and (4) 1 M NaOH, 24 h, 100 °C. This procedure resulted in an almost complete removal of proteins and destruction of cell structures. Such deproteinized cuticle pieces (= chitin flakes) were extensively washed in water and successively in 2 changes of ethanol and then air-dried. In order to get the chitin-protein complexes the flakes were incubated for about 20 h in 3 changes of (a) 5 mg/ml purified arylphorin solution [4], and (b) 10 mg/ml of larval haemolymph in 50 mM phosphate-buffered saline (PBS) as described earlier [5].

Four chitin- and chitin-protein flakes were washed in 50 mM NaH_2–Na_2HPO_4, pH 6.8 and incubated in 3 ml phosphate buffer containing 4 mM N-β-alanyl-dopamine (NBAD) and 75 units of mushroom tyrosinase (Serva, Heidelberg) for 20 h at 27 °C. The incorporation of quinone was observed as brown colouring which was measured by densitometry. The slit of the densitometer was masked by a black film with a central square window (4 × 6 mm) to scan the same area in all preparations.

Results

Oxidation of NBAD by tyrosinase in the presence of chitin- or chitin-protein flakes resulted in various degrees of cross-linking of the oxidation products (NBAD-quinones). Inspection of the flakes for brown colour and densitometric scanning are expressed in Table I. The maximum colouring (comparable to the colour of untreated

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Table I. Binding of oxidation products of NBAD to chitin flakes and chitin-protein flakes.

Untreated chitin flake	Colourless	Adjusted to 100% (blank value)
Chitin flake + NBAD + T	slight brown	79%
Chitin-arylphorin + NBAD + T	light-brown	61%
Chitin-haemolymph + NBAD + T (haemolymph from young 3rd instar larvae)	brown	53%
Chitin-haemolymph + NBAD + T (haemolymph from late larvae)	dark-brown	32%
Untreated puparium	dark-brown	22%

puparia) was in the chitin flakes complexed with haemolymph from late wandering larvae which is supposed to contain high amounts of tanning factors.

Discussion

Very little is known about the characteristics of the cuticular proteins of insects and the nature of their cross-links with chitin and sclerotizing agents. There are some reports on *in vitro* formation of cuticle and the processes of sclerotization and melanization. Because of the limited solubility of the cuticle components, particularly of proteins, cell-free models are useful in demonstrating the molecular interaction and the mechanism of bonding during tanning.

Incubation of chitin flakes with arylphorin and haemolymph resulted in the formation of a chitin-protein complex as we have also shown earlier with purified chitin [5]. Since the enzymatic oxidation of NBAD produces a brown solution and a brown pellet which co-sediments with chitin, it is difficult to assess the actual binding. Therefore, we used chitin flakes instead of purified chitin for our experiments.

N-Acetyl-dopamine has been considered the principal catecholamine metabolite in sclerotization. In insects that form brown cuticles in puparia, pupae, and adults, NBAD has been shown to play a central role (for review, see [11]). Enzymatic oxidation of NADA *in vitro* results in a complex product mixture, irrespective of the presence of aliphatic amines [12]. The resin obtained after drying of the oxidation products shows an increased hydrophobicity. The nascent quinones generated from catechols, protecatechuid acid and DOPA, are capable of precipitating unspecified proteins from cuticle extracts of lepidopteran species [13]. Cuticle proteins from *Calliphora* larvae bind with vegetable tannins to form insoluble complexes [14]. Chitin selectively binds to cuticle proteins of *Calliphora* [15, 16]. These findings deal with cuticle proteins in general without any mention of arylphorins. Grün and Peter [8] described that arylphorins of *Calliphora, Drosophila* and *Manduca,* but not bovine serum albumin (BSA) or ferritin, cross-link with sclerotizing agents. On the other hand chitin has been shown to bind specifically (a) both covalently and non-covalently to *Calliphora* arylphorin, but not to BSA [5], and (b) oxidized NADA [17]. From differential solubility extraction of larval and puparial cuticle proteins of *Calliphora* and electrophoretic analysis it has also been suggested that arylphorin forms both covalent and non-covalent bounds within the cuticle [4].

The present study confirms that during sclerotization, the nascent quinones generated by oxidation of phenolic substrates (NADA, NBAD), attach to chitin, and better to the chitin-protein complex, f.i. chitin-arylphorin. The solubility is decreased due to hydrophobic coating of the protein surface [8, 18]. This is the first report demonstrating insect cuticle sclerotization in a cell-free system.

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