Structural Proteins of Sarcophagid Larval Exoskeleton

COMPOSITION AND DISTRIBUTION OF RADIOACTIVITY DERIVED FROM [7-14C]DOPAMINE*

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In the cyclorrhaphid flies, exoskeletal proteins from the last larval instar cross-link by arylation and glycosylation to form the sclerotized puparial case. Cuticular proteins from maggots killed just prior to tanning were resolved into 21 soluble components by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide electrophoresis. Isoelectric points ranged from pH 4.5 to 6.0, molecular weights were distributed between M, = 16,000 and 24,000. Aspartic and glutamic acids, glycine, serine, valine, and lysine were abundant in all the proteins while sulfur-containing residues were uniformly absent. Heterogeneity was manifest among NH2 termini of the soluble fractions, while the insoluble chitin-linked protein showed only aspartic acid in this position. The sclerotized matrix was assembled by a concerted bridging of protomers without accumulation of di-, tri-, or higher n-mers in the urea-soluble fraction. This mechanism was also favored by uniform distribution of the bridge precursor, [7-14C]dopamine, among the individual larval protomers including the polypeptide bound to chitin. Following administration of isotopic catecholamine 2 to 10 h prior to sclerotization, unbridged larval cuticle retained 3% of the radioactivity, puparial and adult integument 7% and 18%, respectively. Proteolytic digestion afforded labeled peptides with molecular weights in register with the degree of cross-linking. Nonradioactive larval proteins did not incorporate labeled dopamine and exchange incubation of labeled proteins with nonisotopic precursor failed to diminish recoveries of 14C. Since protein synthesis was low as assessed by minimal incorporation of [3H]leucine, metabolites derived from dopamine may have been added after translation in the course of presclerotinal activation of the polypeptides destined for cross-linking.

The transformation of unconjugated soluble polypeptides and chitin-bound protein of maggot procuticle to the hard pigmented puparial sheath coincides with uptake of phenolic intermediates derived from tyrosine, increased covalent bonding between chitin and protein, and reduction in water content (1-3). In the higher Diptera, hardening does not require major synthesis of new structural protein or polysaccharide. Thus the cyclorrhaphid system serves as a good model for the study of tanning (4, 5). The molecular weight of the soluble proteins destined for polymerization is in the range of 12,000 to 26,000, isoelectric points lie between pH 4 and 6, and 60% of the amino acid titer consists of aspartic acid, glutamic acid, glycine, valine, and lysine (6). In contrast to the larval integument, the sclerotized puparium is resistant to proteolytic or chitinolytic digestion, to chemical cleavage with NBS,1 sodium-ammonia, or alkaline borohydride, and to dispersal in chaotropic solvents containing urea, SDS, or alkylamines (2, 7-10). The scheme currently favored for assembly of the arylated matrix features addition of polypeptides to the ring or side chain of catecholamines or phenolic acids. Linkage is affected via ε-amino of lysine or unidentified NH2 termini. Presumably, the benzenoid precursor is activated affording a transient quinone prior to conjugation since molecular oxygen and a tissue-specific phenol oxidase are also required for polymerization (1). The order of assembly of polypeptide protomers also remains to be established in the Cyclorrhapha. Although long lived di- and trimer intermediates have been described from lower orders of insects, the occurrence of partially condensed n-mers has not been examined in the model maggot-pupal system (11, 12).

Enhancement of chitin-protein bonding is also conjectural since chemical evidence is limited to increased recoveries of peptidochitodextrins from chitinase-protease limit digests of puparial integument treated with NBS (2). Post-translational modification of protein and polysaccharide prior to complex formation is likely from evidence provided by tissue analysis and studies with cell-free systems. Isolated cuticle proteins from larvae fail to polymerize in the presence of homologous polyphenol oxidase, Cu2+, and a variety of activators (6). β-Alanine has been recovered from puparial hydrolysates but not from untanned precursor proteins (13). As a preliminary to the investigation of the mechanisms of sclerotization, per se, this contribution describes the isolation and partial characterization of the unmodified larval structural proteins together with observations on the fate of labeled dopamine administered prior to the onset of hardening. A preliminary account of this work has appeared in abstract form (14).

MATERIALS AND METHODS AND RESULTS2

1 The abbreviations used are: NBS, N-bromosuccinimide; PMSF, phenylmethylsulfonyl fluoride; PTTU, phenylthiourea; SDS, sodium dodecyl sulfate; dopa, 3,4-dihydroxyphenylalanine.
2 Portions of this paper (including "Materials and Methods," "Results," Figs. 1 to 3, and Tables 1 to 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 950 Rockville Pike, Bethesda, MD, 20014. Request Document No. 80M-2193, cite author(s), and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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DISCUSSION

Borate-soluble proteins from the procuticle of Sarcophaga bullata have been resolved into 21 homogeneous polypeptides as assessed by isoelectric focusing and SDS-polyacrylamide gel electrophoresis. Attempts to demonstrate heterogeneity by column chromatography on DEAE-cellulose or by electrophoresis on urea gels failed to reveal secondary components within the individual focused bands. Evidence favoring purity of the individual isolates, to be reported in detail in a subsequent publication, has been obtained by identification of a single NH₂ terminus for five of the components (24). The NH₂ termini were not identical, however. Most soluble cuticle proteins from arthropods range from Mₐ = 16,000 to 24,000 and are usually rich in acidic amino acids, glycine, valine, and lysine with somewhat lesser amounts of alanine and serine. The minor variations in these six residues impose heterogeneity of a low order to this large group of functionally related polypeptides. Hackman (25) ascribed this variation to genetic polymorphism. Fristrom et al. (26) examined integumental proteins of Drosophila for separate coding regions and for individual variants in natural populations; neither criterion was in accord with this proposal. Sarcophaga, Calliphora, and Lucilia from the same suborder of Diptera have cuticle proteins similar with respect to isoelectric points, molecular weight range, ratios of hydrophobic to hydrophilic residues, and absence of sulfur-containing amino acids. Drosophila, another cyclorrhaphid, may lack several proteins common to the other two species (26). Similarities in amino acid composition suggest regions of sequence homology, but the variation is sufficient to restrict putative sequence identities to short internal stretches. Restricted homology has been reported for feather keratins from chick embryo (27) and may be common to cross-linked structural matrices comprised of many bridged proteomers. The identification of hydroxylysine in Protein 12 suggests that remnants of the collagenous cuticular basement were also soluble in borate-SDS; hydroxyproline and bound hexose were absent, in common with other insect collagen (28). Proteins 2, 4, 5, 16, and 19 contained single residues of β-alanine, an indicator of the onset of parrunation in this species (29). This amino acid is absent from the cuticle of less advanced stages, suggesting that these proteins are either deposited early during the postfeeding stage or represent an unusual post-translational modification of existing protein. On the basis of the variability in amino acid composition, none of the remaining 14 proteins could serve as precursors for the modified polypeptides by addition of a single residue of β-alanine. In some dipterans and lepidopterans, β-alanine is a constituent of melanoprotein rather than of support substance. Proteins modified by the insertion of β-alanine, therefore, may constitute putative chromoproteins rather than soluble presclerotal initiators of cross-linking (30, 31). Since radioactivity of the β-alanine-enriched components was of the same order as the other proteins acquiring label from 14C]-dopamine (Table III), it is probable that these five entities were modified at the same time as companion proteins, all subsequently participating in the formation of the sclerotized matrix and covalent of the papparium (32, 33).

Proteins ranging from Mₐ = 16,000 to 24,000 would afford di- and trimer intermediates from Mₐ = 32,000 to 70,000 in the course of step-wise cross-linking to sclerotized products. Soluble intermediates were not detected on urea gels (Fig. 2); hence partially tanned structural members either do not exist or accumulate elsewhere than the urea- or borate-soluble fractions. Provided the assumed intermediates occur in measurable amounts, inability to observe their presence may originate in reduced staining following alylation, in declining solubility, or to extensive proteolysis immediately preceding polymerization. However, a search for partially polymerized materials of higher molecular weight on molecular sieves gave no evidence of the presence of larger species with uv-absorption as the criterion of assembly (6). The ubiquity of the dopamine metabolite within the individual larval isolates (Fig. 2) is also in keeping with a concerted assembly rather than the preliminary joining of specific proteins. When borate-extracted larval or sclerotized cuticle was exposed to 0.25 M NaOH in an atmosphere of N₂, low levels of material were solubilized that were excluded from 10% gels, indicative of a molecular mass of 200,000 daltons or greater. Since strong base catalyzes nonspecific cross-linking of tyrosyl and related substituents even in an oxygen-poor environment, evidence favoring these larger oligomers requires further investigation.

On the other hand, diversity of function between structural proteins and an ordered progression of cross-linking may be indicated by the wide variation in amino acid composition, particularly for β-alanine. Following modification by a metabolite of dopamine (Table III), addition of β-alanine to a number of the proteins may initiate concerted bridging.

One-third of the integumental protein fails to dissolve in borate buffer, or cold dilute sodium hydroxide, suggesting covalent linkage to chitin. Although initially unresponsive to digestion by polysaccharases, this fraction is susceptible to proteolysis with the generation of glutamic acid-rich peptidoehitodextrins accessible to chitinase (2, 7). The chitin-peptide bonds are not cleaved by NBS, sulfite addition, alkaline borohydride, Birch reduction, nitrous acid, or dilute sodium hydroxide under conditions inductive to splitting of aspartamido or ester linkages, suggesting atypical joining of the two macromolecules (9, 32–34). The recovery of a single NH₂ terminus, aspartic acid, from the intact chitin-protein complex followed by equivalent yields of the second (valine) and third (alanine) residues introduces several possibilities concerning the distribution of chitin and protein within this complex. Only a single protein species may be involved, or, if the protein component is heterogeneous, companion proteins may be blocked at this locus. The possibility exists that chitin per se or an unidentified bridging group occupies unreactive NH₂ termini rather than the conventional acyl or pyrrolotiamyl groups in blocked proteins. On the other hand, occupation of the COOH terminus seems probable, since prolonged incubation with three carboxypeptidases failed to release amino acids from this position. In view of the resistance of the chitin-protein linkage to alkali, however, acylation of the C3 or C6 hydroxyl of N-acetylglucosamine may be unlikely. Occasional substitution of amino N of the hexosamine in place of N-acetyl remains a possibility.

The incorporation of 14C from the side chain of dopamine during the larval stage when protein synthesis is depressed and catabolism of C7 and C8 of the labeled precursor to 14CO₂ is barely detectable suggests presclerotal activation of the soluble unpigmented proteins. The protein adducts are not formed in vitro, either in the presence or absence of homologous cuticle polyphenol oxidase, a situation in accord with a requirement for modification of the monomers prior to incorporation of the dopamine-derived metabolite (6). The labeled proteins and peptides did not bind to affinity columns specific for o-diphenols either before or after acid hydrolysis, and this property was unchanged by initial treatment with sodium borohydride. These properties introduce the possibility that a portion of the phenol or quinone functions are modified by O-alkylation or by the generation of an open chain structure.

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Sarcophaga Structural Proteins

The housefly's complex neural circuitry allows it to perception of environmental cues in order to navigate effectively in its environment. This involves specific receptors in the antennae that detect airborne ions, such as carbon dioxide and water vapor, as well as visual cues from the ground. The antenna contains sensillae specialized for each type of information. Information from these primary neurons is transmitted to higher brain centers, where it is integrated with other sensory inputs. This complex processing allows the fly to detect and avoid obstacles, and to plan its flight path efficiently.
**Sarcophaga Structural Proteins**

![Image](https://via.placeholder.com/150)

**Table I**

| Protein No. | pI | Molecular Weight | Relative Abundance |
|-------------|----|------------------|-------------------|
| 1           | 4.32 | 127.2            | 1                 |
| 2           | 4.60 | 171              | 4                 |
| 3           | 4.68 | 17               | 3                 |
| 4           | 4.73 | 21               | 1                 |
| 5           | 4.80 | 16               | 5                 |
| 6           | 4.80 | 20               | 7                 |
| 7           | 4.90 | 21               | 6                 |
| 8           | 4.97 | 20               | 13                |
| 9           | 5.01 | 24               | 3                 |
| 10          | 5.08 | 21               | 2                 |
| 11          | 5.13 | 21               | 2                 |
| 12          | 5.18 | 21               | 9                 |
| 13          | 5.31 | 24               | 5                 |
| 14          | 5.33 | 20               | 3                 |
| 15          | 5.35 | 19               | 7                 |
| 16          | 5.50 | 17               | 1                 |
| 17          | 5.53 | 17               | 1                 |
| 18          | 5.75 | 17               | 3                 |
| 19          | 5.80 | 18               | 5                 |
| 20          | 5.90 | 19               | 5                 |
| 21          | 5.96 | 16               | 1                 |

**Table II**

| Protein Name | Values in Residues per Mol of Protein Relative to the Value in Sarcophaga|
|--------------|-----------------------------------------------------------------------|
| Azepinic acid| 25.25                                                                  |
| Glutamic acid| 30.16                                                                  |
| Lysine       | 33.16                                                                  |
| Alanine      | 36.16                                                                  |
| Aspartic acid| 39.16                                                                  |
| Serine       | 42.16                                                                  |
| Proline      | 45.16                                                                  |
| Methionine   | 48.16                                                                  |
| Valine       | 51.16                                                                  |
| Isoleucine   | 54.16                                                                  |
| Leucine      | 57.16                                                                  |
| Tyrosine     | 60.16                                                                  |
| Phenylalanine| 63.16                                                                  |

Fig. 1. SDS-polyacrylamide electrophoresis of sarcophaga proteins stained initially by isoelectric focusing. Lane IEF, homogenized, alcohol precipitable proteins (HPP) and were focused in 1% ampholytes from pH 4.0 to 6.0. Bands were stained with Coomassie Blue. For IEF-electrophoresis, focused bands were visualized by immersion in 0.2% Coomassie Blue R-250. The gel was scanned and then stained with Millipore standards. Dye migration was recorded with a densitometer and a distance of migration was calculated with a membrane scanner. L, carboxyl, 5% white paper; R, white paper stained.

Fig. 2. One-gel electrophoresis of sarcophaga proteins from larvae, with standard and bar graphs and bar charts. Bands from the appropriate lanes were excised with an amorphous pen. bp, on SDS-polyacrylamide gel (SSP) loaded with 25 to 100 mg protein. Molecular weights determined on separate gels with standards described in MATERIALS AND METHODS.
### Table II

| PROTEIN INDEX | PER CENT OF TOTAL ACTIVITY | cpn/mg |
|---------------|---------------------------|--------|
| 1             | 1.0                       | 34     |
| 4             | 1.0                       | 44     |
| 3             | 1.6                       | 34     |
| 5             | 1.4                       | 34     |
| 7             | 2.1                       | 56     |
| 0             | 3.1                       | 44     |
| 6             | 3.1                       | 72     |
| 10            | 6.0                       | 26     |
| 11            | 1.3                       | 28     |
| 12            | 7.7                       | 70     |
| 13            | 7.1                       | 53     |
| 17            | 7.2                       | 49     |
| 14            | 1.0                       | 37     |
| 15            | 1.4                       | 31     |
| 18            | 1.6                       | 30     |
| 19            | 1.8                       | 29     |
| 20            | 3.4                       | 29     |
| 21            | 5.6                       | 18     |
| 22            | 2.1                       | 19     |
| **Control**   |                           | 0.6    |

**Sarcophaga Structural Proteins**

| **Table IV**

| **EXTRACTION MEDIA** | Recovery | Partial | Use | Back | Inadaptable |
|-----------------------|----------|---------|-----|------|-------------|
|                       | %        |         |     |      |             |
| Larval                | 1.2      | 68      | 2,600 | 3   | 3,200       |
| Partial               | 7.7      | 27      | 3,000 | 8   | 4,100       |
| Adult                | 18.5      | 66      | 334,000 | 83  | 1,16,000  |

**Note:**

1. Partial extraction of pupal cuticle also did not exceed 95 recovery.
2. The total extraction of adult was 90.
3. Per cent recovered from outside of total 124, administered.
4. Mean adjusted.
5. Adult abdominal cuticle, thorax and wing.

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Fig. 3. Bioanalog chromatography of proteolytic digest of cuticle from larval and pupal containing [14C] leucine prior to papillation. Digests extracts of the two integralities were digested separately with a, cuticle 14C protease, b, cuticle 14C protease, c, cuticle 14C protease, d, cuticle 14C protease, e, cuticle 14C protease. After digestion the extracts was chromatographed on 3% amyl cellulose at 37°C in a solvent, 50:50:50, 100 ml of water for purification. Porphyrin and peptones were removed under these conditions (Purification). Fractions of 1 cm were assayed at 250 cm and re-processed for determination of radioactivity. a, larval cuticle; b, water papular cuticle.