Chitin-Bound Protein of Sarcophagid Larvae: Metabolism of Covalently Linked Aromatic Constituents†

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ABSTRACT: The borate-insoluble chitin–protein complex, CB-I, from prepuparial sarcophagid larvae was cleaved with chymotrypsin and trifluoromethanesulfonic acid releasing a polypeptide fragment of M_r 68 000. The intact glycoprotein was blocked at the C terminus; the N-terminal sequence of Asp-Val-Ala-His-Tyr was not homologous with seven of the borate-soluble nonglycosylated structural proteins. Bityrosine was identified as a component of the primary chain, both half-residues occupied in peptide linkages. Sclerotization initiated a decline in bityrosine coincident with the addition of soluble proteins to the tanned matrix. The chitin–protein complex also included bound peroxidase, propolyphenol oxidase, and an *o*-diphenol subject to oxidation on activation of the zymogen. In the course of the oxidation N termini declined in accordance with the formation of 1,4 quinonoid cross-links.

At the conclusion of the third larval instar of cyclorrhaphous dipterans, integumental polypeptides cross-link to form the sclerotized puparial case. Structural proteins destined for the tanned matrix consist of a group of 21 borate–NaDod-SO_4[1]-soluble components devoid of carbohydrate and an insoluble fraction, 38% (w/w), linked to chitin (Strout et al., 1976; Lipke et al., 1981). In the course of pupariation, soluble proteins are joined by the insertion of aryl bridges between lysyl and histidyl residues and by an increase in chitin–protein bonds (Kimura et al., 1976; Sugumaran & Lipke, 1982a). In *Sarcophaga bullata* the soluble components do not cross-link when incubated with exogenous dopamine and phenolase and are diverse with respect to termini (Strout & Lipke, 1974; Lipke et al., 1981). The chitin-bound insoluble protein, fraction CB-I, includes benzenoid conjugates that oxidize on activation of a prophenolase incorporated in the chitin–protein complex (Sugumaran et al., 1981). The amino terminus of the complex is uniquely aspartic acid with the carboxyl terminus blocked. The ability of CB-I to developed brown chromophore on activation of the phenolase and exposure to oxygen in the absence of exogenous phenol suggests that sclerotization is initiated by the bound components. Kinetics of solubility loss by the extractable proteins indicate arylation, and addition to the tanned insoluble matrix occurs later in pupariation by concerted reaction with activated CB-I. Thus the two processes differ because arylation of the soluble fraction requires posttranslational modification of a relatively large number of polypeptides and provision of phenolic bridge precursors from the circulation, whereas CB-I utilizes a cuticle-bound phenol and sclerotization is confined initially to a single protein.

In this paper the time course of *o*-diphenol and bityrosine utilization is described for fraction CB-I. Exoskeletal sheaths prepared without activation of phenolase afford an unblocked amino terminus. In accord with a 1,4 nucleophilic addition to quinone, terminal residues become unreactive to phenyl isothiocyanate when the latent phenolase is functional. Recovery of bityrosine also declines as the puparium develops at the expense of both half-bityrosyl residues of the primary chain. When the independent time courses of these aromatic species are considered in relation to the diversity of bridges recovered from tanned puparia, several distinct arylations appear obligatory for completion of the sclerotized integument.

Materials and Methods

Insect Cultures. Sarcophaga bullata and *Periplaneta americana* were cultured according to Lipke & Geoghegan (1971). Dr. G. Goodwin and Dr. J. Stoffalano donated cerambycid and *Tabanus nigrovittatus* larvae from field collections. Proteins from *Drosophila melanogaster* were gifts of Dr. J. Fristrom. *Aedes aegypti*, *Manduca sexta*, and *Spodoptera littoralis* were contributed by Drs. A. Spielman, M. Bade, and A. Navon, respectively.

Preparation of Cuticle Proteins. Borate-soluble proteins and fraction CB-I were prepared in the presence of phenylthiourea and phenylmethanesulfonyl fluoride as described (Lipke et al., 1981). Soluble proteins were resolved by electrofocusing (Lipke et al., 1981), followed by HPLC on an AX-300 aquapore anion-exchange support (Brownell Laboratories) with an ammonium bicarbonate gradient from 0.05 to 0.2 M. The molecular weight of CB-I was estimated by $NaDodSO_4$ electrophoresis in 12% acrylamide (Laemmli, 1970) following cleavage from chitin with 4% (w/w) chymotrypsin. Bound propolyphenol oxidase was inactivated by heating the borate-extracted skins in water at 100 °C for 5 min. When required, the enzyme was activated in unheated skins by partial digestion with Pronase in 0.1 M ammonium bicarbonate, pH 8.1 at 30 °C for 30 min at 70 millitorr, substrate:enzyme = 25. Endogenous substrate oxidation was initiated by the introduction of air. Peroxidase was assayed with antipyrene, and catalase levels were measured with hydrogen peroxide as substrate by the method recommended by the supplier (*Worthington Biochemical Corp. Manual*, 1978). Aromatic constituents in the cuticle were labeled by administration of L-[U-^{14}C]tyrosine to synchronized third instar maggots by injection at a level of 1.5×10^5 dpm/larva.

Analytical Procedures. Hydrolysis of protein and amino acid analysis were as previously described (Lipke et al., 1981). The bound catecholamines were assayed according to Arnow

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; L-dopa, L-3,4-dihydroxyphenylalanine; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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Table I: Amino and Carboxyl Terminal Sequences of Sarcophagid Larval Integumental Proteins[a]

protein[b]	Edman method[c]	termini	
		amino	carboxyl
insoluble			
CB-I	A, M	D-V-A-H-Y-	blocked
soluble			
5-61.48	A	A-L-Q-T-A-N-	nd[d]
5-64.68	A	N-E-D-A-N-V-	nd
5-13.44	A	I-K-A-D-Q-E-	nd
3A-4.4	M	G-H-D-A-G-	-I-V-A
9A-11He[e]	M	D-S-H-P-D-D-V-H-A-G-	-I-A-H-L
12A[e]	M	Y-Y-Y-Y-	nd
14A	M	L-G-H-X-G-G-H-X-G-A-	-H

[a] Amino terminus analyzed by Edman degradation; carboxyl termini assessed with carboxypeptidases A, B, and Y. [b] Borate-soluble proteins resolved by isoelectric focusing according to Lipke et al. (1981) and by HPLC on Aquapore AX-300 as described in the text. [c] Edman procedure: (A) automated; (M) manual. [d] nd, not determined. [e] From Lipke & Henzel (1981).

(1937) following extraction of soluble proteins from the isolated cuticles with tetraborate–$NaDodSO_4$. The residue was washed with water and 1 M HCl and suspended in 1 mL of 0.5 M HCl and 1 mL of 10% sodium nitrite–sodium molybdate. Release of chromogen to the solvent and color development were initiated by addition to 1 mL of 1 M NaOH. Affinity chromatography of catecholamines on borylcellulose followed the method of Sugumaran & Lipke (1982b). Bityrosine and tertyrosine were prepared by the method of Gross & Sizer (1959) and purified by the procedure of Andersen (1966). Isotertyrosine was a gift of Dr. Fujimato, Hamamatsu School of Medicine, Osaka, Japan. Bityrosine was assayed in hydrolysates by the accelerated amino acid program for the Beckman 119C analyzer, eluting in the pH 6.4 buffer between lysine and histidine and on a Phoenix PA-35 cation-exchange resin with a pyridine–acetate gradient (Liebster et al., 1961). Bityrosine in proteolytic digests was also identified by fluorescence and by ultraviolet spectrophotometry, an extinction coefficient of 5.4×10^3 M^{-1} cm^2 for the peak at 283.5 nm being used (Andersen, 1966). Manual sequencing was by a micromodification of the Edman procedure with yields of 90% for cycles 1 and 2 and 55–75% for cycles 3–5 (Zalut et al., 1980). Automatic sequencing was performed by Dr. J. Mole of the University of Massachusetts—Worcester according to Bhown et al. (1981) with repetitive yields in excess of 95%. Amino acid phenylthiohydantoins were identified by HPLC and verified by back-hydrolysis (Mendez & Lai, 1975). Dr. Catherine Costello of the Mass Spectrometer Facility, Massachusetts Institute of Technology, established the molecular mass of bityrosine-containing peptides.

Reagents. Carboxypeptidases A and B, chymotrypsin, Pronase, and peroxidase were products of Worthington, Inc. Carboxypeptidase Y was purchased from Boeringer-Mannheim. Proline endopeptidase and *Staphylococcus aureus* protease V8 were purchased from Miles Laboratories. Bonded C_{18} cartridges (SEP-PAK) were supplied by Waters Associates. New England Nuclear furnished L-[U-^{14}C]tyrosine, specific radioactivity 400 mCi/mmol.

Results

Properties of Fraction CB-I. Development of chromophore on partial proteolysis, amino acid composition, and the presence of a single amino terminus indicated the insoluble component differed from the heterogeneous soluble fraction in structure as well as function. Accordingly, 7 of the 21 soluble proteins were resolved by focusing and HPLC and the first five residues

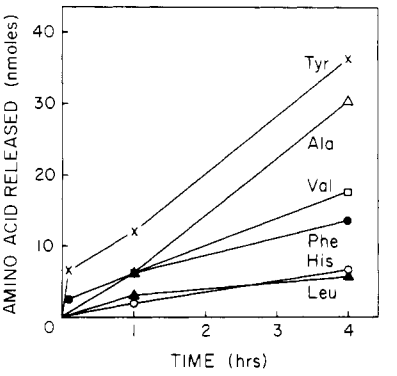

FIGURE 1: Carboxyl terminal sequence of residual chitin-bound peptides after removal of CB-I protein with chymotrypsin. Fraction CB-I (4 mg) was heated to 100 °C in water for 5 min and digested with chymotrypsin for 24 h in 0.1 M ammonium bicarbonate, pH 8.0. The residue was washed with 4 M urea and water prior to sequencing with carboxypeptidase A.

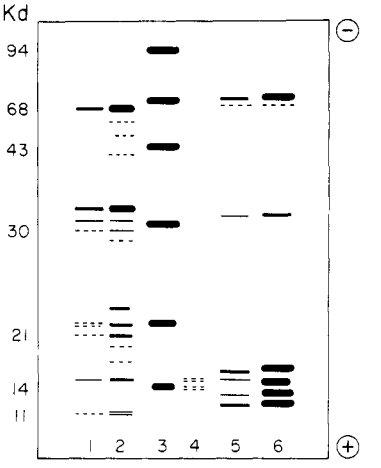

FIGURE 2: Polyacrylamide–$NaDodSO_4$ gel electrophoresis of polypeptides released from CB-I by proteolytic enzymes. Following incubation at 37 °C for the time indicated (CB-I:protease = 25), the soluble portion of the digest was held at 100 °C for 5 min in 2% $NaDodSO_4$–0.015% mercaptoethanol–Tris, pH 7.1. Electrophoresis was performed in 12% acrylamide, pH 8.3, 0.2-mm thick, stained with Coomassie Brilliant Blue: (lanes 1 and 2) trypsin for 8 and 24 h, respectively, (lane 3) molecular weight markers, (lane 4) chymotrypsin only, (lanes 5 and 6) chymotrypsin for 8 and 24 h, respectively.

compared with CB-I. Table I demonstrates heterogeneity at both termini among the soluble proteins as well as between the bound and unbound compartments, CB-I clearly differing from other structural proteins. Extended sequences and homologies with other dipterans will be reported elsewhere. Fraction CB-I was susceptible to partial digestion with chymotrypsin cleaving at a site terminating in the sequence Val-Ala-Tyr-OH as established with carboxypeptidase A (Figure 1). The identical sequence was obtained with carboxypeptidase Y. The molecular weight of the chymotrypsin fragments was estimated by gel electrophoresis following dissociation in $NaDodSO_4$. In the course of digestion, a polypeptide was released of molecular mass 67–68 kdaltons along with smaller fragments of 11–14 kdaltons (Figure 2). At the time of maximum solubilization of the 68-kdalton component, 20% of the total amino acid titer remained attached to the chitin. Identical electrophoretic patterns were obtained in the presence and absence of air. Deglycosylation with trifluoromethanesulfonic acid also released a 68-kdalton component as the largest fragment (results not shown). Acid hydrolysates of proteins and peptides released from the matrix by chymotrypsin were devoid of *N*-acetylglucosamine and neutral sugars.

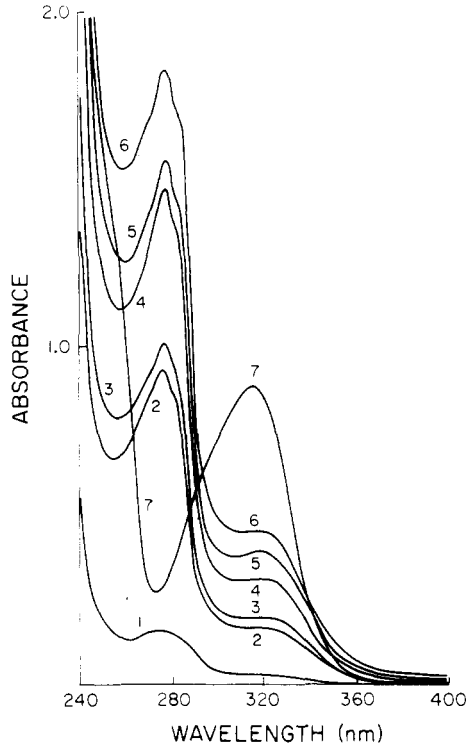

FIGURE 3: Ultraviolet absorbtion spectra of peptides released from CB-I by proteolytic enzymes. Skin (25 mg) was heated to 100 °C in water for 5 min and digested in 2 mL of 0.2 M ammonium bicarbonate, pH 7.8, for 2 h prior to spectrophotometry. Enzymes used for digestion were (1) *Staphylococcus aureus* protease V-8, (2) α-chymotrypsin, (3) trypsin, (4) subtilisin, (5) thermolysin, and (6) Pronase. For comparison the UV spectrum of bityrosine in 0.2 M ammonium bicarbonate (7) is given.

o-Diphenolase activity was present in CB-I at 6 milliunits/mg. Peroxidase was active at 2.7 mEU/mg.

Isolation and Identification of Bityrosine and Tertyrosine. Phenolase activity associated with CB-I was destroyed by heating for 5 min at 100 °C. The stabilized tissue was digested with proteolytic enzymes, and the liberated peptides were examined under ultraviolet light. Figure 3 shows that a mixture of substances was released with absorption maxima at 280–283 nm and a shoulder at 321 nm in accordance with the presence of a mixture of tyrosine, bityrosine, and tertyrosine. Tyrosine is devoid of a secondary peak at 320 nm. The same UV spectrum was evident when intact CB-I was treated with 0.1 M NaOH at 100 °C to destroy *o*-diphenols. The phenolic amino acids were labeled by administration of L-[U-^{14}C]tyrosine to maggots; the Pronase digest was hydrolyzed with HCl at 110 °C and chromatographed on a polystyrene cation-exchange resin with a pyridine–acetate elution program (Figure 4). The first three radioactive and UV-absorbing peaks cochromatographed with authentic tyrosine, bityrosine, and tertyrosine. No isotertyrosine was observed in this species. The last two peaks eluting between fractions 59 and 66 were not identified. The individual peaks were collected and subjected to paper chromatography in three systems: 1-butanol–acetic acid–water, 55:15:30; 1-butanol–formic acid–water, 75:10:15; and 2-propanol–ammonia–water, 8:1:1. In all three systems and on HPLC (C_{18} Ultrasphere ODS, 5 μm) with a linear gradient of 0.1% trifluoroacetic acid–50% acetonitrile, migration corresponded to the putative metabolites. The radioactive areas were excised from the paper chromatograms and eluted with 0.1 N HCl for further identification. The material corresponding to bityrosine showed a fluorescence excitation maximum at 329 nm and an emission

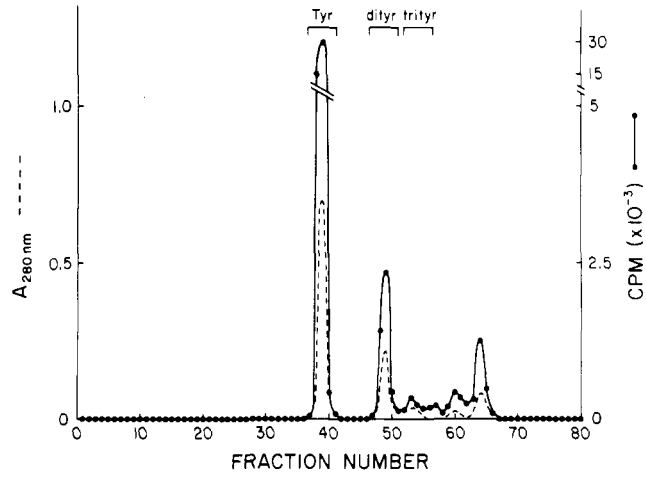

FIGURE 4: Chromatography of ultraviolet-absorbing components of CB-I labeled with L-[U-^{14}C]tyrosine. Maggots were administered labeled tyrosine early in the third instar and sacrificed when wandering. Fraction CB-I (10 mg) was hydrolyzed in 5.8 M HCl and chromatographed on a polystyrenesulfonic acid cation-exchange column (Phenix PA-35) with a pyridine–acetate gradient according to Liebster et al. (1961). Elution volumes of markers were determined on the same column. Bityrosine, dityr; tertyrosine, trityr.

maximum at 412 nm with ultraviolet maxima at 284 nm in acidic solvents and 316 nm in strong base. For tertyrosine, fluorescence excitation and emission maxima were at 320 and 412 nm, respectively; ultraviolet absorption maxima were at 286 nm in acid and 316 nm in alkali. These properties agree with published values (Andersen, 1966; Keeley & Labella, 1972). The isolates fluoresced when exposed to ammonia vapors but not when in HCl and reacted strongly with ninhydrin reagent for α-amino acids and with diazotized *p*-nitroaniline, indicative of phenols, but not with the molybdate reagent for *o*-diphenols or (2,4-dinitrophenyl)hydrazine. Neither desmosine nor 3-hydroxypyridinium fluorescent cross-links found in other structural proteins were observed. Bityrosine and tertyrosine were not oxidized by sarcophagid or mushroom phenolase but polymerized on prolonged incubation with peroxidase and hydrogen peroxide. Analysis of individual or pooled components of the borate-soluble proteins failed to reveal either of the two conjugated tyrosine derivatives.

Bityrosine and tertyrosine were identified in hydrolysates of insoluble proteins from cuticles of three dipterous cyclorrhaphid larvae, *Sarcophaga bullata*, *T. nigrovittatus*, and *D. melanogaster*, but were undetectable in the mosquito, *A. aegypti*. The discontinuous occurrence of bityrosine within the life cycle of a given species was evidenced by good recovery from aedine eggs but not aedine larval cuticle, from *Sarcophaga* larvae but not from empty pupal cases, and from the larvae of *M. sexta* but not from eggs of pupal cuticle. Tanned spermatophores from *Spodoptera littoralis*, skins from cerambycid (coleopteran) larvae and adult cuticle and oöthecae from *P. americana* were devoid of bityrosine.

Sequence of Bityrosyl Peptides. Isolation of bityrosine from acid hydrolysates did not ascertain if both side chains were in peptide linkages. The question was resolved by isolation of two peptides with bityrosine as part of the primary structures. Fragments were generated from CB-I by digestion with Pronase; amino acids and large hydrophilic peptides were removed by passage through bonded C_{18} (SEP-PAK) in 0.1 M acetic acid. Oligopeptides were eluted with methyl alcohol–0.1 M ammonium bicarbonate, 1:1, and chromatographed on Sephadex G-25. The major component with absorbance at 280 nm was collected and chromatographed a second time

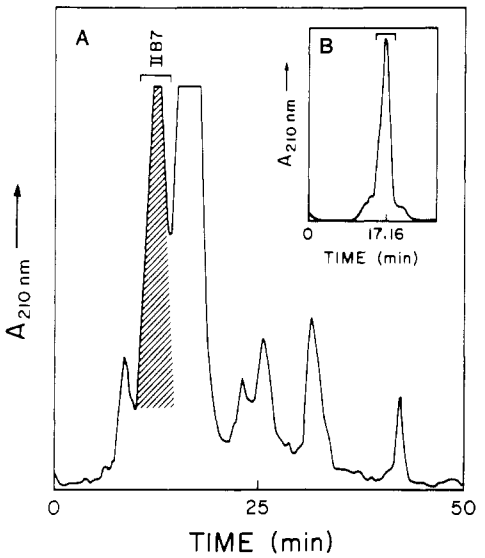

FIGURE 5: HPLC purification of bityrosine–peptide IIB7. A Pronase digest of CB-I was processed on Sephadexes G-25, G-75, and LH-20 for initial purification. Peak fractions from LH-20 were assayed for bityrosine; component IIB was selected for resolution on HPLC. (A) Elution pattern on Altex ODS, 5 μm, reverse phase with a linear gradient of 10% acetonitrile–0.1% trifluoroacetic acid to 25% acetonitrile in 50 min; the flow rate was 2 mL/min. (B) Elution pattern of purified subfraction IIB-7.

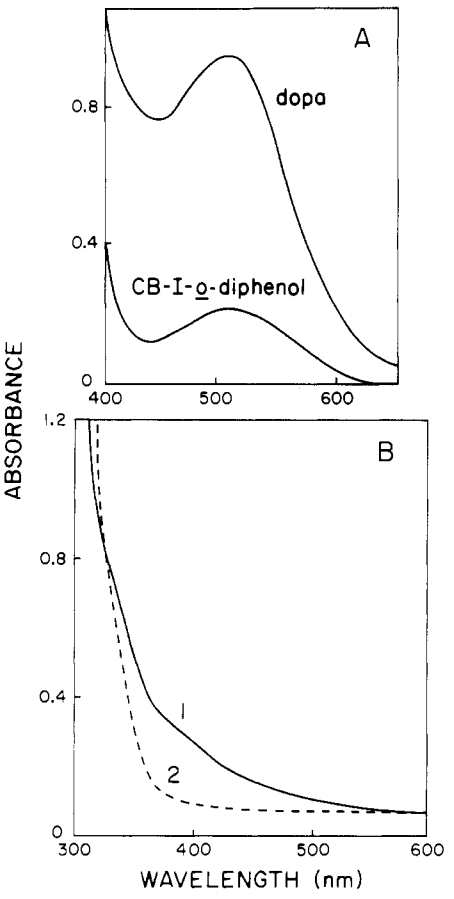

FIGURE 6: (A) Visible absorbtion spectra of nitrite–molybdate complexes of L-dopa and bound *o*-diphenol of CB-I. The sample was washed with 1 M HCl and treated with molybdate–nitrite and sodium hydroxide according to Arnow (1937). The catecholic chromophore was released into the reaction mixture on addition of alkali. (B) Absorbtion spectrum of Pronase digest following incubation at 25 °C for 24 h in the presence (curve 1) and absence of air (curve 2).

on Sephadex G-75 and then on Sephadex LH-20 with 0.25 M formic acid–2% 2-propanol to afford peaks IIB and IIC. The most abundant, IIB, was subjected to HPLC to reveal seven fractions one of which, IIB7, contained bityrosine (Figure 5A). The peptide was collected and rechromatographed prior to sequencing (Figure 5B). The preparation was devoid of free amino acids and released 1 equiv of aspartic acid, serine, proline, glycine, and bityrosine on acid hydrolysis. Mass spectrometry in the fast atom bombardment mode for $C_{32}H_{40}N_6O_{13}Na$ revealed the most abundant *m/e* at 739 at low resolution and at 739.3532 at high resolution by peak matching. Theory requires 739.2551. Table II describes the course of substrative sequencing and specific cleavage with proline-specific endopeptidase and carboxypeptidase Y. The combined enzymatic and chemical probes were required because bityrosyl phenylthiohydantoins generated from bityrosyl peptides did not afford bityrosine on back-hydrolysis. Small peptides containing bityrosine were not hydrolyzed by *Staphylococcus aureus* protease V8 or leucine aminopeptidase; hence, the data permit two sequence isomers at the amino termini of the bridged peptide. Sequencing of IIC, the second component from the LH-20 column, followed a purification scheme essentially as that described for IIB. Assignment of C terminals assumes the cross-linked material resembles authentic prolylvaline in resistance to cleavage by proline-specific endopeptidase.

Bound o-Diphenols of CB-I. When the polyphenol oxidase of CB-I was inactivated by heat treatment of the proenzyme, the presence of a bound *o*-diphenol was indicated by a strong response to molybdate–nitrate (Figure 6A). Incubation with Pronase in vacuo released a material that was retained by a

Table II: Sequence of Bityrosine Peptides from CB-I

	IIB-7	IIC
mole ratio[a]	bityr (1), Asp (1), Gly (1), Pro (1), Ser (1)	bityr (1), Asp (1), Gly (1), Pro (2), Val (1)
Edman degradation		
cycle 1	Asp (1), Gly (1)	Asp (1), Gly (1)
cycle 2	no residue[b]	Pro (1)
cycle 3	Pro (1)	
carboxypeptidase Y	Ser (1)	Val (1)
proline-specific endopeptidase[c]		
HPLC peak 1A[d]	Ser (1)	
HPLC peak 4A[d]	bityr (1), Asp (1), Gly (1), Pro (1)	
HPLC peak 5B[d]		Asp (1), Pro (1), Gly (1)
sequence	Asp-Tyr-Pro-Ser (Gly-Tyr) or Gly-Tyr-Pro-Ser (Asp-Tyr)	Asp-Pro-Tyr-Pro-Val (Gly-Tyr) or Gly-Pro-Tyr-Pro-Val (Asp-Tyr)

[a] Free amino acids were absent before acid hydrolysis. [b] Residual peptide from cycle 1 contains 1 mol equiv of bityrosine. No phenylthiohydantoin formed from bityrosyl peptides. [c] 5-μm ODS column (0.1% trifluoroacetic acid–50% acetonitrile gradient). [d] Peaks 1A and 4A were derived from IIB-7, and 5B was from IIC. For details see text.

Table III: Amino Termini during Oxidation of Endogenous *o*-Diphenol by CB-I[a]

enzyme	time (h)	treatment: vacuum 1	vacuum 2	air 1	air 2
none	0	44.0 (Asp)	38.4 (Val)	44.2 (Asp)	39.1 (Val)
thermolysin[b]	3	120.4[c] (Asp, Gly, Val, Ala)	nd	0.0	0.0
trypsin[d]	24	nd	nd	0.0	0.0
phenolase[e] (ΔA_{420nm}/h)		0.0		0.360	

[a] Values in nmol/10 mg of CB-I. Termini assessed by the Edman reaction as modified by Zalut et al. (1980). [b] Digestion with 1980 units, pH 8.0, 37 °C. [c] Principal residues exposed. [d] Digestion with 6 units, pH 8.0, 37 °C. [e] Phenolase activity with dopamine.

borylcellulose affinity support specific for catecholic substances. The phenol was displaced from the matrix with acetic acid and the *o*-dihydric structure verified by the method of Arnow (1937). Proteolysis of unheated CB-I activated prophenolase and also liberated a phenol that darkened rapidly in air at pH 6.5, 37 °C (Figure 6B). The UV absorption spectrum of the dark-reaction medium was indistinguishable from the quinone–amino acid adducts described in tanned puparia (Sugumaran & Lipke, 1982a). Chromophore production induced by proteolysis of unheated CB-I could be inhibited with 10^{-2} M phenylthiourea, with 10^{-1} M β-mercaptoethanol, or by degradation of the *o*-diphenol with 0.1 M NaOH at 100 °C for 5 min. Alkali treatment inactivated phenolase and degraded the *o*-diphenol as verified by failure to produce chromophore on incubation with sarcophagid phenolase or crystalline mushroom tyrosinase.

Sclerotization is dependent on oxygen, polyphenol oxidase, a phenol, and monomeric structural polypeptides receptive to arylation. In the course of cross-linking, available amino groups add to the catecholic ring via 1,4 addition. Fraction CB-I retained all these components when subjected to mild proteolysis under aerobic conditions since brief treatment with Pronase or thermolysin activated the prophenolase for attack on the phenol. The phenol of heat-treated CB-I was available to exogenous mushroom tyrosinase without prior proteolysis. The presence of a functional sclerotizing system in CB-I was established by titration of the N-terminal aspartic acid with the Edman reagent during the course of mild proteolytic activation of the phenolase. Table III shows that partial proteolysis by added thermolysin or trypsin did not mask the N termini of the residual bound peptides if oxygen was excluded from the reaction. In the presence of oxygen a sharp decline was evident with both proteases. Newly generated amino functions were completely blocked by arylation and were refractive to the Edman reagent for the second cycle as well as the first. In this respect, behavior of isolated CB-I is comparable to sclerotization in the intact organism.

Metabolism of Aromatic Constituents during Pupariation. Solubility in tetraborate affords a clear distinction between the soluble and chitin-bound proteins of the maggot stage. With the onset of pupariation the compartments pool due to the linking of soluble components to CB-I. Bityrosine and *o*-diphenols may act in concert during joining of promoters or may act independently. Figure 7 illustrates separate time courses for the catechol and the diamino acid as well as the relative abundance of each phenolic species. Bityrosine declined as the cuticle darkened and was no longer detectable

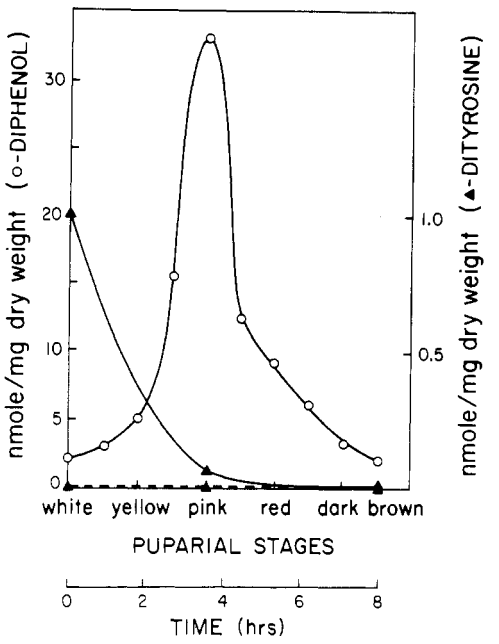

FIGURE 7: Bityrosine and *o*-diphenol metabolism during pupariation. Cuticles were separated from viscerae by dissection. Bityrosine was assessed following acid hydrolysis and paper chromatography. (Solid line) Bityrosine level in cuticle; (dashed line) bityrosine level in viscerae. Diphenol was assayed as in Figure 5.

in the completed puparial case. Bityrosine did not accumulate in the viscerae or body fluids as the cuticular level dropped, but was present in the integument of second as well as third instar larvae.

The *o*-diphenol was present initially at a level only 10% of that of the catechol titer, increasing 10-fold halfway through puparial development at the 4-h interval (Figure 7). During the later stages the dihydric phenol declined precipitously. No *o*-diphenol was detected in material corresponding to CB-I in second instar larvae. The darkened puparium was examined for products of bityrosine and tertyrosine metabolism by paper chromatography. No radioactive material cochromatographing with the markers was observed.

Discussion

During the transformation of the soluble cuticle proteins of the maggot stage to the rigid puparial case chitin synthesis is arrested, and the preexisting polysaccharide reorients following partial depolymerization (Strout et al., 1976). Chitin–protein links are enhanced 6-fold, with a significant difference in the composition of the polypeptide portion of the proteoglycan before and after sclerotization (Kimura et al., 1976). The protein linked to the polysaccharide late in the developmental program is of uncertain origin, although protein synthesis is depressed; at least one gene coding for a soluble structural protein is activated late in the final instar, and marked heterogeneity characterizes the mRNA transcribed in the cuticle at this stage (Fragouli-Fournogeraki et al., 1978; Chihara et al., 1982). In addition to temporal variability with respect to gene expression during the final instar, major differences in structure distinguish the soluble and the residual proteins. Polypeptides extracted with urea or tetraborate–$NaDodSO_4$ are functionally inert and fail to cross-link when incubated with a homologous phenolase and dopamine (Strout & Lipke, 1974). Within this group of proteins major heterogeneity is encountered, at least 21 components have been identified in *Sarcophaga* with relatively small differences between the members in isoelectric point, molecular weight,

and amino acid composition (Lipke et al., 1981). When labeled dopamine is administered early in the final instar, relatively minor levels of radioactivity can be recovered in the tetraborate extract compared to those in the chitin–protein complex. The soluble proteins and derived hydrolysis products have no affinity for borylcellulose and do not respond to the molybdate–nitrite test for catechols. These characteristics differ strikingly from the CB-I complex and suggest that the soluble compartment requires not only posttranslational modification prior to arylation and glycosylation but also conjugation with CB-I. When the metabolic properties of CB-I are considered in detail, auto activation of the chitin-bound polypeptide by substituents associated with the protein moiety becomes the critical event in the initiation of sclerotization.

Evidence in favor of CB-I constituting a single polypeptide chain is based on recovery of aspartic acid in high yield by the Edman procedure (Table III). Cycle 1 afforded 4.4 nmol of this residue followed by 3.8 nmol of valine for the second cycle. The following residues by the manual procedure were present in the indicated amounts: alanine, 3.6 nmol; histidine, 1.02 nmol; followed by tyrosine, 3.5 nmol. The possibility that additional proteins with homologous N termini and blocked C termini are also present is a clear possibility calling for new approaches to the separation of chitin from the protein component(s). In this respect, cleavage with trypsin, chymotrypsin, or trifluoromethanesulfonic acid severed a single large fragment, 68 kdaltons, from the complex with the retention of 20% of the total protein by the polysaccharide. If one assumes a single polypeptide chain, a molecular mass of about 90 kdaltons for the native protein is implied, a dimension 5 times greater than the average for the soluble components that fall between 16 and 24 kdaltons (Lipke et al., 1981). Following treatment with chymotrypsin for separation of the 68-kdalton component, a new C terminus was generated at the cleaved locus (Figure 2). In accord with the specificity of chymotrypsin, the chain was split at a tyrosyl residue, preceded by alanine and then by valine (Figure 1). The pattern of amino acid cleavage by carboxypeptidase A speaks for a chitin-bound peptide stub of unique primary structure and discounts heterogeneity within the bound protein complement.

The presence of propolyphenol oxidase and peroxidase introduces added uncertainties if it is assumed these enzymes are present in amounts below the sensitivity of the Edman and carboxypeptidase probes for heterogeneity. On the other hand, the two activities may be associated with the major component as implied by the loss of phenol oxidase activity from the chitin peptide following removal of the 68-kdalton protein. Substrates for both enzymes, namely, *o*-diphenol and tyrosyl residues, are apparently associated with the main chain, affording some proximity advantage to commonality of location when the system is activated at the conclusion of the third larval instar. In this respect, the gene transcript for CB-I is probably unique for the third instar since the analogous preparation from the second instar showed no detectable phenolase or catechol and is incapable of pupariation under normal circumstances.

Recovery of bityrosine with both tyrosyl residues occupied in peptide linkage is strong evidence that this bridging unit is required for CB-I conformation prior to the onset of pupariation (Table II). Bityrosine declined with the onset of hardening and was undectable in fully sclerotized puparia on the basis of unit weight of cuticle (Figure 7) or per puparial case. Failure to detect bityrosine in noncuticular tissues during disappearance from the integument suggests modification in the course of addition of soluble proteins and circulating catecholamines to CB-I. Since compounds corresponding to bi- and tertyrosine were absent from puparial hydrolysates prelabeled with [^{14}C]tyrosine, further substitution on the ring is probable in the course of the decline. On the basis of a molecular mass of 90 kdaltons for the structural polypeptide component (see above) and a protein content of 38%, a value of 4.2 nmol of protein is present in 1 mg of CB-I together with 2 nmol of bityrosine, or two half-residues per chain. Yet to be determined is the location of the bridges, intra- or interchain, and the contribution of tertyrosine, present in much lower amounts. Coincidence of bityrosine and peroxidase in CB-I favors a role for the latter in tanning. Bi- and tertyrosine are prepared by oxidation of tyrosine with hydrogen peroxide and peroxidase. Prolonged exposure of tyrosine-rich proteins to these reactants affords cross-linked gels analogous to tanned cuticle (Labella et al., 1968; Aeschbach et al., 1976). Enhanced levels of peroxidase are reported for cells secreting resilin in locust (Coles, 1966) and in hardened regions of the lepidopteran proleg (Locke, 1969). The occurrence of bityrosine among the species surveyed was sporadic with no obvious correlation with life stage, cuticle texture, or phylogenetic position.

Following activation by mild proteolysis the polyphenolase zymogen incorporated in CB-I is inhibited by thiols and copper ligands; in the absence of inhibitors the enzyme oxides *o*-diphenols bound to the matrix as well as those transferred from the viscera early in pupariation (Figure 6B). These properties agree with previous reports dealing with several dipterans (Yamazaki, 1969; Hughes & Price, 1975; Barrett & Anderson, 1981). On generation of *o*-quinones by the enzyme, rapid coupling with available amino groups was observed with complete disappearance of the original terminal aspartyl residues as well as new amino groups exposed by thermolysin or trypsin (Table III). Coincidence of amino substitution and quinone formation is a strong indication of Michael 1,4 coupling, the major route to interprotein bridges in *Sarcophaga* (Sugumaran & Lipke, 1982a; Lipke et al., 1983). Occupation of the N termini protected the polypeptides from attack by phenyl isothiocyanate for two cycles of the Edman procedure, corresponding to formation of covalent bonds between the amine nitrogen and the quinonoid residue. In the case of isolated CB-I, quinones add to residues preexisting within the matrix. In the intact organism, however, a substantial increase in reactive centers arises from the presence of the soluble structural proteins comprising about 60% of the polypeptide complement. The recovery of soluble proteins is about 30 nmol/mg of cuticle on the basis of an average molecular mass of 20 kdaltons. Lysine, histidine, asparagine, and glutamine are abundant, providing additional loci for aryl bridges (Lipke et al., 1981). In this circumstance quinone linked to CB-I would couple not only to endogenous amino groups but also to soluble proteins as well, generating cross-links fixing the soluble proteins to the chitin–protein matrix. The introduction of aryl bridges and increased carbohydrate–protein bonding would transform the cuticle into a construct of unusual stability to enzymatic degradation corresponding to the general properties of sclerotized tissue in the intact organism.

These features are shown in the scheme presented in Figure 8. The model sets forth the reactions transpiring during the initial steps of sclerotization, before the bulk of the soluble proteins are polymerized and pigmented (Lipke & Henzel, 1981). The scheme provides for the decline in bityrosine and *o*-diphenol by cross-linking to chitin-bound and unconjugated structural protein, for the presence of the polyphenol oxidase zymogen in proximity to endogenous substrate, and for the

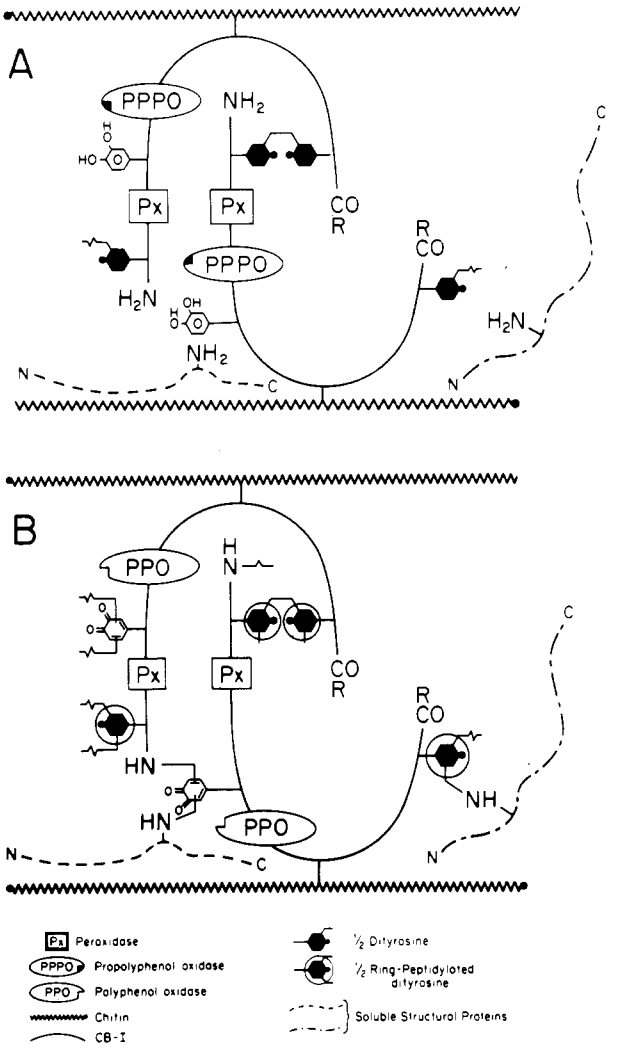

FIGURE 8: Distribution of chitin, protein, and phenols in CB-I. (A) At the commencement of the third larval instar. The catechol is unsubstituted and proximal to propolyphenol oxidase. Bityrosine is in close juxtaposition to peroxidase. Soluble proteins are not linked to the chitin–protein matrix. (B) White puparial and wandering larvae late in the third instar. Activated phenolase oxidizes *o*-diphenol to quinone, introducing a bridge between amino termini of CB-I and soluble proteins via 1,4 addition. Additional soluble proteins attack bityrosine by a separate peroxidase-dependent mechanism.

synthesis of bi- and tertyrosine via a fixed peroxidase. The peroxidase not only generates these two bridges but may function in the subsequent sclerotal coupling of bityrosine to soluble proteins (Locke, 1969). In this respect the model justifies the occurrence of more than one arylation mechanism in the arthropod integument (Lipke et al., 1983). Thus, the preliminary stages of sclerotization are dependent on synthesis of enzymes (a) converting propolyphenol oxidase to the active species, (b) generating hydrogen peroxide, and (c) coupling proteins to bityrosyl residues. The diversity of these requirements speaks against contructs invoking dopa decarboxylase as the initial and unique object of ecdysone secretion (Fragoulis & Sekeris, 1975).

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