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Involvement of tyrosine residues in the tanning of proteins by 3-hydroxyanthranilic acid

(cocoons/oxidation/benzocoumarin/radical/insect)

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ABSTRACT **The binding of oxidized phenolic compounds to proteins is of importance in a number of biological systems, including the sclerotization of insect cuticle and the tanning of cocoons. 3-Hydroxyanthranilic acid (3HAA), an aminophenol, is a tryptophan metabolite that undergoes autoxidation readily, and proteins incubated in the presence of 3HAA and oxygen become colored and oxidized. Some moth species are thought to employ this reactivity of 3HAA with proteins for the tanning of cocoons, but the detailed mechanism of this process has not been studied previously. We show that one reaction pathway involves the covalent coupling of 3HAA with tyrosine to form a benzocoumarin derivative, a dibenzo[*b,d*]pyran-6-one. The stability of the benzocoumarin to conditions of acid hydrolysis normally used for protein digestion has enabled the isolation of the tyrosine adduct from bovine serum albumin that had been incubated with 3HAA. The adduct was also isolated from cocoons of *Samia cynthia* and *Hyalophora gloveri*, two species of moths reported to utilize 3HAA for cocoon tanning. These findings indicate that one mechanism of interaction of 3HAA with proteins involves a radical–radical coupling with tyrosine residues.**

Phenolic compounds and their quinonoid (1) oxidation products are widespread in nature. As discussed in a comprehensive review of these reactions (1), the biological activity of many quinonoids consists ultimately in the chemical modification of biopolymers. Aside from their role as defensive secondary metabolites in, for example, fungi, plants, and marine organisms (2, 3), the interaction of quinonoid compounds with proteins is important in melanoprotein synthesis in animals and sclerotin formation in insects.

Indeed, many stages in the life cycle of insects are dependent on such reactions taking place. The sclerotization (hardening) of the insect cuticle, for instance, with the consequent formation of a rigid yet light exoskeleton confers both protection and the potential for flight to these arthropods. A common feature of cuticle sclerotization, cocoon tanning, and ootheca (egg case) hardening is the modification of structural proteins by phenolic chemicals that have been rendered reactive as a result of enzymic oxidation (4). Most commonly, substituted *o*- or *p*-hydroxyphenols are employed, but the use of *o*-aminophenols such as 3-hydroxyanthranilic acid (3HAA) has also been reported: for example, the North American robin moth (*Hyalophera gloveri*) and the Indian Tree of Heaven silkmoth (*Samia cynthia*) use 3HAA for cocoon tanning (5).

In the present work we have explored the interaction of 3HAA with proteins under oxidizing conditions. We have demonstrated that covalent binding of 3HAA to tyrosine residues of proteins occurs under oxidizing conditions with the formation of a benzocoumarin derivative. This benzocoumarin was also isolated from cocoon proteins of *H. gloveri* and *S. cynthia*. These data indicate that free radical reactions involving the tyrosine residues of proteins occur during the tanning process.

MATERIALS AND METHODS

Cocoons of the silkmoth species *S. cynthia advena* and *H. gloveri* were obtained from Worldwide Butterflies, Sherborne, Dorset, England. Poly(L-tyrosine), bovine serum albumin (BSA), and 3HAA were obtained from Sigma. The benzocoumarin 7-amino-2-(2-amino-2-carboxy)ethyl-8-hydroxy-6*H*-dibenzo[*b,d*]pyran-6-one (Fig. 1*a*) was synthesized according to Manthey *et al.* (6). The glucoside of 3HAA, 2-amino-3-(β-D-glucopyranosyloxy)benzoic acid (Fig. 1*b*), was synthesized by a slight modification of the method of Brunet and Coles (5), employing the methyl ester of 2-nitro-3-hydroxybenzoic acid. A melting point of 220–221°C was recorded for the glucoside.

Analytical HPLC was carried out on a Knauer HPLC system employing a Resolve 10-μm C_{18} Radial-Pak cartridge (10 cm × 8 mm, Waters) or on a semipreparative scale employing a stainless steel μBondapak C_{18} column (30 cm × 13 mm, Waters). The mobile phase consisted of solvent A (40 mM sodium acetate buffer, adjusted to pH 5.0 with glacial acetic acid) and solvent B (acetonitrile). The solvent program consisted of isocratic elution with solvent A for 15 min followed by gradient elution for a period of 25 min to 40% solvent B. Isocratic elution was maintained for a further 20 min. The flow rate was 1.2 ml/min, and detection was carried out fluorimetrically (Waters model 420E fluorescence detector; excitation wavelength, 338 nm; emission wavelength, 425 nm).

Half of one cocoon from each of the silkmoth species *H. gloveri* and *S. cynthia* was sliced into small segments and extracted with aqueous 80% methanol at 80°C for 16 hr. The extract was filtered free of insoluble material, which was retained for subsequent acid hydrolysis as described below. TLC of the filtrate [silica gel; butanol/acetic acid/water, 4:2:1, and 1-propanol/water, 4:1 (vol/vol)] against the synthetic 3HAA glucoside indicated that this was the major fluorescent compound in both extracts. Reversed-phase HPLC revealed a major fluorescent peak whose retention time was identical to that of the glucoside. Purification of the major fluorescent chemical in the extracts from both species was carried out by semipreparative reverse-phase HPLC. The resultant UV spectra of collected peaks showed absorption maxima at 311 nm and 214 nm, in agreement with the fluorescent species being the O-glucoside of 3HAA. Incubation of the cocoon component for 20 min at 100°C in aqueous HCl at pH 1 resulted in its disappearance (as judged by TLC) and the formation of 3HAA.

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Abbreviations: 3HAA, 3-hydroxyanthranilic acid; BSA, bovine serum albumin.

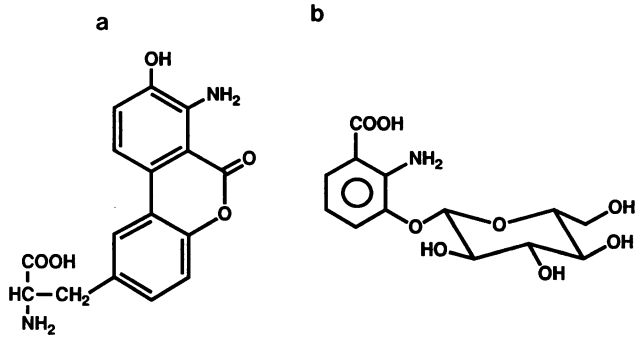

FIG. 1. The benzocoumarin derivative (*a*) and the O-β-D-glucoside (*b*) of 3HAA.

3HAA (330 μmol) was autoxidized in 67 mM sodium phosphate buffer (pH 7.0) in the presence of BSA (10.1 μmol). Oxygen was bubbled (≈1 ml/min) through the solution to maintain saturation. At predetermined intervals, 2-ml aliquots of the oxidation medium were removed and chromatographed on a Sephadex G-25 column (17 cm × 2.7 cm) with water as the eluent. The brown band that eluted at the void volume was collected and either made up to volume (25 ml) and examined spectrophotometrically or lyophilized. The lyophilized BSA or cocoon proteins (≈100 mg), which had been homogenized in 8 M urea and dialyzed against water, were subjected to acid hydrolysis *in vacuo* with 1.5 ml of 6 M HCl for 24 hr at 110°C. The hydrolysate was dissolved in 0.1 M sodium phosphate buffer (pH 4.5) prior to HPLC. A major fluorescent compound in the HPLC profile of cocoon corresponded to the benzocoumarin in Fig. 1*a* (R_t = 32.5 min). This was purified by semipreparative HPLC and the desalted compound was lyophilized and further dried at 100°C for 12 hr prior to silylation and GC/MS. Silylation was carried out using dry pyridine (50 μl), bis(trimethylsilyl)trifluoroacetamide (100 μl), and 4-Å molecular sieve at 100°C for 60 min. GC/MS analyses were performed on a Varian 3700 GC interfaced directly to a MAT-44 quadrupole mass spectrometer equipped with an electron-impact source. The GC was fitted with a 12-m BP-5 fused silica capillary column (Scientific Glass Engineering, Austin, TX). Hydrogen at a flow rate of 30 ml/min was used as the carrier gas, and the temperature was programmed from 50°C to 280°C at 10°C/min.

RESULTS

The autoxidation of 3HAA is complex with competing pathways yielding a number of products, which are often colored (7). To simplify the identification of amino acid residues that are capable of reacting with 3HAA, amino acid homopolymers were exposed to 3HAA in the presence of oxygen. After reaction, the products were separated by gel filtration on Sephadex G-10 and the excluded fraction was examined by UV–visible spectroscopy for evidence of homopolymer modification.

Two amino acid homopolymers, poly(L-lysine) and poly(L-tyrosine), were found to become visibly colored under these conditions. This communication describes the modification of tyrosine residues.

The UV–visible spectrum of poly(L-tyrosine) that had been incubated in the presence of 3HAA under autoxidizing conditions exhibited a new absorbance peak centered at 340 nm. This was subsequently used as a marker to follow the extent of modification of poly(L-tyrosine) by 3HAA as a function of time. The extent of tanning increased significantly with time (data not shown).

The prior identification of a dimer in autoxidized solutions of 3HAA apparently formed by ortho–para radical–radical coupling (6) prompted speculation that the interaction with tyrosine residues may take place via a free radical mechanism. A tyrosine adduct analogous in structure to the 3HAA radical dimer was therefore synthesized (6). This benzocoumarin derivative (Fig. 1*a*) was found to be stable in 6 M HCl at 110°C, conditions used for the hydrolysis of proteins, thus allowing its quantitation in proteins that had been exposed to 3HAA.

When the tanning of BSA was followed as a function of time, and samples were removed for acid hydrolysis and subsequent quantification of the benzocoumarin by HPLC, a similar time course was observed initially for both the increase in A_{340}/A_{280} ratio (Fig. 2*a*) and the appearance of the covalently modified tyrosine derivative (Fig. 2*b*). By 20 hr, ≈3% of the total tyrosine residues had been converted to benzocoumarin derivatives. After 20 hr of incubation, the content of benzocoumarin gradually decreased (Fig. 2*b*). On the basis of the behavior of the authentic compound, this decrease may have been the result of slow autoxidation of the protein-bound benzocoumarin. Although this compound is an *o*-aminophenol, the autoxidation rate is quite slow: the chemical stability may possibly arise from an approach to planarity of the three-ring system, which confers a degree of aromaticity on the lactone ring.

The overall rate of protein tanning continued to increase after 20 hr, probably due to the interaction of 3HAA with lysine residues (8). No dityrosine could be detected in any of the hydrolysates examined.

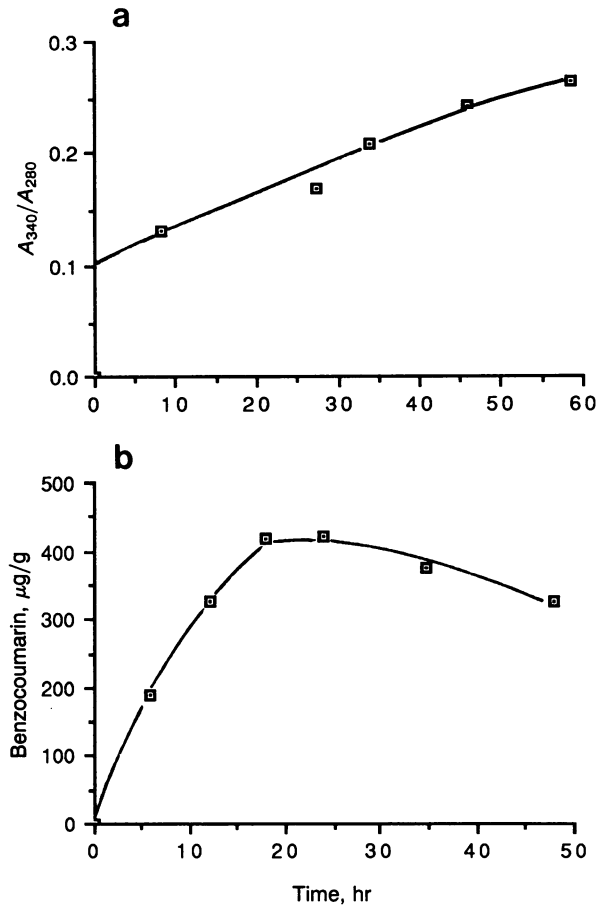

FIG. 2. (*a*) Time course of modification of BSA by 3HAA as judged by the increase in absorbance at 340 nm relative to that at 280 nm. (*b*) Time course of formation of the benzocoumarin derivative in BSA modified by 3HAA. The content of benzocoumarin was determined by HPLC after acid hydrolysis of the modified protein.

Two species of moths (*H. gloveri* and *S. cynthia*) have been described that apparently employ 3HAA for the modification of their cocoons (5). Cocoons of both of these species were obtained from moths bred in captivity at Worldwide Butterflies. Cocoons were first extracted in aqueous methanol to remove low molecular weight chemicals. The methanolic extracts of both cocoons were found to contain 3HAA glucoside (5), which was identified by comparison with a sample of authentic material synthesized in our laboratory. This confirms the previous finding of Brunet and Coles (5).

Proteins were extracted from 40–100 mg of insoluble cocoon material into 8 M urea and then thoroughly dialyzed to remove noncovalently bound material prior to lyophilization and hydrolysis as described for BSA. As judged by HPLC (Fig. 3), significant quantities of the benzocoumarin adduct were found in cocoons of both species. *Samia* cocoons appeared to contain consistently more of the modified tyrosine derivative (22 ± 8 μg/g of cocoon) than did *Hyalophora* cocoons (18 ± 4 μg/g of cocoon); $n = 4$ cocoons for each species. The isolated HPLC component was found in each case to have the same R_f value as the synthetic compound by TLC on silica gel in two solvent systems (butanol:acetic acid:water, 4:2:1, and pyridine:water, 4:1). To confirm the identity of the cocoon component as the dibenzo [*b,d*]pyran-6-one, the HPLC peak was collected, dried, and examined by GC/MS as the silyl derivative. The mass spectrum (Fig. 4) and GC retention time of the cocoon component were in agreement with those obtained from the authentic benzocoumarin.

DISCUSSION

Despite the widespread occurrence of reactions between oxidized phenolic compounds and proteins, the chemistry underlying these fundamental biological processes is poorly understood. To a large extent, this lack of knowledge concerning the exact mechanism of reaction of the tanning agents with proteins has been due to the general inability of researchers to isolate, from the tanned protein, covalently modified amino acids that would ideally incorporate part or all of the phenolic moiety. In one of the few such studies, cysteinyl dopas have been isolated by digestion of proteins exposed to 3,4-dihydroxyphenylalanine (dopa) (9).

Autoxidation of 3HAA at neutral pH leads to the formation of a variety of colored self-condensation compounds, and recent studies support the idea that these different products may arise through a common 3-hydroxyanthranilyl radical intermediate (7). 3HAA reacts with proteins in the presence of oxygen to yield polypeptides that are colored and contain oxidized amino acids (10, 11).

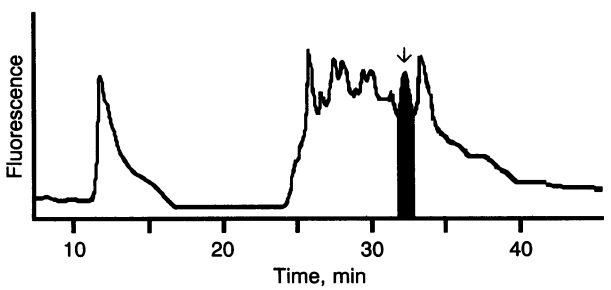

FIG. 3. HPLC profile of the 6 M HCl hydrolysate of *H. gloveri* cocoon. The eluate was monitored for fluorescence (excitation, 338 nm; emission, 425 nm). The elution position of authentic benzocoumarin is indicated by the arrow and the region of the chromatogram collected for subsequent structural investigation is shaded. The HPLC profile of the *S. cynthia* digest was qualitatively similar to that shown here.

The glucoside of 3HAA has been isolated from secretory glands of *S. cynthia* and *H. gloveri* (5). In this study we also found 3HAA glucoside in the cocoons themselves. It has been proposed that the 3HAA released by glycosidase activity may play a role in the tanning of the cocoons of these species (5); however, the mechanism of this interaction has not been understood.

In this paper we describe the reaction of 3HAA with tyrosine residues of proteins and demonstrate that the tyrosine–3HAA adduct, which is formed simply by incubation of proteins such as BSA with 3HAA in the presence of oxygen, is also present in the cocoon proteins of *S. cynthia* and *H. gloveri*. This finding indicates that the process of cocoon tanning involves covalent attachment of 3HAA to protein and that an enzyme-catalyzed oxidation is not a prerequisite for formation of the adduct. The most likely mechanism for the formation of the observed tyrosine–3HAA adduct involves a radical–radical coupling reaction. The phenoxyl radical of 3HAA has been observed by ESR experiments (12), and such a process would be consistent with the isolation of an analogous ortho–para radical dimer from autoxidized solutions of 3HAA (7).

The mechanism of formation of the tyrosine radical is less clear. It may arise as a result of oxidation by the quinonimine form of 3HAA. Activated oxygen species such as superoxide, which are also formed during the course of 3HAA autoxidation (13, 14), may also be implicated. Stable tyrosine radicals have been demonstrated in proteins such as ribonucleotide reductase (15), prostaglandin H synthase (16, 17), and components of photosystem II (18).

Similar chemical processes seem to underlie the sclerotization of insect cuticle, the hardening of egg cases, and the tanning of cocoons (1, 4, 5). In each case a soft, predominantly proteinaceous material is hardened and often colored by the application of a phenolic solution that is secreted onto the protein matrix together with an oxidase enzyme whose role is to convert the catechol or aminophenol into a reactive intermediate. The exact role of these oxidized chemicals in the mechanism of sclerotization is still in dispute.

Most discussions on the molecular mechanism of sclerotization have focused on nucleophilic processes involving the addition of protein amino or sulfhydryl groups to either the ring or side chain of oxidized catechols (e.g., refs. 19 and 20). Experimental observations supporting the role of protein amino groups in sclerotization include the isolation of aryl–lysine and aryl–histidine adducts from the cuticles of insects (21, 22) and solid-state ^{13}C and ^{15}N NMR studies (23) that have provided evidence for covalent linkages between the ring nitrogens of histidine residues and ring carbons derived from dopamine. Although some authors (1, 22) have speculated that free radical addition reactions, specifically those involving catechols, may also take place during biological sclerotization, here we present experimental evidence that such processes do indeed occur. It should be emphasized that the tanning reaction described herein involves an aminophenol and not a catechol and that, as yet, no aminophenols have been implicated in the tanning of insect cuticle. However, the similar chemical reactivities of catechols and *o*-aminophenols (24, 25) suggest that analogous adducts may well be formed from tyrosine residues that have been exposed to oxidized catechols. It is of interest that cuticle proteins are often rich in tyrosine and that these aromatic regions have been proposed to serve as initiation centers for cross-linking (26).

In agreement with earlier quinone model systems (27) reaction between oxidized 3HAA and lysine residues of BSA was also observed, which may involve the formation of adducts similar to those characterized previously (8). This is, however, the first direct evidence, to our knowledge, for the occurrence of covalent interaction between the tanning agent and tyrosine residues of proteins in biochemical tanning.

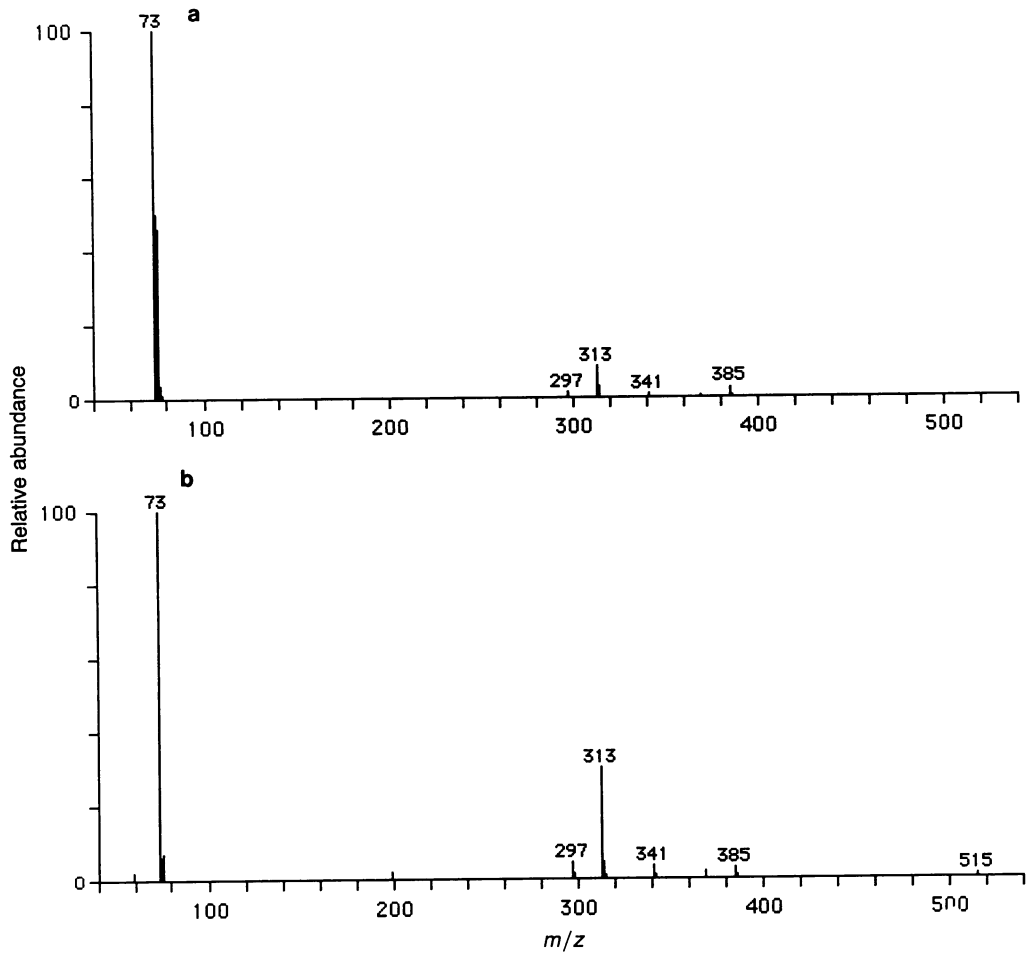

FIG. 4. (*a*) The electron-impact mass spectrum of the fluorescent component isolated from a hydrolysate of *Samia* cocoon protein purified by HPLC and then silylated. (*b*) The electron-impact mass spectrum of the silylated derivative of authentic benzocoumarin. The GC retention time of the trisilyl derivative was 29.53 min in each case.

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