Cysteic Acid is the Chemical Mediator of Automotive Clearcoat Damage Promoted by Dragonfly Eggs

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Received 24 February 2000; accepted 25 January 2001

ABSTRACT: The damage caused by dragonfly eggs on automotive clearcoats exposed to sunlight occurs by a chemical mechanism similar to that caused by acid rain. Cysteine and cystine residues present in dragonfly eggs are oxidized during the egg hardening process, which releases hydrogen peroxide, to a cysteic acid derivative, a strong acid capable to catalyze the hydrolysis of acrylo/melamine clearcoat polymer. Cysteic acid was indeed identified and quantified by ion-exchange HPLC in dragonfly egg extracts submitted to oxidation by H_2O_2 followed by acid digestion. Moreover, H_2O_2 concentration, temperature, and exposure time profiles of cysteic acid formation as well as an apparent activation energy for cysteine (in dragonfly eggs) oxidation to cysteic acid by H_2O_2 (32 ± 2 kJ/mol) were determined. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 81: 1549–1554, 2001

Key words: automotive resin; clearcoat; cysteic acid; dragonfly; melamine; sclerotization

INTRODUCTION

Recently, we reported that dragonfly eggs can damage automotive acrylo/melamine clearcoats at high temperatures (50–90°C) of sunlight-exposed cars.[1] Based on visual, scanning electron microscopy (SEM) and profilometry patterns, we proposed that the clearcoat degradation mechanism should be similar to that caused by acid rain, i.e., by acid hydrolysis.[2] The putative acids responsible for the acid clearcoat hydrolysis were sulfinic and sulfonic acids formed by the oxidation of cysteine (Cys–SH) and cystine (Cys–S–S–Cys) residues[3] present in the egg protein. Surface Enhanced Raman Spectroscopy (SERS)[4] indirectly confirmed the presence of cysteic acid (Cys–SO_3H) in egg protein after oxidation with H_2O_2, therefore reinforcing the acid hydrolysis hypothesis.[5]

In this study, we detect and quantify Cys–SO_3H formed in dragonfly eggs treated with H_2O_2, by ion-exchange HPLC on an amino acid analyzer. The profiles of Cys–SO_3H formation with H_2O_2 concentration, temperature, and exposure time were also characterized. That Cys–SO_3H can indeed efficiently catalyze the hydrolysis of the clearcoat was demonstrated by submitting a clearcoat panel to authentic Cys–SO_3H at 90°C for 4 h. (It must be pointed out here that the acid responsible for the clearcoat hydrolysis is not exactly cysteic acid, as cysteic acid residues are attached to other amino acids in the egg protein structure.) This study confirmed our former hypothesis[1,5] that the mechanism of dragonfly egg-promoted degradation of automotive clearcoat is by acid hydrolysis, due to the formation of a strong acid (cysteic acid-like) formed upon H_2O_2 oxidation of cysteine under sunlight heat.

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Contract grant sponsors: FAPESP, CNPq, PRONEX, and the Alexander von Humboldt Foundation.

Journal of Applied Polymer Science, Vol. 81, 1549–1554 (2001)

EXPERIMENTAL

L-cysteic acid (Aldrich), L-cysteine, (Aldrich) and H_2O_2 (30%, Merck) were used as received. Eggs of dragonflies were obtained as described before.[1] They were collected from three species [namely *Miathyria* sp., *Tauriphila* sp., and *Erythemis* sp. (Libellulidae: Libellulinae)] commonly found at the margins of the Tietê River in the municipality of Novo Horizonte (State of São Paulo, SP, Brazil).

Treatments with varying temperatures were conducted in a thermostated oven WTB Binder FD 115. Amino acid analyses of the dragonfly egg hydrolysates were performed on a Beckman analyzer 7300 as follows. Dragonfly egg (ca. 30 mg) homogenates were prepared with 1.0 mL of deonized water in a Potter-Elvehjem homogenizer. The standard procedure consisted in adding 100 μL of the homogenate to 4.90 mL of 50 mmol/L H_2O_2. The resulting suspension was heated at 90°C for 24 h. Samples of H_2O_2-treated homogenate were then hydrolyzed with HCl (6 mol/L) in the presence of phenol under N_2 atmosphere for 24 h at 110°C, using a Pico Tag Waters workstation. The amino acids were separated by ion-exchange liquid chromatography and detected after postcolumn derivatization with ninhydrin at 440 and 570 nm. The molar fraction of each amino acid present was determined using a standard curve obtained prior to the analyses. The results were normalized for the fraction of L-alanine (Cys–SO_3H/Ala) determined in each run.

The clearcoat panels were generously provided by Renner DuPont (Guarulhos, Brazil). They were prepared by crosslinking two acrylic copolymers with melamine resin, at the following weight ratio: 40% of acrylate copolymer A, 30% of acrylate copolymer B, and 30% of melamine. The monomer package used in the preparation of acrylate copolymer A was methacrylic acid, butyl acrylate, styrene, methyl methacrylate, and 2-hydroxyethyl methacrylate. Acrylate copolymer B was made using butyl acrylate, styrene, acrylic acid, hydroxypropyl acrylate, and butyl methacrylate. Melamine resin was the Cymel 1158 (American Cyanamid). The clearcoat also contained 1% (w : w) of UV Absorber Tinuvin 1130 (a hydroxyphenyl-benzotriazole derivative), and 0.7% (w : w) of HALS Tinuvin 292 (bis-1,2,2,6,6-pentamethyl-4-piperidyl sebacate). The crosslinking of the resin was obtained by baking the clearcoat for 30 min at 140°C.

The dependence of Cys–SO_3H formation in dragonfly eggs on H_2O_2 concentration (0.50, 1.0, 5.0, 10, 50, 250, 600, and 1000 mmol/L), temperature (50, 60, 70, 80, and 90°C) and exposure time (1, 4, 8, 16, and 24 h) was determined here. Conversion of pure cysteine (20 μmol/L) into cysteic acid by H_2O_2 (50 mmol/L) oxidation at several temperatures (50–90°C) was also studied.

RESULTS AND DISCUSSION

The procedure used to determine the Cys–SO_3H concentration in dragonfly eggs must be well qualified to avoid misinterpretation. First, a determined mass of eggs was weighed and homogenized in deonized water. This homogenate was then treated with H_2O_2 at a certain temperature for a given time, followed by hydrolysis with HCl at 110°C for 24 h. The latter released oxidized cysteine and cystine present in the sample, and does not interfere in the determination of Cys–SO_3H formed upon H_2O_2 treatment. Finally, formed Cys–SO_3H was detected and quantified by ion-exchange HPLC using a standard curve (Fig. 1).

We show here that cysteic acid is indeed capable of damaging the acrylo/melamine clearcoat tested at 90°C. The damage is quite similar to that caused by sulfuric acid and by dragonfly eggs.[1] Treating the clearcoat panels with a 100 μmol/L solution of Cys–SO_3H, initial lost of gloss (ca. 2 h at 90°C) occurs and the clearcoat begins to blister after another 2 h (Fig. 2).

The dependence of Cys–SO_3H formation on H_2O_2 concentration at 90°C and 24 h exposure time is shown in Table I. These experimental conditions are the same as those used in our former studies;[1,5] 90°C is about the highest temperature reached by a black car surface exposed to sunlight during the summer.

Because the fresh mass of eggs used could not be accurately weighed, the results obtained with several batches of eggs (triplicates) were normalized for the amount of alanine (Ala), a quite stable amino acid when submitted to drastic pH and temperature treatments. As depicted in Figure 3, the Cys–SO_3H/Ala dependence on H_2O_2 concentration describes a saturation curve. No detectable cysteic acid has been observed either in absence of H_2O_2 or at concentrations below 0.50 mmol/L under heating. At H_2O_2 concentration above 50 mmol/L (with ca. 30 mg eggs), the reaction has practically reached saturation upon heating for 24 h at 90°C. The amount of Cys–SO_3H/

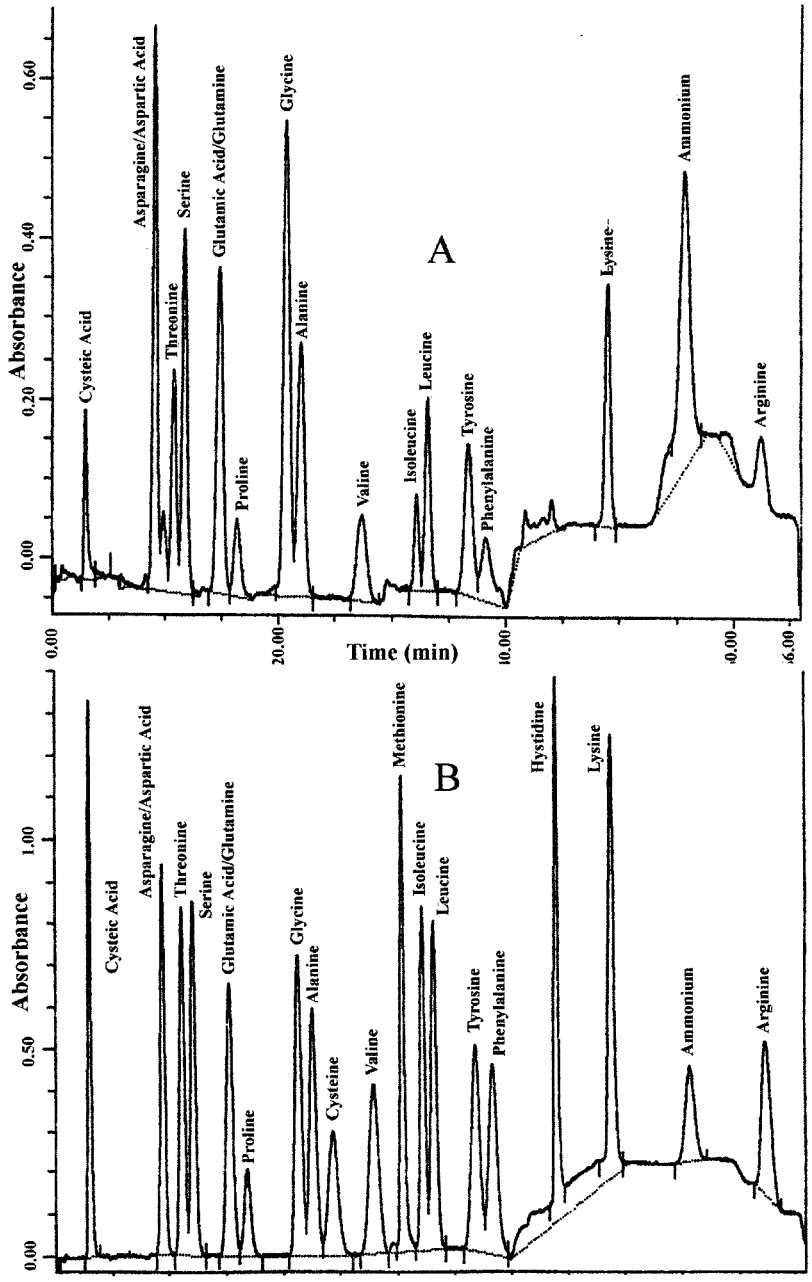

Figure 1 Aminograms of the hydrolyzed dragonfly egg homogenates monitored at 440 and 570 nm after treatment with H_2O_2 (50 mmol/L) at 90°C for 24 h (A). Calibration curve obtained with authentic Cys–SO_3H and amino acids (B).

Ala reached a practically constant value in the range of 0.50–1.0 mol/L H_2O_2 (i.e., 0.37 ± 0.05).

It is difficult to know whether the H_2O_2 concentrations used are compatible or not with the amount actually generated by dragonfly eggs during sclerotization. It is well known that dragonfly eggs can be collected and stored in water for years without change.[1,6] However, they become harder and darker when laid directly exposed to air and light (sclerotization). This means that it is impossible to measure H_2O_2 produced by eggs collected in water, as sclerotization does not occur in these conditions. Anyway, as can be seen in Figure 3, a small amount of hydrogen peroxide suffices to initiate Cys–SO_3H production when dragonfly eggs are exposed long enough to 90°C. Although there is no visual change in eggs stored in water for years, the time required for damaging activity

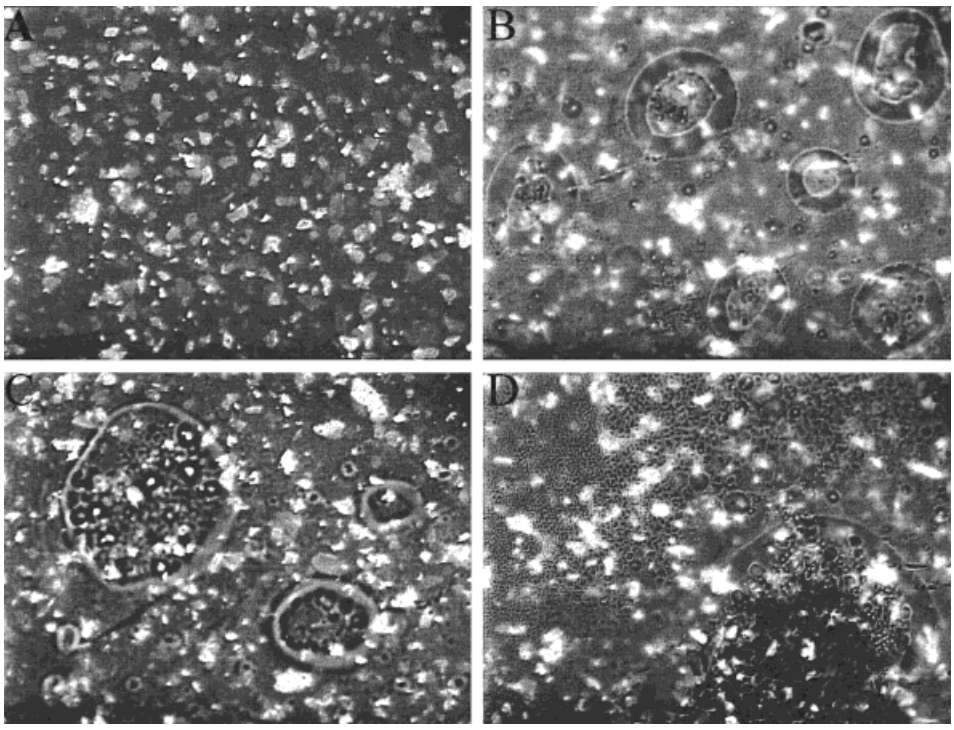

Figure 2 Acrylo/melamine clearcoat panel (A) after Cys–SO_3H attack (B–D) at 90°C for 4 h. The white spots are the metallic pigment of automotive painting. Micrographs magnified 16×.

to the clearcoat panel used is much longer for stored eggs (at least 1 year) than for freshly collected ones.[5]

The temperature dependence of Cys–SO_3H formation in eggs was investigated using 50 mmol/L H_2O_2 for 24 h (Table I). Temperatures above 90°C were not examined, as this is the maximum temperature reached by cars directly exposed to sunlight. An apparent exponential dependence of Cys–SO_3H/Ala formation on the temperature was observed (Fig. 4). An Arrhenius plot of the data gives a roughly straight line, from which is possible to obtain a value for the apparent activation energy of the oxidation of cysteine and cystine residues of egg protein by H_2O_2, E_a = 32 ± 2 kJ/mol (Fig. 4, inset).

To confirm whether the activation energy value obtained is related to the oxidation of egg protein cysteine residues to cysteic acid, the temperature profile of authentic cysteine to cysteic acid oxidation by H_2O_2 was also studied. In a first approach, 10 μmol/L of Cys–SH and 50 mmol/L of H_2O_2 were allowed to react at 50–90°C for 24 h. This exposure was found to be too long as the cysteine oxidation reached saturation above 50°C. Upon shorter exposure (4 h), using ca. 20 μmol/L of

Table I Normalized Values of Cysteic Acid (nmol)/Alanine (nmol) Found in Dragonfly Eggs upon Heating in the Presence of H_2O_2 at Several Exposure Times

[H_2O_2] (mmol/L)[a]	0.50	1.0	5.0	10	50
Cys–SO_3H/Ala	0.19 ± 0.02	0.20 ± 0.01	0.22 ± 0.03	0.28 ± 0.03	0.32 ± 0.05
Temp. (°C)[b]	50	60	70	80	90
Cys–SO_3H/Ala	0.10 ± 0.01	0.14 ± 0.03	0.17 ± 0.07	0.28 ± 0.01	0.35 ± 0.05
Exp. Time (h)[c]	1	4	8	16	24
Cys–SO_3H/Ala	0.13 ± 0.01	0.15 ± 0.03	0.24 ± 0.04	0.26 ± 0.04	0.26 ± 0.01

[a] At 90°C for 24 h.
[b] [H_2O_2] = 50 mmol/L for 24 h.
[c] [H_2O_2] = 50 mmol/L at 90°C.

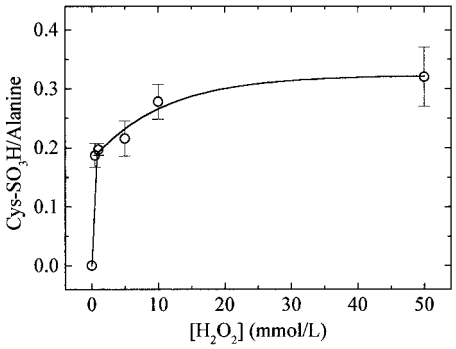

Figure 3 Cysteic acid formation during oxidation of dragonfly egg homogenates by H_2O_2 at 90°C for 24 h.

Table II Temperature Dependence of Cysteine Oxidation to Cysteic Acid on Hydrogen Peroxide

Temperature (°C)	[Cys–SH][a] (μmol/L)	[Cys–SO_3H][a] (μmol/L)
25	22 ± 1	0
50	18 ± 1	4 ± 1
60	13.5 ± 0.4	6.4 ± 0.9
70	11.7 ± 0.9	7.2 ± 0.2
80	5.8 ± 0.3	14.5 ± 0.3
90	0	22 ± 1

[a] Cys–SH recovered and Cys–SO_3H formed after reaction of ca. 20 μmol/L Cys–SH with 50 mmol/L H_2O_2 for 4 h.

Cys–SH and 50 mmol/L of H_2O_2, an exponential dependence of Cys–SO_3H formation on temperature was observed (Table II, Fig. 5). As expected, formation of Cys–SO_3H occurs concomitantly with Cys–SH disappearance. An Arrhenius plot of the data (Fig. 5, inset) also gives a roughly straight line, from which an apparent value for H_2O_2 oxidation of cysteine to cysteic acid can be calculated (E_a = 40 ± 4 kJ/mol). This value agrees very well with that determined by studying direct egg oxidation by H_2O_2, arguing in favor of our proposition that Cys–SO_3H may indeed be formed in the latter process.

Finally, the yield of Cys–SO_3H formed by treatment of eggs with H_2O_2 at 90°C as a function of exposure time was studied. Figure 6 shows saturation in Cys–SO_3H formation after 8–10 h treatment. Nevertheless, cysteic acid can already be detected just after 1 h of exposure to 50 mmol/L of H_2O_2 at 90°C. This result is in agreement with our former study,[1] where damage could be visually detected on clearcoat panels treated with fresh eggs at 80°C for 4 h.

CONCLUSIONS

The present work reinforces our hypothesis on the mechanism of acrylo/melamine clearcoat degradation by dragonfly eggs postulated when we compared the similar effect of acid rain and dragonfly oviposition on clearcoat panels. Either clearcoat acid hydrolysis or nucleophilic or radical attack to the resin promoted by cysteine/cystine residues were first elected as possible causes for the damage.[1] Further study indirectly detected the presence of cysteic acid derivatives on H_2O_2-treated eggs by the SERS technique,[5] and reinforced the belief that cysteic acid was the actual agent of clearcoat hydrolysis. Finally, the data presented here are consistent with the notion that cysteic acid derivatives may be responsible for the acrylo/

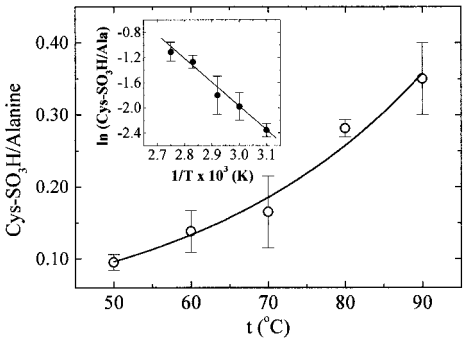

Figure 4 Temperature dependence of cysteic acid formation in the oxidation of dragonfly egg homogenates by H_2O_2 (50 mmol/L) for 24 h. Inset: Arrhenius plot of data.

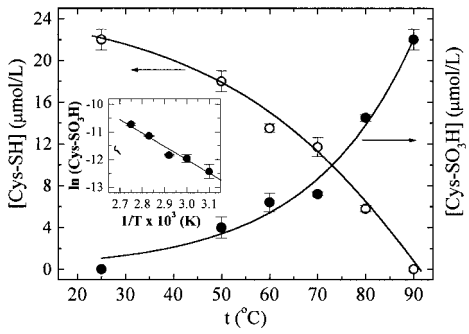

Figure 5 Temperature dependence of cysteic acid formation (and cysteine recovered) in the oxidation of cysteine (~20 μmol/L) by H_2O_2 (50 mmol/L) for 4 h. Inset: Arrhenius plot of data.

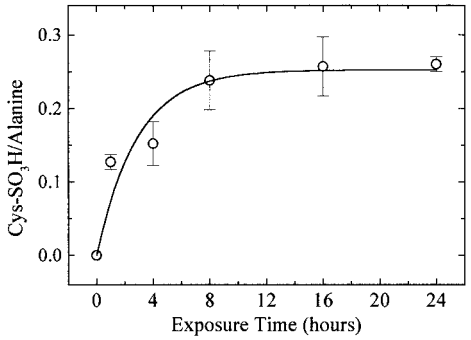

Figure 6 Effect of exposure time on cysteic acid formation in the oxidation of dragonfly eggs with H_2O_2 (50 mmol/L) at 90°C.

melamine clearcoat degradation, as cysteic acid could be unambiguously detected and quantified in egg samples treated with H_2O_2 at high temperatures.

In summary, H_2O_2 is long known to be produced during the "respiratory burst" required for egg sclerotization (tyrosine polymerization). When dragonflies lay eggs on the car surface reflecting the sunlight,[7] a chemical reaction between egg cysteine/cystine residues and H_2O_2 takes place concomitantly, which is certainly accelerated by hot surfaces. The amino acids are oxidized to the correspondent cysteic acids in the egg protein structure, which are strong enough to catalyze the acid hydrolysis of the clearcoat. Being a fixed acid and bearing an organic tail, cysteic acids are expected to be more efficient than sulfuric acid as a catalyst of acrylo/melamine polymer hydrolysis, as is the case of methanesulfonic acid.[1]

We thank the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Programa de Apoio a Núcleos de Excelência (PRONEX), and the Alexander von Humboldt Foundation for financial support and Renner DuPont for the resin samples and technical support.

REFERENCES

1. Stevani, C. V.; Porto, J. S.; Trindade, D. J.; Bechara, E. J. H. J Appl Polym Sci 2000, to appear.
2. Rodgers, W. R.; Garner, D. P.; Cheever, G. D. J Coat Technol 1998, 70, 83.
3. Cremlyn, R. J An Introduction to Organosulfur Chemistry; John Wiley & Sons Ltd.; Chichester, 1996; Darkwa, J.; Mundoma, C.; Simoyi, R. H. J Chem Soc Faraday Trans 1998, 94, 1971; Gilbert, B. C.; Laue, H. A. H.; Norman, R. O. C.; Sealy, R. C. J Chem Soc Perkin Trans 2 1975, 892.
4. de Faria, D. L. A.; Temperini, M. L. A.; Sala, O. Quim Nova 1999, 22, 541.
5. Stevani, C. V.; de Faria, D. L. A.; Porto, J. S.; Trindade, D. J.; Bechara, E. J. H. Polym Degrad Stabil 2000, 68, 61.
6. Kawasaki, H.; Sato, H.; Suzuki, M. Insect Biochem 1975, 4, 99.
7. Horváth, G.; Zell, J. Nature 1996, 379, 303; Kriska, G.; Horváth, G.; Andrikovics, S. J Exp Biol 1998, 201, 2273.