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Identification of the Heme Compound Copurified with Deoxyribonucleic Acid (DNA) from Bloodstains, a Major Inhibitor of Polymerase Chain Reaction (PCR) Amplification

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ABSTRACT: The heme compound found in deoxyribonucleic acid (DNA) extracted from bloodstains, which is regarded as a major inhibitor of polymerase chain reaction (PCR), was characterized in comparison with alkaline and acid hematin, histidine and ammonia hemochromogens, and globin and serum albumin hemochromogens digested by proteinase K. Alkaline and acid hematin were almost completely removed by phenol/chloroform treatment and ethanol precipitation, so as not to be copurified with DNA from the specimens. Spectrophotometric results indicated that the contaminant was likely to be the product of proteinase K digestion of some heme-blood protein complex, which was not completely extracted by organic solvents and remained in the ethanol precipitates of DNA. The results of polyacrylamide gradient gel electrophoresis and intensity of the inhibition of PCR suggested that the ligand of the contaminant was a somewhat large molecule, resistant to the proteolysis by proteinase K. The addition of bovine serum albumin to the reaction mixture prevented the inhibition of PCR by the heme compounds, probably by binding to the heme. This showed that the inhibition was not due to the irreversible inactivation of the enzyme.

KEYWORDS: pathology and biology, deoxyribonucleic acid (DNA), polymerase chain reaction (PCR), bloodstain, hematin, hemin

Because the polymerase chain reaction (PCR) analysis of deoxyribonucleic acid (DNA) can detect trace amounts of target genes it can be a powerful method for forensic practices such as personal identification, parentage testing, sex determination, and species identification. From bloodstains, however, a contaminant is sometimes copurified with

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DNA by phenol/chloroform treatment and ethanol precipitation, that interferes with PCR amplification [*1–4*]. The contaminant has been suggested to be a heme compound such as hematin [*2–4*]. We have also shown a heme *b* derivative was present in an inhibitory sample from the putrefied liver tissue of a cadaver [*5*]. This study demonstrates characteristics of the heme compound found in bloodstain extracts and compares it with hematin and various hemochromogens in order to improve PCR analysis using such contaminated forensic specimen extracts. To investigate the inhibition of PCR by heme compounds, the mitochondrial DNA (mtDNA) D-loop region was amplified. This target region shows considerable DNA variation, and it is easily amplified since it exists in multiple copies in a cell [*5*], making it suitable for personal identification [*6,7*].

Materials and Methods

Extraction of DNA and Contaminant

DNA was extracted using organic solvents [*8,9*] from fresh peripheral leukocytes as well as a 6 cm × 2 cm bloodstain on the surface of an electric pole at the traffic accident scene. The bloodstain was cut into 5 mm × 5 mm pieces. Each specimen was treated with 50 μg/mL proteinase K in 4 ml SE buffer [0.5% N-lauroylsarcosine, 10 mM tris(hydroxymethyl)aminomethane (Tris)/hydrochloride (HCl), 50 mM ethylene-diaminetetraacetic acid (EDTA), pH 7.4] at 37°C for either 2 h or overnight. To each sample was added 4 mL of phenol/chloroform (1:1, water-saturated, pH 7–8) which was then mixed by inverting for 10 min. Following centrifugation at 3000 rpm for 10 min, the aqueous phase was transferred to a fresh tube, and the extraction with phenol/chloroform was repeated. The aqueous phase was added to 4 mL of chloroform/isoamyl alcohol (24:1), mixed by inversion for 10 min and centrifuged. The aqueous phase was transferred to a fresh tube, mixed with 8 mL of cold ethanol, and stored at −20°C for 2 h. Following centrifugation at 7000 rpm for 20 min at 4°C, the precipitate was dried under vacuum, and dissolved in an appropriate volume of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.4).

The color of the bloodstain extract was yellowish, while the leukocyte DNA solution was transparent. The bloodstain extract was dialyzed using a Centricon® 100 (Amicon, Beverly, MA) that was centrifuged at 2500 rpm for 20 min, separating DNA and the colored dialysate. This colored dialysate was regarded as the contaminant in this study, since Centricon treatment can eliminate the inhibitor in the forensic specimen extracts [*5*]. The absorbance spectrum at 350 to 670 nm of the latter was recorded using a Hitachi 220A double beam spectrophotometer. A small amount of sodium hydrosulfite ($Na_2S_2O_4$) was added to the dialysate and its spectrum was also recorded.

Preparation of Hematin and Hemochromogens

All of the standard heme compounds used in this study were prepared from ferriprotoporphyrin chloride (hemin) extracted from peripheral blood [*10*]. Briefly, 12.5 mL of fresh whole blood was added dropwise to 75 mL of sodium chloride (NaCl)-saturated glacial acetic acid stirred at 95 to 100°C. Three drops of HCl was added and the mixture was boiled for 1 h. The formed hemin crystals were collected by centrifugation at 2500 rpm for 5 min, and washed twice successively with 50% acetic acid, water, ethanol and diethylether. The dried residue was dissolved in 0.5 mL pyridine, and diluted with 4 mL chloroform. The solution was filtered using qualitative filter paper (Advantec, Tokyo, Japan) and added to 30 mL glacial acetic acid saturated with NaCl. After boiling to evaporate chloroform, 6 drops of HCl was added and the solution was stored at room temperature overnight. Recrystallized hemin was centrifuged and washed as described

above. Hemin was then dissolved in 1 mL of 1 N sodium hydroxide (NaOH), and the formed ferriprotoporphyrin hydroxide (alkaline hematin) solution was diluted with 9 mL of distilled water. The content of hematin was quantified as pyridine hemochromogen of heme *b* [*11*].

From the alkaline hematin solution, histidine, ammonia, globin and human serum albumin (HSA) hemochromogens were prepared [*12*].

An aliquot of 10 mM L-histidine monohydrochloride, monohydrate (Wako, Osaka, Japan) was prepared and its pH was adjusted to 7.4 by the addition of NaOH. Reduced histidine or ammonia hemochromogen was produced by the mixture of either 600 μL of 10 mM histidine or a 0.25% ammonia solution (Wako), 100 μL of 5 mM alkaline hematin, 100 mL of 1 N NaOH, and a small amount of $Na_2S_2O_4$.

Globin protein was prepared from fresh hemoglobin [*13*]. Globin or HSA hemochromogen was produced by the mixture of 600 μL of 10 mg/mL globin or HSA (Wako) in distilled water, 100 μL of 5 mM alkaline hematin, 100 μL of 1 N NaOH, and a small amount of $Na_2S_2O_4$.

Characterization of the Heme Compounds

Each reduced hemochromogen (0.8 mL) was diluted with 3.2 mL of TE buffer, and exposured to air using a vortex mixer to form oxidized hemochromogen [*14*]. For use in experiments, 100 μL of alkaline and acid hematin solutions were also diluted with 3.9 mL of TE buffer. These solutions has pHs from 7 to 8. Globin and HSA hemochromogens were digested by proteinase K in SE buffer at 37°C overnight, purified with phenol/chloroform and chloroform/isoamyl alcohol, and precipitated by cold ethanol in the same fashion that DNA is purified. The absorbance spectra of hematin and oxidized hemochromogens were recorded using 100 μL each of the solutions diluted in 1 mL of distilled water. Then, these solutions were reduced by addition of a small amount of $Na_2S_2O_4$ and the spectra were again recorded.

Using 12.5 and 125 μM solutions of the heme compounds diluted with TE buffer, the solubilities of alkaline hematin and oxidized hemochromogen solutions in organic solvents were investigated. Globin and HSA hemochromogens were digested by proteinase K in SE buffer at 37°C overnight before the treatments as follows. These solutions were subjected to phenol/chloroform, chloroform/isoamyl alcohol treatment and/or ethanol precipitation as above. The specific absorbances of the solutions were determined and compared with those of untreated samples.

Polyacrylamide gradient gel electrophoresis of the heme compounds was performed using a Polyacrylamide Gradient Gels PAA 4/30 gel and a Gel Electrophoresis Apparatus GE-2/4 (Pharmacia LKB Biotechnology, Uppsala, Sweden). Following pre-electrophoresis at 100 V for 30 min at 10°C, 62.5 μM of the heme compounds in 20% sucrose solution were electrophoresed at 100 V for 2 h at 10°C. The gel was then soaked in 1 M acetic acid-0.05 M EDTA buffer (pH 4.4) containing a small amount of benzidine and a few drops of hydrogen peroxide for 30 min, and stored in 7% acetic acid.

PCR Amplification and the Inhibition by Heme Compounds

The mtDNA D-loop region was amplified by PCR using primers, 5′-TTCGTACATTACTGCCAG-3′ and 5′-TTCACGGAGGATGGTGGT-3′ [*15*]. The PCR reaction mixture (50 μL) consisted of 10 mM Tris/HCl, pH 8.3, 50 mM potassium chloride, 2.0 mM magnesium chloride, 200 μM each of deoxyribonucleoside-5′-triphosphates (dNTP) and 0.5 μM each of primers. The mixtures also contained 100 ng/reaction of template DNA from fresh leukocytes and 1 unit/reaction of AmpliTaq® DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). To this reaction mixture, 0.25 or 2.5 μM of hematin, hemochrom-

ogen or the contaminant was also added. Since the contaminant is a heme *b* compound [*5*], the content of the contaminant was also quantified as pyridine hemochromogen of heme *b* [*9*]. Following a preheating at 94°C for 2 min, consecutive incubations at 94°C for 1 min, 55°C for 0.5 min and 72°C for 2 min were performed 25 times. After a final incubation at 72°C for 7 min, PCR products were electrophoresed in an ethidium bromide-stained, 1.5% agarose gel.

To explore the effect of bovine serum albumin (BSA) on the inhibition of PCR by digested hemochromogens, samples prepared as above containing 2.5 μM of the digested hemochromogens and/or 160 μg/mL BSA were prepared, preheated, and consecutively incubated for 10 PCR cycles as above. These samples were then rapidly chilled in ice-water. Immediately after the addition of 10 μL of the PCR buffer (free from Taq DNA polymerase, template DNA and the heme compounds) containing 960 μg/mL BSA or not, 20 more cycles of PCR and the final incubation were performed. This examination demonstrates reversibility of inhibition of PCR. The inhibitors interfere with the amplification during 10 initial cycles of total 30 PCR cycles. If the inhibition was encountered by BSA, the gene was amplified during 20 more cycles of PCR; 20 PCR cycles is required to visualize the PCR product in the gel.

Results

Spectrophotometric Analysis

The contaminant (colored dialysate) in bloodstain extracts showed spectral peaks at 390 to 400, 500, and 570 to 580 nm, and the absorbances at 416–420 and 550–555 nm appeared in the reduced form (Fig. 1). Each of oxidized and reduced forms of this compound had a small specific peak at 470 nm. Alkaline hematin showed specific absorbances at 390, 490, and 613 nm, and the reduced form at 385, 540, and 580 nm (Fig. 2). The spectral peaks of oxidized and reduced forms of acid hematin were 380, 550 and 640 nm, and 395 nm, respectively. Histidine, ammonia and globin hemochromogens had spectral peaks at 402 to 406 nm and spectral shoulder at 520 to 530 nm in their oxidized forms, and peaks at 410 to 420, and 520 to 555 nm in their reduced forms (Figs. 2 and 3). However, oxidized HSA hemochromogen showed a peak at 390 nm and

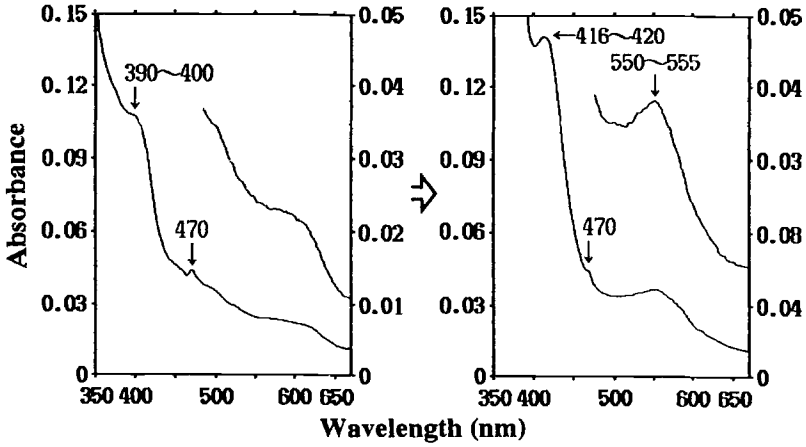

FIG. 1—*Spectra at 350–670 nm of the contaminant in bloodstain extract* (left) *and its reduced form* (right).

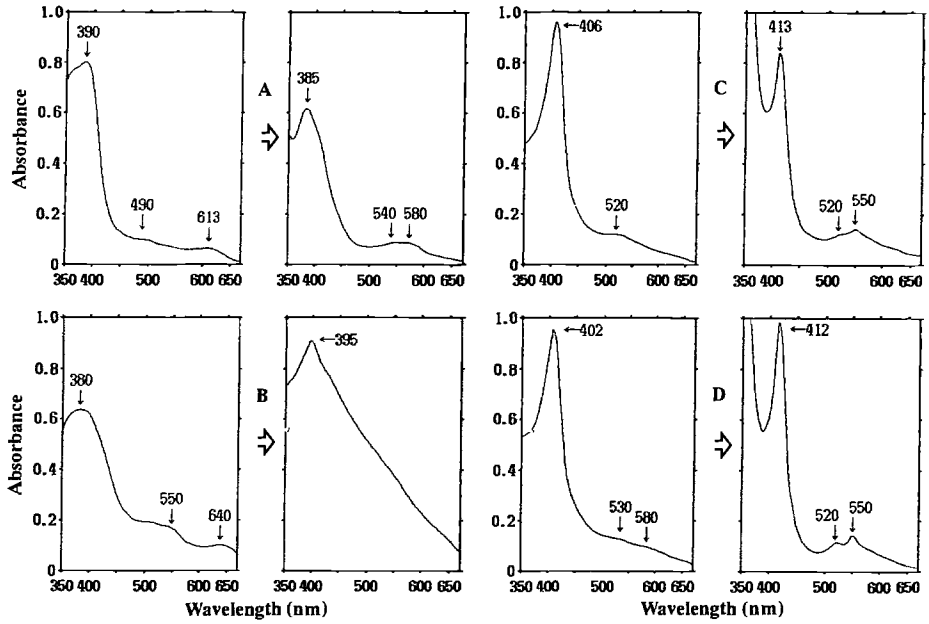

FIG. 2—*Spectra at 350–670 nm of oxidized* (left) *and reduced* (right) *forms of alkaline hematin* (A), *acid hematin* (B), *histidine hemochromogen* (C) *and ammonia hemochromogen* (D).

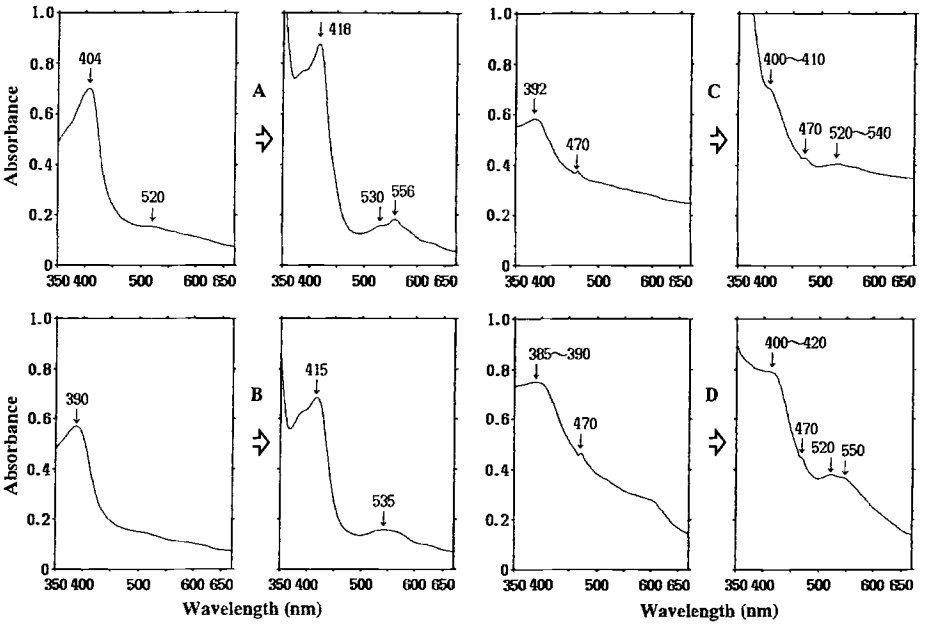

FIG. 3—*Spectra at 350–670 nm of oxidized* (left) *and reduced* (right) *forms of globin hemochromogen* (A), *HSA hemochromogen* (B), *digested globin hemochromogen* (C) *and digested HSA hemochromogen* (D).

the reduced form at 415 and 510 to 540 nm. The spectra of digested globin and HSA hemochromogens were different from their original compounds (Fig. 3), showing the peaks at 390 to 395 nm in the oxidized forms and 400 to 420 and 510 to 540 nm in the reduced forms. Each form of these digested compounds also had a small peak at 470 nm, as did the contaminant in bloodstain extracts.

Solubility in Water and Organic Solvents

The recovery rates of the heme compounds from the organic solvent treatments are shown in Table 1. When alkaline hematin was treated with phenol/chloroform or chloroform/isoamyl alcohol or ethanol, 40 to 60, >95 or >99% of the substance was transferred to each of the organic solvents, respectively. By successive treatments with these organic solvents, almost all alkaline hematin was removed from the samples. Acid hematin was more soluble in organic solvents. These results indicate that neither alkaline nor acid hematin should be copurified with DNA. Binding of any ligand to iron heme was likely to lessen its solubility in organic solvents: About 1.5% of histidine, 8% of ammonia, 40 to 60% of digested globin and 50 to 70% of digested HSA hemochromogens remained following three successive treatments with the organic solvents.

Polyacrylamide Gradient Gel Electrophoresis

The order of the electrophoretic mobilities of the contaminant and the standard heme compounds was: ammonia hemochromogen ≥ histidine hemochromogen > digested globin hemochromogen > digested HSA hemochromogen ≥ alkaline hematin > acid hematin > the contaminant > globin hemochromogen > HSA hemochromogen (Fig. 4).

TABLE 1—*Recovery rates (%) of the heme compounds from organic solvent treatments.*

Porphyrin compounds (μM)	Treatments			
	(1)Phenol/ chloroform	(2)Chloroform/ isoamyl alcohol	(3)Ethanol precipitation	(1)+(2)+(3) (successive)
Alkaline hematin (OD_{390})				
12.5	43.7	2.80	0.0510	<0.01
125	58.8	5.01	0.676	<0.01
Acid hematin (OD_{380})				
12.5	5.01	2.68	2.46	<0.01
125	2.30	0.882	8.21	<0.01
Histidine hemochromogen (OD_{406})				
12.5	44.5	78.0	4.25	1.39
125	41.6	83.5	4.83	1.86
Ammonia hemochromogen (OD_{402})				
12.5	52.9	82.8	15.4	7.63
125	62.2	96.2	3.82	8.89
Digested globin hemochromogen[a] (OD_{390})				
12.5	68.0	85.5	49.9	46.4
125	97.1	86.4	84.5	65.2
Digested HSA hemochromogen[a] (OD_{390})				
12.5	75.3	89.8	55.9	54.1
125	84.8	83.5	87.5	75.1

Specific absorbances of oxidized forms of the heme compounds were present in parentheses.
[a]Hemochromogens digested by 50 μg/mL proteinase K in SE buffer at 37°C overnight.

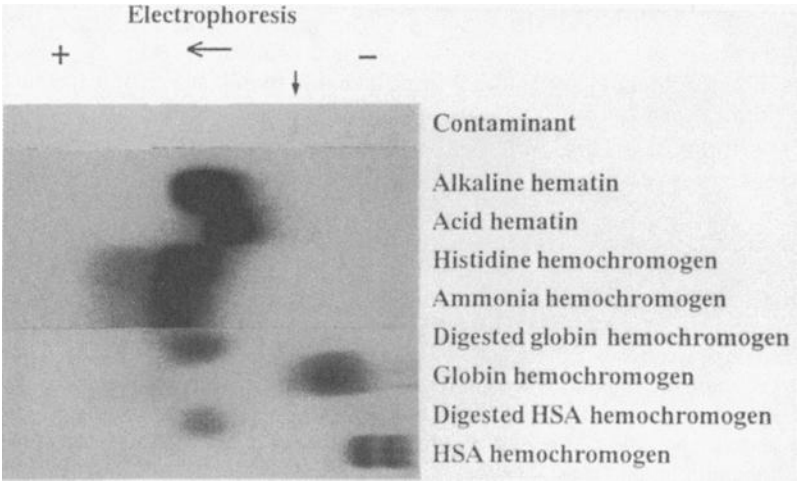

FIG. 4—*Polyacrylamide gradient gel electrophoresis of the contaminant and heme compounds. The arrow indicates the stained band of the contaminant.*

Inhibition of PCR Amplification

As little as 0.25 μM of alkaline or acid hematin inhibited PCR amplification of the mtDNA D-loop region (Fig. 5). This inhibitory action of Taq DNA polymerase was weakened by the binding of any ligand to iron heme; When 0.25 μM of histidine hemochromogen was added, the PCR product was faintly observed in the gel (almost invisible in the photograph). The addition of 0.25 μM of ammonia or globin hemochromogen,

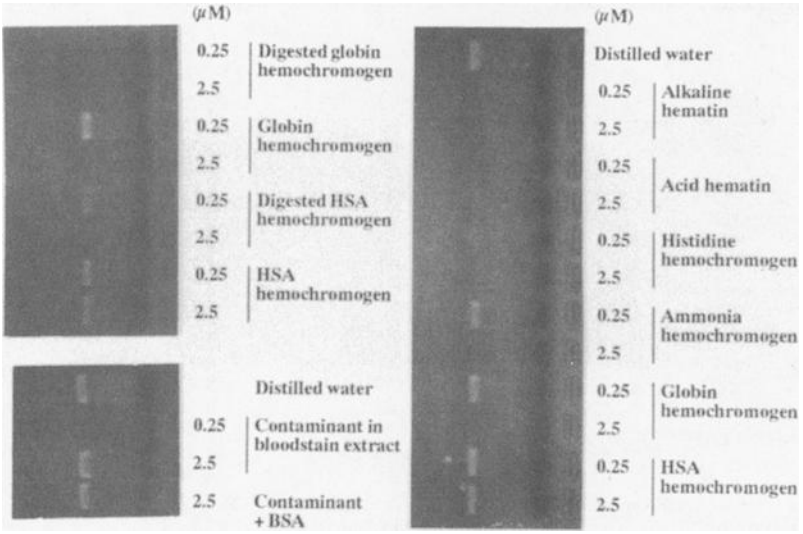

FIG. 5—*Inhibition of PCR amplification of mtDNA D-loop region by the heme compounds and the contaminant in bloodstain extract. Numbers indicate final concentrations of the heme compounds in the PCR mixture.*

or even 2.5 μM of HSA hemochromogen interfered little with PCR amplification. When globin or HSA hemochromogen was digested by proteinase K, the inhibition was strengthened; The addition of 0.25 μM of digested globin suppressed PCR amplification completely, and 0.25 μM HSA hemochromogen suppressed it to some extent. When BSA was added to Taq DNA polymerase solution either before or after the addition of an inhibitor, the inhibitory action was completely blocked (Fig. 6). The addition of BSA even after 10 cycles of PCR reversed the enzyme inhibition and the region was amplified to some extent, during 20 more PCR cycles. The inhibition by the contaminant was obviated by addition of BSA as well (Fig. 5).

Discussion

Hematin has been postulated as a major contaminant in bloodstain extracts, that is it copurified with DNA from forensic specimens and inhibits Taq DNA polymerase activity in PCR [*1–5*]. However, alkaline and acid hematin (ferriprotoporphyrin hydroxide) are both very soluble in both water and organic solvents (Table 1), so that DNA prepared using organic solvents and ethanol precipitation is unlikely to contain these compounds. In addition, a contaminant found in a forensic bloodstain extract of DNA was purified and its spectra examined are found to be very different from acid or alkaline hematin (Figs. 1 and 2). Inhibitory contaminants are little copurified with DNA from freshly prepared bloodstain or fresh whole blood in our experience, suggesting that such contaminant may be derived from degenerated hemoglobin. It is also known that "Serumhemätin," the complex of ferric heme and serum proteins, is produced in putrefied blood and tissues [*16,17*]. This "Serumhemätin" may be digested by proteinase K during the extraction of DNA, probably resulting in the formation of a lower molecular weight compound. To clarify whether such digested compound is the contaminant, we investigated the characteristics of hematin and various hemochromogens in comparison with the contaminant.

As ligands of hemochromogens, nitrogenous substances such as pyridine, nicotine, cyanide, ammonia, imidazole compounds and proteins are known [*12,14*]. Pyridine, nicotine or cyanide hemochromogen was not investigated because these ligands are not always contained in bloodstains and tissues. In human hemoglobin, heme iron contacts

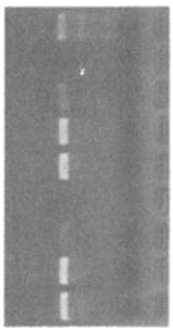

FIG. 6—*Reversibility of PCR inhibition by addition of BSA. [a]Addition of BSA to PCR buffer containing both Taq DNA polymerase and hemochromogen, [b]addition of hemochromogen to PCR buffer containing both Taq DNA polymerase and BSA. Following 10 cycles of PCR were performed, 10 μL of PCR buffer containing BSA or not was added to each samples, and 20 more cycles were performed.*

with imidazole side chains of two histidine residues of globin proteins [*18*]: Proximal histidine (F8) forms ligands with iron heme, and distal histidine (E7) touch the heme but is not bonded to it. This chemical structure resembles that of histidine hemochromgen, which is ferric heme liganded with the imidazole side chain of histidine. The fact that free amino acids are formed in postmortem blood by enzymatic breakdown of proteins [*19*] also suggests that L-histidine is a likely ligand of the contaminant, although it is unknown whether histidine hemochromogen is formed in forensic specimens. Ammonia also increases in postmortem blood [*19*]. Histidine and ammonia hemochromogens were therefore compared with the bloodstain contaminant.

Globin and HSA hemochromogens were prepared as models for the complex of heme and blood proteins. The spectrum of the latter was almost identical with that of "Methämalbumin" (Fig. 3) [*17*], one component of "Serumhemätin." Denatured globin can also be rebonded to heme [*12*]. These hemochromogens were digested by proteinase K: This enzyme is used for the isolation of DNA from tissues since it rapidly destroys deoxyribonuclease (DNase) [*20,21*]. Since proteinase K also shows strong proteolytic activity on denatured proteins such as hemoglobin [*20*], the ferric heme ligands from digested globin and HSA hemochromogens were likely to be amino acids or oligopeptides.

The contaminant found in our bloodstain extracts was yellowish, which is different from the colors of dark green alkaline and orange-colored acid hematin solutions. Colors of the hemochromogens prepared in this study were brownish, and the diluted solutions were yellowish. Bonding some nitrogenous ligands to iron heme lessened its solubilities in organic solvents (Table 1), such that it is reasonable that the hemochromogens could be copurified with DNA. Because of a small amount of such contaminants, it was difficult to identify the ligand from its spectra. However, existence of small peaks at 470 nm in the spectra of the contaminant, digested globin and HSA hemochromogens strongly suggested that the contaminant was likely to be the proteinase K-digest of some heme-blood protein complex. It was unknown what structure of these compounds caused the specific absorbances at 470 nm.

Although histidine, ammonia, digested globin and digested HSA hemochromogens are of greater molecular weights than alkaline and acid hematin, the electrophoretic mobilities of the formers in polyacrylamide gradient gel were larger than those of the latters (Fig. 4). Since the mobility in polyacrylamide gel is affected by both molecular weights and charges of the samples, positive charge of heme iron in alkaline or acid hematin was likely to suppress the electrophoretic movement from anode to cathode side. Ligands such as histidine and ammonia were expected to neutralize the charge of heme iron more than hydroxide ligands of hematin, resulting in increase of the mobility. The mobilities of globin and HSA hemochromogens were smaller because of their greater molecular weights (about 17 000 and 70 000, respectively). Proteolysis of these hemochromogens thus increased the mobilities. The contaminant had less mobility than hematin and digested hemochromogens, and slightly greater mobility than globin hemochromogen. This result suggested that the contaminant was much greater in molecular weight than those of the digested hemochromogens.

Hemin is known to suppress the activities of restriction endonucleases, DNase I, DNA and ribonucleic acid (RNA) polymerases, and reverse transcriptase [*22–27*]; the term "hemin" used in these reports means hemin dissolved in alkaline solution, which is equal to "alkaline hematin" used in this study. PCR using Taq DNA polymerase was also inhibited by hemin (alkaline hematin) (Fig. 5). According to the fact that the hemin inhibition of DNA polymerase was reversed by globin (molecular weight, 16 500) but not by non-heme-containing proteins such as carbonic anhydrase (29 000) [*22*], the intensity of hemin inhibition was affected by the chemical structure of hemeligand complex, possibly in association with the neutralization of the heme iron charge. Thus, the

inhibition by digested HSA hemochromogen was weaker than alkaline or acid hematin, and HSA hemochromogen shows the weakest inhibition. The inhibitory activity of the contaminant was no more than globin hemochromogen and less than histidine and the digested hemochromogens (Fig. 5). From this result and the electrophoretic mobility, the ligand of the contaminant was likely to be somewhat larger molecule than those of the digested hemochromogens, and, therefore, might be resistant to proteolysis by proteinase K.

Addition of BSA to PCR buffer effectively blocked the inhibition of PCR by digested globin and HSA hemochromogens and by the contaminant (Figs. 5 and 6), as demonstrated in our last study [*5*]. Possibly, heme irons of the digested hemochromogens were partly neutralized by their ligands, so that BSA molecule could be bonded to it. Then, HSA hemochromogenlike complex was likely to be formed, preventing the inhibition completely. Since the inhibition of PCR was encountered by BSA even after that 10 PCR cycles were performed, the inhibition was reversible as same as the hemin inhibition of DNA polymerase [*22*].

This study demonstrated characteristics of the contaminant in bloodstain extract in comparison with several heme compounds, suggesting that the contaminant was likely to be the heme-blood protein complex. A further investigation is required to identify the structure for the establishment of more reliable PCR technique in various forensic practices.

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