PCR inhibitor: water-soluble melanin, which inhibits DNA polymerases and DNases

T. Yoshii, K. Akiyama, K. Tamura and I. Ishiyama
Department of Legal Medicine, Teikyo University School of Medicine, Kaga 2-11-1, Itabashi, Tokyo, Japan

With the development of methods in current molecular biology, individual identification and paternity testing have been carried out by typing DNA polymorphisms. Analytical methods are divided into two groups. One group includes Southern blot hybridization which detects RFLPs, VNTR polymorphisms and DNA-fingerprint. The other group includes PCR amplification of a polymorphic DNA region, followed by typing the amplified DNA by probe hybridization, RFLP analysis, VNTR detection or sequence determination. In cases when the materials contain only highly degraded DNAs and/or a trace amount of DNA, DNA typing with PCR amplification have provided good resolution. As indicated by various investigations, regions effectively analyzed are HLA-class Ⅱ genes, several short VNTR regions and noncoding D-loop region of mitochondrial DNA (mtDNA). Among them, the mtDNA D-loop region is the most sensitive, because the region can be amplified very efficiently because of numerous copies of mtDNA more than several hundreds per cell.

A PCR system was used to amplify the noncoding 333-bp region of mtDNA (mt333DNA) from single human hairs, and the following results were obtained: 1) Using natural black hairs in the length over 30 cm, mt333 DNA was always amplified from a 5-cm length of hair shaft sampled within a region 11 cm from the hair root, but it was not always amplified from a 5-cm region adjacent to this 11-cm region, and was not amplified in almost all cases when a 5-cm length of hair shaft was sampled from a region more than 16 cm distant from the hair root (Table 1). DNA preparations not responding to PCR were colored dark brown. 2) Using natural white hairs, mt333DNA was amplified from almost all specimens even up to a length of 31 cm (Table 1). 3) When natural black hairs were stained with an oxidation-type hair-staining agent (Bigen-5Ge), mt333DNA could not be amplified always even from the hair root portion, whereas the same treatment of white hairs gave no effect on the amplification (Table 2). In the cases showing no response on PCR, DNA preparations were also colored dark brown. 4) These dark brown DNA preparations inhibited completely the amplification of mt333DNA even after addition of purified DNAs. These results suggest that the dark brown substance in the DNA preparations inhibits the PCR. We therefore investigated the mechanism responsible for the development of this inhibitor. It was found that hydrogen peroxide (a component of hair-staining agent) produced water-soluble melanins from insoluble melanins. Consequently, it is expected that the water-soluble melanins, which occurs spontaneously and gradually in black hairs, may be provoked by oxidation with air.

We confirmed that water-soluble melanins act as an inhibitor

Table 1. PCR-amplification of mt333DNA from natural hair shafts at 5-cm intervals without hair root, from five women

Case No.	Color	region (cm) 1～6	6～11	11～16	16～21	21～26	26～31	31～36	36～41	41～46	46～51
1	black	+	+	+	−	−	−	−	−	−	
2	black	+	+	+	+	−	−	−	−	−	
3	black	+	+	−	−	−	−	−	−	−	
4	black	+	+	+	+	−	−	−	−	−	
5	black	+	+	−	−	−	−	−	−	−	
6	black	+	+	−	−	−	−	−	−	−	
7	black	+	+	+	−	−	−	−	−	−	
8	black	+	−	−	−	−	−	−	−	−	−
9	black	+	+	−	−	−	−	−	−		
10	black	+	+	−	−	−	−	−	−	−	
11	black	+	+	+	−	−	−				
12	black	+	+	+	+	−	−				
13	white	+	+	+	+	+	+				
14	white	+	+	+	+	+	−				
15	white	+	+	+	+	+	+				
16	white	+	+	+	+	+	+				

+ : amplified, − : not amplified

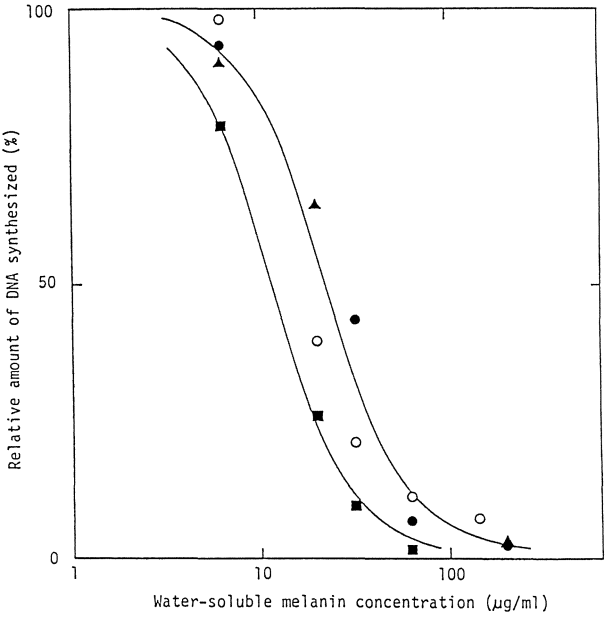

Fig. 1. Inhibition effects of water-soluble melanins (chemically synthesized and natural) on DNA synthesis by *Taq* DNA polymerase. The amount of DNA synthesized was monitored in terms of [α-^{32}P] dCMP incorporated into activated calf thymus DNA. The 100% values correspond to the amount of DNA synthesized in the absence of melanins under each reaction condition. The solid symbols represent the synthesized melanin and the clear symbols natural eumelanin. The circles, triangles and squares represent data obtained at 72℃, 60℃ and 37℃, respectively.

Table 2. PCR-amplification of mt333DNA from hairs stained with an oxidation-type hair-staining agent (Bigen-5Ge). Samples of hair shafts 5 cm long were sampled within a region 6 cm from the hair root

original color	staining time (h)						
	0	0.5	1	2	3	4	5
black	+	−	−	−	−	−	−
white	+	+	+	+	+	+	+

+ : amplified, − : not amplified

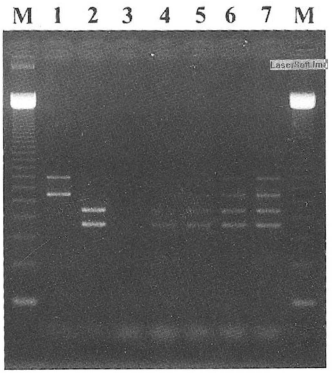

Fig. 2. A possible misdiagnosis of PCR-amplified VNTR (MCT118 locus) fragments by contamination of melanins. Each lane is as follows: (M) 123-bp ladder marker, (1, 2) two heterozygotes, (3～7) mixture of the two which was contaminated by melanin at concentration of 1.6 μg/ml(3), 1.2 μg/ml(4), 0.8 μg/ml(5), 0.4 μg/ml(6) and 0 μg/ml(7), respectively.

of DNA polymerases and DNases, based on the following results: 1) Water-soluble preparations made from chemically synthesized melanin (Sigma products), as well as natural black eumelanins, inhibited the PCR amplification of mt333DNA at concentrations of more than 2 μg/ml. 2) Quantitative measurement of *Taq* and *E. coli* DNA polymerase-catalyzed DNA synthesis in terms of the amount of [α-^{32}P]dCMP incorporated into activated calf thymus DNA showed that both of the water-soluble melanins had the same inhibition activity as represented by the sigmoidal curve derived from a quadratic equation of melanin concentration (Fig. 1). This observation suggested that DNA polymerases combined with two molecules of melanin to form an inactivated complex. 3) Water-soluble melanins gave no effect on neither the thermostability of *Taq* DNA polymerase at 94 ℃, nor the step of primer-annealing to templates. 4) Restriction digestion of λ DNA was inhibited by adding the melanins at concentrations of more than 1 μg/ml in the case of Hind Ⅲ and of more than 4 μg/ml in the case of Hae Ⅲ (the enzymes were at 11 units in 100 μl).

Water-soluble melanins exhibited a stepwise inhibition of DNA amplification of polymorphic DNA with VNTR. In the case of MCT118 (D1S58) locus, amplification degree of individual alleles depended on the numbers of VNTR; i.e. amplification of DNA fragments with a larger VNTR was likely to be more inhibited in comparison with those with a smaller one in the same PCR condition (Fig. 2). Hence, it should be possible that the heterozygous types are misdiagnosed as homozygous ones in DNA typing of criminal cases in the presence of PCR inhibitors.

We established a simple and useful method for removal of water-soluble melanins contaminating DNA preparations from hairs. The method was based on the adsorption of melanins to Bio-Gel. When a Bio-Gel P-60 minicolumn was equilibrated with 10 mM sodium acetate buffer, pH 4.2, water-soluble melanins were completely adsorpted to it whereas DNAs passed through, although the melanins showed incomplete adsorption to the gel when it was equilibrated with TE (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA). The filtrates of DNA preparetions gave always excellent response in amplifying mt333DNA by PCR procedure.

References

Yoshii, T., Tamura, K., Ishiyama, I. (1992) Presence of a PCR-inhibitor in hairs. Jpn J Legal Med 46:313-316

Yoshii, T., Tamura, K., Taniguchi, T., Akiyama, K., Ishiyama, I. (1993) Water-soluble eumelanin as a PCR-inhibitor and a simple method for its removal. Jpn J Legal Med 47:323-329

Prota, G. (1988) Progress in the chemistry of melanins and related metabolites. Med Reseach Reviews 8:525-556

Kasai, K., Nakamura, Y, White, R. (1990) Amplification of a variable number of tandem repeats (VNTR) locus (pMCT118) by the polymerase chain reaction (PCR) and its application to forensic science. J Forens Sci 35:1196-1200

Prinz, M., Berghaus, G. (1990) The effect of various stain carriers on the quality and quantity of DNA extracted from dried bloodstains. Z Rechtmed 103:191-197

Franchis, R., Cross, N. C. P., Cox, T. M. (1988) A potent inhibitor of *Taq* polymerase copurifies with human genomic DNA. Nucl Acids Res 16:10355

8. DNA sequencing