

Purification and Identification of a Serum Protein Increased by Anthelmintic Drugs for *Dirofilaria immitis* in Dogs

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ABSTRACT. Polyacrylamide gel electrophoretic analysis of canine serum protein has revealed that the administration of anthelmintics elicits an increase in a certain serum protein. This protein, named PT60, was partially purified by ammonium sulfate fractionation and preparative electrophoresis. The purified PT60 gave a single band with the molecular size of 53 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions. After reduction with 2-mercaptoethanol, two bands appeared at 35 kDa and 17 kDa, indicating that PT60 consists of two subunits which are linked with each other by disulfide bonds. PT60 had the capacity to bind to hemoglobin. In an immunodiffusion test, an antiserum against PT60 cross-reacted with canine haptoglobin (Hp). N-terminal amino acid sequences of two PT60 subunits were identical to those of α and β subunits of canine Hp, respectively. Thus, PT60 was identified as Hp.—**KEY WORDS:** acute phase protein, anthelmintic, *Dirofilaria immitis*, dog, haptoglobin.

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Mammalian plasma contains more than 200 kinds of protein, most of which are synthesized in the liver. Although the plasma level of the individual proteins is kept rather constant under normal physiological conditions, it changes in specific diseases, and hence is often measured for diagnostic purpose. Some drugs widely used for companion animals, such as anthelmintics, are known to be, more or less, toxic to the liver. Because the liver is the major organ for drug metabolism as well as plasma protein synthesis, it may be expected that plasma protein analysis is also useful for monitoring the effectiveness of drug treatment. We have therefore examined the pattern of serum proteins by polyacrylamide gel electrophoresis in dogs after treatment with anthelmintics for *Dirofilaria immitis*, and found a remarkable increase in certain protein bands. In the present study, this protein was purified and characterized as the initial step in investigating the effects of drug treatment on serum proteins.

MATERIALS AND METHODS

Sera and chemicals: Sera were obtained from male and female mongrel dogs suffering from heartworm, (4–6 kg, 6–12 months old), these were treated with anthelmintics, potassium melarsonyle (5.8 mg/kg, i.m. Rhone Poulenc, France) or levamisole hydrochloride (11 mg/kg, p.o., Nihon Lederle, Tokyo). Sera were also obtained from dogs free from

heartworm before and after the injection of another anthelmintic, milbemycin oxime (0.25 mg/kg, p.o., Sankyo, Tokyo). Serum samples were stored at -20°C until used.

High-performance liquid chromatography (HPLC) columns packed with TSK Phenyl-5PW RP were purchased from Tosoh (Tokyo). The molecular size markers, bovine serum albumin (68 kDa), ovalbumin (43 kDa), α -chymotrypsinogen A (25 kDa), soybean trypsin inhibitor (20 kDa) and cytochrome c (12.4 kDa) were purchased from Sigma (St. Louis, U.S.A.). Canine Hp was kindly provided by Dr. T. Kumazaki (Faculty of Pharmaceutical Sciences, Hokkaido University). Canine hemoglobin (Hb) was prepared according to the method of Makimura and Suzuki [11].

Detection of PT60: Detection of PT60 was performed by controlled electrophoresis [16]. Briefly, 10 μl of serum was mixed with 50 μl of 0.02 M borax-phosphate buffer (pH 8.0) containing 10% glycerol, and 10 μl of the mixture was applied to a 4.5–15.5% polyacrylamide gradient gel (column size, 0.35 \times 14 cm). After electrophoresis with a constant current of 0.55 mA/gel for 3 hr, protein bands were stained with Coomassie brilliant blue R-250, and a band of 60 kDa was referred to as PT60.

Partial purification of PT60: Serum rich in PT60 (20 ml) was fractionated by 50–70% saturated ammonium sulfate. The precipitate was collected by

centrifugation, dissolved into and dialyzed against distilled water. After insoluble materials were removed by centrifugation at $15,000 \times g$ for 20 min, the resulting supernatant was further fractionated by preparative electrophoresis: that is, the supernatant (1.3 mg protein in 60 μ l) was mixed with 30 μ l of 0.02 M borax-phosphate buffer (pH 8.0) and 10 μ l of 50% glycerol, and applied to a 7% polyacrylamide gel (column size, 1.5 \times 3.5 cm) prepared by the method of Davis [2]. After electrophoresis with a constant current of 3 mA/gel for 3 hr, the gel was cut into 3 mm slices, and proteins corresponding to PT60 were extracted from the slices with 7 ml of 0.02 M borax-phosphate buffer (pH 8.0) overnight. The extract was concentrated with cellulose tubing under suction, and used for experiments as a partially purified PT60 sample.

Isolation of subunits: The partially purified PT60 (170 μ g protein) was reduced in 100 μ l of reducing buffer (1% 2-mercaptoethanol, 6 M guanidine-HCl, 0.1 M Tris-HCl, pH 8.5) for 2 hr at room temperature in the dark. After the addition of 150 μ l of glacial acetic acid, the mixture was dialyzed against 0.05% trifluoroacetic acid and applied to reverse-phase HPLC on a TSK Phenyl-5PW RP column (4.6 \times 75 mm) according to the previous method [13]. Elution was carried out with a linear gradient of 5–80% acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 0.5 ml/min, and two major peaks were taken as those for subunits of PT60.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE): The molecular size of PT60 was determined by the method of Laemmli [10] with 15%-polyacrylamide gel.

N-terminal amino acid sequence analysis: The N-terminal amino acid sequence was determined with a model 477A protein sequencer on-lined with a model 120A PTH-amino acid analyzer (Applied Biosystems, Foster City, U.S.A.).

Preparation of antisera: PT60 (100 μ g) was mixed with Freund's complete adjuvant, and injected subcutaneously into a rabbit. Booster injections were carried out 2 and 3 weeks later, and 2 weeks after the last injection, the rabbit was bled to prepare antiserum.

Detection of Hb-PT60 complex: Formation of Hb-PT60 complex was analyzed by controlled electrophoresis [16] followed by peroxidase activity staining; that is, 8 μ l of the partially purified PT60 (1 mg/ml) was mixed with 2 μ l of 0.1% Hb and 5 μ l of 0.02 M borax-phosphate buffer (pH 8.0) containing

10% glycerol, and applied to the gel. After electrophoresis, free Hb and Hb-PT60 complex were stained with 0.05% diaminobenzidine and 0.03% hydrogen peroxide in 50 mM Tris-HCl (pH 7.4).

RESULTS

Serum PT60 after administration of anthelmintics: Figure 1 shows typical electrophoretograms of serum protein after the administration of anthelmintics. In the controlled electrophoresis used in this study, more than 20 protein bands could be detected. Although hardly any protein bands were changed by the anthelmintic treatment, there was a band that showed an apparent increase. This protein migrated to the pre-transferrin position corresponding to a molecular size of about 60 kDa, and was thus tentatively named PT60. When dogs suffering from heartworms (*Dirofilaria immitis*) were given potassium melarsonyle or levamisole hydrochloride, PT60 increased within 2–6 days (Fig. 1, A, B). A remarkable increase in PT60 was found even when an anthelmintic (milbemycin oxime) was given to dogs free from heartworms (Fig. 1, C).

Partial purification and molecular properties of PT60: PT60 was partially purified from dog serum according to the procedure described in MATERIALS AND METHODS. Figure 2 shows the electrophoretogram of the partially purified PT60. A major and a minor bands appeared close to each other and their ratio was about 95:5 as determined by densitogram.

When the partially purified PT60 was analyzed by SDS-PAGE under non-reducing conditions, its molecular size was estimated to be 53 kDa (Fig. 3A). After reduction with 2-mercaptoethanol, two bands appeared at 35 kDa and 17 kDa (Fig. 3B). These results suggest that PT60 consists of small (17 kDa) and large (35 kDa) subunits that are linked by disulfide bonds. This subunit structure of PT60 is similar to that reported in canine Hp [8, 9]. We then further investigated whether PT60 was canine Hp or not.

Identification of PT60 as canine Hp: The Hb-binding activity of PT60 was analyzed by controlled electrophoresis followed by peroxidase activity staining of Hb (Fig. 4). When purified PT60 and Hb were mixed and run on the gel, a new band with peroxidase activity appeared in the region corresponding to a molecular size much larger than PT60 and Hb. This result indicates that PT60 can associate

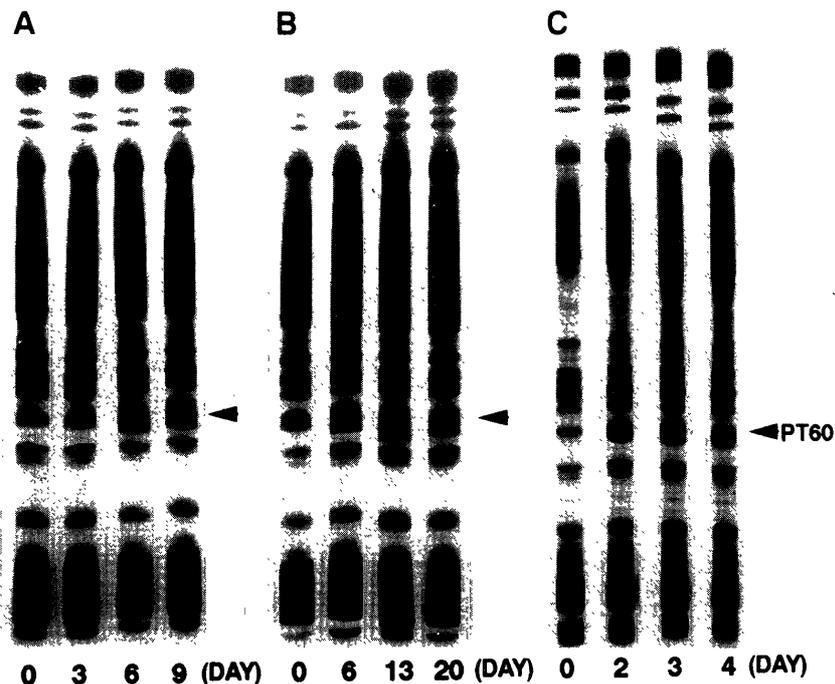


Fig. 1. Electrophoretogram of serum proteins after the administration of anthelmintics in mongrel dogs. A: Potassium melarsonyle (5.8 mg/kg) was given intramuscularly on DAYS 1 and 2. B: Levamisole hydrochloride (11 mg/kg) was given orally on DAYS 4-18. C: Milbemycin oxime (0.25 mg/kg) was given orally on DAY 1.

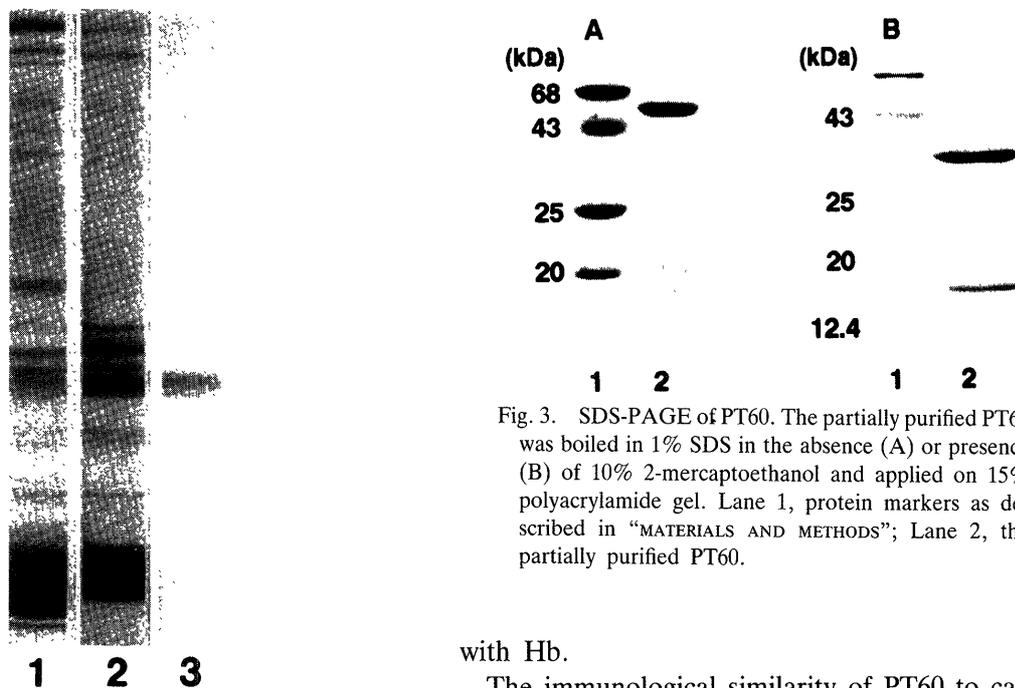


Fig. 2. Electrophoretogram of partial purification of PT60. Lane 1, serum; Lane 2, fraction precipitated by 50-70% saturated ammonium sulfate; Lane 3, extract from polyacrylamide gel.

Fig. 3. SDS-PAGE of PT60. The partially purified PT60 was boiled in 1% SDS in the absence (A) or presence (B) of 10% 2-mercaptoethanol and applied on 15% polyacrylamide gel. Lane 1, protein markers as described in "MATERIALS AND METHODS"; Lane 2, the partially purified PT60.

with Hb.

The immunological similarity of PT60 to canine Hp was also examined. As shown in Fig. 5, the partially purified PT60 and canine Hp formed a completely fused precipitin line against anti-PT60 serum.

Two subunits (35 kDa and 17 kDa in Fig. 3B) of PT60 were purified by reverse-phase HPLC after

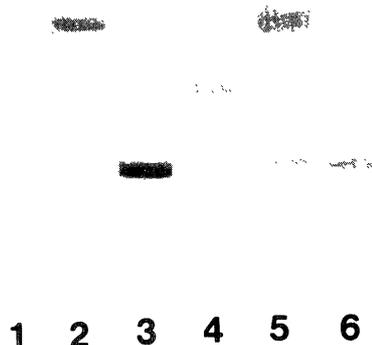


Fig. 4. Detection of Hb-PT60 complex in PAGE. Lanes 1-3, diaminobenzidine staining; Lanes 4-6, protein staining; Lanes 1 and 4, the partially purified PT60; Lanes 2 and 5, the partially purified PT60 mixed with canine Hb; Lanes 3 and 6, canine Hb.

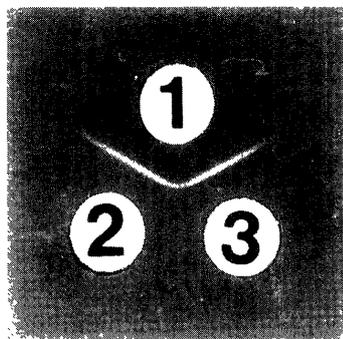


Fig. 5. Double-diffusion test of PT60 and canine Hb. Well 1, anti-PT60 serum; Well 2, the partially purified PT60; Well 3, canine Hb.

reducing PT60 with 2-mercaptoethanol as described in MATERIALS AND METHODS. The N-terminal amino acid sequence of 35 kDa subunit was identical with that of the β chain of Hp (Table 1). Similarly, the sequence of 17 kDa subunit was identical to that of the Hp α chain.

DISCUSSION

In this study, we partially purified a serum protein of dogs that was increased after the administration of anthelmintics, and named it PT60. Our results clearly demonstrate that PT60 is canine Hp.

SDS-PAGE analysis revealed that PT60 consists of small (17 kDa) and large (35 kDa) subunits. This subunit structure of PT60 is quite similar to those of canine Hp reported by Kurosky *et al.* [9]. In fact, PT60 showed Hb-binding activity. Moreover, an antiserum to PT60 cross-reacted with Hp that was independently purified from dog serum. All these results suggest that PT60 obtained in this study is canine Hp. This was confirmed further by amino acid sequence analysis of the N-terminal regions of two subunits. Twenty amino acids of the N-terminal region of 35-kDa and 17-kDa subunits were identical to those of β and α subunits of canine Hp, respectively [7].

It has generally been recognized that Hp is one of the acute phase proteins produced in the liver, and that its serum level increases with inflammation, infection and tissue damage [1, 3, 4, 6, 14]. In this study, we found that the administration of anthelmintics elicited an increase in the serum Hp level in dogs. It was reported that the dose of a drug used in this experiment did not induce an inflammatory response, such as leukocytosis or fever [15]. Moreover, the increase in serum Hp was observed even in dogs without microfilariae. Therefore, it is unlikely that the serum Hp response to anthelmin-

Table 1. Comparison of the N-terminal amino acid sequences of PT60 and canine Hp subunits

Protein	Amino acid																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
PT60 17-kDa subunit	Glu	-Asp	-Thr	-Gly	-Ser	-Glu	-Ala	-Thr	-X ^{b)}	-Asn	Thr	-Glu	-Val	-Ser	-Leu	-Pro	-Lys	-Pro	-Pro	-Val
Canine Hp α -chain ^{a)}	Glu	-Asp	-Thr	-Gly	-Ser	-Glu	-Ala	-Thr	-Ala	-Asn	-Thr	-Glu	-Val	-Ser	-Leu	-Pro	-Lys	-Pro	-Pro	-Val
PT60 35-kDa subunit	X	-Met	-Gly	-Gly	-Ser	-Val	-Asp	-Ala	-Lys	-Gly	X	-Phe	-Pro	X	-X	-Ala	X	-Met	-Val	X
Canine Hp β -chain	Ile	-Met	-Gly	-Gly	-Ser	-Val	-Asp	-Ala	-Lys	-Gly	Ser	-Phe	-Pro	-Trp	-Gln	-Ala	-Lys	-Met	-Val	-Ser

a) Data on canine Hp are from reference No. 7.

b) Not determined.

tics was due to some inflammation and/or dead microfilariae. It is thus more likely that Hp may increase in the active phase of liver functions such as detoxication of drugs. Further studies with other types of drugs are needed to confirm this. In inflammation, interleukin-6 (IL-6) is the most efficient mediator of Hp induction [5, 12], but at present it is not clear whether this is also the case in the drug-induced Hp response.

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