Purification, Electrophoretic Characterization and Plasma Concentrations of Feline or Canine Antithrombin III

Fumihiko SUGIYAMA, and Toshinori FURUKAWA¹⁾

Laboratory Animal Research Center, and ¹⁾Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Sakura-mura, Niihari-gun, Ibaraki 305, Japan

(Received 11 July 1985/Accepted 20 September 1985)

ABSTRACT. Feline or canine antithrombin III (AT III) was purified from plasma by salt precipitations and heparin-attached Sepharose CL-6B. For the isolation of canine AT III, an additional ion exchange chromatography was needed for the final purification. The two purified proteins showed similar characteristics with cellulose acetate, agarose, polyacrylamide gel or immunoelectrophoresis, having the molecular weight of approximately 62,000. The isoelectric points were 4.70 and 4.75 for feline: 4.70, 4.75 and 4.80 for canine AT III. Thrombin inhibition activity of the purified proteins was reinforced in the presence of heparin. Anti-feline and anti-canine AT III sera formed precipitin lines against feline and canine plasma, but not against human, Japanese monkey, rat or mouse plasma. Concentrations of AT III and levels of heparin cofactor activity in plasma were measured by Laurell's electroimmuno assay and enzymatic assay technics, respectively. Values for feline plasma were: 0.50 ± 0.1 (mean $\pm$ standard deviation) g/l; 198.57 ± 33.1 NIH U/ml; and for canine plasma: 0.49 ± 0.1 g/l; 178.51 ± 26.0 NIH U/ml.—**KEY WORDS:** antithrombin III, canine, feline, heparin cofactor activity, plasma

Jpn. J. Vet. Sci. 47(6): 987–994, 1985

Antithrombin III (AT III) is a glycoprotein produced in the liver [7], and migrates to α 2-fraction in electrophoresis [23]. It is also known as a serine proteinase inhibitor as it inhibits thrombin [6], plasmin [21], kallikrein [25], and the coagulant factors IXa, Xa, XIa, or XIIa [4, 6]. It has been isolated from some mammalian species for comparative biophysics and biochemistry. In human, AT III is importantly related to thrombosis, and its plasma level is routinely determined to aid the diagnosis and treatment of thrombosis. Information on the relation of feline and canine AT III to thrombosis is lacking. In the present study, we attempted to purify feline and canine AT III from plasma, observed the migrations of the purified AT III in several types of electrophoresis, and examined their inhibition activities against thrombin. Moreover, the concentrations of AT III and heparin cofactor activity were measured in healthy feline and canine plasma by an electroimmuno assay and enzymatic assay technics,

respectively.

MATERIALS AND METHODS

Blood samples from twenty Japanese domestic cats and twelve Beagles were collected into plastic syringes containing acid-citrate-dextrose solution. Feline or canine AT III was purified principally by the method of Koide [14] as illustrated in Fig. 1.

Cellulose acetate electrophoresis was performed at a constant 300 V/membrane for 35 min using a High Resolution Electrophoresis Kit (Gelman Sciences Inc.). Methods for agarose gel electrophoresis, immunoelectrophoresis, polyacrylamide gel electrophoresis (PAGE), and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) have been described previously [9, 10, 11]. Isoelectric focusing was done with a procedure described by Hirabayashi [13]. The purified protein was electrophoresed at 500 V/gel for 24 hr at 4°C.

For amino acid analysis, the purified pro-

Fig. 1. The purification procedure of antithrombin III.

tein was hydrolyzed for 24 hr at 105°C in thoroughly evacuated glasstubes using 5.7 N HCl and 4% β -mercaptoethanol. The hydrolysate was analyzed with an amino acid analyzer (MLC-203 system IV, Atto Co., Ltd.).

The concentration of protein was determined by dye-binding assay (Bio-rad Co.) with mouse IgG as standard.

Antithrombin and heparin cofactor activity were measured with a Test Teem AT III kit (Daiichi Pure Chemicals Co., Ltd.). Purified protein or plasma was mixed with human thrombin (when measuring heparin cofactor activity, AT III or plasma was mixed with heparin before the addition of thrombin),

incubated at 37°C for 5 min. Then the substrate S-2238 was added, and the mixture was incubated again and absorbance was read at 405 nm.

Rabbit anti AT III sera were obtained by subcutaneous immunization at two times with purified protein emulsified with Freund's complete adjuvant. These sera were stored at -80°C after inactivation for 30 min at 56°C.

Immunological cross-reactivity was detected by Ouchterlony's double diffusion method with 1% agarose in 0.01 M barbital buffer, pH 8.6. Antiserum against feline or canine AT III was placed in a center well and the surrounding wells were filled with human,

Japanese monkey, canine, feline, rat, and mouse plasma.

Concentration of AT III in plasma was determined by Laurell's electroimmuno assay technics [19]. Three diluted samples were applied to holes in agarose gel containing 1% antiserum, and electrophoresed at 10 V/cm for 6 hr in 0.01 M barbital buffer at pH 8.6.

RESULTS

Methods for the purification of AT III is illustrated in Fig. 1. For canine AT III, the eluate from the affinity column (Heparin Sepharose CL-6B, Pharmacia A.B.) was dialyzed against 0.05 M Tris-HCl buffer at pH 8.3, and applied onto a Mono-Q column (Pharmacia A.B.) equilibrated with the same buffer. The column was then eluted by a linear gradient between the above buffer and 0.1 M Tris-HCl buffer at pH 6.0. After the contaminant was eluted at this condition, canine AT III was eluted with 0.1 M Tris-HCl buffer (pH 6.0) in a pure form.

The patterns of purified AT III on electrophoresis are shown in Fig. 2. The two isolated proteins showed similar electrophoretical characteristics. These proteins moved about one third the distance of the migration of albumin from the origin on the cellulose acetate electrophoresis, and formed only a single band. On the agarose gel electrophoresis, a single band at the α 2-fraction was observed with both AT III, similarly, in immunoelectrophoresis, single lines were observed at the α 2-fraction. These proteins were located between albumin and hemoglobin on 7.5% PAGE. There were also two bands on the cathodal side of albumin on 5–20% gradient PAGE. The electrophoretic mobility of feline AT III was greater than that of canine AT III.

The molecular weight of both feline and canine AT III was about 62,000 dalton as a single polypeptide chain as determined by 12% SDS-PAGE with the molecular weight

markers kit (Pharmacia A.B.).

Isoelectric points were 4.70 and 4.75 for feline AT III, and 4.70, 4.75, and 4.80 for canine AT III with pI markers (Pharmacia A.B.).

The two purified proteins had similar amino acid compositions in Table 1.

The antithrombin activity was 119.28 NIH U/mg for the feline protein and 85.77 NIH U/mg for the canine protein. The heparin cofactor activity was 417.50 NIH U/mg in feline protein and 336.32 NIH U/mg in canine protein. Thrombin inhibition by the purified proteins was reinforced just as in human AT III.

Anti-feline and -canine AT III sera were tested against human, monkey, canine, feline, rat, and mouse plasma by Ouchterlony's method for immunological cross-reactivity. Anti-feline AT III serum formed a strong precipitin line with feline plasma and a weak one with canine plasma. The reaction of anti-canine AT III was strong to both feline and canine plasma. But the two antisera had no reactivity against human, monkey, rat, and mouse plasma.

Antithrombin III levels were 0.50 ± 0.1 g/l in feline plasma and 0.49 ± 0.1 g/l in canine plasma as measured by electroimmuno assay (Fig. 3), and heparin cofactor activity was 198.57 ± 33.1 NIH U/ml in feline plasma and 178.51 ± 26.0 NIH U/ml in canine plasma as measured by the enzymatic assay. These levels were obtained with samples taken from ten cats and eleven dogs.

DISCUSSION

Many plasma proteins of human origin have been purified for analysis of physicochemical characters and metabolism. These proteins are useful in clinical diagnosis and treatment. Similar analysis, however, have not frequently been done with feline and canine plasma proteins. In the present study, we purified and characterized feline and ca-

Fig. 2. Several electrophoretograms of feline and canine antithrombin III (a; agarose gel electrophoresis, b; cellulose acetate electrophoresis, c; 7.5% polyacrylamide gel electrophoresis, d; 5–20% gradient polyacrylamide gel electrophoresis, e; SDS-polyacrylamide gel electrophoresis, f; isoelectric focusing, g; immunoelectrophoresis). Abbreviation: FA; feline antithrombin III, FP; feline plasma, CA; canine antithrombin III, CP; canine plasma, MWMs; molecular weight markers, A/FP; anti-feline serum, A/CP; anti-canine serum.

Table 1. Amino acid compositions of feline and canine antithrombin III

Amino acid	Estimated number of residues	
	Feline	Canine
Asparatic acid	47.2	46.9
Threonine	22.7	21.1
Serine	30.2	27.1
Glutamic acid	49.6	46.0
Proline	24.7	21.0
Glycine	18.8	19.7
Alanine	28.8	28.8
Half-cystine	Not investigated	
Valine	25.5	25.7
Methionine	9.6	11.3
Isoleucine	17.9	17.1
Leucine	39.1	38.9
Tyrosine	9.5	8.8
Phenylalanine	24.0	25.2
Tryptophan	Trace	3.4
Lysine	30.9	31.4
Histidine	6.5	5.0
Arginine	19.2	23.1

nine AT III, which act as a blood coagulant inhibitor and accounts for 75–80% of total thrombin inactivator in the human plasma [6].

The precipitation, ion exchange, and gel filtration chromatography were used for purification of AT III [1, 2] before the introduction of affinity chromatography. Miller-Anderson *et al.* [20] established the purification method for AT III with heparin coupled to cyanogen bromide-activated agarose. Since then, the characteristics of AT III purified by the affinity chromatography have been reported for some animal species. For instance, Koide [14] isolated human, pig, rabbit, and rat AT III. In our study with this affinity method for canine AT III, ion exchange chromatography was needed because a impurity still remained after affinity chromatography.

The purified AT III showed the properties similar to the AT III of other animals. First, the molecular weight of our purified AT III, 62,000 dalton, was close to those of the AT III purified from other animals: human, 59,000 [14]; bovine, 61,000 [18]; horse, 58,000 [18]; porcine, 58,000 [14]; rabbit,

Fig. 3. Electroimmuno assay of feline antithrombin III. Samples in three dilutions were electrophoresed in 1% agarose containing 1% rabbit anti-feline antithrombin III serum at 10 V/cm in 0.01 M barbital buffer (pH 8.6) for 6 hr at 10°C.

63,000 [14]; rat, 63,000 [14]; chicken, 60,000 [15]. Secondly, the isoelectric point of our purified AT III was approximate to those in rat (4.85) [17] and human (5.11) [22]. Thirdly, the migration patterns on cellulose acetate and agarose gel electrophoresis, and 5–20% gradient PAGE were similar to those reported by others [5, 8, 23]. Lastly, our purified AT III had the same heparin-dependent inhibition of thrombin as shown by Koide [14, 15] with those from other animals.

Damus & Wallace [5] reported that canine AT III had a molecular weight of 82,000 determined by gel filtration or 77,000 by SDS-PAGE. The values are larger than that of our purified canine AT III or of those of other animals previously reported. Tollefsen *et al.* [24] reported the existence of heparin-dependent thrombin inhibitor without AT III in human plasma. This protein, named HC-II by them, had a molecular weight of 72,000 dalton by SDS-PAGE, and was larger than human AT III. Judging from the molecular weight, it is surmised that the canine AT III isolated by Damus and Wallace is similar to HC-II.

In our affinity chromatography using phosphate buffer containing 2 M NaCl, the eluate from feline plasma contained only AT III, while the eluate from canine plasma at the same stage had one more unknown protein. The two proteins from canine plasma could bind heparin. There are two possible reasons for this phenomenon. The unknown protein

might be either a HC-II-like protein or a phenotype of AT III.

In studying immunological cross-reactivity among human, pig, rabbit, rat, and chicken AT III, Koide [14, 15] noted an immunologic partial cross reaction between human and pig AT III. In our experiments, immunological cross-reactivity was found between feline and canine AT III. Therefore, this protein have a species specific on almost mammals. The reason for this could not be deduced from our analysis of amino acid composition in Table 1.

Electroimmuno assay demonstrated that dogs and cats carry similar concentrations of AT III in their plasma. These concentrations were about twice as large as the concentrations in rat (0.22 g/l) [16] or human (0.19 g/l) [3]. Hence, concentrations of human AT III may not be used for the diagnosis and treatment of AT III related disease in feline or canine species. We also demonstrated that heparin cofactor activity had similar levels in feline and canine plasma. Similar results were obtained ($115 \pm 14\%$ for feline plasma and $104 \pm 12\%$ for canine plasma) by Green [12]. This activity, however, was expressed as a percentage of thrombin inhibition activity of a normal plasma pool. When other workers used Green's normal human plasma pool as a standard, data could be compared with his. But it would be difficult for others to obtain the same normal plasma pool. Therefore, the level of heparin cofactor activity should be represented as thrombin units of inhibited human thrombin.

ACKNOWLEDGEMENTS. We wish to thank Dr. K. Yasuraoka, and Dr. M. Fukui for critical suggestions and reading this manuscript, and dedicate our best thanks to Mr. M. Yamaguchi for this photographic assistance. The work was partly supported by the grant No. 57771558 from the Scientific Research Fund of the Japanese Ministry of Education, Science and Culture.

REFERENCES

1. Abildgaard, U. 1967. Purification of two progressive antithrombins of human plasma. *Scand. J. Clin. Lab. Invest.* 19: 190-195.
2. Abildgaard, U. 1968. Highly purified antithrombin III with heparin cofactor activity prepared by disc electrophoresis. *Scand. J. Clin. Lab. Invest.* 22: 89-91.
3. Chan, V., Chan, T.K., Wong, V., Tso, S.C., and Todd, D. 1979. The determination of antithrombin III by radioimmunoassay and its clinical application. *Brit. J. Haemat.* 41: 563-572.
4. Damus, P.S., Hicks, M., and Rosenberg, R. 1973. Anticoagulant action of heparin. *Nature.* 246: 355-357.
5. Damus, P.S., and Wallace, G.A. 1974. Purification of canine antithrombin III-heparin cofactor using affinity chromatography. *Biochem. Biophys. Research Commun.* 61: 1147-1153.
6. Davie, E.W., and Hanahan, D.J. 1977. Blood coagulation proteins. pp. 421-544. In: *The Plasma Proteins*. Vol. III (Putnam, F.W. ed.), Academic Press, New York.
7. Denny, G.P., and Minot, G.R. 1915. The origin of antithrombin. *J. Physiol.* 38: 233.
8. Eketorp, R. 1982. Purification of antithrombin III by affinity chromatography. pp. 184-187. In: *Method of Plasma Protein Fractionation* (Curling, J.M.), Academic Press, New York.
9. Furukawa, T., and Sugiyama, F. 1984. Isolation of feline plasma plasminogen. *Jpn. J. Vet. Sci.* 46: 867-870.
10. Furukawa, T. 1984. Simple isolation procedure of transferrin in feline plasma. *Jpn. J. Vet. Sci.* 46: 871-875.
11. Furukawa, T., and Sugiyama, F. 1984. Isolation of feline plasma hemopexin. *Jpn. J. Vet. Sci.* 46: 877-880.
12. Green, R.A. 1984. Clinical implication of antithrombin III deficiency in animal diseases. *The compendium on continuing education.* 6: 537-545.
13. Hirabayashi, T. 1981. Two-dimensional gel electrophoresis of chicken skeletal muscle proteins with agarose gels in the first dimension. *Anal. Biochem.* 117: 443-451.
14. Koide, T. 1979. Isolation and characterization of antithrombin III from human, porcine and rabbit plasma, and rat serum. *J. Biochem.* 86: 1841-1850.
15. Koide, T., Ohta, Y., Odani, S., and Ono, T. 1982. Chicken antithrombin. Isolation, characterization, and comparison with mammalian antithrombins

- and chicken ovalbumin. *J. Biochem.* 91: 1223–1229.
16. Koj, A., Regoeczi, E., Toews, C.J., Leveille, R., and Gauldie, J. 1978. Synthesis of antithrombin III and alpha-1-antitrypsin by the perfused rat liver. *Biochem. Biophys. Acta.* 539: 496–504.
 17. Kumada, T., and Abiko, Y. 1980. Preparation and immunological determination of rat antithrombin III. *Thromb. Res.* 17: 789–799.
 18. Kurachi, K., Schmer, G., Hermodson M.A., Teller, D.C., and Davie, E.W. 1976. Characterization of human, bovine, and horse antithrombin III. *Biochemistry* 15: 368–372.
 19. Laurell, C.B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Analytical Biochem.* 15: 45–52.
 20. Miller-Anderson, M., Borg, H., and Anderson, L.O. 1974. Purification of antithrombin III by affinity chromatography. *Thromb. Res.* 5: 439–452.
 21. Rosenberg, R.D. 1974. The effect of heparin on factor XIa and plasmin. *Thromb. Diath. Haemorrh.* 33: 51–62.
 22. Rosenberg, R.D., and Damus, S.D. 1973. The purification and mechanism of action of human antithrombin-heparin cofactor. *J. Biol. Chem.* 248: 6490–6505.
 23. Thaler, E., and Schmer, G. 1975. A simple two-step isolation procedure for human and bovine antithrombin II/III (heparin cofactor): a comparison of two methods. *Brit. J. Haematol.* 31: 233–243.
 24. Tollefsen, D.M., Majerus, D.W., and Blank, M.K. 1982. Heparin cofactor II. Purification and properties of heparin-dependent inhibitor of thrombin in human plasma. *J. Biol. Chem.* 257: 2163–2169.
 25. Vennerod, A.M., and Laake, K. 1975. Inhibition of purified plasma kallikrein by antithrombin III and heparin. *Thromb. Res.* 7: 223–226.

要 約

ネコ・イヌのアンチスロンビンⅢの電気泳動的解析，血漿中濃度および活性について：杉山文博・古川敏紀¹⁾（筑波大学動物実験センター，¹⁾基礎医学系）——小出の方法 [14] に従って，ネコ・イヌ血漿アンチスロンビンⅢ（AT Ⅲ）の分離・精製を試みた。イヌの場合には，イオン交換クロマトグラフィーを必要とした。ネコおよびイヌ AT Ⅲ の電気泳動像は近似し，分子量はネコ・イヌとも約 62,000，等電点はネコで 4.70 と 4.75，イヌで 4.70，4.75 および 4.80 であった。これら AT Ⅲ はヘパリン存在でスロンビンの抑制を促進させた。両種の AT Ⅲ に対する特異抗血清はイヌおよびネコ血漿と反応したが，ヒト，ニホンザル，ラットおよびマウス血漿とは反応しなかった。Laurell の electroimmuno assay で測定した血漿中 AT Ⅲ 濃度はネコでは 0.50 ± 0.1 g/l，イヌでは 0.49 ± 0.1 g/l であり，また酵素活性法で測定した heparin cofactor activity はネコでは 198.57 ± 33.1 NIH U/ml，イヌでは 178.51 ± 26.0 NIH U/ml であった。