Kasetsart J. (Nat. Sci.) 44 : 1144 - 1152 (2010)

Identification and Characterization of Hemoglobin in Thai Bangkaew Dogs using Chromatographic, Electrophoretic and Mass Spectrometric Techniques

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ABSTRACT

The present study was aimed to measure the hematocrit (Ht) and the hemoglobin (Hb) concentration of Thai Bangkaew dogs, to investigate Hb phenotypes based upon the electrophoretic pattern of the Hb and to estimate molecular weights of the Hb (tetramer) and Hb subunits using gel filtration chromatography, SDS-PAGE and MALDI-TOF/TOF MS.

The results showed that 30 dogs had a mean Ht value (± standard error of the mean) of 36.38 ± 0.77% and a mean Hb concentration (± standard error of the mean) of 13.10 ± 0.32 g/dL. In addition, the fraction obtained from gel filtration of Sephadex G-100, at pH 7.4, corresponding to the isolated Hb protein was estimated at a molecular weight of 65,956 Dalton. The Hb fraction was used for analysis by native polyacrylamide gel electrophoresis (native-PAGE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS). The Hb electophoretic mobility in native-PAGE showed that all samples had one two-banded phenotype, consisting of a low mobility major band (94%) and a minor fast band (6%). In addition, SDS-PAGE showed two distinct bands of approximate molecular weights 12,980 and 14,820 Dalton, respectively. Moreover, the MALDI-TOF/TOF MS analyzes on the purified Hb fraction exhibited two abundant mass peaks at the molecular weights of 15,194.78 and 15,946.66 Dalton and three minor peaks at the molecular weights of 32,020.53, 48,326.29 and 64,545.95 Dalton.

Keywords: hemoglobin, chromatography, electrophoresis, mass spectrometry, Thai Bangkaew dog

INTRODUCTION

Hemoglobins (Hbs) are a group of important respiratory proteins with the major function of transportation of oxygen from the lungs to body tissues and facilitation of the return transport of carbon dioxide (Sittivilai *et al.*, 2004; Reece, 2005; Boonprong *et al.*, 2007). The

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Received date : 10/05/10 Accepted date : 20/07/10

hemoglobin (Hb) molecule is an assembly of four globular protein subunits. Each subunit is composed of a protein chain tightly associated with a non-protein heme group (Jain, 1996; Reece, 2005). In solution, mammalian Hbs are tetramers. The tetrameric molecules are composed of two pairs of heme composed of α- and β-subunits. The monomers (α or β), dimers (αβ) and tetramers ($\alpha_2\beta_2$) are in equilibrium (Wright and Douglas, 2009). Hb variations in dogs have been studied with the application of immobilized isoelectric focusing on polyacrylamide gels (Braend, 1988). The results revealed three phenotypes and the Hb types were consistent with polymorphism, controlled by two codominant genes, Ht_{83} and Ht_{85} (Braend, 1988). Moreover, genetic polymorphism of Hb in dogs indigenous to Japan revealed the phenotypic variation of Hb (controlled by one autosomal locus), with two codominant alleles, HbA and HbB (Tanabe *et al*., 1978). On the basis of cellulose acetate electrophoresis mobility, one Hb phenotype was detected in Thai ridgeback dogs, as only one band moved in the electrical field from the cathode to the anode (Noosud *et al*., 2007b). Noosud *et al*. (2008) reported previously that Hb from Thai ridgeback dogs revealed two bands by SDS-PAGE, which had molecular weights of 12,472 and 14,256 Dalton. Moreover, the molecular weight of Hb from Thai ridgeback dogs was determined using MALDI-TOF mass spectrometry. The molecular weights of the monomer were 15,194.56 and 15,946.60 Dalton and for the tetramer was 63,786.23 Dalton (Noosud *et al*., 2008).

Therefore, the aim of the present study was to measure the Ht and the Hb concentration of Thai Bangkaew dogs, to investigate Hb phenotypes based upon the electrophoretic pattern of the Hb and to estimate the molecular weights of the Hb (tetramer) and Hb subunits using gel filtration chromatography, SDS-PAGE and MALDI-TOF/TOF MS.

MATERIALS AND METHODS

Blood collection and hemolysate preparation

A total of 30 clinically normal Thai Bangkaew dogs from a small-animal teaching hospital were used in this study. Blood samples were collected via a saphenous or cephalic venipuncture through a 23-gauge needle, with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Each blood sample was used to measure the Ht by the microhematocrit centrifuge method (Biomed Group Co., Ltd. Thailand) and the Hb concentration by the cyanmethemoglobin method (Bio-Medical Laboratory, Thailand). The hemolysates were prepared according to Tamburrini *et al*. (2001) and Sittivilai *et al*. (2004). Briefly, blood was centrifuged (3,500 rpm, 10 min, 4°C) to remove acellular hemoglobin, plasma proteins, white blood cells and stroma. The precipitated cells were washed three times with an isotonic saline solution and centrifuged repeatedly until the supernatant was clear. The packed red blood cells were hemolysed by the addition of hemolysate reagent containing 0.005 M EDTA and 0.01% potassium cyanide (1 part packed red blood cells to 5 parts hemolysate reagent) as described by Helena Laboratories, USA (2001). The cell debris and cell membrane were removed by centrifugation (11,500 rpm, 5 min, 4°C) according to Bachmann *et al*., 1978. Hemolysates or Hb solutions were collected from the aqueous layer and stored at -20°C for further purification of the Hb. The total protein concentration was determined by the Biuret method.

Isolation of Hb

Hb was purified from the hemolysate using Sephadex gel filtration column chromatography as described by Sittivilai *et al*. (2004). Briefly, hemolysates were applied to a Sephadex G-100 column (1.5 × 75 cm) equilibrated with 0.05 M Tris HCl, pH 7.4 and

0.02% sodium azide, at a flow rate of 0.4 mL/min (Vandergon and Colacino, 1989). The elution was performed with the same buffer. Hb elution fractions and molecular weight protein standards (Sigma, USA) elution fractions were monitored at 415 nm for heme detection and at 280 nm for protein standards detection. The Sephadex G-100 column for the molecular weight estimation was calibrated with blue dextran (2,000,000 Dalton), bovine serum albumin, BSA (68,000 Dalton), ovalbumin (45,000 Dalton), cytochrome c (12,384 Dalton) and DNP-aspartate (299.5 Dalton). A plot of the linear relationship between the elution volumes of the protein standards and the logarithm of their molecular weights was used to estimate the molecular weight of the Hb (tetramer). Hb fractions from the gel filtration column chromatography were kept at -20°C for further analysis by native-PAGE, SDS-PAGE and MALDI-TOF/TOF MS.

Polyacrylamide gel electrophoresis (PAGE)

Native-PAGE and SDS-PAGE were performed on a Bio-Rad Mini Protean III system as previously described (Sittivilai *et al*., 2004; Thongsarn *et al*., 2006) with some modifications. In brief, phenotypes of the Hb were determined by means of native-PAGE using a 10% T and 2.6% C gel with the condition at a constant 200 V and 60 mA per gel for 45 min (Thongsarn *et al*., 2006). In addition, SDS-PAGE was performed in order to examine the subunit size and protein heterogeneity of the denatured globins. A 4% T and 2.6% C stacking gel along with a 15% T and 2.6% C resolving gel was assembled on a vertical discontinuous slab gel. The electrophoresis was set at a constant of 200 V and 60 mA per gel for 45 min in a Laemmli buffer system (Laemmli, 1970; Sittivilai *et al*., 2004).

After electrophoresis, protein bands were visualized by Coomassie brilliant blue R-250 staining. The Quality One software (Bio-Rad Laboratory, USA) was used to estimate molecular weights and for quantitative determination of the bands in native-PAGE and SDS-PAGE. All values were reported as mean ± standard error of the mean, (SEM), with n = 30.

MALDI-TOF/TOF MS

The purified Hb was analyzed using Ultraflex III MALDI-TOF/TOF MS (Bruker, USA) at the Genome Institute, National Science and Technology Development Agency, Pathumthani, Thailand. The molecular weights of Hbs from Thai Bangkaew dogs were determined by the application of MALDI-TOF/TOF MS with sinapinic acid in 100% saturated acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) as a matrix and exposure to a nitrogen laser. Mass spectra were acquired with 20 kV accelerating voltage and 900 Da low mass gate. All spectra were externally mass calibrated with ProteoMass™ peptide and a Protein MALDI-MS Calibration Kit (Sigma-Aldrich, USA) (Nowwarote, 2006; Veenstra, 2006; Kraj and Macht, 2008).

RESULTS AND DISCUSSION

Hematocrit and Hemoglobin concentration

The sample of 30 Thai Bangkaew dogs had a mean Ht value of 36.38 ± 0.77% and a mean Hb concentration of 13.10 ± 0.32 g/dL, which were within the range of reference values (Jain, 1986; Meinkoth and Clinkenbeard, 2000). The results obtained were similar to Noosud *et al*. (2007a) who reported a mean Ht value and Hb concentration of Thai ridgeback dogs of 38.56 ± 1.18% and 13.93 ± 0.34 g/dL, respectively. The interpretation of hematological values requires careful analysis. A wide range of physiological variations, such as severe dehydration, could influence the analyzed values for osmolality, the hematocrit and plasma proteins. The variations of hematological elements might be influenced by the husbandry techniques, health status and environmental conditions, as well as by factors, such as gender, age, origin, feeding

and the breeding system (Brunk, 1969; Jain, 1996; Meinkoth and Clinkenbeard, 2000).

Sephadex G-100 gel filtration

The red cell tetrameric Hbs of Thai Bangkaew dogs were fractionated by a Sephadex G-100 column as a single peak with a molecular weight of approximately 65,956 Dalton that remained stable in buffer pH 7.4 (Figure 1).The molecular weight of the native Hbs was somewhat lower than that of Whitaker (1963) who reported a molecular weight of approximately 68,000 Dalton. Moreover, the elution behavior might have been a result of the buffer system employed, since it has been shown that the types of buffer, as well as the ionic strength and pH of the buffers, can affect the molecular weight determination when using the the gel filtration method (Whitaker, 1963; Sittivilai *et al.*, 2004; Voet and Voet, 2004).

Native- and SDS-PAGE analysis of purified hemoglobin

Native-PAGE of purified Hb solution (hemolysates purified by Sephadex G-100 column chromatography) showed one phenotype with two migrating bands toward the anode, one slow migrating major band (94%) and one fast migrating minor band (6%) of Hb (Figure 2). However, Braend (1988) reported three hemoglobin phenotypes with immobilized isoelectric focusing on polyacrylamide gels. For Thai ridgeback dogs, one Hb phenotype was detected, as only one band moved in the electrical field from the cathode to the anode using cellulose acetate electrophoresis (Noosud *et al*., 2007b). Based on present knowledge, this is the first report on Hb phenotypes in Thai Bangkaew dogs, with more information to be obtained from further investigations.

Discontinuous SDS-PAGE with a 15% T separating gel and a 4% T stacking gel was performed to analyze the molecular weights of the Hb subunits and the resulting gel image is displayed in Figure 3. Two bands with molecular weights of 12,980 and 14,820 Dalton were detected. This finding was similar to a previous report of Noosud *et al*. (2008) for Thai ridgeback dog Hb subunits, which had molecular weights of

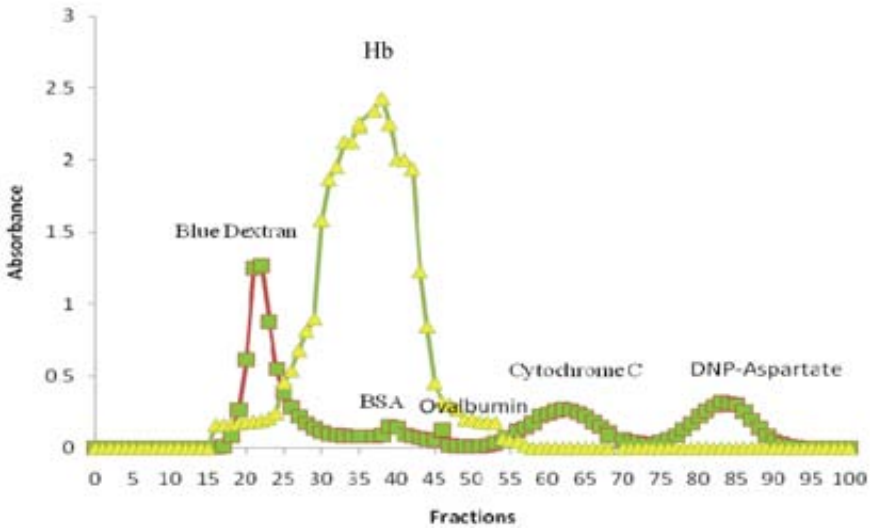

Figure 1 Elution pattern of Hb of Thai Bangkaew dogs on a Sephadex G-100 Grade fine. Hb elution profile was determined at 415 nm and molecular weight standards elution profile determined at 280 nm: blue dextran (2,000,000 Dalton), bovine serum albumin, BSA (68,000 Dalton), ovalbumin (45,000 Dalton), cytochrome c (12,384 Dalton) and DNP-aspartate (299.5 Dalton).

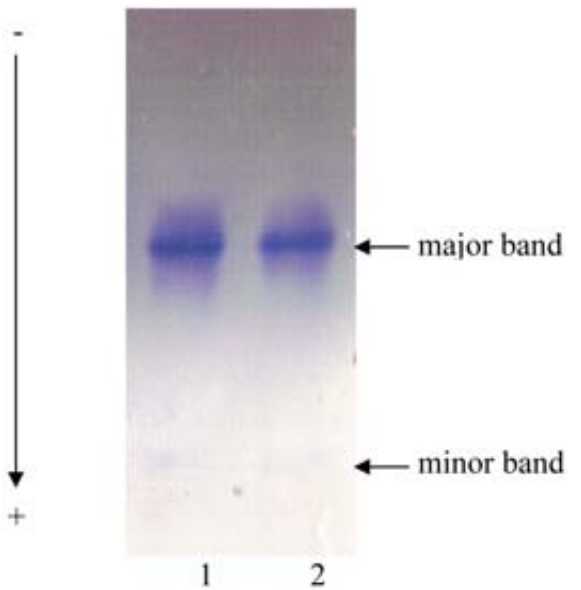

Figure 2 Phenotype of Hbs by native-PAGE, pH 8.3, 10% T and 2.6% C (200 V constant, 60 mA, 45 min). Lanes 1 and 2 = Hbs of Thai Bangkaew dogs from Hb fractions.

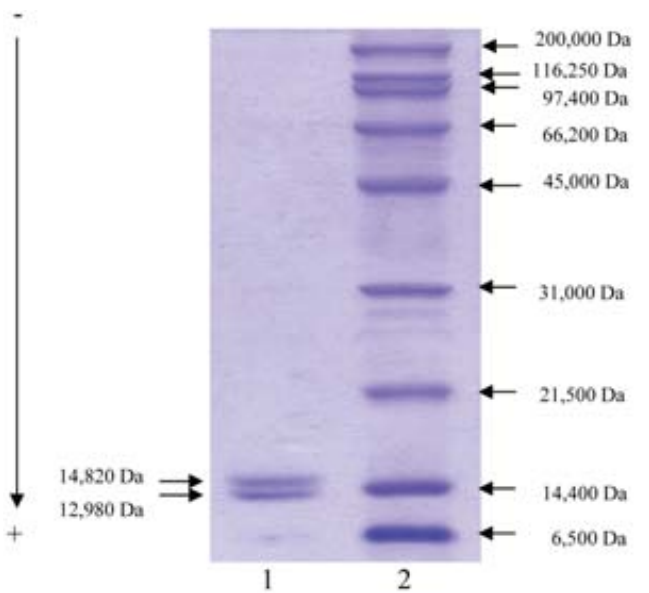

Figure 3 SDS-PAGE of denatured Hb fractions of Thai Bangkaew dogs from Sephadex column, exhibiting two bands with average molecular weights of 14,820 Dalton and 12,980 Dalton. Lane 1 = Hb subunits of Thai Bangkaew dogs; Lane 2 = Protein standards (prestained broad range M.W. standard, Bio-Rad, U.S.A.): myosin, 200,000 Dalton; β-galactosidase, 116,250 Dalton; phosphorylase b, 97,400 Dalton; bovine serum albumin (BSA), 66,200 Dalton; ovalbumin, 45,000 Dalton; carbonic anhydrase, 31,000 Dalton; soybean trypsin inhibitor, 21,500 Dalton; lysozyme, 14,400 Dalton; and aprotinin, 6,500 Dalton.

12,472 and 14,256 Dalton. On the basis of the molecular weight analyzes and in agreement with what had been observed by Adamczyk and Gebler (1997), it was suggested that the higher molecular weight band observed in SDS-PAGE was a β-globin chain, and the other band with the higher mobility was an α-globin chain.

MALDI-TOF/TOF MS analysis

MALDI-TOF/TOF mass spectrometer measurements were performed to determine the molecular weight of the Hb obtained from the Bangkaew dogs. MALDI-TOF/TOF MS revealed that there were at least five forms with molecular weights of 15,194.78, 15,946.66, 32,020.53, 48,326.29, 64,545.95 Dalton (Figure 4). Mass spectrometry peak detections were similar to those in a previous report of Noosud *et al*. (2008) for Hb from Thai ridgeback dogs, which showed molecular weights of 15,194.56 and 15,946.60 Dalton for the monomer subunits and 63,786.23 Dalton for the tetramer subunit. Adamczyk and Gebler (1997) reported previously that by using electrospray ionization mass spectrometry (ESI-MS) the Hbs of dogs were composed of molecular weights of α-I (15,217.3 Dalton) or α-II (15,247.3 Dalton) for the α-globin chain and 15,996.3 Dalton for the β-globin chain. Moreover, the deconvoluted ESI-MS in humans showed two intense peaks at 15,126 and 15,867 Dalton, corresponding to the normal α- and β-globin chains, respectively (Troxler *et al*., 2002). In the present study, the results from mass spectrometry appeared to suggest that the molecular weights of the monomer subunits of Hb were 15,194.78 Dalton for the α-globin chain and 15,946.66 Dalton for the β-globin chain of Thai Bangkaew dogs. Moreover, the molecular weights of the dimer and trimer were 32,020.53 and 48,326.29 Dalton, respectively. According to the results of the present research,

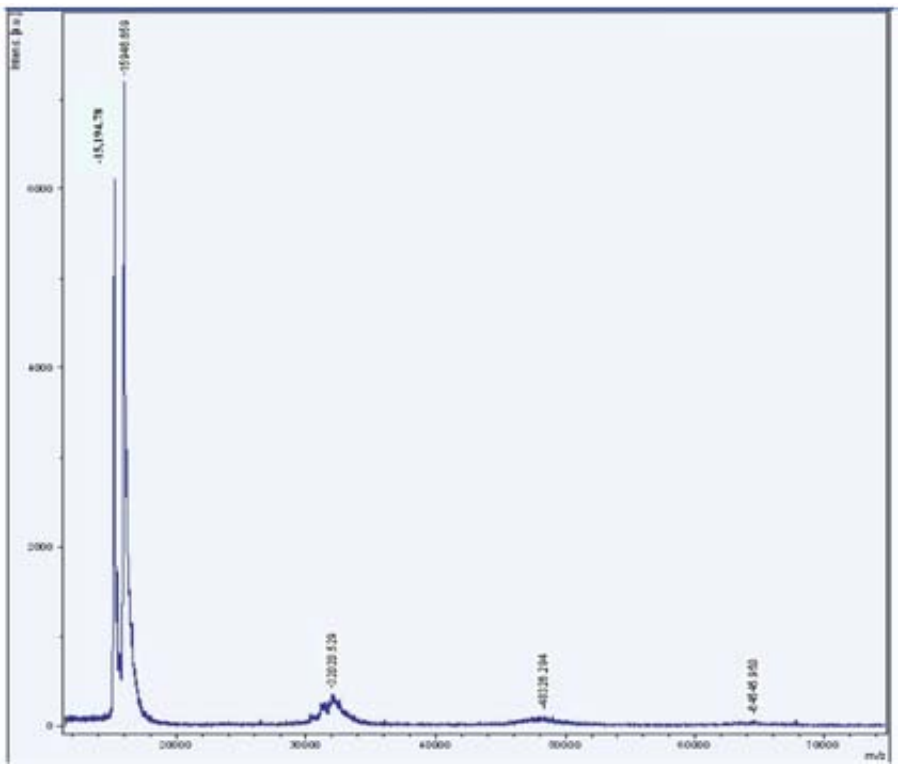

Figure 4 MALDI-TOF/TOF MS spectrum of Hb fractions obtained from Sephadex column.

the molecular weight of the native Hb (tetramer) was 64,545.95 Dalton (Noosud *et al*., 2008).

However, no comparable data are available at the present time for the native dog Hb and their Hb subunits determined by gel filtration and SDS-PAGE, respectively. Traditionally, gel electrophoresis has been performed to verify the molecular weight, but its limited resolution was not suitable for primary sequence verification (Adamczyk and Gebler, 1997). Moreover, detailed studies of mutations, amino acid modifications or protein homogeneity cannot be confirmed accurately by electrophoresis (Adamczyk and Gebler, 1997). The combination of the isoelectric focusing and the electrophoresis of dissociated globin chains of Hb (two-dimensional electrophoresis) exhibiting the primary structure of all globin chains present in the Hbs requires further investigation by ESI-MS.

CONCLUSION

This was the first study that described the mean values of Ht and the Hb concentration of Thai Bangkaew dogs. The study showed also that gel filtration column chromatography was one of the most useful methods for the separation and the purification of the Hb from Thai Bangkaew dogs. The Hb phenotype patterns of intact Hb tetramers were visualized by native-PAGE. Analysis of the purified Hb by SDS-PAGE and MALDI-TOF/TOF MS revealed numbers of Hb subunits and their molecular weights. In conclusion, the results of this work outline basic information on the hemoglobin of the Thai Bangkaew dog breed.

ACKNOWLEDGEMENTS

This research was supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office (PERDO), the Commission on Higher Education, Ministry of Education and the Kasetsart University Research and Development Institute (KURDI), Kasetsart University. The author expresses his gratitude to the Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC) for providing facilities and equipment crucial to the experiment.

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