RESEARCH ARTICLE

Hemoglobins in Thai Ridgeback Dogs: Chromatographic, Electrophoretic and Mass Spectrometric Studies

Jatuporn Noosud[1,2,3], Sittiruk Roytrakul[4], Chaiwat Boonkaewwan[5], Apassara Choothesa[6*]

Abstract

Objectives — To study the hemoglobin phenotypes and to determine the molecular weights of the hemoglobins (tetramer) and the hemoglobin subunits in Thai ridgeback dogs.

Materials and Methods — Blood samples were collected from 30 clinically normal Thai ridgeback dogs. After hemolysate preparation, hemoglobin phenotypes were separated by cellulose acetate electrophoresis. To purify and determine an approximate molecular weight of the hemoglobin, we used Sephadex gel filtration. To study the subunit size, we used SDS-PAGE under denaturing conditions. Additionally, to determine the accurate molecular weights, we used MALDI-TOF mass spectrometer.

Results — We found one phenotype of the hemoglobin moving towards the anode by cellulose acetate electrophoresis. All of the elution patterns, by Sephadex gel filtration, showed a single peak with a molecular weight of 64,000 D. In the result from SDS-PAGE, the denatured hemoglobin migrated separately into two bands with the molecular weights of 15,060 D and

KKU Vet J. 2010;20(2):133-142 *http://vet.kku.ac.th/journal/*

Keywords: Hemoglobin; Chromatography; Electrophoresis; Mass spectrometer; Thai ridgeback dog

[1]Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, 73140, Thailand.

[2]Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok, Thailand.

[3]Department of Companion Animals Clinical Sciences, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, 10900, Thailand.

[4]National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Klongluang, Pathumthani, 12120, Thailand.

[5]Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok, 10900, Thailand.

[6]Department of Physiology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, 10900, Thailand.

***Corresponding author** E-mail: fvetapsc@ku.ac.th

13,790 D. By MALDI-TOF mass spectrometry, the accurate molecular weights of hemoglobin subunits were 15,194.56 D for α-globin chain and 15,946.60 D for β-globin chain. Moreover, the molecular weights of dimer, trimer and tetramer (native) hemoglobins were 32,123.70 D, 48,160.17 D and 63,785.23 D, respectively.

Conclusion — The combination of mass spectrometry with chromatographic and electrophoretic techniques is suitable for studying hemoglobin phenotypes and their biochemical properties in Thai ridgeback dogs.

Introduction

Hemoglobin (Hb) is the predominant biochemical constituent of the vertebrate red blood cells [1]. Hbs are a group of transport proteins and their major functions are to carry oxygen and to facilitate the return transport of carbon dioxide [1-4]. Each Hb is composed of four single polypeptide chains and heme prosthetic groups [1,4]. The tetrameric protein comprises four globular protein chains, 2 α and 2 β subunits in a tetrahedral arrangement [4,5].

The chromatographic and electrophoretic techniques have been widely used for Hb analysis [6,7]. Chromatography was used for purifying Hb, but still contained several impurity proteins [7-9]. Gel filtration chromatography separates proteins on the basis of differences in molecular size and can be used to estimate the molecular weight of these proteins [9]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate blood proteins according to their size and to purify Hb in particular [3,6]. Also, reducing PAGE was performed to study heterogeneity and the subunit size which separated the Hb into the alpha and beta globin subunits [3,9]. Cellulose acetate is the major support medium used and this method is based on the complex interactions of the Hb with the electrophoretic buffer [5,10]. Moreover, there are many reports of Hb electrophoresis on cellulose acetate electrophoresis which is simple, reliable, and gave consistently satisfactory identifications of abnormal Hbs in human [10,11]. Mass spectrometry was commonly used for determination of the molecular weight of Hb [6,12,13]. The application of mass spectrometry improves the sensitivity of Hb analysis [6].

Originally from Asia, the Thai ridgeback dog is an ancient breed of Thai domestic dog for several hundred years. Due to the ancient history, the Thai ridgeback dog has raised ears, sword-like tail and a ridge of hair that runs along its back in the opposite direction to the rest of the coat. The Thai ridgeback dogs have just become a member of the Asian Dogs Organization in 1987 and then as breed number 338 by the Federation Cynologique Internationale in 1993.

Today, the purpose of developing the Thai ridgeback dogs is to preserve the original breed for the people in the next generations [14].

Therefore, this work was designed to study Hb phenotypes and to determine the molecular weights of the Hbs (tetramer) and the Hb subunits of the Thai ridgeback dog by the application of the chromatographic, electrophoretic and mass spectrometric techniques.

Materials and Methods

Animals and blood collection procedures

Blood samples were collected from 30 clinically normal Thai ridgeback dogs. Two milliliters of blood were obtained from each animal by saphenous or cephalic vein puncture through a 23-gauge needle, using EDTA as an anticoagulant.

Hemolysate preparation

Immediately after sampling, whole blood (1 ml) was centrifuged (3,500 rpm, 10 min, 40C) to remove any remaining blood plasma proteins and acellular Hb. The supernatant including white blood cells and stroma were removed after centrifugation. Before lysing, the red blood cells were washed three times with an isotonic saline solution and centrifuged repeatedly until the resultant supernatant was clear. The packed red blood cells were hemolysed by 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, U.S.A. (2001). The cell debris and cell membrane were removed by centrifugation (11,500 rpm, 5 min, 4°C) [2,3,15]. Hemolysates were collected from the aqueous layer and stored at -20°C for further studies of phenotype, purification of Hb and total protein concentration by Biuret method.

Cellulose acetate electrophoresis

Hb phenotypes were identified from hemolysates by cellulose acetate electrophoresis (Helena Laboratory, U.S.A.) using TITAN III-H with Tris-EDTA-boric acid buffer, pH 8.2-8.6 and AFSA2 Hemo control as Hb standard [5,10,16]. The result was analyzed by Molecular Analysis Program version 1.4 (Bio-Rad Laboratory, U.S.A.).

Gel filtration column chromatography

Sephadex gel filtration was performed to determine an approximate molecular weight for the Hb. Hemolysates were applied to a column of SephadexG-100 grade fine (1.5 cm x 75 cm) which equilibrated with 0.05 M Tris buffer, pH 7.4 containing 0.02% sodium azide, at a flow rate of 0.4 ml/min [17]. The elution was carried out with the same buffer. Hb elution fractions

and molecular weight standards elution fractions were monitored at 415 nm and 280 nm, respectively. The Hb fractions were kept at -20°C for further analysis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity and subunit size of Hb were assessed via gel electrophoresis using a Mini-PROTEAN III Cell (Bio-Rad, U.S.A). SDS-PAGE under denaturing conditions. Molecular weight determinations were performed on a vertical slab of discontinuous gel. 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 slab gel apparatus and each lane loaded with a concentration of 4-8 μg protein/band. The electrode buffer was consisted of Tris base, glycine and SDS, pH 8.3. Partial purified Hb fractions from Sephadex column and protein standards were dissolved in a SDS reducing buffer of Tris-HCl, glycerol, SDS, 2-mercaptoethanol and bromophenol blue, pH 6.8 and then boiled for 10 minutes. A prestained broad range molecular weight marker (Bio-Rad, U.S.A.) comprised proteins with molecular weights of 6,500 to 200,000 D, in a Laemmli buffer system [18]. The electrophoresis was set at a constant of 200 V at 60 mA per gel for 45 minutes. The gel was stained with Coomassie Brilliant Blue R-250 for 30 minutes ensuring bleaching applying destaining buffer (10% acetic acid and 25% ethanol) after the electrophoresis.

MALDI-TOF mass spectrometry

Molecular weights of Thai ridgeback dog Hbs were determined by the application of the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with a Bruker Reflex IV MALDI-TOF mass spectrometer (Bruker, U.S.A) using sinapinic acid in a 100% saturated acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) as a matrix and striking with nitrogen laser [13,19].

Results

Cellulose acetate electrophoresis

The Hb phenotypes of Thai ridgeback dogs are shown in Figure 1. It was found that one phenotype (one band) of the dog Hb moved more slowly towards the anode (+) than that of human Hb standard (Hb A and Hb F) **(Figure 1).**

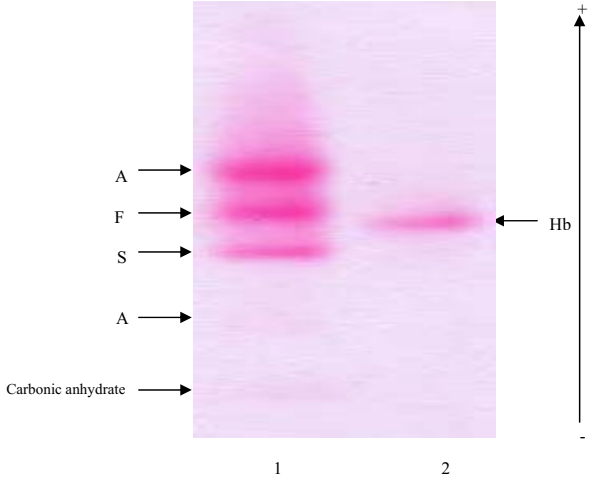
Gel filtration column chromatography

Red cell Hbs of Thai ridgeback dogs were eluted on the Sephadex G-100 column as a single peak, and molecular weight of 64,000 D was determined for the tetrameric molecules which remained stable with buffer pH 7.4 **(Figure 2).**

SDS-PAGE

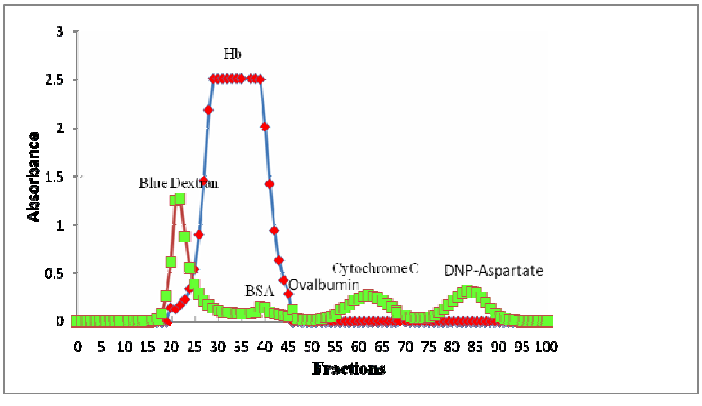
The globin chains of Hb were analyzed by SDS-PAGE on a discontinuous gel in the denaturing condition. Figure 3 shows the result of the electrophoretic pattern of the globins from Hbs of Thai ridgeback dogs. The denatured Hbs appeared as two distinct bands with Rf values corresponding to the molecular weights of 15,060 D and 13,790 D **(Figure 3).**

Figure 1. Hemoglobin Phenotype by Cellulose Acetate Electrophoresis



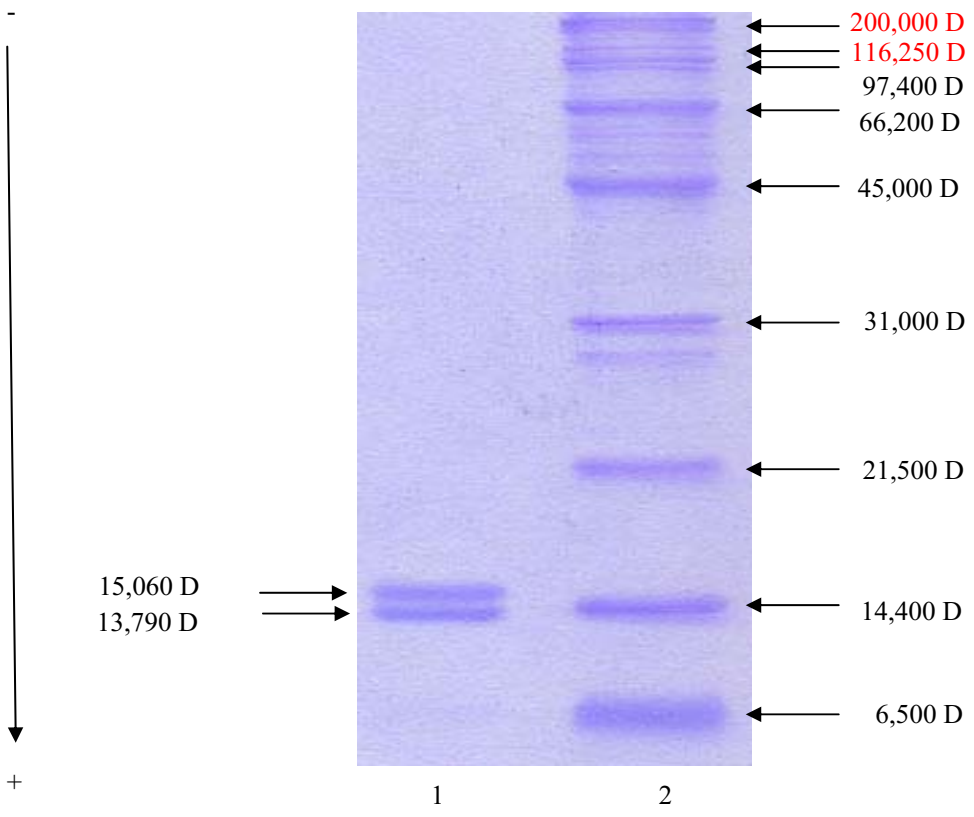
This result was run on pH 8.2-8.6 at 350 volts for 25 mins. Lane assingments: lane 1 = $AFSA_2$ Hemo Control of Helena Laboratory, U.S.A. and lane 2 = Hbs of the Thai ridgeback dog from hemolysates

Figure 2. An Elution Pattern of the Hemoglobin in Thai Ridgeback Dogs



The elution pattern was obtained from a Sephadex G-100 grade fine, equilibrated with 0.05 M Tris buffer pH 7.4, at a flow rate of 0.4 ml/min. Hb elution profile determined at 415 nm and molecular weight standards elution profile determined at 280 nm: Blue Dextran (2,000,000 D), Bovine Serum Albumin, BSA (68,000 D), Ovalbumin (45,000 D), Cytochrome C (12,384 D) and DNP-Aspartate (299.5 D)

Figure 3. SDS-PAGE of Denatured Hemoglobin from Sephadex Column of Thai Ridgeback Dogs

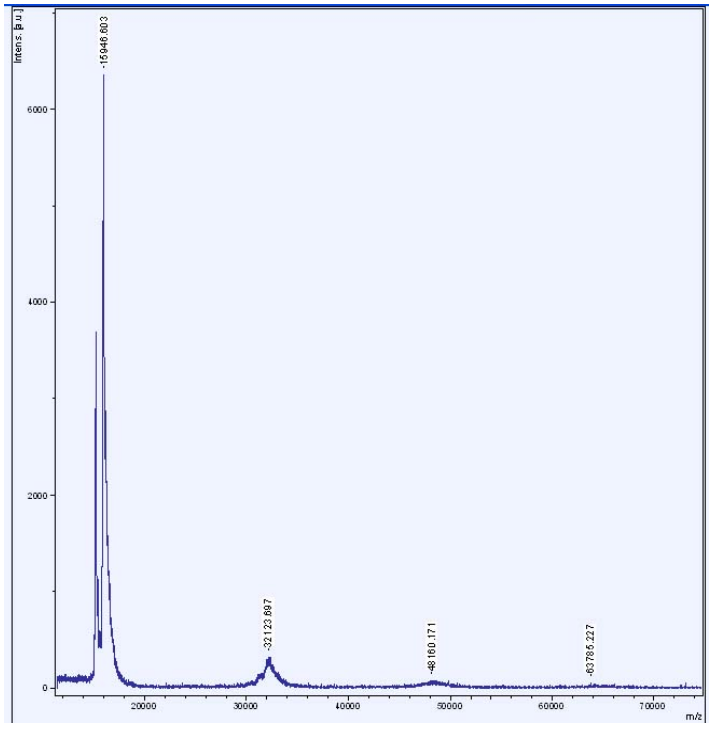


This photograph shows two bands with the average molecular weight of 15,060 D and 13,790 D. Lane assignments: lane 1 = Hb subunits of Thai ridgeback dogs and lane 2 = protein standards (including myosin 200,000 D, β-galactosidase 116,250 D, phosphorylase b 97,400 D, bovine serum albumin 66,200 D, ovalbumin 45,000 D, carbonic anhydrase 31,000 D, soybean trypsin inhibitor 21,500 D, lysozyme 14,400 D and aprotinin 6,500 D) (prestained broad range M.W. standard, Bio-Rad, U.S.A).

MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometer was performed to determine the molecular weights of the Thai ridgeback dog Hb. The molecular weights of the subunits revealed 15,194.56 D for the α-globin chain and 15,946.60 D for the β-globin chain. Moreover, the molecular weights of dimer and trimer were 32,123.70 D and 48,160.17 D, respectively. The molecular weight of the Hb tetramer was 63,785.23 D **(Figure 4)**.

Figure 4. Mass Spectrometry of Hemoglobins in Thai Ridgeback Dogs



The accurate molecular weights of globin chains in hemoglobins of native Thai ridgeback dogs were determined with different peaks.

Discussion

This study was initiated by the observation that Hb phenotype of Thai ridgeback dogs was detected as only one type (one band) by cellulose acetate electrophoresis which moved in the electrical field from the cathode to the anode. However, Braend (1988) [20] reported that the Hb variations of domestic dogs as determined by immobilized isoelectric focusing on polyacrylamide gels revealed three phenotypes and the Hbs types were consistent with polymorphism [10,11].

Gel filtration column chromatography and SDS-PAGE were conducted to determine the molecular weight of native Hbs (tetramer) and denatured Hbs (Hb subunits) of Thai ridgeback dogs. For molecular weight determination by gel filtration, the elution profile patterns of Hbs obtained from Sephadex G-100 column are exhibited in one peak with a molecular weight of

about 64,000 D. The result of the molecular weight was somewhat lower than would have been expected as reported by Reece (2005) [4] with a molecular weight of about 67,000 D. Thus, 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 pH and the ionic strength of the buffers can affect the molecular weight determination by the application of the gel filtration method [3,9,21].

The reducing SDS-PAGE of Hbs fractions from a single peak of Sephadex G-100 column demonstrated heterogeneity of the subunits with Coomassie Brilliant Blue R-250 staining, allowing visualization of the separated proteins. Following electrophoresis, the molecular weights of the single polypeptide chains are determined by SDS-PAGE together with molecular markers of known molecular weights in separate lane in the gel. The electrophoresis of the Hb under dissociating conditions revealed the presence of two bands with the molecular weights of 15,060 D and 13,790 D. Analysis of purified Hb by SDS-PAGE is the most common method of assessment of the molecular weight of protein subunits due to its reliability and ease [3,9].

Under the experimental conditions employed, the MALDI-TOF mass spectrometry circumstances were found to measure the molecular weight of 15,194.56 D for the α-globin chain and 15,946.60 D for the β-globin chain. MALDI-TOF mass spectrometry is a relative novel technique which is used for the identification and the characterization of Hb molecule. It is a very sensitive method which allows the detection of low (10^{-15} to 10^{-18} mole) quantities of sample with an accuracy of 0.1-0.01% [21]. The mass accuracy of MALDI-TOF MS could be sufficient to characterize proteins. However, Adamczyk and Gebler (1997) [22] reported the difference of the molecular weight that were α-I 15,217.8 ±1.8 D and α-II 15,247.2 ±1.0 D for α-globin chain and 15,995.8 +1.0 D for β-globin chain in dogs by the application of electrospray mass spectrometry (ESI-MS). ESI-MS has become a powerful tool for the identification of peptides and proteins with a high-sensitivity analysis of Hb molecules [13,19,22].

As a result, gel electrophoresis can be used to separate and verify the Hb molecular weight but its restricted resolution is not optimal for the amino acid sequence verification [22,23]. Moreover, SDS-PAGE cannot separate two proteins of the same molecular weight from each other [9,22,23]. Therefore, detailed studies of amino acid modifications, mutations, or protein homogeneity cannot be accurately confirmed by electrophoresis [22]. The combination of the isoelectric focusing and the electrophoresis of dissociated globin chains of dog Hb exhibiting the primary structure of all the globin chains present in the Hbs requires further investigation by ESI-MS. ESI-MS has been used for detailed studies of Hbs and their variants and it can determine the molecular weights of Hbs and their amino acid sequences.

Acknowledgements

This research is supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office (PERDO), 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 Department of Physiology, Faculty of Veterinary Medicine, Kasetsart University and the National Center for Genetic Engineering and Biotechnology (BIOTEC) for providing the facilities, as well as the Creative Thai Dog Club for the assistance in the collection of the prerequisite dog blood.

References

1. Guyton AC, Hall JE. Red Blood Cells, Anemia, and Polycythemia. In: Guyton AC, Hall JE, editor. *Textbook of Medical Physiology.* 9th ed. W.B. Saunders, Pennsylvania; 1996. p.425-433.
2. Boonprong S, Choothesa A, Sribhen C, Parvizi N, Vajrabukka C. Relationship between haemoglobin types and productivity of Thai indigenous and Simmental x Brahman crossbred cattle. *Livest Sci.* 2007;111: 213-217.
3. Sittivilai R, Sribhen C, Isariyodom S, Songserm T, Choothesa A. A chromatographic and electrophoretic study of hemoglobin of domestic fowl. *Kasetsart J.* 2004;38(6):132-136.
4. Reece WO. *Functional anatomy and physiology of domestic animals.* 3rd ed. Philadalphia, Lippincott Williams & Wilkins, 2005. 513p.
5. Helena Laboratories, U.S.A. *Hemoglobin Electrophoresis Procedure.* Instruction manual. 2001.
6. Kleinert P, Schmid M, Zurbriggen K, Speer O, Schmugge M, Roschitzki B, Durka SS, Leopold U, Kuster T, Heizmann CW, Frischknecht H, Troxler H. Mass spectrometry: a tool for enhanced detection of hemoglobin variants. *Clin Chem.* 2008;54(1):69-76.
7. Sun G, Palmer AF. Preparation of ultrapure bovine and human hemoglobin by anion exchange chromatography. *J Chromatogr B.* 2008;867:1-7.
8. Prisco GDi, Giardina B, Weber RE. *Hemoglobin function in vertebrates: molecular adaptation in extreme and temperate environments.* Springer, 2000. 123p.
9. Voet D, Voet JG. *Biochemistry.* 3rd ed. John wiley & Sons, Inc., New Jersey, 2004. 1591p.
10. Ueda S, Schneider RG. Rapid differentiation of polypeptide chains of hemoglobin by cellulose acetate electrophoresis of hemolysates. *Blood.* 1969;34(2):230-235.
11. Rosenbaum DL, Capt MC. Hemoglobin electrophoresis on cellulose acetate. *Am J Med Sci.* 1996;252(6):130.
12. Zanella-cleon I, Joly P, Becchi M, Francina A. Phenotype determination of hemoglobinopathies by mass spectrometry. *Clin Biochem.* 2009;42(18):1807-17.

13. Kraj A, Macht M. Introduction to protein and peptide mass spectrometry: matrix-assisted laser desorption/ionization. In: Kraj A, Macht M, editor. *Proteomics: Introduction to Methods and Applications.* John Wiley & Sons, Inc. New Jersey; 2008. p.89-99.
14. Sterling J. The Thai Ridgeback Dogs. [cited 2010 Jul 29]. Available from: http://www.thaidog.com/libraryhistory/00000099e7012c703/index.html.
15. Bachmann AW, Campbell RSF, Yellowlees D. Haemoglobins in cattle and buffalo: haemoglobin types of *Bos taurus*, *Bos indicus*, *Bos bangteng* and *Bubalus bubalis* in Northern Australia. *Aust J Exp Biol Med Sci.* 1978;56(5):623-629.
16. Thongsarn K, Worawattanamateekul W, Tunkijjanukij S, Sribhen C, Choothesa A. Biochemical properties of nile tilapia (*Oreochromis niloticus*) hemoglobin. *Kasetsart J.* 2006;40:69-73.
17. Vandergon TL, Colacino JM. Characterization of hemoglobin from *Phoronis Architecta* (Phoronida). *Comp Biochem Physiol.* 1989;94B:31-39.
18. Laemmli UK. Cleavage of Structural Protein during the Assembly of the Head of Bacteriophage T4. *Nature.* 1970;227:680.
19. Veenstra TD. Mass spectrometry. In: Veenstra TD, and Yates JR III, ed itor. *Proteomics for Biological Discovery.* John Wiley & Sons, Inc. New Jersey. 2006. p.3-17.
20. Braend M. Hemoglobin polymorphism in the domestic dog. *J. Hered.* 1988;79:211-212.
21. Motoyama N, Dauterman WC. Comparative studies on the molecular weight of glutathione S-transferases from mammalian livers and an insect. *Comp Biochem Physiol.* 1979;63B:451-454.
22. Adamczyk M, Gebler JC. Electrospray mass spectrometry of α - and β - chains of selected hemoglobins and their TNBA and TNB conjugates. *Bioconjug Chem.* 1997;8:400-406.
23. Barinaga M. Protein chemists gain a new analytical tool. *Science.* 1989;246:32.