Kasetsart J. (Nat. Sci.) 38 : 132 - 136 (2004)

A Chromatographic and Electrophoretic Study of Hemoglobin of Domestic Fowl

Raksak Sittivilai[1], Choosri Sribhen[1], Supaporn Isariyodom[2], Thaweesak Songserm[3] and Apassara Choothesa[1]

ABSTRACT

Gel filtration column chromatography and sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) were used to study the chemical properties of hemoglobins for 2-week-old broilers, adult broilers and adult layer hen as well as adult ducks. All of the elution patterns obtained with 0.05 M Tris buffer pH 7.4 containing 0.02% sodium azide and monitored at 415 nm for hemoglobin, was consisted of one peak which was estimated as a molecular weight of 51,784 daltons. As compared with the result from SDS-PAGE, the denatured hemoglobin migrated into two bands with the molecular weight of 12,519 daltons and 10,984 daltons.

Key words: hemoglobin, chromatography, electrophoresis, domestic fowl

INTRODUCTION

Hemoglobin (Hb) is the major oxygen transport protein found in all vertebrates with the exception of a few Antarctic deep-sea fishes (Abbasi and Lutfullah, 2002). Hb is composed of polypeptide chains called globin, and iron protoporphyrin heme groups. Avian Hbs have four heme groups containing iron as do mammals but their protein moieties are different (Sturkie, 1976). The blood of adult chicken, as in most other birds, is composed of two major Hbs, HbA and HbD that have identical β chains but differ in the amino acid sequences of the α chains. HbA ($\alpha^{A}{}_{2}\beta_{2}$) and HbD($\alpha^{D}{}_{2}\beta_{2}$) are expressed in a 3:1 ratio in adult chickens (Knapp *et al.*, 1999).

Both α chains have the same number (141) of amino acids, but they are arranged in different sequences within and between species. The two β chains contain the same number (146) and sequence of amino acids for the same species, but vary in sequence between species. There are mutant types of Hb and some of the types are inherited. The mutations occur in the α and β chains in such a way that the rate of mutation is low in the β chains, higher in the α^{A} chain and highest in the α^{D} chain (Sturkie, 1976).

Besides these major Hbs there are minor Hbs, HbA$^{/}$ is the met-derivative of HbA and HbK. HbK differs from the two major HbA and HbD in the α chain. HbK α chain (α^{k}) is similar to α^{A} chain of HbA except that the C-terminal amino acid Arg 141 is lacking (Cirotto and Parente, 1987). Cirotto and Parente (1987) have suggested that chicks HbK may be an intermediate product in the Hb turnover and from their data it is more

1 Department of Physiology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand.
2 Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.
3 Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand.

plausible to believe that α^k derives from α^A by a post-translation modification rather than the existence of a new α^k gene.

In avian embryos, at least four Hbs have been identified: two major types, HbP and HbP / and two minor types, HbE and HbM. These types undergo changes and develop into the adult types before hatching time, and this changes the affinity of the Hbs for oxygen (Sturkie, 1976).

Gel filtration chromatography is a special type of partition chromatography in which separation is based on molecular size and shape. It is therefore a valuable analytical tool to purify the globular proteins and to estimate the molecular weight of these proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is the most common way of estimating the molecular weight of protein subunits in use today. The molecular weights of the single polypeptide chains are determined in SDS-PAGE by electrophoresing them together with several protein markers of known molecular weights (Voet and Voet, 2004).

The present work was designed to determine the molecular weights of the Hbs (tetramer) and the Hb subunits of 2-week-old and adult domestic fowl, within and between species, using the chromatographic and electrophoretic techniques.

MATERIALS AND METHODS

Preparation of Hb solutions

Blood samples from 2-week-old broilers (Cobb), adult broilers (Cobb), adult ducks (Cherry) and layer hen (Heysac Brown) were collected in tubes containing heparin as an anticoagulant. The blood was centrifuged (3,000 rpm for 10 min) to remove the plasma. The packed red blood cells were lysed by addition of hemolysate reagent containing 0.005M EDTA and 0.01% potassium cyanide (1 part packed red blood cells to 6 parts hemolysate reagent) as described by Helena Laboratories, U.S.A. (2001). The cell debris was removed by centrifugation at 11,500 rpm for 5 min (Washburn, 1968). Hemolysates or Hb solutions were collected from the aqueous layer and stored at – 80°C for futher analyses. All the preparation steps were performed at 4°C.

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 Sephadex G-100 fine (1.5 cm × 75 cm), equilibrated with 0.05 M Tris buffer containing 0.02% sodium azide, pH 7.4 (Vandergon and Colacino, 1989). The elution was carried out with the same buffer. Hb elution fractions were monitored at 415 nm, while other molecular weight standards elution fractions were monitored at 280 nm.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE (Laemmli buffer system)was used to study the subunit size and heterogeneity. Molecular weight determinations were performed on a vertical slab gel (linear gradient 4-20%T 2.6%C). The electrode buffer consisted of Tris base, glycine and SDS, pH 8.3. Samples and protein standards (SDS-PAGE Standard, Low Range, BioRad, U.S.A. and ColorMeRanger Unstained Protein Molecular Weight Marker Mix, Pierce, U.S.A.), dissolved in a SDS reducing buffer of Tris-HCl, glycerol, SDS, 2-mercaptoethanol and bromophenol blue, pH 6.8, were loaded and the electrophoresis was run at a constant 200 V and 60 mA per gel for 45 min. The gel was stained with Coomassie Brilliant Blue R-250 (Laemmli, 1970).

RESULTS

Gel filtration column chromatography

Red cell Hbs of domestic fowl eluted on the Sephadex G-100 column as a single peak with an approximate molecular weight of 51,784 daltons (Figure 1)

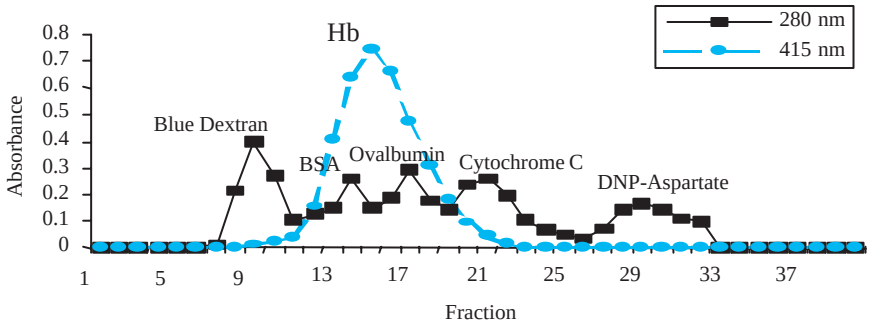

Figure 1 An elution pattern of Hb from domestic fowl on a Sephadex G-100 fine column. Hemoglobin elution profile determined at 415 nm. Molecular weight standards elution profile determined at 280 nm :
Blue Dextran (2,000,000 daltons)
Bovine Serum Albumin, BSA (68,000 daltons)
Ovalbumin (45,000 daltons)
Cytochrome C (12,384 daltons)
DNP-Aspartate (299.5 daltons)

SDS-PAGE

The globin chains constituting each Hb tetramer were analysed by SDS-PAGE on a linear gradient gel in denaturing conditions. Figure 2 shows the electrophoretic pattern of the globins from Hbs of domestic fowl. The denatured Hbs appeared as two distinct bands with Rf values corresponding to the molecular weights of 12,519 daltons and 10,984 daltons.

DISCUSSION

Abbasi and Lutfullah (2002) reported that the molecular weights (tetramer) of the two components of tufted duck Hb as determined by ESI-MS (Electrospray Ionization Mass Spectroscopy) are 63,426.34 daltons and 64,131.78 daltons for HbA and HbD, respectively. When analysing the Hbs of 2-week-old broilers, adult broilers and adult layer hen as well as adult ducks by Sephadex G-100 column chromatography, the same elution patterns of Hbs were found. During gel filtration, the domestic fowl hemolysates fractionated in a single peak with a molecular weight of about 51,784 daltons. The result was somewhat lower than would be expected. Thus, the elution behaviour may be a result of the buffer system employed, since it has been shown that the types of buffer as well as the ionic strength of the buffers can affect the molecular weight determination by gel filtration method. However, extremely low values obtained could be due to dissociation of the subunits of protein (Motoyama and Dauterman, 1979). This in turn could provide the explanation for the anomolous results of gel filtration chromatography.

SDS-PAGE of fractions containing Hbs from a single peak of Sephadex G-100 column demonstrated heterogeneity of the subunits with Coomassie staining. Electrophoresis of all the Hbs under dissociating conditions showed the presence of two bands with the molecular weights of 12,519 daltons and 10,984 daltons. These results show that the Hbs of all the experimental animals are composed of subunits with apparently different molecular weights.

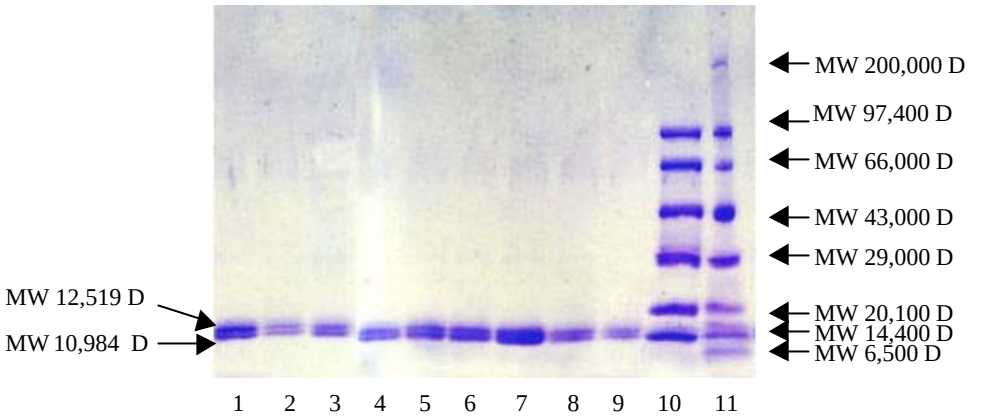

Figure 2 SDS-PAGE of denatured Hbs from the hemolysates of domestic fowl. All exhibited two bands with molecular weights of 12,519 daltons and 10,984 daltons.

Lane 1, 7 Hb subunits of adult broiler.
Lane 2, 8 Hb subunits of adult layer hen.
Lane 3, 9 Hb subunits of adult ducks.
Lane 4, 6 Hb subunits of 2-week-old broilers.
Lane 10, 11 protein standards :
Myosin (200,000 daltons)
Phosphorylase b (97,400 daltons)
BSA (66,000 daltons)
Ovalbumin (43,000 daltons)
Carbonic Anhydrase (29,000 daltons)
Soybean Trypsin Inhibitor (20,100 daltons)
Lysozyme (14,400 daltons)
Aprotinin (6,500 daltons)

A difference in molecular weight has been reported that ESI-MS revealed a molecular weight of 15,391.98 ±1.56 daltons for α^A chain and 16,321.19 ±1.16 daltons for β chain (Abbasi and Lutfullah, 2002). At present, however, no comparable data is available on the molecular weight determination of the native Hb (tetramer) and the molecular weight determination of the Hb subunits by gel filtration method and by SDS-PAGE under dissociation condition, respectively. From these studies, it is interesting to note that by gel filtration chromatography and SDS-PAGE, the resuts obtained are not different with age and species of domestic fowl. The combination of the isoelectric focusing and the electrophoresis of dissociated globin chains of domestic fowl Hbs showing the primary structure of all the globin chains present in the Hbs requires further investigation.

CONCLUSION

Gel filtration column chromatography and SDS-PAGE were conducted to determine the molecular weight of native Hbs (tetramer) and the molecular weight of denatured Hbs (Hb subunits) of domestic fowl. The elution patterns of Hbs obtained from Sephadex G-100 column showed a single peak with a molecular weight of about 51,784 daltons. Two bands with the molecular weight of 12,519 daltons and 10,984 daltons from SDS-PAGE under denaturing conditions of all Hbs were obtained. The conclusion from these studies is that there is no difference with age and species in the molecular weights of the Hbs and the Hb subunits determined by gel filtration chromatography and SDS-PAGE, respectively.

ACKNOWLEDGEMENTS

The author would like to thank the Graduate School of Kasetsart University for thesis and research station support fund and the Kasetsart University Research and Development Institute for the research fund.

LITERATURE CTTED

Abbasi, A. and G. Lutfullah. 2002. Molecular Basis of Bird Respiration: Primary Hemoglobin Structure Component from Tufted Duck (*Aythya fuligula, Anseriformes*) –Role of a 99 Arg in Formation of a Complex Salt Bridge Network. **Biochem. Biophys. Research. Com.** 291:176-184.

Cirotto, C. and A. Parente. 1987. A Koelliker Hemoglobin in Chick Erythrocytes. **Comp. Biochem. Physiol.** 87B (4):1025-1032.

Helena Laboratories, U.S.A. 2001. **Hemoglobin Electrophoresis Procedure.** Instruction manual.

Knapp, J.E., M.A. Oliveira, Q. Xie, S.R. Ernst, A.F. Riggs and M.L. Hackert. 1999. The Structural and Functional Analysis of the Hemoglobin D Component from Chicken. **J. Biol. Chem**. 274(10): 6411-6420.

Laemmli, U.K. 1970. . Cleavage of Structural Protein during the Assembly of the Head of Bacteriophage T4. **Nature.** 227 : 680.

Motoyama, N. and W.C. Dauterman. 1979. Comparative Studies on the Molecular Weight of Glutathione S-Transferases from Mammalian Livers and an Insect. **Comp. Biochem. Physiol.** 63B:451-454.

Sturkie, P.D. 1976. **Avian Physiology**. 3rd ed. Springer-Verlag, New York, Heidelberg, 400p.

Vandergon, T.L. and J.M. Colacino. 1989. Characterization of Hemoglobin from *Phoronis Architecta* (Phoronida). **Comp. Biochem. Physiol.** 94B:31-39.

Voet , D. and J.G. Voet. 2004. **Biochemistry.** 3rd ed. John Wiley & Sons, Inc., New Jersey, 1591p

Washburn, K.W. 1968. Effects of Age of Bird and Hemoglobin Type on the Concentration of Adult Hemoglobin Components of the Domestic Fowl. **Poult. Sci**. Jul.47(4):1083-1089.