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Biochemical Properties of Nile Tilapia (*Oreochromis niloticus*) Hemoglobin

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ABSTRACT

Hemoglobins of *O. niloticus* (averaged standard length of 18.07 ± 0.89 cm and weight of 216.86 ± 27.13 g) were analysed of some biochemical properties. Average hematocrits were 25.80 ± 3.71% and average hemoglobin concentrations were 7.05 ± 1.08 g/dl. It was found that there were 9 – 11 types of hemoglobin by cellulose acetate electrophoresis. The anodic and cathodic hemoglobins were revealed by native polyacrylamide gel electrophoresis with approximate amounts of 45.72 and 54.28% of total hemoglobin, respectively. In addition, the cathodic hemoglobin was classified by cellulose acetate electrophoresis as cathodic I and cathodic II with approximate amounts of 47.62 and 6.66% of total hemoglobin, respectively. Mass spectrometry was performed to determine molecular weights of hemoglobin. It was found that the molecular weights of native hemoglobin was 63.0 kDa and the molecular weights of subunits were 15.3, 15.6 and 16.1 kDa. The pIs of anodic and cathodic hemoglobins ranged 5.28 – 6.80 and 7.27 – 9.71 , respectively.

Key words: Nile Tilapia, fish hemoglobin, Tilapia hemoglobin

INTRODUCTION

Sripanitan (1983) reported that the oxygen affinity of hemoglobin was dependent on temperature, pH, organic phosphates and pCO_2. The increase in pH or decrease in organic phosphates and temperature result in higher oxygen affinity and decreased P_{50}. In addition, hemoglobin greatly increases the carrying capacity of oxygen, CO_2 and H^+ as a result of reversible binding of the distal histidine ligand of the hemoglobin molecule and appropriate allosteric interactions between the binding sites (Perry and Tufts, 1998).

Variation of water parameters in fish habitats such as O_2 availability, salinity, ionic compounds, pH and temperature and their different gas exchange organs such as gills, lungs, skin, swim–bladders, pharyngeal surface and gut surface cause functional heterogeneity of fish hemoglobin (Fago *et al*., 1995; Weber, 2000). As in mammals, fish hemoglobin is globular tetrameric proteins that carry oxygen for using in energy production process of the tissues (Horton

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et al., 2002). In contrast to mammals which commonly have several "isoHbs" that may be polymorphic, they often show marked functional differentiation (Weber, 2000).

Weber (2002) indicated that fish hemoglobins were separated as anodic and cathodic hemoglobins by electrophoresis. The anodic hemoglobin's properties of Salmonids had low oxygen affinity, high Bohr and Root effects while the cathodic hemoglobin had high oxygen affinity, no or reverse Bohr effect. They believed that, the cathodic hemoglobin might have function as O_2 reserve and O_2 carrier under hypoxia.

Autoxidation is the spontaneous oxidation of hemoglobin (Fe^{2+}) to methemoglobin (Fe^{3+}) by molecular oxygen. Methemoglobin cannot bind oxygen and functional inert with respect to oxygen transport (Perry and Tufts, 1998). It is believe that fish hemoglobin autoxidation is related to fish storage time. Therefore, this work was designed to study some biochemical properties of hemoglobin of nile tilapia (*O. niloticus*) as the basic information to study fish hemoglobin autoxidation for development as fish freshness indicator.

MATERIALS AND METHODS

Fish samples

Thirty–five live *O. niloticus* (averaged standard length of 18.07 ± 0.89 cm and weight of 216.86 ± 27.13 g) were obtained from Kamphaengsaen Fisheries Institute, Faculty of Fisheries, Kasetsart University.

Blood and hemoglobin preparation

Thirty–five blood was collected by cardiac puncture with heparinized syringes and kept on ice. Each sample was separated to two parts. The first part was used to investigate the hematocrit by microhematocrit centifuge method (Biomed Group Co., Ltd. Thailand) and hemoglobin concentration by cyanomet-hemoglobin method (Bio–Medical Laboratory, Thailand). The second part was prepared for hemolysate as described by Tamburrini *et al*. (2001) and analysed total protein concentration by Biuret method. These hemolysates were kept at –20°C for further investigation of hemoglobin phenotype.

Ten hemolysates were purified by gel filtration chromatography using Sephadex G–100 (fine grade) column and eluted by 0.05 M Tris–HCl buffer (containing 0.02% sodium azide), pH 7.4 with flow rate of 1.8 ml/min at 8°C. Five hemolysates were classified of hemoglobin groups by anion–exchange chromatography of DEAE–cellulose column as described by Tamburrini *et al*. (2001). The hemoglobin fractions from gel filtration column chromatography and anion–exchange column chromatography were desalted by ultrafiltration with centricon–30 (Amicon, U.S.A.), centrifuged at 3,000 rpm at 4°C for 30 min and kept at –20°C to determine molecular weight and pI of hemoglobin.

Hemoglobin phenotype

Hemoglobin phenotype were identified from thirty–five 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 $AFSA_2$ Hemo control as hemoglobin standard. The result was scanned with GS–670 densitometer and analysed by Molecular Analysis Program version 1.4 (Bio–Rad Laboratory, U.S.A.). Native polyacrylamide gel electrophoresis was used for studying hemoglobin phenotype of fourteen hemolysates. The running condition was at 200 volts, 120 mA for 40 min with electrode buffer, pH 8.3 and the gel was stained with Coomassie blue R–250.

Hemoglobin molecular weight

Molecular weights of hemoglobin were determined by mass spectrometry with Bruker Reflex IV MALDI–TOF mass spectrometer (Bruker, U.S.A.) using sinapinic acid as matrix and hitting with nitrogen laser. The samples were

fractioned from gel filtration chromatography of Sephadex G–100 column.

Hemoglobin pI

Isoelectric focusing (IEF) (Bio–Rad Laboratory, U.S.A.) was used to determine the pI (isoelectric point) of hemoglobin. The IEF gel (pH ranges of 3 – 10) and broad range IEF standards consisted of a mixture of native proteins with pIs ranges of 4.45 – 9.69 were used. Electrode buffers contained 20 mM lysine and 20 mM arginine for cathode and 7% phosphoric acid for anode. The gels were scanned by Genius Bio Imagine System Gel Documentation from Lab Focus (U.S.A.) and were investigated with Gene Tool Analysis Program version 3. 03. 03. (Syngene, U.S.A.).

RESULTS AND DISCUSSION

Identification of hematocrit and hemoglobin of *Oreochromis niloticus*

Thirty–five *O. niloticus* with average standard length of 18.07 ± 0.89 cm and average weight of 216.86 ± 27.13 g were identified for hematocrits and hemoglobin concentrations. It was found that average hematocrits were 25.80 ± 3.71% (ranging from 15.50 – 34.00%) and average hemoglobin concentrations were 7.05 ± 1.08 g/dl (ranging from 4.46 – 9.49 g/dl). These results were similar to those of Sun *et al*. (1995) showing the hematocrits and hemoglobin concentrations of *O. niloticus* at 28.40 ± 0.90% and 7.02 ± 0.20 g/dl, respectively.

Phenotype of *Oreochromis niloticus* hemoglobin

The hemoglobin phenotypes of *O. niloticus* are shown in Figure 1. It was found that there were 9 – 11 types of hemoglobin which moved more slowly to anode (+) than human hemoglobin standard (A and F). These results might be affected by the function of fish hemoglobin that was different from mammal.

Weber (2000) reported that fish hemoglobins had multiplicity and similarity was called IsoHbs such as hemoglobins of Salmonids group had 8 – 19 isomers that differed at genetic

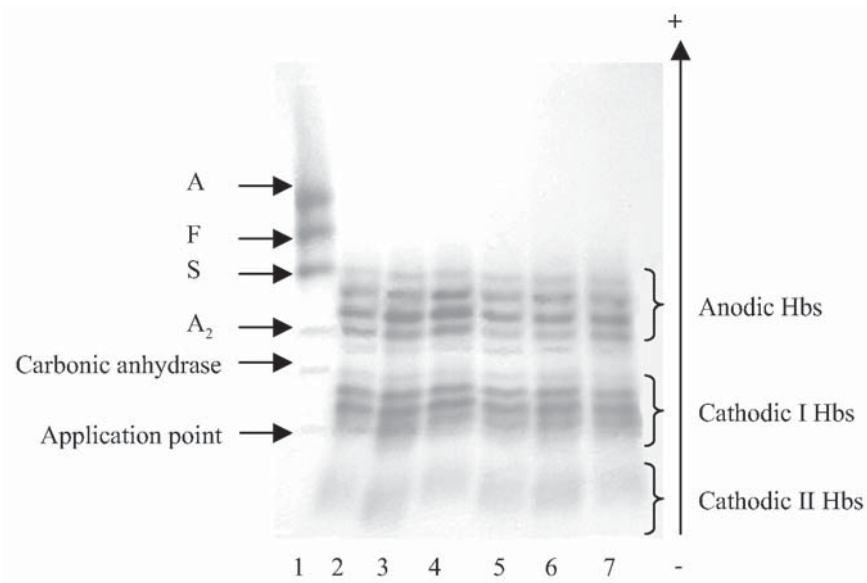

Figure 1 Phenotypes of hemoglobins by cellulose acetate electrophoresis, pH 8.2 – 8.6 (350 volts, 25 mins). Lane 1 was $AFSA_2$ Hemo Control of Helena Laboratory, U.S.A. and lane 2 – 7 were hemoglobins of *O. niloticus* from hemolysates.

level and showed marked polymorphism. The hemoglobins of Elasmobranch had several isomers (12 – 13 isomers) called IsoHbs and they were different (Weber, 2000). The seven polymorphisms of hemoglobins of Atlantic Croaker (*Micropogon undulatus*) from Chesapeake Bay in Virginia were found by native polyacrylamide gel electrophoresis (Shelly and Mangum, 1997).

There were two hemoglobin phenotypes of *O. niloticus* as presented in Figure 2. The first one called anodic hemoglobin with approximate amount of 45.72% of total hemoglobin and the second one called cathodic hemoglobin with approximate amount of 54.28% of total hemoglobin. In addition, the cathodic hemoglobin was classified as two groups by cellulose acetate electrophoresis, pH 8.2 – 8.6. The first group called cathodic I with approximate amount of 47.62% of total hemoglobin and the second group called cathodic II with approximated amount of 6.66% of total hemoglobin as presented in Figure 1.

Molecular weight of *Oreochromis niloticus* hemoglobin

MALDI–TOF mass spectrometer was used to determine the molecular weights of *O. niloticus* hemoglobin. The molecular weight of native hemoglobin was 63.0 kDa and molecular weights of subunits were 15.3, 15.6 and 16.1 kDa. Falk *et al*. (1998) indicated that hemoglobins of five tilapia species of genera *Oreochromis* and *Serotherodon* were composed of 2 major alpha chains and 4 (*O. andersonii, S. gallaeus* and *S. melanotheron*) or 5 (*O. aereus* and *O. niloticus*) beta chains. Molecular weights of hemoglobin subunits were estimated ranging from 16.3 – 17.6 kDa (kilodaltons) and the molecular weight of native hemoglobin with approximated value of 65.0 – 70.0 kDa. They believed that these hemoglobins and globin chains might be applied to fish taxonomy and population studies.

pI of *Oreochromis niloticus* hemoglobin

The IEF, pH 3–10 were used to determine pIs of *O. niloticus* hemoglobins. The pIs of anodic and cathodic hemoglobins ranged from 5.28 – 6.80 and 7.27–9.71, respectively. Falk *et al*. (1998) reported that pIs of hemoglobins of 5 tilapia species of genera *Oreochromis* and *Serotherodon* were found range from 5.94–8.06 and they differed by their globin chains.

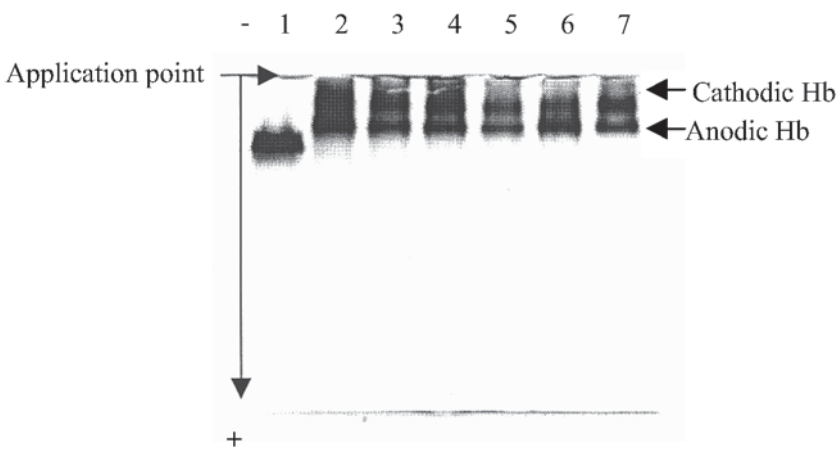

Figure 2 Phenotypes of hemoglobins by native polyacrylamide gel electrophoresis, pH 8.3, 12%T and 4%C (200 volts constant, 120 mA, 40 min). Lane 1 was $AFSA_2$ Hemo Control of Helena laboratory (U.S.A.) and lane 2 – 7 were hemoglobins of *O. niloticus* from hemolysates.

CONCLUSION

Averaged hematocrits were 25.80 ± 3.71% (ranging from 21.00 – 34.00%) and average hemoglobin concentrations were 7.02 ± 0.2 g/dl. There were 9 – 11 types of hemoglobin by cellulose acetate electrophoresis. The anodic and cathodic hemoglobins were revealed by native polyacrylamide gel electrophoresis. The cathodic hemoglobin was classified by cellulose acetate electrophoresis as cathodic I and cathodic II. The molecular weight of native hemoglobin was 63.0 kDa and the molecular weights of subunits were 15,3, 15,6 and 16,1 kDa. The pIs of anodic and cathodic hemoglobins ranged from 5.28 – 6.80 and 7.27–9.71, respectively by isoelectric focusing.

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LITERATURE CITED

Fago, A., V. Carratore, G. D. Prisco, R. J. Feuerlein, L. Sottrup-Jensen and R. E. Weber. 1995. The cathodic hemoglobin of *Anguilla anguilla*: amino acid sequence and oxygen equilibria of a reverse Bohr effect hemoglobin with high oxygen affinity and high phosphate sensitivity. **J. Biol. Chem.** 270 (32): 18897 – 18902.

Falk, T. M., E. K. Abban, W. Villwork and L. Renwrantz. 1998. Hemoglobin variations in some tilapiine species (Teleostei, Cichildae) of the genera *Oreochromis* and *Sarotherodon*. **Genetics and Aquaculture in Africa, Actes de Collogue Genetique et Aquaculture en Afrique, Abidjan, Cute d' lvoire, Apr. 1-4**: 147 – 152.

Horton, H. R., L. A. Moran, R. S. Ochs, J. D. Rawn and K. G. Scrimgeour. 2002. **Principle of Biochemistry**. 3rd ed. Prentice–Hall, Inc., Upper Saddle River. 862 p.

Murakami, K. and S. Mawatari. 2003. Oxidation of hemoglobin to methemoglobin in intact erythrocyte by a hydroperoxide induces formation of glutathionyl hemoglobin and binding of α–hemoglobin to membrane. **Arch. Biochem. Biophys**. 417: 244 – 250.

Shelly, D. A. and C. P. Mangum. 1997. Hemoglobin polymorphism in the Atlantic croaker, *Micropogon undulatus*. **Comp. Biochem. Physiol**. 118A (4): 1419 – 1428.

Sripanitan, R. 1983. **Structural Characterization of the Four Component of Hemoglobin Constant Spring**. M.S. Thesis, Mahidol University. Bangkok.

Sun, L. G., G. Chen and C. Chang. 1995. Acute responses of blood parameters and comatose effects in salt–acclimated tilapias exposed to low temperatures. **J. Therm. Biol**. 20 (3): 299 – 306.

Tamburrini, M., C. Verde, A. Olianas, B. Giardina, M. Corda, M. T. Sanna, A. Fais, A. M. Deiana, G. D. Prisco and M. Pellegrini. 2001. The hemoglobin system of the brown Moray *Gymnothorax unicolor*: structure/ function relationships. **Eur. J. Biochem**. 268: 4104 – 4111.

Weber, R. E. 2000. Adaptations for oxygen transport : lessons from fish hemoglobin, pp. 22 – 37. *In* G. D. Prisco, B. Giardina and R.E. Weber (eds.). **Hemoglobin Function in Vertebrates: Molecular Adaptation in Extreme and Temperate Environments**. Springer, Verlag Italia, Milano.