Songklanakarin J. Sci. Technol.
30 (Suppl.1), 19-23, April 2008

Songklanakarin Journal of Science and Technology
http://www.sjst.psu.ac.th

Original Article

Effect of pH and ATP on lipid oxidation in unwashed and washed seabass mince (*Lates calcarifer*) mediated by hemoglobin

Chaiwut Buaneow, Worapong Usawakesmanee, Sunisa Siripongvutikorn, and Chakree Tongraung*

Department of Food Technology, Faculty of Agro-Industry,
Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand.

Received 29 December 2006; Accepted 2 August 2007

Abstract

Hemoglobin-mediated lipid oxidation was studied by using washed and unwashed seabass mince with and without added hemoglobin. Three pH values of postmortem fish were examined (physiological pH (6.5), 6.2, and 7.0). The lag time prior to thiobarbituric acid reactive substance development in the unwashed mince without added hemoglobin decreased greatly as the pH was reduced ($p<0.05$). Addition of hemoglobin decreased the lag time especially at the pH 6.2. In addition, the level of deoxyhemoglobin was found sharply increase with pH reduction in the range of pH 7.0 to 5.0. This suggested a potential role for deoxyhemoglobin as a catalyst. ATP lowered hemoglobin oxygenation at all pHs studied. Washing effectively prevented lipid oxidation in the washed mince without added hemoglobin. However, by addition of hemoglobin significant development of TBARS at low pH values except at pH 7.0. The results proved that ATP and low pH values increased oxidative activity of seabass hemoglobin. This investigation suggests that deoxyhemoglobin was a potent pro-oxidant.

Keywords: ATP, seabass hemoglobin, deoxyhemoglobin, TBARS

1. Introduction

Lipid oxidation is a major cause of quality deterioration in muscle-based foods where flavor, color, texture, and nutritional values can be also negatively affected (Kanner, 1994; Gandemer and Meynier, 1995). Heme pigments such as hemoglobin and myoglobin are believed to contribute to lipid oxidation of tissue (Koizumi *et al.*, 1987; Chan *et al.*, 1997). It was reported that human hemoglobin stimulated peroxidation of linoleic acid only slightly at pH 7.4, but the rate increased considerably at pH 6.5 (Gutteridge, 1987). Values of pH below neutrality are typical of post mortem muscle systems. Thus, knowledge about the role of pH below neutrality is critical in understanding lipid oxidation in post mortem meat and fish.

Acceleration of lipid oxidation by pH reduction could be due to enhanced autoxidation of hemoglobin at reduced pH (Tsuruga *et al.*, 1998). Hemoglobin autoxidation causes the production of superoxide anion radical and methemoglobin from ferrous oxyhemoglobin (Misra and Fridovich, 1972). Dismutation of superoxide anion radical will produce hydrogen peroxide, which activates methemoglobin as an initiator of lipid peroxidation (Harel and Kanner, 1986). Lowering the oxygenation of hemoglobin was found to enhance the autoxidation of hemoglobin (Balagopalakrishna *et al.*, 1996; Richard and Hultin, 2000). This is relevant since binding of oxygen by hemoglobins in rainbow trout and cod decreased in air atmospheres at post mortem pH values (Binotti *et al.*, 1971; Richard and Hultin, 2000; Thongraung *et al.*, 2006).

Seabass is one of the high economical value fish species of Thailand. It has white and lean flesh and is highly accepted due to its good texture and low fishy ordor. However, there are complaints about strong painty odor in fillet of the fish especially after storage in refrigeration. Most of the fish arriving the market are live fish or dead fish in the pre-rigor state. Thus, filleting the fish while they still very

*Corresponding author.
Email address: chakree.t@psu.ac.th

fresh leads to blood spread over the fillet surface. Consequently, there is a need to verify association between deoxygenation of seabass hemoglobin and painty flavor development in refrigerated fillet due to lipid oxidation. The main objectives of this work was to investigate the effect of pH and ATP on deoxygenation of seabass hemoglobin and to verify the possible role of deoxyhemoglobin on lipid oxidation.

2. Materials and methods

2.1 Materials

Live cultivated seabass (*Lates calcarifer*) weight between 0.8 to 1.2 kg were purchased from a fish farm in Songkhla lake, Songkhla Province. The fish were transported on ice to Faculty of Agro-Industry, Prince of Songkla University, within 1 h and used for an experiment within 2 h.

2.2 Reagents

Bovine hemoglobin, heparin, streptomycin sulfate sodium phosphate (monobasic and dibasic), and sodium potassium tartrate and other chemicals of analytical grade were procured from Sigma Chemical (St. Louis, MO, USA).

2.3 Methods

1) Hemolysate preparation

Seabass blood was collected from the caudal vein according to the method of Rowley (1990) using aminobenzoic acid ethyl ester as an anesthetic. Hemolysate was prepared immediately after the blood was drawn according to the method of Fyhn *et al.* (1979). Heme protein content of the seabass hemolysate was quantified according to the method of Evelyn and Malloy (1938).

2) Unwashed and washed Seabass mince preparation

To prepare unwashed mince the light muscle of a prerigor seabass was minced by using a food mincer (Kitchen Aid Inc., St. Joseph, MI). Washed minced fish was prepared by washing the mince three times with deionised water using a water to mince ratio of 3:1 (v/w). During washing, the mince slurry was occasionally stirred with a plastic rod and allowed to stand for 10 min and then drained on cheesecloth. A solution of NaCl (0.3% w/v) was used in the third wash to aid in dewatering. The minced was adjusted to 6.2 and 7.0 by addition of 1 M NaOH or 1 M HCl. An appropriate volume of the hemoglobin stock was added to a final concentration of 5.8 μmol per kg mince and stirred with a plastic spatula to distribute the heme protein. Streptomycin sulfate (200 ppm) was added to inhibit microbial growth during storage. The pH of samples was rechecked just after addition of hemoglobin. The final moisture content of the samples was adjusted to 90% with deionized water if necessary.

3) Effect of pH and ATP on deoxygenation of Seabass hemoglobin

The seabass hemoglobin (6.5 mM) in 50 mM phosphate buffer at pH 5.5, 6.0, 6.2, 6.5, and 7.0 was scanned from 630 to 450 nm using a double-beam spectrophotometer model U-3110 (Hitachi Instruments, Inc., San Jose, CA) against phosphate buffer. The difference between the absorbance at the peak (575 nm) and the valley (560 nm) was used for calculation of the relative oxygenation of the hemoglobin. Oxygenation was assigned a value of 100% at pH 7.0. Effect of ATP on hemoglobin deoxygenation was assessed by using Seabass hemoglobin (5.8 μM) in 50 mM phosphate buffer at pH 7.0 with 0, 0.5, 1.0, and 1.5 mM ATP.

4) Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined according to a modified procedure of Buege and Aust (1978). Fifty percent trichloroacetic acid (TCA) containing 1.3% thiobarbituric acid (TBA) was heated to 65°C and used to dissolve the TBA before analysis. Approximately 150 mg of sample was added to 1.1 ml of the TCA-TBA mixture and incubated for 1 h at 65°C. After centrifugation (2500 *g* for 10 min), the absorbance of the supernatant at 532 nm was determined. A standard curve was constructed using 1,1,3,3-tetraethoxypropane.

2.4 Moisture determination

Moisture content was quantified by using a Cenco rapid moisture determination balance (CSC Scientific Co., Inc., Fairfax, VA) equipped with an infrared lamp.

2.5 pH measurement

The pH values of the samples were obtained by homogenizing 1 g of sample with 10 ml of deionised water and determining pH using an Accumet pH/conductivity meter model 20 (Fisher Scientific, Fair Lawn, NJ, USA) equipped with a thermocouple to compensate for temperature.

2.6 Statistical analyses

Data were subjected to analysis of varince (ANOVA) and mean comparisons were performed using Duncan New Multiple range test. Statistical analyses were carried out using the SAS statistical software (SAS, 1996).

3. Results and discussion

3.1 Effect of pH and ATP on deoxygenation of seabass hemoglobin

The absorbance spectra (350-630 nm) of seabass hemoglobin at pH 7.0 (Figure 1) composed of three absor-

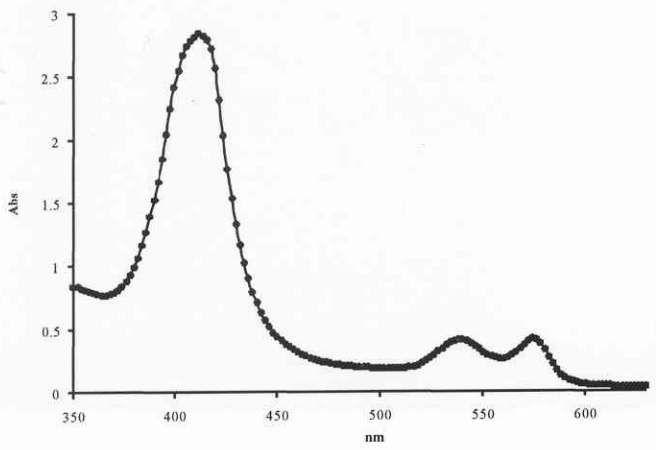

Figure 1. Absorbance spectrum of seabass hemoglobin (5.8 μmol) in 50 mM phosphate buffer at pH 7.0.

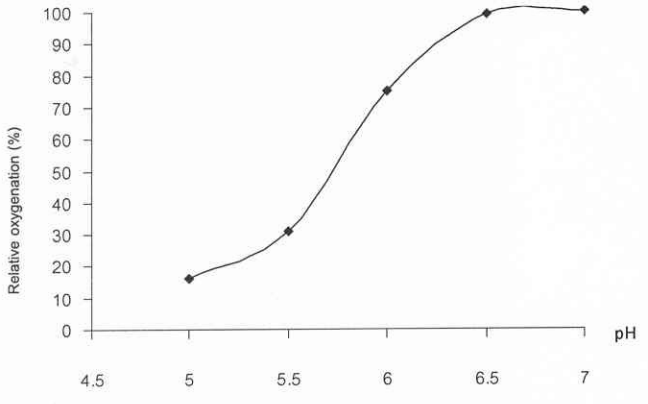

Figure 2. Relative oxygenation of seabass hemoglobin at various pH values.

bance peaks at 412, 540, and 575 nm corresponding to the general characteristics of hemoglobin from other animals reported elsewhere. The absorbance peak at 412 nm is a typical strong absorption band in the blue region of the optical absorption spectrum of a heme protein (Jensen *et al.*, 1998). Two other peaks, at 540 and 575 nm, associate with hemoglobin oxygenation state. It was suggested that a large difference between absorbance at the peak (574 nm) and at its valley between 575 and 540 nm indicate a high relative content of oxyhemoglobin (Pelster and Weber, 1991). Based on this fact, the result suggested that oxygenation of seabass hemoglobin was determined by pH as it increased with increasing pH between pH 6.0 and pH 7.0 in a sigmoidal fashion (Figure 2). Deoxygenation is a predominant property of the anodal hemoglobins to release bind oxygen to the cell (Jensen *et al.*, 1998). This result was in agreement with the observation made with cod, herring, and trout hemoglobin (Thongraung *et al.*, 2005; Richard and Hultin, 2000). Furthermore, it was found that at pH 5.5 the absorbance spectra of the seabass hemoglobin corresponded to a general characteristic of methemoglobin (Jensen *et al.*, 1998) (data not showed).

Existence of ATP as low as 0.5 mM induced deoxygenation of the hemoglobin at pH 7.0 and caused a further significant reduction in the relative oxygenation of the hemoglobin at other lowers pHs (Table 1). The result pointed to the significant role of ATP on hemoglobin deoxygenation. The finding corresponded with the reports mentioning that ATP and ADP decreased oxygenation of trout and cod hemoglobin (Thongraung *et al.*, 2005; Richard and Hultin, 2000). It is believed that these nucleotides bind at the entrance to the central cavity between the two α chains in the T form of hemoglobin. In addition, organic phosphates are known to be potent allosteric effectors of hemoglobins in fish and decrease their oxygen affinity (Jensen *et al.*, 1998).

3.2 Effect of pH and hemoglobin on lipid oxidation of washed and unwashed Seabass mince

Figures 3 and 4 show TBARS development of washed and unwashed seabass mince with and without added hemoglobins at various pHs. It was found that lipid oxidation of the unwashed mince at pH 6.2 and 6.5 occurred more rapidly than that of the mince at pH 7.0 on the basis of TBARS values. Addition of hemoglobin into these fish minces significantly reduced the lag time prior to TBARS ($p<0.05$) development. The lag time was considered the time it took for TBARS to exceed 8 mgMDA/kg of tissue (Richards and Hultin, 2000). Washing of fish mince was an effective means to prevent lipid oxidation within 57 h of this study. However, the development of TBARS occurred after addition of the hemoglobin with a comparable lag time with that of the unwashed mince at the same pH except the washed mince at pH 7.0.

4. Discussion

Lipid oxidation resulting in off-odors, off-flavors, color problems and texture defects is the great concern of food scientist (Kanner,1994). Although seabass is considered a white and lean fish, there are complains about development of strong rancid/painty odors especially in the fish fillet stored in refrigeration. Deoxyhemoglobin and methemoglobin were proposed to be potential pro-oxidation in muscle foods

Table 1. Effect of pH and ATP on relative oxygenation of seabass hemolysate. (mean ± SD)

ATP (mM)	pH		
	6.20	6.50	7.00
0.00	59.09±2.38 ^{aw}	92.47±1.52 ^{bw}	100.00±0.00 ^{cw}
0.50	54.90±2.58 ^{aw}	88.76±2.21 ^{bx}	93.57±2.59 ^{cx}
1.00	52.92±1.97 ^{aw}	88.14±1.02 ^{by}	91.09±3.28 ^{bcy}
2.00	52.32±2.14 ^{aw}	86.94±2.09 ^{bz}	89.65±5.50 ^{cz}

The means followed by difference letters in the same row (a-c) or column (x-z) are significantly difference ($P<0.05$).

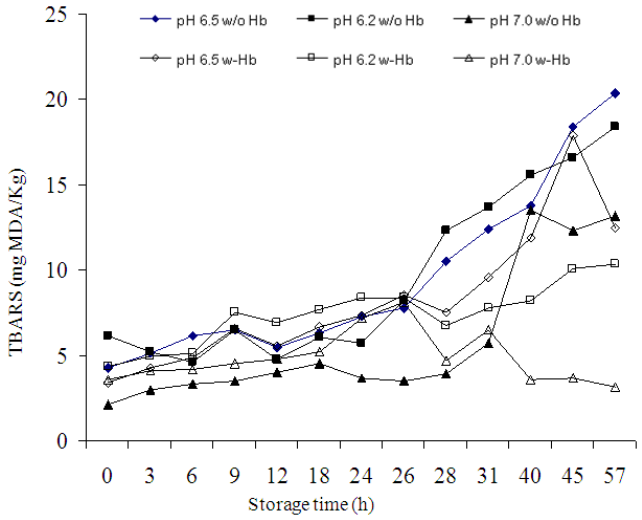

Figure 3. TBARS development of unwashed mince with and without added seabass hemoglobin (w-Hb and w/o-Hb) at pH 6.5, 6.2, and 7.0.

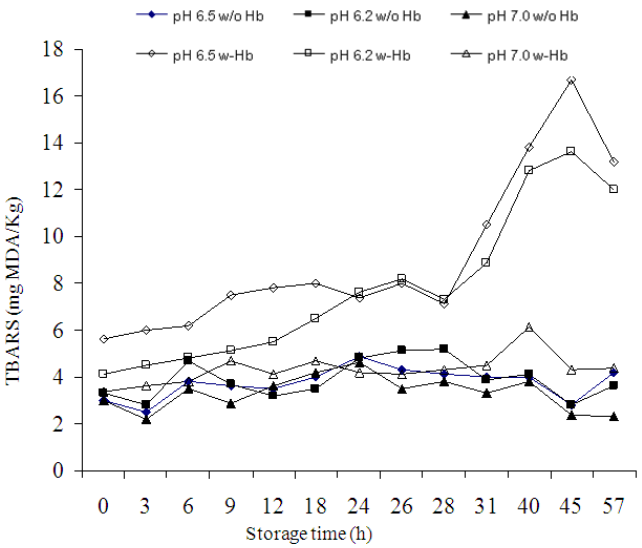

Figure 4. TBARS development of washed mince with and without added seabass hemoglobin (w-Hb and w/o-Hb) at pH 6.5, 6.2, and 7.0.

(Gutteridge, 1987). Recently, it was reported that trout hemoglobin stimulated lipid oxidation at pH 6.0 corresponding to increasing of its deoxyhemoglobin at this pH (Richards *et al.*, 2002; Richards *et al.*, 1998). Although hemoglobin is normally compartmentalized in erythrocytes confined in an intact capillary system, there are many possible situations leading to deoxygenation of hemoglobin. A rough handling of fish on board vessels and/or filleting just-after death or pre-rigor, for instance, lead to exposure of hemoglobin to deoxygenation-inducing factors. This study proved that deoxygenation of seabass hemoglobin was stimulated by lowering of pH and exposure to ATP (although with less efficacy). Thus, increasing of seabass deoxyhemoglobin in the fillet sold in a fresh market is likely since live or a pre-rigor seabass with high content of nucleotide compounds is generally used.

Lipid oxidation, according to the TBARS values, occurred readily in the unwashed mince regardless to an addition of hemoglobin (Figure 3). Although, the fish used for the study was a pre-rigor fish, the postmortem stage of fish that was well balance of pro-oxidant and antioxidant capacity (Richards *et al.*, 1998). It was expected that lipid oxidation would be enhanced by fish mincing. This was due to damaging of fish muscle tissue that would allow leaking of either pro/antioxidant from muscle cell and incorporation of air into the damaged tissue. The lag time before the unwashed mince develops a TBARS value exceeding 8 mg MDA/kg of tissue was reduced significantly ($p<0.05$) after hemoglobin addition suggested an increase of pro-oxidant in the unwashed minces. If in fact deoxyhemoglobin accelerates lipid oxidation, the higher ratio of deoxyhemoglobin to oxyhemoglobin at pH lower than 7.0 would be expected to stimulate lipid oxidation readily. Indeed, at pH 6.2 and 6.5 added hemoglobin decreased the lag time of the washed minces from 26 and 28 h to 24 and 26 h, respectively, whereas at pH 7.0 the same treatment reduced the lag time of the washed minces from over 60 h to 26 h suggested the added hemoglobin at pH 7.0 had a higher pro-oxidative activity. As fish muscle endogenous constituents could confound lipid oxidation in the unwashed mince due to added hemoglobin, washing the fish mince was performed to remove aqueous antioxidants and pro-oxidants from the muscle. Non-noticeable development of TBARS in the washed mince without added hemoglobin compared to that of the unwashed mince without added hemoglobin proved that washing was an effective mean to remove interference of lipid oxidation. Consequently, the acceleration of lipid oxidation in the washed mince with added hemolysate at pH 6.2 and 6.5 associated with increasing of deoxyhemoglobin suggested that this state of hemo-globin was a potent pro-oxidant. One explanation for the limited reduction of the lag time prior the development of TBARS at pH 6.2 and 6.5 relative to that of pH 7.0 when hemoglobin was added to unwashed mince may be because decreasing of pH caused imbalance of endogenous pro-oxidant and antioxidant resulting in content of pro-oxidant over the threshold required to catalyze lipid oxidation. Increasing deoxyhemoglobin in these systems, thus, had marginal effect to mediate the lipid oxidation. Since pH 7.0 had none significant effect on the increased amount of deoxyhemoglobin and no significant TBARS development at pH 7.0 was observed in washed mince with and without added hemoglobin. The results thus confirmed that at pH 7.0 the hemoglobin had no pro-oxidative activity. The decrease in lag time of TBARS development at pH 7.0 in unwashed with added hemoglobin relative to that of unwashed without added hemoglobin was, therefore, likely due to the elevated level of deoxyhemoglobin caused by aqueous endogenous compounds. Based on the result of Table 1, nucleotides especially ATP, are likely to be

involved. Mincing of just-killed fish, for instance, could increase the concentration of ATP available for hemoglobin from 0.4 to 9.9 mM since the hemoglobin in the plasma would be mixed with the nucleotide-rich sarcoplasm (Richard and Hultin, 2000). Future work should continue to investigate the role of hemoglobin in fish at various post mortem stages associated with changing endogenous pro- and antioxidant on in lipid oxidation mediated by its hemoglobin.

5. Conclusion

The results revealed that lowering the pH within post-mortem pH from 7.0 to 5.0 caused deoxygenation of seabass hemoglobin. ATP was able to accelerate deoxygenation at pH 7.0 and other lower pH values. Thus, pH of the post mortem seabass and ATP in the pre-rigor fish could elevate the levels of deoxyhemoglobin resulting an acceleration of lipid oxidation in the seabass fillet.

Acknowledgements

This work was supported by Prince of Songkla University, Thailand.

References

Balagopalakrishna, C., Manoharan, P. T., Abugo, O. O., and Rifkind, J. M. 1996. Production of superoxide from hemoglobin-bound oxygen under hypoxic conditions. Biochemistry. 35: 6393-6398.

Binotti, I., Giovenco, S., Giardina, B., Antonini, E., Brunori, M., Wyman, J. 1971. Studies on the functional properties of fish hemoglobins. II. The oxygen equilibrium of the isolated hemoglobin components from trout blood. Arch. Biochem. Biophys. 142: 274-280.

Buege, J.A. and Aust, S.D. 1978. Microsomal lipid peroxidation. *In*: S. Fleischer and L. Packer, Editors, Methods in enzymology (V 52). Academic Press. New York. p. 302.

Chan, W. K. M., Faustman, C., Yin, M., and Decker, E. A. 1997. Lipid oxidation induced by oxymyoglobin and metmyoglobin with involvement of H2O2 and superoxide anion. Meat Sci. 46: 181-190.

Evelyn, K.A. and Molloy, H.T. 1938. Microdetermination of oxyhemoglobin , methemoglobin, and sulfhemoglobin in a single sample of blood. J. Biol. Chem. 126: 655-662.

Fyhn, U. E., Fyhn, H. J., Davis, B. J., Powers, D. A., Fink, W. L., and Garlick, R. L. 1979. Hemoglobin heterogenity in amazonian fishes. Comp. Biochem. Physiol. 62A: 39-66.

Gandemer, G., and Meynier, A. 1995. The importance of phospholipids in the development of flavour and off-flavour in meat products. *In*: Composition of meat in relation to processing, nutritional and sensory quality: from farm to fork. Lundstrom, K., Hansson, I., Wiklund, E., Eds., pp 119-128.ECCEAMST: Utrecht, The Netherlands.

Gutteridge, J. M. C. 1987. The antioxidant activity of haptoglobin towards haemoglobin-stimulated lipid peroxidation. Biochim. Biophys. Acta. 917: 219-223.

Harel, S., and Kanner, J. 1986. Hydrogen peroxide-activated methaemoglobin and other haemoproteins as initiators of membranal lipid peroxidation. *In* Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine, Rotilo, G., Ed., pp 25-28. Elsevier Science B.V. (Biomedical Division): New York.

Jensen, F.B., Fago, A., and Weber, R.E. 1998. Hemoglobin structure and function. *In*: Fish respiration. Perry S.F. and Trufs B.L., ed. p. 1-40. Academic Press, San Diego.

Kanner, J. 1994. Oxidative processes in meat and meat products: quality implications. Meat Sci. 36: 169-189.

Koizumi, C., Wada, S., and Ohshima, T. 1987. Factors affecting development of rancid odor in cooked fish meats during storage at 5°C. Nippon Suisan Gakkaishi. 53: 2003-2009.

Misra, H. P. and Fridovich, I. 1972. The generation of superoxide during autoxidation of hemoglobin. J. Biol. Chem. 247: 6960-6962.

Pelster, B. and Weber, R.E. 1991. The physiology of the Root effect. *In*: Advances in comparative and environmental physiology. Vol. 8. Gilles R. ed. p. 51-77. Springer-Verlag, Berlin.

Richards, M. P., Modra, A.M., and Li, R . 2002. Role of deoxyhemoglobin in lipid oxidation of washed cod muscle mediated by trout, poultry and beef hemoglobins. Meat Sci. 62: 157-163.

Richards, M. P. and Hultin, H. O. 2000. Effect of pH on lipid oxidation using trout hemolysate as a catalyst: a possible role for deoxyhemoglobin. J. Agric. Food Chem. 48: 3141-3147.

Richards, M. P., Kelleher, S. D., and Hultin, H. O. 1998. Effect of washing with or without antioxidants on quality retention of mackerel fillets during refrigerated and frozen storage. J. Agric. Food Chem. 46: 4363-4371.

SAS. 1996. Statistical Analysis System Software. Version 6.08. Cary, NC: SAS Institute, Inc.

Thongruang, C., Benjakul, S., and Hultin, H.O. 2006. Effect of pH, ADP and Muscle soluble components on cod hemoglobin characteristics and extractabilty. Food Chem. 97-567-576.

Tsuruga, M., Matsuoka, A., Hachimori, A., Sugawara, Y., and Shikama, K. 1998. The molecular mechanism of autoxidation for human oxyhemoglobin. J. Biol. Chem. 273: 8607-8615.