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Research Article

A fast protein liquid chromatography method for purification of myoglobin from different species

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ABSTRACT

The aim of this study was to describe a fast method for the purification of high-purity myoglobin for mass spectroscopy analyses and to use it as standard grade material. A three-step fast protein liquid chromatography (FPLC) method was used to produce high-purity myoglobin. SEC 650 gel filtration followed by ENrich Q anion exchange chromatography was used to produce myoglobin in acceptable purity for most research methods. A second filtration step was carried out with narrow field SEC 70 gel to prepare high-purity myoglobin at standard-grade purity and suitable for mass spectroscopy analyses. At least 90% pure myoglobin was obtained by applying two chromatography steps to the samples of three species, and over 99% pure myoglobin was obtained in standard material quality and suitable for mass spectroscopy when the additional narrow field SEC 70 chromatography step was applied. The proposed method provided higher purity compared to other methods and takes less time. FPLC columns significantly reduced the duration of the chromatography steps. At the same time, the use of solid extraction columns instead of dialysis reduced the long overnight dialysis process to a few minutes.

INTRODUCTION

Myoglobin, a cytoplasmic protein found in cardiac and skeletal muscle, is a one-polypeptide chain of 154 amino acids with a molecular weight between 14 and 18 kDa (Ordway and Garry, 2004). Myoglobin's primary structure varies among species. These differences in amino acid sequences affect the secondary structure as well as the chemical behavior of proteins like polarity and isoelectric point. The characterization of myoglobin from various animals has become an important subject in nutrition, food science, and biology. The amino acid sequence of myoglobin from various species and edible animals was identified previously. The primary structure of myoglobin from livestock and edible animals like cattle, sheep, pigs, horses, rabbits, goats, bison, buffalo, musk oxen, chickens, turkeys, pheasants, tuna, mackerel, and sardine were established and generally used for food safety and meat adulteration and authentication(Di Giuseppe et al., 2018; Dosi et al., 2006; Joseph et al., 2011; Joseph, et al., 2010a; Mottola et al., 2022; Nurilmala et al., 2018; Ragucci et al., 2019; Saud et al., 2019; Suman et al., 2009; Tan et al., 2021)containing the heme pigment, is present in large amount in meat, thus influencing meat color and consumer choice. Here, the primary structure of Mb isolated from muscle of Phasianus colchicus L. was determined by using a comparative peptide mapping approach based on MALDI-TOF mass spectrometry. This strategy allowed the determination of common pheasant Mb primary sequence, which resulted identical to the chicken Mb, as also confirmed by intact molecular mass determination by ESI/Q-TOF mass spectrometry. Indeed, the accurate molecular mass (17,290.50 Da. Suman et al. compared the amino acid sequence of goat myoglobin with other ruminant myoglobin sequences (sheep, cattle, buffalo, and deer). It was similar to other ruminant myoglobin by having 153 residues. However, the amino acid sequence in goat myoglobin was quite different from that in sheep and other ruminants. The similarity between the myoglobin of the five tested animals was between 95.4% and 99.3% (Suman et al., 2009)(Table 1).

Table 1. Percentage sequence similarities among myoglobins from different meat-producing ruminant species. Goat (Capra hircus); Sheep (Ovis aries); Cattle (Bos Taurus); Buffalo (Bubalus bubalis); Deer (Cervus elaphus) (Suman et al., 2009).

Species	Goat	Sheep	Cattle	Buffalo	Deer
Goat	100				
Sheep	98.7	100			
Cattle	97.4	98.7	100		
Buffalo	95.4	96.7	98.0	100	
Deer	98.0	99.3	98.0	96.1	100

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In addition to health and food safety prospects in myoglobin studies, it is also used widely in biological science as a marker in evolution and taxonomy research (Di Giuseppe et al., 2017; Enoki et al., 2008; Ragucci et al., 2022; Romero-Herrera et al., 1976). Purification of myoglobin is the first and main step in research based on the characterization of myoglobin either for health, nutrition, and food sciences or for biological research. Cellulose and Sephadex-based columns are widely used for the purification of myoglobin in research laboratories. Yamazaki et al. described a two-column method at alkaline pH for purification of oxymyoglobin with DEAE-cellulose and another column that was not indicated (Yamazaki et al., 1964). Similar time-consuming two-column methods were developed in the following years (Gotoh and Shikama, 1974; Shikama and Sugawara, 1978; Suzuki et al., 1980). Later Renerre et al. described a two-step chromatographic method with DEAE-Sepharose and TSK gel filtration columns (Renerre et al., 1992) and Gatellier used a one-step chromatographic method with an HPLC column for the purification of myoglobin (Gatellier et al., 1993). Two-step purification methods were still used for the purification of myoglobin with different columns and buffers (Chotichayapong et al., 2016; Dosi et al., 2012; Enoki et al., 2008; Giaretta et al., 2013). Although these methods are generally effective, they are time-consuming, with approximately two or three days needed to purify myoglobin from one species.

Herein we describe an FPLC method that took less than one day to produce high-purity myoglobin from different animals for mass spectroscopy (MS) analyses and standard preparation. In addition, we present a two-dimensional SDS-PAGE method for characterizing and distinguishing myoglobin of different animals from each other.

MATERIAL and METHODS

Materials

Cattle and chicken hearts were obtained from the local market. A pig's heart was obtained from a pork market in Istanbul. ENrich SEC 650 and ENrich SEC 70 gel filtration chromatography and ENrich Q anion exchange chromatography preparative FPLC columns, IEF strips, precast gradient electrophoresis gels, and chemicals for IEF and electrophoresis were obtained from Bio-Rad, USA. Amicon ultra-15 centrifugal filters were obtained from Merck Millipore, USA. PD-10 desalting columns were obtained from GE Healthcare, USA. Pure horse myoglobin, size exclusion chromatography standards, and other chemicals were obtained from Sigma-Aldrich, USA.

Methods

Heart homogenization and myoglobin extraction

The animal hearts were cut into small pieces and suspended in 50 mM potassium phosphate buffer containing 0.5 mM EDTA (pH 7.4) at a ratio of 10 ml of buffer to 1 ml of meat and homogenized using a blender/homogenizer on ice. The resulting homogenate was then centrifuged at 20,000 × *g* for 40 minutes. The supernatant was filtered with Miracloth to remove fats and then centrifuged at 15,800 × *g* for 15 minutes and the pellet was discarded. Supernatant fractions containing myoglobin were pooled for the further steps.

SEC 650 size exclusion chromatography

All chromatography assays were carried out at 4 °C. An ENrich SEC 650 (10 × 300 mm) gel filtration column was used for the first step of purification. The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.6) at a flow rate of 0.4 ml/min prior to the experiment and all SEC 650 experiments were carried out under the same conditions.

Preparation of a standard chromatogram for the ENrich SEC 650 size exclusion column

Prior to the application of homogenate supernatant to the column, the standard plot for the ENrich SEC 650 column was prepared. A mixture of six pure protein fractions, namely amylase (200 kDa), alcohol dehydrogenase, (150,000 kDa), cattle serum albumin (66 kDa), carbonic anhydrase (29 kDa), horse

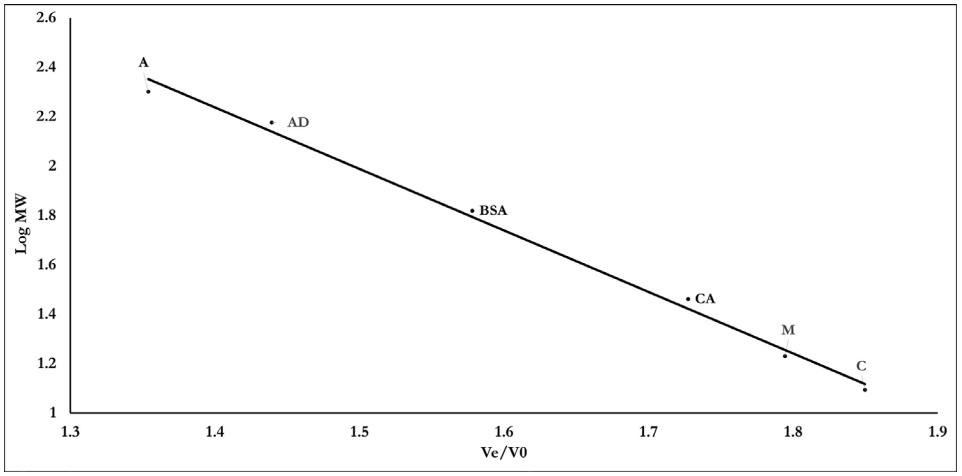

Figure 1. Standard plot for SEC 650 Gel Filtration chromatography column. Amylase 200 KDa(A), Alcohol Dehydrogenase, 150,000 KDa (AD), Cattle Serum Albumin 66KDa (BSA), Carbonic Anhydrase 29KDa (CA), Horse myoglobin (M) 17 KDa, Cytochrome C 12,4 KDa.(C)

myoglobin (17 kDa), and horse cytochrome C (12.4 kDa), was applied to the column. The void volume (V0) of the column and retention volumes (Ve) of the standards were determined and a standard plot was drawn as a logarithm of molecular weight (LogMW) versus V0/Ve. The retention volume of the myoglobin fraction determined was 15.55 ml (Figure 1).

SEC 650 size exclusion chromatography of meat samples

Supernatant from each animal heart was concentrated using Amicon Ultra-15 centrifugal filters with a cutoff membrane of 10 kDa to discard the light proteins below 10 kDa and reduce the volume of the sample to 1 ml, which is essential for gel filtration applications. Next 1 ml of the concentrated materials was applied to the column and the output of the column was monitored at 280 nm for total protein and 527 nm for myoglobin detection. The output was collected in 1-ml fractions and fractions near the retention time of 16 min with high absorbance at 527 nm were collected for the subsequent steps (Figure 2).

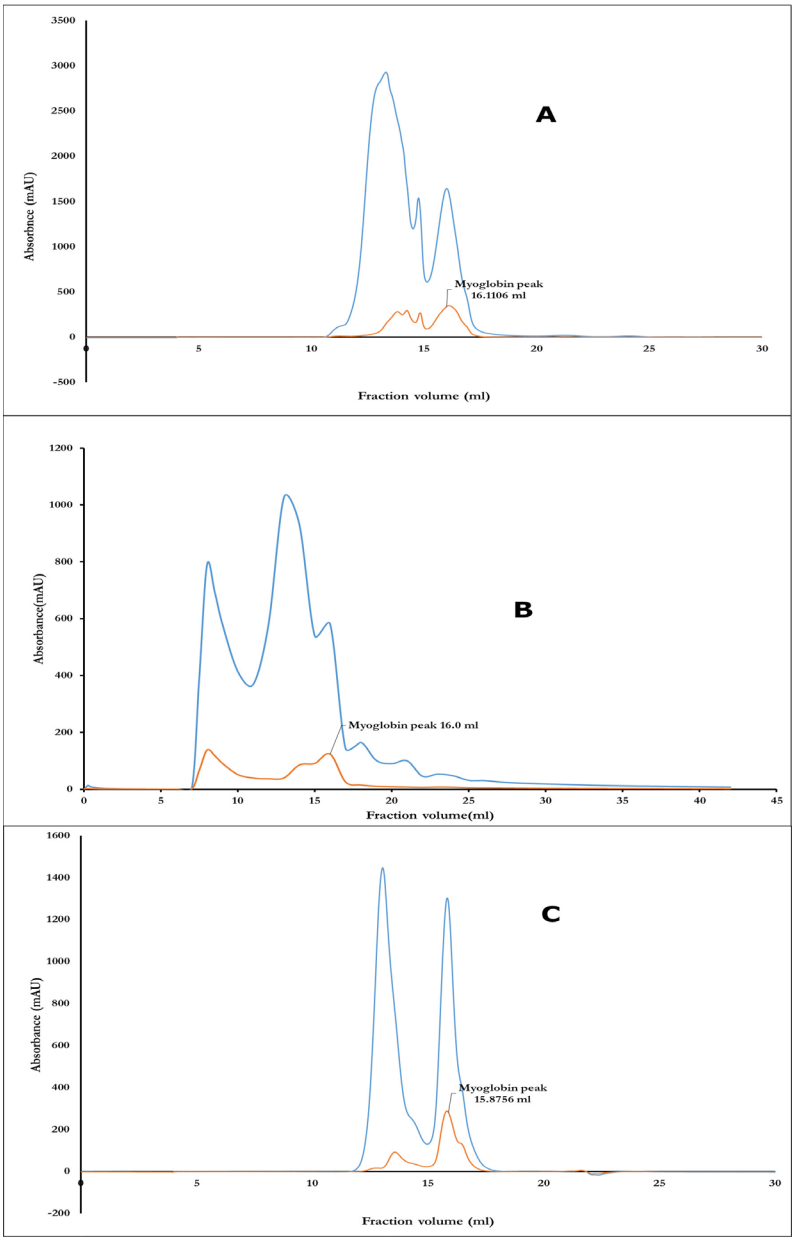

Figure 2. Size exclusion chromatogram of (A) cattle, (B) pork and (C) chicken myoglobin by SEC 650 FPLC column. (-Blue line: A280 , Brown line: A527)

ENrich Q anion exchange chromatography

Fractions collected from the size exclusion chromatography were then subjected to a PD-10 desalting column and the bonded material was eluted with 10 mM Tris-HCl buffer (pH 8.6) and applied to an ENrich Q anion exchange column (10 × 100 mm) equilibrated with the same buffer at a flow rate of 1 ml/min. The column was washed with the same buffer until A280 reached the baseline. Myoglobin was eluted from the column with a linear gradient of NaCl (0-1.0 M) in Tris-HCl (pH 8.6) buffer. The absorbance of the elusions at 280 nm and 527 nm was checked and fractions with high 527 nm absorbance were collected. Pure horse myoglobin was also subjected to

the same procedure for the determination of elution characteristics. Pig, cattle, horse, and chicken myoglobin were eluted at 0.20 M, 0.212 M, 0.256 M, and 0.370 M NaCl concentration of elution gradient, respectively. The myoglobin fractions were collected and stored for further steps (Figure 3).

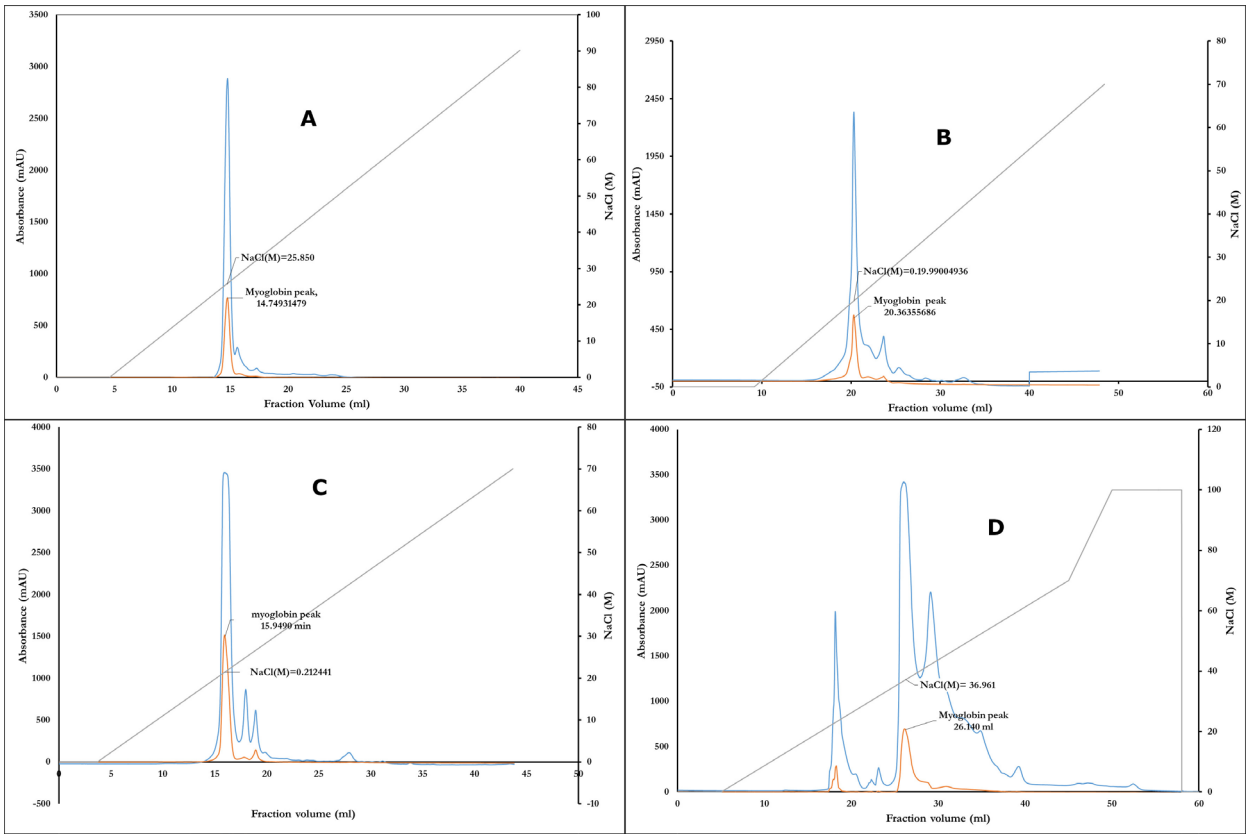

Figure 3. Anion Exchange chromatogram of (A) horse, (B) pork, (C) cattle and (D) chicken myoglobin by Enrich Q FPLC column. (¬Blue line: A280, Brown line: A527)

SEC 70 size exclusion chromatography of meat samples

The fractions collected from the IEX steps were applied to an ENrich SEC 70 column to remove the trace impurities and to prepare samples for the analytical steps. The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.6) at a flow rate of 0.4 ml/min prior to the experiment and all SEC 70 experiments were carried out under the same conditions. Horse myoglobin was used as standard and all myoglobin fractions were eluted with 11.97-12.00 ml of elution buffer (Figure 4).

SDS-PAGE purity tests

The purity of samples was tested using SDS-PAGE. Samples were run on Bio-Rad "TGX any kD" precast gradient gels using the Laemmli buffer system (Laemmli, 1970).

Protein assay

Protein amounts in each myoglobin fraction were determined by the BCA method before mixing and application to the UHPLC system.

Characterization of myoglobin from different species by HPLC

UHPLC analysis of myoglobin was performed using a Thermo Fisher Dionex 3000 UHPLC system with a diode array detector. Myoglobin was characterized by the method described by Giaretta et al. with some modifications(Giaretta et al., 2013). A Protein-Pak Hi-Res Q column [4.6 × 100 mm, 5 µm (Waters SpA)] was used to separate myoglobin from different species. To load samples 80% 20 mM glycine NaOH buffer pH 9.2 (mobile phase A) and 20% 20 mM glycine NaOH buffer pH 9.2 containing 0.2 M NaCl (buffer B) were used. The myoglobin was eluted by a linear gradient of buffer B from 20% to 30% B for 7 min, followed by 70% for 2 min, returning to 20% for the following analyses. The analyses were carried out at a flow rate of 0.4 ml/min and 40 °C.

Two dimension electrophoresis of mixed myoglobin

Isoelectric focusing of the myoglobin mixture

IEF was performed using a Bio-Rad Protean i12 IEF system. A mixture of the 4 types of myoglobin studied was used as the sample. Myoglobin samples were dried and resuspended in an IEF rehydration solution containing 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% carrier ampholyte, and 0.001% bromophenol blue to a final concentration of 1 mg protein in 1 ml solution. Next 200 µl of the sample was pipetted into a rehydration/equilibration tray and an IPG strip (pI +5-8) was placed over it side down. The strip was incubated for 12 hours for absorbing the sample. Ready strips were transferred to the IEF system focusing tray for the first dimension of electrophoresis. The IEF program was started with an application of 250 volts for 20 minutes and a gradual increase in voltage to

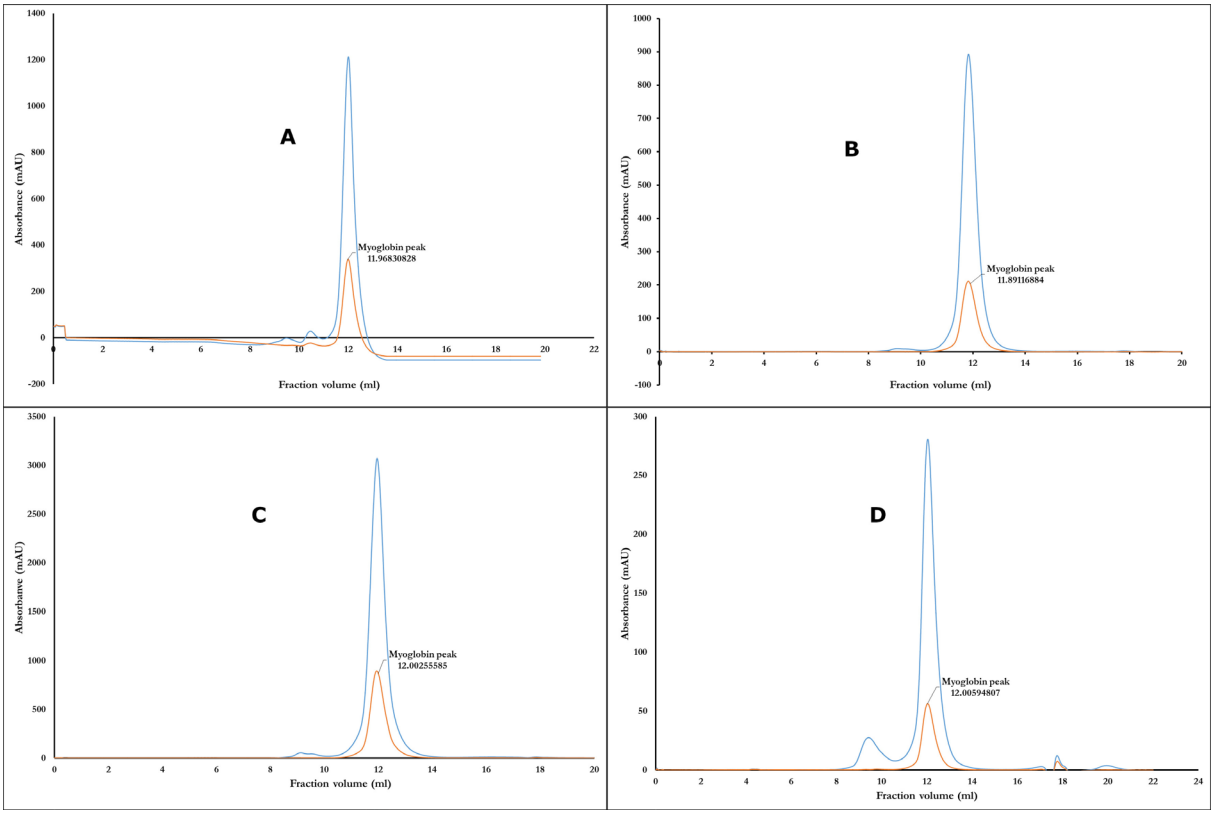

Figure 4. Size exclusion chromatogram of (A) horse, (B) cattle, (C) pork and (D) chicken myoglobin by SEC 70 FPLC column. (¬Blue line: A280, Brown line: A527)

8000 volts over 60 minutes. Power application continued until the total amount of voltage applied reached 26000 volt hours.

Second dimension SDS-PAGE

The IPG strips were equilibrated before the SDS-PAGE application. For this the proposed IPG strips were incubated in equilibration buffers I and II containing 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 2% DTT for buffer I and 2.5% iodoacetamide for buffer II for 10 minutes each. The IPG strips then were placed on top of the SDS-PAGE gels. Continuity was achieved with overlay agarose containing 0.5% low melting point agarose in 25 mM tris, 192 mM glycine, 0.1% SDS, and a trace of bromophenol blue. Electrophoresis was carried out until the bromophenol blue reached the end of the SDS-PAGE gel. The Laemmli method was used for the second dimension electrophoresis (Laemmli, 1970). Gels were removed from their cassettes and stained with Coomassie brilliant blue R-250 stain.

RESULTS

Purification of myoglobin

Myoglobin from 3 species was purified partially by one step of gel filtration and one step of ion-exchange chromatography and purified completely by an additional step of narrow range gel filtration chromatography (Table 2). The purity of myoglobin obtained from the last step was also tested by SDS-PAGE (Figures 5 and 6).

Table 2. Purification steps of different animal myoglobin

	Cattle			Pork			Chicken		
Purification step	**purity (%)**	**(fold)**	**yield (%)**	**purity (%)**	**(fold)**	**yield (%)**	**purity (%)**	**(fold)**	**yield (%)**
Homogenate	25.69	1	100	22.85	1	100	24.72	1	100
Primary gel filtration	77.84	3.03	75.43	80.2	3.51	83.65	80	2.89	80.7
Anyon Exchange	90.18	3.51	58.68	98.96	4.33	72.03	92.26	3.73	45.31
Secondary gel filtration	99.81	3.89	48.44	100	4.38	71.27	99.1	4	30.7

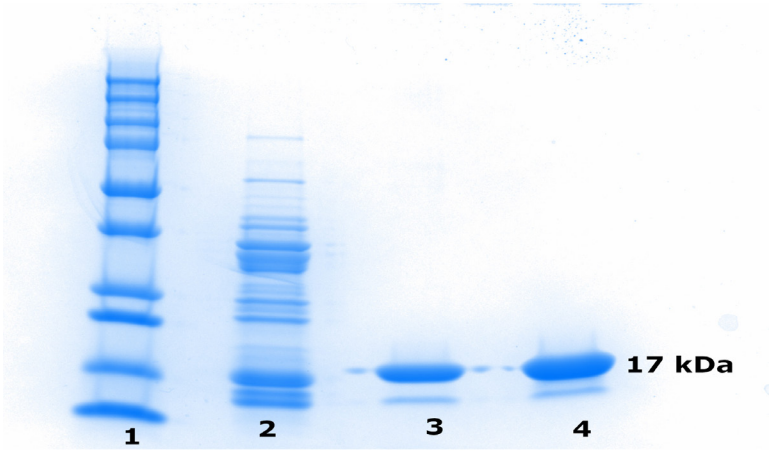

Figure 5. SDS-PAGE of myoglobins after anion exchange chromatography. 1: molecular wight Marker. 2: Chicken. 3: Cattle and 4: pork myoglobins

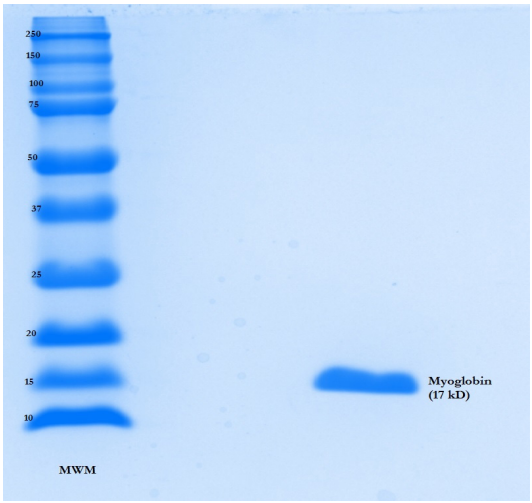

Figure 6. SSDS-PAGE of chicken myoglobin SEC 70 size exclusion chromatography. Left: molecular wight Marker. Right: Chicken myoglobin.

Characterizing of myoglobin samples

Myoglobin from 4 different species was thoroughly separated with HPLC. These results indicate that HPLC analyses of myoglobin could be used as an effective method for determining the species (Figure 7). Myoglobin fractions in the mixed

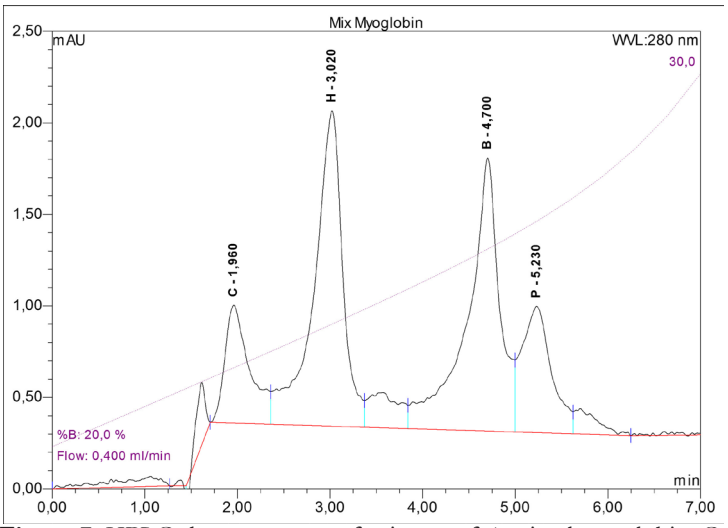

Figure 7. HPLC chromatogram of mixture of 4 animal myoglobin. C: Chicken, H: Equine, B: Bovine and p: porcine myoglobin peaks.

samples were also thoroughly separated and characterized by 2-dimensional gel electrophoresis due to differences in their iso-electric points (Figure 8) which also could be extracted from 2-dimensional gel electrophoresis (Table 3)

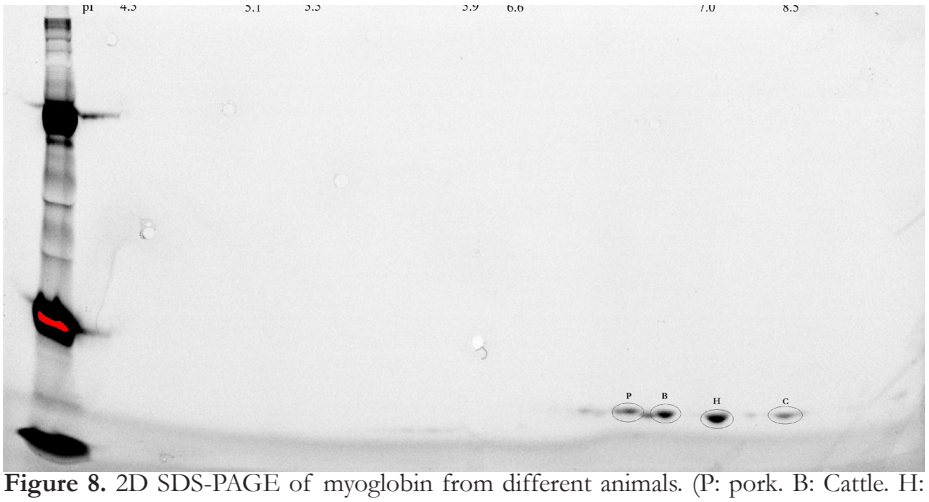

Figure 8. 2D SDS-PAGE of myoglobin from different animals. (P: pork. B: Cattle. H: Horse. C: chicken).

Table 3. Iso-electric points of myoglobin from pork, cattle, horse and chicken

Myoglobin	Isoelectric point
Pork	6.85
Cattle	6.89
Horse	7.12
chicken	7.95

DISCUSSION

Considering the results of the different purification steps, the purity of myoglobin for analysis in HPLC or 2D electrophoresis is acceptable after one step of gel filtration and one step of ion-exchange chromatography. These myoglobin fractions had trace impurities, so this level of purity is not sufficient for sequence analyses because of probable interference by extraneous peptides. The second gel filtration step with a narrow range gel filtration column was required for high-purity myoglobin production suitable for MS or sequence analyses. The results of both the HPLC and 2D SDS-PAGE analyses indicate that the products of this method meet the purpose of the study and could be used as standard materials for the determination of species in meat analyses in food safety and meat adulteration or any biological analyses.

CONCLUSION

The preparation of pure proteins is a major topic in biochemistry and many studies have been carried out to find an easy, feasible, and effective method for the purification of various proteins. In the present study, we introduced a fast and effective method for the purification of myoglobin as standard material for use in further analyses in species determination. The existing methods based on ammonium sulfate fractionation, dialysis and conventional low-pressure gel filtration, and ion exchange chromatography steps take days to prepare pure myoglobin(Chaijan et al., 2007; Joseph et al., 2010b), whereas in our method the purification time is reduced to a few hours. The repeatability and applicability of the procedure to different animal species are other advantages of our method. Unequal retention times in HPLC and separate points in 2D gel electrophoresis make myoglobin a favorable material in species determination. After specifying the position of myoglobin from each animal species by using standard myoglobin, a whole protein fraction of meat could be used after a simple protein isolation step. Special 2D gel analysis and comparison software is required for this purpose. The lack of this type of software limited our study in the case of studying mixed meat samples.

DECLARATIONS

Ethics Approval

There was not any human participant in this study.

Conflict of Interest

The authors have no conflicts of interest to declare

Consent for Publication

There was not any human participant in this study so no Consent for Publication is needed

Author contribution

Idea, concept and design: BS, NSM, ÖE

Data collection and analysis: BS, NSM, ÖE

Drafting of the manuscript: BS, NSM, ÖE

Critical review: BS, NSM, ÖE

Data Availability

Data were available on request from Correspondence author

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REFERENCES

1. Chaijan, M., Benjakul, S., Visessanguan, W., & Faustman, C. (2007). Characterisation of myoglobin from sardine (Sardinella gibbosa) dark muscle. Food Chemistry, 100(1), 156–164. https://doi.org/10.1016/j.foodchem.2005.09.030

2. Chotichayapong, C., Sattayasai, N., Kanzawa, N., Tamiya, T., Tsuchiya, T., & Chanthai, S. (2016). Purification, peptide mapping and spectroscopic characterization of myoglobin from striped snake-head fish (ophicephalus striatus). Oriental Journal of Chemistry, 32(1), 181–194. https://doi.org/10.13005/OJC/320119

3. Di Giuseppe, A. M. A., Russo, R., Ragucci, S., Landi, N., Rega, C., Chambery, A., & Di Maro, A. (2018). Myoglobin from common pheasant (Phasianus colchicus L.): Purification and primary structure characterization. Journal of Food Biochemistry, 42(2), 1–9. https://doi.org/10.1111/jfbc.12477

4. Di Giuseppe, A. M. A., Russo, L., Russo, R., Ragucci, S., Caso, J. V., Isernia, C., Chambery, A., & Di Maro, A. (2017). Molecular characterization of myoglobin from Sciurus vulgaris meridionalis: Primary structure, kinetics and spectroscopic studies. Biochimica et Biophysica Acta - Proteins and Proteomics, 1865(5), 499–509. https://doi.org/10.1016/j.bbapap.2017.02.011

5. Dosi, R., Carusone, A., Chambery, A., Severino, V., Parente, A., & Di Maro, A. (2012). Rapid primary structure determination of myoglobins by a complementary approach based on mass spectrometry and Edman degradation. Food Chemistry, 133(4), 1646–1652. https://doi.org/10.1016/j.foodchem.2012.02.042

6. Dosi, R., Di Maro, A., Chambery, A., Colonna, G., Costantini, S., Geraci, G., & Parente, A. (2006). Characterization and kinetics studies of water buffalo (Bubalus bubalis) myoglobin. Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology, 145(2), 230–238. https://doi.org/10.1016/j.cbpb.2006.07.006

7. Enoki, Y., Ohga, Y., Ishidate, H., & Morimoto, T. (2008). Primary structure of myoglobins from 31 species of birds. Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology, 149(1), 11–21. https://doi.org/10.1016/j.cbpb.2007.07.006

8. Gatellier, P., Anton, M., & Renerre, M. (1993). A rapid method of oxymyoglobin purification. Meat Science, 33(3), 401–407. https://doi.org/10.1016/0309-1740(93)90011-6

9. Giaretta, N., Di Giuseppe, A. M. A., Lippert, M., Parente, A., & Di Maro, A. (2013). Myoglobin as marker in meat adulteration: a UPLC method for determining the presence of pork meat in raw beef burger. Food Chemistry, 141(3), 1814–1820. https://doi.org/10.1016/J.FOODCHEM.2013.04.124

10. Gotoh, T., & Shikama, K. (1974). Autoxidation of native oxymyoglobin from bovine heart muscle. Archives of Biochemistry and Biophysics, 163(2), 476–481. https://doi.org/10.1016/0003-9861(74)90504-9

11. Joseph, P., Suman, S. P., Li, S., Beach, C. M., & Claus, J. R. (2010). Mass spectrometric characterization and thermostability of turkey myoglobin. LWT - Food Science and Technology, 43(2), 273–278. https://doi.org/10.1016/J.LWT.2009.08.019

12. Joseph, P., Suman, S. P., Li, S., Beach, C. M., Steinke, L., & Fontaine, M. (2010). Characterization of bison (Bison bison) myoglobin. Meat Science, 84(1), 71–78. https://doi.org/10.1016/j.meatsci.2009.08.014

13. Joseph, P., Suman, S. P., Li, S., Claus, J. R., Fontaine, M., & Steinke, L. (2011). Primary structure of turkey myoglobin. Food Chemistry, 129(1), 175–178. https://doi.org/10.1016/j.foodchem.2011.04.024

14. Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature, 227(5259), 680–685. https://doi.org/10.1038/227680a0

15. Mottola, A., Piredda, R., Catanese, G., Lorusso, L., Ciccarese, G., & Di Pinto, A. (2022). Species authentication of canned mackerel: Challenges in molecular identification and potential drivers of mislabelling. Food Control, 137(January), 108880. https://doi.org/10.1016/j.foodcont.2022.108880

16. Nurilmala, M., Ushio, H., Watabe, S., & Ochiai, Y. (2018). A streamlined isolation method and the autoxidation profiles of tuna myoglobin. Journal of Food Science and Technology, 55(5), 1641–1647. https://doi.org/10.1007/s13197-018-3068-3

17. Ordway, G. A., & Garry, D. J. (2004). Myoglobin: An essential hemoprotein in striated muscle. Journal of Experimental Biology, 207(20), 3441–3446. https://doi.org/10.1242/jeb.01172

18. Ragucci, S., Russo, R., Landi, N., Valletta, M., Chambery, A., Esposito, S., Raundrup, K., & Di Maro, A. (2019). Muskox myoglobin: purification, characterization and kinetics studies compared with cattle and water buffalo myoglobins. Journal of the Science of Food and Agriculture, 99(14), 6278–6286. https://doi.org/10.1002/jsfa.9901

19. Ragucci, S., Woodrow, P., Clemente, A., Russo, R., Valletta, M., Landi, N., Russo, L., Chambery, A., & Di Maro, A. (2022). Myoglobin from Atlantic and Tinker mackerels: Purification, characterization and its possible use as a molecular marker. International Journal of Biological Macromolecules, 214(June), 459–469. https://doi.org/10.1016/j.ijbiomac.2022.06.128

20. Renerre, M., Anton, M., & Gatellier, P. (1992). Autoxidation of purified myoglobin from two bovine muscles. Meat Science, 32(3), 331–342. https://doi.org/10.1016/0309-1740(92)90096-M

21. Romero-Herrera, A. E., Lehmann, H., Castillo, O., Joysey, K. A., & Friday, A. E. (1976). Myoglobin of the orangutan as a phylogenetic enigma. Nature 1976 261:5556, 261(5556), 162–164. https://doi.org/10.1038/261162a0

22. Saud, S., Li, G., Kong, H., Khan, M. I., Qiang, Z., Sun, Y., Liu, W., Ding, C., Xiao, H., Wang, Y., Li, H., & Cao, C. (2019). Identification of chicken meat quality via rapid array isoelectric focusing with extraction of hemoglobin and myoglobin in meat sample. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 1128(August), 121790. https://doi.org/10.1016/j.jchromb.2019.121790

23. Shikama, K., & Sugawara, Y. (1978). Autoxidation of native oxymyoglobin. Kinetic analysis of the pH profile. European Journal of Biochemistry, 91(2), 407–413. https://doi.org/10.1111/J.1432-1033.1978.TB12693.X

24. Suman, S. P., Joseph, P., Li, S., Steinke, L., & Fontaine, M. (2009). Primary structure of goat myoglobin. Meat Science, 82(4), 456–460. https://doi.org/10.1016/j.meatsci.2009.02.017

25. Suzuki, T., Sugawara, Y., Satoh, Y., & Shikama, K. (1980). Human oxymyoglobin: isolation and characterization. Journal of Chromatography A, 195(2), 277–280. https://doi.org/10.1016/S0021-9673(00)96820-1

26. Tan, B., Sun, B., Sun, N., Li, C., Zhang, J., & Yang, W. (2021). Structure, functional properties and iron bioavailability of Pneumatophorus japonicus myoglobin and its glycosylation products. International Journal of Biological Macromolecules, 173, 524–531. https://doi.org/10.1016/j.ijbiomac.2021.01.138

27. Yamazaki, I., Yokota, K. N., & Shikama, K. (1964). Preparation of Crystalline Oxymyoglobin from Horse Heart. Journal of Biological Chemistry, 239(12), 4151–4153. https://doi.org/10.1016/S0021-9258(18)91147-6