Bovine Myoglobin: Its Purification and Detection by the Antiserum

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ABSTRACT. Bovine myoglobin (Mb) was purified and specific antiserum was prepared. Myoglobin was purified from bovine skeletal muscle by fractionation with ammonium sulfate and two cycles of crystallization. Reasonably pure crystals of bovine metmyoglobin were obtained. Anti-bovine Mb serum, prepared with the purified Mb, was useful in detecting Mb in blood and urine of a calf with nutritional myopathy.—**KEY WORDS:** anti-myoglobin serum, cattle, myoglobin.

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Myoglobin (Mb) is a hemo-protein responsible for storing and transfer of oxygen to muscle tissues. When muscle fibers collapse, Mb is released into blood and eventually excreted in urine [5]. Studies on the detection of Mb in serum and urine have been reported in human [3, 4, 6, 9, 11, 15] and equine [12, 13] myopathy, but no report has been available on bovine Mb.

In this paper, bovine Mb was purified by crystallization from the skeletal muscle. Anti-Mb serum was prepared against the purified Mb and used for determining bovine myopathy.

Bovine Mb was crystallized by the method of Nakanishi *et al.* [7] with some modification. Fresh bovine skeletal muscle was minced twice and suspended in an equal volume of distilled water. After the suspension was homogenized, the homogenate was centrifuged at 1,500 g for 15 min. Saturated lead subacetate solution was added to the supernatant until no more precipitate was formed, and then centrifuged at 1,500 g for 15 min. Immediately, saturated sodium pyrophosphate solution was added to the supernatant until no more precipitate was formed, and centrifuged at 1,500 g for 15 min. After the supernatant was adjusted to pH 7.0 with

0.1 N NaOH, ammonium sulfate was added up to 55% (W/V) saturation. After removal of the precipitate by filtration, the concentration of ammonium sulfate was gradually increased up to 95% (W/V) saturation. The precipitate was collected, dissolved in distilled water, and then dialyzed against distilled water for 48 hrs at 5°C. Thereafter, an equimolar mixture of NaH₂PO₄ and K₂HPO₄ was gradually added to the solution to give the concentration of 3 M. After filtration the phosphate mixture was added up to 4.5 M concentration. The precipitate was collected and dissolved in distilled water. After another filtration, the phosphate mixture was gradually added to the solution to a final concentration of 3.8 M, and the filtrate was stored at 5°C. After one or two weeks, three dimensional crystals based on the hexahedron appeared in the upper layer and its photomicrograph is shown in Fig. 1. The crystallization was repeated twice, and the sample was used for the examination after freeze-drying.

Absorption spectrum in the visible region of purified Mb was examined by the method of Bowen [1]. Mb was dissolved in phosphate buffer (pH 6.6, μ 0.1) and absorption from 400 nm to 700 nm was measured by

Fig. 1. Crystals of purified bovine myoglobin. $\times 560$.

Fig. 2. The absorption spectrum of purified bovine myoglobin in the visible region.

Hitachi 557 spectrophotometer in a silica cell of 1 cm light path. The molar extinction coefficients were calculated on the basis of 11.3×10^3 at 540 nm [2] as the molar extinction coefficient for metcyanmyoglobin which was prepared by mixing an equal volume of 0.4% KCN with the mixture of 2 mM potassium ferricyanide and Mb solution (1:3). The absorption curve is shown in Fig. 2. The peaks were found at 500 nm and 630 nm, and the corresponding millimolar extinction coefficients were 9.7 and 3.7, respectively. The results agreed with those of horse metmyoglobin by Bowen [1] and porcine metmyoglo-

Fig. 3. SDS-PAGE of purified myoglobin (Mb) and bovine hemoglobin (Hb). Hemoglobin was denatured to monomer by the SDS treatment.

bin by Nakanishi *et al.* [7].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified Mb and bovine hemoglobin (Hb) purchased from Sigma Chemical Company were performed in 10% acrylamide gel according to the method of Weber *et al.* [14]. A single band of purified Mb was observed (Fig. 3) and no contaminating protein was found.

The purified Mb (10 mg) was dissolved in physiological saline, emulsified in an equal volume of Freund's complete adjuvant and administered to rabbit foot pads. After 3 weeks, a booster dose of 5 mg was injected to the animal and given repeatedly at weekly intervals. Only one precipitate arc was observed with immunoelectrophoresis [10] between a serum sample obtained after 5 weeks and as shown Fig. 4.

The specificity of anti-bovine Mb serum

Fig. 4. Immunoelectrophoretic patterns of purified myoglobin (Mb) and crude muscle extract (Crude Mus). The troughs contained anti-myoglobin serum (anti-Mb) and anti-bovine muscle serum (anti-Mus).

Fig. 5. Double immunodiffusion of anti-myoglobin serum. (Ab) Anti-myoglobin serum; (Mb) Solution of myoglobin; (Hb) Solution of hemoglobin; (MH) Extract from homogenate of bovine skeletal muscle; (S), (U) Normal serum and urine; and (MS), (MU) Serum and urine of a calf with nutritional myopathy.

was confirmed by Ouchterlony method [8]. Anti-Mb serum reacted with the purified Mb and extract from the bovine skeletal muscle homogenate (Fig. 5a), and its precipitin line was fused completely. But bovine Hb, serum and urine of a healthy cow did not react with anti-Mb serum (Fig. 5a, 5b). Using anti-bovine Mb serum, serum and urine from a calf with nutritional myopathy, which was diagnosed by clinical symptoms and marked elevation of serum CPK activity, were examined by Ouchterlony method. A precipitin line was observed between anti-Mb serum and urine or serum of the calf as shown in Fig. 5c. Furthermore, the precipitin line fused completely with the line between anti-

Mb serum and the purified Mb. Thus, a calf affected with nutritional myopathy contained Mb in blood and urine.

The results of this study indicate that the anti-Mb serum against purified bovine Mb is useful for determining bovine myopathy.

REFERENCES

1. Bowen, W. J. 1949. *J. Biol. Chem.* 179: 235-245.
2. Drabkin, D. L. 1947. *J. Biol. Chem.* 171: 409-417.
3. Hällgren, R., Karlson, F. A., roxin, F. A., and Venge, P. 1978. *J. Lab. Clin. Med.* 91: 246-254.
4. Hiasa, M. 1975. *Shikoku. Acta. Med.* 31: 193-204.
5. Miyoshi, K., Kawai, H., Hiasa, M., and Iwasa,

- M. 1977. pp. 581-594. *In: The Function and Structure of Muscle.* (Sakai, T., Endo, M., and Sugita, H. ed.), Igaku Shoin Ltd., Tokyo, Japan (in Japanese).
6. Miyoshi, K., Saito, S., Kawai, H., Kondo, A., Iwasa, M., Hayashi, T., and Yagita, M. 1978. *J. Lab. Clin. Med.* 92: 341-352.
7. Nakanishi, T., and Izumimoto, M. 1972. *Agr. Biol. Chem.* 36: 1505-1510.
8. Ouchterlony, O. 1953. *Acta. Path. Microbiol. Scand.* 32: 231-240.
9. Reynafarje, B. 1963. *J. Lab. Clin. Med.* 61: 138-145.
10. Scheidegger, J. J. 1955. *Int. Arch. Allergy.* 7: 103-110.
11. Stone, M. J., Willerson, J. T., Sanchez, C.E.G., and Waterman, M. R. 1975. *J. Clin. Invest.* 56: 1334-1339.
12. Watanabe, H., Yamaoka, S., Ikeda, S., and Kameya, T. 1975. *Exp. Rep. Equine Hlth. Lab.* No. 12: 64-73.
13. Watanabe, H., Ikeda, S., Yamaoka, S., Kameya, T., Nagasawa, Y., Takizawa, I., and Hasegawa, M. 1978. *Exp. Rep. Equine Hlth. Lab.* No. 15: 79-90.
14. Weber, K., and Osborn, M. 1969. *J. Biol. Chem.* 244: 4406-4412.
15. Yakulis, V. J., and Heller, P. 1962. *Am. J. Clin. Path.* 37: 253-256.

要 約

ウシミオグロビンの精製と免疫学的方法によるミオグロビンの検出について(短報): 納 敏・一条 茂 (帯広畜産大学家畜内科学教室)——分離・精製したウシミオグロビンを抗原として抗ウシ Mb 血清を作成し、免疫学的方法による Mb の検出の可否について検討した。牛の骨格筋から硫酸アンモニウム分画法によって分離し、リン酸緩衝液中で結晶化操作を2回行った結果、純度の高いメトミグロビンの結晶が得られた。作成した抗ウシ Mb 家兎血清は Mb と特異的に反応し、かつ仔牛の栄養性ミオパチーにおける血中および尿中のを検出することができた。