Quantification of NADH–Cytochrome b₅ Reductase (Metmyoglobin–Reducing Enzyme) in Bovine Skeletal Muscle by an Immunoblotting Assay

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Abstract Quantitative determination method of NADH-cytochrome b₅ reductase (metmyoglobin-reducing enzyme) in bovine skeletal muscle was developed by combining SDS-PAGE and immunoblotting techniques. Recoveries of the enzyme by this assay ranged from 90.0 to 94.4%. The content of NADH-cytochrome b₅ reductase in bovine skeletal muscle was estimated to be 13.8±2.6 (9.0-18.2) µg/g tissue (n=19). Also, a quantitative assay for determining cytochrome b₅ (an electron transfer mediator for metmyoglobin reduction) was similarly established and cytochrome b₅ content in bovine skeletal muscle was estimated to be 59.0±20.9 µg/g tissue. The quantitative data of NADH-cytochrome b₅ reductase in this study supported our previous hypothesis that the enzyme plays a critical role in preventing metmyoglobin accumulation in muscles.


Key words: NADH-cytochrome b₅ reductase, Metmyoglobin-reducing enzyme, Bovine skeletal muscle, Immunoblotting, Quantitative assay

Myoglobin is responsible for oxygen storage and transport in living muscles[4]. However, it is easily oxidized to metmyoglobin, resulting in the impairment of physiological functions of myoglobin. Also, myoglobin plays an important role in post mortem change of meat color[2,8,10], an important sensory property affecting consumer acceptability, because meat color is dependent on the amount and the chemical state of myoglobin. Desirable cherry red color of fresh meat turns brown due to the accumulation of metmyoglobin during storage. Because of these reasons, preventing metmyoglobin formation is critical in keeping normal physiological functions of muscle and in keeping fresh meat color.

In a series of our studies[2-7] concerning metmyoglobin-reducing enzyme systems in muscles, we have demonstrated the significance of NADH-cytochrome b₅ reductase (NADH : cytochrome b₅ oxidoreductase, EC 1.6.2.2) for preventing metmyoglobin accumulation. Until recently, this enzyme in muscle was also called metmyoglobin reductase[2,3,11]. We have proposed a new pathway that NADH-cytochrome b₅ reductase reduces metmyoglobin to myoglobin via either OM cytochrome b or cytochrome b₅ as an electron transfer mediator[2,5,6]. This pathway would contribute to keep the chemical state of the heme of myoglobin in ferrous state in maintaining normal physiological functions of myoglobin in muscles. Therefore, this pathway would play a very important role.

Thus, it is desirable to establish a reliable method to determine NADH-cytochrome b₅...
reductase content in order to make a thorough investigation in proving the pathway. This enzyme is present as a tightly membrane-bound form in most tissues (e.g. liver, brain, placenta, lung, muscle)\(^{15,16}\), and its activity is relatively unstable and inactivated during extraction procedures\(^{9}\). Since it seems likely that the concentration of endogenous intermediate acceptor (cytochrome \(b_5\) or OM cytochrome \(b\)) is not saturated in those tissues, differences in its concentration could be a cause of the differences in the activity of the enzyme at equal enzyme concentrations.

Also, physiological intermediate acceptors for the assay of enzyme activity are not available commercially. Because of these reasons, it has been difficult to examine the exact activity and quantity of the enzyme, in muscles. In this study, we determined the content of NADH-cytochrome \(b_5\) reductase and cytochrome \(b_5\) in bovine skeletal muscle by immunoblotting techniques.

**Materials and Methods**

**Bovine skeletal muscle**:

Bovine thigh muscle (biceps femoris) used for establishing a quantitative method was obtained immediately after slaughter with the assistance of the laboratory of veterinary pathology staff, Kitasato University. The muscle was stored at \(-20^\circ C\) until use. For the determination of NADH-cytochrome \(b_5\) reductase content in beef, fresh thigh muscle samples were purchased from a local supermarket.

**NADH-cytochrome \(b_5\) reductase and cytochrome \(b_5\)**:

NADH-cytochrome \(b_5\) reductase and cytochrome \(b_5\) for immunization described below and standard solutions for the assay were prepared from bovine erythrocytes according to the procedures described in previous papers\(^{3,4,6}\).

**Antibodies**:

Antisera against bovine NADH-cytochrome \(b_5\) reductase and cytochrome \(b_5\) previously raised in rabbits\(^{6}\) were used in this study. Antibodies in the serum were purified by affinity chromatography on CNBr-Sepharose 4B coupled to the purified bovine NADH-cytochrome \(b_5\) reductase or cytochrome \(b_5\) according to the manufacturer's protocols (Pharmacia Fine Chemicals, Uppsala, Sweden). The specificity of antibodies was confirmed by an immunoblotting technique.

**SDS-PAGE and Immunoblotting**:

Determination of NADH-cytochrome \(b_5\) reductase and cytochrome \(b_5\) was carried out as follows. SDS-PAGE was performed according to the method of Laemmli\(^{10}\), using 12.5% acrylamide separating gel. Muscle samples (0.1 g) were minced with scissors and dispersed in Laemmli's sample buffer (1.9 ml) with Potter's homogenizer for 1 min before heating (100°C, 3 min). The homogenate was centrifuged at 5,000 \(\times g\) for 5 min and its supernatant (10 \(\mu l\)) was used for SDS-PAGE. Following the fractionation by SDS-PAGE, proteins were transferred from the gel to PVDF membranes (Clearblot P-membrane, Atto Co., Tokyo), as outlined by Kyhse-Andersen\(^{9}\), using a homemade horizontal electrophoresis apparatus. Blotting was carried out with 25 mM Tris-192 mM glycine-20% (v/v) methanol buffer (pH 8.3) at 100 mA for 60 min. After electrophoretling, blots on the membrane were immunostained using the primary antibody (anti-NADH-cytochrome \(b_5\) reductase or anti-cytochrome \(b_5\), approximately 1 \(\mu g\) IgG/ml) described above and an Bio-Rad Immun-Blot Assay Kit (Bio-Rad Laboratories, Richmond, CA, USA; secondary antibody, horseradish peroxidase-labelled goat anti-rabbit IgG; development reagent, 4-chloro-1-naphthol). All immunostaining procedures were performed according to the manufacturer's protocols. Pre-stained SDS-PAGE standards and SDS-PAGE low-molecular-weight standards (Bio-Rad) were used to monitor the efficiency of electrophoretic transfer of the enzyme and to determine the molecu-
Muscle Metmyoglobin-Reducing Enzyme

lar weight of the enzyme, respectively. Absorbance and area of immunostained bands on the membrane was measured by reflection mode at 570 nm with a Shimadzu (Kyoto, Japan) chromato-scanner CS-930.

Results and Discussion

After SDS-PAGE and immunoblotting, NADH-cytochrome $b_5$ reductase in bovine skeletal muscle was well recognized as a single band on the immunostained membrane in accordance with the results obtained in the previous paper$^6)$. Thus, the immunological detection method of the present study was proved to be highly specific for the enzyme. The molecular weight value (35,000) of NADH-cytochrome $b_5$ reductase, based on the position of the immunostained band, was identical to that of a previous paper$^6)$. The optimum heating period and SDS concentration for the preparation of SDS-PAGE samples were 3 min and 2%, respectively.

Quantification of the amount of the enzyme present in muscles was made by preparing a standard calibration curve by plotting the absorbance of the purified NADH-cytochrome $b_5$ reductase preparation versus protein concentration. From the standard curve shown in Fig. 1, the assay for this enzyme was quantitative over the entire range examined (4-128 ng).

As shown in Table 1, the recoveries of the component added to bovine skeletal muscle homogenate demonstrated that the quantification procedure of the present study is highly reliable and gives satisfactory values. The determination method for cytochrome $b_5$ was also established similarly with that for NADH-cytochrome $b_5$ reductase. These results strongly indicate that the present quantitative method based on the immunoblotting procedure is applicable for the determination of NADH-cytochrome $b_5$ reductase.

Table 1. Recoveries of NADH-cytochrome $b_5$ reductase added to bovine skeletal muscle homogenates

<table>
<thead>
<tr>
<th>Enzyme added$^1$ (ng)</th>
<th>Found$^2\pm$SD (ng)</th>
<th>Recovery (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>5.2±0.7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>12.4±0.9</td>
<td>90.0</td>
</tr>
<tr>
<td>16</td>
<td>20.0±1.2</td>
<td>94.4</td>
</tr>
<tr>
<td>32</td>
<td>34.6±2.5</td>
<td>91.9</td>
</tr>
</tbody>
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$^1$ Purified enzyme preparation was added to 10 μl of muscle homogenate sample for SDS-PAGE.

$^2$ Average of three replicates.

Fig. 1. Calibration curve for standard NADH-cytochrome $b_5$ reductase solution. A, calibration curve drawn from the peak area of densitogram (B); B, densitogram of the membrane immunostained by anti-NADH-cytochrome $b_5$ reductase antibodies.
Table 2. Contents of NADH-cytochrome b₅ reductase and cytochrome b₅ in bovine skeletal muscle determined by the immunoblotting procedure

<table>
<thead>
<tr>
<th>Contents (µg/g muscle)</th>
<th>Mean±SD</th>
<th>Range</th>
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<tbody>
<tr>
<td>NADH-cytochrome b₅ reductase (n = 19)</td>
<td>13.8± 2.6</td>
<td>9.0~18.2</td>
</tr>
<tr>
<td>Cytochrome b₅ (n = 12)</td>
<td>59.0±20.9</td>
<td>22.8~96.0</td>
</tr>
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1 Average of three replicates of each sample was used.

Averages of the contents of NADH-cytochrome b₅ reductase and cytochrome b₅ content in muscle.

Averages of the contents of NADH-cytochrome b₅ reductase and cytochrome b₅ (electron-transfer mediator for metmyoglobin reduction) in bovine skeletal muscle (thighs) were estimated to be 13.5 and 59.0 µg/g muscle, respectively, by the present immunoblotting procedure (Table 2).

In erythrocytes, it has been recognized that sufficient quantity of NADH-cytochrome b₅ reductase (water-soluble form) is present in the cells to prevent the accumulation of methemoglobin (an oxidized form of hemoglobin) in bovine skeletal muscle (thighs) as well as in human erythrocytes. Hemoglobin and myoglobin have common properties in the structure, derivative formation and physiological functions. The weight ratio of NADH-cytochrome b₅ reductase : hemoglobin was calculated to be approximately 1 : 75,000 from those contents in human erythrocytes. On the other hand, the ratio in bovine skeletal muscle, at least in thigh, NADH-cytochrome b₅ reductase : myoglobin is approximately 1 : 145~360 from the calculation using the content of the enzyme of present study and the myoglobin content of bovine skeletal muscle (2-5 mg/g tissue). From the comparison of these values, sufficient quantity of NADH-cytochrome b₅ reductase exists to prevent metmyoglobin accumulation in muscle. In conclusion, the data in this study supported our previous hypothesis that NADH-cytochrome b₅ reductase plays a critical role in reducing metmyoglobin to myoglobin in muscles.

Acknowledgements

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References

8) Faustman C, Cassens RG. The biological basis


