Purification of Methionine Sulfoxide Reductase from Cattle Liver

Takafumi NAGAMINE, Takashi NAGASAWA
and Ryoji ONODERA

Animal Science Division, Faculty of Agriculture,
Miyazaki University, Miyazaki-shi 889-21

(Received September 12, 1991)

Abstract Methionine sulfoxide reductase has been purified from extracts of cattle liver. The purification procedure was acid treatment, Butyl-Toyopearl 650 S chromatography, DEAE-Toyopearl 650 M chromatography and hydroxylapatite chromatography. This enzyme was molecular weight of about 20,000 and dependent on thioredoxin system.

Anim. Sci. Technol. (Jpn.) 63 (2) : 134-140, 1992

Key words : methionine sulfoxide reductase, cattle liver, thioredoxin system

In our previous report, cattle and swine were originally shown to have the ability to reduce free methionine sulfoxide (MSO) to methionine using crude enzyme solutions prepared from liver and kidney⁹⁾. This suggests that MSO is nutritionally effective as a methionine source in cattle and swine as well as in rat²⁾ and rabbit⁵⁾. The enzyme has been purified from *E. coli*⁴⁾ and yeast³⁾. The enzyme of animal tissue, has not been purified. The present paper reports a purification of MSO reductase from cattle liver.

Materials and Methods

1. Materials

Livers were obtained from cattle (3 years old, Japanese Black, castrated males) slaughtered in a meat plant in Miyazaki City, Japan. Butyl-Toyopearl 650 S and DEAE-Toyopearl 650 M were purchased from Tosoh Co. Ltd., Japan. Hydroxylapatite was from Bio-Rad Laboratory. Ultrafree-20 was from Millipore, USA. NADPH was from Kohjin Co. Ltd. Dithiothreitol (DTT) and bovine albumin, guanidine hydrochloride, ethylenediamine-

tetraacetic acid disodium salt dihydrate (EDTA), 5, 5'-dithiobis (DTMB) were from Nacalai Tesque, Inc., Japan. Insulin (from bovine pancreas) was from Wako Pure Chemical Industries Ltd., Japan.

2. Assay for the activity of MSO reductase

The assay mixture contained 0.2 ml of enzyme solution and 0.2 ml of substrate solution which contained 0.01 M potassium phosphate buffer (pH 7.0), 5 mM EDTA and 1 mM DTT. The mixture was incubated at 37°C for 3h. After incubation, 0.1 ml of 27% (w/v) sulfo-salicylic acid was added to the incubation mixture. The mixture was, then, centrifuged (27,000 × g, 30 min, at 4°C) and the methionine concentration of supernatant fluid was determined by an amino acid analyzer (High Speed Amino Acid Analyzer, MLC-203, ATTO, Japan).

3. Assay for the activity of thioredoxin reductase and thioredoxin

Thioredoxin reductase activity with DTNB and thioredoxin activity with insulin were assayed using the methods described by HOLMGREN (1977)⁶⁾.

4. Polyacrylamide gel electrophoresis

The purity of the enzyme was examined by polyacrylamide gel electrophoresis under non-denaturing conditions without sodium dodecyl sulfate in 8% gels. Proteins in gels were detected by coomassie brilliant blue and silver staining.

5. Concentration of protein

Protein concentrations were determined by the method of LOWRY *et al*⁽⁸⁾.

Results

1. Purification of MSO reductase and preparation of thioredoxin and thioredoxin reductase

1) Outline of the purification of MSO reductase and the preparation of thioredoxin and thioredoxin reductase

MSO reductase, thioredoxin and thioredoxin reductase were obtained from the same lyophilized powder made from cattle liver. The lyophilized powder was dissolved in 0.01 M potassium phosphate buffer and fractionated by Butyl-Toyopearl 650 S chromatography. Thus fraction A (MSO reductase and thioredoxin) and fraction B (thioredoxin reductase) were obtained. Fraction A was divided into fraction C (thioredoxin) and fraction D (MSO reductase) by DEAE-Toyopearl 650 M chromatography. Fraction C and fraction D were further fractionated by hydroxylapatite chromatography and more purified MSO reductase and thioredoxin were obtained. Fraction B was also fractionated by DEAE-Toyopearl 650 M chromatography and more purified thioredoxin reductase was obtained.

The details of the purification of MSO reductase and the preparation of thioredoxin and thioredoxin reductase are described in the following items.

2) Acid treatment of crude extract and lyophilization

At first, lyophilized powder of crude extract was made out of cattle liver. Cattle liver (50 g) was added to 250 ml of deionized water

containing 1 mM EDTA and 1 mM DTT, and homogenized with Homo Blendor, Sakuma Seisakusho, Ltd., Japan. The homogenate was adjusted at pH 5.0 with HCl and centrifuged ($27,000 \times 30$ min, at 4°C). The supernatant fluid was adjusted at pH 7.0 with NaOH and then lyophilized with a freeze-dryer (Labconco FD-5, USA). Lyophilized powder was stored at -20°C . When the powder was stored at 4°C , the MSO reductase activity gradually decreased.

3) Butyl-Toyopearl 650 S chromatography

The lyophilized powder was dissolved in 0.01 M potassium phosphate buffer (pH 7.0) which included 1 mM EDTA and 1.3 M ammonium sulfate, and centrifuged for 30 min at $27,000 \times g$. The supernatant was applied on a column of Butyl-Toyopearl 650 S (1.5×15 cm) equilibrated with the same potassium phosphate buffer. The enzymes were eluted with a linear gradient from 1.3 to 0 M ammonium sulfate. One peak of MSO reductase activity (fraction A) and another peak of thioredoxin reductase (fraction B) were obtained in the chromatogram (Fig. 1). The peak of MSO reductase was eluted at a salt concentration of around 0.8 M and the peak of thioredoxin reductase was at 0.6 M.

4) DEAE-Toyopearl 650 M chromatography

Fraction A was dialyzed against 0.02 M potassium phosphate buffer, pH 7.4, 1 mM DTT and applied on a column of DEAE-Toyopearl 650 M (1.5×15 cm) equilibrated with the same buffer. The enzymes were eluted with a linear gradient from 0 to 1 M sodium chloride. Two peaks of MSO reductase activity were obtained in the chromatogram (Fig. 2). The first peak (fraction C) was eluted at 0.09 M sodium chloride and the second peak (fraction D) was at 0.13 M. Fraction C was thioredoxin and fraction D was MSO reductase. It was reported that thioredoxin was reduced by DTT⁽⁷⁾ and MSO was reduced by thioredoxin⁽¹⁾. Fraction C showed thioredoxin activity with insulin, but fraction D did not.

Fig. 1. Chromatography of methionine sulfoxide reductase activity and thioredoxin reductase on a column of Butyl-Toyopearl 650 S (1.5×15 cm). Fraction of 4.5 ml were collected. —, methionine sulfoxide reductase activity; ----, thioredoxin reductase activity; ·····, absorbance at 280 nm; — (crossing straight line), ammonium sulfate.

Fig. 2. Chromatography of methionine sulfoxide reductase on a column of DEAE-Toyopearl 650 M (1.5×15 cm). —, methionine sulfoxide reductase activity; ·····, absorbance at 280 nm; — (crossing straight line), sodium chloride.

Fraction B was also applied on a column of DEAE-Toyopearl 650 M (1.5×15 cm) equilibrated with 0.02 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. The enzyme was eluted with a linear gradient from 0 to 1 M sodium chloride. The thioredoxin reductase was eluted at 0.15 M sodium chloride.

5) Hydroxylapatite chromatography

Fraction D was applied on a column of hydroxylapatite (1.0×20 cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM DTT. The MSO reductase was eluted with a linear gradient from 0.02 to 0.4 M phosphate. The MSO reductase was

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Fig. 3. Chromatography of methionine sulfoxide reductase on a column of hydroxylapatite (1.0 × 20 cm). —, methionine sulfoxide reductase activity; ·····, absorbance at 280 nm; — (crossing straight line), potassium phosphate.

Table 1. Purification of methionine sulfoxide reductase from cattle liver

	Total protein (mg)	Specific activity* (nmol/mg protein/h)	Total activity* (nmol/h)	Recovery (%)
Crude extract	3201	0.344	1072	100
Acid treatment	977.0	1.05	997	93
Lyophilization	339.7	2.87	975	91
Butyl-Toyopearl 650S	266.5	2.77	740	69
DEAE-Toyopearl 650M	11.78	12.7	149	14
Hydroxylapatite	0.188	105.7	20	2

* Enzyme activity was expressed with the amount of formed methionine.

eluted at 0.06 M phosphate (Fig. 3).

Fraction C was also applied on the same column and eluted with the same gradient. The thioredoxin was eluted at 0.07 M phosphate.

Table 1 summarizes the purification procedure for MSO reductase. The enzyme was purified about 307-fold with an overall recovery of 2%. Figure 4 shows a polyacrylamide gel electrophoresis pattern of the purified MSO reductase. One major band is observed on the electrophoresis pattern. The molecular weight of the enzyme was about 20,000 with Sephadex G-100 chromatography (data not shown).

2. Characteristics of MSO reductase

Characteristics of MSO reductase was examined under the presence of 1 mM DTT, 1 mM MSO and the absence of the thioredoxin system. DTT was employed in this study, although not its physiological condition, because MSO reductase activity was not detected under the presence of NADPH or NADH. Under these conditions, MSO reductase was stable between pH 4.2 and pH 10.6. Optimum pH of this enzyme was 8.8 (Fig. 5). This enzyme activity was not inhibited by 1 mM EDTA, 200 mM KCl, 200 mM NaCl, 200 mM Na₂SO₄ and 10 mM MgSO₄, but inhibited by 10 mM CaCl₂, FeSO₄, CuSO₄ and CuCl₂.

Fig. 4. Slab polyacrylamide gel electrophoresis of the purified enzymes. The purified enzyme were electrophoresed on 8% polyacrylamide gel. Proteins in gels were detected by coomassie brilliant blue and silver staining.

MSO reductase activity was not obtained under the presence of 1 mM NADPH and 1 mM MSO as mentioned above. However, high MSO reducing activity was observed when thioredoxin and thioredoxin reductase was added (Table 2).

Discussion

There have been some reports concerning the relationship between MSO reductase and thioredoxin system. The MSO-reducing activity of yeast required three protein fractions³⁾ and

two of them were thioredoxin and thioredoxin reductase¹⁰⁾. It was reported that MSO reductase of *E. coli* also depended upon the thioredoxin system⁴⁾. In the present experiment, MSO reductase prepared from cattle liver itself did not show any activity under the condition with only NADPH or NADH present, but it showed high activity when determined with thioredoxin and thioredoxin reductase (Table 2). Therefore, MSO reductase from cattle liver seemed to require the thioredoxin system like those from yeast¹⁰⁾ and *E. coli*⁴⁾.

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Fig. 5. Effect of pH on methionine sulfoxide reductase. The methionine sulfoxide reductase was examined under the conditions of 1 mM DTT, 1 mM MSO and absent thioredoxin system.

Table 2. Effect of combination of methionine sulfoxide reductase, thioredoxin and thioredoxin reductase on the reducing activity of methionine sulfoxide to methionine

Methionine formed (%)*	
Methionine sulfoxide reductase only	0.0
Thioredoxin only	6.0
Thioredoxin reductase only	0.0
Methionine sulfoxide reductase + thioredoxin	5.7
Methionine sulfoxide reductase + thioredoxin reductase	10.2
Thioredoxin + thioredoxin reductase	4.6
Mixture of three proteins	100.0

* The percentage of methionine formed from methionine sulfoxide under each condition to that under the condition of mixture of three proteins (methionine sulfoxide reductase+thioredoxin+thioredoxin reductase). The total volume of incubate mixture was 0.4ml and contained 1 mM MSO and 1 mM NADPH.

The molecular weight of MSO reductase purified from cattle liver was about 20,000 and was almost similar to the enzyme purified from *E. coli*⁴⁾. Optimum pH of the purified MSO reductase was 8.8 in the present experiment which was different from the value, 6.0, of a crude enzyme of cattle liver obtained before⁹⁾. The reasons for the difference of optimum pH are thought that (1) purified MSO reductase activity was assayed by the reducing ability with DTT, or (2) thioredoxin reductase may be a rate-limiting step in the process of MSO reduction.

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牛肝臓のメチオニンスルホキシドレダクターゼの精製

長峰孝文・長澤孝志・小野寺良次

宮崎大学農学部, 宮崎市 889-21

本実験は, 牛の肝臓の MSO レダクターゼを精製することを目的とした. 精製には pH 処理, 疎水クロマトグラフィー, 陰イオンクロマトグラフィー, ヒドロキシアパタイトクロマトグラフィーを用いた. 精製された酵素は, ポリアクリルアミド電気泳動で, バンドが1本であることを確認した. また, 同時にチオレドキシンおよびチオレドキシンレダクターゼも部分精製した.

精製 MSO レダクターゼは, ジチオトレイトールの存在下では MSO をメチオニンに還元するが, NADPH では還元が起らなかった. しかし, チオレドキシンおよびチオレドキシンレダクターゼを加えると NADPH で高い MSO 還元活性が得られた. このことから, 大腸菌や酵母などと同様に, 牛の肝臓の MSO レダクターゼもチオレドキシン系が必要であると考えられた.

日畜会報, 63 (2): 134-140, 1992