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Rapid and Large-scale Isolation of Microsomal Fraction of Mouse Liver by Lyophilization and Low Speed Centrifugation

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Iwata, N., Mukai, T., Hara, S., Endo, T. and Tomoda, A. *Rapid and Large-scale Isolation of Microsomal Fraction of Mouse Liver by Lyophilization and Low Speed Centrifugation.* Tohoku J. Exp. Med., 1996, **180** (1), 65-71 —— We could prepare the microsomal fraction of mouse liver, without using an ultracentrifuge but with a low speed centrifuge. The procedure includes 1) lyophilization of post-mitochondrial fraction (9,000×g supernatant) of mouse liver, 2) powdering of the lyophilized sample, 3) the addition of 1.15 per cent potassium chloride solution or distilled water, which afforded microsomal aggregates, 4) sedimentation of microsomal fraction by low-speed centrifugation (20,000×g, 20 min). The sedimented microsomal fraction showed normal contents of cytochrome P-450 and cytochrome b_5, and gave a normal pattern on SDS polyacrylamide gel electrophoresis and normal electron microscopic feature. This method should be convenient for rapid and large-scale preparation of microsomes, especially for the preparation of cytochrome b_5 and cytochrome P-450. ——— rapid isolation; microsomal fraction; mouse liver; lyophilization; low speed centrifugation

Liver microsomes, which contain various enzyme activities, cytochrome b_5, NADPH cytochrome c reductase, and cytochrome P-450, are conventionally prepared by ultracentrifugation of the post-mitochondrial fraction (9,000×g supernatant) of the liver of various animals (Lowry et al. 1951; Omura and Sato 1964; Omura and Takesue 1970). However, it can take more than 90 min to obtain the microsomal fraction by ultracentrifugation (105,000×g), and only small volumes can be handled.

Concerning rapid preparation of microsomes, acid precipitation of microsomes (Karler and Turkanis 1968) and Ca^{++} precipitation of microsomes (Kamath and Rubin 1972) are reported. The enzyme activity of microsomes prepared by these methods have been shown to be comparable to that prepared by ultracentrifugation (Karler and Turkanis 1968; Kamath and Rubin 1972). However, these methods seem not to be popularly used for the rapid preparation of microsomes, probably due to need for the addition of acid or Ca^{++}.

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We recently found that the lyophilized post-mitochondrial fraction is instantly aggregated in water or in 1.15% KCl solution, and we utilized this property to prepare large amounts of microsomal fraction using a vacuum freeze-dry equipment and a low-speed centrifuge. The method is presented here.

Materials and Methods

Liver microsomes were prepared from male ICR strain mice (>20 g, Japan SLC, Shizuoka). Mice were killed by decapitation, and the livers were quickly removed, perfused with 1.15% KCl solution, and homogenized with four volumes (w/v) of ice-cold 1.15% KCl solution in a Potter-Elvehjem homogenizer (Fig. 1). The 20% homogenate (w/v) was centrifuged at 9,000×g for 20 min and the supernatant (S9) was divided into two parts. One was ultracentrifuged at 105,000×g for 1 hr, 4°C, and the microsomal fraction (105,000×g pellet) was resuspended in the original homogenization solution (1.15% KCl solution). The other part was quickly frozen in dry ice-acetone and lyophilized overnight (a vacuum freeze-dry equipment, Yamato Co., Ltd., Tokyo). Next day, the lyophilized microsomes were ground, and 1.9 g of the powder was added to 45 ml KCl solution (1.15%) or to 45 ml distilled water. The resultant microsomal aggregates were collected by centrifugation at 20,000×g for 20 min. Then, the pellets were suspended in 45 ml KCl solution (1.15%) or in 45 ml distilled water, and were centrifuged at 20,000×g for 10 min. The pellets were suspended in the same solution as stated above. This suspension was used as the "washed lyophilized microsomal fraction".

Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as as standard.

Cytochrome P-450 and cytochrome b_5 contents were determined after dilution of the microsomes in 20% glycerol, 1.0 mM EDTA, 0.2% Emulgen 913 (Kao Chemicals, Tokyo) and 100 mM potassium phosphate (pH 7.25) and reduction with dithionite according to Omura and Sato (1964) and Omura and Takesue (1970). The activity of lactate dehydrogenase was measured for the cytosol fraction, which was obtained either by centrifugation at 20,000× for 20 min, of the suspension of lyophilized powder with 1.15% KCl solution or by centrifugation at 20,000×g for 20 min of the resuspension of 20,000×g pellet with 1.15% KCl solution. The activity of lactate dehydrogenase was also determined for the washed lyophilized microsomal fractions (Washed 20,000×g pellet in Fig. 1). The activity of lactate dehydrogenase in these fractions was determined according to the method of Lee et al. (1982).

Sodium dodecyl sulfate (SDS)-polyacraylamide gel electrophoresis was performed as described by Laemmli (1970) using 7.5% or 10% polyacrylamide gel. Protein bands were visualized by Coomassie brilliant blue staining.

For the morphological studies, each fresh microsomal preparation was fixed in 1% OsO_4 in phosphate buffer (pH 7.4), 2 hr. Then, thin sections of epoxe embed-

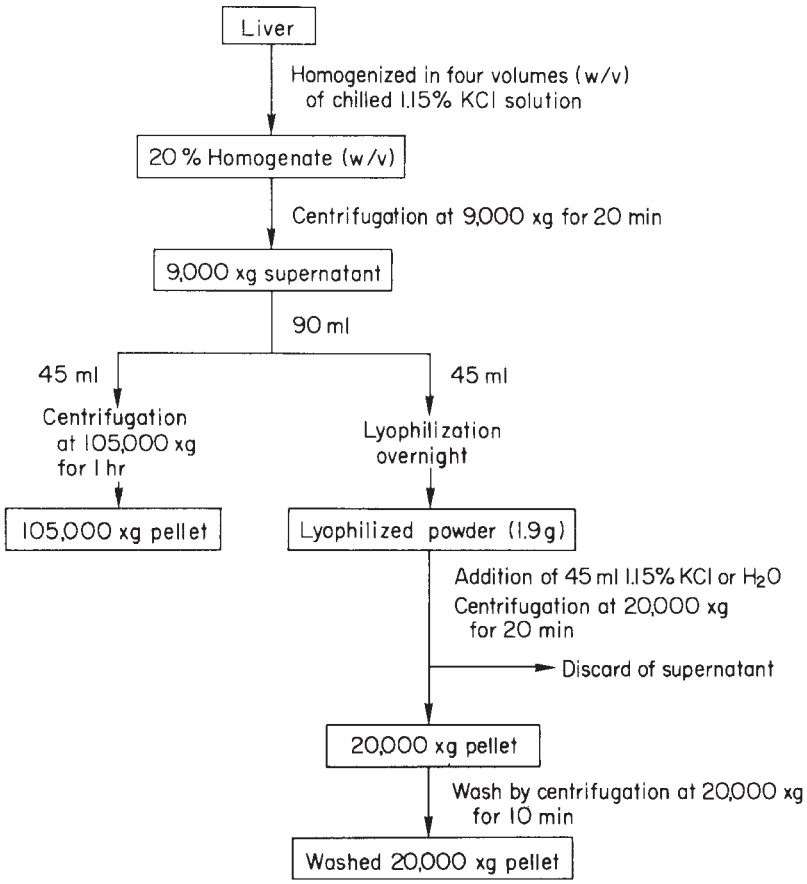

Fig. 1. Protocol for preparation of microsomal fractions.

ded sample were examined by a JEM-1200 transparent electron microscopy (JEOL Co., Ltd., Tokyo).

Results and Discussion

The 9,000×g supernatant of liver homogenate is often referred to as the post-mitochondrial fraction, and is used as the source material for the preparation of microsomes by ultracentrifugation (Lowry et al. 1951; Omura and Sato, 1964; Omura and Takesue, 1970). However, we found that when the post-mitochondrial fraction was lyophilized, powdered by grinding, and suspended in 1.15% KCl solution or in distilled water, microsomes were instantly aggregated, and could be easily collected as the 20,000×g pellet, (lyophilized microsomal fraction) (Fig. 1). The recovery rate of the lyophilized microsomal fraction in 1.15% KCl solution or in distilled water was more than 75% of the microsomal fraction which is obtainable by ultra-centrifugation, as determined by total proteins. The aggregation of the microsomal fraction may be explained in terms of the hydrophobicity of phospholipids, contained abundantly in the microsomes. When the lyophilized microsomal fraction (20,000×g pellet) was washed with 1.15% KCl solution (centrifugation at 20,000×g, 20 min), the microsomal fraction was almost completely sedimented.

The morphology of the lyophilized microsomal fraction showed normal smooth endoplasmic reticulum and rough endoplasmic reticulum, though these components were packed compactly due to aggregation when lyophilized powder was dissolved in 1.15% KCl solution (Fig. 2).

Fig. 3 compares the contents of cytochrome b_5 and cytochrome P-450 of mouse liver microsomes prepared by the ultracentrifugal method (105,000×g, 60 min), by the ultracentrifugal method followed by storage at −40°C for 24 hr, and by the present method (lyophilization and low-speed centrifugation). Since cytochromes P-450 and b_5 are unstable and are denatured by several repeated freezing and thawing or by long term storage at −40°C, we also studied the contents of cytochrome b_5 and cytochrome P-450 of mouse liver microsomes, which were prepared by ultracentrifugal method followed by storage at −40°C for 24 hr (Fig. 3). There was little difference in the contents of cytochrome P-450 or cytochrome b_5 among these microsomes prepared by different methods.

Fig. 4 shows the SDS gel electrophoresis patterns of microsomes prepared by the different methods (25 μg and 10 μg of proteins) on 7.5 per cent acrylamide gel. The electrophoretic patterns of both microsomal samples (No. 1: samples prepared by ultracentrifugation; No. 2: samples prepared by the present lyophilization

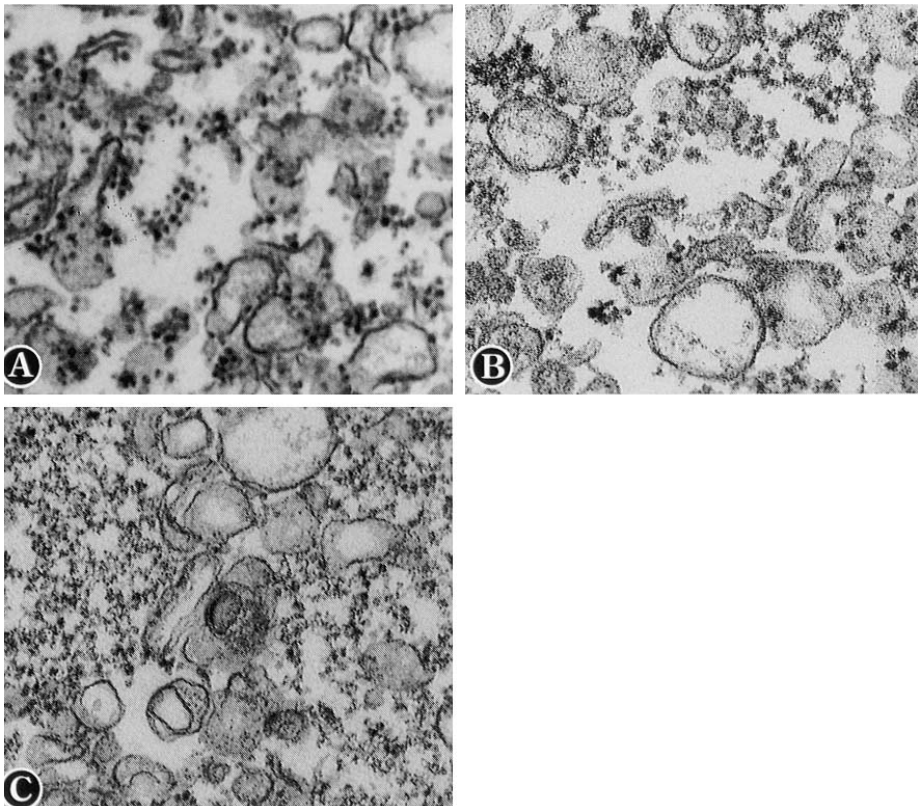

Fig. 2. Electron microscopy of freshly obtained microsomal fractions (×40,000). Each sample was examined by an transparent electron microscope. (A) Control: 105,000×g pellet; (B) 20,000×g pellet (suspended in H_2O); (C) 20,000×g pellet (suspended in 1.15% KCl solution).

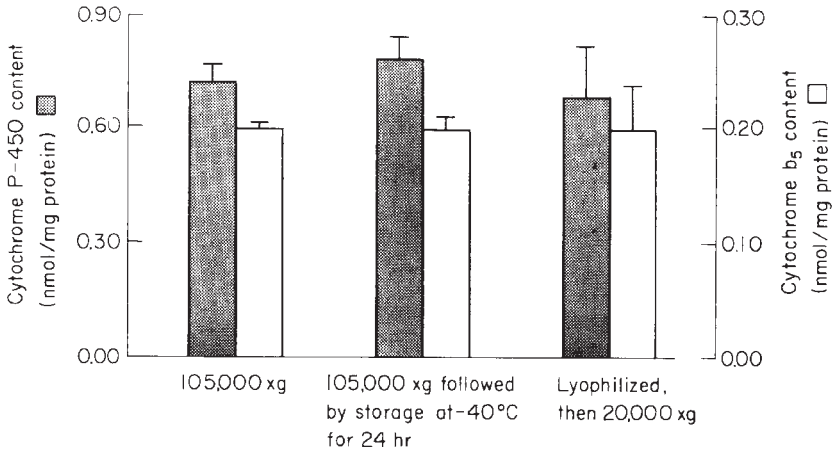

Fig. 3. Contents of cytochrome b_5 and cytochrome P450 in microsomal fractions prepared by the different methods. The washed lyophilized 20,000×g pellet in Fig. 1 was subjected to the measurement of cytochrome b_5 and cytochrome P450. Each column shows means±S.D. of 3 determinations.

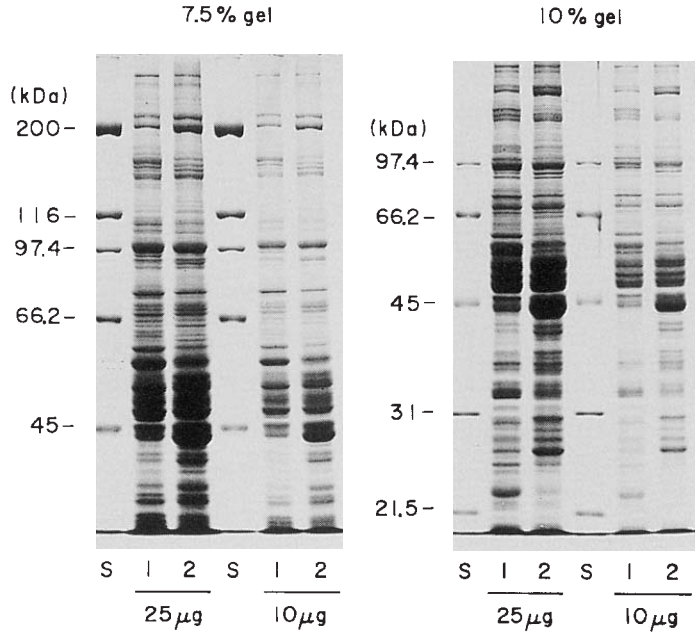

Fig. 4. SDS gel electrophoresis patterns of microsomes prepared by different methods (25 μg and 10 μg proteins). S: standards; 1 : 105,000×g procedure; 2: lyophilized and dissolved in 1.15% KCl solution, followed by 20,000× procedure.

method) were essentially identical. Similar patterns were also obtained on 10 per cent acrylamide gel, though the density of protein bands was higher for the lyophilized microsomes than for the ultracentrifuged microsomes, except for the proteins near 22 kDa.

In order to eliminate the possibility that the cytosol enzymes might be contaminated into the washed lyophilized microsomal fractions, we checked the activity of lactate dehydrogenase in the cytosol and the washed lyophilized

TABLE 1. *Activity of lactate dehydrogenase in cytosol and washed lyophilized microsomal fractions*

	Lactate dehydrogenase (mmol/mg • protein/min)
Cytosol 1[a]	3.315±0.59
Cytosol 2[b]	1.618±0.27
Washed lyophilized microsomal fraction	0.077±0.012

[a]obtained by centrifugation at 20,000×g, 20 min of the suspension of lyophilized powder with 1.15% KCl solution.

[b]obtained by centrifugation at 20,000×g, 20 min of the resuspension of the 20,000×g pellet with 1.15% KCl solution.

The livers were obtained from 12 mice, and were subjected to the homogenization and treatment by centrifugation as described in Fig. 1. The activity of lactate dehydrogenase was expressed by the means±S.E.

microsomal fractions (Table 1). Consequently, the activity of lactate dehydrogenase in the washed lyophilized microsomal fractions was extremely low (0.077±0.012) compared with that in cytosol 1 (3.315±0.59) and cytosol 2 (1.618±0.27), showing that there was little contamination of cytosolic enzymes in the washed lyophilized microsomal fractions.

The present method should be extended to large-scale preparation of microsomes for the purification of microsomal enzymes such as cytochrome b_5 and cytochrome P-450. About 3,000 ml of post-mitochondrial fraction (9,000×g supernatant) can be treated at the same time by a vacuum freeze-dry equipment, according to our present method. It is important for efficient aggregation of microsomes that water should be removed (by lyophilization) from the post-mitochondrial fraction (9,000×g supernatant) as completely as possible. Kamath and Rubin (1972) reported that rat liver microsomes are precipitated rapidly by the addition of 8 mM Ca^{++} to a dilute post-mitochondrial supernatant, and described a method for rapid and preparative isolation of microsomes. Our method may be alternative for their method, because of no need for the addition of Ca^{++}.

Acknowledgments

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