SHORT COMMUNICATIONS

BBA 73088

Gel filtration of marker enzymes of plasma membrane and endoplasmic reticulum from rat liver

In studies of subcellular particulates, activities of marker enzymes have been used as indicators of purity of the isolated fractions. The indicators are particularly useful in the isolation of membranous structures, since morphological studies do not provide enough evidence for assessing the purity. Thus, 5'-nucleotidase[1] (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) and glucose-6-phosphatase[2] (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) are commonly used to characterize plasma membrane and endoplasmic reticulum of liver cells, respectively. Generally, however, each membranous structure contains several marker enzymes (for plasma membrane, see ref. 3). One wonders if these marker enzymes are indeed bound to membranes as possible structural proteins or are merely embedded into membrane organizations.

By solubilizing hepatic microsomes containing both plasma membrane and endoplasmic reticulum with sodium deoxycholate and by fractionating by gel filtration on Sepharose columns, it was found that the marker enzymes could be separated readily, suggesting that locations of marker enzymes within the membrane are different.

5'-Nucleotidase (Enzyme I) and nucleoside triphosphate pyrophosphatase[4] (Enzyme II) were used as marker enzymes of plasma membrane, and the behavior of glucose-6-phosphatase (Enzyme III), a marker enzyme for endoplasmic reticulum, was also investigated.

Enzyme I was assayed by incubating 0.5 ml of 0.1 M sodium barbital–HCl buffer (pH 8.5), 0.2 ml of 0.025 M inosinic acid (IMP), 0.2 ml of 0.05 M $MgCl_2$ and 0.5 ml of enzyme solution or suspension at 37° for 1 h. Enzyme II was assayed according to LIEBERMAN *et al.*[4], except that the reaction mixture contained 0.02 M $CaCl_2$ and its pH was 10.5 instead of 12. Under these conditions, pyrophosphatase activity of endoplasmic reticulum could be minimized. The external pyrophosphatase preparation used was prepared according to KUNITZ[5]. For Enzyme III, the assay system contained 0.6 ml of 0.1 M Tris–HCl buffer (pH 6.5), 0.1 ml of 0.1 M glucose-6-phosphate and 0.2–1.0 ml of enzyme solution or suspension, incubated at 30° for 30 min.

In these assays, liberated P_i was estimated according to NAKAMURA[6].

Protein was determined by the procedure of LOWRY *et al.*[7]. When L-tyrosine and EDTA were used for stabilizing the activity of Enzyme III (see below), protein was determined after precipitating it with 5 % trichloroacetic acid, washing it with ethanol and dissolving it in 0.1 M NaOH.

Isolation of microsomes from rat liver and subsequent fractionation thereof using sodium deoxycholate into clear supernatant (C-fraction), and membrane fraction (M-fraction), were carried out according to ERNSTER *et al.*[8]. For Enzyme III, however,

microsomes were separately treated with sodium deoxycholate in the presence of 0.001 M L-tyrosine and 0.001 M EDTA, which were effective in stabilizing the enzyme as described by HERS *et al.*[9].

About 70 % of Enzyme I and 50–60 % of Enzyme III were recovered in the M-fraction, with increases in specific activities of 6-fold and 4-fold for Enzyme I and Enzyme III, respectively. M-fraction was then submitted to gel filtration. It should be noted that this fraction did not show the activities of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) and acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) under the conditions used for the assays of Enzymes I, II and III.

M-fraction was dispersed in 0.25 M sucrose containing 0.5 % sodium deoxycholate and 0.01 M sodium barbital–HCl buffer (pH 7.8, about 5 mg protein per ml), and a 5-ml portion thereof was placed on a column (1.5 cm × 99 cm) of Sepharose 4B (Pharmacia, Uppsala, Sweden) equilibrated with the same solvent. Elution with the same solvent resulted in a pattern, shown in Fig. 1, in which most of the proteins (more than 95 %) were found in the retarded fractions (Peak II) and 1–3 % of the proteins passed through the column (Peak I). All the Enzyme II activities and a smaller part of Enzyme I were found in Peak II, while Peak I still retained Enzyme III together with the greater part of Enzyme I. It should be noted that the pattern of protein distribution on gel filtration in the presence of tyrosine and EDTA shown in Fig. 2 was essentially the same as in Fig. 1.

Recoveries of individual enzyme activities were 75–85 % for Enzyme I, 85 % for Enzyme II and 40–60 % for Enzyme III. Purification of Enzyme I by this fractionation was noticeable, and the specific activity in Peak I reached 2–5 units/mg

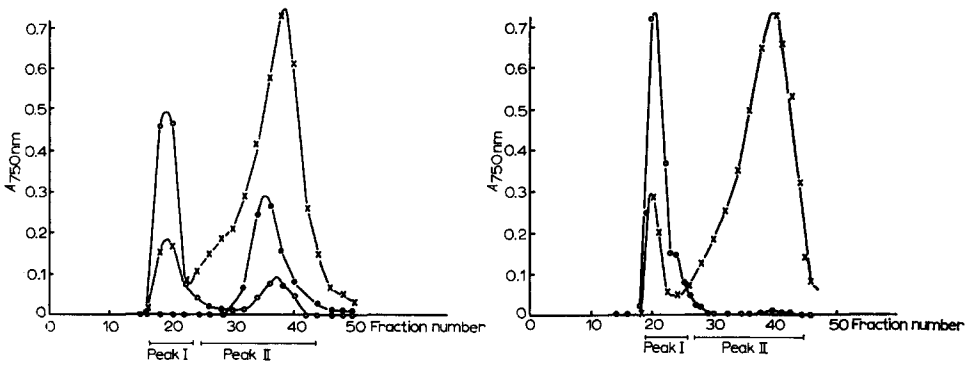

Fig. 1. Gel filtration of M-fraction on Sepharose 4B. 25 mg of M-fraction dissolved in 5 ml of 0.01 M sodium barbital–HCl buffer (pH 7.8) containing 0.25 M sucrose and 0.5 % sodium deoxycholate was placed on a column (1.5 cm × 99 cm) equilibrated and eluted with the same buffer at 0°. Flow rate was 5 ml/h and 3-ml fractions were collected. Enzyme activities were assayed as described in the text and 0.5 ml of each fraction was used for protein determination. O—O, Enzyme I; ●—●, Enzyme II; ×—×, protein.

Fig. 2. Gel filtration of M-fraction on Sepharose 4B in the presence of tyrosine and EDTA. 25 mg of M-fraction dissolved in 5 ml of 0.01 M sodium barbital–HCl buffer (pH 7.8) containing 0.25 M sucrose, 0.5 % sodium deoxycholate, 0.001 M L-tyrosine and 0.001 M EDTA was placed on a column (1.5 cm × 97 cm) equilibrated and eluted with the same buffer at 0°. Flow rate and fraction volume were the same as in Fig. 1. Enzyme activity was assayed as described in the text and 0.5 ml of each fraction was used for protein determination after precipitating the protein as described in the text. O—O, Enzyme III; ×—×, protein.

protein compared to about 0.2 in the M-fraction (1 unit is the amount of enzyme that will liberate 1 μmole of P_i from IMP per min and corresponds to 3 units when AMP is used as substrate), the fluctuation of the values being due to unavoidable inactivation during the fractionation. It is also noteworthy that the increase in specific activity of Enzyme III was considerable, about 1 (sometimes 2 or 3) units/mg protein being obtained compared to about 0.2 in the M-fraction.

Peak I was turbid, suggesting that this fraction still possessed an aggregated form with extremely large particle size. Rechromatography of Peak I under conditions used in the initial chromatography did not result in any change in the pattern of protein and enzyme distributions, indicating that Peak I represents a particular fraction of membrane structure and that at least two forms of Enzyme I exist.

From these results it may be deduced that Enzyme I and Enzyme II are differently located within plasma membrane, only the former being tightly bound to membrane.

Peak I was then submitted to gel filtration on a column of Sepharose 2B, eluting with the same solvent as that in Figs. 1 and 2. However, most of the proteins and practically all the activities of both Enzymes I and III were recovered in a peak appearing in the void volume, giving 1.5-fold purification of Enzyme I and 2-fold purification of Enzyme III.

Partial separation of these two enzymes occurred when Peak I was centrifuged at 105000 $\times$ *g* for 30 min. By this centrifugation, 90% of the proteins precipitated and the supernatant contained about 20% of Enzyme I, whereas practically all the Enzyme III activities were in the precipitate. It should be noted that no inactivation of the enzyme activities occurred during the centrifugation. Thus, this separation is compatible with the fact that these two enzymes are of different subcellular origins.

On density gradient centrifugation of the supernatant obtained above, three peaks possessing the Enzyme I activities were obtained as shown in Fig. 3. Thus, it appears that there are altogether four forms of Enzyme I. Centrifugation also resulted in considerable purification of Enzyme I, but the specific activity of the most active peak was again only about 5 units/mg protein, due to inactivation during the centrifugation procedure.

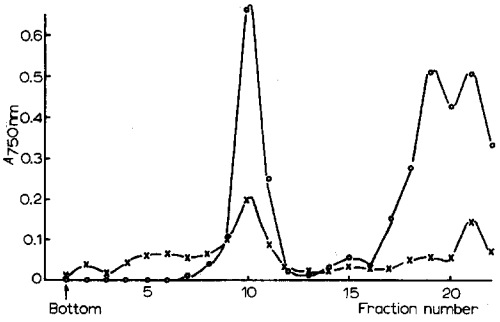

Fig. 3. Density gradient centrifugation of Peak I from the Sepharose 4B gel filtration (Fig. 1). 0.2 ml of the supernatant of Peak I after centrifugation at 105000 $\times$ *g* for 30 min was layered on 5 ml of 20–50% linear sucrose gradient in 0.01 M sodium barbital–HCl buffer (pH 7.8) containing 0.5% sodium deoxycholate. The tubes were centrifuged at 130000 $\times$ *g* in a Hitachi RPS 40 swinging bucket rotor for 16 h at 0°. Fractions from three tubes were combined, 0.24 ml from each tube in one fraction, and 0.2 ml and 0.3 ml thereof were used for enzyme assay and protein determination, respectively. O—O, Enzyme I; ×—×, protein.

The results of the experiments reported here demonstrate, firstly, that two marker enzymes of hepatic plasma membrane could be solubilized in different ways by a detergent, sodium deoxycholate. This may not be characteristic only of the marker enzymes investigated, since various enzymes or functional proteins have been solubilized in different manners and purified from microsomes or mitochondria. However, it is remarkable that Enzymes I and III are contained in a very small fraction of the microsomal proteins and that this small fraction was very resistant to further solubilization with sodium deoxycholate. This resistance may rationalize the use of Enzymes I and III as marker enzymes for membranes. Secondly, at least four forms of Enzyme I were found to exist in the sodium deoxycholate-solubilized microsomes. It is not known whether the multiple forms are due to the presence of different enzyme proteins. WEAVER AND BOYLE[10] also found recently that 5'-nucleotidase activity of the isolated plasma membrane fraction from rat liver is separable into two peaks on zonal centrifugation. However, recent studies on the purification of Enzyme I from rat liver microsomes showed that a single lipoprotein containing only one phospholipid, sphingomyelin, can be prepared[11]. This may imply that the multiple forms are due to the formation of complexes with different sizes between Enzyme I and membranous components and represent a status of membrane-bound enzymes within membrane organization.

Department of Biological Chemistry,
Faculty of Pharmaceutical Sciences, Kyoto University,
Kyoto (Japan)

KENJI NAKAI
SHIGEHIDE TAKEMITSU
TOSHISUKE KAWASAKI
IKUO YAMASHINA*

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Received July 23rd, 1969

* Inquiries should be sent to Dr. Yamashina.