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Isolation of peroxisome subpopulations from rat liver by immune free-flow electrophoresis

Introduction

Peroxisomes (POs) are ubiquitously distributed, heterogeneous cell organelles which, in mammals, are most abundant in liver and kidney. The functional significance of PO in man is stressed by the existence of a group of inherited diseases in which one or more of their functions is impaired. The heterogeneity of POs is a wellknown phenomenon, but it is quite difficult to purify a definite PO subpopulation by conventional gradient centrifugation because of their high fragility, sensitivity to mechanical stress and the very similar sedimentation properties among the subsets and to other subcellular organelles, particularly microsomes. Free-flow electrophoresis in combination with an immune reaction (so-called immune free flow electrophoresis (IFFE) offers a powerful solution to this problem, because it allows the convenient isolation of different subpopulations of POs from pre-separated heavy, light, and postmitochondrial fractions (HM, LM, and PM, respectively) of rat liver homogenates. IFFE combines the advantages of electrophoretic separation with the high selectivity of an immune reaction. It makes use of the fact that the electrophoretic mobility of peroxisomes complexed to antibodies against the cytoplasmic domain of the integral PO membrane protein PMP 70 is greatly diminished, provided that the pH of the electrophoresis buffer is adjusted to pH ~ 8.0, the pI of IgG molecules. Because of this reduced electrophoretic mobility, IgG-coupled organelles can be separated in an electric field from those that don't carry IgGs and hence are more mobile.

Methods

Sample preparation

Rat livers were drained of blood by perfusion with saline (0.9% NaCl,w/v), subsequently excised, dried with filter paper, and weighed. After removal of the connective tissue, they were minced in icecold homogenization buffer (HB: 250 mM sucrose, 5 mM MOPS, 1 mM EDTA,0.1% ethanol, 2 mM PMSF, 1 mM DTT, 1 mM e-aminocaproic acid; 5 ml/g) using a cooled Potter–Elvehjem tissue grinder equipped with a motor-driven teflon pestle (1000 rpm).

The homogenate was then sub-fractionated in consecutive steps (100 g, 1950 g, 25500 g), giving rise to pellets (1950 g and 25500 g) that were resuspended by means of a hand-driven teflon pestle in ice-cold HB (4 and 1 ml/g liver). The suspensions thus obtained correspond to heavy (HM) and light (LM) mitochondrial fractions, respectively.

The supernatant recovered after the LM pelleting was layered on a cushion, prepared by dissolving Nycodenz in 5 mM MOPS, 1 mM EDTA,0.1% ethanol, 2 mM PMSF, 1 mM DTT, 1 mM eaminocaproic acid (r = 1.20 g/cm3). It was spun in a fixed-angle type rotor (Ti 45; Beckman) at 37000 _ g for 20 min and the fluffy layer banding on top of the cushion, comprising microsomes, lysosomes, and peroxisomes was re-loaded onto a fresh Nycodenz cushion and re-centrifuged at 55000 _ g for 20 min in the same rotor.

The pellet collected was re-suspended in ice-cold HB (1.2 ml) using a glass rod and was designated the postmitochondrial fraction (PM). In addition, highly purified POs were isolated from a LM fraction by metrizamide density gradient centrifugation and were designated LMgrad-PO. 1 ml-aliquots of the HM, LM, and PM fractions

corresponding to about 0.25–1 g of liver were mixed with 1 ml HB containing 1 mg polyclonal anti-PMP 70 antibody, 1 mg of BSA, and leupeptin (1 µM), and the mixtures were incubated at room temperature for > 1 h to immunocomplex the POs of these fractions. Immunoprecipitates and non-complexed particles were collected by centrifugation for 20 min at 1950 x g (HM), 25500 x g (LM), and 55000 x g (PM), and the pellets were suspended in 2 ml separation buffer (SB), pH 8.0, containing 250 mM sucrose, 10 mM triethanolamine, 10 mM acetic acid, and 0.25 mg/ml BSA.

Free-flow electrophoresis

Just before each PZE-run, the separation chamber was washed with SB and electrophoresis was subsequently conducted at 4°C in the same buffer with a field of 1000 V and 100 mA and a flow rate of ~ 5 ml fraction/h. Samples were perfused into the separation chamber at ~ 2 ml/h. Individual runs took approximately 60–90 min. Consecutive fractions were pooled in pairs, numbered 1 (anode) through 45 (cathode), and assayed for protein (OD280). The fractions were concentrated by centrifugation for 20 min at g forces stated above. Pellets were suspended in HB and stored at -80°C for enzyme analyses, SDS-PAGE, and immunoblotting.

Data analysis Protein was assayed using the Coomassie Blue binding method, with BSA as a standard. SDS-PAGE was performed under reducing conditions using 12.5% polyacrylamide gels. Polypeptides resolved were transferred to nitrocellulose, which was incubated for 1 h at 37°C with 5% nonfat milk/ 10 mM Tris buffer, pH 7.4/0.05% Tween 20 to block unspecific binding sites. For peroxisome- specific immunocomplexing, 10 µg/ml of urate oxidase (UOx) antibodies were mixed with the same amount of one of the other antibodies used (catalase (Cat) and acyl-CoA oxidase (AOx)), and incubation was performed at 4°C overnight in the same buffer containing 1% nonfat milk.
After repeated washing, a peroxidase-conjugated goat anti-rabbit antibody (1:10000) was added for 1 h at room temperature and enhanced chemiluminescence (ECL) was used to visualize the immune complexes. Specific signals revealed by ECL, representing the immune complexes of matrix and membrane proteins of PO derived from LM, HM, and PM fractions as well as from an LM density gradient centrifugation (LMgrad-PO),were analyzed by densitometry and ratios of intensities were calculated, with the signal of insoluble PO matrix enzyme UOx serving as reference for the soluble PO matrix enzymes Cat and AOx.

Results

The electropherogram shown in Figure 1 exemplifies the fractionation of an LM fraction by means of IFFE. The respective peroxisomes isolated, LMPO1 collected in fractions 32-34 and LM-PO2 in fractions 37-39, are indicated by red dots.

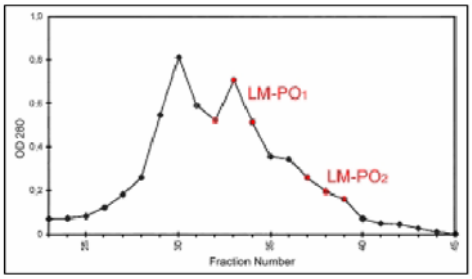

Figure 1: Distribution of proteins across IFFE fractions (for further explanation see text).

The distribution of protein across all fractions, reflected by the optical density at 280 nm (OD280) and the determination of PO matrix enzyme activities (Cat, AOx, and UOx, data not shown) consistently reveal a clear-cut separation of nonperoxisomal organelles (first peak) and peroxisomes (second peak and shoulder).

This is in accordance with the corresponding HM and PM electropherograms: A major peak comprising the bulk of organelles not immunocomplexed (mainly mitochondria for HM and microsomes for PM) and a minor one containing POs as identified by marker enzyme activities (not shown). These findings demonstrate the feasibility of the IFFE technique for isolation of POs in general and of PO subpopulations in particular:

To figure out whether distinct physicochemical properties account for the distribution of POs to the hepatic subfractions investigated or whether they truly differ in their protein composition, HM-, LM-, and PM-POs in comparison to traditionally purified LM-POs (LMgrad-PO; obtained by density gradient centrifugation of the same LM preparation) were analyzed by SDS-PAGE (Figure 2A) and subsequentWestern blotting (Figure 2B) using polyclonal antibodies directed against peroxisomal matrix proteins. Whereas the polypeptide patterns of POs immunoisolated from the LM and PM fractions largely coincide with the LMgrad- PO, that of HM-POs does not (Figure 2A).

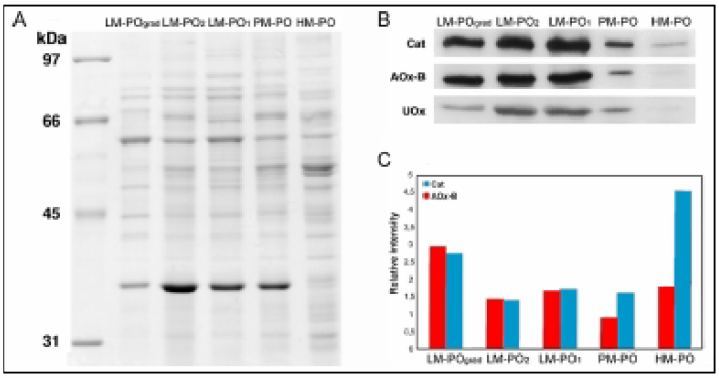

Figure 2: Analysis of fractions by A SDS-PAGE; B Immunoblotting; C Densitometric analysis of immunoblots (for further explanation see text).

This might be due to polypeptides from contaminating organelles, although HM-PO might also represent a unique PO subpopulation. Immunoblotting using antibodies against PO matrix enzymes (Cat, AOx, UOx) revealed differences between the protein- specific signals in a given subpopulation (Figure 2B). More importantly, however, each protein investigated specifically varied in its signal intensity between LM-, HM-, PM-, and LMgrad-PO, suggesting a different composition of these PO populations.

This interpretation is substantiated by the densitometric analysis of the blots and the calculation of relative intensities with the UOx signals serving as reference. As is indicated by the data depicted in the graph related to the blot, individual PO subpopulations indeed differ in the ratios of the soluble matrix enzymes Cat and AOx to the insoluble matrix enzyme UOx (Figure 2C) as well as in the ratios of various membrane proteins (data not shown). These data provide strong evidence that HM-PO, LM-PO1, LMPO2, and PMPOs are distinct subpopulations. Therefore, the ratios of the soluble matrix enzymes Cat and AOx to the insoluble UOx are quite similar in LM-PO1 and LM-PO2, as well as in LMgrad-PO, yet markedly differ in PM- and HM-POs (Figure 2).

Conclusion

Exemplified by the purification of distinct subpopulations of POs from crude subfractions of rat liver homogenates, IFFE is a powerful technique for the fractionation of cell organelles and corresponding subpopulations not to be separated by isopycnic or rate-dependent density gradient centrifugation. An appropriate antibody may be a polyclonal but hast to be monospecific, and directed against epitopes located at the outer aspect of the antigenic membrane protein of the subcellular particles to be isolated. To effectively diminish the electrophoretic mobility of particles coupled to an antibody from others not immunocomplexed, a pH of the separation media is required, approximating the pI of the antibody used.

References

1) Völkl, A.,Mohr, H.,Weber, G. and Fahimi, H. D. (1997) Isolation of rat hepatic peroxisomes by means of immune free flow electrophoresis, Electrophoresis 18(5) 774-780.
2) Völkl, A.,Mohr, H.,Weber, G. and Fahimi, H. D. (1998) Isolation of peroxisome subpopulations from rat liver by means of immune free-flow electrophoresis, Electrophoresis 19(7) 1140-1144.
3) Völkl, A.,Mohr, H. and Fahimi, H. D. (1999) Peroxisome subpopulations of the rat liver. Isolation by immune free flow electrophoresis, J Histochem Cytochem 47(9) 1111-1118.

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