Determining the topology of peroxisomal proteins using protease protection assays

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i. Abstract

Protease protection assays are powerful tools to determine the topology of organelle proteins. Their simplicity, together with the fact that they are particularly suited to characterize endogenous proteins, are their major advantages and the reason why these assays have been in use for so many years. Here, we provide a detailed protocol to use with mammalian peroxisomes. Suggestions on how these assays can be controlled, and how to identify some technical pitfalls are also presented.

ii. Keywords

Peroxisome, peroxisomal proteins, protease protection assay, protein topology, proteinase K.

1. Introduction

Knowledge on the topology of peroxisomal proteins is crucial to understand their function. This is valid not only for enzymes, but also for all proteins involved in the biogenesis/structure of the organelle. Only then can one know whether a given enzyme acts on the cytosolic or organelle pools of its substrate or on which side of the peroxisomal membrane does a given protein-protein interaction take place.

Many different strategies can be used to define the topology of these proteins. For instance, in the case of mammalian peroxisomes immunofluorescence techniques using semi-permeabilized cells have provided useful information [1–3]. However, whenever an antibody directed to a protein of interest is available, a simple protease protection assay

using an organelle suspension can also provide valuable information [3–5]. The advantages of this approach are several-fold. First, it is very simple. Indeed, besides requiring just a few specific reagents (*i.e.*, a protease and a protease inhibitor) and common protein analysis equipment (*e.g.*, SDS-PAGE and Western-blotting apparatus), the whole procedure can be completed in a few hours. Second, any antibody that recognizes the protein of interest upon Western-blotting can be used in this approach. Finally, although organelle suspensions obtained from transfected cells expressing epitope-tagged versions of a protein can surely be used in these assays, the technique is particularly suited to characterize endogenous proteins. The latter situation is always preferred because all the potential problems associated with the use of transfected cells (*i.e.*, protein missorting/incorrect topology due to abnormal expression levels or to the presence of tags) are avoided.

Rationale and limitations of the method

From a conceptual point of view, a protease protection assay could not be simpler. Essentially, it relies on the fact that an exogenously added soluble protease will hydrolyze/degrade a given protein in an organelle suspension as long as the protein is accessible to the protease; if the protein (or a domain of it) is inside an intact organelle, then it will resist proteolysis (*see* **Figure 1**).

Despite its simplicity, several aspects need to be considered when performing a protease protection assay. A critical one regards the protease susceptibility of the protein of interest – the protein has to be susceptible to a selected protease under native conditions and in the absence of intact organelle membranes; otherwise no conclusions regarding its topology can be drawn from these experiments. Although this is generally not a problem – many

peroxisomal proteins are at least partially susceptible to proteases – there are several exceptions. For instance, mammalian catalases and soluble urate oxidase from mouse liver peroxisomes are all extremely resistant to proteolysis, even when using aggressive proteases such as proteinase K (PK) or pronase ([6, 7], unpublished results). Thus, it is always advisable to determine the protease susceptibility of the protein of interest before proceeding with a protease protection assay. A simple way to do this is described below (*see subheading 3.1.1*). In essence, an aggressive protease (*e.g.*, PK, pronase or trypsin) is added to Triton X-100-solubilized organelles, aliquots are removed at different time points and analyzed by SDS-PAGE/Western-blot. Ideally, the protein of interest should be completely digested (or converted into smaller fragment(s)) within the first minutes of digestion.

A protease protection assay always comprises three different types of samples. One sample simply contains intact/untreated organelles; another contains protease-treated intact organelles; and the third contains organelles that were disrupted before protease treatment. Two methods to disrupt organelles are provided in this protocol. One, the most commonly used, consists in adding a mild detergent (*e.g.*, Triton X-100) to the organelle suspension to solubilize the peroxisomal membrane. The other consists in sonicating organelles in the presence of the exogenously added protease. In principle, the two methods will yield the same result, *i.e.*, proteins (or protein domains) that resist proteolysis in the sample containing intact organelles will now be accessible to the protease and thus they may be cleaved. However, for membrane proteins or subunits of detergent-sensitive protein complexes the results may differ. For instance, PEX14, a 40-kDa peroxisomal intrinsic membrane protein [8], is cleaved into a 16-kDa fragment by PK when intact or sonicated

organelles are used in these assays, but is completely digested by the protease when the organelles are first solubilized with Triton X-100 [9]. Seemingly, membrane lipids or some PEX14-interacting protein protect the 16-kDa fragment of PEX14 from proteolysis by steric hindrance.

A protease protection assay is only conclusive when both the activity of the exogenously added protease and the intactness of organelle membranes are monitored. This is achieved by analyzing the behavior of organelle protein(s) of known topology. A first-choice mammalian peroxisomal protein to control these assays is PEX13, a 44-kDa transmembrane peroxin [3]. PEX13 is readily accessible to PK or trypsin in intact organelles yielding a protected fragment of *ca.* 28-30 kDa. However, upon disruption of the peroxisomal membrane, either by solubilization with mild detergents or by sonication, this fragment is completely degraded by these proteases [4]. Thus, by following the behavior of just one peroxisomal protein it is possible to monitor both the activity of the protease and the integrity of the peroxisomal membrane. If an anti-PEX13 antibody with the capacity to recognize the intraperoxisomal domain of PEX13 is not available, then there are other options. A good one consists in using an antibody directed to ABCD3 (also known as PMP70) and another directed to sterol carrier protein x (SCPx). ABCD3 is a 70-kDa transmembrane protein which is largely exposed at the cytosolic face of the peroxisomal membrane and is highly susceptible to most proteases [10]. Thus, it will be digested by the protease even when using intact organelles. SCPx is a 59-kDa peroxisomal matrix protein, which is partially processed upon import into the peroxisome, yielding two polypeptides of 46 and 13 kDa, respectively (reviewed in [11]). However, processing of SCPx is a very slow process *in vivo* and thus intact/unprocessed SCPx protein is detectable in most (if not

all) organs/tissues [12]. Unprocessed SCPx will only be cleaved by the protease when the peroxisomal membrane is disrupted [6]. Note, however, that peroxisomes are quite fragile organelles and tend to release a fraction of their matrix proteins during tissue homogenization and even during simple manipulations such as sedimentation and resuspension [13]. Thus, a fraction of intact SCPx (and other matrix proteins) will actually be accessible to the protease when using intact organelles. The pool of intact SCPx that is relevant in these assays is thus the one that resists protease digestion in the sample containing intact organelles and is cleaved when the peroxisomal membrane is disrupted.

The protocol described here uses very large amounts of an aggressive protease to avoid misleading situations of partial proteolysis. Naturally, the protease should remain active during incubation with the organelle proteins but should be completely inactivated at the end of that incubation. Two strategies are used in tandem to inactivate the protease: 1) treatment with an irreversible protease inhibitor and 2) a fast denaturing protein precipitation step using trichloroacetic acid. Despite this, it is important to note that reactivation of the protease in subsequent steps (*e.g.*, during SDS-PAGE) can still occur. Although this is a rare problem, it must always be considered; otherwise wrong conclusions will be drawn from these assays. This is actually quite easy to achieve – a comparison of protein patterns after Western-blotting should reveal several major bands, some of which run in the high molecular mass region of the gel, in all samples, including those containing protease-treated disrupted organelles. These bands correspond to proteins that are intrinsically resistant to proteolysis (*e.g.*, catalase). Only Western-blots passing this simple quality control check should be probed with antibodies.

2. Materials

2.1. Protease protection experiments:

1. Organelle suspension – Any organelle suspension prepared in an isotonic buffer (*e.g.*, SEM buffer 1X; see below) can be used in this protocol. Excellent results are obtained with both purified peroxisomes and post-nuclear supernatants from rat/mouse liver (PNS; *see Note 1*) but other subcellular fractions (*e.g.*, a peroxisome enriched “light mitochondria” fraction; [14]) can also be used. Rat/mouse liver is a first-choice organ to obtain these fractions for two reasons: 1) peroxisomes are very abundant in this organ (they comprise 2-3% of total protein; [14]), and 2) hepatocytes can be disrupted using very gentle conditions thus yielding organelles of good quality ([14]). Other organs such as rat/mouse kidney, testis, adrenals, and spleen also yield excellent PNSs having a large fraction of intact organelles. Note, however, that with the exception of rat/mouse kidney [15, 16], it is still not possible to obtain highly pure peroxisomes from these organs. It is not necessary to use freshly prepared organelle suspensions for this protocol. PNS or purified peroxisome aliquots from rat/mouse liver that were snap frozen in liquid N₂, stored at -70 °C for several months, and rapidly thawed just before use also yield good results (*see Note 2*). Ultimately, the type of subcellular fraction selected for these assays depends on the sensitivity of the antibodies that will be used to detect the protein of interest and the peroxisomal proteins which will be used to control these assays (*see Note 3*).
2. SEM buffer 3X: 0.75 M sucrose, 60 mM MOPS-KOH pH 7.2, 3 mM EDTA-NaOH pH 8.0 (keep at 4 °C);

3. 20 mM MOPS-KOH pH 7.2 (keep at 4 °C);
4. 20 mg/mL Proteinase K in 20 mM MOPS-KOH pH 7.2 (store as single-use aliquots at -70 °C);
5. 50 mg/mL phenylmethylsulfonyl fluoride (PMSF) in 100% ethanol (store at -20 °C; Hazardous! Handle with care.);
6. 20% (w/v) Triton X-100 (TX-100);
7. Ultrasonic homogenizer (*e.g.*, SONOPULS HD2200-BANDELIN equipped with an MS 73 microtip);
8. 100% (w/v) trichloroacetic acid (TCA) (TCA is very corrosive. Hazardous! Handle with care.);
9. Acetone;
10. Microcentrifuge.

2.2. SDS-PAGE and Western-blotting:

1. Standard equipment and solutions required to perform SDS-PAGE/Western-blotting;
2. Laemmli sample buffer 1X: 50 mM Tris pH 8.8, 2 mM EDTA-NaOH pH 8.0, 2% (w/v) SDS, 0.02% (w/v) bromophenol blue;
3. 0.45 µm nitrocellulose membrane (*e.g.*, Amersham, 10600002);
4. Ponceau S stain: 0.2% (w/v) Ponceau S in 3% (w/v) TCA;
5. Antibodies – Besides one antibody directed to the protein being characterized, it is necessary to apply antibodies directed to peroxisomal proteins of known topology. As stated above an anti-PEX13 antibody [4, 17] that recognizes the intraperoxisomal domain of this protein in Western-blots is sufficient to control

both the protease activity and the intactness of the peroxisomal membrane.

Alternatively, use antibodies directed to SCPx (*e.g.*, ProteinTech™, 19182-1-AP) and ABCD3 (*e.g.*, Sigma-Aldrich®, SAB4200181).

3. Methods

3.1. Protease protection experiments:

3.1.1 Preliminary experiment: Intrinsic protease susceptibility of the protein of interest

1. Assemble the reaction on ice according to Table 1. Mix well by inverting the tube a few times.
2. Remove a 196 µL aliquot (0') and keep on ice.
3. Add 16 µL of 20 mg/mL PK (400 µg/mL final concentration) to tube 1 (*see* **Notes 2 and 4**) and incubate on ice. Remove aliquots of 200 µL at different time points (*e.g.*, 5', 10', 20' and 40') and, immediately, add 2 µL of 50 mg/mL PMSF to each aliquot to inhibit PK (*see* **Note 5**). Mix gently, spin down for 1 sec to collect the content at the bottom of the tube and incubate for 2 min on ice.
4. As soon as the previous 2-min incubation is finished precipitate proteins in samples with 10% TCA (final concentration). For this, add 22 µL of 100% TCA to each aliquot, vortex and incubate for at least 30 min on ice.
5. Centrifuge at 11,300 x g for 15 min at 4 °C and discard the supernatant.
6. Add 1 mL of acetone to the pellets. Vortex vigorously and centrifuge at 11,300 x g for 15 min at 4 °C.

7. Discard the supernatant. Spin down for 1 sec and remove all traces of the acetone supernatant. Dry the pellets at 37 °C (2-5 min) (*see Note 6*).
8. Resuspend protein pellets in Laemmli sample buffer 1X, incubate at 65 °C for 10 min and then at 90 °C for 5 min, with shaking. Proceed to “SDS-PAGE and Western-Blotting” (*see Subheading 3.2*).

3.1.2 Protease protection assay:

1. Prepare samples according to Table 2 (Mix the first two components well before adding the organelle suspension).
2. Immediately after adding PK, sonicate tube 4 for 20-30 sec (10% output power, 40% duty cycle) (*see Note 7*).
3. Incubate all tubes for 40 min on ice.
4. Add 2 µL of 50 mg/mL PMSF to each tube. Mix gently, spin down samples for 1 sec, and incubate for 2 min on ice (*see Note 5*).
5. Proceed as in steps 4-8 of the preliminary experiment (*see Subheading 3.1.1*).

3.2. SDS-PAGE and Western-Blotting

1. Subject samples to SDS-PAGE/Western-blot.
2. Stain the nitrocellulose membrane with a reversible stain (*e.g.*, Ponceau S) and keep a digital record (*see Note 8*).
3. Probe the membrane with relevant antibodies (*see Note 9*).

4. Notes

1. When preparing a PNS, the total cell homogenate should be centrifuged twice (~600 x g for 10 min at 4 °C) to ensure that intact cells and large plasma-membrane vesicles containing cytoplasm are completely removed.
2. Both the organelle suspensions and the protease stock solution (*e.g.*, PK) should be quickly thawed in your hands, gently flicking the tubes to homogenize the suspensions/solutions, placed on ice and promptly used.
3. The amount of protein to be used depends on the nature of the organelle suspension and has to be adjusted accordingly. When using purified peroxisomes, 10-30 µg of protein per reaction are sufficient, whereas for PNS, 80-150 µg of total protein may be necessary.
4. PK is used throughout this protocol, but other proteases, such as pronase or trypsin at a final concentration of 400 µg/mL should also be tested.
5. PMSF in the stock solution precipitates on ice; keep at room temperature and ensure all PMSF is in solution before using it.
6. Make sure that all acetone evaporates.
7. Sample heating must be avoided. Sonication is performed on ice and for short periods of time (*e.g.*, cycles of 5 sec). Also, be careful to avoid protein foaming during sonication.
8. The nitrocellulose membrane should be stained with Ponceau S to assess protein loadings and a digital record of it should be kept. Please note that the overall protein pattern does not change extensively after proteolysis. This is true even for samples

containing protease-treated disrupted organelles. Indeed, only minor alterations are observed since many abundant proteins are intrinsically resistant to proteolysis (*e.g.*, catalase and urate oxidase; please *see* Refs. [6] and [7], respectively).

9. As it will be necessary to probe the membrane with several antibodies and since some proteins may co-migrate or present a complex proteolysis pattern, it is generally necessary to run several sample sets of the same assay. Just scale-up the reactions accordingly.

5. References

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Figure caption:

Fig. 1- Graphic representation of the different experimental conditions used to assess the topology of peroxisomal proteins.

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Table 1. Sample preparation:

Components	Per time point	Tube 1
ddH ₂ O	To 200 µL	(603 – 5x) µL
SEM buffer 3X	1X	327 µL
Organelle suspension	10 µg - 150 µg (see Notes 2 and 3)	5x µL
20% (w/v) TX-100	1%	50 µL

Table 2. Reactions assembly:

		Tube 1	Tube 2	Tube 3	Tube 4
Components	Per reaction	–PK	+PK	+PK / +TX-100	+PK /+sonication
ddH₂O	To 200 µL	(129.3 – x) µL	(129.3 – x) µL	(119.3 – x) µL	(129.3 – x) µL
SEM buffer 3X	1X	66.7 µL	66.7 µL	66.7 µL	66.7 µL
Organelle suspension	10 µg - 150 µg (see Notes 2 and 3)	x µL	x µL	x µL	x µL
20% (w/v) TX-100	1%	-	-	10 µL	-
20 mM MOPS pH 7.2	4 µL	4 µL	-	-	-
20 mg/mL PK	400 µg/mL (see Notes 2 and 4)	-	4 µL	4 µL	4 µL

Protease protection assay

● - cytosolic protein,
 ● - membrane protein,
 ● - matrix protein,
 ✂ - protease