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A rapid method to separate endosomes from lysosomal contents using differential centrifugation and hypotonic lysis of lysosomes

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Abstract

Here we describe a fast and efficient subcellular fractionation procedure that permits lysosomes to be separated from endosomes. Differential centrifugation is used to isolate a subcellular fraction containing both endosomes and lysosomes. Because lysosomes are sensitive to osmotic stress, hypotonic conditions destroy them, whereas endosomes, which are osmotically insensitive, stay intact. We demonstrate that hypotonic lysis of an endosome–lysosome-pool releases 85% of the lysosomes into the supernatant as measured by the activity of the lysosomal marker enzyme *N*-acetyl-β-D-glucosaminidase (β-AGA). The endosomal fraction is thoroughly characterised using a variety of subcellular markers. After pulsing cells with fluorescein isothiocyanate labelled transferrin (FITC-Tf), only about 12% of the marker is released under hypotonic conditions. A typical fractionation procedure takes about 1–2 h from initial cell homogenisation. The fractionation gives a pure lysosomal fraction (fraction L) containing high activities of lysosomal enzymes and an endosomal fraction (fraction E) reflecting different stages of endosomes.

Keywords: Endosomes; Lysosomes; Subcellular fractionation

Abbreviations: β-AGA, *N*-acetyl-β-D-glucosaminidase; DH, dehydrogenase; EBV, Epstein–Barr virus; FITC, fluorescein isothiocyanate; FITC-Tf, fluorescein isothiocyanate labelled transferrin; fraction C, cytosolic fraction; fraction De, debris, undestroyed cells; fraction E, endosomal fraction; fraction L, lysosomal fraction; fraction Mi, microsomal fraction; fraction PN, plasma membrane and nuclear fraction; HPSEC, high performance size exclusion chromatography; LDH, lactate dehydrogenase; MHC, major histocompatibility complex; PAGE, polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; *Q*, relative specific activity (specific activity of fraction/specific activity of total cell lysate); RIA, radioimmunoassay; SDS, sodiumdodecyl sulfate; tris(hydroxymethyl)aminomethane

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1. Introduction

Lysosomes are the final degrading organelles of both endocytic and autophagic pathways (Bohley, 1995; Bowers, 1998). Containing over 15 proteolytic enzymes as well as numerous other hydrolases, lysosomes are well equipped to degrade the variety of proteins and other macromolecules which are continually transported to these organelles (Seglen and Bohley, 1992). It has been notoriously difficult to determine whether molecules that are transported towards the lysosomes by the endocytic or autophagic route are present in lysosomal or prelysosomal organelles (Berg et al., 1994). It is similarly difficult to prove whether certain proteolytic processes occur in endosomal or in lysosomal compartments. Endosomal proteolysis is a very important process, e.g., in antigen processing (Fineschi and Miller, 1997), activation and inactivation of hormones or the activation of lysosomal proteases (Berg et al., 1995). Studies on the proteolytic activity of endosomes can only be done if the high protease activity of lysosomes can be efficiently separated from the endosomes. Because of their high hydrolytic activity, lysosomes contain many products of hydrolysis in the form of small molecules such as amino acids. Therefore, lysosomes are sensitive to osmotic lysis (de Duve et al., 1955), whereas endosomes are osmotically insensitive (Park et al., 1988). Several authors have used special hydrolase substrates, so-called lysosome disrupting substrates, which further increase the lysosomal concentration of small molecules, to rupture specifically lysosomes by osmotic lysis (Goldman and Kaplan, 1973; Jadot et al., 1984; Berg et al., 1994). A more direct approach to the isolation of lysosomal proteases was described in 1969 (Bohley et al., 1969). Bohley et al. used hypotonic lysis in combination with differential centrifugation to isolate lysosomes. Subcellular fractionation by differential centrifugation was first described by de Duve et al. (1955) and has subsequently been the method of choice for isolating cell organelles from various tissues and cultured cells. Compared to subcellular fractionation by density gradient centrifugation using Percoll (Harding and Geuze, 1993; Max et al., 1993), sucrose (Maric et al., 1994; Ferrari et al., 1997) or iodinated density gradient material (Ford and Rickwood, 1982; Rickwood et al., 1982), differential centrifugation, has the great advantage of being very fast. A typical fractionation procedure takes about 1–2 h from initial cell homogenisation. In addition, interference of the gradient forming material with the activity of enzymes can be ruled out. Limitations concerning the number of cells which can be used per fractionation, as with density gradients, are not valid for differential centrifugation.

Here we describe a rapid subcellular fractionation procedure to separate lysosomes from endosomes and prelysosomal compartments using differential fractionation and hypotonic lysis. This approach results in a pure lysosomal fraction (fraction L) containing high specific activities of lysosomal enzymes and an endosomal fraction (fraction E) containing different stages of endosomes.

The fractionation scheme is inexpensive and applicable to a variety of different human cell lines such as Epstein–Barr virus (EBV)-transformed B-cells, renal carcinoma cells or keratinocytes. Also, a range of cell numbers can be used for the fractionation. The procedure was performed using 1×10^8–7×10^9 cells without any detrimental effects on the quality of the isolated fractions.

2. Materials and methods

2.1. Biochemicals

RPMI 1640, penicillin and streptomycin were purchased from Gibco (Eggenstein, Germany). Enzyme substrates were obtained from Boehringer Mannheim (Mannheim, Germany). The anti-cathepsin B antibody, anti-protein disulfide isomerase (PDI) antibody (microsomes) and secondary antibodies were obtained from Dianova (Hamburg, Germany). The anti-mitochondria serum was a gift from Dr. R. Klein, Department of Internal Medicine, Universitätsklinikum Tübingen. The serum was obtained from a primary biliary cirrhosis patient and was characterised as containing anti-α-ketoglutarate dehydrogenase and anti-pyruvate dehydrogenase antibodies. The ECL-system was obtained from Amersham (Buckinghamshire, England). Fetal calf serum and other chemicals were purchased from Sigma (Deisenhofen, Germany).

2.2. Cells

The EBV-transformed human B-cell lines BSM and Cox (gifts by C.A. Müller, Tübingen) and the human keratinocyte cell line HaCaT (a gift by H.-W. Boehncke, Frankfurt) were used. The human renal epithelial carcinoma cell line HTB44 (ATCC A498) was purchased from ATCC (Manassas, VA, USA). Cells were cultured at 37°C, 5% CO_2 in RPMI 1640 medium with HEPES containing 10% heat-inactivated fetal calf serum and antibiotics. For large-scale experiments, 5–20 l of BSM cells were grown in roller bottles to densities of $0.5–0.75 \times 10^6$ cells/ml.

2.3. Differential fractionation

All procedures were carried out at 4°C or on ice. Centrifugations were performed in a Beckman TL100 ultracentrifuge (Beckman, Palo Alto, USA). Unless otherwise stated, the fractionation buffer (10 mM tris(hydroxymethyl)aminomethane/acetic acid pH 7.0, 250 mM sucrose) was used throughout the fractionation. $1 \times 10^8–7 \times 10^9$ cells were harvested and washed three times. The cell pellet was resuspended with 2–5 ml and homogenized using 10 strokes in a 5-ml Potter–Elvehjem-homogenisor with a 60-μm gap at 500 rpm. The cell homogenate was then centrifuged at $2000 \times g$ for 2 min to pellet debris and undestroyed cells (fraction De). The supernatant was collected in a new tube and the pellet washed with twice the pellet weight of fractionation buffer and centrifuged again. The supernatants were combined and centrifuged at $4000 \times g$ for 2 min to pellet a crude fraction showing a high content of plasma membrane and nuclei (fraction PN). The supernatant was transferred to a new tube and the pellet washed with twice the pellet weight of fractionation buffer. The combined supernatants were centrifuged at $100\,000 \times g$ for 2 min to pellet mitochondria, endosomes and lysosomes (fraction EL). Finally, the supernatant was collected in a new tube and centrifuged at $400\,000 \times g$ for 12 min to pellet microsomes (fraction Mi). The final supernatant contained highly purified cytosol (fraction C).

2.4. Separation of endosomes and lysosomes

Lysosomes were isolated from the fraction EL by a 10-min hypotonic lysis according to Bohley et al. (1969) using three to five times the pellet volume of distilled water, depending on the cells used. The amount of water necessary for optimal lysis of lysosomes without a high percentage destruction of endosomes was tested for every cell type: most of the lysosomal marker, but little or no endosomal marker should be found in the supernatant after the lysis. Five times the pellet volume was necessary for EBV-transformed B-cells, whereas three times the pellet volume was sufficient for HTB44 and HaCaT. After another centrifugation step with $100\,000 \times g$ for 2 min, lysosomal material remained in the supernatant (fraction L), while mitochondria and endosomes were in the pellet (fraction E).

2.5. Characterisation

Lysosomes were characterized by the activity of *N*-acetyl-β-D-glucosaminidase (β-AGA; EC 3.2.1.30) determined by a fluorometric assay according to Schmid et al. (1993). β-AGA was considered to be suitable for distinguishing between endosomes and lysosomes, because it is active only in terminal lysosomes (Casciola-Rosen and Hubbard, 1991). Plasma membrane was marked chemically according to Amigorena et al. (1994) using the fluorescence label fluorescein isothiocyanate (FITC) instead of biotin and the following modifications: 1.5×10^9 cells were incubated with 0.6 mg FITC in 10 ml PBS pH 7.4 on ice for 2.5 min. The reaction was stopped by adding 20 ml of fractionation buffer and the cells were washed three times. After fractionation, the specific content of protein-bound fluorescence was detected in the different fractions by high performance size exclusion chromatography (HPSEC) according to Kalbacher et al. (1996). Cytosol was characterized by the activity of lactate dehydrogenase (LDH; EC 1.1.1.27) according to Storrie and Madden (1990). Early endosomes were marked by pulsing the cells with FITC-labelled transferrin (FITC-Tf) and detection of the specific content of protein-bound fluorescence by HPSEC (Max et al., 1993; Kalbacher et al., 1996). DNA was assayed as a nuclear marker according to Karsten and Wollenberger (1977). All signals were finally calculated as relative specific activities *Q*, i.e., the specific activity of a given fraction divided by the specific activity of total cell lysate (de Duve et al., 1955).

The anti-PDI-antibody (microsomal marker) was used to detect microsomal material using immunoblots as described by Max et al. (1993) using 13% sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10 μg of protein per lane. Mitochondrial material was detected using the polyclonal anti α-mitochondria serum in immunoblots. The ECL-system was used for final detection.

2.6. Immunological markers

Using antigen presenting cells, molecules necessary for major histocompatibility complex (MHC) class II-restricted antigen presentation were detected as endosomal markers by immunoblotting. MHC class II molecules were detected using the monoclonal anti β-chain antibody TÜ39 (Ziegler et al., 1986; Klohe et al., 1988) and invariant chain was detected using the monoclonal antibody $SD_3$253.74 (Max et al., 1993).

2.7. Determination of protease distribution

Cathepsin D distribution was determined using radioimmunoassay (RIA) from CIS (Deisenhofen, Germany). Cathepsin B was detected by immunoblot analysis.

3. Results

3.1. Optimal homogenisation for the B-cell line BSM

The fractionation method we describe here was developed using the EBV-transformed B-cell line BSM. As can be seen in Table 1, about 40% of the total protein was collected into fraction De after homogenisation with 10 strokes at 500 rpm in a 5-ml Potter–Elvehjem-homogenisor with a 60-μm gap. As the percentages of organelle markers found in fraction C reveal, this homogenisation procedure led to about 10% of destroyed lysosomes. More rigorous homogenisation conditions decreased the percentage of total protein in fraction De, but increased the percentage of destroyed organelles.

3.2. Distribution of organelle markers in the different subcellular fractions of BSM cells

As can be seen in Table 1, the highest activities of plasma membrane and nuclear markers were found in fractions De and PN. Most of the endosomal marker activity was in fraction E, most of the lysosomal marker was in fraction L and the cytosol marker was mainly found in fraction C. Fig. 1 shows that mitochondria were found in fractions De, PN and E, whereas fraction Mi did in fact contain microsomes

Table 1
Distribution of protein and *Q* of subcellular markers in the different fractions of BSM cells
After differential fractionation and hypotonic lysis (see Section 2), different parameters and organelle markers were determined: protein content, distribution of fluorescence after labelling the cell surface with FITC on ice for 2.5 min, DNA as a nuclear marker, protein-bound fluorescence after pulsing with FITC-Tf as a marker for endosomes, β-AGA as a lysosomal marker and LDH as a cytosolic marker. The distribution of the protein in the different fractions is given as percentages of the total protein. For the markers, *Q* in the respective fractions is given (specific activity of a given marker in a particular fraction divided by the specific activity of this marker in the total cell lysate; de Duve et al., 1955). All markers were measured at least three times except for the plasma membrane marker and FITC-Tf, which were only measured twice.

Fraction	Percentage of total protein	*Q*				
		Plasma membrane marker	DNA (nuclei)	FITC-Tf (endosomes)	β-AGA (lysosomes)	LDH (cytosol)
De	35.9 ± 1.9	1.0 ± 0.2	2.2 ± 0.3	0.5 ± 0.04	1.0 ± 0.1	0.6 ± 0.1
PN	6.8 ± 1.8	5.8 ± 0.6	2.4 ± 0.3	1.8 ± 0.8	2.4 ± 0.2	0.3 ± 0.1
E	4.3 ± 0.5	0.3 ± 0.02	0.4 ± 0.07	6.6 ± 0.3	1.4 ± 0.1	0.1 ± 0.07
L	3.9 ± 0.8	0.7 ± 0.03	0.0 ± 0.0	0.8 ± 0.15	9.0 ± 1.4	0.1 ± 0.02
Mi	18.6 ± 1.6	0.4 ± 0.05	0.1 ± 0.05	0.6 ± 0.05	0.2 ± 0.1	0.6 ± 0.06
C	30.5 ± 3.5	0.4 ± 0.1	0.0 ± 0.0	0.9 ± 0.14	0.5 ± 0.1	2.1 ± 0.2

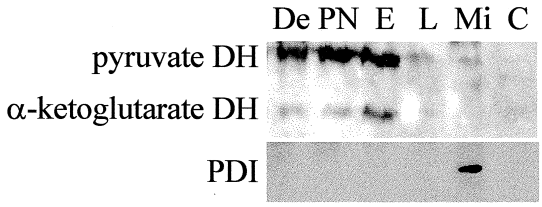

Fig. 1. Distribution of mitochondria and microsomes in subcellular fractions of BSM cells. Immunoblot analysis of the mitochondrial proteins pyruvate dehydrogenase and α-ketoglutarate dehydrogenase and of PDI (microsomal marker) in the different subcellular fractions of the BSM cell line.

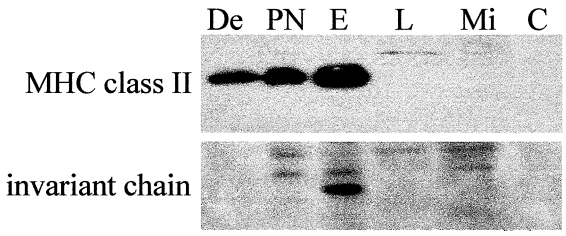

Fig. 2. Immunoblot analysis of MHC class II and Ii. The MHC class II-β-chain and invariant chain were detected using the monoclonal antibodies TÜ39 and $SD_3$253.74 as described in Section 2.

as detected with the respective antibodies on immunoblotting.

Fractions E and L, the fractions of main interest, contained low percentages of cytosolic, nuclear and plasma membrane markers, but significant amounts of their own markers (see Table 1). 85% of the β-AGA activity was released into the supernatant (fraction L) by hypotonic lysis using five times the pellet volume of distilled water, whereas only 12% of the FITC-Tf was found in the supernatant.

3.3. Q of organelle markers for BSM cells

In Table 1, *Q* of the different subcellular markers (activity of a given marker in a particular subcellular fraction divided by the activity of that marker in total cell lysate) and the relative protein content of the fractions are shown. The *Q* of the respective markers in endosomes and lysosomes were high. The graphs for the markers of cytosol and nuclei show that fractions E and L were clearly depleted of cytosolic and nuclear material. The absence of cytosolic compounds is very important for the activity of lysosomal proteases, since the cytosol contains a number of strong protease inhibitors, e.g., cystatins (Abrahamson, 1994).

3.4. Distribution of proteases, MHC class II molecules and invariant chain

As B-cells are antigen presenting cells, molecules involved in antigen presentation can also be used as organelle markers to characterize further the subcellular fractions. The distribution of MHC class II molecules and invariant chain are shown in Fig. 2. Fraction E showed strong signals, whereas no signal was observed for fraction L. The strong signals for MHC class II molecules in fractions De and PN probably reflect the MHC molecules on the plasma membrane.

To determine whether proteases occur in both fractions E and L, cathepsins B and D were detected by RIA and immunoblotting, respectively. As Fig. 3 shows, both proteases, the aspartic protease cathepsin D and the cysteine protease cathepsin B were present in fractions E and L.

3.5. Transfer of the fractionation method to other cell lines

The renal carcinoma cell line HTB44, the keratinocyte cell line HaCaT and the EBV-transformed cell line Cox were used for fractionations to test whether this method would also prove effective for cell lines other than BSM. As can be seen in Table 2, all fractionations showed a very similar distribution of the lysosomal marker enzyme β-AGA after differ-

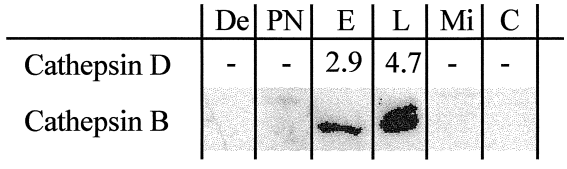

Fig. 3. Distribution of cathepsins B and D in subcellular fractions of BSM cells. The numbers represent nanogram cathepsin D per milligram protein [ng/mg] as determined by RIA (-cathepsin D < 0.2 ng/mg). The blot shows cathepsin B as determined by immunoblot analysis.

Table 2
Q of the lysosomal marker enzyme β-AGA for fractionations of HaCaT, HTB44 and Cox cell lines
In addition to the EBV-transformed B-cell line BSM, the keratinocyte cell line HaCaT, the renal carcinoma cell line HTB44 and the EBV-transformed B-cell line Cox were submitted to the fractionation procedure. *Q* (specific activity of β-AGA of a given fraction divided by the specific β-AGA activity of the total cell lysate) is given for the subcellular fractions of those cell lines.

Fraction	HTB44	HaCaT	Cox	BSM
De	0.9±0.5	1.0±0.0	1.0±0.1	1.0±0.1
PN	2.8±0.2	1.0±0.0	1.2±0.1	2.4±0.2
E	2.0±0.1	2.0±0.1	2.4±0.2	1.4±0.1
L	11.0±3.2	10.0±2.1	16.1±2.5	9.0±1.4
Mi	0.8±0.4	0.5±0.2	0.3±0.1	0.2±0.1
C	0.1±0.1	0.4±0.1	0.8±0.1	0.5±0.1

ential centrifugation and hypotonic lysis using three times the pellet volume of distilled water for HaCaT and HTB44 and five times the pellet volume of distilled water for Cox. Lysosomes showed high *Q* of β-AGA in all cases.

4. Discussion

The fractionation procedure described here is based on a combination of two methods. Differential centrifugation as first described by de Duve et al. (1955) was followed by hypotonic lysis of lysosomes as described by Bohley et al. (1969) to isolate lysosomal enzymes. Homogenisation of the cells was done using a Potter–Elvehjem-homogenisor with a 60-μm gap, as this homogenisation method efficiently destroys the plasma membrane, but largely preserves the other subcellular organelles except for the ER and Golgi. B-cells are very small, which makes them difficult to homogenise completely without destroying the organelles. Homogenisation was therefore performed carefully and a loss of organelle markers and of total protein into the first fraction (fraction De, see Table 1) was considered to be a minor problem compared to destroyed organelles. Therefore, the loss of total protein into fraction De was tolerated in order to retain a high percentage of intact organelles. If too many lysosomes were destroyed during homogenisation, the recovery of lysosomal marker in fraction L would decrease dramatically.

The scheme of differential fractionation employed here gave very good results for the *Q* of the major organelle markers (see Table 1). Plasma membrane and nuclei were almost completely found in fractions De and PN. In particular, fractions E and L were almost devoid of markers for the plasma membrane and nuclei. Cytosol could also be efficiently separated from other subcellular compartments and, once again, fractions E and L showed the lowest contamination of cytosolic compounds.

As the distribution of endosomal and lysosomal markers revealed, it was possible to collect endosomes and lysosomes into one fraction by differential centrifugation and to separate lysosomes from endosomal compartments by hypotonic lysis (see Table 1 and Fig. 2). Fraction E contained mitochondria, whereas fraction L was completely devoid of mitochondrial material (see Fig. 1). Depending on the organelles of interest, fraction E could in fact be designated a mitochondrial fraction. As mitochondria are fundamentally different from endosomes as far as structure and enzymatic equipment is concerned, contamination of these organelles with one another can be tolerated for most investigations. For example, studies on endocytic uptake and processing would not be severely disturbed by mitochondrial enzyme activities.

To investigate whether late endosomes can be found in fraction E or fraction L, several experiments were carried out to determine which types of endosome would be collected into fraction E. Uptake of FITC-Tf was used as a specific marker for early endosomes. In addition, EBV-transformed B-cells are antigen presenting cells and therefore, MHC class II molecules and invariant chain are also suitable markers for endosomal compartments (Neefjes and Ploegh, 1992). All endosomal markers are found in fraction E, but not in fraction L (see Table 1 and Fig. 2). Pulse chase experiments using fluorescence-labelled peptides showed that after about half an hour, the fluorescence reached the lysosomes, but the peptide was completely degraded and only fluorescence-labelled single amino acids could be detected (data not shown). All these data suggest that hypotonic lysis destroys lysosomes and, at the most, some late endosomes, so that almost all stages of endo-

somes are collected into fraction E. This raises the question of the protease content of fractions E and L. Cathepsin B, a thiol protease, and cathepsin D, an aspartic protease, have been detected immunochemically. As expected, the greatest amount of both proteases can be found in fraction L (see Fig. 3), but fraction E also contains significant amounts of these proteases.

To show that our method is generally applicable, the EBV-transformed B-cell line Cox as well as the renal carcinoma cell line HTB44 and the keratinocyte cell line HaCaT were also fractionated. As the results for differential fractionation and hypotonic lysis in Table 2 show, the fractionation procedure works well for other cell types also. For the two EBV-transformed cell lines, five times the pellet volume of distilled water was needed for effective hypotonic lysis. The other two cell types required only three times the pellet volume of distilled water to destroy lysosomes. The reason for this may be that keratinocytes and the renal carcinoma cells are substantially larger than the EBV-transformed B-cells and therefore contain larger, more osmotically sensitive lysosomes.

A great advantage of the method presented here is that both differential centrifugation and hypotonic lysis can be preformed with large amounts of material. This means that large numbers of cells can be fractionated on a preparative scale. The method has been tested in our laboratory with up to 7×10^9 cells in one fractionation. This permits the isolation of huge quantities of endosomes and lysosomes or of any other particular subcellular fraction. Large-scale fractionations are necessary for the isolation of proteins and especially precursors from certain subcellular compartments. Furthermore, studies on endocytosis or proteolytic processing within endosomes may require large-scale preparations.

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