Short Communication

Rapid Isolation of Lysosomal Membranes from Cultured Cells

(lysosomes / lysosomal membrane / methionine methyl ester / gradient centrifugation)

D. MUŠÁLKOVÁ, J. LUKÁŠ, F. MAJER, O. HŘEBÍČEK, E. SVOBODOVÁ, L. KUCHAŘ, J. HONZÍKOVÁ, H. HŮLKOVÁ, J. LEDVINOVÁ, M. HŘEBÍČEK

Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic

Abstract. We present a simple method for enrichment of lysosomal membranes from HEK293 and HeLa cell lines taking advantage of selective disruption of lysosomes by methionine methyl ester. Organelle concentrate from postnuclear supernatant was treated with 20 mmol/l methionine methyl ester for 45 min to lyse the lysosomes. Subsequently, lysosomal membranes were resolved on a step sucrose gradient. An enriched lysosomal membrane fraction was collected from the 20%/35% sucrose interface. The washed lysosomal membrane fraction was enriched 30 times relative to the homogenate and gave the yield of more than 8 %. These results are comparable to lysosomal membranes isolated by magnetic chromatography from cultured cells (Diettrich et al., 1998). The procedure effectively eliminated mitochondrial contamination and minimized contamination from other cell compartments. The enriched fractions retained the ability to acidify membrane vesicles through the activity of lysosomal vacuolar ATPase. The method avoids non-physiological overloading of cells with superparamagnetic particles and appears to be quite robust among the tested cell lines. We expect it may be of more general use, adaptable to other cell lines and tissues.

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Corresponding author: Martin Hřebíček, Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Ke Karlovu 2, Prague 2, Czech Republic. Phone: (+420) 2 2496 7208; Fax: (+420) 2 2496 7119; e-mail: martin.hrebicek@lf1.cuni.cz

Abbreviations: EDTA – ethylenediamine tetraacetic acid, HRP – horseradish peroxidase, LAMP1 – lysosomal associated membrane protein 1, LM – lysosomal membranes, MME – methionine methyl ester, PBS – phosphate-buffered saline, SDH – succinate dehydrogenase, TEA – triethanolamine-EDTA-acetic acid.

Introduction

Lysosomal membranes (LM) are often isolated from biological material for proteomic studies (Schröder et al., 2007a,b; Callahan et al., 2009) or for the study of individual lysosomal membrane proteins (Meikle et al., 1995; Taute et al., 2002). Hypotonic lysis of lysosome-enriched fractions from isopycnic centrifugation on density gradients is a frequently used method for preparation of LM (Meikle et al., 1995). Lysosomes can be purified to high purity by well-established procedures from some animal tissues, for instance from rat liver, which was the principal source of lysosomes for most of the structural and biochemical studies of the organelle. Isolation from other tissues may require procedures tailored to achieve the required enrichment or yield (which are almost as a rule inversely related variables). The ability to isolate the organelles from readily available tissues is especially important in the study of human cells and general isolation procedures may need to be optimized for a specific tissue or for preservation of lysosomal functions (Graham, 2009) – hence the number of papers describing isolation of lysosomes from different tissues or cell lines. We have aimed to develop a simple method for isolation of human lysosomal membranes, which would allow us to perform biochemical studies on lysosomal ghosts – lysosomal membrane vesicles without lysosomal matrix proteins.

Lysosomes, mitochondria, and peroxisomes have similar and partially overlapping densities in sucrose and to a lesser extent in other gradient media, making their full separation based on density alone very difficult. The resolution of lysosomes, however, can be significantly improved by several techniques. Density perturbation of lysosomes in gradients can greatly enhance their separation from other organelles (Graham, 2009). Highly purified lysosomes were isolated from animal tissues by density shift of lysosomes after treatment of animals with Triton WR1339 (Leighton et al., 1968) or dextran (Arai et al., 1991). Mitochondria swell in the presence of calcium ions and become less dense, and addition of $CaCl_2$ in 1 millimolar final concentration to postnuclear supernatant improves their separation from lysosomes in Percoll gradients (Arai et al., 1991).

LM were also successfully isolated by magnetic chromatography after treatment of cultured skin fibroblasts with superparamagnetic magnetite/dextran nanoparticles; lysosomes containing endocytosed particles were retained on the magnetic column and LM were obtained after on-column hypotonic lysis of lysosomes (Diettrich et al., 1998).

Lysis of lysosomes and separation of lysosomal membranes by centrifugation is another technique suitable for enrichment of LM even from complex organelle fractions. Ohsumi et al. (1983) directly treated postnuclear supernatant from rat liver with a hypotonic buffer to lyse lysosomes and collected enriched LM by a four-step differential centrifugation procedure. Also, methyl esters of certain amino acids can be used for selective rupture of lysosomes (Goldman and Kaplan, 1973). They readily cross the lysosomal membrane and enter lysosomes, where they are converted to free amino acids, presumably by lysosomal hydrolases. Accumulation of free amino acids leads to lysosomal swelling and rupture across a wide concentration range. This property was used for disruption of the lysosomal function in tissues (Reeves et al., 1981) and for enrichment of LM from lysosomes (Symons and Jonas, 1987; Schröder et al., 2007a).

Here we present a simple method for isolation of LM from HEK293 and HeLa cell lines taking advantage of selective disruption of lysosomes by methionine methyl ester, which produces lysosomal membrane vesicles retaining the ability to acidify their content.

Material and Methods

HEK293 or HeLa cells from 12–22 confluent 75 cm^2 flasks were washed twice by PBS, collected by scraping, washed once in isotonic TEA buffer (10 mmol/l triethanolamine, 1 mmol/l EDTA Na_2, 10 mmol/l acetic acid, pH 7.2) with 250 mmol/l sucrose and homogenized in the total volume of 8 ml of the same buffer by 10 strokes of tight-fitting pestle B in the glass Dounce homogenizer (Kontes, Kimble Chase Kontes, Vineland, NJ). The homogenization and all further manipulations were performed at 4 °C unless specified otherwise. The homogenate was centrifuged for 10 min at 1,000 × *g* in a swing-out rotor in a tabletop centrifuge; the supernatant was collected and the pellet was re-homogenized by three strokes in the Dounce homogenizer in a total volume of 5 ml of TEA and centrifuged in the same conditions. Both portions of postnuclear supernatant were combined and centrifuged for 15 min at 11,000 × *g* to collect the organelle pellet. The organelle pellet was re-suspended in 8 ml of isotonic HEPES buffer (10 mmol/l HEPES, pH 7.2, 250 mmol/l sucrose, 1 mmol/l EDTA Na_2) and 20 mmol/l methionine methyl ester (MME, Sigma-Aldrich, St. Louis, MO) (Schröder et al., 2007a) and incubated for 45 min at room temperature with stirring. After that the suspension was placed on ice, protease inhibitors were added (Complete, Roche Diagnostic, Mannheim, Germany) to a 1× final concentration, and the suspension was centrifuged at 20,000 × *g* for 20 min. The pellet was resuspended in 8 ml of isotonic HEPES buffer.

The degree of lysosomal lysis was estimated from the amount of hexosaminidase activity released into the supernatant. Samples (200 µl) were taken at 15 min intervals, stored on ice after the addition of protease inhibitors (Complete, Roche), and centrifuged at 25,000 × *g* for 25 min. Supernatants were collected and hexosaminidase and glucocerebrosidase activity was measured as described below.

A linear sucrose gradient was prepared from 15 ml of 32.5 % (w/v) sucrose and 15 ml of 55.5% (w/v) sucrose using a gradient mixer. The gradient was overlaid with 8 ml of MME-treated organelle suspension. Alternatively, a step sucrose gradient was constructed in the following manner: 6 ml of 41% or 35% (w/v) sucrose in 10 mmol/l HEPES buffer was overlaid with 5 ml of 20% sucrose in the same buffer and, finally, by 5 ml of methionine methyl ester-treated organelle suspension. The gradients were centrifuged at 112,700 × g_{max} in SW 32 or SW 32.1 (Beckman-Coulter, München, Germany) overnight without braking and sixteen 1 ml fractions were collected from the top of the step gradient or the band at the 20/41% sucrose interface was collected by a cannula. The linear gradient was fractionated into nineteen 2 ml fractions from the top. The fractions with the highest glucocerebrosidase specific activity were diluted 10 or more times with 10 mmol/l Tris buffer pH 7.2, pelleted by ultracentrifugation at 250,000 × g_{max} for 2 h in 70 Ti rotor (Beckman-Coulter), and flash frozen in liquid nitrogen.

ATP-dependent acidification of lysosomal ghosts prepared by MME-dependent lysis of organelle suspension was determined by the acridine orange absorbance decrease assay as described previously (Dell' Antone, 1979; Moriyama et al., 1982). The reaction solution (1 ml) contained 20 mmol/l HEPES buffer, pH 7.2, 0.2 mol/l sucrose, 50 mmol/l kalium chloride, and 20 µmol/l acridine orange (Sigma-Aldrich). The absorbance of acridine orange was followed at 492 nm at room temperature using a Shimadzu UV-2550 photometer (Schimadzu, Duisburg, Germany) with slits set to 5 nm. Lysosomal membranes (10 µg of protein) were added and the absorbance at 492 nm was allowed to stabilize before the addition of ATP (sodium salt, Sigma-Aldrich) and $MgCl_2$, both to a final concentration of 2 mmol/l. Decrease of the absorbance at 492 nm was followed for more than 2 minutes, after which ammonium sulphate was added to a final concentration of 10 mmol/l and absorbance changes were followed for at least another minute.

The amount of lysosomal membrane protein LAMP1 in fractions during purification was determined by Western blotting. Ten µg of protein from each fraction were separated on a 7–15% gradient SDS-PAGE gel according to Laemmli (1970) and transferred onto PVDF Immobilon-P membrane (Millipore, Bedford, MA) by semi-dry blotting. The membrane was blocked with 3%

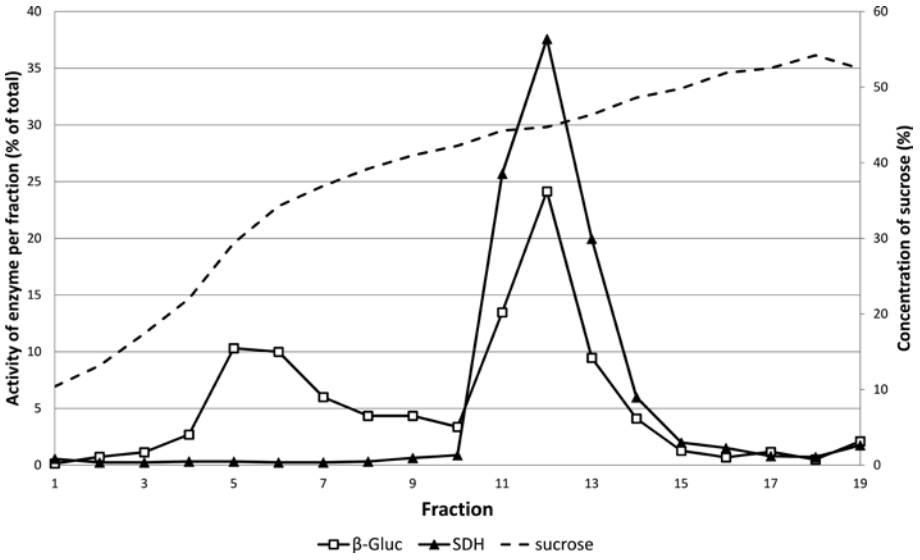

Fig. 1. Resolution of LM fraction from HeLa cells on the linear sucrose gradient
Organelle concentrate prepared from postnuclear supernatant was treated with 20 mmol/l MME for 45 min, overlaid onto linear 32.5%–55.5% sucrose gradient and centrifuged overnight at 112,700 × g_{max}. In fractions the activity of glucocerebrosidase (β-Gluc) and succinate dehydrogenase (SDH) was determined and expressed as percents of the total activity. Sucrose concentration is shown in percents (w/v).

BSA and 0.05% Tween 20-phosphate-buffered saline. The membrane was probed with anti-LAMP1 rabbit polyclonal antibody (1 : 5000, a kind gift of Dr. Carlsson, University of Umea, Sweden) at room temperature for 1 h, washed four times with PBS-Tween and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1 : 3000, Thermo Scientific, Rockford, IL) in PBS-Tween containing 1% BSA. After washing, the blot was developed with an enhanced chemiluminescence (ECL) detection kit (Thermo Scientific).

The activities of marker enzymes succinate dehydrogenase, acid and alkaline phosphatase, catalase, and NADPH – cytochrome *c* reductase were determined as described by Graham (1993). Glucocerebrosidase and total hexosaminidase activity was measured fluorimetrically (Wenger and Williams, 1991). Glucocerebrosidase, which does not have any transmembrane domains, is considered a peripheral lysosomal membrane protein and was used as a marker of lysosomal membrane (Schröder et al., 2007b). Sucrose concentrations in gradient fractions were determined by refractometry. Protein concentrations were measured using the Bradford method (Bradford, 1976).

Results and Discussion

We first attempted to obtain enriched LMs from MME-treated postnuclear supernatant by differential centrifugation according to Ohsumi et al. (1983). The fractions we obtained, however, contained multiple marker enzyme activities and specific activities of lysosomal markers did not suggest enrichment of LM (data not shown). We have therefore fractionated the MME-lysate on a linear 32.5%–55.5% sucrose gradient. There were two peaks of glucocerebrosidase activity – the first at about 30–41% and the second, which also contained significant mitochondrial contamination, at approximately 45% (Fig. 1). We have designed a step gradient (lysate/20% sucrose/41% sucrose) and collected a glucocerebrosidase-enriched membrane fraction from the 20%/41% sucrose interface. The fraction still contained some mitochondrial contamination, which was essentially eliminated by lowering sucrose concentration from 41% to 35% in the gradient – at the same time leading to a decreased yield of enriched LM (Fig. 2).

Lysosomal marker enzyme activity was enriched in the LM fraction recovered from the 20%/35% sucrose interface. We have also followed the amounts of prototypical lysosomal membrane protein LAMP1 (Schröder et al, 2010) in fractions by Western blotting, showing enrichment of the antigen during the purification process (Fig. 3). Glucocerebrosidase activity was enriched on average 15 times (7–22 times, 7 experiments in total); washing of the fraction in 10 mmol/l Tris further increased its specific activity approximately twice (Table 1). In a typical experiment, the postnuclear supernatant contained 89 % of the glucocerebrosidase activity, while organelle pellet retained 77 % activity. The highest of the 20%/35% interface fractions (fraction 11, enrichment relative to the homogenate 14×) contained 12 % of the homogenate glucocerebrosidase activity. The washed pellet from this fraction represented 8.8 % of the initial activity. Contaminating activities were generally low (less than 1 %) with the exception of catalase (2.6 %), suggesting mild contamination with peroxisomes (Table 1). While highly purified LM preparations contain plasma membrane marker proteins (Schröder et al., 2010), presumably originating from the plasma membrane portions entering the endosomal/lysosomal system via endocytosis, it should be noted that

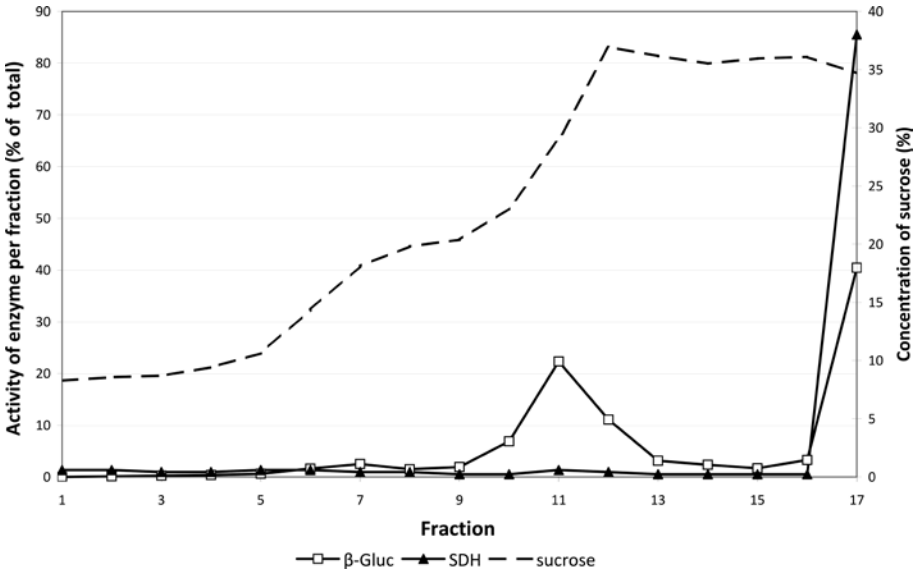

Fig. 2. Resolution of LM fraction from HeLa cells on the step sucrose gradient
MME-treated organelle concentrate was resolved on the step gradient created by overlaying 20% sucrose over 35% sucrose. The gradient was centrifuged overnight at 112,700 × g_{max}. In fractions the activity of glucocerebrosidase (β-Gluc) and succinate dehydrogenase (SDH) was determined and expressed as percents of the total activity. Concentration of sucrose is shown in percents (w/v).

some plasma membrane fragments may also focus on the 20%/35% (41%) sucrose interface (Scott et al., 1993) as LM, as was noted in some experiments. Spurious contamination of the LM fraction by plasma membrane arguably may occur as a result of the more vigorous homogenization.

We have determined activities of total hexosaminidase, a lysosomal matrix enzyme, in 25,000 × *g* supernatants of samples taken during MME treatment of the organelle concentrate as a measure of lysosomal lysis. The supernatant of the sample taken after 15 min contained 36.7 % of total hexosaminidase activity of the sample subjected to MME. The samples taken at 30, 45, and 60 min retained 39.0 %, 47.6 %, and 54.4 % of the initial hexosaminidase activity, respectively. At the same time the total hexosaminidase activity in the sample decreased by 13 % (9.81 nmol/ml/min at 0 min to 8.54 nmol/ml/min at 60 min). The increased concentration of MME (50 mmol/l) did not result in higher release of hexosaminidase into the supernatant. Glucocerebrosidase activity did not increase in the supernatant during the MME treatment. On the basis of these results, 45 min were chosen for MME treatment as a compromise between higher degree of lysosomal lysis and the risk of proteolysis.

We have not attempted to further enrich the core lysosomal membrane proteins by removing peripheral membrane proteins or loosely bound matrix proteins by high-salt washing, although these proteins may contaminate the enriched lysosomal fractions to a significant level. It is of interest that matrix proteins may associate, even temporarily, with the lysosomal membrane (Jadot et al, 1979) and some lysosomal proteins, including glucocerebrosidase, apparently exist in luminal and membrane-bound form (Imai, 1985).

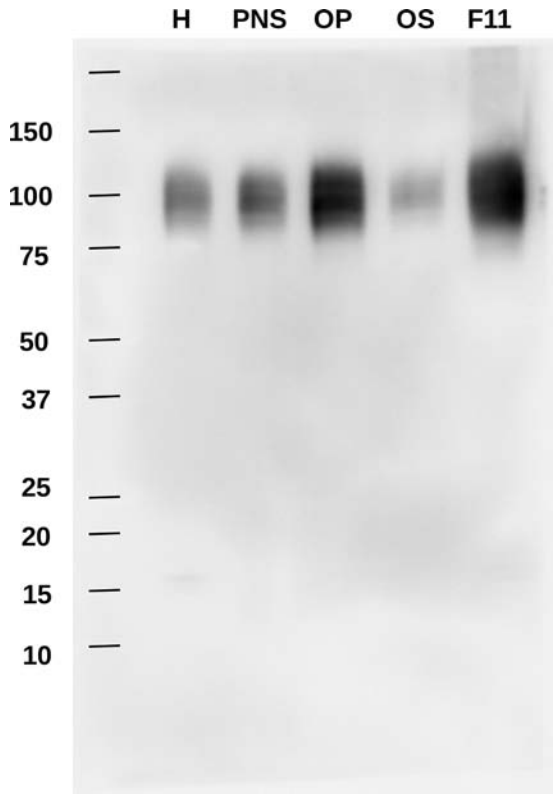

Fig. 3. Western blotting of subcellular fractions with anti-LAMP1 antibody
Ten μg of protein from each fraction were loaded to individual lanes. From the left: homogenate (H), post-nuclear supernatant (PNS), organelle pellet (OP), post-organellar supernatant (OS), and washed lysosomal membranes (F11). Positions of molecular weight markers in kilodaltons are shown on the left. Note the typical blurred appearance of the bands, which is assumed to be due to differential glycosylation.

Table 1. Purification of lysosomal membranes from HeLa cells. Typical values for protein concentration and glucocerebrosidase activity are shown.

Fraction	Protein		Glucocerebrosidase		
	Total (mg)	Yield (%)	Yield (%)	Specific activity (nmol/mg/min)	Purification factor
Homogenate	49.5	100.0	100.0	1.31	1.0
Nuclear pellet	8.1	16.4	8.4	0.67	-
Postnuclear supernatant	37.8	76.3	88.6	1.53	1.2
Organelle pellet	9.7	19.6	77.1	5.17	3.9
Postorganellar supernatant	21.3	43.1	11.2	0.34	-
LM Fraction (Fraction 11 from the sucrose gradient *#)	0.4	0.9	12.0	18.24	13.9
Washed LM fraction	0.1	0.3	8.8	38.5	29.6

* Organelle pellet was divided into two portions overlaid over two identical sucrose gradients. The values here are averaged from both gradients.
# Fraction 11 of the sucrose gradient had the highest specific glucocerebrosidase activity and was considered the LM fraction (see Fig. 2).

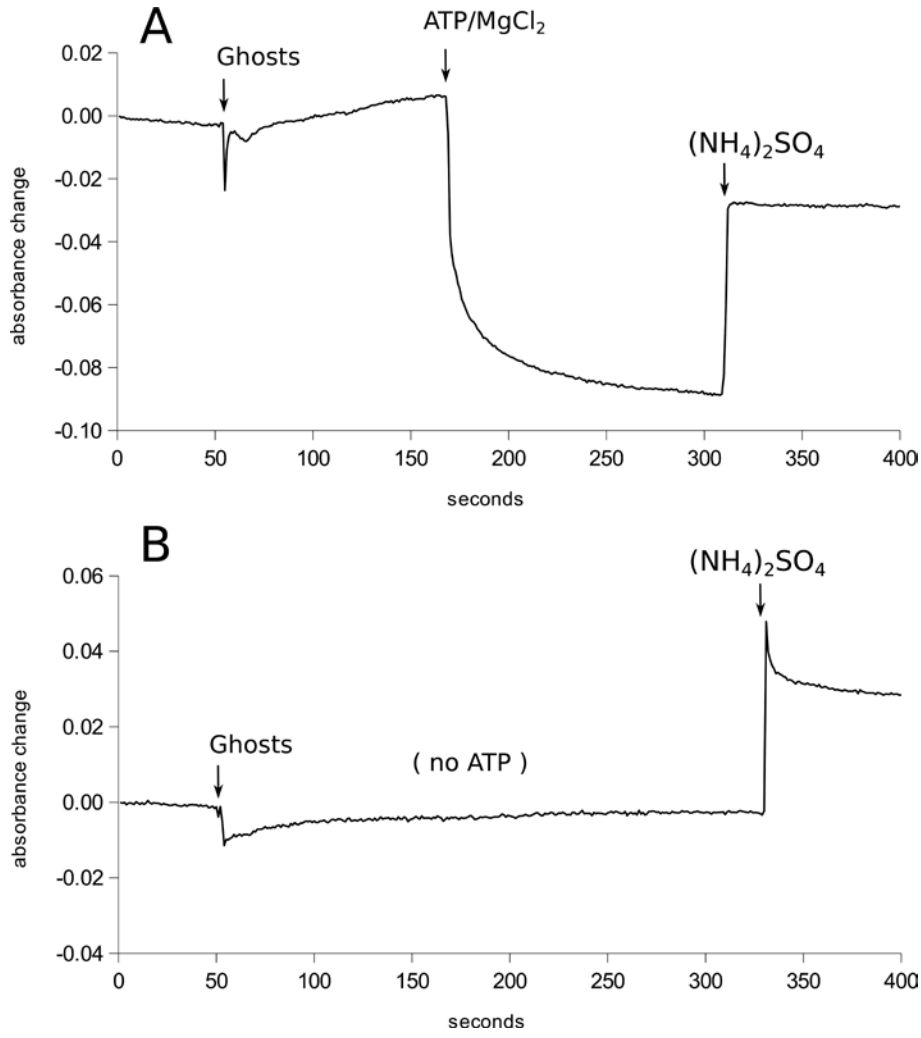

Fig. 4. Acidification of lysosomal ghosts after the addition of ATP
Acidification of washed lysosomal membrane vesicles was measured as the decrease of acridine orange absorbance at 492 nm. The reaction solution (1 ml) contained 20 mmol/l HEPES buffer, pH 7.2, 0.2 mol/l sucrose, 50 mmol/l kalium chloride, and 20 μmol/l acridine orange (Sigma-Aldrich). Lysosomal ghosts (10 μg of protein) were added (Ghosts) and the following reagents were added at the time points depicted by arrows: ATP and $MgCl_2$ (panel A), both to a final concentration of 2 mmol/l, and ammonium sulphate to a final concentration of 10 mmol/l. When ATP was omitted (panel B), the decrease of absorbance did not occur.

The washed lysosomal membranes were acidified after the addition of ATP (Fig. 4). The activity of the multiprotein complex of vacuolar ATPase, the proton pump residing in the lysosomal membrane which is responsible for the acidification of lysosomes, was apparently preserved in samples of enriched lysomal membrane fractions. Addition of ammonium sulphate (final concentration 10 mmol/l) resulted in alkalization of the membrane vesicles (Fig. 4).

We have sought to develop a simple method for the isolation of LM that would not require non-physiological overloading of the lysosomal/endosomal system with particles or detergents as the resulting cells are clearly abnormal. In our hands, the method of Ohsumi et al. (1983), originally optimized for rat liver tissue and based on hypotonic treatment of postnuclear supernatant followed by differential centrifugation, did not provide LM in sufficient yields and purity from HEK293 cells. Osmotic lysis of lysosomes with methyl esters of leucine or methionine, highly specific for the target organelle, was chosen for the release of LM, which were subsequently resolved on the step sucrose gradient. The enriched LM fraction could easily be collected from the 20%/35% interface. The procedure effectively eliminated mitochondrial contamination, minimized contamination from other cell compartments and appeared to be sufficiently robust. While it did not yield LM of very high purity, the enrichment was comparable to the magnetic chromatography technique developed by Diettrich et al. (1998). The method yielded similar results both for HEK293 and HeLa cell lines, suggesting that it may be adapted to other cell lines or possibly tissues.

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