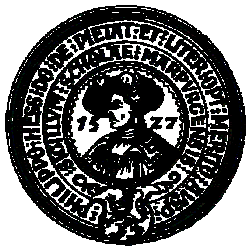
Aus dem
Institut für Physiologische Chemie
der
Philipps-Universität Marburg
Geschäftsführender Direktor: Prof. Dr. Andrej Hasilik

Arbeitsgruppe Biochemie und Pathobiochemie des lysosomalen Apparates
Leiter: Prof. Dr. Andrej Hasilik

Rapid purification of human lysosomal membranes, characterisation of the detergent resistant microdomains, and purification and reconstitution of the vacuolar proton pump (V-ATPase).



INAUGURAL DISSERTATION

Zur Erlangung des Doktorgrades der Humanbiologie

(Dr. rer. physiol.)

dem Fachbereich Humanmedizin

der Philipps-Universität Marburg

vorgelegt

von

Rajesh Chandramohanadas

aus

Haripad, Kerala, Indien

Marburg 2006

Angenommen vom Fachbereich Humanmedizin der Philipps-Universität Marburg
am 30. 03. 2006

Dekan: Prof. Dr. B. Maisch

Referent: Prof. Dr. A. Hasilik

Koreferent: Prof. Dr. Aumüller

Dedicated to my family!

INDEX

Figures

Tables

1 Introduction

Lysosomes, the acidic subcellular compartments serve as one of the major sites for intracellular degradation. This function is attributed to a broad variety of hydrolytic enzymes and transport proteins that function in tandem. The substrates for degradation are targeted to the lysosomes mainly by vesicular transport such as endocytosis and autophagocytosis. Most of the lysosomal matrix proteins are soluble hydrolases whereas the transmembrane proteins are responsible for transport processes across the membranes. They are also involved in communication with the surrounding cytosol and other organelles like late endosomes. These proteins bear one or more membrane spanning segments with their luminal protrusions and loops usually glycosylated as a means of protection against lysosomal self-digestion (Peters and von Figura, 1994).

One of the transport proteins, the vacuolar ATPase (V-ATPase) is in charge of acidification of the lysosomes and the others are concerned with disposal of degradation products. The turn-over of cellular proteins, down-regulation of surface receptors, release of endocytosed nutrients, inactivation of pathogenic organisms, repair of plasma membrane, and processing of antigens to be loaded for antigen presentation are the other functions, these proteins are involved in (Eskelinen *et al.,* 2003).

There exist at least 40 distinct inherited disorders sharing the common name lysosomal storage disorders, caused by a deficiency in a single lysosomal enzyme or a cofactor leading to the accumulation of one or several natural compounds in the lumen of the lysosomes (Winchester *et al.,* 2000). In diseases like Hermansky-Pudlak syndrome (White, 1982), the membrane and morphology of the lysosomes are affected that alter immunological as well as pigmentation functions. The functions of several lysosomal membrane proteins are known as exemplified by the corresponding genetic diseases, those of LAMP II in Danon disease (Nishino *et al.,* 2000), Sialic acid transporter in Salla disease (Verheijen *et al.,* 1999) and Cystine transporter in Cystinosis (Town *et al.,* 1998). If the vacuolar type ATPase on the lysosomal membrane functioning as the proton pump to maintain the acidic environment of the lysosomal lumen is inefficient in either cleaving the ATP or transporting protons, accumulation of lipofuscin derivatives in the retinal pigment epithelial cells results. Accumulation of lipofuscin derivatives is one factor behind the pathology of age-related macular degeneration (Bergmann *et al.,* 2004) that results in a loss of vision.

The lysosomal hydrolases, especially several cysteine proteases which are known to be part of the degradation machinery, are involved in specialised functions within the cells, as suggested by their roles in matrix remodeling, regulating the immune responses, and involvement in certain lung diseases (Wolters and Chapman, 2000). From this perspective, a proteomic investigation of the lysosomal protein complexes, mainly those are associated with the membrane not only provides an overall view of the number and variety of proteins that are part of the organelle, but also of the special interactions among these proteins and with the membrane lipids.

1.1 Lysosome-the organelle

Christian R. de Duve, a Belgian scientist was the first to identify the degradative bags within the cells in 1949, which were later termed lysosomes (de Duve, 2005). He was investigating hepatic enzymes that are involved in diabetes. The experiments for separating the components of cells by centrifugation resulted in the release of acid phosphatase proportionately to the damage of cell compartments during the homogenisation and subfractionation procedures (de Duve *et al.*, 1955). De Duve proposed that the cells contain membranous sacs enclosing acid phosphatase and functioning as `digestive bodies`. In 1974, he was rewarded for this discovery with the Nobel Prize in medicine that he shared with Albert Claude and George Palade.

The polymorphisms among the lysosomal populations have been well studied (Rome *et al.,* 1979, Kelly *et al.,* 1989) where the sub populations differ in their density as well as morphology. The denser lysosomes are rather spherical small bodies while the more buoyant ones are bigger multi-vesciculated structures. Both these populations contain the markers for lysosomes in similar proportions, such as the acid phosphatase. It has also been reported that oxidative stress results in variability among the lysosomal populations not only among different cell types, but also among the cells of the same type (Nilsson *et al.,* 1997).

The lysosomal membrane, a dynamic platform of 7-10 nm thickness (Winchester *et al*., 2000) with a low electron density on the luminal face. It is composed mainly of phospholipids, cholesterol, and the resident membrane proteins. From the rat liver lysosomes, the phospholipid composition of the membrane has been evaluated. The results show high proportions of phosphatidyl choline (41.6%), phosphatidyl ethanolamine (27.3%), phosphatidyl inositol (9.4%) and sphingomyelin (9.1%) (Bleistein *et al.,* 1980). Among the fatty acids in the phospholipids, those with 16-20 carbon atoms were the most abundant

(Henning and Heidrich, 1974). These membranes are also enriched in cholesterol with cholesterol:phospholipid ratio of 0.4-0.5 (mol/mol) reported from tritosomes. They contained LDL-cholesterol, esterified cholesterol, and triglycerides (Johnson *et al.,* 1997, Schoer *et al.,* 2000). Presence of detergent resistant microdomains on the lysosomal membranes prepared from human placenta has been reported (Taute *et al.,* 2002), where special proteins are localised, but more information is yet to be obtained.

1.2 Synthesis and trafficking of lysosomal proteins

The lysosomal membrane proteins such as the acid phosphatase precursor may follow the constitutive secretory pathway via the cell surface and endocytosis (Hunziker and Geuze, 1996). However, the delivery of most of these proteins appears to proceed through a direct pathway after exiting the *trans*-Golgi network in clathrin-coated vesicles and fusing with early and late endosomes (Rouille *et al.,* 2000). The sorting process is highly dependent on specific signals encoded in the cytosolic domain of these proteins. The lysosomal targeting signals are either tyrosine-based or di-leucine- based. These signals are necessary for the binding of clathrin adapter proteins (APs) either at the *trans*-Golgi network (AP-1) or at the plasma membrane (AP-2) (Honing *et al*., 1996). The third member of this family, AP-3 operates through a distinct mechanism where it`s release from the membranes is regulated by a small GTP binding ADP-ribosylation factor, the *Arf*. The GTP hydrolysis by the *Arf* is the key step in detachment of AP-3 complex from the membranes (Simpson *et al*., 1997). The AP-4 is involved in protein sorting from TGN or endosomes to lysosomes (Dell`Angelica *et al*., 1999).

The GGAs (Golgi-localised γ-ear-containing, *Arf* binding proteins) are a group of recently identified (Hirst *et al.,* 2000) highly conserved clathrin adaptor proteins facilitating cargo transport and vesicle formation at the TGN (Boman, 2001). Their involvement in the trafficking of cargo molecules such as mannose-6-phosphate receptors has been reported (Ghosh *et al.,* 2003).

Table. 1.1: Endosomal/lysosomal sorting signals (Bonifacino and Traub, 2003)

Signal type	Recognition protein/domain	Function
NPXY	Clathrin terminal domain	Internalisation
	AP-2 μ^2 subunit	
	PTB domain of Dab-2	
YXX∅	μ subunit of AP complexes	Internalisation

		Lysosomal targeting Basolateral targeting
[DE] XXXL[LI]	μ and ß subunits of AP complexes	Internalisation Lysosomal targeting Basolateral targeting
DXXLL	VHS domain of the GGAs	TGN to endosomes
Acidic cluster	PACS-1	Endosomes to TGN
FW-or P-rich	TIP47	Endosomes to TGN
NPFX(1,2)D	SHD1 domain of *S. cer* S1a1P	Internalisation
Ubiquitin	UBM, UBA and UBC domains	Internalisation Lysosomal/vacuolar targeting

Amino acids are designated based on single letter codes. X stands for any amino acid and ∅ for amino acids with bulky hydrophobic chains. Abbreviations: PTB, phospho tyrosine binding; Dab2; disabled-2; AP, adapter protein; VHS; domain present in vps27p, hrs, stam; GGAs, Golgi-localized γ-ear-containing, *Arf* binding proteins; PACS-1, phosphofurin acidic cluster sorting protein 1; TIP47, tail interacting protein of 47 kDa; SHD1; sla 1p homology domain 1; UBA, ubiquitin associated; UBC, ubiquitin conjugating; UIM, ubiquitin interaction motif

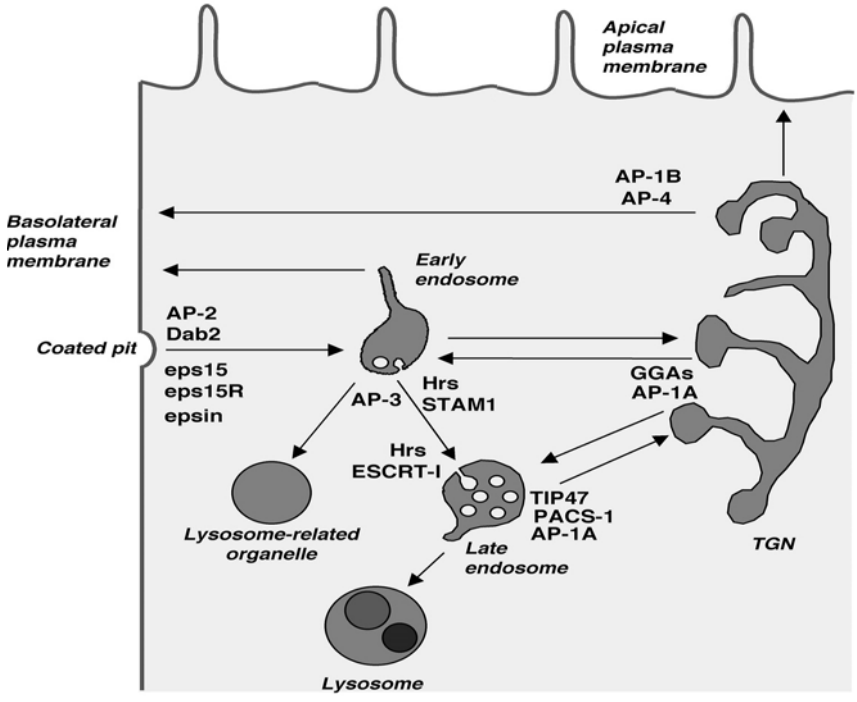


Fig. 1.1: Sorting pathways for lysosomal membrane proteins (Bonifacino and Traub, 2003)

Though these systems for the trafficking of membrane proteins in to the lysosomal compartment are well studied, a possibility of alternate mechanisms operating for special proteins cannot be excluded.

The lysosomal matrix proteins are synthesised in the rough endoplasmic reticulum (ER) with a signal peptide for their targeting to the ER lumen. Removal of this signal peptide is followed by *N*-glycosylation and the proteins are transferred to the *cis*-Golgi complex in transport vesicles. A phophotransferase within the Golgi recognises the proteins and modifies them with N-acetyl glucosaminyl phosphoryl residues (Yaghootfam *et al*., 2003). In *trans*-Golgi, the diester residues are hydrolysed thus uncovering the terminal mannose-6-phosphate residues (Steet *et al*., 2005). These unmasked mannose-6-phosphate moieties are recognised by the mannose 6-phosphate receptors that bind and carry the proteins to early endosomes. From the late endosomes, the receptors are recycled to the *trans*-Golgi network.

1.3 Lysosomal membrane proteins

The lysosomal membrane contains a number of proteins with diverse functions. There are more than 20 transport proteins that are characterised functionally on the lysosomal membranes- like those involved in the transport of amino acids and peptides (Pisoni *et al.,* 1987, Zhou *et al.,* 2000), carbohydrates (Mansini *et al.,* 1990, Jonas and Jobe, 1990), vitamin B_{12} (Idriss and Jonas, 1991), phosphate (Pisoni 1991), sulphate (Jonas and Jobe, 1990) and nucleosides (Pisoni and Thoene, 1989). However, little information regarding these proteins is available to date. In relative abundance, about half of the total membrane proteins in lysosomes are lysosome associated membrane proteins (LAMPs) and lysosomal integrated membrane proteins (LIMPs), which differ in the number of transmembrane segments and glycosylation patterns.

1.3.1 The LAMPs

LAMPs are a group of type I membrane proteins with single membrane spanning segmant and a short cytosolic C-terminus of 10-20 residues (Wickner and Lodish, 1985) where the lysosomal targeting signal is situated. In humans there are 2 forms of LAMP denoted as LAMP-1 (90-120 kDa) and LAMP-2 (110-120 kDa). These proteins carry complex N-linked as well as O-linked oligosaccharides. Their expression in cancer cells are characterised by increased sialylation and ß1-6-linked branching within the complex-type asparagine (Asn)-linked oligosaccharides as compared to the normal cells (Furuta *et al.,* 2001). The functions of LAMP proteins include autophagy (Majeski and Dice, 2004) and lysosomal

biogenesis (Eskelinen *et al.,* 2003). LAMP-1 is considered to be house keeping protein that can be functionally substituted with LAMP-2, whereas a deficiency of LAMP-2 leads to Danon disease characterised by fatal cardiomyopathy and myopathy (Nishino *et al.,* 2000, Saftig *et al.,* 2001). Initially, this disease was biochemically characterised as a vacuolar glycogen storage disease (Uzuki *et al.,* 1994) with normal acid glucosidase activity. Later, it was observed that in affected cells, autophagosomal sequestration is functioning, whereas the delivery of the lysosomal enzymes to autophagosomes is missing (Nishino, 2003).

1.3.2 The LIMPs

The LIMPs are type-III membrane proteins with both the N and C termini oriented towards the cytosolic side (Barriocanal *et al.,* 1986). LIMP-1 also called LAMP-3 (identical to CD 63) is a membrane protein with four transmembrane segments while LIMP-2 or LGP 85 (Tabuchi *et al.,* 1997) has two membrane spanning segments. LIMP-II is reported to have important functions in maintaining endosomal transport as well as a role in lysosomal biogenesis (Eskelinen *et al.,* 2003).

1.3.3 Lysosomal proton pump (vacuolar ATPase)

The lysosomal membrane ATPase complex belongs to the family of vacuolar proton pumps (V-ATPase). It performs the acidification of intracellular compartments such as endosomes, lysosomes and secretory vesicles. For the first time it was purified to homogeneity from rat liver lysosomes (Arai and Shimaya, 1992). The acidification of these subcellular organelles by vacuolar ATPase (V-ATPase) is of high importance for many physiological functions including receptor mediated endocytosis, intracellular traffic, zymogen activation and degradation, homeostasis, neurotransmitter storage and release and entry of certain viruses and toxins from endocytic compartments into cytosol (Nelson, 1992, Nishi and Forgac, 2002). In specialised types of insect and vertebrate cells, V-ATPases are seen in high amounts in the plasma membrane where they pump protons from cytosol out of the cell (Gluck, 1992).

Similar to the mitochondrial enzyme, the vacuolar ATPase consists of two domains (Nelson N, 1989). They are referred to as a water soluble V_1, and a membrane bound V_0 domain. ATP hydrolysis taking place on the V_1 is coupled to proton transport through V_0. The V_1 domain is comprised of 8 subunits with a total molecular weight of 600- 650 kDa and a subunit stoichiometry A_3 B_3 C_1 D_1 E_1 F_1 G_2 $H_{1\text{-}2}$. The membrane inserted V_0 domain forms a complex of 250 kDa with a subunit structure, a_1 d_1 $e_{(x)}$ c_4 c` c `` (Arai *et al.,* 1988). Most of the subunits are conserved to a high extent in all eukaryotes. In addition to the above-

mentioned subunits there are two accessory subunits mentioned as Ac45 (Supek *et al*, 1994) and M8-9 (Ludwig *et al*, 1998) are reported. The vacuolar ATPase subunits share sequence similarities with mitochondrial, chloroplast, and bacterial enzymes reflecting an evolutionary relationship (Kane, 1995). Depending on the tissue, several subunits are represented by alternative forms.

The ATP driven proton transport depends on a functional and structural coupling of the two sectors. A reversible disassembly of the domains seems to be an important regulatory mechanism for silencing the ATPase activity and conserving the ATP (Merzendorfer *et al.*, 1997). It should be mentioned that in eubacteria and plants several subunits that are part of the catalytic domain could dissociate from the complex upon cold incubation rendering the enzyme biologically inactive (Moriyama and Nelson, 1989).

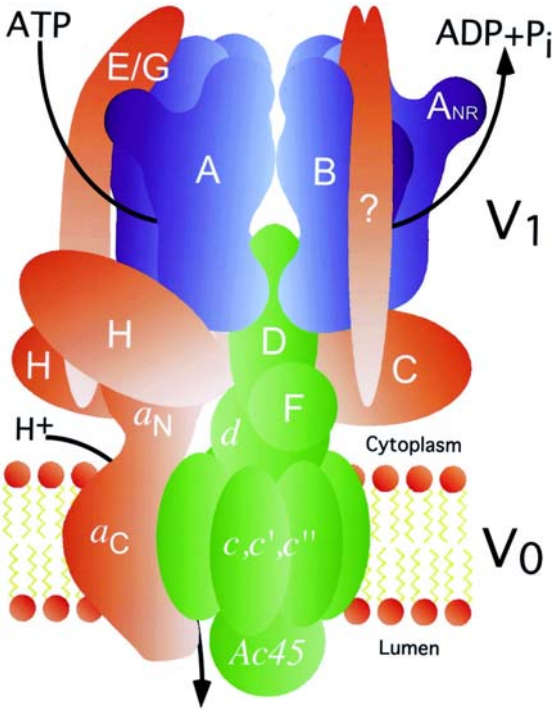


Fig. 1.2: Structure of yeast vacuolar ATPase (*S. cerevisiae*).

(Wilkens *et al*., 2004)
The catalytic V_1 domain is coupled to the membrane-inserted V_0 domain, responsible for proton translocation. The catalytic sites (A_3B_3) are kept in position by the stator subunits (C,E,G,H and a). Upon ATP cleavage the rotatory subunits (D and F) effects a structural re-arrangement and proton translocation is achieved by a combined functioning of a,c,c`and c`` thereby acidifying the luminal compartments.

Many of the vacuolar ATPase subunits are expressed as tissue-specific isoforms. A complete list of vacuolar ATPase subunits in humans and their isoforms is given below (Table. 1.2). The nominal molecular weights and theoretical isoelectric points are calculated using the Expasy proteomics server (http://www.expasy.ch/).

Table. 1.2: Human V-ATPase subunits and isoforms

Domain	Subunit	MW (kDa)	pI	Gene code	Function and location within the complex
V_1	A	70	5.3	ATP6V1A	catalytic ATP binding
	B1	56	5.52	ATP6V1B1	non catalytic ATP binding
	B2	58	5.57	ATP6V1B2	non catalytic ATP binding
	C1	42	7.02	ATP6V1C1	peripheral stator
	C2	42	5.72	ATP6V1C2	peripheral stator
	D	34	9.36	ATP6V1D	central rotor
	E1	31	7.7	ATP6V1E1	peripheral stator
	E2	26	8.79	ATP6V1E2	peripheral stator
	F	14	5.29	ATP6V1F	central rotor
	G1	13	8.93	ATP6V1G1	peripheral stator
	G2	13	10.26	ATP6V1G2	peripheral stator
	G3	13	9.16	ATP6V1G3	peripheral stator
	H	50	6.07	ATP6V1H	peripheral stator
V_0	a1	100	6.02	ATP6V0A1	peripheral stator, H^+ pump
	a2	98	6.18	ATP6V0A2	peripheral stator, H^+ pump
	a3	93	6.58	ATP6V0A3	peripheral stator, H^+ pump
	a4	96	5.75	ATP6V0A4	peripheral stator, H^+ pump
	d1	40	4.89	ATP6V0D1	non integral component
	d2	40	5.18	ATP6V0D2	non integral component
	c	16	7.98	ATP6V0C	rotor, H^+ translocation
	c`	21	7.61	ATP6V0B	rotor, H^+ translocation
	c``	9	8.96	ATP6V0E	rotor, H^+ translocation

There are reported impairment of biological activity of this protein complex in the retinal pigment epithelial cells due to accumulation of lipids and lipid peroxidation products (Kopitz *et al*., 2004). Such events alter the lysosomal pH and accumulation of auto-fluorescent products occur leading to age-related macular degeneration (ARMD) and vision loss. It has been shown that a major lipofuscin derivative A2-E can inhibit the lysosomal proton pump on a dose-dependent manner (Bergmann *et al*., 2004) though there are several environmental as well as genetic factors mentioned playing a role in the pathogenesis of ARMD (Klein *et al.,* 2004, Zurdel and Richard, 2002).

The pyridinium retinoid, A2-E is derived from two molecules of vitamin A, and one molecule of ethanolamine and thus acquired the name.

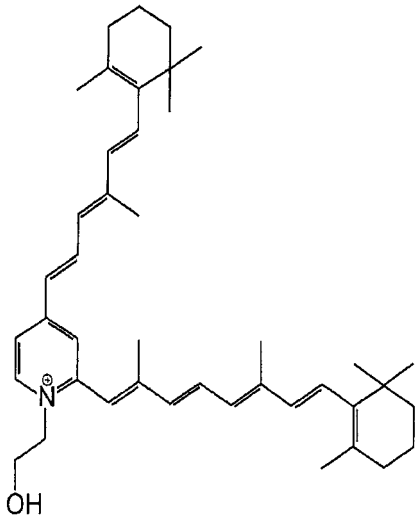


Fig. 1.3: The Structure of A2-E
(Kopitz *et al*., 2004)

1.3.4 Niemann-Pick C1 protein

The Niemann-Pick C1 (NPC-1) protein involved in cholesterol trafficking is composed of 1278 amino acids, bearing 13 potential transmembrane segments and 14 potential glycosylation sites. NPC-1 localises mostly to the endosomes (Ioannou, 2000). The di-leucine motif required for the lysosomal targeting is conserved in a short cytoplasmic tail region. There exist multiple signals for the proper targeting of this protein including the one located at the sterol-sensing domain of the protein (Scott and Ioannou, 2004). A defect in this protein leads to the pathological condition termed Niemann-Pick C disease, characterised by cholesterol accumulation in lysosomes and a severe progressive neurodegeneration (Maxfield and Tabas, 2005). A feedback regulation of cholesterol homeostasis within the cells (Davies *et al.,* 2000) suggests its role in transporting LDL derived-cholesterol. The recent investigations showing an abundance of this protein in the axons of sympathetic neurons and recycling endosomes in the pre-synaptic nerve terminals suggests a relatively uncharacterised role of this protein in the pre-synaptic nerve terminal (Karten *et al*., 2005).

1.3.5 Acetyl CoA: α-glucosaminide N-acetyl transferase

Acetyl CoA: α glucosaminide *N*-acetyl transferase (EC 2.3.1.3) is involved in the degradation of heparan sulphate within the lysosomal compartment. It transfers acetyl groups to the terminal alpha glucosamine residues that become exposed during the step-wise degradation of heparan sulphate (Bame and Rome, 1986). The enzyme is shown to function in a two-step reaction comprising of (1) acetylation of the enzyme and then (2) transferring the acetyl group to glucosamine residue through a ping- pong mechanism. An irreversible and specific inhibition of the enzyme activity by *N*-bromosuccinimide indicates the presence of an active

site histidine residue. Association of this protein with detergent resistant microdomains within the lysosomal membrane has been recently reported by our group (Taute *et al.,* 2002).

A deficiency of this enzyme is the cause of the Sanfilippo- C disease also called mucopolysaccharidosis type IIIC. This disease is characterised by dysmorphic signs including coarse facies, hypertelorism, low-set ears, depressed nasal bridge and coarse hair in the early childhood (Sewell *et al.,* 1988).

At present, there is not much information available about either the structure of the protein or the gene that codes the enzyme. A genome wide scans on 44 MPS IIIC patients and their unaffected relatives from 31 families were carried out recently. Several of the families were consanguineous. An analysis of excess homozygosity in patients and identity in state of genotypes among affected relatives indicated the gene to be localised on chromosome 8. From a linkage analysis (Ausseil *et al.,* 2004) it was suggested that an 8.3-cM interval in the pericentromeric region of chromosome 8 encodes this protein.

Using biochemical techniques, an acetylated transmembrane protein from the placental lysosomal membranes was isolated, which was missing in the MPS type IIIC (Ausseil *et al*., 2006). The specific activity of this candidate protein of 240 kDa molecular weight was highly dependent on the concentration suggesting an oligomeric nature of the protein complex.

1.3.6 The CLN proteins

The neuronal ceroid lipofuscinoses are a group of recessively inherited storage disorders in which autofluorescent inclusions of lipofuscin are observed in the lysosomes. The 8 different forms of this disease share similar clinical and pathological abnormalities. The main features of this disease are failure of psychomotor development, impaired vision, seizures, and premature death. The proteins deficient in this disorder are either transmembrane proteins (CLN3 and CLN8) or lysosomal hydrolases (CLN1, CLN2 and CLN5) (Mitchison and Mole, 2001, Zhong *et al.,* 2000).

The CLN3 gene product is a 438 amino acid long peptide with 5 transmembrane segmants and a dileucine motif at the C terminal domain. This protein is defective in the juvenile form of ceroid lipofuscinosis (Boriack and Bennet, 2001). There are recent reports that both CLN6 (Mole *et al.,* 2004) and CLN8 (Lonka *et al.,* 2000) are resident ER membrane proteins.

The CLN1 protein is a palmitoyl protein thioesterase that is localised in the lysosomal matrix. The CLN2, a lysosomal matrix serine protease with a tripeptidyl peptidase-1 activity is defective in the late infantile neuronal ceroid lipofuscinosis. This is characterised by accumulation of the subunit c of ATP synthase within the lysosomes (Ezaki *et al.,* 1999). It has been reported to possess interaction with the matrix protein CLN3 as well as the membrane protein CLN5 (Vesa *et al.,* 2002). However the recent results suggest the CLN5 protein of 60 kDa molecular weight to be a lysosomal matrix glycoprotein (Isosomppi *et al.,* 2002).

1.3.7 Other transport proteins in the lysosomal membrane

In addition to the above-mentioned, there are a few relatively uncharacterised proteins located on the lysosomal membrane. Among them is an anion transporter, the mutation of which causes an autosomal recessive neurodegenerative disorder called sialic acid storage disease. The lysosomes are enlarged and abnormal amounts of sialic acid are excreted through urine in the affected individuals. The gene coding for the protein was designated *SLC17A5* generating a polypeptide of 57 kDa (Verheijen *et al.,* 1999).

Cystinosis is another common lysosomal storage disorder caused by a defect in the transport of cystine out of the lysosomes (Town *et al.,* 1998). The gene responsible for this defect, mentioned *CTNS* is mapped to chromosome 17p13 and codes a 367aminoacid long polypeptide that does not require ATP for transporting cystine (Gahl *et al.,* 1993). The pathology is associated with less opacities and renal tubular dysfunction leading to a fatal electrolyte imbalance.

The ATP-binding cassette transporters are involved in the energy-dependent transport of a variety of substrates across the organellar membranes. A few members of this category have been identified to be residing in the lysosomal membrane including ABCA2 (Zhou *et al.,* 2000), which was cloned and characterised recently (Vulevic *et al.,* 2001). Another transmembrane protein, designated ABCB9 with a lysosomal localisation was cloned (Zhang *et al.,* 2000). This protein with an unknown function is phylogenetically connected to TAP1 and TAP2 found in the endoplasmic reticulum. The new member of this group is ABCA5, a protein composed of 1642 amino acids. An experimentally created defect of this protein showed symptoms of several lysosomal diseases in mouse (Kubo *et al.,* 2005), but the function is not yet known.

2 Aims and objectives

The lysososomal membrane carries a number of highly hydrophobic proteins involved in the transport of nutrients and cleaning up of the degradation products. The transport processes these proteins are involved in are of high importance as a defective protein can lead to accumulation of bio-molecules within the lysosomes with lethal consequences. Though functionally characterised, there are several proteins that are not isolated or cloned. The main goal of this study is to purify the lysosomal membrane proteins from placental preparations and analyse the protein complexes associated with the compartment.

The lysosomal membrane provides a platform where interactions among proteins and in between proteins and lipids take place forming supra-molecular complexes. Presence of detergent resistant microdomains (DRM) on the lysosomal membranes have been published (Taute A *et al.*, 2003), where special proteins are found to be enriched including the Acetyl-CoA: α-glucosaminide N-acetyl transferase, a mutation of which causes Sanfilippo- C syndrome. Though the pathology is known, there is not much information currently available about neither the protein nor the gene involved. At this perspective, a proteomic analysis of the microdomains on the lysosomal membrane will provide a new insight into the number and variety of proteins associated with the DRMs, the kind of interactions the proteins possess with the lipids and a possibility to identify and characterise the Sanfilippo C protein.

The vacuolar ATPase required for the acidification of intra-cellular compartments have been mentioned to be DRM associated (Galli *et al.*, 1996, Blonder *et al.*, 2004, Yoshinaka *et al.*, 2004), and characterisation of DRMs from the lysosomal membrane might improve the current information regarding this observation. Isolation of the V-ATPase complex can be helpful in understanding the role of special lipids on the biological activity by reconstitution experiments. These investigations can be of importance as the lipid and lipid peroxidation products are reported inhibitors of V-ATPase *in vivo*, resulting in pathological conditions such as age-related macular degeneration. Isolation and reconstitution of this protein complex can be thus useful as some *in vitro* activity studies can be performed in presence of synthetic compounds that are relevant to this disease.

3 Materials and methods

3.1 Materials

3.1.1 Chemicals

All chemicals were stored as per manufacturer's instructions. Doubly deionised water from a water purifying system (Milli Q UF from Millipore, Erkrath) was used for dissolving the chemicals in all experiments, unless otherwise specified.

Acetic acid	Merck	Darmstadt
Acetyl coenzyme A	Sigma	Mannheim
Acetone	Merck	Darmstadt
Acetonitrile	Merck	Darmstadt
ß-N-Acetyl glucosaminidase	Sigma	Taufkirchen
Adenosine diphosphate	Boehinger	Mannheim
Adenosine triphosphate	Roche	Baton Rouge, USA
Adenosine monophosphate	Boehinger	Mannheim
Ala-Ala-Phe-Ala-7-amido-4-methyl coumarine	Sigma	Taufkirchen
ATP-Binders resin™	Merck	Darmstadt
Agarose Sea Plaque	Biozyme	Heidelberg
ß-Aminocaproic acid	Riedel-de Haen	Seelze
Ampholytes (Servalyte 3-7, 4-7, 5-7, 7-9, 3-10)	Serva	Heidelberg
Ammonium molybdate	Sigma	Taufkirchen
Ammonium peroxisulphate	Bio-Rad	Munich
Ascorbic acid	Merck	Darmstadt
ASB-14	Calbiochem	Bad Soden
Bafilomycin-A1	LC laboratories	MA, USA
Boric acid	Merck	Darmstadt
Cetyltrimethylammonium bromide (CETAB)	Merck	Darmstadt
3-[(3-Cholamidopropyl)-dimethylammonio]-propanesulfonate (CHAPS)	Calbiochem	Bad Soden
Chloroform	Riedel-de Haen	Seelze
Cholesterol	Sigma	Taufkirschen
Coomassie blue (Brilliant blue G 250)	Serva	Heidelberg

Diaminobenzidine	Sigma	Taufkirschen
Diethyl ether	Roth	Karlsruhe
Diphenyliodonium chloride	Fluka	Taufkirschen
Dimethyl sulphoxide	Roth	Karlsruhe
Dithiotheitol	ICN	Heidelberg
ECL detection kit	Amersham	Freiburg
Ethanol	Roth	Karlsruhe
Ethylene diamine tetraacetate	Sigma	Taufkirchen
Formaldehyde	Merck	Darmstadt
Glycerol	Merck	Darmstadt
HEPES	Serva	Heidelberg
Iodixanol (Optiprep™)	Axis-Schield	Oslo, Norway
Iodoacetamide	Serva	Heidelberg
Iodonitrophenyl tetrazolium chloride	Sigma	Taufkirchen
Leupeptin	Boehinger	Manheim
L-Phenyl alanine	Gibko BRL	Karlsruhe
Lubrol	Sigma	Taufkirschen
Magnesium chloride	Baker	Freiburg
Methionine methyl ester	Sigma	Taufkirschen
Methylene blue	Fluka	Taufkirschen
4-Methylumbelliferyl-ß-D-glucosamine	Moscerdam	Netherlands
Methyl-ß-cyclodextrin	Sigma	Taufkirschen
NADH	Substrates	Rotterdam
N-Ethylmaleimide	ICN	Heidelberg
Octyl thioglucoside	Calbiochem	Bad Soden
Octyl glucopyranoside	Calbiochem	Bad Soden
p-Nitrophenyl-N-acetyl-ß-D-glucosaminide	Sigma	Taufkirschen
p-Nitrophenyl-ß-D-glucopyranoside	Sigma	Taufkirchen
p-Nitrophenyl phosphate	Merck	Darmstadt
Pepstatin A	Sigma	Taufkirchen
Percoll©	Amersham	Freiburg
Phenyl methyl sulphonyl fluoride	Merck	Darmstadt
Phosphatidyl choline (from egg yolk)	Sigma	Taufkirschen
Potassium cyanide	Sigma	Taufkirschen

Pitassium sulphate	Sigma	Taufkirschen
Protein molecular weight marker (Pre-stained)	NEB	Frankfurt
Protein molecular weight marker (broad range)	Bio-Rad	Munich
Pyruvate	Sigma	Taufkirchen
Roti-Blue (colloidal Coomassie blue)	Roth	Karlsruhe
Sucrose	Roth	Karlsruhe
Sodium azide	Merck	Darmstadt
Sodium bicarbonate	Merck	Darmstadt
Sodium dodecyl sulphate	Roth	Karlsruhe
Triethanolamine	Merck	Darmstadt
Thiourea	Merck	Darmstadt
Trifluroacetic acid	Sigma	Taufkirschen
Triton X-100	Merck	Darmstadt
Trypsin	Promega	Mannheim
Valinomycin	Sigma	Taufkirchen
ZFF (Z-Phe-Phe-diazomethyl ketone)	Bachem	Heidelberg

3.1.2 Antibodies

Table. 3.1: Antibodies used and their dilutions

Antibody	**Organism**	**Source**	**Dilution**
Anti-LAMP-2	Mouse (Monoclonal)	Raised in Prof. Hasilik`s laboratory	1: 2000
Anti-CLN2	Rabbit	Donated by Dr. E Kominami, Japan	1: 2000
Anti-NPC1	Rabbit	Raised in Prof. Hasilik`s laboratory	1: 4000
Anti-V-ATPase (Subunit `a`)	Rabbit	Santa Cruz Biotechnology, Heidelberg	1: 1000
Anti-Mouse IgG HRP conjugate)	Goat	Bio-Rad, Munich	1: 8000
Anti-Rabbit IgG (HRP conjugate)	Goat	Bio-Rad, Munich	1: 4000

3.1.3 Apparatus

Blotting chamber	TRANS-BLOT^{R} SD	Bio-Rad, Munich
Preparative centrifuge	RC 5B +	Sorvall, Bad Homburg
ELISA-Reader	MRX	Dynatech Laboratories, Denkendorf
Fluorometer	Fluostar Galaxy	BMG Labtechnologies, Offenburg
Gel filtration column	Superdex-200	Amersham Pharmacia
Liposome extrusion device	LiposoFast	Avestin, Ottawa, Canada
Maldi-TOF	Reflex III	Bruker Daltonic, Leipzig
Rotors for cooling centrifuge	GSA	Sorvall, Bad Homburg
	SS 34	Sorvall, Bad Homburg
Rotors for ultracentrifuge	TV 860	Sorvall, Bad Homburg
	Ti 60	Sorvall, Bad Homburg
	SW 40	Beckman, Munich
Spectrophotometer	Pharmacia	Freiburg
Speed-vac	Centrivac	Heraeus, Hanau
Ultracentrifuges	Combi Plus	Sorvall, Bad Homburg
	L8-70 M	Beckman, Munich
Tissue homogeniser	T 45	Ultrathurrax

3.2 Methods

3.2.1 Purification of lysosomal proteins from human placenta

The lysosomes were isolated from human placenta as per Diettrich *et al*. (1996). Fresh human placentas were collected from local hospitals and were kept on ice until processed. All the buffers used were ice-cold.

3.2.1.1 Tissue homogenisation and removal of debris and nuclei

Using a sharp knife, the hard tissue of the amniotic sac and the umbilical cord were removed. The trophoblastic material thus collected was washed with 0.9 % NaCl for 3 times. The tissue was passed in a regular household mincer. After this step, about 300 g of the tissue was mixed with 250 ml homogenisation buffer and treated 4 times with a T 45 Ultrathurrax each time for 5 sec. The post nuclear supernatant was collected by centrifuging 600 ml homogenate in a Beckmann JA 10 rotor for 10 min at 4,000 rpm. The latter was then aliquoted into 6 portions

each containing 50 ml, to which 4 ml Percoll© solution (I) was underlayed. A centrifugation for 15 min at 20,000 rpm was carried out in an SS-34 rotor. After separating from the sediment, the organelle concentrates from different tubes were pooled. The pool was made up to a total volume of 40 ml with the homogenisation buffer.

Homogenisation buffer:	250 mM	Sucrose
	10 mM	Acetic acid
	10 mM	Triethanolamine
	10 mM	EDTA
	pH adjusted to 7.2 with NaOH	

65 % (w/v) Sucrose in homogenisation buffer

Homogenisation buffer-I (1.075 density):

For 50 ml:	18.87 g	Percoll© in
	250 mM	Sucrose
	10 mM	Acetic acid
	10 mM	Triethanolamine
	10 mM	EDTA
	pH adjusted to 7.2 with NaOH	

Homogenisation buffer-II (1.095 density):

For 50 ml:	27.56 g	Percoll© in
	250 mM	Sucrose
	10 mM	Acetic acid
	10 mM	Triethanolamine
	10 mM	EDTA
	pH adjusted to 7.2 with NaOH	

3.2.1.2 Subcellular fractionation

A self-forming density gradient of polyvinylpyrollidine coated silica particles (Pertoft *et al.,* 1978) was used for the separation of lysosomes from other sub cellular compartments (Rome *et al.,* 1979). Gradients were made in four 35 ml ultra thin tubes made of polyallomer. Step gradients were created in each tube with 3 ml 65 % (w/v) sucrose, 8 ml Percoll© solution (I), 14 ml Percoll© solution (II), and on the top 10 ml of organelle concentrate was layered. Tubes

were sealed tightly as per description, placed in a vertical TV-860 rotor and centrifuged for 30 min at 21,000 rpm (42000 × g_{max}). Opening of the tubes was done carefully at the bottom for collecting the fractions using a fraction collector. From each tube, 17 fractions of 2 ml were collected. All the fractions as well as the post nuclear supernatant were analysed for the protein content and marker enzyme activities. Fractions showing the highest specific activities of lysosomal markers (2-5) were pooled. The well-mixed dense lysosomal vesicles (`Dense pool`) thus prepared were dropped into liquid nitrogen and stored at -80 °C.

3.2.1.3 Isolation of membranes by ultracentrifugation

The dense lysosomal vesicles obtained by differential centrifugation in the Percoll© gradient was subjected to hypotonic lysis, soluble proteins were washed away, and the membranes were sedimented by heavy speed centrifugation for the analyses. Protease inhibitors were included to limit the degradation of membrane proteins by the acid hydrolases released during the process of vesicle disruption by ultrasonication.

Washing buffer (I): 10 mM Tris
pH adjusted to 7.4 with HCl

Table. 3.2: The protease inhibitors and concentrations

Protease inhibitors	Final concentration	Proteases affected
Iodoacetamide	0.5 mM	-SH hydrolases
Leupeptin	10 µM	Serine and cystine proteases
Pepstatin A	1 µM	Aspartic proteases
Phenyl methyl sulphonyl fluoride	1 mM	Serine proteases
Z-Phe-Phe-diazomethylketone	1 µM	Cathepsins

After thawing the `dense pool` lysosome fraction in an ice bath, protease inhibitors were added to make the preferred end concentrations. After ultrasonication for 10 sec, ice cold washing buffer (I) was used to dilute the disrupted vesicles to 10 fold for effecting the hypotonic lysis. Ultracentrifugation for 2 h at 4 °C was carried out in a Ti-60 rotor at 50,000 rpm (250,000 × g_{max}). The pelleted membranes were collected carefully with a pasteur pipette and re-suspended in washing buffer for another 10-fold dilution. Repeated the centrifugation step, pellets were collected, aliquoted and stored at -70 °C for long-term use.

3.2.1.4 Immuno-affinity purification

Eupergit C1Z beads coupled to the monoclonal antibody (2D5) against lysosomal marker protein LAMP-2 was used for the specific enrichment of lysosomes from dense pool fraction by immuno- affinity adsorption. Experiments were carried out in 1.5 ml Eppendorf vials, with 500 µl of dense pool lysosomes for each trial. Protease inhibitors were included in the system to get the preferred end concentrations. After sonication for 5 sec, the samples were centrifuged in a Jouan-centrifuge for 35 sec to reach the highest acceleration. Undisrupted sub cellular compartments, especially a major portion of mitochondria were sedimented by this speed. Supernatant obtained was incubated with 40 µl of pre-washed 2D5-Eupergit C1Z beads, at 4 °C with an end-to-end rotatory wheel for 4-12 h for maximum binding.

Unbound proteins were removed accordingly by centrifuging in a Jouan-centrifuge with 30 sec acceleration at full (12,000 rpm). The pellet was washed gently using 1 ml washing buffer followed by another centrifugation for 30 sec. Supernatant was discarded and the pellet suspended in 20 µl washing buffer for storage at -20 °C.

Washing buffer (II):	10 mM	Tris
	150 mM	NaCl
	pH adjusted to 7.4 with HCl	

3.2.1.5 Purification of dense pool by MME treatment

A substrate induced selective disruption of lysosomes with amino acid esters (Goldman and Kaplan, 1973, Reeves, 1979) was carried out to obtain one more level of purification. Methionine methyl ester was found to be the most suitable for placental lysosomes.

Homogenisation buffer:	250 mM	Sucrose
	10 mM	Acetic acid
	10 mM	Triethanolamine
	5 mM	EDTA
	pH adjusted to 7.5	
Substrate:	100 mM	Methionine methyl ester in homogenisation buffer
$MgCl_2$:	1 M	solution in water

A suspension of 32 ml of dense pool lysosomes was thawed on ice, diluted to 6-8 fold and centrifuged using a Ti-60 rotor for 90 min at 35,000 rpm (110,000 × g_{max}). Sedimented

organelles were collected using a pasteur pipette and resuspended in 50 ml homogenisation buffer to repeat the centrifugation at 32,000 rpm (100,000 × g_{max}) for another 60 min. Sediment was collected, suspended in 10 ml homogenisation buffer and MME was added to get a final concentration of 100 mM. Incubation at room temperature for 40 min was followed by addition of required amounts of protease inhibitors for terminating the MME-induced lysis and protection from other hydrolases.

From the lysate obtained after MME treatment, aliquotes of 1 ml were serially pipetted in to eppendorf caps to which $MgCl_2$ stock solution was added to get a final concentration of 10 mM for aggregation of contaminating membranes (Stoner and Sirak, 1978) (Morris *et al.,* 1979) (Kwan, 1986). The mixture was allowed to stand for 12 h at 4 °C after mixing thoroughly. Non-disrupted contaminating organelles were removed by a short step centrifugation for 5 min at 4 °C in a Sorvall centrifuge with 11,000 rpm (10,000 × g_{max}). Supernatant obtained containing enriched lysosomal vesicles was sedimented with the help of eppendorf adaptors in a Ti-60 rotor at 50,000 rpm (250,000× g_{max}) for 90 min, and stored at -70 °C for further analyses.

3.2.1.6 Separation of membrane complexes by gel filtration

Gel filtration for separating high molecular weight complexes from the lysosomal membrane was performed using a Superdex-200 column (Amersham Pharmacia, Freiburg). Proteins from MME treated lysosomal membranes were extracted for 20 min in 200 µl extraction buffer at either 4 °C or room temperature with occasional gentle shaking. The detergent treated samples were centrifuged for 10 min at 14,000 rpm to remove unsolublilised material. From the supernatant obtained, 200 µl was loaded on a 4.6 X 150 mm gel filtration column connected to an HPLC apparatus with the help of a fine syringe. The flow rate was adjusted to 250 µl/min and fractions of 250 µl were collected at uniform intervals of time using a fraction collector connected to the apparatus. Samples obtained were stored directly at 4 °C or frozen for further analyses.

Extraction buffer:	20 mM	Tris-HCl
	150 mM	NaCl
	1 % (v/v)	Triton X-100 (reduced)
	0.25 % (w/v)	CHAPS
	Adjusted to pH 7.5 with HCl	

Gel filtration buffer:	20 mM	Tris-HCl
	150 mM	NaCl
	0.1 % (v/v)	Triton X-100 (reduced)
	0.025 % (w/v)	CHAPS
	Adjusted to pH 7.5 with HCl	

3.2.1.7 Separation of floating material in Optiprep™ density gradient

The lysosomal membranes obtained from dense pool after MME-treatment were extracted with 450 µl of extraction buffer at 4 °C for 20 min. Sample was centrifuged at a speed of 14,000 rpm for 10 min at 4 °C and the extract was mixed with 2 volumes of Optiprep™ (Veldhoven *et al.,* 1996) for layering on the top of a 60 % (v/v) iodixanol cushion. A step gradient of iodixanol was made as mentioned below in a 4.5 ml plastic tube.

Extraction buffer	100 mM	Tris-HCl
	150 mM	NaCl
	10 mM	EDTA
	1 % (w/v)	Triton X-100
	pH adjusted to 7.4 with HCl	

Table. 3.3: Preparation of the iodixanol gradient

Step Gradient (from the top)	Iodixanol concentration (%)	Volume (µl)
1	0	300
2	30	2100
3	40	1350
4	60	500

Centrifugation was done in a SW 60 rotor with adaptors for the plastic tubes that contain the sample. For the separation of floating material, 50,000 rpm (250,000 × g_{max}) for 5 h was applied. After the centrifugation, fractions of 300 µl were collected carefully from the top of the gradient using a yellow pipette tip.

3.2.1.8 Assays of enzyme activities

3.2.1.8.1 Assay of N-Acetyl-ß-D-glucosaminidase; EC 3.2.1.52 (von Figura 1977)

All chemicals including the substrate *p*-Nitrophenyl-2-acetamido-2-deoxy-ß-D-glucopyranoside were purchased from Sigma unless otherwise mentioned.

Substrate buffer:	100 mM	Citrate
	0.2 % (w/v)	Bovine serum albumin
	0.2 % (w/v)	Triton X-100
	0.04 % (w/v)	Sodium azide
	adjusted to pH 4.6 with NaOH.	
Substrate solution:	10 mM	*p*-Nitrophenyl-2-acetamide-2-deoxy-ß-D-glucoside in substrate buffer
Stop solution:	0.4 M	Glycine/NaOH, pH 10.4

Microtitre plates were used for the enzymatic assays. Samples to be analysed were diluted in 0.9 % NaCl to 25 µl, mixed with an equal volume of the substrate solution and incubation was performed for 30 or 60 min at 37 °C. After the addition of 250 µl stopping buffer, the extent of *p*-nitrophenol liberated was measured at 405 nm with the help of a microplate ELISA reader. Activity of ß-hexosaminidase was evaluated as

$$\frac{\text{Measured volume x net absorption}}{\text{Absorption coefficient x sample volume x incubation time x path length (1 cm)}}$$

3.2.1.8.2 Assay of acid ß-Glucocerebrosidase; EC 3.2.1.45 (Gatt 1969)

The activity of membrane bound ß-Glucosidase was estimated colorimetrically by following the degradation of *p*-Nitrophenyl-ß-D-glucopyranoside by the enzyme. This was followed by measurement of the generated *p*-nitrophenol at 405 nm.

Substrate solution:	4 mM	*p*-Nitrophenyl-ß-D-glucopyranoside
	0.2 % (v/v)	Triton X- 100
	0.2 % (w/v)	Na-Taurocholate
	0.2 M	Sodium acetate buffer, pH 5.0
Stop buffer:	0.4 M	Glycine/NaOH, pH 10.4

Samples in Eppendorf caps were diluted with 0.9 % NaCl to 25 µl, added equal volume of the substrate and incubated at 37 °C for 60-120 min and the assay was stopped by adding 300 µl stop buffer. The supernatants after centrifugation at 14,000 rpm for 10 min were collected in microtitre plates for measuring the absorption at 405 nm.

3.2.1.8.3 Assay of Acetyl-CoA: α-glucosaminide N-acetyl transferase; EC 2.3.1.78 (Voznyi *et al*., 1993)

Acetyl-CoA: α-glucosaminide N-acetyl transferase was measured fluorimetrically using a coupled enzyme assay**.** A fluorogenic substrate, 4-methylumbelliferyl-β-D-N-glucosaminide was converted by the enzyme to an N-acetylated intermediate, which was cleaved by an externally provided ß-N acetyl glucosaminidase (EC 3.2.1.52). The resulting fluorescent product was measured with the use of a fluorometer.

Substrate buffer:	0.25 % (w/v)	Triton X-100
	0.02 %	Sodium azide
	200 mM	Sodium phosphate/100 mM Citrate, pH 5.5
Coenzyme (stock):	100 mM	Acetyl coenzyme A in water
ß-hexosaminidase (stock):	20 m units	ß-N acetyl glucosaminidase in water
Substrate solution:	3 mM	4-Methylumbelliferyl-alpha-D-N-Sulphoglucosaminide in buffer
Stop buffer:	0.4 M	Glycine/NaOH, pH 10.4

The assay was performed in microtitre plates in a reaction volume of 30 µl. Samples to be analysed were diluted with water to 14 µl. The reaction was started by adding 16 µl substrate reagent providing final concentrations of 2 mM acetyl CoA, 2 m units ß-N acetyl glucosaminidase and 3 mM substrate. After mixing well, samples were incubated at 37 °C up to 2 h and the reaction was terminated by adding 70 µl stop buffer. The fluorescence was measured at excitation and emission wavelengths of 355 and 460 nm respectively using a fluorescence-ELISA-reader.

3.2.1.8.4 Assay of placental alkaline phosphatase; EC 3.1.3.1

PLAP, a DRM-associated protein is mainly localised in the plasma membrane and is probably a contamination in the lysosomal preparations. Liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate by the PLAP was measured at 405 nm using a microplate reader.

Substrate solution:	5 mM	*p*-Nitrophenyl phosphate
	250 mM	Sucrose
	5 mM	$MgCl_2$
	0.1 % (w/v)	Triton X-100
	50 mM	Tris-HCl, pH 9.0

Samples were diluted in 0.9 % NaCl to 50 µl, and 250 µl of the substrate was pipetted in to the wells for incubation at 37 °C. Cleavage of the substrate to *p*-Nitrophenol was observed by the development of yellow colour and was measured accordingly.

3.2.1.8.5 Assay of tripeptidyl peptidase-1; EC 3.4.14.9 (Junaid *et al*., 2001)

Lysosomal tripeptidyl peptidase-1 activity was traced by the cleavage of it`s synthetic substrate Ala-Ala-Phe-7-amido-4-methyl coumarine by the enzyme so as to generate a fluorescent product 7-amino-4-methyl coumarine.

Substrate solution:	0.25 mM	Ala-Ala-Phe-7-amido-4-methyl coumarine
	100 mM	Sodium acetate pH 3.5
	0.1 % (v/v)	Triton X-100
Stop buffer:	0.4 M	Glycine/NaOH, pH 10.4

Samples were diluted with water to 15 µl and mixed with 15 µl doubly concentrated substrate solution to effect production of fluorescent product at room temperature. After an incubation period of 20 min the reaction was stopped by the adding 70 µl stop buffer. Liberated 7-amino-4-methyl coumarine was measured fluorometrically with an excitation maximum at 360 nm and emission at 460 nm.

3.2.1.8.6 Assay of succinate dehydrogenase; EC 1.3.5.1

As a mitochondrial marker enzyme, succinate dehydrogenase was followed on the course of purification strategy using a colorimetric system.

Isotonic saline:	150 mM	NaCl
Substrate mixture:	122 mM	Sodium succinate
	61 mM	Sodium phosphate, pH 7.4
	0.09 % (w/v)	Iodonitrophenyl tetrazolium chloride
	0.05 % (w/v)	Sodium azide
Stop solution:	10 % (w/v)	Trichloroacetic acid
Extraction solution:	50 % (v/v)	Ethyl acetate
	50 % (v/v)	Ethanol

The sample to be assayed was made up to 50 µl with isotonic saline to which 82 µl of the freshly made substrate was added. Reaction was allowed to proceed at 37 °C for several hours, and terminated by the addition of 150 µl stop solution. The water insoluble end product was extracted with 400 µl extraction solution, with thorough vortexing, and centrifuged at 14,000 rpm for 10 min. In to a 96 well microplate, 300 µl supernatant was pipetted for measuring the absorption at 490 nm using an ELISA plate reader.

3.2.1.8.7 Assay of inorganic phosphate

ATPase activity of lysosomal preparations were measured using the conventional inorganic phosphate assay. Degradation of ATP by the enzyme was followed by coupling of generated inorganic phosphate to ammonium molybdate. The resultant coloured complex was measured at 630 nm using a regular colorimeter.

Substrate (stock):	100 mM	Adenosine triphosphate-Na salt
Substrate buffer:	25 mM	HEPES, pH 7.5
	25 mM	KCl
	5 mM	$MgCl_2$
	5 mM	KCN
Coupling solution:	0.5 % (w/v)	Ammonium molybdate in 0.7 N H_2SO_4
Anti-oxidant:	10 % (w/v)	Ascorbic acid

ATPase assay was carried out in 1.5 ml eppendorpf vials at 37 °C with 200 µM ATP as the substrate. Samples to be analysed were diluted with water to 50 µl and added 50 µl sample buffer containing the prescribed amount of ATP. Reaction was terminated after 2-4 h by heating the sample for 30 sec at 95 °C. Care was taken to make sure that heating doesn't effect in ATP degradation. Samples were allowed to cool down, centrifuged for 1 min at 2000 rpm at 4 °C and added 42 µl coupling solution. To prevent oxidation of the complex, 8 µl of 10 % ascorbic acid was added and mixed thoroughly. Heated at 95 °C for 90 sec and the absorbance were measured at 630 nm after transferring in to a micro titre plate. Vacuolar ATPase activity corresponding to the lysosomes was calculated by the extent of inhibition of ATP hydrolysis using *N*-ethyl maleimide or bafilomycin-A1 as specific inhibitors of vacuolar ATPase. Trace amounts of detergents in the sample, especially NP-40 and Triton X-100 resulted in turbidity of the coloured complex, which was efficiently removed by mixing the complex with equal amount of chloroform followed by its removal after a centrifugation. Controls were maintained to assure that, this process does not affect either the stability of the complex or the optical density.

3.2.1.9 Estimation of total protein by Bradford method

Protein concentration was determined by the method by Bradford (1976) using bovine serum albumin as the standard. Samples were diluted in 0.25 M NaOH/0.025 % (v/v) Triton X-100 for the solubilisation of membrane proteins and centrifuged for 10 min. From the supernatant, 20 µl was used for the estimation of protein. The absorbance was measured at 595 nm in a microtitre plate, after adding 300 µl Bradford reagent to the sample.

3.2.1.10 SDS-PAGE

For sample characterisation, proteins in the purified lysosomal fractions were separated by SDS-PAGE according to Laemmli (1970) with modifications as described below. A range of proteins with molecular weights was used as standards from Myosin (200 kDa) to Aprotinin (8.5 kDa). Solubilisation buffer containing the anionic detergent sodium dodecyl sulphate was used for extraction and denaturation of the proteins, which were then separated on a polyacrylamide gel on the based on molecular weight.

3.2.1.10.1 Preparation of acrylamide gels

Gels with a thickness of up to 1mm, length 13.5 cm and width 14 cm were used for the separation. Plastic combs with 20, 22 or 33 wells were used. Gels were casted with a low percentage stacking gel on the top, which makes the migration of proteins easy and sharp followed, by higher percentage gel for the adequate separation of proteins. Gels were stable at

4 °C up to 1 week in a plastic sheet. The top and bottom of the gels were kept wet for the gel not to dry or broken.

Table. 3.4: Gel solutions

Stacking gel	**Final acrylamide concentration**	
Acrylamide (% (w/v))	3 %	4.8 %
N,N`-Methylene bisacrylamide (% (w/v))	0.11 %	
Tris-HCl, pH 8.8	0.125 M	
Sodium dodecyl sulphate	0.1 % (w/v)	
TEMED	0.1 % (v/v)	
APS	0.1 % (w/v)	

Separation gel	**Final acrylamide concentration**			
Percentage	8,1 %	10.7 %	12.6 %	15.2 %
Acrylamide (% (w/v))	8 %	10.6 %	12.5 %	15 %
N,N`-Methylene bisacrylamide (% (w/v))	0.08 %	0.11 %	0.13 %	0.16 %
Tris-HCl, pH 8.8	0.375 M			
Sodium dodecyl sulphate	0.1 % (w/v)			
TEMED	0.1 % (v/v)			
APS	0.1 % (w/v)			

Separation gel overlay buffer:	0.375 M	Tris-HCl, pH 8.8
	50 % (w/v)	Isopropanol
Stacking gel overlay buffer:	0.125 M	Tris-HCl, pH 6.8
	0.1 % (w/v)	SDS

Glass plates were washed repeatedly with millipore water and ethanol. A spacer was used between the glass plates and the plates were stuck together using a clamp. Separation gel mixture was prepared and poured in between the glass plates carefully without any air bubbles and separation gel overlay was applied on the top. After 30 min, the overlay was washed off. A filter paper was used to remove the water. The stacking gel mixture was prepared meanwhile and poured on the top of the separation gel and well combs were inserted. The polymerisation was completed within 30 min after which the gel was either used fresh or stored.

3.2.1.10.2 Sample preparation

Samples containing the proteins to be separated were mixed with sample buffer in 1:1 ratio with the end volume being not more than 30 µl. Samples in the form of pelleted proteins were solubilised with 1X solubilisation buffer. After the addition of solubiliser, samples were kept at 37 °C with gentle shaking. Iodoacetamide was added to a final concentration of about 75 mM for carbamidomethylation of the reduced proteins and incubated for another 15 min at 37 °C. A centrifugation was carried out to sediment insoluble aggregates at 14,000 rpm for 5 min.

2X Solubilisation buffer:	2 % (w/v)	SDS
	20 mM	Dithiothreitol
	0.25 M	Tris-HCl, pH 6.8
	20 % (v/v)	Glycerol

3.2.1.10.3 Electrophoresis

All the buffers were stored at 4 °C. For one trial of electrophoresis, 5 litres of anode buffer and 250 ml cathode buffer were required. Electrophoresis was performed at 15 mA/gel during the migration though the stacking gel, and at 30 mA once it reached the separation gel. Bromophenol blue in the sample as well as cathode buffer migrated just behind the salt boundary and marked end of the separation after approximately 3.5 h.

Cathode buffer:	384 mM	Glycine
	23 mM	Tris
	0.01 % (w/v)	SDS
	0.001 % (w/v)	Bromophenol blue
Anode buffer:	384 mM	Glycine
	23 mM	Tris

3.2.1.10.4 Staining of proteins by silver nitrate (modified from Heukeshoven, 1988)

Proteins on the analytical scale were visualised by silver staining method due to its sensitivity, rapidity and easiness in handling.

Fixative:	30 % (v/v)	Ethanol
	10 % (v/v)	Acetic Acid

Thiosulphate solution:	0.1 M	Sodium acetate, pH 6
	30 % (v/v)	Ethanol
	0.1 % (w/v)	Sodium thiosulphate
	(used for one dimensional analyses)	
	0.2 % (w/v)	Sodium thiosulphate
	(used in two dimensional analyses)	
Silver nitrate solution:	0.1 % (w/v)	Silver nitrate
	0.01 % (w/v)	Formaldehyde
Developer:	2.5 % (w/v)	Sodium carbonate
	0.02 % (w/v)	Formaldehyde

Incubations for silver staining were done at room temperature in cleaned glass trays with continuous gentle shaking. For each step 100 ml solution was used. To start with, the gel was kept in fixative for 30 min, followed by another 30 min incubation in thiosulphate solution. In the case of two-dimensional gels 0.2 % thiosulphate solution was used with an extended incubation to over night for the better staining. Gels were washed 3 times each for 10 min and kept in silver nitrate solution for 25 min. Formaldehyde was added to the silver nitrate solution as well as developer just before it being applied to the gel. After a short washing step for 30 sec, 100 ml developer solution was used for staining. Once the protein signals were visible, 7-8 ml concentrated acetic acid was dropped into the tray so as to stop the staining procedure. Gels can be washed and stored after drying in between two layers of cellophane membrane.

3.2.1.10.5 Coomassie staining of proteins

Protein staining with colloidal Coomassie blue solution was applied in the case of preparative gels, as silver staining is not compatible with mass spectrometry normally though otherwise reported.

Fixative:	1 % (v/v)	*o*-Phosphoric acid 85 % (w/v)
	20 % (w/v)	Methanol
Dye solution:	20 % (v/v)	Roti-blue (concentrate)
	20 % (v/v)	Methanol
Washing:	25 % (v/v)	Methanol

The gel was incubated in the fixative solution (100 ml/gel) for 1 h. Freshly prepared dye solution was added to the gel for overnight incubation. Next day, the gel was transferred to a new clean glass tray for washing with 25 % methanol 3 times, each spanning 2 min. Washing step allows the elimination of unspecific binding of dye in order to decrease the background for better visualisation. Gel was kept in deionised water for storage at cold.

3.2.1.11 Two-dimensional electrophoresis (O` Farrell, 1975)

Two-dimensional electrophoresis (2DE) was performed with isoelectric focusing using carrier ampholytes for the first dimensional separation of proteins based on their isoelctric points and with SDS-PAGE for the separation in second dimension.

3.2.1.11.1 Isoelectric focusing of proteins

Glass tubes (16 X 3.1 mm inner diameter) were used for casting the tube gels for isoelectric focusing. The bottom end of the tube was sealed tightly with parafilm and a mark was made at 12.5 cm from this end. Composition of the tube gel varied with the kind of detergent system used for solubilisation of membrane proteins.

Acrylamide solution:	28.4 % (w/v)	Acrylamide
	0.8 % (w/v)	N, N`-Methylene bisacrylamide
Sample overlay:	7 M	Urea
	0.8 % (v/v)	40 % (w/v) Ampholytes, pH 4-7
	0.2 % (v/v)	40 % (w/v) Ampholytes, pH 3-10
Cathode electrolyte:	200 mM	NaOH
Anode electrolyte:	100 mM	H_3PO_4

3.2.1.11.2 Urea/CHAPS gel and solubilisation system

Composition of one capillary gel

0.48 gm	Urea
107 µl	40 % (w/v) Ampholytes, pH 4-7
27 µl	40 % (w/v) Ampholytes, pH 3-10
143 µl	Acrylamide solution
107 µl	20 % (w/v) CHAPS
357 µl	de-ionised water

The gel mixture was degassed for 10 min followed by addition of 0.4 µl TEMED and 8 µl of 10 % (w/v) APS for the polymerisation to be induced. A long Pasteur pipette was used for transferring the gel mix to the already prepared clean capillary tubes up to the 12.5 cm marking. 130 µl of 6.5 M Urea solution was used as an overlay and allowed to stand at room temperature for 1 h to commence complete polymerisation. Capillary gels thus made were stored at 4 °C for a maximum period of 2 weeks.

Protein samples were solubilised in the lysis buffer containing urea and CHAPS at 37 °C. Composition of the lysis buffer was as follows.

9M	Urea
4 % (w/v)	CHAPS
2 % (v/v)	40 % (w/v) Ampholytes, pH 4-7
0.5 % (v/v)	40 % (w/v) Ampholytes, pH 3-10
10 mM	Dithiotheitol

Protein samples were used either directly (≤ 20 µl) or after a precipitation with 4 volumes of cold acetone when a large volume was to be used. They were dissolved in 100 µl lysis buffer and incubated at 37 °C for 2 h. A centrifugation was carried out for 10 min prior to loading the solubilised proteins on the capillary gel.

3.2.1.11.3 Thiourea/ASB 14 gel and solubilisation system

Inclusion of thiourea in the isoelectric focusing system together with urea and substitution of CHAPS with ASB-14 is reported to be better for either the solubilisation and resolution of membrane proteins (Shaw and Riederer, 2003). Instead of TEMED and APS-induced polymerisation of the acrylamide in capillary gel, a photopolymerisation with methylene blue in presence of sodium toluene sulphinate and diphenyliodonium chloride was applied as the former strategy being not compatible with thiourea.

Thiourea considerably increases the solubilisation of hydrophobic membrane proteins, though it has the disadvantage that at a pH range of 8.5-9 during the equilibration step, the sulphur atom of thiourea is highly reactive and consumes the iodoacetamide resulting in poor alkylation of proteins.

Composition of one capillary gel is given in the table below.

Urea	6 M
Thiourea	2 M
40 % (w/v) Ampholytes, pH 3-7	10.2 % (v/v)
40 % (w/v) Ampholytes, pH 3-10	2.6 % (v/v)
Acrylamide	4.1 % (w/v)
N, N´-Methylene bisacrylamide	0.11 % (w/v)
ASB-14	2 % (w/v)
Triton X-100	0.5 % (w/v)

The gel mix was degassed for 10 min. Photo polymerising agents were then added to obtain the following end concentrations.

Sodium-*p*-toluene sulfinate	500 µM
Diphenyliodonium chloride	25 µM
Methylene blue	50 µM

The gel mixture for polymerisation was filled into the capillary tubes as in the case of urea/ CHAPS system and was hung in a vertical upright position with a source of visible light kept as close as 35 cm from the capillary tubes for inducing photo polymerisation. For complete polymerisation, 12 h were sufficient after which gels were stored at 4 °C up to 2 weeks.

The composition of the solubilisation buffer was

6 M	Urea
2 M	Thiourea
2 % (w/v)	ASB-14
0.5 % (w/v)	Triton X-100
2.5 % (v/v)	40 % Ampholytes, pH 7-9
10 mM	DTT

The sample solubilisation of proteins with this buffer was carried out as described for the urea/CHAPS system (3.2.1.9.3).

3.2.1.11.4 Performing isoelectric focusing

The capillary gels were allowed to thaw to the room temperature, overlayed sequentially with 100 µl corresponding lysis buffer and 50 µl de-ionised water. After 1 h at room temperature, the bottom sealing of the gel was replaced by a dialysis membrane stabilised by a rubber ring. The solubilised samples were loaded on to the equilibrated capillary gel and overlayed with 20 µl sample overlay containing 7 M urea. Before starting the isoelectric focusing, the tubes were filled with cathode buffer avoiding air bubble trapping.

In the bottom buffer reservoir, 4.5 L of 0.01M H_3PO_4 was filled and 1.25 L of 0.02 M NaOH was poured in to the upper cathode chamber. With the urea/CHAPS and thiourea/ASB-14 systems temperature was maintained at 4 °C and 37 °C respectively.

Table. 3.5: The voltage settings during isoelectric focusing

Step	Voltage (V)	Duration (h)
1	200	0.5
2	750	15.5
3	850	1
4	1000	4

In both systems, the voltage settings were as described in Table. 3.5

3.2.1.11.5 Equilibration prior to the second dimension SDS-PAGE

After the completion of isoelectric focusing, the gels were displaced using a plastic syringe filled with water in to a small dish. Maximum care was taken while pressing out the gels from the tubes to avoid breaking. The alkaline end of the gel was marked with 10 % (w/v) bromophenol blue.

25 ml equilibration buffer I and II (see below) were used for the urea/CHAPS system each for 30 min at 37 °C respectively with gentle shaking. For thiourea/ASB-14 system, first equilibration with buffer I was carried out at 37 °C followed by a second equilibration with the same buffer at 50 °C. The step was repeated with equilibration buffer III for 2 times at 50 °C each with 25 ml fresh buffer, which was pre-incubated at the same temperature.

Equilibration buffer I	0.05 M	Tris-HCl, pH 6.8
	4 % (w/v)	SDS
	0.25 % (w/v)	DTT
	15 % (w/v)	Glycerol

Equilibration buffer II	0.05 M	Tris-HCl, pH 6.8
	4 % (w/v)	SDS
	25 mM	Iodo acetamide
	30 % (w/v)	Glycerol
	6 M	Urea
Equilibration buffer III	0.05 M	Tris-HCl, pH 6.8
	4 % (w/v)	SDS
	25 mM	Iodo acetamide
	30 % (w/v)	Glycerol

3.2.1.11.6 The second dimension SDS-PAGE

An SDS-polyacrylamide gel was overlaid with 500 µl of warm molten agarose solution for fixing the capillary gels. An equilibrated capillary gel was transferred to a cellophane sheet, and the liquid was sucked out. The blotted tube gel was placed on the top of the SDS-acrylamide gel with the alkaline side on the right end and overlaid with molten agarose.

Agarose solution	0.5 % (w/v)	Agarose
	125 mM	Tris-HCl, pH 6.8
	0.1 % (w/v)	SDS

Once the gel is placed, a second layer of agarose was applied on the top of it, and any air bubbles were removed. After standing at room temperature for 10 min molecular weight standards were introduced. The separation was started at an electric current of 15 mA per gel. The current was raised to 30 mA per gel once the bromophenol blue reached the separation gel. The run was stopped when bromophenol blue band reached 2 mm from the bottom end of the gel. The gels containing 20-40 µg proteins were stained with silver nitrate whereas those with 200-400 µg were stained with colloidal Coomassie blue.

3.2.1.12 CETAB-Diagonal electrophoresis (CETAB/SDS-PAGE)

A cationic detergent, CETAB (Cetyltrimethylammonium bromide) at an acidic pH was used for the solubilisation and separation of proteins in the first dimension followed by SDS-PAGE in the second dimension (Penin *et al*, 1984). The gels were casted as before (3.2.1.10.1). For the separation, an 8 % gel was used that decreased the duration of separation.

Table. 3.6: The gel composition for CETAB electrophoresis

Components	**Stacking gel (pH 5.1)**	**Separation gel (pH 4.0)**
Acrylamide	4 % (w/v)	7.5 % (w/v)
N,N´-Methylene bis acrylamide	0.34 % (w/v)	0.26 % (w/v)
KOH	64 mM	43 mM
Acetic acid	94 mM	280 mM
Urea	6 M	6 M
CETAB	0.1 % (w/v)	0.1 % (w/v)
Ascorbic acid	4 mM	4 mM
$FeSO_4$	4.25 µM	8 µM
H_2O_2	0.0015 %	0.001 %

Stacking gel buffer:	43 mM	KOH
	280 mM	Acetic acid
	0.1 % (w/v)	CETAB
	50 % (w/v)	Isopropanol
Separation gel buffer:	64 mM	KOH
	94 mM	Acetic acid
	0.1 % (w/v)	CETAB
1.5 X Solubilisation buffer (pH 5.1)	4.5 M	Urea
	1.5 % (w/v)	CETAB
	96 mM	KOH
	141 mM	Acetic acid
	15 mM	DTT
	7.5 % (v/v)	Glycerol
	0.01 % (w/v)	Pyronin Y

Samples were diluted with water to 15 µl and mixed with 30 µl of 1.5X solubilisation buffer to obtain 45 µl for loading. For solubilising the membrane pellets, 1X buffer was prepared by diluting the stock buffer and effective solubilisation was achieved at 60 °C when incubated for 5 min. The solubilised material was subjected to a centrifugation for 5 min at 14,000 rpm to remove aggregates and undissolved debris.

Anode buffer for CETAB electrophoresis (pH 4.0)	40 mM	ß-Alanine
	70 mM	Acetic acid
	0.1 % (w/v)	CETAB
Cathode buffer (pH 5.7)	50 mM	KOH
	56 mM	Acetic acid

The anode and cathode buffer chambers were filled with 250 ml and 5 L of the corresponding buffers, respectively. Buffers used were pre-warmed to 25 °C to avoid precipitation of CETAB. Electrophoresis was started at a current of 10 mA per gel and continued at 20 mA once the dye front reached the separation gel.

After the CETAB electrophoresis, the sample lanes were cut out using a sharp blade and incubated in 25 ml equilibration buffers I and III each for 20 min at 37 °C. The strips were transferred on top of the second dimension gel as mentioned previously using pre-molten agarose for fixing the strip in between the glass plates on the top of the SDS polyacrylamide gel. For the migration of proteins in the stacking gel zone, 15 mA was applied which was increased accordingly to 30 mA once the dye front reached the separation gel. Samples were stained with either silver nitrate (20-40 µg protein) or using colloidal Coomassie blue (200-400 µg protein).

3.2.2 Identification of proteins

The samples were characterised either by western blotting using specific antibodies or by mass spectrometric analyses of the tryptic peptides.

3.2.2.1 Western blotting

Samples to be analysed by Western blotting were first separated on an SDS-PAGE. Low percentage gels (8 %) were mostly used for separating high molecular weight proteins as it enables the easy migration. This also enables a better transfer of proteins. Once the run was completed, gel was equilibrated with washing buffer for 20 min while shaken.

A PVDF membrane soaked in methanol was equilibrated with the washing buffer. Two pairs of blotting paper with the size of the gel were soaked, in the anode buffer and the cathode buffer for 20 min respectively. A semi-dry blotting chamber was used. The anode platform wet with anode buffer and two blotting papers soaked in anode buffer were gradually descended to avoid any trapping of air bubbles. This was followed by the soaked PVDF

membrane, the gel and the wet cathode blotting papers. A test tube was rolled over the layers to remove the excess liquid. The blotting was performed at 1 mA/cm^2 for 2 h at 4 °C. After the run all the lanes and the corners of the gel as well as dye front were marked with a ballpoint pen.

Along with the samples for Western blotting, molecular weight markers that are either pre- stained or of broad range molecular weights in sufficient amounts for Coomassie blue staining were used. These lanes were cut out and either dried or developed with Coomassie staining respectively.

The labelled PVDF membrane was placed in blocking buffer containing ovalbumin for at least 2 h at room temperature or overnight at 4 °C in order to saturate the binding sites and to decrease unspecific binding of the immunoglobulins.

Washing buffer (W.B.):	23 mM	Tris
	384 mM	Glycine
Anode buffer:	40 mM	ε-Amino caproic acid
	192 mM	Glycine
Cathode buffer:	25 mM	Tris
	192 mM	Glycine
Blocking buffer:	7 % (w/v)	Skimmed milk powder in W.B.

After blocking for at least 4 h at room temperature, the buffer was replaced with a new aliquot containing the primary antibody. Different dilutions were used as specified before (3.1.2). The binding was allowed to take place for 2 h at room temperature or overnight at 4 °C. The excess antibody was washed away using the wash buffer and then with the wash buffer containing 0.1 % Triton X-100. This was repeated for 3 times with each solution. The secondary antibody an HRP conjugate diluted in the blocking buffer was poured on the washed membrane and incubation was performed for another 2 h at room temperature. The washing was repeated.

ECL detection kit (ECL Plus, Amersham, Freiburg) was used to develop the blot. The PVDF membrane was kept on a thin layer of plastic on the top of which ECL mixture was poured and spread uniformly. Within 2 min, the blot was transferred in to a cassette and exposed to a photographic film (Kodak D-19, Kodak, Paris) for a specified time. The film was

placed in a developer solution, fixed in acidofix (Agfa, Leverkusen) for 5 min, and was dried in air.

3.2.2.2 Identification of proteins by mass spectrometry

Mass spectrometry was performed on tryptic digests of proteins. Samples containing the proteins to be analysed were separated on polyacrylamide gels and stained with colloidal Coomassie blue. Protein spots were cut out from the gels using a capillary device of 1.5 mm diameter. Prior to the digestion with trypsin, gel pieces were washed with different solutions at 37 °C each time for 30 min as described below.

Table. 3.7: The washing steps of gel pieces prior to tryptic digestion

Step	Solution
1	Distilled water
2	25 mM NH_4CO_3
3	25 mM NH_4CO_3/50 % (w/v) acetonitrile
4	25 mM NH_4CO_3/50 % (v/v) acetonitrile
5	Acetonitrile

Each time, washing was performed with 100 µl of the solution with gentle shaking of the vials. After the final acetonitrile washing, gel pieces were kept at room temperature for 5 min to remove all the organic solvents.

Trypsin stock solution 0.5 µg/µl in 0.01 % TFA/water (stored at -20 °C)

Working solution 20 ng/µl in 25 mM NH_4CO_3/water (made fresh from the stock every time)

The dried gel pieces were soaked in 6 µl diluted trypsin (total 120 ng) solution on ice. After swelling for 15 min, the digestion was performed at 37 °C for 12 h. The bottom of the tube was pierced with a syringe for collecting the peptides by a short centrifugation within a larger vial. The gel pieces were incubated for 20 min with 20µl TFA/water mixture and the centrifugation was repeated. The combined extracts were dried completely in a vacuum concentrator.

For the identification of proteins, Matrix Assisted Laser Desorption Ionisation-Time of flight spectroscopy was carried out with the help of Dr. Bernhard Schmidt at Max Planck

Institute for Biophysics- Goettingen. Spectral data obtained were processed with the help of Bruker X tof 5.1.1 programme in our laboratory. Spectral peak obtained from trypsin peptide at 842.51 was used as an internal control for calibrating the sample signals. The analysis of evaluated data was performed by Mascot analysis tools (http://www.matrixscience.com) with defined modifications. A peptide tolerance of 0.1 Da was kept normally during the search with carbamidomethylation and methionine oxidation being the user defined modifications.

3.2.3 Reconstitution of proteins

Reconstitution of proteins was performed for the enzymatic characterisation and transport studies.

Liposome buffer:	20 mM	HEPES, pH 7.4
	1 mM	EDTA
	120 mM	K_2SO_4
Triton X-100 (stock):	20 % (w/v)	in liposome buffer

Bio Beads SM-2

3.2.3.1 Preparation of liposomes

Triton X-100 solubilised proteins were incorporated in to detergent-saturated liposomes (Paternostre *et al*., 1988). For the preparation of liposomes, egg yolk lecithin and cholesterol were used. Egg yolk lecithin (50 mg) and cholesterol (11.25 mg) were solubilised in chloroform and the organic solvent was later evaporated under a stream of nitrogen. The material remaining was dried under vacuum for 30 min and the lipid film obtained was re-dissolved in 3 ml of diethyl ether. To the mixture, 1 ml of the liposome buffer was added and sonicated for 2 min at 4 °C. Removal of diethyl ether was performed under a steam of nitrogen. Normally, 15 min are required for the formation of multilamellar vesicles. After addition of further 2 ml of liposome buffer, evaporation of the organic solvent was continued for another 30 min. An extrusion device, LiposoFast (Avestin, Ottawa, Canada) was used for the preparation of unilamellar vesicles (MacDonald *et al*., 1991) of about 100 nm diameters.

3.2.3.2 Solubilisation of proteins and reconstitution

Proteins to be reconstituted were extracted with a final concentration of 0.5 % Triton X-100. The membrane pellets were extracted with 100 µl liposome buffer containing 0.5 % Triton X-100 with occasional vortexing for 30 min at 4 °C. The sample was centrifuged for 10 min at 14,000 rpm at 4 °C to remove insoluble material. Supernatant obtained was used for reconstitution. The molar ratio of phospholipid to protein was maintained at 8000 mol/mol corresponding to 80:1 (w/w) for a 100 kDa protein (Rigaud and Levy, 2003). The protein extract was mixed with a corresponding amount of freshly prepared liposomes and mixed by vortexing for 5-10 sec. The sample was allowed to stand on ice for 60 min with vortexing for5-10 sec after every 20 min.

SM-2 Bio Beads were used (10 g dry beads/g of Triton X-100) for the slow removal of detergents to obtain proteoliposomes. The beads were washed 3 times with methanol, 3 times with water and finally with liposome buffer prior to its addition to the sample. The beads were added to the protein-lipid mixture in 4 equal portions and incubated on ice for at least 2 h per every addition (Rigaud *et al*., 1995).

Beads were spun down from the sample with a short centrifugation for 10 sec. The resulting supernatant was centrifuged at 14,000 rpm for 15 min at 4 °C to remove insoluble material. Proteoliposomes were collected by ultracentrifugation at 4 °C for 60 min with a speed of 50,000 rpm in a Ti 60 rotor. The proteoliposomes obtained were suspended in a volume of 100 µl, stored on ice and analysed within 7-10 days.

4 Results

4.1 Purification of lysosomes

4.1.1 Isolation of lysosomal membrane by ultracentrifugation

For conducting a biochemical study of the lysosomal membrane complexes, several strategies were adopted to purify placental lysosomes. Purification of human lysosomes was done from placental preparations since the availability of tissue was met from the local hospitals. Centrifugation on a Percoll® density gradient for isolating dense lysosomal vesicles- the `dense pool` (DP) was already standardised in the laboratory (Diettrich *et al.,* 1996). In every preparation (Fig. 4.1), specific marker enzymes, membrane bound ß-glucosidase for the lysosomal membrane and ß-hexosaminidase for the matrix were followed and protein pattern analysed by two-dimensional electrophoresis (2DE). Comparison of specific activities showed a 50-55-fold enrichment of lysosomal vesicles through this procedure.

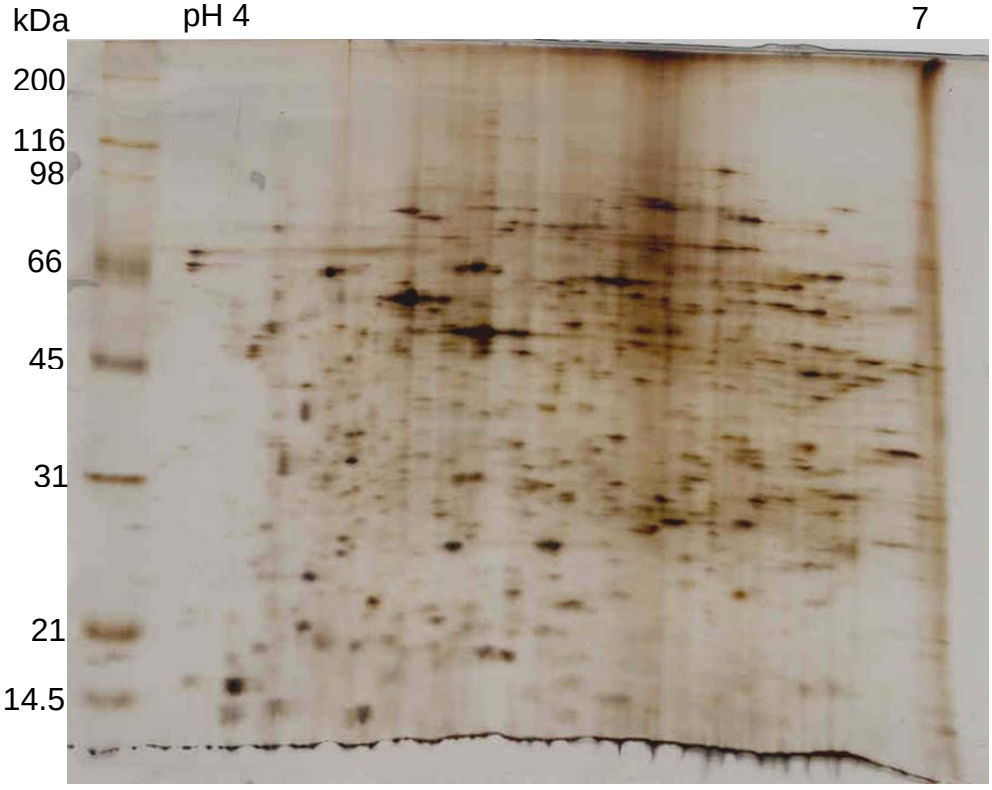


Fig. 4.1: Two-dimensional separation of the dense pool lysosomal fraction. The proteins (20 µg) from dense pool fraction of the percoll® gradient were solubilised in lysis buffer containing urea and CHAPS. Isoelectric focusing was performed for 20 h followed by separation of proteins on a 15 % SDS-PAGE. The polypeptides were stained using silver nitrate.

The lysosomal membranes (`UC membranes`) were prepared from the dense pool lysosomes by ultracentrifugation at 250,000 × g_{max} after a hypotonic lysis (see 3.2.1.3). The

soluble proteins of the lysosomal matrix were recovered in the `UC supernatant` fraction. The pellet prepared by ultracentrifugation contained approximately 40 % of the initial protein was enriched in lysosomal membrane proteins as indicated by an average 1.5 fold increase (n=3) in the specific activity of the non-integrated membrane bound ß-glucosidase (Table.4.1).

Table: 4.1: Enrichment of lysosomal membrane proteins by ultracentrifugation

Material	Volume (ml)	Protein (mg/ml)	ß-glucosidase (mU/mg)	ß-hexosaminidase (mU/mg)
Dense pool	32	1.53+/-0.28	15.5+/-2.4	1361+/-26.5
UC membrane	1	20+/-8.5	23.5+/-7.8	233+/-17
UC Supernatant	200	0.11+/-0.05	5.2+/-0.5	2295+/-21.4

A specific enrichment of lysosomal membrane proteins in the `UC membrane` fraction was indicated on comparison of Fig. 4.2 with Fig. 4.1. The intensities of lysosomal marker proteins LAMP-2, LIMP-2 and ß-glucosidase were enriched by the ultracentrifugation step. Interestingly, a tentatively lysosomal matrix protein TPP-1 (Hoffmann *et al*., 2002) was co-sedimented with the membrane fraction suggesting a probable membrane interaction of this protein. The lysosomal membranes enriched in resident membrane proteins (Fig. 4.2) obtained were used for the initial experiments where purity was not the primary concern.

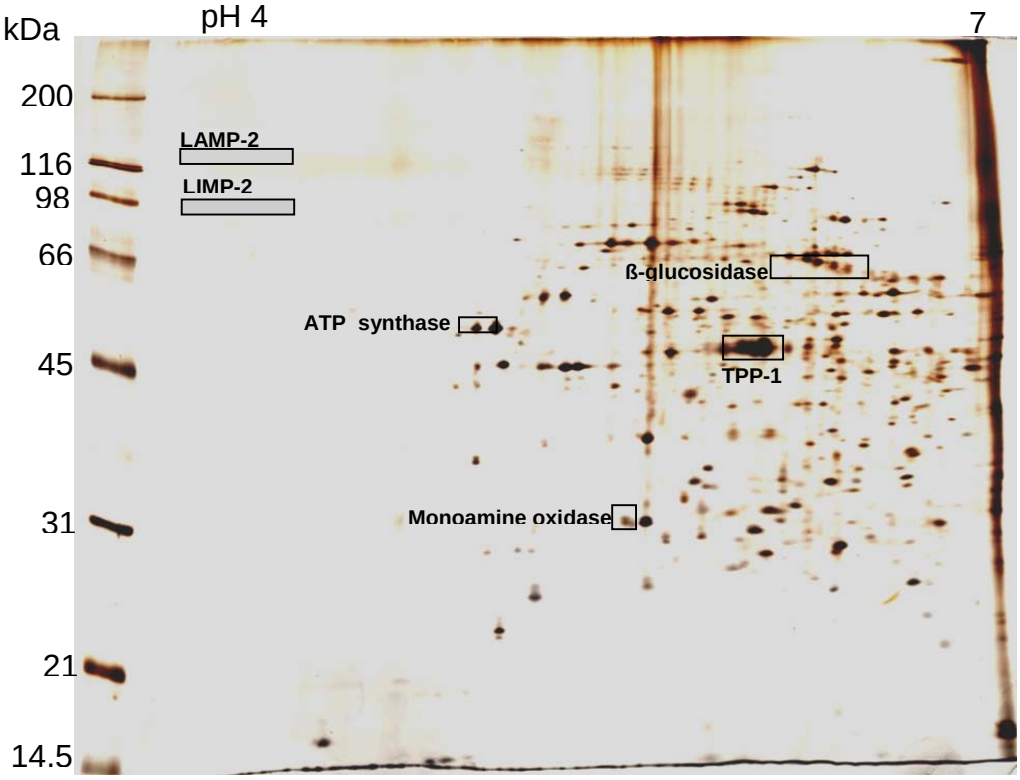


Fig. 4.2: Two-dimensional separation of ultracentrifuged lysosomal membrane proteins. UC membrane proteins were (20 µg) solubilised in lysis buffer containing urea and CHAPS. After isoelectric focusing for 20 h, proteins were separated in second dimension on a 15 % acrylamide gel and stained with silver nitrate**.**

The Percoll® that usually interfere with protein solubilisation and recognition of proteins by antibodies, was partly removed by this centrifugal step. The high-speed centrifugation resulted in the sedimentation of the membranes present in the sample on the top of a lucid Percoll® layer. Mass spectrometric analysis showed a contamination with mitochondrial proteins including monoamine oxidase, ATP synthase as well as a few proteins of plasma membrane or endoplasmic reticulum localisation (Dr. Buttgereit, personal communication). The lysosomes contribute to a small percentage of the cell contents. A small fraction of mitochondria co-sedimenting with lysosomes was sufficient to mask the lysosomal proteins of low abundance at later stages of analyses. This made further steps in the purification of lysosomes mandatory.

Several strategies were adopted to solve this problem. Attempts to purify the lysosomes from the dense pool using an immobilised antibody against the lysosomal membrane protein LAMP-2 (see 4.1.2) and alternatively a substrate-induced lysis of the lysosomes in combination with centrifugation (see 4.1.3) yielded good results. Several of the other strategies adopted were not efficient in purification and are not described.

4.1.2 Immuno-affinity purification of lysosomes

Since the lysosomal membrane protein LAMP-2 is expressed in high abundance, a monoclonal antibody raised against the luminal tail of this protein was used for the specific purification of the lysosomal vesicles from the DP. The antibodies were covalently coupled on the surface of Eupergit C1Z beads to perform immuno-affinity adsorption (see 3.2.1.4) after disrupting the lysosomes by sonication. The lysosomal vesicles thus produced allowed the binding of antibody to LAMP-2.

The DP lysosomes were ultrasonicated and a short-step centrifugation was performed prior to the incubation with the immuno-affinity beads. This centrifugation resulted in the sedimentation of aggregates (pellet) and intact organelles, mainly the contaminating mitochondria (Fig. 4.3). Most of the lysosomal proteins were not sedimented by this centrifugation suggesting a considerable enrichment of lysosomes by this step.

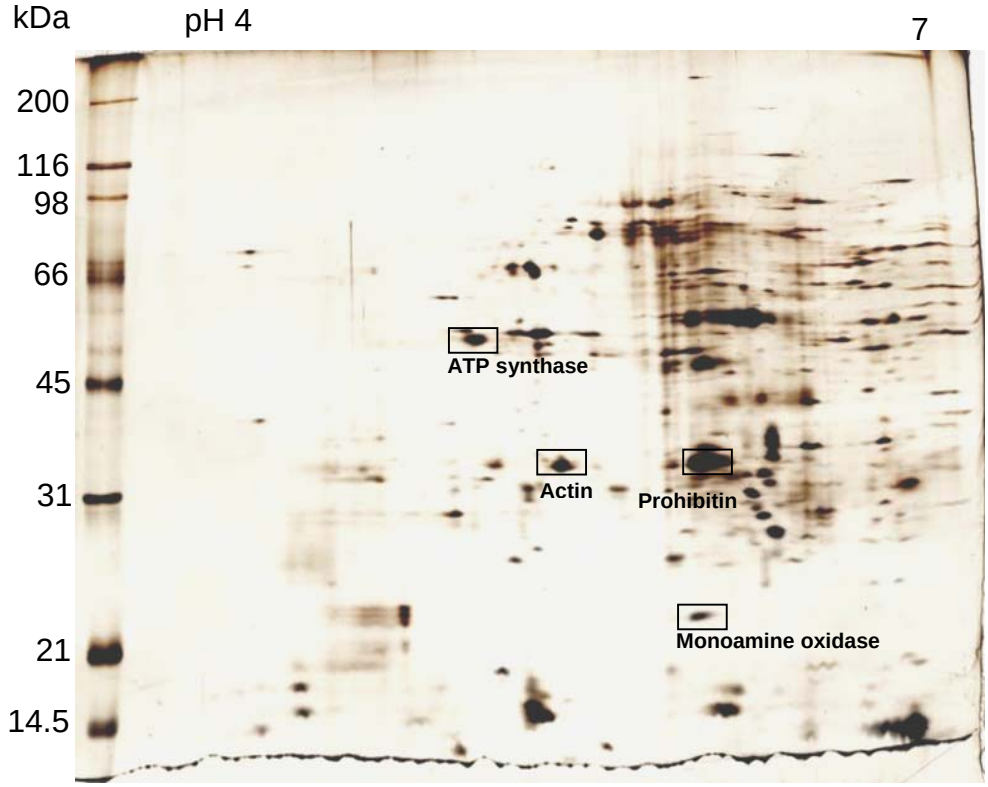


Fig. 4.3: Two-dimensional separation of the pellet containing mitochondrial proteins. The proteins (20 µg) pelleted by centrifugation before immuno-affinity purification. The sample was solubilised in 100 µl lysis buffer containing urea and CHAPS. After isoelectric focusing for 20 h, proteins were separated on a 15 % SDS-PAGE and stained with silver nitrate.

Marker enzymes for the lysosomal compartment- the membrane bound ß-glucosidase (Table. 4.2) and membrane integrated acetyl-CoA:α-glucosaminide N-acetyltransferase were enriched through this procedure. A large amount of ß-hexosaminidase activity was observed in the unbound fraction (Supernatant). This could be the portion of the enzyme released from lysosomes during the disruption by sonication. The purification was also confirmed by the separation of samples in two-dimensional electrophoresis (Fig. 4.4).

Table. 4.2: Purification of lysosomal membrane proteins by immuno-adsorption

Material	Protein (mg/ml)	ß-glucosidase (mU/mg)	ß-hexosaminidase (mU/mg)	Purification factor
Dense pool	1.53+/-0.28	15.3+/-2.4	1361+/-26.5	1
Pellet	0.9+/-0.23	4.4+/-0.7	211+/-15.5	0.29
Supernatant	1.27+/-0.17	7.87+/-0.85	7480+/-271	0.51
Affinity purified vesicles	0.93+/-0.35	48.4+/-10.3	1536+/-49.5	3.2

As shown in the table, affinity purification resulted in a 3.2 fold enrichment of lysosomal ß-glucosidase activity (average of 3 independent experiments.)

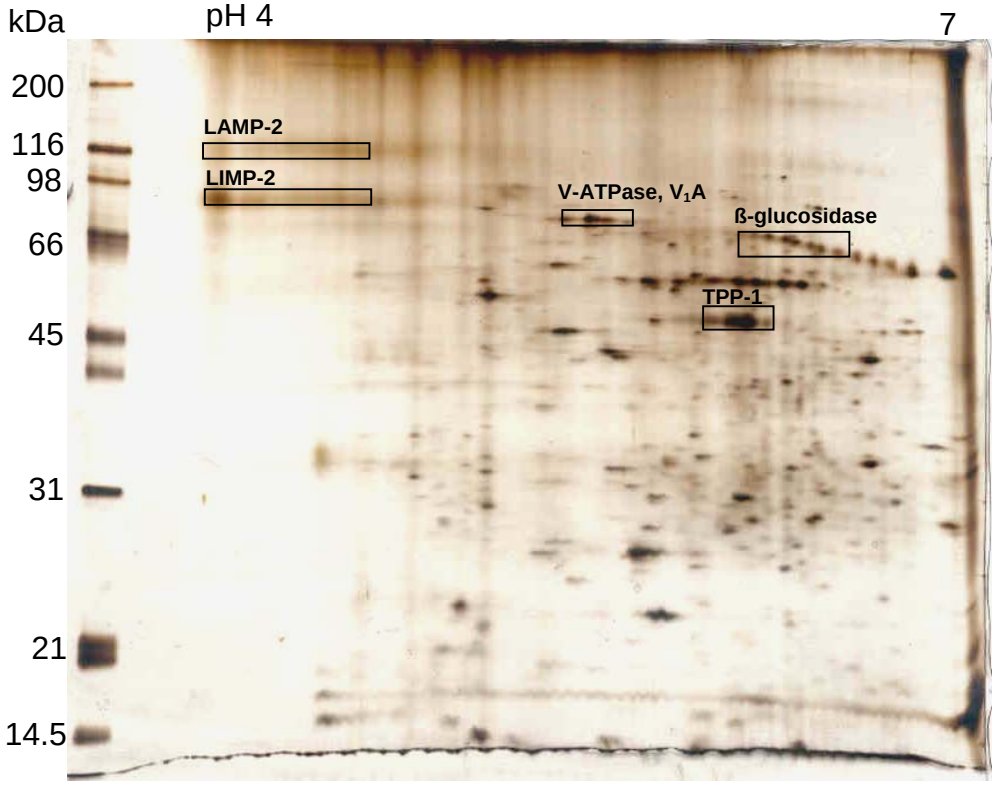


Fig. 4.4: Two-dimensional separation of the lysosomal membrane proteins after immuno-affinity purification. The lysosomal proteins (20 µg) purified by immuno-affinity adsorption were solubilised in lysis buffer containing urea and CHAPS. After isoelectric focusing for 20 h, second dimensional separation was carried out on a 15 % acrylamide gel and stained with silver nitrate.

The affinity purification resulted in a significant purification of lysosomal vesicles as shown by enrichment of ß-glucosidase activity. The signals for the integral membrane proteins LAMP, LIMP, TPP-1, vacuolar ATPase subunits and ß-glucosidase were enriched as observed from the 2D analysis (Fig. 4.4) in comparison to the signals from whole dense pool (Fig. 4.1).

Though the purification by immuno-affinity adsorption was quite successful and resulted in considerable enrichment of lysosomal proteins, the samples became contaminated with the antibody that was released from the beads. Another limitation of this procedure was regarding the recovery of the bound proteins from the beads in a native form. Detergents such as NP-40 and CHAPS were efficient for the extraction procedure. However, the antibodies from the beads contaminated the extracts. When the proteins were extracted with glycine/NaOH at pH 9, the antibody was not released but the efficacy of the subsequent binding was reduced to only 30-40 %, of the first time. On the preparative level this caused a limitation since a large-scale preparation could not be performed with the available antibody stock.

4.1.3 Purification of lysosomes by MME treatment

A selective substrate-induced disruption of lysosomes was achieved (see 3.2.1.5) in a buffer containing methionine methyl ester (MME). This amino acid ester was taken up by the lysosomes, and degraded by the hydrolases contained within. Accumulation of methionine and methanol produced by the hydrolysis were likely to cause an osmotic imbalance and bursting out of the vesicles. The sub-lysosomal vesicles thus obtained were separated by centrifuging in a linear sucrose gradient. As a result, the lysosomal marker enzymes fractionated in fractions of lower density compared with mitochondria.

An alternative method was sought to improve the removal of the contaminating vesicles. After the treatment of the lysosomes with MME, the incubation was continued in the presence of 10 mM $MgCl_2$ at pH of 7.4. An aggregation of membranous materials was observed that could be removed by a low-speed centrifugation (5 min at 11,000 rpm). The aggregates contained a portion of the mitochondrial marker enzyme, succinate dehydrogenase depending on the concentration of $MgCl_2$ used (Fig. 4.5). It was found that, to selectively sediment the mitochondrial contaminants, 10 mM $MgCl_2$ was the optimal concentration. The major portion of the lysosomal proteins (ß-glucosidase) remained in the solution.

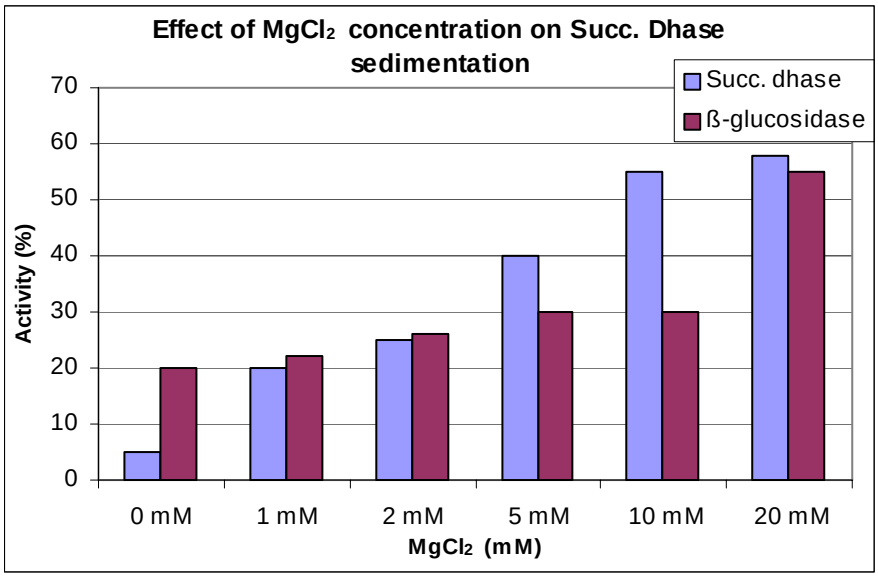


Fig. 4.5: The effect of $MgCl_2$ concentration on the sedimentation of succinate dehydrogenase. Samples disrupted with MME and incubated without $MgCl_2$, but centrifuged at 11,000 rpm were used as controls and the activities measured from this sample without centrifugation were taken as 100 %.

The proteins sedimented by centrifugation were analysed by 2D electrophoresis using thiourea/ASB-14 solubilisation system (Fig. 4.6) and was found to contain a high-intensity signal corresponding to the mitochondrial ATP synthase which was almost invisible in the

supernatant. There were also weak signals for LAMP-2, LIMP-2 and TPP-1, but were weakly stained as compared with the one in the supernatant fraction.

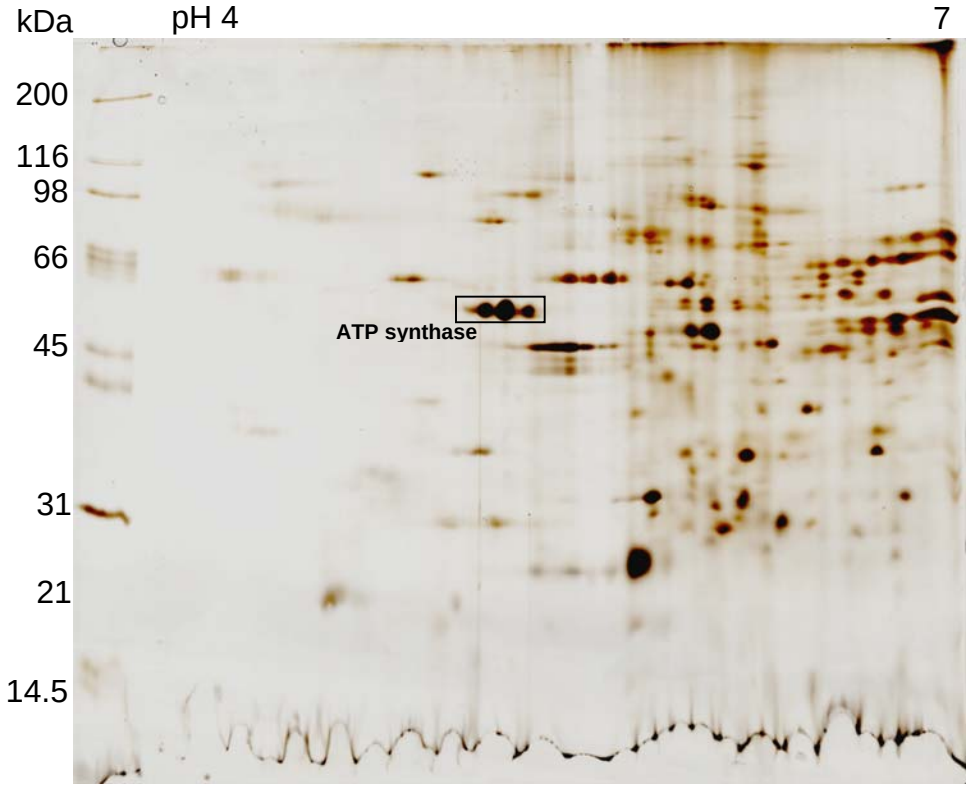


Fig. 4.6: The two-dimensional electrophoresis of the aggregated proteins after the $MgCl_2$ treatment. The aggregated proteins (20 µg) obtained by $MgCl_2$ treatment was solubilised in lysis buffer containing thiourea and ASB-14. After isoelectric focusing for 14 h at 37°C, proteins were separated on a 15 % acrylamide gel and stained with silver nitrate.

Table. 4.3: Disruption of lysosomes with MME and removal of contaminants by incubation with 10 mM $MgCl_2$ for 12 h, followed by centrifugation at 11,000 for 5 min.

Material	ß-glucosidase (mU/mg)	ß-hexosaminidase (mU/mg)	Succinate dehydrogenase(mU/mg)
Dense pool (DP)	42+/-12.3	845+/-35	6.3+/-1.8
Lysate of DP by MME	63+/-9.5	412+/-16	6.48+/-1.67
Supernatant fraction after MME-induced lysis of DP lysate and $MgCl_2$ incubation	124+/-16.2	950+/-28	1.6+/-0.45
Aggregate after the $MgCl_2$ treatment	21+/-4.2	75+/-7.4	8.27+/-0.44
Lysosomal matrix fraction	Not detectable	1732+/-45.2	Not detectable
Lysosomal membrane fraction	241+/-47.8	Not detectable	Not detectable

(Values given are the averages of 3 independent experiments)

The supernatant and pellet obtained after the $MgCl_2$-induced aggregation was analysed on a Western blot after separation on a 10 % SDS-PAGE (Fig. 4.7). A polyclonal antibody against the subunit `a` of the vacuolar ATPase complex was used for the detection.

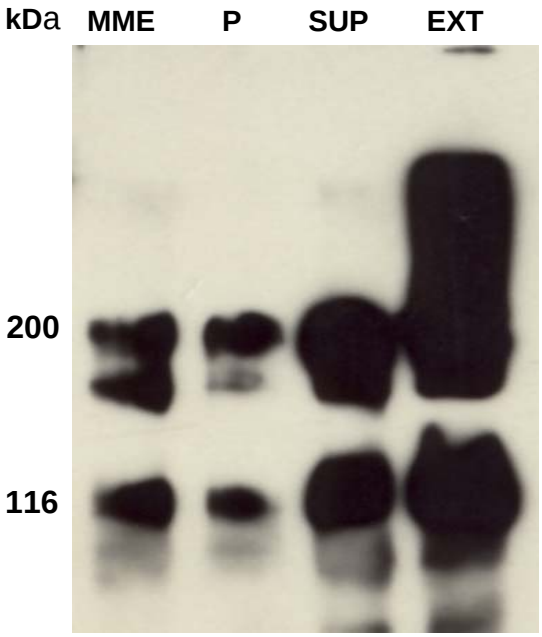


Fig. 4.7: Effect of $MgCl_2$-induced sedimentation on vacuolar ATPase. The MME-lysed lysosomes (MME), pellet obtained after $MgCl_2$ treatment (P), supernatant after $MgCl_2$ treatment (SUP) and the membrane extracted with 1 % (w/v) Triton X-100 (EXT), each 5 µg were solubilised in SDS-buffer by incubating at 37 °C and separated on a 10 % acrylamide gel. After blotting to a PVDF membrane, antibody against the subunit `a` of V-ATPase was used for the detection. Binding of peroxidase conjugated secondary antibody was visualised by the ECL method.

All examined samples contained the equal amount (5 µg) of protein. In comparison to the starting material (MME), the signal for vacuolar ATPase subunit `a` was more prominent in the supernatant fraction obtained after cleaning with $MgCl_2$ (SUP). In the pellet fraction- where mostly contaminating proteins were observed, the signal was week. This suggests that a good degree of purification can be achieved by the $MgCl_2$ treatment. When the supernatant was sedimented by ultracentrifugation and the sediment extracted with a detergent (1 % Triton X-100), a strong antibody signal was found in the soluble fraction.

In addition to the signal corresponding to the nominal molecular mass (116 kDa), there was an additional signal around 200 kDa. Before the detergent extraction, this signal was weak. Its origin is not clear. One possibility would be a protein precipitation and aggregation preventing it from the downward movement along the gel.

The supernatant obtained after $MgCl_2$ treatment was analysed by two-dimensional electrophoresis. A specific enrichment of lysosomal marker proteins including LIMP-2, ß-glucosidase, TPP-1 and the subunits from V-ATPase were observed in these cleaner membranes upon visualisation by silver staining (Fig. 4.8).

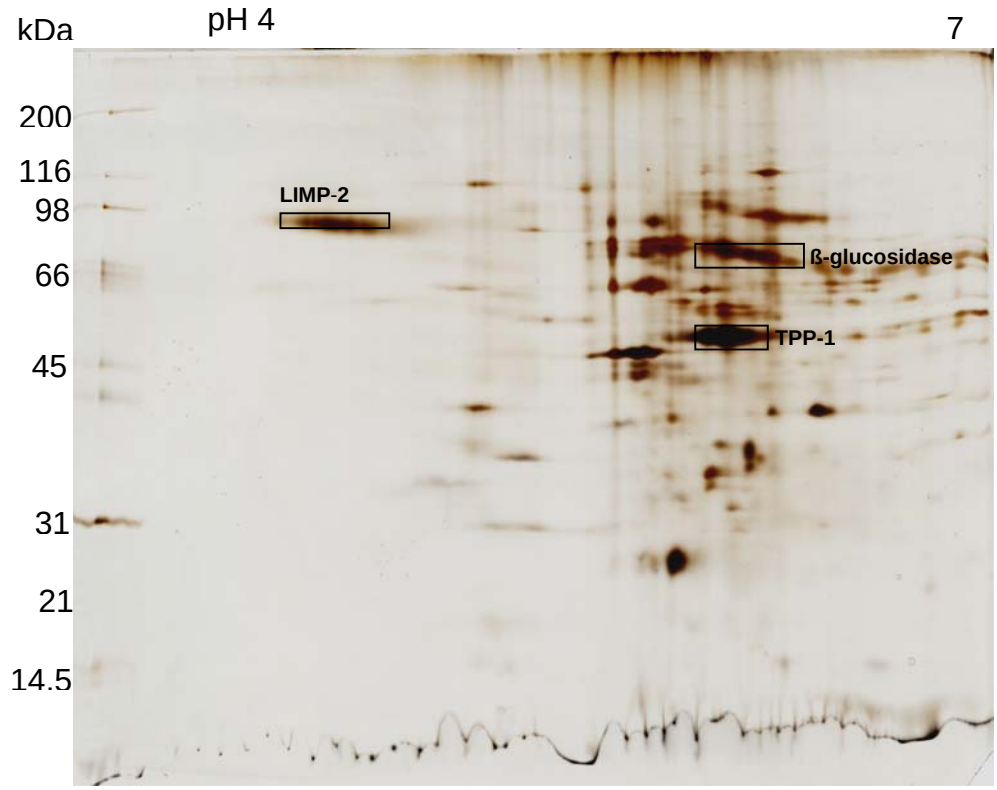


Fig. 4.8: Two-dimensional separation of lysosomal vesicles in the supernatant after $MgCl_2$-induced aggregation. The supernatant (20 µg) obtained after $MgCl_2$ treatment was solubilised with lysis buffer containing thiourea and ASB-14. Sample was subjected to isoelectric focusing for 20 h at 37 °C and separated on a 15 % acrylamide gel. The polypeptides were visualised by silver staining.

Ultracentrifugation of these vesicles provided lysosomal membranes (Fig. 4.9) with a high purity as shown by enrichment of membrane bound ß-glucosidase activity up to 6 fold (Table. 4.3). Activity of mitochondrial markers were too less to be calculated, which again suggested a good degree of lysosomal membrane enrichment.

The number and complexity of protein spots observed in the cleaned membranes after the removal of aggregates was decreased as compared to the preparations made by other procedures. A spot resembling the NPC-1 protein was visible upon silver staining as a faint band on a high molecular weight region of the gel. The appearance of this hydrophobic protein with 13 tentative transmembrane segments in the membrane preparations not only indicated a good quality of the membranes, but also an effective solubilisation in the thiourea/ASB-14 system as compared to the urea/CHAPS system.

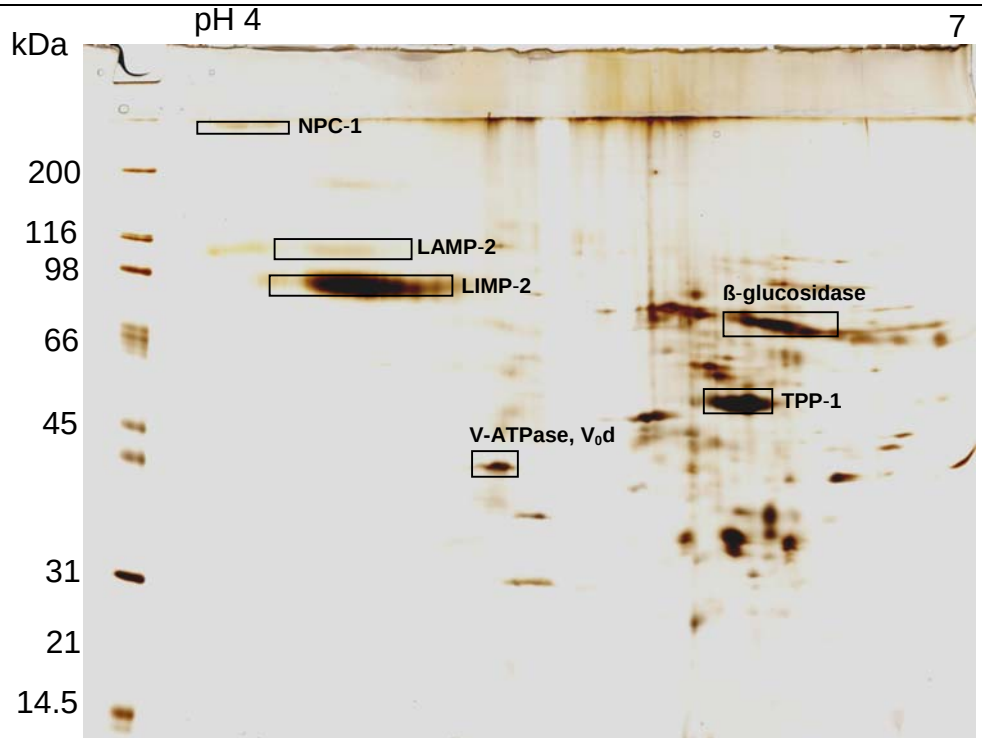


Fig. 4.9: Two-dimensional separation of the lysosomal membranes obtained after the removal of $MgCl_2$-induced aggregates and the matrix proteins. The membrane preparation (20 µg) obtained by the ultracentrifugation of supernatant fraction after removal of aggregates was solubilised in 100 µl lysis buffer containing thiourea and ASB-14 and subjected to isoelectric focusing for 20 h at 37 °C. Proteins were separated in the second dimension on a 15 % acrylamide gel, and stained with silver nitrate.

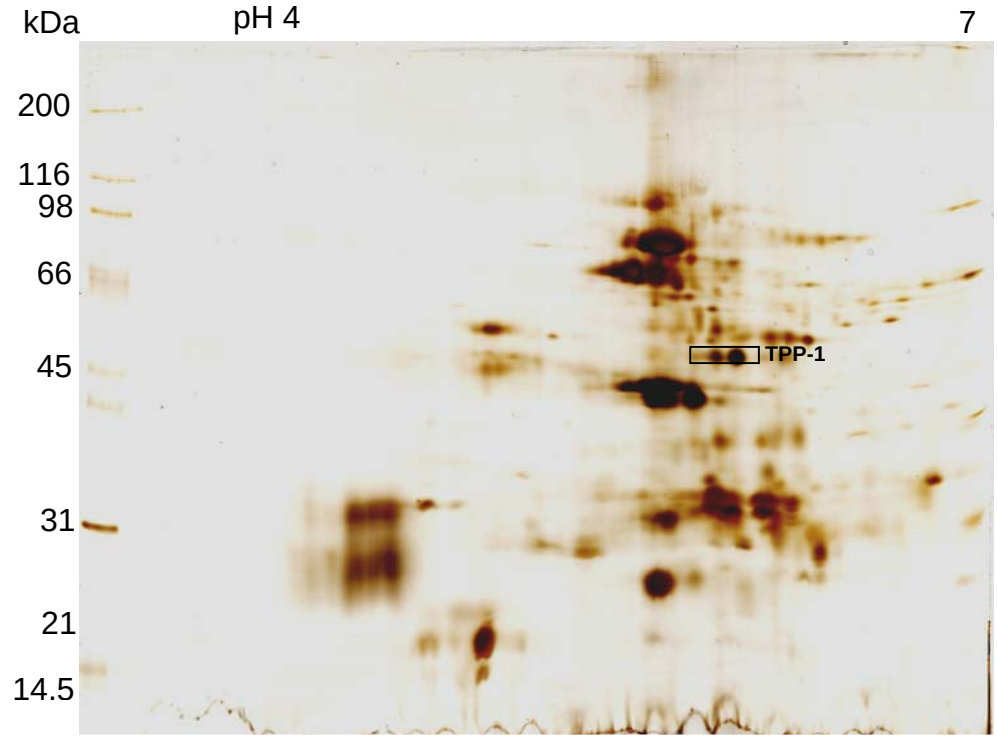


Fig. 4.10: Two-dimensional electrophoresis of the lysosomal matrix protein fraction. The matrix proteins (20 µg) in the supernatant fraction of the lysosomal vesicles made by MME-treatment and $MgCl_2$-induced cleaning. The sample was solubilised in 100 µl lysis buffer containing thiourea and ASB-14 and subjected to isoelectric focusing for 20 h at 37 °C. Proteins were separated on a 15 % acrylamide gel, and stained with silver nitrate.

The supernatant obtained after the ultracentrifugation step contains the matrix proteins from the lysosomal compartment with an enrichment of up to 2 fold as shown by ß-hexosaminidase activity measurement. Proteins in this fraction were separated by two-dimensional electrophoresis and visualised by silver staining (Fig. 4.10). The sample was found to contain several glycosylated proteins. A major observation was the relative abundance of tripeptidyl peptidase-1 (TPP-1) in the matrix fraction. In comparison with the membrane fraction, the intensity of the spot in the matrix fraction was decreased. This suggested that during the separation of matrix proteins by the ultracentrifugation a larger portion of TPP-1 was sedimented with the membrane components.

The pellet and supernatant obtained after $MgCl_2$ treatment were analysed on separate sucrose density gradients, and activities were followed. Upon centrifugation in a self-forming sucrose density gradient, almost all the proteins from the supernatant (Fig. 4.11) were localised in the low-density zone of the gradient, whereas the proteins from the pellet sample were observed on a more dense area of the gradient (Fig. 4.12).

Such an observation suggested that the pellet obtained after $MgCl_2$ treatment was predominantly contaminating proteins. A small portion of the proteins- but distinct in its pattern from the general profile was found in the low-density area of the gradient. The protein pattern was similar to the supernatant obtained after $MgCl_2$ treatment. This could be the portion of lysosomal vesicles that was trapped within the aggregates, and set freed by the dilution during the density gradient centrifugation.

In the sucrose gradient, where the supernatant fraction obtained after the $MgCl_2$-induced was separated, most of the proteins were belonging to the lysosomes, and a very small portion of the total protein was found in the higher-density zone of the gradient. The observation was similar in the sucrose gradient where the aggregates were separated, but most proteins were located on the area where mitochondrial proteins would be expected in such a gradient.

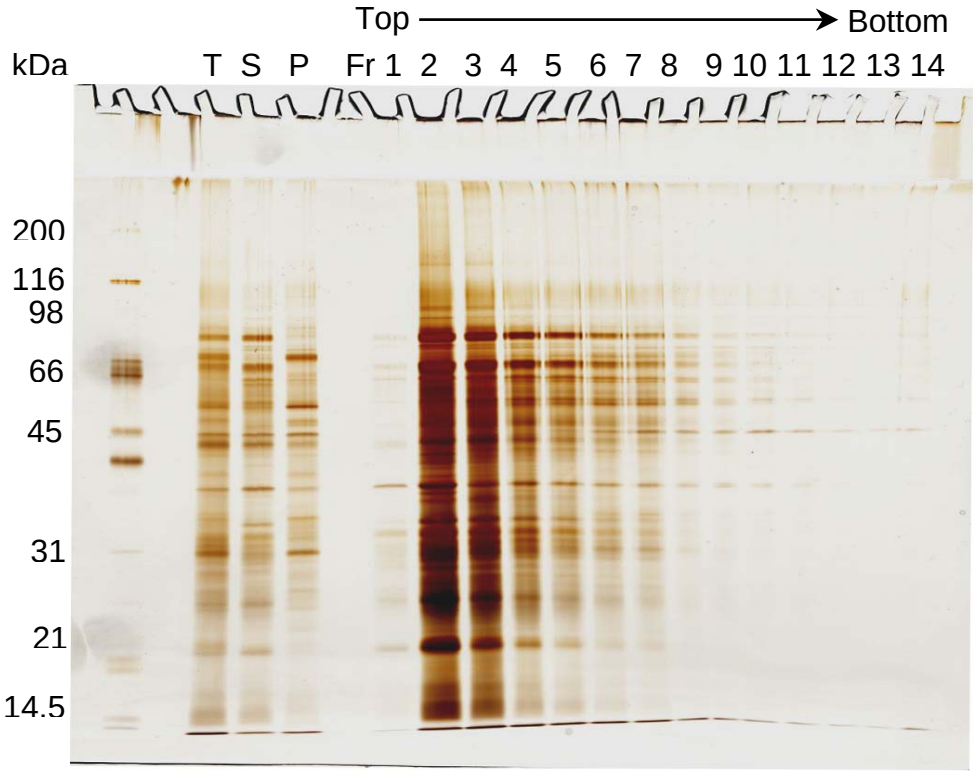


Fig. 4.11: The separation of membranes that were not aggregated in the presence of $MgCl_2$. The lysate after MME treatment (T) was separated by centrifugation into supernatant (S) and pellet (P) fractions after $MgCl_2$ treatment. The supernatant fraction was separated by centrifuging at 100,000 × g_{max} for 10 h on a linear gradient of sucrose (30 %-55 % sucrose) and collected into 14 fractions of equal volumes. From each fraction, 0.5 µg protein was incubated with SDS solubiliser, separated on a 15 % SDS-PAGE and stained with silver nitrate.

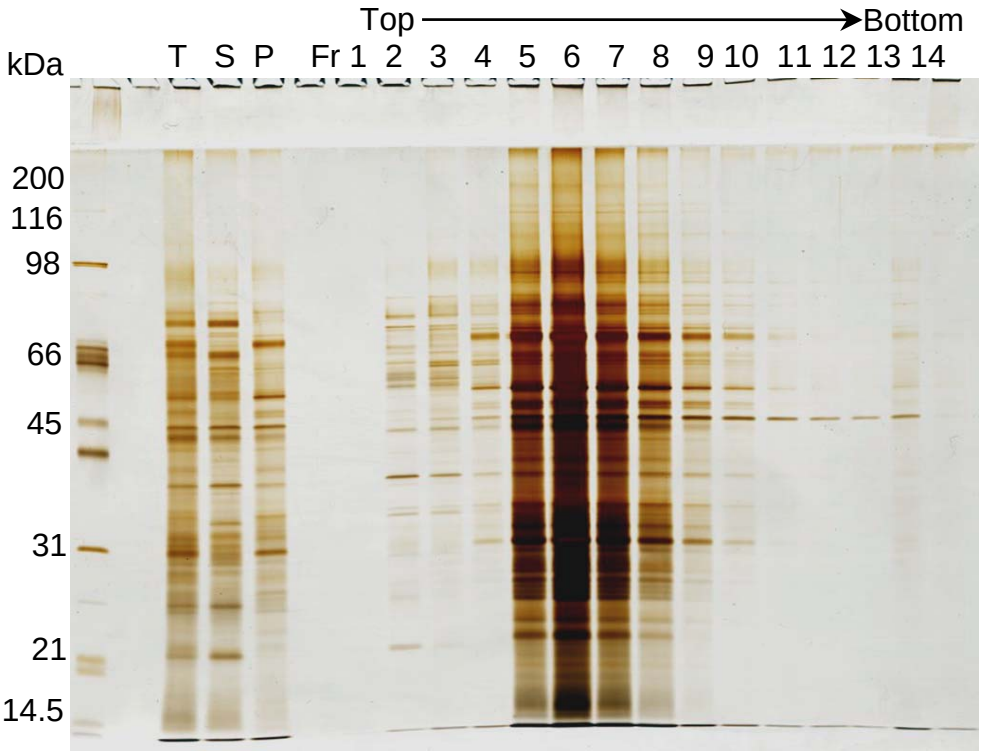


Fig. 4.12: The separation of membranes aggregated in the presence of $MgCl_2$. The lysate after MME treatment (T) was separated by centrifugation into supernatant (S) and pellet (P) fractions after $MgCl_2$ treatment. The pelleted fraction was separated by centrifuging at 100,000 × g_{max} for 10 h on a linear gradient of sucrose (30 %-55 % sucrose) and divided into 14 fractions of equal volumes. From each fraction, 0.5 µg protein was incubated with SDS solubiliser, separated on a 15 % SDS-PAGE and stained with silver nitrate.

4.2 Analysis of detergent resistant microdomains on the lysosomal membrane

4.2.1 Flotation experiments of detergent resistant microdomains

The presence of detergent resistant micodomains (DRM) on the lysosomal membrane has been reported (Taute *et al.,* 2002). These micro domains were found to be enriched in the lysosomal membrane acetyl-CoA: alpha-glucosaminide N-acetyl transferase (AcTf) activity. A proteomic approach was carried out for the identification of proteins associated with a DRM fraction from lysosomal membrane. This was done by extracting the whole proteins from the lysosomal membrane under specific conditions of detergent concentration (1 % Triton X-100) and temperature (4 °C). The extract was mixed with iodixanol to obtain a concentration of 40 % followed by layering it on the bottom of the gradient. After an ultracentrifugation for 5h the floating low-density material was collected.

All the fractions obtained from the gradient were analysed by separating on an SDS-PAGE and visualising by silver staining (Fig. 4.16). There were a number of proteins that showed a uniform distribution throughout the gradient. On the other hand, a specific enrichment of a small population of polypeptides was observed in the fractions containing the DRMs. Most of them were distinct from the majority of proteins. A few proteins were present only in the upper fractions while a few others, though present in all fractions most abundantly visible in the low-density fractions.

The lysosomal preparations showed PLAP activity. This GPI-anchored protein is localised mainly in the plasma membrane and associated with DRMs (Snyers *et al.,* 1999). It provided an internal control for the flotation experiments. Initial experiments were performed using UC membranes of less purity, which showed an enrichment of AcTf and PLAP (Fig. 4.13) in the floating fractions though the recovery of AcTf was only around 10 %.

As described by others, the floating properties of these enzymes were highly dependent on the temperature of extraction. When the extraction was carried out at a higher temperature (37 °C) the floatation was no longer observed (Fig. 4.14). The DRMs are known to disintegrate in the detergent solutions at higher temperatures.

The recoveries of both PLAP and AcTf were very low when the extraction was done at 37 °C (Fig. 4.14). Similar results were obtained when the extraction was carried out at 4 °C after a pre-incubation with 20 mM of the cholesterol-depleting agent, methyl-ß-cyclodextrin (MBCD). Cholesterol is an important component of the DRMs. Its selective depletion resulted

in a decrease in activity of the proteins associated with the DRMs and the floatation was no longer observed.

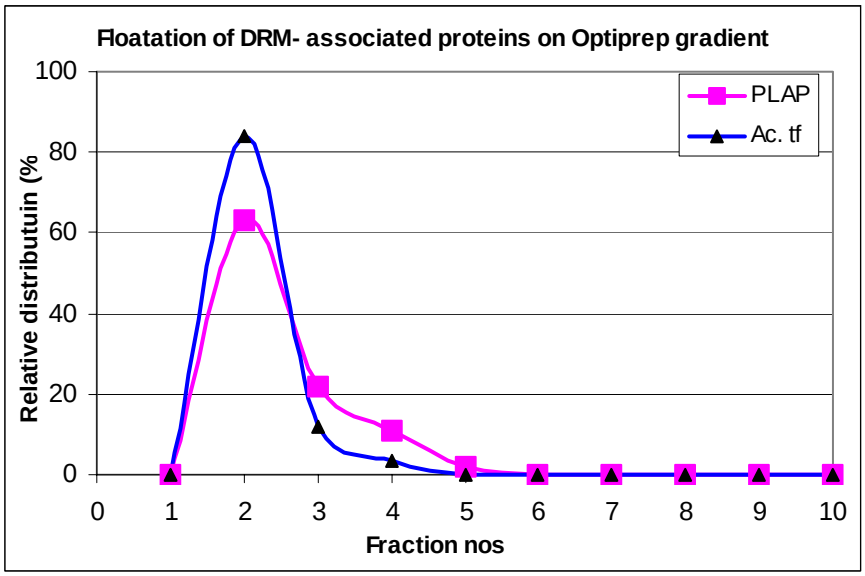


Fig. 4.13: The distribution of AcTf and PLAP on iodixanol gradient. The membranes were extracted at 4 °C, and separated on a step-gradient of iodixanol. (Activities expressed as percentage of recovered)

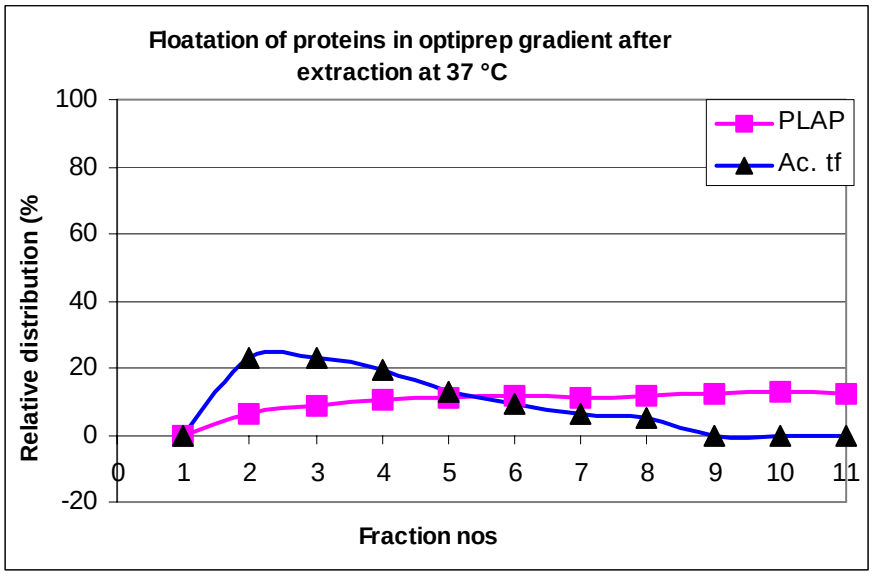


Fig. 4.14: The Distribution of AcTf and PLAP when extracted at 37 °C. (Activities expressed as percentage of recovered)

The detergent used for extraction was critical in obtaining the DRMs. Experiments carried out with CHAPS, NP-40, octyl thioglucoside and Lubrol did not show any specific floating of either PLAP or AcTf irrespective of the temperature of extraction (data not shown). All experiments in the preparative level were thus performed with Triton X-100 and the extraction was performed at 4 °C.

A significant portion of the lysosomal V-ATPase was found in the low buoyant density fractions 2 and 3 (Fig. 4.15). It has been reported recently that the DRMs isolated from plasma membranes contain several subunits of V-ATPase (Galli *et al.,* 1996, Blonder *et al.,* 2004, Yoshinaka *et al.,* 2004) and that in yeast such an assembly of vacoular ATPase is necessary for the surface delivery (Bagnat *et al.,* 2000). However, the recovery of V-ATPase activity after the iodixanol gradient was very low. It is not clear if the activity of the V-ATPase was decreased as a whole or due to a selective detachment of one or more subunits diminished the activity.

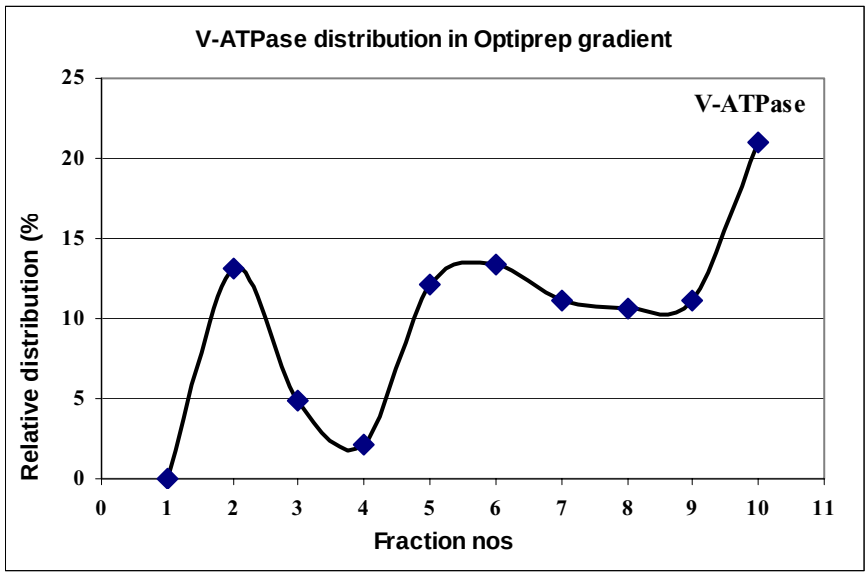


Fig. 4.15: The distribution of V-ATPase activity in fractions of Optiprep™ density gradient. The membranes were extracted at 4 °C with 1 % (w/v) Triton X-100 and separated on a step-gradient of Iodixanol (Activities expressed as percentage of recovered)

4.2.2 Identification of floating components of the lysosomal membrane

To identify proteins that are associated with the lysosomal DRMs, mass spectrometric analysis was carried out after pooling fractions from 3 separate Optiprep™ density gradients. The lysosomal membranes that were disrupted by treatment with MME, and cleaned by $MgCl_2$ were used for the analyses. Separation of proteins was done on a 15 % SDS-PAGE (Fig. 4.16) for analytical level and stained with colloidal Coomassie blue (Fig. 4.17) for the mass spectrometric analysis as mentioned (see 3.2.1.10.5). An aliquot of the sample was run simultaneously for silver staining as a control. Protein bands were cut out from the Coomassie blue-stained gel, and tryptic digests were used for Maldi-TOF and corresponding proteins were identified (Table. 4.4).

Out of the 9 protein bands analysed, 5 subunits of V-ATPase were identified to be present in the DRM fractions. These observations are correlating to the findings obtained. Placental alkaline phosphatase was a major protein identified in the band numbered 3 with the highest mowse score of 180. Though the floating fractions showed activity for AcTf, none of the bands identified gave corresponding results. This might be due to the very low content of this protein in the fractions analysed.

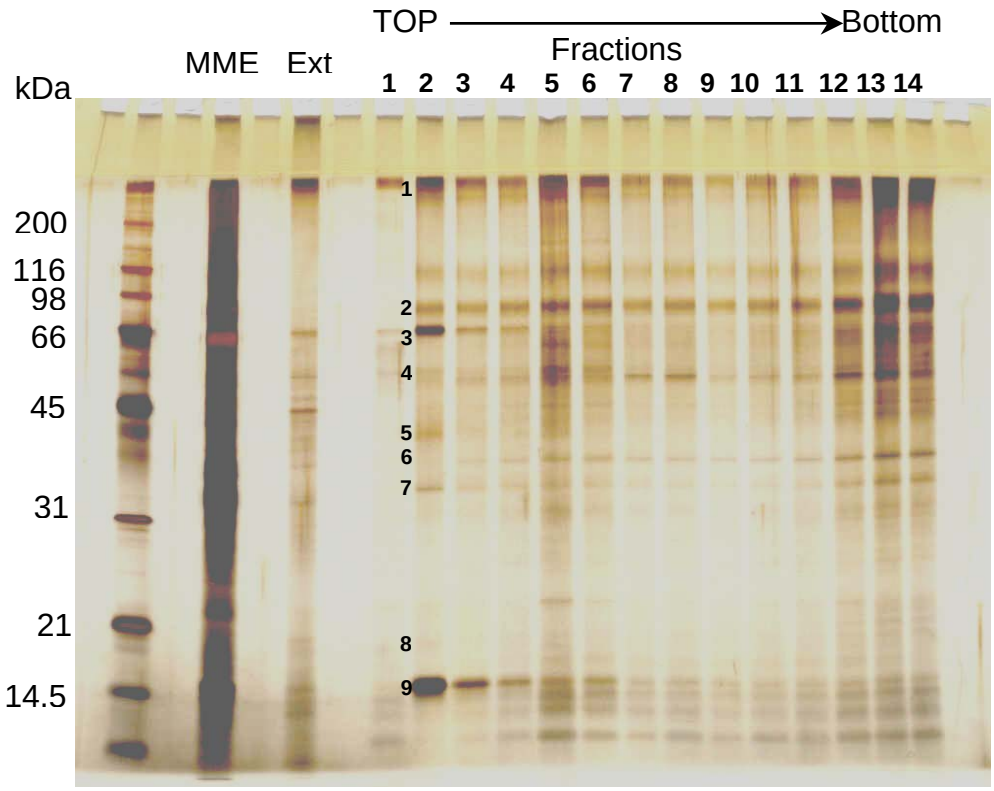


Fig. 4.16: Optiprep™ gradient fractions prepared from the MME lysed lysosomal membranes. Lysosomes after MME-mediated lysis (MME), and extraction with Triton X-100 at 4 °C (Ext) were separated on an optiprep™ gradient into 14 fractions. From each sample, an aliquot containing 0,5 µg protein were solubilised and separated on a 15 % SDS-PAGE, for staining with silver nitrate.

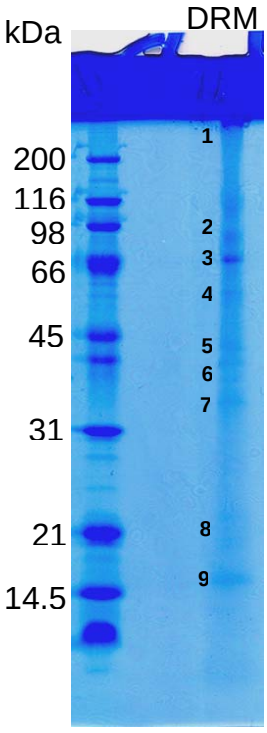


Fig. 4.17: Optiprep™ gradient fractions prepared from MME lysed lysosomal membranes for mass spectrometric analysis. For the identification of proteins associated with DRMs, low-density fractions (fractions 1and 2) from three different gradients were pooled, concentrated (DRM) by precipitating with 4 volumes of cold acetone and separated on a 15 % SDS-PAGE after solubilisation. The separated protein was stained by colloidal Coomasssie blue and the protein bands were analysed by Maldi-TOF after digesting with trypsin.

Table. 4.4: Proteins identified by Mass spectrometry from lysosomal DRMs

Spot number	Protein identified	Mowse score	Modification	Mass tolerance (Da)	GRAVY score
1	Vacuolar ATPase subunit a (V_0a)	83	Carbamidomethyl	0.1	0.009
2	LIMP-2	64	Carbamidomethyl	0.1	-0.043
3	Placental alkaline phosphatase (PLAP)	180	Carbamidomethyl	0.1	-0.207
4	Vacuolar ATPase subunit B (V_1B)	74	Carbamidomethyl	0.1	-0.174
5	Vacuolar ATPase subunit D (V_1D)	120	Carbamidomethyl Oxidation (M)	0.2	-0.444
6	Annexin I	75	Carbamidomethyl	0.2	-0.426
7	Vacuolar ATPase subunit E (V_1E)	76	Carbamidomethyl Oxidation (M)	0.2	-0.532
8	Neural adhesion molecule NB-3 (fragment)	66	Carbamidomethyl	0.25	-0.255
9	No match (V-ATPase-V_0c 5 peptides found)	Not significant	Carbamidomethyl Oxidation (M)	0.5	1.04

Identification of lysosomal membrane vacuolar ATPase subunits as components of the DRMs was of relevance as the same observations have been published by others, but from other organelles and tissue types. Isolation and functional reconstitution of this proton pump could provide a system to understand the role of these lipid domains on the enzymes` stability and biological activity as well as to study the inhibitory effects of a few chemically derived agents, which are relevant to the pathology of lysosomes.

4.3 Isolation of vacuolar ATPase

4.3.1 Affinity purification of V-ATPase

For the isolation of the vacuolar ATPase, biochemical techniques utilising its affinity to ATP were checked. The $(AB)_3$ cluster in the V_1 domain is responsible for ATP cleavage. For isolating the protein complex, immobilised ATP, coupled to Sepharose was used as a ligand. The ATP in the resin was coupled through its γ-phosphate group so that there cannot be any cleavage of the substrate upon protein binding (manufacturer's instructions). Though there was a clear binding of ATP-binding proteins (Fig. 4.18), it was found that the resin bound proteins including the contaminating ATPase of mitochondrial origin. The elution procedure

with a higher concentration of ATP (20mM) interfered with the enzyme activity measurements, based on Pi generation using 200 µM ATP as the substrate even after dialysis.

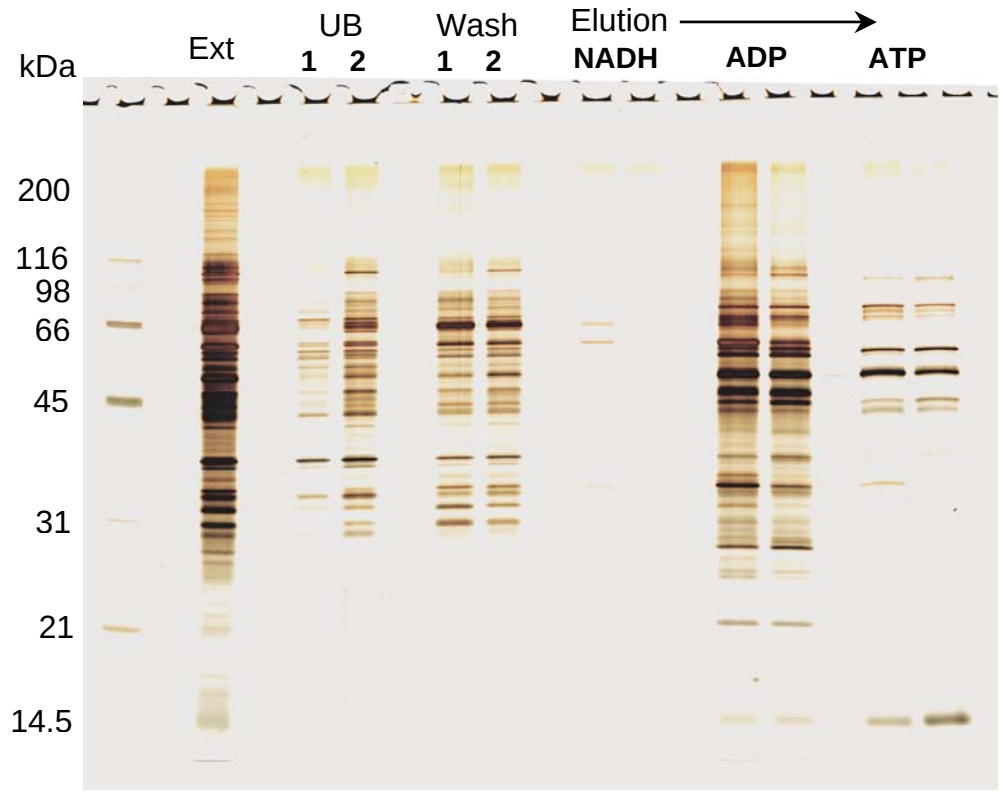


Fig. 4.18: Purification of ATP-binding proteins by affinity chromatography. The lysosomal membranes prepared by MME-induced lysis and ultracentrifugation was extracted with 1 % (w/v) Triton X-100 (Ext). This was incubated with the ATP-acrylamide resin and unbound proteins (UB) removed by centrifugation followed by a step-wise elution of bound proteins using NADH, ADP and ATP. Aliquots containing 0.5 µg protein from each fraction were incubated with solubiliser, separated on a 15 % SDS-PAGE, and visualised by silver staining.

4.3.2 Isolation of V-ATPase by gel filtration

The V-ATPase is a high molecular weight protein complex, which made its separation possible by gel filtration. The gel filtration was performed using the lysosomal membranes prepared by MME-induced disruption of dense pool and removal of contaminating proteins through a cleaning step by incubation with $MgCl_2$ and removal of the aggregates by centrifugation. The proteins were extracted with 1 % Triton X-100 and 0.25 % CHAPS (see 3.2.1.6) in presence of 20 mM methyl ß-cyclodextrin, to disrupt the lipid mediated assembly of proteins in the DRMs. The extraction was done at room temperature for 30 min. Fractions from the gel filtration were collected and various enzymes were assayed. Multiple ATPase activity peaks were observed in the fractions (Fig. 4.19). Only the activity near the void volume was sensitive to *N*-ethylmaleimide (NEM). These fractions (Fractions 9-12) from the gel filtration corresponded to 950-1000 kDa. They were pooled and used for further analysis.

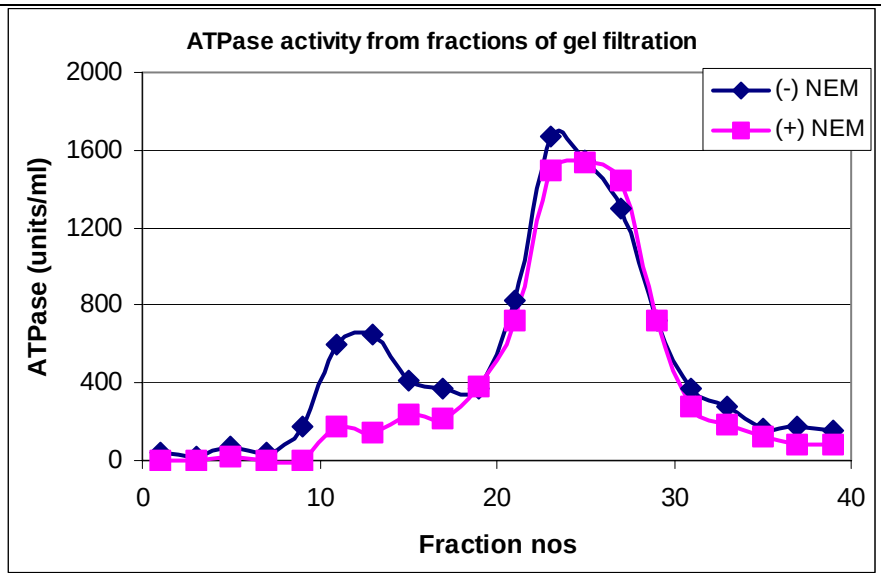


Fig. 4.19: ATPase activity from the fractions of gel filtration. The lysosomal membranes were prepared from dense pool by the MME-induced disruption followed by $MgCl_2$ mediated aggregation of mitochondrial contamination and its removal by centrifugation. The membranes (150-200 µg) obtained were extracted with 1 % Triton X-100 and 0.25 % CHAPS in presence of 20 mM methyl-ß-cyclodextrin at room temperature so as to get a protein concentration of 75-100 µg/ml. The extract was separated by gel filtration using a Superdex-100 column. Fractions were collected and ATPase activity was measured in the presence and absense of NEM. *(One unit of ATPase activity is defined as the amount of inorganic phosphate in micromoles generated by the enzyme during one hour of incubation with the substrate-ATP.)*

The later fractions (13-16) were enriched in the activity of AcTf. This suggests either the complex molecular structure of the protein or the possibility of a supra-molecular assembly the protein is involved in, which was not disrupted by the treatment with MBCD.The activity of PLAP was also found in the fractions of V-ATPase activity. To confirm that the ATPase activity from these fractions was attributed to V-ATPase, the ATPase activity fractions were assayed in the presence of L-Phenylalanine, an inhibitor of PLAP (Fishman, 1987). L-Phenylalanine in millimolar concentrations affected the activity of PLAP, while concentrations up to 50 mM did not influence the activity of V-ATPase at all (Fig. 4.20).

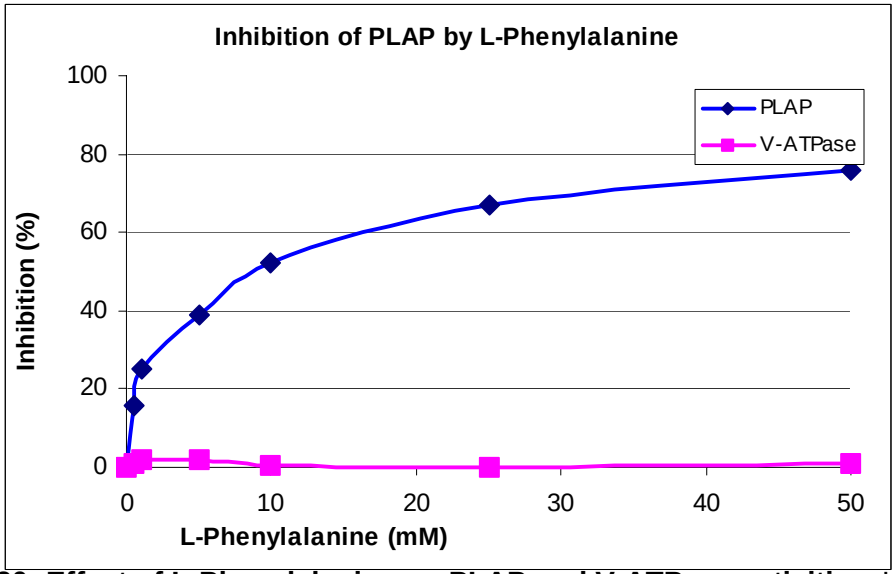


Fig. 4.20: Effect of L-Phenylalanine on PLAP and V-ATPase activities. L-phenylalanine at 50 mM concentration completely inhibits PLAP, but had little effect on V-ATPase activity.

The fractions showed V-ATPase activity obtained from the gel filtration were analysed on SDS-PAGE (Fig. 4.21).

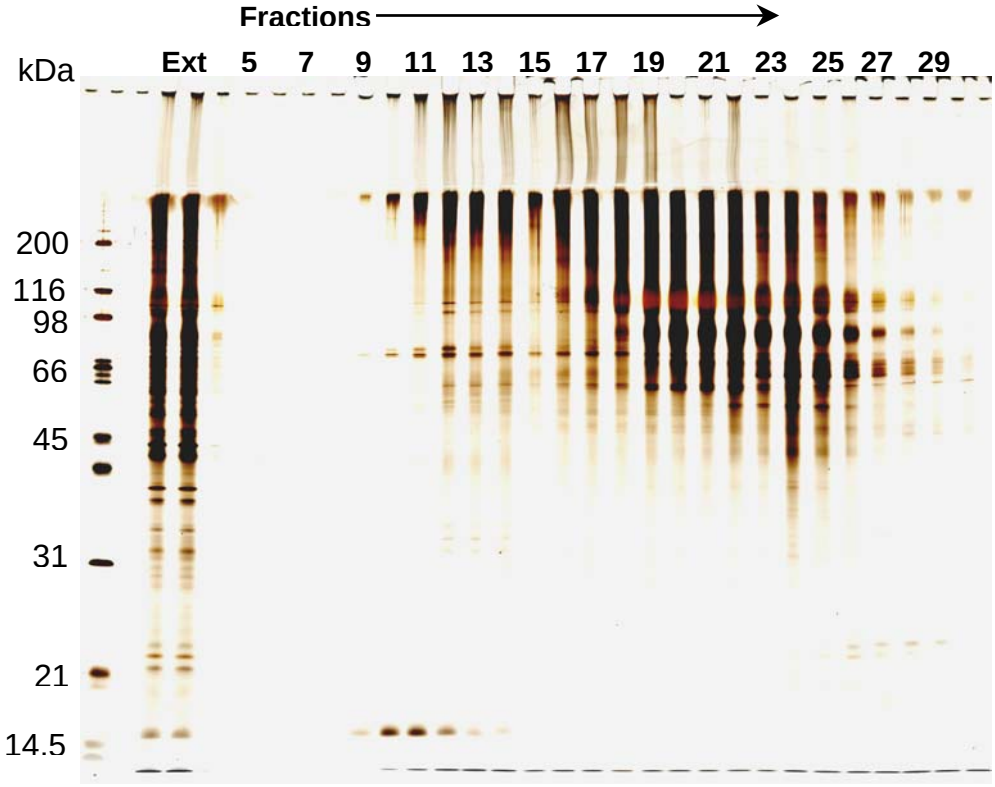


Fig. 4.21: Characterisation of proteins in the fractions of gel filtration.
The lysosomal vesicles prepared by MME-induced lysis of dense pool were incubated with $MgCl_2$. After removing the aggregated proteins by centrifugation, lysosomal membranes were prepared by ultracentrifugation and extracted at room temperature (Ext) with 1 %Triton X-100 and 0.5 % CHAPS in presence of 20 mM methyl-ß-cyclodextrin.The extract was separated by gel filtration and the fractions were solubilised in SDS solubiliser for separating on a 15 % SDS-PAGE. The proteins were visualised by silver staining.

The ATPase activity fractions were separated on two-dimensional electrophoresis (Fig. 4.22) using thiourea/ASB-14 solubilisation system after concentrating the samples by precipitating with cold acetone. A protein spot corresponding to the accessory subunit of V-ATPase (Ac 45) was found to be enriched in the fractions analyesd. Since several of the V-ATPase subunits have a basic isoelectric point (Table. 1.2), they could not be detected in the regular two-dimensional systems. Furthermore, the resolution of protein spots were not satisfactory, possibly due to the more hydrophobic proteins present in these samples or the lipids that are part of this complex.

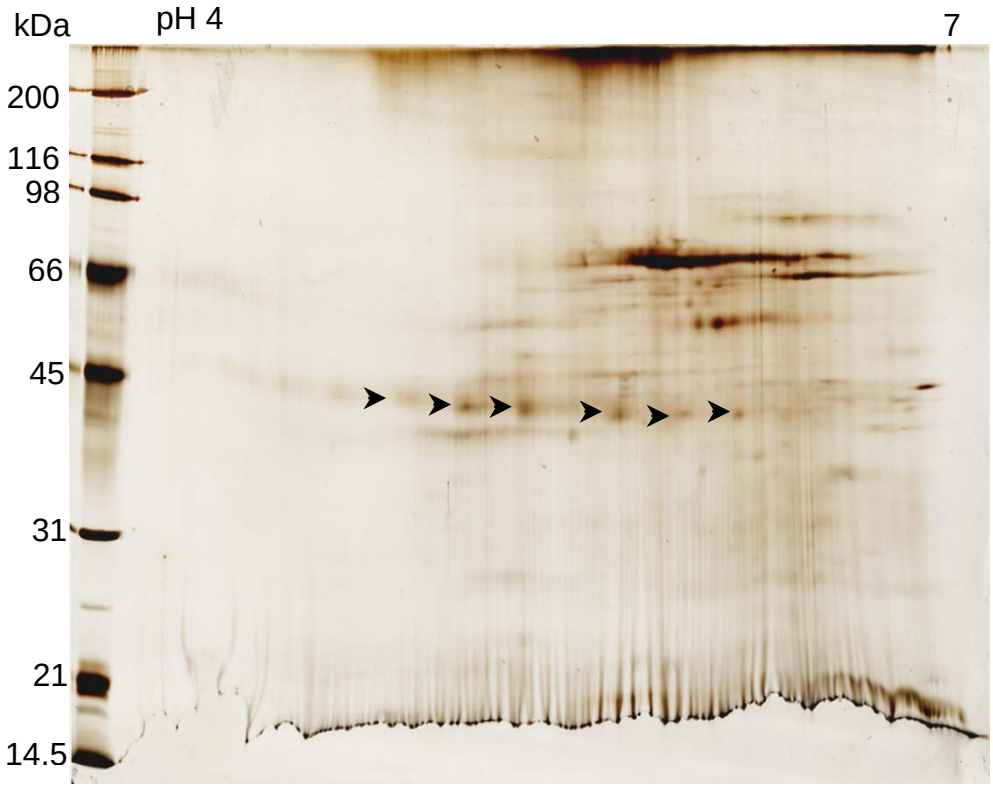


Fig. 4.22: Two-dimensional separation of the V-ATPase activity fractions prepared by gel filtration. The fractions of gel filtration (9-12) were solubilised in lysis buffer containing thiourea and ASB-14 and subjected to isoelectric focusing for 20 h. Proteins were separated on a 15 % SDS-PAGE in the second dimension and visualised by silver staining. The arrowheads indicate a series of protein spots resembling an ATPase accessory protein (Ac45) that has been identified in a separate experiment in our group (Bernd Schroeder, personal communication)

As an alternative method for the separation of vacuolar ATPase polypeptides, a diagonal electrophoresis was performed with the cationic detergent CETAB for the first dimensional separation followed by second dimensional SDS-PAGE (see 3.2.1.12). This was efficient in resolving the hydrophobic proteins and the basic polypeptides of V-ATPase complex.

For a comparison of the protein profile, the lysosomal membrane extracts used for gel filtration was also subjected to CETAB-PAGE. On staining with silver nitrate, this sample showed complicated pattern with several protein signals (Fig. 4.23). The fractions of gel filtration containing the NEM-sensitive ATPase activity were pooled, concentrated by precipitating with 4 volumes of ice-cold acetone and separated on a CETAB-PAGE. On silver staining, these fractions showed characteristic protein pattern with less complexity (Fig. 4.24), suggesting a high degree of purification with respect to V-ATPase. The majority of proteins, which were clearly visible in the starting material, were removed by the gel filtration.

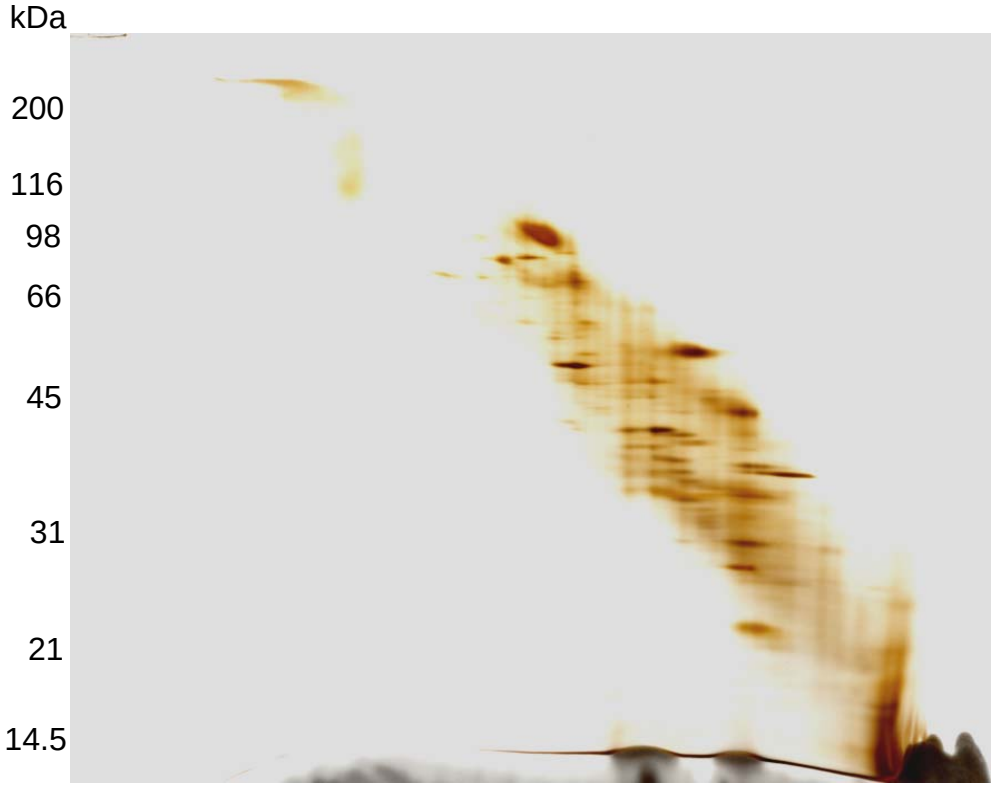


Fig. 4.23: Two-dimensional separation of detergent-extracted lysosomal membrane, separated on CETAB/ SDS-PAGE. The purified lysosomal membranes were extracted with 1 % Triton X-100 and 0.25 % CHAPS for gel filtration and an aliquot of 10 µg protein was separated on a 8 % CETAB gel. After equilibration, the proteins were separated on a 15 % SDS-PAGE and visualised by silver staining.

Fig. 4.24: Two-dimensional separation of the V-ATPase activity fractions from gel filtration separated on a CETAB/SDS-PAGE. The MME- treated lysosomal membranes were extracted with detergents and separated by gel filtration. NEM-sensitive ATPase activity fractions were separated on a 8 % CETAB gel. After equilibration, the proteins were separated on a 15 % acrylamide gel and stained with silver nitrate.

To identify the proteins present in these fractions, a preparative two-dimensional electrophoresis (CETAB/SDS) was performed with 100-µg of the sample, and stained with colloidal Coomassie blue (Fig. 4.25). The protein spots were excised, digested with trypsin, and mass spectrometric analysis was carried out.

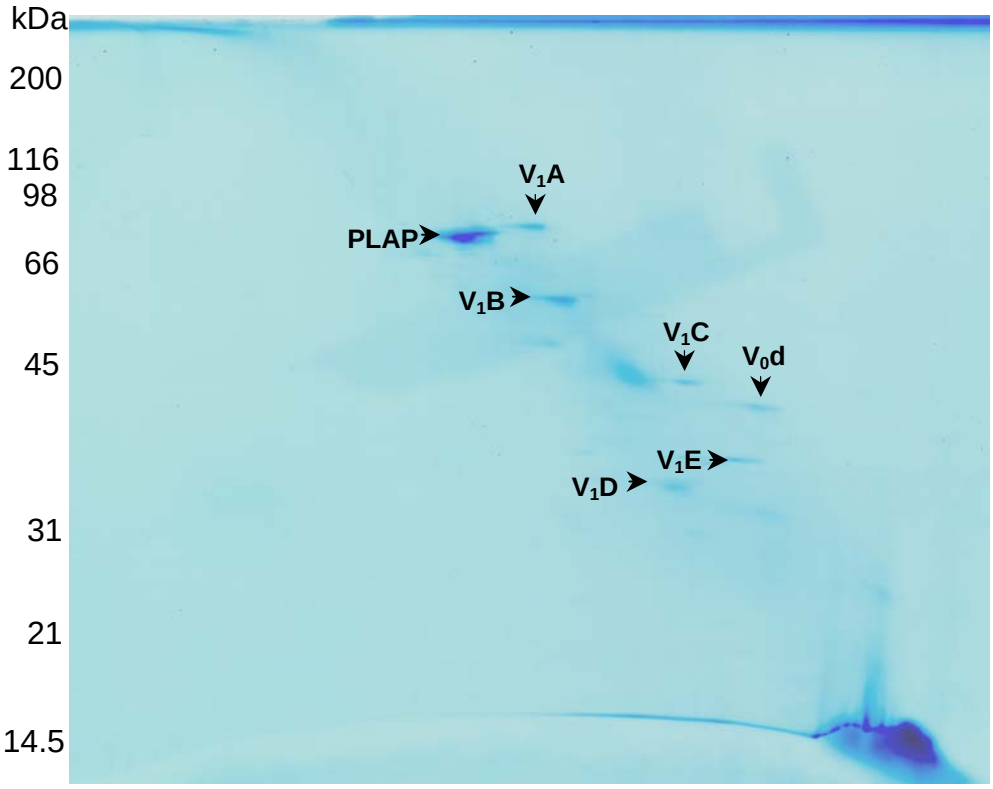


Fig. 4.25: Two-dimensional separation of the V-ATPase activity fractions separated by CETAB/SDS-PAGE and analysed by mass spectrometry. The lysosomal membranes purified by MME treatment and incubation with $MgCl_2$ were extracted with detergents and separated by gel filtration. NEM-sensitive ATPase activity fractions were separated on a 8 % CETAB gel. After equilibration, the proteins were separated on a 15 % acrylamide gel and stained with colloidal Coomassie blue.The spots were identified by Maldi-TOF.

The protein spots when analysed by mass spectrometry showed subunits of vacuolar ATPase, which were mostly from the V_1 domain. There were several spots that could not be identified by Maldi-TOF. From the V_0 domain, only one subunit-V_0d was identified in the V-ATPase activity fractions. The subunits that are part of the V_0 domain are hydrophobic and yield only few peptides upon tryptic digestion. The signal corresponding to the accessory subunit of V-ATPase- Ac 45 was quite evident in the two-dimensional gels with thiourea/ASB-14 solubilisation system. This polypeptide was also not identified from the spots analysed by mass spectrometry. There were no other proteins with nucleotidase activity identified from this sample confirming that the ATPase activity found was entirely due to the V-ATPase.

4.4 Reconstitution of vacuolar ATPase

The V-ATPase activity fractions prepared through gel filtration was reconstituted into uni-lamellar liposomes in presence of 0.5 % Triton X-100. The proteoliposomes obtained was suspended in the defined volume of reconstitution buffer. The reconstitution provided an opportunity to examine the effects of V-ATPase inhibitors in the presence of defined lipids.

The reconstitution was performed using egg yolk lecithin (see 3.2.3) and the reconstituted proteins were suspended in reconstitution buffer. The ATPase activity was measured by estimating the liberation of inorganic phosphate by the colorimetric assay. As a control, proteoliposomes were incubated in the substrate buffer without ATP to assure that the inorganic phosphate measured was not endogenous, but resulted from ATP hydrolysis by the enzyme. It was found that inclusion of cholesterol in the liposomes resulted in a better recovery of most of the lysosomal membrane enzymes in the reconstituted fraction (Christian Wrocklage, personal communication). Taking this into account, the subsequent experiments for reconstituting the V-ATPase were performed with cholesterol contributing 22.5 % (mg/mg) of total lipid in the liposomes.

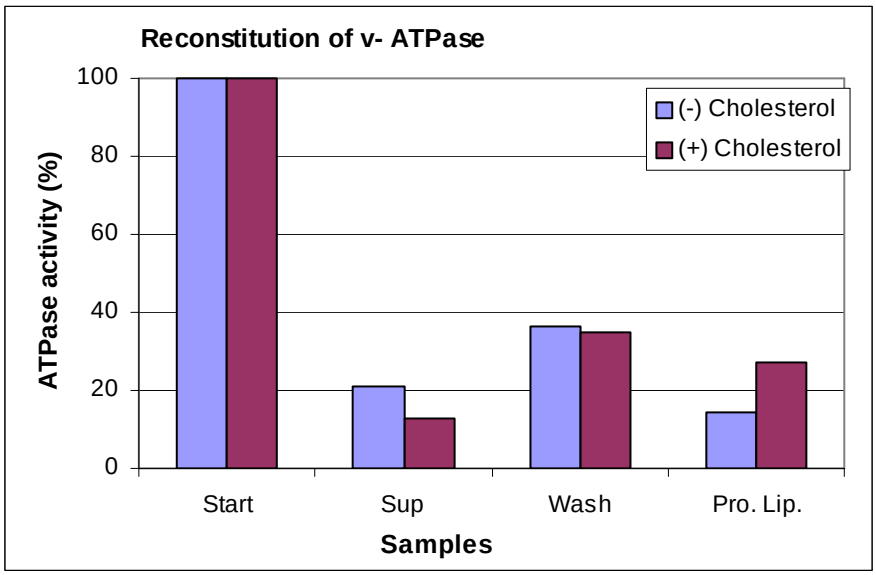


Fig. 4.26: The reconstitution of vacuolar ATPase. Reconstitution of V-ATPase into liposomes was done in presence and absence of cholesterol. The total activity from the extract (Start) is considered 100 %. The non-incorporated (Sup), wash (Wash) and incorporated V-ATPase activities (Pro. Lip.) are expressed as percentage of total.

The amount of V-ATPase activity in the proteoliposomes was 14.5 % of the initial activity when the reconstitution was performed with unilamellar vesicles made of solely lecithin. The incorporation of cholesterol into the vesicles increased the reconstitution efficiency by a factor of 2 (Fig. 4.26). A corresponding decrease in ATPase activity in the

non-incorporated fraction was also observed in reconstitutions done in the presence of cholesterol while there was no significant difference between the aliquots of washes.

The protein pattern of the reconstituted vesicles was analysed on diagonal electrophoresis. The solubilised V-ATPase used for the reconstitution (Fig. 4.27) was analysed as a control for comparison. The major protein spots ranged from 20 to 100 kDa.

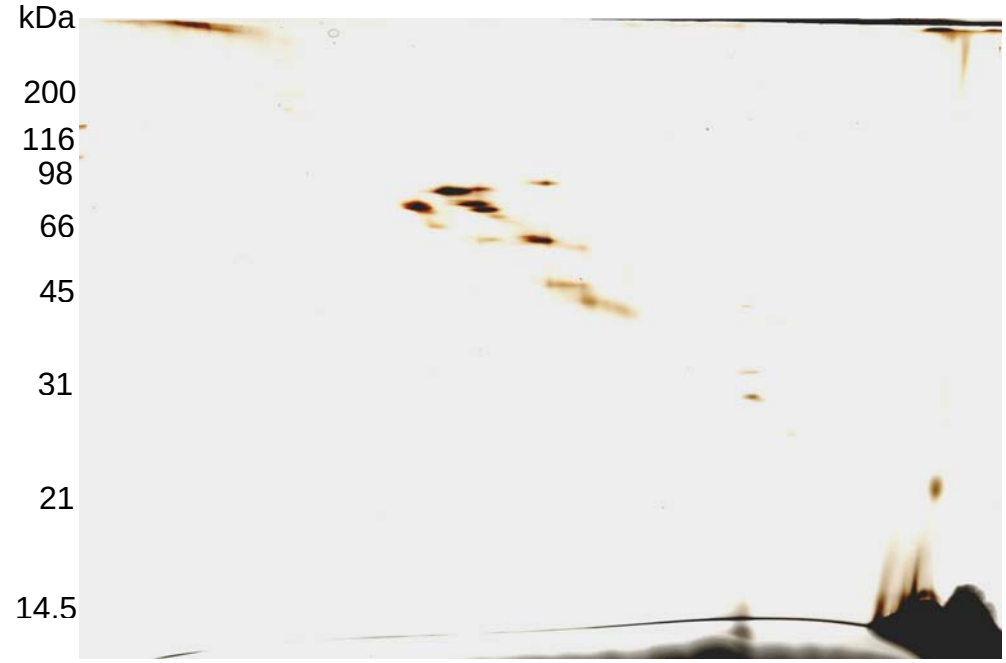


Fig. 4.27: Two-dimensional separation of the V-ATPase used for reconstitution on a CETAB/SDS-PAGE. The solubilised material (5 µg) used for reconstitution was separated on a 8 % CETAB gel. After the equlibration the proteins were separated on a 15 % SDS-PAGE and visualised by staining with silver nitrate.

After reconstitution and removal of the detergent, the aggregated material was sedimented by centrifuging the sample at 14,000 rpm for 15 min. The aggregation is possibly due to a denaturation of a small portion of proteins during the procedure of reconstitution. The pellet obtained containing the aggregated proteins was analysed on a CETAB/SDS-PAGE and visualised by staining with silver nitrate (Fig. 4.28). The protein pattern resembled to the staring material (V-ATPase activity fraction from gel filtration) in that the prominent spots were the same.

The reconstituted proteins were separated from the remainder by sedimenting the liposomes by ultracentrifugation. The supernatant containing the non-incorporated proteins was analysed by diagonal electrophoresis. This fraction was enriched in proteins that were predominantly of low molecular weight (Fig. 4.29).

Fig. 4.28: Two-dimensional separation of the proteins that were aggregated during the reconstitution on a CETAB/SDS-PAGE. The non-incorporated and aggregated proteins (5 µg) were removed by centrifugation. The aggregated protein sample was separated on an 8 % CETAB-PAGE followed by a 15 % SDS-PAGE and stained with silver nitrate.

Fig. 4.29: Two-dimensional separation of the non-reconstituted proteins separated by centrifugation on a CETAB/SDS-PAGE. The proteins that were not reconstituted were separated as the supernatant fraction by ultra centrifugation. An aliquot containing 5 µg proteins was solubilised and separated on an 8 % CETAB gel. After the equilibration step, the proteins were separated on a 15 % SDS-PAGE followed by visualising the polypeptides by silver staining.

The proteoliposomes obtained after ultra centrifugation were resuspended in the reconstitution buffer and the protein pattern analysed on a CETAB-PAGE (Fig. 4.30). The protein pattern showed most of the major spots that were present in the starting material.

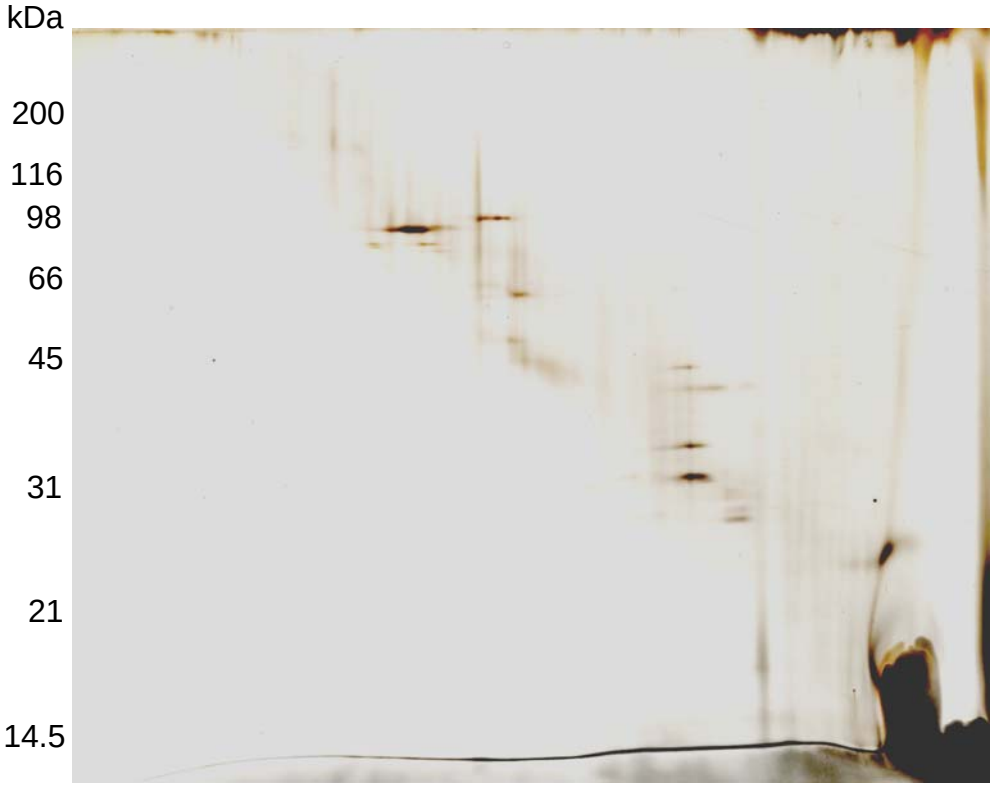


Fig. 4.30: Two-dimensional separation of the proteoliposomes containing the reconstituted V-ATPase on CETAB/SDS-PAGE. The proteoliposomes obtained by ultra centrifugation containing the reconstituted V-ATPase (5 µg) was separated on an 8 % CETAB gel. After the equlibration, the proteins were separated on a 15 % SDS-PAGE and stained with silver nitrate.

The orientation of reconstituted vacuolar ATPase was analysed by examining the accessibility of V_1 domain components to the substrate-ATP. The ATPase activity from the proteoliposomes was measured in the presence and absence of 0.5 % Triton X-100 at a final concentration. As controls, fractions from the starting material, washes and the non-incorporated material were analysed too for the comparison (Fig. 4.31).

The ATP cleaving domain of the vacuolar ATPase is oriented on the cytosolic side of the lysosomes. If the reconstitution resulted in the correct orientation of the complex, the V_1 domain of the ATPase complex should have an access to ATP in the medium. If there were populations of proteoliposomes with the protein in both orientations, only partial ATP cleavage would occur depending on the percentage of properly oriented enzyme. After the detergent treatment, the vesicles were disrupted providing both orientations an access to ATP. There was no major increase in the activity due to the detergent. This suggested that after the

reconstitution, the V_1 portion was present at the outer surface of the liposomes as it would, in the case of the protein complex in the lysosomal membrane.

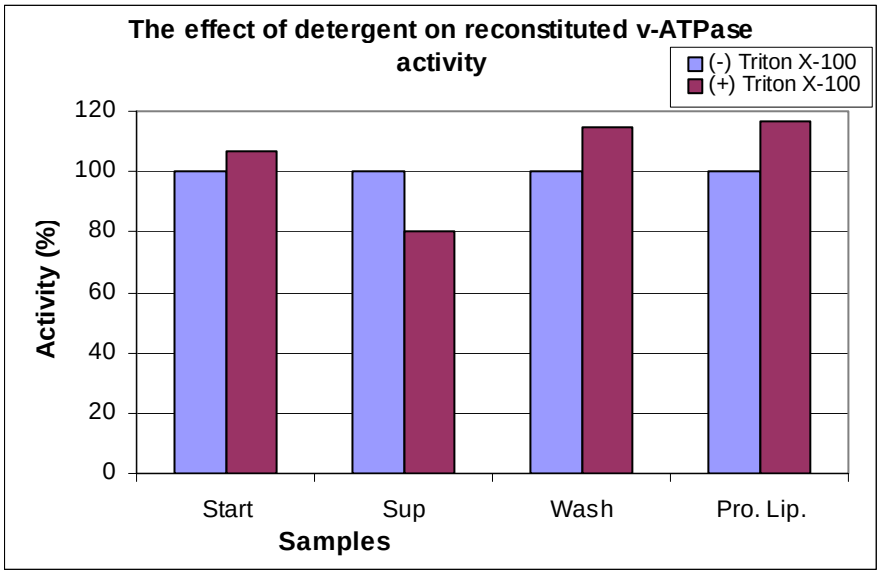


Fig. 4.31: The Effect of detergent on reconstituted V-ATPase activity. Samples including the starting material (Start), non-incorporated portion (Sup), wash (wash) and proteoliposomes (Pro. Lip) were assayed for ATPase activity in presence and absence of 0.5 % Triton X-100. Activity of the samples in the absence of Triton X-100 is expressed as 100 % in each case.

4.5 Inhibitory studies on reconstituted V-ATPase

After reconstitution, the V-ATPase was subjected to inhibitory studies to check the effects of several chemical agents. The isolated enzyme was found to be sensitive to *N*-ethylmaleimide (NEM) and bafilomycin-A1 in a dose-dependant manner, both of which are reported inhibitors of vacuolar type ATPases along with a lipofuscin derivative designated as A2-E.

N-ethylmaleimide, the sulphhydryl modifying agent is a well-known inhibitor of vacuolar type ATPases. To analyse the inhibitory effects of NEM, different concentrations were used. Reconstituted vacuolar ATPase was diluted with water and incubated with NEM upto 2 mM concentration at room temperature (25 °C) for a period of 15 min. After this time, the solution containing the substrate was added. The extent of ATP hydrolysis by the enzyme within a period of 3 h was measured. The activity obtained with the non-treated proteoliposomes was considered to be 100 % (no inhibition).

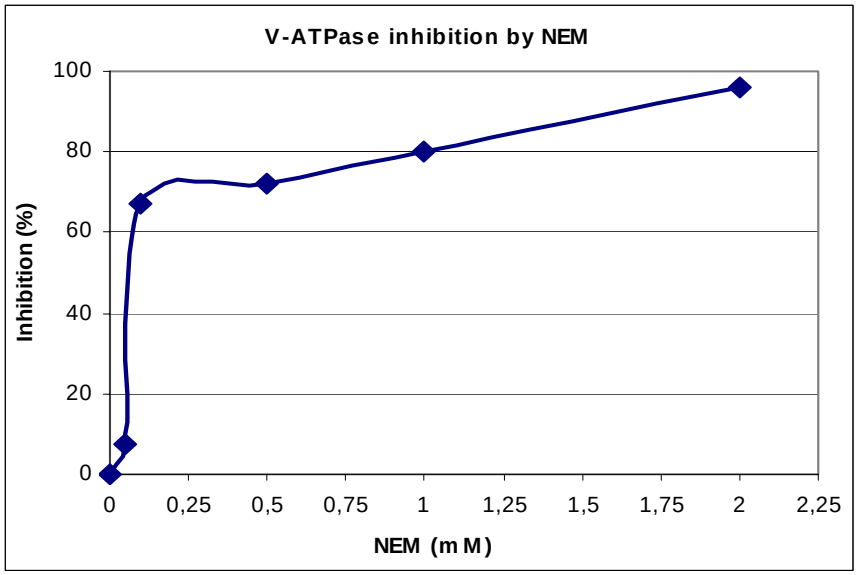


Fig. 4.32: The effect of NEM on the reconstituted lysosomal ATPase. An aliquot (5 µl) of the reconstituted V-ATPase corresponding to 50-55 units of enzyme activity were used in every trial. The activity from the untreated sample was considered to be 100 % (no inhibition) and activities obtained after the pre-treatment with different NEM concentrations are expressed (average of 2 independent experiments) as the net activity.

Inhibition of v type ATPase by NEM has been reported to be complete when used at a concentration of 1 mM (Arai *et al.,* 1993). However, in our experiments with reconstituted lysosomal ATPase, only 80 % inhibition was observed at the same concentration. When a concentration of 2 mM NEM was used for inhibition, 95 % of the ATPase was inhibited (Fig. 4.32).

Another inhibitor used was bafilomycin-A1, which is reported to inhibit the vacuolar ATPase at a concentration of 100 nM completely. In the experiment shown in Fig. 4.33, at an inhibitor concentration of 1 nM, > 30 % inhibition was observed. At 100 nM concentrations, the inhibition was more than 90 %.

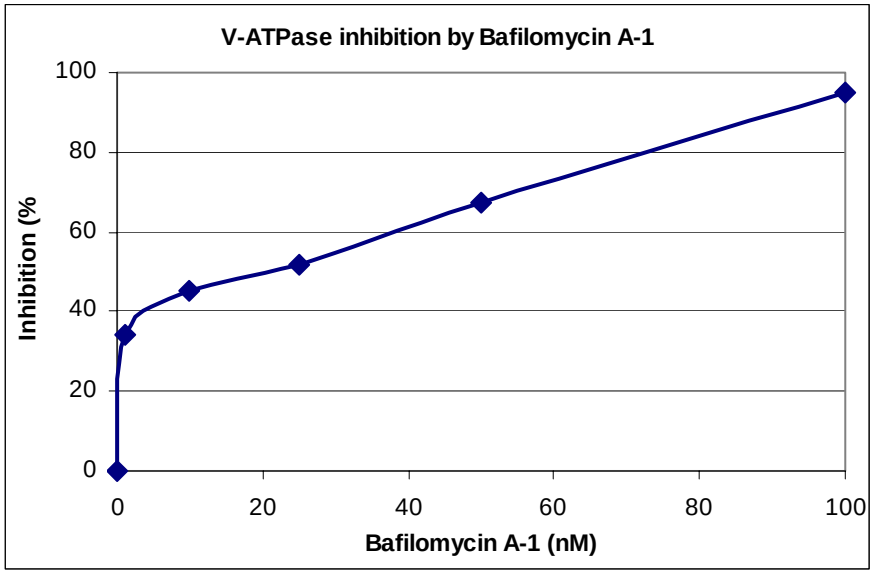


Fig. 4.33: Inhibition of V-ATPase by bafilomycin A-1after reconstitution. To a 5 µl aliquot of reconstituted V-ATPase corresponding to 50-55 units of the enzyme activity, different concentrations of bafilomycin A-1 were added. The activity obtained from the untreated sample was considered to be 100 % (no inhibition), for comparing the concentration-dependence of inhibition and an average of two independent measurements are shown for each value.

The effect of sodium azide, an inhibitor of F-type ATPases was also investigated on the proteoliposomes. There was no significant inhibition observed with sodium azide when a maximum concentration of 10 µM was used (Data not shown).

The inhibitory effect of synthetic A2-E was analysed on the isolated and reconstituted vacuolar ATPase. This lipofuscin derivative is a reported inhibitor of vacuolar proton pump in retinal pigment epithelial cells and contributes to the pathology of age-related macular degeneration and vision loss. A2-E is shown to affect both the ATP-hydrolysis and proton translocation in a dose-dependent manner in the intact organelles (Bergmann *et al*., 2004). These studies showed a dose-dependent inhibition of the ATPase activity by the A2-E (Fig. 4. 34).

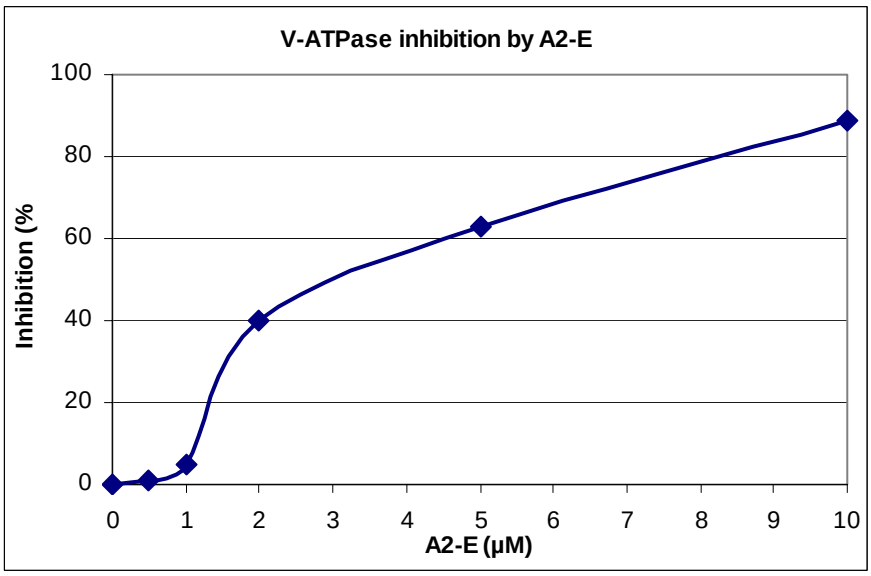


Fig. 4.34: Inhibition of reconstituted vacuolar ATPase by A2-E. Different concentrations of A2-E were added to 5 µl aliquots of the reconstituted ATPase and ATPase activity was measured after the pre-treatment. The activity obtained from untreated sample was considered to be 100 %. An average of two independent experiments is given in the graph.

As a control, the purified lysosomal membranes were extracted with 0.5 % Triton X-100 and reconstituted. The effect of A2-E on these vesicles was analysed for a comparison (Fig. 4.35). The extent of inhibition was considerably low when compared to the purified ATPase. This suggests that the amount of V-ATPase in the membranes is only a small percentage of the total ATPase activity. Such an observation is compatible with the partial sensitivity (Fig. 4.18) of the ATPase activity fractions in the low-molecular area of the gel filtration.

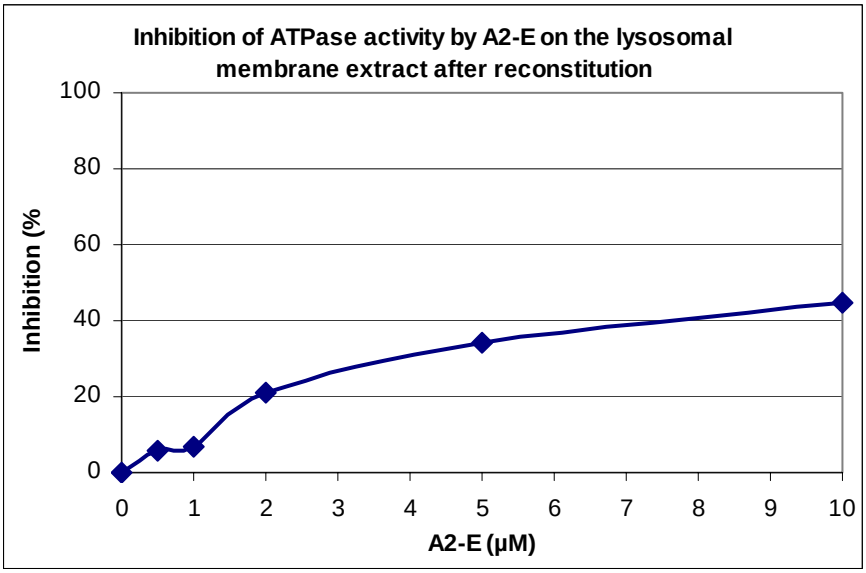


Fig. 4.35: Effect of A2-E on the purified lysosomal membrane extract after reconstitution. Different concentrations of A2-E were checked on the lysosomal membrane extracts after reconstitution. Every trial of the experiment was performed with 50-55 units of ATPase activity. The activity obtained from untreated sample was considered to be 100 %.

5 Discussion

5.1 Purification of lysosomal membrane proteins

A biochemical study on the membrane protein complexes from the lysosomal compartment demanded a convenient and efficient strategy to prepare lysosomal membranes of good quality to work with. Though there were several attempts for the purification and a proteomic analysis of the lysosomal membrane proteins with medical relevance, they are far from complete (Journet and Ferro, 2004, Bagshaw *et al.*, 2003). This is largely due to the low-abundance and high hydrophobicity of the lysosomal membrane proteins in addition to the contamination of lysosomal preparations with non-lysosomal proteins. In the current study, several biochemical techniques were employed for the purification and analysis of the lysosomal membrane proteins isolated from human placenta and the scheme adopted for the study is shown in the chart.

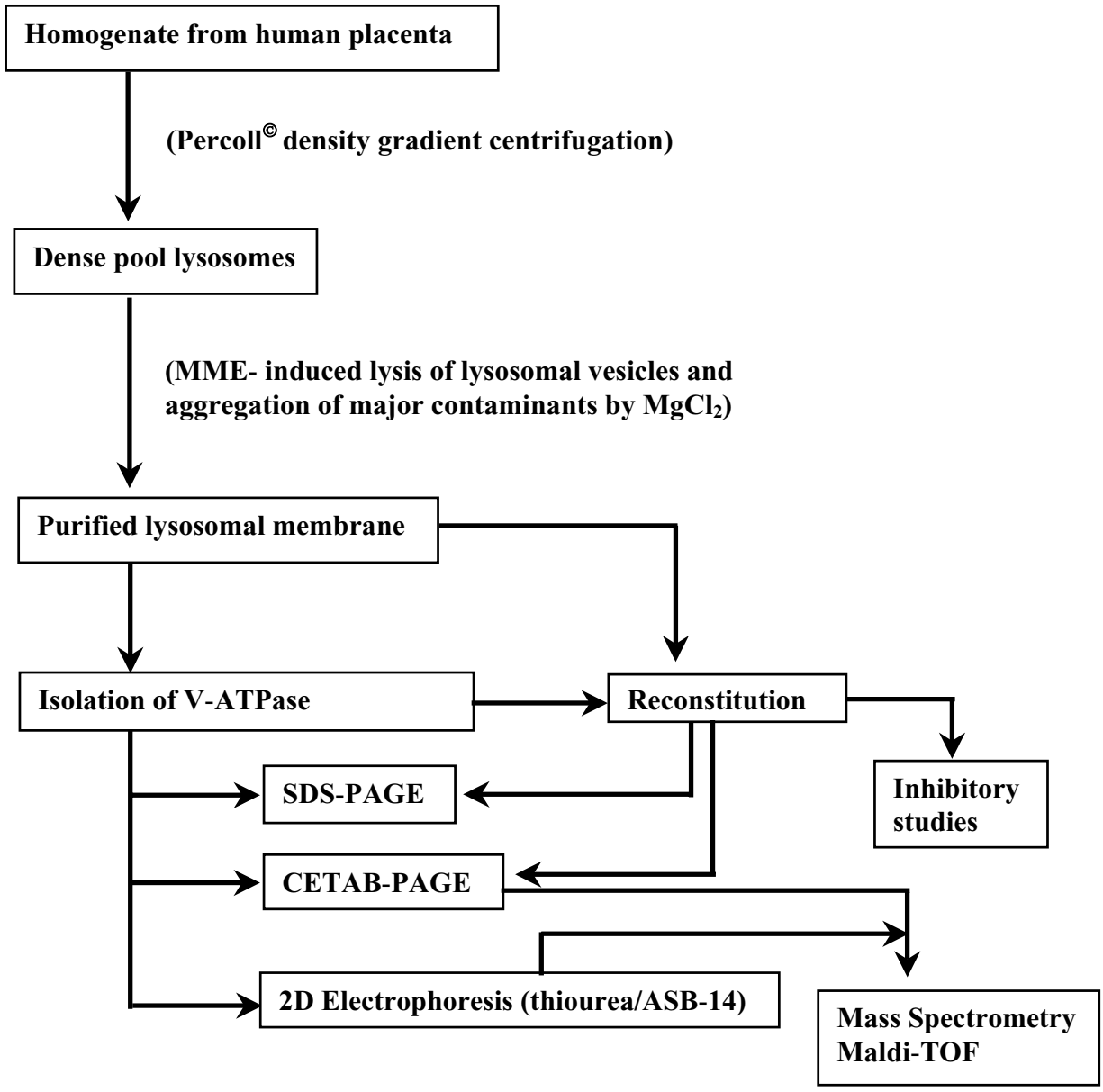


Fig. 5.1: The isolation and characterisation of lysosomal membrane proteins

The isolation of lysosomes from placenta homogenates by Percoll© density gradient centrifugation resulted in a 50-55-fold enrichment as suggested by an increase in the specific activity of the membrane bound ß-glucosidase. This has been reported by Diettrich *et al.* (1996). Though the enrichment of lysosomes and separation of a major fraction of mitochondria was achieved by this procedure, the samples still contained non-lysosomal proteins, as is known from other reports as well (Chatway *et al.*, 1998). There are reported improvements in the further purification by a second step of Percoll© gradient centrifugation (63+/-12 fold purification) from rat liver lysosomes (Symons and Jonas, 1987) but with low yields.

As an alternative procedure, purification of lysosomal vesicles using immuno-affinity adsorption was employed. A monoclonal antibody recognising the lysosomal membrane protein, LAMP-2 coupled to C1Z-Eupergit beads was used for this purpose. This resulted in purification of lysosomal vesicles as shown by 3.2-fold enrichment in the membrane bound ß-glucosidase activity. However, upon elution of the bound proteins from the beads, the detachment of antibody chains from the support contaminated the proteins. Moreover the detachment of antibody from the beads limited the re-use of the beads, demanding high amounts of antibody reserves for the complete analyses.

Separation of vesicles based on polyethylene glycol phase-separation procedure was examined, but without much success. A substrate-induced lysis of the lysosomes by methionine methyl ester (Goldman and Kaplan, 1973, Reeves, 1979) yielded vesicles that could be separated from the bulk of mitochondria. The lysosomal vesicles thus obtained were of relatively low buoyant density as compared to the mitochondria and this made the enrichment of these vesicles possible using density gradient centrifugation in a linear sucrose gradient. A good degree of purification was observed and the lysosomal vesicles were collected from the low-density fraction of the gradient (fractions 3-5) whereas the mitochondrial proteins were present in higher density fractions (fractions 6-9). Though, this purification strategy resulted in a high degree of purification (6-8-fold enrichment of the membrane-associated ß-glucosidase activity, (Bernd Schroeder, personal communication) the procedure was time-consuming and laborious.

An alternative strategy was explored to shorten this procedure. It has been reported that the divalent cation Mg^{2+} induced aggregation of the mitochondrial membranes (Stonet *et al.*, 1978, Morris *et al.*, 1979, Kwan, 1986). Therefore an incubation with $MgCl_2$ was

performed after the disruption of the lysosomes by MME treatment. The aggregated membranes, mainly mitochondria were sedimented by centrifugation and this resulted in an additional 3-fold purification of lysosomal membranes in the supernatant fraction. The lysosomal membranes were then prepared by ultracentrifugation. The specific activity of the lysosomal membrane-bound ß-glucosidase (250-300 mu/mg) was increased several folds due to the separation of a large portion of the contaminants and of the matrix proteins. Taken together, there was a 6-fold purification of lysosomal membranes from the dense pool, yielding 300-fold purification from the placental homogenate level. A similar purification of the human placental lysosomal membranes has not been reported before.

An improvement that was achieved by treating the MME-disrupted vesicles with $MgCl_2$ appeared to be the removal of a major fraction of the mitochondrial contamination. Such a step allowed a convenient, rapid and efficient method of purifying the vesicles from disrupted lysosomes, and thus the membranes. On an average, the yield of protein obtained in the purified lysosomal membrane fraction was only 3.5-4 % of the amount of proteins that were present in the dense pool lysosomes. Nevertheless, 25-28 % of the activity of the lysosomal membrane associated ß-glucosidase activity was recovered after the final stage of purification. The efficiency of purification was confirmed by two-dimensional analyses of proteins from all the fractions, as well as by the activity measurements of marker enzymes of both lysosomal (the membrane-bound ß-glucosidase) and the mitochondrial (succinate dehydrogenase) compartments. In the two-dimensional analyses, the relative intensities of the mitochondrial protein spots were strongly reduced as compared to the marker proteins from the lysosomal membrane.

An interesting observation during the isolation of lysosomal membranes using the current strategy was the co-sedimentation of a tentatively matrix protein (Golabek *et al*., 2002) tripeptidyl peptidase-1 (CLN2) along with the membranes. The possibility of an ionic interaction involved in this was examined by washing the membranes with 0.5 M NaCl. The washing step resulted in the removal of only 30-35 % of the biologically active TPP-1. Another possibility would be the occurrence of multiple forms of the protein leading to different levels of interaction with the lysosomal membrane. To check this, the protein samples from the membrane and matrix fractions were resolved separately on two-dimensional electrophoresis and checked for their detection by an antibody. The antibody recognised the protein from both fractions at the same area of molecular weight and isoelectric point, suggesting that the protein form present in the matrix is probably the same as

that co-sedimenting with the membranes. There are interactions among the different proteins of the CLN family, especially the lysosomal pH regulating protein battenin (CLN3) and a soluble lysosomal protein of unknown function that is defective in the late infantile variety of the CLN disease (CLN5) with TPP-1 which is also called CLN2 (Vesa *et al*., 2002), studied using co-immuno precipitation. This enlightens a possible protein-protein interaction responsible for keeping a fraction of the CLN2 in the lysosomal membrane, though attempts to cross-link *in vitro* with DSP (data not shown) were not successful.

The lysosomal membrane carries a number of yet uncharacterised proteins performing important functions as evident from the diseases caused by mutations affecting these proteins. These include the Niemann-Pick C1 type protein involved in the cholesterol metabolism, the Sanfilippo type C protein required for the heparan sulphate degradation, sialic acid transpoter, cystine transporter, transport proteins for ions and small molecules etc. to name a few. Though many of them are characterised with respect to their gene loci, very little information is currently available regarding the protein as such. The lysosomal membranes prepared by MME and $MgCl_2$ treatment can be proposed for the enrichment, isolation and characterisation of these proteins.

5.2 Analysis of DRMs from the lysosomal membrane

The lysosomal membrane is a highly dynamic environment where specific interactions among proteins and between proteins and lipids occur. These interactions are necessary for the proper functioning of the lysosomal apparatus for allowing the passage of molecules into and out of the lysosomes. Interestingly, presence of detergent resistant microdomains (DRM) on the lysosomal membrane that are enriched in particular proteins has been reported by our group (Taute *et al.,* 2002). These domains are of low buoyant density, and very resistant to non-ionic detergents such as Triton X-100; the characteristics exploited to isolate and study the raft complexes.

The DRMs are enriched in cholesterol and sphingolipids. In the lysosomes, the mobilisation of cholesterol depends on Niemann-Pick proteins C-1 and C-2 that are components of the membrane and matrix respectively. Defects in these proteins cause lysosomal storage disorders characterised by an accumulation of lipids, especially sphingomyelin and cholesterol in the degradative compartments. These mutations cause the C1 and C2 type Niemann-Pick syndromes. It was postulated that one sequela of these

lipidoses might be an accumulation of DRMs in endosomes and lysosomes (Simons and Ehehalt, 2000).

The detergent resistant microdomains were isolated from the purified lysosomal membranes as shown in the following chart. The fractions containing the DRMs were enriched in characteristic proteins that made a proteomic profiling of these domains interesting to look at. Among the proteins enriched in the low-density fractions, several membrane proteins of the lysosomal origin were observed by activity measurements including the lysosomal membrane acetyl transferase and the V-type ATPase.

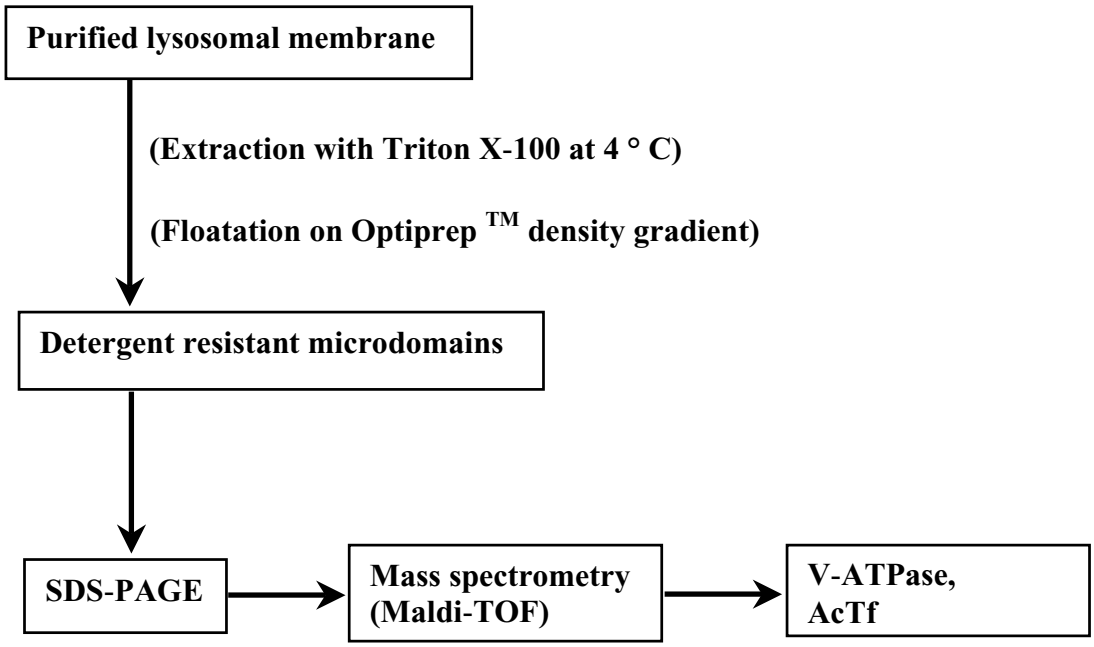


Fig. 5.2: Analysis of DRM-associated proteins from the lysosomal membrane

Though the low-density DRM fractions showed enrichment in the activity of AcTf, the total yield was as low as 10-12 %. The detergent, Triton X-100 used for the DRM analyses was found to diminish the activity of this enzyme. Concerning the low yield, it is not known if the detergent inhibits the enzyme or causes the separation of an yet unknown subunit of the enzyme. Use of other detergents including CHAPS, NP-40 or Lubrol was not suitable for the DRM isolation since no floatation of the protein was obtained under these conditions.

Identification of the human lysosomal membrane V-ATPase as a component of DRMs is novel though there are earlier reports regarding the localisation of several plasma membrane ATPases (Sepulveda *et al*., 2005, Pang *et al*., 2005) as well as the yeast vacuolar

ATPase (Opekarova *et al*., 2005) in these domains. It has been experimentally proven that cholesterol depletion by methyl-ß-cyclodextrin, on V-ATPase complex isolated from synaptic vesicles of rat brain resulted in the decrease of the enzyme activity (Yoshinaka *et al.*, 2004). In the current experiments for isolating the DRMs by floatation experiments, a correlating result was obtained, as there was no floatation of V-ATPase or any other proteins after the MBCD treatment. The cholesterol-rich DRMs might be a critical factor for the proper subunit structure and or the activity the V-ATPase.

Though the DRM fractions showed V-ATPase activity only 4 of the subunits were identified in these low-density fractions (subunits V_1B, V_1D, V_1E and V_0a). The extreme hydrophobicity of the subunits that are part of the V_0 domain might be a limiting factor for a good solubilisation and resolution of these subunits in the gel system used for the current experiments. When the mass spectrometric analysis was carried out to identify the proteins present in the lysosomal DRMs, the subunit of V-ATPase mentioned V_0a was identified at the junction between the stacking gel and separation gel with a significant mowse score of 83. In the Western blotting experiments it was observed that upon detergent solubilisation of the membrane proteins the V_0a tend to precipitate thereby preventing its entry into the gels during protein separation (see Fig. 4.7). This could occur with the other subunits of the complex as well.

The association of V-ATPase with the detergent resistant microdomains of the lysosomal membrane made the isolation and reconstitution the protein complex interesting to look at. This attempt provided a system to study the effect of lipids on the biological activity of the complex upon reconstitution and the effect of chemical derivatives that are likely to influence the biological activity.

5.3 Isolation of V-ATPase

A major problem faced during the purification procedure was that the dense pool contained both V-ATPase and mitochondrial ATPase (F-type) activities. This was solved to a very high extent by removing the mitochondrial contamination by disruption of the dense pool with MME and the subsequent incubation with $MgCl_2$. In evaluating the purification procedure, an advantage was taken from the differential sensitivities of the ATPases towards NEM. Several strategies were examined for the isolation of V-ATPase from the detergent solubilised lysosomal membranes. The protein complex has 13 major subunits and 2 accessory polypeptides (Smith *et al*., 2003), and any treatment resulting in either the removal of one or

several subunits or depletion of lipid moieties resulted in considerable loss of activity making the enzyme difficult to isolate.

Several biochemical techniques including ion-exchange chromatographic procedures, blue-native electrophoresis, glycerol density gradient and affinity chromatography (using immobilised ATP) were implemented for isolating the complex, but with limited success. Separation of detergent solubilised lysosomal membranes by gel filtration resulted in a good degree of purification of the NEM-sensitive ATPase activity from majority of the proteins as well as from the contaminating ATPases. During the course of purification, the separation and enrichment of V-ATPase was confirmed by checking the reactivity to inhibitors of V-type ATPases, either *N*-ethyl maleimide (Hunt and Sanders, 1996) or bafilomycin A-1 (Schuhmann and Grond, 2004).

In the initial gel filtration experiments, there were several polypeptides present in the vacuolar ATPase activity fractions (fractions 9-13). It was found that several of them could be removed if the membranes were treated with 20 mM methyl-ß-cyclodextrin prior to the extraction. This could well be explained on the basis of depletion of cholesterol by MBCD. Due to the treatment, the activity of V-ATPase was partially quenched. However, the decrease in the activity was out-weighed by the subsequent increase in the purity. For example, the activity for the lysosomal AcTf, which in the preliminary experiments was co-localised with V-ATPase was shifted to a lower molecular weight area (fractions 13-17) upon the treatment with MBCD. The membranes are likely to contain different kinds of DRMs that may be enriched in different sets of proteins of different properties. Interestingly, the fractions showing AcTf activity obtained by gel filtration were devoid of all the predominant proteins, though the yield of this enzyme was only around 15 %. It should be feasible to optimise the system to improve the recovery by altering the temperature and the detergent compositions and enhance the chances to isolate and characterise this interesting protein.

After the purification of V-ATPase by gel filtration, the samples were analysed in acrylamide gels. The accessory subunit of the enzyme -designated as Ac-45-was a major spot found in the two-dimensional electrophoretic pattern as compared to previous mass spectrometric results. However, several of the subunits (C, D, E, G, c, c`, c``) that are part of the complex possess a basic isoelectric point, and this might explain why these were not detected in the two-dimensional systems used. As an alternative, a diagonal separation was introduced with a cationic detergent CETAB for the solubilisation and separation of proteins

in the first dimension followed by SDS-PAGE in the second dimensional separation. In this system, unlike in the regular two-dimensional systems, it was also possible to recover extremely hydrophobic polypeptides (Bernd Schroeder, personal communication).

The purification of V-ATPase was confirmed by separating the activity fractions in a preparative diagonal electrophoresis and mass spectrometric identification of the obtained polypeptides. Though the fractions showed NEM-sensitive ATPase activity, only 6 subunits of the protein complex were identified from the gel with significant MOWSE score. They were V_1A, V_1B, V_1C, V_1D and V_1E in addition to one subunit from the V_0 domain- V_0d. These results correlated to the subunit stoichiometry of the complex, as the subunits that are present in the high copy numbers and low hydrophobicity are identified through mass spectrometry. It is likely that several unidentified spots contain hydrophobic polypeptides of the V-ATPase complex that do not provide sufficient number of peptides by tryptic digestion making the identification by the Maldi-TOF method inefficient.

Apart from the identified V-ATPase subunits, these fractions of gel filtration also contained placental alkaline phosphatase and stomatin. Presence of both these proteins- known members of the DRM-associated protein family (Snyers *et al*., 1999, Mairhofer *et al*., 2002) in the V-ATPase activity fractions suggested that MBCD treatment resulted in only a partial depletion of DRMs. The cyclodextrin treatment is likely to disrupt the cholesterol-rich DRMs only.

An ideal strategy for the complete exploration on the vacuolar ATPase complex and its interaction partners might be a combination of blue-native electrophoresis with LC-nanospray-MS/MS analysis as reported for the hydrophobic membrane complexes from mitochondria (Fandino *et al*., 2005). Unfortunately, such a technique was not available to us. The glycolytic enzyme aldolase (Lu *et al*., 2001, Lu *et al*., 2004) and the cytoskeletal protein actin (Vitavska *et al*., 2005) were reported to interact with V-ATPase. A complex molecule-RAVE has been reported to be required for the assembly of the V-ATPase complex in yeast (Samrdon *et al*., 2002) but no such molecule has been characterised from the human system so far. An attempt to find human homologues of this protein by BLAST performance could not yield any match. On this perspective, a complete proteomic analysis of the V-ATPase activity fractions obtained through gel filtration would enlighten the subunit composition of the lysosomal ATPase and the kind of polypeptides it interacts with.

5.4 Reconstitution of V-ATPase

The vacoular proton pump residing in the lysosomal membrane has an important role in maintaining the acidic mileu in the lysosomal lumen, which is required by the lysosomal hydrolases. If the proton translocation or ATP hydrolysis by the V-ATPase is impaired, the lysosomal homeostasis is altered with lethal effects. A major pathological sequela is the age-related macular degeneration (ARMD). Lipids and several products of lipid peroxidation have been found to play a critical role in the development of this disease (Kopitz *et al*., 2004). The pathogenesis of ARMD is characterised by deposition of auto-fluorescent bodies of lipofuscin in the retinal pigment epithelial (RPE) lysosomes. A major lipofuscin derivative designated as A2-E has been suggested to take part in the development of ARMD through an inhibition on the V-ATPase (Bergmann et al., 2004). The purification of V-ATPase provided an opportunity for reconstituting the protein into liposomes in order to study the *in vitro* inhibitory effects of A2-E among a few other inhibitors, in a defined lipid environment.

The V-ATPase fractions from gel filtration were reconstituted into uni-lamellar liposomes made of egg yolk lecithin. A lipid:lipid ratio of 1:1 (w/w) and lipid:protein ratio of 1:3 (w/w) has been reported to be ideal for the reconstitution of Na, K-ATPase (de Lima Santos *et al*., 2005) using phosphatdyl choline and phosphatidyl ethanolamine. It is also known that presence of cholesterol (40 mole %) during the reconstitution decreased the optimal chain length of the phospholipids from 22 to 18 (Cornelius, 2001) referring to the importance of the hydrophobic matching of the bilayer. However, an attempt to reconstitute the lysosomal membrane proteins showed good results when a concentration of 22.5 % (mg/mg) cholesterol was used, which was adopted for the V-ATPase reconstitution from thereon. Presence of cholesterol during the reconstitution improved the recovery of V-ATPase as measured by the inorganic phosphate determination. In the absence of cholesterol, only 14.5 % of the initial activity was incorporated into the vesicles, whereas 30-32 % of the total activity was recovered in the experiments using 22.5 % (mg/mg) cholesterol.

The proteins incorporated into the liposomes were analysed in a two-dimensional (diagonal) electrophoresis. After silver staining, in the reconstituted fraction a protein profile was observed that was comparable to that of the starting material. The protein spots ranged in apparent molecular weights between 30 and 100 kDa. A group of polypeptides (50-100 kDa) that were aggregated due to a possible denaturation during the reconstitution of the vesicles corresponded to the major signals in the starting material. A small portion of the protein

remained in the final soluble fraction and contained mainly polypeptides of relatively low apparent molecular weight (20-30 kDa).

5.5 Inhibition studies on the reconstituted V-ATPase

The reconstituted V-ATPase was used for analysing the effect of various inhibitors on the ATPase activity. In the initial phase of this study, the ATPase activity from dense pool preparations was checked for its sensitivity towards sodium azide, and was found to show a partial inhibitory effect. Sodium azide inhibits generally the F-type ATPases, but has been reported to inhibit the proton translocation by V-ATPases at 0.2-0.4 mM concentrations (Vasilyeva and Forgac, 1998). However, the toxic compound showed no effect on the ATP hydrolysis in the experiments using the purified and reconstituted ATPase.

Several inhibitors were introduced in the assay system to understand their influence of ATPase activity after reconstitution. The chemical *N*-ethylmaleimide, which modifies the -SH groups adjacent to the ATP binding pocket, is a well-studied inhibitor of V-type ATPases (Hunt and Sanders, 1996). NEM can also affect the P-type ATPase activity whereas the F-type ATPases are resistant to it. In the current study, the reconstituted V-ATPase was observed to be sensitive to NEM. At a concentration of 2 mM, the ATPase activity was inhibited 95 % as reported in the case of V-ATPase from the rat liver lysosomes (Arai *et al*., 1992). Thus, the inhibition of the reconstituted enzyme is similar to that of the native form.

Bafilomycin A-1 is a macrolide antibiotic with a high specificity to V-type ATPases (Gagliardi et al., 1999). It has been known that the macrolactone ring of the molecule contributes to the inhibitory effects (Drose and Altendorf, 1996) and is likely to exert the effect through a non-competitive mechanism. As most other V-ATPases, the reconstituted ATPase from the lysosomal membrane was sensitive to bafilomycin A-1. The inhibition was almost complete when a bafilomycin concentration of 100 nM was used, as was the case with the rat liver enzyme.

N-Retinylidene-*N*-retinylethanolamine (A2-E, see Fig. 1.3), a major lipofuscin derivative is one among the compounds deposited in the retinal pigment epithelial cells in cases of age-related macular degeneration (ARMD) resulting in irreversible vision loss. It has been reported that the fluorogenic material, A2-E, an inhibitor of the lysosomal proton pump (Bergmann *et al.,* 2004) might be one factor linked to the pathological condition. A2-E is also shown to affect the membrane integrity due to a detergent-like effect and is shown to affect the intactness of lysosomal and mitochondrial membranes (Schutt *et al.,* 2002).

The effect of A2-E was analysed on the reconstituted vacuolar ATPase in the current study. The synthetic A2-E was a kind gift from Dr. Kopitz, University of Heidelberg. This vitamin A derivative is known to have detergent-like properties, which could be one factor behind its inhibitory effects. A2-E dissolved in DMSO was used for the analyses. Unfortunately, DMSO interfered with the inorganic phosphate estimation method. However, using proper controls the inhibitory effects of A2-E could be unequivocally demonstrated.

Upon incubation with A2-E, the ATPase activity of the proteoliposomes was reduced significantly in a dose-dependent manner. The ATPase activity was inhibited to 80 % when an A2-E concentration of 10 µM was introduced during the pre-incubation. When the extracted lysosomal membranes without separation of the NEM-sensitive ATPase by gel filtration were incorporated into the liposomes, the inhibition by A2-E was only partial. At a concentration of 10 µM, the inhibition was around 40 % in contrast to the 80 % inhibition from the purified protein. Apparently, A2-E is less or nr not inhibitory towards the F-type ATPase. The result demonstrates the separation of the A2-E-insensitive and sensitive ATPase by the step of solubilisation and gel filtration.

The results shown here is the first report on the isolation of vacuolar ATPase from human tissues and its reconstitution into liposomes. The inhibitory studies using A2-E on the reconstituted ATPase strengthens a possible role of the lipofuscin-derived products on the pathology of age-related macular degeneration, as was proposed by others from studies using the cultured cells (Eldred, 1995, Kopitz *et al*., 2004).

The ARMD is one among the major causes of vision loss in elderly worldwide. Though there are environmental (Seddon *et al*., 2005) and genetic factors (Tuo *et al*., 2004, Guymer *et al*., 2005) known to contribute to the development of this disease, further investigations on the effect of lipofuscin flurophores on the acidification of lysosomes can give a new insight into the lysosomal impairments by these compounds. Such information may contribute to a better understanding of the age-related retinal disorders and their management or prevention.

6 Literature

Arai, K., Shimaya, A., Hiratani, N., Ohkuma, S. (1993): Purification and characterization of lysosomal H(+)-ATPase. An anion-sensitive v-type H(+)-ATPase from rat liver lysosomes. *J. Biol. Chem.* **268**, 5649-60.

Arai, H., Terres, G., Pink, S., Forgac M. (1988): Topography and subunit stoichiometry of the coated vesicle proton pump. *J. Biol. Chem.* **263**, 8796-802.

Ausseil, J., Loredo-Osti, J.C., Verner, A., Darmond-Zwaig, C., Maire, I., Poorthuis, B., van Diggelen, O.P., Hudson, T.J., Fujiwara, T.M., Morgan, K., Pshezhetsky, A.V. (2004): Localisation of a gene for mucopolysaccharidosis IIIC to the pericentromeric region of chromosome 8. *J. Med. Genet.* **41**, 941-5.

Ausseil, J., Landry, K., Seyrantepe, V., Trudel, S., Mazur, A., Lapointe, F., Pshezhetsky, A.V. (2005): An acetylated 120-kDa lysosomal transmembrane protein is absent from mucopolysaccharidosis IIIC fibroblasts: A candidate molecule for MPS IIIC. *Mol. Genet. Metab.* **87**, 22-31.

Bagnat, M., Chang, A., Simons, K. (2000): Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast. *Mol. Biol. Cell.* **12**, 4129-38.

Bagshaw, R.D., Pasternak, S.H., Mahuran, D.J,, Callahan, J.W. (2003): Nicastrin is a resident lysosomal membrane protein. *Biochem. Biophys. Res. Commun.* **300**, 615-8.

Bame, K.J., Rome, L.H. (1986): Genetic evidence for transmembrane acetylation by lysosomes. *Science*. **233**, 1087-9.

Barriocanal, J.G., Bonifacino, J.S., Yuan, L., Sandoval, I.V. (1986): Biosynthesis, glycosylation, movement through the Golgi system, and transport to lysosomes by an N-linked carbohydrate-independent mechanism of three lysosomal integral membrane proteins. *J. Biol. Chem.* **261**, 16755-63.

Bergmann, M., Schutt, F., Holz, F.G., Kopitz, J. (2004): Inhibition of the ATP-driven proton pump in RPE lysosomes by the major lipofuscin fluorophore A2-E may contribute to the pathogenesis of age-related macular degeneration. *FASEB. J.* **18**, 562-4.

Bleistein, J., Heidrich, H.G., Debuch, H. (1980): The phospholipids of liver lysosomes from untreated rats. *Hoppe. Seylers. Z. Physiol. Chem*. **361**, 595-7.

Blonder, J., Hale, M.L., Lucas, D.A., Schaefer, C.F., Yu, L.R., Conrads, T.P., Issaq, H.J., Stiles, B.G., Veenstra, T.D. (2004): Proteomic analysis of detergent-resistant membrane rafts. *Electrophoresis*. **25**, 1307-18.

Boman, A.L. (2001): GGA proteins: new players in the sorting game. *J. Cell. Sci*. **114**, 3413-8.

Bonifacino, J.S., Traub, L.M. (2003): Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu. Rev. Biochem.* **72**, 395-447.

Boriack, R.L., Bennett, M.J. (2001): CLN-3 protein is expressed in the pancreatic somatostatin-secreting delta cells. *Eur. J. Paediatr. Neurol.* **5 Suppl A**, 99-102.

Bradford, M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-54.

Cornelius, F. (2001): Modulation of Na,K-ATPase and Na-ATPase activity by phospholipids and cholesterol. I. Steady-state kinetics. *Biochemistry.* **40**, 8842-51.

Davies, J.P., Chen, F.W., Ioannou, Y.A. (2000): Transmembrane molecular pump activity of Niemann-Pick C1 protein. *Science*. **290**, 2295-8.

De Duve, C., Pressman, B.C., Gianetto, R., Wattlaux, R., Appelmans, F. (1955): Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60**, 604-17.

de Duve ,C. (2005): The lysosome turns fifty. *Nat. Cell. Biol.* **7**, 847-9.

de Lima Santos, H., Lopes, M.L., Maggio, B., Ciancaglini, P. (2005): Na,K-ATPase reconstituted in liposomes: effects of lipid composition on hydrolytic activity and enzyme orientation. *Colloids. Surf. B. Biointerfaces.* **41**, 239-48.

Dell'Angelica, E.C., Mullins, C., Bonifacino, J.S. (1999): AP-4, a novel protein complex related to clathrin adaptors. *J. Biol. Chem.* **274**, 7278-85.

Diettrich, O., Gallert, F., Hasilik, A. (1996): Purification of lysosomal membrane proteins from human placenta. *Eur. J. Cell. Biol.* **69**, 99-106.

Edidin, M. (2003): The state of lipid rafts: from model membranes to cells.
Annu. Rev. Biophys. Biomol. Struct. **32**, 257-83.

Eldred, G.E. (1995): Lipofuscin fluorophore inhibits lysosomal protein degradation and may cause early stages of macular degeneration. *Gerontology.* **41**, 15-28.

Elina, I. (2001): Roles of lipid rafts in membrane transport.
Current opinion in Cell biology. **13**, 470-7.

Eskelinen, E.L., Tanaka, Y., Saftig, P. (2003): At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends. Cell. Biol.* **13**, 137-45.

Ezaki, J., Tanida, I., Kanehagi, N., Kominami, E. (1999): A lysosomal proteinase, the late infantile neuronal ceroid lipofuscinosis gene (CLN2) product, is essential for degradation of a hydrophobic protein, the subunit c of ATP synthase. *J. Neurochem.* **72**, 2573-82.

Fishman, W.H. (1987): Clinical and biological significance of an isozyme tumor marker--PLAP. *Clin. Biochem.* **20**, 387-92.

Furuta, K., Ikeda, M., Nakayama, Y., Nakamura, K., Tanaka, M., Hamasaki, N., Himeno, M., Hamilton, S.R., August, J.T. (2001): Expression of lysosome-associated membrane proteins in human colorectal neoplasms and inflammatory diseases. *Am. J. Pathol.* **159**, 449-55.

Gahl, W.A., Bernardini, I., Tietze, F., Kohn, L.D. (1993): Effects of cycloheximide and tunicamycin on lysosomal cystine transport in rat FRTL-5 cells.
Biochem. Med. Metab. Biol. **49**, 243-54.

Galli, T., McPherson, P.S., De Camilli, P. (1996): The V0 sector of the V-ATPase, synaptobrevin, and synaptophysin are associated on synaptic vesicles in a Triton X-100-resistant, freeze-thawing sensitive, complex. *J. Biol. Chem.* **271**, 2193-8.

Garver, W.S., Heidenreich, R.A., Erickson, R.P., Thomas, M.A., Wilson, J.M. (2000) Localization of the murine Niemann-Pick C1 protein to two distinct intracellular compartments. *J. Lipid. Res*. **41**, 673-87.

Gatt, S. (1969): ß-Glucosidase from bovine brain. *Methods in Enzymology.* **14**, 152-155.

Ghosh, P., Griffith, J., Geuze, H.J., Kornfeld, S. (2003): Mammalian GGAs act together to sort mannose 6-phosphate receptors. *J. Cell. Biol.* **163**, 755-66.

Gluck, S. (1992): V-ATPases of the plasma membrane. *J.Exp.Biol.* **172**, 29-37.

Golabek, A.A., Kida, E., Walus, M., Wujek, P., Mehta, P., Wisniewski, K.E. (2002): Biosynthesis, glycosylation, and enzymatic processing in vivo of human tripeptidyl-peptidase I. *J. Biol. Chem.* **278**, 7135-45.

Goldman, R., Kaplan, A. (1973): Rupture of rat liver lysosomes mediated by L-amino acid esters. *Biochim. Biophys. Acta.* **318**, 205-16.

Guymer, R.H., Chiu, A.W., Lim, L., Baird, P.N. (2005): HMG CoA reductase inhibitors (statins): do they have a role in age-related macular degeneration?
Surv. Ophthalmol. **50**, 194-206.

Heukeshoven, J., Dernick, R. (1988): Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels.
Electrophoresis. **9**, 28-32.

Hirst, J., Lui, W.W., Bright, N.A., Totty, N., Seaman, M.N., Robinson, M.S. (2000): A family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome. *J. Cell. Biol.* **149**, 67-80.

Hofmann, S.L., Atashband, A., Cho, S.K., Das, A.K., Gupta, P., Lu, J.Y. (2002): Neuronal ceroid lipofuscinoses caused by defects in soluble lysosomal enzymes (CLN1 and CLN2).
Curr. Mol. Med. **2**, 423-37.

Honing, S., Griffith, J., Geuze, H.J., Hunziker, W. (1996): The tyrosine-based lysosomal targeting signal in lamp-1 mediates sorting into Golgi-derived clathrin-coated vesicles.
EMBO. J. **15**, 5230-9.

Hunziker, W., Geuze, H.J. (1996): Intracellular trafficking of lysosomal membrane proteins.
Bioessays. **18**, 379-89.

Idriss, J.M., Jonas, A.J. (1991): Vitamin B12 transport by rat liver lysosomal membrane vesicles. *J. Biol. Chem.* **266**, 9438-41.

Ikonen, E. (2001): Roles of lipid rafts in membrane transport.
Curr. Opin. Cell. Biol. **13**, 470-7.

Ioannou, Y.A. (2000): The structure and function of the Niemann-Pick C1 protein.
Mol. Genet. Metab. **71**, 175-81.

Isosomppi, J., Vesa, J., Jalanko, A., Peltonen, L. (2002): Lysosomal localization of the neuronal ceroid lipofuscinosis CLN5 protein. *Hum. Mol. Genet.* **11**, 885-91.

Journet, A., Ferro, M. (2004): The potentials of MS-based subproteomic approaches in medical science: the case of lysosomes and breast cancer. *Mass. Spectrom. Rev.* **23**, 393-442.

Junaid, M.A., Brooks, S.S., Pullarkat, R.K. (2001): Specific substrate for CLN2 protease/tripeptidyl-peptidase I assay. *Eur. J. Paediatr. Neurol.* **5**, 63-8.

Johnson, W.J., Phillips, M.C., Rothblat, G.H. (1997): Lipoproteins and cellular cholesterol homeostasis. *Subcell. Biochem*. **28**, 235-76.

Jonas, A.J., Jobe, H. (1990): N-acetyl-D-glucosamine countertransport in lysosomal membrane vesicles. *Biochem. J.* **268**, 41-5.

Jonas, A.J., Jobe, H. (1990): Sulfate transport by rat liver lysosomes.
J. Biol. Chem. **265**, 17545-9.

Kane, P.M. (1995): Disassembly and reassembly of the yeast vacuolar H(+)-ATPase in vivo.
*J. Biol. Chem***. 270**, 17025-32.

Karten, B., Campenot, R.B., Vance, D.E., Vance, J.E. (2005): The Niemann-Pick C1 protein in recycling endosomes of pre-synaptic nerve terminals. *J. Lipid. Res.* Ahead of print.

Kelly, B.M., Waheed, A., Van Etten, R., Chang, P.L. (1989): Heterogeneity of lysosomes in human fibroblasts. *Mol. Cell. Biochem.* **87**, 171-83.

Klein, R., Peto, T., Bird, A., Vannewkirk, M.R. (2004): The epidemiology of age-related macular degeneration. *Am. J. Ophthalmol.* **137**, 486-95.

Kopitz, J., Holz, F.G., Kaemmerer, E., Schutt, F. (2004): Lipids and lipid peroxidation products in the pathogenesis of age-related macular degeneration. *Biochimie.* **86**, 825-31.

Kubo, Y., Sekiya, S., Ohigashi, M., Takenaka, C., Tamura, K., Nada, S., Nishi, T., Yamamoto, A., Yamaguchi, A. (2005): ABCA5 resides in lysosomes, and ABCA5 knockout mice develop lysosomal disease-like symptoms. *Mol. Cell. Biol.* **25**, 4138-49.

Kwan, C.Y. (1986): Aggregation of smooth muscle membranes and its use in the preparation of plasma membrane enriched fraction from gastric fundus smooth muscle.
Biochem. Cell. Biol. **64**, 535-42.

Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**, 680-5.

Lonka, L., Kyttala, A., Ranta, S., Jalanko, A., Lehesjoki, A.E. (2000): The neuronal ceroid lipofuscinosis CLN8 membrane protein is a resident of the endoplasmic reticulum. *Hum. Mol. Genet.* **9**, 1691-7.

Lu, M., Holliday, L.S., Zhang, L., Dunn, W.A. Jr., Gluck, S.L. (2001): Interaction between aldolase and vacuolar H+-ATPase: evidence for direct coupling of glycolysis to the ATP-hydrolyzing proton pump. *J.Biol. Chem.* **276**, 30407-13.

Lu, M., Sautin, Y.Y., Holliday, L.S., Gluck, S.L. (2004): The glycolytic enzyme aldolase mediates assembly, expression, and activity of vacuolar H+-ATPase. *J. Biol. Chem.* **279**, 8732-9.

Ludwig, J., Kerscher, S., Brandt, U., Pfeiffer, K., Getlawi, F., Apps, D.K., Schagger, M. (1998): Identification and characterization of a novel 9.2-kDa membrane sector-associated protein of vacuolar proton-ATPase from chromaffin granules. *J. Biol. Chem.* **273**, 10939-47.

MacDonald, R.C., MacDonald, R.I., Menco, B.P., Takeshita, K., Subbarao, N.K., Hu, L.R. (1991): Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta.* 1061, 297-303.

Majeski, A.E., Dice, J.F. (2004): Mechanisms of chaperone-mediated autophagy. *Int. J. Biochem. Cell. Biol*. **36**, 2435-44.

Marley, K., Mooney, D.T., Clark-Scannell, G., Tong, T.T., Watson, J., Hagen, T.M., Stevens, J.F., Maier, C.S. (2005): Mass tagging approach for mitochondrial thiol proteins. *J. Proteome. Res.* **4**, 1403-12.

Maxfield, F.R., Tabas, I. (2005): Role of cholesterol and lipid organization in disease. *Nature.* **438**, 612-21.

Merzendorfer, H., Graf, R., Huss, M., Harvey, W.R., Wieczorek, H. (1997): Regulation of proton-translocating V-ATPases. *J. Exp. Biol.* **200**, 225-35

Mitchison, H.M., Mole, SE. (2001): Neurodegenerative disease: the neuronal ceroid lipofuscinoses (Batten disease). *Curr. Opin. Neurol.* **14**, 795-803.

Mole, S.E., Michaux, G., Codlin, S., Wheeler, R.B., Sharp, J.D., Cutler, D.F. (2004): CLN6, which is associated with a lysosomal storage disease, is an endoplasmic reticulum protein. *Exp. Cell. Res*. **298**, 399-406.

Moriyama, Y., Nelson, N. (1989): H+-translocating ATPase in Golgi apparatus. Characterization as vacuolar H+ -ATPase and its subunit structures. *J. Biol. Chem.* **264**, 18445-50.

Morris, S.J., Chiu, V.C., Haynes, D.H. (1979): Divalent cation-induced aggregation of chromaffin granule membranes. *Membr. Biochem*. **2**, 163-201.

Muller, M.L., Irkens-Kiesecker, U., Kramer, D., Taiz, L. (1997): Purification and reconstitution of the vacuolar H+-ATPases from lemon fruits and epicotyls. *J. Biol. Chem.* **272**, 12762-70.

Nelson, N. (1989): Structure, molecular genetics, and evolution of vacuolar H+-ATPases. *J. Bioenerg. Biomembr.* **21**, 553-71.

Nelson, N. (1992): Structure and function of V-ATPases in endocytic and secretory organelles. *J. Exp. Biol.* **172**, 149-53.

Nilsson, E., Ghassemifar, R., Brunk, U.T. (1997): Lysosomal heterogeneity between and within cells with respect to resistance against oxidative stress. *Histochem. J.* **29**, 857-65.

Nishi, T., Forgac, M. (2002): The vacuolar (H+)-ATPases--nature's most versatile proton pumps. *Nat. Rev. Mol. Cell. Biol.* **3**, 94-103.

Nishino, I. (2003): Autophagic vacuolar myopathies. *Curr. Neurol. Neurosci. Rep.* **3**, 64-9.

Nishino, I., Fu, J., Tanji, K., Yamada, T., Shimojo, S., Koori, T., Mora, M., Riggs, J.E., Oh, S.J., Koga, Y., Sue, C.M., Yamamoto, A., Murakami, N., Shanske, S., Byrne, E., Bonilla, E., Nonaka, I., DiMauro, S., Hirano, M. (2000): Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease). *Nature.* **406**, 906-10.

O`Farrel, P.H. (1975): High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007-4021.

Opekarova, M., Malinska, K., Novakova, L., Tanner, W. (2005): Differential effect of phosphatidylethanolamine depletion on raft proteins: further evidence for diversity of rafts in Saccharomyces cerevisiae. *Biochim. Biophys. Acta.* **1711**, 87-95.

Pang, Y., Zhu, H., Wu, P., Chen, J. (2005): The characterization of plasma membrane Ca2+-ATPase in rich sphingomyelin-cholesterol domains. *FEBS. Lett.* **579**, 2397-403.

Paternostre, M.T., Roux, M., Rigaud, J.L. (1988): Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 1. Solubilization of large unilamellar liposomes (prepared by reverse-phase evaporation) by triton X-100, octyl glucoside, and sodium cholate. *Biochemistry.* **27**, 2668-77.

Penin, F., Godinot, C., Gautheron, D.C. (1984): Two-dimensional gel electrophoresis of membrane proteins using anionic and cationic detergents. Application to the study of mitochondrial F0-F1-ATPase. *Biochim. Biophys. Acta.* **775**, 239-45.

Peters, C., von Figura, K. (1994): Biogenesis of lysosomal membranes. *FEBS. Lett.* **346**, 108-14.

Pertoft, H., Laurent, T.C., Laas, T., Kagedal, L. (1978): Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll). *Anal. Biochem.* **88**, 271-82.

Pisoni, R.L., Flickinger, K.S., Thoene, J.G, Christensen, H.N. (1987): Characterization of carrier-mediated transport systems for small neutral amino acids in human fibroblast lysosomes. *J. Biol. Chem.* **262**, 6010-7.

Pisoni, R.L. (1991): Characterization of a phosphate transport system in human fibroblast lysosomes. *J. Biol. Chem.* **266**, 979-85.

Pisoni, R.L., Thoene, J.G. (1989): Detection and characterization of a nucleoside transport system in human fibroblast lysosomes. *J. Biol. Chem.* **264**, 4850-6.

Pralle, A., Keller, P., Florin, E.L., Simons, K., Horber, J.K. (2000): Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells.
J. Cell. Biol. **148**, 997-1008.

Reeves, J.P. (1979): Accumulation of amino acids by lysosomes incubated with amino acid methyl esters. *J. Biol. Chem*. **254**, 8914-21.

Rigaud, J.L., Pitard, B., Levy, D. (1995): Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins.
Biochim. Biophys. Acta. **1231**, 223-46.

Rigaud, J.L., Levy, D. (2003): Reconstitution of membrane proteins into liposomes.
Methods. Enzymol. **372**, 65-86.

Henning, R., Heidrich, H.G. (1974): Membrane lipids of rat liver lysosomes prepared by free-flow elctrophoresis. *Biochimica. at. Biophysica. Acta.* **345**, 326-335.

Rome, L.H., Garvin, A.J., Allietta, M.M., Neufeld, E.F. (1979): Two species of lysosomal organelles in cultured human fibroblasts. *Cell*. **17**, 143-53.

Rouille, Y., Rohn, W., Hoflack, B. (2000): Targeting of lysosomal proteins.
Semin. Cell. Dev. Biol. **11**, 165-71.

Saftig, P., Tanaka, Y., Lullmann-Rauch, R., von Figura, K. (2001): Disease model: LAMP-2 enlightens Danon disease. *Trends. Mol. Med*. **7**, 37-9.

Samsonov, A.V., Mihalyov, I., Cohen, F.S. (2001): Characterization of cholesterol-sphingomyelin domains and their dynamics in bilayer membranes. *Biophys. J*. **81**, 1486-500.

Sangiorgio, V., Pitto, M., Palestini, P., Masserini, M. (2004): GPI-anchored proteins and lipid rafts. *Ital. J. Biochem*. **53**, 98-111.

Schoer, J.K., Gallegos, A.M., McIntosh, A.L., Starodub, O., Kier, A.B., Billheimer, J.T., Schroeder, F. (2002): Lysosomal membrane cholesterol dynamics. *Biochemistry*. **39**, 7662-77.

Schutt, F., Davies, S., Kopitz, J., Holz, F.G., Boulton, M.E. (2000): Photodamage to human RPE cells by A2-E, a retinoid component of lipofuscin.
Invest. Ophthalmol. Vis. Sci. **41**, 2303-8.

Schutt, F., Bergmann, M., Holz, F.G., Kopitz, J. (2002): Isolation of intact lysosomes from human RPE cells and effects of A2-E on the integrity of the lysosomal and other cellular membranes. *Graefes. Arch. Clin. Exp. Ophthalmol*. **240**, 983-8.

Schuhmann, T., Grond, S. (2004): Biosynthetic investigations of the V-type ATPase inhibitors bafilomycin A1, B1 and concanamycin A. *J. Antibiot. (Tokyo).* **57**, 655-61.

Scott, C., Ioannou, Y.A. (2004): The NPC1 protein: structure implies function.
Biochim. Biophys. Acta. **1685**, 8-13.

Seddon, J.M., Cote, J., Page, W.F., Aggen, S.H., Neale, M.C. (2005): The US twin study of age-related macular degeneration: relative roles of genetic and environmental influences. *Arch. Ophthalmol.* **123**, 321-7.

Sepulveda, M.R., Berrocal-Carrillo, M., Gasset, M., Mata, A.M. (2005): The Plasma Membrane Ca2+-ATPase Isoform 4 Is Localized in Lipid Rafts of Cerebellum Synaptic Plasma Membranes. *J.Biol.Chem.* **281**, 7-53.

Sewell, A.C., Pontz, B.F., Benischek, G. (1988): Mucopolysaccharidosis type IIIC (Sanfilippo): early clinical presentation in a large Turkish pedigree. *Clin. Genet*. **34**, 116-21.

Sharom, F.J., Lehto, M.T. (2002): Glycosylphosphatidylinositol-anchored proteins: structure, function, and cleavage by phosphatidylinositol-specific phospholipase C. *Biochem. Cel.l Biol.* **80**, 535-49.

Shaw, M.M., Riederer, B.M. (2003): Sample preparation for two-dimensional gel electrophoresis. *Proteomics.* **3**, 1408-17.

Shogomori, H., Futerman, A.H. (2001): Cholesterol depletion by methyl-beta-cyclodextrin blocks cholera toxin transport from endosomes to the Golgi apparatus in hippocampal neurons. *J. Neurochem.* **78**, 991-9.

Simons, K., Ikonen, E. (1997): Functional rafts in cell membranes. *Nature.* **387**, 569-72.

Simons, K, Gruenberg, J. (2000): Jamming the endosomal system: lipid rafts and lysosomal storage diseases. *Trends. Cell. Biol*. **10**, 459-62.

Simons, K., Ehehalt, R. (2002): Cholesterol, lipid rafts, and disease. *J. Clin. Invest*. **110**, 597-603.

Simons, M., Kramer, E.M., Thiele, C., Stoffel, W., Trotter, J. (2000): Assembly of myelin by association of proteolipid protein with cholesterol- and galactosylceramide-rich membrane domains. *J. Cell. Biol*. **151**, 143-54.

Simpson, F., Peden, A.A., Christopoulou, L., Robinson, M.S. (1997): Characterization of the adaptor-related protein complex, AP-3. *J.Cell.Biol.* **137**, 835-45.

Smith, A.N., Lovering, R.C., Futai, M., Takeda, J., Brown, D., Karet, F.E. (2003): Revised nomenclature for mammalian vacuolar-type H+ -ATPase subunit genes. *Mol. Cell.* **12**,801-3.

Snyers, L., Umlauf, E., Prohaska, R. (1999): Association of stomatin with lipid-protein complexes in the plasma membrane and the endocytic compartment. *Eur. J. Cell. Biol*. **78**, 802-12.

Steet, R., Lee, W.S., Kornfeld, S. (2005): Identification of the minimal lysosomal enzyme recognition domain in cathepsin D. *J. Biol. Chem.* **280**, 33318-23.

Stoner, C.D., Sirak, H.D. (1978): Magnesium-induced inner membrane aggregation in heart mitochondria. *J. Cell. Biol.* **77**, 417-26.

Supek, F., Supekova, L., Mandiyan, S., Pan ,Y.C., Nelson, H., Nelson, N. (1994): A novel accessory subunit for vacuolar H(+)-ATPase from chromaffin granules. *J. Biol. Chem.* **269**, 24102-6.

Symons, L.J., Jonas, A.J. (1987): Isolation of highly purified rat liver lysosomal membranes using two Percoll gradients. *Anal. Biochem.* **164**, 382-90.

Tabuchi, N., Akasaki, K., Sasaki, T., Kanda, N., Tsuji, H. (1997): Identification and characterization of a major lysosomal membrane glycoprotein, LGP85/LIMP II in mouse liver. *J. Biochem. (Tokyo)*. **122**, 756-63.

Taute, A., Watzig, K., Simons, B., Lohaus, C., Meyer, H., Hasilik, A. (2002): Presence of detergent-resistant microdomains in lysosomal membranes. *Biochem. Biophys. Res. Commun*. **298**, 5-9.

Town, M., Jean, G., Cherqui, S., Attard, M., Forestier, L., Whitmore, S.A., Callen, D.F., Gribouval, O., Broyer, M., Bates, G.P., van't Hoff, W., Antignac, C. (1998): A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis. *Nat. Genet*. **18**, 319-24.

Tuo, J., Bojanowski, C.M., Chan, C.C. (2004): Genetic factors of age-related macular degeneration. *Prog. Retin. Eye. Res.* **23**, 229-49.

Usuki, F., Takenaga, S., Higuchi, I., Kashio, N., Nakagawa, M., Osame, M. (1994): Morphologic findings in biopsied skeletal muscle and cultured fibroblasts from a female patient with Danon's disease (lysosomal glycogen storage disease without acid maltase deficiency). *J. Neurol. Sci.* **127**, 54-60.

Van Veldhoven, P.P., Baumgart, E., Mannaerts, G.P. (1996): Iodixanol (Optiprep), an improved density gradient medium for the iso-osmotic isolation of rat liver peroxisomes. *Anal. Biochem*. **237**, 17-23.

Verheijen, F.W., Verbeek, E., Aula, N., Beerens, C.E., Havelaar, A.C., Joosse, M., Peltonen, L., Aula, P., Galjaard, H., van der Spek, P.J., Mancini, G.M. (1999): A new gene, encoding an anion transporter, is mutated in sialic acid storage diseases. *Nat. Genet*. **23**, 462-5.

Vesa, J., Chin, M.H., Oelgeschlager, K., Isosomppi, J., DellAngelica, E.C., Jalanko, A., Peltonen, L. (2002): Neuronal ceroid lipofuscinoses are connected at molecular level: interaction of CLN5 protein with CLN2 and CLN3. *Mol. Biol. Cell*. **13**, 2410-20.

Vulevic, B., Chen, Z., Boyd, J.T., Davis, W. Jr., Walsh, E.S., Belinsky, M.G., Tew, K.D. (2001): Cloning and characterization of human adenosine 5'-triphosphate-binding cassette, sub-family A, transporter 2 (ABCA2). *Cancer. Res.* **61**, 3339-47.

von Figura, K. (1977): Human alpha-n-acetylglucosaminidase. 2. Activity towards natural substrates and multiple recognition form. *Eur. J. Biochem.* **80**, 535-42.

Voznyi Ya,V., Karpova, E.A., Dudukina, T.V., Tsvetkova, I.V., Boer, A.M., Janse, H.C., van Diggelen, O.P. (1993): A fluorimetric enzyme assay for the diagnosis of Sanfilippo disease C (MPS III C). *J. Inherit. Metab. Dis.* **16**, 465-72.

Walker, J.E., Collinson, I.R., Van Raaij, M.J., Runswick, M.J. (1995): Structural analysis of ATP synthase from bovine heart mitochondria. *Methods. Enzymol.* **260**, 163-90.

Wang, H., Voznyi, Y.A., Huijmans, G.M., Geilen, G.C., Karpova, E.A., Dudukina, T.V., Zaremba, J., Van Diggelen, O.P., Kleijer, W.J. (1993): Prenatal diagnosis of sanfilippo disease type-C using a simple fluorometric enzyme assay. *Prenat. diag*. **14**, 17-22.

Warren, M., Smith, J.A., Apps, D.K. (1992): Rapid purification and reconstitution of a plant vacuolar ATPase using Triton X-114 fractionation: subunit composition and substrate kinetics of the H(+)-ATPase from the tonoplast of Kalanchoe daigremontiana.
Biochim. Biophys. Acta. **1106**, 117-25.

White, J.G. (1982): Membrane defects in inherited disorders of platelet function.
Am. J. Pediatr. Hematol. Oncol. **4**, 83-94.

Wickner, W.T., Lodish, H.F. (1985): Multiple mechanisms of protein insertion into and across membranes. *Science.* **230**, 400-7.

Wilkens, S., Inoue, T., Forgac, M. (2004): Three-dimensional structure of the vacuolar ATPase. Localization of subunit H by difference imaging and chemical cross-linking.
J. Biol. Chem. **279**, 41942-9.

Winchester, B., Vellodi, A., Young, E. (2000): The molecular basis of lysosomal storage diseases and their treatment. *Biochem. Soc. Trans*. **28**, 150-4.

Winchester, B. (2001): Lysosomal membrane proteins. *Eur. J. Paediatr. Neurol.* **5** Suppl A:11-9.

Wolters, P.J., Chapman, H.A. (2000): Importance of lysosomal cysteine proteases in lung disease. *Respir. Res.* **1**, 170-7.

Yaghootfam, A., Schestag, F., Dierks, T., Gieselmann, V. (2003): Recognition of arylsulfatase A and B by the UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-phosphotransferase. *J. Biol. Chem.* **278**, 32653-61.

Yoshinaka, K., Kumanogoh, H., Nakamura, S., Maekawa, S. (2004): Identification of V-ATPase as a major component in the raft fraction prepared from the synaptic plasma membrane and the synaptic vesicle of rat brain. *Neurosci. Lett.* **363**, 168-72.

Zhang, F., Zhang, W., Liu, L., Fisher, C.L., Hui, D., Childs, S., Dorovini-Zis, K., Ling, V. (2000): Characterization of ABCB9, an ATP binding cassette protein associated with lysosomes. *J. Biol. Chem.* **275**, 23287-94.

Zhong, N.A., Wisniewski, K.E., Ju, W., Moroziewicz, D.N., Jurkiewicz, A., McLendon, L., Jenkins, E.C., Brown, W.T. (2000): Molecular diagnosis of and carrier screening for the neuronal ceroid lipofuscinoses. *Genet. Test*. **4**, 243-8.

Zhou, X., Thamotharan, M., Gangopadhyay, A., Serdikoff, C., Adibi, S.A. (2000): Characterization of an oligopeptide transporter in renal lysosomes.
Biochim. Biophys. Acta. **1466**, 372-8.

Zurdel, J., Richard, G. (2002): Genetic studies of age related macular degeneration. *Ophthalmologe.* **99**, 636-41.

7 Summary

The lysosomal membrane is a dynamic environment where specific interactions among proteins and between proteins and lipids occur. These interactions are necessary for the proper functioning of the lysosomal apparatus that allows the passage of molecules into and out of the lysosomes for the degradation and recycling. From the previous studies on human placental lysosomes, occurrence of detergent resistant microdomains, in which special proteins including the acetyl-CoA: α-glucosaminide N-acetyl transferase are localised has been reported by Taute *et al.* (2002).

In an attempt to characterise the proteins that are part of such microdomains from the lysosomal membranes, the V-ATPase was found to be enriched in fractions containing the DRMs. This protein complex catalyses an ATP dependent proton translocation and is responsible for the acidification of the lysosomal lumen. It is known that lipids and lipid peroxidation products influence the biological activity of the V-ATPase that may contribute to age-related macular degeneration, a major cause of vision loss as reported by Kopitz *et al*. (2004). In this work a method was established for the isolation and reconstitution of the human V-ATPase which should allow further studies on the role of special lipids and lipid derivatives on the macular degeneration.

To isolate the V-ATPase, a simple and efficient system for purifying the lysosomal membrane proteins was sought as the commonly used procedures yield lysosomal preparations that are contaminated with mitochondria. To reduce the contamination, a substrate-induced selective disruption of the lysosomal vesicles followed by aggregating and sedimenting the bulk of contaminating membranes, especially those from the mitochondria, was developed. A purification of the lysosomal membrane proteins up to 300 fold as compared to the placental homogenate, based on the specific activity of the membrane-associated ß-glucosidase was achieved using this novel and convenient procedure. The lysosomal membranes thus prepared may also be used for the selective isolation and characterisation of lysosomal membrane proteins that are not yet studied in detail, like the acetyl-CoA: α-glucosaminide N-acetyl transferase and Niemann- Pick C1 protein known to be involved in the degradation of heparan sulphate and in cholesterol transport, respectively.

From the purified lysosomal membranes, the V-ATPase was extracted using Triton X-100 and CHAPS and further purified by gel filtration. This is the first report on the purification of V-ATPase from a human tissue. Methyl-ß-cyclodextrin was included during

the extraction step to deplete cholesterol and thereby to disrupt the microdomains. Since a number of the V-ATPase subunits possess a basic isoelectric point, and as such are difficult to be analysed by the common two-dimensional electrophoretic systems, a novel CETAB/SDS-PAGE system was used for the proteomic characterisation of the purified V-ATPase. The V-ATPase activity fractions were devoid of all the major proteins present in the lysosomal preparation used for gel filtration, though two other DRM-associated proteins, placental alkaline phosphatase and stomatin were identified pointing towards the possibility of different types of DRMs on the lysosomal or the contaminating membranes, that were not removed during the purification steps.

The purified protein complex was successfully reconstituted into unilamellar vesicles. When the vesicles containing lecithin and cholesterol were used for the reconstitution, a recovery of upto 30 % was observed in the incorporated fraction, as compared to 14 % when the experiment was performed with cholesterol-free vesicles. Finally, the dose-dependant inhibitory effects of the lipofuscin componenent, A2-E were confirmed with the reconstituted V-ATPase. The ATP hydrolysis by the enzyme was completely inhibited at an A2-E concentration of 10 μM. This result extends the previous findings using cell cultures on the inhibition of V-ATPase by A2-E described by Bergmann *et al.* suggesting a role of A2-E in the pathogenesis of age-related macular degeneration. The inhibition of ATP hydrolysis and thereby of the lysosomal acidification may cause an accumulation of lipofuscin within the lumen of the lysosomes and thus contribute to the degeneration of the retinal epithelium. The purified and reconstituted enzyme may facilitate further studies on its inhibitory and protective agents.

7 Zusammenfassung

Eine Reinigung von lysosomalen Membranen aus humaner Plazenta, bei der sich die spezifische Aktivität der membranassoziierten ß-Glucocerebrosidase gegenüber dem Homogenat 300-fach anreichern ließ, wurde erzielt. Aus diesen Membranpräparationen konnte die vakuoläre H^+-ATPase extrahiert und durch Gelfiltration weiter gereinigt werden. Dies ist die erste Beschreibung einer Solubilisierung und Anreicherung der V-ATPase aus humanem Gewebe. Weiterhin konnte gezeigt werden dass sich der Enzymkomplex aus detergenzresistenten Mikrodomänen (DRMs, rafts) durch Methyl-ß-cyclodextrin freisetzen ließ. Neben einigen V-ATPase Untereinheiten ließen sich die alkalische Phosphatase und Stomatin als weitere DRM-assoziierte Proteine durch Kombination von CETAB- und SDS-PAGE mit anschießendem MALDI-Massen-Fingerprint identifizieren, die unter Methyl-ß-cyclodextrin-Behandlung jedoch nicht freigesetzt wurden. Des Weiteren konnten in Rekonstitutionsversuchen 30 % der gereinigten V-ATPase-Aktivität in Cholesterol-haltigen und 14 % in Cholesterol-freien Liposomen erhalten werden. Die Aktivität der V-ATPase in den gereinigten lysosomalen Membranen und ebenso diejenige des in Liposomen rekonstituierten Enzyms erwies sich als vollständig mit dem Retinalderivat A2-E (10 µM) hemmbar. Dadurch wurde eine Basis für weitere Untersuchungen des pathogenetischen Potentials des A2-E geschaffen.

8 Appendix

8.1 Abbreviations and expressions

ABC	**A**TP **B**inding **c**assette
Ac-45	**Ac**cessory protein-**45**
AcTf	**Ac**etyl **t**rans**f**erase
A2-E	*N*-Retinylidene-*N*-retinylethanolamine
APS	**A**mmonium **p**eroxi**s**ulphate
AP	**A**daptor **p**rotein
ASB-14	**A**mido**s**ulfo**b**etaine-**14**
Arf	**A**DP-**r**ibosylation **f**actor
ARMD	**A**ge-**r**elated **m**acular **d**egeneration
ATP	**A**denosine **t**ri**p**hosphat**e**
BLAST	**B**asic **l**ocal **a**lignment **s**earch **t**ool
BSA	**B**ovine **s**erum **a**lbumin
CHAPS	3-[(3-**ch**ol**a**mido**p**ropyl)-dimethylammonio]- propane**s**ulfonate
cDNA	**c**omplementary **DNA**
CLN-proteins	**N**euronal **c**eroid **l**ipofuscinos-associated **proteins**
cm	**C**enti**m**eter
CoA	**Co**enzyme **A**
Da	**Da**lton
°C	Degree celcius
DP	**D**ense **p**ool
DNA	**D**eoxy ribo**n**ucleic **a**cid
DMSO	**Di**methyl **s**ulph**o**xide
DTT	**Di**thio**t**hreitol
ECL	**E**nhanced **c**hemi **l**uminiscence
EC	**E**nzyme **c**ommission
ER	**E**ndoplasmic **r**eticulum
EDTA	**E**thyline**d**iamine **t**etra**a**cetate
et al.	and others (Latin)
GRAVY score	**Gr**and **a**verage h**y**drophobicity score
GGA	**G**olgi-localized γ-ear-containing, ***Arf*** binding proteins
g	Gram

ß-Gluc	**ß-Gluc**ocerebrosidase
HPLC	**H**igh **p**erformance **l**ipid **c**hromatography
HRP	**H**orse **r**adish **p**eroxidase
h	**H**our
IgG	**I**mmuno**g**lobulin
IEF	**I**so**e**lectric **f**ocusing
kDa	kilo Dalton
LAMP	**L**ysosome **a**ssociated **m**embrane **p**rotein
MS	**M**ass **s**pectrometry
LIMP	**L**ysosomal **i**ntegral **m**embrane **p**rotein
Maldi-TOF	**M**atrix-**a**ssisted **l**aser **d**esorption/**i**onisation- **T**ime-**o**f-**f**light
µl	**Mi**cro**l**iter
min	**Min**ute
mA	**M**illi **a**mpere
ml	**M**illi**l**iter
mm	**M**illi**m**eter
MME	**M**ethionine **m**ethyl **e**ster
MBCD	**m**ethyl-**ß**-**c**yclo**d**extrin
MW	**M**olecular **w**eight
M	**M**olar
nm	**n**ano **m**ole
NCBI	**N**ational **C**enter for **B**iological **I**nformation
NEM	***N*-e**thyl**m**aleimide
NADH	**N**icotinamide **a**denine **d**inucleotide (reduced)
NPC1	**N**iemann-**P**ick-**C1**
NPC2	**N**iemann-**P**ick-**C2**
NP-40	**N**onidiet **P**-**40**
ORF	**O**pen **r**eading **f**rame
Pi	**I**norganic **p**hosphate
pI	**I**solectric **p**oint
pH	**-**log (**H^+**)
PAGE	**P**oly**a**crylamide **g**el **e**lectrophoresis
PC	**P**hosphatidyl **c**holine
PMSF	**P**henyl **m**ethyl **s**ulphonyl **f**luoride

PLAP	**Pl**acental **a**lkaline **p**hosphatase
PVDF	**P**oly**v**inyli**d**ene **f**luoride
rpm	**r**evolutions **p**er **m**inute
SDS	**S**odium **d**odecyl **s**ulphate
SDH	**S**uccinate **d**e**h**ydrogenase
TEMED	N' N' N' N'-**Te**tra**me**thylene **d**iamine
TGN	**T**rans-**g**olgi-**n**etwork
TCA	**T**ri**c**hloro**a**cetic acid
TPP-1	**T**ri**p**eptidyl **p**eptidase-**1**
2DE	**T**wo-**d**imensional **e**lectrophoresis
UC	**U**ltra **c**entrifuge
UV	**U**ltra **v**iolet
V-ATPase	**V**acuolar **ATPase**
v/v	**v**olume/**v**olume
w/v	**w**eight/**v**olume
ZFF	**Z**-**P**he-**P**he-diazomethyl ketone

8.2 Curriculum vitae

Name: Rajesh Chandramohanadas
Gender: Male
Birthdate: 28-12-1978
Nativity: Haripad, Kerala, India

Educational Qualifications:

Primary Education:

Pothappally LPS, Kumarapuram.
(1984-93) Govt. UPS, Haripad.
Govt. HSC, Haripad.

Secondary Education:

(1994-96) T.K.M.M. College, Nangiarkulangara, Haripad.
(University of Kerala)

Bachelor of Science:

(1996-99) N.S.S. College, Pandalam.
(University of Kerala)

Master of Science:

(1999-2001) Dept. of Biochemistry, Kariavattom, Thiruvananthapuram.
(University of Kerala)

PhD student:

(Feb.2002- Jan.06) Institute for Physiological Chemistry,
Philipps University, Marburg.
(Lysosomal Proteomics- research group- Prof. Dr. Hasilik)

Rapid purification of human lysosomal membranes, characterisation of the detergent resistant microdomains, purification and reconstitution of the vacuolar proton pump (V-ATPase).

8.3 Acknowledgment

I would like to express sincere gratitude to my Ph.D. adviser Prof. Dr. Andrej Hasilik for giving me an opportunity to conduct this study in his laboratory. I am thankful for his support during my stay and research in Germany and for the suggestions and constant encouragement.

Thanks to Dr. Bernhard Schmidt and his colleagues (Max-Planck Institute for Biophysical Chemistry, Göttingen) for performing the mass spectrometric analysis and Dr. Jürgen Kopitz (University of Heidelberg) for providing the synthetic A2-E used for the inhibitory studies.

I am grateful to Dr. Christian Wrocklage for his help in protein reconstitution experiments and to Dr. Bernd Kösters for being part of the scientific discussions. Their critical suggestions were highly influential in my research and their help admirable. My thanks also go to Dr. Peter Lemansky and Dr. Lankat Buttgereit for their support and help.

I thank Bernd Schröder, Eva Smolenova and Dr. Hans-Gerhard Löffler for being very friendly co-workers and for the scientific discussions we shared. Dr. Madanan Madathilparampil Gopalakrishnan was very helpful during the early stages of my research and I am indebted to him for introducing me to the laboratory.

A lot of thanks to Irena Müller, and Evelin Heinemann for their help in the official matters and to Traudel Jarosch, Thomas Stein, Heinrich Kaiser, Renate Gondrum, Eva Becker, Ute Beck, KarheinzBurk and Charlotte Jung for their efficient technical support.

I thank Prof. Dr. Klaus-Heinrich Röhm and Dr. Mechthild Röhm for lending me the helping hands whenever required and the affection they showed to me, in addition to the scientific suggestions.

I am grateful to Prof. Dr. P.R. Sudhakaran (University of Kerala, Kerala, India) for his continuous motivation. I appreciate all my Indian friends especially Sunil, Elam, Srinivas, Ramesh, Vijay, Avinash and Beerendra for the cheerful environment they created and for their help in one way or the other.

The financial support by BMBF is gratefully acknowledged.

Finally, I am deeply indebted to all my family members for their care and continuous support without which it would not have been possible to accomplish the targets.

8.4 Declaration

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin zur Promotionsprüfung eingereichte Arbeit mit dem Titel `Rapid purification of human lysosomal membranes, characterisation of the detergent resistant microdomains, purification and reconstitution of the vacuolar proton pump (V-ATPase)` im Institut für Physiologische Chemie, Arbeitsgruppe Lysosomale Proteomik unter der Leitung von Herrn Prof. Dr. Hasilik ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angegebenen Hilfmittel benutzt habe. Ich habe bicher an keinem in- und ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Posterpräsantationen

7th Young Scientists Meeting (Deutsche Gesellschaft für Zellbiologie).
22-24th September, 2005, Jena, Germany.
Rajesh Chandramohanadas, Christian Wrocklage, Bernd Schröder, Bernhard Schmidt and Andrej Hasilik: *Proteomic analysis of lipid rafts from lysosomal membranes with special emphasis on vacuolar ATPase.*

Scientific Meeting on Niemann-Pick Type C disease (The Ara Parseghian Medical Research Foundation), 3-4th June, 2005, Tucson, Arizona, USA.
Bernd Schröeder, Fengzhi Jin, **Rajesh Chandramohanadas**, Bernd Kösters and Andrej Hasilik: *Human Lysosomal Membrane Proteome- Initial results on the extraction of NPC1 from human placental lysosomes.*

Marburg, den January 2006

Rajesh Chandramohanadas