Screening for lysophosphatidylserine lipases in mouse tissues

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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Date: 10 April, 2023

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From May 2022 to Mar 2023

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled **Screening for lysophosphatidylserine lipases in mouse tissues** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Prajwal Punnamraju** at Indian Institute of Science Education and Research under the supervision of **Dr. Siddhesh S. Kamat, Associate Professor, Department of Biology**, during the academic year 2022-2023.

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Name of Your TAC: Dr. Thomas Pucadyil

This thesis is dedicated to IISER Pune

Declaration

I hereby declare that the matter embodied in the report entitled **Screening for lysophosphatidylserine lipases in mouse tissues** are the results of the work carried out by me at the **Department of Biology, Indian Institute of Science Education and Research, Pune**, under the supervision of **Dr. Siddhesh S. Kamat** and the same has not been submitted elsewhere for any other degree

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Abstract

Lysophosphatidylserines (Lyso-PSs) are immunomodulatory signalling lipds whose metabolism is of growing importance. Known enzymes linked to synthesis and degradation of lyso-PS are directly associated with pathophysiological conditions. ABHD12 is known to be the primary lyso-PS lipase in brain, while lyso-PS lipases in other tissues are unknown. Through this project, progress has been made towards identifying lipases that degrade lyso-PS in murine tissues other than brain. Using protein separation techniques, we developed protocols to obtain protein fractions with distinct protein profiles, which are important to narrow down candidate enzymes capable of lyso-PS lipase activity. MS-based lipid profiling experiments were performed, which revealed the translation of lyso-PS lipase activity upon detergent treatment. We also studied distributions of lyso-PS and ABHD12 across tissues using untargeted lipidomics and western blotting respectively, and the results obtained further strengthen the basis of the project.

Acknowledgments

I am extremely grateful to Dr. Siddhesh Kamat for accepting me as a master's student as part of an exciting project, and for providing the valuable guidance and resources that were crucial to the completion of this thesis. I am thankful to Dr. Thomas Pucadyil for agreeing to be on my thesis advisory committee, and for giving me his time, comments, and feedback.

I am highly obliged to Arnab for his valuable mentorship and calming influence throughout the thesis. I am indebted to Kamat lab members Aakash, Arnab, Karthik, Kaveri, Kundan, Ojal, Sonali, Anisha, Rohith, Mihika, Chaitanya, Sreedev, Abhishek, Kavita and Pooja for always maintaining a friendly and constructive lab environment which made it an enjoyable workplace. I want to thank Aakash for his help during mass spectrometry-based experiments. I also want to thank members of National Facility for Gene Function in Health and Disease for their assistance in maintaining animal models.

I am grateful to my friends KC, Ratan, Vara, Madhesh, Thushar, Lalith, Lokesh, Parag, Sreyas, and many other batchmates for always making me feel at ease in IISER through their company. I extend my thanks to IISER and its community for enabling me to explore and develop new interests apart from academic growth. Most importantly, I would like to thank my family, especially my parents, for their constant support and encouragement during my studies. Finally, I want to thank the Department of Biology at IISER Pune for estabilishing a system that provided me with all the resources I needed, and KVPY for their financial support.

Contributions

Contributor name	Contributor role
Dr. Siddhesh Kamat, Arnab	Conceptualisation Ideas
Arnab, Aakash, Prajwal	Methodology
-	Software
Prajwal	Validation
Prajwal	Formal analysis
Prajwal	Investigation
Dr. Siddhesh Kamat, Apurbo (G3 lab)	Resources
Prajwal	Data Curation
Prajwal	Writing - original draft preparation
Arnab	Writing - review and editing
-	Visualisation
Dr. Siddhesh Kamat	Supervision
-	Project administration
Dr. Siddhesh Kamat	Funding acquisition

Please add – (dash symbol) if nobody needs to be attributed for a category. Do not remove any of the categories. You may add a category if somebody contributed to the research whose criteria do not match the ones above. For an explanation read the CRediT taxonomy explanation. [Remove the italics text once done].

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

Chapter 1 : Introduction

Lipids are a crucial class of biomolecules that are associated with roles in cell structure maintenance, energy storage and signalling. With the advances in separation science, mass spectrometry and lipid imaging techniques, their roles as capable signalling molecules are becoming clearer.[1]

The most abundant group of lipids are phospholipids, which make up >75% of total lipid molecules in a typical mammalian cell.[2] There are numerous types of phospholipids that carry out a variety of essential physiological roles. Phospholipids with glycerol as the backbone are termed glycerophospholipids, and they typically have two fatty acyl chains attached to the glycerol backbone through ester bonds. These lipids make up most of the membrane lipidome, and their assembly is key to the existence of the membrane bilayer. Phosphatidylserines (PS) are the most abundant class of anionic phospholipids in eukaryotic cells with well-characterised roles in apoptosis and blood clotting.[3]

Phospholipids are constantly degraded into numerous degradation products by lipases. A class of degradation products of phospholipids are lysophospholipids (LPLs), which are formed upon the cleavage of an ester bond that links a fatty acyl group to the backbone molecule. As a result, lipids with only one fatty acyl chain are obtained, thus enhancing their water solubility. This allows them to transcend roles limited to the cell membrane, to act as lipid mediators in numerous cellular processes. [4] Lysophosphatidylserines (Lyso-PS) are a class of signalling lysophospholipids that have been an active research focus since the latter part of the twentieth century. It is now known that they influence a variety of important biological processes such as mast cell degranulation, macrophage efferocytosis and calcium signalling. [5,6] Steps towards lyso-PS discovery began when it was hypothesized that the anticoagulation properties were influenced by a lipid, and a lipid fraction rich in PS was isolated[7,8] which displayed this activity. It was eventually understood that the activity was being facilitated by a product resulting from the degradation of PS, which we now know as lyso-PS.

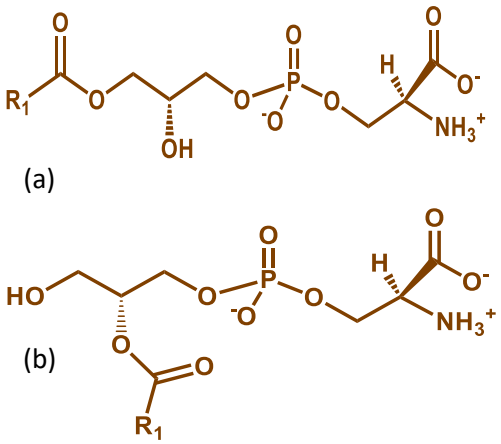


Figure 1.1

Structure of lysophosphatidylserines (a)sn-1 isomer, (b) sn-2 isomer

Structurally, they occur in two types: *sn*-1 and *sn*-2 lyso-PS, depending on the position of the acyl chain. Although both the forms are present in murine tissues, the *sn*-1 form is significantly more abundant, and it has been shown that *sn*-2 lyso-PS convert non-enzymatically to the *sn*-1 form via an intramolecular acyl transfer reaction at physiological conditions.[3]

They are biosynthesized both intracellularly and extracellularly, and various enzymes have been identified that are responsible for their biosynthesis. [3–5]

[9].Deregulation[10–12] in lyso-PS metabolism has been linked to various human pathophysiological conditions.[5] Mainly, it was shown that lyso-PS stimulates mast cell degranulation, leading to the rapid release of histamine, which acts as an immune signal against allergic antigen.[13,14] It is this release of histamine that immunologically stimulated the anti-coagulative properties that were initially being studied.[14,15]

It was also shown that lyso-PS was the only class of lysophospholipids whose levels were heightened during macrophage efferocytosis.[16] Putative receptors involved in these physiological roles have also been identified, although their interactions with lyso-PS aren't yet well characterised.[17–19] Lyso-PS was also understood to interact with numerous GPCR receptors, thereby playing a role in calcium signalling and chemotactic migration of cells.[20] When Lyso-PS synthesis was disturbed by generating mice with genetically knocked out ABHD16A, it was seen that a partial embryonic lethality developed, along with shunted animal growth, suggesting that lyso-PS metabolism has a role in embryonic development associated with the central nervous system and immune system.[21,22]

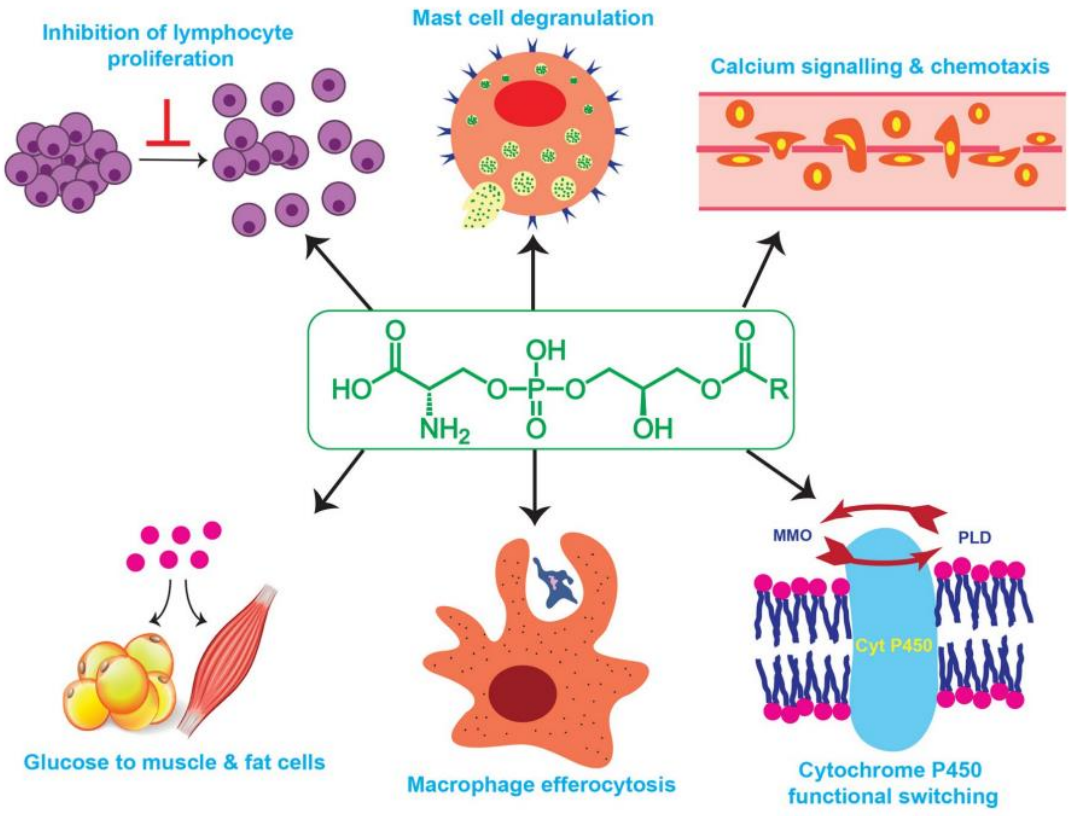


Figure 1.2 – Illustration of various cellular roles of lyso-PS[5] (adapted with permission from Shanbhag *et al*)

The degradative pathway of lyso-PS is characterized only in the brain tissue of mouse models thus far. The intracellular membrane protein ABHD12 was shown to play a key role in this process by Cravatt and coworkers. Although ABHD12 was first shown to have *in vitro* monoacylglycerol lipase activity[23,24], when the levels of various phospholipids were measured in ABHD12 +/+ and -/- mouse brains, and this revealed that the levels of Lyso-PS, and no other phospholipid or neutral lipid were elevated significantly in ABHD12 -/- mice. Thereby, ABHD12 was characterised as the principal lipase that metabolises Lyso-PSs in mammalian brain cells.[23] Moreover, it was shown that patients suffering from the human neurodegenerative disorder PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract) had mutations in their ABHD12 gene, which lead to loss of ABHD12 activity. Hence, loss of ABHD12 activity is understood to essentially cause PHARC. [25–27]

At a molecular level, ABHD12 cleaves the ester linkage at the sn-1 position, thus leading to the formation of glycerophosphoserine, and the corresponding free fatty acid.

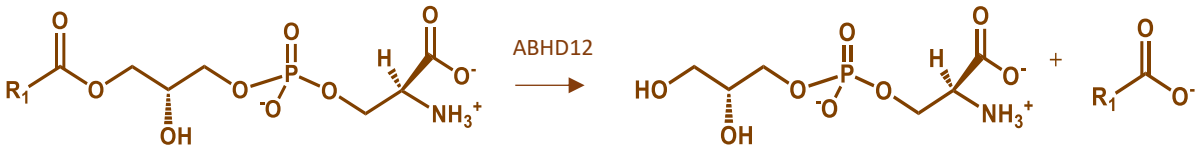


Figure 1.3 – Degradative reaction of lyso-PS

However, when lyso-PS levels were measured in ABHD12 +/+ and -/- mice in various tissues using untargeted lipidomics methods, it was found that while in brain, there is a significant increase in lyso-PS levels, there is no such difference in case of other tissues.[23,28,29] This suggests that while ABHD12 is the primary lyso-PS lipase in brain, other tissues have a different enzyme that is responsible for the lyso-PS lipase activity. Given the importance of Lyso-PS metabolism and the disease-linked nature of its key regulator in brain tissues, understanding Lyso-PS flux in other tissues (such as kidney, liver, lungs and heart) is of significance. This project aims to identify the principal Lyso-PS lipase(s) in peripheral mouse tissues.

Towards that aim, experiments were performed in the lab, by Theja Sajeevan, an alumnus of the lab, during her M.S. thesis. The soluble and membrane fractions of various tissues from ABHD12 +/+ and -/- mice were tested for lyso-PS lipase activity, and this data supports the hypothesis that ABHD12 is not the principal lyso-PS lipase in tissues other than brain. Moreover, it was revealed that only the membrane fractions of the tissues showed significant activity, which suggests that the lyso-PS lipases are all membrane proteins. ABHD12 belongs to a family of enzymes called Serine hydrolases. Serine hydrolases are a group of enzymes that have a conserved set of amino acid residues in their active site, namely the Ser-his-asp catalytic triad. As a result the active site serine is highly nucleophilic, and serine hydrolases function as adept nucleophile in various physiological processes. Experiments were done by Theja incubating the membrane fractions of various tissues with the serine hydrolase specific fluorophosphonate (F.P.) inhibitor[30]. Interestingly, all the fractions showed a loss of lyso-PS lipase activity upon F.P. incubation. Hence, this suggests that the Lyso-PS lipases were are interested in are membrane proteins that belong to the serine hydrolase family.

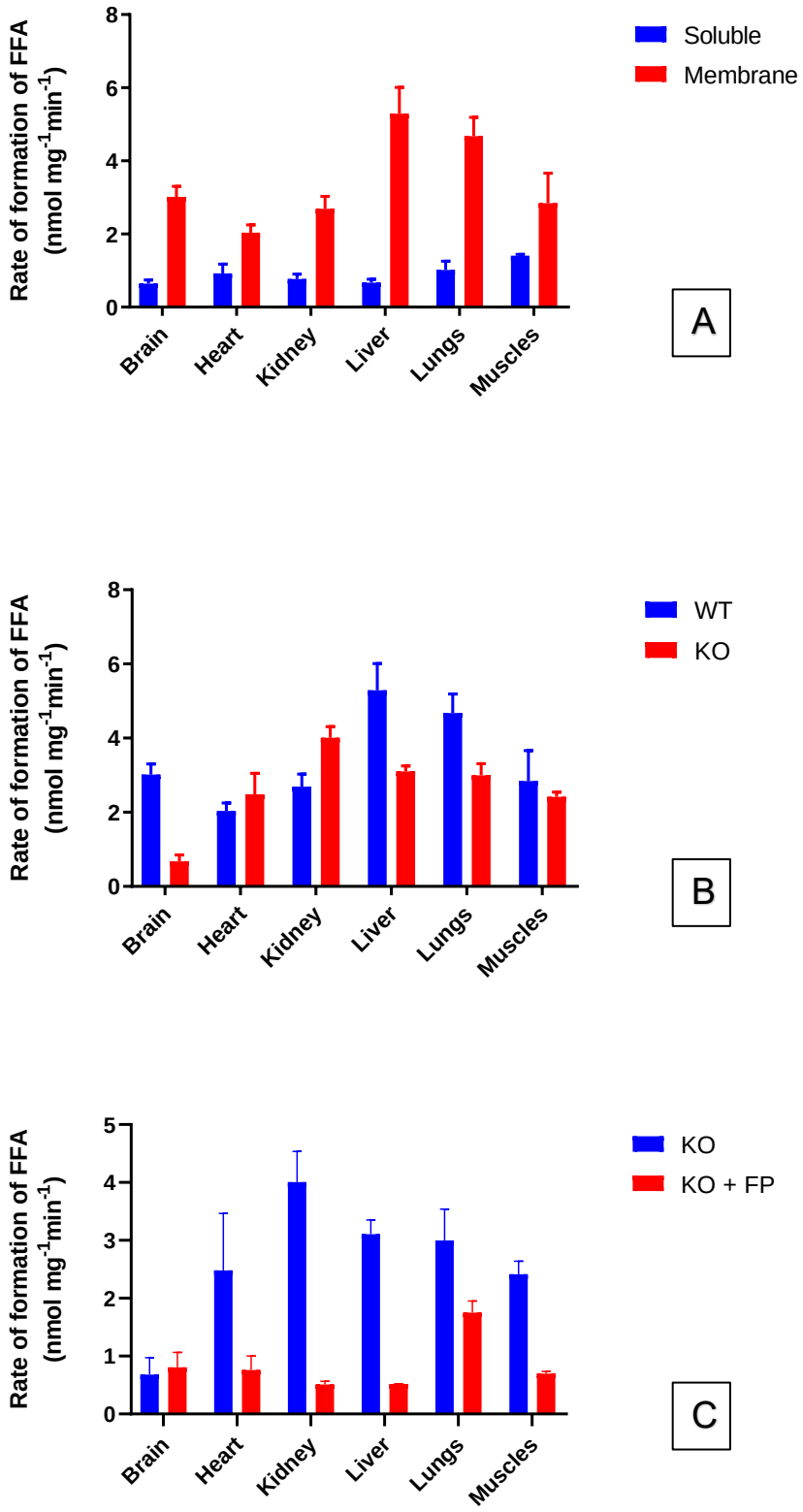


Figure 1.4 – Comparision of Lyso-PS lipase activities of various fractions A: Soluble and membrane fractions of various tissues; B: WT and KO. comparision; C: Effect of FP inhibitor on activity

Towards this end, serine hydrolases profiled in various mouse tissues[31] were studied proteomics to narrow down candidate enzymes. However, a large number of proteins were picked up, and it was not possible to identify a small number of candidate enzymes despite an extensive literature search. Hence, narrowing down the number of candidate enzymes is needed to make progress towards identifying Lyso-PS lipases.

Given this information, the following research plan has been employed:

1. Fractionation of membrane proteome:
   For a given tissue, we wish to separate its membrane proteins in order to segregate them into different fractions. This could be done using protein separation techniques such as sucrose density gradient centrifugation.[32]

2. Activity assays to determine fractions containing candidate lipases:
   Each fraction resulting from step 1 will be tested to see if they contain Lyso-PS lipase activity using a Mass Spectrometry-based substrate assay. Steps 1 and 2 will greatly reduce the number of proteins from which the lipase needs to be identified.

3. MS-based Activity-based protein profiling (ABPP) proteomics to separate candidate lipases from other proteins in the active fractions:
   Since we are looking to probe for serine hydrolases, we will employ fluorophosphonate probes in proteomics techniques to fish out serine hydrolases from the active fractions and predict their identities using M.S. methods.

4. Then, additional experiments can be planned to express the candidate lipases in cell models to gain more insight into their action *in vitro* and to target the candidate lipases *in vivo* using inhibitors to observe changes in metabolome.

Chapter 2 : Materials and methods

Table 2.1 Materials and resources table

Material/ Instrument/animal model/software	**Source**	**Identifier**
Chemical/Reagent		
15:0 Free Fatty acid	Sigma Life Science	P-6125
C17:1 Lyso-PS	Avanti Polar Lipids	858141
IGEPAL CA-630	Sigma Life Science	8896-50ML
FP-Rhodamine	Scripps Research	NA
Methanol (MS grade)	JT Baker	BAKR9830-03
Chloroform (MS grade)	Sigma	650498-1L
Isopropanol (MS grade)	Fisher Chemical	A4614
Anti-ABHD12	Abcam	Ab87048
BCA Reagents A and B	Thermo Fisher Scientific	-
Animal model		
Mouse: ABHD12 null	Scripps Research	NA
Mouse: C57BL/6J	The Jackson Laboratory	RRID: IMSR_JAX:000664
Software/Instrumentation		
Prism 8 (version 8.0e)	GraphPad	https://www.graphpad.com/scientific-software/prism/
ImageJ v1.52u	NIH	https://imagej.nih.gov/ij/
Agilent MassHunter Qualitative Analysis 10.0	Agilent Technologies	https://www.agilent.com/en/product/software-informatics/mass-spectrometry-software/data-analysis/qualitative-analysis

Agilent LC-QTOF 6545 B	Agilent Technologies	-
MS PCDL manager B.08.00	Agilent Technologies	-
Agilent Infinity 1290 II	Agilent Technologies	-
Beckmann ultra tubes	Beckmann Coulter	Ref# 357448
FPLC	GE AKTA Prime Plus	-

1. Mouse organ harvesting

All mouse studies have received formal approval from the Indian Institute of Science Education and Research, Pune – Institutional Animal Ethics Committee (IISER-P IAEC) (application nos: IISER_Pune IAEC/2016_02/01, and IISER_Pune IAEC/2019_2/07) constituted as per the guidelines and norms provided by the Committee for the Purpose of Control and Supervision of Experiments in Animals (CPCSEA), Government of India. 2-8 month old mice housed in the National Facility for Gene Function in Health and Disease (NFGFHD), IISER Pune, were used for all experiments. All mice had *ab libitum* access to food and water. Mice were anesthetised using carbon dioxide treatment, after which they were euthanised through cervical dislocation. Organs were then harvested one by one, stored in Eppendorf tubes and flash frozen using liquid nitrogen.

2. Homogenisation of tissues

Tissue was taken from -80°C freezer and cut into two halves on an ice-cold metal plate, and the halves were transferred into Tarsons 1.5 mL eppendorfs Tissue was thawed on ice, and 500µL of ice-cold DPBS was added into the eppendorfs. One scoop of beads (glass beads for brain; stainless steel beads for liver, heart and spleen; zirconium beads for other tissues) was added into each tube

Tubes were then kept in bead beater for 6 minutes (3 minutes x 2) at speed 8. 500µL DPBS was further added to the resultant homogenates. The homogenates were probe sonicated for 1 minute 30 seconds (with a 2 second gap every 2 second pulse) using a medium sized probe for cell lysis. Resulting lysates were centrifuged for 5 minutes at 1500 rcf to separate beads, debris and unlysed cells. The supernatant was transferred into new ultracentrifuge-compatible tubes and kept for ultracentrifugation at 1,00,000 rcf for 1 hour. This protocol was derived with slight modifications to earlier reported protocol.

3. Detergent solubilisation

After ultracentrifugation, the soluble fraction (supernatant) was aspirated, and ~950µL DPBS was added to make up the final volume ~1mL (950µL DPBS + membrane pellet volume). The pellet was resuspended through sonication at 60 amplitude for 5-10 pulses of 2 seconds each.

5 µL of IGEPAL CA-630 was added in 1 mL resuspended membrane , and the mixture was mixed using pipette till detergent is dissolved. The mixture was centrifuged at 21,100 rcf for 2 hours. The supernatant was transferred to a new Eppendorf, resulting in separated solubilised membrane Alternatively, membrane was resuspended in 500µL buffer, and 500µL of 1% detergent solution was added to make final concentration 0.5% (v/v).

4. BCA Protein estimation

Reagents A and B (Fischer Scientific Pierce BCA Assay Kit) were mixed in 50:1 ratio. 200µL of the prepared mixture was added to 20µL protein and incubated at 37°C for 25 minutes. Absorbance was then measured, and concentration was obtained using equation generated by absorbances of protein standards of known concentration.

5. Sucrose density gradient ultracentrifugation

Protocol:

- Solutions corresponding to different sucrose concentrations were prepared in separate 15 mL falcons with the following components:
- Usually 5-6 different concentrations of sucrose were prepared, ranging from 5-40 %

Component (stock solution)	Volume added (µL)	Final concentration of component
60% sucrose (w/v)	1000/x	x% (w/v)
60mM $CaCl_2$	50	3mM
50mM DTT	40	2mM
1M Tris	50	50mM
MilliQ	860-(1000/x)	-

Table 2.2- Components of x% sucrose in a sucrose gradient

- Prepared solutions were carefully added drop by drop in decreasing order of concentration using 1 mL pipette ensuring that there is minimal diffusion across fractions, into ultracentrifuge compatible 12 mL tubes (compatible with SW-41 Ti rolor of Beckmann-Coulter)
- Final sum of volume of sucrose containing fractions was kept 9 mL, on which 1 mL of solubilised membrane was added to make the total volume 10 mL (solubilised membrane must be in 50 mM tris buffer instead of DPBS or others)
- The tube was carefully transferred into the SW-41 swinging bucket rotor, and balance tube was also added when required
- The gradient was ultra-centrifuged at 4°C for 18 hours at 38,000 rcf under vacuum
- After centrifuge, fractions were slowly pipetted out as 1 mL volumes, and stored in new eppendorfs

6. Analysis of fractions using SDS PAGE and ABPP

Protocol:

Stacking and resolving gels were prepared in falcons by adding appropriate volumes of all required reagents except APS and TEMED. All gels used were 10% polyacrylamide gels

Component	**Volume added for 10% resolving gel**	**Volume added for stacking gel**
Milli Q	4 mL	3mL
Tris pH 8.8	2.5 mL	-
Tris pH 6.8	-	1.25 mL
30 % Acrylamide – bis acrylamide	3.3 mL	0.67 mL
10 % SDS	100µL	50µL
Ammonium persulphate	100µL	50µL
TEMED	10µL	5µL

Table 2.3 - Components of a 10% SDS PAGE gel

Using BIORAD gel apparatus, gels were kept in place and 1x running buffer was poured.

Component	**Amount**
Tris base	58g
Glycine	293g
dH_2O	Made upto 2L

Table 2.4 Components of 2L 10x Running buffer

Samples were loaded in the wells using a 20µL thin tip pipette, and the gel was run at 80 V for 20 minutes followed by 120 V afterward.

Sample preparation:

Brief theory – Activity based protein profiling is a technique used to understand a group of enzymes that are bound selectively by an activity based probe. In the context of this project, Fluorophosphonate (F.P.) probes are used, which selectively bind serine hydrolases. A fluorescent reporter tag is attached to the F.P. probe in the form of Rhodamine (Figure E). When proteins are incubated with FP-Rhodamine and visualised fluorescently, we are able to selectively visualise serine hydrolase enzymes.

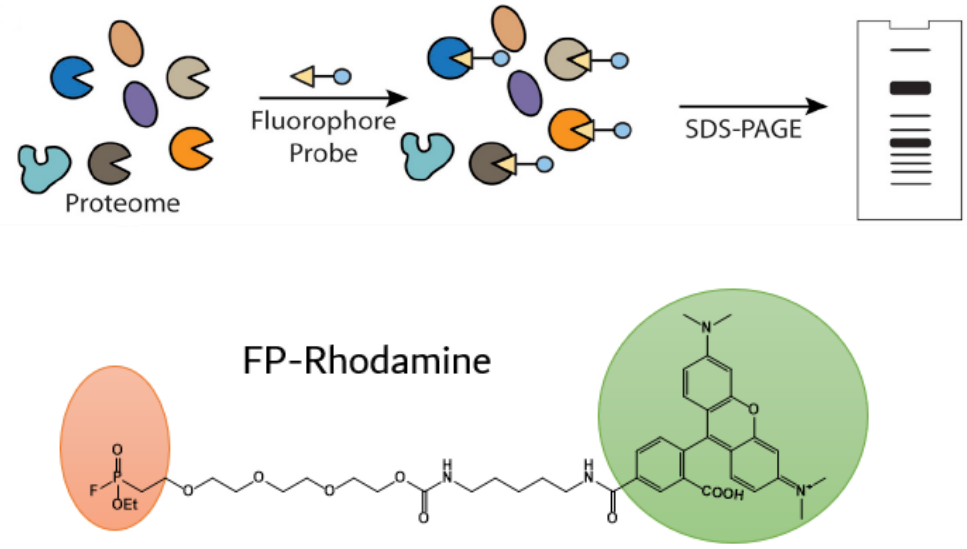


Figure 2.1 – Overview of Activity Based Protein Profiling (ABPP) for in-gel fluorescence using FP-Rhodamine probe (adapted from Fauscher *et al*)[33]

Protocol:

- Protein fractions were aliquoted into 50µL aliquots in eppendorf tubes., and FP-Rhodamine was added (1 µL of 100µM FP-Rhodamine- final concentration = 2µM)
- Samples were incubated for ABPP reaction at 37 °C at 1000 rpm mixing on a thermomixer for 45 minutes
- After reaction, 17µL 4x Loading dye was added to each tube, and the samples were kept at 95°C for 15 min

- ~40 µL sample was loaded in each well for a 10-well gel, or ~30µL for a 15 well gel

7. Anion exchange chromatography

Required before run: Start and elution buffer, column, milli Q water, protein, 2 mL Eppendorf tubes, FPLC

- Tubes A1 and A8 were washed with start buffer (50mM tris pH 8, 0.5% IGEPAL CA-630) with 10 mL / min flow rate for 2-3 min
- Tube B was washed with elution buffer (50mM tris pH 8, 0.5% IGEPAL CA-630, 1 M NaCl)
- Flowrate was lowered to 3 mL/min and insert column using correct technique
- Start buffer was passed through column until equilibration, which was monitored using U.V. and conductance measurements
- After equilibration, solubilised protein was passed using A8 tube ensuring that no air bubbles were allowed to enter the column
- If air bubbles were formed, the run was paused and milliQ was pipetted into the tube (kept inverted) till the bubbles were removed
- Flow through was collected during the protein binding process
- Once protein was bound, inlet was changed from A8 to A1, and gradient was set up (0 $\longrightarrow$ 1M NaCl, length = 40 mL)
- Elution was started, and protein fractions were collected in 2 mL Eppendorf tubes
- After elution, column was washed with 100 % B
- Column was then washed with milli Q, along with each of the tubings

8. Substrate assay

Protein samples to be assayed were prepared in 1 mg/mL dilutions. 17:1 Lyso PS (substrate) solution in $CHCl_3$ was made in a 5 mL glass vial such that it contains required amount of lipid for all the samples to be assayed (~5.5 µg per sample). $CHCl_3$ was completely dried from the aliquot using N_2 stream (2-3 minutes at full pressure). After drying, lipid was resuspended in DPBS (volume of DPBS added =

n*80 µL, n= number of samples). Solution was thoroughly vortexed till there are no visible deposits of Lyso-PS. Reaction was set up in Eppendorf tubes by adding 20 µL protein and 80 µL lipid for each sample, and the tubes were placed on thermomixer at 37°C at 1000 rpm mixing for 30 minutes.

Internal standard solution was prepared such that there is 200 µL $CHCl_3$, 100µL MeOH, and 0.5 nM of 15:0 free fatty acid (i.e. 0.2424 µL of 0.5 mg/mL FFA solution) per sample. Extraction was then done using Folch method.[34] After reaction completion, 300 µL of extraction mixture was added to each tube. The mixtures were then vortexed and spun at 2300 x g for 10 minutes using bench top centrifuge. After spin, 150 µL of lower layer was transferred into new tube for each sample. Samples were dried using N_2 stream, and each sample was resuspended in 300µL $CHCl_3$. 80µL from the 300µL was added into fresh insert along with 40 µL MeOH, and run in LC-QTOF.

Method was established with modifications to previously reported protocol.[35]LC separation was done using Gemini 5U C18 column (Phenomenex, 5µm, 50x4.6mm) coupled to a C18 Gemini guard column (Phenomenex, 4x3 mm, Phenomenex security cartridge). Gradient used- Solvent A= 95:5 Water:Methanol ; Solvent B= 60:35:5 IPA:Methanol:Water. Gradient was first equilibrated with A for 1.5 minutes, followed by linear increase on B% from 0 to 100 till 6 minutes. 100% B was passed through column till 11.5 minutes, after which gradient was re-equilibrated with 100% A. Flowrate used was 0.5 mL/min.

Samples were run in negative mode, using zero collision energy. The fragmentor voltage and ion source voltage were -150 and -4000 V respectively. The drying gas temperature was 320°C and flowrate was 10 L/min. Nebulizer pressure was 50 psi for these experiments.

Analysis was done similar to reported protocol[35], using the ratio of area under the curve obtained of internal standard and product fatty acid.

9. Lipid extraction from tissues

Portion of a tissue was homogenised using bead homogenisation in 500 µL DPBS. 500µL more was added post homogenisation, and the tube was spun at 1500g for 5

minutes. 1 mL supernatant (homogenate) was taken and transferred into a 5 mL glass vial. 2:1 $CHCl_3$: MeOH containing 1 nmol internal standard was added (3 mL volume). The vial was vortexed thoroughly and spun at 2000g for 15 minutes. 1.5mL from $CHCl_3$ layer (lower layer) was transferred to a new 5 mL glass vial. In old vial, 50 µL HCOOH was added, followed by addition of 2 mL $CHCl_3$. Vial was vortexed, and another spin was given at 2000g for 15 minutes. 2 mL $CHCl_3$ layer was transferred post spin into the new vial containing 1.5 mL transferred layer from previous spin. Vial was dried under N_2 spin, and 1 mL $CHCl_3$ was added for resuspension, and the resuspended lipids were transferred to new 2 mL glass vial. The 1 mL $CHCl_3$ was dried, and 200 µL 2:1 $CHCl_3$: MeOH was added for resuspension. 100 µL of resuspended lipids was transferred into fresh inserts, and the samples were run in LC-QTOF.

L.C. separation was done using Gemini 5U C18 column (Phenomenex, 5µm, 50x4.6mm) coupled to a Gemini guard column (Phenomenex, 4x3 mm, Phenomenex security cartridge). Lipids were ionised in the negative ionisation mode. Solvent systems used were: solvent A: 95:5 (v/v) H2O: methanol (MeOH) + 0.1% ammonium hydroxide; and solvent B: 60:35:5 (v/v) Isopropanol: MeOH: H2O + 0.1% ammonium hydroxide using an established L.C. method on an dual AJS (Agilent Jet Stream) ESI source. The [M–H]– of the lipid standard and m/z values of the endogenous lipids were comparatively assessed by LC-MS/MS analysis. The total scan time for both the MS1 and MS2 spectra was 3.016 s, and the collision energy (volts) of 20 and 30 for lyso-PS. The declustering potential and ion source voltage were set at –100 and –4000 volts respectively. The drying gas temperature was 320 °C, drying gas flow rate was 10 L/min, and the nebuliser (ion source gas) pressure was 45 psi for this lipid fragmentation study. For analysis lyso-PS library was employed in the form of a Personal Compound Database Library (PCDL), and the peaks were validated based on relative retention times and fragments obtained. The lipid levels were quantified using area under the curve relative to that of internal standard.

10. Western Blotting

Transfer buffer was prepared by mixing 200 mL 10x Transfer buffer, 200 mL MeOH and 1600 mL dH_2O. The buffer was cooled at 4°C. Overnight transfer was set up at

4°C at 80 mA. After transfer, blot was removed from holder, and placed in ponceau solution for 5 minutes incubation.

Composition	**Amount**
Tris base	58g
Glycine	293g
dH_2O	Made upto 2L

Table 2.5- Components for preparation of 2L 10x Transfer buffer

For 1x transfer buffer preparation, 10x was diluted by ten times with 8:1 dH2O:MeOH. Ponceau blot was imaged, and ponceau was washed off with PBST (0.1% Tween-20). Blot was then placed in 5% milk solution for 1 hour on rocker for blocking. Primary antibody (Abcam Anti-ABHD12) was prepared in 1:3000 dilution, and added to the blot for primary incubation at 4°C for 12 hours. After primary antibody incubation, the blot was removed and three washes were given with PBST at 5 minute intervals. Apppropriate secondary antibody (Anti-rabbit IgG HRP) was diluted 1:10000 of milk, and blot was placed in the solution using empty tip box, and kept for 1 hour. Blot was then removed and three PBST washes were given at 5 minute intervals. Luminol and Hydrogen peroxide reagents were mixed in 2mL Eppendorf (1 mL volume each), and added on to the blot such that the mixture is in contact with the entire side of the blot. The blot was then imaged using gel doc.

Chapter 3 : Results and discussion

3.1 Protein fractionation

With the purpose of dividing the membrane proteins of various tissues into fractions with different protein profiles, two techniques were mainly tried at a variety of settings. Mainly, sucrose density gradient ultracentrifugation was employed which is a method that separates proteins based on mainly their size and density. This technique was previously reported to have given different fractions for proteins from brain tissue[32]. Given this literature precedence, it was used to try to fractionate proteins from other tissues from mice. In attempts to get better fractionation, reported protocols were changed in various aspects such as sucrose distribution across gradient, centrifugation conditions, homogenization conditions, and nature of load protein. As an alternate method, anion exchange chromatography was tried for kidney, liver and brain tissues. The obtained results have been discussed below.

3.1.1 Sucrose density gradient ultracentrifugation

Membrane proteins from tissues of interest were extracted using homogenization and ultracentriugation procedures. Proteins were then solubilised with detergent (IGEPAL CA-630 at 0.5% v/v) and loaded onto a prepared sucrose density gradient. The protein separation was visualised by running resultant sucrose fractions on a 10% SDS-PAGE gel after serine hydrolase-specific fluorescent labelling using FP-Rhodamine. According to reported protocol, gradient of sucrose concentrations from 0-40% was recommended.[32] Initial separation protocol for brain tissue was developed in the lab by Arnab, and was subsequently reproduced. A 10 mL gradient volume, with sucrose concentrations going from 0 to 25% in a non-linear fashion was used for brain, run at 38,000 x g for 18 h at 4°C, vacuum (Figure 3.1A). Using BCA protein estimation, load protein concentration was kept near 5 mg/mL.

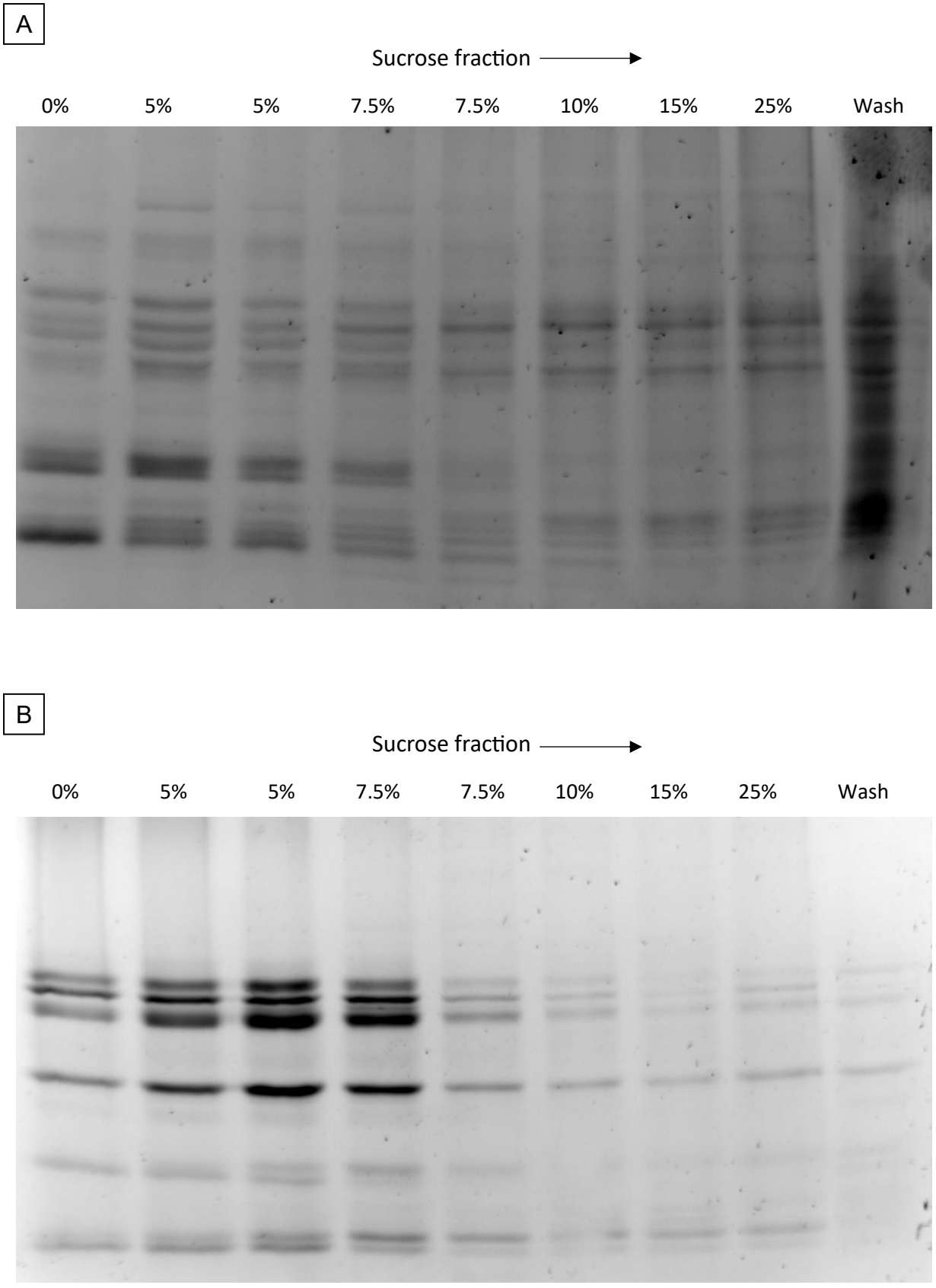


Figure 3.1 – Serine hydrolase protein profiles of sucrose fractions collected after ultracentrifugation at 38,000 x g for 18 hours, visualised using 10% SDS PAGE gel post ABPP (2µM FP-Rhodamine). A: Brain tissue; B: Liver tissue

In brain, protein bands of significant intensity were observed in most fractions, and differential localisation of multiple proteins can be seen i.e. there are bands of protein present in specific fractions that are absent in other fractions, indicating decent separation. However, there are many bands that are present in all fractions at similar intensities, indicating scope for development.

The same gradient was tested for liver solubilised membrane (Figure 3.1B). In case of liver, it can be seen that there are only few bands that can be seen at very high intensities. Initially, it was hypothesised that this is because of protein aggregation or improper homogenisation of liver tissue, which is leading to the extraction of only few proteins. Hence, homogenisation time was increased to nine minutes, along with increase in solvent volume used for homogenisation and decrease in amount of liver used. However, no significant change was seen in protein profiles, and only few bands are visible (Figure 3.2A). It was then hypothesised that the intense bands correspond to carboxylesterase enzymes that are highly expressed in liver which require a very low exposure time to be captured fluorescently, and this makes other proteins invisible.

Observation was made from figure 3.1B that most of the protein band intensity comes from the initial fractions of 0-7.5% sucrose. Hence, gradient distributions were tried with more fractions having lower sucrose concentrations, with fractions at intermediate concentrations of 1 and 3%, as opposed to just 0 and 5% concentrations used previously. During fraction collection, the intermediate fractions between 0 and 5% were collected as 500µL fractions as opposed to 1mL fractions to further improve resolution of separation visualisation. Two such gradients were tried with slightly different sucrose distributions, and it was seen that protein intensity was coming from more number of fractions compared to previous experiments with liver. However, no visible separation was seen, as most of the bands were present in all fractions, and no band was present differentially in a few particular fractions (Figure 3.2B and 3.2C).

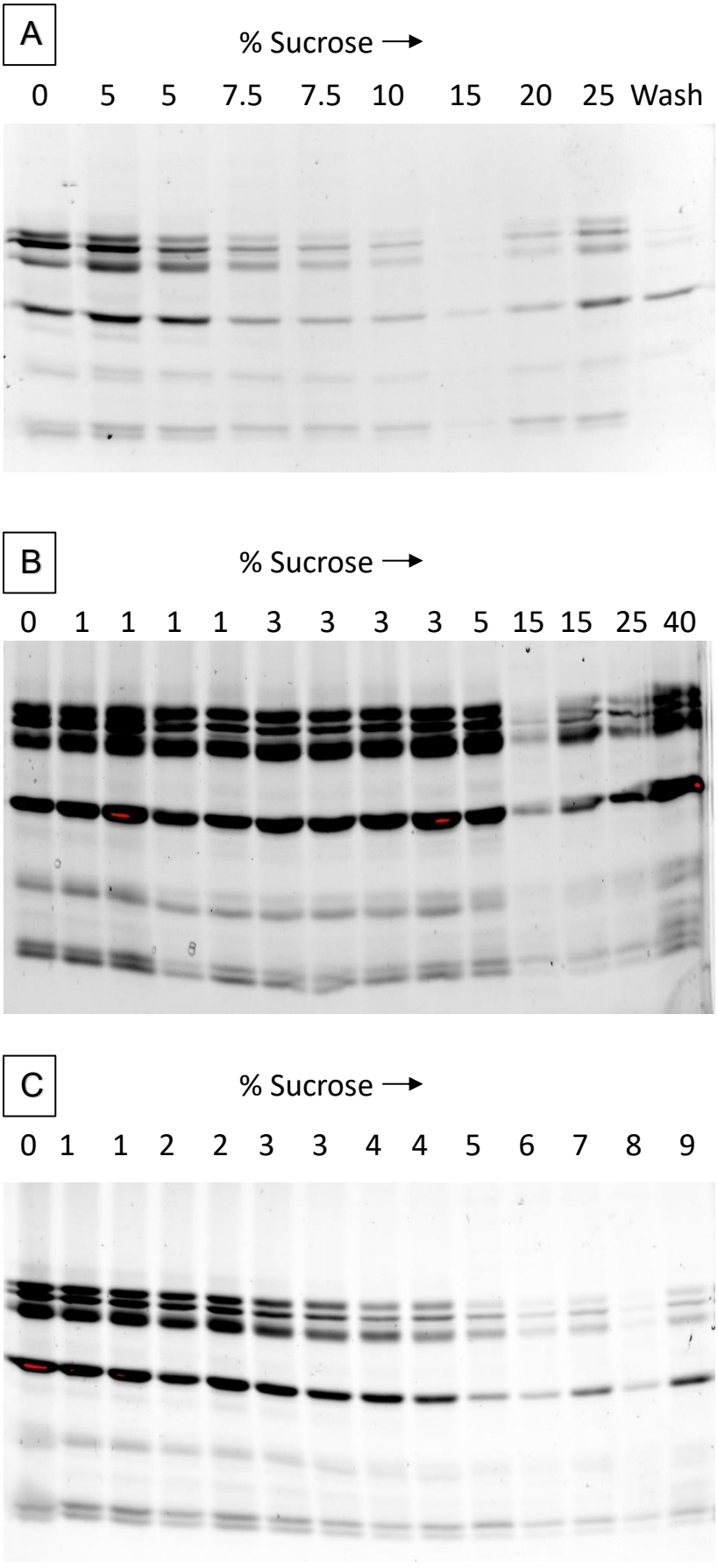


Figure 3.2

Liver solubilized membrane: Serine hydrolase protein profiles of sucrose fractions collected after separation at 38,000 x g for 18 hours, visualized using 10% SDS PAGE gel post ABPP (2μM FP-Rhodamine). Deviations from starting settings are:

A: Changed tissue homogenization conditions – increased time of ultracentrifugation, increased amount of starting protein

B: Gradient prepared to have more volume of low sucrose concentrations

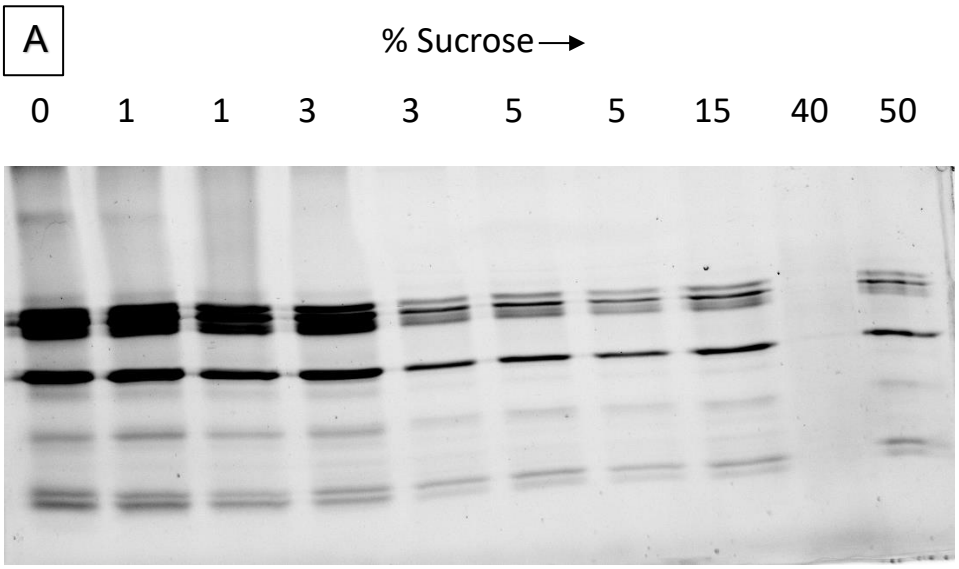
C: Gradient prepared to only have low sucrose concentrations

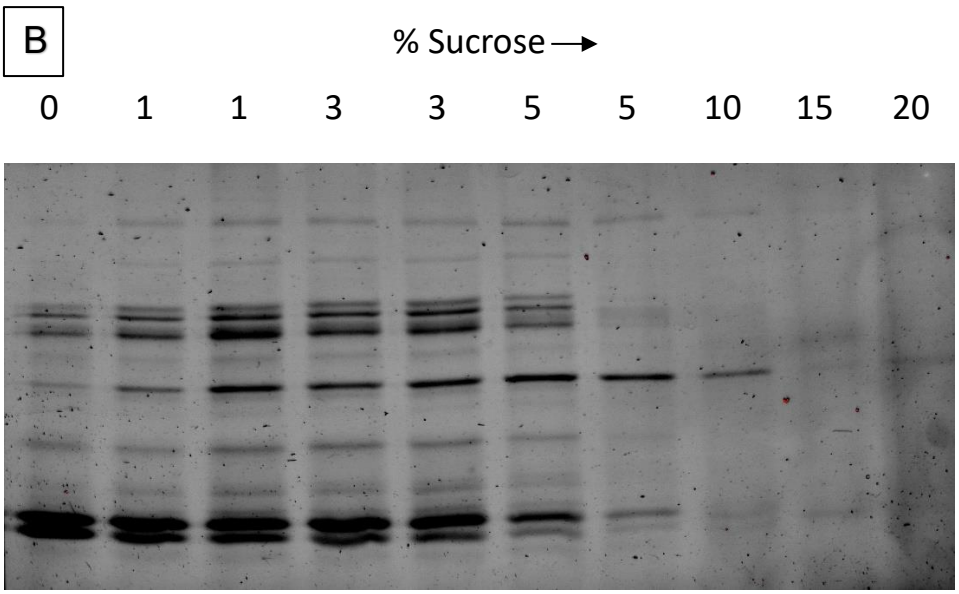
In an effort to improve separation, an alternate centrifugation protocol was tried, involving a spin at 1,00,000x g for two hours, which was reported in literature for brain.[32] However, the obtained result (figure 3.3A) was similar to the previous results with liver, and most of the band intensity was observed in the initial bands with no differential localisation of bands across fractions.

Considering that the initial fractions have the most protein, one such fraction (a 5% fraction from figure 3.1B) was taken and loaded onto another sucrose density gradient for further separation. Since it was a 5% fraction being loaded, most of the fractions in the gradient were kept ≤ 5%, and the centrifugation setting was kept as 1,00,000xg for 2 hours. Interestingly, it was observed (figure 3.3B) that there were differential intensities of bands across fractions. The upper bands were present more in the fractions with higher sucrose fractions, and the lower bands were present more in the initial fractions, indicating a better separation.

A similar gradient was tried with a different load – a 1% fraction from figure 3.2B (figure 3.3C). However, this did not give any differential separation, maybe because the density of the proteins loaded was already too low to pass through the gradient. Fractionation was also tried for kidney proteins after membrane separation and solubilisation. Solubilised protein was loaded onto the same gradient distribution developed for brain at 38,000 x g for 18 hours. Resultant fractions (figure 3.4A) showed differential localisation of few bands, but most of the protein intensity was coming from the initial fractions, as observed for liver. More number of bands were visible as compared to liver, possibly because there are no proteins with high relative expression as was likely the case with carboxylesterases in liver.

Subsequently, the 5% fraction was taken and loaded onto another gradient with containing concentrations between 0 and 5%, and run at 1,00,000 x g for 2 hours (figure 3.4B). This revealed enrichment of few protein bands in the 3% fractions, while remaining bands were present in all fractions at similar intensity. The experiments with liver and kidney suggest that a double centrifugation, i.e. initial fractionation of solubilised membrane followed by further fractionation of 5% fraction, was required for better protein enrichment.





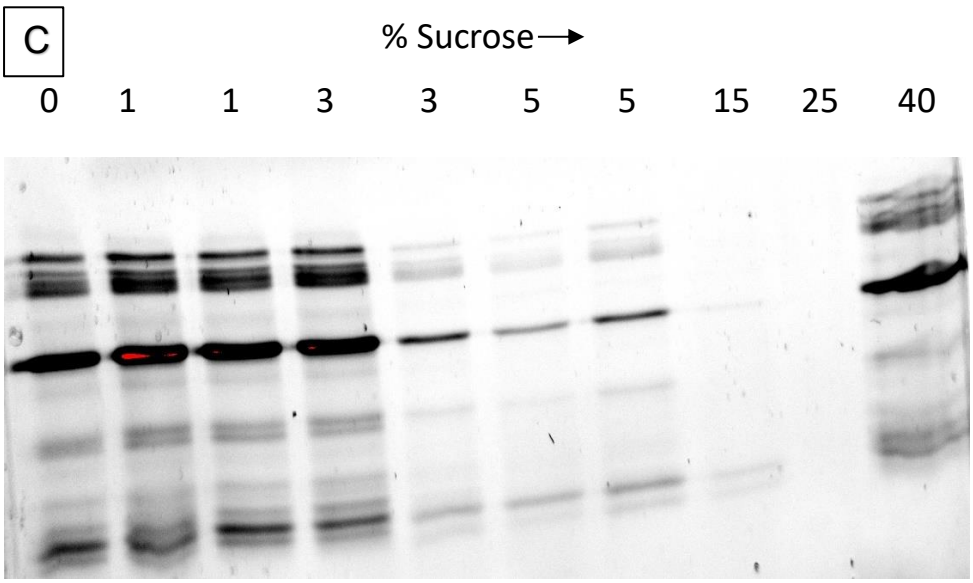


Figure 3.3

Serine hydrolase protein profiles of sucrose fractions collected after separation at 1,00,000 x g for 2 hours, visualized using 10% SDS PAGE gel post ABPP (2µM FP-Rhodamine).

A: Loaded protein: Liver solubilized membrane

B: Loaded protein: 5% fraction from figure 2 A

C: Loaded protein: 1% fraction from figure 2 B

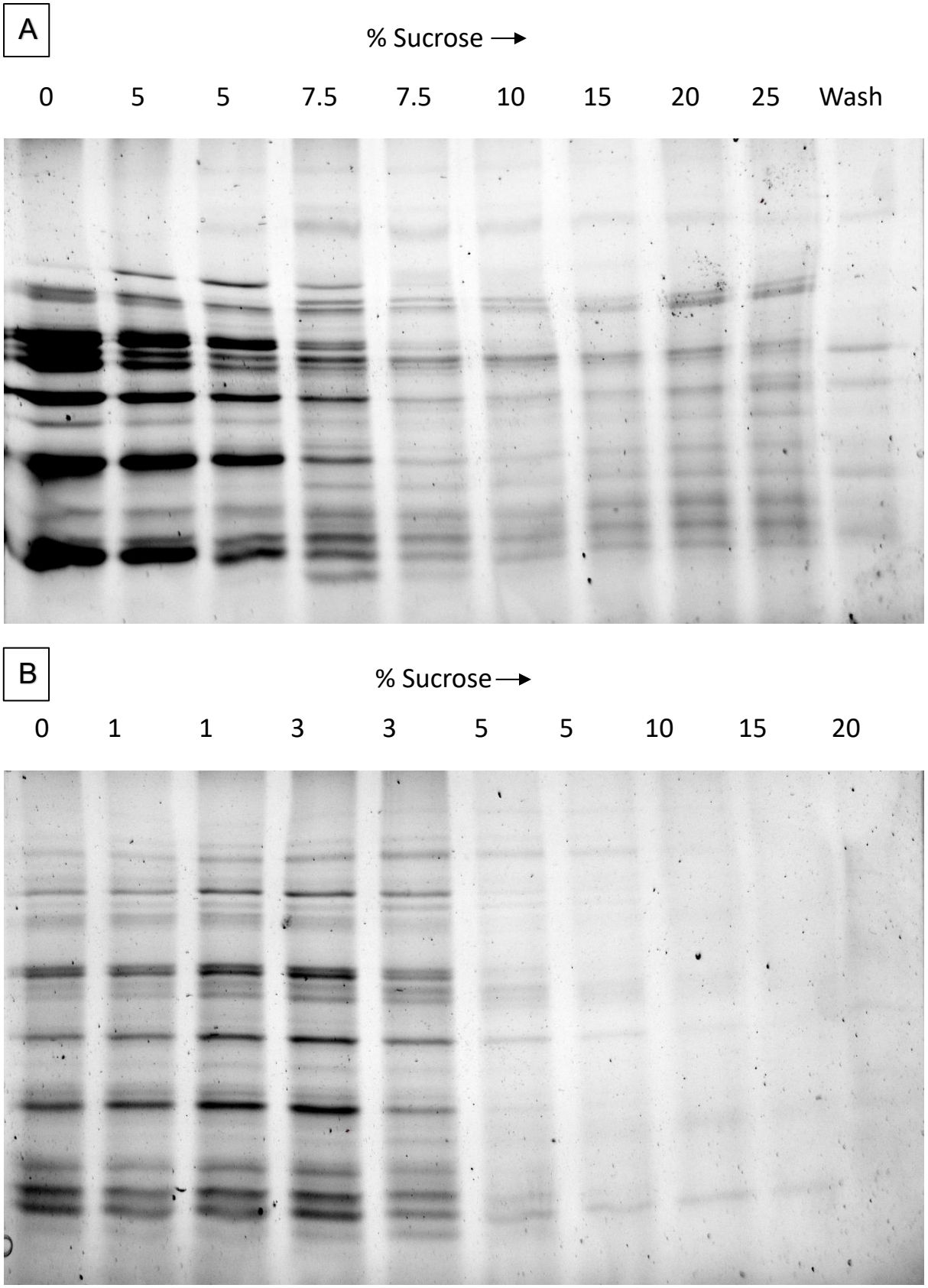


Figure 3.4 – Kidney tissue: Serine hydrolase protein profiles of sucrose fractions collected after ultracentrifugation, visualised using 10% SDS PAGE gel post ABPP (2μM FP-Rhodamine). A: Loaded protein= kidney solubilized membrane, run settings: 38,000 x g for 18h; B: Loaded protein= 5% fraction from figure 4A, run settings: 1,00,000 x g for 2h

Detergent screening:

Solubilisation of membrane proteins using detergent was required to reduce protein aggregation and lipid interference that could affect subsequent steps. For this purpose, two commercially available detergents were tested at different concentrations to identify the best detergent and concentration for membrane solubilisation (Figure 3.5).

While both detergents gave similar results, it was seen that IGEPAL CA-630 at concentration was slightly better in the solubilisation of few bands especially in liver. Also, it was observed that 1% v/v detergent concentration led to loss of protein activity as compared to 0.5%. Hence, IGEPAL CA-630 at 0.5% v/v was used for all fractionation attempts.

3.1.2 Anion exchange chromatography

As an alternate method to fractionate proteins, anion exchange chromatography was employed. As an initial test, kidney solubilised membrane (~15 mg in 10 mL) was passed through the column, and an elution gradient of 0 to 1M NaCl (25mL length) was set up. Fractions were collected in 3mL volumes and run using SDS PAGE (Figure 3.6A). In both the Coomassie and the ABPP gel, it is evident that there is protein separation across fractions, as there are significantly different protein profiles in each of the fractions. Protein elution was monitored using U.V. absorbance and conductance measurements (figure 3.6C).

To further develop the method to obtain more number of distinct fractions, the elution gradient length was increased to 40 mL and fraction size was reduced to 2mL, so as to obtain 20 fractions during elution, as opposed to 8 obtained using starting settings. This was done for liver solubilised membrane (figure 3.7), as well as kidney and brain solubilised membrane (figure 3.8). In each case, a coomassie stained gel was used to determine the fractions with a significant amount of protein, and the ABPP gel was performed for those fractions to analyse further. As expected, more number of distinct fractions were obtained, indicating much better protein fractionation as compared to gradient ultracentrifugation used previously.

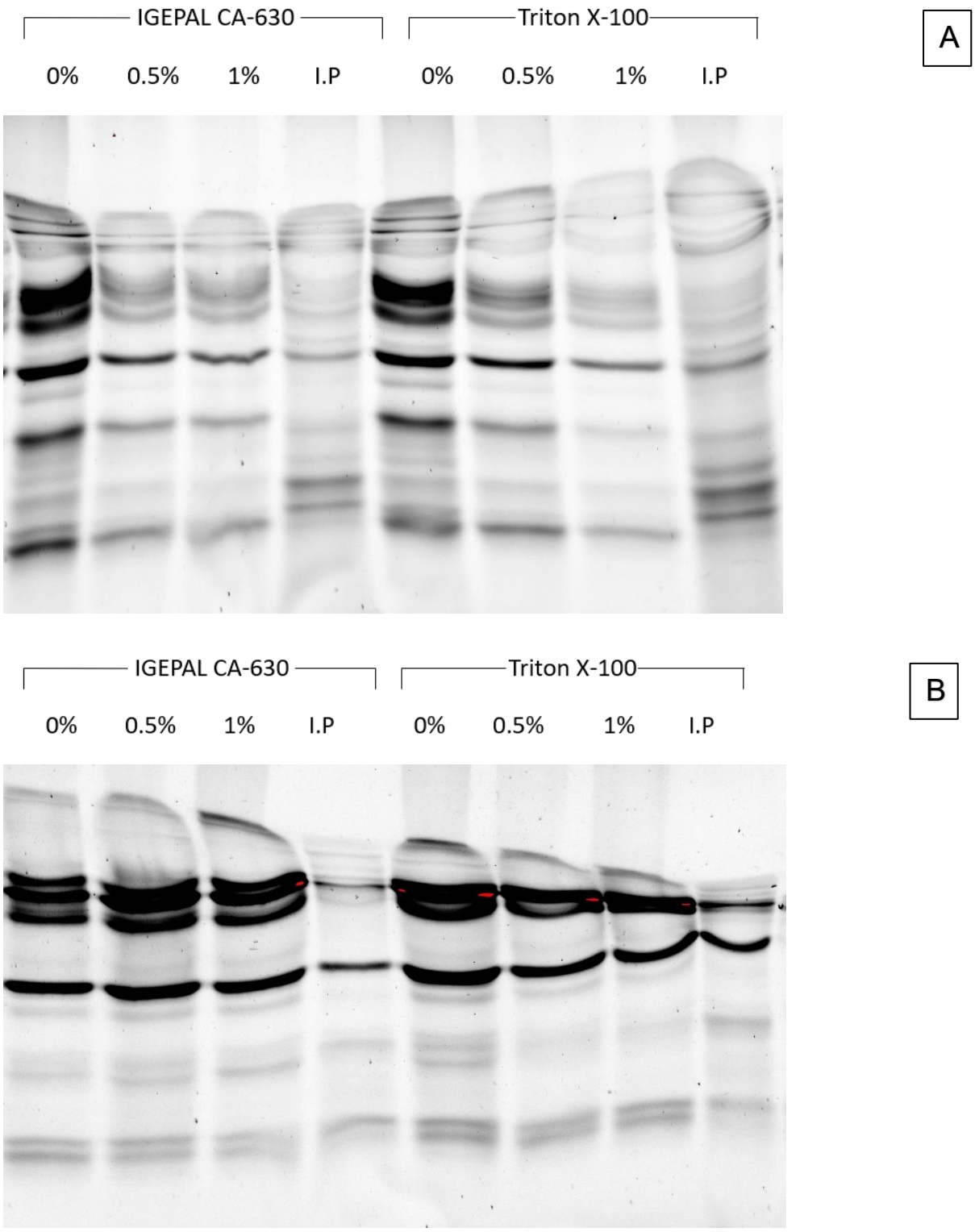
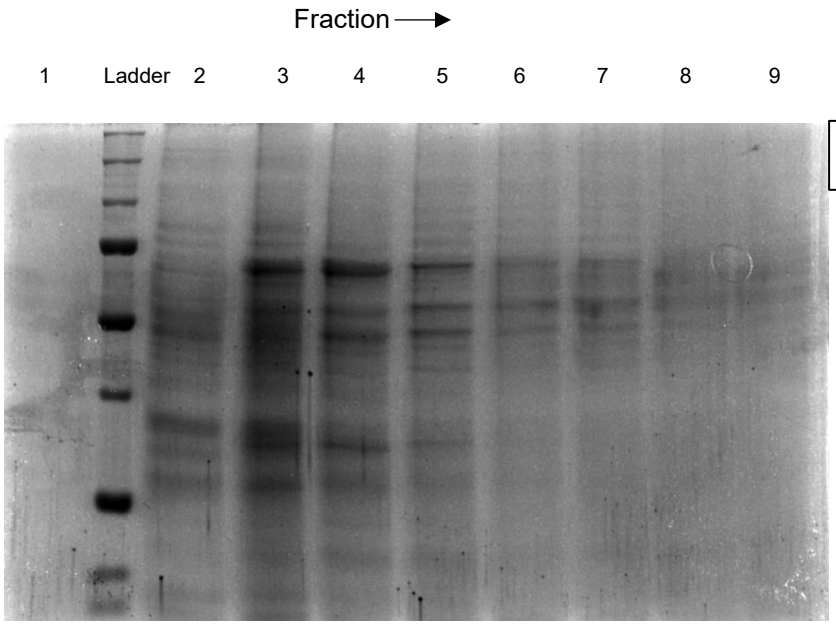
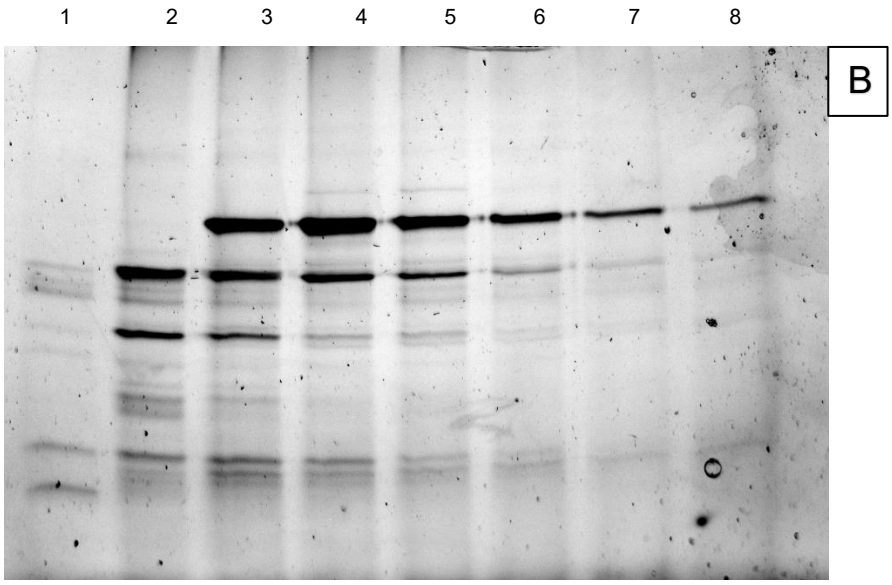


Figure 3.5 –Detergent concentration screening for membrane solubilisation, visualised using 10% SDS PAGE gel post ABPP (2μM FP-Rhodamine). A: kidney tissue; B: Liver tissue; I.P = Insoluble proteins, i.e. proteins that pelleted during solubilisation at 0.5% detergent conc.





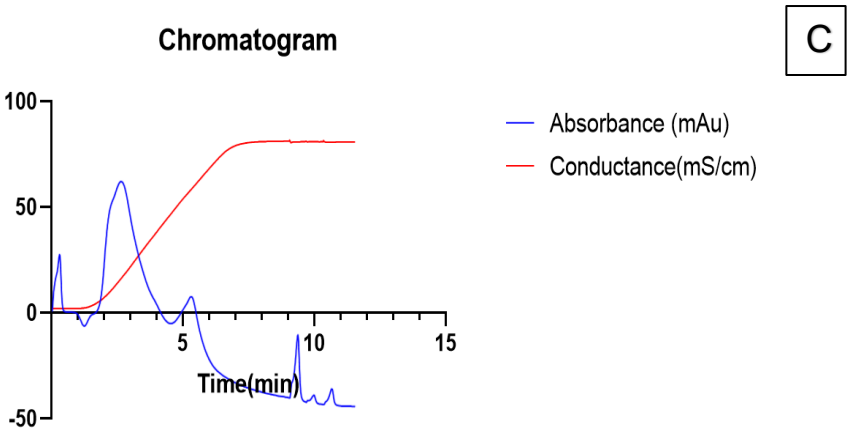


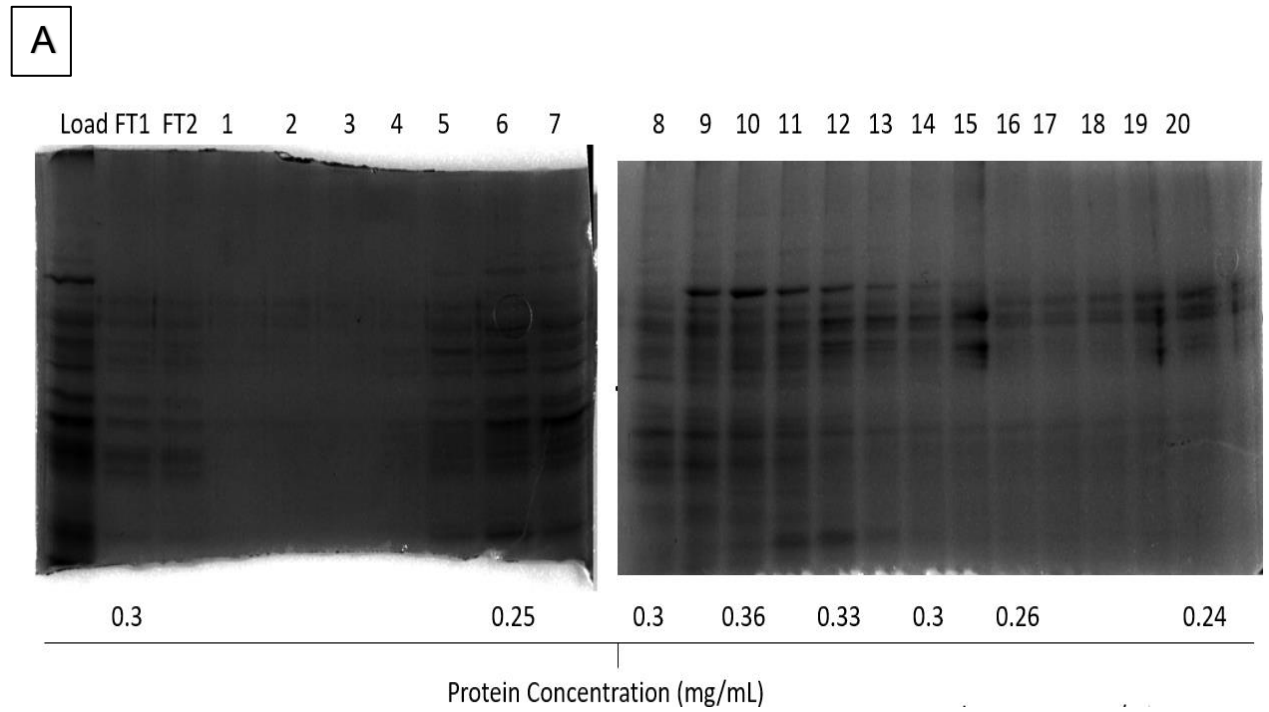
Figure 3.6

Kidney solubilized membrane fractionated using anion exchange chromatography. Elution settings: 0 to 1M NaCl, length = 25 mL, fraction size = 3 mL, flow rate= 5mL/min

A: Coomassie stained proteins from resultant fractions (10% SDS PAGE gel)

B: ABPP gel image of proteins from resultant fractions (2μM FP-Rhodamine, 10% SDS PAGE gel)

C: Conductance and absorbance at 280nm during protein elution



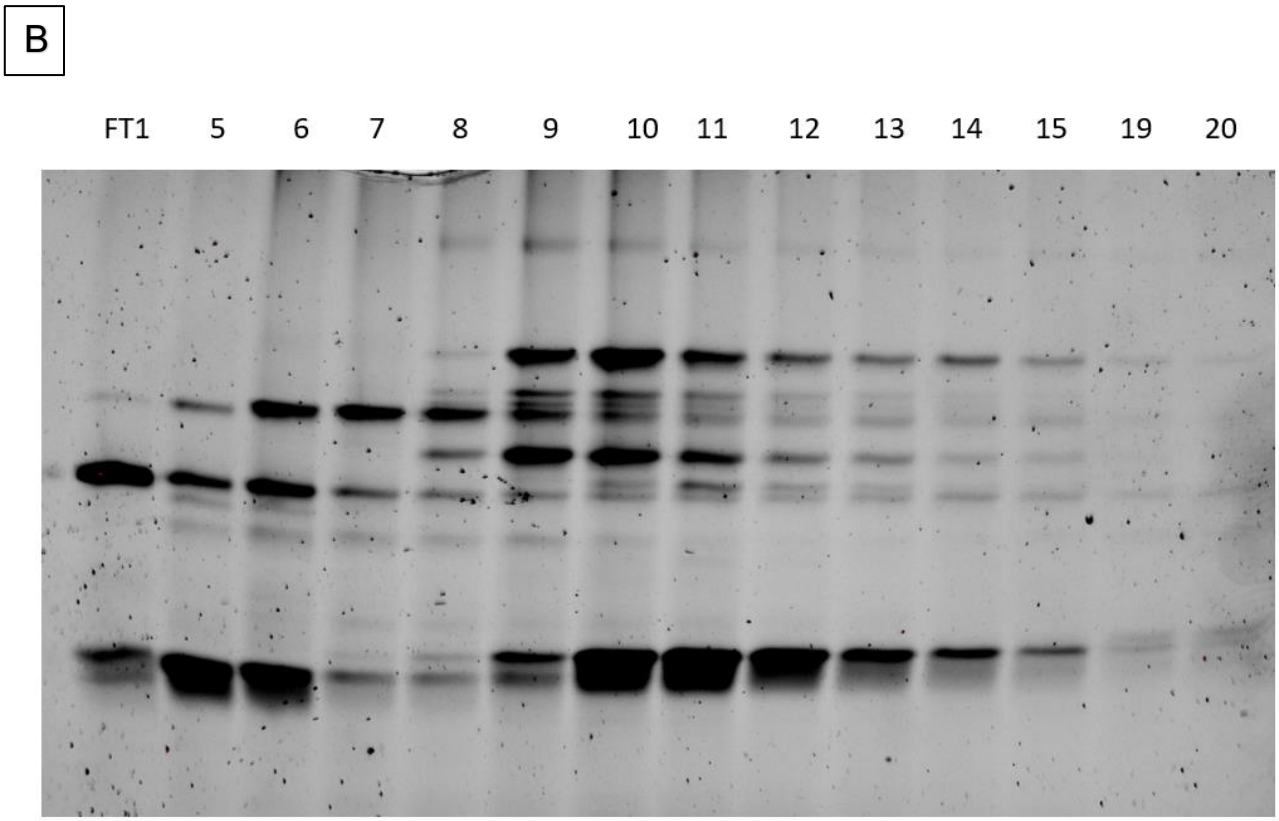


Figure 3.7 – Liver solubilised membrane fractionated using anion exchange chromatography. Elution settings: 0 to 1M NaCl, length = 40 mL, fraction size = 2 mL, flow rate= 3 mL/min. A: Coomassie gel (FT= Flowthrough); B: ABPP gel of protein rich fractions

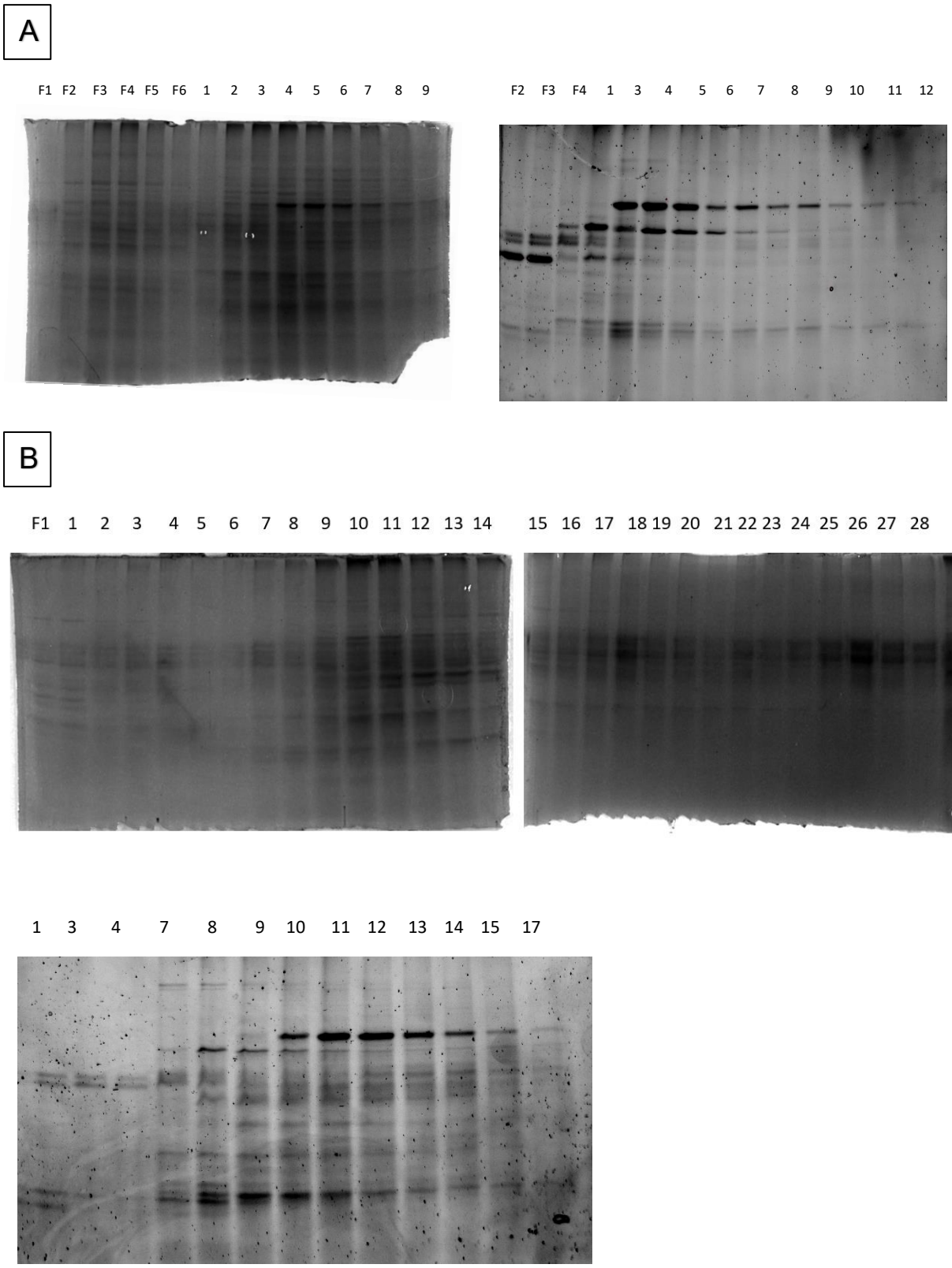


Figure 3.8 – Coomassie and ABPP gels of solubilised membrane fractionated using anion exchange chromatography. Elution settings: 0 to 1M NaCl, length = 40 mL, fraction size = 2 mL, flow rate= 3 mL/min. A: Kidney tissue; B:Brain tissue

3.2 Substrate assays

As a next step, activity assays were planned for the fractions obtained using anion exchange chromatography. Initial control experiments were performed to confirm the successful application of the assay, and to test the performance of a new mass spectrometer. Activity was measured for ABHD12 overexpressed in HEK cells (transfected by Arnab), and 3-4 fold change was observed between mock and overexpressed protein, with PBS showing minimal activity (figure 3.9A).

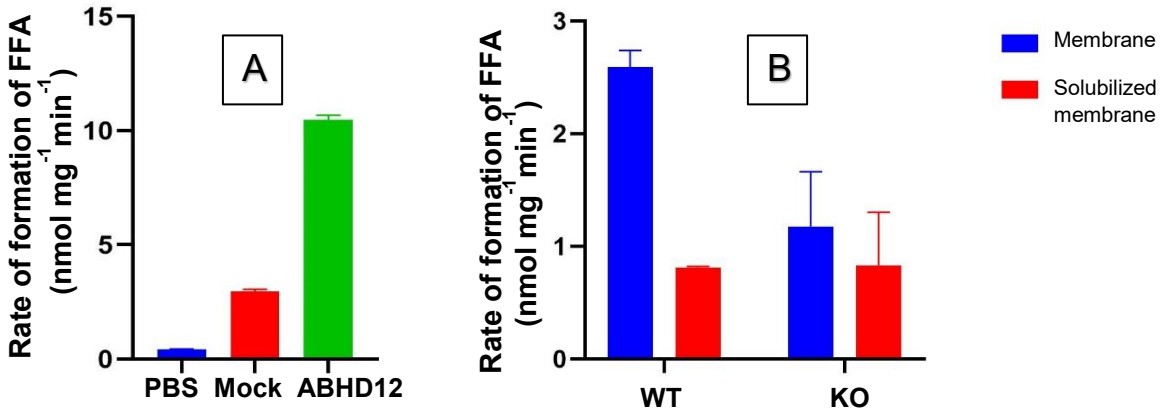


Figure 3.9 – Activity assays measuring lyso-PS lipase activity. A: Comparison of mock and transfected ABHD12; B: Comparison of ABHD 12 W.T. and K.O. fractions in brain, Sol mem = membrane solubilised by 0.5% IGEPAL CA-630. n=2 (technical replicates), central tendency measure: Standard deviation

Assays were also done for W.T. and ABHD12 KO mice for proteins of mouse brain membranes, and the proteins solubilised by detergent (figure 3.9B). It can be clearly seen that a 2-3 fold change exists between W.T. and K.O. membrane fractions, which is close to the literature reported value[23]. However, the detergent solubilised membrane doesn't have significant activity, which suggests that ABHD12 is not being solubilised by detergent in the applied solubilisation protocol. It is also possible that the protein was successfully solubilised but the detergent treatment lead to loss of enzyme activity. It was concluded from these control experiments that the assay is being applied correctly, and the method developed for the assay in the mass spectrometer is performing in an acceptable manner.

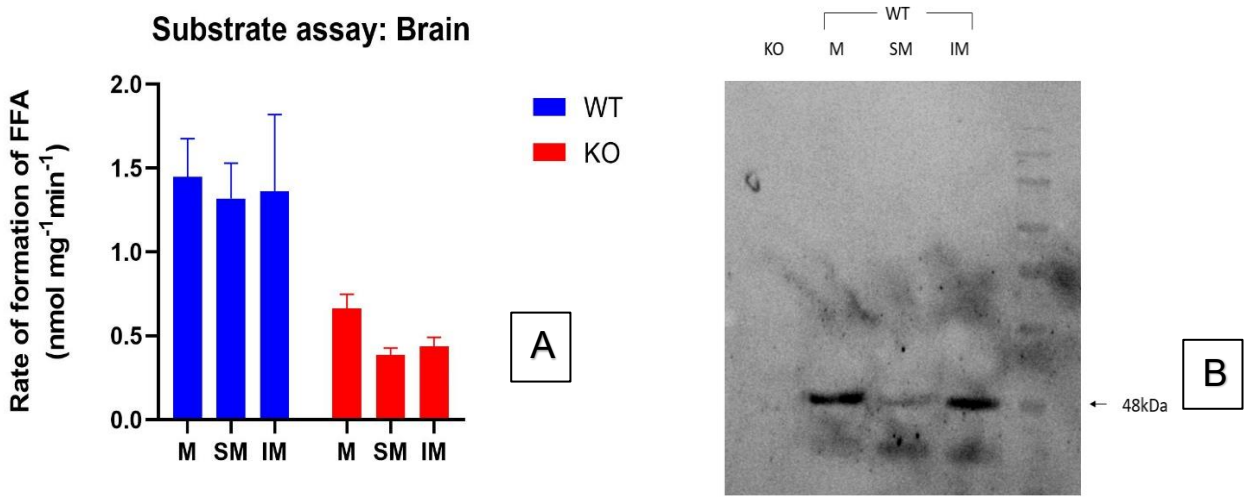


Figure 3.10 – A: Activity comparison of fractions derived from detergent treatment (M=membrane before detergent treatment, SM= membrane proteins solubilised by detergent, IM= proteins insoluble despite detergent treatment). n=3 (biological replicates), central tendency : standard deviation. B: Western blot for ABHD12 of relevant fractions

Following the initial experiments, activity comparisons were done to understand the translation of activity from membrane fraction upon detergent treatment. The detergent incubation time for the experiment was kept 1 hour with gentle mixing at 4°C. In brain (figure 3.10). Firstly, it can be seen that K.O. fractions have 2-3 fold lesser activity than corresponding W.T. fractions, which supports the idea that ABHD12 is the principal lyso-PS lipase in brain. It can also be observed that both the W.T. solubilised membrane and insoluble membrane showed activity similar to the membrane, suggesting that ABHD12 was half solubilised by detergent, while the other half was insoluble. However, upon doing a western blot (figure 3.10B), it was found that ABHD12 is present in the insoluble fraction in much higher amount compared to the solubilised membrane. This suggests that the solubilised fraction is showing activity due to the *in vitro* activity of another enzyme, or because the presence of detergent affects antibody binding to ABHD12.

The same experiment was then done for liver and kidney. For these tissues, unlike brain, it can be seen that K.O. fractions have similar activity as their corresponding W.T. fractions, which supports the idea that ABHD12 plays a minimal role in the lyso-PS hydrolysis activity in these tissues.

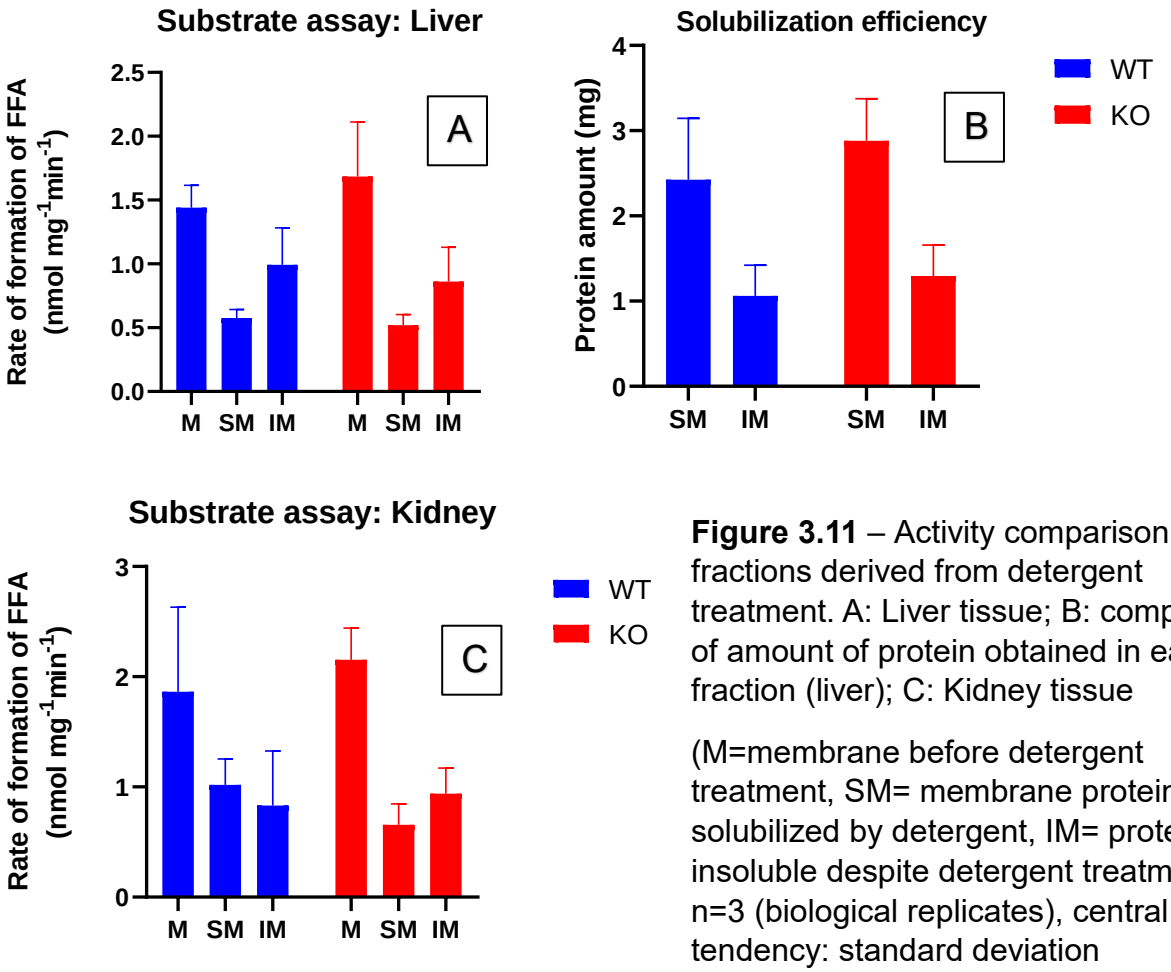


Figure 3.11 – Activity comparison of fractions derived from detergent treatment. A: Liver tissue; B: comparison of amount of protein obtained in each fraction (liver); C: Kidney tissue

(M=membrane before detergent treatment, SM= membrane proteins solubilized by detergent, IM= proteins insoluble despite detergent treatment). n=3 (biological replicates), central tendency: standard deviation

For both liver and kidney, it can be seen that there is a loss of activity being caused as a result of detergent addition, because the activity of S.M. and I.M. fractions is significantly lower compared to membrane fraction. It can also be seen that the I.M. fraction generally has a higher activity than the S.M. fraction, suggesting that the detergent is unable to solubilise the lyso-PS lipase in these tissues (figure 3.11A and C) at the used concentration. However, it can be seen from the solubilisation efficiency graph (figure 3.11B) that 60-70% of the protein is being solubilised by the detergent. Hence, the fractions showing activity have only about 30% of total membrane protein, and it may be worthwhile to perform proteomics experiments on these fractions to understand how many candidate enzymes have been filtered out by the detergent treatment.

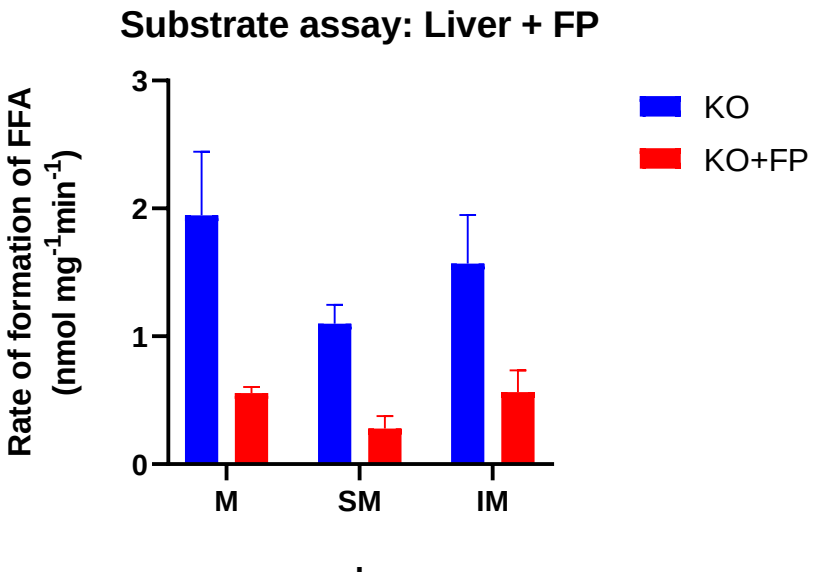


Figure 3.12 –Activity comparison between fractions with and without fluorophosphonate. n=3 (biological replicates), central tendency : standard deviation, with 1.5 hour detergent incubation

To understand the effect of fluorophosphonate on the lyso-PS lipases in liver and kidney, detergent treated fractions from ABHD12 KO liver and kidney were incubated with 20μM FP-Biotin for ABPP reaction, and their activities were later observed using substrate assay. In an attempt to increase the solubility of the lipase, the detergent incubation time was increased to 1.5 hours. Interestingly, it can be seen that the relative activities of S.M. and I.M. fractions w.r.t membrane fraction have increase compared to previous attempt. However, the activity of I.M. is still significantly higher than that of S.M., which suggests that the protein has still not been solubilised.

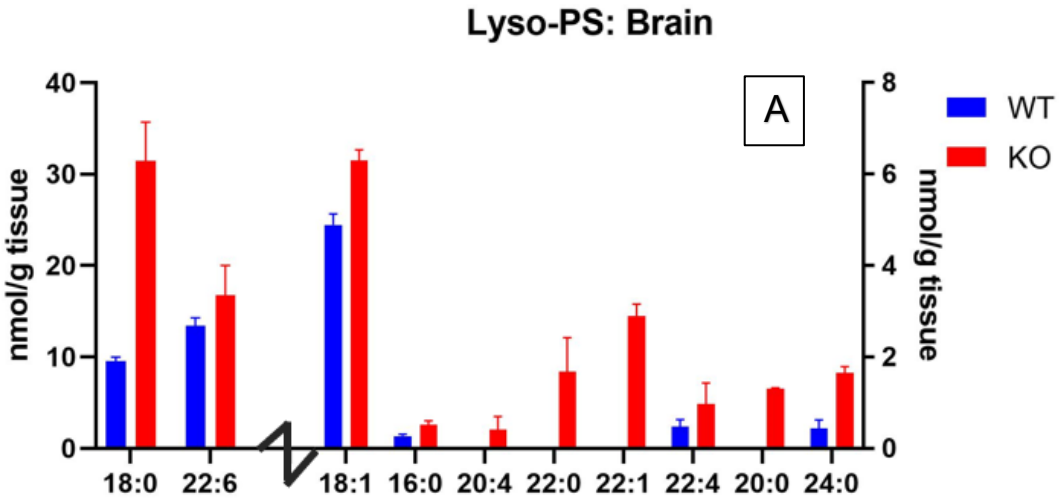
It can also clearly be seen that the addition of FP leads to a 4-5 fold decrease in activity, which strongly suggests that the lyso-PS lipase in these tissues is a serine hydrolase. This enables us to use FP-Biotin as a probe for proteomics experiments to selectively pick up serine hydrolases, instead of analysing the entire membrane proteome.

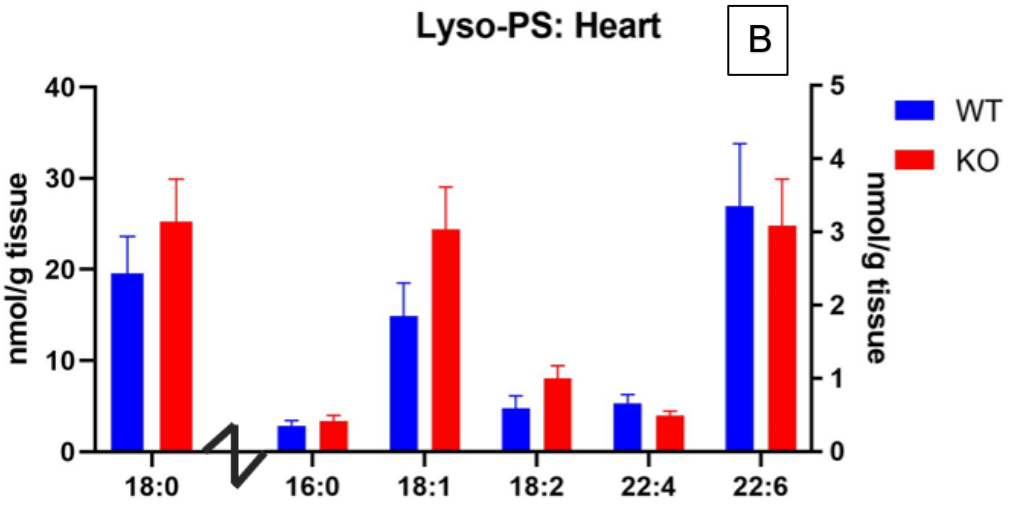
3.3 Distribution of Lyso-PS and ABHD12 across tissues

Although Lyso-PS levels have been measured in mouse tissues numerous times in the past[23], since there was a new mass spectrometer with advanced detection ability, remeasuring the lyso-PS levels was of interest. Lyso-PS levels were measured in six tissues using untargeted lipidomics measurements (Figure 3.13).

In all the tissues, it can be seen that there are only even chain length fatty acids, because mammalian systems rarely have odd chain length lipids, and given that lyso-PS are low abundant lipids, it is even more challenging to detect odd chain length species even if there were any. It can also be observed that 18:0 lyso-PS is by far the most abundant species of lyso-PS in almost all tissues, which is consistent with previously reported trends. In brain, we can observe that the lyso-PS levels increase significantly upon knocking out ABHD12, with ~3 fold change in the most abundant species (18:0), and over 1000 fold change in some low abundant species.

However, in other tissues, such fold changes are not found, and it can clearly be seen in most other tissues that lack of ABHD12 doesn't drive any significant increase in lyso-PS levels.




Lyso-PS: Heart
B
WT
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2
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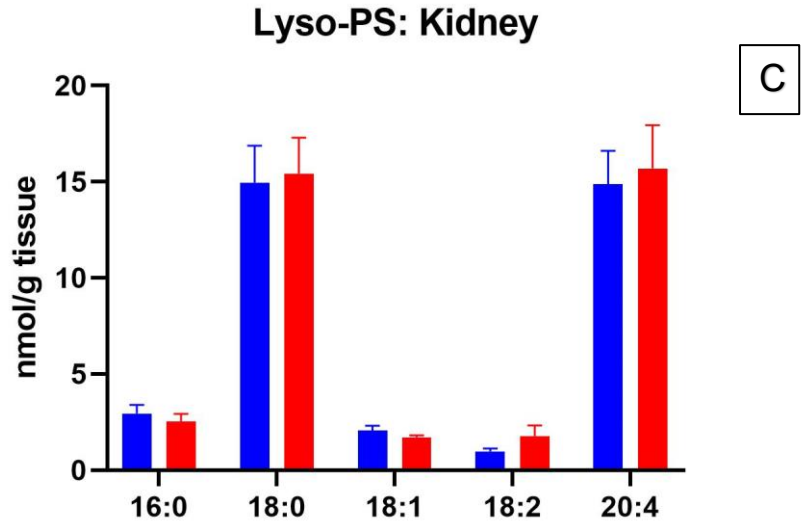
Lyso-PS: Kidney
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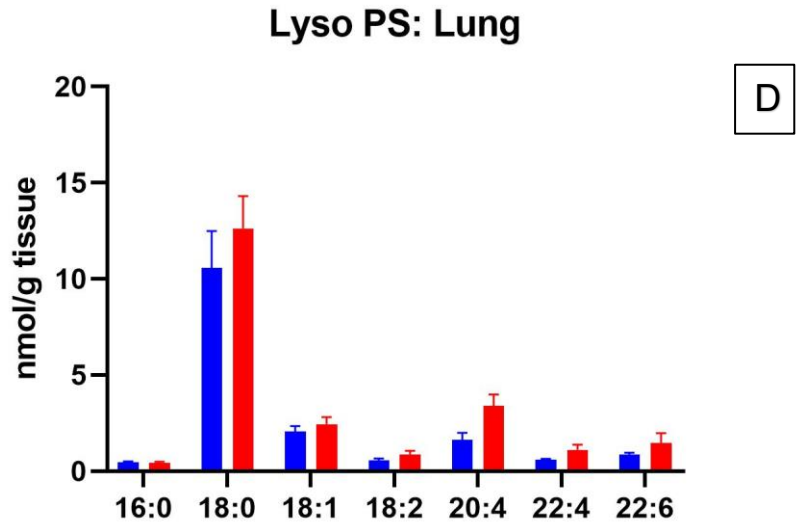
Lyso PS: Lung
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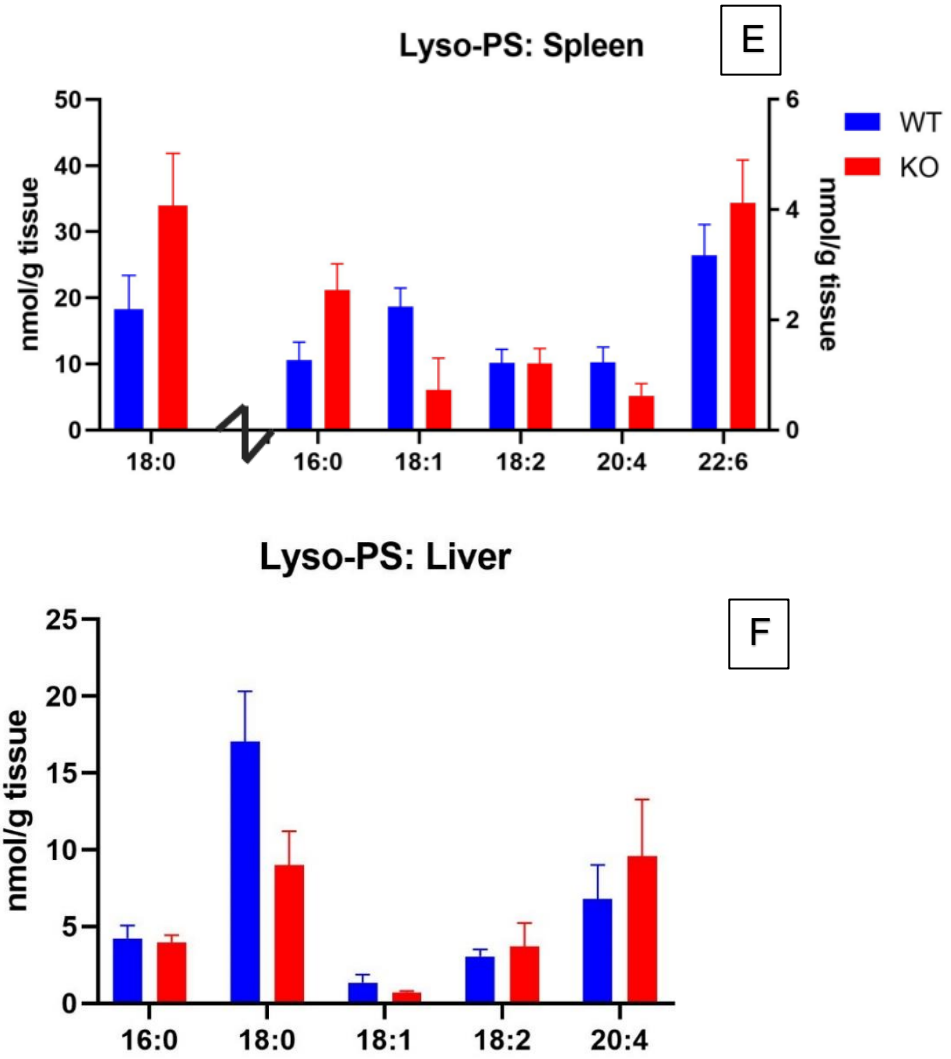

Figure 3.13 –Lyso-PS levels in tissues. Central tendency- Brain: Standard deviation; Others: Standard error of mean. Biological replicates n=5 (Heart, Kidney, Lung); n=4 (Liver, Spleen); n=3 (Brain)

Apart from lyso-PS levels, measuring the ABHD12 expression levels in these tissues is also of significance. Western blot analysis was done after preparation of 1mg/mL membrane protein fractions from various tissues which were probed using ABHD12 antibody (Figure 3.14) after SDS PAGE and overnight transfer.

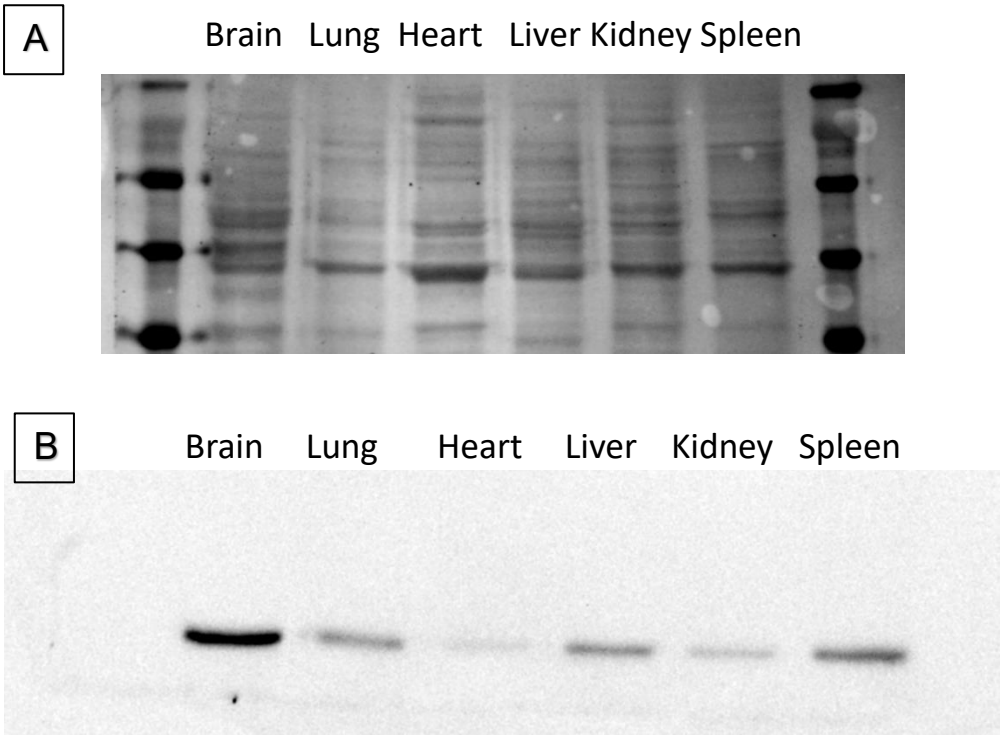

Figure 3.14 – A: Ponceau image of protein samples run on a 10% SDS PAGE gel, blotted using nitrocellulose membrane. B: Western blot image showing ABHD12 expression levels

Firstly, from the ponceau image of the blot, it was concluded that there was near equal loading in the wells, which adds validity to the blot. The experiment clearly reveals that ABHD12 has a very high relative expression level in brain, as compared to other tissues. The band intensities of ABHD12 seen are correlating closely with previously reported ABHD12 expression study using Bio-GPS.[25] This further corroborates the hypothesis that ABHD12 is not the principal lyso-PS lipase in other tissues. This result, combined with the substrate assay experiments that revealed that other tissues had comparable *in vitro* lyso-PS lipase activity, and lipdomics measurements, adds to the basis of this project.

3.4 Final conclusions

Membrane protein fractionations tried using sucrose density ultracentrifugation led to limited success, especially in the case of liver proteins. However, anion exchange chromatography was applied to successfully fractionate the solubilised membrane to yield fractions with significantly distinct protein profiles. While the reasons why the

former technique did not produce good separation are not completely known, we hypothesized that it could be due to a combination of multiple factors such as low centrifugation time used, interactions of sucrose with lipoproteins[36] and fluorophosphonate probes, and the highly manual error-prone nature of the technique. The fractions obtained using the latter technique are promising for the next steps in the project, as 5-10 fractions with significant differences in their protein profiles were obtained for each tissue processed, which could be tested for activity. Moreover, the developed protocol has been the same for the three tissues tried, which suggests that it can easily be applied to other tissues such as heart and lung when needed.

Substrate assays mainly revealed that the proteins responsible for lyso-PS lipase activity in liver and kidney are only partially soluble upon the applied usage of detergent. This is evident because most of the activity from the membrane fraction is not being translated to the solubilised fraction. This may be due to reasons such as compatibility of the protein with the detergent used for solubilisation, and the solubilisation conditions employed. This suggests that there is a need to perform an extensive detergent screening by trying various other detergents and solubilisation conditions that could solubilise the protein to a large extent. From the substrate assays done for liver at 1.5 hour detergent incubation, it can be seen that while majority of activity is coming from the insoluble fraction upon solubilisation, there is still significant activity from the solubilised fraction, which can therefore be tried for fractionation using developed protocol. Also, the insoluble fraction contains only ~30% of membrane proteins by mass, but shows activity near 70-80% that of membrane fraction, and this suggests that it maybe worthwhile to perform proteomics to analyse the number of serine hydrolase hits that are obtained after detergent treatment, to understand the degree to which the number of candidate enzymes is reduced.

Finally, the measurements of lyso-PS and ABHD12 distribution across tissues are in-line with trends reported earlier[23,25], and they further strengthen the basis for the project. If active fractions with small pools of proteins are obtained, proteomic study of those fractions would greatly narrow down the list of candidate enzymes that are capable of being *in vivo* lyso-PS lipases in the respective tissues. The candidates

can then be analysed and biochemically characterised, which are the future directions in this project to understand disease associated lyso-PS metabolism.

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