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MASTER'S THESIS

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I. Abstract

During photosynthesis light energy in used to make ATP, during this process cytochrome b6f acts as a link between photosystem I and II. Cytochrome b6f received electrons from photosystem II and passes them to photosystem I, and play a key role in the formation of ATP form ADP. The p-side quinol deprotonation-oxidation reactions within the cytochrome b6f complex were found to be involved in generating reactive oxygenic species. Within the quinol oxidation site, a chlorophyll molecule has been found. This Chlorophyll molecule have been suggested to perform a structural, non-photochemical function by enhancing the rate of formation of the oxygen species. Similarities between unique features in Cytochrome bc1 and have been found in cytochrome b6f. These features could provide the key information to understand the evolution and regulation of electron transfer process in the oxygenic photosynthesis. One of the objectives of this thesis is to investigate the exchange of protochlorophyllide bound to cytochrome b6f complex in etiolated plants against chlorophyll. Previous instigations have shown that cytochrome b6f isolated in the dark can bind to chlorophyll a *in vitro*, however it is still unknown if cytochrome b6f can bind to chlorophyll b. Special focus in this thesis is to investigate the binding of chlorophyll b to cytochrome b6f complex.

The main focus of this thesis however, has been to develop a method that can be used in order to investigate the binding of chlorophyll b to cytochrome b6f. The method for solubilisation were in the developmental phases at the start of this thesis, and one of the objectives have been to optimize and standardize the method of solubilisation of dark etioplasts.

II. Acknowledgement

The last twelve months have been intensive and difficult with many ups and downs. I have had the opportunity to work independently and through a lot of work and many errors I have gained new knowledge. I would like to take this opportunity to thank everyone who has helped me throughout this period.

I would like to thank Lutz Andreas Eichacker for the opportunity to work on this project with his group. I would also like to express my heartfelt thanks to Ann Kristin Bue, whom without this thesis would not have happened. She has been there throughout the whole process and have helped with the lab work, and answers all questions that I have had. I would also like to thank friends and family for all the support they have given these last twelve months, it has been hugely appreciated and necessary in order to get through to the other end.

III. Abbreviations

APS - Ammonium persulfate

ATP – Adenosine triphosphate

Chl a – Chlorophyll a

Chl b – Chlorophyll b

Cyt b6f – Cytochrome b6f

dH_2O – Destilled Water

DNA - Deoxyribonucleic acid

DM - n-Decyl-β-D-Maltoside

ECL - Enhanced chemiluminescence

EDTA - Ethylenediaminetetraacetic acid

GGR - Geranylgeranyl Diphosphate Synthase, Type II

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

KCl - potassium chloride

KOH - Potassium hydroxide

LDS - Lithium dodecyl sulfate

Lil 3 - Light harvesting like protein called Lil3

$MgCl_2$ - Magnesium chloride

MS – Mass spectroscopy

NaCl - Sodium chloride

NADPH - Nicotinamide adenine dinucleotide phosphate

NADP+ - Nicotinamide adenine dinucleotide phosphate oxidised form of NADPH

NH_4HCO_3 - Ammonium bicarbonate

PAGE – Polyacrylamide Gel Electrophoresis

PChl - Protochlorophyllide

PChla - Protochlorophyllide a

Photosystem I - PSI

Photosystem II - PSII

POR – Protochlorophyllide oxidoreductase

Rpm - Revolutions per minute

SDS – Sodium dodecyl sulfate

TBS - Tris-buffered saline buffer

TEMED – Tetramethylethylenediamine

TLC – Thin Layer Chromatography

List of Content

1. Introduction

1.1. Background

The cytochrome b6f complex is an enzyme, which is isolated from the thylakoid membrane of chloroplasts, cyanobacteria and green algea. The Cyt b6f complex acts as a link between photosystem I and II, which are the reaction centres that capture light energy that drive the electron redox reactions of oxygenic photosynthesis. Cyt b6f receives electrons from PSII and passes them to PSI, and have a key role in the formation of ATP from ADP. The Cyt b6f complex provides the structural and functional link between the Photosystems I and II (PSI and II) which are the reaction centres that capture light energy in order to drive the electron redox reactions of oxygenic photosynthesis. The complex receives electrons from PSII and passes them to PSI, and contributes to creating the electrochemical gradient that is the basis for the formation of ATP from ADP. (Berg, Tymoczko et al. 2015)

The p-side quinol deprotonation-oxidation reactions within the Cyt b6f complex were found to be involved in the generation of reactive oxygen species. A Chl molecule has been identified within the quinol oxidation site. This Chl molecule have been suggested to preform a structural, non-photochemical function by enhancing the rate of formation of the oxygen species. This could be used as a potential redox-pathway for intra cellular communication. (Baniulis, Hasan et al. 2013, Hasan, Proctor et al. 2014)

The Cyt b6f complex have the ability to switch to a cyclic mode of electron transport in PSI using a unknown pathway. When looking at an X-ray structure of the Cyt b6f from alga, *Chlamydomonas reinhardtii*, has been solved at 3.1 Å. Showing the similarities to the Cyt bc 1 and its unique features, like Chl, ß-carotene and harm sharing a quinone site that is bound by one thioether linkage without an axial amino acid ligand. These cofactors could provide the key information to understand the evolution and regulation of the electron transfer process in the oxygenic photosynthesis. (Stroebel, Choquet et al. 2003)

1.2. Objectives

The objectives of this thesis is to develop a standard method for solubilisation, and to investigate the exchange of PChl bound to the Cyt b6f complex in etiolated plants against Chl. Special focus is to investigate binding of Chl b to Cyt b6f complex. The work is based on previous investigations showing that Cyt b6f isolated in the dark can bind to Chl a *in virto*, however whether the Cyt b6f could also bind to Chl b is still unclear.

1.3. Plant Cell

All beings on earth, humans, animals, plants are structured by cells. More specifically eukaryote cells. One thing that separates animals from plants are the fact that plants are made up of plant cells, while animals are made up of animal cells.

There are some structural differences between an animal and plant cell. A lot of the structures are similar, both types of cells have a nucleus, mitochondria, and ribosomes, but one of the structures that differs between them are plastids.

1.3.1. Plastids

Plastids are the organelle in the cell where plants harbour photosynthesis, and store polymerizes glucose as starch for short term energy storage. There are different types of plastids that reflect the developmental state of the plant, and each have a different function for plant survival. (Raven, Evert et al. 2005)

1.3.1.1. Chloroplast

Plastids are classified by the type of pigmentations. Typically plastids contain carotenoids. Chloroplasts are specified by containing Chl which makes chloroplasts the site for photosynthesis. (Berg, Tymoczko et al. 2015)

Chlorophyll is responsible for the green colour of plants. A single plan cell can have as many as 40 to 50 chloroplasts, and they are free to orient inside the cell based on the influence of light quality and quantity. (Berg, Tymoczko et al. 2015)

The internal structure of chloroplasts are complex and contain different complexes. One of the complexes substructuring a chloroplast are called the thylakoid. Chlorophyll and carotenoid pigments are found at high concentration in the thylakoid, and this is the place where photosynthesis takes place.(Berg, Tymoczko et al. 2015)

1.3.1.2. Chromoplast

Chromoplasts are also pigmented plastids, but they do not contain chlorophyll, only carotenoid pigments. These pigments are responsible for the red and yellow colour seen in plants. (Raven, Evert et al. 2005)

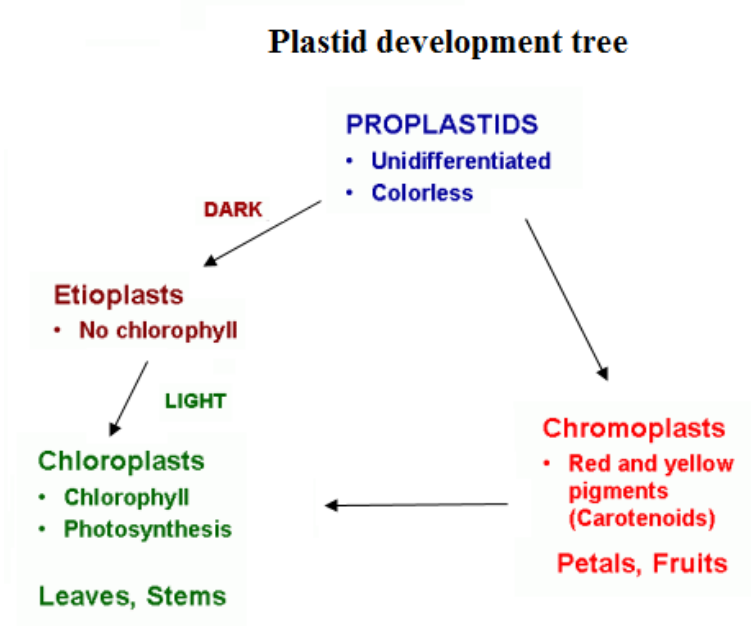


Figure 1.3.1.2. Plastid development tree.

Etioplasts are developed from proplastids in the dark, and when exposed to light will transform into chloroplasts. Proplastids may also develop into chromoplasts, which in turn can develop chloroplasts. This figure have been modified by Marianne Stokka, and were found at (Kantharaj)

Chromoplasts are transformed from chloroplasts and the transformation is accompanied by a degradation of chlorophyll and other internal structures and accumulation of carotenoids (Raven, Evert et al. 2005)

One of the ecological functions of chromoplasts could be the attraction of insects and other animals in order to help with cross-pollination and distribution of seeds. (Raven, Evert et al. 2005)

1.3.1.3. Proplastids

Proplastids are colourless or pale green, small and undifferentiated plastids that occur in cell division in roots and shoots. They are the precursor to other specialised plastids such as chloroplasts and chromoplasts. (Raven, Evert et al. 2005)

1.3.1.4. Etioplast

When the development of proplastids are arrested ie. in darkness, prolamellar bodies may form. These are semicrystalline bodies composed of tubular membranes, and they are called etioplasts. (Raven, Evert et al. 2005)

Etioplasts form in plants that grow in the dark. When such plants are exposed to light etioplasts will develop into chloroplasts, and the prolamellar bodies develop into thylakoids. (Raven, Evert et al. 2005)

Robertson and Laetsch have shown (Robertson and Laetsch 1974), that barley grown in the dark is characterized by a specific rate of growth, and the leaves show a succession of leaf regions containing different developmental states of etioplasts.

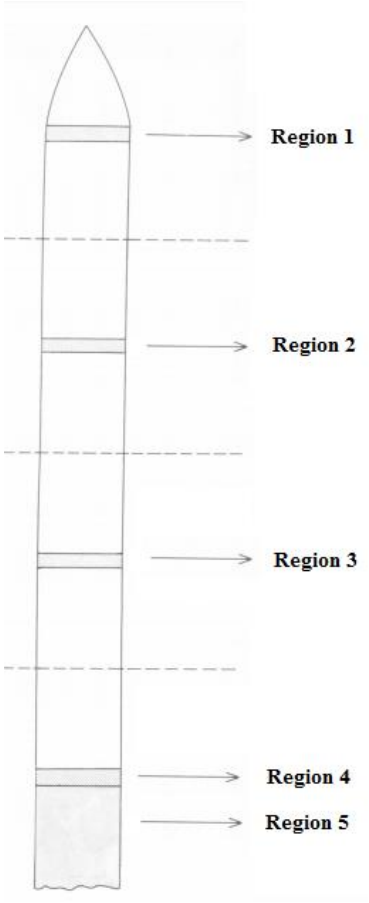


Figure 1.3.1.4.A Division of a single barley strand.

Robertson and Laetsch (1974) divided a single strand of barley into five different regions. The different regions have different amount of etioplasts, and through a series of experiments they optimized the regions from where dark etioplasts is found in highest density. The figure have been modified by Marianne Stokka and the original were found in (Robertson and Laetsch 1974)

They also found that from base to tip, etioplasts in the dark grown plants were able to continue dividing and enlarging as the leaf and its regions were growing. Results showed that it is in the three top regions of the barley leaf that etioplasts were developed from the base to the tip, and at the tip can be found in larger quantities with a larger diameter. In the two lower levels, 4 and 5, the etioplasts were found to be very small and in low quantity. The three top regions of the barley leaves therefor are attractive to isolate etioplasts. (Robertson and Laetsch 1974)

1.4. Cytochrome

Cytochrome was discovered as early as 1880s. Charles A. MacMunn discovered something that he concluded had to have something to do with the respiratory system, and gave it the names myoheamating and histohematin. It was not until early in the nineteen-hundreds that David Kailin gave the name known today as cytochrome. Through a series of experiments done by different scientists, it has been found that there are in fact many different groups of cytochromes. (Cramer, Kallas et al. 2016) At one point in time there existed seven different groups of cytochromes; a, b, c, e, f, h and o. (Cramer, Kallas et al. 2016) However today it seems to be the first three sub groups discovered that are the main groups that can encompass all differences in the different cytochromes. (Cramer, Kallas et al. 2016)

Cytochrome plays an important role in the electron transport chain. It is a major class of electron transport metalloproteins. (Cramer, Kallas et al. 2016) Cytochrome always contain at least one heme group, and help to move electrons via redox reactions. (Cramer, Kallas et al. 2016) It is believed that cytochrome played an important evolutionary role when plants went from being anoxygenic to oxygenic. (Cramer, Kallas et al. 2016) This evolutionary step could happened due to the plants needs to evolve towards the oxygenic environment. (Cramer, Kallas et al. 2016) In the photosynthetic pathway, different groups of cytochrome can be found. (Cramer, Kallas et al. 2016)

1.4.1. Cytochrome b6f

One of the many cytochrome complexes in existence is the cytochrome b6f complex. Cytochrome b6f is a dimer, and both of its two monomers have eights subunits. (Cramer, Kallas et al. 2016) Cytochrome b6f is an enzyme that can be found in the thylakoid membrane in plant cells, and it aids the photosynthesis in making ATP. (Berg, Tymoczko et al. 2015)

This complex takes part in the electron transport chain of the oxygenic photosynthesis. Cytochrome b6f is engaged in the proton coupled transfer reactions. (Cramer, Kallas et al. 2016) More precisely it is responsible for linking the two light trapping photosystem reaction centres in

the electron transport chain together. (Cramer, Kallas et al. 2016)
It is only recently that scientists have started to discover the complex role cytochrome b6f plays in the electron transport chain. (Cramer, Kallas et al. 2016) New technology, like x-ray crystallopraphic analysis, have helped in understanding the structure of the complex and the role it plays in the electron transport chain. (Cramer, Kallas et al. 2016)

1.5. Photosynthesis

All organisms have a metabolic process, in plants this process is called photosynthesis. In photosynthesis plants take energy in form of sunlight and use it to convert carbondioxide (CO2) and water (H2O) into sugar (C6H12O6) and oxygen (O2). This process had been known since the early eighteen hundreds. (Sadava 2010)
The metabolic process mentioned is the main overall overview of photosynthesis, but is not the only process in photosynthesis. (Sadava 2010) Photosynthesis is mainly divided into two reactions, the light reaction and the light independent reaction. (Sadava 2010) As their names suggest one need light in order to work, while the other does not. In light reaction sunlight is converted into ATP and NADPH. (Sadava 2010) While the light independent reaction use the ATP and NADPH, made from the light reaction, together with CO2 to produce carbohydrates. (Sadava 2010)
Many different pigments found in photosynthetic organisms absorb the energy collected from sunlight, and in plants two of them are chlorophyll a and chlorophyll b. (Sadava 2010) These pigments that can be found in all photosynthetic organism, are working as an energy absorbing antenna; the light harvesting complex. (Sadava 2010)

In the photosynthetic system, there are two electron transport chains; Non-cyclic electron transport and cyclic electron transport. (Sadava 2010) The difference between these two is that in the non-cyclic, both NADPH and ATP are produced, while the cyclic electron transport only ATP is produced. (Sadava 2010) In the non-cyclic electron transport system we find photosystem I and II, both of these systems can be found in the thylakoid membrane. (Sadava 2010)

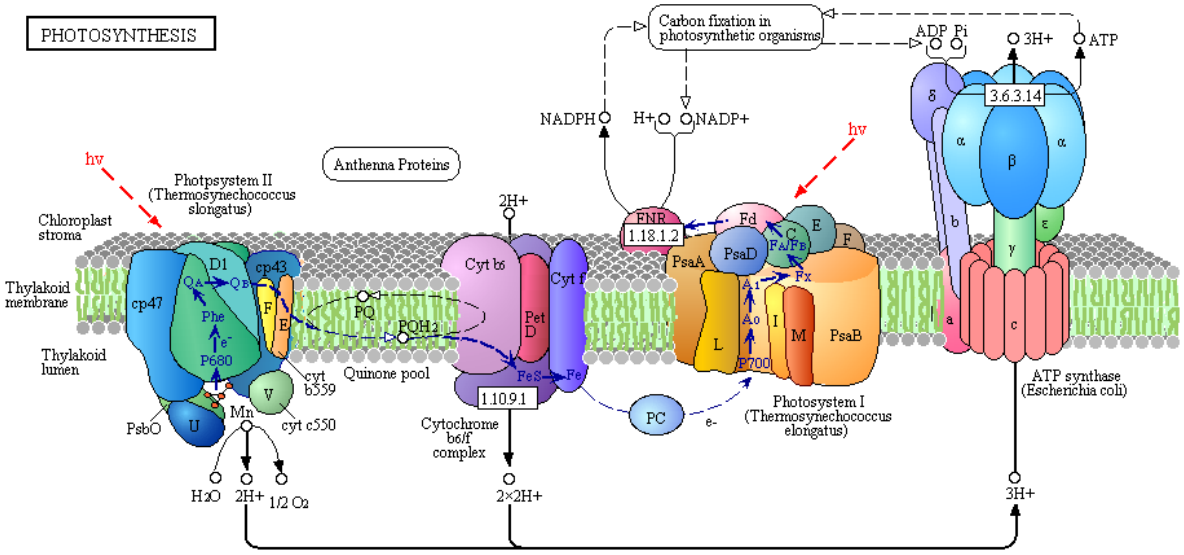


Figure 1.5.A Photosynthesis.

Photosyntesis is a complex pathway, where light energy from the sun is transformed into ATP thorugh a series of different pathways. Photosystesis is made up of different units, photosystem I and II, the Cytb6f complex as well as the ATP synthase unit. This figure have been taken from (Laboratories 2014)

1.5.1. Photosystem I

Photosystem I is actually the second system in this reaction. Photosystem I was the one of the two that was first discovered, but photosystem II actually starts the energy producing process. (Sadava 2010)

The reaction centre in photosystem I, which is responsible for the uptake of light energy, contains two chlorophyll a, called P700. (Sadava 2010) They have gotten this name due to them absorbing light with a wavelength of 700nm. (Sadava 2010)

After absorption of energy in the reaction centre in photosystem I, ATP is produced. (Sadava 2010) This happens through a series of events where the light energy in itself is directly responsible for the creation of NADPH from NADP+, which in turn aids in the ATP synthesis. (Sadava 2010)

1.5.2. Photosystem II

Photosystem II uses light energy to oxidize water molecules, and as a result produce electrons, protons (H+) and O2. (Sadava 2010) The reaction centre of photosystem two contain two chlorophyll a called P680. (Sadava 2010) Much the same as for photosystem I, the reaction centre gets its name based on at which wavelengths it absorbs light energy. (Sadava 2010) Photosystem II absorbs light that is more energetic than what is absorbed in photosystem I. (Sadava 2010)

The electrons produced in photosystem II will pass though a series of transfer reactions in the electron transport, before it reaches photosystem I, where it will be used to create NADPH. (Sadava 2010)

1.5.3. Chlorophyll a

Chlorophyll a can be found in plants all over the world, and is the reason why plants look green. Chlorophyll a will absorb all colours such as blue/violet and red/orange, but will reflect the wavelength equivalent to green, which in turn will give the plant its green colour. (Młodzińska 2009)

During photosynthesis, chlorophyll a helps with the electron transport chain, where it serves as the main electron donor. (Sadava 2010) Chlorophyll a can be found in the reaction centre of both photosystem I and II, and have the specificity to absorb light with wavelengths of 680 and 700 nm in photosystem II and I respectively. (Sadava 2010)

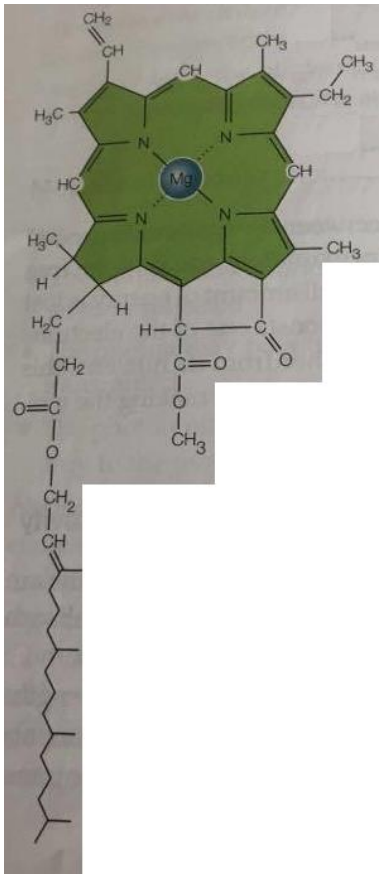


Figure 1.5.3.A: Structure of clorophyll a

The molecular structure of Chl a consists of a ring structure called a chlorin and a side chain. The chlorin ring have four nitrogen atoms faced inwards where they bind to a magnesium atom. (Sadava 2010)

Chlorophyll a is a component that is essential to all photosynthetic organisms due to its partaking in the energy creating photosynthetic process. (Sadava 2010)
When we look at the molecular structure of chlorophyll a we can see a ring which contain four nitrogen atoms, which is on the inside and turned toward each other. (O'Neal, Roberts et al. 2006) In the center of these four nitrogen atoms a magnesium atom can be placed (much similar to iron in hemoglobin in human blood). (O'Neal, Roberts et al. 2006) This ring structure is known as a chlorin, which is a heterocyclic compound derived from pyrrole. (O'Neal, Roberts et al. 2006) In chlorophyll a, the chlorin structure have side chains, and it is these chains that makes chlorophyll a different from chlorophyll b (Figure 1.5.3.A). (Sadava 2010) The chlorin ring also have a long hydrophobic tail, which acts as an anchor, and attaches it to the thylakoid membrane of the chloroplast (Figure 1.5.3.A). (Sadava 2010)

The absorption of light by Chl can be extended, meaning the plant can absorbe more light (a broader spectra) is more than one pigment is used. (Berg, Tymoczko et al. 2015) Chl a and b can well explain this.(Berg, Tymoczko et al. 2015) With Chl b added to Chl a, the spectra increases absorbance in the central region of the two maxima of the Chl a spectrum. (Berg, Tymoczko et al. 2015) An example of such a spectra can be seen in figure 1.5.3.B.

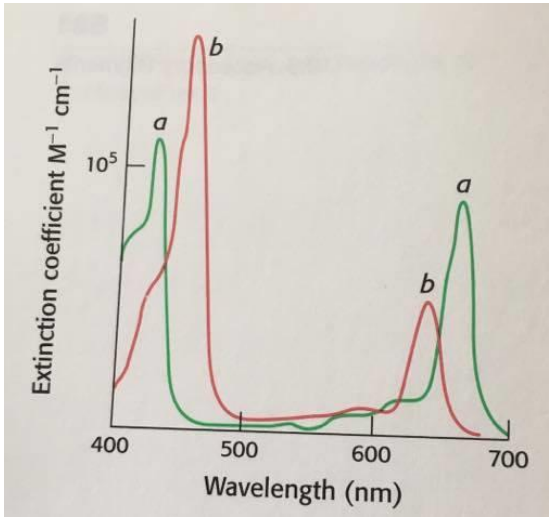


Figure 1.5.3.B. Absorption spectra of chlorophyll a and b.
The absorption spectra of Chl a can be extended, which means that a plant can absorb more light. The spectra of Chl a can be extended in such a way with the addition of Chl b. The a marked Chl a and b markes Chl b. (Berg, Tymoczko et al. 2015)

1.5.4. Chlorophyll b

The difference between chlorophyll a and b structurally is not very large, it is only one of the side chains in the chlorin ring that differ. Chl b is characterized by an aldehyde group instead of a methyl group attached to the C7 carbon atom. (Berg, Tymoczko et al. 2015)

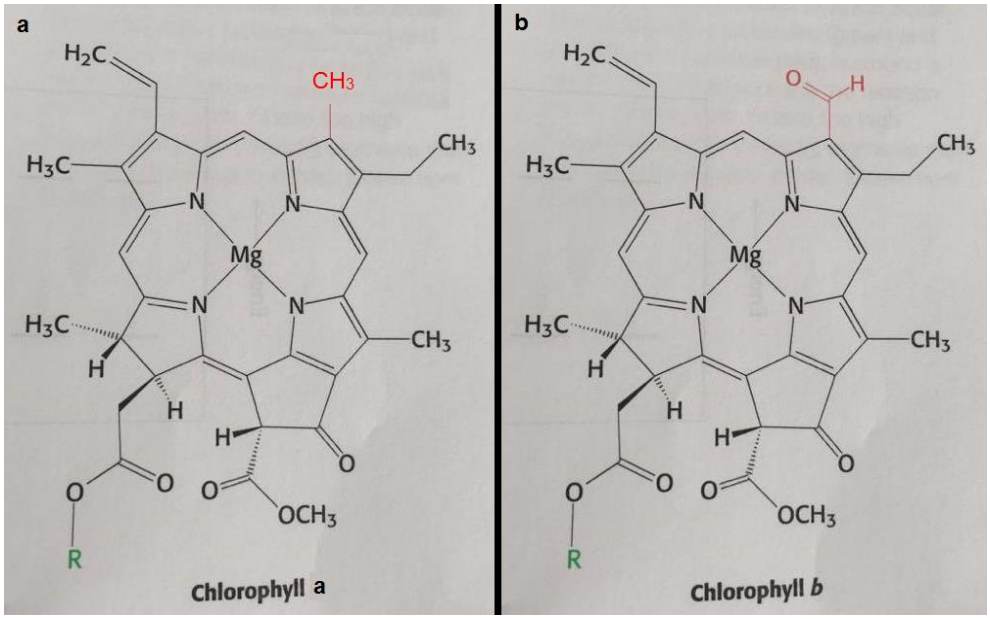


Figure 1.5.4.A. Structure of the chlorin ring of chlorophyll a and chlorophyll b.

The structure of both Chl a (a) and Chl b (b) both have four nitrogen atoms faced inwards, where they bind to a nitrogen atom. The difference between Chl a and Chl b is the functional group found at C-7. Chl a have a methyl group at C-7 while Chl b have a formal group. The functional groups at C-7 are marked by a red Colour. The figure for Chl a (a) is a modified version of Chl b (b), which were taken from (Berg, Tymoczko et al. 2015).

Chlorophyll b is essential for photosynthesis, since it has been found that without chlorophyll b present, plants have a relatively low photosynthetic capacity. (Eggink, Park et al. 2001) Chlorophyll b will reflect light with wavelength equivalent to yellow green. (Młodzińska 2009) In plants grown under normal light, chlorophyll a is 2-4 times as abundant at chlorophyll b, but in plants grown in shaded places less Chl a, and more Chl b is present and leaves appear more yellow in colour to the eye. (Młodzińska 2009) A yellow pigmentation that is also found in dark grown plants. (Młodzińska 2009) Here, plastids are devoid of Chl a and b, but contain carotenoids. (Młodzińska 2009) For synthesis of Chl a and B plants have to be exposed to light. (Młodzińska 2009)

1.6. Etiolation

Etiolation is growth characteristic of flowering plants growing in the absence of light within a developmental program termed photomorphogenisis in which develop plastids as etioplasts. (Farrar 2003)Typically plants will get a lighter colour when growing in the dark, plants will also have fewer leafs growing together and the cell wall will also be weakened. (Farrar 2003) A plant that grows in the absence of light will be highly sensitive to light and the developmental program of photomorphogenesis will be induced if light becomes available. (Farrar 2003)

1.7. Deetiolation

Deetiolation is when a plant grown in darkness is exposed to light. Many processes are initiated within a plant during this transfer. The morphogenetic changes prepare the plant to initiate photosynthesis. (Symons, Smith et al. 2008) One of the changes that happens is that etioplasts formed in the dark will transform and develop into chloroplasts. (Raven, Evert et al. 2005)

2. Material and methods

2.1. List of chemicals

• Ɛ-Aminohexaniocacid	Merck
• p-Coumarsäure	Sigma Aldrich
• Acetone	Marck
• Acetonitrile	Marck
• Acrylamide 30%	Panreac Applichem
• Antirabbit	Sigma Aldrich
• Antirabbit 800 cw	Li-COR
• Antirabbit lgG 550	Agrisera
• APS	Serva
• Bis-Tris	VWR
• Cyt b	Agrisera
• Digitoinin	Sigma Aldrich
• DM	Calbiochem
• EDTA	Amresco
• Glycerol	Panreac Applichem
• Glycine	Merck
• HEPES	VWR
• Hydrogen Peroxide	VWR
• KCl	Ridel-deHaën
• KOH	Merck
• LDS	Sigma Aldrich
• Lil 3	Agrisera
• Luminol	Sigma Aldrich
• Methanol	Methanol
• Milkpowder	Panreac Applichem
• $MgCl_2$	Alfa Aesar
• NaCl	VWR
• NH_4HCO_3	Applichem
• Percoll	GE Healthcare
• Ponceau S	Sigma Aldrich

• POR	Agrisera
• SDS	Merck
• Sorbitol	VWR
• TEMED	Fluka
• Tricine	Panreac Applichem
• Tris	VWR
• Trypsin	Sigma Aldrich
• Urea	Applichem

2.2. Solutions

2.2.1. Isolation

1M HEPES*KOH, pH 8, 1000 mL

- 238.4g, HEPES
- dH_2O
- pH adjusted with KOH

Isolation medium, 1000 mL

- 78.88 g, 0.4 M Sorbitol
- 50 mL, 50 mM HEPES*KOH pH 8.0
- dH_2O

Percoll 40 %, 120 mL

- 48 mL Percoll
- 6 mL, 1 M HEPES*KOH pH 8
- 8.74 g, 1M Sorbitol
- 280 µL, 0.5 M EDTA pH 7.5
- 4 mL, 2mM EDTA
- dH_2O

Percoll 80%, 60 mL

- 48 mL Percoll
- 3 mL, 1 M HEPES*KOH pH 8
- 4.37 g, 1M Sorbitol
- 120 µL, 0.5 M EDTA pH 7.5

1M Sorbitol, 100 mL

- 0.4 M Sorbitol
- dH_2O

Wash medium, 500 mL

- 36.4g, 0.4 M Sorbitol
- 25 mL, 50mM HEPES*KOH, pH 8
- dH_2O

2.2.2. Solubilisation

2D SDS gel, 5 gels

- 7.21g, Urea
- 12.50 mL, 30% Acrylamide
- 3.75 mL, 8X Tris pH 8,8
- 9 mL, dH_2O
- 50 µL,10% APS
- 15 µL, TEMED

2D SDS Stacking gel, 5 gels

- 0.80 mL, 30% Acrylamide
- 2.48 mL, 2x Tris pH 6.8
- 1.60 mL, dH_2O
- 50 µL, 10 % APS
- 5 µL, TEMED

Anode Buffer (10x), 500 mL

- 52.3 g, 500mM Bis-Tris pH 7.0
- dH_2O

Cathode buffer (10x), 500 mL

- 44.8 g, 800 mM Tricine
- 15.59 g, 150 mM Bis-Tris
- dH_2O

Gel Buffer (6x), 100 mL

- 39.36, 3 M Ɛ-Aminohexaniocacid
- 30 mL, 0,3 M Bis-Tris pH 7.0
- dH_2O

LDS cathode buffer (5x)

- 50 mL Cathode buffer
- 1 mL 5%/0,05g , LDS
- 13.425 g, Tricine
- dH_2O

LDS cathode buffer 0.02mM, 200mL

- 200 mL cathode buffer
- 21.8 µL LDS

LDS cathode buffer 0.04mM, 200mL

- 200 mL cathode buffer
- 42.6 µL LDS

LDS Sample Buffer (4x), 10 mL

- 0.666g, Tris HCl
- 0.682g, Tris Base
- 0.800g LDS
- 0.006g EDTA
- 4g Glycerol
- dH2O

Native LDS-Polyacrylamide Gel 7.5%, 5 gels

- 7.875mL Acrylamide
- 5.25 mL, Gel Buffer (6x)
- 18.375 mL, dH_2O
- 20 µL, TEMED
- 80 µL, 10% APS

Native LDS-Polyacrylamide Stacking gel, 5 gels

- 0.656 mL, Acrylamide
- 0.833 mL, Gel Buffer (6x)
- 3.445 mL, dH_2O
- 5 µL, TEMED
- 50 µL, 10% APS

Running buffer (10x), 1000 mL

- 144.13g, 1.92 M Glycin
- 30.3g, 0.25 M Tris
- 10 g, 1% SDS
- dH_2O

TMK-buffer (10x), 100 mL

- 10 mL Tric HCl pH 8.5
- 10 mL 1M $MgCl_2$·
- 10 mL 2M KCl
- dH_2O

2.2.3. Western blott

5% Milk in TBS, 200 mL

- 10 g Milkpowder
- TBS (1x)

Ponceau, 100 mL

- 0.2 g, 0,2% (w/v) Ponceau S
- 1 mL, 1% Hydrogen Peroxide
- dH_2O

TBS (10x), 1000 mL

- 100 mL, 10 mM Tris/HCl pH 7,5
- 300 mL, 150mM NaCl
- dH_2O

Towbin, 1000mL

- 7.2g, 96mM Glycin
- 1.21g, 10mM Tris
- 100 mL, Methanol
- dH_2O

Tabell 2.2.2.A. Overview of primary and secondary antibodies.

All antibodies have been made using the 5% Milk in TBS solution. The antibodies have been made with different dilutions.

Antibody	Dilution
Primary Cyt b6f 15mL	1:10 000
Primary Cyt b6f 800	1:10 000
Primary POR	1:2500
Primary Lil 3	1:7500
Secondary Antirabbit	1:10 000
Secondary Antirabbit 800 cw	1:15 000
Secondary Antirabbit lgG 550	1:2500

2.2.4. TLC

Initiation Solution, 20 µl

- 16 µl Acetonitril
- 4 µl, dH_2O

TLC Run Solution

- 30 mL Methanol
- 20 mL Acetone
- 1 mL dH_2O

Trypsin Working Solution (TWS) 1/10 dilution

- 2 µl Trypsin
- 18 µl, 50mM NH_4HCO_3 (pH = 8.0)

2.3. Isolation of plastids

The method described by (Eichacker, Müller et al. 1996) was used as a basis when developing the method used in this experiment. Small changes were made to the method due to some trouble with making the isolation work.

All equipment and solutions were cooled down before use. The isolation solution was cooled down to the point of crystallization, and the centrifuge to about -4 ºC

The top area (2-3 cm from the top) barley was cut and placed in the isolation media, where the pieces continued to be cut into smaller pieces in order to release as much etioplasts as possible.

The isolation media with the barley pieces were filtered and the solution were spun down for 6 min at 4 ºC at 5000 rpm.

Most of the isolation medium was poured out (~2mL left in the beaker), and the pellet were resuspended. The solution were filtered into a tube containing 40% and 80% percoll solutions, and the tubed were spun down for 10 min at 4 ºC at 5000 prm.

The top layer were taken out and the lower visible layer of etioplasts were transferred into a new tube. The new tube was filled up with wash medium and spun down for 6 min at 4 ºC at 6500 rpm.

Most of the supernatant were pored out, and the pellet were resuspended in ~1mL of the supernatant. The liquid was transferred to an Eppendorf tube.

998 µL of wash medium and 2µL of the sample was transferred to an eppendorf tube for quantification. A microscope was used to count and quantify the cells.

The non-diluted sample can be stored in the freezer before use.

The changes made to the method from (Eichacker, Müller et al. 1996) are small, but had to be made due to some trouble with a loose pellet. With the last spinning step the rpm was raised in order to make the pellet slightly firmer, and less likely to dissolve on its own. In some of the isolations one extra step had to be added in order to be able to isolate any etioplasts. After the last spinning, more of the supernatant had to be kept to be certain there was no etioplasts lost. The eppendorf tubes containing the cells were spun down again in a fourth spin step, for 6 min at 4 ºC at 6500 rpm. Some of the supernatant were removed and the pellet resuspended, and joined with any other eppendorf tubes.

2.4. Solubilisation

2.4.1. Gel electrophoresis

Gel Electrophoresis relies on the known fact that opposite charges attract each other. The forces associated with the attraction can be used to separate proteins, DNA and other macromolecules. During electrophoresis these molecules are placed in an electric field and drawn to the opposite polar site. (Berg, Tymoczko et al. 2015)

Electrophoresis is typically conducted in a porous gel. The gel have pores that will help give a better separation, where small molecules move through the gel with no problem, while larger molecules will have more resistance. The electric field is oriented in such a way that proteins will migrate from the negative side to the positive. In a gel, proteins are most often separated based on their molecular weight or isoelectric point. (Berg, Tymoczko et al. 2015)

2.4.1.1. Native LDS-Polyacrylamide Gel Electrophoresis

Proteins can be separated using a gel without denaturing it by using Native LDS-Polyacrylamide Gel Electrophoresis. The system is applied with the intention to keep the three dimensional structure of proteins intact during the analysis. Detergents can be used to solubilise the lipid membrane, and maintain complexes intact. Also folding should remain intact to maintain proteins in their native state, hence the name Native. (Arnold, Shapiguzov et al. 2014)

2.4.1.2. Making Native LDS-Polyacrylamide Gel

Assemble the gel chamber and add the Native LDS-Polyacrylamide Gel 7,5% solution, add water saturated 2 butanol to the top of each gel to make the gel evenly distributed. After ~1,5 hour the gel will be polymerized. Add some agarose to the sides of the gel to close it off. A comb can be added to create wells, and the Native LDS-Polyacrylamide Stacking Gel can be added to create the stacking gel. Polymerazation will take about 10 min. The finished gels can be stored in the fridge in a moist plastic bag for about 1 week.

2.4.2. Solubilisation

The following procedure are to be done in a dark room.

If the dark etioplasts have been isolated at an earlier stage you need to defrost them on ice. It is very important in order to not destroy the sample that it is kept cool during the whole procedure. The centrifuge needs to be cooled down to 10 ºC before use.

The necessary amount of sample needs to be taken out and mixed with samplebuffer, sorbitol,

dH_2O and heated Digitonin. The sample then needs to incubate on top of the ice (10 ºC) for 10 min before spinning down for 10 min 10 ºC at 21800g. The supernantant is then transferred to another tube and divided onto as many tubes as necessary, be careful not to get any of the pellet into any of the samples. The procedure is finished by adding red glycerol right before loading onto the gel.
Chl a or b can be added in a step before digitonin is added, before spinning. It is also possible to add Chl a or b after the samples have been spun down, right before adding glycerol and loading onto the gel.

2.4.3. Spectroscopy

In many different fields of science spectroscopy is a widely used analytical method. This is a method that can be used in many different forms, but mainly what happens is that light either produced from a sample or sent through a sample can be analysed. Spectroscopy can be used to measure bacteria in a sample by sending light through it or by measuring the fluorescent light sent out from a sample.

2.4.3.1. Fluorescence

Fluorescence is the emission of light from a substance showing the property to release the excitation energy from absorption of electromagnetic radiation by light. A substance giving off fluorescent light will stop doing so of the radiation stops. (Hage and Carr 2010)

2.4.3.2. Scanning gel

The gels are scanned using two different machines, the Typhoon and the Odysse. These two machines scan at different wavelengths and will therefor show slightly different results.
The typhoon scan at 633nm while the odyssey scan at 700nm.

2.4.4. 2D SDS Polyacrylamide Gel Electrophoresis

One of the differences between SDS and Native gel is that in a SDS gel proteins are denaturated, while in a Native one all organelles are still intact. (Berg, Tymoczko et al. 2015)
Using a 1D Native Page upfront of a 2D SDS Page allows a combination of native and denaturing analysis. (Berg, Tymoczko et al. 2015)
In the first dimension, a separation of native protein complexes is achived, while in the second dimension protein complexes will be denatured and protein subunits of the complexes are

separated. (Berg, Tymoczko et al. 2015)

2.4.4.1. Making and running 2D PAGE

A strip from a Native LDS-Polyacrylamide Gel can be cut out and run on a 2D SDS gel.
The method for making the gels are the same as for a Native LDS-Polyacrylamide Gel, but the recepi for the gel is different, and can be found under 2.2.2.Solubilisation. Use agarose to seal of the gel and make the stacking gel. Do not fill up completely to the top with the stacking solution. A small portion of the top needs to be clear in order to fit a gel strip from the native gel. Add water solubilised butanol to the top of the stacking gel to make it even.
The gel strip is cut out from the Native LDS-Polyacrylamide Gel and placed into a LDS Sample Buffer. Incubate for about 10 min before transferring the strip to the 2D gel. A ladder can be loaded onto a small piece of filter paper and added next to the gel strip, most preferably at the running from side of the strip. Use Agarose to seal of the top of the gel, and assemble the 2D SDS gel in a chamber and run for 1-1.5 hour. Run the gel at 1200 V, 30 mM and 24W
After running the gel can be scanned and used for western blot.

2.4.5. Western blot

Western blot is a widely used method, used to detect specific proteins. In order to start, one first need to use gel electrophoresis to separate proteins in a sample. (Mahmood and Yang 2012) The proteins in the gel is then transferred to a membrane that can be treated with several different antibodies that are specific to a certain protein, and thus identifying it. (Mahmood and Yang 2012) The method utilizes the force of an electric current to transfer proteins on to the membrane. (Mahmood and Yang 2012) Proteins are transferred onto a membrane with the ability to bind proteins. (Mahmood and Yang 2012) In order to prevent any interactions between the membrane and the antibodies that will be used to detect the proteins, the membrane needs to be blocked from further protein binding after the transfer is completed. (Mahmood and Yang 2012) The membrane can easily be blocked by placing it in non-fat dry milk in tris-buffered saline (TBS). (Mahmood and Yang 2012) The proteins in the milk attaches to the membrane in all places where the target proteins have not attached, and thus further protein binding is not possible. (Mahmood and Yang 2012) The only binding that can happen is antibody binding to the specific proteins on the membrane, and thus specific proteins can be detected. (Mahmood and Yang 2012)
To be able to detect a protein on a membrane, two antibodies must be used, a primary antibody and a secondary one. (Mahmood and Yang 2012) The primary will be used to bind directly to the protein on the membrane, while the secondary one will bind to the primary antibody. (Mahmood

and Yang 2012) There are different methods available for detection of the antibodies, one of them is to utilize a fluorescent primary or secondary antibody. (Mahmood and Yang 2012)

2.4.5.1. Method for western blot

The method for western blott used in this experiment is; semi-dry electroblotting. This method for transferring proteins from a gel to a membrane was done as described by (Towbin 2009)
Take 3 filter papers and one membrane and soak it in towben (seperatly), and place the gel on top, add another 3 soaked filter papers on top and run a glass rod over the sandwich to get out any air bubbles. Electricity is applied, and the proteins should move from the gel down to the membrane. The filters and gel are removed, and the membrane needs to be placed into a container where the membrane will be covered with ponceau and placed on a shaker for 1 min. Pour back the ponceau and wash with dH_2O 3 times. Add 1x TBS and have it on a shaker until it is destained. It is possible to leave the gel in TBS on the counter overnight.
Pour out the TBS and add 5% milk, let it stand on shaker for 1 hour. This is to block the gel.
Remove the milk and have 5 mL of primary antibody, and let it incubate on shaker for 1 hour.
Take the antibody back to its container (can be used 3 times), and wash with 5 mL 1x TBS 3X5 min on shaker.
Add 5 mL of secondary antibody and incubate on shaker for 1 hour.
Put the antibody back into the container and wash 3x5 min 5mL TBS
During the last washing step (or before) prepare two solutions Ecl1 and Ecl2. The Ecl1 needs to be covered with aluminium foil due to it being light sensitive.
In the empty box mix the Ecl1 and Ecl2 solutions and soak the membranes in it for about 1 min.
Put the wet membranes onto a plastic folder and place in machine for photographing.
It is possible to use the blot several times, and to stain it with different antibodies.
In this thesis the method utilized to look at the blot is by using a fluorescent primary or secondary antibody. This can be scanned with the Odysse at 800nm. The preparation method of the membrane is the same as described above, except the primary or secondary antibody is fluorescent and needs to be kept in the dark during shaking and before scanning.

2.4.6. Pigment determination

Calculate the amount needed from the sample acquired from the isolation step. Take out that amount and freeze it in liquid nitrogen. Add acetone -80 ºC, and wait for the sample to thaw, and store the sample at -80 ºC. The sample can the next day be thawed and Chl a added.

To know how much chlorophyll to add, chlorophyll concentration needs to be calculated. This is done spectrophotometrically. Chlorophyll is added to a solution of 80% acetone/20% HEPES. Once the concentration needed is calculated the sample can be spun, and the supernatant transferred to another tube. The Chl a can then be added to the supernatant.
The sample then needs to be dried, this is done my utilizing a centrifuge that is put under vacuum conditions and the sample is slowly dried.
After drying the sample can be stored at -80 ºC.

2.5. Thin Layer Chromatography

Chromatography is a method existing in many different forms, in general the principal idea with this method is to separate molecules in a sample. Thin layer chromatography (TLC), accoarding to its name, is a thin layer on top of a glass plate. A sample is applied to the plate and will due to capillary force, move up on the plate. A sample can be visible to the naked eye, moving upward, or a plate can be scanned after running to see the result.

Figure 2.5.A: TLC plate.
A TLC plate can excist in many different forms. The one used in this thesis have a glass plate on the back, as well as two layers. One thinner layer where the sample is applied at the bottom part of it, and a thicker layer on the top. The sample applied to the plate will move upwards through capillary forces.

The plate used in this experiment have two parts, the first part where the sample is applied is much thinner than the top part. The sample will be working its way up on the plate, meeting more resistance in the beginning due to a thinner plate, and when moving into the second part will have less resistance due to more space, and will move faster.

2.5.1. Pigment extraction

The pigments were extracted from whole plastids, and were extracted according to (Mork-Jansson, Bue et al. 2015).

3. Results

3.1. Solubilisations

3.1.1. One; Test of detergent added in both samples and cathode buffer, and pH of buffer

In order to get an overview of the method, the first solubilisations preformed in this thesis was done using etioplasts isolated from plants grown in the dark, but isolated in light environment. The first solubilisation were preformed with some changes to the standard (2.4. Solubilisation) in order to acquire the results in this part (Figure 3.1.1.A-3.1.1.F). In this experimental part digitonin was added as a detergent with different concentrations, as well as another detergent, DM. DM was added with the same concentrations as digitonin.

Most of the detergents were also added in two steps, once before spinning, and once after spinning, the total amount of the detergent added will be explained.
The different samples were all taken from L2, this was the second sample of etioplasts isolated. There were in total 7 samples and one ladder loaded onto the gel:
1: Ladder, 2: L2, 3: Di 1 mM, 4: Di 2mM, 5: Di 2,6mM, 6: DM 1mM, 7: DM 2mM, 8: DM 2.6 mM.
The L2 sample in itself had 4mM digitonin added before spinning, while all the other samples have extra detergent added in a step after spinning. The samples were All samples were spun for 2 min at 2000 rpm and then at 30 000 rpm for 30 min at 10 °C. The name of the samples 3-8 was fashioned after the concentration of detergent added after spinning. Sample 3 ha 1 mM digitonin added after spinning, giving it a total concentration of 5 mM Digitonin. The same goes for all the other samples, where sample 4 and 5 have 2 and 2.6mM digitonin added after spinning, giving them a total concentration of 6 and 6.2mM digitonin. The concentration of DM for sample 6-8 is the same as the digitonin concentration for sample 3-5.

4 different cathode buffers were used in this part.

1. One cathode buffer, 80mM Tricine and 15 mM Bis-Tris. pH 7.07
2. Cathode buffer, 80mM Tricine and 15mM Bis-Tris pH adjusted; pH 6.74.
3. Cathode buffer 80mM Tricine and 15 mM Bis-Tris with a LDS concentration of 0.04mM, and pH adjusted; pH 6.74.
4. Third was a cathode buffer 50mM Tricine and 15mM Bis-Tris with a LDS concentration of 0.04mM and a pH 6.74.

One of the buffers (80mM Tricine and 15mM Bis-Tris) were used as the base for 3 of the cathode

buffers. While the fourth cathode buffer used was a separate one.

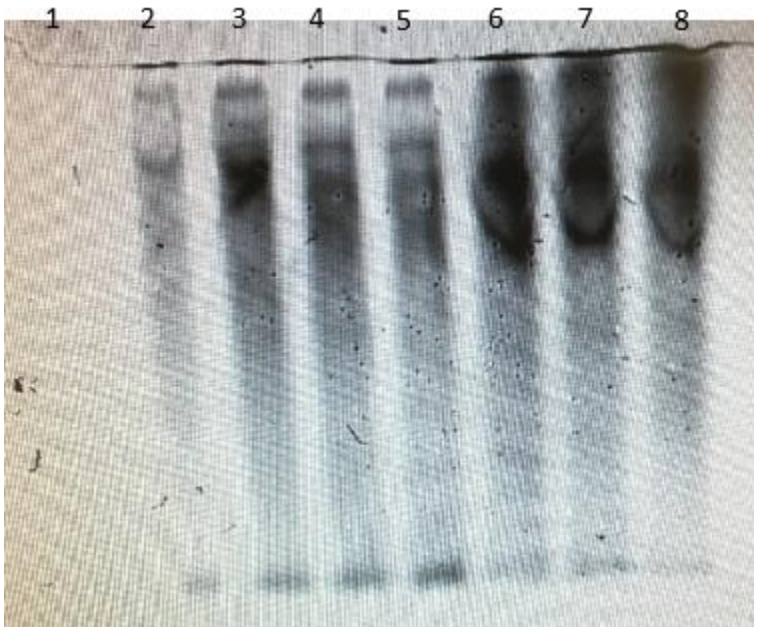


Figure: 3.1.1.A Result from running a gel with a cathode buffer with tricine concentration of 80mM and Bis-Tris concentration of 15mM pH 7.

Etioplasts were isolated and solubilized in the dark and applied to the gel. The samples were prepared with different concentration of detergent, and two different detergents were used, digitonin and DM. The samples are as follows: 1: Ladder, 2: L2, 3: Di 1 mM, 4: Di 2mM, 5: Di 2,6mM, 6: DM 1mM, 7: DM 2mM, 8: DM 2.6 mM. Sample 2 have 4 mM digitonin added in one step, sample 3 have 5 mM digitonin added in two steps, sample 4 have 6 mM digitonin added in two steps and sample 5 have 6.2mM digitonin added in two steps. Sample 6 have 5 mM DM added in two step, sample 7 have 6 mM DM added in two steps, sample 8 have 6.2 mM DM added in two steps. The sample 2 have detergent added in one step before spinning, while the other samples have detergent added in two steps before and after spinning. The gel were scanned at 700nm using the Odysse.

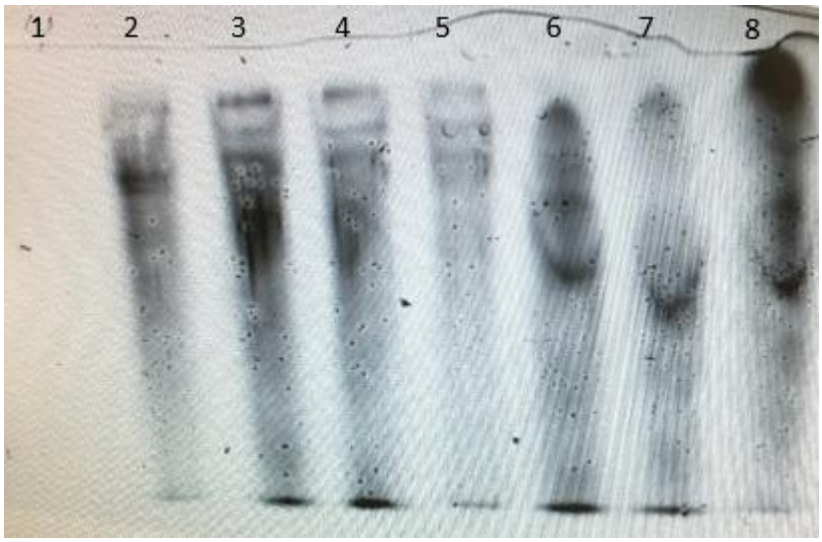


Figure: 3.1.1.B Result from running a gel with a cathode buffer with tricine concentration of 80mM and Bis-Tris concentration of 15mM pH 6.74.

Etioplasts were isolated and solubilized in the dark and applied to the gel. The samples were prepared with different concentration of detergent, and two different detergents were used, digitonin and DM. The samples are as follows: 1: Ladder, 2: L2, 3: Di 1 mM, 4: Di 2mM, 5: Di 2,6mM, 6: DM 1mM, 7: DM 2mM, 8: DM 2.6 mM. Sample 2 have 4 mM digitonin added in one step, sample 3 have 5 mM digitonin added in two steps, sample 4 have 6 mM digitonin added in two steps and sample 5 have 6.2mM digitonin added in two steps. Sample 6 have 5 mM DM added in two step, sample 7 have 6 mM DM added in two steps, sample 8 have 6.2 mM DM added in two steps. The sample 2 have detergent added in one step before spinning, while the other samples have detergent added in two steps before and after spinning. The gel were scanned at 700nm using the Odysse.

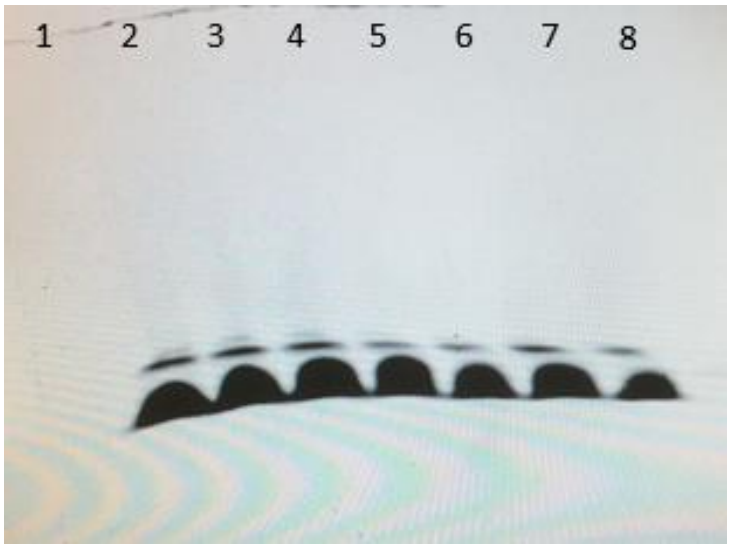


Figure: 3.1.1.C Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.04mM, pH 6.74.

Etioplasts were isolated and solubilized in the dark and applied to the gel. The samples were prepared with different concentration of detergent, and two different detergents were used, digitonin and DM. The samples are as follows: 1: Ladder, 2: L2, 3: Di 1 mM, 4: Di 2mM, 5: Di 2,6mM, 6: DM 1mM, 7: DM 2mM, 8: DM 2.6 mM. Sample 2 have 4 mM digitonin added in one step, sample 3 have 5 mM digitonin added in two steps, sample 4 have 6 mM digitonin added in two steps and sample 5 have 6.2mM digitonin added in two steps. Sample 6 have 5 mM DM added in two step, sample 7 have 6 mM DM added in two steps, sample 8 have 6.2 mM DM added in two steps. The sample 2 have detergent added in one step before spinning, while the other samples have detergent added in two steps before and after spinning. The gel were scanned at 700nm using the Odysse. The scanning of the gel were interruptes when the computer froze and shut down, therefor the scan in incomplete.

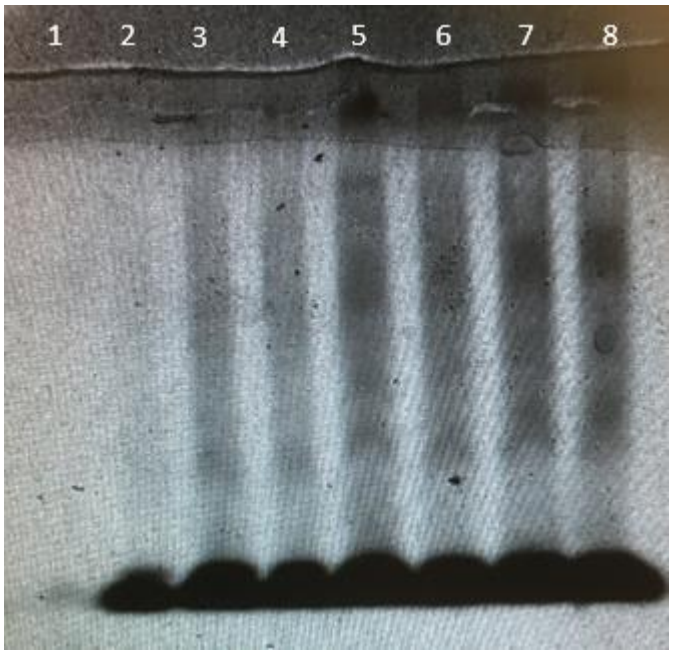


Figure: 3.1.1.D Result from running a gel with a cathode buffer with tricine concentration of 50mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.04mM, pH 6.74.
Etioplasts were isolated and solubilized in the dark and applied to the gel. The samples were prepared with different concentration of detergent, and two different detergents were used, digitonin and DM. The samples are as follows: 1: Ladder, 2: L2, 3: Di 1 mM, 4: Di 2mM, 5: Di 2,6mM, 6: DM 1mM, 7: DM 2mM, 8: DM 2.6 mM. Sample 2 have 4 mM digitonin added in one step, sample 3 have 5 mM digitonin added in two steps, sample 4 have 6 mM digitonin added in two steps and sample 5 have 6.2mM digitonin added in two steps. Sample 6 have 5 mM DM added in two step, sample 7 have 6 mM DM added in two steps, sample 8 have 6.2 mM DM added in two steps. The sample 2 have detergent added in one step before spinning, while the other samples have detergent added in two steps before and after spinning. The gel were scanned at 700nm using the Odysse.

The samples were loaded the same on all four gels. The four different cathode buffers used have given different result on each gel.
The effect of the detergent LDS added to the cathode buffer can be seen quite clearly on the gels (figure 3.1.1.A-3.1.1.F). When looking that the bottom of each gel it is quite clear that there is a higher signal given from figure 3.1.1.C and 3.1.1.F. LDS can be helpful when added in the right concentration, but it also can “wash out” the samples, for this reason the concentration of LDS added to the cathode buffer has been changed throughout the thesis. For figure 3.1.1.C the scan was not completed, therefor the image of the gel is not complete. The computer froze mid-scan, but the washed out effect can still be seen by the intense signal given off from the front of the gel. There were two buffers that had LDS added to the, where the tricine concertation were the difference between them. The same washed out effect can be seen for both buffers.

Comparing the buffer used for that gel, we have another buffer with 0.04mM LDS. The same complete washed out effect can not be see, as some bands are visible, but a large compiling of sample at the bottom can be seen in both figure 3.1.1.D, and the gel on the bottom of figure 3.1.1.E.

The two other buffers used do not have an addition of LDS, but have a difference in the pH of them. It is possible in figure 3.1.1.A and 3.1.1.B to see that pH of the cathode buffer can also affect the samples. The samples appear to also get a washed out effect by lowering the pH of the buffer. When comparing the bands of all the samples, especially sample 6-8 in figure 3.1.1.A and 3.1.1.B we can see this washed out effect. Where the bands do get move visible, as more of the "background noise" is washed out. The same effects can also be seen on gel a and b of figure 3.1.1.E.

There is also a noticeable difference between the different detergents used. Digitonin and DM was the two detergents chosen for this particular experiment, in order to determine which of them gave the best result. It is quite clear from looking at figure 3.1.1.A and 3.1.1.B that digitonin give the samples clearer bands that DM. Although using DM together with LDS seems to help give clearer bands to look at, this effect can be seen in figure 3.1.1.C, where bands similar to the bands given with digitonin can be observed. Even though bands can be detected with DM in combination with LDS, it can also be seen in figure 3.1.1.C that high concentrations of digitonin in addition to LDS gives more bands, this effect can be seen looking at sample 5, where a total amount of 5 bands can be counted, compared to 3 bands seen on all the other samples 2-4 and 6-8.

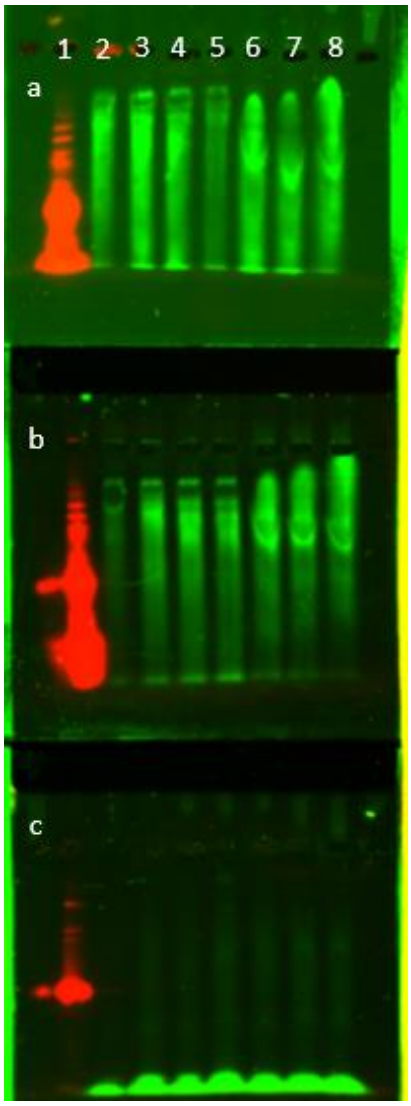


Figure: 3.1.1.E Result from running three gels. Gel a with a cathode buffer with tricine concentration of 80mM and Bis-Tris concentration of 15mM pH 6.74. Gel b with a buffer with tricine concentration of 80mM and Bis-Tris concentration of 15mM pH 7. Gel c with a buffer with tricine concentration of 50mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.04mM, pH 6.74.

Etioplasts were isolated and solubilized in the dark and applied to the gel. The samples were prepared with different concentration of detergent, and two different detergents were used, digitonin and DM. The samples are as follows: 1: Ladder, 2: L2, 3: Di 1 mM, 4: Di 2mM, 5: Di 2,6mM, 6: DM 1mM, 7: DM 2mM, 8: DM 2.6 mM. Sample 2 have 4 mM digitonin added in one step, sample 3 have 5 mM digitonin added in two steps, sample 4 have 6 mM digitonin added in two steps and sample 5 have 6.2mM digitonin added in two steps. Sample 6 have 5 mM DM added in two step, sample 7 have 6 mM DM added in two steps, sample 8 have 6.2 mM DM added in two steps. The sample 2 have detergent added in one step before spinning, while the other samples have detergent added in two steps before and after spinning. The gel were scanned at 633 nM using the Typhoon scanner.

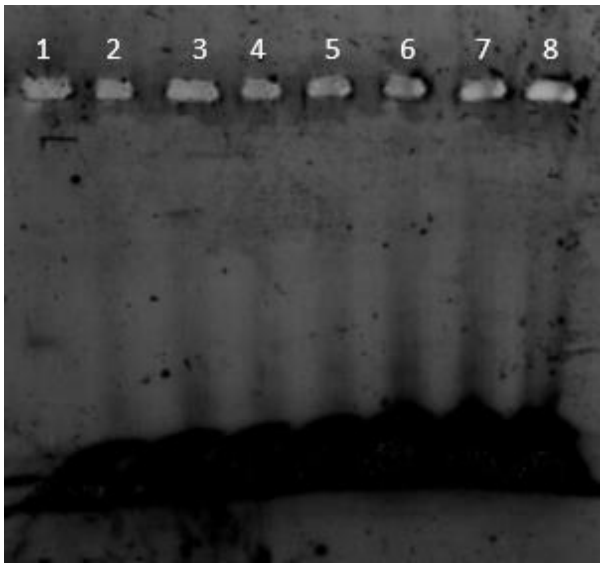


Figure: 3.1.1.F Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.04mM, pH 6.74.

Etioplasts were isolated and solubilized in the dark and applied to the gel. The samples were prepared with different concentration of detergent, and two different detergents were used, digitonin and DM. The samples are as follows: 1: Ladder, 2: L2, 3: Di 1 mM, 4: Di 2mM, 5: Di 2,6mM, 6: DM 1mM, 7: DM 2mM, 8: DM 2.6 mM. Sample 2 have 4 mM digitonin added in one step, sample 3 have 5 mM digitonin added in two steps, sample 4 have 6 mM digitonin added in two steps and sample 5 have 6.2mM digitonin added in two steps. Sample 6 have 5 mM DM added in two step, sample 7 have 6 mM DM added in two steps, sample 8 have 6.2 mM DM added in two steps. The sample 2 have detergent added in one step before spinning, while the other samples have detergent added in two steps before and after spinning. The gel were scanned at 633 nM using the Typhoon scanner.

3.1.2. Two; Test of different speed during spinning, detergent added to the samples and tricine concentration of the cathode buffer

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. During this second solubilisation different parameters were tested, speed and concentrations. During the spinning step, some of the samples (sample A) were spun with 16 000 rcf, while the others (sample B) were spun with 30 000 rcf. All samples were spun for 2 min at 2000 rpm and then at a higher speed for 30 min at 10 °C. Thee different cathode buffers were also used, with different Tricine concentration and different pH adjustments.

In total 10 samples were prepared:

Sample A divided into:

1: L2, 2: Di 2mM, 3: Di 2,6 mM, 4: Di + β-DM 2mM, 5: Di + β-DM 2.6 mM

Sample B divided into:

6: L2, 7: Di 2mM, 8: Di 2.6mM, 9: Di + β-DM 2mM, 10: Di + β-DM 2.6Mm.
Samples 1 and 6 have 4 mM digitonin added in one step, samples 2 and 7 have 6 mM digitonin added in two steps. Samples 3 and 8 have 6.2 mM digitonin added in two steps. Samples 4 and 9 have 6 mM of β-DM added in two steps, and samples 5 and 10 have 6.2 mM β-DM added in two steps. Samples 1 and 6 have detergent added in one step before spinning while the other samples have detergent added in two steps, before and after spinning.

Cathode buffers:
A: 1x Cathode buffer
85mM Tricine
15mM Bis-Tris
pH 7,0
B: 1x Cathode buffer
80mM Tricine
15 mM Bis-Tris
pH 7.0
C: 1x Cathode buffer
80mM Tricine
15mM Bis-Tris pH 7,0.

The three buffers all have the same pH, but buffer C was made with a solution of 15mM Bis-Tris that had a pH of 7.0 before adding it to the a solution of 80Mm Tricine. Buffers A and B had the pH of the whole buffer after mixing measured to pH 7.0. The concentration of Tricine have also been adjusted to see how it will affect the samples.

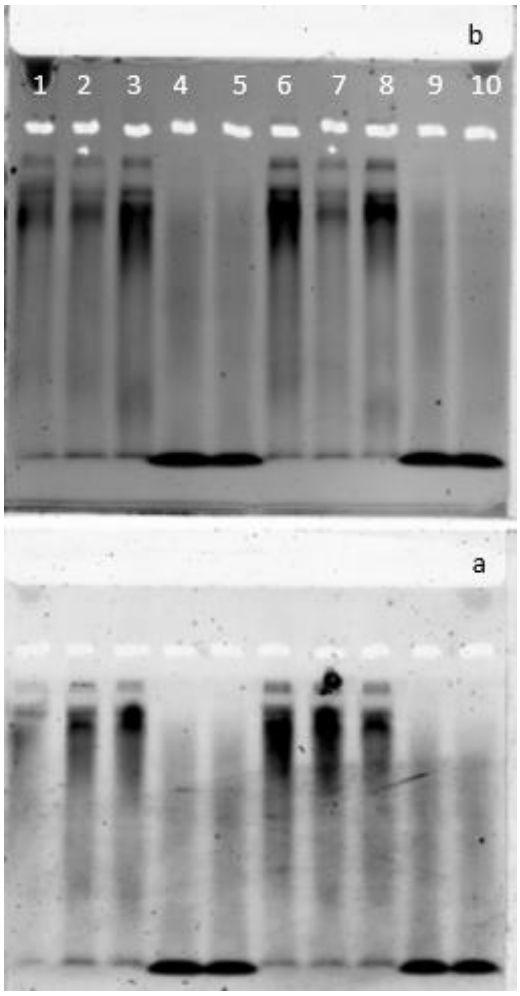


Figure: 3.1.2.A Result from running two gels. Gel a with a cathode buffer with tricine concentration of 85mM and Bis-Tris concentration of 15mM pH 7. Gel b with a buffer with tricine concentration of 80mM and Bis-Tris concentration of 15mM pH 7.

The samples on the gel were divided into two, samples A and samples B. During the spinning step, samples A were spun with 16 000 rcf, while samples B were spun with 30 000 rcf. All samples were spun for 2 min at 2000 rpm and then at a higher speed for 30 min at 10 °C. The samples on the gel: Samples A: 1: L2, 2: Di 2mM, 3: Di 2,6 mM, 4: Di + β-DM 2mM, 5: Di + β-DM 2.6 mM. Samples B: 6: L2, 7: Di 2mM, 8: Di 2.6mM, 9: Di + β-DM 2mM, 10: Di + β-DM 2.6Mm. Samples 1 and 6 have 4 mM digitonin added in one step, samples 2 and 7 have 6 mM digitonin added in two steps. Samples 3 and 8 have 6.2 mM digitonin added in two steps. Samples 4 and 9 have 6 mM of β-DM added in two steps, and samples 5 and 10 have 6.2 mM β-DM added in two steps. Samples 1 and 6 have detergent added in one step before spinning while the other samples have detergent added in two steps, before and after spinning. The gel were scanned at 633nm with the Typhoon.

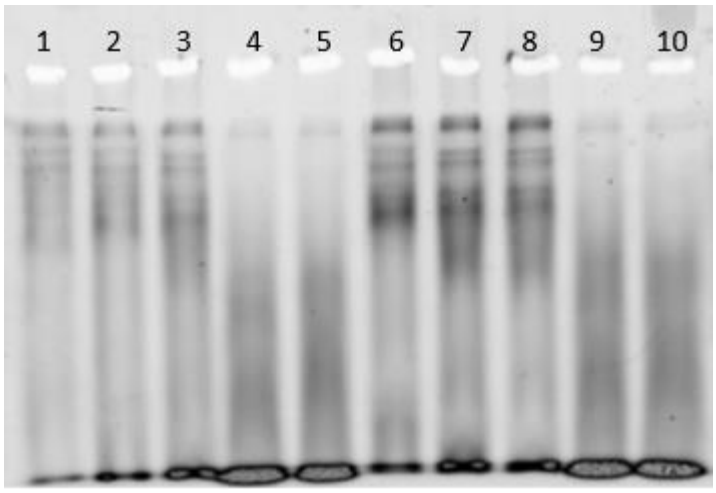


Figure: 3.1.2.B Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM that were pH adjusted to a pH 7 before mixing it with Tricine.

The samples on the gel were divided into two, samples A and samples B. During the spinning step, samples A were spun with 16 000 rcf, while samples B were spun with 30 000 rcf. All samples were spun for 2 min at 2000 rpm and then at a higher speed for 30 min at 10 °C. The samples on the gel: Samples A: 1: L2, 2: Di 2mM, 3: Di 2,6 mM, 4: Di + β-DM 2mM, 5: Di + β-DM 2.6 mM. Samples B: 6: L2, 7: Di 2mM, 8: Di 2.6mM, 9: Di + β-DM 2mM, 10: Di + β-DM 2.6Mm. Samples 1 and 6 have 4 mM digitonin added in one step, samples 2 and 7 have 6 mM digitonin added in two steps. Samples 3 and 8 have 6.2 mM digitonin added in two steps. Samples 4 and 9 have 6 mM of β-DM added in two steps, and samples 5 and 10 have 6.2 mM β-DM added in two steps. Samples 1 and 6 have detergent added in one step before spinning while the other samples have detergent added in two steps, before and after spinning. The gel were scanned at 633nm with the Typhoon.

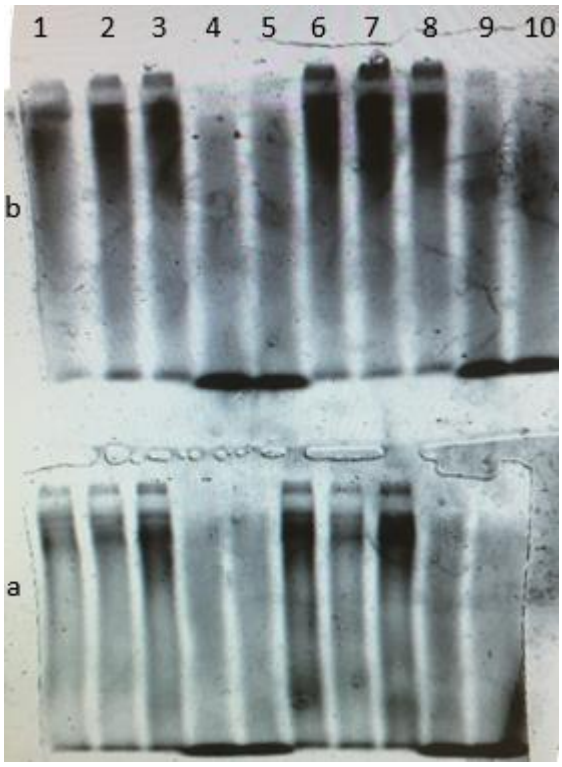


Figure: 3.1.2.C Result from running two gels. Gel a with a cathode buffer with tricine concentration of 85mM and Bis-Tris concentration of 15mM pH 7. Gel b with a buffer with tricine concentration of 80mM and Bis-Tris concentration of 15mM pH 7.

The samples on the gel were divided into two, samples A and samples B. During the spinning step, samples A were spun with 16 000 rcf, while samples B were spun with 30 000 rcf. All samples were spun for 2 min at 2000 rpm and then at a higher speed for 30 min at 10 °C. The samples on the gel: Samples A: 1: L2, 2: Di 2mM, 3: Di 2,6 mM, 4: Di + β-DM 2mM, 5: Di + β-DM 2.6 mM. Samples B: 6: L2, 7: Di 2mM, 8: Di 2.6mM, 9: Di + β-DM 2mM, 10: Di + β-DM 2.6Mm. Samples 1 and 6 have 4 mM digitonin added in one step, samples 2 and 7 have 6 mM digitonin added in two steps. Samples 3 and 8 have 6.2 mM digitonin added in two steps. Samples 4 and 9 have 6 mM of β-DM added in two steps, and samples 5 and 10 have 6.2 mM β-DM added in two steps. Samples 1 and 6 have detergent added in one step before spinning while the other samples have detergent added in two steps, before and after spinning. The gel were scanned at 700nm with the Odysse.

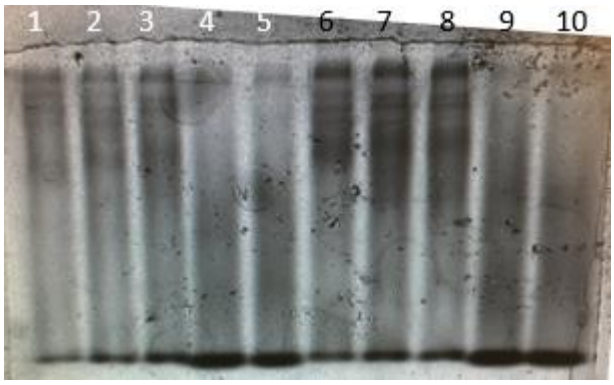


Figure: 3.1.2.D Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM that were pH adjusted to a pH 7 before mixing it with Tricine.
The samples on the gel were divided into two, samples A and samples B. During the spinning step, samples A were spun with 16 000 rcf, while samples B were spun with 30 000 rcf. All samples were spun for 2 min at 2000 rpm and then at a higher speed for 30 min at 10 °C. The samples on the gel: Samples A: 1: L2, 2: Di 2mM, 3: Di 2,6 mM, 4: Di + β-DM 2mM, 5: Di + β-DM 2.6 mM. Samples B: 6: L2, 7: Di 2mM, 8: Di 2.6mM, 9: Di + β-DM 2mM, 10: Di + β-DM 2.6Mm. Samples 1 and 6 have 4 mM digitonin added in one step, samples 2 and 7 have 6 mM digitonin added in two steps. Samples 3 and 8 have 6.2 mM digitonin added in two steps. Samples 4 and 9 have 6 mM of β-DM added in two steps, and samples 5 and 10 have 6.2 mM β-DM added in two steps. Samples 1 and 6 have detergent added in one step before spinning while the other samples have detergent added in two steps, before and after spinning. The gel were scanned at 700nm with the Odysse.

Figure 3.1.2.A show a difference in the Tricine concentration. Gel b have a tricine concentration of 85mM while gel a have a Tricine concentration of 80mM. Gel a is not quite as clear as gel b, but it does have clearer bands. By lowering the Tricine concentration bands have become more visible, without having the same washed out effect that was seen by the addtition of LDS. This same effect of clear bands can be seen when comparing gel a and b in figure 3.1.2.C.
Figure 3.1.2.B and 3.1.2.D show the gel run with the buffer that had a Bis-Tris solution pH adjusted before adding Tricine to it. This variation of the buffer also gives off clear bands, with no washed out effect.
From all figures 3.1.2.A-3.1.2.D it can quit clearly be seen that the last two samples, samples 9-10, have a strong washed out effect, caused by the addition of DM to the sample containing digitonin. Most of the bands that can clearly be seen in the other samples 1-8 is no longer visible in these two samples, and it was determined that the addition of DM was not contributing to any positive result.
Samples 1-5 were spun at 30 000 rpm while sample 6-10 were spun at 16 000 rpm. Looing at all the samples in all figures 3.1.2.A-3.1.2.D there is no clear difference between the samples spun a

different speed. It was expected to see a certain difference between the two, but alas no such difference can be distinguished.

3.1.3. Three; Test of different speed during spinning and detergent added to the samples and cathode buffer

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. During this solubilisation a new detergent along with digitonin was used. Although β-DM was determined to be to strong of a detergent to use with the samples, during this part it was still decided to try and see what effect α-DM would have on the samples. Like the last solubilisations two sets of samples were made, A and B, where the A samples were spun at 30 000 rpm and the B samples at 16 000 rpm. All samples were spun for 2 min at 2000 rpm and then at a higher speed for 30 min at 10 °C. The samples were prepared the same for the fourth solubilisations except instead of β-DM α-DM was used as the second detergent. The detergent for sample 2-6 and 7-10 were added in two steps, before and after the spinning step and the total detergent concentration for the samples were; 1: 4Mm digitonin, 2: 6mM digitonin 3: 6.2mm digitonin 4: 6mM α-DM, 5: 6.2mM α-DM, 6: 4mM digitonin, 7: 6mM digitonin, 8: 6.2mM digitonin, 9: 6mM α-DM and 10: 6.2mM α-DM.
Sample A divided into:
1: M2, 2: Di 2mM, 3: Di 2,6 mM, 4: α-DM 2mM, 5: α-DM 2.6 mM
Sample B divided into:
6: M2, 7: Di 2mM, 8: Di 2.6mM, 9: α-DM 2mM, 10: α-DM 2.6Mm.

Two buffers were used in this part, or with and one without LDS. During the previous experiments the buffer with a tricine concentration of 80mM and a concentration of 15mM bis-tris were determine to be the best one. One of the buffers had 0.02mM LDS added to it, the effect of giving clearer bands can not be ignored, even if the use of LDS gives a chance of washing out the samples, and therefore one buffer with LDS was used as well in this part.
A
Tricine 80 mM
Bis-Tris 15mM
B
Tricine 80 mM
Bis-Tris 15mM
0.02mM LDS

The etioplasts used in this solubilisation were taken from M2. The difference between this sample and the one previously used in the two first solubilisations is that this one was isolated in the dark. The plants were grown in the dark, and the etioplasts were completely isolated in the dark. The only exposure to light that they have had is by the green light used in the dark room, and a very brief exposure to white light when loading on to the gel. The gel was run in a light room, but were placed inside a container, which unfortunately was not completely light proof. It is expected to see a difference between these samples from dark etioplasts and from the light etioplasts. This was the first of many solubilisations preformed using dark etioplasts.

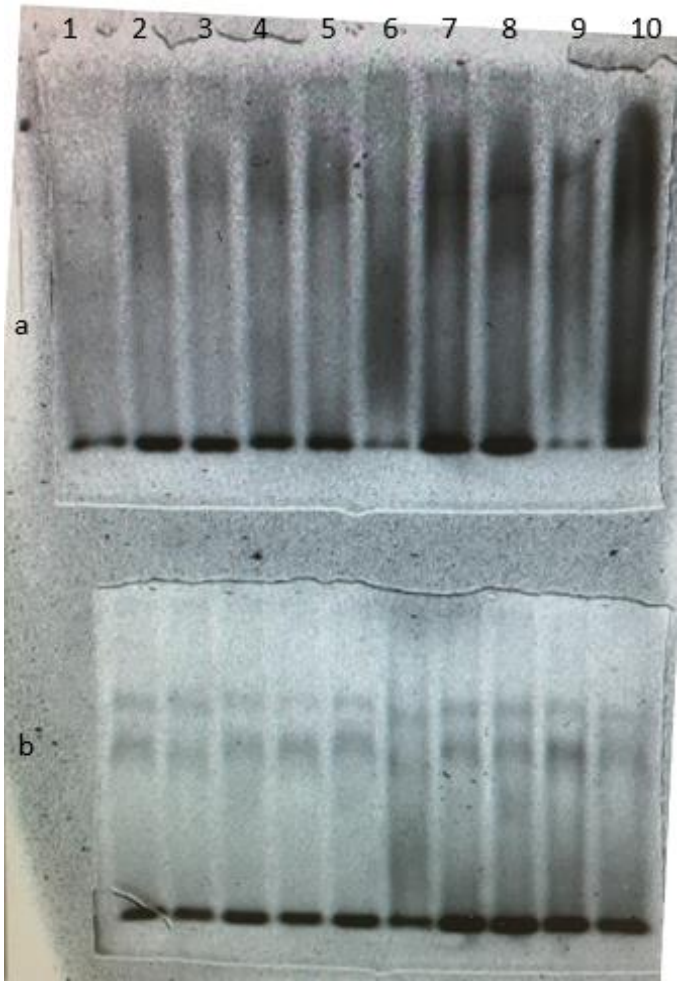


Figure: 3.1.3.A Result from running two gels. Gel a with a cathode buffer with tricine concentration of 80mM and Bis-Tris concentration of 15mM pH 7. Gel b with a buffer with tricine concentration of 80mM, Bis-Tris concentration of 15mM and LDS concentration of 0.02 mM pH 7.

The samples on the gel were divided into two, samples A and samples B. During the spinning step, samples A were spun with 16 000 rcf, while samples B were spun with 30 000 rcf. All samples were spun for 2 min at 2000 rpm and then at a higher speed for 30 min at 10 °C. The samples on the gel: Samples A: 1: M2, 2: Di 2mM, 3: Di 2,6 mM, 4: α-DM 2mM, 5: α-DM 2.6 mM. Samples B: 6: M2, 7: Di 2mM, 8: Di 2.6mM, 9: α-DM 2mM, 10: α-DM 2.6Mm. Samples 1 and 6 have 4 mM digitonin added in one step, samples 2 and 7 have 6 mM digitonin added in two steps. Samples 3 and 8 have 6.2 mM digitonin added in two steps. Samples 4 and 9 have 6 mM of α-DM added in two steps, and samples 5 and 10 have 6.2 mM α-DM added in two steps. Samples 1 and 6 have detergent added in one step before spinning while the other samples have detergent added in two steps, before and after spinning. The gel were scanned at 700nm with the Odysse.

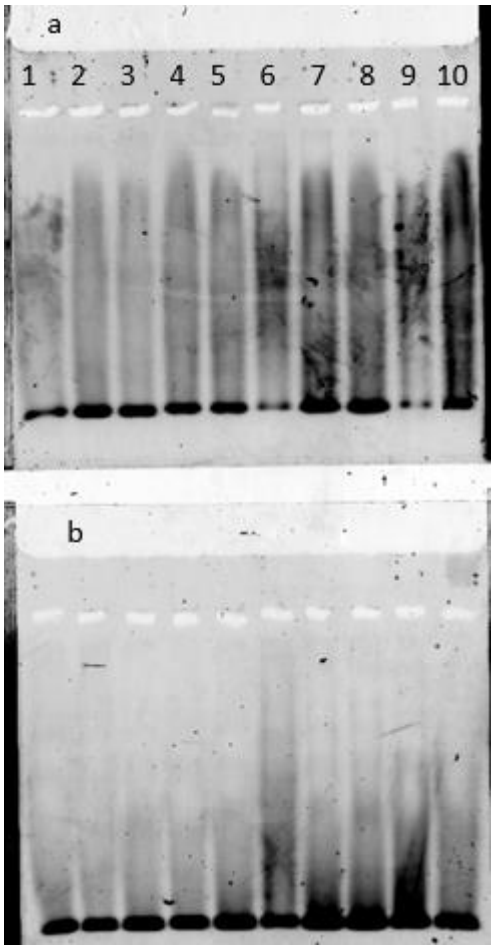


Figure: 3.1.3.B Result from running two gels. Gel a with a cathode buffer with tricine concentration of 80mM and Bis-Tris concentration of 15mM pH 7. Gel b with a buffer with tricine concentration of 80mM, Bis-Tris concentration of 15mM and LDS concentration of 0.02 mM pH 7.

The samples on the gel were divided into two, samples A and samples B. During the spinning step, samples A were spun with 16 000 rcf, while samples B were spun with 30 000 rcf. All samples were spun for 2 min at 2000 rpm and then at a higher speed for 30 min at 10 °C. The samples on the gel: Samples A: 1: M2, 2: Di 2mM, 3: Di 2,6 mM, 4: α-DM 2mM, 5: α-DM 2.6 mM. Samples B: 6: M2, 7: Di 2mM, 8: Di 2.6mM, 9: α-DM 2mM, 10: α-DM 2.6Mm. Samples 1 and 6 have 4 mM digitonin added in one step, samples 2 and 7 have 6 mM digitonin added in two steps. Samples 3 and 8 have 6.2 mM digitonin added in two steps. Samples 4 and 9 have 6 mM of α-DM added in two steps, and samples 5 and 10 have 6.2 mM α-DM added in two steps. Samples 1 and 6 have detergent added in one step before spinning while the other samples have detergent added in two steps, before and after spinning. The gel were scanned at 633 nm with the Typhoon.

In figure 3.1.3.B it is quite clear that the buffer with LDS (gel b) makes the bands a whole lot more clear than the one without (gel a). With the contrast settings of figure 3.1.3.B it is harder to see the bands as clearly, but they can still be seen faintly giving of a signal on gel b. The washing out effect is not quite as prominent during this solubilisation as it has been for the other ones, but

parts of the sample have been washed out making the bands visible.
Samples 1-5 were spun at 30 000 rpm, they quite clearly look very different than samples 6-10 that were only spun at 16 00 rpm. In figure 3.1.3.A gel a samples 6-10 have a much darker look than samples 1-5, the samples spun only at 16 000 rpm have not been spun at a high enough speed in order to separate the same way that samples 1-5 that were spun at 30 000 rpm. This difference is what was expected to be seen in the previous solubilisation (3.1.2 Two). When comparing the use of digitonin to the use of α-DM, there is no clear difference between them.

3.1.4. Four; Test of different speed during spinning and concentration of detergent added to the samples and cathode buffer

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. During this run the same procedure of having one sample with digitonin added in one step together with several samples where its added in two steps is followed. One of the parameters which were investigated during this part is how an increase in digitonin will affect the samples. The samples in this part was spun at 20 800 rpm, it was clear from previous solubilisations that a higher speed than 16 000 is needed, and during this part a speed of 20 800 rpm was tested. All samples were spun for 2 min at 2000 rpm and then at 20 800 for 30 min at 10 °C. The samples are as follows: 1: M2, 2: 2mM Digi, 3: 2.6mM Digi, 4: 6.7mM Digi and 5: 8mM digi.
Sample 1 have digitonin added in one step, while sample 2-5 have it added in two steps, before and after spinning. The total concentration of digitonin in the samples are: 1: 4mM, 2: 6mM, 3: 6.2mM, 4: 10.7mM and 5: 12mM.
The effect of LDS concentration was tested once again, where the buffers have LDS added in concentrations 0mM, 0.01mM, 0.02mM and 0.04mM.

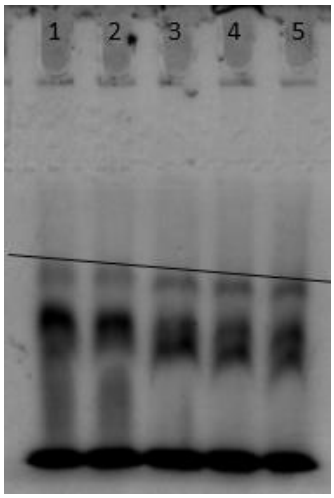


Figure: 3.1.4.A Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM pH 7.

The samples were spun for 2 min at 2000 rpm at 10 °C and then at 20 800 rpm for 30 min at 10 °C. The samples on the gel: 1: M2, 2: 2mM Digi, 3: 2.6mM Digi, 4: 6.7mM Digi and 5: 8mM digitonin. Sample 1 have 4 mM digitonin added in one step, while sample 2-5 have digitonin added in two steps. The total concentration of digitonin in the samples 2-5 are: 2: 6mM, 3: 6.2mM, 4: 10.7mM and 5: 12mM. The detergent were added once before spinning for sample 1, and were added in two steps for sample 2-5, before and after spinning. The gel were scanned at 700nm by the Odysse. The line across the gel shows how the samples have run further on the gel with higher concentration of digitonin.

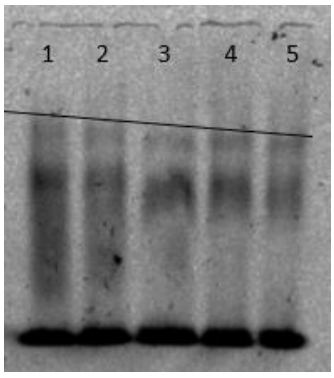


Figure: 3.1.4.B Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and LDS concentration of 0.01mM, pH 7.

The samples were spun for 2 min at 2000 rpm at 10 °C and then at 20 800 rpm for 30 min at 10 °C. The samples on the gel: 1: M2, 2: 2mM Digi, 3: 2.6mM Digi, 4: 6.7mM Digi and 5: 8mM digitonin. Sample 1 have 4 mM digitonin added in one step, while sample 2-5 have digitonin added in two steps. The total concentration of digitonin in the samples 2-5 are: 2: 6mM, 3: 6.2mM, 4: 10.7mM and 5: 12mM. The detergent were added once before spinning for sample 1, and were added in two steps for sample 2-5, before and after spinning. The gel were scanned at 700nm by the Odysse. The line across the gel shows how the samples have run further on the gel with higher concentration of digitonin.

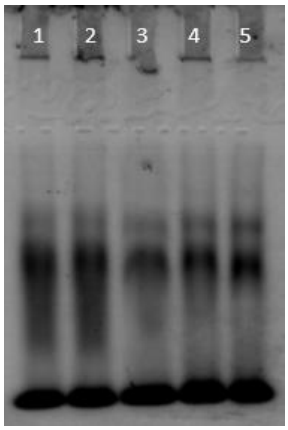


Figure: 3.1.4.C Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and LDS concentration of 0.02mM, pH 7.

The samples were spun for 2 min at 2000 rpm at 10 °C and then at 20 800 rpm for 30 min at 10 °C. The samples on the gel: 1: M2, 2: 2mM Digi, 3: 2.6mM Digi, 4: 6.7mM Digi and 5: 8mM digitonin. Sample 1 have 4 mM digitonin added in one step, while sample 2-5 have digitonin added in two steps. The total concentration of digitonin in the samples 2-5 are: 2: 6mM, 3: 6.2mM, 4: 10.7mM and 5: 12mM. The detergent were added once before spinning for sample 1, and were added in two steps for sample 2-5, before and after spinning. The gel were scanned at 700nm by the Odysse.

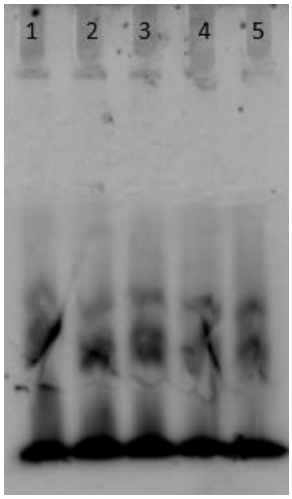


Figure: 3.1.4.D Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and LDS concentration of 0.04 mM, pH 7.

The samples were spun for 2 min at 2000 rpm at 10 °C and then at 20 800 rpm for 30 min at 10 °C. The samples on the gel: 1: M2, 2: 2mM Digi, 3: 2.6mM Digi, 4: 6.7mM Digi and 5: 8mM digitonin. Sample 1 have 4 mM digitonin added in one step, while sample 2-5 have digitonin added in two steps. The total concentration of digitonin in the samples 2-5 are: 2: 6mM, 3: 6.2mM, 4: 10.7mM and 5: 12mM. The detergent were added once before spinning for sample 1, and were added in two steps for sample 2-5, before and after spinning. The gel were scanned at 700nm by the Odysse. The gel had a unruly surface when removed from the gel chamber, which can be seen interfering with the samples visual outlook.

Through the three previous solubilisations, the buffer with 80mM Tricine and 15mM Bis-Tris has been chosen to be the cathode buffer that gives the most optimal bands. One of the parameters tested in this part was the addition of higher and higher concentrations of LDS in the cathode buffer. By studying the gels (figure 3.1.4.A-3.1.4.D) it can be seen that the sample gets more washed out, making some of the bands more visible, but also creating a larger deposit at the bottom of the gels.
The gel in figure 3.1.4.D a swirly pattern can be seen, this is not due to the samples itself, but rather to the composition of the gel. After the gel was run and taken out of the gel chamber, it had an unruly surface, which matches the pattern seen on the gel. It can also be seen here that most of the sample have gone into the gel, it is only in figure 3.1.4.C that there are any signs of some of the sample left in the wells, parts that most likely were too big to go into the pores of the gel.
The addition of more and more digitonin is not quite as prominent at the first glance, but the samples are distinguishable more lower on the gel for the samples with higher digitonin concentrations than the ones with low concentrations, with the helping line in figure 3.1.4.A and 3.1.4.B this difference can more clearly be seen.

3.1.5. Five; test of spin time and detergent concentration in cathode buffer

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. For this part one of the parameters testes was spin time. One of the parameters to perfect in a method is time, to find the best possible method that take the least of time. One other parameter that was continued to be investigated were the addition of LDS, having buffers with a LDS concentration of: 0mM, 0.02mM, 0.04mM and 0.05mM. One last parameter that was looked at was how etioplast volume would affect the solubilisation, when decreasing the etioplast volume, more water was added to the water to keep the total volume the same, and vice versa for when adding extra plastid volume.
Five samples were prepared for this part; 1: 2mM* spin 10 min, 2: 2mM* spin 30 min, 3: 6mM (added right away), 4: 1|2 plastid volume, 5: 1.5 pastids volume
Sample 1 had 6mM digitonin added in two steps, 4 mM + 2mM, before and after spinning, the sample were spun for 10 min at 20 800 rpm at 10°C. Sample 2 was prepared with the same concentration of digitonin as sample 1, and was spun for 30 min at 20 800 rpm at 10°C. Sample 3 instead of having 6mM digitonin added in two steps, have 6mM digitonin added in one step, before spinning, and were spun for 30 min at 20 800 rpm at 10°C. The two next sample, sample 4 and 5 have less and more etioplasts added. Sample 4 have half the volume while sample 5 have a

volume of 1.5 of the etioplast level of sample 1-3.

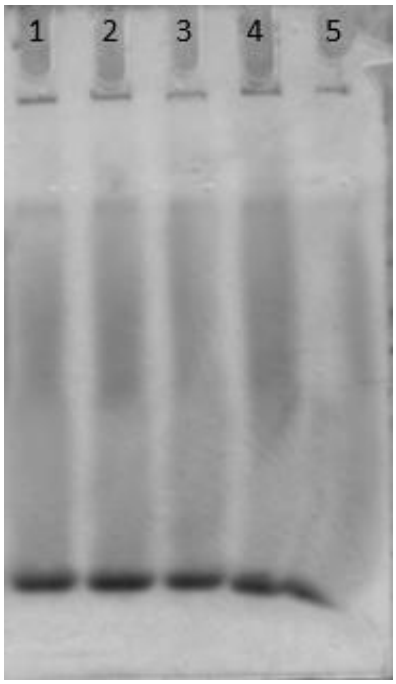


Figure: 3.1.5.A Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7.

The samples on the gel: 1: 2mM* spin 10 min, 2: 2mM* spin 30 min, 3: 6mM (added right away), 4: 1|2 plastid volume, 5: 1.5 plastids volume. Sample 1 have 6mM digitinon added in two steps, before and after spinning (4mM + 2mM), and the sample were spun 10 min at 20 800 rpm at 10°C. Sample 2 have 6 mM digitonin added in two steps, the same as sample 1, and were spun for 30 min at 20 800 rpm at 10°C. Sample 3 have 6mM digitonin added in one step, before spinning, and were spun for 30 min at 20 800 rpm at 10°C. Sample 4 have half the volume of etioplasts added, and sample 5 have 1.5 the volume of etioplasts added. The gel were scanned at 700nm with the Odysse.

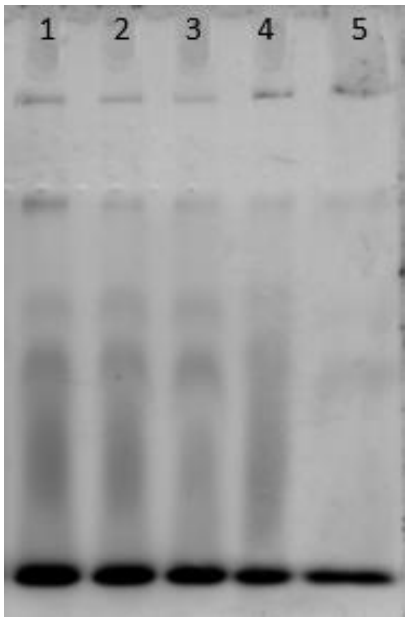


Figure: 3.1.5.B Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

The samples on the gel: 1: 2mM* spin 10 min, 2: 2mM* spin 30 min, 3: 6mM (added right away), 4: 1|2 plastid volume, 5: 1.5 plastids volume. Sample 1 have 6mM digitinon added in two steps, before and after spinning (4mM + 2mM), and the sample were spun 10 min at 20 800 rpm at 10°C. Sample 2 have 6 mM digitonin added in two steps, the same as sample 1, and were spun for 30 min at 20 800 rpm at 10°C. Sample 3 have 6mM digitonin added in one step, before spinning, and were spun for 30 min at 20 800 rpm at 10°C. Sample 4 have half the volume of etioplasts added, and sample 5 have 1.5 the volume of etioplasts added. The gel were scanned at 700nm with the Odysse.

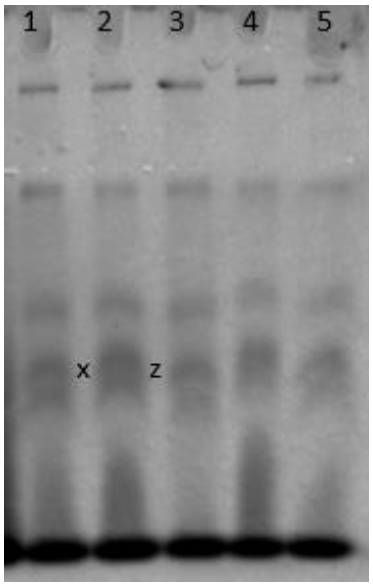


Figure: 3.1.5.C Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.04mM, pH 7.

The samples on the gel: 1: 2mM* spin 10 min, 2: 2mM* spin 30 min, 3: 6mM (added right away), 4: 1|2 plastid volume, 5: 1.5 plastids volume. Sample 1 have 6mM digitinon added in two steps, before and after spinning (4mM + 2mM), and the sample were spun 10 min at 20 800 rpm at 10°C. Sample 2 have 6 mM digitonin added in two steps, the same as sample 1, and were spun for 30 min at 20 800 rpm at 10°C. Sample 3 have 6mM digitonin added in one step, before spinning, and were spun for 30 min at 20 800 rpm at 10°C. Sample 4 have half the volume of etioplasts added, and sample 5 have 1.5 the volume of etioplasts added. The gel were scanned at 700nm with the Odysse. The x marks the spot where a band is more separated than the band marked by a z.

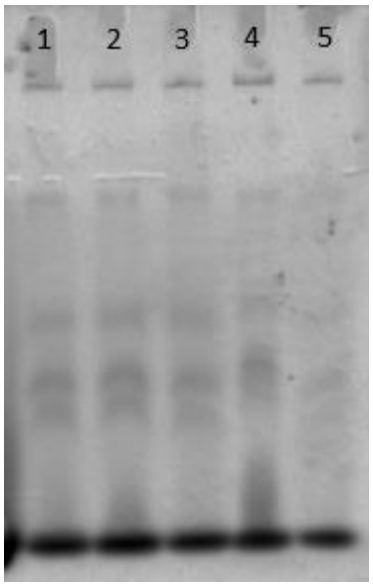


Figure. 3.1.5.D Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.05mM, pH 7.

The samples on the gel: 1: 2mM* spin 10 min, 2: 2mM* spin 30 min, 3: 6mM (added right away), 4: 1|2 plastid volume, 5: 1.5 plastids volume. Sample 1 have 6mM digitinon added in two steps, before and after spinning (4mM + 2mM), and the sample were spun 10 min at 20 800 rpm at 10°C. Sample 2 have 6 mM digitonin added in two steps, the same as sample 1, and were spun for 30 min at 20 800 rpm at 10°C. Sample 3 have 6mM digitonin added in one step, before spinning, and were spun for 30 min at 20 800 rpm at 10°C. Sample 4 have half the volume of etioplasts added, and sample 5 have 1.5 the volume of etioplasts added. The gel were scanned at 700nm with the Odysse.

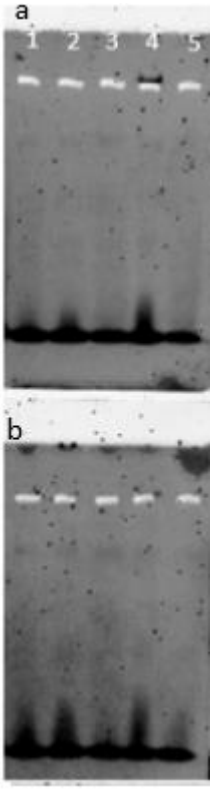


Figure: 3.1.5.E Result from running two gels. Gel a with a cathode buffer with tricine concentration of 80mM, Bis-Tris concentration of 15mM and a LDS concentration of 0.05mM, pH 7. Gel b with a buffer with tricine concentration of 80mM, Bis-Tris concentration of 15mM and LDS concentration of 0.04 mM pH 7. Gel c with a buffer with tricine concentration of 80mM, Bis-Tris concentration of 15mM and LDS concentration of 0.02 mM pH 7. Gel a with a buffer with tricine concentration of 80mM, and Bis-Tris concentration of 15mM and LDS, pH 7.

The samples on the gel: 1: 2mM* spin 10 min, 2: 2mM* spin 30 min, 3: 6mM (added right away), 4: 1|2 plastid volume, 5: 1.5 plastids volume. Sample 1 have 6mM digitinon added in two steps, before and after spinning (4mM + 2mM), and the sample were spun 10 min at 20 800 rpm at 10°C. Sample 2 have 6 mM digitonin added in two steps, the same as sample 1, and were spun for 30 min at 20 800 rpm at 10°C. Sample 3 have 6mM digitonin added in one step, before spinning, and were spun for 30 min at 20 800 rpm at 10°C. Sample 4 have half the volume of etioplasts added, and sample 5 have 1.5 the volume of etioplasts added. The gel were scanned at 633nm with the typhoon.

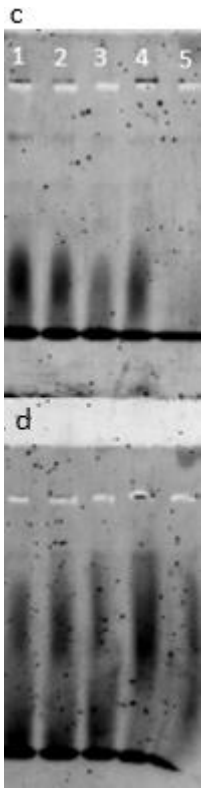


Figure 3.1.5.F. Result from running two gels. Gel c with a buffer with tricine concentration of 80mM, Bis-Tris concentration of 15mM and LDS concentration of 0.02 mM pH 7. Gel a with a buffer with tricine concentration of 80mM, and Bis-Tris concentration of 15mM and LDS, pH 7. The samples on the gel: 1: 2mM* spin 10 min, 2: 2mM* spin 30 min, 3: 6mM (added right away), 4: 1|2 plastid volume, 5: 1.5 plastids volume. Sample 1 have 6mM digitinon added in two steps, before and after spinning (4mM + 2mM), and the sample were spun 10 min at 20 800 rpm at 10°C. Sample 2 have 6 mM digitonin added in two steps, the same as sample 1, and were spun for 30 min at 20 800 rpm at 10°C. Sample 3 have 6mM digitonin added in one step, before spinning, and were spun for 30 min at 20 800 rpm at 10°C. Sample 4 have half the volume of etioplasts added, and sample 5 have 1.5 the volume of etioplasts added. The gel were scanned at 633nm with the typhoon.

One of the parameters looked at was spin time, when comparing sample 1 and 2 to each other in all figures 3.1.5.A-3.1.5.E there is virtually no difference between the two, except it seems that the separation of the bands from sample 1 seems to be better than for sample 2, this can be seen in figure 3.1.5.C where the x marks where two bands stands much closer together for the 30 min spin sample than for the 10 min spin sample.

Comparing the gels to each other, the same washing out effect as seen previously can also be noted in this part. The gel in figure 3.1.5.A there is no clear bands visible, but these bands start to show up the moment some LDS is added to the buffer. With a higher concentration of LDS the samples seem to “clear up”, giving another example of the washing out effect seen in previous

parts.
The effect of adding more or less etioplasts can not really be seen, but it is possible to see an improvement of separation in sample 3. Sample 3 had 6mM digitonin added in one step, before spinning, and not in two like the others. When looking at sample 3 it is possible to se that the band marked by the z is also separated better into two bands just as the bands marked by the x for sample 1.

3.1.6. Six; test of incubation time of digitonin and freezing of solubilized sample

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. During this part, it was decided that since there were really no difference between spinning for 10 min and 30 min, to only spin the samples for 10 min. One other parameter that can be changed in order to make the whole method go faster is the incubation time. Up until this part, the samples have always been incubated for 10 min with the digitonin, this is a parameter that was changed and tested. There were 3 samples that were made; 1: M4 1min, 2: M4 10min, 3: M2 6mM. Sample 1 were only incubated with digitonin for 1 min before spinning, and sample 2 were incubated for the normal 10 min before spinning. The third sample were added as an experiment to see how well a sample can handle being frozen and thawed before running it on a gel. Sample 3 used in this part is the same sample that can be seen in figure 3.1.5.A-3.1.5.E as named sample 3. All samples have a digitonin concentration of 6mM added in one step before spinning, and they were spun for 2 min at 2000g at 10°C, then for 10 min at 20100g at 10°C.

Only one buffer were used for this part:
80 mM Tricine
15 mM BisTris
0,02mM LDS
pH 7.0

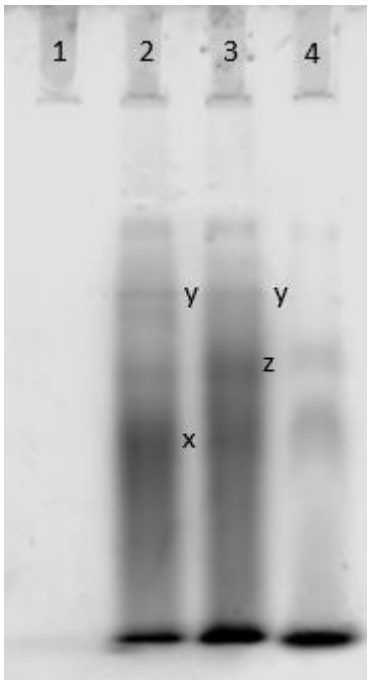


Figure: 3.1.6.A Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

The samples on the gel are: 1: M4 1min, 2: M4 10min, 3: M2 6mM. Sample 1 were only incubated with digitonin for 1 min before spinning, and sample 2 were incubated for the normal 10 min before spinning. Sample 3 is the same sample 3 as can be seen in figures 3.1.5.A-3.1.5.E. This sample have been frozen and thawed before applying on the gel. All samples have a digitonin concentration of 6mM added in one step before spinning, and they were spun for 2 min at 2000g at 10°C, then for 10 min at 20100g at 10°C. The gel were scanned at 700nm with the Odysse. The y markes the a band that only show up in sample 1 and 2. The z markes the band where sample 2 have a darker signal than sample 1, and the x markes the band which have a darker signal for sample 1 than for sample 2. Both sample 1 and 2 have a much darker signal than sample 3.

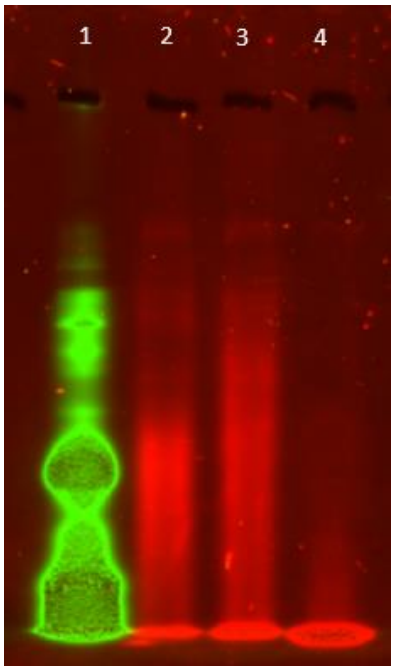


Figure: 3.1.6.B Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

The samples on the gel are: 1: M4 1min, 2: M4 10min, 3: M2 6mM. Sample 1 were only incubated with digitonin for 1 min before spinning, and sample 2 were incubated for the normal 10 min before spinning. Sample 3 is the same sample 3 as can be seen in figures 3.1.5.A-3.1.5.E. This sample have been frozen and thawed before applying on the gel. All samples have a digitonin concentration of 6mM added in one step before spinning, and they were spun for 2 min at 2000g at 10°C, then for 10 min at 20100g at 10°C. The gel were scanned at 633nm with the typhoon.

When looking at the three samples in figure 3.1.6.A, there is a clear difference between sample 1-2 and 3. Sample 3 is very clearly more faded than the other two, which is possibly a caused by the freezing of the sample. There is also a noticeable difference between sample 1 and 2. There is a difference in incubation time for these two samples. Sample 1 seems to have a darker signal coming from the lower parts of the gel marked by x, while sample 2 have a darker signal coming from a little higher up marked by z.

In figure 3.1.6.A there is one band, marked by y, that only show up in samples 1 and 2.

3.1.7. Seven; test of light exposure and addition of GGPP and NADPH

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. In this part one of the parameters looked at was the difference between a sample exposed to light and kept in the dark. One of the samples also had GGPP and NADPH added to it as well as being exposed to light.

3 samples were prepared;

1: M4D, 2: M4L and 3: M4L+.

Sample 1 had digitonin added in one step, before spinning, at a concentration of 6mM. It was incubated for 10 min and spun for 10 min at 20 800 rpm at 10°C. Sample 2 was exposed to light for 10 seconds before adding digitonin and incubating it for 10 min, and was spun at 10 min for 20 800 rpm at 10°C. Sample 3 had GGPP and NADPH added to it before exposing it to light for 10 seconds, digitonin was added and the sample were spun with the others for 10 min at 20 800rpm at 10 °C.

One buffer were used in this part; 80mM Tricine, 15 Mm Bis-Tris, 0.02mM LDS.

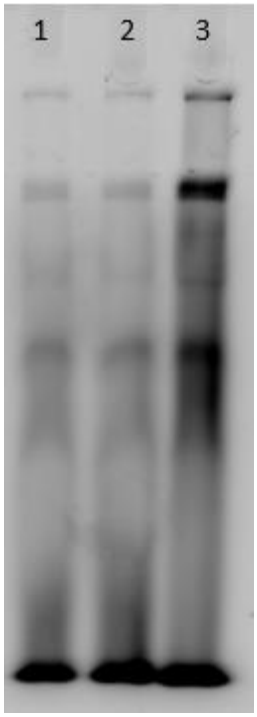


Figure: 3.1.7. Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

Sample 1 have 6mM digitonin added before spinning. Sample 2 were exposed to light for 10 sec, and 6 mM digitonin added before spinning. Sample 3 had GGPP and NADPH added before exposing it to light for 10 sec, 6mM digitonin were added to the sample before spinning. All samples were incubated for 10 min before spinning for 10 min for 20 800 rpm at 10°C. The gel were scanned at 700nm with the Odysse.

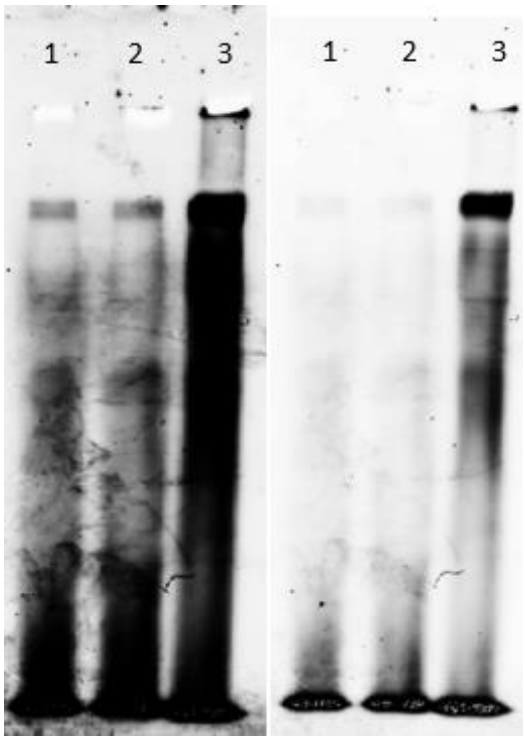


Figure: 3.1.7.B Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

Sample 1 have 6mM digitonin added before spinning. Sample 2 were exposed to light for 10 sec, and 6 mM digitonin added before spinning. Sample 3 had GGPP and NADPH added before exposing it to light for 10 sec, 6mM digitonin were added to the sample before spinning. All samples were incubated for 10 min before spinning for 10 min for 20 800 rpm at 10°C. The gel were scanned at 633nm with the typhoon.

In figures 3.1.7.A and 3.1.7.B when comparing sample 1-2 to sample 3 there is a clear difference between them, whereas sample 3 are quite a bit darker than the sample 1 and 2. Beside the signal given off from the samples are not quite as different as expected. All the bands can be seen in all of the three samples. In figure 3.1.7.B two pictures of the same gel have been included, the contrast of colour on the scan had to be adjusted to be able to see bands from sample 1 and 2, and adjusted again to be able to see the bands from sample 3.

3.1.8. Eight; test of light exposure and addition of GGPP and NADPH

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. In this part the light exposure was further investigated. 7 samples were made; 2: M4D-, 3: M4D, 4: M4DDM, 5: M4L, 6: M4L+, 7: M4DL+, 8: M4L+10.

All the samples except for sample 2 and 4, had digitonin added to them with a concentration of 6mM. Digitonin was added and the samples were incubated for 10 min before spinning. Sample 2

had no detergent added to it. Sample 4 had another detergent added to it, a combination of two detergents, digitonin and DM. This detergent were added to sample 4 and incubated for 10 min before spinning. All samples 2-8 were spun for 10 min at 20 800 rpm at 10 °C.
Both sample 3 and 4 were solubilized as according to the standard method, with no extra additives except for the detergent. Sample 5-6 and 8 were all exposed to light for 10 seconds, while sample 2-4 and 7 were not exposed to light.
Sample 5 were made mostly accoarding to the standard method, but it was exposed to light for 10 seconds before digitonin was added. Sample 6-8 had GGPP and NADPH added to it, it was added to sample 6 and 8 right before light exposure, and to sample 7 right before digitonin was added. Sample 8 were first exposed to light for 10 seconds, were incubated for 10 min before digitonin was added to it.
Two buffers were used for this part; A: 80mM Tricine, 15mM BisTris pH 7, B: 80mM Tricine, 15mM BisTris and 0.02mM LDS, pH 7.

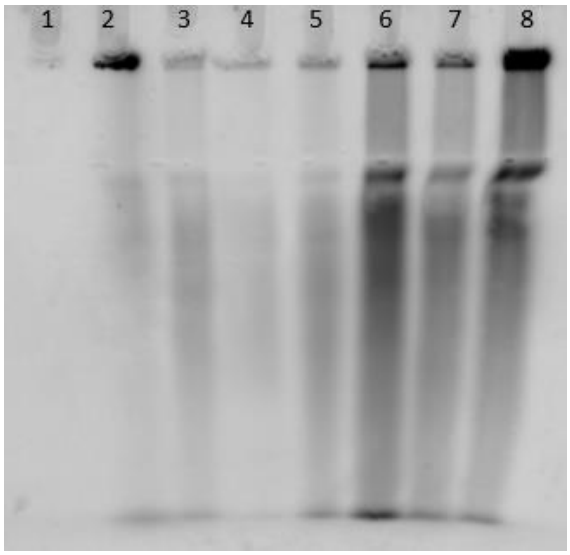


Figure. 3.1.8.A Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7.

The samples on the gel: 1: ladder 2: M4D-, 3: M4D, 4: M4DDM, 5: M4L, 6: M4L+, 7: M4DL+, 8: M4L+10. Sample 2 have no digitonin added to it. Sample 3 have 6mM digitonin added before spinning. Sample 4 have a detergent mix of digitonin and DM added before spinning. Sample 5 were exposed to light for 10 seconds before 6mM detergent were added before spinning. Sample 6 had GGPP and NADPH added to it before it was exposed to light for 10 sec, 6mM digitoin were added before spinning. Sample 7 had GGPP and NADPH added, with no light exposure, and 6mM digitonin added before spinning. Sample 8 had GGPP and NADPH added before it was exposed to light for 10 sec, incubated for 10 min before digitonin were added before spinning. All samples with detergent added, were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

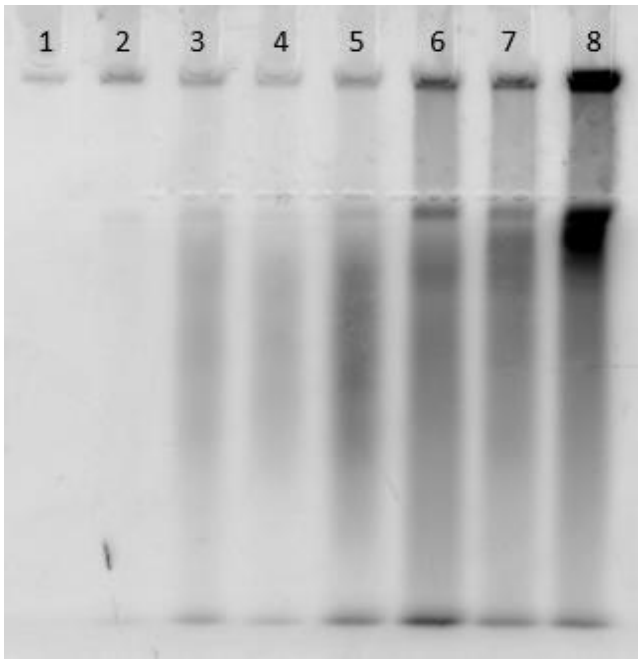


Figure. 3.1.8.B Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

The samples on the gel: 1: ladder 2: M4D-, 3: M4D, 4: M4DDM, 5: M4L, 6: M4L+, 7: M4DL+, 8: M4L+10. Sample 2 have no digitonin added to it. Sample 3 have 6mM digitonin added before spinning. Sample 4 have a detergent mix of digitonin and DM added before spinning. Sample 5 were exposed to light for 10 seconds before 6mM detergent were added before spinning. Sample 6 had GGPP and NADPH added to it before it was exposed to light for 10 sec, 6mM digitoin were added before spinning. Sample 7 had GGPP and NADPH added, with no light exposure, and 6mM digitonin added before spinning. Sample 8 had GGPP and NADPH added before it was exposed to light for 10 sec, incubated for 10 min before digitonin were added before spinning. All samples with detergent added, were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

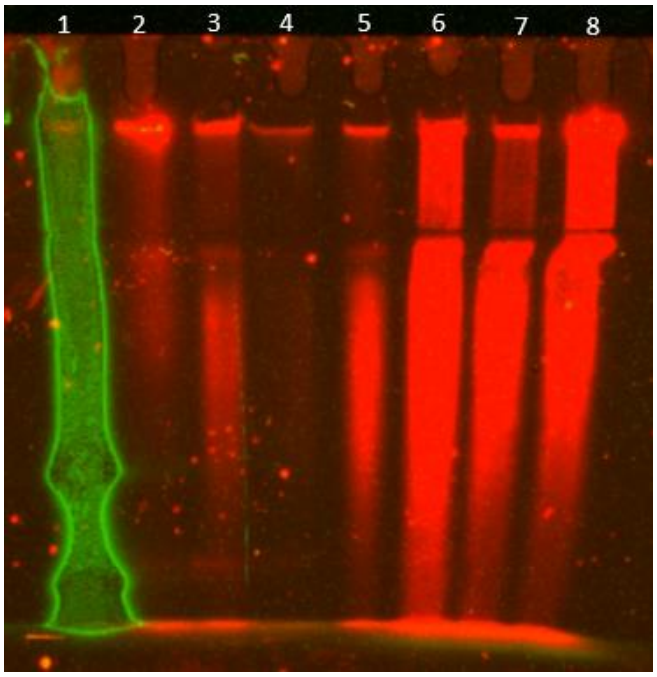


Figure. 3.1.8.C Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM and a LDS, pH 7.

The samples on the gel: 1: ladder 2: M4D-, 3: M4D, 4: M4DDM, 5: M4L, 6: M4L+, 7: M4DL+, 8: M4L+10. Sample 2 have no digitonin added to it. Sample 3 have 6mM digitonin added before spinning. Sample 4 have a detergent mix of digitonin and DM added before spinning. Sample 5 were exposed to light for 10 seconds before 6mM detergent were added before spinning. Sample 6 had GGPP and NADPH added to it before it was exposed to light for 10 sec, 6mM digitoin were added before spinning. Sample 7 had GGPP and NADPH added, with no light exposure, and 6mM digitonin added before spinning. Sample 8 had GGPP and NADPH added before it was exposed to light for 10 sec, incubated for 10 min before digitonin were added before spinning. All samples with detergent added, were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon.

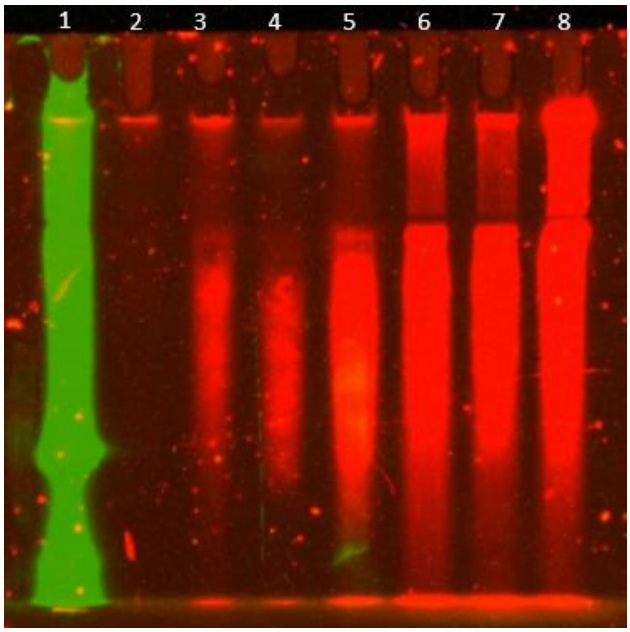


Figure. 3.1.8.D Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

The samples on the gel: 1: ladder 2: M4D-, 3: M4D, 4: M4DDM, 5: M4L, 6: M4L+, 7: M4DL+, 8: M4L+10. Sample 2 have no digitonin added to it. Sample 3 have 6mM digitonin added before spinning. Sample 4 have a detergent mix of digitonin and DM added before spinning. Sample 5 were exposed to light for 10 seconds before 6mM detergent were added before spinning. Sample 6 had GGPP and NADPH added to it before it was exposed to light for 10 sec, 6mM digitoin were added before spinning. Sample 7 had GGPP and NADPH added, with no light exposure, and 6mM digitonin added before spinning. Sample 8 had GGPP and NADPH added before it was exposed to light for 10 sec, incubated for 10 min before digitonin were added before spinning. All samples with detergent added, were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon scanner.

Comparing the two buffers used to one another there is a difference, but not quite the same distinguishable difference that have been seen in the previous results. The bands do not get clearer in the same way that they have previously, however it is possible to see that what seems to be left in the top of the gels (sample 2) , in figure 3.1.8.A is not seen in figure 3.1.8.B. It seems like more of the sample left in the wells of sample 6 and 7 have also gone into the gel with the help of LDS added to the cathode buffer.

Sample 2 in figure 3.1.8.A and 3.1.8.C there is a signal given, this signal was not expected to be seen, seeing as no detergent were added. It seems like its only in figures 3.1.8.A and 3.1.8.C that there is a signal for this sample, seeing as it is not possible to see anything in figures. 3.1.8.B and 3.1.8.D.

Comparing samples 2-4 and 7 with 5-6 and 8, it is possible to see a difference between the light exposed samples and the ones not. The biggest difference is seen between the dark samples that do not have any additives except for detergent.
The one sample that gave a surprising result were sample 8. Looking at sample 8 in figure 3.1.8.A, there is a much darker signal given for this sample compared to the others. This sample had GGPP and NADPH added to it before exposing it to light and letting it incubate for 10 min. These 10 minutes of incubation seems to have affected the sample quite a bit, compared to sample 6 that had digitonin added to it right after light exposure.
In the wells of sample 7-9 there is still sample left in the wells, all these samples have NADPH and GGPP added to them.

3.1.9. Nine; Test of addition of Chl a and b to freshly isolated dark etioplasts

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. In this part, chlorophyll a and b were added to the samples. 4 Samples were made from freshly isolated etioplasts; 1: M17, 2: M17Chlb, 3: M17Chla1, 4: M17Chla10
All samples have a concentration of digitonin of 6mM, and digitonin was added before spinning the samples. Sample 1 have no chlorophyll addition, but act as a blank and have HEPES 50mM added to it. Sample 2 have Chl b added to it. The sample were incubated for 10 min after Chl b was added and before loading onto the gel. Sample 3 have Chl a added to it. The sample were incubated for 1 min after adding Chl a and before loading onto the gel. Sample 4 had Chl a added to it. The sample were incubated for 10 min after adding Chl a and before loading onto the gel.
Two buffers were used;
A: 80mM Tricine, 15mM Bis-Tris pH 7
B: 80mM Tricine, 15mM Bis-Tris pH 7 0.02mM LDS

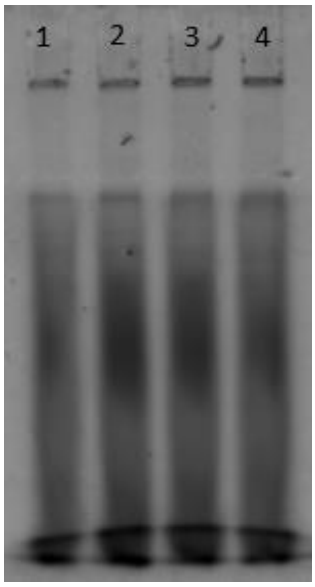


Figure. 3.1.9.A Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7.

The samples on the gel; 1: M17, 2: M17Chlb, 3: M17Chla1, 4: M17Chla10. All samples 1-4 were incubated for 10 min with digitonin before spinning. Sample 1 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 2 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 3 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

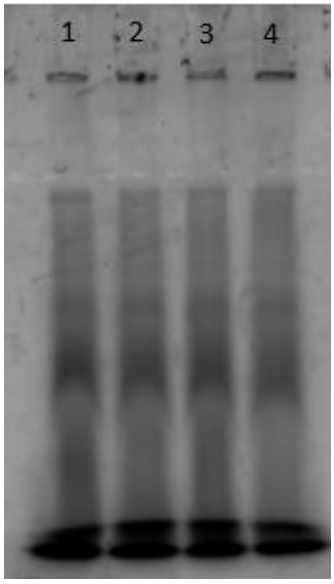


Figure. 3.1.9.B Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentratio of 0.02mM, pH 7.

The samples on the gel; 1: M17, 2: M17Chlb, 3: M17Chla1, 4: M17Chla10. All samples 1-4 were incubated for 10 min with digitonin before spinning. Sample 1 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 2 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 3 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

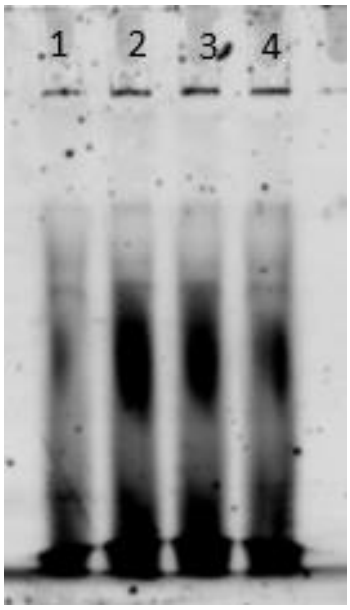


Figure. 3.1.9.C Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7.

The samples on the gel; 1: M17, 2: M17Chlb, 3: M17Chla1, 4: M17Chla10. All samples 1-4 were incubated for 10 min with digitonin before spinning. Sample 1 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 2 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 3 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon.

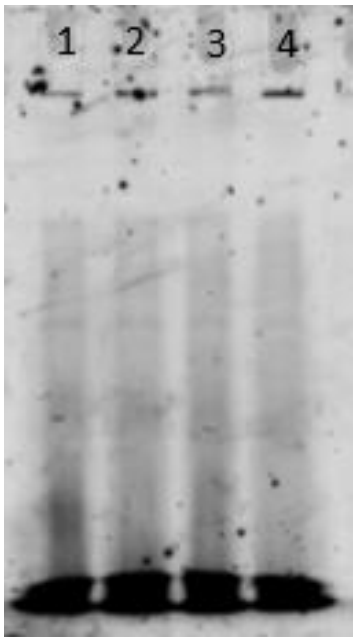


Figure. 3.1.9.D Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentratio of 0.02mM, pH 7.

The samples on the gel; 1: M17, 2: M17Chlb, 3: M17Chla1, 4: M17Chla10. All samples 1-4 were incubated for 10 min with digitonin before spinning. Sample 1 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 2 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 3 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon.

Two buffers were used, and the same washing out effect as seen previously in the other parts is also apparent in this part. Comparing figure 3.1.9.A and 3.1.9.B band that can clearly be seen in figure 3.1.9.B are not as prominent in figure 3.1.9.A. The LDS in the buffer have worked by washing out samples part of the sample, and the bands became visible. How much of the sample have been washed out, can be seen when comparing the front of both gel in figure 3.1.9.A and 3.1.9.B. In figure 3.1.9.B there is a much darker signal coming from the front than in figure 3.1.9.A.

When comparing the samples themselves, there are not much difference between the four. They are giving of almost the exact same signal for all the bands. It is only in figure 3.1.9.C that there is any form of obvious difference between them. When looking closer the same difference can be distinguished in figure 3.1.9.A, but it is not as clear of a difference.

3.1.10. Ten; Test of reproduction and freezing of samples after solubilisation

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. In this gel, the same samples as for part 3.1.9 Nine were made. They all have 6mM digitonin, were all spun at 20 800 rpm for 10 min at 10 °C and they all have an addition of HEPES or Chl a/b added right before loading.

However what should be noted is that in between the ninth and tenth gel running, there was one extra gel run. This gel was run using samples from M18. Due to some technical difficulties, the samples did not run, and seems to have diluted into the buffer, and the gel after scanning was completely blank. These samples had thankfully been saved in the freezer at -20'C, and was loaded alongside the samples prepared for the tenth solubilisation. This gave a good opportunity to again look at how well the samples handled being frozen and thawed before solubilizing.

Eight samples were loaded onto the gel; 1: M19, 2: M19Chlb, 3: M19Chla1, 4: M19Chla10, 5: M18, 6: M18Chlb, 7: M18Chla1, 8: M18Chla10.

Two buffers were used:

A: Tricine 80mM, BisTris 15mM pH 7

B: Tricine 80mM, BisTris 15mM 0.02mM LDS pH 7

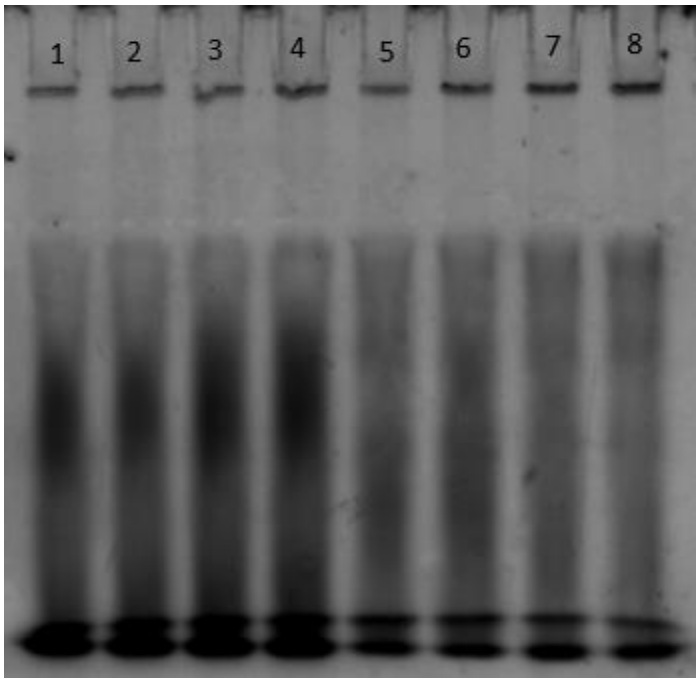


Figure. 3.1.10.A Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7.

Samples on the gel: 1: M19, 2: M19Chlb, 3: M19Chla1, 4: M19Chla10, 5: M18, 6: M18Chlb, 7: M18Chla1, 8: M18Chla10. All samples 1-8 were incubated for 10 min with digitonin before spinning. Sample 1 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 2 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 3 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 10 min before applying to the gel. Sample 5 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 6 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 7 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 8 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

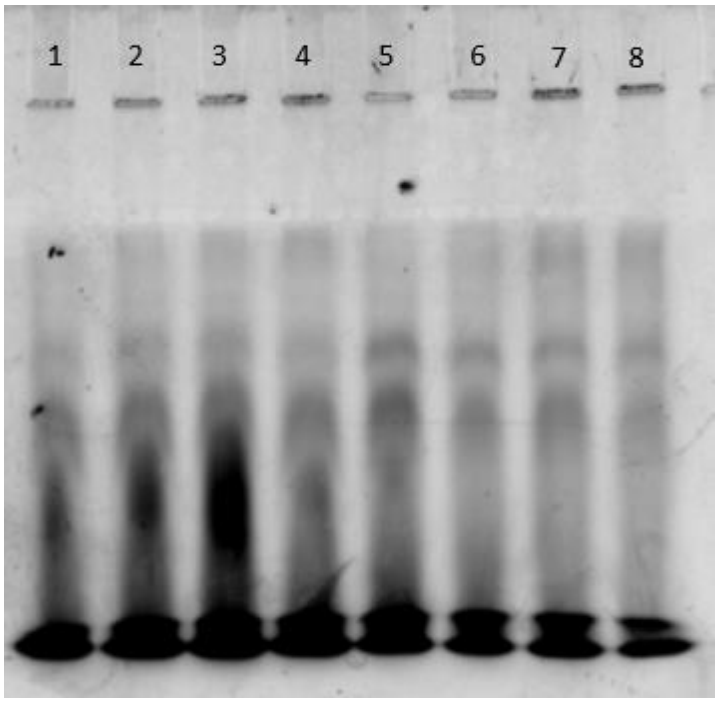


Figure. 3.1.10.B Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

Samples on the gel: 1: M19, 2: M19Chlb, 3: M19Chla1, 4: M19Chla10, 5: M18, 6: M18Chlb, 7: M18Chla1, 8: M18Chla10. All samples 1-8 were incubated for 10 min with digitonin before spinning. Sample 1 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 2 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 3 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 10 min before applying to the gel. Sample 5 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 6 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 7 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 8 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

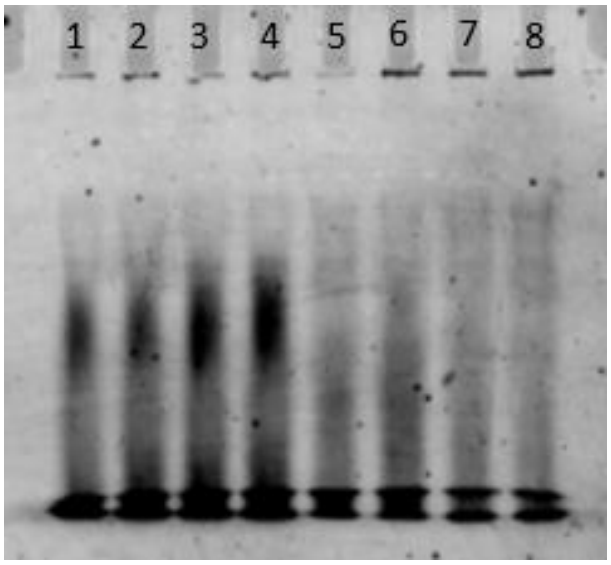


Figure. 3.1.10.C Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7.

Samples on the gel: 1: M19, 2: M19Chlb, 3: M19Chla1, 4: M19Chla10, 5: M18, 6: M18Chlb, 7: M18Chla1, 8: M18Chla10. All samples 1-8 were incubated for 10 min with digitonin before spinning. Sample 1 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 2 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 3 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 10 min before applying to the gel. Sample 5 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 6 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 7 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 8 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon.

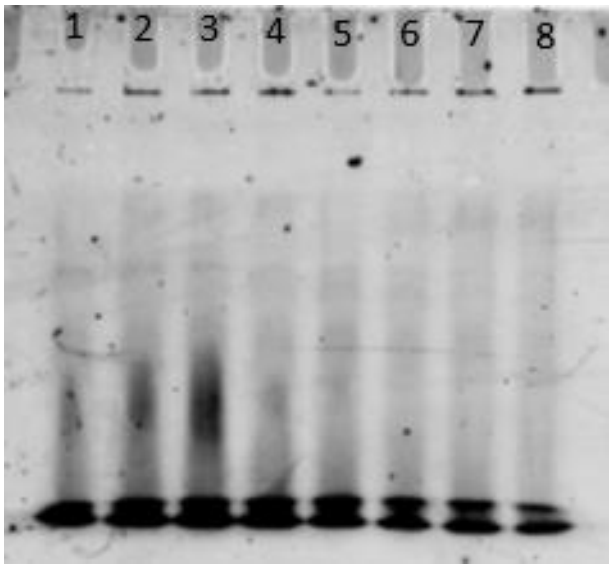


Figure. 3.1.10.D Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.
Samples on the gel: 1: M19, 2: M19Chlb, 3: M19Chla1, 4: M19Chla10, 5: M18, 6: M18Chlb, 7: M18Chla1, 8: M18Chla10. All samples 1-8 were incubated for 10 min with digitonin before spinning. Sample 1 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 2 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 3 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 10 min before applying to the gel. Sample 5 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 6 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 7 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 8 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

Looking at the two buffers used, LDS seems to again prove that it can make bands become more clear with its washing out effect. One thing that is very interesting is that samples 5-8 in figure 3.1.10.A can barely be seen, while the same samples in figure 3.1.10.B where LDS was present in the buffer, bands are suddenly apparent.
The samples look more or less the same as for part 3.1.9. nine, where the samples look almost identical to one another. Some differences can be seen, the samples are different, for example sample 3 with Chl a added to it have a stronger signal than sample 2 that have Chl b added to it. The bands in figure 3.1.10.B they look very much the same but in figure 3.1.10.D it is very clear that there is a much intense signal given for sample 3.

3.1.11. Eleven; test of solubilizing and running in the dark

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. One parameter that was tried during this part was to use samples that were not only prepared in the dark, but also run on a gel in the dark.

Due to having trouble isolating a sample of high density etioplasts, only a amount of 10^7 etioplasts have been used. In this experiment one other parameter that was changed was that the amount needed to get a amount of etioplasts equal to 10^8 was taken out and spun down. The supernantant was taken out and the pellet was resuspended in the normal solubilisation combination of water, 1M sorbitol, sample buffer as well as 6mM digitonin. The sample were incubated for 10 min before spinning, the samples were spun at 20 800 rpm for 10 min at 10 °C. After spinning ChlA/B/HEPES were added to their respective samples.

The samples on the gel; 1: Native Marker 2: M20, 3: M20Chlb, 4: M20Chla1, 5: M20Chla10. The samples were prepared the same as for the last two solubilisations.

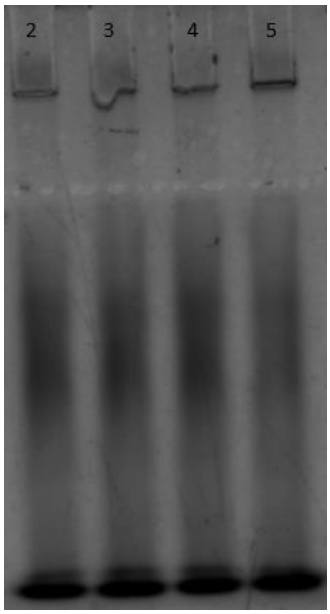


Figure. 3.1.11.A Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7.

The samples on the gel; 1: Ladder, 2: M17, 3: M17Chlb, 4: M17Chla1, 5: M17Chla10. All samples 2-5 were incubated for 10 min with digitonin before spinning. Sample 2 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 3 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 5 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

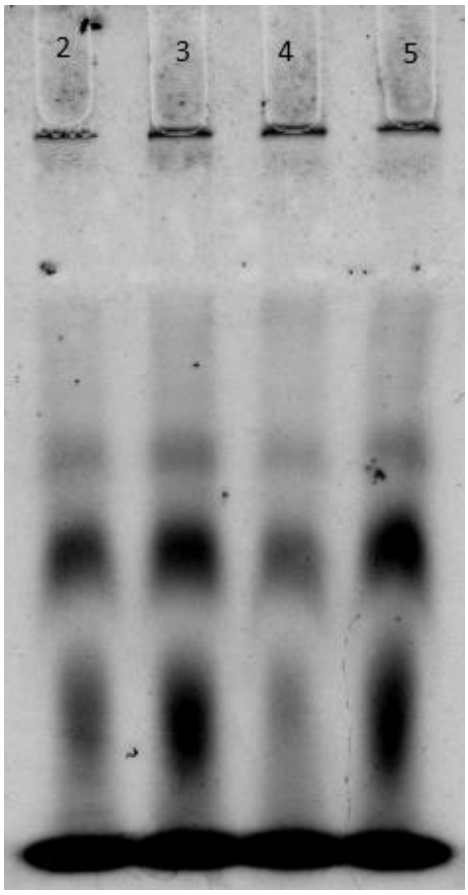


Figure. 3.1.11.B Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

The samples on the gel; 1: Ladder, 2: M17, 3: M17Chlb, 4: M17Chla1, 5: M17Chla10. All samples 2-5 were incubated for 10 min with digitonin before spinning. Sample 2 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 3 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 5 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

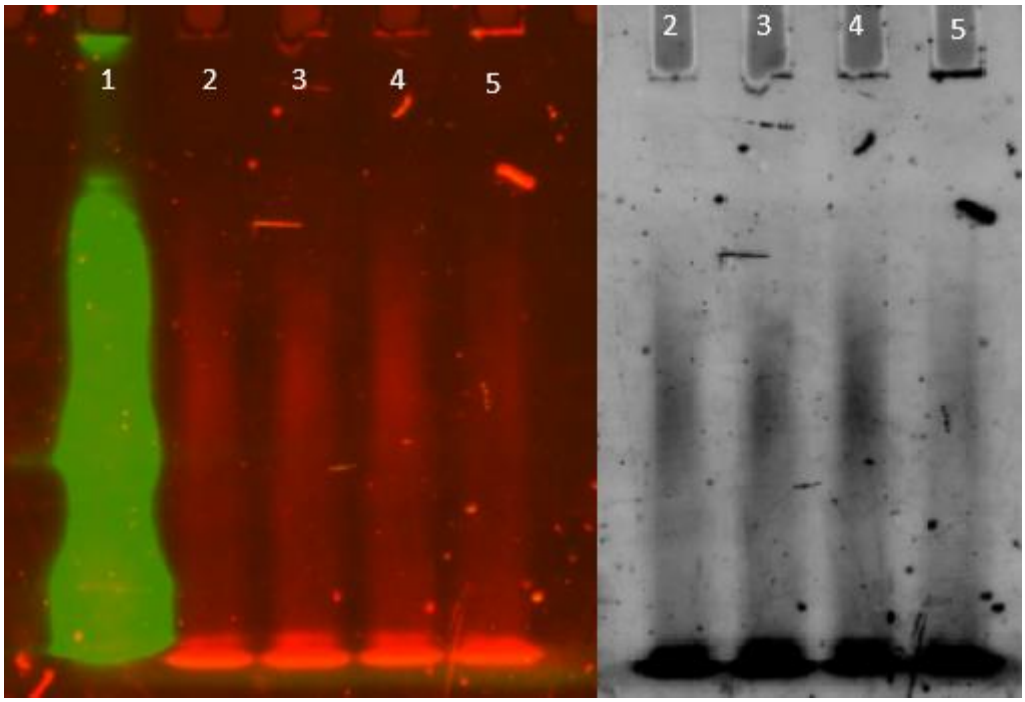


Figure. 3.1.11.C Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7.

The samples on the gel; 1: Ladder, 2: M17, 3: M17Chlb, 4: M17Chla1, 5: M17Chla10. All samples 2-5 were incubated for 10 min with digitonin before spinning. Sample 2 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 3 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 5 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon. The part of the figure that is with colours have the ladder, while the white and black parts have no ladder.

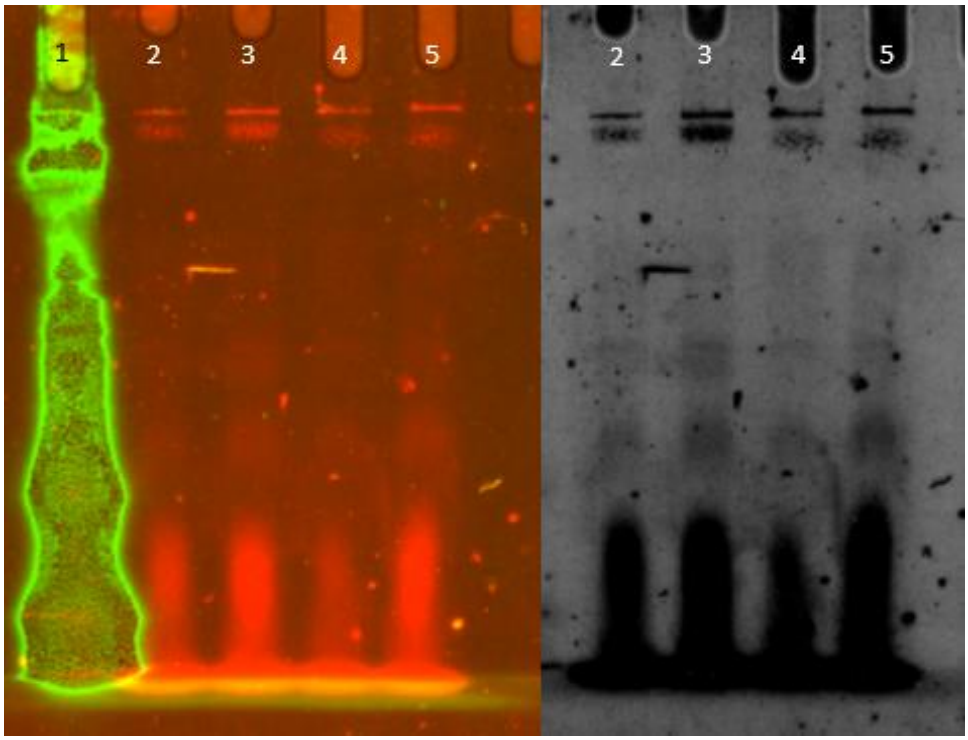


Figure. 3.1.11.D Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.
The samples on the gel; 1: Ladder, 2: M17, 3: M17Chlb, 4: M17Chla1, 5: M17Chla10. All samples 2-5 were incubated for 10 min with digitonin before spinning. Sample 2 have 50mM HEPES added instead of any Chl, and were incubated for 1 min before applying to the gel. Sample 3 have Chl b added, and were incubated for 1 min before applying to the gel. Sample 4 have Chl a added, and were incubated for 1 min before applying to the gel. Sample 5 have Chl a added, and were incubated for 10 min before applying to the gel. All samples were spun for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon. The part of the figure that is with colours have the ladder, while the white and black parts have no ladder.

Comparing the gel in figure 3.11.1.A and 3.1.11.C with 3.1.11.B and 3.1.11.D we see the difference between using a buffer without and with LDS. In figure 3.1.11.B and 3.1.11.D the bands on the gel become visible after LDS have washed out parts of the sample.
One of the interesting tings that can be seen in figure 3.1.11.B is that sample 4, the sample with Chl a added and incubated for 1 min, is actually giving off a different signal than sample 5 that was incubated with Chl a for 10 min. This difference have not been seen in the two previous parts. The other samples are also slightly different in the signal intensity on the gel, but the bands looks very much the same.

3.1.12. Twelve; test of adding Chl before and after spinning.

In this part the standard solubilisation (2.4. Solubilisation) was followed with the addition to a few changes. In this part, the etioplasts sample were up concentrated just as for the last one, to be 10^8 amount of etioplasts. One problem that was encountered was a very loos pellet. So in this experiment is that the supernantant removed in the up concentrating step was also applied to the gel as a control, to see if some parts of the pellet were still present.

All samples were prepared in complete darkness, but applied to the gel and run outside the dark room. All the samples have 6mM digitonin and incubated for 10 min before spinning. Building on the last experiment, half the samples, samples A have chlorophyllA/B/HEPES added before digitonin was added and before spinning. While samples B have chlorphyllA/B/HEPES after digitonin was added and after spinning right before loading onto the gel.

Ten samples were prepared and loaded onto the gel: 1: M20A0, 2: M20A, 3: M20ChlbA, 4: M20Chla1A, 5: M20Chla10A, 6: M20B0, 7: M20B, 8: M20ChlbB, 9: M20Chla1B, 10: M20Chla10B.

Sample 1 and 6 were made as a control, to see if there were any etioplasts left in the supernantant removed from the upconcentrated sample of etioplasts.

Sample 2 and 7 have HEPES added instead of chlorophyll. Sample 2 have HEPES added 1 min before adding digitonin, and sample 7 had it added 1 min before loading onto the gel.

Sample 3 and 8 have Chl b added to them. Sample 3 have Chl b added 1 min before adding digitonin, and sample 8 had it added 1 min before loading onto the gel.

Sample 4 and 9 have Chl a added to them. Sample 4 have Chl a added 1 min before adding digitonin, and sample 9 had it added 1 min before loading onto the gel.

Sample 5 and 10 have Chl a added to them. Sample 4 have Chl a added 10 min before adding digitonin, and sample 9 had it added 10 min before loading onto the gel.

There were two buffers used;

A: 80 mM Tricine, 15mM Bis-Tris pH 7

B: 80 mM Tricine, 15mM Bis-Tris, 0.02 mM LDS pH 7

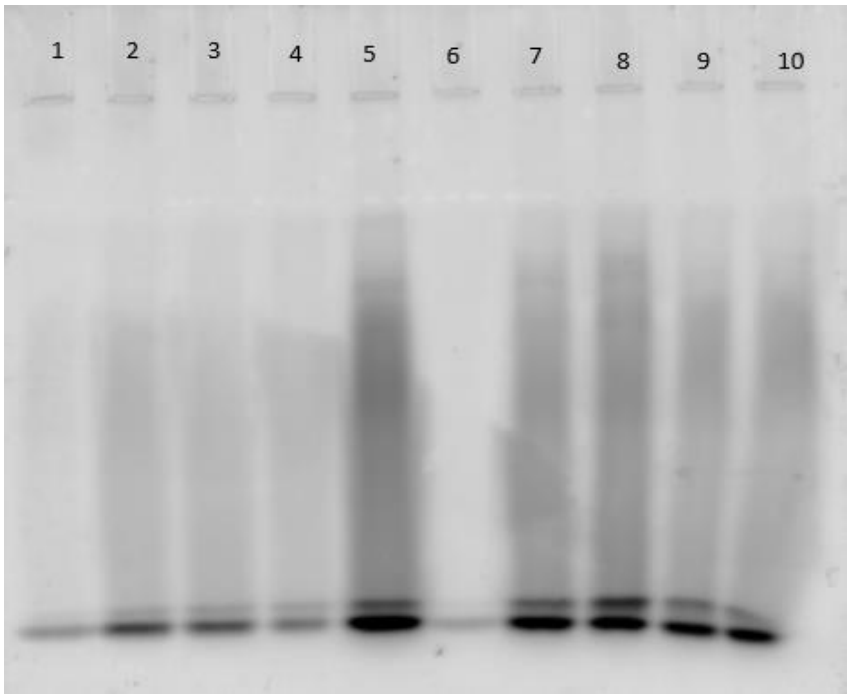


Figure. 3.1.11.A Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7.

The samples on the gel: 1: M20A0, 2: M20A, 3: M20ChlbA, 4: M20Chla1A, 5: M20Chla10A, 6: M20B0, 7: M20B, 8: M20ChlbB, 9: M20Chla1B, 10: M20Chla10B. The sample of etioplasts were up-concentrated before use. Sample 1 and 6 are the supernatant removed during the up-concentration step. Sample 2 and 7 have HEPES added and were incubated for 1 min, Sample 3 and 8 have Chl b added and were incubated for 1 min, Sample 4 and 9 have Chl a added and were incubated for 1 min, and sample 5 and 10 have Chl a added and were incubated for 10 min. Sample 1-5 have addetives added before digitonin and before spinning. Sample 6-10 have addetives added after spinning, right before applying to the gel. All samples have 6mM digitonin added to them before spinning, the samples were incubated for 10 min before spinning 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

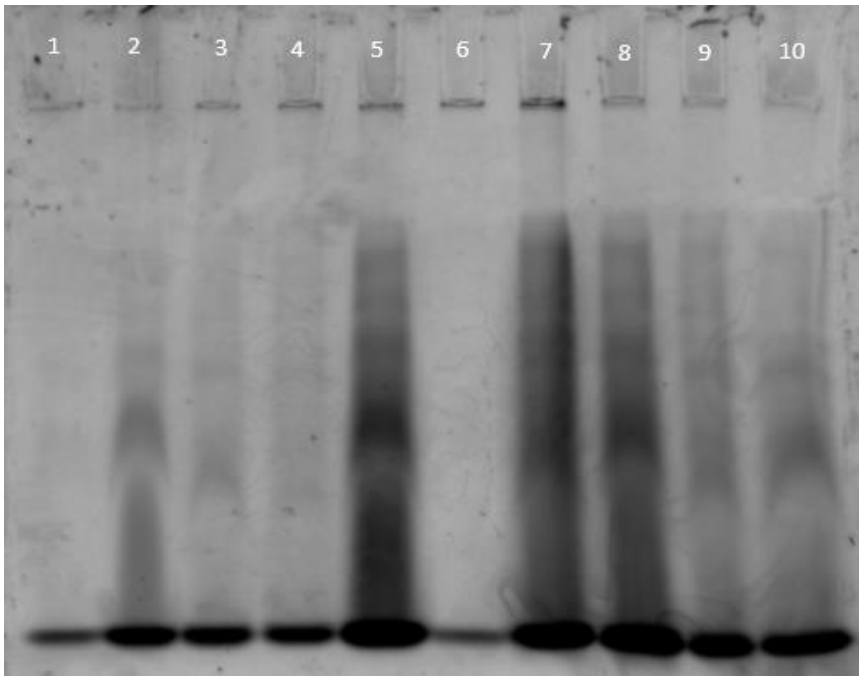


Figure. 3.1.12.B Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

The samples on the gel: 1: M20A0, 2: M20A, 3: M20ChlbA, 4: M20Chla1A, 5: M20Chla10A, 6: M20B0, 7: M20B, 8: M20ChlbB, 9: M20Chla1B, 10: M20Chla10B. The sample of etioplasts were up-concentrated before use. Sample 1 and 6 are the supernatant removed during the up-concentration step. Sample 2 and 7 have HEPES added and were incubated for 1 min, Sample 3 and 8 have Chl b added and were incubated for 1 min, Sample 4 and 9 have Chl a added and were incubated for 1 min, and sample 5 and 10 have Chl a added and were incubated for 10 min. Sample 1-5 have addetives added before digitonin and before spinning. Sample 6-10 have addetives added after spinning, right before applying to the gel. All samples have 6mM digitonin added to them before spinning, the samples were incubated for 10 min before spinning 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

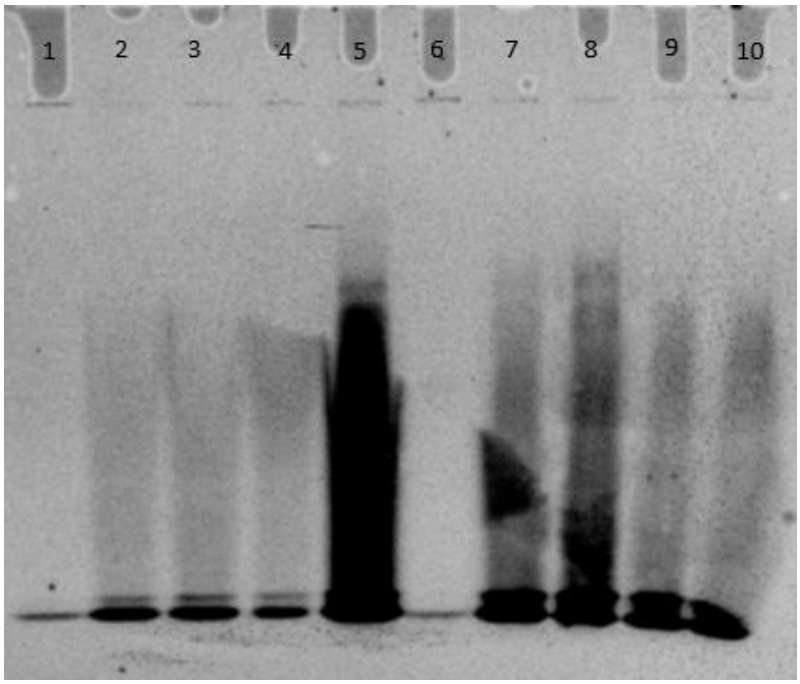


Figure. 3.1.12.C Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7.

The samples on the gel: 1: M20A0, 2: M20A, 3: M20ChlbA, 4: M20Chla1A, 5: M20Chla10A, 6: M20B0, 7: M20B, 8: M20ChlbB, 9: M20Chla1B, 10: M20Chla10B. The sample of etioplasts were up-concentrated before use. Sample 1 and 6 are the supernatant removed during the up-concentration step. Sample 2 and 7 have HEPES added and were incubated for 1 min, Sample 3 and 8 have Chl b added and were incubated for 1 min, Sample 4 and 9 have Chl a added and were incubated for 1 min, and sample 5 and 10 have Chl a added and were incubated for 10 min. Sample 1-5 have addetives added before digitonin and before spinning. Sample 6-10 have addetives added after spinning, right before applying to the gel. All samples have 6mM digitonin added to them before spinning, the samples were incubated for 10 min before spinning 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon.

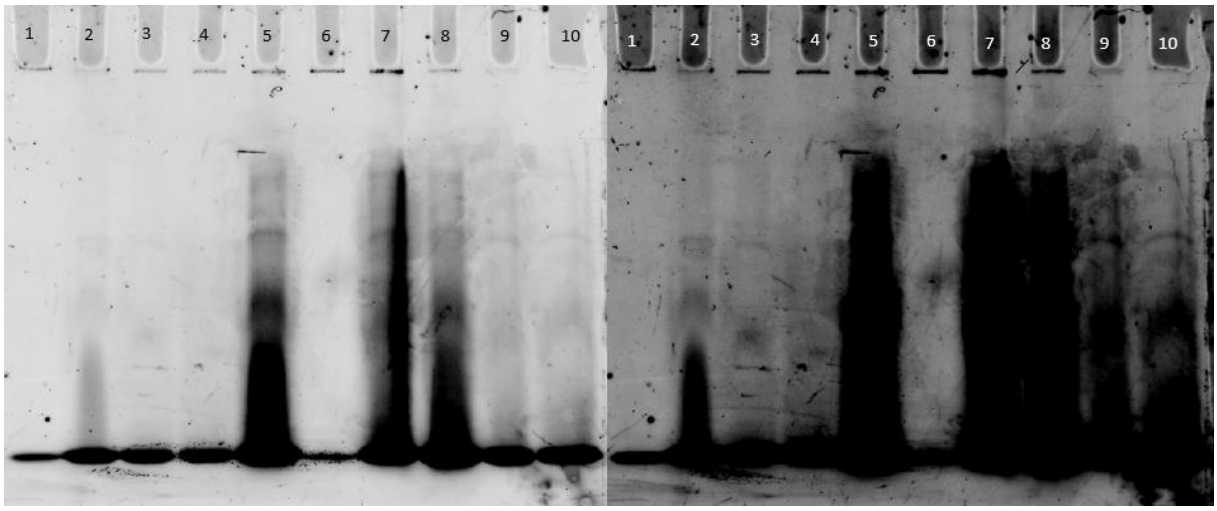


Figure. 3.1.12.D Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.
The samples on the gel: 1: M20A0, 2: M20A, 3: M20ChlbA, 4: M20Chla1A, 5: M20Chla10A, 6: M20B0, 7: M20B, 8: M20ChlbB, 9: M20Chla1B, 10: M20Chla10B. The sample of etioplasts were up-concentrated before use. Sample 1 and 6 are the supernatant removed during the up-concentration step. Sample 2 and 7 have HEPES added and were incubated for 1 min, Sample 3 and 8 have Chl b added and were incubated for 1 min, Sample 4 and 9 have Chl a added and were incubated for 1 min, and sample 5 and 10 have Chl a added and were incubated for 10 min. Sample 1-5 have addetives added before digitonin and before spinning. Sample 6-10 have addetives added after spinning, right before applying to the gel. All samples have 6mM digitonin added to them before spinning, the samples were incubated for 10 min before spinning 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon. The two pictures side by side are of the same gel at different contrasts.

When looking at the effect of the two different buffers, it is still clear that the buffer with LDS, buffer B, does wash out parts of the sample, making it possible to see bands in the gel. This effect can be seen when comparing figures 3.1.12.A and 3.1.12.C with 3.1.12.B and 3.1.12.D. Figures 3.1.12.B and 3.1.12.D bands in the gels can be seen.
As was expected to se is that sample 1 and 6 do not show anything on the gel. If any etioplasts had been left in the supernantant, there would be a signal coming from these two samples. When comparing the samples to one another samples 1-5 that had chlorophyll added before spinning and samples 6-10 that had chlorophyll added after spinning. Sample 2-4 are much fainter than sample 7-9, while comparing sample 6 to 10 this effect is the opposite. Sample 6 has a much darker signal than sample 10.

3.2. Western blots

3.2.1. First Solubilisation; test of fluorescent primary antibody

The gel from figure 3.1.1.A was blotted onto a membrane and treated with antibodies. The two holes that are present on the blot is from where a OMX tube was used to pick parts of a band. The blot was treated with a fluorescent primary antibody, this was a new technique that was tested out during this thesis.

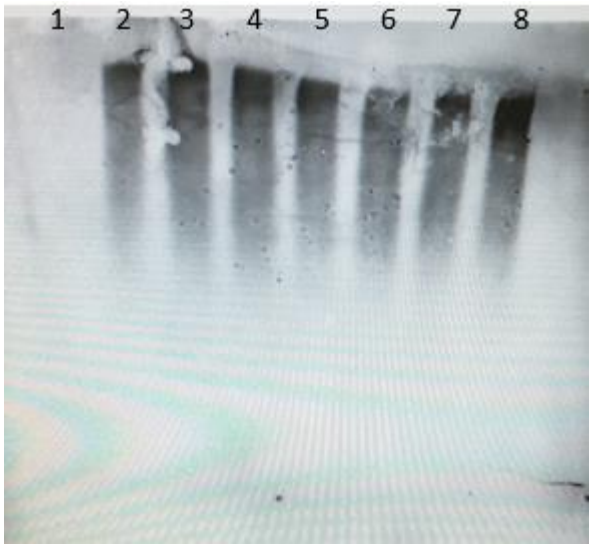


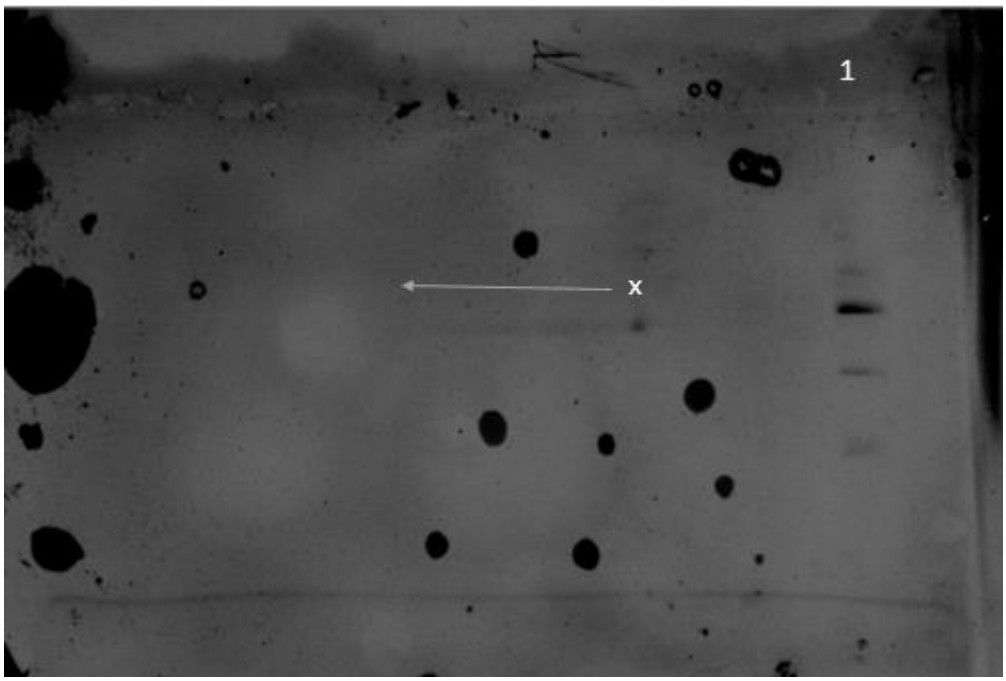
Figure: 3.2.1.A Result from blotting of the gel from figure 3.1.1.A

The blot was treated with a fluorescent primary antibody, Cyt b 800. Due to the fluorescent abilities of the primary antibody, the blot were not treated with any secondary antibody. The blot were scanned with the odyssey at 800nm.

As can be seen, there is a lot of signal coming from the blot, seemingly showing Cyt b binding in the whole top parts of the gel. The blot were not treated with any secondary antibody due to the fact that the primary antibody is fluorescent.

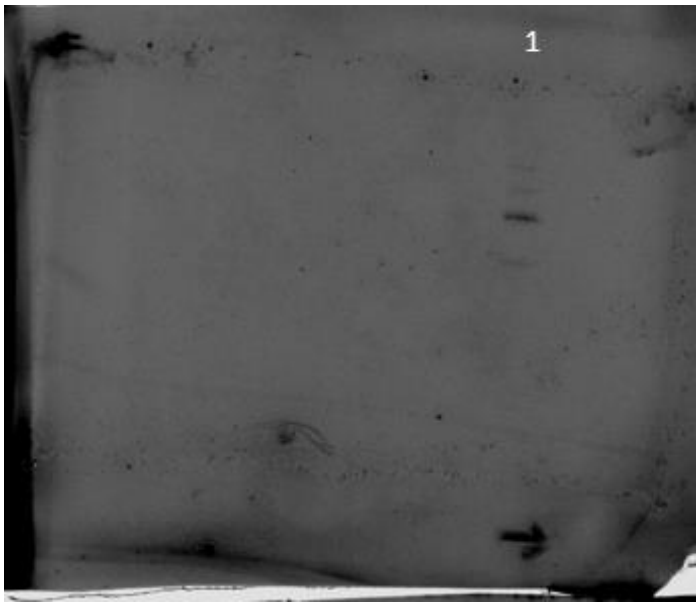
3.2.2. Sixth Solubilisation; Blotting of 2D SDS gel

The gel shown in figure 3.1.6.A was divided up into 3 strips, one for each sample. The strips were run on a 2D SDS gel and the gel blotted onto a membrane with antibodies. The blots were treated with different primary antibodies, and with a fluorescent secondary antibody; anti rabbit 800. All 2D SDS gels were run with a magic marker.



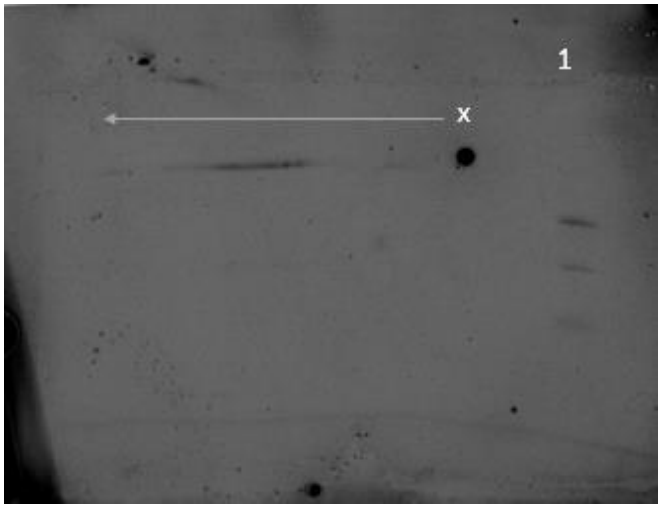
Figur. 3.2.2.A Result from blotting of sample 3 from the gel in figure 3.1.6.A, treated with POR
The blot were treated with the primary antibody POR, and with a fluorescent secondary antibody antirabbit lgG 550. The strip cut from the gel were sample 3 from figure 3.1.6.A. The marker used on the 2D SDS gel were a magic marker, and it is visible on the right side of the figure. The x and line on the blot marked where a signal from the antibody can be seen. The blot were scanned with the odyssey at 800nm.

The blot in figure 3.2.2.A were treated with POR primary antibody. The magic marker used is very clearly visible in the right side of figure 3.2.2.A marked with 1. There seems to be some markings from a signal in the middle of the blott, marked with an x and an arrow. The front of the strip were put against the magic marker.



Figur. 3.2.2.B Result from blotting of sample 1 from the gel in figure 3.1.6.A, treated with Cyt b6f
The blot were treated with the primary antibody Cyt b6f, and with a fluorescent secondary antibody antirabbit lgG 550. The strip cut from the gel were sample 1 from figure 3.1.6.A. The marker used on the 2D SDS gel were a magic marker, and it is visible on the right side of the figure. The blot were scanned with the odyssey at 800nm.

The blot in figure 3.2.2.B were treated with Cyt b. The magic marker used is clearly visible and is marked with 1 in the figure. There does not seem to be any other signal given off from the blot.



Figur. 3.2.2.C Result from blotting of sample 2 from the gel in figure 3.1.6.A, treated with Lil 3
The blot were treated with the primary antibody Lil 3, and with a fluorescent secondary antibody antirabbit lgG 550. The strip cut from the gel were sample 2 from figure 3.1.6.A. The marker used on the 2D SDS gel were a magic marker, and it is visible on the right side of the figure. The x and line on the blot marked where a signal from the antibody can be seen. The blot were scanned with the odyssey at 800nm.

The blot in figure 3.2.2.C were treated with Lil 3 primary antibody. The magic marker marked by 1 is clearly visible in the figure. A signal from the sample on the strip can be seen marked with an x and an arrow.
Comparing all the three blots to one another they all give off different signals. Unfortunatly figure 3.2.2.B does not seem to have any other signal other than the magic marker, while both figure 3.2.2.A and 3.2.2.C that were treated with POR and Lil3 respectively is giving off a signal, and at different parts of the membrane.

3.2.3. Eleventh solubilisation; Fluorescent secondary antibody

The gel from the eleventh solubilisation were blotted onto a membrane and treated with two different primary antibodies. Both membranes were treated with a fluorescent secondary antibody; anti rabbit lgG 550.

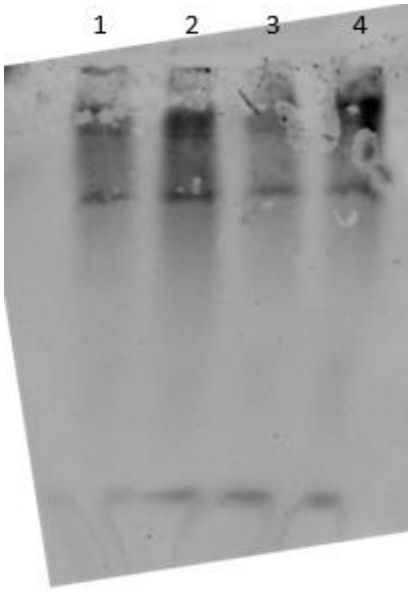


Figure 3.2.3.A Result from blotting of sample 2 from the gel in figure 3.1.11.B treated with Cyt b6f
The blot were treated with the primary antibody Cyt b6f, and with a fluorescent secondary antibody antirabbit lgG 550. The blot were scanned with the odyssey at 800nm.

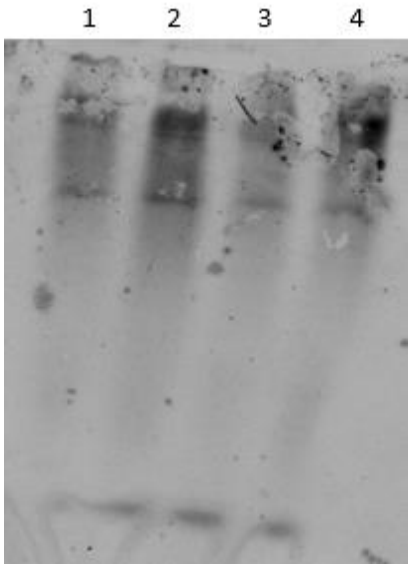


Figure 3.2.3.B Result from blotting of sample 2 from the gel in figure 3.1.11.B treated with POR
The blot were treated with the primary antibody POR, and with a fluorescent secondary antibody antirabbit lgG 550. The blot were scanned with the odyssey at 800nm.

One ting that should be noted is that there were gel bits left on the membrane when treating it with antibodies and scanning it, and this is the reason for why the figures are not as clear as would have been preferred. Figure 3.2.3.A were treated with primary antibody Cyt b and figure 3.2.3.B were treated with POR. The blot were treated with antibodies on two separate occasions, and are of the same blot. Comparing the two both figures give of much the same signal, and are not very different from one another. Figure 3.2.3.B seems to have a little fainter signal, but the same bands are visible on both figure 3.2.3.A and 3.2.3.B even though they are treated with two separate primary antibodies.
Comparing the blots in figure 3.2.3.A and 3.2.3.B to the gel in figure 3.1.11.B, they are not similar in the signal showing. The gel have bands more in the lower half while the blot have signals in the top parts of the figure. The blot were scanned with the odyssey at 800nm.

3.2.4. Twelfth solubilisation; Fluorescent secondary antibody

The gel from the twelfth solubilisation were blotted onto a membrane and treated with two different primary antibodies. Both membranes were treated with a fluorescent secondary antibody; anti rabbit 800.

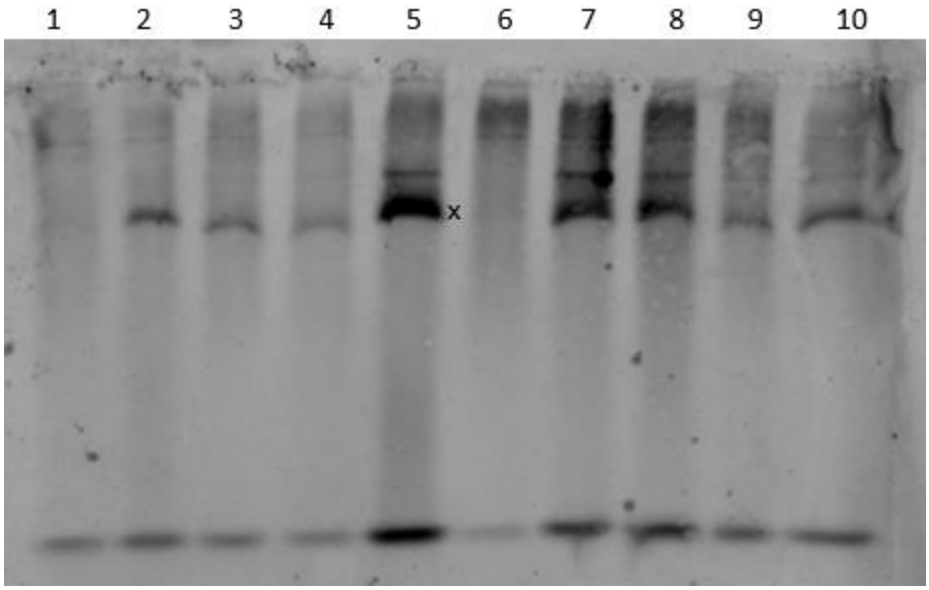


Figure 3.2.4.A Result from blotting of sample 2 from the gel in figure 3.1.12.B treated with Cyt b6f
The blot were treated with the primary antibody Cyt b6f, and with a fluorescent secondary antibody antirabbit lgG 550. The blot were scanned with the odyssey at 800nm.

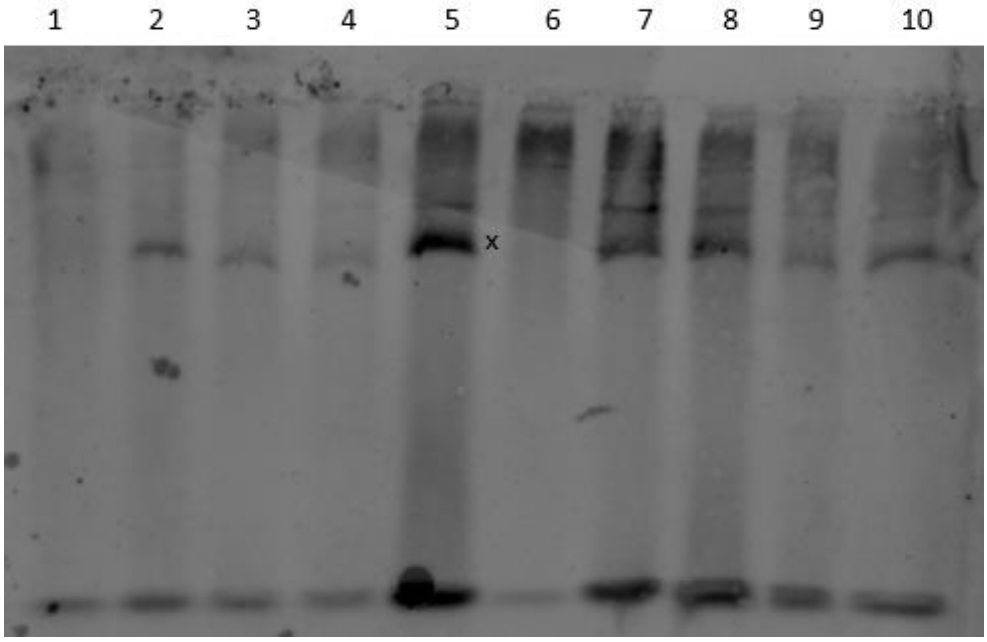


Figure 3.2.4.B Result from blotting of sample 2 from the gel in figure 3.1.11.B treated with POR
The blot were treated with the primary antibody POR, and with a fluorescent secondary antibody antirabbit lgG 550. The blot were scanned with the odyssey at 800nm.

The two figures 3.2.4.A and 3.2.4. were treated with two different primary antibodies at two separate occations. The blot were treated with Cyt b in figure 3.2.4.A and POR in 3.2.4.B. When comparing the two figures to one another, there seems to be much the same signals given for both

antibodies, however when treating the blot with Cyt b there is a stronger signal given, this effect can be seen when looking at the band in sample 5 marked with x.
Comparing the blots in 3.2.4.A and 3.2.4.B to the gel in figure 3.1.12.B, they are not similar in the signal showing. The gel have bands more in the lower half while the blot have signals in the top parts of the figure. The blot were scanned with the odyssey at 800nm.

3.3. TLC Plate

The last part of this thesis is to determine the pigment and be able to conclude if the band suspected to be cytochrome b6f is in actuality cytocome b6f.

3.3.1. TLC one; Test of method

The first TLC plate were run with 3 samples, the sample used had 10^7 dark etioplasts. One sample were prepared and run on a gel. The sample were prepared as according to the standard solubilisation method mentioned in 2.4. Solubilisation. 6mM digitonin were used as a detergent in the sample, and the buffer used to run the gel had a tricine concentration of 80mM and 15mM Bis-Tris, 0.02mM LDS and pH 7. The gel were scanned and three bands were choosen to run on a TLC plate. The three samples; 1: Top band, 2: middle band, 3: bottom band. The three bands have been marked on figure 3.3.1.A
The samples that can be seen in figure 3.3.1.C and 3.3.1.D are as follows;
1: Top band, 2: middle band, 3: bottom band, 4: Chla1:100, 5: Chla1:500, 6: Chla1:1000, 7: Chlb1:50, 8: Chlb1:100, 9: Chlb1:500, 10: PChl1:500, 11: Pchl1:5000, 12: Pchl1:50000
Many different standards were chosen for this first TLC run since it was yet to be seen how strong of a signal the samples applied to the plate would be.

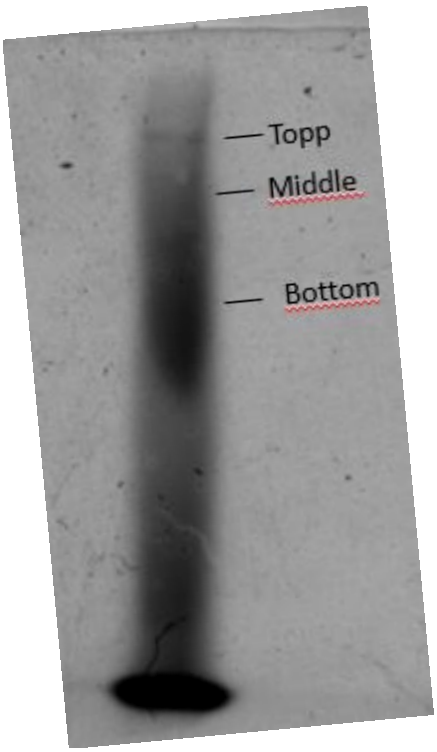


Figure 3.3.1.A: Result before cutting from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

The sample run on the gel had 6mM digitonin added to it before spinning, it were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The three bands marked by Topp, Middle and Bottom are the three bands picked and applied to the TLC plate.

The top band can clearly be seen on the figure while the middle and bottom band are not. The middle band is very faintly seen on figure 3.3.1.A, and the bottom band can not be seen as an actual band, but more a large deposit of sample.

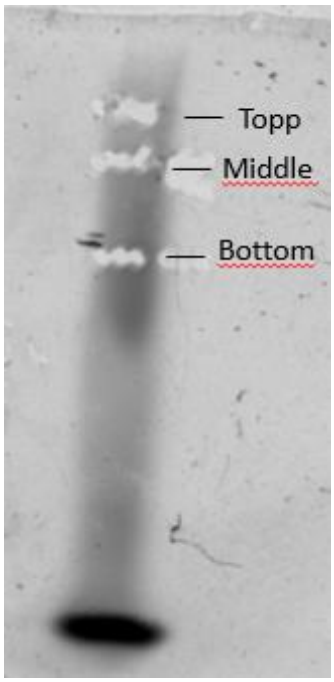


Figure 3.3.1.B. Result after cutting from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

The sample run on the gel had 6mM digitonin added to it before spinning, it were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The three bands marked by Topp, Middle and Bottom are the three bands picked and applied to the TLC plate.

Figure 3.3.1.B show the gel scanned after the bands were picked with an OMX tube. The bands were chosen; 1: Topp, 2: Middel, 3:Bottom. The bands picked were placed on a TLC plate.

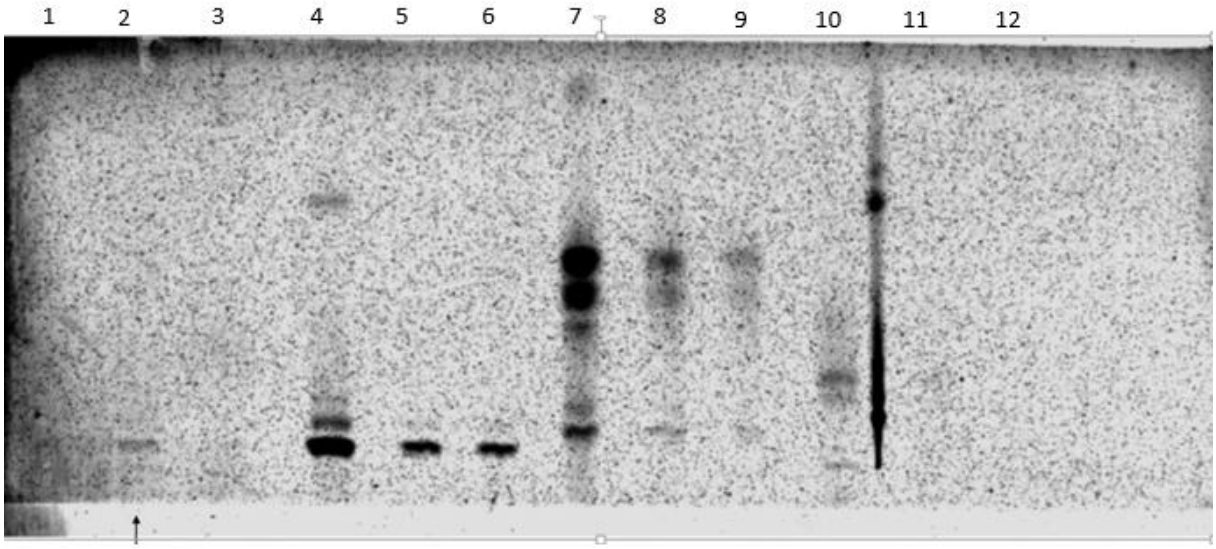


Figure 3.3.1.C Results from the TLC plate, the plate were left in the running buffer for 30 min before scanning with the Odysse

The three samples Topp, Middle and Bottom were applied to the TLC plate marked by 1-3, and the middle sample is visible and marked with an arrow. The standards used can be seen on the plate marked by 4-12: 4: Chla1:100, 5: Chla1:500, 6: Chla1:1000, 7: Chlb1:50, 8: Chlb1:100, 9: Chlb1:500, 10: PChl1:500, 11: Pchl1:5000, 12: Pchl1:50000

The 3 samples and all the standards used on the TLC plate have been marked 1-12 on figure 3.3.1.C and 3.3.1.D. The three samples applied to the plate is not very visible. The middle band have given a signal, but it is very faint. The standard can clearly be seen, except for sample 11 and 12.

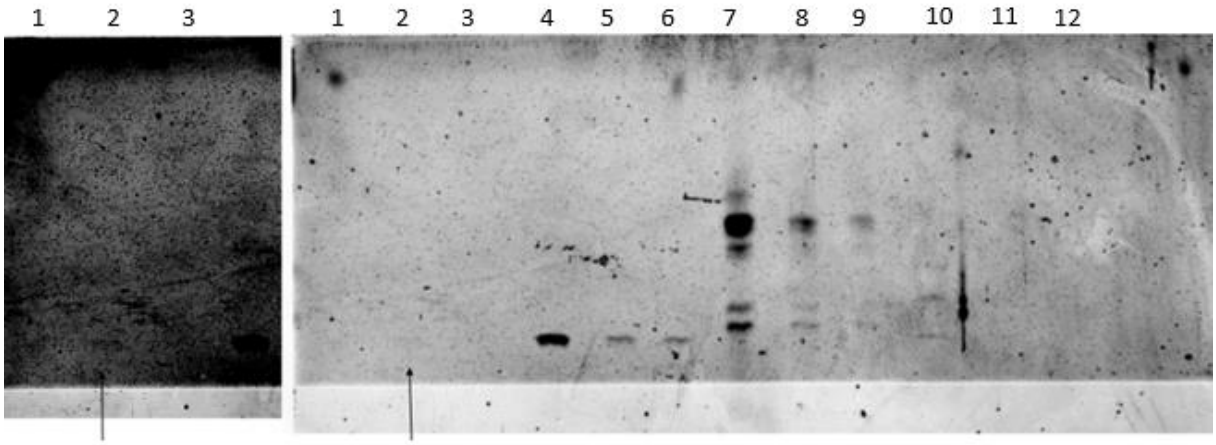


Figure 3.3.1.D Results from the TLC plate, the plate were left in the running buffer for 30 min before scanning with the typhoon.

The three samples Topp, Middle and Bottom were applied to the TLC plate marked by 1-3, and the middle sample is visible and marked with an arrow. The standards used can be seen on the plate marked by 4-12: 4: Chla1:100, 5: Chla1:500, 6: Chla1:1000, 7: Chlb1:50, 8: Chlb1:100, 9: Chlb1:500, 10: PChl1:500, 11: Pchl1:5000, 12: Pchl1:50000. The darker part of the figure shows the three samples added to the plate at a darker contrast in colours.

Figure 3.3.1.D shows the TLC plate scanned with the typhoon scanner. On this scanner sample 3 that were slightly visible on figure 3.3.1.C, can almost not be seen here. The arrow marks where a faint signal can be seen on the plate, and the darker part of the figure it is a little easier to see this signal. However it was too faint to be possible to determine the pigment in the sample.

3.3.2. TLC Two; Test of method

The second TLC plate run in this thesis had more samples, 4 samples were prepared and run on a gel, the samples were applied twice to the gel. The samples prepared this time had 10^8 dark etioplasts, which should give a stronger signal on the TLC plate. The samples on the gel; 1: M19Chla1 2: M19Chla1, 3: M19Chla10 4: M19Chla10, 5: M19Chlb 6: M19Chlb, 7: M19 8: M19 The samples were prepared as according to the standard method described in 2.4. Solubilisation. 6mM digitonin were added to the samples. Sample 1 and 2 have Chl a added and were incubated for 1 min before applying to the gel. Sample 3 and 4 have Chl a added and were incubated for 10 min before applying to the gel. Sample 5 and 6 have Chl b added and were incubated for 1 min before applying to the gel, and sample 7 and 8 have HEPES 50mM added and were incubated for 1 min before applying to the gel. The gel were run with a buffer that had a tricine concentration of

80mM, 15mM Bis-Tris and pH 7. Three standard were used for this TLC plate, based on the previous. The standards used: 13: Chl a 1:100, 14: Chl b 1:50 and 15: PChl a 1:500.

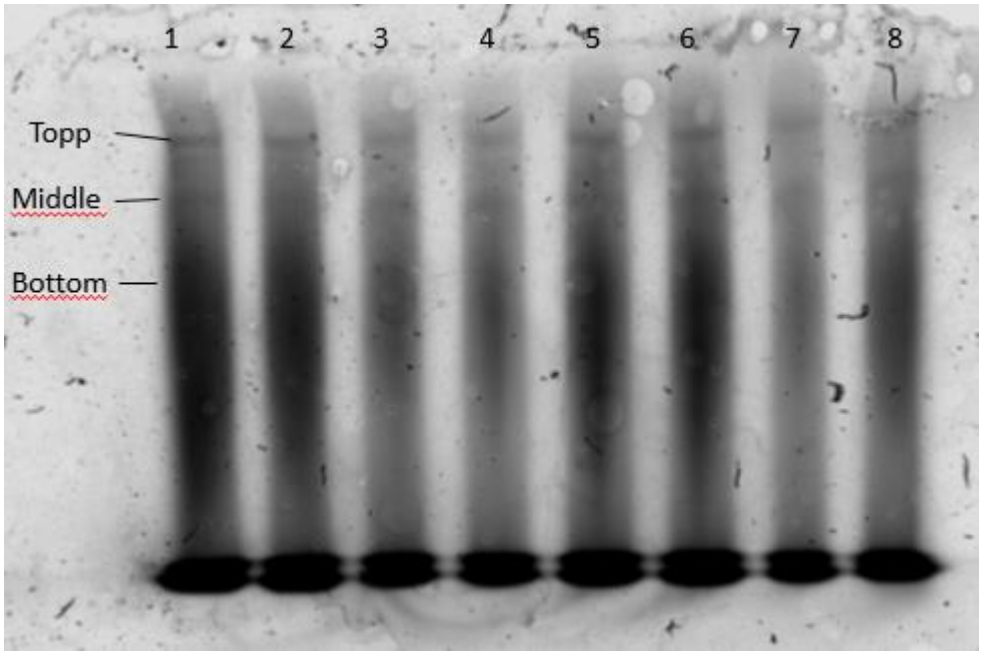


Figure 3.3.2.A. Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7, before cutting.

The samples on the gel: 1: M19Chla1 2: M19Chla1, 3: M19Chla10 4: M19Chla10, 5: M19Chlb 6: M19Chlb, 7: M19 8: M19. Sample 1 and 2 have Chl a added and were incubated for 1 min before applying to the gel. Sample 3 and 4 have Chl a added and were incubated for 10 min before applying to the gel. Sample 5 and 6 have Chl b added and were incubated for 1 min before applying to the gel. Sample 7 and 8 have 50mM HEPES added and were incubated for 1 min before applying to the gel. All samples have 6mM digitonin added before spinning, the samples were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

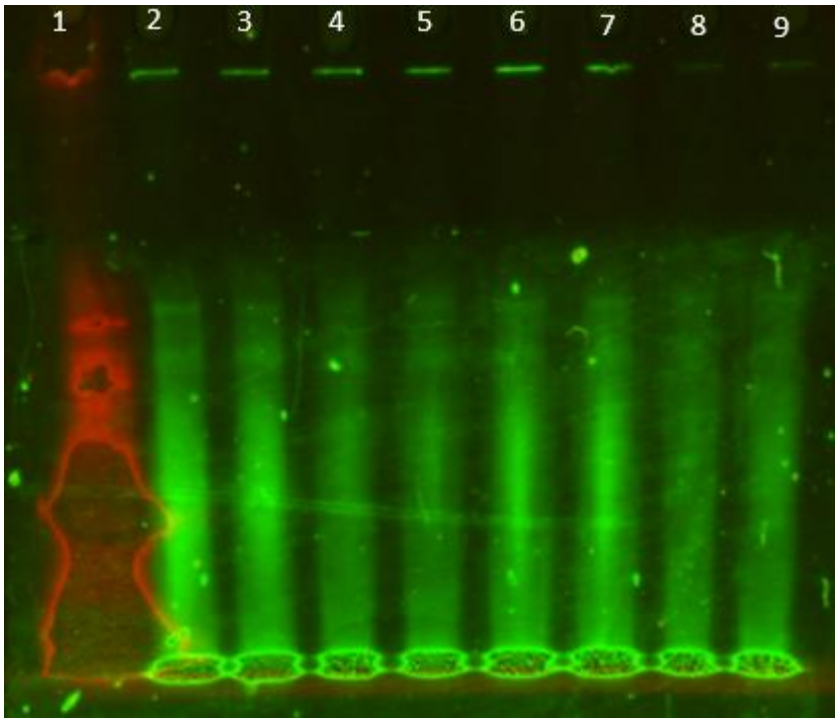


Figure 3.3.2.B. Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7, before cutting, with ladder.

The samples on the gel: 1: M19Chla1 2: M19Chla1, 3: M19Chla10 4: M19Chla10, 5: M19Chlb 6: M19Chlb, 7: M19 8: M19. Sample 1 and 2 have Chl a added and were incubated for 1 min before applying to the gel. Sample 3 and 4 have Chl a added and were incubated for 10 min before applying to the gel. Sample 5 and 6 have Chl b added and were incubated for 1 min before applying to the gel. Sample 7 and 8 have 50mM HEPES added and were incubated for 1 min before applying to the gel. All samples have 6mM digitonin added before spinning, the samples were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon.

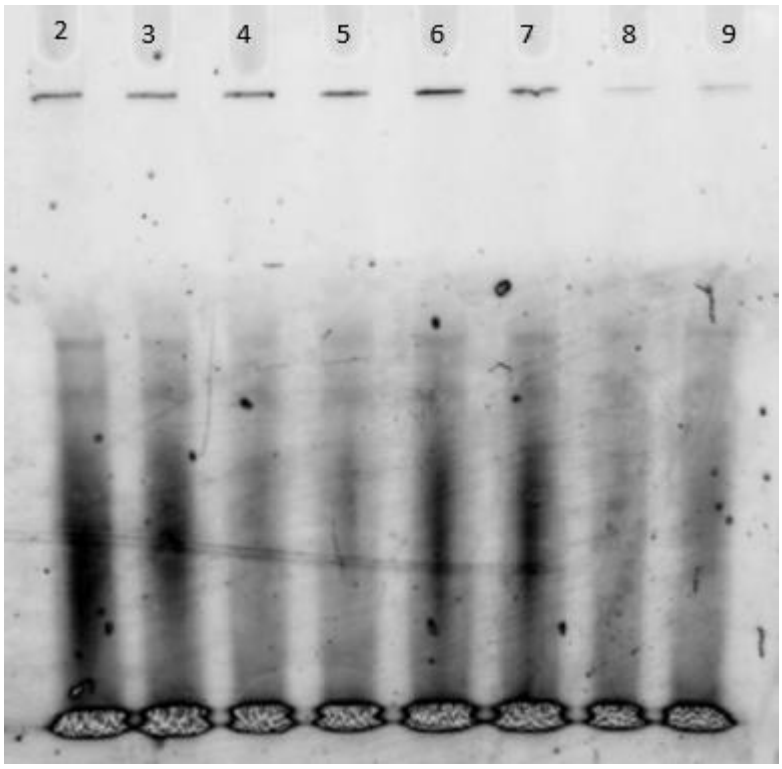


Figure 3.3.2.C. Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7, before cutting, without ladder.

The samples on the gel: 1: M19Chla1 2: M19Chla1, 3: M19Chla10 4: M19Chla10, 5: M19Chlb 6: M19Chlb, 7: M19 8: M19. Sample 1 and 2 have Chl a added and were incubated for 1 min before applying to the gel. Sample 3 and 4 have Chl a added and were incubated for 10 min before applying to the gel. Sample 5 and 6 have Chl b added and were incubated for 1 min before applying to the gel. Sample 7 and 8 have 50mM HEPES added and were incubated for 1 min before applying to the gel. All samples have 6mM digitonin added before spinning, the samples were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon.

Figure 3.3.2.A-3.3.2.C show the gel scanned before the bands were picked. In figure 3.3.2.A the three bands chosen to be picked, are marked.

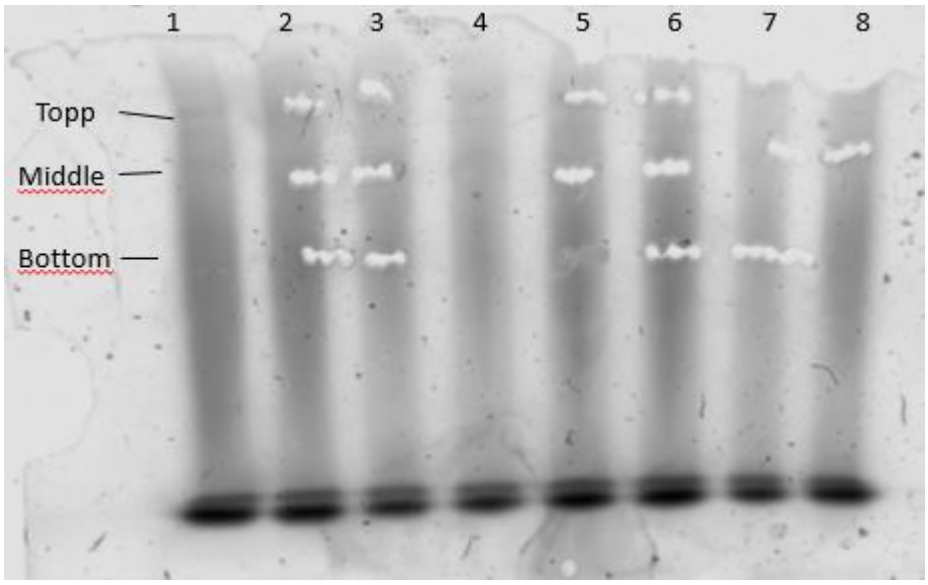


Figure 3.3.2.D. Result from running a gel with a cathode buffer with tricine concentration of 80mM, and a Bis-Tris concentration of 15mM, pH 7, after cutting.

The samples on the gel: 1: M19Chla1 2: M19Chla1, 3: M19Chla10 4: M19Chla10, 5: M19Chlb 6: M19Chlb, 7: M19 8: M19. Sample 1 and 2 have Chl a added and were incubated for 1 min before applying to the gel. Sample 3 and 4 have Chl a added and were incubated for 10 min before applying to the gel. Sample 5 and 6 have Chl b added and were incubated for 1 min before applying to the gel. Sample 7 and 8 have 50mM HEPES added and were incubated for 1 min before applying to the gel. All samples have 6mM digitonin added before spinning, the samples were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse. The three bands marked by Topp, Middle and Bottom are the three bands picked and applied to the TLC plate.

The three bands were taken from sample 2-3 and 5-8, and sample 2-3, 5 and 7 were applied to the TLC plate. In total 12 samples from the gel were applied to the TLC plate as well as three standards.

The samples applied to the TLC plate as follows: 1: M19Chla1 Top, 2: M19Chla1 Middle, 3: M19Chla1 bottom, 4: M19Chla10 Top, 5: M19Chla10 Middle, 6: M19Chla10 Bottom, 7: M19Chlb Top, 8: M19Chlb Middle, 9: M19Chlb Bottom, 10: M19 Top, 11: M19 Middle, 12: M19 Bottom, 13: Chla 1:100, 14: 1:Chlb 1:50 and 15: PChla 1:500

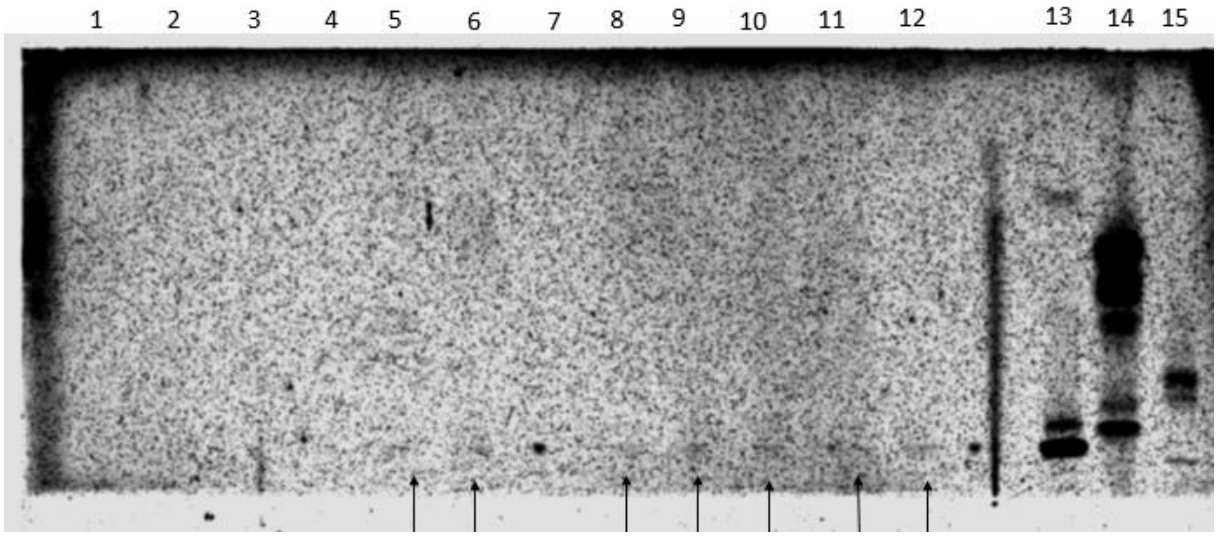


Figure 3.3.2.E. Results from the TLC plate, the plate were left in the running buffer for 30 min before scanning with the Odysse

The samples applied to the TLC plate as follows: 1: M19Chla1 Top, 2: M19Chla1 Middle, 3: M19Chla1 bottom, 4: M19Chla10 Top, 5: M19Chla10 Middle, 6: M19Chla10 Bottom, 7: M19Chlb Top, 8: M19Chlb Middle, 9: M19Chlb Bottom, 10: M19 Top, 11: M19 Middle, 12: M19 Bottom, 13: Chla 1:100, 14: 1:Chlb 1:50 and 15: PChla 1:500. The samples where signal can be seen have been marked with arrows.

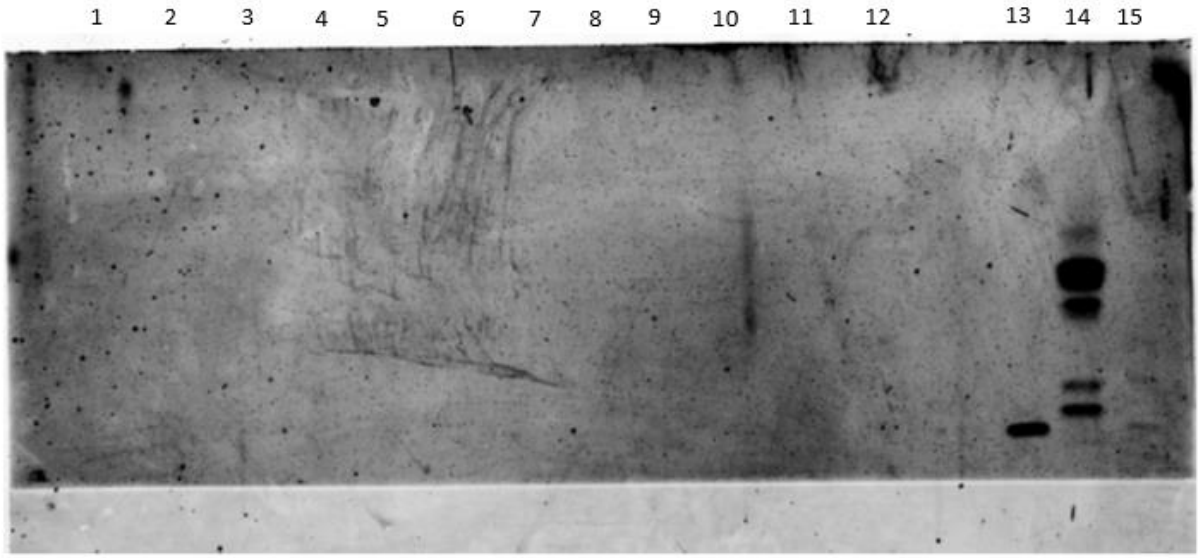


Figure 3.3.2.F. Results from the TLC plate, the plate were left in the running buffer for 30 min before scanning with the typhoon.

The samples applied to the TLC plate as follows: 1: M19Chla1 Top, 2: M19Chla1 Middle, 3: M19Chla1 bottom, 4: M19Chla10 Top, 5: M19Chla10 Middle, 6: M19Chla10 Bottom, 7: M19Chlb Top, 8: M19Chlb Middle, 9: M19Chlb Bottom, 10: M19 Top, 11: M19 Middle, 12: M19 Bottom, 13: Chla 1:100, 14: 1:Chlb 1:50 and 15: PChla 1:500.

The result of the TLC run can be seen in figure 3.3.2.E and 3.3.2.F. On this plate not much can be seen. The standard are visible on the end makred by 13-15, but all the samples marked by 1-12 are not very visible. On figure 3.3.2.F it is impossible to see any of the samples, even with different contrasts, the samples were still not visible. On figure 3.3.2.E however, you can see something. It is very faint signal, but for sample 5-6 and 8-12 it is possible to see something. However the signal on this signal TLC plate is still to faint to try for a pigment determination.

3.3.3. TLC three; Test of method

For the third TLC the samples were prepared the same as for the second TLC. The same amount of dark etioplast (10^8) were used to once again try and get a signal on the TLC plate. The samples on the gel are: 1: Ladder, 2: M19Chla1, 3: M19Chla10, 4: M19Chlb, 5: M19.
The buffer used for this gel had 80 mM tricine, 15 mM Bis-Tirs, 0.02mM and pH 7.
Sample 2 have Chl a added and were incubated for 1 min before applying to the gel. Sample 3 have Chl a added and were incubated for 10 min before applying to the gel. Sample 4 have Chl b added and were incubated for 1 min before applying to the gel, and sample 5 have HEPES 50mM added and were incubated for 1 min before applying to the gel.

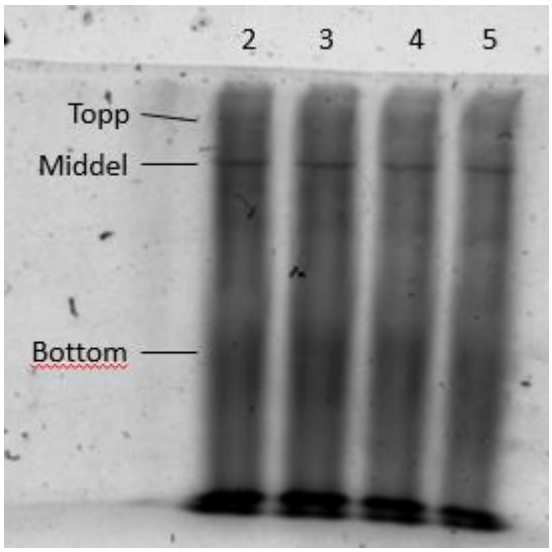


Figure 3.3.3.A. Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7, before cutting.

The samples on the gel: 1: M19Chla1, 2: M19Chla10, 3: M19Chlb, 4: M19 . Sample 1 have Chl a added and were incubated for 1 min before applying to the gel. Sample 2 have Chl a added and were incubated for 10 min before applying to the gel. Sample 3 have Chl b added and were incubated for 1 min before applying to the gel. Sample 4 have 50mM HEPES added and were incubated for 1 min before applying to the gel. All samples have 6mM digitonin added before spinning, the samples were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse.

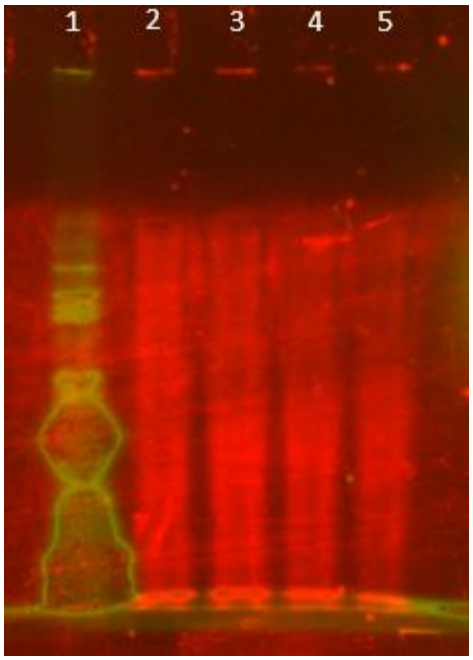


Figure 3.3.3.B. Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7, before cutting, with a ladder.

The samples on the gel: 1: M19Chla1, 2: M19Chla10, 3: M19Chlb, 4: M19 . Sample 1 have Chl a added and were incubated for 1 min before applying to the gel. Sample 2 have Chl a added and were incubated for 10 min before applying to the gel. Sample 3 have Chl b added and were incubated for 1 min before applying to the gel. Sample 4 have 50mM HEPES added and were incubated for 1 min before applying to the gel. All samples have 6mM digitonin added before spinning, the samples were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon.

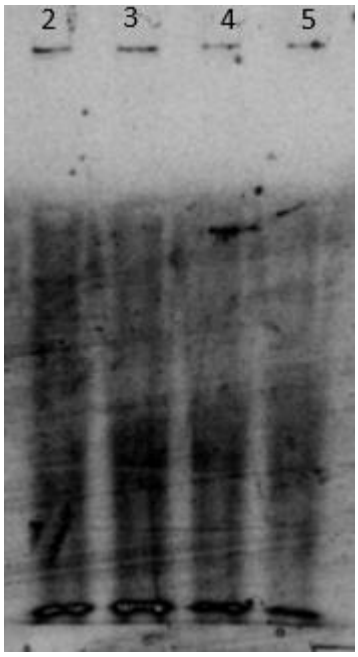


Figure 3.3.3.C. Result from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7, before cutting, without a ladder.

The samples on the gel: 1: M19Chla1, 2: M19Chla10, 3: M19Chlb, 4: M19 . Sample 1 have Chl a added and were incubated for 1 min before applying to the gel. Sample 2 have Chl a added and were incubated for 10 min before applying to the gel. Sample 3 have Chl b added and were incubated for 1 min before applying to the gel. Sample 4 have 50mM HEPES added and were incubated for 1 min before applying to the gel. All samples have 6mM digitonin added before spinning, the samples were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 633nm with the typhoon.

Figure 3.3.3.A-3.3.3.C show the gel scanned before any bands were picked. The three bands chosen to pick from the gel are marked in figure 3.3.3.A. The bands were picked from samples 2-5.

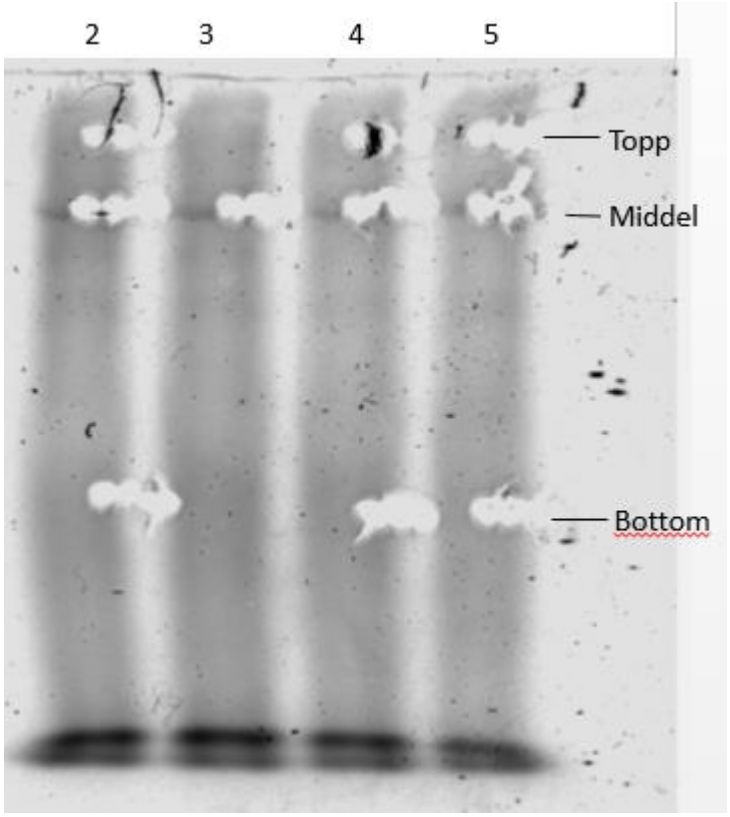


Figure 3.3.3.D Result after cutting from running a gel with a cathode buffer with tricine concentration of 80mM, a Bis-Tris concentration of 15mM and a LDS concentration of 0.02mM, pH 7.

The samples on the gel: 1: M19Chla1, 2: M19Chla10, 3: M19Chlb, 4: M19 . Sample 1 have Chl a added and were incubated for 1 min before applying to the gel. Sample 2 have Chl a added and were incubated for 10 min before applying to the gel. Sample 3 have Chl b added and were incubated for 1 min before applying to the gel. Sample 4 have 50mM HEPES added and were incubated for 1 min before applying to the gel. All samples have 6mM digitonin added before spinning, the samples were incubated for 10 min before spinning for 10 min at 20 800 rpm at 10 °C. The gel were scanned at 700nm with the Odysse. The three bands marked by Topp, Middle and Bottom are the three bands picked from samples 2 + 4-5 and applied to the TLC plate.

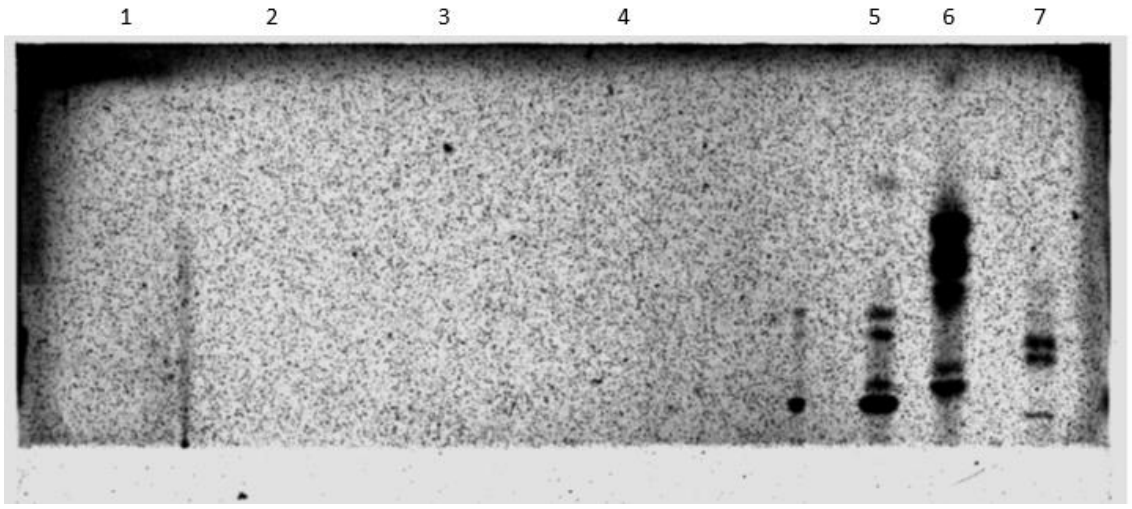


Figure 3.3.3.E. Results from the TLC plate, the plate were left in the running buffer for 30 min before scanning with the Odysse

The samples on the TLC plate are as follows; 1: Chla1min middle, 2: Chla10min middle, 3: Chlb middle, 4: M19 middle, 5: Chl a 1:100, 6: Chl b 1:50, 7: PChl a 1:500.

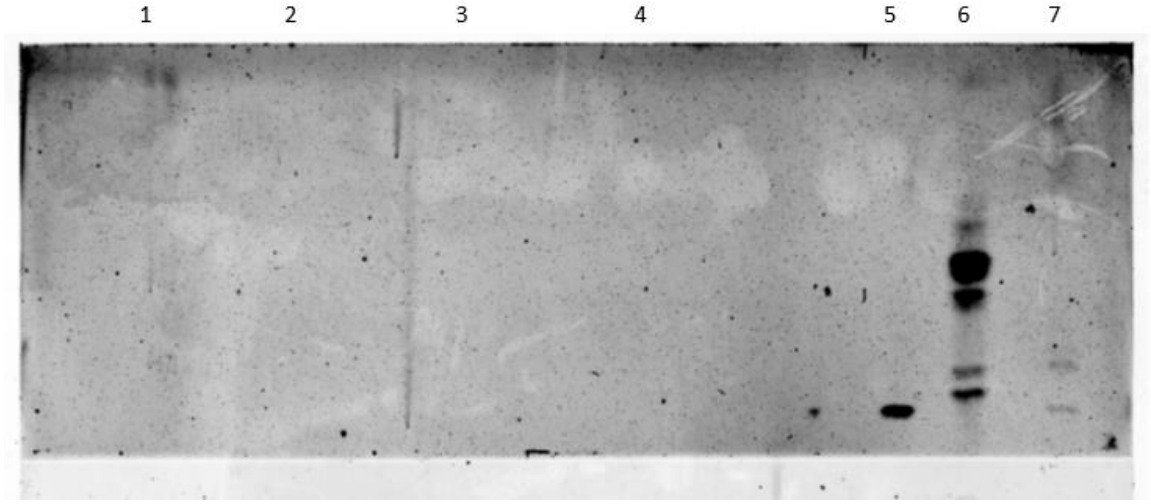


Figure 3.3.3.F. Results from the TLC plate, the plate were left in the running buffer for 30 min before scanning with the typhoon.

The samples on the TLC plate are as follows; 1: Chla1min middle, 2: Chla10min middle, 3: Chlb middle, 4: M19 middle, 5: Chl a 1:100, 6: Chl b 1:50, 7: PChl a 1:500.

When preparing the samples some technical difficulties arose, and as a result only the middle band for sample 2-5 were applied to the gel. The samples on the TLC plate are as follows; 1: Chla1min middle, 2: Chla10min middle, 3: Chlb middle, 4: M19 middle, 5: Chl a 1:100, 6: Chl b 1:50, 7: PChl a 1:500.

Looking at the TLC plate in figure 3.3.3.E. and 3.3.3.F no signal can be seen. There is no signal for any of the samples applied to the plate, except for the standards. The three standards applied to the plate can be see marked by sample 5-7.

4. Discussion

4.1. Solubilisation

At the start of the practical part of this thesis, the method was still in a developmental phase. Different parameters needed to be tried and tested, and this development became a large part of this thesis.

One of the parameters that has taken up a lot of the time is testing of the best possible cathode buffer to use with the Native LDS-PAGE.

The buffers and the detergents used in the method needed to be tested and perfected. This is because detergents have different effect on proteins. When too much LDS is added to a buffer, the lipids will be removed, but parts of the proteins can also be removed by LDS denaturating it, while too little detergent used can have the opposite effect, where lipids are not removed enough from the protein, and hides the bands. LDS have the effect that it creates vesicles, this means that the lipids are taken away and one gets clearer bands on a gel. The perfect LDS concentrations will allow for the bands to be clearly seen on the gel, but not the proteins to be denaturised. LDS can also help with changing the size of the proteins, by removing lipids. This can help the proteins fit into the pores of a gel, and move through it.

Digitonin is a detergent added to the samples. With too low concentration, nothing will happen. Digitonin will not denaturate the membrane, but will help stabilize the protein by binding to it, this binding will protect the protein and can hinder protein-protein interaction. Digitonine can also make the protein too big to enter the gel, by aggregation. Digitonin can aggregate together, creating a large unit, if this binds to the protein it can become too big to enter the pores in the gel. Stability of the protein also rests on salts binding to it, salts together with digitonin will help protect and keep its shape.

It can clearly be seen in all of the supposed dark samples, that they have been exposed to some light. When looking at the gels that have samples both dark and exposed to light, they do look similar, with all the bands. Although all the dark samples were isolated and solubilized in the dark, almost all the gels were run with exposure to light, the gels were kept in a dark container, but had some exposure time when the samples were applied to the gel. This could contribute to the similarities between the light and dark samples. Mork-Jansson et al. (2015) isolated dark etioplasts from barley, and they also experimented with light exposed and dark samples of etioplasts. Their results shows how they light exposed samples have two bands determined to be Lil3 in between two bands of high molecular and low molecular bands of Cyt b6f. These four bands together can be seen in most of my gels. However figure 3.1.11.B is from a gel that have

samples both isolated, solubilized and run in the dark, and here we find three bands, one of which looks to be Cyt b6f low molecular density, and one that seems to be Lil3. There are several possible answers to why the samples on the gel look like they have been exposed to light. As the freezer where the samples are stored, are not in a dark room, and the container that the sample were stored in can not be 100% light tight, even with extra protection around the sample itself, there is a possibility that the sample have been exposed to some light during transportation from the freezer to the dark room, which would count for the likeness to the light exposed samples.

During the experimental phase of this thesis the cathode buffer as well as method was slightly changed in order to optimize the method. The method that was developed during the couse of this thesis has become the standard way to preform a solubilisation. Any changes that have been made will be explained as the results are give.

4.1.1. Concentration of tricine and pH in the cathode buffer

During all the twelve solubilisations a standard method was developed. When this parameters looked at and perfected were the spinning time. The standard spin time at the beginning of this thesis were 30 min. One of the important factors of developing a method is getting the best possible result, but also to try an minimize the waiting time. Reducing spinning from 30 min to 10 min was tried. In the fifth solubilisation spin time was investigated, and the result showed that spinning 10 min vs. 30 min had next to no difference, and spin time as a result of this part, were reduced in the standard method.

The first parameter looked at during this thesis were the Tricine concentration and pH of the buffer. The first two parts the concentration and the pH of the cathode buffer were tested and tried, and after comparing the results of those parts, one buffer were chosen to be the standard cathode buffers used. Looking at the results in part 3.1.1-3.1.2, there are clear differences between the use of the different cathode buffers.

Buffers can have different effect with different concentration and pH. Buffers can have a major impact on the structure of proteins, and it is important to find just the right buffer to use in an experiment. It is important not to use a buffer with too high or too low pH and concentration, because a slight difference in such parameters can denature and otherwise change the proteins structure.

In parts one comparing the results comparing the figures to one another, the gel in figure 3.1.1.B-3.1.1.D is much lower than for the buffer used with gel 3.1.1.A, and it’s the gel in figure 3.1.1.A

that has the best separation of bands. The gel in figure 3.1.1.D had a buffer with a much higher tricine concentration, a concentration of 50mM, the separation of this gel is good, but there is an addition of LDS to also consider with that cathode buffer. In part two the tricine concentration were investigated further, a buffer with 80mM Tricine and 85mM Tricine were tried. The two gels in figure 3.1.2.A there is a clear difference between the gel on top and on the bottom. As marked in the figure, the gel on top is gel B, that used cathode buffer B that had a tricine concentration of 80mM, while the gel on bottom utilized cathode buffer A which had a Tricine concentration of 85mM. It is quite clear from looking at these two gels, that it is much more optimal for the results when running a gel to use a cathode buffer that have a tricine concentration of 80mM. This seems to be the best possible concentration to use. This conclusion is also backed up when looking at the gel in figure 3.1.2.B, this gel also use a buffer that have a tricine concentration of 80mM. The cathode buffer that was chosen to be used for the reminding parts had a Tricine concentration of 80mM and a Bis-Tris concentration of 15mM. The pH was also looked at during these two parts. The gels in figures 3.1.1.B-3.1.1.D have a lower pH than the other buffers used in parts one and two. Comparing a pH of 6.75 to the use of a buffer with a pH of 7, there is also a difference here. Comparing figure 3.1.1.A and 3.1.1.B get a clear picture of how well a pH of 7 works compared to a pH of 6.75. The gel in figure 3.1.1.B were run with a cathode buffer that had a pH of 6.75, this is not quite as clear and well separated as the gel in figure 3.1.1.A that were run with a buffer with a pH of 7. The standardized buffer with a tricine concentration of 80mM and 15mM Bis-Tris have a pH of 7, and this wer used for the rest of the thesis.

4.1.2. The concentration of LDS in the cathode buffer

The addition of a detergent to a buffer can be useful, but also harmful to a sample. It is very important to be careful when using a detergent. Too much can seem to give clear bands, but one might end up washing out much of the sample on the gel. LDS were the detergent used in the cathode buffer. In parts 4 and 5 of the solubilisation LDS tests were run. Gels were run with buffers that had different LDS concentration.

The best effect of the LDS can be seen when comparing figures 3.1.5.A-3.1.5.D. Here there is a clear difference between the gels as the LDS concentration in the cathode buffer rises. The band marked by x and z in figure 3.1.5.C starts out as one, and as the LDS concentration gets higher the bands start to separate into two bands. This is one of the good uses of LDS, but how much of the sample is washed out? The sample deposit at the bottom of the gel gets larger and the signal stronger as the LDS concentration rises. This increase in the deposit of the sample in the front of the gel can also be seen in figures 3.1.4.A-3.1.4.D. The bottom of the gel also get an increase in

size and signal given off. It has not been determined exactly how much of the sample applied to the sample were lost in wish washing out, but due to the result seen, it was decided that it was more optimal for the sample to use less LDS, than higher, even if more bands were exposed with higher concentration of LDS. It was determined that it would be better to use only a small concentration of LDS, or even no LDS. Since the gel chamber has space for two gels, in parts eight to twelve both a buffer without and one with 0.02mM LDS were used, just to continue to compare the result to one another.

4.1.3. Detergent in sample

One of the parameters investigated were the usage of detergent in the sample. There are many different detergents that can be used, one of the milder ones when it comes to affecting the sample negatively is digitonin, but due to it being a milder detergent, stronger ones were also tried.
Two other detergents beside digitonin were tried, α-DM and β-DM, as well as one combination detergent with both digitonin and α-DM. In figure 3.1.2.A and 3.1.2.B it can clearly be seen that β-DM is too strong of a detergent to use with these samples. Sample 4-5 and 9-10 have β-DM added and almost at the whole sample have been washed out, and a large deposit at the bottom of the gel can be seen. In figure 3.1.3.A looking at sample 4-5 and 9-10 that have α-DM added, it does not look much different than the other samples that have digitonin added. α-DM does not appear to be as strong of a detergent as β-DM, but digitonin was found to still be the better detergent and was chosen as the standard detergent to add to the sample. However as a last experiment on detergents, in part 8 a combination detergent with both digitonin and β-DM were added to sample 4. Looking at figure 3.1.8.A there is almost nothing to be seen for this sample. In figure 3.1.8.B the sample does not seem to be washed out, but there is no clear bands that can be seen, and this combination detergent were also dismissed as not optimal to use.
The concentration of digitonin have been investigated. In the beginning of this thesis the standard were to add digitonin in two steps. First one time before spinning the sample, and then add even more after spinning. Looking at figure 3.1.5.C sample 3 have 6mM digitonin added in one step, before spinning. Sample 1 have 6mM digitonin in two steps. These two samples have the same concentration of digitonin and both show a good separation. The band marked with a x and z have for both these two samples separated into two bands. It was descided after this fifth part to make 6mM digitonin the standard addition of detergent to the sample, and to only add it in one step, since this seemed to be just as effective as adding it in two steps. Adding digitonin to the sample only in one step will also optimize the method, where one step is removed and a little time is saved.

4.1.4. Light exposure

Light exposure was also looked at during the experiment. In figure 3.1.7.A there are three samples, sample one that were kept in the dark, sample 2 that were exposed to light and sample 3 that were also exposed to light and had addetives of GGPP and NADPH. The difference between sample 1 and 2 can not be seen. It were expected to see a difference between these two samples, since one were exposed to light while the other was not, but they look suspiciously the same. One reason for this is that the samples were applied to the gel and run outside the dark room, where they were exposed to light. The gel chamber were kept in a dark box, but the damage would have been done already when applying the sample to the gel, therefor the results seen is expected as it is. Sample 3 in figure 3.1.7.A is giving off a much stronger signal than the two other samples, and this was expected to be seen.

In figure 3.1.8.A there are samples where some were exposed to light and some kept in the dark. Like for part 7 there is no difference between the light exposed samples and the ones kept in the dark. Looking at figure 3.1.8.A at sample 3 and 5, these two were prepared almost the same, with the exception that sample 5 were exposed to light. They are giving off almost the exact same signal. Sample 6-8 had addetives of NADPH and GGPP. These samples appear darker than the rest. Sample 6 and 8 were exposed to light while sample 7 were not. Sample 6 and 8 are darker than sample 7, but sample 7 again is darker than the sample 5 that were also kept in the dark.

4.1.5. Chlorophyll addition to the sample

In parts nine to twelve, as well as for TLC parts two and three, Chl a and Chlb were added to the etioplast sample. There is not as big of a difference between Chl a and Chl a begin added to the sample. It had been expected that there would be some distinguished difference between the two, but they look almost identical when looking at the bands in the gel. In figure 3.1.10.A there is some difference between sample 2 with Chl b and sample 3 and 4 that have Chl a added, but not a very distinguishable difference can be seen. One thing that is interesting is in figure 3.1.11.B there is a difference between sample 4 and 5. These are the two sample that both have Chl a added, but sample 5 were incubated for 10 min before applying to the gel while sample 4 were only incubated for 1 min. This shows that there is something happening in the sample whle its incubating.

One other interesting fact seen is in figure 3.1.12.A and B, where sample 5 is much darker than sample 10. These two samples are almost the same, the only difference between then is that sample 5 have Chl a added before digitonin is added and before spinning, while sample 10 after spinning, right before loading onto the gel. Samples 1-5 all have addetives added before digitonin

and spinning, and with the exception of sample 5 they are all much lighter than samples 6-10. Sample 5 however is much darker than its cousin sample 10, the reason why is still unknown.

4.1.6. Cytochrome b6f

One of the goals with this thesis was to look at how Cyt b6f bind to chlorophyll. It was a goal to not only look at the interaction between Cyt b6f and Chl a, but also if it was possible to see a binding between Cyt b and Chl b happening. Mork-Jansson et all 2015 found that cytochrome b6f can be seen on a native gel as two bands. They concluded that cytochrome b6f shows up in two bands, one low molecular weight monomeric and the other high-molecular weight dimeric, where the low molecular weight monomeric can be found as the band that have travelled furthest on the gel. In the same experiments they also ran a gel with etioplasts exposed to light, and Lil3 were found in two bands in-between he two Cyt b6f bands. In figure 3.3.3.A the top or middle band is believed to be the high molecular weight dimeric Cyt b6f bands while the bottom band to be the low molecular weight monomeric. It was hoped that with running these bands on a TLC plate that it would be possible to determine whether or not this suspicion were in fact true. However due to time running out and the failed TLC experiements, it was not possible to get to the point where any pigment determination could be preformed.

4.2. Western blot

There were a few western blots run before the ones that were included in this thesis. These blots did not show much of any signal, and have therefor been chosen to be excluded from this thesis. The method used for those blots were the method described in 2.4.5. Western blot. Instead of using the fluorescent antibodies, chemiluminescent solutions were used to get a signal from the blots. The blots in figure 3.2.4.A and 3.2.4.B were some of the blots that were subjected to both methods to be able to compare the two methods. There were no signal given off from the blot when using both POR and Cyt b as primary antibodies. It is not completely clear why there were not signal given of, but one of the possibilities is that there were simply too low concentration of sample transferred from the gel to the membrane. The blots in figure 3.2.2.A-3.2.2.C were also subjected to both western blot detection methods, and the chemiluminescent method were unsuccessful the give any signal with this blot as well.

The method that have been used for all of the blots included in this thesis is a new and untried method for detection of antibodies. A fluorescent antibody were used, both a fluorescent primary antibody and secondary antibody were tried, and signal is given off from the blots when scanning. The blots were scanned at 800nm.

When comparing the result in figure 3.2.4.A and 3.2.4.B as well as comparing 3.2.3.A and 3.2.3.B to each other there is not a large difference between them. Figure 3.2.3.A and 3.2.4.A show the use of Cyt b as a primary antibody, while 3.2.3.B and 3.2.4.B show the use of POR as a primary antibody. The figures of the same blot with a different antibody show signal for all the same bands, there are no parts where one have signal and the other do not. One thing that does separate them form one another is that the blot in figure 3.2.3.A and 3.2.4.A that were treated with Cyt b, seems to have a stronger signal, which one can see due to them being darker in colour for the bands.

The blot in figure 3.2.2.A-3.2.2.C are not of the same blot, but are from three strips that were taken from the same gel and run on a 2D SDS gel. These three blots were treated with three different antibodies, and show different signals for them. However only two of the blots show any signal or the primary antibody. The blot in figure 3.2.2.B that were treated with Cyt b, do not show a signal. It is possible that due to this being a 2D gel that was blotted that there are not enough material to give a signal. Figure 3.2.2.A and 3.2.2.C do show signal for POR and Lil3 respectively. This signal is marked in the figures with an x and an arrow.

4.3. TLC

During the experiment, three TLC plates were run. These plates can be seen in figures 3.3.1.C, 3.3.1.D, 3.3.2.E, 3.3.2.F, 3.3.3.E and 3.3.3.F. When comparing these, the results is much the same. Some very faint signals for the samples can be seen in two of the plates. The faint signal can simply due to the fact that too low concentration of etioplasts were used. It can also be due to the fact that not enough material were picked from the bands on the gel. Without enough material, no signal will show up on the TLC plate. It is also possible that material were lost during the preparation of samples. Due to there not being enough time, there were only time to try this method three times, and it is also a very likely possibility that there were simply not enough time to completely understand and perfect the method, and that is the reason behind the faint signals on the TLC plate.

5. Conclusion

As a conclusion having looked at all the result, it has been found that adding too much LDS to a sample can have adverse effects, and LDS should only be added in small quanteties to ensure that parts of a sample one wished to have on the gel is not washed out. Detergent use in the sample is also important for a successful solubilisation, but to use a too strong detergent will wash out the sample and not give the wanted result. The right amount of detergent to add to a sample needs to be determined in order to get the best result. Digitonin have been shown to be a good detergent, that is kind to the samples, and can be added in a higher concentration than other detergent s without getting the same adverse effect of washing out parts of the sample.
Further investigations needs to be done in order to determine wheter or not it is possible for Cyt b6f to bind to Chl b.

6. Future Projects

This thesis could have been taken further, if there had been more time. I would have wanted to go through the process all over form the start. The future plans for this thesis would have been to do more isolations and manage to get a sample with a higher concentration of etioplasts. These etioplasts would then have been used to run on a Native LDS-PAGE and a OMX tube would have been used to pick some of the bands. The sample would have been run on a TLC plate, and due to a higher concentration of etioplasts present, the pigment could have been determined. The OMX tubes would have also been used to pick bands that would have been run on a MS machine. This was something that was talked about, but alas, time ran out.

7. References

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