Annika Väntänen

PURIFICATION OF TAP TAGGED YEAST PROTEINS ASSOCIATED WITH MITOCHONDRIAL FATTY ACID SYNTHESIS

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ABSTRACT

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Author(s): Annika Väntänen
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This thesis was done in the research groups of Kalervo Hiltunen and Alexander Kastaniotis at the Oulu University in the Department of Biochemistry. The aim of the study was to purify TAP tagged Rpm2p protein from yeast cell extracts or mitochondrial extracts with IgG columns with a mildly modified procedure in wildtype and the mtFAS deficient *etr1Δ* strains to asses Rpm2p palmitoylation and physical interactions.

The first purification was made using solubilised extract from 2-3 g of cells and the second using purified mitochondria. Both were then continued in a similar manner by binding the TAP tagged target proteins in IgG columns and then eluting them with self made TEV protease. Finally the proteins were analysed with SDS-PAGE and MALDI-TOF mass spectrometry.

Neither of these purifications worked out for the protein of main interest. Only the self made TEV protease and the Gcv3p protein of the control strain could be identified. Nevertheless, the fact that the Gcv3p could be purified proves that the purification should work in general and this work will provide a good starting point for optimization and further investigations.

Keywords:
Mitochondria, protein purification, mtFAS, TAP purification, affinity chromatography, MALDI-TOF, TEV protease

TIIVISTELMÄ

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Tekijä(t): Annika Väntänen
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Tämä opinnäytetyö tehtiin Kalervo Hiltusen ja Alexander Kastaniotiksen tutkimusryhmissä Oulun yliopiston biokemian laitoksella. Työn tavoitteena oli puhdistaa mitokondriaalinen Rpm2p proteiini TAP-affiniteettikromatografisesti kahta erilaista uutetta käyttäen. Ensimmäinen puhdistus tehtiin uuttamalla proteiinit 2-3 grammasta hiivasoluja ja toinen puhdistus tehtiin puhdistetuista mitokondrioista. Molempia puhdistuksia jatkettiin tästä samalla tavoin sitomalla kohdeproteiinit IgG-kolonniin ja eluoimalla ne itse tuotetulla TEV proteaasilla. Proteiinit analysoitiin vielä SDS-PAGElla ja MALDI-TOF -massaspektrometrillä.

Kumpikaan puhdistusmenetelmistä ei toiminut kohdeproteiinin osalta ja ainoastaan kontrollihiivakannan proteiini Gcv3p sekä itse tuotettu TEV proteaasi saatiin tunnistettua massaspektrometrillä. Koska Gcv3p onnistuttiin kuitenkin puhdistamaan, voidaan päätellä, että puhdistusmenetelmä on kehityskelpoinen. Tämä työ toimiikin hyvänä lähtökohtana puhdistusmenetelmän optimoimiselle ja tuleville tutkimuksille.

Asiasanat:
Mitokondrio, proteiinien puhdistus, mtFAS, affiniteettikromatografia, MALDI-TOF, TEV proteaasi

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1 INTRODUCTION

Although the mitochondrion is usually regarded as the "power plant" of the cell it is a lot more. One of the recently recognised significant features of mitochondria is their ability to synthesise fatty acids. The enzyme cofactor lipoic acid is one of the end products of this mitochondrial fatty acid synthesis (mtFAS) pathway. Other products are long-chain fatty acids which may be used for the acylation of proteins.

The target protein, Rpm2p, is a membrane associated mitochondrial protein that is predicted to be covalently modified by acylation with palmitic acid, one of the end products of the mitochondrial FAS pathway. Hence, it is a protein of interest in the research of the physiological function of mtFAS. This protein is encoded in the nucleus, translated in the cytoplasm and then imported into the mitochondria where it forms functional complex with an RNA subunit and has been reported to interact with the mitochondrial translation machinery (Daoud – Forget – Lang 2011).

The aim of the study was to purify TAP tagged Rpm2p from yeast cell extracts or mitochondrial extracts with IgG columns with a mildly modified procedure in wildtype and the mtFAS deficient *etr1Δ* strains to assess Rpm2p palmitoylation and physical interactions. The procedure was modified by lowering the pH in order to maintain the possible palmitoylation of the protein. TAP-tagged Gcv3 was used as a control. As Gcv3p is lipoylated and required for the lipoylation of other enzyme complexes, it is also a protein of interest for the research of the Hiltunen group where the work was conducted. The cleavage of the TAP tags was done with self made TEV protease, an enzyme produced in and purified from an *Escherichia coli* strain with a plasmid encoding the protein.

The work was conducted in the research groups of Kalervo Hiltunen and Alexander Kastaniotis at the Oulu University in the Department of Biochemistry. The main areas of interests within the research group are the mitochondrial fatty acid synthesis and its physiological significance, the role of peroxisomes and mitochondria in the metabolism of fatty acids and their derivatives, and acyl-

thioester binding enzymes in metabolism and their structural enzymology. Most of the experiments are done using yeast and mouse as a model organism.

2 REVIEW OF LITERATURE

Theoretical background on the main topics of the thesis is presented on the review of the literature section. General information on mitochondria, mitochondrial fatty acid synthesis, and covalent modification of proteins emphasizing on palmitoylation and myristylation are presented.

2.1 Mitochondria and their structure

The mitochondrial contribution to cell physiology goes far beyond its function as a cellular power plant that it was regarded as until the late 1980s when the assumption that everything important of the organelle was already known was questioned. In the current view, the organelle rather appears like a cellular conglomerate involved in various processes, generating a large number of essential products. The observations in recent research include aspects of mitochondrial fusion and fission events, linkage of mitochondrial fusion events to the progression of the cell cycle, mitochondrial-nuclear crosstalk, mitochondrial DNA replication, transcription and translation, iron-sulphur cluster biogenesis and the role of mitochondria in apoptosis. (Hiltunen – Chen – Haapalainen – Wierenga – Kastaniotis 2010b, 28)

The mitochondrion is a very important eukaryotic organelle and has been essential for the evolution of complex animals. The metabolism of sugars is completed in the mitochondria and without them animal cells would be dependent on anaerobic glycolysis for all their ATP. Mitochondria also have their own DNA and their own apparatus for synthesis of RNA and proteins. (Alberts 2002, 769; Campbell 1999, 37)

The size of higher eukaryote mitochondria varies from 2 μm to 8 μm in length and the diameter is typically 1 μm, which is approximately the size of many bacteria (Campbell 1999, 28), while yeast mitochondria are somewhat smaller (Shinigawa – Inouye – Onishi – Hagihara 1966). They are mobile and plastic organelles, constantly changing their shape and also fusing with each other and then separating again (Alberts 2002, 769). The number of mitochondria varies

significantly from cell type to cell type (Scheffler 2008,16). A schematic of a mitochondrion is presented in figure 1.
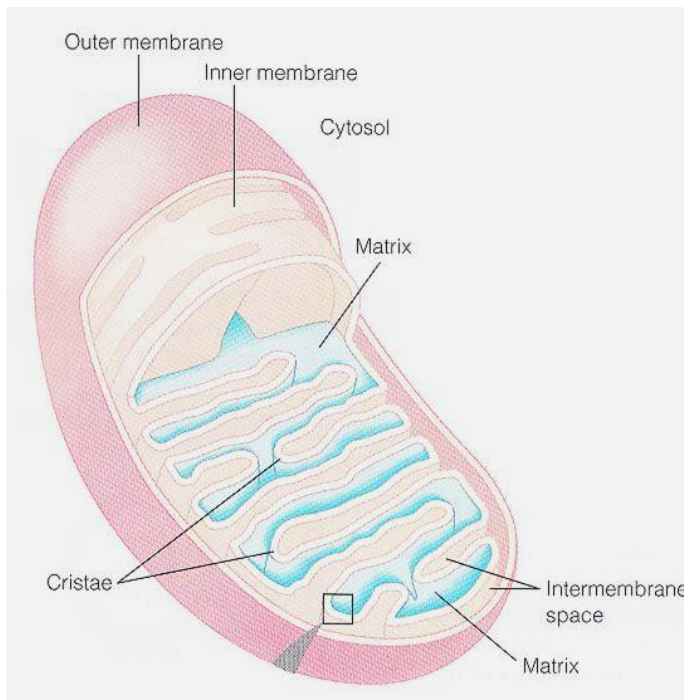
FIGURE 1. Structure of mitochondrion. (Mathews – van Holde – Ahern 1999)

As seen in figure 1 mitochondria have a double membrane: an outer membrane, which has a fairly smooth surface and an inner membrane with many folds called cristae increasing the area of the membrane. These membranes together create two compartments inside the mitochondria, the internal matrix and an intermembrane space, which is much narrower than the inner space in the mitochondria. The two mitochondrial membranes have very different functions. The outer membrane contains a large amount of a transport protein called porin and is more permeable to molecules than the inner membrane. The intermembrane space therefore is chemically equivalent to the cytosol in terms of solutes. (Alberts 2002, 771)

The matrix and the inner membrane that surrounds it is the scene for the major events in the mitochondria. By completing the metabolism of sugars in the mitochondria, 36 molecules of ATP are produced for each molecule of glucose oxidized which is remarkably more compared to the 2 molecules of ATP produced by glycolysis alone. Although *S. cerevisiae* respiration is much less efficient (only 16 ATP total yield from complete oxidation of glucose the yield is

still 8 fold higher than from glycolysis) (Rodrigues – Ludovico – Leão 2006). The matrix therefore contains many enzymes involved in the metabolism of pyruvate and fatty acids to produce acetyl-CoA and enzymes involved in the citric acid cycle. (Alberts 2002, 771)

Mitochondria are able to use both pyruvate and fatty acids as fuel. These molecules are transported through the inner membrane and converted into acetyl-CoA by enzymes in the matrix. The acetyl-CoA enters then the citric acid cycle by which the acetyl groups are oxidized. This most importantly produces high-energy electrons carried by the activated carrier molecules NADH and $FADH_2$. These molecules are then transported into the electron-transport chain which is located in the inner membrane of the mitochondrion. Via the electron-transport chain 34 molecules of ATP is produced. (Alberts 2002, 771–773)

2.2 Mitochondrial fatty acid synthesis

Among the recently recognised features of mitochondrial functions is their ability to synthesise fatty acids. This mitochondrial fatty acid synthesis pathway proceeds in an acyl carrier protein-dependent manner through a discrete set of monofunctional enzymes (Hiltunen et al. 2010b, 28). It takes place in the mitochondrial matrix and follows the process of fatty acid synthesis in general (Kursu 2010, 17).

The first committed step of fatty acid synthesis is the generation of malonyl-CoA via the condensation of acetyl-CoA and bicarbonate which is performed by acetyl-CoA carboxylase (ACC) (Hiltunen et al. 2010b, 38). The phosphopantetheine transferase (PPT) activates the apo-ACP into holo-ACP which is then attached to the malonyl group of malonyl-CoA by malonyl-CoA:ACP transacylase (MCAT). The formed Malonyl-ACP is then extended to 3-ketoacyl-ACP by an acetyl group from acetyl-CoA, acetyl-ACP or a longer saturated fatty acid attached to ACP. This reaction is catalysed by 3-ketoacyl-ACP (KAS). (Hiltunen et al. 2010b, 31; Kursu 2010, 17) The 3-keto group is reduced to a 3-hydroxy group by 3-ketoacyl reductase (KAR) and a water molecule is removed by 3-hydroxyacyl-ACP dehydratase (HTD). In the last step the resulting enoyl group from the trans-2-enoyl-ACP is reduced to acyl-ACP

which is catalysed by enoyl-thioester reductase (ETR). (Kursu 2010, 17–18) The steps of the fatty acid synthesis in mitochondria are presented in figure 2.
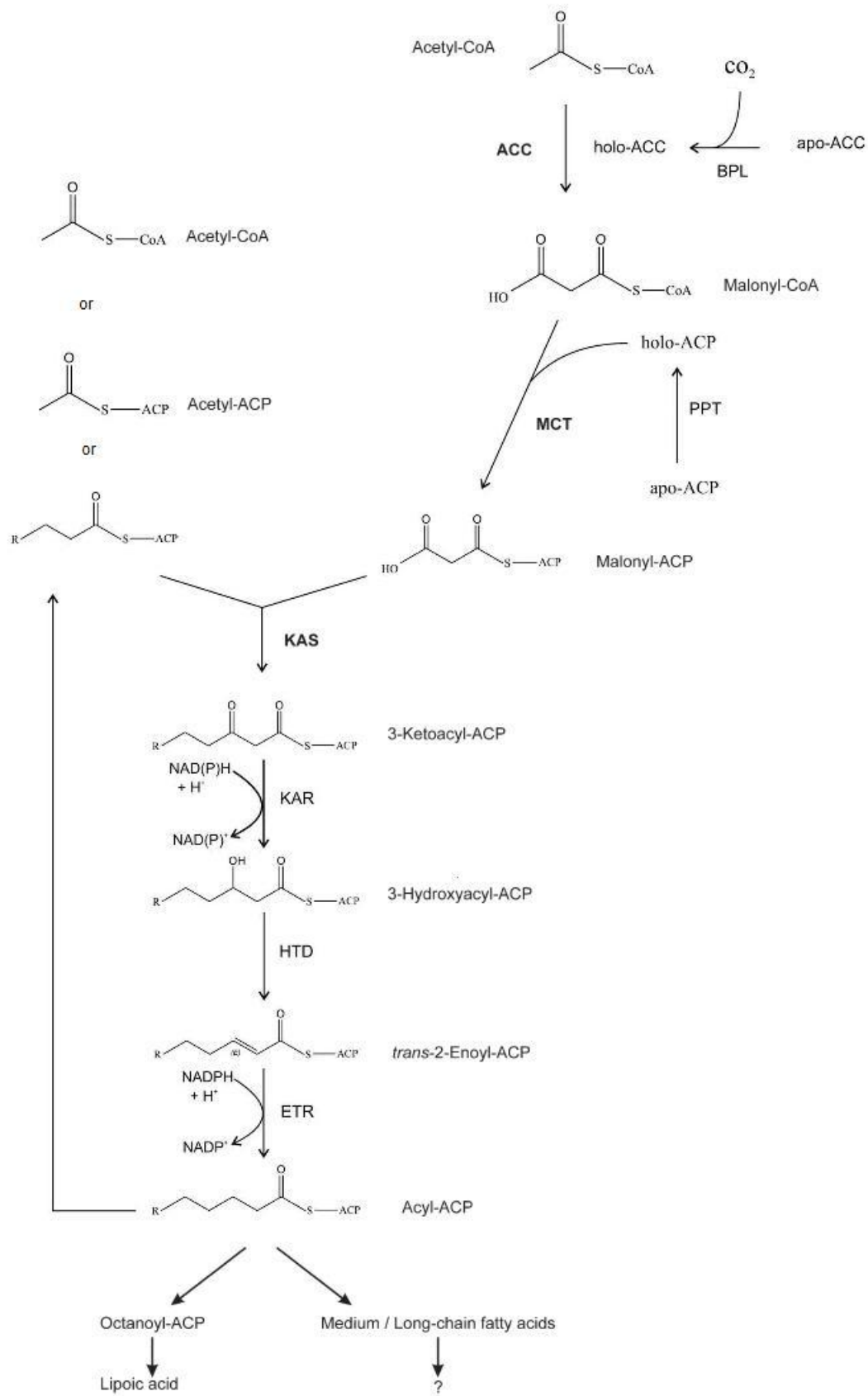
FIGURE 2. Mitochondrial fatty acid synthesis. (Hiltunen et. al 2010a,1196; Hiltunen et al. 2010b, 29)

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The end products of the mitochondrial FAS are octanoyl-ACP, medium- and long-chain fatty acids. These mitochondrially synthesised long-chain fatty acids, for example 3-hydroxymyristoyl-ACP, are possibly used for protein acylation which is one type of lipid modifications of proteins. The best characterised physiological function of the pathway is to provide the octanoyl chain (octanoyl-ACP), a substrate, for lipoic acid synthesis which seems to be an essential process in mammals. The octanoyl-ACP is also one of the major products of this mitochondrial fatty acid synthesis pathway. (Hiltunen – Autio – Schonauer – Kursu – Dieckmann – Kastaniotis 2010a, 1195; Kursu 2010, 69)

The mitochondrial pathway is best described in S*accharomyces cerevisiae* and it is the first organism in which all the mitochondrial fatty acid synthesis enzymes have been characterized completely (Hiltunen et al. 2010b, 28; Kursu 2010, 18). With the help of the research on the mitochondrial FAS in *S. cerevisia* evidence have been found to support the idea that this pathway is essential in many ways. If any member of the mitochondrial FAS pathway is deleted in *S. cerevisiae* it leads to a respiratory deficient phenotype, lack of cytochromes, and a decrease in lipoic acid, which indicates that this pathway is essential for mitochondrial function. (Hiltunen et al. 2010b, 28) There is also a possible link between the mitochondrial FAS and RNA processing in vertebrates. (Hiltunen et al. 2010a, 1200)

Mitochondria of yeast cells lacking functional mitochondrial FAS appear small and rudimentary and exhibit a fragile, more highly branched morphology than mitochondria of wild type cells which indicates that the mitochondrial FAS has an effect on the morphology of mitochondria. It is also possible that mitochondrial FAS dysfunction leads to the development of disorders in mammals. (Hiltunen et al. 2010a, 1200) A first case of a patient suffering from lipoic acid deficiency has been recently published (Mayr – Zimmermann – Fauth – Meierhofer – Raydmayr – Zschocke – Koch – Sperl 2011).

As mentioned before mitochondrial FAS is essential for the synthesis of lipoic acids, as the synthesis of the cofactor starts from octanoic acid moieties produced by the mtFAS pathway. According to the existing model in yeast, an octanoyl group/lipoic acid is transferred to Gcv3p (the H-protein) of the glycine

cleavage system followed by lipoic acid synthesis. The lipoylated Gcv3p is then required for lipoylation of other lipoic acid-dependent enzyme complexes – pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KDH). (Hiltunen et al. 2010a, 1196–1198)

Kursu (2010, 70) states in his thesis that the mitochondrial FAS pathway could act as the master regulator of respiratory growth via synthesis of lipoic acid precursors and longer fatty acids required for post-transcriptional gene expression. Kursu (2010, 76) also drew a conclusion that the pathway could be the sensor for the nutritional status of the cell by connecting acetyl-CoA availability to functional fatty acids. But despite all the current knowledge of the mitochondrial FAS pathway, not all physiologically relevant end products and their functions have been discovered and, thus, the physiological significance of the mitochondrial FAS pathway is still partly unsettled (Kursu 2010, 27).

2.3 Lipid modifications of proteins

Proteins are covalently modified with lipids in many ways (Nadolski & Linder 2007, 5202). These modifications play important roles inside and outside of the cell and they are characterized by the identity of the lipid moiety attached, the nature of the covalent bond, the attachment site of the lipid on the protein and the enzymes catalysing the reaction involved. There are four major types of known lipid modifications; cholesteroylation, prenylation, glypiation and fatty acylation. (Martin – Beauchamp – Berthiaume 2010, 18)

Fatty acylation mainly consists of the addition of palmitic or myristic fatty acids covalently to proteins (Martin et al. 2010, 18). These long-chain fatty acids used in the acylation are the products of the mitochondrial FAS pathway and therefore emphasis will be put only to palmitoylation and myristoylation.

2.3.1 Palmitoylation

The attachment of the 16-carbon saturated fatty acid palmitate to an S-terminal cysteine residue via a thioester bond is typically referred to as palmitoylation. In some cases the palmitoyl moiety is linked to the N-terminal cysteine on the protein via an amide bond. (Martin et al. 2010, 18) S-terminal palmitoylation is

reversible, which makes it unique among lipid modifications (Nadolski & Linder 2007, 5203). It can also occur spontaneously, without enzymes involved in the reaction (Martin et al. 2010, 18).

The roles of palmitoylation are diverse. According to Nadolski & Linder (2007, 5204) the most commonly described function of palmitoylation is to increase the affinity of a soluble protein for membranes, which can thereby affect the localisation of the protein. Other functions for palmitoylation are regulation of protein trafficking and modulating protein stability by quality control and regulating protein degradation. (Nadolski & Linder, 5203–5205)

2.3.2 Myristoylation

Typically, myristoylation consists of the covalent addition of the 14-carbon saturated fatty acid myristate to the N-terminal glycine residue of the protein through a stable amide bond catalysed by myristoyl-CoA:protein N-myristoyltransferase (NMT). Also various saturated and unsaturated 14-carbon fatty acids are found on the N-terminal glycine of proteins in myristoylation.

Myristoylation is required for membrane binding and many myristoylated proteins play key roles in cellular signalling pathways. The myristoyl moiety of these proteins has been shown to mediate subcellular targeting, protein-protein and protein-membrane interactions required for the activities of these proteins. Another role for myristoylation is that it can participate in differential targeting to membranes and sub-membrane domains. (Martin et al. 2010, 18–19)

3 PURIFICATION AND CHARACTERISATION OF PROTEINS

Proteins are very important molecules for life. Enzymes, which in most cases are proteins also, catalyse all the chemical reactions essential for life. Proteins are also involved in the following important functions: the import of molecules in and out of the cell, building of cell organelles, contracting of muscles, transportation of cells, the identification of the surface structure of cell, hormonal functions, and the formation of antibodies. (Turpeenoja 2005, 85)

3.1 Purification of proteins in general

Proteins not only differ by their function but also by their size, solubility, charge and their biological affinity. These differences are utilized when purifying proteins from cells or tissue. (Turpeenoja 2005, 100)

When purifying proteins, methods that do not denature the proteins quaternary and tertiary structure should be used. Matters that have to be taken account, are the working temperature, the pH and the ion strength of the solutions used. Most of the proteins are very sensitive to heat: most of the soluble proteins are denatured when the temperature rises above +50 ^{o}C. In order to maintain the native structure of the protein and prevent degradation, they should be isolated and purified near the temperature of 0 ^{o}C. Also, repeated freezing and melting denatures proteins.

Proteins are maintained native at the pH range of 5-9 and therefore buffers used in the purification of proteins. When purifying enzymes ethylenediaminetetraacetic acid (EDTA) is often added to bind divalent cations to inactivate cation-dependent proteases.

In order to prevent oxidation of proteins, compounds that protect the thiol groups are often added, such as dithiothreitol (DTT). Also compounds, like saccharose and albumin that stabilise proteins can be used in the buffers. In cell and tissue preparations there are proteolytic enzymes that degrade proteins. The activity of these enzymes can be reduced by protease-inhibitors and by working at low temperatures.

At the early stages of protein purification, different types of precipitation methods are often used (e.g. ammonium sulphate- and acetone precipitation). The precipitation is based on the different solubilities of different proteins. Precipitations with organic solvents are done quickly and at low temperature. (Turpeenoja 2005, 170)

As there are many different purification methods, an overall purification strategy helps to get started with the purification. A three phase purification strategy can be applied when purifying proteins (see figure 3). It is used as an aid for development of different purification processes.
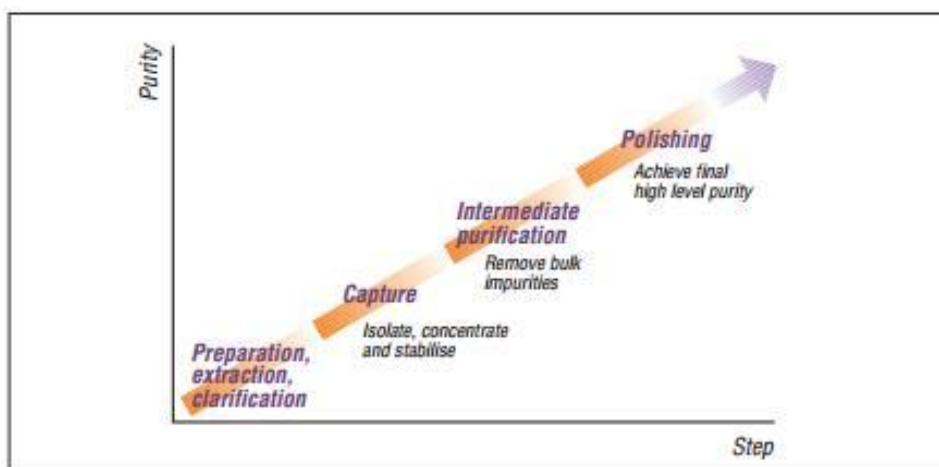
FIGURE 3. Preparation and three phase purification strategy (Amersham Pharmacia Biotech 1999)

A specific objective is assigned to each step in the three phase strategy. The objectives in the *capture* phase are to isolate, concentrate and stabilise the proteins of interest. Most of the bulk impurities are removed in the *intermediate purification* phase. At this phase most of the impurities have already been removed except for trace amounts or substances that are closely related. These are removed in the last step, the *polishing* phase, in which the objective is to achieve final purity.

Even though there are three steps in the three phase strategy, it should be noted that not all the strategies must have three purification steps. Some purifications might need less than three steps because of the high purity or the starting material or because the purity demands are low. On the other hand,

sometimes a fourth or a fifth step is required because of high purity and safety demands. Therefore, this is only an overall strategy which is easily modified to different to cases. (Amersham Pharmacia Biotech 1999, 19–20)

3.2 Affinity chromatography

The ability of molecules to bind to each other specifically, is used in the purification of biomolecules by affinity chromatography. Affinity chromatography is a unique purification method since it is the only technique that enables the purification of a biomolecule on the basis of its biological function or its individual chemical structure. In addition, the method has high selectivity, hence high resolution, and usually high capacity for proteins of interest. (Amersham Pharmacia Biotech, 7)

The interaction between a protein and a specific ligand coupled to a chromatographic matrix is reversible. The interaction can be a result of electrostatic or hydrophobic interactions, van der Waals' forces and/or hydrogen bonding. Typical examples of specific interaction partners are: enzyme-substrate complex, antigen-antibody reaction, hormone-receptor binding, base pairing of nucleotides and metal ions with histidine tagged proteins. (Turpeenoja 2005, 180; Amersham Pharmacia Biotech, 9)

Because the interaction between the target proteins and the affinity matrix is specific, all the contaminating molecules will run through the chromatography column leaving only the target proteins into the column. Depending of the wash condition, also proteins specifically interacting with the target may be retained. The target proteins can be then eluted from the affinity medium by reversing the interaction, either specifically using a competitive ligand or non-specifically, by changing the pH, ionic strength or polarity. (Amersham Pharmacia Biotech, 9; Puig – Caspary – Rigaut – Rutz – Bouveret – Bradago – Nilsson – Wilm – Séraphin 2001) The main stages of affinity chromatographic purification can be seen in figure 4 below.
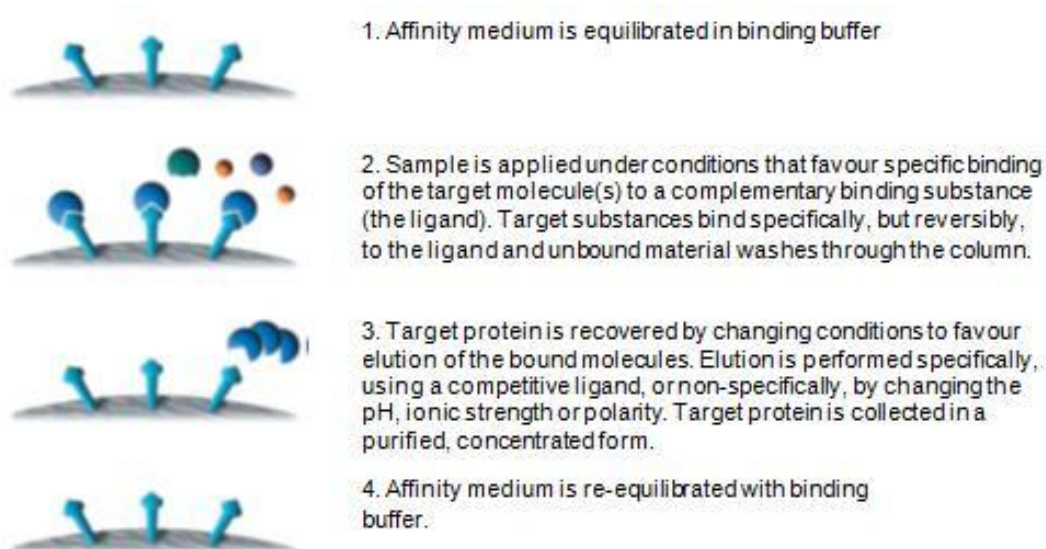
1. Affinity medium is equilibrated in binding buffer

2. Sample is applied under conditions that favour specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.

3. Target protein is recovered by changing conditions to favour elution of the bound molecules. Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.

4. Affinity medium is re-equilibrated with binding buffer.

FIGURE 4. Typical affinity purification. (Amersham Pharmacia Biotech)

3.3 Tandem affinity purification

Tandem affinity purification (TAP) allows rapid purification with high yield of protein complexes under native conditions, even when expressed at their natural level. The purified complex can be used for protein identification, functional, or structural studies. (Puig et al. 2001, 218–219)

The TAP method requires the fusion of the TAP tag to the protein of interest and the introduction of the construct to the host cell or organism. The composition of the TAP tag is two IgG binding sites of *Staphylococcus aureus* protein A (ProtA) and a calmodulin binding peptide (CBP). These binding sites are separated by a TEV protease cleavage site (see figure 5 A). (Puig et al. 2001, 219)

Various cell extraction procedures can be used depending on the target protein. The fusion protein as well as associating partners is then recovered from the extract by two specific affinity purification steps (see figure 5 B). The ProtA domain binds tightly to an IgG matrix and, thus, requires the use of TEV protease in order to elute material under native conditions. In the second affinity purification, the eluate of this first purification step is incubated with calmodulin-coated beads in the presence of calcium. The bound material is then washed, which removes contaminants and the remaining TEV protease, and released

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under mild conditions with EGTA (ethylene glycol tetraacetic acid). (Puig et al. 2001, 219)
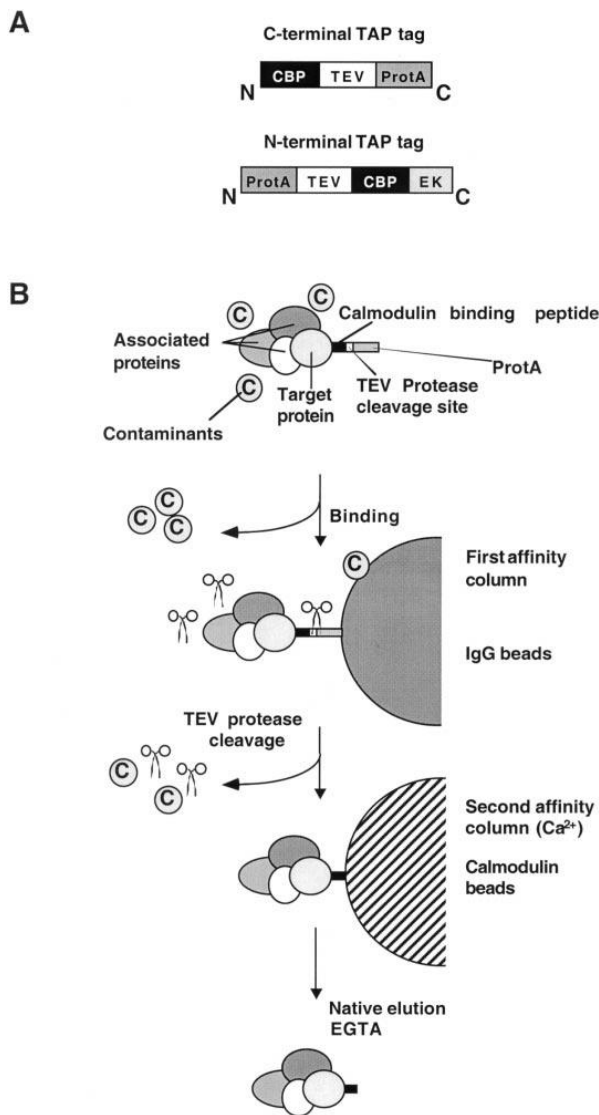
FIGURE 5. (A) The C- and N-terminal TAP tag. (B) Brief overview of the TAP method. (Puig et al. 2001, 220)

The material achieved by TAP method can be analysed in several ways. The TAP method can be used to purify recombinant proteins that are expressed at low levels. It can be used to analyse the activity of the purified complex or the structure providing that the purified complex is stable, sufficiently large and concentrated. However, the major application is to identify the proteins functionally interacting with the target protein. (Puig et al. 2001, 222–223)

3.4 Characterisation of proteins

There are a lot of different types of methods to characterise proteins. It needs to be determined case by case which method to use and, therefore, only relevant methods are presented below.

3.4.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique to characterise proteins by separating them on a gel according to their size with the help of an electrical current. It is a very course technique and precise results cannot be achieved – the molecular weight can only be estimated. Very small amounts of protein can be analysed with it and, therefore, it will not waste the sample.

Proteins are treated with SDS (sodium dodecyl sulphate) in order to make them negatively charged. The denaturing of the proteins is enhanced with fast heating and mercaptoethanol which breaks the disulphide bridges. The proteins are then run on a polyacrylamide gel. An electrical current is applied and the molecules move towards the oppositely charged pole. The smaller the molecule, the faster it moves and the further it gets in the gel. Finally, the protein bands are visualised by staining the gel. Usually Coomassie Brilliant Blue and silver staining is used. With Coomassie, staining bands containing 2 µg of protein can be made visible. Silver staining is a more sensitive technique which allows bands containing few nanograms of protein to be made visible.

A molecular marker is usually run along with the samples. It contains proteins with known masses which allow the approximate determination of the sizes of the sample proteins. (Turpeenoja 1999, 178–190) The proteins can also be further analysed for example with western blotting or mass spectrometry.

3.4.2 Western blotting

After separating proteins using SDS-PAGE, specific proteins can be identified by western blotting. The proteins are transferred from the gel onto a nitrocellulose or an alternative polymer membrane with the help of an electrical

current. Once transferred onto the membrane, proteins can be examined using antibodies that interact specifically with the protein(s) of interest. The membrane is first incubated in a protein solution which will block all the areas on the membrane where no proteins from the sample are present. The membrane is then incubated in a solution containing the primary antibody directed against the protein(s) of interest. After this, the membrane needs to be incubated further with a secondary antibody against the primary antibody. This secondary antibody contains a label in order for the reaction to be detectable. Usually enzyme-linked antibodies are used. The membrane is incubated with a substrate which is converted into a coloured product or fluorescence by the enzyme-label. The coloured band therefore indicates that the protein(s) of interest is present. (Wilson – Walker 2010, 419–420)

3.4.3 Mass spectrometry

Mass spectrometry is an analytic technique with which the masses of individual molecules and atoms can be determined. It probably is the most versatile and comprehensive analytic technique used by chemists and biochemists. By the lack of a suitable ionization technique, there were not applications of mass spectrometry for samples of a biological origin in the past. But the situation changed as several gentler modes of ionisation were developed including fast-atom bombardment (FAB), electrospray ionisation (ESI) and matrix assisted laser desorption/ionisation (MALDI). These ionisation techniques allowed the production of ions from non-polar compounds, compounds of large molecular mass and compounds of biological relevance. (Dass 2001, 2–3)

The first essential step in a mass spectrometry analysis is to convert the analyte molecules into gas-phase ions in order to be detectable. The excess energy presented into the analyte during ionisation leads to fragmentation of the ion. These molecular ions and their charged fragments are then separated according to their m/z (mass/charge) ratio and their ion current is detected by a suitable detector. A high vacuum is applied under all these steps to enable the ions to move freely without colliding or interacting with other species. Finally, a mass spectrum is displayed on the basis of the information achieved. (Dass 2001, 3)

There are numerous different ionisation techniques available but in this work the MALDI technique was used. In the MALDI technique the sample is ionised using an intense laser beam. It deposits a large amount of energy into the sample molecules for their desorption into the gas phase. The use of a matrix allows a large amount of energy to be absorbed efficiently by the matrix and subsequently transferred into the sample in a controlled manner. This leads to the evaporation of the matrix and the analyte with the matrix and as a consequence the analyte molecules become ionised. (Dass 2001, 26–27)

The most important property of the matrix is that it absorbs energy at the wavelength of the laser radiation. Other desirable characteristics are the ability to form microcrystals with the sample and a low sublimation temperature. Time of flight (TOF) instruments are commonly used in the applications of MALDI because the pulsed nature of the laser beam matches well with the pulsed scanning mode of TOFMS. (Dass 2001, 29) Also in this work, TOF was used with MALDI.
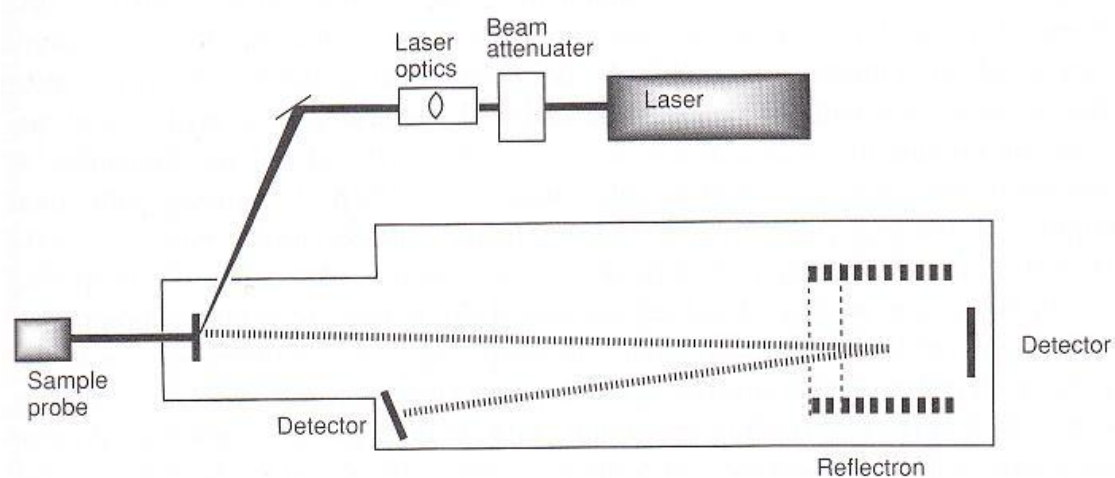

FIGURE 6. A simple illustration of MALDI-TOFMS (Dass 2001, 29)

4 EXPERIMENTAL PART

The aim was to purify Rpm2p protein which is a yeast protein associated with the mitochondrial FAS. The first purification was made using yeast cell extracts and the second purification using mitochondrial extracts. By using a mildly modified procedure, recovery of the protein and associated factors from the extracts with IgG columns eluting with self-made tobacco etch virus (TEV) protease was performed (van den Berg – Löfdahl – Berglund 2006). A control construct encoding tagged glycine cleavage system H protein Gcv3p was run in parallel to test specificity of the purification. Interactions of Gcv3p with other proteins were also a topic of interest.

4.1 Production and purification of TEV protease

First, the His-tagged TEV protease was produced in a BL21(DE3)pLysS RARE pTH24:TEVsh *E. coli* strain, isolated and then purified using affinity chromatography (van den Berg – Löfdahl – Berglund 2006). The self made protease was then later used for the cleavage of the TAP tags of the target proteins by TEV elution. The gene expression was induced with IPTG and the cells were then broken by sonication.

4.1.1 Growing cultures and the induction of the gene expression

First, 50 ml of LB (see appendix 1) with chloramphenicol (34 mg/ml) and carbenicillin (50mg/ml) was inoculated with BL21(DE3)pLysS RARE pTH24:TEVsh and cultured overnight at $+37^o$C. 10 ml of this overnight culture was then used to inoculate a litre culture the next day. M9ZB medium (see appendix 1) with chloramphenicol (34 mg/ml) and carbenicillin (50 mg/ml) was used in the litre culture. The culture was incubated at $+37^o$C until the OD_{600} reached 0.420 and was then transferred into a $+20^o$C incubator. As the OD_{600} reached 0.610, the gene expression was induced by addition of IPTG to a final concentration of 0.1 mM. The induction was continued for ~20 hours at $+20^o$C.

4.1.2 Harvesting and breaking the cells

The cells were harvested by centrifuging for 25 minutes at 4200 rpm, +4°C (rotor 4.2). The supernatant was discarded and the cells were washed with 50 ml of 1 x PBS (see appendix 1). The cell suspensions were then divided in two 50 ml Falcon tubes, centrifuged for 10 minutes at full speed with a table top centrifuge and the supernatants were discarded. The cells were then frozen at -70 ^{o}C until the experiment was continued.

The frozen cell pellets were thawed out on ice and the approximate pellet weight was determined. The pellet was re-suspended in 5ml/g of cold lysis buffer (see appendix 1). Lysozyme was added to a concentration of ~0.3 mg/ml and the suspensions were then incubated 30 min on ice. The cell suspensions were sonicated 7x20 seconds holding 10 second breaks in between while cooling in ice water. Cell debris was removed by centrifugation at 15000 x g, +4°C for 30 min using the SS-34 rotor.

4.1.3 Purification of TEV protease

The supernatant achieved from the breaking of cells was mixed gently with 8 ml of equilibrated 50 % Ni-NTA Superflow slurry (Qiagen) for 1 h at +4°C. The resin was packed into a washed column and the column flow through was collected. The column was washed with the wash buffer (see appendix 1) to remove contaminating *E. coli* proteins and the His-tagged TEV protease was eluted with the elution buffer (see appendix 1).

EDTA and DTT were added into the eluate to the final concentration of 2 and 10 mM, respectively. 10 ml of the eluate was then dialysed against 1 l of the dialysis buffer (see appendix 1) overnight at +4°C. After removal of any precipitate by centrifugation at 14000 x g for 20 min the protease concentration was determined with NanoDrop 1000. For storage glycerol was added to a final concentration of 10 %. The samples were then flash frozen in liquid N$_2$ and stored at -70°C.

All the samples collected during the purification procedure were run on a 15 % SDS-PAGE gel at 150 V for 55 min to analyse the purity of the protease.

Fermentas PageRuler^{TM} Prestained Protein Ladder was run along with the samples (see appendix 2). The gel was then stained with Coomassie Brilliant Blue and dried.

4.2 Detection of Rpm2p

Before starting the purifications of the target protein, it is needed to test if the protein Rpm2p is detectable. In order to test it, 100 ml cultures were grown and samples taken at certain OD_{600} values. The proteins were then extracted by TCA-precipitation (trichloroacetic acid) and the samples were run on a SDS-PAGE identified by western blotting.

4.2.1 Growing cultures

At first, 5 ml cultures of W1536 5B (control strain) in SCD medium (see appendix 1), W1536 5B+YCpIIIRPM2+TAPtag (wt) in SCD-LEU medium (see appendix 1) and BY4741Δrpm2+YCpIIIRPM2+TAPtag in SCD-LEU medium were grown overnight at +30 ^{o}C and used to inoculate 100 ml cultures the next day. The 100 ml cultures were grown overnight and a 10 ml sample of each culture was taken for protein extraction. 5 ml of the overnight cultures were used to inoculate 100 ml cultures. Samples were taken at certain OD_{600} values: a 50 ml sample at OD_{600} ~1 and a 25 ml sample at OD_{600}~2. Later it was noticed that the measurements of the OD_{600} were done incorrectly and therefore the OD_{600} values of the samples were probably much greater (1.5-2x) than intended.

4.2.2 Protein extraction from yeast by TCA-precipitation

The cells were harvested by centrifugation at 3 000 x g, +4 ^{o}C for 5 min with a table top centrifuge and washed with 25 ml of sterile water. 1 ml of sterile water was added and the cell suspensions were transferred into pre-weighed Eppendorf tubes. After centrifuging for ~10 seconds at full speed, the supernatant was discarded. The centrifugation was repeated and the rest of the supernatant was carefully withdrawn with a pipette and discarded. The pellet weight was then determined and 1 ml sterile water was added per 100 mg of cells. 300 µl (~30 mg) of the cell suspensions were transferred into fresh

Eppendorf tubes and 15 µl of 1 M KPi (potassium phosphate) buffer pH 7.4 was added and mixed. 100 µl of 50 % TCA was added and mixed and the tubes were stored at -70 ^{o}C until use. Freezing in TCA aids the cell disruption.

The cells were thawed and centrifuged for 10 min at full speed. After discarding the supernatant the pellets were washed with ice-cold 80 % acetone and centrifuged for 5 min at full speed. The supernatant was discarded and the pellets were re-suspended in 60 µl 1 % SDS/ 0.1 M NaOH -solution. 20 µl of 4 x SDS-PAGE loading buffer containing 10 % mercaptoethanol was added and the samples were boiled 5 min at +95 ^{o}C.

10 µl of each sample was run on a BIO-RAD Mini-Protean® TGX^{TM} Any kD^{TM} gel (10-well comb, 30 µl/well, Cat. #456-9033) at 150 V for 50 min. Fermentas PageRuler^{TM} Prestained Protein Ladder was run along with the samples (see appendix 2). The proteins were then transferred onto a 0.2 µm nitrocellulose membrane (BIO-RAD Trans-Blot® Turbo^{TM} Transfer Pack, Mini Format, 0.2 µm Nitrocellulose, Single Application, Cat.#170-4158), using BIO-RAD Trans-Blot® Turbo^{TM} Transfer system.

4.2.3 Western blotting

The western blotting was done using Millipore Snap i.d. blotting system. The membrane was first blocked with 30 ml of Sigma's 1 x blocking buffer (Cat# B6429-500ml). 3 ml of the 1:500 diluted primary antibody, rabbit-anti-goat-HRP, was incubated for 10 minutes on the membrane and washed three times with 30 ml of TBS-Tween pH 7.4 (see appendix 1). Then the 1:5000 diluted secondary antibody, goat-anti-rabbit-HRP, was incubated for 10 minutes on the membrane and the membrane was then washed twice with 30 ml of TBS-Tween pH 7.4 and once with 30 ml of TBS pH 7.4 (see appendix 1). 1.5 ml of peroxide solution and luminal/enhancer solution of BIO-RAD Immun-Star^{TM} WesternC^{TM} Kit (Cat.#170-5070) was mixed and incubated 5 minutes on the membrane. The detection was done using BIO-RAD Molecular Imager® Chemidoc^{TM} XRS+ with Image Lab^{TM} Software.

4.3 First purification using solubilised extract

The first purification of the proteins was made using solubilised total cell extract. The membrane protein complexes were first isolated from 2-3 g of cells using three different yeast strains. The isolated proteins were made soluble using Triton X-100, then bound to an IgG column and eluted with the self made TEV protease.

4.3.1 Isolation of yeast proteins and solubilisation

Cultures of 10 ml of the following yeast strains were grown overnight at +30°C shaker in SCD-LEU medium.

- W1536 5BΔgcv3+YCpIIIGCV3+TAPtag (control)
- W1536 5B+YCpIIIRPM2+TAPtag (wildtype)
- W1536 5B+YCpIIIΔetr1+YCpIIIRPM2+TAPtag (Δetr1)

These cultures were used for inoculating 50 ml cultures the next day. The 50 ml cultures were grown approximately 7 hours and 1 litre cultures were inoculated with these cultures and grown overnight.

The cells were harvested centrifuging 30 min at 3000 x g, +4°C (rotor 4.2). Supernatant was discarded and the cells were re-suspended in the residual liquid. The cell suspensions were transferred into pre-weighed Falcon tubes and centrifuged for 5 min at 4100 x g, +4°C. The cells were washed twice with 25 ml of sterile water and centrifuged as previously. The wet weights of the pellets were determined and 2-3 g of cells per each yeast strain was taken for the extraction.

The pellets were suspended in 9 ml of buffer 2 (see appendix) and 12 g of glass beads was added. The cells were homogenized in a bead beater and the beads were spun down with a table top centrifuge. 9 ml of the supernatant was transferred into ultracentrifuge tube and centrifuged for 1 hour at 38 000 rpm, +4°C (rotor 70.1 Ti). The pellet was suspended in 1 ml of buffer 2 with a Potter homogenizer (15 strokes, 500 rpm). The absolute protein concentration of the solution was determined with NanoDrop (Thermo Scientific) and approximately

100 mg of protein per yeast strain was transferred into ultracentrifuge tubes. The volume was brought up to 4.25 ml with buffer 4 (see appendix 1) and 4.25 ml of buffer 5 (see appendix 1) was added. The suspensions were incubated 1 hour in a rotating shaker at +4°C and then centrifuged for 1 hour at 38 000 rpm, +4°C (rotor 70.1 Ti). The supernatants were transferred into fresh Falcon tubes.

4.3.2 Affinity chromatography and TEV elution

In order to bind the proteins to the IgG beads, 70 µl of IgG-sepharose (GE Healthcare) slurry equilibrated in buffer 4 per tube was added and incubated overnight at +4°C rotating shaker. The beads were spun down and the supernatant was discarded carefully. The sepharose pellets were re-suspended in the residual liquid and transferred into Mobicol spin columns (Mobitech GmbH, Göttingen, Germany) equilibrated with buffer 5. The columns were washed with 250 µl and 200 µl of buffer 5 which is the flow through and again with 10 x 350 µl.

Then 3 µl of the self-made TEV protease and 73 µl of buffer 5 was added to each column and mixed carefully. After incubating 2 hours at +16°C, the columns were centrifuged and washed twice with 40 µl of buffer 5. The fractions of each column were frozen in liquid N$_2$ as 4 aliquots of 39 µl and stored at -70 ^{o}C (total volume ~156 µl). After centrifuging the columns, 350 µl of 1 x SDS-sample buffer was added to each column and centrifuged. This was repeated with 350 µl.

All the samples collected were run on a BIO-RAD Mini-Protean® TGX^{TM} Any kD^{TM} gel (10-well comb, 30 µl/well, Cat. #456-9033), control samples at 200 V for 28 min and the rest of the samples at 200 V for 45 min. The gels were then silver stained and dried. Fermentas PageRuler^{TM} Prestained Protein Ladder was run along with the samples (see appendix 2).

4.4 Second purification using purified mitochondria

The second purification was carried out using purified mitochondria. First, the crude mitochondrial fraction was prepared. In order to remove contamination by ER or vacuole, the mitochondrial fraction was purified using a sucrose gradient.

The purified mitochondria were then solubilised using Triton X-100 and the proteins bound to IgG-sepharose column. The proteins of interest are eluted with the self-made TEV protease.

4.4.1 Isolation of crude mitochondrial fraction

Pre-cultures of 10 ml of yeast strains W1536 5BΔgcv3+YCpIIIGCV3+TAPtag (control) and W1536 5B+YCpIIIRPM2+TAPtag (wildtype) were inoculated and cultured overnight in SCD-LEU medium at +30°C shaker. With these 10 ml cultures 50 ml cultures were inoculated and cultured overnight. The next day 1 litre cultures were inoculated and cultured for approximately 10 hours until they had reached OD$_{600}$ values of 3.48 (gcv3) and 3.31 (rpm2).

The cells were harvested in 1 litre centrifuging bottles at 4 000 rpm, +4 ^{o}C for 30 min. The cell pellets were then transferred into pre-weighed Falcon tubes and washed with 25 ml of sterile water. After centrifuging at 3 000 x g for 5 min the wet pellet weight was determined. The pellets were re-suspended in 2 ml of DTT buffer per g of cells and incubated 20 minutes in a +30 ^{o}C shaker (slow speed ~100 rpm). The cells were then centrifuged for 5 min at 3 000 x g and the supernatant was discarded. Zymolyase buffer (see appendix 1) was added up to 25 ml and in which the cells were re-suspended. The supernatant was discarded after centrifuging 5 min at 3 000 x g and the cells were re-suspended in 7 ml of zymolyase buffer per g of cells. Also 7 mg of Zymolyase-20T (Seikagaku Kogyo Co.) per g of cells was added and mixed carefully. The cells were then incubated 45 min at +30 ^{o}C shaker (100 rpm).

The cell suspensions were centrifuged for 5 min at 3 000 x g and the supernatant was discarded. From this point on all the steps were done on ice in order to prevent the degradation of the protein. Ice-cold homogenisation buffer (see appendix 1) was added up to 25 ml and after adding 250 µl of PMSF (phenylmethylsulfonyl fluoride) to the concentration of 1mM the cells were broken immediately in a glass potter with 15 strokes at 500 rpm. The cell suspensions were transferred back into the Falcon tubes and centrifuged for 5 min at 1 500 x g, +4 ^{o}C. The supernatant was then transferred into a fresh Falcon tube and centrifuged for 10 min at 3 000 x g, +4 ^{o}C. The supernatant

was transferred into ultracentrifuge tubes and centrifuged for 15 min at 12 000 x *g*, +4 ^{o}C. The pellet was re-suspended into 10 ml of ice-cold SEM buffer (see appendix 1) and then treated with 10 strokes in a glass potter at 500 rpm.

4.4.2 Sucrose gradient

The sucrose gradients were loaded into tubes using a pump to slowly drain the layers on top of each other. The homogenate was then carefully loaded on top of the sucrose gradients using the pump. The gradients were consisted of the following layers (starting from the bottom):

- 9 ml 60 % sucrose in EM buffer (see appendix 1)
- 24 ml 32 % sucrose in EM buffer
- 9 ml 23 % sucrose in EM buffer
- 9 ml 15 % sucrose in EM buffer

The tubes were then balanced carefully and centrifuged for 1 hour at 41 400 rpm, +2 ^{o}C. After centrifugation the purified mitochondria were collected from the 60 % and 32 % interface. Also the mitochondria on the walls were collected. The volume of the mitochondria was assessed and an equal amount of SEM buffer was added. The mixture was then centrifuged for 15 min at 10 000 x *g*, +4 ^{o}C. The supernatant was discarded and the pellet was re-suspended into 1 ml of SEM buffer. The mitochondria suspension was transferred into a pre-weighed Eppendorf tube and centrifuged for 10 min at full speed at +4 ^{o}C. The wet pellet weight was determined and the pellet was frozen in liquid N_2. The pellet was stored at -70 ^{o}C.

4.4.3 Solubilisation

The mitochondrial pellets were thawed on ice and re-suspended in 1 ml of buffer 4. The absolute protein concentration was determined with NanoDrop and approximately 40 mg of protein was transferred into ultracentrifuge tubes. The volume was brought up to 8.5 ml with buffer 4 and the tubes were incubated 1 hour at +4 ^{o}C in a rotating shaker. After balancing the tubes they were centrifuged for 1 hour at 38 000 rpm, +4 ^{o}C (rotor 70.1 Ti).

4.4.4 Affinity chromatography and TEV elution

At first 35 µl of the IgG-sepharose slurry per 15 ml Falcon tube was equilibrated with buffer 4. The supernatant achieved from the solubilisation was then transferred into the tube containing equilibrated IgG-sepharose. The tubes were incubated overnight at +4 ^{o}C in a rotating shaker.

In order to remove the supernatant the tubes were centrifuged for 5 min at 1 500 x g. Approximately 500 µl of the supernatant was left for re-suspension. The sepharose pellets were transferred into Mobicol columns equilibrated with buffer 5 and centrifuged for 1 min at 200 rpm. The columns were washed 10 times with 175 µl of buffer 5.

Then 1.5 µl of the self-made TEV protease and 73.5 µl of buffer 5 was added into the column and carefully mixed. The columns were incubated 2 hours at +16 ^{o}C. After the incubation the columns were centrifuged (1 min at 200 rpm) and washed twice with 37.5 µl of buffer 5. The fractions were frozen in liquid N_2 as four aliquots of 37.5 µl each (total volume 150 µl) and stored at -70 ^{o}C. Lastly, the column was eluted with 170 µl and 100 µl of 1 x SDS-sample buffer.

All the samples collected were run on a BIO-RAD Mini-Protean® TGX^{TM} Any kD^{TM} gel (10-well comb, 30 µl/well, Cat. #456-9033) at 200 V for 27 min. The gels were then silver stained and dried. Fermentas PageRuler^{TM} Plus Prestained Protein Ladder was run along with the samples (see appendix 2).

4.5 Preparation of samples for MALDI-TOF mass analyser

The samples from the first purification and the second purification were prepared in a similar way for the mass analyser. Only different matrix solutions were used.

Bands of interest and a blank were cut from the silver stained SDS-PAGE gel. The gel pieces were transferred into Eppendorf tubes and destained by adding 100 µl of a solution containing 1 part of sodium thiosulphate and 1 part of potassiumhexacyanoferrat and incubating 10 min at room temperature. After

removing the destaining solution they were washed three times with 200 μl of sterile water until they were no longer yellow.

100 μl of 40 % acetonitrile (AcN), 0.2 M NH_4HCO_3 and 2 μl of 1 M DTT was added and incubated 1 hour at +37 ^{o}C heating block. The solution was removed and 100 μl of 40 % AcN, 0.2 M NH_4HCO_3 and 5 μl of 1M IAA was added and incubated 1 hour at a +37 ^{o}C heating block. The gel pieces were then washed with 200 μl of 40 % AcN, 0.2 M NH_4HCO_3 and dehydrated with 50 μl of 100 % AcN. After removing the AcN solution they were dried and cooled on ice. The gel pieces were soaked with trypsin solution (20 ng of trypsin per sample) and first incubated 30 min on ice and then overnight at a +37 ^{o}C heating block.

The next day 20 μl of 30 % AcN/ 0.5 % TFA (trifluoroacetic acid) was added and incubated 5 min in an ultrasonic bath. The 20 μl was transferred into a fresh Eppendorf tube and 20 μl of 70 % AcN/0.1 % TFA was added on the gel piece and incubated in an ultrasonic bath as previously. The two 20 μl extractions were pooled and dried under vacuum in a speed-vac for 30 min. 5 μl of TA30 was added to each sample and incubated 2 hours in a shaker at room temperature.

4.6 Analysing samples with MALDI-TOF mass analyser

The samples from the second purification were analysed on a different plate than the samples from the first purification. A ground steel plate was used for samples from the first purification and an anchored-chip-plate for samples from the second purification.

4.6.1 Samples from the first purification

Samples were mixed 1:1 with HCCA (α-cyano-4-hydroxy cinnamic acid) matrix solution. Each sample was then pipetted on a ground steel plate and analysed with the mass analyser using dried droplet protocol which enables fast, simple and robust MALDI preparation of virtually all types of samples.

4.6.2 Samples from the second purification

Samples were first pipetted on the anchor chip plate, which concentrates the sample on the plate and, thus, enhances the analysing of samples of low concentration. The samples were dried and then the TFA matrix was pipetted on top of the sample. All the samples were then analysed with the mass analyser.

5 RESULTS

In this section, results from the experimental part of the thesis are presented. Pictures of SDS-PAGE gels, western blots etc. from the experiments are explained and only the results significant for this thesis are summarised.

5.1 Production and purification of TEV protease

The purity of the TEV protease was monitored by taking samples before, during and after the purification procedure and running them on a SDS-PAGE gel. As seen on the Coomassie stained gel (figure 7), the purity of the protease increases significantly along the way. TEV is the size of 34 kDa. The band of TEV protease after dialysis is very intense and there are hardly any contaminants.

In order to know whether the protease is active, it should have been tested using a suitable substrate to this enzyme. Because of lack of a substrate, the protease was not tested before use. However, the TEV protease has been purified in this manner numerous times by Dr. Tiila Kiema in the Wierenga laboratory at the Biochemistry Department Oulu.
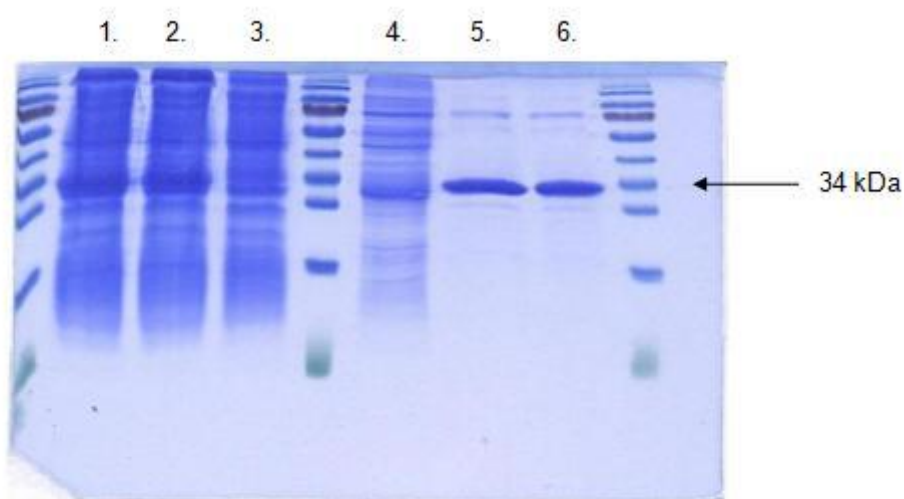

FIGURE 7. Samples of the purification of TEV protease on a SDS-PAGE gel. (1.) Lysate supernatant (2.) lysate supernatant (3.) flow through (4.) wash (5.)eluate (6.) dialysed TEV.

5.2 Detection of Rpm2p

The protein was extracted from the cultures by TCA precipitation and separated on a SDS-PAGE gel. A western blot was then done for the protein using antibodies against the Prot-A of the TAP tag on the protein. The detection was remarkably difficult as the protein is of low abundance but after several western blots a weak band of the Rpm2p protein was detected. As seen in figure 8, the band for the wt is poorly noticeable but it can be confirmed that the protein is detectable and the project could be continued. The control strain was later changed to another strain and therefore the results related to the control strain used in this experiment are not important.
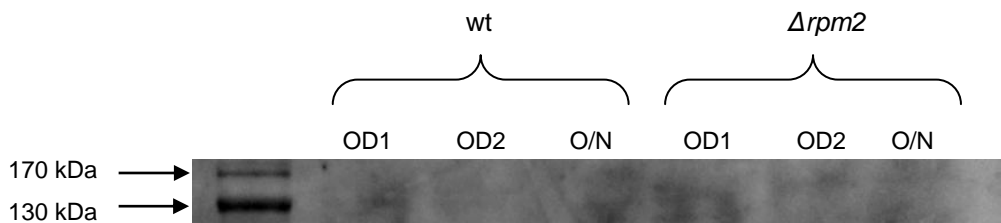

FIGURE 8. Western blot of the Rpm2p protein.

5.3 First purification using solubilised extract

Samples were collected during the protein extraction and the purification of the protein. These samples were then run on SDS-PAGE gels and the gels were silver stained. In figure 9 are the samples of the control strain, in figure 10 samples of the *Δetr1* strain and in figure 11 samples of the wildtype strain. The samples of wildtype and *Δetr1* strains were run for a bit too long and therefore the three last bands of the molecular weight marker and some of the proteins of the samples (smaller than 26 kDa in size) were run off the gel. The highest band of the molecular weight marker is 170 kDa. The strong band in lanes 8 and 9 is the TEV protease in all the three gels.
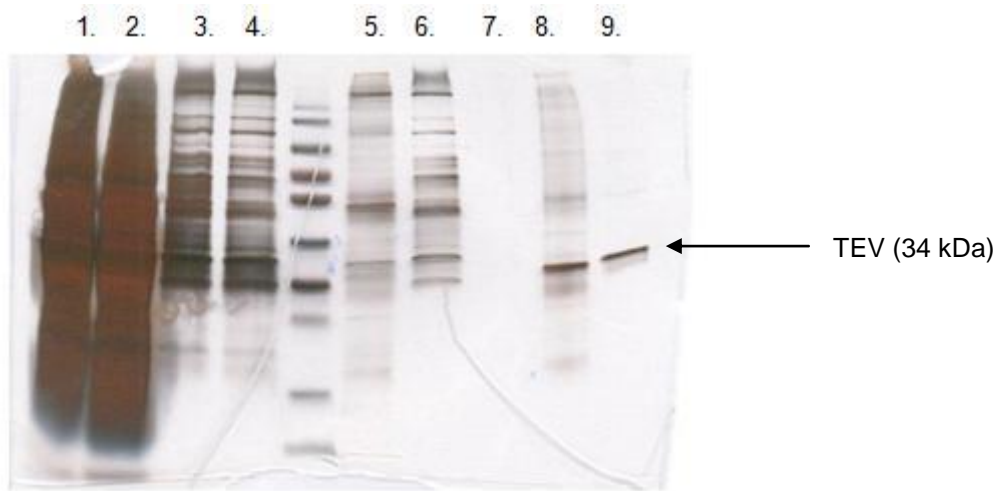
FIGURE 9. Samples of the control strain on a silver stained SDS-PAGE gel. (1.) homogenate (2.) supernatant after the first ultracentrifugation (3.) pellet after the first ultracentrifugation (4.) supernatant after the second ultracentrifugation (5.) pellet after the second ultracentrifugation (6.) flow through (7.) wash (8.) SDS-elution (9.) fraction from the TEV elution.

FIGURE 10. Samples of the Δetr1 strain on a silver stained SDS-PAGE gel. (1.) homogenate (2.) supernatant after the first ultracentrifugation (3.) pellet after the first ultracentrifugation (4.) supernatant after the second ultracentrifugation (5.) pellet after the second ultracentrifugation (6.) flow through (7.) wash (8.) SDS-elution (9.) fraction from the TEV elution.
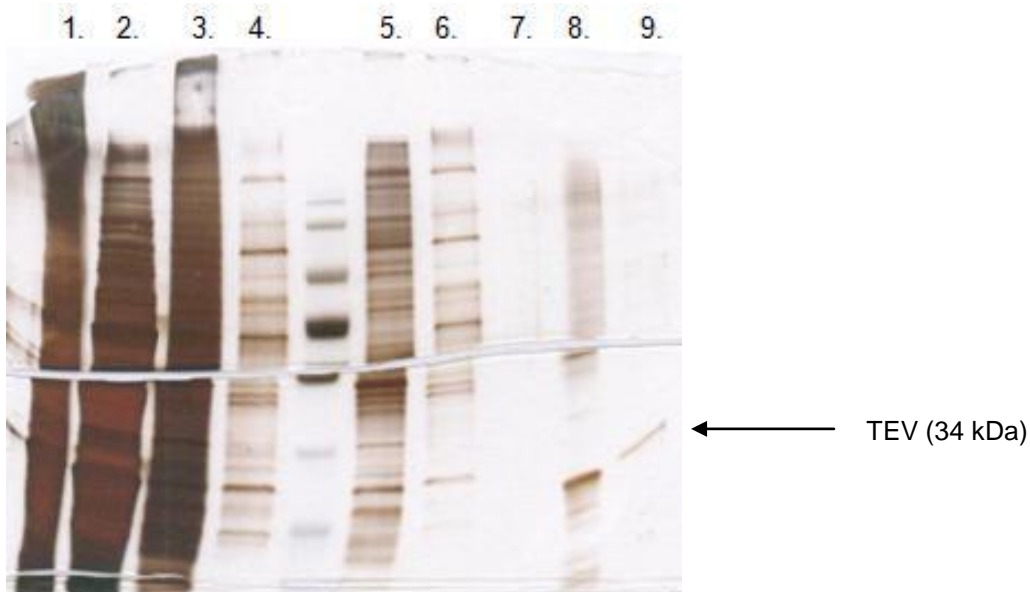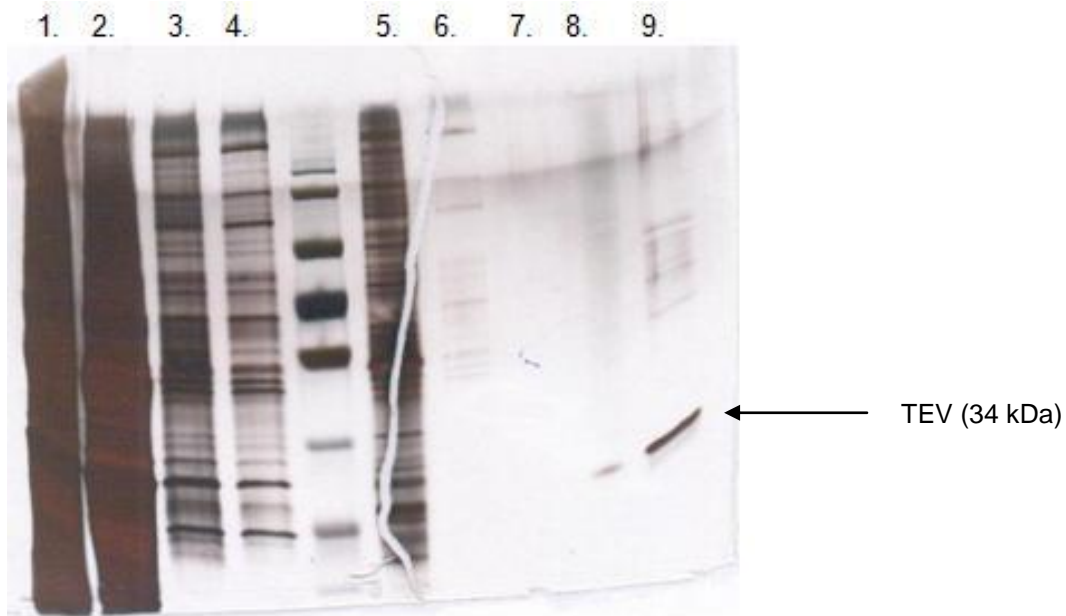
FIGURE 11. Samples of the wildtype strain on a silver stained SDS-PAGE gel. (1.) homogenate (2.) supernatant after the first ultracentrifugation (3.) pellet after the first ultracentrifugation (4.) supernatant after the second ultracentrifugation (5.) pellet after the second ultracentrifugation (6.) flow through (7.) wash (8.) SDS-elution (9.) fraction from the TEV elution.

Fractions of all of the strains from the TEV elution were run on a bigger SDS-PAGE in order to get better separation of the proteins and more clear bands which could be then further analysed. The gel was then silver stained. The lane 1 in figure 12 of the silver stained gel is the control strain, lane 2 is the wildtype strain and the lane 3 is the *Δetr1* strain. The bands found most interesting were then analysed with the MALDI-TOF mass analyser. The bands of the three different strains were compared with each other and the bands with differences between the strains were chosen. The bands identified can be seen in figure 12.
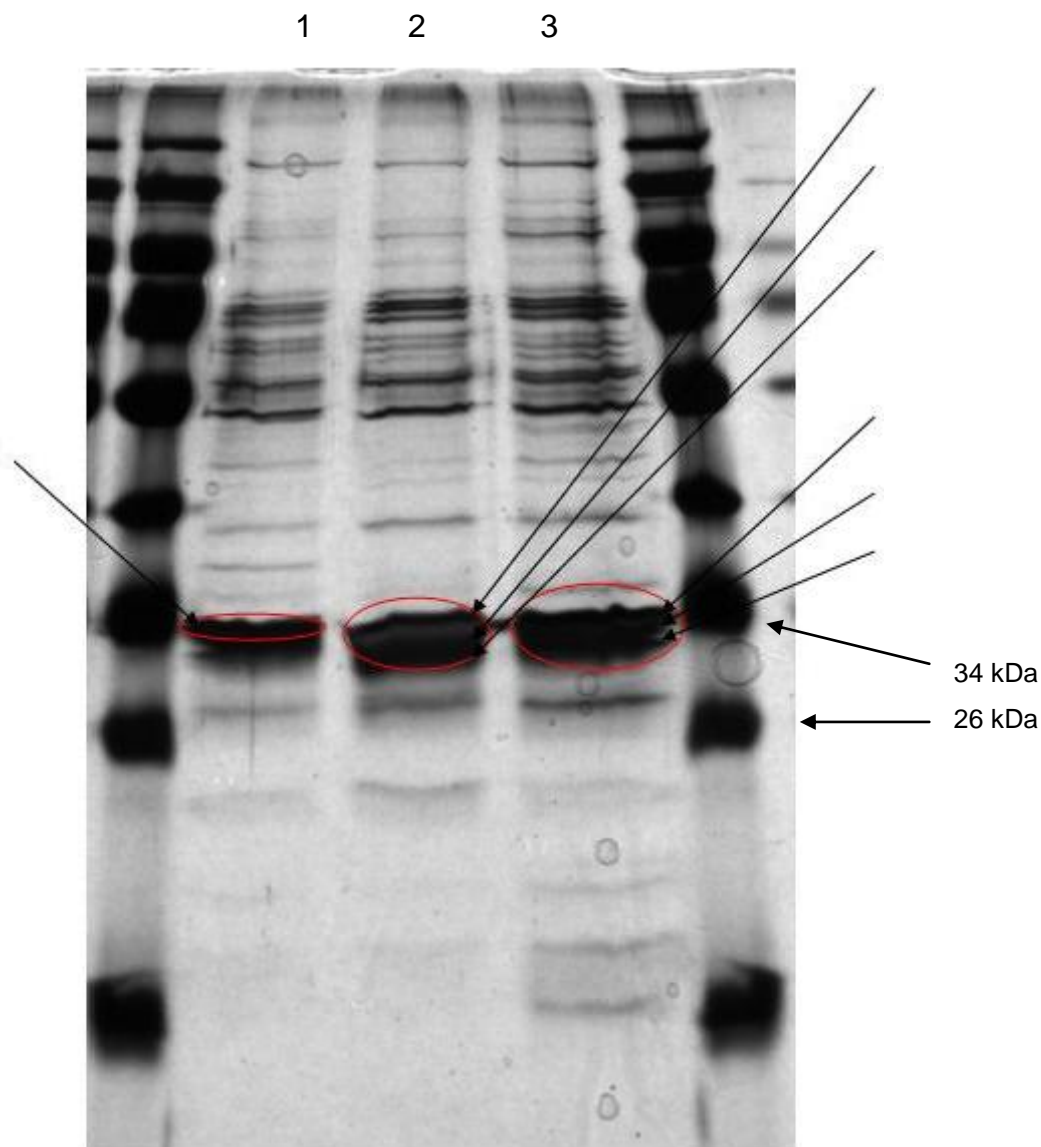
FIGURE 12. Silver stained SDS-PAGE gel of all the fractions from TEV elution. The bands indicated were further analysed with MALDI-TOF. (1.) control strain, (2.) wildtype strain (3.) Δetr1 strain.

The proteins of interest could not be traced with the mass analyser. The only discovery was the TEV protease which was found on the bands expected. All of these bands seen in the picture were identified as catalytically active Tobacco etch virus protease complexed with the product. The sizes of these bands on the gel are approximately around 34 kDa which is exactly the size of TEV protease.

5.4 Second purification using purified mitochondria

Only the control strain and the wildtype strain were used for the second purification. Therefore the *Δetr1* strain was left out because this strain was not the main interest and the other strains had to be concentrated on.

The bands identified with the mass analyser can be seen in figure 13. Lane 1 is the SDS-elution sample and lane 2 is the fraction sample of the wt strain. Lane 3 is the SDS-elution sample and lane 4 is the fraction sample of the control strain. The band b2 was identified to be Gcv3p. The size of the protein on the gel is approximately 36 kDa which is relatively big for Gcv3p because the evaluated size of it is 19 kDa. The size would perhaps better be equivalent to the size of Gcv3p with the TAP tag which is ~35 kDa or a Gcv3p dimer. But, in any case, the Gcv3p protein is present, it cannot be merely a coincidence. The high molecular weight band just above 130 kDa in lane 4 seems to be specific for the Gcv3p but, unfortunately, it could not be identified.

The band b4 was identified to as catalytically active Tobacco etch virus protease complexed with the product. The size of the protein on the gel is between 28 kDa and 36 kDa which would correspond to the size of TEV protease which is 34 kDa.
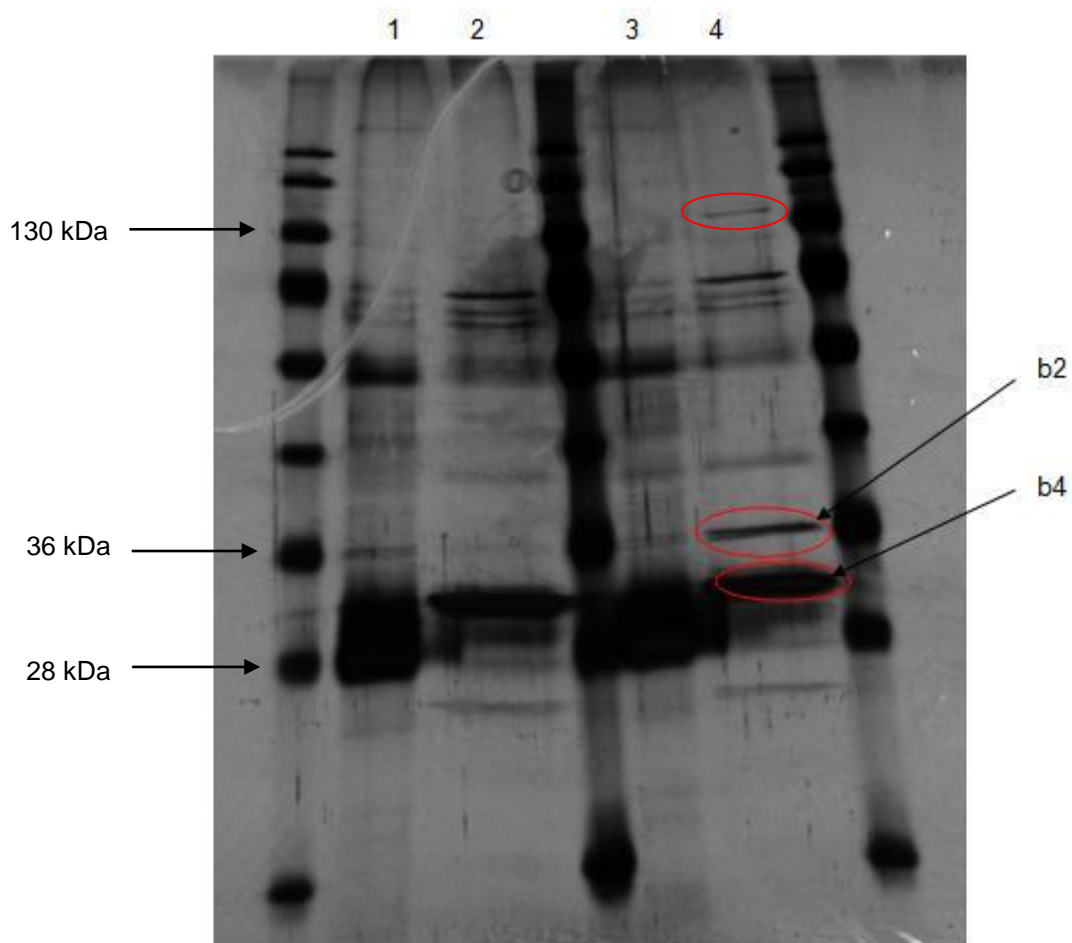
FIGURE 13. The silver stained SDS-PAGE gel of the fractions from the TEV elution and SDS-elution of both the control and the wildtype strain. (1.) SDS-elution of wt strain, (2.) fraction of wt strain, (3.)SDS-elution of control strain, (4.) fraction of control strain.

6 DISCUSSION

The purification of the produced TEV protease was successful in general. A very clear band was achieved in the Coomassie stained SDS-PAGE gel. There were hardly any contaminating proteins and if there were, the bands were very light commensurated to the bands of TEV protease. Even though the protease could not be tested properly, due to the lack of a substrate, it could be identified with the mass analyser.

There were difficulties in detecting the Rpm2p protein. It could not be detected at all at the first attempts but after several western blots a weak signal was finally achieved. The reason why the signal was so weak might be because of the low abundance of the protein under the conditions examined.

The first purification did not go as expected as the target proteins could not be tracked with MALDI-TOF. The low abundance of the Rpm2p and Gcv3p might be one reason also for this. The amount of the proteins produced in the cells is so small and therefore the purification method is not effective enough – too much contaminating protein is interfering and the target proteins are lost in the mix. This means that a more effective purification protocol must be used and therefore a second purification was done. Even though not being able to identify the target proteins the TEV protease was identified, as mentioned before, which confirmed it was what it should be and would compensate the failure of not been able to test it with substrate.

The second purification was a bit more successful than the first purification as the control protein Gcv3p could be identified from the silver stained SDS-PAGE gel with the MALDI-TOF mass analyser. Although the target protein Rpm2p could not be purified and identified, the fact that the control protein could be identified would therefore state that the purification should work in general. It is interesting that Gcv3p ran at a size of about 36 kDa. This size corresponds to the TAP tagged Gcv3p monomer, but would also fit a Gcv3p dimer. As the protein A part was not identified in the Gcv3p band by mass spectrometry, and the protein was released in the TEV elution, it seems unlikely that the band

represents tagged Gcv3p. On the other hand, it is not clear how a Gcv3p dimer could resist the harsh conditions of SDS PAGE. Remarkably, a high molecular weight band apparently specific for the Gcv3p purification appeared in the Gcv3 sample. Unfortunately, it was not possible to identify the protein represented by this band.

The failure to isolate Rpm2p in the procedure leads to the question of why Rpm2p could not be purified even though a purified mitochondrial fraction was used. It might be that the protein is low abundant and gets lost in the mix of contaminating proteins. As in the western blots for the TAP tag of the Rpm2p, the signal achieved is very pale which tells there is not much protein in question in the sample. Nevertheless, there is Rpm2p in the sample. It is also possible that despite the use of detergent in the purification, a large part of the protein may remain in pelleted fractions as Rpm2p has been reported to be membrane associated.

Another factor possibly interfering the purifying of Rpm2p, is the modification in the protocol as the pH was lowered down from 8 to 7. It could be that the lower pH is interfering the binding of the protein A to the IgG-matrix. This would mean that most of the protein would be left in the supernatant when incubating the solubilised extract with the IgG-beads. Of course the protein should then be able to be detected in the supernatant after taking the IgG-beads but the concentration of the protein in it is so low because of the high volume of the supernatant that it might not possible to be detected at all. In addition, the success of purifying Gcv3p would indicate that the tag is still bound even at pH 7. It may be worthwhile to investigate growth conditions that allow detection of clearer amounts of Rpm2p.

As Gcv3p can be isolated in this procedure, it may be possible to find and identify proteins physically interacting with Gcv3p. The fact that the other components of glycine cleavage system were not found, may indicate that the purification has to be optimized for the identification of interacting factors (e.g. variation of the detergent used). An improvement of this purification may yield in answers on what the role of Gcv3p is in lipoylation, for example if interactions with lipoyl transferases could be shown. This work will provide a good starting

point for optimization and further investigations while it was not possible to yield concrete results on Rpm2p modifications or Rpm2 and Gcv3p interactions.

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Solutions used

LB medium

10 g Tryptone

5 g Yeast extract

10 g NaCl

Add distilled water up to 1 litre and autoclave.

SCD medium

6.7 g Yeast Nitrogen base w/o amino acids (DIFCO)

1. 92 g Synthetic Drop Out media supplement without Uracil (SIGMA)

0.08 g Uracil

20 g Glucose

Add distilled water up to 1 litre and autoclave.

SCD-LEU medium

6.7 g Yeast Nitrogen base w/o amino acids (DIFCO)

1.6 g Synthetic Drop Out media supplement without Leucine

20 g Glucose

Add distilled water up to 1 litre and autoclave.

10 x M9-SALT

10 g NH_4Cl

30 g KH_2PO_4

31.77 g Na_2HPO_4

Add distilled water up to 1 litre and autoclave.

M9ZB medium

10 g Casein hydrolysate

5 g NaCl

Add 890 ml distilled water and autoclave.

Before use add:

100 ml 10 x M9-SALT

10 ml sterile 85 % glycerol

2 ml sterile 1 M $MgSO_4$

Lysis buffer

50 mM NaH2PO

300 mM NaCl

10 mM imidazole (added just before use)

Adjust pH to 8.0 using NaOH

Wash buffer

50 mM NaH_2PO

300 mM NaCl

20 mM imidazole (added just before use)

Adjust pH to 8.0 using NaOH

Elution buffer

50 mM NaH_2PO

300 mM NaCl

250 mM imidazole (added just before use)

Adjust pH to 8.0 using NaOH

Dialysis buffer

25 mM Tris, pH 8.0 at 4 ^{o}C

150 mM NaCl

2 mM EDTA

2 mM DTT

10 x Lysis buffer (buffer 1)

0.2 M HEPES

1 M Potassium acetate

50 mM Magnesium acetate

Adjust pH to 7.0 with KOH at 4 ^{o}C

1 x Lysis Buffer + inhibitors (buffer 2)

1 x Lysis buffer

Inhibitors (1 tablet per 10 ml)

1 mM PMSF

10 mM EDTA

Chech pH at 4 ^{o}C

Solubilisation buffer (buffer 3)

1 x Lysis buffer

10 % (w/v) Glycerol

10 mM EDTA

Chech pH at 4 ^{o}C

Solubilisation buffer + inhibitors (buffer 4)

1 x Lysis buffer

Inhibitors (1 tablet per 10 ml)

1 mM PMSF

10 mM EDTA

10 % (w/v) Glycerol

Chech pH at 4 ^{o}C

Solubilisation buffer + inhibitors + Triton X-100 (buffer 5)

1 x Lysis buffer

Inhibitors (1 tablet per 10 ml)

1 mM PMSF

10 mM EDTA

1 % Triton X-100

10 % (w/v) Glycerol

Chech pH at 4 ^{o}C

PBS

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

Adjust pH to 7.4

TBS

20 mM Tris-HCl, pH 7.6

137 mM NaCl

TBS-Tween

20 mM Tris-HCl, pH 7.6

137 mM NaCl

0.1 % Tween 20

Zymolyase buffer

1.2 M Sorbitol

20 mM Potassium phosphate, pH to 7.4

Homogenisation buffer

0.6 M Sorbitol

10 mM Tris-HCl, pH 7.4

1 mM EDTA

0.2 % (w/v) BSA

SEM

250 mM Sucrose

1 mM EDTA

10 mM Mops, pH 7.2

EM

10 mM Mops, pH 7.2

1 mM EDTA

Molecular weight markers

Fermentas
LIFE SCIENCES

CERTIFICATE OF ANALYSIS

PageRuler™
Prestained Protein Ladder

#SM0671   2 x 250 µl

(for 100 mini gel applications 5 µl per well or 50 large gel applications 10 µl per well)

Lot: **Expiry Date:**

Store at -20°C
(or at 4°C for 3 months)

ISO ISO
9001 14001
www.fermentas.com

Description

PageRuler™ Prestained Protein Ladder is a 3-color protein molecular weight standard containing 10 prestained recombinant proteins covering a wide range molecular weights from 10 kDa to 170 kDa. The ladder contains one orange reference band of ~70 kDa and one green reference band of 10 kDa.
Lot-to-lot variation of the apparent molecular weight of prestained proteins is ~5%.
The ladder is supplied in gel loading buffer and is ready-to-use: no heating, further dilution or addition of a reducing agent is required.
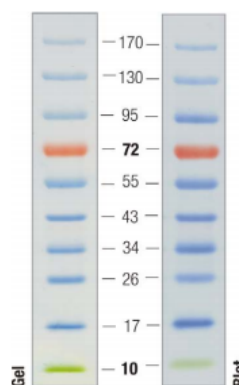
Applications

- Monitoring of protein separation during SDS-PAGE (1).
- Verifying Western transfer efficiency (2, 3).
- Approximate sizing of proteins on SDS-polyacrylamide gels and Western blots.
- Locating a protein of interest for excision from an unstained preparative gel.

Storage Buffer

62.5 mM Tris-H_3PO_4 (pH 7.5 at 25°C), 1 mM EDTA, 2% (w/v) SDS, 10 mM DTT, 1 mM NaN_3 and 33% (v/v) glycerol).

Recommendations for Loading

1. Thaw the ladder at room temperature for a few minutes to dissolve precipitated solids. **Do not boil!**
2. Mix gently, but thoroughly, to ensure the solution is homogeneous.
3. Load the following volumes of the ladder on an SDS-polyacrylamide gel:
   – 5 µl per well for mini gel,
   – 10 µl per well for large gel.
   Use the same volumes for Western blotting.
   The loading volumes listed above are recommended for gels with a thickness of 0.75-1.0 mm. The loading volume should be doubled for 1.5 mm thick gels.

Note

- Each lot of the PageRuler™ Prestained Protein Ladder is calibrated against PageRuler™ Unstained Protein Ladder and calculated apparent molecular weights are reported in the picture.
- Prestained proteins can have different mobilities in various SDS-PAGE-buffer systems. However, they are suitable for approximate molecular weight determination when calibrated against unstained standards in the same system. *See* reverse page for migration patterns in different electrophoresis conditions.
- For precise molecular weight determinations use PageRuler™ Unstained Protein Ladder, #SM0661, *see* www.fermentas.com.
- In 8 or 10% gels low molecular weight proteins may migrate with the dye front.
- PageRuler™ Prestained Protein Ladder can be used in Western blotting with all common membranes: PVDF, nylon and nitrocellulose.
- Longer transfer times or higher transfer voltages may be required for Western blotting of large (>100 kDa) proteins.

Representative lot of PageRuler™ Prestained Protein Ladder, apparent MW, kDa

4-20% Tris-glycine SDS-PAGE

QUALITY CONTROL

5 µl of PageRuler™ Prestained Protein Ladder provide 10 bands of equal intensities in 4-20% SDS-PAGE (Tris-glycine buffer) and after electrotransfer onto PVDF membrane.

Quality authorized by: Jurgita Zilinskiene

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LIFE SCIENCES

CERTIFICATE OF ANALYSIS

IMPROVED
green 10 kDa band

PageRuler™ Plus Prestained Protein Ladder

#SM1811 2 x 250 µl

(for 100 mini-gel applications 5 µl per well or 50 large gel applications 10 µl per well)

Lot: **Expiry Date:**

Store at -20°C
(or at 4°C for 3 months)

ISO ISO
9001 14001
www.fermentas.com

Description

PageRuler™ Plus Prestained Protein Ladder is a mixture of prestained 9 recombinant proteins covering a wide range molecular weights from 10 kDa to 250 kDa. The ladder contains two orange reference bands at ~70 kDa and 25 kDa and one green reference band of 10 kDa. Lot-to-lot variation of the apparent molecular weight of prestained proteins is ~5%.
The ladder is supplied in gel loading buffer and is ready-to-use: no heating, further dilution or addition of a reducing agent is required.

Applications

- Monitoring of protein separation during SDS-PAGE (1-3).
- Verifying Western transfer efficiency (1-3).
- Approximate sizing of proteins on SDS-polyacrylamide gels and Western blots.
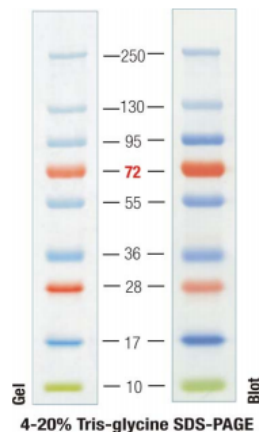- Locating a protein of interest for excision from an unstained preparative gel.

Storage Buffer

62.5 mM Tris-H_3PO_4 (pH 7.5 at 25°), 1 mM EDTA, 2% (w/v) SDS, 10 mM DTT, 1 mM NaN_3 and 33% (v/v) glycerol.

Rev.20

|||
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Recommendations for Loading

1. Thaw the ladder either at room temperature or at 37-40°C for a few minutes to dissolve precipitated solids. **Do not boil!**
2. Mix gently, but thoroughly, to ensure that the solution is homogeneous.
3. Load the following volumes of the ladder on SDS-polyacrylamide gel:
   – 5 µl per well for mini gel,
   – 10 µl per well for large gel.

Use the same volumes for Western blotting.
The loading volumes listed above are recommended for gels with a thickness of 0.75-1.0 mm. The loading volume should be doubled for 1.5 mm thick gels.

Note

- Prestained proteins can have different mobilities in various SDS-PAGE-buffer systems. However, they are suitable for approximate molecular weight determination when calibrated against unstained standards in the same system. *See* reverse page for migration patterns in different electrophoresis conditions.
- In 8 or 10% gels low molecular weight proteins may migrate with the dye front.
- Longer transfer times or higher transfer voltages may be required for Western blotting of large (>100 kDa) proteins.

Representative lot of PageRuler™ Plus Prestained Protein Ladder, apparent MW, kDa

Gel | Blot

—250—
—130—
—95—
—72—
—55—
—36—
—28—
—17—
—10—

4-20% Tris-glycine SDS-PAGE

QUALITY CONTROL

5 µl of PageRuler™ Plus Prestained Protein Ladder provide 9 bands of equal intensities in SDS-PAGE (Tris-glycine buffer) and after electrotransfer onto a PVDF membrane.

Quality authorized by: Jurgita Zilinskiene