universität wien

DIPLOMARBEIT

Proteomics of cyst formation in the free-living amoeba Acanthamoeba castellanii

angestrebter akademischer Grad

Magister/Magistra der Naturwissenschaften (Mag. rer.nat.)

Verfasserin / Verfasser:	Andrea Deutsch
Matrikel-Nummer:	0007520
Studienrichtung (lt. Studienblatt):	Molekulare Biologie
Betreuerin / Betreuer:	Prof. Dr. Wolfgang Löffelhardt (Universität Wien) Prof. Dr. Günther Allmaier (TU Wien)

Wien, am 15.07.2008

Zusammenfassung

Die Amoebe Acanthamoeba castellanii gehört zu den frei lebenden Amoeben der Familie Acanthamoebidae und ist weltweit aus vielen verschiedenen Quellen zu isolieren. Wie alle Amoeben, leben auch diese räuberischen eukaryotischen Mikroorganismen von Bakterien, Algen und Hefen und sind auch in der Lage menschliche Zellen zu lysieren. *A.castellanii* ist sowohl als Auslöser von Krankheiten als auch Überträger von humanpathogenen Bakterien nicht zu unterschätzen, da besonders bei immunsupprimierten Menschen schwere Infektionen, teilweise mit sehr fatalen Verläufen, beobachtet werden können. Die Amoebe ruft schwere Infektionen der Augen (Acanthamoeben-Keratitis) des Gehirns (granulomatöse Acanthamoeben-Encephalitis) und auf Haut und in der Lunge hervor. Die Behandlung einer *A. castellanii* Infektion ist nicht immer einfach, da sich der Eukaryot einer Heilung durch Zystenbildung entziehen kann. Im Zystenstadium ist *A.* castellanii in der Lage, einer Vielzahl von Bioziden zu trotzen und auch für ihn sehr lebensunwirklichen Umweltbedingungen unbeschadet zu überstehen. Die danach aufflammenden Infektionen haben meist einen schwereren Verlauf und können bei geschwächten Personen auch zum Tod führen.

Der molekularbiologische Mechanismus und auch die an En- und Exzystierung beteiligten Proteine sind noch weitgehend unerforscht. Der erste Teil dieser Arbeit beschäftigte sich mit der Isolierung der Proteine des Mikroorganismus in den verschiedenen Zelldifferenzierungsstadien während der En – und Exzystierung mittels 2D Gelelektrophorese (2D PAGE). Die nach der Elektrophorese erhaltenen Proteinspots wurden einem tryptischen in-Gel Verdau unterworfen und mit MALDI RTOF MS und MALDI RTOF MS/MS analysiert. Die Auswertung der PMFs (Peptide mass fingerprints) und PSD MS/MS Daten erfolgte mit der Proteindatenbank Mascot. Im zweiten Teil der Arbeit wurde eine neue nano high-performance chromatography (nHPLC)-Methode entwickelt um eine Vortrennung der manchmal aus mehreren Proteinen bestehenden Proben zu ermöglichen. Die aufgetrennten Proben wurden in verschiedenen Fraktionen auf ein MALDI Target gespottet und mit MALDI RTOF MS und MALDI RTOF MS/MS analysiert. Es konnten den Proteinspots „Enc 2“ das Protein „Actin“ und dem Spot „Alle 6“das Protein „Elongationsfaktor 2“ einwandfrei zugewiesen werden.

Abstract

The amoeba *Acanthamoeba castellanii* belongs to the free living amoeba in the family of Acanthamoebidae, which can be isolated from a lot of sources all over the world. Like all other amoeba species this microorganism lives manly on bacteria, alge and yeast and is able to lyse human cells. *A. castellanii* is on the one hand elicitor of diseases and on the other hand carrier for humanpathogen bacteria which can cause harmful diseases that are very dangerous for immunsuppressed patients. The chances for immunsuppressed patients to get cured from such an infection are not high. *A .castellanii* causes infections in the eyes (acanthamoeba ceratitis) in the brain (amoeba encephalitis), also in the lungs and on the skin. A therapy of this kind of infection is not easy because the amoebas ability to form stable cysts. In its cyst stadium the amoeba can resist a lot of biocides so that medication can be ineffective. When the medication stops the infection starts again, but this time the infection becomes more serious and frequently life-threatening for immunsuppressed patients.
The molecular mechanism and the proteins of the en and excystment are not identified yet. The aim of the first part of my thesis was to identify the proteins of A. castellanii during en- and excystment. The proteins were separated with 2D gelelectrophoresis (2D PAGE) and single protein “spots” were achieved. With these “spots” a tryptic digest was performed and the tryptic peptide mix was analysed by MALDI RTOF MS and MALDI RTOF MS/MS. The obtained PMFs (peptide mass fingerprints) and PSD MS/MS fragment data were evaluated by means of Mascot database search.
In the second part of the thesis a protocol has to be developed for a nano high-performance liquid chromatography spotter device (nHPLC-Spotter), to get better results and make it also possible to separate protein mixtures before the MALDI analysis. As a result the spot Enc 2 was identified as protein “actin” and the spot “Alle 6” was identified as protein “ elongation factor 2”.

Acknowledgement

First of all I want to say thank you to Prof. Allmaier for giving me the opportunity of doing the research for my diploma thesis in his group.

Next I want to especially thank my mother and Gerald, who made my study possible and for their support through this very hard time.

I also want to thank the people that were part of Prof. Allmaier´s group during the time I was working there: Birigit, Jasmin, Martina, Wolfgang, Tom, Gerald, Ernst, Alex, Evita and Pelin.

Last but not least thanks are going to all my friends and to all my colleques I had the pleasure to work with/for.

I. Introduction

1. Biological background

Amoebae are the most prevalent protozoa found in the environment. They are distributed worldwide and have been isolated from soil, dust, air, natural and treated water, seawater, swimming pool water, hospital surfaces, contact lenses and as a contamination in bacterial, yeast and mammalian cell cultures (1). All amoebae are single cell eukaryotic organisms belonging to the big group of protozoa. The life cycle of amoebae consist of two stages, an activated feeding stage (trophozoite stage) and a dormant cyst stage. The formation of cysts (encystment) occurs when the environmental conditions like pH or temperature change to the worse. The double walled wrinkled cyst consists of an ectocyst (first cyst wall) and an endocyst (inner cyst wall) and varies in size between 13-20 µm. Sizes varies from species to species. These cysts are resistant to biocides, chlorination and antibiotics and can survive over long periods at low temperatures (0℃ to 2℃). When the environmental conditions become more suitable the degradation of the cyst initiated (excystment) resulting in an activated trophozoite amoeba. The active amoeboid cell is characterised by a central less viscous portion of the cytoplasm (endoplasm) and an outer portion of a continuous phase of cytoplasm (ectoplasm) and the formation of pseudopodia, used for their movement. This amoeboid kind of movement is a general characteristic of all amoebae (1).

All amoebae are heterotrophic, they feed on bacteria, algae and yeasts in the environment, but can also take up small nutrical particles by pinocytosis and some pathogenic strains can also lyse human cells. Digestion in the free living species is normally done by hydrolases. *Entamoeba histolytica* as a member of pathogenic intestinal amoebae, ferments carbohydrates producing thereby ethanol and CO_2. The waste removal in all amoebae species is done by pulsing vacuoles which can fuse with the outer membrane. Reproduction is taking place during the trophozoite state by mitosis.

The large group of amoebae is a very heterogenous group. It is now generally accepted that the amoebae belong to different phylogenetic groups and do not represent a monophylum.

Acanthamoeba was first described by Castellani in 1930. He found amoeba in his fungi cell cultures and named it *Hartmannella* (2). A year later Volkonsky subdivided the genus *Hartmannella* in 3 genera classified by cyst formation and cyst appearance (3).Sawyer & Griffin finally established the family Acanthamoebidae in 1975 (4). The currently accepted taxonomy of *Acanthamoeba* and other free living amoebas is shown in figure 1.

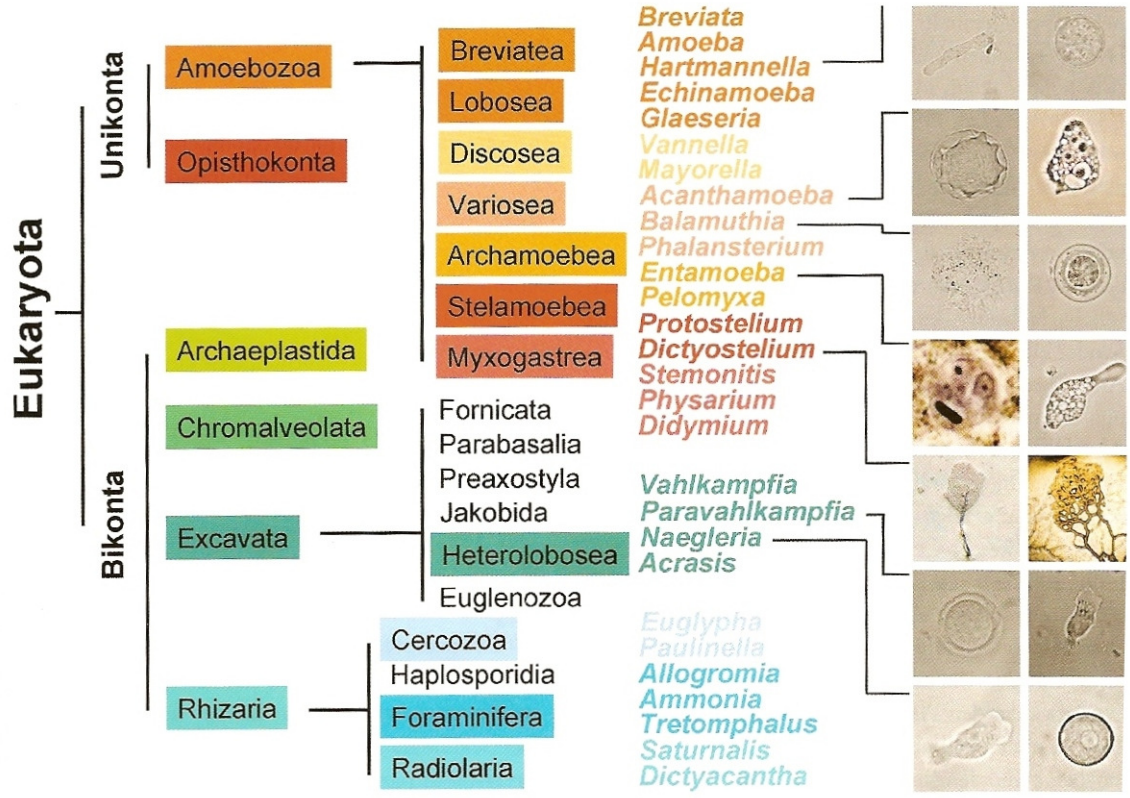

Figure 1. The scheme of free living amoebae (Light microscopic image of column A: trophozoite states and column B: cyst states) (1)

Modern molecular genetic studies confirm the classification done by Pussard & Pons in 1977 which is based on the comparison of morphological appearances of cysts in free living amoebas. Pussard & Pons subdivided the different species of *Acanthamoeba* to 3 cyst morphological groups (6).

Group 1	Group 2	Group 3
A. astronyxis	***A. castellanii***	*A. culbertsoni*
A. comandoni	*A. divionensis*	*A. lenticulata*
A. tubiashi	*A. griffin*	*A. royreba*
	A. hatchetti	*A. palestinensis*
	A. lugdunensis	
	A. mauritaniensis	
	A. polyphaga	
	A. quina	
	A. rhysodes	
	A. triangularis	

Table 1. Groups of *Acanthamoebas (6)*

The species belonging to group 1 form relatively big trophozoites and cysts with diameters over 18 µm. The smooth endocyst and the star shaped ectocyst are clearly separated (Figure 2a).*Acanthamoeba castellanii* belongs to the Group 2 of Acanthamoebae characterised by a diameter of less than 18 µm. The ectocyst can be thick or thin but it is clearly separated from the endocyst. The shape of the endocyst can vary from star shape over triangular to round (figure 2b). The cysts of the *Acanthamoeba* species belonging to Group 3 have diameters under 18 µm and their exo- and endocyst are round and closely together (figure 2c).

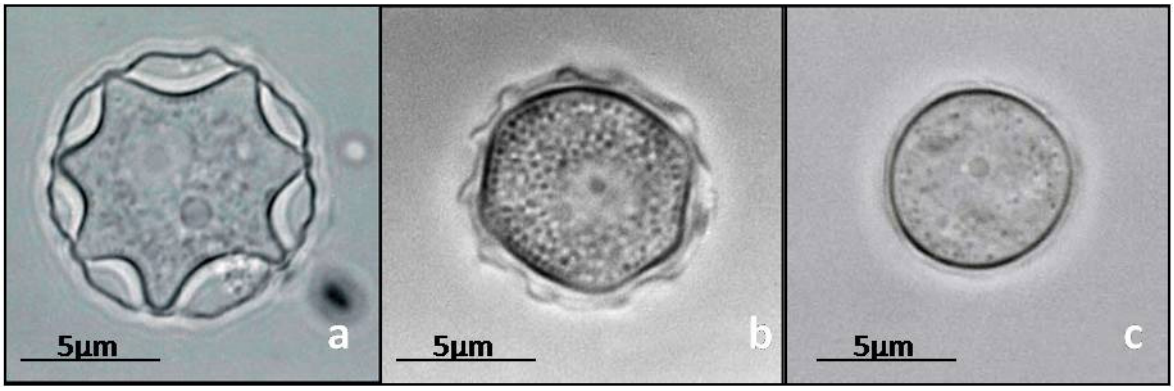

Figure 2a-c. Light microscopic image of cysts of *Acanthamoeba* Gr. 1 (a), Gr. 2 (b), Gr. 3 (c) (5)

Another very important classification of *Acanthamoeba* was established by Gast et al. (6) in 1996. He developed a classification schema based on nuclear r RNA gene sequences (18rDNA). The complete gene sequence of nuclear small ribosomal subunit RNA (Rns) was determined. Using this approach Stothard et al. (7) classified 53 isolates of *Acanthamoeba* species on the basis of 12rDNA sequence types (Rns genotypes) designated typing units T1 to T12. Current classification schemes integrate the morphological groups which were established by Pussard and Pons (6) with the 12 sequence types (Rns genotypes) such that Group 1 includes sequence types of T7, T8 and T9 ,Group 2 includes sequence types T3, T4 and T11, and Group 3 includes sequence types T1, T2, T5, T6, T10 and T12. The majority of *Acanthamoeba castellanii* clinical isolates which has been analysed belonging to sequence type T4.

The trophozoites of *Acanthamoeba* are normally flat and without any typical shape (figure 3a-c). Their size is between 15-45 µm and the cell surface is covered with long spiny protectors made of hyaline called acanthopodia. The same organelles found in Acanthamoeba were also found in other higher eukaryotic cells like Golgi complex, smooth and rough endoplasmatic reticula, free ribosomes, mitochondria, digestive vacuoles and microtubules.

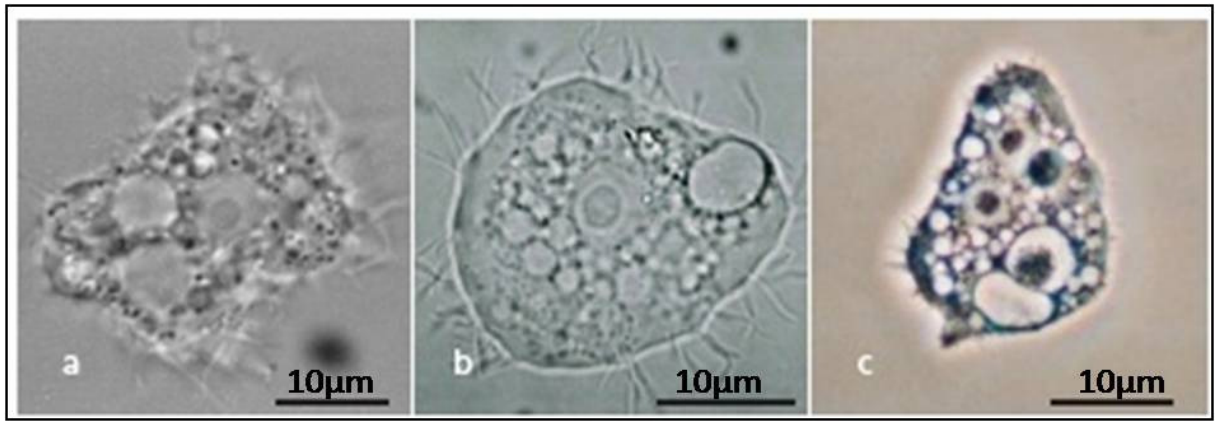

Figure 3 a-c. Light microscopic image of trophozoites of free living *Acanthamoeba* (5)

2. Medical Relevance

In the past years *Acanthamoeba* became of special interest for human medicine due to fact that *Acanthamoeba* cysts are very robust. They not only survive drug treatments, but also survive treatments with disinfectants. Moreover, amoebas are able to intracellular protect bacteria in their cysts. Especially, for people with suppressed immunsystems, resulting e.g. from special medical treatment, *Acanthamoeba* infections are very serious. They can cause illnesses like Granulomatous *Acanthamoeba* Encephalitis (GAE), *Acanthamoeba* keratitis and infections of lungs and skin. Special interest has been drawn on *Acanthamoeba* keratitis which is common under contact lens users all over the world. This small amoeba lives in the contact lenses fluid and can infect the cornea through micro lesions. The infection is very painful and needs a very long complicated treatment.
GAE is a chronical or sub-acute processing illness of the brain with an incubation time from a few weeks to a few months normally ending with the death of the affected patient. Several pathogens of the genus *Acanthamoeba* were found in immunosuppressed patients. The affected individuals infect themselves by inhaling the cysts or getting into contact with contaminated water. They start to replicate in the human body and the cysts can be carried with the bloodstream and are able to finally cross the blood brain barrier. In the brain the amoebae start to lyse the cells and cause damage of large parts of the tissue. Normally, the treatment of this kind of infection is not successful even when the illness is recognised in its early state (8-11).

3. Proteomics

3.1. Protein

In 1940 it was well known that proteins were built up by amino acids but it was not possible to determinate the sequences of peptides. In 1955, Sanger was able to identify the whole amino acid sequence of Insulin (12). In the beginning of 1950 a Swedish scientist Pehr Edman developed a more efficient way of amino acid determination known as Edman degradation which is still used nowadays (13). The information about the sequence of protein becomes more and more important in the field of biochemistry.Proteins are made of amino acids, molecule that contains both amine and carboxyl functional groups. Twenty common amino acids are used by

cells in protein biosynthesis specified by the general genetic code. All organisms differ in the amino acids they can synthesize and the ones they have to obtain through their nutrition. Those amino acids that cannot be synthesized by an organism are called essential amino acids. Amino acids are the basic structural building units of proteins. They are linked by an amide bond, also called peptide bond, to build up proteins. The sequence in which the amino acids are arranged is called primary structure of a protein. But just the correct folding into repeating local, (secondary structure), and a protein specific global (tertiary) structure, enables biological activity. The interaction of more than one protein subunit (quaternary structure) guarantees its full function. If proteins are treated with heat or chemicals they lose their native quaternary structure and as a consequence their biological functions as very often active centers are destroyed. This so called denaturation is in many cases irreversible so it is not possible to reconstitute the protein. This can be explained by the different characteristics of the functional side chains of the amino acid. The side chains are the variable part of an amino acid and are the reason for the differences in size, chemical reactivity, charge and molecular weight (Table 2) (14,15).

Amino Acid	3-Letter	1-Letter	Side chain polarity	Side chain acidity or basicity
Alanine	Ala	A	nonpolar	neutral
Arginine	Arg	R	polar	basic (strongly)
Asparagine	Asn	N	polar	neutral
Aspartic acid	Asp	D	polar	acidic
Cysteine	Cys	C	polar	neutral
Glutamic acid	Glu	E	polar	acidic
Glutamine	Gln	Q	polar	neutral
Glycine	Gly	G	nonpolar	neutral
Histidine	His	H	polar	basic (weakly)
Isoleucine	Ile	I	nonpolar	neutral
Leucine	Leu	L	nonpolar	neutral
Lysine	Lys	K	polar	basic
Methionine	Met	M	nonpolar	neutral
Phenylalanine	Phe	F	nonpolar	neutral
Proline	Pro	P	nonpolar	neutral
Serine	Ser	S	polar	neutral
Threonine	Thr	T	polar	neutral
Tryptophan	Trp	W	nonpolar	neutral
Tyrosine	Tyr	Y	polar	neutral
Valine	Val	V	nonpolar	neutral

Table 2. Overview over the 20 most common amino acids (15)

Amino acids are molecules that contain both an acidic group and a basic group, and are therefore ampholytic, which means that they are chemically neutral but have formal positive and negative charge on different atoms. A mixture of ampholytic substances can develop a pH gradient in an electric field and when amino acids were added they start to move to their specific isoelectric point (pI). The pI is the point where the netto charge of the amino acid is zero. This specific nature of amino acids and proteins is used in the first step of 2D electrophoresis (isoelectric focusing) one of the most important methods in protein analytics nowadays.

3.2. Proteome

The first very important invention for the research field of proteomics was published in 1975 from O´Farrel (23) and Klose (24). They made it possible to separate complex mixtures of proteins using a combination of isoelectric focusing (IEF) and SDS gel electrophoresis. This new method, the 2D gel electrophoresis, started to become one of the most important methods in biochemistry. This new method has the advantage to be a separation technique with a high resolution. At the time most of the analytical methods for protein characterisation (sequence analysis, amino acid sequencing) were not able to analyse small amounts of protein coming from the 2D electrophoresis. Another reason was the fact that the proteins were imbedded in a gel matrix which was incompatible with the protein chemical techniques during this time. This problem changed after new developments in protein chemistry. A lot of methods were improved or become more sensitive, furthermore it was possible to cleave proteins in the gel matrix and to transfer it onto a chemically inert membrane and have the opportunity to analyse them.

The biological vocabulary has been expanded with a series of “omes”: the genome (DNA sequence of an organism), the transcriptome (the messenger RNA expressed at a given time in the cell) and in 1995 Marc Wilkins (16) created the word proteome (the protein equivalence of a genome) as the study of proteins.

In contrast to genomic analysis proteomics is much more complex because the genome of an organism is more or less constant; a proteome differs from cell to cell and during its biochemical interactions with the environment and other cells. Another very important difference is the complexity of proteins because of mechanisms such

as alternative splicing, protein modification (glycosylation, phosphorylation) and protein degradation.
The identification and quantification of a complete set of proteins from organs or cells at any time requires powerful analytical techniques. Proteomics is a rapidly expanding field of research which requires sensitive and selective technologies.
The following sets of techniques are commonly used in proteomic experiments.

- Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)
- Liquid high performance chromatography (HPLC)
- Matrix assisted laser desorption/ionisation mass spectrometry (MALDI-MS)
- Electrospray ionization mass spectrometry (ESI-MS)

Over the years of research on the field of proteomics a lot of new technologies e.g. nanotechnology and methods e.g. protein microassays were established helping the scientists to identify proteins and protein interactions within different cells, organs and organisms (17).

4. Tools for Proteomics

4.1. Sample pre-treatment

Most of the samples in protein chemistry are a mixture of more than one kind of bio molecule. In a lot of cases it is not possible to identify the proteins in this mixture because of contaminations, complexity of the mixture and other disturbing agents. In proteomics it is necessary to pretreat the sample either to get rid of contaminations to enrich the amount of protein or to fractionate the proteome.

There are 5 commonly used sample preparation methods:

- Precipitation: For this fast and easy sample preparation mostly salts and organic solvents were used. A disadvantage of this method is the relatively high protein loss during purification. Therefore, this method cannot be used when protein concentrations are very low. It has turned out that the efficiency can be improved by freezing but this protocol should not be done with temperature sensitive proteins (26).

- Ultrafiltration: This separation process is used to purify and concentrate protein solutions. The basic of ultrafiltration is membrane filtration; a solution is pressed against a semi permeable membrane by hydrostatic pressure forces. Molecules of high molecular weight will be retained while water and other small solutes pass through the membrane (26).

- Dialysis: Dialysis is used for desalting and to change buffers. A semi permeable membrane, e.g. cellulose acetat or nitrocellulose, is placed between the sample solution and the exchange medium. Due to the osmotic pressure, the salts diffuse through the membrane, whereas proteins are retrained on one side (26).

- Chromatographic methods are based on the distribution of the analyte between a mobile and a stationary phase. This principle of reversed phase liquid chromatography (RPLC) is used in ZipTip Technology (18) .These Zip Tips are polypropylene micropipette tips with an expanded bed of 15 µm silica particles. The particles can be modified with C_4- or C_{18}- alkyl residues and therefore this type of ZipTips can be compared to a micro reversed phase column. For various sample preparation questions other types of ZipTips are available e. g.: filled with metal chelat resin (IMAC) or strong cation resin (IEX). All of these materials allow a bi-directional flow and therefore easy handling is possible. The tip is soaked with protein solution and thereby the protein is bound to the silica particles. In the next step the protein is washed and then eluted with an organic solvent. Due to the easy handling ZipTips are commonly used for desalting, concentration and sample preparation before MALDI-MS.

4.2. Enzymatic protein cleavage

In proteomics the complexity of native proteom has to be reduced to analyse them first by means of a protein separation technology (e.g. 2D PAGE).After the reduction of complexity specific enzymes were applied which cut the protein on specific cleavage sites. Every enzyme has its specific cleavage sites that are well known. There are 2 possibilities to digest a protein with an enzyme; first the digestion takes place in solution or second in a gel matrix.

Some sample preparation steps have to be done before starting an enzymatic digest. At first the proteins have to be reduced and alkylated. In this step disulfide bonds are broken and the free generated cysteins are alkylated to avoid recombination. So the enzyme can reach all its target bonds. In a further crucial step all contaminates have to be eliminated e.g. alkylation reagents, to guarantee reaction conditions suitable for the enzyme as the enzyme itself might get reduced or alkylated. To establish perfect conditions for the enzymatic cleavage the solvent has to be exchanged by a convenient digestion buffer. After adding the enzyme the enzymatic cleavage is carried out under “enzyme-friendly” conditions (correct pH, optimum temperature). After successful digestion the peptides can be washed out of the gel matrix and after additional washing steps, to remove unwanted buffer salts, peptides can be used for further analyses e.g. MALDI-MS or ESI-MS.

Trypsin is the most commonly used enzyme for a lot of different analysis. Trypsin is an endoprotease; it hydrolyses peptide bonds C-terminal of lysine and arginine. Trypsin, a serine protease is found in the digestive system of many different species, including humans, but mostly bovine or pig trypsin is used for biochemical applications. It has its highest activity between pH from 7.5 to 9 and a temperature of 37°C. In all experiments peptide digestion were carried out with a chemically modified Trypsin (This enzyme is chemically protected against self digestion).

4.3. Electrophoresis

The first electrophoresis was conducted by the Swedish scientist called Arne Tiselius in 1926. He was able to separate the main constituents of the human serum albumin, α-, β- and γ-globulin and therefore was awarded the Nobel Prize in 1948 (19). In 1959 Raymond and Weintraub invented polyacrylamide gels which are used for separation of DNA and protein mixtures by their different molecular weights (20). The next milestone in electrophoresis was the invention of agarose gels in 1961, which are used for analysis of DNA fragments (21).With the introduction of carrier ampholytes, which form stable pH gradients, in 1964 by a Swedish scientist, the separation of analytes by their different pIs (isoelectric focussing,[IEF]) became possible (22). Nevertheless electrophoresis had its breakthrough in 1975 when O´Farrell and Klose applied a new outstanding method called 2D electrophoresis (23, 24). 2D electrophoresis is a combination of IEF and Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) and they were able to separate total cell lysates. Based on that they gained information on the proteome of a whole cell. In 1988 a further improvement of 2D electrophoresis immobilised pH gradient (IPG) was introduced (25). The advantages of 2D electrophoresis are its good reproducibility, high loading capacity and highest resolution achievable for protein mixtures.

4.3.1. Principles of electrophoresis (26)

Electrophoresis is based on the migration of charged particles in an electrical field. Because of their different charges and molecular weights the particles have different electrophoretic velocities during migration in the electrical field.

Three types of electrophoresis are commonly known:

- Zone electrophoresis, uses a homogenous buffer system, thus gel electrophoresis belongs to this group.
- Isotachophoresis uses a discontinuous buffer system.

- Isoelectric focussing uses a pH gradient to separate the substances due to their pI values.

Media for electrophoretic separations:

- Solution, where separation is based on charge differences.
- Stabilising matrix, like membranes or gels. In these matrices separation is based on charge and size of applied particles.

4.3.2. Sample preparation

All kind of electrophoretic methods are sensitive against high salt and buffer concentrations and therefore buffer concentration are limited to 50 mmol/L. To obtain this level of salt and buffer concentration, desalting steps are often necessary, like gel filtration, dialysis, precipitation ect. Complete dissolvation of the sample is necessary because small solid particles, lipid droplets or insoluble proteins may interrupt separation. To avoid this problem filtration or centrifugation of the sample are recommended. Nevertheless it can happen that some proteins are insoluble or are getting insoluble. For this kind of proteins special sample solvents are available.

The sample for 2D gel electrophoresis has to be converted from its native form to a more suitable and stable conformation for IEF while preserving the native charge and molecular weight (M_r) of the protein of interest. Several sample preparation steps have to be done like solubilisation, disaggregation, denaturation and reduction of the sample (27). Complete denaturation has to be done to make sure that the protein is present in only one conformation and that no aggregation and intermolecular interactions will occur.

4.3.3. Isoelectric Focusing (IEF) -1.Dimension

IEF separates the proteins according to their isoelectric points (pI). On its pI the net charge of a protein and therefore the velocity in an electrical field is zero. The net

charge of a protein is the total of all formal positive and formal negative charges of the amino acid side chains and is influenced by the protein configuration and modifications like phosphorylation or glycosylation. There is also a focussing effect acting on the proteins which concentrates them at their pI values. This effect appears when the protein diffuses away from the pI, then it gains charge again and migrates back to the pI.

Types of pH gradient:

- Carrier ampholytes are small aliphatic molecules with different pI values. The advantage of the carrier ampholytes are high buffer capacities, high solubilities and constant conductivities near the pI. Its disadvantage is low reproducibility of separation as well as of producing the pH gradient, low stability of the gradient and tendency to drift towards the cathode.
- Immobilised pH gradient (IPG): Immobilizes are derivatives of acryl amide and are weak acids or bases that are polymerised within a gel matrix. The advantages of IPGs are that they are stable throughout the separation, its high reproducibility and high loading capability. The disadvantages are the complex production of IPGs and the long separation times.

Several types of sample application in IEF:

- Rehydration loading: The sample is a component of the rehydration solution. Advantages of this method are application of lager sample quantities, no precipitation on the point of application and easy handling.
- Cup loading: The sample is placed in so called sample cups. These cups should contact the gel to avoid leakage and should not destroy it. The application point on the IPG strip depends on the nature of the sample. Disadvantages are lower sample loads and the possibility of protein precipitation.
- Paper-bridge loading: The paper-bridge is soaked with the sample solution and then placed onto the IPG strip.

4.3.4. SDS-PAGE – 2.Dimension

In the second dimension SDS PAGE is used for separation. This separation is based on the different molecular weights of the proteins and peptides in a mixture. Before separating the proteins by mass, high concentrations of sodium dodecyl sulphate (SDS) has to be added to sample and buffers. SDS denatures the proteins and binds a number of SDS molecules roughly proportional to the protein's length. Because a protein's length (when unfolded) is roughly proportional to its mass, this is equivalent to saying that it attaches a number of SDS molecules roughly proportional to the protein's mass. Since the SDS molecules are negatively charged, the result of this is that all of the proteins will have approximately the same mass-to-charge ratio as each other. In addition, proteins will not migrate when they have no charge therefore the coating of the protein in SDS allows migration of the proteins in the second dimension

4.3.5. Gels for electrophoresis

- Polyacrylamide gels (PA): PA gels are made of acryl amide monomers and polyacrylamide. After adding ammonium persulfate and Tetramethylethylenediamine (TEMED) the polymerisation of polyacrylamide and the acryl amide monomers starts leading to a stable, transparent and chemically inert gel matrix.

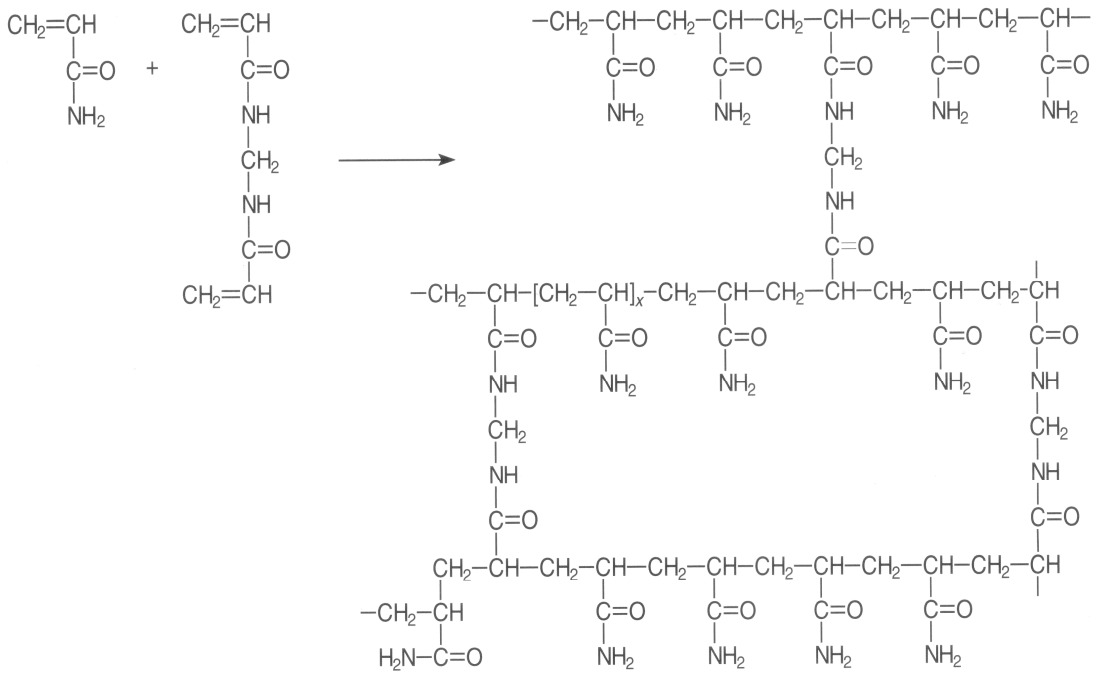

Figure 4. Chemical reaction, which builds up polyacrylamide.

- Agarose gels: are made of Agarose, a polysaccharide extracted from sea weed. These kinds of gels are widely used in biosciences nowadays, because of their easy handling practice and their relatively wide application area.

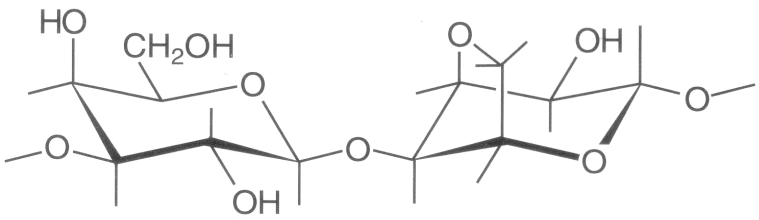

Figure 5. Chemical structure of the disaccharide, which is the basic unit of agarose.

4.3.6. Visualisation of the proteins on the gel

Detection of the protein bands can be done immediately on the gel.

- Coomassie (triphenylmethane dye) G and R staining: A common visualisation method is the staining with Coomassie G and R, both with high affinity to the protein, high extinction coefficients and good detection limits (~ 1 µg) (28). The gels are soaked in dye, the dye is absorbed by the protein and excess stain is then washed away with a solvent ("destaining").

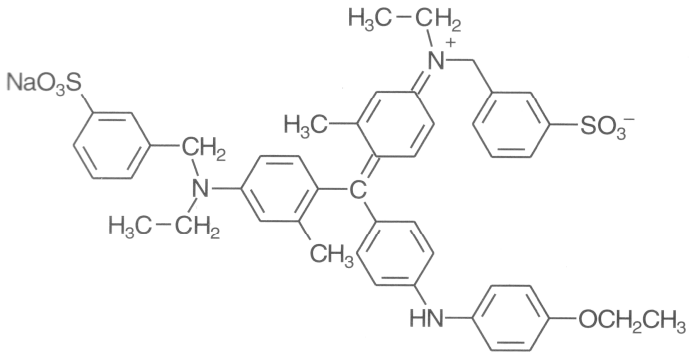

Figure 6. Chemical structure of Coomassie Brilliant Blue G 250

- Silver staining: This kind of staining is important especially to show small amounts of proteins and glycoproteins on a gel. This staining protocol is more sensitive than Coomassie staining and allows visualization of proteins below 1ng. The basic mechanism underlaying silver staining of proteins in the binding of silver ions to the amino acid side chains, primary the sulfhydril and carboxyl groups of proteins followed by reduction to free metallic silver. The protein bands are visualized as brown to black spots. (29)

5. High Performance Liquid Chromatography (HPLC)

5.1. Introduction

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. This situation changed during 1970's were most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. A lot of these chromatographic techniques were inadequate for quantification of compounds and similar compounds. These methods were very time consuming so that high pressure liquid chromatography was used to decrease experimental time. Liquid chromatography was able to reduce the separation time of a compound leading to the wide application of HPLC in the mid 1970´s (30). Not only the chromatographic technique was improved but a lot of new improvements in the fields of column packing materials, on-line detectors and new sample preparation

techniques prior to the separation made HPLC become one of the most important bioanalytical instruments. In the next years a lot of stationary phases like reverse phase liquid chromatography were improved helping to make the HPLC more useful in more and more analytical fields (31).

Nowadays, HPLC is widely used and is a main technique for biotechnological and biomedical research and is regularly used in the pharmaceutical industry (32).

The main parts of a modern HPLC system are: (schema is seen in figure 7)

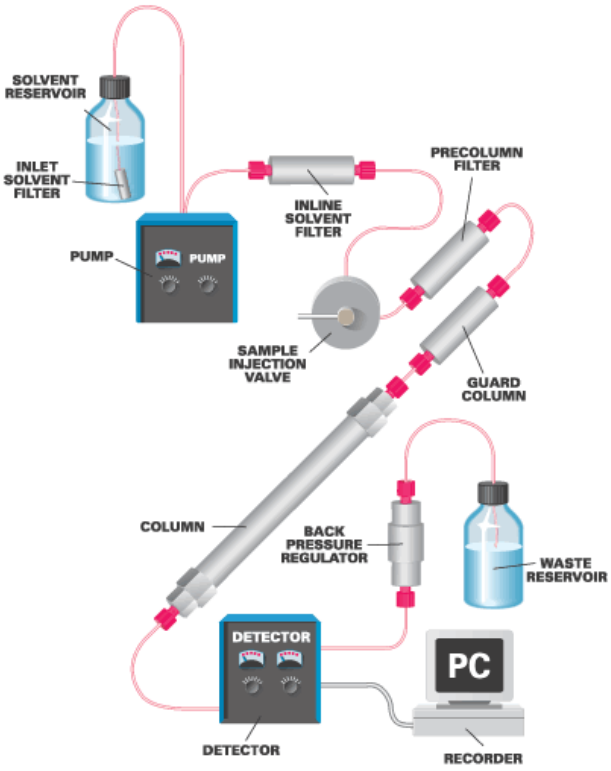

Figure 7. Schema of an HPLC System (**www.upchurch.com/TechInfo/**)

5.2. Solvents (mobile phase) reservoir

The reservoirs are containing the solvents that are continuously applied to the stationary phase in a column. The solvents are called mobile phase and are the carrier of the sample through the HPLC system. The chemical interaction of mobile phase and sample, with the stationary phase the migration behavior and final

separation of components contained in the sample. A change in the mixture of the mobile phase can lead to a change in the interactions of the sample with the stationary phase. There are two kinds of elution techniques: Isocratic and gradient elution.

Isocratic elution: This is the most common elution technique in HPLC; a mobile phase of a fixed composition is used during the whole experiment.

Gradient elution: The composition of the solvent changes during the whole experiment leading to better separation than isocratic elution. Normally, the sample is injected while a mobile phase with water content (polar solvent) is being applied to the system. The mixture of the mobile phase is changed stepwise or linear raising the organic solvent fraction resulting in elution of retained components (33).

5.3. Pumps

Pumps for use with HPLC analysis have to fulfill the following requirements:

- Be able to create pressures up to 15 MPa
- Chemical resistance
- Constant delivery rate

There are several types of pumps available:

- **Reciprocating Piston Pumps** consist of a small motor driven piston which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400 µL in volume. On the back stroke, the separation column valve is closed, and the piston pulls in solvent from the mobile phase reservoir. On the forward stroke, the pump pushes solvent out to the column from the reservoir. A wide range of flow rates can be attained by altering the piston stroke volume during each cycle, or by altering the stroke frequency. Dual and triple head pumps consist of identical piston-chamber units which operate at 180 or 120 degrees out of phase. This type of pump system is significantly smoother than other types of pumps because one pump is filling while the other is in the delivery cycle.
- **Syringe Type Pumps** are most suitable for small bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled.

These pumps have a volume between 250 to 500 mL. The pump operates by a motorized lead screw that delivers mobile phase to the column at a constant rate. The rate of solvent delivery is controlled by changing the voltage on the motor.

- In **Constant Pressure Pumps** the mobile phase is driven through the column with the use of pressure from a gas cylinder. A low-pressure gas source is needed to generate high liquid pressures. The valving arrangement allows the rapid refill of the solvent chamber whose capacity is about 70 mL. In contrast to the other pumps these kinds of pumps have low level of pulsations which provides continuous mobile phase flow rates.

5.4. Injection port

Samples are injected into the HPLC via an injection port. The injection port of an HPLC commonly consists of an injection valve and the sample loop. The sample is typically dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a syringe and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 1 µl to over 500 µl (34).

5.5. Columns

Nowadays, in bioscience most of the HPLC analysis uses the reversed phase method (reversed phase HPLC). In RP-HPLC the stationary phase consists of an unpolar material and the mobile phase is made of a polar solvent (reversed phase). The typical column for RP-HPLC is made of stainless steel or fused silica filled with small sized particles made of modified silica gel or polymer material (stationary phase). The inner diameter of a typical column ranges from 3-5 mm whereas the internal diameter of a typical capillary column ranges from 3 to 200 µm. The particle size of the packing material ranges from 3-10 µm in standard columns and from 2-5 µm in capillary columns. In most of the HPLC systems precolumns are used to

purify the sample from unwanted contaminations and to protect the analytical column to extend the lifetime of the column. This precolumns are normally much shorter, have larger inner diameters and higher particle sizes than the standard columns.

5.6. Detectors

The detector for an HPLC is the component that generates a response due to the eluting sample compound and subsequently forms a peak in the chromatogram. It is positioned immediately posterior to the column in order to detect the compounds as they elute from the column. The width and height of the peaks may usually vary significantly, and the detection as well as sensitivity parameters have to be adopted. There are many types of online-detectors that can be used with HPLC. Some of the more common detectors include: Ultra-Violet (UV), Fluorescence and Mass Spectrometry (MS).

5.7. Nano HPLC (35-37)

Miniaturisation of an HPLC-system implies that all system components should be downscaled, including column, connecting tubing, injector and detector or detection interface. LC columns of 75 µm inner diameter or less require flow rates of 200nL /min or less. To reach the very high sensitivity often required in proteomics analysis, it is necessary to deliver a stable and reproducible flow at rates at which miniaturised electrospray interfaces perform best. Flow splitting systems, in which the flow is split between the pump and the injector, are often used but have the disadvantage that high losses of sample can occur. A better solution is the use of dual syringe pumps that can generate low flow gradients and accommodate high back pressures. As the flow is not split with this technique, solvent consumption is very low, allowing for continuous automated analysis even if the syringes can only contain a limited volume. Exponential and sigmoidal gradients can be generated using two syringe pumps, a relatively large mixing chamber and two 4 port valves. Miniaturised LC systems can only accommodate nL to µL injection volumes. However, sensitivity is

proportional to the absolute amount of sample loaded on to the column. Therefore, a lower injection volume results in diminished sensitivity and thus in a higher limit of detection.

6. Mass Spectrometry

Mass spectrometry is an analytical technique to determine the m/z values of ions present in a high vacuum. A mass spectrometer consists of an ion source to produce ions, a mass analyser to separate the ions due to their different m/z ratio and a detector to detect and quantify the ions.
Matrix assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI) are the most commonly used desorption/ionisation techniques in bioscience nowadays, mostly because high and polar molecular substances can be desorbed/ionised, in a very gentle way, leading to very low fragmentation and unproblematic molecular weight determination. ESI is commonly coupled with quadrupole or ion-trap analyzers, whereas MALDI-MS is mostly coupled with time of flight (TOF) analyzers.

6.1. Matrix assisted laser desorption/ionisation – mass spectrometry (MALDI-MS)

6.1.1. Introduction

To reach direct desorption and ionisation of organic molecules out of condensed phases lasers were already applied in the 1970s. Therefore thin sample layers were applied on a metallic surface and then irradiated with pulsed laser beams. Mass spectra with low peak intensity and high sample fragmentation were the result. Only ions with low molecular masses up to 2 kDa could be detected, that was the reason why laser desorption/ionisation mass spectrometry had very low importance for analyses of biomolecules.
In 1987 Karas, Hillenkamp and Tanaka (Nobel Prize 2002) invented MALDI-MS (38-40). With a new sample preparation method they were able to produce mass spectra

with higher peak intensity and hardly any fragmentation. The sample molecules were embedded into a solid/liquid matrix. These matrices were small organic molecules with high absorption at the laser's wave-length. UV absorbing matrices are used for UV laser, like N_2-laser (337 nm) and Nd:YAG-laser (355 or 266 nm), whereas IR absorbing matrices are used for IR lasers, like Er:YAG-laser (2,94 µm) and CO_2-laser (10 µm).

6.1.2. MALDI matrices

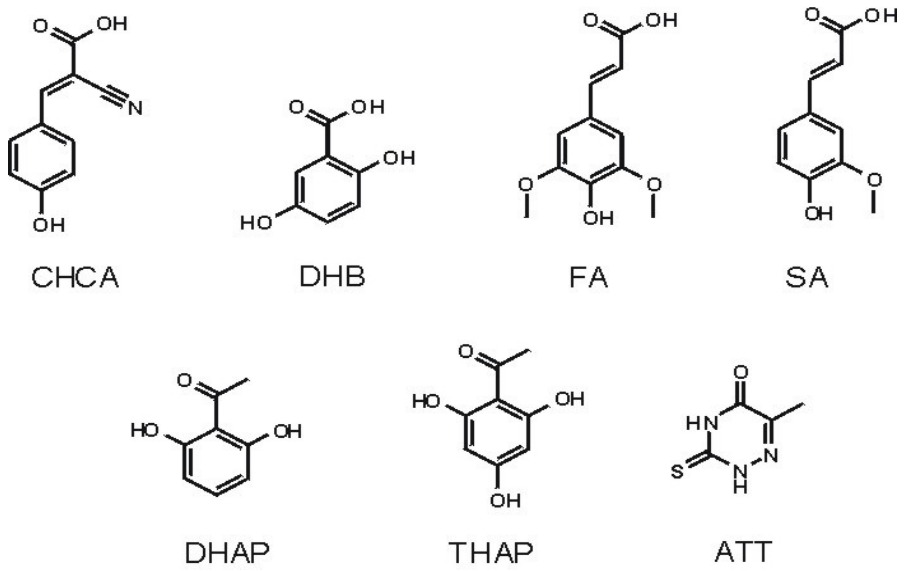

Figure 10. Chemical structures of commonly used MALDI matrices for UV lasers.

The most important properties for a MALDI matrix to fulfil:

- Absorption of the laser light with a particular emitted wave-length of the laser
- Isolation of the molecules via a solid crystalline or liquid viscous matrix
- Assembly of the analyte into the crystal lattice, so no intermolecular interactions can occur
- Providing support for ionisation (e.g. proton –transfer, adduct formation)

6.1.3. Sample preparation

The crucial point of MALDI-MS is the sample preparation. There are two important requirements to the sample. When measuring low concentrated samples the ion currents may reach the limit of detection and samples with higher concentrations may result in saturation of the detector and this causes broad, not useful signals. High sample concentration often leads to decreasing peak intensities and low resolution due to peak broadening. The standard sample amount actually needed is in the range of pico mol and femto mol.
The second request is high purity, because high concentrations of salt, buffer and detergents can interfere with the co-crystallisation of analyte and matrix. Additionally these contaminants are also visible in the mass spectra, because they are easy to desorb and ionise or are already charged. A crucial point is that the liquid containing the matrix and the liquid containing the sample have to be mixable.

There are four different main types of sample preparation (41):

- Dried-droplet method: 0,5-2 µL sample and 0,5-1 µL matrix solution are mixed on the MALDI target and allowed to air-dry.
- Volume method: When using the method mentioned above sample and matrix solution are mixed directly on the target. When using the volume method sample and matrix solutions are pre-mixed in an Eppendorf tube and then 1-1,5 µL of the mixture are applied on the target and dried under a gentle stream of air.
- Thin-layer method: A thin homogeneous layer of small matrix crystals is produced by applying 0,5 µL matrix solution whereas the solvent is a fast evaporating solvent on the target. The droplet is allowed to dry. Then 0,5-1 µL sample solution is applied on the matrix layer and the spot was dried under a gentle stream of air.
- Sandwich method: The first step of this method is analogous to the thin-layer method, a thin homogeneous matrix-layer is applied on the target and on this layer the sample is applied and allowed to air-dry. An additionally layer of matrix is applied on top of the already dried spot and dried under a gentle stream of air.

6.1.4. Laser

As the mostly used MALDI instruments are equipped with an UV-laser, the following chapter deals primarily with the UV-laser. The laser beam spot with a diameter of about 150 µm for UV lasers is focused over an optical system onto the sample plate. Commonly used laser intensities are 10^6 to 10^7 W/cm^2, if higher intensities are used this can result in fragmentation of the analyte.

Wavelength and laser pulse duration are dependent on the type of laser. The standard N_2-laser has a pulse duration of 3-5 ns and a wavelength of 337 nm whereas the pulse of a Nd:YAG-laser lasts 5-15 ns and works with 355 or 266 nm.

6.1.5. The Desorption/Ionisation Processes in UV-MALDI

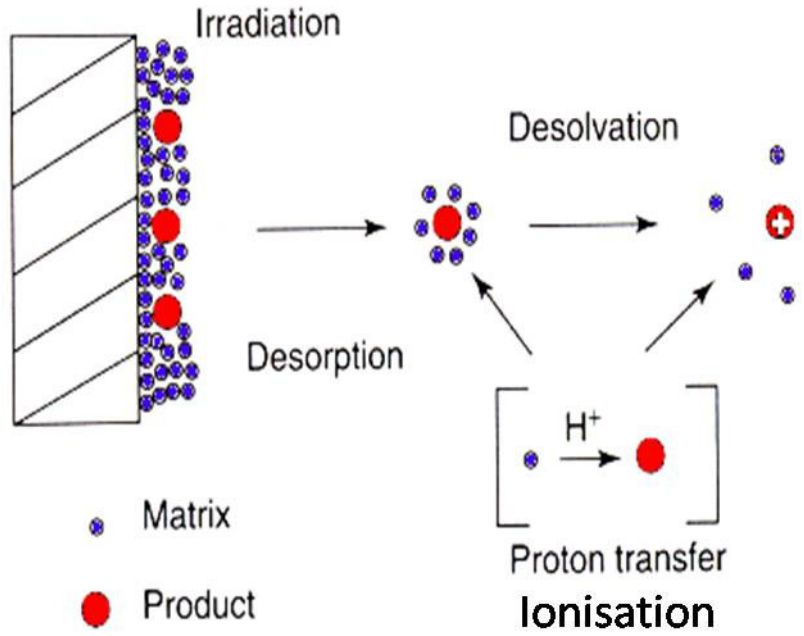

Figure 11. Principle of MALDI process

Until now the mechanisms of MALDI desorption/ionisation process is not completely understood.

Proposed mechanisms for primary desorption/ionisation (42):

- Multiphoton ionisation: A good explanation for ion generation with laser excitation is the ionisation of an absorbing organic molecule by more than one photon ($M^{+\circ}$).

$$M \rightarrow M^{+\circ} + e^-$$

For a long time this process was considered to be the key to all other MALDI ions. These directly excited matrix molecules (M*) have a few nanoseconds lasting life time, but nevertheless another photon could be absorbed to create the matrix radical cation.

$$M \rightarrow M^* \rightarrow M^{+\circ} + e-$$

This two photon process is very unlikely due to the usually used laser intensities.

- Energy pooling and multicenter models: Another possibility to generate matrix radical cations or highly excited matrix molecules is that two or more separately excited matrix molecules (M*M*) pool their energy.

$$MM \rightarrow M^*M^* \rightarrow M + M^{+\circ} + e^-$$

$$M^*M^* + A \rightarrow MM + A^{+\circ} + e^-$$

- It is statistically more likely that a neighbouring molecule of an excited molecule absorbs a photon rather than that the already excited molecule will be hit by a second photon. Thus the energy pooling mechanism will be favoured, if it can occur.

- Disproportionation reactions: It is a fact that some matrices will often perform ionisation in positive and as well in negative ion modes. A good correlation to this phenomenon gives the disproportionation reaction model.

$$2M \rightarrow (MM)^* \rightarrow (M - H)^- + MH^+$$

- Excited-state proton transfer (ESPT): ESPT is a very common ionisation model. It requires only one photon for ionisation. A singly excited matrix molecule is assumed to become more acidic than one being in the ground state. Thus neighbouring analyte or matrix molecules become protonated and the excited matrix molecule relaxes.

$$M + hv \rightarrow M^*$$

$$M^* + A \rightarrow (M - H)^- + AH$$

$$M^* + M \rightarrow (M - H)^+ + MH$$

Proposed mechanisms for secondary ionisation (42):

- MALDI plume: Due to molecular dynamics simulations the UV-MALDI plume can be described as a very rapid solid to gas phase transition where single molecules, clusters and matrix aggregates are ejected. To minimize the generation of these clusters the laser intensity has to be kept at the threshold of ion generation. The maximum amount of free molecules and small clusters can be reached at intensities close to the threshold for ion production.

- Gas phase proton transfer: The possibility that many matrix-matrix reactions in the plume can occur is obvious. Primary ions normally are radical cations, whereas proton transfer can lead to protonated matrix ions.

$$M^{+\circ} + M \rightarrow MH^+ + (M - H)^\circ$$

Whereas protons are mobile in hydrogen-bonded networks, like water, they are less mobile in matrix clusters, but still mobile enough to migrate over some distance to be trapped by an analyte with higher proton affinity, such as peptides and proteins.

- Gas phase cationisation: Cationized molecules are mainly generated through ion-molecule reactions in gas phase, like several studies have documented.

6.2. MALDI-TOF-MS

6.2.1. Reflectron-TOF

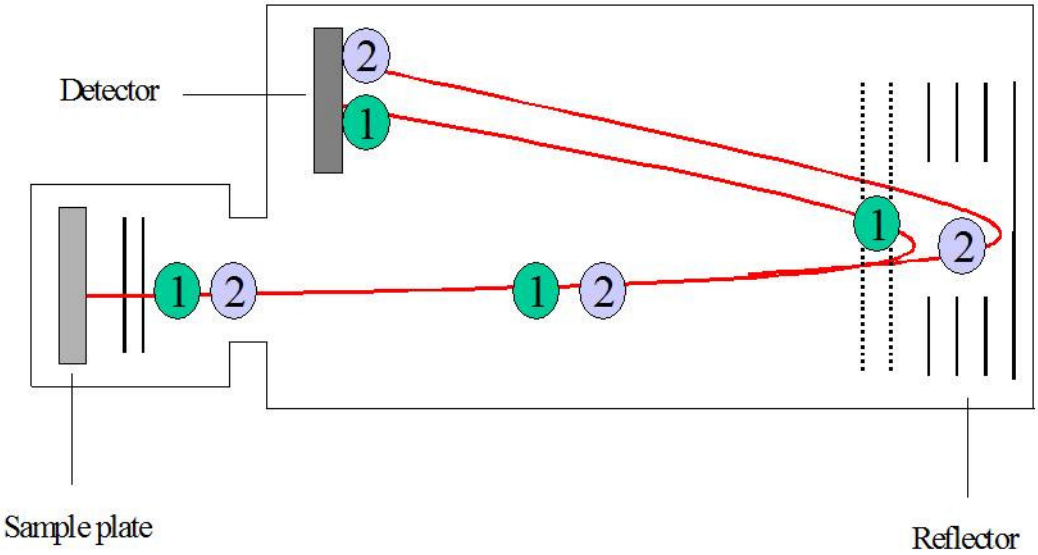

Figure 12. Schematic construction of a reflectron time of flight mass analyser

Due to the desorption/ionisation process the ions are already moving and therefore possess different velocities before acceleration takes place. This results in energy and spacial spreading, different velocities of the ions, different flight times and finally in peak broadening at the detector. One solution for this problem is using a reflectron-TOF analyzer. It possesses an ion reflector, which possesses an electric potential gradient and is called “reflectron”. The ions are decelerated in the reflectron and passes a turning point of their flight path at different locations in the reflector. So ions with higher kinetic energy spend a longer time in the mirror as ions with lower kinetic energy. When the geometry and voltages of the reflectron are optimised, the spreading of the arrival times can be corrected which leads to an increased mass spectrometric resolution (43).

6.2.2. Time delayed extraction

This method is used with a time-of-flight mass spectrometer in which the accelerating voltage pulse is applied after a time delay following pulsed desorption ionization from a surface. The extraction delay can produce energy focusing and improve mass resolution and prevent peak broadening.

6.3. Detector

The detector used in MALDI TOF MS is a channel electron-multiplier Array (CEMA) which consists of a honeycomb arrangement of many channels, each having a diameter on the order of 25 µm. The internal surface of each channel is coated with a resistive conductor much like that of the nonmagnetic electron multiplier. A potential difference applied to opposite ends of the channels cause a gradient along the resistive conducting surface (44).

7. Literature

1. Walochnik J. & Aspock H., J Lab Med (2005),29(6):446–456.

2. Castelani A., An.de l'Inst. Pasteur (1955), 89(1), 1-7.

3. Volkonsky M., Arch. Zool. Exp. Gen.7, (1933),2:317-339.

4. Sawyer T.K., & Griffin J.L. , Trans. Am. Microsc. Soc., (1975), 94:93-98.

5. Khan N. A., FEMS Microbiology Rev., (2006),30, 564-595.

6. Pussard M. & Pons, R.,. Protistologica, (1977), 8, 557-598.

7. Gast R.J., Ledee D.R., Fuerst P.A. & T. Byers, J. Euk. Microbiol. (1996), 43: 498-504.

8. Stothard D.R., Schroeder-Diedrich J.M., Awwad M.H., Gast R.J. , Ledee D.R., Rodriguez-Zaragoza S., Dean C.L., Fuerst P.A. & Byers T.J., J.Eukaryot. Microbiol., (1998), 45: 45-54.

9. Marciano-Cabral F., Puffenbarger R., and. Cabral G. A.. J. Eukaryot. Microbiol., (2000), 47:29-36.

10. Schuster F., Visvesvara G.S., , Int. J. for Parasit., (2004), 34, p 1001-1027.

11. Marciano-Cabral F. & Cabral G.A., Clinic. Microbiol. Reviews, (2003), p 273-307.

12. Brown H., F. Sanger and R. Kitai; Biochemical Journal, (1955), 60(4): p. 556-565

13. Edman P., Acta Chemica Scandinavica, (1950),**10,** pp. 761–768.

14. Lottspeich F., Zorbas H. Bioanalytik. Spektrum Verlag, München (1998); p. 150 – 1050.

15. Löffler G., Petro E. Petrides, und Heinrich P. C. ,Springer Verlag, München (2006), p 30 – 850.

16. Wilkins M.R. et al, Biotechnology & Genetic Engineering Reviews, (1996), 13: p.19 – 50

17. Peng J. & Gygi S.P., Journal of Mass Spectrometry, (2001), 36 (10): p.1083 – 91.

18. Pluskal, M.G., Nature Biotechnology (2000),18(1) p.104-5.

19. Putnam F., Perspectives in Biological Medicine, (1993), 36:323.

20. Raymond S. & Weintraub L., Science, 1959; 130:711.

21. Hjertén S., Biochimica et Biophysica Acta, 1961; 53:514.

22. Vesterberg O., Svensson H., Acta Chemica Scandinavica, (1966), 20 (3):820.

23. O´Farrell P., Journal of Biological Chemistry, (1975), 250:4007.

24. Klose J.,Humangenetik, (1975), 26:231.

25. Görg A., Postel W., Gunther S., Electrophoresis, (1988), 89:531.

26. Lottspeich F., Zorbas H. Bioanalytik. Spektrum Verlag, München (1998), p 219pp

27. Shaw M., Riederer B., Proteomics, (2003); 3:1408

28. Rabilloud T., Proteome research: Two-dimensional gel electrophoresis and identification methods. Springer Verlag, Berlin, Heidelberg, New York (2000), p15 – 35.

29. Celis, J.E., Carter N., Hunter T., Simons K., Small J.V. and Shotton, Cell Biology: A Laboratory Handbook. 3rd Edition, Elsevier, Academic Press, Amsterdam, The Netherlands (2006) p 342 - 355

30. Synder, L.R.; Stadalius, M.A.; Quarry, M.A. Analytical Chemistry, (1983), Vol. 55, pp. 1412-30.

31. Brown, P.R. Analytical Chemistry, (1990), Vol. 62, p. 995.

32. Simpson, R.C. and Brown, P.R.; J. Chromatogr., (1987), No. 400, p. 297.

33. DiCesare J.L.; Dong, M.W.; Vandermark, F.L.; Am. Lab., (1981), No. 13, p. 52.

34. Willard H., Merritt L., Dean J.A., Settle Jr., F.A. Inst. Methods of Analysis , 7th ed. , Wadsworth Publishing Co.,New York, United States (1988)

35. Kromidas St., HPLC made to measure, WILEY-VCH Verlag, Weinheim (2006) page 467 pp

36. Vissers J. P. C.; Journal of Chromatography A, (1999), 856,p 117 - 125.

37. Meiring H. D., van der Heeft E., ten Hove G. J., de Jong A. P. J. M.; Journal of Separation Sciences, (2002), 25,p 557- 570.

38. Tanaka K., Waki H., Ido Y., Akita S., Yoshida Y., Yohida T., Rapid Communication in Mass Spectrometry, (1988), 2, p 151- 166

39. Karas M., Bachmann D., Bahr U., Hillenkamp F., International Journal of Mass Spectrometry and Ion Processes, (1987), 78,p 53 -60.

40. Karas M., Hillenkamp F.,Analytical Chemistry, (1991), 63 (24),p 1193 – 1210.

41. Kussmann M., Nordhoff E., Rahbek-Nielsen H., Haebel S., Rossel-Larsen M., Jakobsen L., Gobom J., Mirgorodskaya E., Kroll-Kristensen A., Palm L., Roepstorff P., Journal of Mass Spectrometry, (1997), 32, p 593- 605.

42. Zenobi R., Knochenmuss R., Ion Mass Spectrometry Reviews, (1999), 5, p 337-360.

43. Kellner R., Lottspeich F., Meyer H. E., VCH Verlag (1994); page 152 pp.

44.Throck W.J. Raven Press, New York, (1985),page 249

II. Materials and Methods

1. Chemicals

Acetone (p.a.), acetonitrile (p.a.), acidic acid (glacial, anhydrous; 100%), formic acid (98-100 %), and methanol (p.a.) were obtained from Merck (Darmstadt, Germany).
ACTH (fragment 18-39), angiotensin II, bradykinin (fragment 1-7), N-acetyl renin substrate, 1, 4 dithioerythriol (DTT; min 99, 0 %), iodoacetamide (IAA; min 99%), ammoniumhydrogencarbonate (NH_4HCO_3 p.A.) and α-cyano 4-hydroxy cinnamic acid (min 99%,CHCA) and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (Steinheim, Germany)
Ultra pure H_2O (UHQ) was prepared from distillate H_2O using Simplicity® Millipore system ($\Omega_m \leq 18\mu S/cm$)
Trypsin (modified sequence grade) was obtained from Roche (Basel, Switzerland)

2. 2D gel electrophoresis

The 2D gel electrophoresis was prepared by Dr. David Leitsch during his Ph.D thesis (1) at the Clinical Institute for Hygiene and Medical Microbiology, Medical University Vienna.

2.1. Sample preparation

After cell-harvest (600 g), pellets were always washed once in 1xPBS followed by another washing step in UHQ to remove residual serum proteins from the culture medium (and salts from 1x PBS). When studying whole cell lysates, disruption of cells is not a critical step, as the 2DE solubilisation buffer thoroughly and quickly lyses *A.castellanii* trophozoites. However, repeated freezing and thawing or disruption in a Dounce homogenizer can be applied if deemed necessary. Because it is important to keep salt concentrations as low as possible, due to the fact that salts interfere with isoelectric focussing (IEF), the pellets are normally resuspended in an

appropriate volume of UHQ. After that a solution of 12.5% tetrachloroacetic acid (TCA) in acetone (-20°C) was added until a TCA concentration of 10% was attained. Proteins were precipitated at -20°C for at least one hour. Afterwards, the precipitated proteins were centrifuged at 20,000 g for 20 min (4°C) and washed twice with icecold 90% acetone/ 10% UHQ (v/v). These washing steps are essential because residual TCA would severely disturb IEF. After washing, pellets were air-dried for 30 minutes and resuspended in an appropriate amount of 2DE solubilisation buffer (7M urea, 2M thiourea, 1% DTT, 1% ampholytes, 4% 3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS)). Resolubilization should be given enough time (2h or more) under mild shaking, e.g. in a thermo-mixer. However, temperature must not rise above 32°C, as the high urea concentrations might otherwise lead to carbamylation of proteins. After resolubilization 1h centrifugation at 100,000 g is recommended to remove residual nucleic acids or cell debris. More details are available from the PH.D thesis of David Leitsch (1).

2.2. Isoelectric focusing

The IEF was performed on a Tube Cell 175 together with a 1000/500 Power Supply (both devices from Biorad, Munich)

The applied program for IEF consisted of following steps:

1. Rehydration 12h (50 mV)
2. 250 V, 1h, rapid slope
3. 500 V, 1h, rapid slope
4. 2000 V, 2h, linear slope
5. 5000 V, 2h, linear slope
6. 10000 V, 5h, rapid slope

2.3. Dimension SDS-PAGE

It is essential that the pH of the resolution buffer (1.5 M Tris.HCl) is adjusted to pH 8.6 as carefully as possible. If the pH is higher, protein spots tend to diffuse during 2D-PAGE; lower pH intolerably slows down 2D-PAGE due to the elevated salt concentrations (pH is adjusted with hydrochloric acid). The agarose overlay-solution should be made with proteinase-free agarose, otherwise in-gel digestion of proteins can occur. The gels were run at 4℃ at a constant current of 20 mA.

2.4. Visualization

Visualization by Coomassie Blue Brilliant was done after following protocol:
The gel was fixed for 30 minutes in fixing solution (45 % MeOH, 5 % acetic acid, UHQ). In the second step the gel was stained for 1 hour (or shorter when the spots are visible earlier) with staining solution (0,1 % Coomassie R 250, 45 % MeOH, 5 % acetic acid, UHQ). Destaining was done with solution 1 (40 % MeOH, 7 % acetic acid, UHQ) and 2 (5 % MeOH, 7 % acetic acid, UHQ) till the background appears transparent. More details are available from the PH.D thesis of David Leitsch (1).

The spots of interest were cut out of the 2D gel and stored at -80℃.

3. Tryptic in-gel digestion

The gel spots were cut into small cubes and put into 0,5 µl Eppendorf tubes and washed two times with UHQ and UHQ/ acetonitrilie (1:1, v/v) for 15 min each. After the washing step the liquid is removed and the gel pieces were covered with acetonitrilie (ACN) for a few minutes and after the pieces are shrunk the ACN was removed and the gel was rehydrate in 50 mM ammoniumbicarbonate buffer (NH_4HCO_3) for 5 min. An equal volume of ACN was added to the Eppendorf tube and slightly mixed and incubated for 15 min at room temperature (RT). After the incubation all the liquid was removed and the gel pieces were dried in the vacuum centrifuge. After that the gels were swollen with 10 mM DTT (15,4 mg DTT in 1 mL 50 mM NH_4HCO_3) and incubated for 45 min at 56℃ on the Thermo mixer. The tube

was chilled to RT and all liquid was again removed from the gel pieces. After adding 55 mM iodoacetamide solution (10,2 mg iodoacetamide in 1 mL NH_4HCO_3) the tube was slightly mixed and incubated for 30 min at RT in the dark. This procedure is followed by another washing step with 50 mM NH_4HCO_3 and ACN (1:1, v/v) and an incubation of another 20 minutes. The gel pieces were dried again in the vacuum centrifuge and afterwards rehydrated in digestion buffer .The digestion buffer consists of 12,5 ng trypsin/µl prepared in 50µl 50 mM NH_4HCO_3 .To be sure that the gel pieces will not dry during digestion the pieces were covered with a small volume of 50 mM NH_4HCO_3. The digestion was performed over night at 37°C on the Thermo mixer. On the next day ACN was added to the tube and incubated for 15 min. The supernatant was transferred to a new 0,5ml Eppendorf tube and the remaining gel pieces were covered with a mixture of 0,1 % TFA and ACN (1:1,v/v). This procedure was repeated twice and followed by a final extraction step with ACN. The supernatants from all extraction steps were pooled. In the end the gel pieces were discarded and all the pools were lyophilised in the vacuum centrifuge.

4. Desalting of protein mixtures

The desalting of the sample was performed using Zip Tips (Millipore, Bedford, MA, USA). The sample is dissolved in 10 µL 0,1 % TFA prior to the desalting step.
For all experiments C_{18} tips were used.
The ZipTip was used according to the customer's protocol, supplied by Millipore as follows:

- The ZipTip was wetted three times with 10 µL wetting solution (0,1 % TFA/ACN (1:1,v/v))
- Further the bed was equilibrated three times with 10 µL equilibration solution (0,1 % TFA)
- The tryptic peptides were bound to the chromatographic material by sucking in the solution three times
- The bound peptides were washed three times with 10 µL wash solution (0,1 % TFA)

The peptides were eluted from the Zip Tip by sucking up 1.5 µl of a freshly prepared CHCA solution (3 mg/ml in 0,1% TFA/ACN (1:1,v/v)). Then the tip was brought onto contact with the target. The liquid was partly deposited on the target and removed again. This step was repeated twice and finally the complete solution was deposited. In the end the spots were dried in a gentle stream of air.

5. Nano HPLC/MS (offline LC-MALDI)

5.1. Sample preparation

Directly after digestion the samples were diluted in 5µl ACN/0,1%FA (1:1,v/v) and briefly sonificated. The samples were pipetted directly in a 96 well microtiter plate and put on the cooler of the LC autosampler.

5.2. Instrumentation

The nano LC experiments were performed using a nano HPLC connected to a MALDI Spotter device (EASY-nLC Spotter, Proxeon Biosystems, Odense M, Denmark). Matrix was delivered by a syringe pump.

Figure13. Easy-nLC Spotter

The chromatographic separations were performed using a Proxeon fused silica trapping column (filled with 5µm silica C18 particles, ID 0,75 µm, length 35 mm) and a Proxeon analytical column (filled with 3µm silica C18 particles ,ID 0,75 µm, length 10 mm).

5.2.1. Nano LC settings

Injection volume: 2µl
Solvent A: H_2O + 0,1% FA (v/v)
Solvent B: ACN + 0,1% FA (v/v)
Solvent flow: 500nl/min

- Gradient:

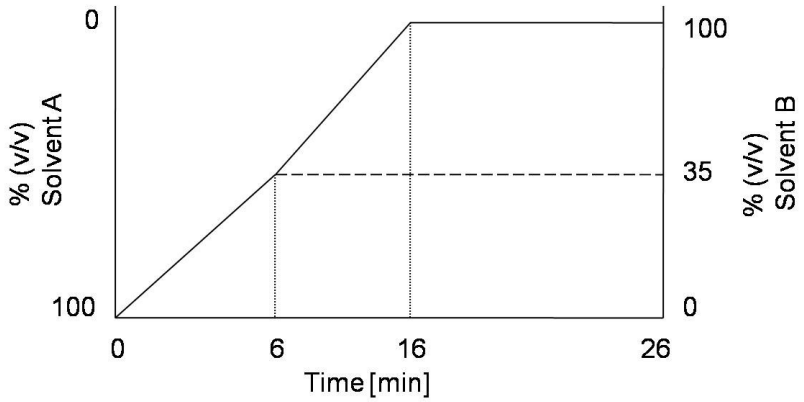

Figure14. Gradient used in all the experiments

5.2.2. MALDI-Spotter settings

Matrix solution: CHCA in ACN/0,1 % TFA (1:1,v/v)
Matrix flow rate: 0,8 µl/min
Spot volume: 2,0 µl

6. MALDI-TOF-MS

6.1. Sample preparation

Sample preparation was performed on stainless steel MALDI targets by using the following preparation technique.

- CHCA applied in thin-layer technique (2)

  0,5 µL CHCA (6 mg in 1 mL acetone) solution were applied on the target. The solution spread immediately and the acetone was evaporated in a few seconds. This resulted in a thin homogenous layer of small crystals.
  On top of this layer 1 µL of ZipTip desalted sample solution was applied and dried in a gentle stream of air.

6.2. Instrumentation

The mass spectra were recorded on an AXIMA-CFR+ (Shimadzu Biotech Kratos Analytical, Manchester, UK). The AXIMA-CFR+ was equipped with a pulsed nitrogen laser (λ = 337 nm, 4 ns pulse width), a curved field reflector, an integrated 1 GHz recorder and a monochrome CCD camera for monitoring the sample inside the instrument.
All peptide mass fingerprints (PMF) were recorded in positive ion reflectron mode applying 20 kV acceleration voltage. The setting of delayed extraction was set for ions with m/z 2000 to get the best results for the relevant peptide m/z range.
Mass spectra of the relevant for PMF experiments were acquired by accumulating 200-1000 single unselected laser shots, whereas for PSD MS/MS experiments of characteristic tryptic peptides 1000 – 2500 laser shots were averaged.

6.3. Calibration

Mass calibration was performed with a mixture of four standard peptides in the m/z range between 750 and 3500 Da.

Protein	MW monoisotopic [Da]
Bradykinin	757,3997
Angiotensin II	1046,5423
N-acetyl renin substrate	1800,3941
ACTH	2465,1989

Table 2. List of peptides used for external calibration from 750 to 3500 Da.

6.4. Database search and bioinformatics

All mass spectra and PSD spectra were evaluated with the software supplied with the mass spectrometer called Shimadzu Biotech Launchpad 2.7 to get lists of monoisotopic or average m/z values of the detected peptides or fragment ions. For optimal data presentation PSD spectra were baseline subtracted and smoothed with the Savitsky Golay algorithm (3) (Smoothing filter width: 7 channels), whereas mass spectra of peptide PMFs were neither baseline subtracted nor smoothed. Each m/z data set was submitted to the search engine, either for PMF search or for MS/MS search. In some cases it was possible to search with several sequence tags obtained from the same spot.

Searches were run without a limitation or exclusion of species or genera in the public Databases MSDB (2006/08/30), NCBInr (2007/06/23), SWISS-PROT (version 53.0) and the following search engines Mascot (4), Profound (5) and Aldente (6).

The following values where used in all searches:

<u>Peptide mass fingerprint (PMF):</u>

- Databases: NCBInr, SWISS PROT, MSDB
- Search engine: Mascot, Profound, Aldente
- Enzyme: trypsin (max. 1 missed cleavage)
- Fixed modification: carbamidomethyl (C)
- Variable modification: oxidation (M)

- Monoisotopic $[M+H]^+$ ions
- Peptide tolerance ± 0,2 Da

MS/MS search:

- Databases: NCBInr, SWISS PROT, MSDB
- Search engine: Mascot
- Enzyme: trypsin (max. 1 missed cleavage)
- Fixed modification: carbamidomethyl (C)
- Variable modification: oxidation (M)
- Average masses and tolerance ± 0,8 Da

Only proteins which gave a significant hit in terms of the probability based MOWSE Score (significance threshold $p<0,5$) as result of PMF and MS/MS search were considered as identified.
In some cases it was necessary to evaluate the MS/MS spectra manually. Therefore the differences of the m/z values of all peaks were compared with the known masses of all 20 common amino acids. This de novo procedure allowed the identification of possible sequence tags from the MS/MS spectra. This sequence tags were blasted (NCBI Blast; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi (7)) to get information about homologies of these sequence tags.

7. Literature

1. D.Leitsch Ph.D thesis, (2007) Univ. of Vienna, Vienna, Austria

2.M.Kussmann, E.Nordhoff, Henrik R.Nielsen,S.Haebel, M.Rossel-Larsen, L.Jakobsen, J.Gobom, E. Mirgorodskaya, A.Kroll-Kristensen, L. Palm, P. Roepstorff, Journal of Mass Spectrometry, (1997), 32, p 593-601.

3. Savitzky, Abraham, and Marcel J.E. Golay,(1964) Analytical Chemistry 36 (8), p 1627-1639.

4.M.J. MacCoss, C.C. Wu, and J.R. Yates, (2002) Analytical Chemistry 74 (21), p 5593-5599.

5. W. Zhang and B.T. Chait (2000) Analytical Chemistry 72 (11), p 2482-2489.

6. M. Tuloup, C. Hernandez, I. Coro, C.Hoogland, P. A. Binz and R.D. Appel (2003) Swiss Proteomics Society Congress: Understanding Biological Systems through Proteomics, (2003), December 2-4, Basel

7. S.F. Altschul, T.L. Madden, A.A. Schaffer, J.Zhang, Z.Zhang, W.Miller and D.J. Lipman (1997) Nucleic Acids Research 25(17), p 3389-3402.

III. Results

This study was focused on the proteome of *Acanthamoeba castellanii*. This organism causes a lot of serious infections in humans and because of its ability to form resistant cysts the main goal of this work was to identify proteins which are involved in cyst formation. First an infectious strain of *A. castellanii* was isolated from a patient, cultured in different cell culture media and studied over the whole period of en- and excystment in cell cultures. It was supposed that some characteristic changes in the protein pattern in the different stages of cyst formation and degradation can be observed. The proteins that changes during the continuous cystation process where analysed by the means of a proteomic approach based on MALDI-MS and MALDI-MS/MS.

In addition a nano liquid high performance chromatography MALDI spotting system (nLC) was established to perform a separation of the tryptic peptide mix directly after in gel digestion of a single spot before analysing with MALDI-MS.

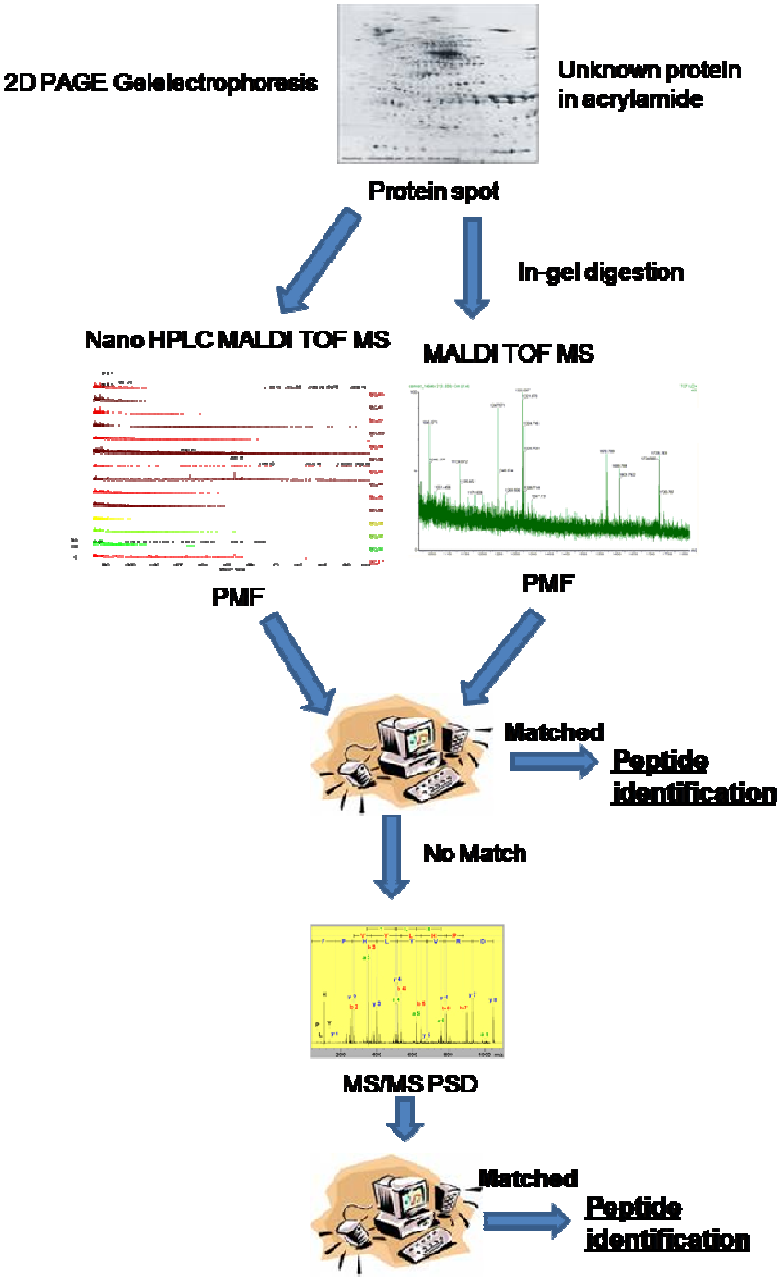

Figure 15. Schema of proteomic workflow

1. 2D SDS PAGE Gels (Figures 16-19)

The proteins in Figure 16 show the ensemble proteome of *A. castellanii* in its trophozoite stage. In Figure 17 and further in Figure 18-19 the protein pattern of the amoebae changes because of the beginning and ongoing encystment. Some proteins disappear from the gel whereas other proteins become more visible. The

proteins which were regulated during encystment were of special interest and picked for further analysis.

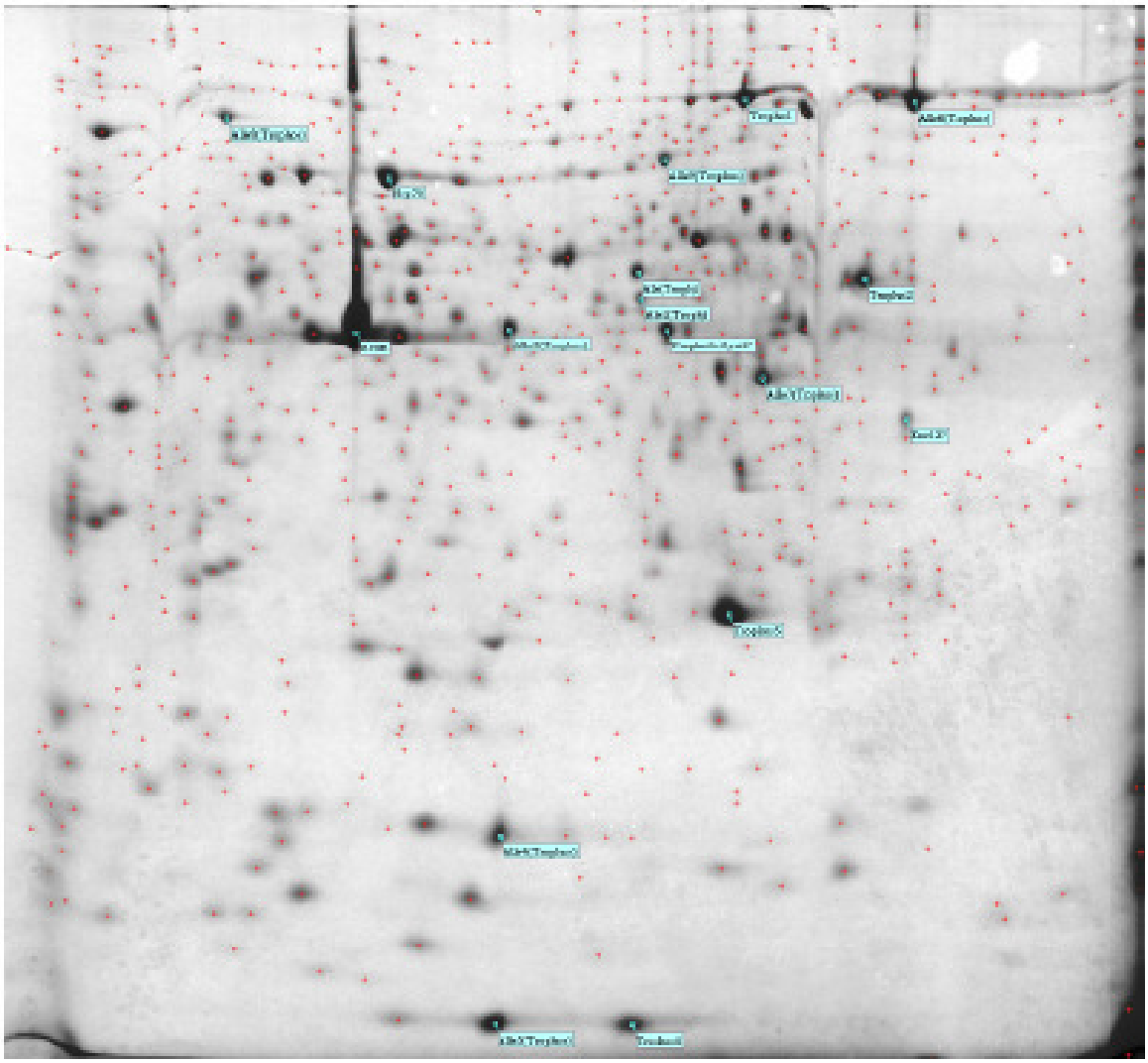

Figure 16. 2D gel of *A. castellanii* in its trophozoite state (gel was stained with Coomassie blue, + proteins detected by Bio-Rad GS-800 scanner)

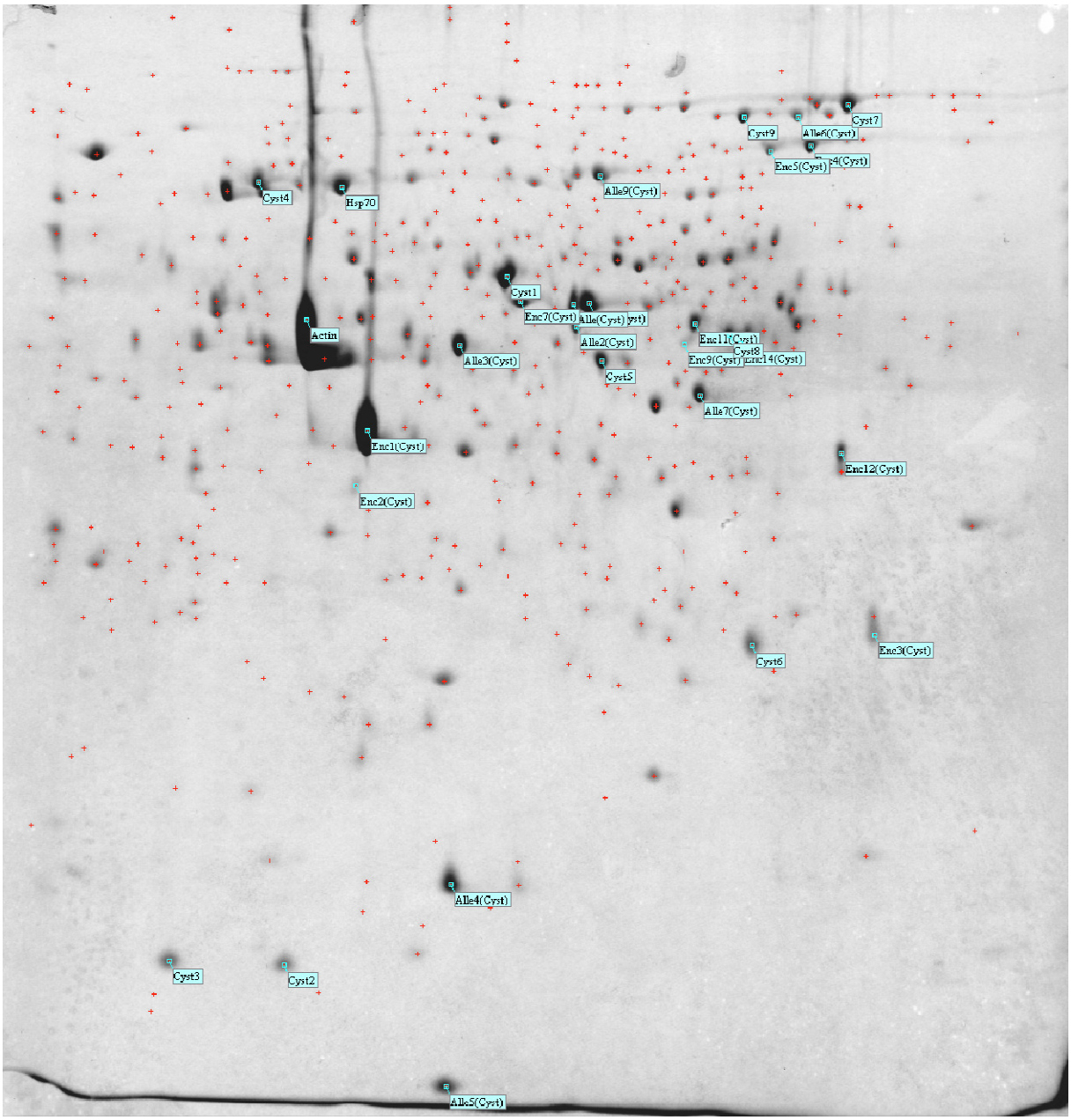

Figure 17. 2D Gel of an *A. castellanii* cell culture extract in the early stage of its cyst stage (gel was stained with Coomassie blue, + proteins detected by Bio-Rad GS-800 scanner)

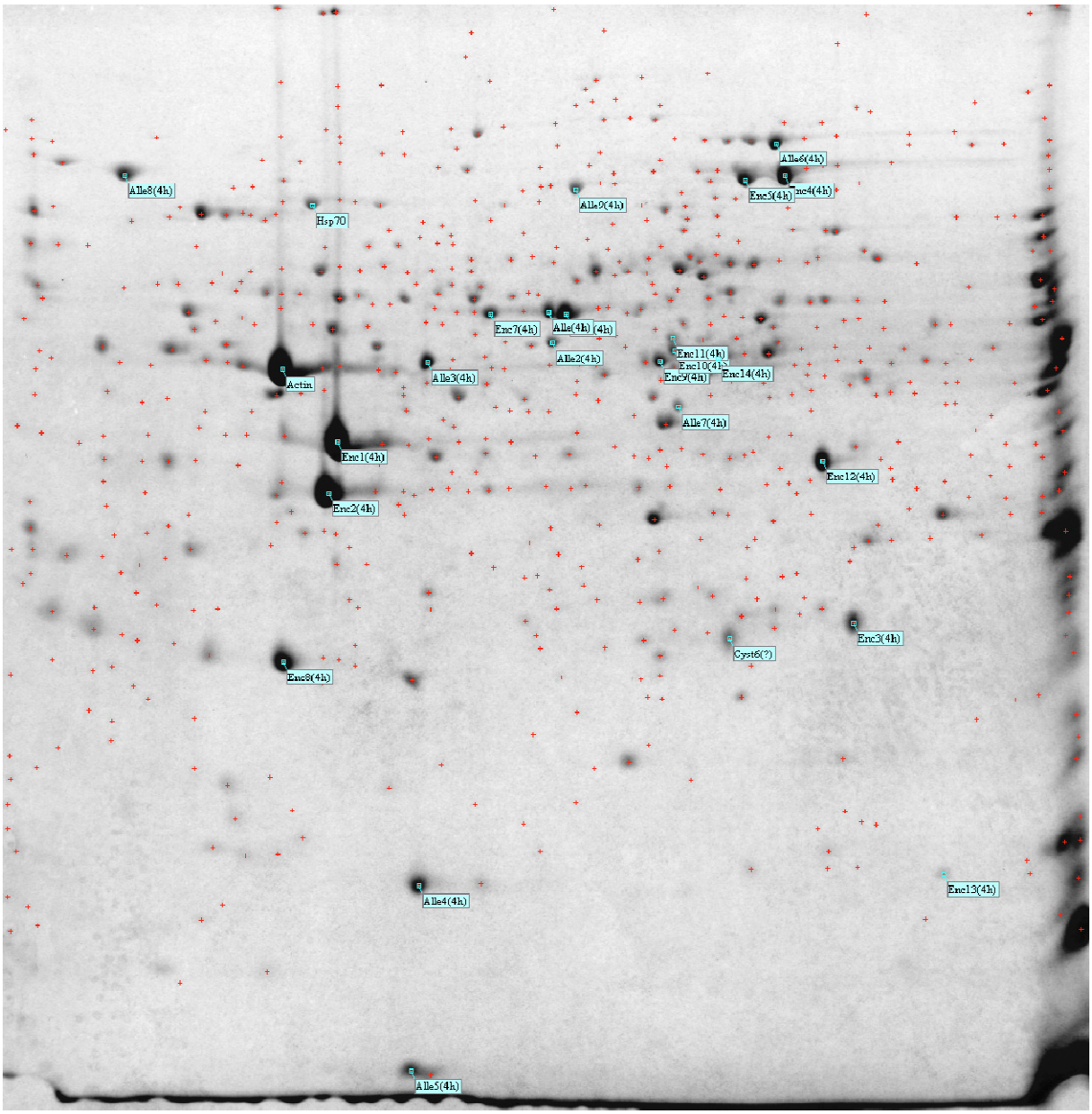

Figure 18. 2D gel of *A. castellanii* after 4 hours of incubation in its cyst stage (gel was stained with Coomassie blue, + proteins detected by Bio-Rad GS-800 scanner)

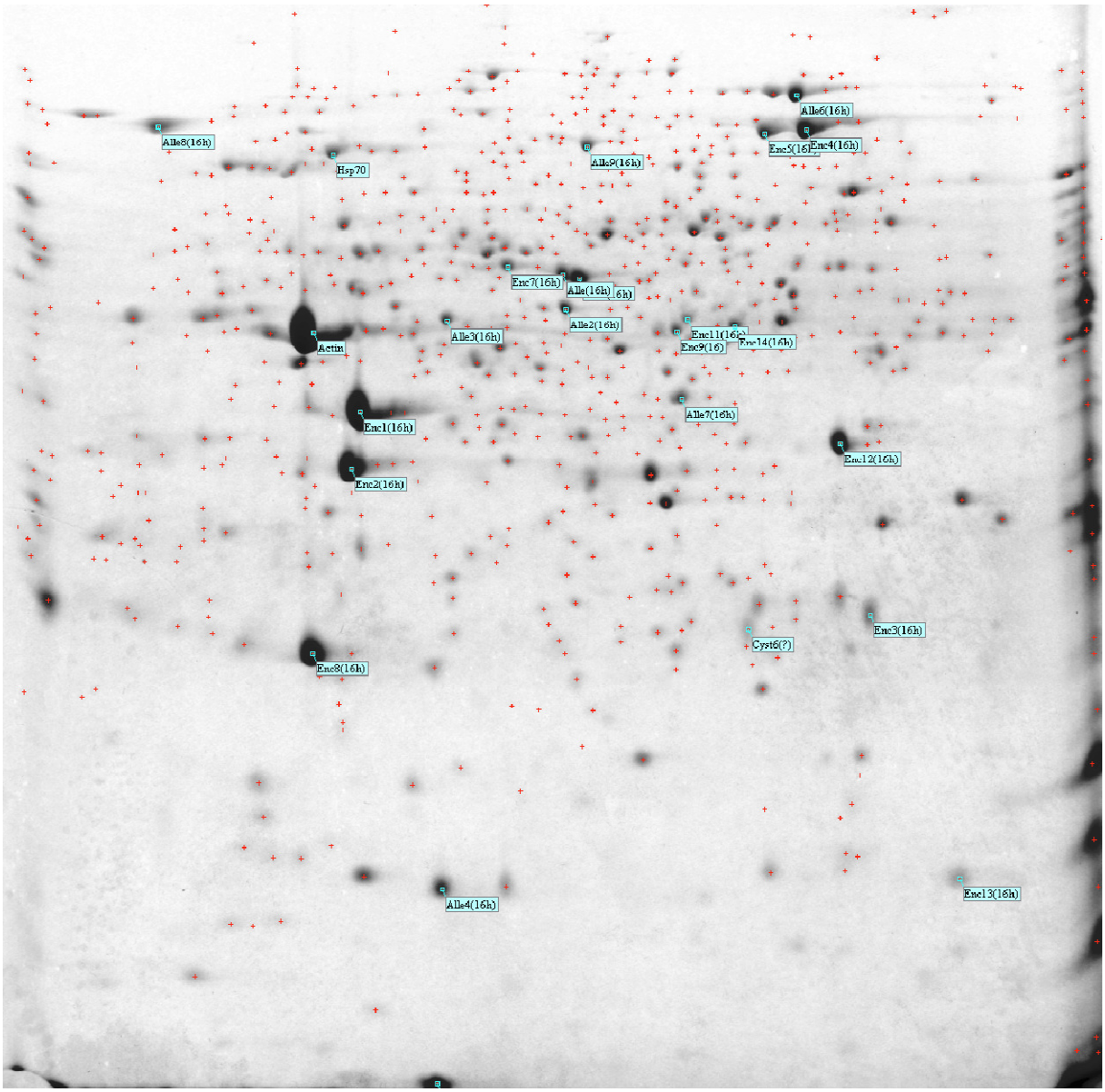

Figure 19. 2D gel of an *A. castellanii* culture after 16 hours of incubation in its cyst stage (gel was stained with Coomassie blue, + proteins detected by Bio-Rad GS-800 scanner)

2. Identification of protein spots from 2D gels

Spot Alle 1

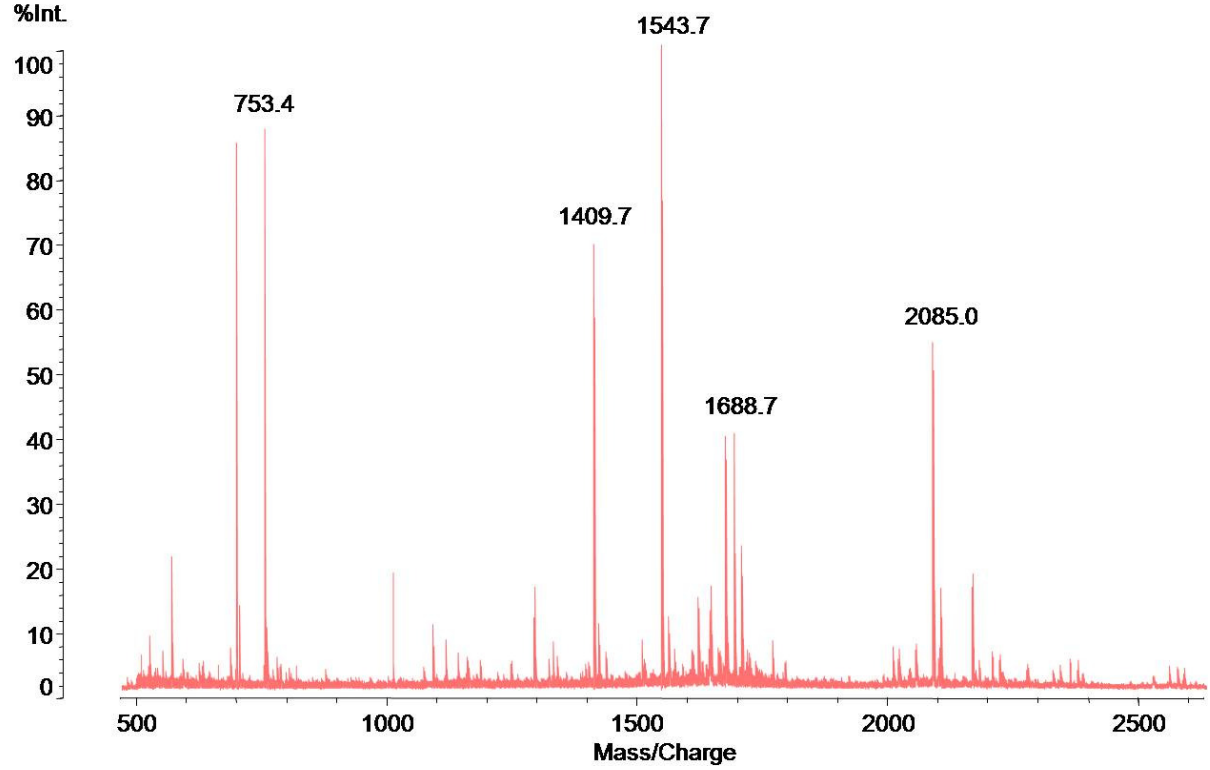

Figure 20. Peptide mass fingerprint of Alle 1 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Alle 1 as a protein of approximately 50 kDa with a pI of 6.6 (Figure 15-18). The mass list (see Appendix chapter 6) of the PMF (Figure 20) was submitted to Mascot resulting in a significant hit (Score: 81, Threshold: 80) for ramosa 3 of *Setaria veridis* with a calculated molecular mass of 37,728 kDa and a calculated pI value of 6.29. Thirty-two peptides were used for database search where 9 values matched to the proposed protein, giving sequence coverage of 32% (figure 21). This significant protein hit was only found in the NCBInr database, in all the other databases (Swiss Prot, MSDB) no significant protein hit could be obtained.

```
  1 MTKHAGYATD EAVTAVAAPA PAGLHFSPFP PPKVAARDCK KVAALHVDRA
 51 APGGGGGGSW FESMKASSPR RAADAEHGDWEKHPSALTWEPALAAAKG
101 KQIVMFLDYD GTLSPIVEDP DRAVMSEEMR DAVRRVAEQF PTAIVSGRCR
151 DKVFNFVKLT ELYYAGSHGM DIEGPAKQSN KHVQANAEEAVHYQAGSEFL
201 PIIEEVYRTL TAKMESIAGA KVEHNKYCLS VHFRCVQEEE WKAVEEEVRS
251 VLKEYPDLKL THGRKVLEIR PSIKWDKGKA LEFLLKSLGY AGRSDVFPIY
301 IGDDRTDEDA FKVLRGMGQG IGILVSKFPK ETAASYXLRD
```

Figure 21. Sequence coverage (Matched peptides shown in **Bold Red**)

Two PSD spectra were generated to confirm the PMF result. Peptide 1 (m/z 1409.71) and Peptide 2 (m/z 1543.69) gave no significant hit in none of the databases. It was not possible to manually obtain sequence tags from the mass spectra. This means that it was not possible to confirm the PMF result with a PSD spectrum. Therefore this spot has to be considered as “not identified”, but the PMF gives a hint of a potential protein.

Spot Alle 4:

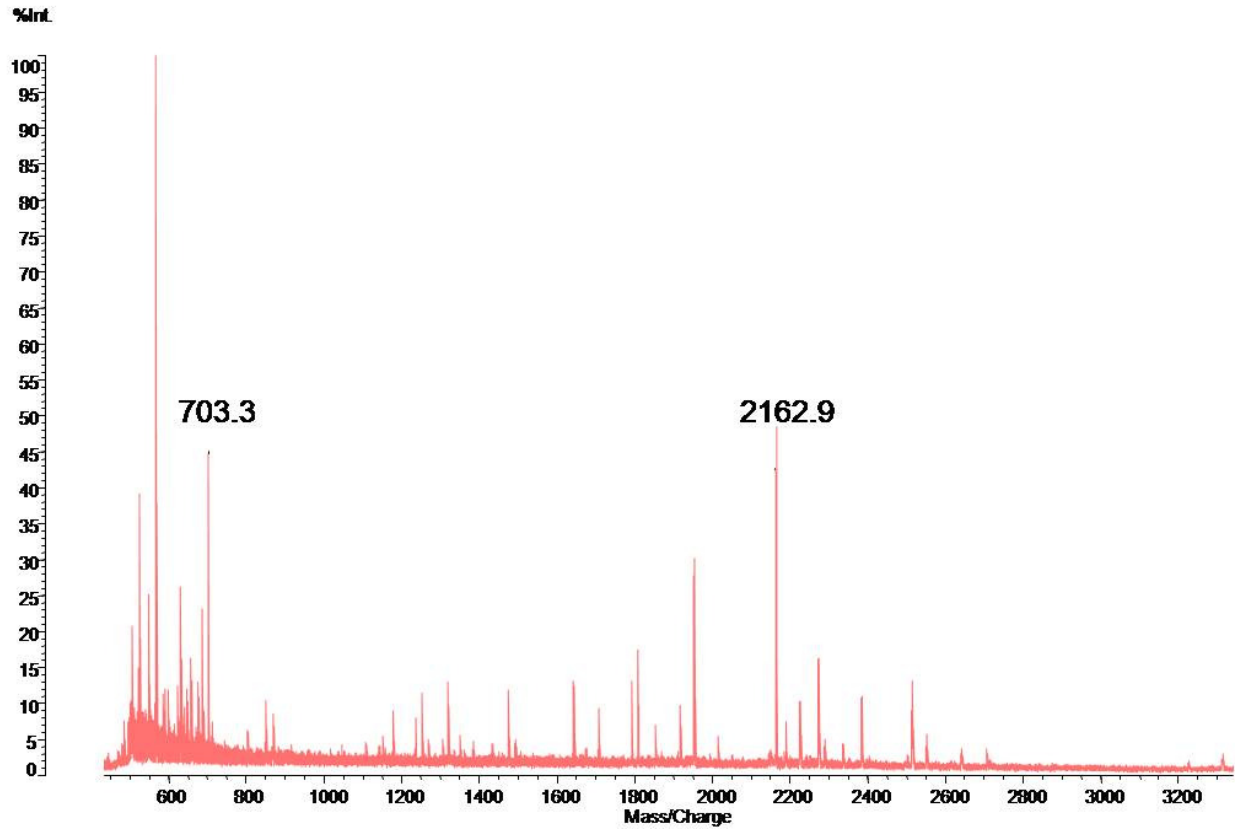

Figure 22. Peptide mass fingerprint of Alle 4 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Alle 4 as a protein of approximately 19 kDa with a pI of 6.0 (Figure 18-19). The PMF is shown in Figure 22. The resulting peptide m/z list (see Appendix chapter 6) was submitted to the Mascot search engine and a significant hit (Score: 81, Threshold: 67) was found. The protein found in SWISS-PROT and NCBI is “Sporulation Specific Chitinase 2 precursor” in yeast with sequence coverage of 18% (Figure 23). To achieve this result 17 m/z value were submitted whereas 7 matched leading to this protein with a calculated molecular mass from 59,366 kDa and a calculated pI of 8.28.

```
  1 MVGHSAQHRS KSSLVSHLLI LLIFITIIIE MCLYNKIFKN QRSDDIRDNF
 51 NNGGHRVPSN VQNHGTHIRD EAFISGVYYS NWSPYKPRFH FPHDINLKQV
101 SHIYYAFFKI NSRTGGIENT DSWSDLEMNL YKSLAIKNSE LIKESSNNSV
151 QNILPLGCIG ELFYLKNTCS DKKFKVIMSI GGWSDSENFK IIIKDDKLLQ
201 NFVDSSVETM FRLGFDGIDL DWEFPGNNES EPRGYLKLVR MLRLKLNSLE
251 SQIFGKRTED HFQLSIAAPA FKDKLFYLPI TEIDQYVDYW NMMTYDYYGS
301 WSETTGYHSN LFSETELNGN FAMHYMIDRF GVNSRKLVLG MAAYGRSFHI
351 KDNKFEPFNQ NTVLINKIFK GVGKPTKEID KADGKEGIWP YKNLPKIGTI
401 EQYDPKYVSA YCFDEKNSIF ISYDNTKSVK TKAEYVTHNN LGGGFWWESC
451 GEAYANESRS LINAFNEGLH FNVSSKPSIF QDVRVKKYYL NKYGDGGFLS
501 PYLKHLDSRK Q
```

Figure 23. Sequence coverage (Matched peptides shown in **Bold Red**)

The search with the PSD fragment ion list of the chosen peptide (m/z 1951.76) gave no significant protein hit in all databases and therefore did not confirm the protein hit found with PMF. Due to the fact that the chosen PSD spectrum contains not much fragment peaks; it was not possible to evaluate the PSD spectra manually. Therefore this spot has to be considered as “not identified”, but the PMF gives a hint of a potential protein.

Spot Alle 6: Elongations factor 2 (Dictyostelium discoideum)

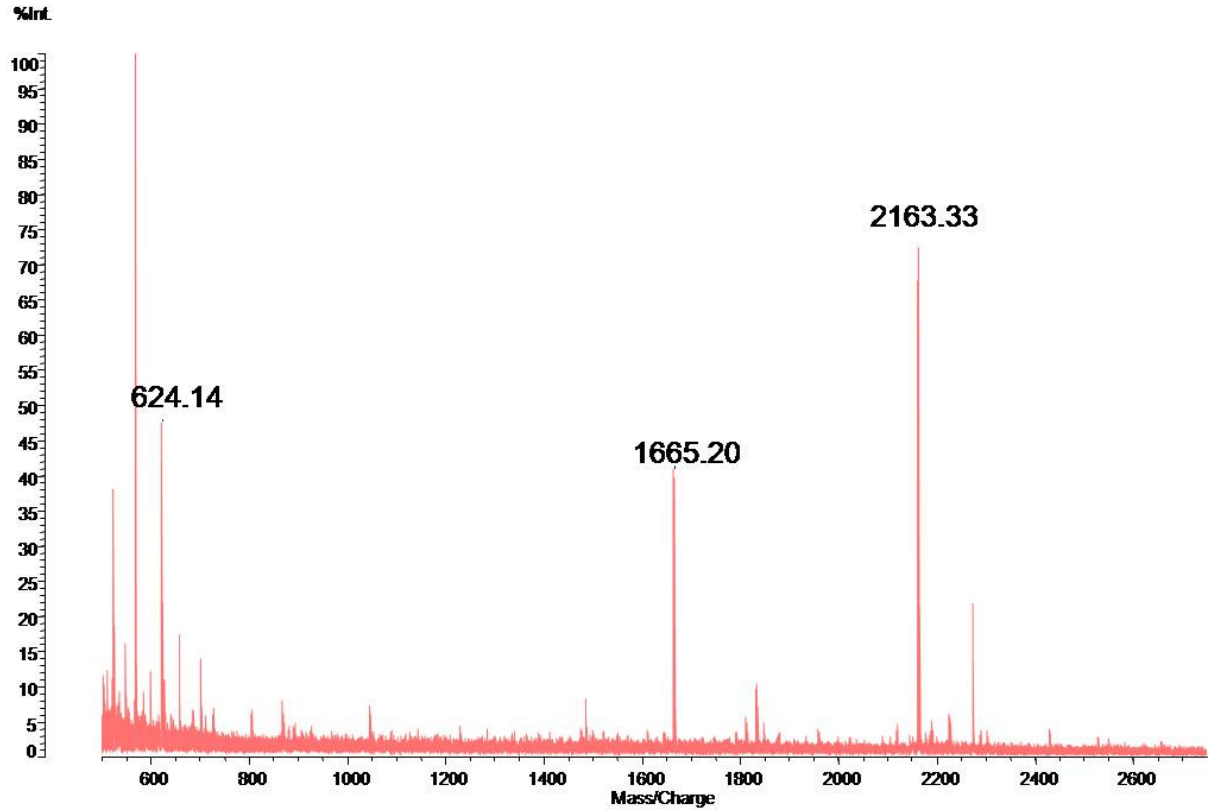

Figure 24. Peptide mass fingerprint of Alle 6 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Alle 6 as a protein of approximately 100 kDa with a pI of 6.5 (Figure 16-19). The PMF is shown in Figure 24.The resulting m/z list (see Appendix chapter 6) was submitted to Mascot search and a significant hit (Score 77, Threshold: 67) was obtained. The protein found was"Elongations factor 2" of *Dictyostelium discoideum*, a fungus related to *Acanthamoeba castellanii* (see chapter 1) with a molecular mass of 92,498 kDa and a calculated pI of 6.22. This hit was the result of a search with 24 m/z values whereas 11 values matched and gave sequence coverage of 12% (Figure 25).

```
  1 MVNFTIDQIR AIMDRRENIR NMSVIAHVDH GKTTLSDSLI QRAGIIADKV
 51 SGDMRYMSCR ADEQERGITI KSSSVSLHFE MPKEDKLPAG CTSHEFLINL
101 IDSPGHVDFS SEVTAALRVT DGALVVIDCV EGVCVQTETV LRQAVAERIK
151 PVLFVNKVDR FLLELQLNTE EAYLSFRRAI ESVNVIVGNT EDKEFGDVTV
201 SPEKGTVAFG SGLHGWGFTL GRFAKLYAAK FGDPEDKLMG RLWGDSYFDA
251 TAKKWTSNPQ SADGKALPRA FCQFVLEPIY QLTRAIVDED AVKLEKMMKT
301 LQITLAPEDA EIKGKQLVKA VMRKFLPAAD AILSMIVTHL PSPLVAQKYR
351 CANLYEGPMD DECAVAIQKC DPNGPLMMYV SKMVPTSDKG RFYAFGRVFS
401 GIIVPVKRSE LWVSTYVPGK KDDLFLKSIQ RTVLMMGRKT EQIEDCPCGN
451 IVGLVGVDQF LVKSGTITTS EVAHNIRVMK FSVSPVVRVA VEPKNPSDLP
501 KLVEGLKRLA KSDPCVLCYS EESGEHIVAG AGELHLEICL KDLAEDHAGI
551 EIKTTDPVVS FRESVKASPI SMELQDLIEA GSDISSKDDP KARANYLADN
601 HEWDKNDAMN IWSFGPEGNG ANLLVNVTKG VQYLNEIKDS FVGAFQWATK
651 EGVVCDENMR GIRFNLYDVT LHTDAIHRGG GQIIPTARRV LYAAELTASP
701 TLLEPIYLVE ITAPENAIGG IYSVLNRRRG IVIGEERRIG SPLFSVKAHL
751 PVLESLRFTA DLRSHTAGQA FPQCVFDHWA SIGVVNKDKK ATEVALATRK
801 RKGLAPEIPA LDKFHRKTIN NLSHTLSFQI
```

Figure 25. Sequence coverage (Matched peptides shown in **Bold Red**)

To confirm the PMF result a PSD MS/MS experiment was performed on one peptide (m/z 760.46) and the resulting fragment ion list was submitted to Mascot search.

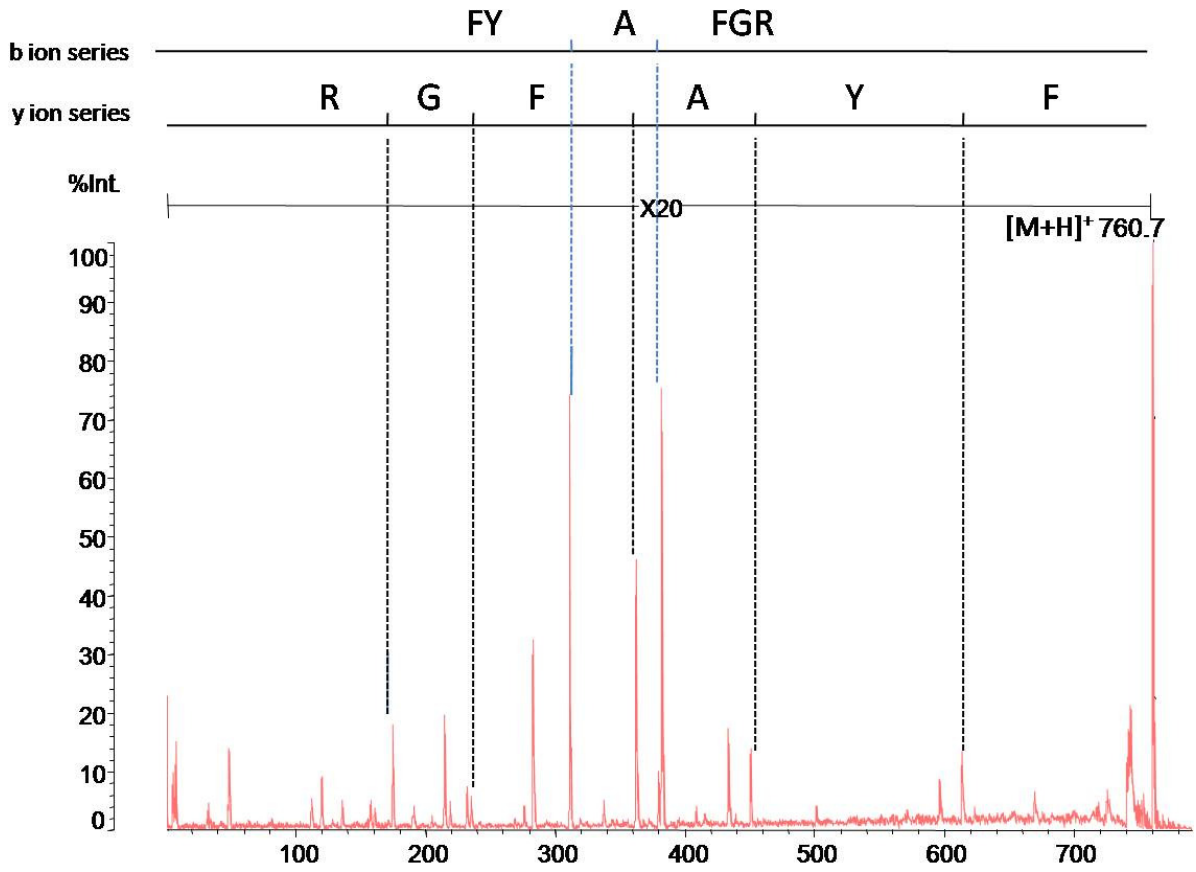

Figure 26. PSD spectrum of precursor ion m/z 760.7 of the PMF Alle 6

The Mascot search of this peptide led to a significant peptide hit based on homology with a Mascot ion score of 25 (threshold: ≥24 indicates homology; threshold ≥ 41 indicates intensity). The 31 most intensive peaks were used leading to 21 fragment ions and 13 matched fragment ions and in the end to the following sequence: FYAFGR (Figure 26).

The spot Alle 6 was identified as “Elongations factor 2” but because of the missing database entries of this protein in *Acanthamoeba castellanii* it was only possible to find the protein in *Dictyostelium discoideum.* The identified protein sequence (Figure 26) belongs to a high conserved region of the second domain of elongation factor (EF) EF-2 which can be found in eukaryotes and archea.

Spot Alle 7

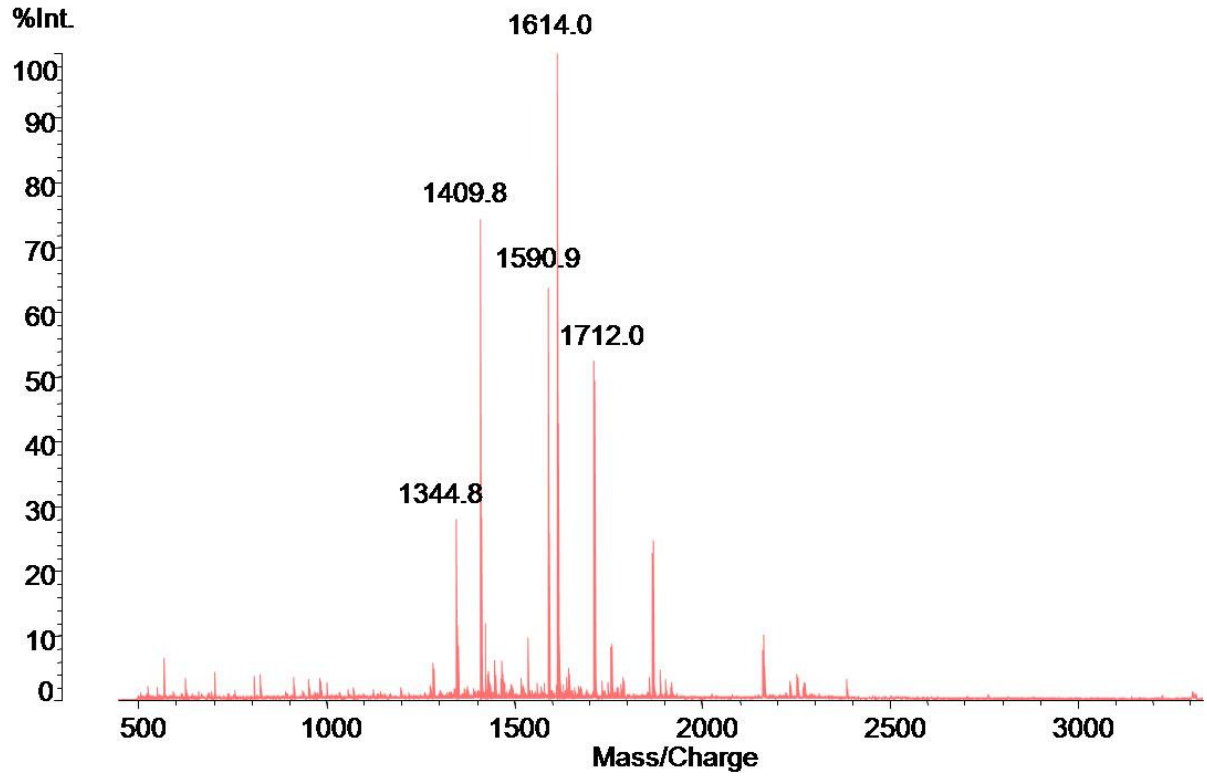

Figure 27. Peptide mass fingerprint of Alle 7 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Alle 7 as a protein of approximately 35 kDa with a pI of 7.0 (Figure 16-19). Its PMF is shown in figure 27. The list of monoisotopic peptide masses (see Appendix chapter 6) was submitted to Mascot search, but no significant protein hit was obtained. Therefore MS/MS experiments were carried out on 3 peptides (peptide 1: m/z 1410.6, peptide 2: m/z 1591.8 and peptide 3: m/z 1713.3) and the resulting fragment ion lists were submitted to Mascot search. No peptide sequence or protein hit was obtained by this method, so manual interpretation was carried out.

Peptide 1:

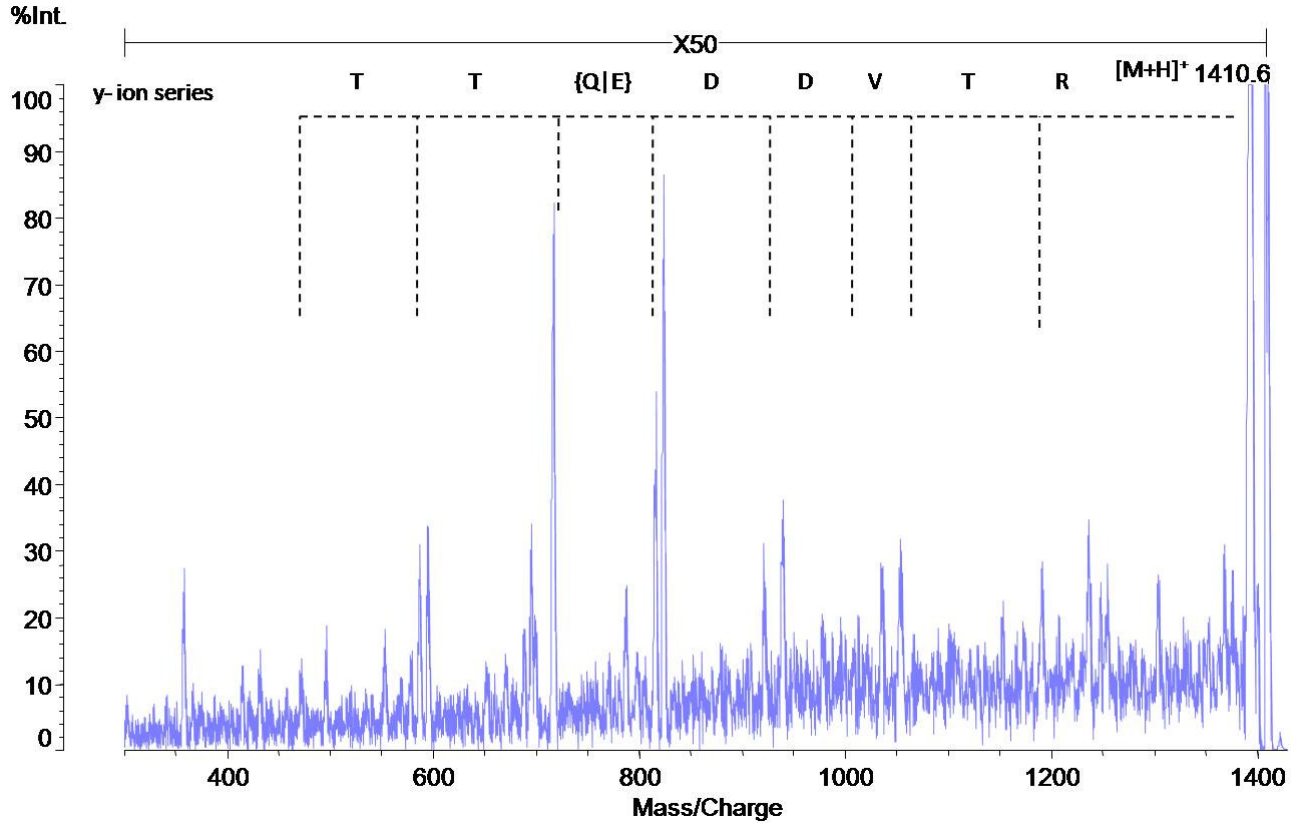

Figure 28. PSD spectrum of peptide 1 of precursor ion m/z 1410.6 of the PMF Alle 7

Manual interpretation of the spectra allowed the following conclusions:

- y-ion series sequence: (495,07) –TT{E/Q}DDVZTR-(1409.80)

The sequence tag gave no result in any of the databases by any search algorithm applied. Therefore a homology search was performed with the sequence to see if this sequence allows a result in sequences of closely related organism. The homology search was successful and a protein sequence of *Entamoeba histolytica* was found. But this protein is an unknown protein and so no more information can be obtained by this data.

Peptide 2:

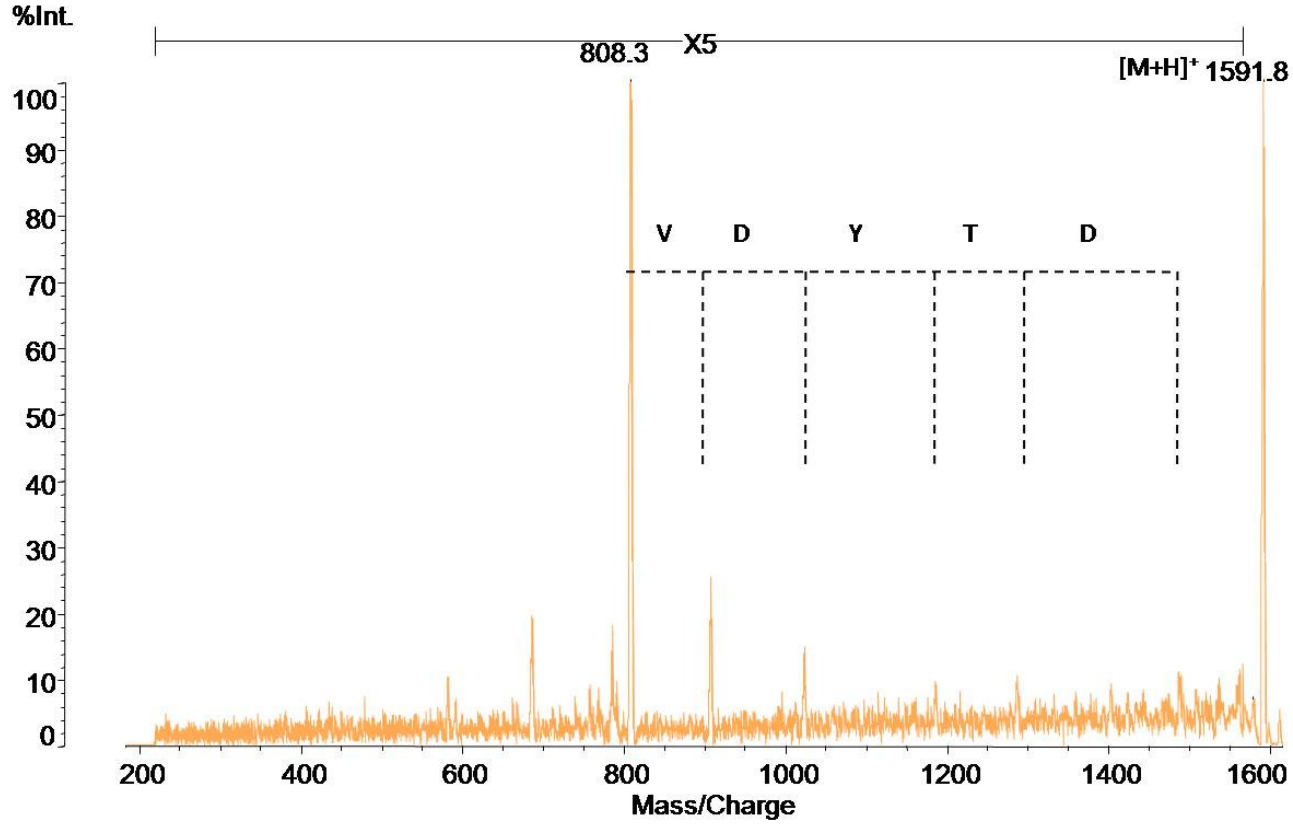

Figure 29. PSD spectrum of peptide 2 of precursor ion m/z 1591.8 of the PMF Alle 7

The spectrum of peptide 2 was evaluated manually and lead to the resulting sequence:

- b-ion series sequence: (808.27)-**DTYDV**-(1402.45)

This short sequence tag gave no result in the Mascot search so that we had to do a homology search again. This sequence gave a hit (peptide score:21) for the putative protein serine/threonine kinase of *Dictyostelium Discoideum*. The two sequences differ in only one amino acid as shown here:

DTYD**V**.....peptide 2 spot Alle 7

DTYD**K**.....*Dictyostelium Discoideum*

This sequence tag is very short and the protein found by homology search gave no usefull result.

Peptide 3:

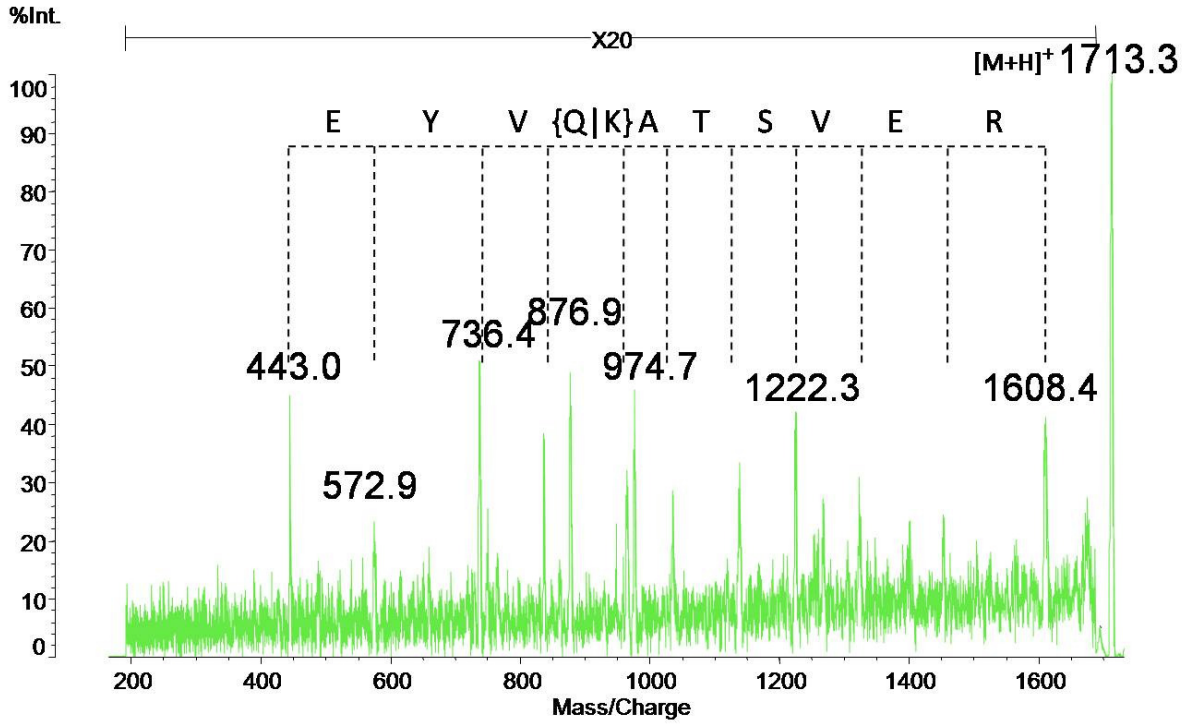

Figure 30. PSD spectra of peptide 3 of precursor ion m/z 1713.3 of the PMF Alle 7

The manually evaluation of the spectra of peptide 3 results in a 10 amino acids long sequence: (444.36)-EYV{Q/K}ATSVER-(1610.10). It was not possible to evaluate from the spectrum whether it is a b- or a y-ion series sequence tag.

The sequence tag gave no result in any of the databases. Therefore a homology search was performed with the sequence to see if this sequence is present in closely related organism. The evaluated sequence did not lead to any homology result.

All manually acquired sequence tags were not only submitted separately but also as a combined sequence tag to Mascot using all databases. None of the results included more than one of the sequence tags. It is therefore not possible to identify this protein with the currently available databases although the mass spectrum of the PMF and all PSD spectra were of high quality. Therefore this spot has to be considered as “not identified”.

Spot Alle 9

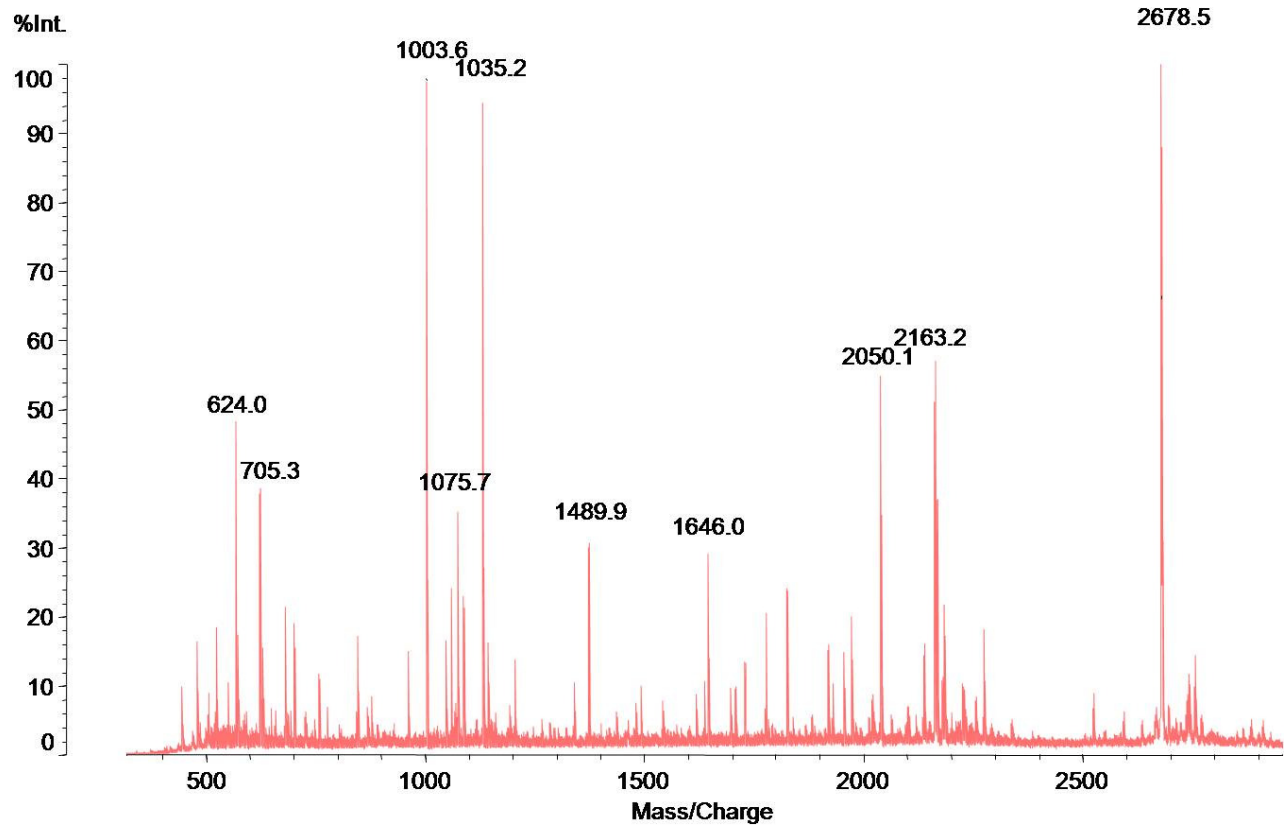

Figure 31. Peptide mass fingerprint of Spot Alle 9 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Alle 9 as a protein of approximately 75 kDa with a pI of 6.7 (Figure 15-18). The m/z list (see Appendix chapter 6) of the peptide mass fingerprint (Figure 31) was submitted to Mascot search, and a significant protein result was obtained. The significant result (Score: 69, Threshold: 67) was the protein Anthranilate phosphoribosyltransferase in *Sulfolobus acidocaldarius*, a protein with a calculated mass of 37,471kDa and a calculated pI of 8,73. From 50 m/z values submitted, 9 values matched but and 39% sequence coverage was gained. In comparison of the found and the proposed (calculated) m/z values, it was astonishing that a lot of the most intense peaks of the spectra did not match to the protein found by Mascot search. Therefore MS/MS experiments were carried out leading to fragment mass lists (see Appendix chapter 6) for 3 peptides (m/z 1003.6, m/z 2038.1 and m/z 2681.4). None of the peaks used for the MS/MS experiment was found in the mass list of the significant protein hit found by PMF. All

mass lists were submitted separately to Mascot search and none of the searches allowed the identification of a peptide or protein.

Peptide 3:

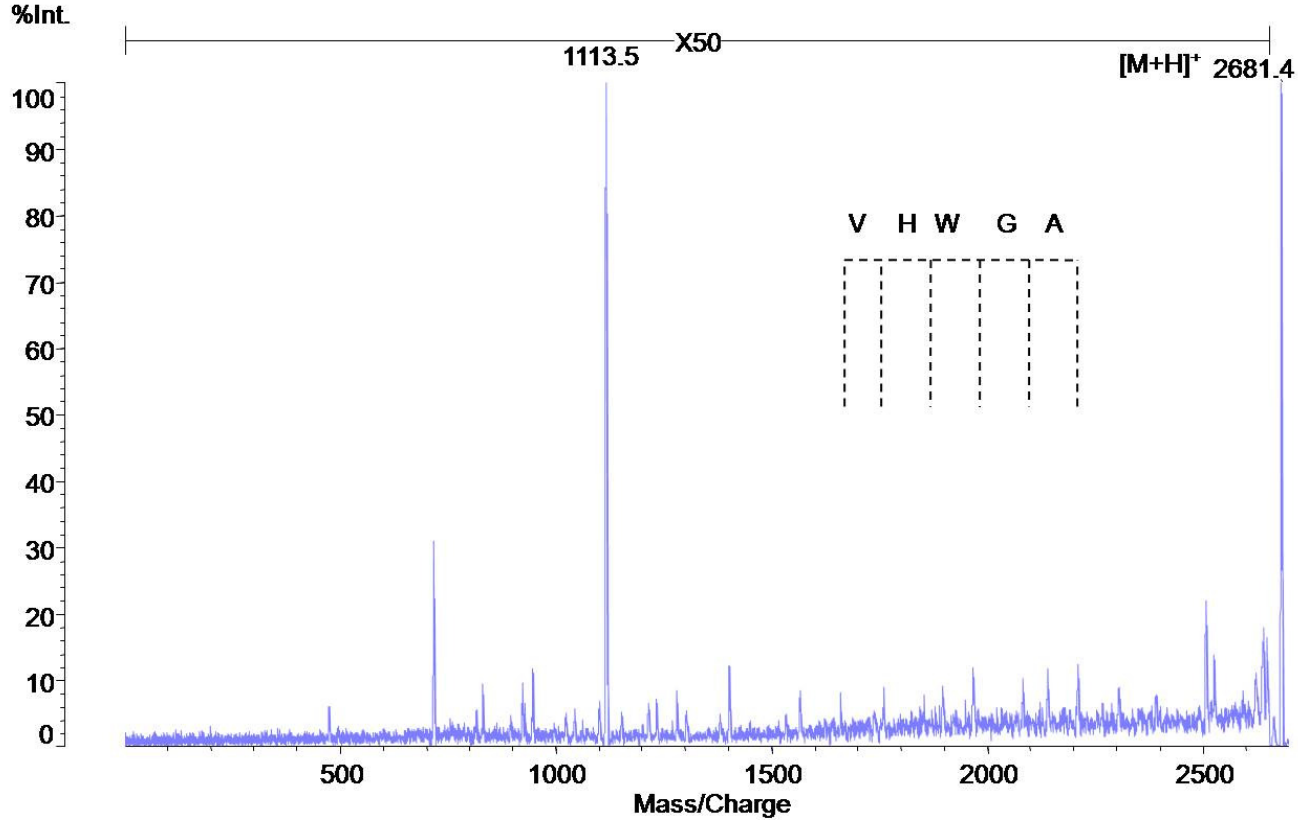

Figure 32. PSD of peptide 3 of precursor ion m/z 2681.4 of the PMF Alle 9

The spectra of peptide 3 allowed a manual interpretation leading to the following sequence:

- (1660.33)-VHWGA-(2209.91).

It was not possible to evaluate whether it is a b- or y-ion series sequence.
The sequence was submitted to Mascot and a homology search was performed but did not lead to an identification of spot Alle 9.
Due to the fact that the spectrum of the PMF of spot Alle 9 and the protein Anthranilate phosphoribosyltransferase show a lot of differences in the mass spectra and the MS/MS data did not give significant results, the conclusion is that this spot cannot be considered as “identified”.

Spot Cyst 1

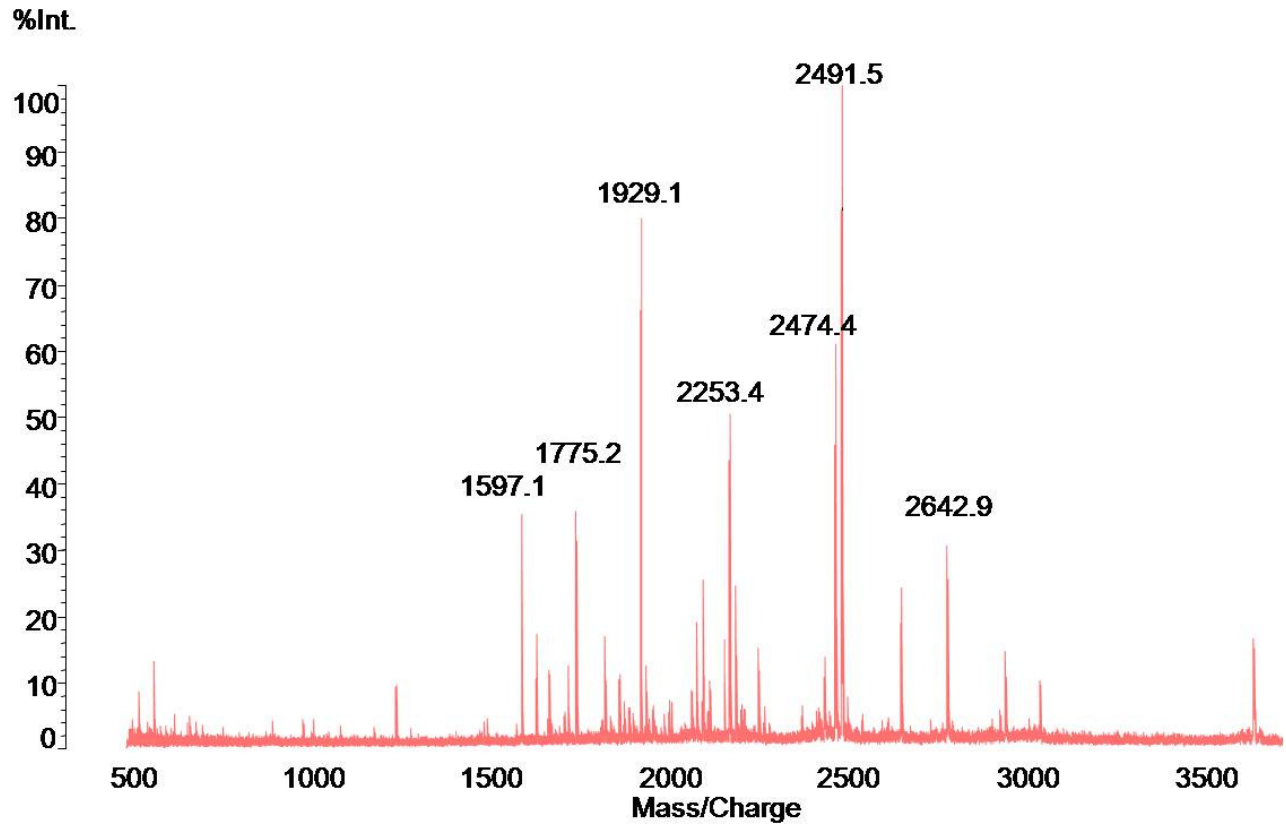

Figure 33. Peptide mass fingerprint of Spot Cyst 1 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Cyst 1 as a protein of approximately 55kDa with a pI of 6.3 (Figure 16). The m/z list (see Appendix chapter 6) of the peptide mass fingerprint (Figure 33) were submitted to Mascot search, and a significant protein result for the protein vacuolar ATP synthase subunit B 1 from Hordeum vulgare (Score: 74, Threshold 67) was obtained. This protein has a molecular mass of 54,107 kDa and a calculated pI of 5.12. For this hit 31 m/z values were submitted to Mascot whereas 7 m/z values matched giving sequence coverage of 15% (Figure 33).

```
  1 MGLVKEGADM EEGTLEIGME YRTVSGVAGP LVILDKVKGP KYQEIVNIRL
 51 GDGTTRRGQV LEVDGEKAVV QVFEGTSGID NKYTTVQFTG EVLKTPVSLD
101 MLGRIFNGSG KPIDNGPPIL PEAYLDISGS SINPSERTYP EEMIQTGIST
151 IDVMNSIARG QKIPLFSAAG LPHNEIAAQI CRQAGLVKRL EKGKHAEGGG
201 EDDNFAIVFA AMGVNMETAQ FFKRDFEENG SMERVTLFLN LANDPTIERI
251 ITPRIALTTA EYLAYECGKH VLVILTDMSS YADALREVSA AREEVPGRRG
301 YPGYMYTDLA TIYERAGRIE GRTGSITQIP ILTMPNDDIT HPTPDLTGYI
351 TEGQIYIDRQ LHNRQIYPPI NVLPSLSRLM KSAIGEGMTR RDHSDVSNQL
401 YANYAIGKDV QAMKAVVGEE ALSSEDLLYL EFLDKFERKF VAQGAYDTRN
451 IFQSLDLAWT LLRIFPRELL HRIPAKTLDA FYSRDAAH
```

Figure 34. Sequence coverage (Matched peptides shown in **Bold Red**)

It was not possible to perform PSD MS/MS experiments because the molecular ions of interest were lying too close together. Without any MS/MS fragment data to verify the PMF result the spot is cannot be considered to be identified.

Spot Cyst 3

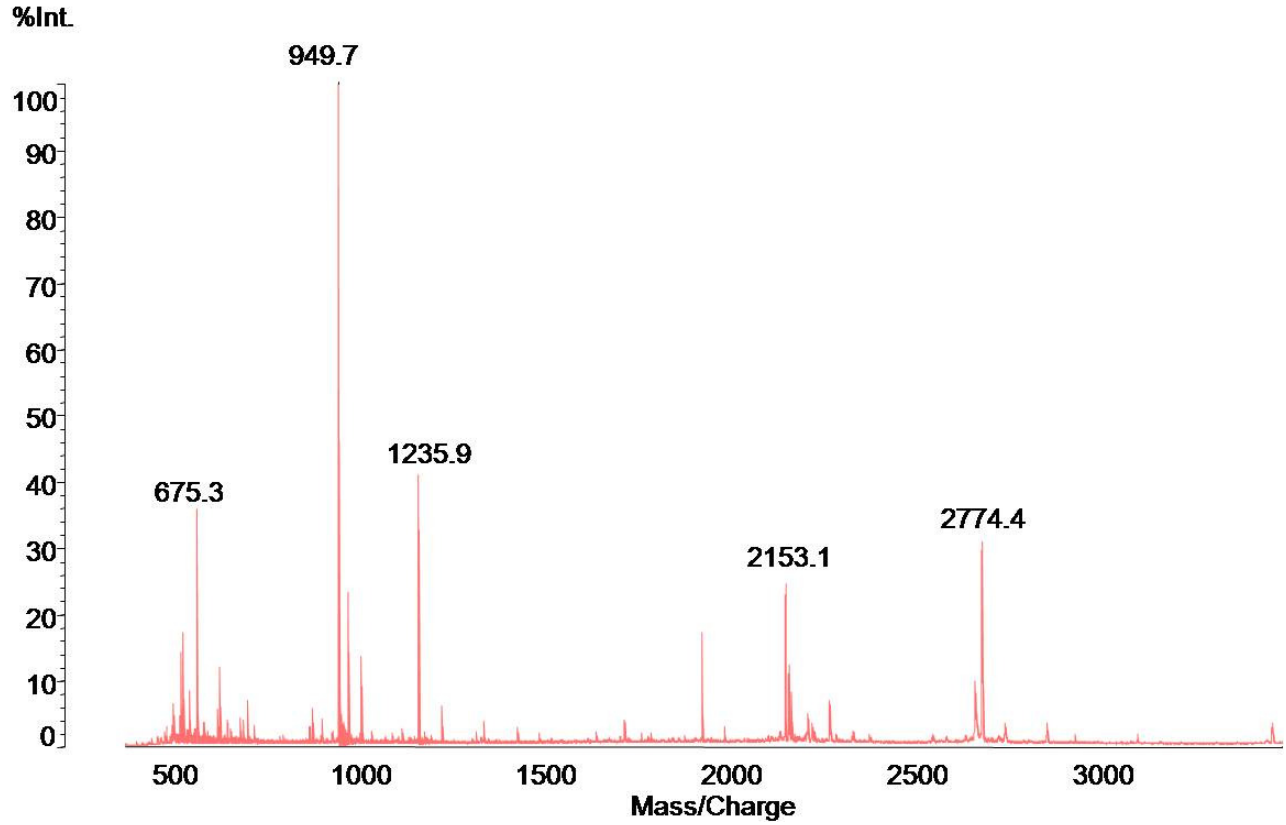

Figure 35. Peptide mass fingerprint of Cyst 3 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Cyst 3 as a protein of approximately 14 kDa with a pI of 5.2 (Figure 16). The m/z list (see Appendix chapter 6) of the PMF (Figure 35) was submitted to Mascot search, and no significant result could be obtained.

Therefore PSD MS/MS experiments were performed giving PSD spectra of 3 peptides (peptide 1: m/z 949.8 [Figure 36], peptide 2: m/z 1164.9[Figure 37] and peptide 3: m/z 2153.5 [Figure 38]).

Peptid 1:

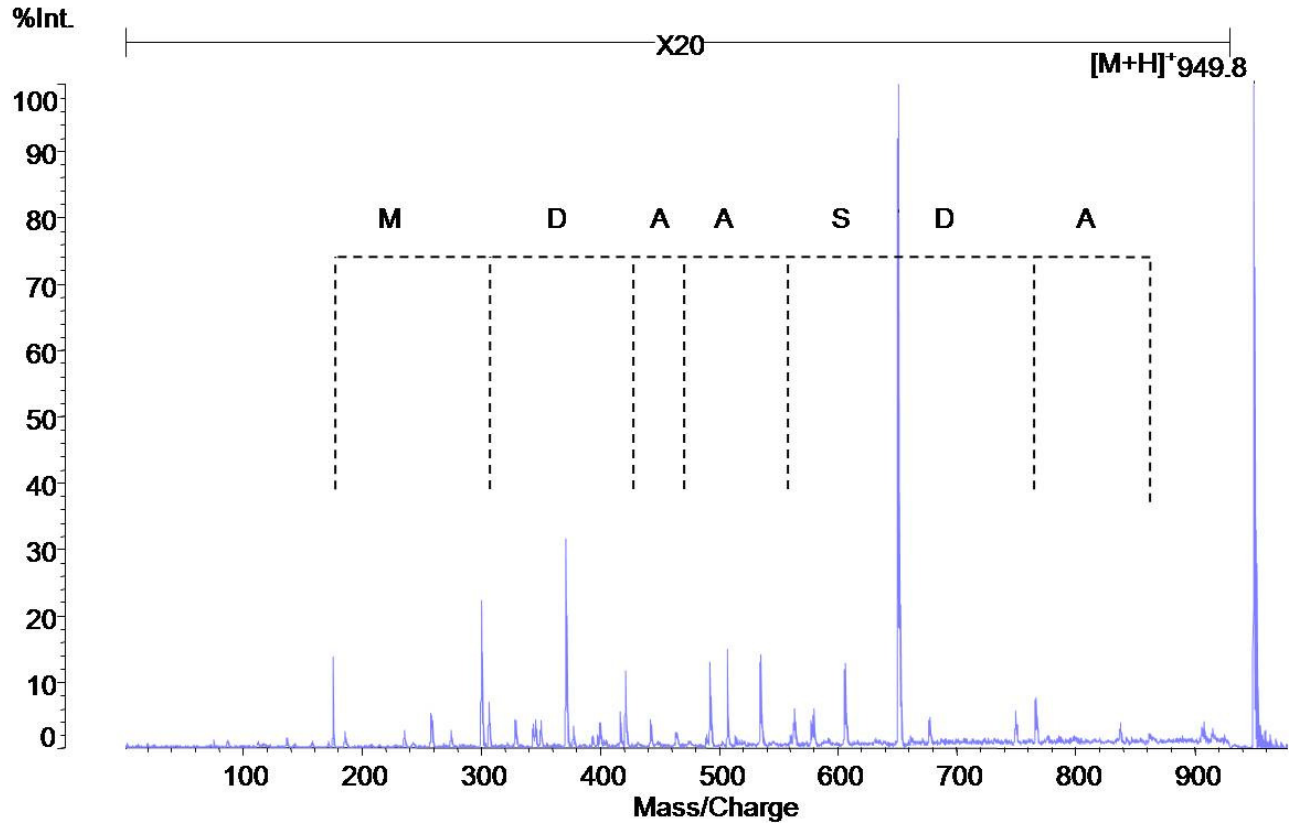

Figure 36. PSD spectrum of peptide 1 of precursor m/z 949.8 of the PMF Cyst 3

The fragment ion list (see Appendix chapter 6) was submitted to Mascot but no result was obtained. Therefore manual evaluation was carried out leading to the following amino acid sequence:

- (0.0)-MDAASDA-(837.06)

It was not possible to evaluate from the spectrum whether it is a b- or a y-ion series sequence. For this sequence tag no homologies were found

Peptide 2:

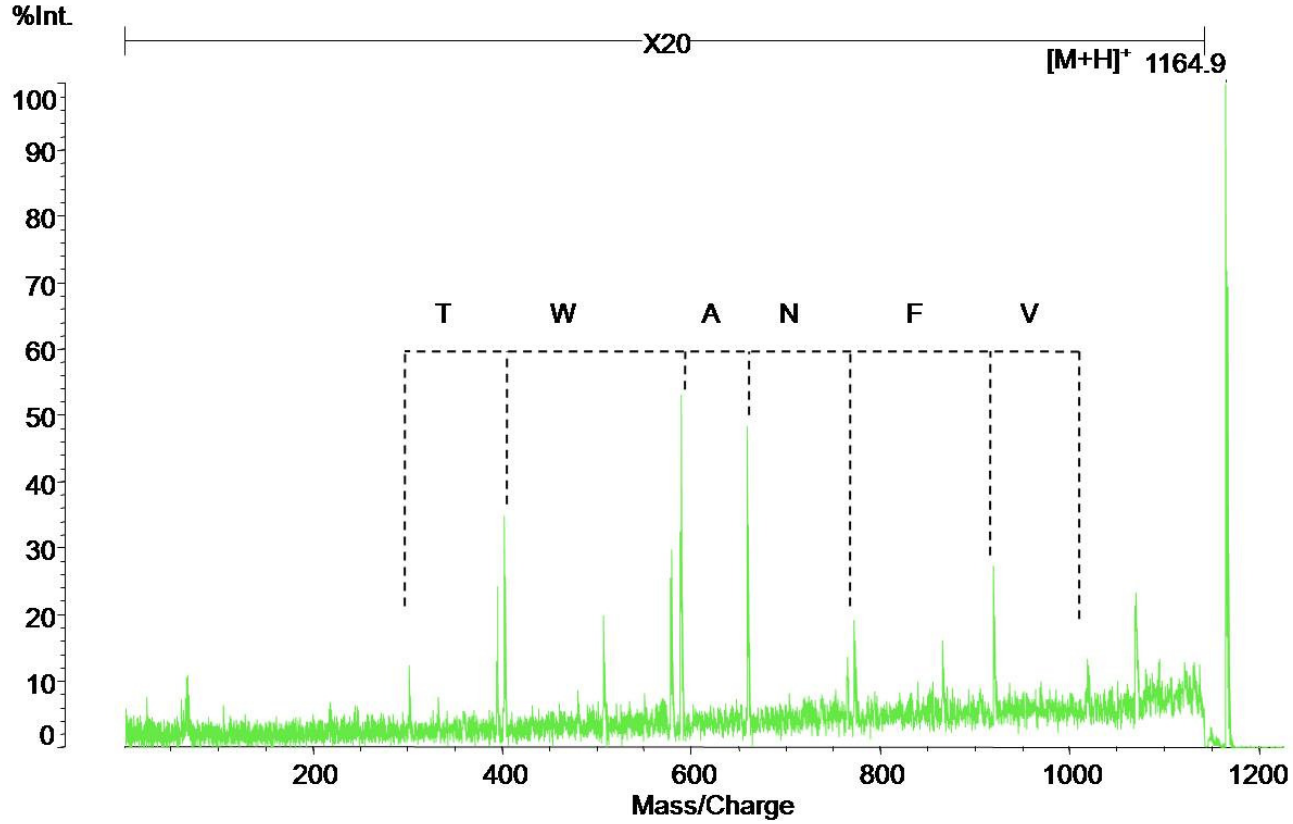

Figure 37. PSD spectrum of peptide 2 of precursor m/z 1164.9 of the PMF Cyst 3

The fragment mass list (see Appendix chapter 6) was submitted to Mascot search engine gave no result therefore manual evaluation was performed leading to the following sequence:

- (301.16)-TWANFV-(1013.14)

It was not possible to evaluate from the spectrum whether it is a b- or a y-ion series sequence. For this sequence tag no homologies were found.

Peptide 3:

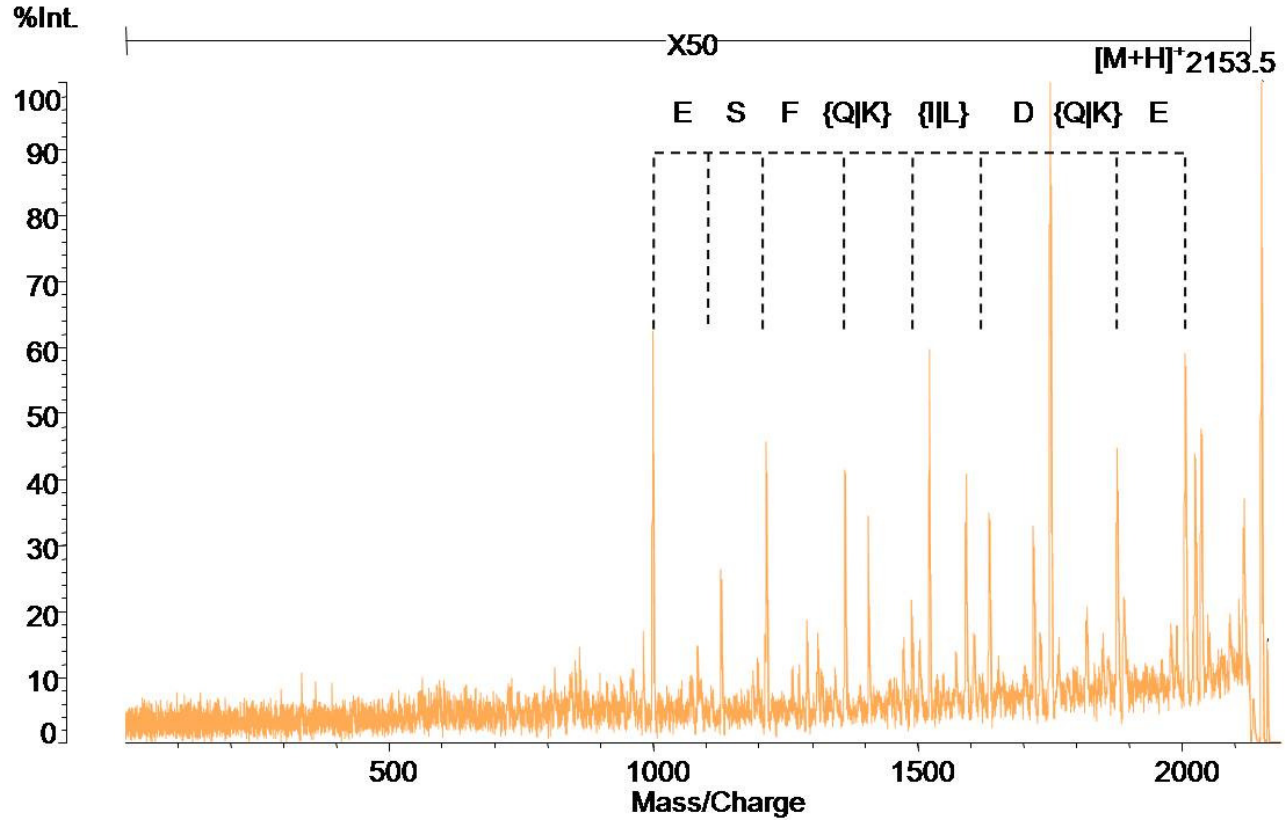

Figure 38. PSD spectrum of peptide 3 of precursor m/z 2153.5 of the PMF Cyst 3

The fragment mass list of peptide 3 (see Appendix chapter 6) gave no result in none of the databases. Due to the fact that there were enough peaks for manually interpretation it was possible to get the following sequence:

- (1000.29)-ESF{Q/K}{I/L}D{Q/K}E-(2008.99)

It was not possible to evaluate from the spectrum whether it is a b- or a y-ion series sequence

Homology search was performed resulting in a hypothetical protein of *Entamoeba hystolytica* with the following sequence:

- KMFQEDQE.

Due to the fact that the protein we found by homology search is not identified yet, we are not able to get an additional hint for the identification of this spot.

Combination of sequence tags:

Three PSD spectra were generated to confirm the PMF result. All peptides gave no significant hit in none of the databases. It was possible to get sequence tags out of three spectra by evaluating the spectra manually. The resulting sequence tags were submitted separately but also together in one Mascot sequence query to all databases. None of the results included more than one of the sequence tags. The logical conclusion is the impossibility of protein identification with this data.

Due to the fact that it was not possible to confirm the PMF result with a PSD spectra this spot has to be considered as “not identified”.

Spot Cyst 4

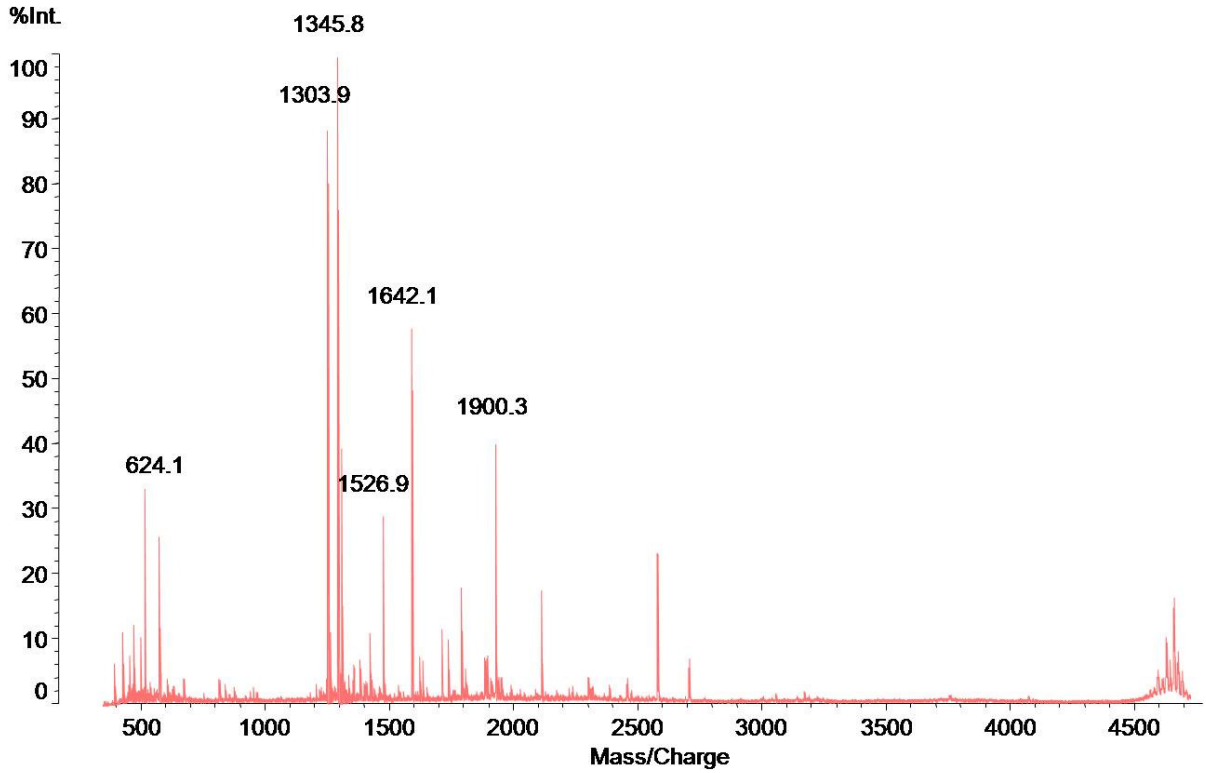

Figure 39. Peptide mass fingerprint of Spot Cyst 4 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Cyst 4 as a protein of approximately 70 kDa and a pI with 5.3 (Figure 16). With the m/z list (see Appendix chapter 6) of the PMF (Figure 39) a Mascot search was done leading to no significant protein hit. A lot of unspecific protein hits were reached but none of them could give us a hint for the identification of spot Cyst 4.

Peptide 1:

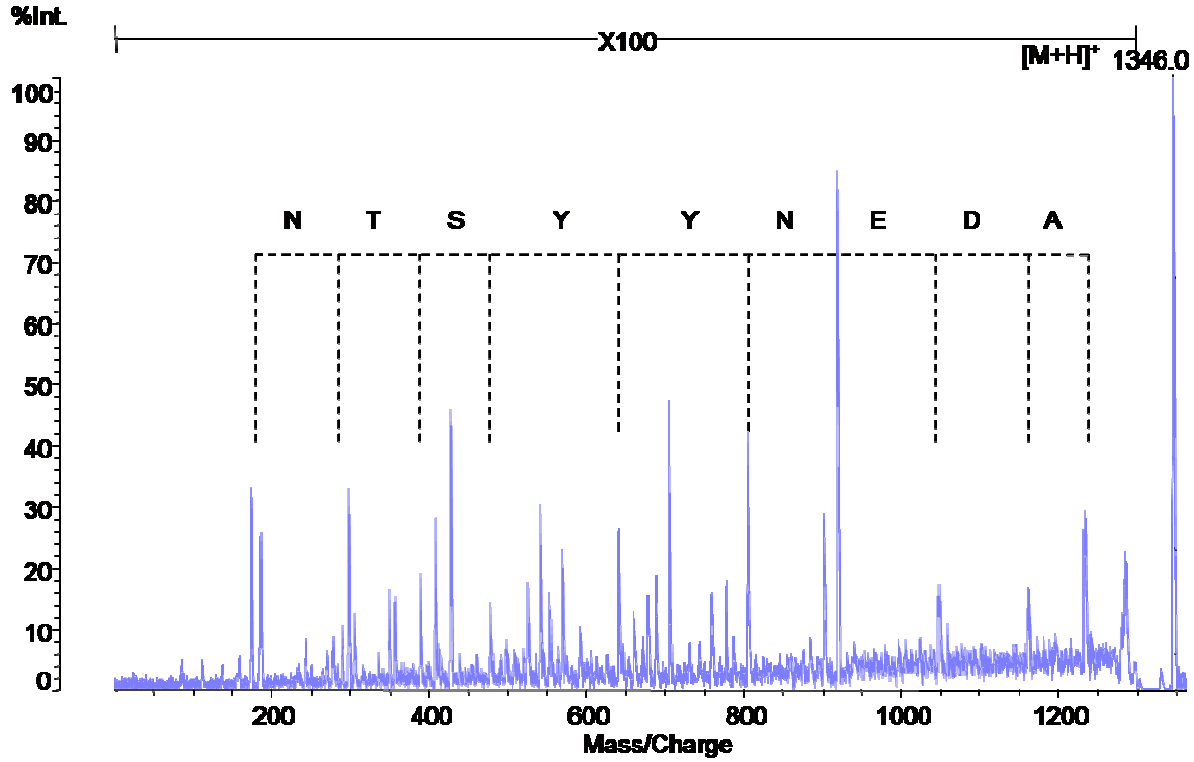

Figure 40. PSD spectrum of peptide 1 of precursor m/z 1345.8 of the PMF Cyst 5

MS/MS experiments were performed and the fragment mass lists were submitted to the databases NCBI and Mascot but do not lead to any results. It was possible to evaluate one spectrum manually, leading to the following sequence:

- (175.15)-NTSYYNEDA-(1232.49)

It was not possible to evaluate from the spectrum whether it is a b- or a y-ion series sequence.

With this sequence we performed a homology search using protein prospector leading to a sequence of Entamoeba histolytica. These two sequences differ in 4 amino acids.

RN**TS**YY**N**ED**A**...Cyst 4

RN**SC**YY**G**ED**K**...Entamoeba histolytica

Due to the fact that the found protein of Entamoeba histolytica is a hypothetical protein, it is not possible to get a hint for identification of this spot.

The performed experiments did not lead to identification of the spot Cyst 4 because neither the PMF nor the MS/MS fragment data did give us certain results.

Spot Cyst 5

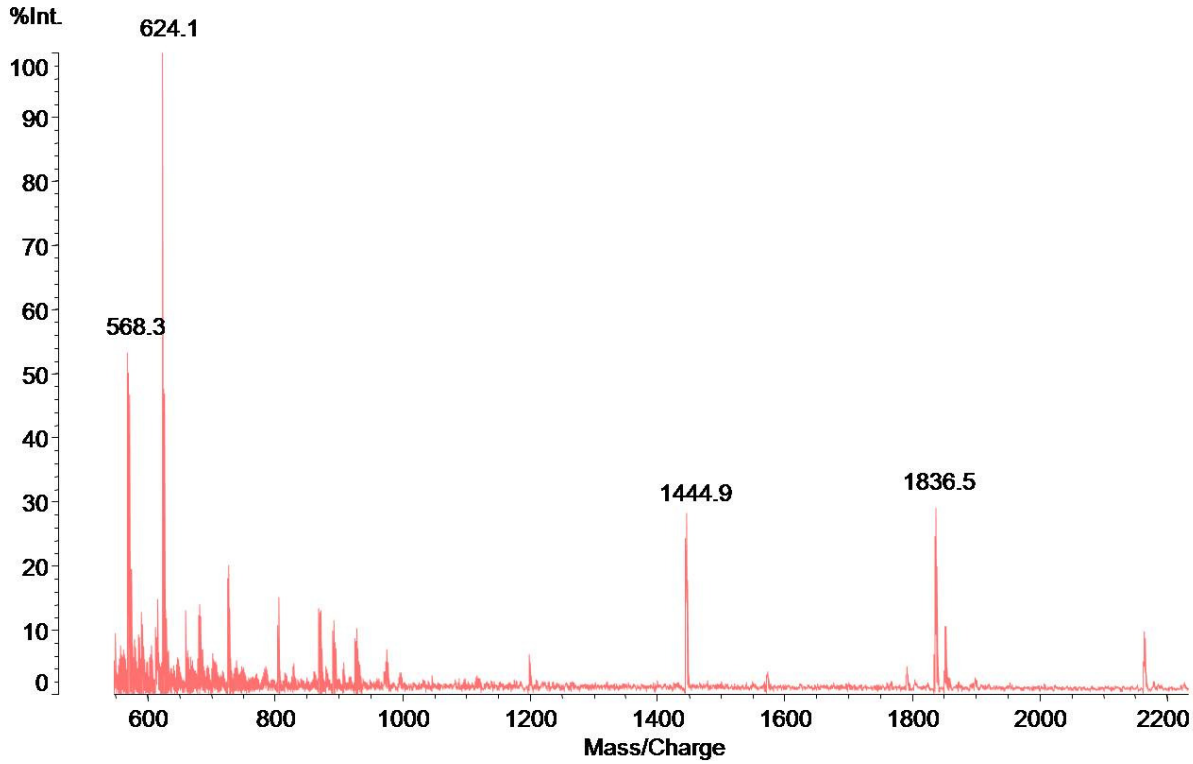

Figure 41. Peptide mass fingerprint of spot Cyst 5 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Cyst 5 as a protein of approximately 40 kDa with a pI of 6.6 (Figure 17). The m/z list (see Appendix chapter 6) from PMF (Figure 41) was submitted to the databases but no significant result was obtained.

Therefore MS/MS experiments were performed. The MS/MS experiment delivered one peptide spectrum (m/z 1836.8) that was submitted to Mascot search.

Peptide 1:

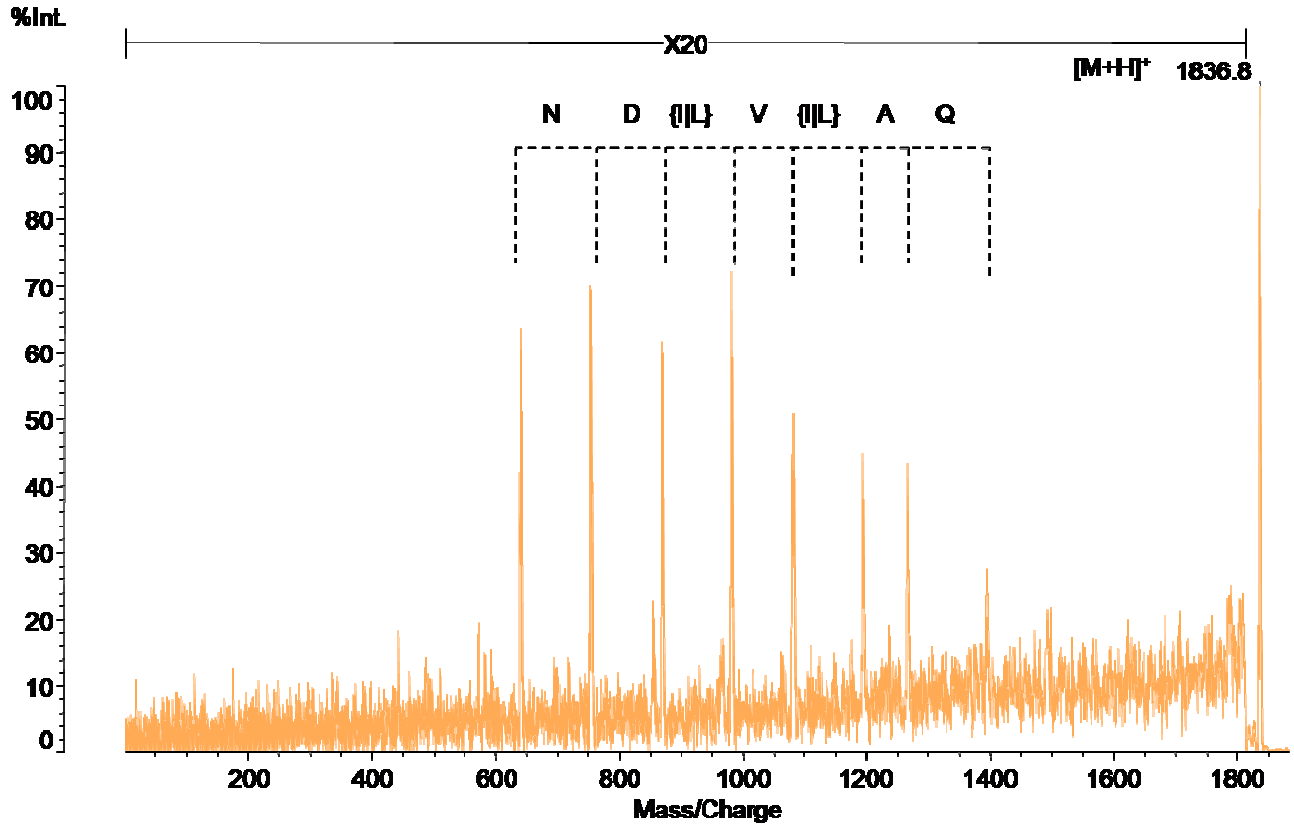

Figure 42. PSD Spectrum of peptide 1 of precursor m/z 1836.8 of the PMF Cyst 5

It was possible to evaluate the spectrum manually leading to the following sequence:

- (641.3)-ND{I/L}V{I/L}AQ-(1394.9).

It was not possible to evaluate from the spectrum whether it is a b- or a y-ion series sequence.

This sequence was found in the databases with high homology to the protein sequence of Phosphofructokinase from Entamoeba histolytica.

ND{I/L}**V**{I/L}AQ.....Cyst 5

ND L **L** I AQ.....Phosphofructokinase Entamoeba histolytica

The protein Phosphofructokinase is an enzyme involved in the main metabolism of all living organisms therefore it can be found in nearly every organism in high amounts. The PMF data did not proof the result of the homology search. The reason is that there were either too few peak values in the PMF or the peak values in the PMF were not specific enough get a significant hit in the databases. In the PMF spectrum (Figure 41) there is a gap between m/z 1200-2163 with very few peaks with low intensities but just in this area the most specific peaks for this enzyme were assumed. Further PMF data of this spot has to be collected to proof the identity.

Spot Cyst 6

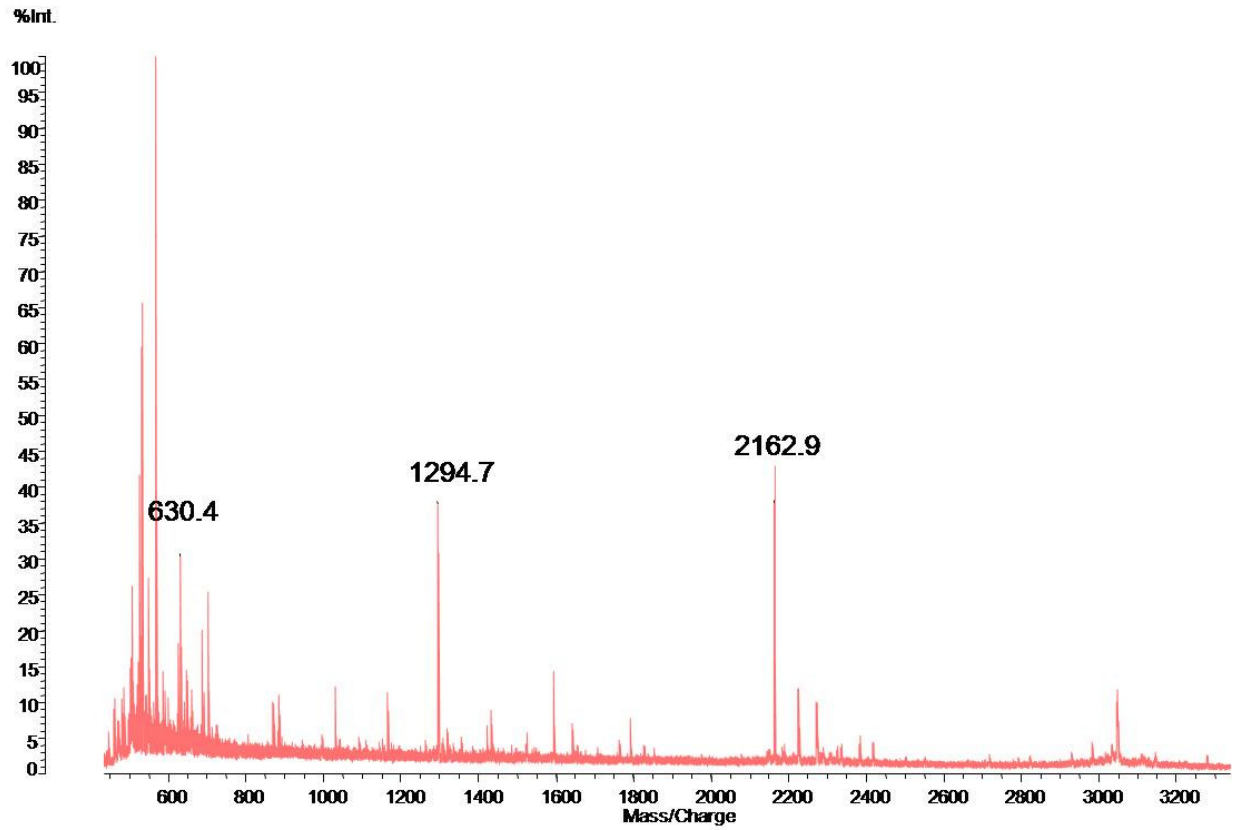

Figure 43. Peptide mass fingerprint of spot Cyst 6 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Cyst 6 as a protein of approximately 25 kDa and a pI of 7.2 (Figure 17). The m/z list (see Appendix chapter 6) of the peptide mass fingerprint (Figure 43) was submitted to Mascot resulting in a significant

hit for Myb family DNA binding protein from *Entamoeba histolytica* (Score:80, Threshold:80). This protein with a mass of 27599 Da and a calculated pI of 7.39 fits very well to the information resulting from its position on the 2D gel. From 14 m/z values submitted to Mascot, 7 values matched leading to sequence coverage of 21% (Figure 44).

```
  1 MSTFGNAAGV IWRNTEDEVL KAGVMKYGKN EWARISSLIA GKSPQQCKAR
 51 WYSWLDPSIK KTEWTSTEEE KLLHLIKIFP SQWQTISKSV GRTPAQCIEK
101 YNQLKDEATG DDCSGLREKE MNEIIPETRP ALKDRVDLDD DEIEMLNEVR
151 ARLANTKGKK AKRKERQKLQ QDTAYATELQ KRRELRAAGI QVSYSHKIKG
201 PDYNSEIPFF REVPQGKFNP QKDRKPPKKP SFIGKEMN
```

Figure 44. Sequence coverage (Matched peptides shown in **Bold Red**)

To get more sequence information MS/MS experiments were performed but none of the chosen peptides gave evaluable PSD spectra. The peaks chosen for PSD did not fragment so that no PSD spectrum could be made. Due to the fact that the protein found with PMF data did not give us a high significant hit and that it was not possible to verify the PMF result with PSD MS/MS results this spot is considered as “not identified”.

Spot Cyst 8

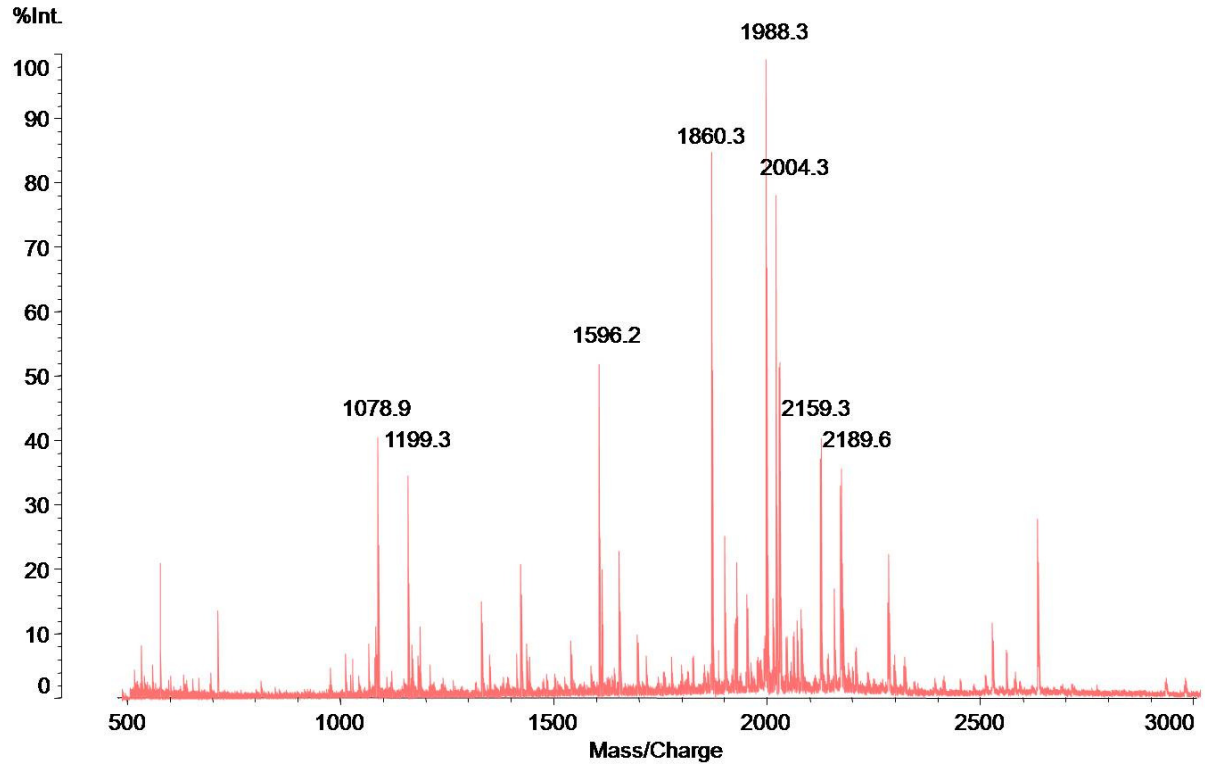

Figure 45. Peptide mass fingerprint of Cyst 8 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify spot Cyst 8 as a protein of approximately 45 kDa and a pI of 7 (Figure 17) was analysed and the m/z list of the peptide mass fingerprint (Figure 45) was submitted to the databases resulting in no significant hit. Subsequent PSD MS/MS experiments were done and fragment m/z lists of one peptide (average m/z 1990.1) was used to make a Mascot query.

Peptide 1:

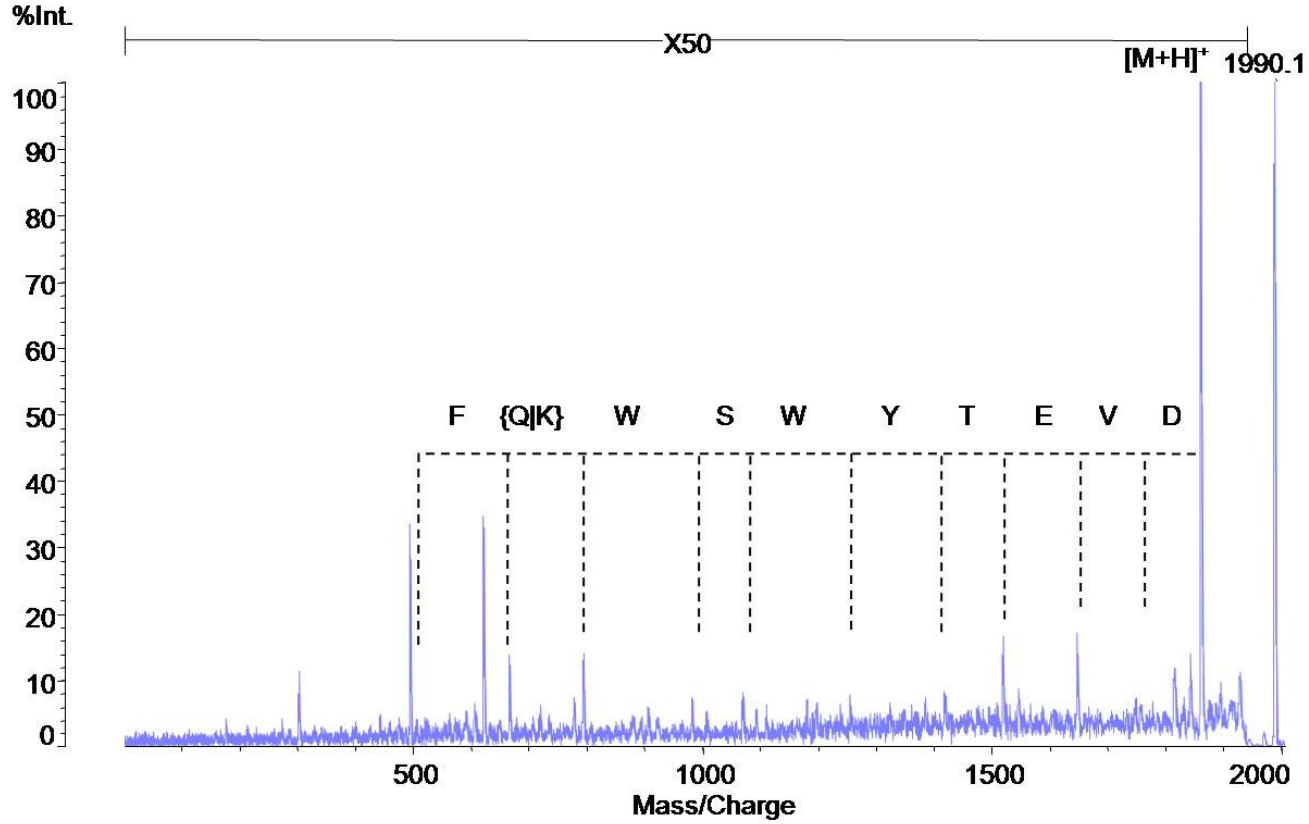

Figure 46. PSD spectrum of peptide 1 of precursor m/z 1990.1 of the PMF Cyst

Manual interpretation leads to the following sequence tag:

- (519.96)-F{Q/K}WSWYTEVD-(1862.53)

The sequence and the fragment mass list were submitted to Mascot search but no significant hit could be obtained. With the sequence a homology search was performed but no homologies were found.

Spot Cyst 9

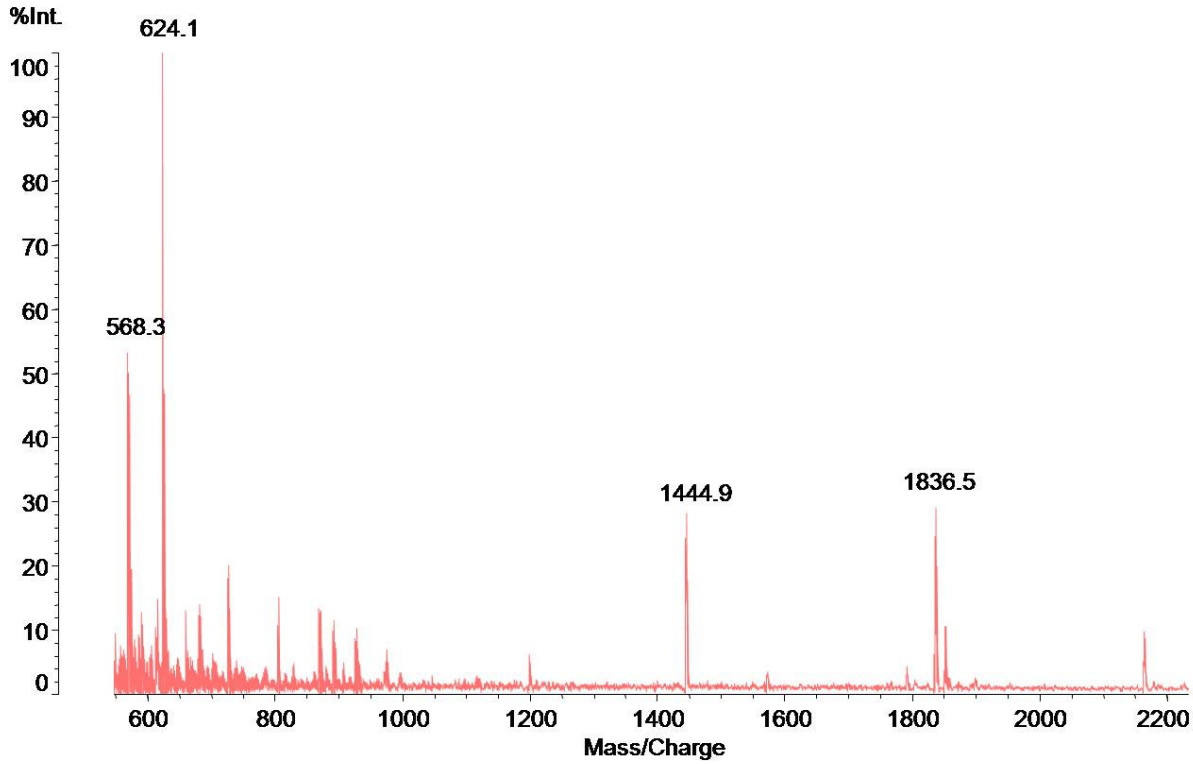

Figure 47. Peptide mass fingerprint of Cyst 9 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify Spot Cyst 9 as a protein of approximately 100 kDa and a pI of 7.2 (Figure 17).Even though that the peptide mass fingerprint consisted of enough m/z values it was not possible to get a significant protein hit in none of the used databases. The mass lists (see Appendix chapter 6) were submitted to SWISS PROT, NCBInr and MSDB and produced a lot of low significant hits with Mascot scores of 80 (Threshold 80) but none of them was fitting in weight and pI with the data from the 2D gel. This situation made it essential to do MS/MS experiments to get more certain results to work with. The peptide mass fingerprint made it not easy to get PSD spectra because the peaks were very close together. It was possible to get an evaluable spectrum from one peptide (average m/z 1087.9, Figure 48). The fragment ion list was submitted to Mascot search but no significant result was obtained.

Peptide 1:

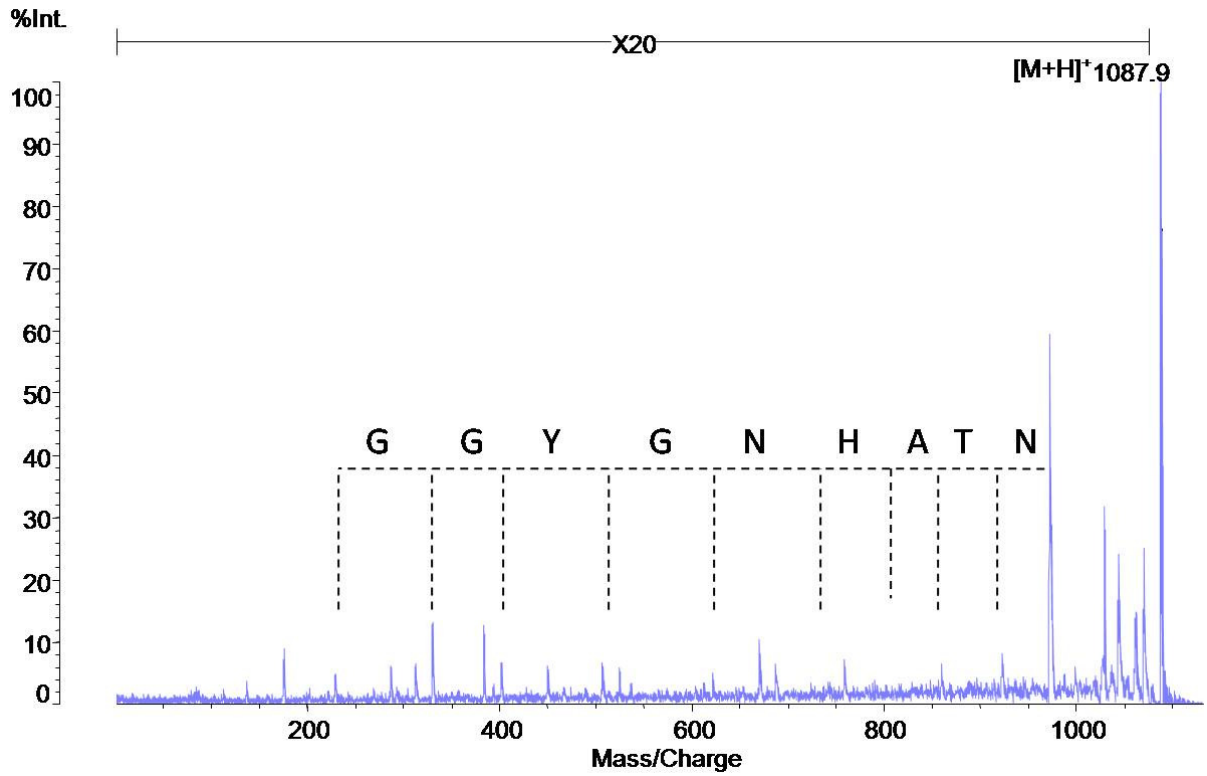

Figure 48. PSD spectrum of peptide 1 of precursor m/z 1087.9 of the PMF Cyst 9

It was possible to evaluate one spectra manually leading to the following sequence:

- (0.0)-(R)GGYGNHATN-(973.78)

A homolog sequence was found after homology search in *Dictyostelium Discoideum*.

RGGYG**NH**A**TN**....Cyst 9
DGGYG**SK**AS**R**....*Dictyostelium Discoideum*

This sequence was described as hypothetical protein so that we are not able to get another hint for the identification of Cyst 9.

Spot Enc 2- Actin 1

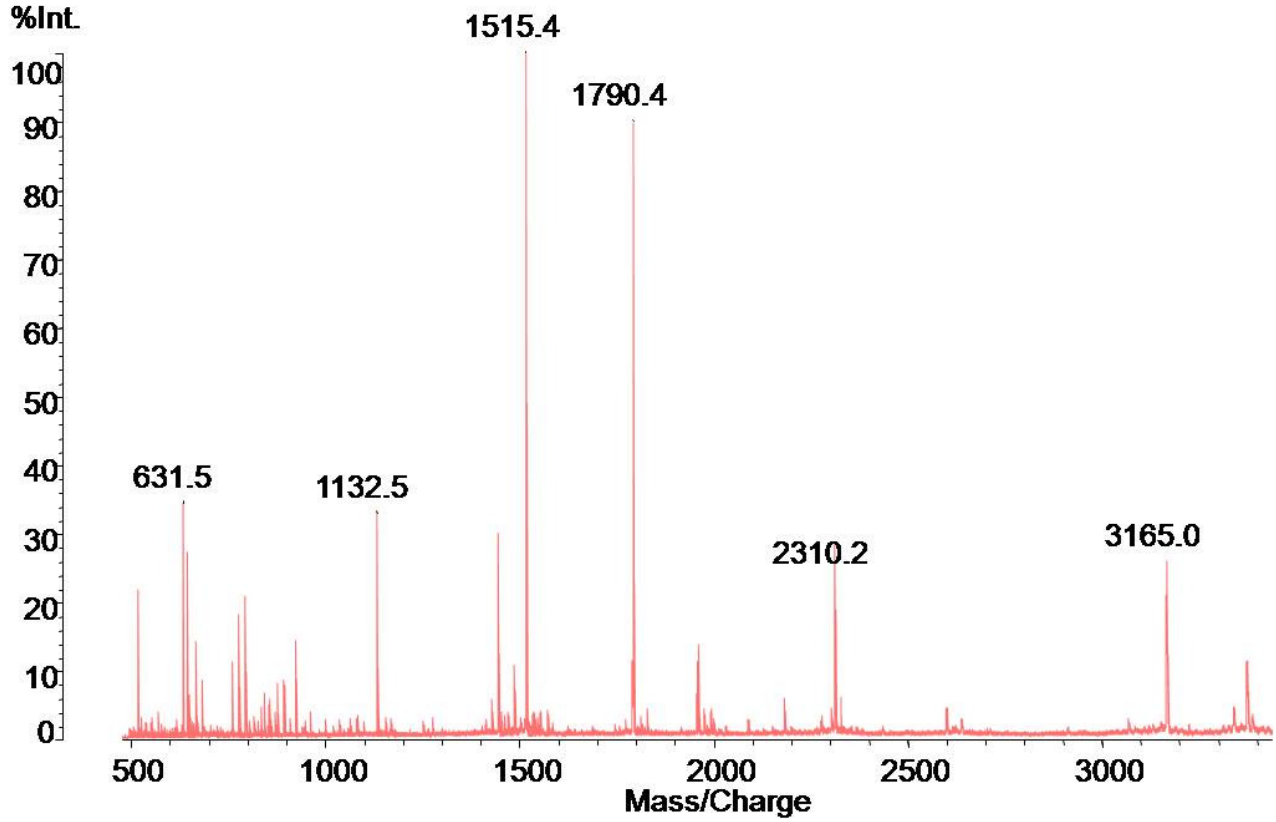

Figure 49. Peptide mass fingerprint of Enc 2 obtained by MALDI RTOF mass spectrometry

Data from 2D gel electrophoresis identify Spot Enc 2 as a protein of approximately 32 kDa and a pI of 5.7 (Figure 17-19). The m/z list (see Appendix chapter 6) of the PMF (Figure 49) was submitted to Mascot resulting in a high significant hit for actin in *Acanthamoeba castellanii* (Score: 109 Threshold: 67) with a calculated molecular mass of 41877 Da and a calculated pI of 5.37. To receive these result 19 m/z values were submitted whereas 12 masses matched giving sequence coverage of 41% (Figure 50).

```
  1 MGDEVQALVI DNGSGMCKAG FAGDDAPRAV FPSIVGRPRH TGVMVGMGQK
 51 DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEKIWHHTF YNELRVAPEE
101 HPVLLTEAPL NPKANREKMT QIMFETFNTP AMYVAIQAVL SLYASGRTTG
151 IVLDSGDGVT HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTERGYSF
201 TTTAERFIVR DIKEKLCYVA LDFEQEMHTA ASSSALEKSY ELPDGQVITI
251 GNERFRAPEA LFQPSFLGME SAGIHETTYN SIMKCDVDIR KDLYGNVVLS
301 GGTTMFPGIA DRMQKELTAL APSTMKIKII APPERKYSVW IGGSILASLS
351 TFQQMWISKE EYDESGPSIV HRKCF
```

Figure 50. Sequence coverage (Matched peptides shown in **Bold Red**)

To proof the result of the PMF, PSD MS/MS experiments of peptide m/z 1790.4 (Figure 51) were performed. The fragment ion list (see Appendix chapter 6) which was submitted to Mascot and a significant hit for actin (Score 54, Threshold: 41) from *Dictyostelium Discoideum* was received. It was not possible to find fragment ion data entries of protein actin from *A.castellanii* in any of the used databases and because of high conserved sequences (Figure 49a) the fragment mass search resulted in a significant hit of the near related organism *Dictyostelium Discoideum.*
The spot Enc 2 was identified as protein actin.

Peptide 1:

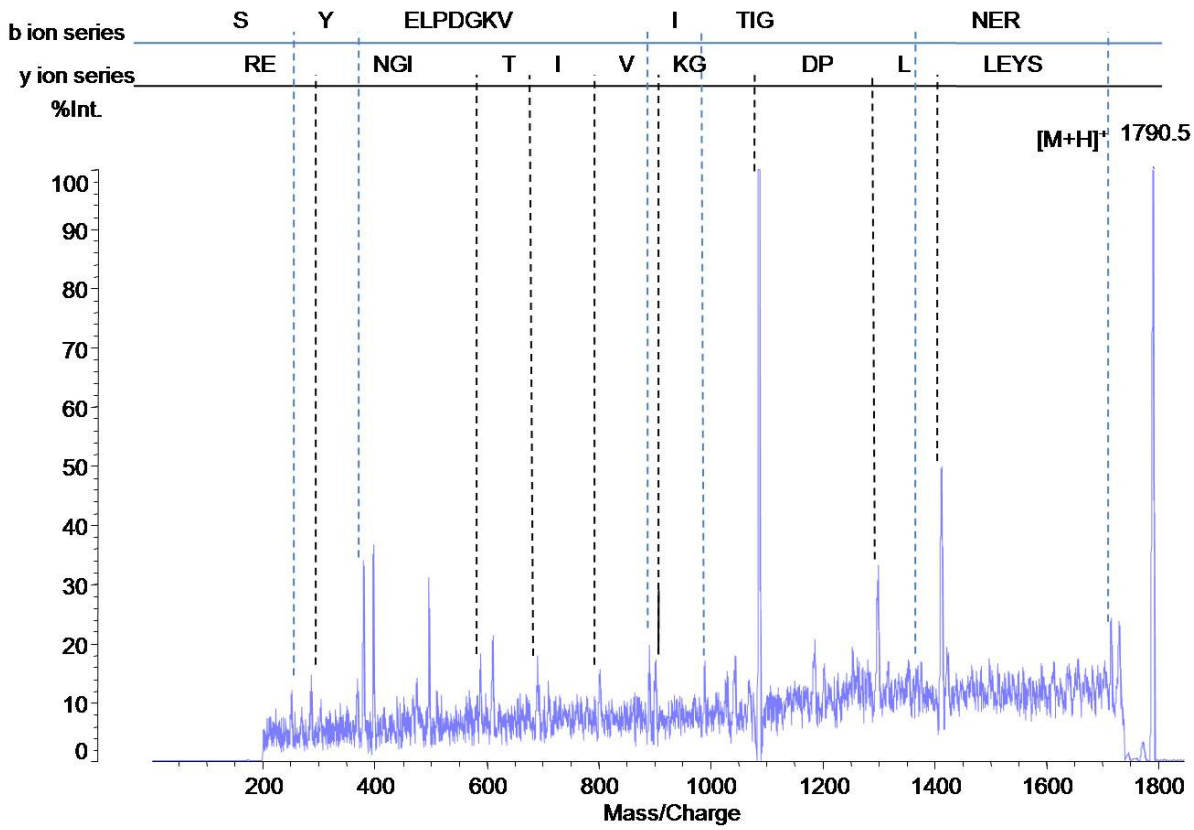

Figure 51. PSD of peptide 1 of precursor m/z 1790.5 of the PMF Enc 2

2.1. Summary

This table shows up all 39 spots which were measured during this diploma thesis. All mass lists for PMF and all fragment mass lists are shown in the appendix section of this work (chapter 6).

Spots (name)	PMF	MS/MS	result
Enc1	yes	no	no identification
Enc 2	yes	yes	actin
Enc 3	yes	no	no identification
Enc 4	no	no	no identification
Enc 5	yes	yes	no identification
Enc 6	yes	yes	no identification
Enc 7	no	no	no identification
Enc 8	yes	no	no identification
Enc 9	yes	yes	no identification
Enc 10	yes	yes	no identification
Enc 11	yes	yes	no identification
Enc 12	no	no	no identification
Enc 13	no	no	no identification
Enc 14	no	no	no identification
Enc15	no	no	no identification
Tropho1	yes	no	no identification
Tropho2	yes	yes	no identification
Tropho3	no	no	no identification
Tropho4	no	no	no identification
Tropho5	no	no	no identification
Cyst1	yes	no	suspision: Vacuolar ATP synthase subunit B1
Cyst2	yes	yes	no identification
Cyst3	yes	yes	no identification
Cyst4	yes	yes	no identification
Cyst5	yes	yes	suspision:Phosphofructokinase
Cyst6	yes	yes	suspision: Myb family DNA binding protein
Cyst7	no	no	no identification
Cyst8	yes	yes	no identification
Cyst9	yes	yes	no identification
Alle1	yes	yes	suspision:Ramosa 3
Alle2	no	no	no identification
Alle3	yes	yes	no identification
Alle4	yes	yes	suspision: Sporulation specific chitinase 2 precursor
Alle5	no	no	no identification
Alle6	yes	yes	Elongation factor 2
Alle7	yes	yes	suspision: protein serine/threonin kinase
Alle8	no	no	no identification
Alle9	yes	yes	no identification
Alle10	yes	yes	no identification

Table 8. Overview over the results of all analysed spots (red: identification; blue: suspicions; green: not identified)

3. Identification of a protein spot using nano HPLC MALDI Spotter (nLC Spotter)

The aim of the second part of the work was to develop a nLC procedure for protein identification after in gel digestion. (Settings see chapter materials and methods)

On this account a prominent protein was chosen to demonstrate the identification using nLC-Spotter device. The chosen protein is actin from *Entamoeba histolytica* (e-actin) with a molecular weight of 42 kDa and a pI of 5.19. The peptide mass fingerprint of this protein without proceeding separation is shown in Figure 52.The peptide mass fingerprint m/z list (see appendix chapter 6) was submitted to Mascot giving a significant hit for actin (score: 91, threshold: 67).To receive this result 12 mass values were submitted whereas 8 values matched giving sequence coverage of 27% (Figure 53).

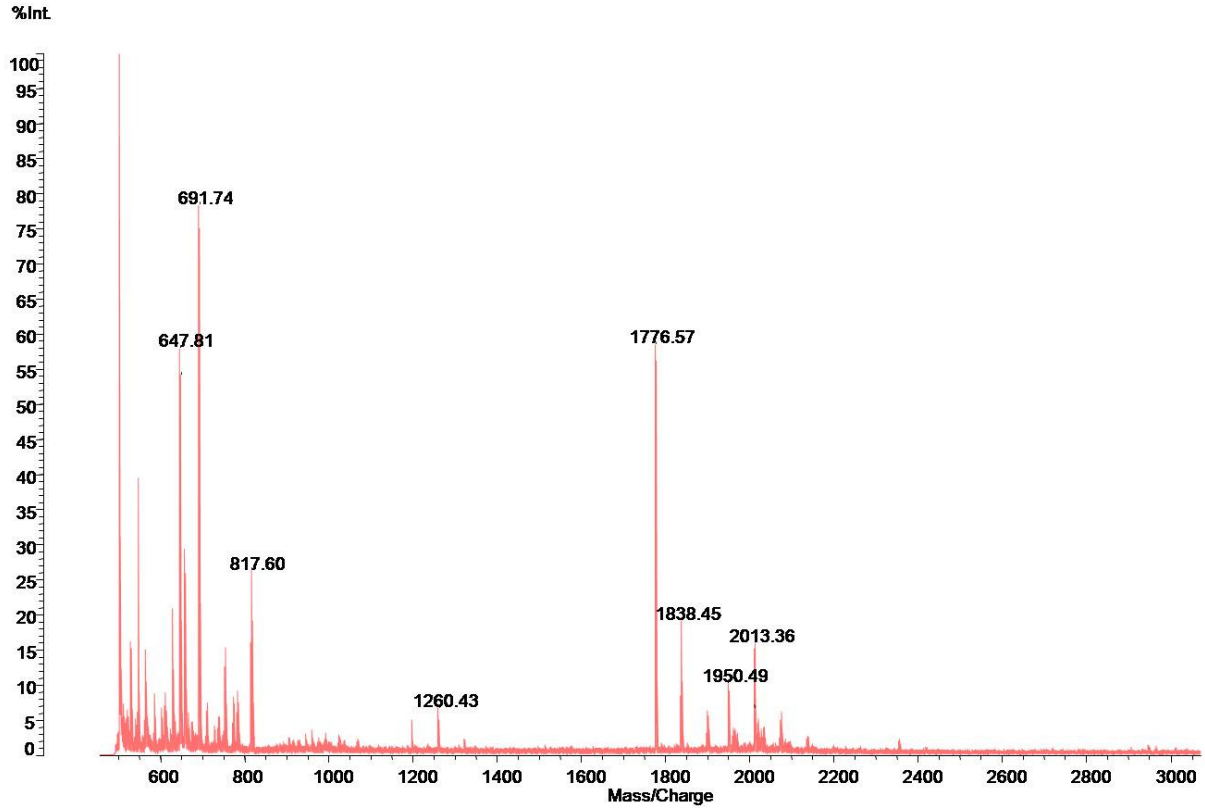

Figure 52. PMF spectrum of spot e-actin (sample preparation: dried droplet)

1 MGDEEVQALV VDNGSGMCK**A GFAGDDAPRA VFPSIVGRPR** HVSVMAGMGQ

51 KDAYVGDEAQ SKRGILTLK**Y PIEHGIVNNW DDMEK**IWHHT FYNELR**VAPE**

101 **EHPVLLTEAP MNPK**ANREKM TQIMFETFNT PAMYVGIQAV LSLYASGRTT

151 GIVMDSGDGV SHTVPIYEGF SLPHAILRLD LAGRDLTDYL MKILTERGYA

201 FTTTAEREIV RDIKEKLCYV AEDFNEEMQK AASSSELEK**S YELPDGQVIT**

251 **VGNER**FRCPE ALFQPSFLGM ECNGIHETTY NSIMKCDVDI RK**DLYGNIVL**

301 **SGGTSMYPGI NTR**LEK**EMIQ LAPPTMKIK**V IAPPERKYSV WIGGSILASL

351 STFQNMWITK EEYDESGPAI VHRKCF

Figure 53. Matched peptides shown in **Bold Red**

In comparison the spectra of the nanoLC experiment (Figure 51).

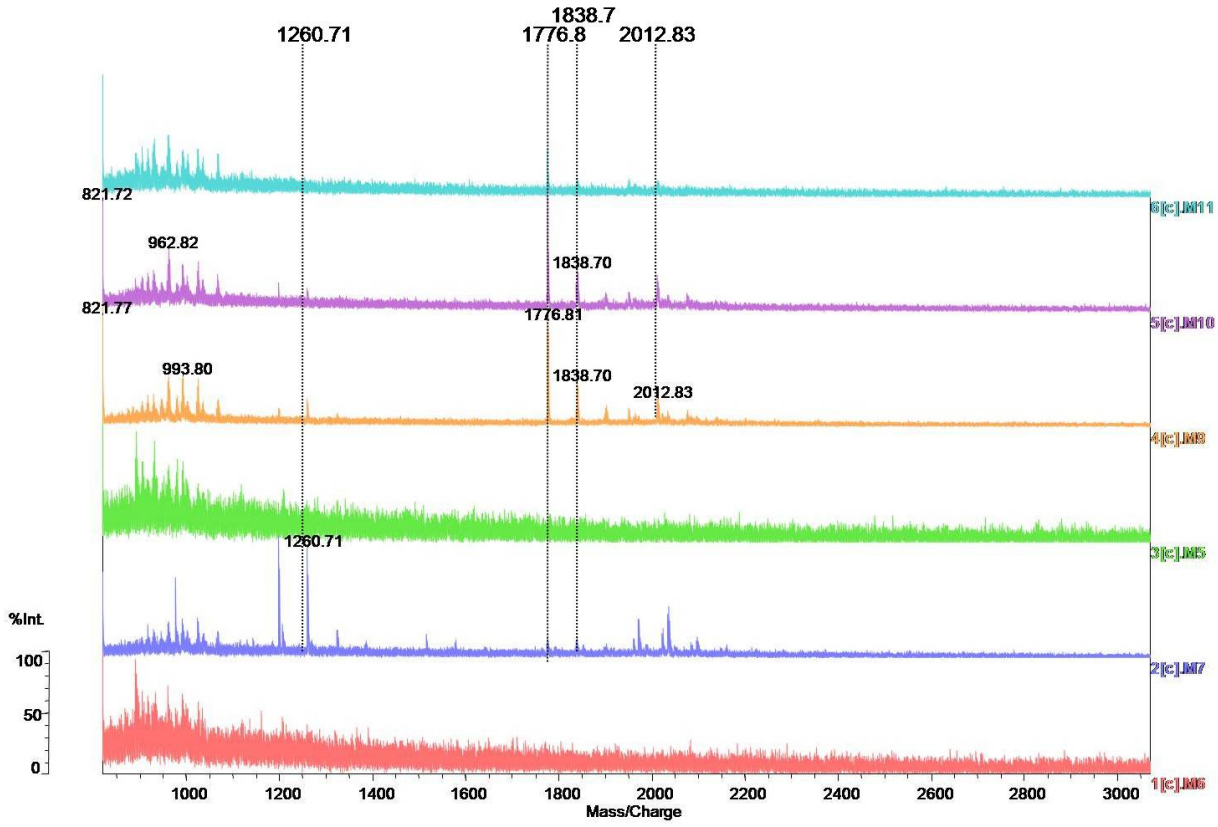

Figure 54. Mass spectra of spotted fractions 6-11 of spot E-actin

Figure 54 shows 5 mass spectra representative from the nLC experiment. These mass spectra and fractions were chosen as they contain the tryptic peptide peaks which are important for the identification of this e-actin. You can see in figure 54 that the intensive peaks with the m/z values 1260.71 and 1776.81 are separated in different spotted fractions. In more complex samples this separation step can help

the identification because of getting rid of contaminations and other troublesome peaks which make it impossible to get reproducible PSD spectra. It is also possible to see peaks that could be suppressed by other more intense peaks and in a lot of cases this small peaks are important for the identification. It can also be observed that peak detection gets improved. For the ion at m/z 1260.71, which is less intensive in Figure 52, the signal improves significant (Figure 54). Therefore problematic peak detection due to ion suppression effect can be avoided.

Peptide 1:

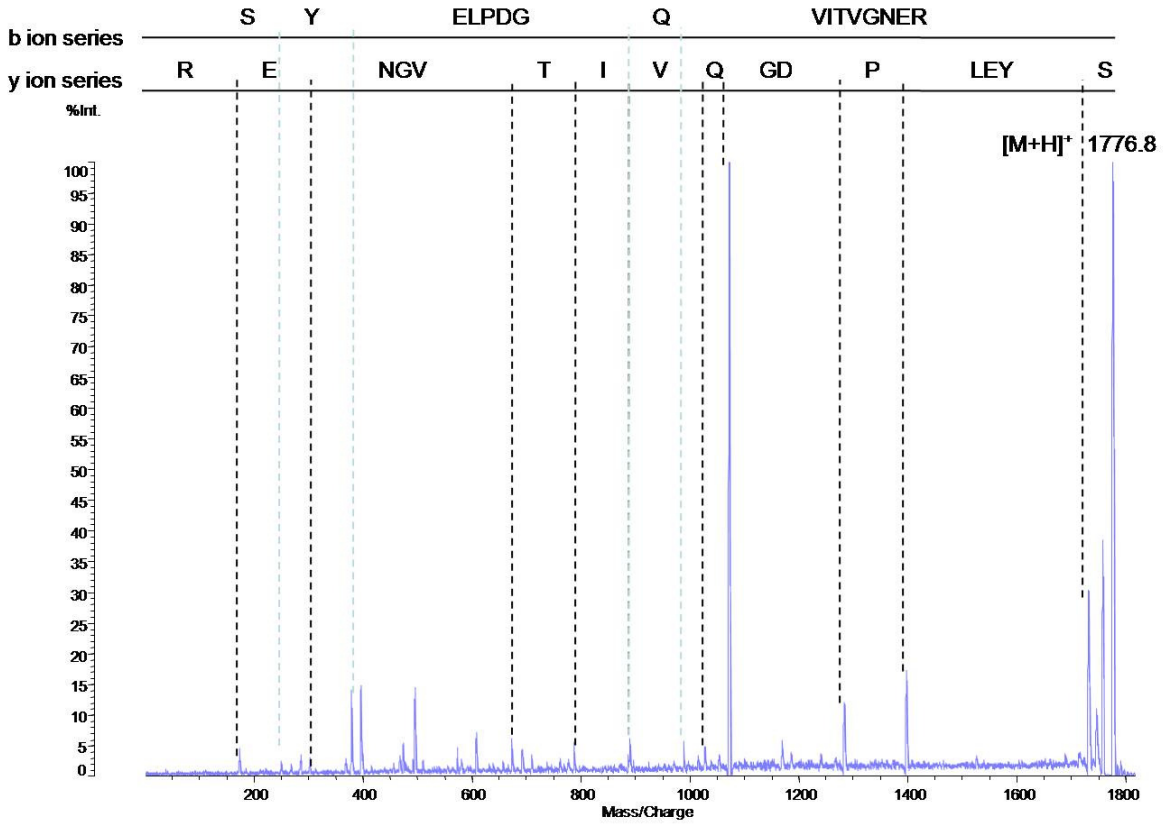

Figure 53. PSD spectrum of spot E-actin

The PSD spectrum was generated from tryptic peptide with a protonated molecular ion m/z 1776.5 of spotted fraction 9 (orange spectrum in figure 54) because of the highest intensity. The resulting fragment mass list (see Appendix chapter 6) was submitted to Mascot search obtaining a significant hit (Score 55, Threshold 41) for the protein actin of *Entamoeba histolytica*.

Sequences of protein actin:

- SYELPDGQVIT**I**GNER..... *Acanthamoeba castellanii* (spot Enc 2)
- SYELPDGQVIT**V**GNER..... *Entamoeba histolytica*

In comparison of the two sequences the actin sequences of peptide m/z 1776.5 (*E. histolytica)* and peptide m/z 1790.4 *(A. castellanii*) differ in one amino acid. The well known protein actin is a protein which can be found ubiquity in every organism with very few changes in its sequence. The identification of this spot with nLC Spotter MALDI was successful. It was possible to show that a separation step can improve the PMF and PSD spectra. This can be seen in the much higher scores and improved PSD spectrum.

IV. Discussion

One part of this work was to analyse 39 2D PAGE Spots from *Acanthamoeba castellanii* by PMF and MALDI PSD MS/MS. The main reason for these experiments was to get more information's about the proteins involved in the cyst formation and degradation. From a large part of proteins identification was not possible because of missing data in all of the used databases. In the database there were only 127 entries for *Acanthamoeba castellanii* proteins and a large part were only fragments of proteins calculated from genetic approaches. One of the main reasons why there is so few information available is that the genome of *A. castellanii* is not sequenced and that the number of people working with this special organism, despite of its medical relevance, is low.

With this small amount of information from the databases it was only possible to get a proposal for most of the proteins through homology search of the sequence tags that were obtained by evaluating the PSD spectra manually. In some cases it was possible to identify a protein because of the conserved sequence of this protein is present in related species.

A second reason for problems with the identification of the protein spots was that some spots have been stored for nearly one year before analysis. In some cases it was not even possible to get A PMF because it was not possible to eluate the tryptic peptides out of the gel matrix. It was not possible to repeat all problematic spots but in some very important cases, there was the opportunity to analyse spots from freshly prepared gels which turned out to work well.

Another problem we were confronted with was a microbiological contamination of the cell culture of *A. castellanii* resulting in a lot of spots which do not only contain *A. castellanii* proteins but also proteins from the other organism. In a few cases there is also the suspicion that the spot contained not only one protein but several. Nevertheless, it was possible to identify unambiguously 2 spots, namely Enc 2 and Alle 6. The spot Enc 2 was identified as actin and the spot Alle 6 was identified as elongation factor 2 .In both cases the PMF and the PSD MS/MS results matched despite it was not possible to get complete identity match by the databases because of the missing *A. castellanii* data entries.

V. Conclusion

The organism *Acanthamoeba castellanii* belongs to the species Amoebae and is distributed worldwide. It is well known for serious infection in humans e.g. Acanthamoeba Encephalitis (GAE) and Acanthamoeba keratitis. The lifecycle of all amoebic species consists of 2 stages: an active dividing trophozoite and a dormant cyst stage. The cysts of *A. castellanii* are of special interest because of its resistance to biocides and disinfectants. Nearly nothing is known about the proteins involved in the formation and degradation of its cysts. The organism was studied over the whole period of encystment in cell culture and in each life stage samples were taken. With these samples 2D gel electrophoresis was performed and the spots of interest were cut out of the gel. The identification of the proteins of the *A. castellanii* was performed by tryptic in-gel digestion. Afterwards the analyses were performed by MALDI RTOF MS and sequence tag determination with MALDI PSD MS/MS and nLC MALDI MS. The experiments lead to the identification of 2 spots Enc 2 and Alle 6. Enc 2 was identified as protein actin and Alle 6 was identified as protein elongations factor 2. All the other data from the experiments, despite there are of good quality, have to be evaluated later on when the databases are updated with more sequence data for *A. castellanii*.

VI. Appendix

Peptide mass fingerprints and PSD of all measured spots.

	m/z values→ intensity
Alle 1_PMF	753.36 95916984.00
	1008.52 18992488.00
	1089.52 8409352.00
	1291.57 12048965.00
	1328.62 6277300.00
	1409.71 60936872.00
	1419.67 7915648.50
	1504.71 6096929.00
	1543.69 82359184.00
	1559.67 9041157.00
	1616.74 9616793.00
	1640.58 14329890.00
	1688.73 33835704.00
	1704.72 18864322.00
	1765.75 6040759.00
	1791.79 4331428.50
	2004.97 6402269.00
	2018.04 5068712.00
	2051.84 1403783.50
	2083.95 6534823.00
	2100.96 16249541.00
	2178.01 4475284.00
	2203.03 6521727.00
	2219.10 4893694.00
	2326.00 3513983.50
	2339.15 4004867.75
	2358.12 7439837.50
Alle 1_1409.71PSD	166.7 5101.1
	175.4 11254.4
	176.6 6052.8
	200.2 5153.6
	234.6 5999.2

	258.3 5527.9 262.6 5575.6 288.4 5144.1 390.6 10002.2 430.5 8434.9 447.7 20473.3 464.5 7827.1 512.5 8100.1 519.1 8357.2 530.6 8396.5 547.0 11164.3 601.1 10885.3 630.6 8538.9 647.9 11764.6 749.3 7462.2 764.7 15011.1 777.2 7069.4 793.9 7296.3 811.3 7447.6 848.0 8134.8 865.1 10721.5 1003.6 7611.7 1021.4 14412.2 1038.0 7870.0 1089.5 7698.8 1092.6 8093.0
Alle1_1543.69 PSD	110.6 6226.5 111.2 5851.8 157.1 6060.8 175.5 6176.7 257.3 11328.1 285.4 18711.9 322.0 6759.2 322.9 6223.8 414.7 9717.6 432.8 16495.9

	457.7 11969.4 474.7 18947.0 519.9 9758.7 533.7 9348.5 544.4 9674.5 561.6 11377.4 661.0 10544.5 704.3 13232.0 721.5 17268.4 825.0 11926.1 857.7 19970.3 867.8 14747.7 885.7 17467.6 911.2 16040.9 957.1 10940.3 985.2 17583.3 1010.5 15594.6 1052.2 11206.3 1066.0 16550.2 1072.4 14537.5 1114.3 10468.8 1204.9 10501.9 1296.8 13885.4 1389.2 13980.5 1408.7 19773.6 1435.5 15229.7 1468.4 18765.5 1495.3 23841.5 1499.8 96654.7 1502.1 97410.7
Alle1_2084.94 PSD	209.4 4664.3 306.1 4230.7 353.6 4808.8 354.4 4483.3 356.0 4205.2 375.5 5056.8

	385.0 4427.8 385.7 5897.1 476.7 6216.3 478.1 5682.8 551.8 36253.9 649.2 6348.5 724.1 87276.3 793.2 8690.5 821.3 7722.7 839.4 7055.0 893.5 5225.6 907.6 4879.0 919.0 19612.0 936.0 15423.2 1049.2 5786.4 1050.8 4858.4 1106.2 9255.8 1227.3 5487.8 1252.4 7615.5 1269.5 93854.6 1366.8 6691.1 1384.3 8535.6 1411.0 5751.7 1605.8 5689.6 1611.8 6136.8
Alle 3_PMF	807.47 1957263.63 976.55 1574529.38 993.52 56699996.00 1019.55 8803821.00 1055.40 9351880.00 1102.60 23506714.00 1134.56 1906066.63 1164.58 4573702.00 1198.75 5777284.50 1235.69 1393060.25 1260.67 1513025.25

	1320.69	4336532.00
	1355.79	3249923.00
	1382.87	36443124.00
	1444.76	2623351.25
	1501.77	26488610.00
	1563.70	3414269.00
	1642.82	1472789.75
	1670.12	2994679.75
	1765.86	5146702.50
	1790.99	17747630.00
	1822.98	6600698.50
	1962.02	9105445.00
Alle 3_1383.79 PSD	623.83	
	640.92	
	744.00	
	1325.39	
	1339.28	
	1348.60	
	1351.77	
	1355.82	
	1366.44	
	1369.53	
	1380.28	
	1383.04	
	1383.44	
	1384.03	
	1385.05	
	1386.01	
	1387.03	
	1388.65	
	1390.85	
	1395.12	
	1396.15	
Alle 4_PMF	852.52	7481221.00
	1109.44	2904700.25
	1141.46	921344.00

	1153.47	2712442.50
	1178.48	5926535.50
	1235.44	5084520.50
	1253.47	9102947.00
	1269.48	2679421.75
	1319.63	9293548.00
	1352.45	2767450.25
	1491.51	2507171.50
	1642.55	10901705.00
	1791.56	12772717.00
	1917.57	9421608.00
	1951.76	37026128.00
	2013.71	3885083.50
	2188.88	7210349.50
Alle 6_PMF	760.46	117694928.00
	822.43	5701727.00
	856.68	20780950.00
	890.68	5613120.50
	969.72	7596339.50
	1021.68	6536302.00
	1096.65	117694928.00
	1105.76	66001744.00
	1236.70	8031189.00
	1344.96	5444233.00
	1378.91	43488472.00
	1487.93	17934558.00
	1534.99	96749336.00
	1596.93	10739001.00
	1622.93	5906586.50
	1683.00	31207192.00
	1910.17	44331764.00
	1964.19	10021415.00
	2024.27	210541936.00
	2086.16	8810368.00
	2180.33	39290700.00
	2237.37	3548004.75

	2242.31 2953927.25 2249.31 2528357.25
Alle 6_760.88 PSD	112.6 56455.0 120.6 96761.3 121.7 16961.6 129.4 17744.8 136.4 54386.5 155.3 16011.9 155.6 19936.0 156.0 19671.0 157.6 15830.8 158.4 53421.5 161.4 38717.6 172.3 16682.0 172.7 15061.9 175.5 192315.0 191.6 42348.7 192.7 14485.1 205.1 13782.6 205.5 24913.2 215.5 209462.3 219.5 52449.1 232.6 78171.5
Alle 6_2026.37	196.3 24084.0 295.0 74864.7 407.8 26680.9 409.4 18527.2 488.3 17350.6 523.9 19575.8 734.9 29587.6 753.9 30988.5 880.8 19388.4 882.2 21801.0 990.2 39233.4 1009.6 18844.3 1011.4 22276.1

	1021.1 37561.1 1038.8 47434.1 1040.4 19504.0 1129.5 25564.0 1146.6 26864.8 1221.2 22991.0 1249.5 23599.7 1265.6 35626.9 1275.0 78396.0 1292.4 20689.3 1293.1 20275.5 1403.7 47162.2 1475.5 25032.3 1532.5 28071.9 1534.6 21622.7 1620.3 28330.4 1638.2 24670.1 1684.3 34488.8
Alle 7_PMF	807.45 5083158.50 824.50 4567702.00 914.51 6028881.00 983.56 2985614.25 1072.67 2641364.00 1124.58 1522152.63 1284.70 5587531.00 1344.77 27749952.00 1409.80 99057544.00 1422.77 12083996.00 1536.93 9459671.00 1590.88 77813528.00 1613.99 123272720.00 1712.04 79674512.00 1759.07 14013740.00 1868.13 37316628.00 1889.05 8347822.00 1903.20 4049071.00

	1919.14 3437363.50 2233.31 3639579.50 2254.40 6252691.50
Alle 7_1591.20 PSD	581.0 7384.3 582.1 7393.8 685.5 13940.0 785.2 12858.3 790.4 6921.5 808.2 117694.2 907.4 18035.6 1022.4 10569.6 1185.8 6862.6 1286.9 7414.2 1485.8 7940.4 1488.6 7312.4 1489.9 7552.1 1535.4 7197.3
Alle 7_1616.37 PSD	381.5 3741.6 683.2 4153.1 782.3 4368.0 799.8 19085.6 805.3 18439.9 904.7 3853.8 1143.9 3897.6 1183.8 4442.4 1200.6 3460.5 1226.5 3615.6 1284.3 4406.5 1328.0 4598.8 1371.2 3798.8 1402.7 3781.9 1407.3 3993.2 1441.6 5660.9 1469.1 5093.1 1486.7 27181.6

	1500.8 3559.8 1502.3 3608.4 1504.8 4227.1 1513.2 3767.2 1526.0 3793.4 1537.0 3622.9 1538.9 3702.4 1540.6 3727.7 1543.8 4340.1 1548.8 3891.3 1555.4 4675.7 1570.5 10953.7
Alle 7_1714.16 PSD	444.8 11164.5 574.3 5756.8 574.7 5116.4 737.1 12671.3 739.6 5395.5 749.5 6327.7 837.6 9546.8 877.5 12121.0 948.3 5686.0 965.2 7968.5 976.5 11391.0 1035.5 7107.5 1138.0 8295.2 1139.2 5696.8 1225.2 10451.4 1253.0 5224.6 1260.2 5455.2 1267.2 6722.5 1268.8 6524.1 1323.0 7655.3 1324.8 6088.3 1335.2 4971.8 1401.7 5820.6 1453.8 6066.1

	1566.0 5010.4 1610.3 10220.5 1613.7 6276.3 1668.2 5200.1 1672.3 6151.7 1673.4 5277.1 1673.8 5608.9
Alle 9_PMF	869.02 4321356.00 877.52 8179296.00 1003.63 108987400.00 1075.74 40233448.00 1088.70 30008460.00 1131.73 100176192.00 1144.66 16367896.00 1203.79 11723379.00 1267.77 2969839.25 1339.75 7495841.50 1373.86 26933128.00 1435.81 3421982.75 1480.87 4299777.00 1490.89 7862177.00 1539.97 5819667.00 1616.91 869571.75 1636.94 7530523.50 1645.95 24123864.00 1697.10 6754672.00 1707.91 7473836.50 1728.08 12417830.00 1777.07 15303065.00 1826.16 38798648.00 1916.06 13518428.00 1920.12 11883484.00 1928.07 4510640.00 1954.20 16291396.00
Alle 9_1004.22 PSD	175.2 5624.9 274.6 8136.2

	276.4 12035.0 356.6 11457.2 375.5 5698.3 403.5 6537.4 455.7 9853.7 587.8 7555.9 594.7 5382.1 612.6 39173.3 629.8 19149.9 631.0 8573.9 632.1 6511.8 666.7 6080.5 686.8 5616.2 693.4 6043.6 699.8 6920.9 703.2 5946.3 710.9 6725.7 711.7 8973.9 729.0 6421.3 731.7 5894.0 736.8 5647.9 740.1 5815.7 748.5 7985.0 754.3 6712.3 760.2 5741.3 763.0 5926.6 767.6 5723.6 794.4 7471.6 818.0 7248.9
Alle 9_2039.25 PSD	710.7 13962.2 854.7 25860.8 975.5 14336.2 975.9 14289.5 1066.3 15331.1 1185.7 45297.5 1253.0 14344.2

	1272.0 17214.2 1273.5 17397.7 1329.3 43139.1 1380.8 20858.9 1450.9 13973.1 1467.5 53286.1 1479.0 14105.4 1496.5 41066.1 1566.5 17349.0 1582.2 16723.6 1608.5 19789.9 1623.3 23770.0 1625.6 17521.3 1680.2 28120.0 1751.3 16586.1 1795.3 33883.4 1810.9 17093.9 1820.2 17366.4 1836.0 16346.1 1867.1 17900.6 1893.1 38502.6 1897.1 17505.7 1911.3 30353.5 1916.5 16768.7 1932.8 16607.1 1959.8 16693.9 1963.5 17539.8 1977.7 42189.5 1990.1 15837.1 1992.8 16404.3
Alle 9_2680.88 PSD	716.7 56097.3 831.6 18331.6 832.9 13698.0 833.2 15078.0 924.4 18962.1 928.9 14504.3

	929.2 13522.0 946.6 22272.2 1100.4 13816.7 1117.4 230272.2 1232.8 13424.5 1233.2 13601.3 1233.7 13629.3 1281.0 16774.8 1403.3 24940.5 1566.7 17519.0 1660.5 17485.7 1760.7 18647.8 1853.8 15978.7 1889.9 14628.6 1896.6 18198.5 1949.7 14653.4 1966.2 21622.6 2083.4 21984.7 2140.1 24248.2 2209.8 23425.8 2304.0 16894.0 2392.3 16673.4 2508.3 42554.7 2525.6 28282.9 2592.0 17134.1
Alle 10_PMF	804.26 12851456.00 884.38 20363022.00 1125.27 79546512.00 1143.29 264116752.00 1466.25 112602296.00 1510.45 1107736448.00 1525.39 194619088.00 1595.54 16353916.00 1602.37 69447544.00 1638.39 67102232.00 1653.36 41463744.00

Cyst 1_PMF	1245.73	457352.50
	1597.05	6244285.00
	1636.02	1343805.63
	1673.94	2370157.75
	1726.99	968488.25
	1748.01	10408959.00
	1830.01	3972141.75
	1869.08	2145102.75
	1885.04	1705207.88
	1929.09	24045180.00
	1945.05	4091605.50
	1963.34	730127.38
	2072.20	2472753.50
	2085.14	6837568.00
	2101.18	436480.00
	2123.28	1241460.75
	2163.24	5448736.00
	2178.39	15921827.00
	2194.33	5922394.50
	2258.29	5111597.50
	2274.33	294437.16
	2474.40	23718286.00
	2483.82	512948.38
	2489.22	94822.40
	2490.40	2592935.00
	2491.48	65584520.00
	2657.55	9419442.00
	2741.51	991648.06
	2785.64	13953695.00
	2948.67	4953807.50
	3046.86	3008956.25
Cyst 2_PMF	784.05	21493578.00
	724.11	21493578.00
	869.20	21493578.00
	949.62	119971672.00
	1062.10	21493578.00

	1064.18 21493578.00 1011.53 14925413.00 1495.57 3074646.75 1642.60 1887645.88 1721.70 90390224.00 1783.62 44874984.00 1847.50 7648141.00 2224.81 13092320.00 2682.04 3160209.25
Cyst 2_950.00 PSD	46.7 3471.6 79.5 3456.2 117.0 8740.3 119.0 3603.2 119.2 4339.8 175.7 5919.8 300.9 8279.7 306.8 4953.5 328.9 3703.4 343.5 3392.5 346.0 3423.8 371.8 11757.8 416.8 3715.9 421.9 5254.2 493.0 6700.6 507.0 7710.6 533.6 3719.8 534.9 6600.1 536.0 3965.3 564.3 4356.3 605.8 7132.5 650.3 40187.8 652.3 16462.4 726.5 5442.0 732.1 6851.8 736.5 6428.8 744.5 5785.8

	746.5 5393.3 768.6 6796.9 787.4 8561.2 795.4 8169.0 797.6 9734.3 800.2 7310.9 907.4 7370.2 914.5 8019.3 918.5 7427.9
Cyst 2_1721.70	301.0 7107.3 430.3 7467.9 650.9 25532.8 951.1 7480.7 974.9 8567.6 1037.1 7996.8 1073.4 38945.2 1152.2 8125.5 1176.2 18230.6 1193.5 32507.2 1275.2 8197.5 1290.6 8592.5 1308.8 8516.6 1477.6 13382.6 1494.8 20582.2 1521.0 17136.7 1549.3 18075.1 1567.2 69203.6 1663.4 47790.6 1677.7 9934.0 1681.0 94983.1
Cyst 3_PMF	722.22 10772750.00 881.57 26742154.00 949.75 422441408.00 975.78 94209560.00 1013.68 35654632.00 1165.82 156552288.00

	1227.74 18999954.00 1342.27 12184053.00 1642.82 5700278.50 1721.92 14377586.00 1926.95 115895224.00 1990.82 8899438.00 2152.05 196677376.00 2170.08 60765388.00 2213.98 20801286.00
Cyst 4_PMF	1303.89 390235712.00 1345.76 435525248.00 1361.78 161775376.00 1526.91 118757288.00 1642.13 221640064.00 1842.08 76772232.00 1980.26 270720384.00 2630.63 511556640.00 2758.70 95051376.00
Cyst 4_1346.41 PSD	175.5 119439.5 186.6 92911.1 278.6 31835.0 299.5 119930.6 349.5 59511.7 357.7 54986.1 390.4 69494.9 407.4 101704.0 428.5 166439.4 478.5 51995.3 525.6 63799.3 542.7 110369.0 553.6 57895.3 570.6 83660.8 641.9 96026.2 688.8 68502.5 706.1 171426.6 760.0 57488.9

	777.0 64440.1 805.0 152703.8 901.9 104615.8 918.9 307807.5 922.8 37448.0 1048.2 62359.1 1050.5 34154.1 1162.2 60654.6 1232.4 106150.9 1280.7 45821.4 1285.1 82522.7 1300.7 94325.5 1302.1 651647.8 1303.4 571763.5 1312.2 57603.0
Cyst 4_1643.12 PSD	417.14 20000 516.74 25646.81 615.07 20000 1372.40 150371.86 1515.55 1309522.75 1599.12 1847325.88
Cyst 5_PMF	726.25 6092046.50 869.07 6203945.00 926.15 2506496.00 927.27 2506496.00 976.61 2237743.50 1119.06 1146957.63 1198.91 2506496.00 1444.91 10576172.00 1572.14 2863197.75 1792.06 1778109.63 1836.05 10310912.00 1852.08 3631995.50
Cyst 5_1445.08 PSD	247.32 4565.99 398.69 31210.55 504.31 4565.99

	575.42 3848536.50 673.28 48004.13 747.27 1629.13 973.14 286392.09 997.72 3361.65 1013.27 72114.90 1029.76 236021.25 1069.86 188187.84 1087.12 244364.78 1165.53 35032.08 1226.79 316222.47 1256.00 45481.43 1323.44 134316.39 1369.13 75367.59 1402.41 5124274.00 1428.51 6124274.00
Cyst 5_1836.19 PSD	443.18 2450.34 571.64 2450.34 641.31 427709.25 755.56 611518.06 870.31 330914.72 983.48 468513.34 1082.23 274197.78 1195.45 127142.59 1266.55 127051.52 1394.93 18029.78 1804.95 245.34 1820.30 868989.94
Cyst 6_PMF	869.24 11271020.00 947.57 2746316.75 1029.66 15320890.00 1166.71 14617464.00 1294.70 32098466.00 1408.63 1351597.00 1422.75 2585857.25 1433.70 5883975.50

	1525.10 3643718.50 1592.85 9949606.00 1642.66 5469033.00 1765.66 3362337.00 1791.65 6928282.50 2224.83 11294165.00
Cyst 6_1294.81 PSD	148.5 5746.6 160.0 5684.6 263.1 8061.8 303.9 7803.8 383.0 6235.5 402.0 7951.6 430.2 6064.0 432.8 6730.0 449.3 12236.7 467.8 5867.6 488.8 5699.5 591.3 6940.1 617.5 7450.8 634.6 6691.4 663.1 7310.5 701.6 6942.0 718.4 11091.6 735.1 6503.8 765.5 6402.2 842.7 7279.2 860.9 7198.7 873.6 7640.6 912.7 7105.4 970.4 7400.9 985.8 7214.7 989.6 7534.0 1007.9 7746.4 1023.4 6974.5 1071.9 7056.6 1080.7 6809.6

	1114.4	8152.5
	1137.4	7463.6
	1152.2	13732.2
	1168.4	59729.9
	1230.2	17600.2
Cyst 8_PMF	1078.83	31924740.00
	1147.85	22382712.00
	1320.87	5391822.50
	1412.98	11877097.00
	1596.15	36487040.00
	1603.20	3676643.00
	1643.11	6870966.00
	1860.22	94425592.00
	1892.18	13220835.00
	1918.39	14194784.00
	1944.32	4062769.50
	1988.37	185602112.00
	2011.53	136331344.00
	2020.35	76969584.00
	2062.47	2190052.50
	2071.57	7956780.50
	2116.51	41727652.00
	2148.49	8221866.00
	2163.65	64097616.00
	2167.68	16863514.00
	2273.76	6272812.50
	2519.78	2667196.25
	2625.10	22417514.00
Cyst 8_1989.40 PSD	175.81	2409.53
	303.46	523338.53
	443.33	38016.34
	494.82	3557053.50
	606.08	202137.39
	623.13	3577897.25
	667.06	1032557.94

	778.53 364680.44 795.50 1253248.13 907.07 79353.52 1069.76 361202.41 1093.68 132125.69 1110.55 129916.65 1256.32 313459.19 1419.46 536644.75 1520.59 1443721.25 1547.82 491613.03 1649.32 1590230.75 1734.02 112252.52 1816.55 1346224.75 1862.53 19761382.00 1946.94 8494557.00 1960.25 1554906.50 1972.84 15744160.00
Cyst 9_PMF	977.53 13912711.00 1039.47 877985.94 1087.53 108416584.00 1125.65 10706384.00 1149.43 852092.56 1183.63 34112552.00 1198.68 31631.66 1200.66 113379824.00 1201.07 45656.94 1204.65 22630.40 1206.72 4632060.50 1208.72 7375546.00 1215.65 185270.86 1232.62 1392369.75 1247.55 58075660.00 1262.59 1582534.63 1309.50 2672937.75 1328.78 7481869.00 1369.69 2141485.25

	1375.65 5559163.00 1385.78 2692295.50 1429.74 31927180.00 1433.74 1176126.75 1457.80 17263456.00 1497.76 1008067.75 1519.72 758065.69 1900.89 2784412.00 2019.90 221960.44 2047.10 3350513.25 2062.02 4960752.00 2163.02 19967598.00 2173.02 33554540.00 2189.02 101549.40 2205.01 97242.06 2224.90 19191.45 2234.95 148046.92 2301.12 33343300.00 2333.11 1659308.13 2363.02 157559.81
Cyst 9_1088.33 PSD	84.6 9219.1 136.7 12210.3 174.5 9112.0 175.5 29798.8 229.3 16293.5 229.9 13809.5 269.1 8642.2 287.4 20196.5 293.1 8827.0 304.8 8668.4 312.7 21549.7 330.7 43774.7 383.9 42482.1 393.4 8793.6 393.9 10698.5 401.7 22070.7

	428.5 8963.4 450.7 20588.6 467.4 8828.3 474.2 9236.4 507.8 22208.0 509.1 12466.6 524.9 19246.4 526.1 9727.1 536.9 11061.0 603.8 9768.8 613.0 11327.7 621.8 16388.1 645.4 8928.7 671.2 34336.6 688.3 21343.4 724.0 11115.3 741.8 11891.8 743.4 11895.7 759.1 23461.5 785.0 9987.4 788.5 11040.9 790.9 12777.9 792.7 9929.3 793.7 9730.3 802.3 10211.7 816.2 10718.1 818.0 10571.5 830.1 11858.4 839.3 10864.8 844.0 10691.5 848.1 12550.0 852.2 10135.6 856.9 12881.2 860.1 21608.1 923.5 27229.8 945.3 14346.7

	973.0 198478.8 974.4 126593.3 987.7 15861.7 999.5 20096.4 1010.2 13976.2 1020.0 15120.0 1029.2 106191.9 1030.9 50699.6 1037.4 20760.0 1039.5 16817.5 1044.0 80615.1 1045.5 55752.2 1052.9 13919.6 1054.8 15187.4 1061.8 49260.0 1063.2 48909.7 1064.5 21084.3
Cyst 9_2302.00 PSD	746.4 15292.9 1033.0 14637.2 1558.8 22356.6 1733.2 30447.1 1845.6 14632.3 1862.6 23852.0 1945.1 29929.9 1988.2 14422.8 2015.1 13766.3 2104.2 13808.6 2104.7 14907.0 2105.4 14440.5 2106.6 14571.7 2129.5 18079.3 2147.0 14180.9 2151.5 34545.7 2155.1 14797.9 2170.6 15957.8 2174.4 17592.4

	2176.5 17356.2 2233.0 16320.9 2235.0 15064.1 2244.5 19168.7 2247.4 25766.7
Enc 2_PMF	631.55 53640972.00 644.56 34255556.00 666.14 15394712.00 682.11 8417404.00 795.58 30821296.00 893.06 9688669.00 923.61 20485528.00 1132.47 41121548.00 1444.59 38287032.00 1487.56 14147940.00 1515.40 132478728.00 1790.36 161272752.00 1953.40 8076526.50 1960.25 24071988.00 2182.18 12111594.00 2310.20 68474736.00 3164.97 74778432.00 3372.88 8071694.00
Enc 2_1791.43 PSD	171.8 10932.8 175.3 12869.5 224.5 8452.5 250.3 11357.7 267.4 8750.8 285.9 13775.2 304.1 9959.5 341.1 9369.4 350.3 8940.0 368.6 13265.3 380.1 32146.3 398.2 34625.8 478.4 13323.9

	496.9 29419.3 512.0 11372.8 588.4 17273.9 610.2 20151.4 689.4 16913.4 694.4 12271.8 710.0 12843.4 760.5 10812.5 803.3 14798.5 862.6 11000.0 890.5 18599.3 901.9 16260.0 989.1 16126.7 1009.1 11061.3 1022.2 13081.3 1031.8 14424.0 1041.9 16885.0 1086.6 231294.2
Enc 3_PMF	930.40 6439680.00 1086.56 9021696.00 1197.60 9300480.00 1433.51 7395328.00 1487.51 71538200.00 1549.43 17339152.00 1643.48 28604720.00 1706.51 11662592.00 1775.50 9200640.00 1813.47 8340736.00 1960.51 84187912.00 2023.51 8637952.00 2116.51 18156900.00 2224.46 12843950.00 2301.64 16857574.00 2336.55 17290810.00 2371.71 32810502.00 2430.59 8063744.00

	2528.71	19339592.00
	2592.71	10361600.00
Enc 5_PMF	780.58	2576442.25
	816.59	1438414.50
	893.10	1713327.00
	930.57	2227245.00
	1002.64	3163719.25
	1130.73	1467568.63
	1197.87	5380491.00
	1284.68	2983466.00
	1392.77	2519176.00
	1465.82	7334817.00
	1487.93	38970472.00
	1521.86	6535932.00
	1545.00	1125500.13
	1644.02	18408274.00
	1703.01	688260.31
	1765.94	684746.88
	1777.05	4571527.50
	1791.86	2244883.75
	1814.03	4345337.50
	1833.04	5735110.50
	1849.02	1160483.13
	1858.10	4562613.00
	1917.07	2345234.00
	1959.04	1312705.25
	1977.14	3300580.75
	1993.11	3599913.75
	2018.08	3419853.75
	2080.24	3126138.75
	2297.14	6744868.00
	2301.20	169881.59
	2384.07	4967957.50
	2430.37	5783489.00
	2455.30	1026548.00
	2534.22	1736664.25

	2593.48 7805887.00
Enc 5_1489.80 PSD	437.0 7063.8 567.8 7721.6 607.3 13608.9 696.8 7412.6 697.8 8733.1 736.6 10943.9 738.3 7612.7 754.5 9177.1 833.9 7754.2 839.9 7200.5 840.9 7100.6 850.2 9528.6 867.0 7567.8 867.7 7229.7 939.0 7490.6 961.1 7792.1 978.8 51817.2 981.9 8210.3 1032.6 7729.0 1075.5 7265.9 1093.2 8250.3 1094.7 8025.2 1103.3 6856.8 1105.6 8180.9 1110.1 7268.1 1115.4 7108.3 1117.6 7019.6 1204.0 7293.0 1207.5 7494.6 1250.5 7469.2 1285.1 8775.9
Enc 6_PMF	804.53 14766314.00 889.58 9713776.00 998.61 20419022.00

	1070.64 11546517.00 1088.61 67646248.00 1091.70 3697966.50 1135.68 21248796.00 1183.84 74045800.00 1395.92 39310968.00 1432.86 103491080.00 1494.81 14230146.00 1504.88 85337904.00 1538.97 16464896.00 1586.95 53699132.00 1602.92 22965160.00 1618.93 23541558.00 1630.01 6069988.00 1660.98 35410652.00 1695.96 7874932.00 1726.94 59546308.00 1937.08 51480080.00 2067.09 31142604.00 2086.17 71630000.00 2108.14 21978660.00 2197.26 14970997.00 2201.23 1976832.13 2488.27 25038460.00 2783.43 717605184.00 3038.51 25636180.00
Enc 6_1432.85 PSD	256.1 21633.5 256.3 20737.5 295.2 32063.8 313.1 22407.6 325.2 23378.5 382.2 31750.8 400.3 22411.3 412.3 25557.2 432.2 21688.3 441.5 123500.2

	458.7 139293.3 514.4 23156.5 522.4 31452.3 523.6 21683.3 531.6 42719.1 541.5 30136.6 550.4 24942.2 555.3 26757.3 559.4 37582.1 572.4 22895.7 580.4 65322.2 589.3 20740.7 597.4 31081.9 607.4 36140.9 614.5 46294.7 635.7 21195.3 678.9 30823.9 706.9 46846.2 710.8 25555.0 720.6 24200.8 727.7 33050.7 774.8 21020.0 791.8 20412.4 802.7 33457.2 819.7 22036.4 831.9 20710.0 832.9 28126.3 859.6 36296.0 874.8 37818.1 931.4 39481.7 948.5 73173.9 959.5 28414.5 975.6 272725.9 977.0 171190.6 994.3 40219.1 1005.2 34569.9

	1021.7 44617.3 1023.1 35804.0 1031.6 29992.9 1055.9 28577.6 1074.4 35605.9 1089.7 37553.5 1091.5 35762.4 1102.9 34895.6 1108.7 33174.9 1120.7 29939.3 1122.4 31571.7 1148.9 107038.6 1230.8 249509.0 1232.6 282209.1 1258.9 936599.2 1260.2 826466.3 1261.5 400452.3 1276.9 4845679.5 1278.2 4304237.0 1279.5 2046824.8 1291.1 65067.2 1293.2 73076.7 1372.9 1035421.3 1374.2 1120788.0 1375.5 628353.3 1388.8 100381.1 1390.8 3354162.5 1392.2 2875412.5 1393.5 1314908.6 1399.2 159361.6 1402.8 153040.6
Enc 6_1505.74 PSD	175.5 45557.5 176.3 21383.2 326.4 38806.7 327.5 21387.3 420.3 20855.0

	420.6 23686.7 437.4 28492.6 455.6 51783.8 569.8 31720.1 608.0 126736.5 699.0 57097.4 737.9 47043.8 756.3 76261.7 834.1 48716.7 840.9 28908.7 842.0 28783.3 851.5 114546.3 869.3 41260.6 938.9 22118.6 956.0 21388.1 979.8 451288.7 981.1 308150.2 1031.8 28262.5 1033.2 26328.1 1069.1 20642.9 1078.3 39352.0 1095.4 47228.6 1141.4 20492.9 1150.0 21530.1 1209.6 24285.7 1215.0 23628.3 1263.3 22808.2 1264.4 22450.4 1274.7 22657.5 1288.7 42915.6 1290.8 22441.1 1306.1 105595.6 1309.1 22894.2 1339.6 28695.9 1391.9 23914.7 1392.6 23765.7

	1393.4 23495.2 1395.2 23552.7 1418.5 38215.9 1432.3 85181.4 1450.5 47064.4 1454.6 37473.1 1461.1 39333.1 1463.4 37597.7
Enc 6_2087.20	627.9 39761.6 644.1 36012.5 742.1 61866.7 840.6 55638.0 856.4 27477.4 857.5 23442.5 859.1 22949.1 860.3 23080.7 968.9 46511.8 985.5 21549.5 986.6 22355.2 1052.4 44644.6 1070.3 32381.5 1115.1 38611.0 1151.4 40835.4 1169.3 25356.1 1232.4 45153.7 1319.2 29006.3 1329.3 23305.2 1346.8 50931.4 1445.77 43319.9 1464.7 25105.2 1466.7 27257.9 1483.9 217160.0 1573.8 29488.2 1597.2 38764.9 1657.5 34838.3 1675.0 37667.5

	1756.5 34933.2 1759.8 30708.2 1774.1 27289.3 1775.9 23568.4 1888.7 49125.3 2027.0 20565.2
Enc 8_PMF	659.29 13629466.00 795.47 205284768.00 821.51 4623886.00 827.11 8310182.00 847.97 1988712.50 857.41 4436958.00 858.91 0.00 859.02 96768.00 859.43 3871481.25 859.77 23296.00 923.58 166748272.00 948.47 11659561.00 985.52 6777086.50 1086.66 180455856.00 1175.68 147395536.00 1230.72 16891984.00 1232.10 254301.09 1232.72 10301819.00 1272.78 14906127.00 1444.90 18149550.00 1460.90 8685528.00 1790.97 172467152.00 1833.11 14352985.00 1850.11 4837197.50 1917.83 4021352.00
Enc 8_1173.66 PSD	505.8 25623.0 540.0 18049.0 540.1 17059.3 540.6 20424.4 564.2 24440.6

	564.7 21893.0 565.6 18261.9 597.8 18569.0 598.2 16979.0 598.7 21795.3 600.0 28083.1 620.6 36565.1 634.1 30985.1 644.4 17373.1 645.2 28717.2 677.9 19121.6 678.9 20391.3 679.6 29537.9 691.7 17536.8 693.5 46612.1 724.3 96313.1 776.8 20493.4 830.3 18579.2 830.7 20561.6 831.9 27952.0 850.4 20479.5 853.6 21177.5 854.2 18246.1 860.4 42452.6 866.8 18551.0 867.9 24145.2 872.1 41169.4 885.3 74829.9 901.1 17578.8 901.9 18638.7 902.7 20292.6 908.7 22128.0 916.7 31948.4 921.0 20082.5 922.2 25669.9 936.0 316326.8

	949.2 156418.2 970.6 43319.2 983.6 22032.2 984.4 22049.3 990.4 38856.8 1003.7 56670.8 1017.2 20720.0 1074.4 106554.9 1088.3 26046.5 1090.3 24527.9 1095.7 48026.0 1097.9 20910.2 1109.1 189624.0 1118.9 21609.4 1128.1 63360.4 1138.5 23569.0 1139.7 29765.4 1141.0 31243.9 1147.6 22326.0
Enc 9_PMF	841.57 847143.88 1034.56 2096352.50 1055.50 14441655.00 1072.52 10529026.00 1098.55 900966.88 1117.42 706880.25 1143.53 762435.25 1162.63 2483855.25 1200.59 7249820.00 1275.69 3685102.25 1371.57 3764028.50 1403.77 2227969.00 1499.73 3008522.25 1516.73 1955654.75 1531.73 11205929.00 1620.81 2591312.75 1689.76 1684591.00

	1748.88 3559473.25 1917.88 23406368.00 2070.95 13174273.00 2314.05 7365880.50 2344.06 7094515.00 2378.96 1000.000 2442.14 7925356.50 2571.16 26734146.00
Enc 9_1052.69 PSD	111.3 7233.8 112.2 3912.1 159.0 3962.1 176.2 26597.2 253.9 7279.8 259.2 7310.5 296.2 24554.3 313.4 287813.2 368.2 5108.9 388.5 41161.2 393.5 5292.5 411.4 32667.1 428.4 11662.7 517.7 29944.4 524.5 10763.9 534.6 6313.5 541.8 22684.4 604.5 6936.8 617.4 6467.6 623.5 5950.3 653.6 9784.7 655.0 8937.4 670.8 11582.2 755.1 9114.9 765.2 9171.9 782.3 8904.8 798.4 11558.5 801.2 9263.0

	855.2 11762.5 883.6 12970.6 901.0 28857.3 928.1 9460.5 998.9 16803.2 1013.7 23529.1 1016.7 18129.2 1023.8 19213.7
Enc 10_PMF	912.51 4143515.00 1055.47 2480529.75 1159.59 2936832.00 1176.61 10149103.00 1341.55 58495572.00 1602.69 99445984.00 1685.68 40012848.00 1698.81 18919548.00 1760.72 1000 1905.56 1000 1995.61 1000 1730.77 6766083.50 1841.64 6666534.50 1917.74 8678654.00 1956.84 8618480.00 2061.70 65446700.00 2104.75 7795500.50 2125.06 698045.69 2151.69 8175161.50 2162.81 1000000.00 2208.70 2926180.25 2224.70 4079467.2
Enc 11_PMF	912.70 7046144.00 1159.91 9953536.00 1176.92 18571392.00 1300.83 602368.00 1308.01 1385472.00 1329.05 247552.00

	1341.93 10985984.00 1403.02 2807040.00 1455.07 1258752.00 1476.12 4118411.75 1538.06 1756928.00 1603.22 47945728.00 1712.25 4474414.50 1843.23 26001920.00 1843.56 80416.00 1971.32 1967587.50 2006.29 1656320.00 2018.31 3483776.00 2063.30 97184512.00 2105.39 7504384.00 2106.43 10316928.00 2120.38 5527040.00 2163.41 3387392.00 2274.49 1881344.00 2324.47 1116416.00 2384.31 3075328.00 2479.62 52626.29 2501.54 2171155.75 2551.55 373248.00 2751.68 8197888.00 2768.76 1003392.00
Enc 11_1603.30	271.4 6870.9 272.1 8665.1 431.8 7485.6 467.8 6863.0 558.8 7069.8 560.1 8301.7 631.6 8579.8 657.7 7770.5 659.5 9480.5 660.8 6517.3 674.5 8236.9

	675.8 7023.6 771.2 6461.5 930.6 35979.9 991.7 7617.1 1002.6 7097.0 1017.9 6483.4 1039.1 7369.7 1058.9 6732.4 1060.4 8037.8 1069.1 6496.9 1074.4 6852.1 1122.9 7027.8 1127.8 6421.5 1141.4 6532.9 1195.8 6554.8 1208.1 6520.2 1231.4 9663.6 1245.0 7078.3 1259.9 6515.4 1268.3 7017.3 1288.6 6922.4 1330.7 8446.8 1333.0 8778.7 1348.3 6840.8 1404.0 6597.9 1431.0 7061.8 1434.5 6710.0 1465.1 7307.2 1467.0 6842.4 1470.0 7659.4 1500.6 7278.7 1504.1 7038.0 1519.8 13913.9 1524.9 6960.5 1535.5 9716.7 1536.7 6965.3

	1537.2 6804.7 1548.7 8169.4 1560.5 58112.8
Enc 11_1688.73 PSD	276.7 15583.0 325.6 16097.9 342.7 19873.0 424.0 32053.5 441.1 11017.4 442.2 13168.5 538.0 10661.8 538.7 13383.9 539.7 10549.1 556.8 10865.5 593.1 10530.6 643.7 12058.5 752.6 11123.9 915.7 11104.1 916.3 10233.6 933.4 11802.3 935.2 13858.5 1043.5 10189.9 1062.5 11329.4 1063.3 10831.8 1064.4 11768.9 1115.0 10220.8 1132.5 11261.4 1150.4 13881.6 1245.3 10207.5 1264.7 10686.0 1346.0 11279.4 1409.2 10337.9 1411.0 10454.5 1411.8 11189.0 1550.6 10717.0 1554.8 10231.8 1572.4 10686.4

	1599.8 41664.1 1625.0 10483.4 1628.0 15756.4 1639.0 26256.1 1643.5 14739.5 1645.3 12640.0
Enc 11_2065.03 PSD	287.7 18065.5 352.5 9547.5 353.9 17370.4 542.1 11653.5 827.9 19710.6 880.9 13112.9 896.3 21404.2 926.2 9992.9 942.4 26573.3 1152.5 9700.1 1168.8 33703.2 1218.5 9278.4 1235.5 14722.2 1279.8 11605.0 1297.2 17519.9 1299.3 9349.3 1396.9 10724.4 1425.2 10418.1 1470.9 9246.9 1503.2 10046.0 1505.6 14604.6 1522.6 48740.7 1555.0 9574.5 1559.5 10844.5 1579.0 9581.3 1607.1 10861.2 1609.0 9649.1 1623.9 14036.0 1680.6 17971.5 1732.9 10270.7

	1759.7 13027.1 1777.1 81911.0 1808.0 10005.4 1810.3 11983.7 1825.1 10684.8 1828.3 15594.8 1857.7 10764.8 1863.6 10857.0 1868.6 10306.7 1890.2 15842.9 1908.3 20212.4 1927.4 17727.4 1944.7 10265.5 1956.8 11380.2 1973.6 47045.3 1986.7 12292.6 2003.8 31628.8 2020.4 195293.2
Tropho 1_PMF	869.12 4269824.00 944.66 6654805.00 1068.51 7015799.50 1175.62 1517476.38 1284.82 26326968.00 1385.78 18241762.00 1447.69 9004502.00 1544.90 12367838.00 1589.82 4599519.00 1603.83 4527624.00 1623.78 5746077.50 1628.86 10043498.00 1685.72 3693897.00 1690.82 6286702.00 1700.96 19305380.00 1762.93 10659343.00 1824.84 3976013.25 1950.91 8183350.00

	2012.83	5160865.50
	2123.02	9524162.00
	2137.02	8363952.50
	2210.04	16630027.00
	2224.05	14497779.00
	2398.11	3679620.00
	2632.45	4538630.00
Tropho 2_PMF	746.13 3733534.25	
	1093.58	8630730.00
	1325.63	1435646.63
	1791.81	2173103.00
	1873.02	7573207.00
	2111.10	16285560.00
	2163.09	5651420.00
	2239.25	62875164.00
	2304.13	1341417.75
	2812.36	2163126.75
	2911.42	2248791.50
	2925.47	5147336.50
	2952.39	14275670.00
	3014.26	1946369.75
	3056.26	2580529.25
	3068.58	0.00
	3152.41	411197.56
	3182.57	1224690.63
	3195.58	19248366.00
	3234.68	347037.16
	3300.67	11754.67
Tropho 2_2111.10 PSD	1042.3 8194.8	
	1042.8 8697.3	
	1566.2 18500.6	
	1655.4 31528.6	
	1681.3 33133.0	
	1883.3 9058.2	
	1981.9 10700.1	
	1983.4 9193.3	

	2000.0 29801.8 2017.2 8351.3
Tropho 2_2239.25 PSD	713.61 8904.7 827.6 8059.1 1030.9 8402.6 1039.6 9078.0 1066.4 12941.5 1083.8 13553.2 1125.6 8591.4 1140.7 10606.8 1167.3 7985.3 1183.4 17282.2 1210.8 10571.0 1223.2 9177.8 1297.5 9653.2 1378.5 8333.9 1413.0 8948.4 1436.8 8319.3 1541.2 9278.4 1578.1 8491.8 1595.96 16091.0 1637.484121 10515.2 1655.0 77876.9 1694.2 46132.3 1711.2 16751.8 1713.7 8652.0 1736.3 13387.1 1752.8 10880.4 1754.6 8844.7 1758.8 8717.1 1767.3 9225.3 1769.6 11277.3 1790.9 9880.6 1809.3 79099.9 1851.1 8676.0 1914.2 9144.6

	1967.8 13957.7 1970.8 9569.9 1980.7 8722.9 2012.3 18206.8 2024.9 9147.5 2082.5 12500.2 2110.6 31053.5 2114.7 9328.8 2123.8 9141.5 2127.7 89230.4 2150.2 9107.3 2168.9 11158.4 2177.2 13283.3 2180.6 10747.3	
Tropho 5_PMF	1404.88 1465.95 1483.84 1490.88 1664.89 1681.92 1707.88 1765.91 1783.95 1809.95 1835.99 1845.87 1866.01 1871.88 1961.07 2156.10 2182.12 2218.03 2244.04 2258.13 2273.22 2281.02	80877456.00 15292626.00 496359.84 2638800.75 1469183.00 3031653.50 22272.00 5458652.50 260527120.00 305725312.00 19758994.00 16222680.00 4251439.50 7248123.00 12593992.00 161396448.00 159149216.00 11018420.00 11619456.00 50924380.00 671149.19 616607.50

	2292.19 788708.50 2384.04 6468378.00 3118.46 5316452.50 3162.54 95719416.00 3224.51 3049469.50
E-actin_PMF	945.71 2878494.00 976.61 3687019.50 1197.85 261632.00 1198.87 21104024.00 1259.78 225024.00 1515.89 6040323.50 1777.01 226685824.00 1960.08 18243762.00 1972.22 6801426.50 1976.07 4587337.00 1981.94 1262398.63 2228.31 2990312.25
E-actin_1776.88 PSD	175.1 64964.1 251.1 34015.3 286.1 51698.2 304.1 42640.6 380.1 211601.5 400.1 218583.0 574.2 64811.3 581.3 37446.7 675.3 86334.2 693.3 62076.6 711.3 47741.0 771.3 37207.0 788.4 76001.0 887.4 48542.4 890.3 89931.7 971.4 32843.7 989.4 80211.5 1015.5 44235.8 1055.5 49975.0

	1072.5 1679289.0 1169.5 84556.4 1186.5 53498.6 1203.5 33369.5 1222.7 33935.6 1239.5 34478.5 1240.5 50743.0 1266.6 42601.3 1284.6 178905.0 1359.6 35663.2 1397.7 257588.5 1689.8 459851.0