

Analyse des Proteoms von *Trypanosoma brucei* unter  
besonderer Berücksichtigung des Stoffwechsels von  
Prostaglandinen

Proteome analysis of *Trypanosoma brucei* with emphasis of  
prostaglandin metabolism

**DISSERTATION**

der Fakultät für Chemie und Pharmazie  
der Eberhard-Karls-Universität Tübingen

zur Erlangung des Grades eines Doktors  
der Naturwissenschaften

2006

vorgelegt von  
Leonard Ehigie

Dekan:

1. Berichtstatter
2. Berichtstatter

Prof. Dr. L. Wesemann

Prof. Dr. M. Duszenko

Prof. Dr. D. Mecke

## Acknowledgement

This study here was carried out at the Interfakultäres Institut für Biochemie in the Department of Chemistry and Pharmacy, University of Tübingen under the direction of Prof. Dr. M. Duszenko.

I am very grateful to Prof. Duszenko for providing laboratory space and facilities to carry out this work and also for his constant encouragement, creative discussion and support endowed upon me.

I would like to express my gratitude to Prof. D. Mecke for his support and critical reading of the manuscript, and also for the discussion and evaluation of the work.

My sincere gratitude to Dipl. Bioinform. Rudolf Koopmann for the proteomics analysis and discussion.

I thank Dr. Bernd Kammerer and Dr. Antje Frickenschmidt from the Institut für Klinische Pharmakologie at Universität Tübingen for the Mass spectrometer peptide analysis.

I would like to express my thanks to my friends and co-workers Björn Bassarak, Björn Buchholz, Viola Denninger, Gordon Eske, Witta Kaiser, Patrick Merkel, Khalid Muhammad, Konstaninos Nouskas, Caroline Schönfeld, and Alexandra Thor for the friendly atmosphere in the laboratory.

Finally I would like to thank my mother and father for the emotional support throughout my stay in Europe.

## Table of contents

|          |  |           |
|----------|--|-----------|
| <b>1</b> | <b>Introduction</b>  | <b>1</b>  |
| 1.1.1    | Trypanosoma brucei as pathogen of sleeping sickness and Magana                   | 1         |
| 1.1.2.   | Classification   | 5         |
| 1.1.3    | Life cycle   | 7         |
| 1.1.4    | Morphology   | 8         |
| 1.2      | Prostaglandins   | 12        |
| 1.2.1    | The prostaglandin pathway  | 13        |
| 1.2.2    | Prostaglandin biosynthesis in parasites  | 14        |
| 1.3      | Apoptosis and programmed cell death  | 16        |
| 1.4      | Proteomics Two-dimensional electrophoresis techniques                            | 18        |
| 1.4.1    | First dimension: isoelectric focussing   | 19        |
| 1.4.2    | Second dimension: Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis | 20        |
| 1.4.3    | Staining and Reproducibility   | 20        |
| 1.4.4    | Protein identification methods   | 21        |
| 1.4.5    | Matrix-assisted laser desorption/ionization time-of-flight mass Spectrometry     | 22        |
| 1.4.6    | Database search based on peptide mass fingerprint spectra                        | 23        |
|          | Mascot search results  | 25        |
|          | Mascot peptide mass fingerprint  | 26        |
|          | Strategy of protein identification   | 27        |
| 1.5      | Objective of this work   | 28        |
| <b>2</b> | <b>Materials and methods</b>   | <b>29</b> |
| 2.1      | Materials  | 29        |
| 2.1.1    | General chemicals  | 29        |
| 2.1.2    | Radiochemicals   | 30        |
| 2.1.5    | Electrophoresis and Western Blotting   | 30        |
| 2.1.6    | Fluorography   | 31        |
| 2.1.7    | Other Consumable Materials   | 31        |
| 2.2      | Media, Buffers and Solutions   | 32        |
| 2.2.1    | Cultivation and isolation of Trypanosomes  |           |
| 2.2.2    | Lysate preparation from <i>T. brucei</i>   | 34        |
| 2.2.3    | Prostaglandin D Synthase assay.  | 34        |
| 2.2.4.   | SDS-PAGE According to Laemmli.   | 34        |
| 2.2.4    | Coomassie Staining   | 35        |

## Table of contents

|            |  |           |
|------------|--|-----------|
| 2.2.4      | Silver Staining  | 35        |
| 2.2.5      | Western- Blotting  | 36        |
| 2.2.6      | Thin Layer Chromatography  | 36        |
| 2.2.7      | 2-Dimensional Gel Electrophoresis  | 37        |
| 2.2.8      | In-gel trypsin digestion.  | 38        |
| 2.2.9      | Desalting and determination of protein concentration   | 39        |
| 2.2.10     | Immunoprecipitation  | 40        |
| <b>2.3</b> | <b>Methods</b>   | <b>40</b> |
|            | General Methods  | 40        |
| 2.3.1.     | Protein quantitation assay   | 40        |
| 2.3.1.2    | SDS-PAGE according to Laemmli  | 41        |
| 2.3.1.3    | Commassie Staining   | 41        |
| 2.3.1.4    | Silver staining  | 41        |
| 2.3.1.5    | Gel drying and preserving  | 42        |
| 2.3.1.6    | In-gel digestion   | 42        |
| 2.3.1.7    | Sample purification Zip Tip (C <sub>18</sub> )   | 43        |
| 2.3.1.8    | Western blotting analyses.   | 43        |
| 2.3.1.9    | Fluorography.  | 44        |
| 2.3.2      | Two-dimensional Gel Electrophoresis  | 44        |
| 2.3.2.1.   | First dimension-isoelectric focussing (IEF)  | 44        |
| 2.3.2.2.   | Preparation of second-dimension gel.   | 44        |
| 2.3.2.3.   | Peptide mass fingerprinting by MALDI-TOF<br>mass spectrometry  | 45        |
| 2.3.2.4    | Protein ladder sequencing of peptide fragments<br>Computer aided analysis of protein mass fingerprints.    | 46        |
| 2.3.3      | Cultivation and Isolation of trypanosomes from rat.  | 49        |
| 2.3.3.1    | Lysis of trypanosomes.   | 49        |
| 2.3.3.2.   | Isolation of cytosolic and membrane protein  | 49        |
| 2.3.3.4    | <i>In vitro</i> cultivation of trypanosomes<br>Cultivation of Bloodstreamform Trypanosomes <i>in vitro</i> | 50        |
| 2.3.4.     | Prostaglandin assay.   | 50        |
| 2.3.4.1    | Prostaglandin D Synthase assay.  | 50        |

## Table of contents

|           |  |           |
|-----------|--|-----------|
| 2.3.4.2.  | Protein Purification (Gel filtration and Ion-exchange chromatography).                                     | 51        |
| 2.3.4.3.  | Immunoprecipitation  | 51        |
| 2.3.4.4.  | Western Blotting and Analysis of Prostaglandin D Synthase in trypanosomal lysate.                          | 52        |
| 2.4       | Sample preparation for MALDI analysis.   | 52        |
| 2.4.1     | Sample purification Zip Tip (C <sub>18</sub> )   | 52        |
| 2.5.      | Special Equipment.MALDI-TOF mass spectrometer (Reflex II from Bruker-Daltonik, Bremen).Procedure           | 53        |
| 2.5.1     | Computer aided analysis of protein mass fingerprints.  | 54        |
| <b>3.</b> | <b>Results</b>   | <b>55</b> |
| 3.1       | Production of Prostaglandins from arachidonic acid by <i>T. brucei</i> .                                   | 55        |
| 3.1.2     | Production of prostaglandins from the cytosolic protein fraction of the <i>T. brucei</i> bloodstream form. | 55        |
| 3.2       | Two dimension gel electrophoresis of <i>T. brucei</i> proteins.  | 57        |
| 3.2.1     | Preparation of protein samples   | 57        |
| 3.2.2     | Two-dimensional electrophoresis  | 58        |
|           | 2D-gel comparison of the same region from <i>T. brucei</i> cytosolic protein gels.                         | 59        |
|           | 2D- gel electrophoresis analysis of membrane protein fraction from <i>T. brucei</i>                        | 61        |
| 3.3       | Gel staining   | 63        |
| 3.4       | Protein (peptide mass fingerprints) analysis.  | 64        |
| 3.4.1.    | MALDI-TOF and MALDI-QTOF tandem mass spectrometry  | 64        |
| 3.4.2     | Enolase  | 66        |
| 3.5       | The strategy of protein identification.  | 67        |

## Table of contents

|           |   |                            |
|-----------|---|----------------------------|
| 3.5.1     | Database search for protein identification  | 67                         |
| 3.6       | Protein identification and functions of the identified<br><i>T. brucei</i> protein  | 68                         |
| 3.6.1     | Prostaglandin F <sub>2α</sub> synthase, VSGs, and glycolytic enzymes<br>in <i>T. brucei</i> .<br>Result from <i>T. brucei</i> sequences of differentially expressed<br>induced cytosolic proteins. Matrix desorption/ionisation-<br>time-of-flight (MALDI-TOF) mass spectrometry of sequenced<br>protein.<br>Result from <i>T. brucei</i> highly expressed protein from membrane<br>fraction.<br>2D-gel of the <i>T. brucei</i> cytosolic protein fraction<br>(without ZnCl <sub>2</sub> ) reference map.<br>Result from <i>T. brucei</i> sequenced highly expressed<br>cytosolic protein fraction. | 70<br>71<br>73<br>74<br>75 |
| 3.6.2     | Comparison of theoretical and the experimentally observed pI<br>and MW<br>Result from <i>T. brucei</i> sequenced protein reference map<br><i>T. brucei</i> proteins identified by MALDI-TOF with known<br>functions grouped into functional categories<br>3.6.3 ADP-ribosylation-factor like protein (ARL1).  | 76<br>77<br>79<br>80       |
| 3.6.4     | <i>T. brucei</i> , Hypoxanthine-guaine phosphoribosyltransferase<br>(HPRT)  | 81                         |
| 3.7       | Ion-exchange chromatography and immunoprecipitation.  | 83                         |
| 3.7.2     | Immunoprecipitation of <i>T. brucei</i> protein fractions using<br>anti-PGDS from sheep.  | 85                         |
| 3.2.2     | Western Blotting with anti-PGDS from sheep.   | 89                         |
| <b>4.</b> | <b>Discussion</b>   | <b>90</b>                  |
| 4.1       | Protein isolation   | 89                         |
| 4.2       | Two-dimensional gel electrophoresis   | 90                         |

## Table of contents

|         |   |            |
|---------|---|------------|
| 4.2.1   | The choice of carrier ampholytes for isoelectric focussing .                  | 90         |
| 4.2.2   | Reproducibility   | 91         |
|         | The number of the visualized proteins on the gel                              | 92         |
| 4.2.3   | Staining of the gel   | 92         |
| 4.2.4   | Protein identification  | 93         |
| 4.3     | The <i>Trypanosoma brucei</i> proteome reference map                          | 94         |
| 4.3.1   | The identified proteins   | 94         |
| 4.3.2   | Multiple spots proteins   | 96         |
| 4.3.3   | Comparison of my results with other <i>trypanosomal</i><br>proteomic projects | 96         |
| 4.3.3   | Comparison of my results with other <i>trypanosomal</i><br>proteomic projects | 97         |
| 4.3.4.2 | Comparison of the experimentally observed MW and the<br>theoretical MW        | 98         |
|         | <b>Summary</b>  | <b>100</b> |
|         | <b>Zusammenfassung</b>  | <b>103</b> |
|         | <b>Literature</b>   | <b>104</b> |
|         | <b>Appendix</b>   | <b>119</b> |



## Table of contents

### List of Abbreviations

|                |  |
|----------------|--|
| A (ma)         | ampere (milliampere)   |
| AA             | Arachidonic acid   |
| Bp             | baes pair  |
| BSA            | bovine serum albumin   |
| <sup>0</sup> C | degree Celcius   |
| CGA            | Citrat glucose anticoagulant   |
| CHAPS          | 3-[(3-cholamidopropyl)-dimethylammonio-propan-sulfonate]             |
| Cm             | centimeter   |
| 1D             | first dimension  |
| 2D             | second dimension   |
| Da (kDa)       | dalton (kilodalton)  |
| DEAE           | Diethylaaminoethyl   |
| DTT            | 1,4-dithiothreitol   |
| EMBL           | European Molecular Biology Laboratory                                |
| g              | gravity (used in centrifugation)                                     |
| GPI            | Glycosylphosphatidylinositol   |
| GPI-PLC        | GPI-specific phospholipase   |
| HRP            | Horseradish peroxidase   |
| h              | hour   |
| IEF            | isoelectric forcussing   |
| l (ml,µl)      | liter (milliter, microliter)   |
| M (mM)         | molar (milli-mollar)   |
| MALDI-TOF      | matrix assisted laser desoption ionisation-time of flight            |
| MALDI-TOF      | matrix assisted laser desoption ionisation-quadrupole/time of flight |
| Min            | minute   |
| MW             | molecular weight   |
| No             | number   |
| PBS            | Phosphate buffered salin   |
| PI             | isoelectric point  |
| rpm            | rotation per minute  |
| SDS-PAGE       | sodium dodecylsulfatepolyacrylamide gel electrophoresis              |
| sec            | second   |
| TBS            | Tris buffered salin  |
| TEMED          | N,N,N`N`-tetramethylethylenediamine                                  |
| TFA            | trifluoroacetic acid   |
| TOF            | time of flight   |
| Tris-base      | tris(hydroxylmethyl)-amonionethan                                    |
| Tris-HCl       | trizma-hydrochloride.  |
| TX 100         | Triton X 100   |
| v/v            | volume/volume  |
| V(mv)          | volt (millivolt)   |
| VSG            | Variant surface glycoprotein   |
| mVSG           | Membrane bound VSG   |
| sVSG           | Soluble VSG  |
| w/v            | weight per volume  |

## Table of contents

### LIST OF INTERNET SITES

| <b>Internet site name</b> | <b>Internet address</b>   |
|---------------------------|---|
| Blast 2 sequences         | <a href="http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.htm">http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.htm</a>   |
| GenBank                   | <a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a>   |
| Genome Database (GDB)     | <a href="http://gdbwww.gdb.org/">http://gdbwww.gdb.org/</a>   |
| NCBI                      | <a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a>   |
| PeptideMass               | <a href="http://www.expasy.ch/tools/peptide-mass.html">http://www.expasy.ch/tools/peptide-mass.html</a>   |
| PeptIdent                 | <a href="http://www.expasy.ch/tools/peptident.html">http://www.expasy.ch/tools/peptident.html</a>   |
| PeptideSearch             | <a href="http://www.mann.embl-heidelberg.de/GroupPage:Link/peptidesearchpage.html">http://www.mann.embl-heidelberg.de/GroupPage:Link/peptidesearchpage.html</a> |
| ProFound                  | <a href="http://129.85.19.192/profound_bin/WebProFound">http://129.85.19.192/profound_bin/WebProFound</a>   |
| Protein Data Bank (PDB)   | <a href="http://mist.rcsb.org/pdb/">http://mist.rcsb.org/pdb/</a>   |
| SOSUI                     | <a href="http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.h">http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.h</a>   |
| Swiss-Prot/ TrEMBL        | <a href="http://www.expasy.ch/cgi-bin/sprot-search-ful">http://www.expasy.ch/cgi-bin/sprot-search-ful</a>   |

# 1 Introduction

## 1.1 Trypanosomes

### 1.1.1 *Trypanosoma brucei* as pathogen of sleeping sickness and nagana.

Trypanosomes are extracellular, eukaryotic flagellated protozoa belonging to the order Kinetoplastida. Some species are responsible for human sleeping sickness in tropical Africa and for a similar disease called ‘nagana’ in cattle. Human African trypanosomiasis (HAT), or sleeping sickness, is an illness endemic to sub-Saharan Africa. It is caused by *Trypanosoma brucei*, which exists in three morphologically identical subspecies of which two are human infectious: *Trypanosoma brucei rhodesiense* – (Stephens and Fantham, 1910) (East African or Rhodesian African trypanosomiasis) and *Trypanosoma brucei gambiense*-(Dutton, 1902) (West African or Gambian African trypanosomiasis). These parasites are transmitted to human hosts by bites of infected tsetse flies (*Glossina* species), which are found only in Africa. Unlike a number of other parasites, African trypanosomes are extracellular parasites throughout their entire life cycles. The reservoir of the infection for these vectors is almost exclusively human in West African trypanosomiasis (Fevre *et al.*, 2006).

Sleeping sickness, primarily affects the poorest rural populations in some of the least developed countries of Central Africa (WHO 2004). The incidence may approach 300,000 to 500,000 cases per year, and it is invariably fatal if untreated. In the past thirty years, failures in control measures and treatment regimes have caused the impact of this parasite to increase markedly, such that it is now the biggest killer in some parts of Africa, surpassing HIV/AIDS in provinces of Angola, Congo and Southern Sudan (<http://www.who.>).

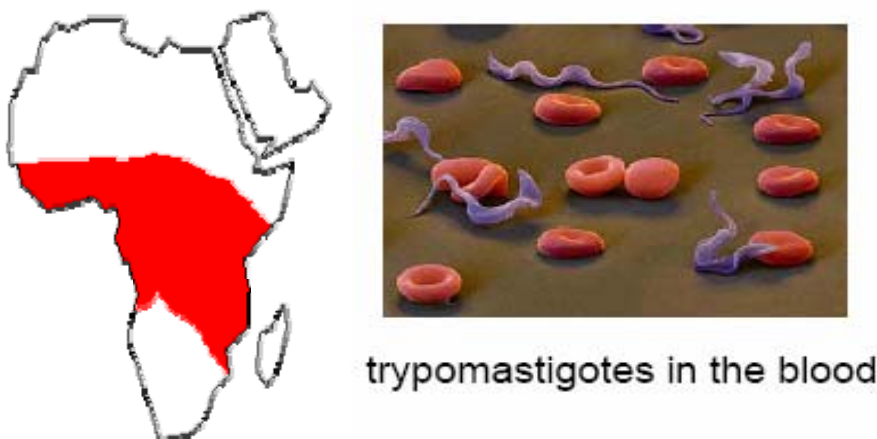


Fig. 1.1: Distribution of *T. brucei* in West Africa (*gambiense*) and *T. brucei rhodesian* in East African.

## Introduction

The third subspecies, known as *Trypanosoma brucei brucei*, is not a pathogen for human, because this parasite is lysed by a component of the human serum (haptoglobin of HDL) (Hajduk *et al.*, 1994). It infects domestic animals (cattle, sheep, dogs, pigs, and goats) and many other wild animals. The trypanosomiasis caused in domestic animals has important economic significance (Nagana). The sleeping sickness and Nagana-plague are restricted to tropical Africa in a breath of 15 degree north and 18 degree South (Tsetse-belt) (Mhlanga, 1996, Donges, 1988). African trypanosomiasis is distinct from American trypanosomiasis, which is caused by *Trypanosoma cruzi* and has different vectors, clinical manifestations, and therapies.

*Trypanosoma cruzi* is the causative agent of Chagas disease, which exhibit a patchy distribution throughout south and Central America. The infective stage of the organism was discovered by chance while Carlos Chagas was studying the vectors. *T. cruzi* is transmitted to the vertebrate host by three different genera of the Triatominae subfamily (*Triatoma*, *Rhodnius*, *Panstrongylus*). Members of this family are blood-feeding insects and are called many different names including: triatomine bugs, reduvid bugs, kissing bugs, and assassin bugs. While taking a blood meal the triatomines will defecate and infective metacyclic trypomastigotes are released in the faeces

In contrast to the African trypanosomes, the trypomastigotes of *T. cruzi* do not replicate within the bloodstream or tissues of the vertebrate host. Instead the trypomastigotes invade macrophages and transform into the amastigote form.

The genetic and evolutionary relationships between *T. gambiense*, *T. rhodesiense*, and *T. brucei* may provide some insight into the evolution of human disease. Molecular techniques show that *T. gambiense* is relatively homogeneous throughout its wide distribution in central and western Africa. In contrast, distinguishing *T. rhodesiense* and *T. brucei* has been much more problematic. Molecular analyses indicate that both of these species exhibit a wide range of sequence heterogeneity and in some cases more homology is observed between *T. rhodesiense* and *T. brucei* than between isolates of the same species obtained from different geographical regions. The discovery of a single gene, called Serum Resistance Associated (SRA) that can confer resistance to trypanosome lytic factor has provided some insight into the evolutionary relationships between *T. rhodesiense* and *T. brucei* (Gibson *et al.*, 2002).

SRA is a mutated VSG (see antigenic variation) with a deletion in the central portion of the gene and is found only in *T. rhodesiense*. Presumably this mutation in the ancestral *T. brucei* conferred human infectivity and thus represented the origin of *T. rhodesiense*. Transfer of this gene to other *T. brucei* isolates with different genetic backgrounds via sexual recombination would account for

## Introduction

the genetic heterogeneity of *T. rhodesiense* and its spread in eastern Africa. In this regard, one might also consider *T. rhodesiense* a host-range variant of *T. brucei*. (MacLean *et al.*, 2004).

Infection with African trypanosomes can result in disease manifestations ranging from asymptomatic or mild to a severe fulminating disease. *T. rhodesiense* is more likely to cause a rapidly progressing and fulminating disease than *T. gambiense*. *T. gambiense* tends to cause a slow progressing disease which may either be self-limiting or develop into a chronic disease involving the lymphatics and the central nervous system (CNS).

The infection is initiated when metacyclic trypomastigotes are introduced from the saliva of the tsetse into the bite wound. Generally there is an asymptomatic incubation period of 1-2 weeks in which the trypomastigotes are replicating within the tissue near the site of the bite. Occasionally, a local inflammatory nodule known as a 'trypanosomal chancre' is observed during this period. In addition, they will invade the capillaries and enter the circulatory system immediately after infection and continue to replicate within the blood of the human host. The establishment of this acute blood stage infection is characterized by irregular episodes of fever and headache. In the case of *T. gambiense* the number of parasites in the blood tends to be very low and often the infected person exhibits no symptoms, whereas most persons infected with *T. rhodesiense* will exhibit a much higher parasitemia and a more pronounced fever sometimes associated with rigour (Turner *et al.*, 2004).

Disease progression is often characterized by invasion of the lymphatics in *T. gambiense* infections. Symptoms during the lymphatic stage include enlarged lymph nodes (particularly post-cervical group), weight loss, weakness, rash, itching, and edema as well as the continued intermittent febrile attacks. Higher parasitemias are often associated with the symptoms. The infection can spontaneously resolve during either the blood stage or the lymphatic stage. There is usually little evidence of lymphatic involvement in *T. rhodesiense* infections (Jannin and Cattand, 2004).

A hallmark feature of African trypanosomiasis is the invasion of the CNS and nervous system impairment. Trypanosomes crossing the blood-brain barrier result in a generalized meningoencephalitis characterized by progressively worsening symptoms. Indications of nervous impairment include: apathy, fatigue, confusion, somnolence, and motor changes (such as tics, slurred speech, and incoordination). The changes in sleep patterns are often characterized by extreme fatigue during the day and extreme agitation at night. Generally it is 6-12 months (or even years) after the infection before the neurological symptoms start to become apparent in the

## Introduction

case of *T. gambiense*. Neurological manifestations can occur within weeks after *T. rhodesiense* infections. If untreated, the CNS stage of the disease will almost always progress to include convulsions or coma followed by death in both *T. gambiense* and *T. rhodesiense* infections.

Diagnosis depends upon detection of trypanosomes in blood, lymph node aspirations, or spinal fluid. Typically few trypanosomes are detected in the blood or other fluids during the *T. gambiense* infections. The trypanosomes are more likely to be detected during symptomatic periods (e.g., during febrile episodes). In the absence of detectable parasites, travel or residence in an endemic area combined with the symptoms discussed above can be used as a presumptive diagnosis. Because of the low parasitemias exhibited during *T. gambiense* infections it is often not possible to detect parasites by standard thin and thick blood smears. Techniques to increase the sensitivity of detection are often needed. For example, looking at fresh whole blood mounts may increase the sensitivity due to the distinctive movement of the parasite. Another method to increase sensitivity is to centrifuge the blood in a microhematocrit tube. The parasites are enriched in the 'buffy coat' which is the band of white cells directly on top of the packed erythrocytes. The tube is then broken at the buffy coat and Giemsa-stained blood smears are prepared from these cells. Blood can also be passed over a small anion exchange column. This removes the red and white blood cells expresses negative surface charge. Trypanosoma have no surface charge due to the VSG coat and pass right through the column and resin. Removing the blood cells obviously makes detection of the parasites easier.

There are 2 recognized stages in the clinical presentation of HAT (Barrett, 1999) the early hemolymphatic stage and the late encephalitic stage, when the central nervous system (CNS) is involved. Successful treatment of the latter stage of the disease has been limited for several reasons. Treatment relies on arsenic compounds for example, melarsoprol, which are associated with a high degree of toxicity (Pepin and Milord, 1994) and the relatively new drug difluoromethylornithine (DFMO), which is effective against the West African form of HAT caused by *T. brucei gambiense* (Bacchi *et al.*, 1994), but not against the East African form, (caused by *T. brucei rhodesiense*). Melarsoprol treatment is followed by post treatment encephalitis in up to 10% of cases, with a fatality rate of about 50%. Moreover, there is evidence of increasing resistance to this drug, with reported rates of treatment failure of 30% (Brun *et al.*, 2001). DMFO, an ornithine decarboxylase inhibitor, is an effective anti-trypanosomal against *Trypanosomes*. It has been nicknamed the 'resurrection drug' because of some spectacular rescue of comatose patients in late stage of HAT sickness. However, it is expensive to produce and the

## Introduction

standard treatment includes consecutive daily injections of high doses due to its rapid metabolism.(WHO, 2005).

Suramin and pentamidine are recommended drugs during the acute stage without CNS involvement; whereas melarsoprol or tryparsamide is recommended if CNS is involved. The prognosis is excellent if treatment starts during the acute stage. Due to severe side effects, these drugs are less than ideal and more drugs need to be developed. For example, melarsoprol, an arsenical based drug, has been used for more than 50 years despite its relatively high toxicity (>10% of patients die from the drug treatment).

Prophylactic drugs are contraindicated since they mask infections and the drugs are somewhat toxic. Vector avoidance (e.g., protective clothing, insect repellents) is the recommended prophylactic measure. Control activities include: surveillance and treatment, traps, insecticides, and habitat alteration.

### 1.1.2. Classification

The genus *Trypanosoma* belongs to the order Kinetoplastida, which belongs to the Euglenozoa phylum. They represent one of the earliest mitochondrial-containing extant branches of the eukaryotic lineage. Within the mitochondrion of Kinetoplastids, mtDNA is located in a specialized area near the flagellar pocket referred to as the kinetoplast. This kinetoplast of trypanosomatids consists of a network of thousands of catenated circular DNA molecules. This DNA is divided into two classes of circles, maxicircles and minicircles. It is located in a capsular region of the single mitochondrion, close to the basal body of the flagellum (Vickerman, 1994).

Within the order Kinetoplastida there are two major subgroups. One subgroup consists of trypanosomatids, the other of the bodonids and related cryptobiids. The trypanosomatids include trypanosomes and several other species, including *Leishmania*, *Crithidia*, *Herpetomonas* and *Blastocrithidia* (Maslov *et al.*, 1994).

Another distinctive characteristic that separates trypanosomes from other Kinetoplastids is the nature of their surface membrane, which has adapted ways to cope with host defence. It is composed by the variant surface glycoprotein (VSG), which evades the host's immune responses through antigenic variation. Through the advancement of molecular biology and the emergence of modern phylogenetic analyses, new findings suggest that trypanosomes are monophyletic rather than paraphyletic, as assumed had been previously thought (Stevens and Gibson, 1999). Some studies

## Introduction

also suggest that the genus split into Salivarian trypanosomes and non- Salivarian trypanosomes occurred about 200-300 million years ago (Stevens *et al.*, 19991). The origin of Salivarian trypanosomes is thought to predate the appearance of placental mammals, and their common vector, the tsetse fly as well as the split between the African continent and South. America (Overath *et al.*, 2001). The likely scenario is therefore that the *T. brucei* clade arose in Africa, parasitizing early mammals, which became isolated when Africa split off from other continents. The following classification is based on (Levine *et al.*, 1980).

|            |                                |                        |
|------------|--------------------------------|------------------------|
| Phylum     | Protozoa                       |                        |
| Subphylum  | Sarcomastigophora              |                        |
| Class      | Mastigophora                   |                        |
| Subclass   | Zoomastigophora                |                        |
| Order      | Kinetoplastida                 |                        |
| Family     | Trypanosomatidae               |                        |
| Genus      | Trypanosoma                    |                        |
| Species    | Trypanosoma brucei             |                        |
| Subspecies | Trypanosoma brucei brucei      | (non human pathogenic) |
|            | Trypanosoma brucei gambiense   | (human pathogenic)     |
|            | Trypanosoma brucei rhodesiense | (human pathogenic)     |



1.1.3 Life cycle

The trypanosome is transmitted between mammalian hosts by the tsetse fly, *Glossina* spp, in which it initially establishes in the midgut after a blood meal but then migrate to the salivary

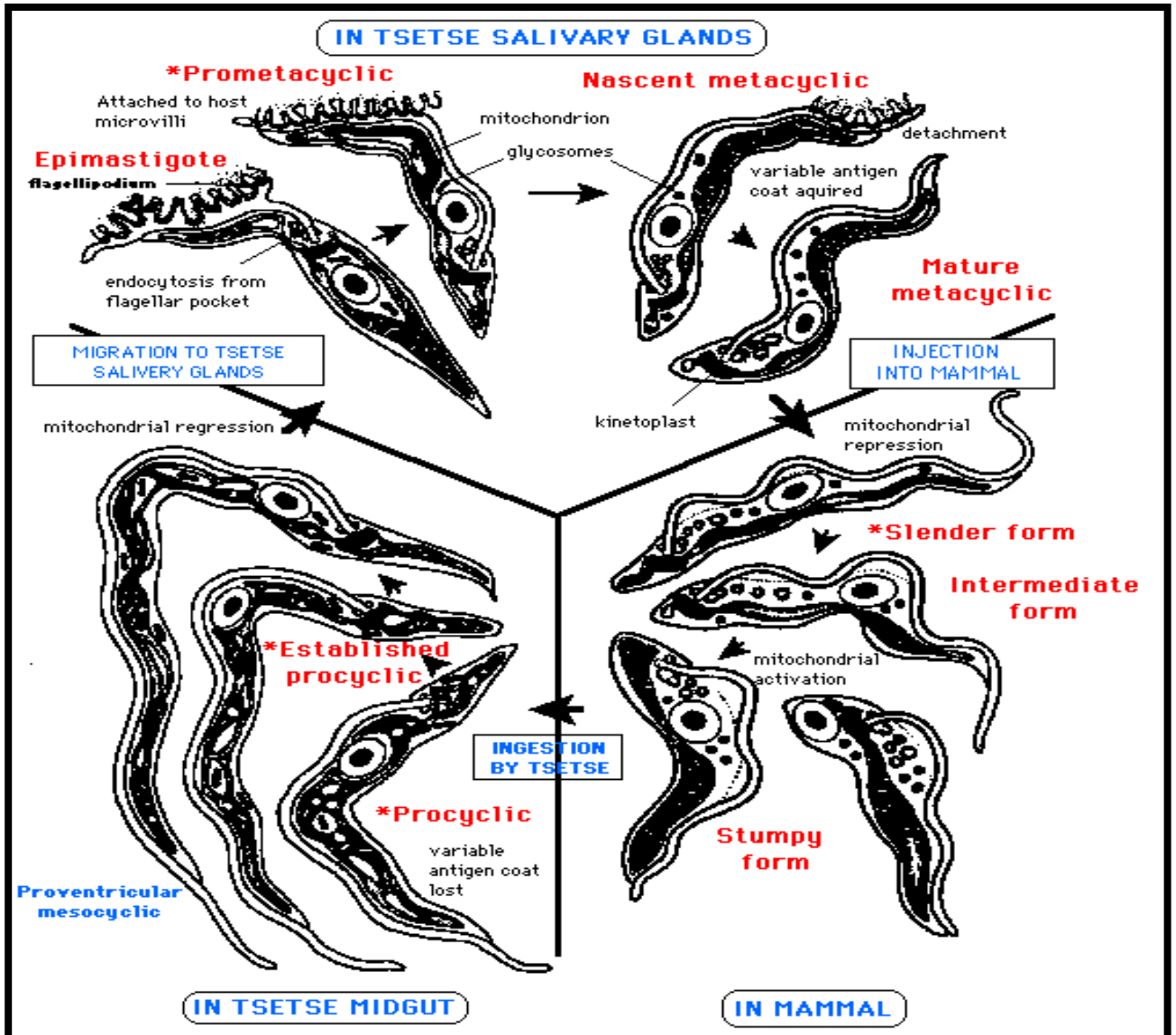


Fig. 1.2: *T. brucei*. Schematic representation of development cycle in a mammal and in the tsetse fly vector. (After Vickerman, 1985)

gland in preparation for transmission to a new mammalian host. Because this mode of transmission is by inoculation during biting this group of trypanosomes are also referred to as salivatype or “Salivarian” (<http://www.who.int/en/>).

## Introduction

In mammals, the parasite survives free in the bloodstream, being able to evade antibody responses through antigenic variation (McCulloch, 2004; Pays *et al.*, 2004). This entails the sequential expression of antigenically distinct variable surface glycoproteins (VSGs), which are linked to the surface membrane by a glycosylphosphatidylinositol (GPI) anchor. Trypanosomes proliferate in the mammalian bloodstream as the morphologically distinct slender forms, which are replaced by the non-proliferative stumpy form as parasite numbers increase (Matthews *et al.*, 2004). This serves two purposes: (1), the accumulation of division-arrested forms limits the increase in parasite numbers and thereby prolongs host survival (and hence the probability of disease transmission). (2), the uniform arrest of the stumpy forms in the G1 phase of the cell cycle ensures that morphological changes occur prior to transmission to the tsetse fly can be coordinated with re-entry into the cell cycle. This is important because correct organelle positioning is crucial for successful completion of the cell cycle of tsetse midgut procyclic forms. The ingested form that is infectious for the fly is termed the short-stumpy blood stream trypomastigote, which is a non-dividing form. Upon uptake by the tsetse, bloodstream trypanosomes replace the VSG coat with a less-dense surface coat composed of procyclin, which is also GPI anchored. After about two weeks some procyclics migrate from the midgut through the hemocoel eventually reaching the salivary glands (Roditi and Liniger, 2002, Acosta-Serrano, *et al.*, 2001).

Energy generation also switches from being exclusively based on glycolysis to a mitochondrion-based respiratory system, which requires structural elaboration and metabolic activation of the organelle. The epimastigote form generated there attach to the gland wall through elaborations of the flagellar membrane. After further multiplication, the parasite undergoes division arrest, reacquires a VSG coat and is released into the salivary gland lumen, in preparation for inoculation into a new mammalian host. Metacyclic trypomastigotes are found in the salivary glands ~ 20 days after the blood meal and there would be about 40000 trypomastigotes/bite, while it takes only one to initiate an infection (<http://www.who.int/en/>).

Most studies on the trypanosome have focused on the bloodstream and procyclic forms of the parasite. This is because these stages can be readily cultured *in vitro* (Matthews, 2005)

### 1.1.4 Morphology

The trypanosome cell is elongated and has a highly polarized microtubule cytoskeleton. A typical Trypanosomae is an elongated organism, 10-15µm in length (including flagellum) and 0.5-2µm width, with a single nucleus containing a large central nucleolus. The nuclear genome of

## Introduction

trypanosomes show an equivalent complexity: *T. brucei* contains 11 ‘mega base’ chromosomes and more than 100 minichromosomes of about 50 kbp in size. These minichromosomes harbour a repertoire of *VSG* genes, each flanked by a 177 bp repeat sequence (Wickstead *et al.*, 2004). Although the minichromosomes segregate with fidelity (Wickstead *et al.*, 2003), this is not through association with the microtubule spindle via kinetochore attachment but rather results from tracking along the microtubules that run from pole to pole (Ersfeld and Gull K, 1997). The cell shape remains intact throughout the cell cycle, the daughter microtubule cytoskeleton being assembled and interdigitated between existing microtubules, such that cytoskeletal inheritance is semiconservative (Sherwin and Gull K, 1989b).

The single-copy organelles in the trypanosome cell (i.e. the flagellar pocket, flagellum, kinetoplast, mitochondrion, including the kinetoplast) are precisely positioned within the cytoskeletal corset and are concentrated between the posterior end and the centre of the cell. The most posterior structure is the mouth of the flagellar pocket. This is the exit point for the flagellum, which is tethered along the exterior length of the parasite. The flagellar pocket is the only site of endo- and exocytose (Overath and Engstler, 2004); this is important in bloodstream forms, in which the surface membrane is densely packed with *VSG* to protect against the alternative pathway of complement activation (Ferrante and Allison, 1983) and to shield common antigenic determinants from immune recognition. This dense packing requires that the GPI-anchored *VSG* is significantly concentrated during trafficking from the endoplasmic reticulum (ER) to the flagellar pocket surface (Grunfelder *et al.*, 2002).

The kinetoplast containing (the mitochondrial genome of the parasite) is located at the posterior end of the cell and mitochondrial activity is relatively repressed. As parasite numbers increase in the blood stream, differentiation to morphologically without stumpy form occurs. This is a division-arrested form preadapted for transmission to the tsetse flies. Endocytosis of the *VSG* and other molecules in the flagellar pocket is clathrin dependent: RNAi directed against the clathrin heavy chain causes rapid death of cells after massive enlargement of the flagellar pocket (Allen *et al.*, 2003). A similar phenotype is seen when actin is targeted by RNAi in bloodstream forms, implicating this protein to be involved in endocytosis and intracellular trafficking. Endocytosis in procyclic cells occurs at a lower rate, but is also clathrin dependent (Allen *et al.*, 2003; Hung *et al.*, 2004). Interestingly, however, RNAi directed against actin is not lethal in this life cycle stage (Garcia-Salcedo *et al.*, 2004).

The small size of the trypanosome, its high rate of trafficking of GPI-anchored *VSG* to the surface and the concentration of the endocytic apparatus into the posterior end of the cell have made bloodstream forms an excellent system for analysis of protein trafficking using

## Introduction

fluorescence and electron microscopy. Moreover, the single Golgi apparatus of the parasite and its restricted positioning between nucleus and flagellar pocket has enabled *T. brucei* to become a model for the biogenesis of this organelle in eukaryotes. A new marker has been derived for the *T. brucei* Golgi (TbGRASP) similar to mammalian GRASP-55 and its been used as photobleaching approach to demonstrate that, during cell division, the new Golgi forms de novo from material source directly from the old Golgi, rather than from the ER (He *et al.*, 2004).

The motility of the trypanosome is dependent upon its single flagellum, which has a conventional axonemal structure plus an associated paraflagellar rod (Vaughan and Gull, 2003). Trypanin (Hutchings *et al.*, 2002), a trypanosome protein related to a subunit of the dynein motor regulatory complex (PF2 in *Chlamydomonas* and Gas11/Gas 8 in mammalian cells), also contributes to motility (Rupp and Porter, 2003).

During cell division, the growing daughter flagellum precisely tracks the old flagellum, such that structural information is transferred from the old to the new flagellum through a novel form of cytotoxic inheritance (Moreira-Leite *et al.*, 2001). This information is imparted through the flagellar connector, a mobile structure that connects the tip of the new flagellum with three of the doublet microtubules of the axoneme of the old flagellum (Briggs *et al.*, 2004). Although, it is apparently fundamental for the division of the procyclic form, there is no evidence for the existence of a similar flagellar connector in the bloodstream stage of *T. brucei*, nor is any related structure obvious in other kinetoplastids (Briggs *et al.*, 2004). This surprising finding highlights very basic differences in cell-cycle control for different life cycle stages of trypanosomes. Perhaps the procyclic trypanosome population can afford fewer mistakes in cell division as it struggles to establish a foothold in the tsetse fly. The trypanosome flagellum originates from a basal body that is, in turn, linked through the mitochondrial membrane to the mitochondrial genome, which comprises a mass of catenated DNA termed the kinetoplast. The kinetoplast and basal body are linked by a tripartite attachment complex that must traverse both the cell and the mitochondrial membranes (Ogbadoyi *et al.*, 2003).

The mitochondrion itself is a single elongated organelle that runs from the posterior to the anterior end of the cell. In bloodstream forms, the mitochondrion is a simple tubular structure nearly devoid of cristae. This reflects the absence of a mitochondrial/respiratory chain during this stage, linking energy generation exclusively on glycolytic reactions compartmentalized within specialized organelles termed glycosomes (Parsons, 2004). However, the procyclic form does not have the luxury of blood glucose as an abundant energy source and has a highly active mitochondrion which atypically contains acetate: Succinate CoA transferase and succinyl-CoA synthetase cycle (Bochud-Allemand and Schneider, 2002; van Weelden *et al.*, 2003), in addition

## Introduction

to the components of the Krebs cycle and the electron transport chain. Acetate: succinate CoA transferase, which also operates in anaerobic mitochondria of some metazoa and anaerobic protists but not in mammals, generates ATP by the conversion of acetyl- CoA to acetate. As in the case of other single-copy organelles, the mitochondrion must segregate with fidelity during cell division, and one contributor to this appears to be a dynamin-like protein (TbDLP), which is involved in the division of the mitochondrial membrane either directly or through recruitment of other effectors of membrane scission (Morgan *et al.*, 2004)

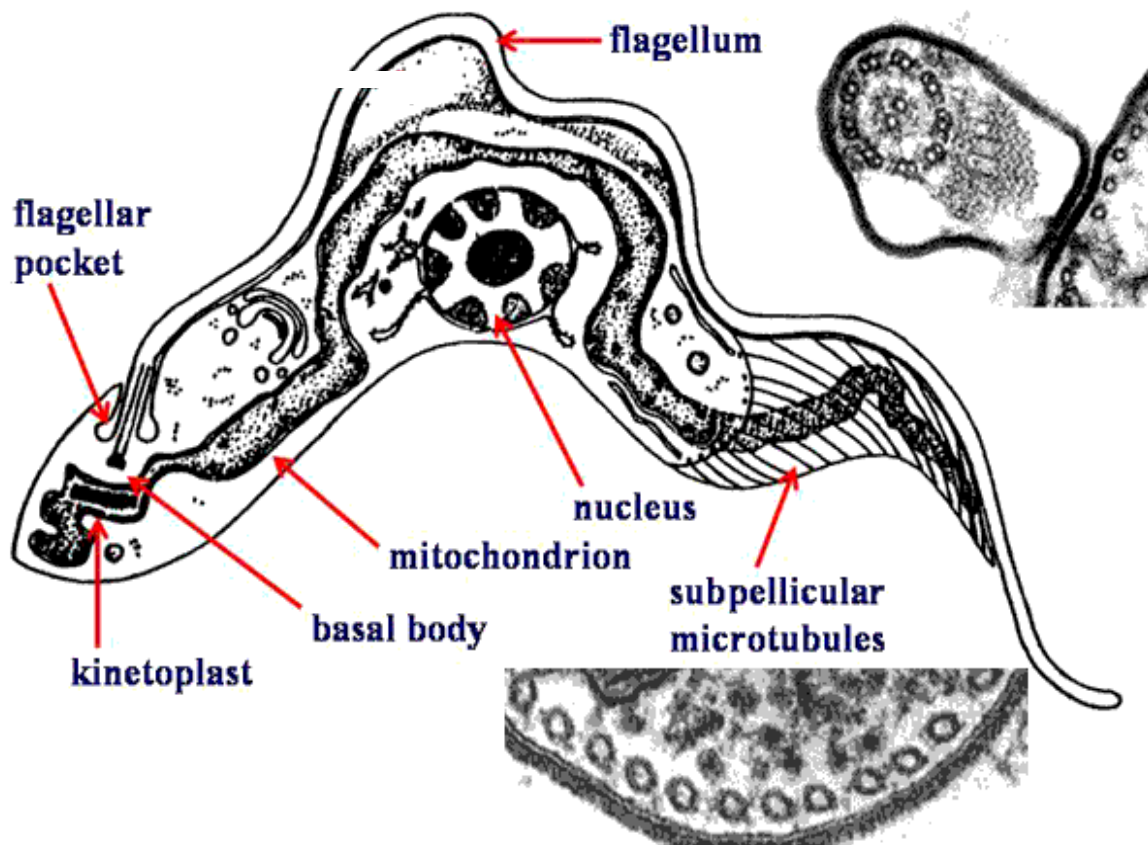


Fig. 1.3: Trypanosome cell architecture

### 1.2 Prostaglandins

The mysterious and complex family of prostaglandins constitutes one of the most intriguing discoveries in the history of modern medicine. Discovered in 1936 by von Euler, prostaglandins derive their name from the fact that they were first detected in human seminal fluid. It was not until the advent of more sophisticated instruments 40 years later that these compounds could be studied in depth. Researchers discovered that the original substance is just one of a family of compounds found in nearly cell of the body; in fact, prostaglandins are found throughout the animal kingdom, even in species as lowly as insects, shellfish and corals

Prostaglandins are a subset of a larger family of substances called eicosanoids. Other subgroups include thromboxanes, leukotrienes, lipoxins and the nearly discovered Levulgalndin. Eicosanoids are localized hormones that seem to be the fundamental regulating molecules in most forms of life. They do not travel in the blood like hormones, but are created in the cells to serve as catalysts for a large number of processes including the movement of calcium and other substances into and out of cells, dilation and contraction, of smooth muscle inhibition and promotion of blood clotting, regulation of secretions including digestive juices and hormones, and control of fertility, cell division and growth. The list of biological functions involving prostaglandins is limited only by our ignorance of their effects. As research continues, our knowledge of these fascinating substances expands and grow (Horrobin and David, 1983).

Prostaglandins are products of the arachidonic acids metabolism released from cell membranes, which is through the action of phospholipase A<sub>2</sub> (PLA<sub>2</sub>). Its conversion to prostaglandins is catalysed by the enzyme cyclooxygenase (COX), which exists in two isoforms (Mattei *et al.*, 2001). Prostaglandins were first extracted from semen, prostate, and seminal vesicles by Goldblatt and von Euler 1930s. Bergstrom and colleagues purified the first prostaglandin isomers during the 1950s and 60s, and in 1964, van Dorp *et al.* and Bergstrom *et al.*, independently identified AA, a 20-carbon tetraenoic fatty acid (C<sub>20</sub>:4 $\omega$ 6) as precursor to prostaglandins. The cyclooxygenase reaction through which AA is enzymatically cyclized and oxygenated to yield endoperoxide-containing prostaglandin (PGG<sub>2</sub>) which was later identified by Samuelsson and colleagues (Hamberg and Samuelsson, 1973; Hamberg *et al.*, 1974) (Fig. 1.3 page 14). The enzyme, cyclooxygenase (COX) that catalyzes this ring formation also reduces one hydroperoxyl group in PGG<sub>2</sub> to a hydroxyl residue thus forming PGH<sub>2</sub> via a separate peroxidase active site

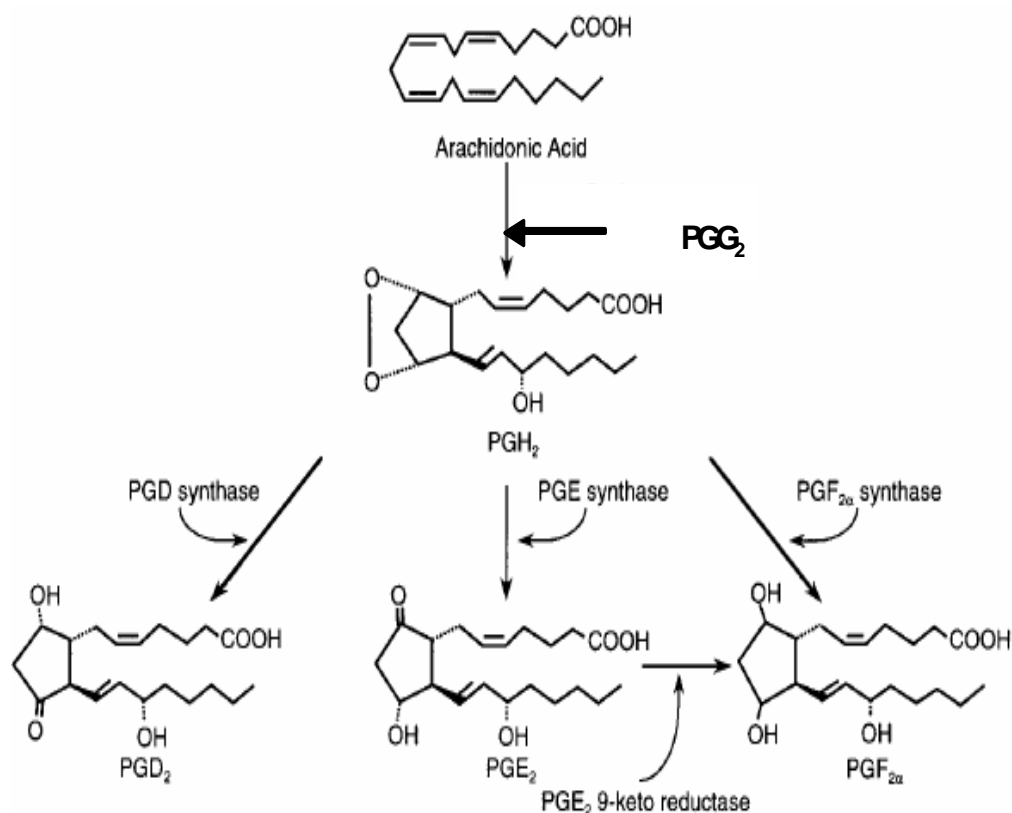
## Introduction

within the same enzyme. Isomerases and oxidoreductases produce various bioactive prostaglandin isomers using PGH<sub>2</sub> as substrate

In 1971, John Vane used a cell-free homogenate of guinea pig lung to demonstrate that aspirin, indomethacin, and salicylate, popular nonsteroidal anti-inflammatory drugs (NSAIDs), were inhibitors of this enzyme PGH<sub>2</sub>, thus defining the mechanism of action of this important class of drugs. Until 1976 (Hemler and Lands, 1976; Miyamoto *et al.*, 1976), when purified COX preparations were first described, tissue homogenates were used as a source of COX enzyme activity, which was frequently referred to at that time as a prostaglandin synthetase. Since the COX enzyme reaction does not require ATP, the nomenclature was later changed to synthase. The COX enzyme, also known as prostaglandin H synthase (PGHS) or prostaglandin endoperoxide synthase (E.C. 1.14.99.1), was identified as the major enzyme in the oxidative conversion of AA to PGG<sub>2</sub> and PGH<sub>2</sub> (Smith and Lands, 1972; Hamberg *et al.*, 1974), using seminal vesicles of sheep, and bovine as a rich enzyme source (Smith and Lands, 1972). Thus, purification of PGHS enzyme to homogeneity was first achieved from the sheep (Van der Ouderaa *et al.*, 1977) and bovine (Miyamoto *et al.*, 1976; Ogino *et al.*, 1978) seminal vesicles. Since detergents such as Tween 20 were needed to solubilize the enzyme, it was classified as an integral microsomal membrane protein.

### 1.2.1 The prostaglandin pathway

The predicted amino acid sequences of COX-2, cloned in chicken and mammals showed about 60% amino acid identity with COX-1 (Simmons *et al.*, 1991). COX-1 and COX-2 were found to be approximately 600 amino acids in size in all species. The COX-1 and/or -2 cDNA sequences from many organisms, including bony and cartilaginous fish, birds, and mammals have been characterized. Furthermore COX genes appear to be expressed in invertebrates, such as coral and sea squirts, where two COX isoform have been identified in two different species of each phylum (Valmsen *et al.*, 2001, Jarving *et al.*, 2004). These data suggest that the cyclooxygenase pathway was present in early invertebrate species and in the animal kingdom. Recently a cyclooxygenase enzyme from the protozoan *Entamoeba histolytica* has been identified that lacks structural similarity with other COXs enzymes, but produce PGE<sub>2</sub> from arachidonic acid (Dey *et al.*, 2003). Therefore, prostaglandin-synthesizing enzymes distinct from the COX lineage characterized in vertebrates, coral, and sea squirts appear to have arisen during evolution of some organisms (Daniel. *et al.*, 2004).



**Fig. 1.4:** The biosynthetic pathways of prostaglandin from arachidonic acid.

### 1.2.2 Prostaglandin biosynthesis in parasites

The biochemical pathways of parasite eicosanoid metabolism are poorly characterized. Parasites require exogenous fatty acids (Liu and Weller, 1970) which may be converted to AA by chain elongation and desaturation (Birke *et al.*, 1988). Most AA is esterified into cell membrane phospholipids but can be liberated from the membranes by phospholipase, in particular phospholipase A<sub>2</sub> (Kuhn and Borngräber, 1998; Maxey and MacDonald, 1998) after mechanical, chemical or hormonal stimulation. However, alternative pathways have been described in invertebrates (De Petrocellis and Di Marzo, 1994). In parasites, COX seems to act primarily in a constitutive manner (Belly and Chadee, 1995).

The biosynthetic pathways of PG synthesis in *ac. castellanii* have been studied in some detail and a range of COX products was indeed detected by thin layer chromatography (TLC) and spectrophotometry (Hadas, 1998b and Mazur, 1997).



## Introduction

Miyares and Hollands (1976) reported the first evidence for the production of substances with PG like properties in *cestodea-moniezia*. The tissue dwelling larvae absorb albumin-bound AA from their environment, which is metabolized to PGE<sub>2</sub> and secreted to contribute to the evasion of host attack (Fukushima *et al.*, 1993).

Filarial worms have attracted special attention with respect to endogenous eicosanoid metabolism because of their medical importance. It has been repeatedly demonstrated that filariae synthesize PGI<sub>2</sub> and PGE<sub>2</sub> (Liu *et al.*, 1990) and to a less degree, PGD<sub>2</sub> and PGF<sub>2</sub>α but not TXA<sub>2</sub> from AA which was either administered exogenously or liberated from an intrinsic phospholipid pool (Liu *et al.*, 1990).

In recent studies, PGH/E-isomerase, an enzyme involved in PG metabolism, has been purified from *Ascarida galli* and partially sequenced (Meyer *et al.*, 1996). Pigs infected with *Toxoplasma* exhibited elevated plasma levels of PGF<sub>2</sub>α, probably related to pathology of the uterus, fetus and subsequent abortion (Baetz *et al.*, 1981)

Leishmaniasis is a major threat to human health in subtropical and tropical climates and it has been extensively studied with respect to immune modulation exerted by the intracellular amastigote stages. Increased production of AA metabolites by peritoneal phagocytes infected with *leish. donovani* has been reported in other studies (Reiner and Malemud, 1984, 1985).

Studies on mice infected with *Tryp. Brucei brucei* have shown that the immune suppression that normally occurs during trypanosomiasis correlates with changes in AA metabolism of macrophages. This hypothesis of PG-mediated immune suppression is supported by the finding that disrupted *Tryp. brucei* induces increased secretion of PGE<sub>2</sub> and PGD<sub>2</sub> by fibroblasts and astrocytes, similar to endotoxin stimulation (Alafiatayo *et al.*, 1994).

In addition to immune suppression, somnolence is another typical feature of (HAT). Both have been suspected to be followed by increased PGD<sub>2</sub> synthesis. Patients that are suffering from HAT caused by *Tryp. Brucei gambiense* had three-fold higher levels of PGD<sub>2</sub> in their cerebrospinal fluid as compared to non-infected patients, while the PGE<sub>2</sub> levels are similar (Pentreath *et al.*, 1990).

In 2000 and 2002, (Kubata *et al.*) showed that trypanosomes produce PGD<sub>2</sub>, PGE<sub>2</sub> and PGF<sub>2</sub>α from arachidonic acid. These PGs led to a broad variety of different physiological effects in higher eukaryotes and their accumulation in serum coincides remarkably with symptoms observed during trypanosomiasis, such as fever, pain, immunosuppression, dysregulation of sleep/wake cycles and others. So far, it is not clear why protozoa produce PGs, but it is tempting to speculate that these parasites may have adopted the formation of PGs to modify host reactions for their own benefit. In addition, (Kubata *et al.*, 2000) also found that PGF<sub>2</sub>α was mainly

## Introduction

produced in fast dividing forms of the parasite such as the slender bloodstream form, and in the procyclic insect form and was scarcely secreted into the media. In contrast PGD<sub>2</sub>, another derivative of the PGs was mainly produced by the nondividing stumpy bloodstream forms and primarily secreted into the media. (Figarella *et al.*, 2005)

Although our current knowledge of the metabolism and function of the parasite derived eicosanoids is still scarce, it becomes evident from the available data that these mediators play an essential role in the physiology of various protozoan and metazoan parasites. The wide variety of parasites that are able to synthesize eicosanoid makes it highly probable that eicosanoid production and excretion is a common feature in a parasitic protozoa, helminths and arthropods (Dauguschies and Joachim, 2000). The relevant biochemical pathways are highly conserved and are also present in non-parasitic invertebrates, indicating that eicosanoid production by parasites was originally evolved for intrinsic physiological purposes.

### 1.3 Apoptosis and programmed cell death

In multicellular organisms, programmed cell death (PCD) and apoptosis is a specific form of PCD to control cell number for proper development and tissue homeostasis, removal of unwanted cells and functional control of the immune, haemopoietic and nervous systems (Duszenko *et al.*, 2006). The process of PCD is activated by genetically controlled cell suicide machinery (Ameisen, 1996). In vertebrate cells, PCD can be activated by external stimuli (ethanol, reactive oxygen species, and receptor ligands) and internal processes (mitotic catastrophe, replication failures, developmental programmed cell death). PCD is also a common response to cell stress caused by different toxins (Vaux 2002). Independent of the stimulus, PCD usually involves alteration in the mitochondrial membrane permeability, caspase activation, phosphatidylserine (PS) exposure, nuclear and cytoplasmic condensation, DNA fragmentation and breakage of the cell into apoptotic bodies, which are engulfed by the surrounding macrophages (Vaux and Strasser, 1996). PCD is a process found in virtually all nucleated metazoan cells, and it has been recently associated with several species of unicellular eukaryotes, notably kinetoplastids (Figarella *et al.*, 2005, 2006, Ameisen *et al.*, 1995, Welburn *et al.*, 1996, Moreira *et al.*, 1996, Ridgley *et al.*, 1999, Arnoult *et al.*, 2002, Lee *et al.*, 2002, Zangger *et al.*, 2002), yeast (Madeo. *et al.*, 1999), and amitochondrion parasites (Chose *et al.*, 2002, Mariante *et al.*, 2003).

In higher organisms, cellular growth and development can be controlled by programmed cell death (PCD), which is defined by a sequence of regulated events. However, PCD is thought to

## Introduction

have evolved not only to regulate growth and development in multicellular organisms but also to have a functional role in the biology of unicellular organisms. In protozoan parasites and in other unicellular organisms, features of PCD similar to those in multicellular organisms have been reported, suggesting some commonality in the PCD pathway between unicellular and multicellular organisms. As a matter of fact, similarities between multicellular organisms and the in the control of very basic mechanisms in cell physiology are not restricted to apoptosis, but extend to different cell biological phenomena such as cell-cycle control, multiplication, and differentiation (Nakhasi *et al.*, 2003).

It has been shown that trypanosomes can be induced to undergo apoptosis after stimulation with Con A and that the process of death is associated with *de novo* gene expression (Welburn *et al.*, 1996, Murphy & Welburn 1997). To provide an overview of the genetic changes occurring during execution of the cell death program in *T. b. rhodesiense*, Welburn & Murphy, 1998 have used a differential display method (Randomly Amplified Developmentally Expressed Sequences-PCR (RADES-PCR) for the identification of genes which are differentially expressed in cells which have been induced to die. These differentially expressed genes and their encoded products may be implicated directly or indirectly in cell death mechanism.

Prostaglandin D<sub>2</sub> induces programmed cell death in *Trypanosoma brucei* bloodstream form (Figarella *et al.*, 2006). Owing to the lack of caspases in *T. brucei*, a caspase-dependent apoptosis or paraptosis was excluded. Interestingly, we discovered five metacaspase genes in trypanosomes, which have all been cloned and heterologously expressed in yeast. One of the gene products led to a petite phenotype and induced clonal cell death in *Saccharomyces cerevisiae* (Szallies *et al.*, 2002).

It is interesting to note that caspase inhibitors (Webster *et al.*, 1990), was used to control cell density in infected animals. Slender forms produce a so far unknown differentiation factor, (Hamm. *et al.*, 1990) which, depending on cell density, induces differentiation to stumpy forms (Gull *et al.*, 2001) and (Cross, 1996).

Duszenko *et al.*, (2006), findings, indicate that PGD<sub>2</sub>-treated cells can undergo cell death that shares essential characteristics with apoptosis in higher eukaryotes and other protozoa. PGD<sub>2</sub> treatment induced nuclear segmentation, chromatin condensation and DNA degradation, was observed from Transmission electron microscopy (TEM), FACS and TUNEL test. In addition, dilation of the mitochondrion in TEM was equally observed.

It is interesting to note that caspase inhibitors do control cell density in infected animals: (1) slender forms produce a so far unknown differentiation factor, which depending on cell density, induces differentiation to stumpy forms ((Hamm *et al.*, 1990)

## Introduction

Stumpy forms produce PGD<sub>2</sub>, which induces cell death primarily of stumpy form cells. Since PGs have a short half-life and thus work as local mediators only, this model provides a mechanism for a sustained infection, because it would certify that cell density is locally regulated, leading to a controlled parasite density throughout the host. On the other hand, a sustained infection would never be in question, because dividing slender forms would not be affected by PCD. The appearance of specific antibodies would thus provide an additional mechanism to keep the parasitemia low. It should be kept in mind, however, that cell density regulation also occurs in cell culture in the absence of antibodies.

These findings suggest that PGD<sub>2</sub> secretion by trypanosomes is involved in cell cycle regulation and provide evidence for the existence of a caspase-independent Programmed cell death (PCD) in bloodstream forms of African trypanosomes

### **1.4 Proteomics Two-dimensional electrophoresis techniques**

The proteome was defined as the entire protein content expressed by a cell-type, tissue or an organism (Wilkins *et al.*, 1996). The term “proteome” was first advocated by Marc Wilkins in 1996 as a linguistic equivalent to “genome” which indicates all chromosomes and their genes of any cell type of a given organism. Genome research usually refers to sequencing the total genomic DNA of an organism and mapping all genes within these sequences. In contrast, the aim of proteome research focuses on the structural and functional analysis of the proteome and the interaction of proteins with one another. This analysis could lead the way to explain the function of an organism dynamically rather than statically.

Proteome analysis includes the identification and characterization of all proteins encoded by the genome of an organism at a given time. This is important since the protein compositions, concentrations change from cell type to cell type and within sub-cellular compartments. Moreover, they also differ between various stages of development (Abbott, 1999). Proteome analysis can also offer the opportunity to examine entire pathways, or multiple enzymatic pathways simultaneously (Lopez *et al.*, 2000). High throughput two-dimensional protein electrophoresis (Klose, 1975; O’Farrell, 1975) coupled with peptide mass fingerprinting analysis by MALDI-TOF mass spectrometry (Karas *et al.*, 1988) have become the most powerful techniques for proteome analysis.

## Introduction

Proteomics is based on 2D-electrophoresis, in which a complex protein mixture can be separated by two different biochemical principles. In the first dimension isoelectric focussing (IEF) is used, i.e. the proteins are separated according to their isoelectric points (pI). During this separation proteins run in an electric field as long as the surrounding pH differs from their pI and their net charge reaches zero. Then, they stop running in the electric field. In the second dimension, proteins are separated according to their molecular weights (MW) in a SDS-polyacrylamide gel.

The initial methodical approach to separate proteins electrophoretically by two different physio-chemical methods principles was carried out in 1956. Smithies and Poulik developed a two-dimensional (2D) electrophoresis technique combining filter paper electrophoresis (first dimension) and starch gel electrophoresis (second dimension) (Smithies, 1956). In the following years, a number of other 2D-electrophoresis methods were developed by combining various electrophoretic techniques.

Klose (1975) and O'Farrell (1975) developed the current modern 2D-electrophoresis technique. They combined isoelectric focussing (first dimension) with SDS-polyacrylamide gel electrophoresis (second dimension). This method separates proteins firstly according to their isoelectric points (pI) and secondly according to their molecular weights (MW). The 2D-electrophoresis method allows the visualization of thousands of protein-spots at a time, even up to a total of 10,000-15,000 protein spots in a single large gel (Klose *et al.*, 1995). Each protein can then be attributed a pI and a MW.

Boucherie *et al.* (1995) was the first to establish a whole proteome map using two-dimension protein electrophoresis in yeast. Four years later, Perrot *et al.*, (1999) identified more than 400 proteins on the reference proteome map. Few years later, (Garrels *et al.*, 1997), Shevchenko *et al.*, (1996b), Maillet *et al.*, (1996), Lopez. *et al.*, (2000) showed similar results.

### **1.4.1 First dimension: isoelectric focussing**

Isoelectric focussing (IEF) is the separation of proteins according to their isoelectric points (pI). The pI depicts the pH-value at which the net charge of the protein is zero. The mobility of a protein in an electric field depends on the sum of its positive and negative charges. A pH gradient can be established by adding a mixture of ampholytes with different isoelectric points to a polyacrylamide gel. The protein mixture can then be loaded either on the anodic or on the

## Introduction

cathodic end of the gel. Klose, (1975) to loaded protein samples on the acid side (pH 3) of the IEF-gel (Klose, 1995). Since some very basic proteins may not migrate into the gel if the proteins were loaded on the cathodic (pH 10) end of the gel

### **1.4.2 Second dimension: Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis**

The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) utilizes SDS as an anionic detergent. SDS forms complexes with proteins and dissociates them into their individual subunits. In SDS-protein complexes, the sizes of the proteins are directly proportional to their molecular weights. Thus the electrophoretic mobility of the SDS-protein complexes depends only on their molecular weight, i.e. the mobility of the proteins is little influenced by any individual protein feature such as their original charge or conformation (Weber *et al.*, 1969; and Laemmli, 1970). Secondly, the complexes have a high anionic charge density which is much higher than the charge density of an individual protein. Therefore the charge difference between proteins can be ignored. Due to the similar charge/protein ratio at pH > 10, all SDS-protein complexes migrate to the cathode if an electrical field is applied

### **1.4.3 Staining and Reproducibility**

To visualize the protein spots on the gel, the gel has to be stained. If the protein abundance is high (i.e. more than 100 ng), the gel can be dyed with Coomassie brilliant blue. For the detection of lesser protein amounts different silver staining protocols have been developed (Rabilloud, 1990 and 1992; Swain *et al.*, 1995; Klose, 1999). Compared to the commonly used Coomassie brilliant blue staining, silver staining is more sensitive and has an improved detection limit of 1-10 ng. Moreover, the sensitivity of silver staining can be improved further by the use of several sensitizers. These sensitizers act via different chemical mechanisms: increasing the binding of silver (sulfosalicylic acid), creating latent images of spots by precipitation of micro-granules of silver sulfide (sodium thiosulfate, dithiothreitol), promoting silver reduction (glutaraldehyde) and complexing free unbound silver cations (chelators). On the other hand, the silver staining techniques treat proteins with the strong oxidizing agent Ag<sup>+</sup> that may cause oxidative damage to the proteins. This can lead to chemical modification or destruction, which hinders subsequent protein microanalysis impossible. Several sensitizing pre-treatments of the gel with

## Introduction

glutaraldehyde, chromic acid, sodium thiosulfate could also result in covalent modifications of the proteins. Shevchenko *et al.*, (1996a) have tried to solve this problem by modifying the "classic" silver-staining protocols. They omitted fixation and sensitization treatment with glutaraldehyde that is known to attach covalently to the protein through Schiff base formation with the  $\alpha$ - and  $\epsilon$ -amino groups. Additionally they carried out the silver nitrate treatment at 4°C in order to minimize oxidation.

Reproducibility means that if the same sample is run on two or more different two-dimensional gels, each spot on one gel must have its corresponding spot on another gel. This can be influenced by many factors ranging from sample preparation, stability of electrophoresis conditions and temperature to gel staining and drying (Klose, 1975; O'Farrell, 1975). Occasionally, some single spots can change their positions within well-reproduced patterns. This phenomenon can be caused by some proteins with specific properties. The problem may be solved by using optimal conditions, i.e. running the same sample twice, side by side and by using the same batches of solutions at each step (Klose, 1995). The reproducibility of 2D-electrophoresis is reliable enough and can be used to detect genetic variations by demonstrating the qualitative and quantitative changes of protein spots (Klose, 2002; Giavalisco *et al.*, 2003).

### 1.4.4 Protein identification methods

The identification of proteins from the proteome analysis is necessary in order to connect the information from the protein spot to the corresponding gene. Mass spectrometry has a proof capacity for the identification of proteins separated by two-dimensional protein electrophoresis. It is based on the principle of peptide mass fingerprinting by trypsin digestion, in which Trypsin cleaves at the carboxylic side of arginine and lysine residues. The sizes of the peptide fragments obtained after trypsin digestion represent the peptide mass fingerprint and are characteristics for each protein.

Peptide mass fingerprinting is the most commonly used technique for protein identification by mass spectrometry. This method involves the generation of peptides from a protein by a proteolytic enzyme such as trypsin. The masses of the ensuing peptides are determined by mass-spectrometry and are matched against a theoretical list of peptide fragments calculated from databases of known protein sequences (Pennisi, 1998; ExPaSy database

<http://www.ncbi.nlm.nih>). The particular advantage of this technique is that it generally requires a limited amount of material. Sometimes even femtomoles are sufficient (Perrot *et al.*, 1999), since peptide mass fingerprinting has a higher sample throughput than amino acid sequencing. It is especially suitable for rapid protein identification

### **1.4.5 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry**

Peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is one tool for protein identification (Fernandez *et al.*, 1998). The principle of this technique is that the peptides in the sample are ionized and energized by a laser-beam and are accelerated in an electric field. The time of flight (=TOF) of each peptide fragment towards a target is measured. Since the TOF is proportional to the mass/charge ratio of each peptide, the mass of the peptide can thus be calculated. This way the mass spectra of the peptide fragments of a whole protein can be obtained. After in-gel digestion, the protein is cut into several peptide fragments by trypsin treatment. This peptide-mixture is mixed with a matrix of 2,5-dihydroxybenzoic acid or  $\alpha$ -cyano-4-hydroxycinnamic acid and is let to crystallize. Subsequently the surface of the peptide/matrix mixture is evaporated and ionized by the photons of a high-energy laser beam. The ions are then accelerated in an electrical field. The speed and the time of flight depend on the mass/charge ratio of the peptides. The time of flight can be used to exactly measure the molecular weight of each peptide fragment up to the precision of 0.1 Dalton. At first this method was a rather unspecific identification tool but improved rapidly with the advent of machines that were able to measure also high molecular weight fragments with sufficient accuracy. New matrix preparations and higher sensitivity led to higher sequence coverage (Fernandez *et al.*, 1998). In favourable cases a mass coverage of over 90% of the peptide fragments can be achieved. The high accuracy in mass determination is made possible by the “delayed extraction” method (Jensen *et al.*, 1996). Until now, bio-macromolecules with molecular masses up to 300 kDa can be identified by peptide mass fingerprinting (Nielsen *et al.*, 2002). This technique has been developed to an extent that high throughput analyses are possible, and it has a firm place for protein identification in proteomic projects (Chamrad *et al.*, 2003).



### 1.4.6 Database search based on peptide mass fingerprint spectra

The obtained spectra of peptide masses are analyzed further by searching through different databases to find the corresponding protein. Each protein in the databases can be “digested in silico” by trypsin and thus provides a theoretical spectrum of its peptide masses. Comparing the experimental with the bioinformatic data, several candidate proteins with high probability scores can be identified. This task can be performed with the help of several search engines on the internet: MASCOT, ProFound, MS-Fit, PeptIdent, PeptideSearch, and PepSea (see “The list of internet sites”).

Before the search, several parameters including the taxonomy of the specimen, the used protease and the number of accepted missed cleavages, the peptide mass states (usually the monoisotopic mass), the mass deviation tolerance, and possible modifications are chosen. Peptide modifications are important since they influence the peptide masses and might be introduced artificially in the preparation process (e.g. oxidation of methionine-residues). The oxidation of an amino acid (e.g. methionine) in a polypeptide increases its mass by 18 Da. The number of 18 Da deviations should therefore correspond to the number of methionine residues in a certain peptide. On the other hand, this “artifact” may serve as a second independent verification of the identity of a peptide. The search engine gives out a list of best matching proteins. The larger the sequence to be covered by the fragment the higher is the statistical probability of a positive peptide result. In most cases, not all peptide masses in the spectra can be matched to the theoretical digestion of a protein. Deviations between the theoretical peptide mass fingerprint and the experimental one might be due to the following reasons (Nielsen *et al.*, 2002).

- 1 Existing secondary protein structures which would hamper the enzyme to recognize a certain cleavage site even after the use of several detergents like SDS. This may result in the increase of the number of missed cleavages.
- 2 Any kind of modification for example, the modification of cysteine by acrylamide and the oxidation of methionine are the most frequent modifications that would cause a deviation of peptide mass. More than 300 different modification artefacts may be introduced during the whole procedure from protein extraction to identification. The high reactivity of the sulfhydryl group of cysteine would usually combine it with the unpolymerized acrylamide

## Introduction

during the 2D-electrophoresis. The oxidation of methionine results from its easily reducible sulfhydryl side chain.

- 3 The masses of the peptides are best calculated by using the monoisotopic peak of an internal calibration marker such as the auto-digestion product of bovine trypsin (residues 50-69,  $M+H^+ = 2163.06$  Da) and the matrix trimer ion ( $3M+H^+ = 568.14$  Da). The read-outs of the mass values are sensitively affected by the shape of the peak that depends on the mass/charge ratio ( $m/z$ ) and the strength of the reflecting electric field. This electric field has a role to focus the molecules of the same mass in order to generate a slim peak. For larger peptides, however, the peak shape of the mass spectrum usually becomes broadened by unsatisfactory focussing. This might lead to false mass calculations. The use of sodium-containing buffers can also lead to broadened peak shapes since the molecule-ion of ( $M+Na^+$ ), instead of ( $M+H^+$ ), can be detected wrongly. This would cause an increase of the  $m/z$  value.
- 4 When two protein spots lie very close to or overlap each other in the 2D-electrophoresis gel, the overlay of two mass-spectra might disturb the correct identification of the single protein

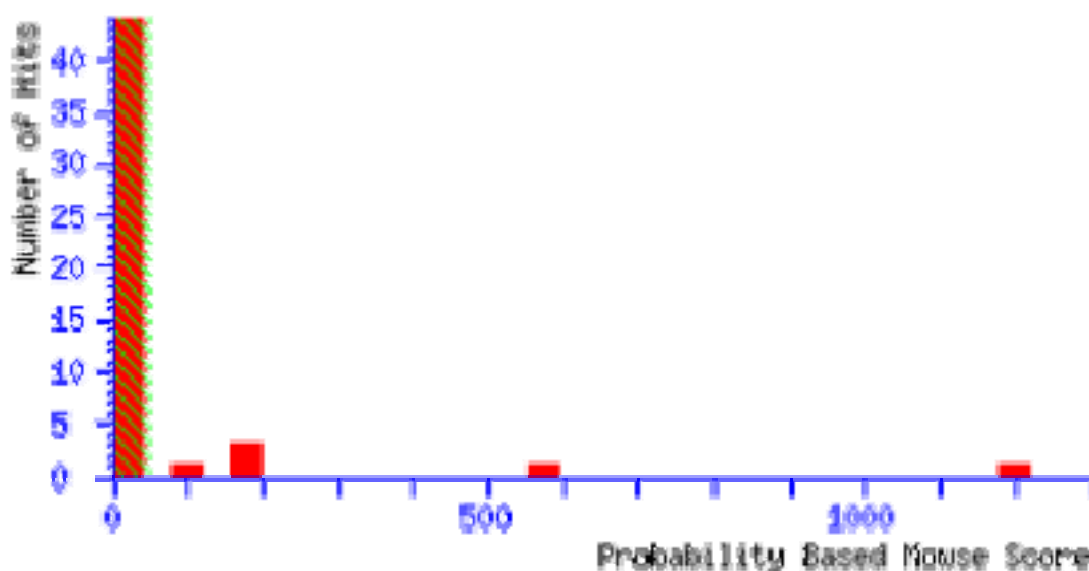
Sometimes, less stringent criteria, such as more than one allowed missed cleavage, few kinds of possible modifications, larger mass deviation tolerances have to be granted in order to match the experimental peptide mass spectra to their theoretical ones. If this is not possible, it is advisable to sequence an abundant peptide in the spectrum by MALDI-QTOF tandem mass spectrometry

## Mascot Search Results

User :  
 Email :  
 Search title : MS/MS Example  
 MS data file : C:\Auto MSMS output\Sample 1.pkl  
 Database : MSDB 20050227 (1942918 sequences; 629040812 residues)  
 Timestamp : 27 Apr 2005 at 18:35:42 GMT  
 Significant hits: [A32800](#) chaperonin GroEL precursor - human  
                   [Q803B0\\_BRARE](#) Hspd1 protein (Heat shock 60 kD protein 1).-  
 Brachydanio rerio (Zebrafish) (Danio rerio).  
                   [O46219\\_9DIPT](#) Heat shock protein 60.- Culicoides variipennis.  
                   [Q6RFF9\\_9CNID](#) Mitochondrial 60 kDa heat shock protein.-  
 Anemonia viridis.  
                   [Q6NR71\\_DROME](#) SD06594p.- Drosophila melanogaster (Fruit  
 fly).  
                   [AAK84594](#) AC084153 NID: - Caenorhabditis elegans  
                   [Q6YRM8\\_ORYSA](#) Hypothetical protein OSJNBa0091F23.13  
 (Hypothetical protein B1203H11.28) (Hypothetical protein OSJNBa0073J19.5).-  
 Oryza sativa (japonica cultivar-group).

## Probability Based Mowse Score

Ions score is  $-10 \cdot \log(P)$ , where P is the probability that the observed match is a random event. Individual ions scores > 46 indicate identity or extensive homology ( $p < 0.05$ ). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



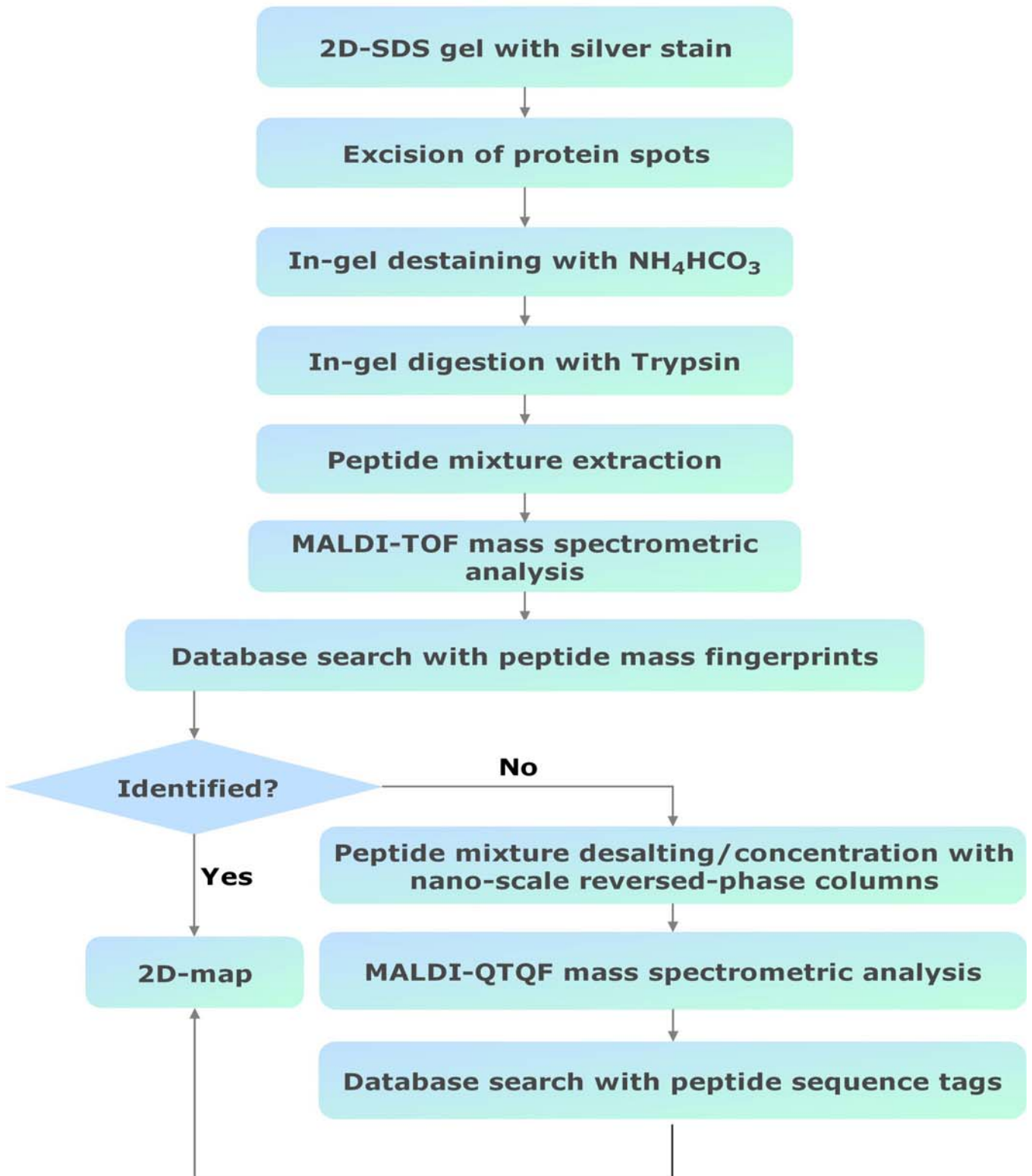
## MASCOT Peptide Mass Fingerprint

|   |   |  |   |
|---|---|--|---|
| <a href="#">Your name</a>                       | <input type="text" value="Leo-Elgie"/>  | <a href="#">Email</a>                  | <input type="text"/>  |
| <a href="#">Search title</a>                    | <input type="text"/>  |  |   |
| <a href="#">Database</a>                        | MSDB  |  |   |
| <a href="#">Taxonomy</a>                        | All entries   |  |   |
| <a href="#">Enzyme</a>                          | Trypsin   | <a href="#">Allow up to</a>            | 1 missed cleavages  |
| <a href="#">Fixed modifications</a>             | <input type="checkbox"/> Acetyl (K)<br><input type="checkbox"/> Acetyl (N-term)<br><input type="checkbox"/> Amide (C-term)<br><input type="checkbox"/> Biotin (K)<br><input type="checkbox"/> Biotin (N-term) | <a href="#">Variable modifications</a> | <input type="checkbox"/> Acetyl (K)<br><input type="checkbox"/> Acetyl (N-term)<br><input type="checkbox"/> Amide (C-term)<br><input type="checkbox"/> Biotin (K)<br><input type="checkbox"/> Biotin (N-term) |
| <a href="#">Protein mass</a>                    | <input type="text"/> kDa  | <a href="#">Peptide tol. ±</a>         | 1.0 Da  |
| <a href="#">Mass values</a>                     | <input checked="" type="checkbox"/> MH <sup>+</sup> <input type="checkbox"/> M <sub>r</sub> <input type="checkbox"/> M-H  | <a href="#">Monoisotopic</a>           | <input checked="" type="checkbox"/> Average   |
| <a href="#">Data file</a>                       | <a href="#">Query</a> <input type="text" value="Trypsin"/><br>NB Contents of this field are ignored if a data file is specified.  |  |   |
| <a href="#">Overview</a>                        | <input type="checkbox"/>  | <a href="#">Report top</a>             | 20 hits   |
| <input type="button" value="Start Search ..."/> |   |  |   |

[Q803B0\\_BRARE](#)    **Mass:** 61158    **Score:** 594    **Queries matched:** 13

|                    |           |           |           |        |     |     |         |   |                         |
|--------------------|-----------|-----------|-----------|--------|-----|-----|---------|---|-------------------------|
| <a href="#">15</a> | 451.2499  | 900.4853  | 900.5280  | 0.0427 | - 0 | 52  | 0.025   | 1 | K.LSDGVAVIK.V           |
| <a href="#">16</a> | 456.7806  | 911.5467  | 911.5803  | 0.0337 | - 0 | 59  | 0.0041  | 1 | K.VGLQVVAVK.A           |
| <a href="#">21</a> | 480.7447  | 959.4748  | 959.5036  | 0.0288 | - 0 | 45  | 0.11    | 1 | R.VTDALNATR.A           |
| <a href="#">27</a> | 617.2857  | 1232.5569 | 1232.5884 | 0.0315 | - 0 | 81  | 2.7e-05 | 1 | K.VGGTSDVEVNEK.K        |
| <a href="#">31</a> | 672.8375  | 1343.6605 | 1343.7085 | 0.0480 | - 0 | 64  | 0.001   | 1 | R.TVIEEQSWGSPK.V        |
| <a href="#">51</a> | 1057.0537 | 2112.0929 | 2112.1322 | 0.0303 | - 0 | 116 | 4.6e-08 | 1 | R.ALMLQGVDLLADAVAVTMGPK |

## Strategy of protein identification



### 1.5 Objective of this work

The present study is to identify prostaglandin-synthases or related enzymes in the arachidonic acid metabolic pathway and to establish a reference proteome map for *T. brucei*. This reference map and database can be later used to compare deviating protein patterns between the different life stages of these parasites and to identify proteins that may be responsible for the cell division and infectious nature in *T. brucei*.

The aim of proteome research focuses on the structural and functional analysis of the proteome and the interaction of proteins with one another. This includes the isolation, identification and characterization of all proteins encoded by the genome of an organism. In contrast, genome research usually refers to sequencing the total genomic DNA of an organism and mapping all genes within these sequences. Proteome analysis could lead the way to explain the function of an organism dynamically rather than statically (Sperling, 2001). This is important since the protein compositions and concentrations change from cell type to cell type, even within sub-cellular compartments. Moreover, they also differ between various stages of development (Abbott, 1999). Proteome analysis can also offer the opportunity to examine entire pathways, or multiple enzymatic pathways simultaneously (Lopez *et al.*, 2000).

Previous studies in our laboratory has, shown that *T. Brucei* produces Prostaglandins which has been shown to play a vital role in the cell cycle regulation and differentiation of these parasites (Figeralla *et al.*, 2005). The synthesis of these proteins increases when *T. brucei* are been induced with arachidonic acid in a growth medium.

The objective of this work was to establish a method to identify *T. brucei* proteins as little as possible from different fractions and equally a reference proteome map for *T. brucei*. The map and database can be later used to compare deviating protein patterns. This might direct the attention to disease-specific proteins, genes, open new ways to diagnose and treatment of “HAT” diseases on proteome level or with a combined genomic and proteomic approach.

## Materials and methods.

### 2.1 Materials

#### 2.1.1 General chemicals

|   |                                      |
|---|--------------------------------------|
| Acetic acid                                 | Roth, Karlsruhe                      |
| Acetonitrile                                | Merck, Eurolab GmbH, Mannheim        |
| Acrylamide (Rotiphoresis Gel 30; 37:5:1)    | Roth, Karlsruhe                      |
| Albumin (BSA)                               | Sigma, Deisenhofen                   |
| Agarose                                     | Sigma, Deisenhofen                   |
| Ammonium persulphate (APS)                  | Merck, Eurolab GmbH, Mannheim        |
| Ampicilin                                   | Roth, Karlsruhe                      |
| Arachidonic acid                            | Sigma, Deisenhofen                   |
| Arachidonic acid ( <sup>14</sup> C)         | Cayman Chemicals, IBL, Hamburg       |
| ATP   | Sigma, Deisenhofen                   |
| BCA protein assay reagent (Reagent A and B) | Pierce                               |
| BenchMark™ prestained protein marker        | Gibco BLR Life Technology, Karlsruhe |
| Bisbenzimidazole                            | Sigma, Deisenhofen                   |
| BMA GelBond PAGfilm                         | Biowhittaker, Walkersville, MD, USA  |
| Bromophenol blue                            | Roth, Karlsruhe                      |
| 3-[(cholamidopropyl)-dimethylammonio]       | Sigma, Deisenhofen                   |
| -1-propanesulfonate (CHAPS).                |                                      |
| Complete*, Protease-Inhibitor               | Roche, Mannheim                      |
| Coomassie brilliant blue G 250              | Roth, Karlsruhe                      |
| DEAE-Sephacel                               | Sigma, Deisenhofen                   |
| Dimethyl sulfoxide (DMSO)                   | Sigma, Deisenhofen                   |
| Dithioerythritol (DTT)                      | Roth, Karlsruhe                      |
| Formaldehyde (37%)                          | Sigma, Deisenhofen                   |
| Glycerol (87%)                              | Merck, Eurolab GmbH, Mannheim        |
| Glycine                                     | Merck, Eurolab GmbH, Mannheim        |
| Hybond-EECL Nitocellulose membrane          | Amersham Bioscience                  |
| Immobilien stripes                          | Amersham Bioscience                  |
| Iodoacetamide                               | Serva, Heidelberg                    |
| MEM vitamin solution                        | Sigma, Deisenhofen                   |
| Pager-Ruler protein ladder                  | Fermentas                            |
| Penicillin/Streptomycin solution            | Sigma, Deisenhofen                   |
| Protein molecular marker                    | Sigma, Deisenhofen                   |
| Protein A/G Agarose                         | Sigma-Aldrich                        |
| Sephadex-G75                                | Pharmacia, Muenchen                  |
| Superdex -75                                | Pharmacia, Muenchen                  |
| Sodium dodecyl sulphate                     | Roth, Karlsruhe                      |
| N,N,N,N-tetramethylaminomethane (TEMED)     | Roth, Karlsruhe                      |
| Trifluoroacetic acid (TFA)                  | Merck, Eurolab GmbH, Mannheim        |
| Tris-hydroxymethyl-aminomethane (TRIS)      | Serva, Heidelberg                    |
| Trypsin                                     | Promega                              |
| Triton X-100                                | Sigma, Deisenhofen                   |

## Materials and methods

|                                    |                               |
|------------------------------------|-------------------------------|
| Urea                               | Roth, Karlsruhe               |
| Vetren* 200                        | Roth, Karlsruhe               |
| Zinc-Chloride (ZnCl <sub>2</sub> ) | Merck, Eurolab GmbH, Mannheim |
| Zip Tip (C <sub>18</sub> )         | Milipore, Germany             |

### 2.1.2 Radiochemicals

|                                       |                                  |
|---------------------------------------|----------------------------------|
| [1- <sup>14</sup> C] Arachidonic acid | Cayman Chemicals, IBL, Hamburg   |
| Prostaglandin Kit                     | Oxford Biomedical Research, USA. |

### 2.1.3 KITs

|                                      |                                 |
|--------------------------------------|---------------------------------|
| 2D gel clean kit                     | Amersham Bioscience.            |
| BCA Protein determination kit        | Pierce                          |
| Prostaglandin (COX) <b>assay kit</b> | Oxford Biomedical Research, USA |
| Amplify™ Western detection kit       | Roth, Karlsruhe                 |

### 2.1.4 Equipments

|   |  |
|---|--|
| Autoclave                               | Müchener Medizin Mechanik GmbH           |
| Biofuge A                               | Heraeus, Osterode                        |
| Electrophoresis power supply (EPS 1001) | Amersham Pharmacia Biotech               |
| MALDI-TOF mass spectrometer             | (Reflex II from Bruker-Daltonik, Bremen) |
| Melanie 2D gel analysis                 | Swiss Bioinformatics                     |
| Microfuge E                             | Beckmann, München                        |
| New Block 2-2503                        | Neo-Lab, Germany                         |
| Sigma 302K Labor Centrifuge             | Sigma, Deisenhofen                       |
| Sonifer cell disruptor B-30             | Schwäbisch-Gmünd                         |
| Suprafuge 22 with Rotor HFA 22.50       | Heraeus, Osterode                        |
| Table Centrifuge Denver Force 13        | Denver Inst. Company, USA                |
| Ultral Centrifuge TL 100                | Beckmann, München                        |

### 2.1.5 Electrophoresis and Western Blotting

|  |   |
|--|---|
| Electrophoresis Minigel system         | Hofer Scientific Instrument, San Francisco, USA |
| ‘Mighty small II’ SE 250               | Biorad, München                                 |
| Semi-Dry Electrophoretic transfer Cell | Amersham Pharmacia Biotech                      |
| Dry Stripes holder                     | Amersham Pharmacia Biotech                      |
| Rehydration chamber                    | Amersham Pharmacia Biotech                      |
| Ettan Dalt six                         | Amersham Pharmacia Biotech                      |
| Ettan-IPGhor                           | Amersham Pharmacia Biotech                      |



## Materials and methods

### 2.1.6 Fluorography

Ad-aware software 6.0  
Fluorescent Imaging Analyzer FLA 3000  
Konica SRX 101A  
Scan Jet 5370 C

Fuji Photo Film Company, Japan  
Fuji Photo Film Company, Japan  
Hannover-Germany  
Germany

### 2.1.7 Other Consumable Materials

6, 24, and 96-well plate  
BioMax MR-1 *scientific imaging film*  
BioMax Light *scientific imaging film*  
Culture flask  
Dialysis tube  
Glasfaser filter GF/C  
HybondECL™ nitrocellulose membrane  
Kryo-tubes  
Microcon 30 ultrafiltration units  
Microplate  
Parafilm  
Sterile tube, Falcon\* 15ml, 50ml  
Whatman 3 MM paper  
Sterile filter SFCA-membrane  
Thin Layer Chromatography sheets  
(Kiesegel 60F DC-platen  
Neubauer Cell-Counter (0.02 mm)  
Sonicator (Transsonic 310)  
Spectrophotometer (MRXTC-Revelation)  
Spectrophotometer (UV-120-01)

Greiner, Frickenhausen  
Kodak, Stuttgart  
Kodak, Stuttgart  
Greiner, Frickenhausen  
Serva, Heidelberg  
Whatman, Maidstone, USA  
Ammersham, Braunschweig  
Greiner, Frickenhausen  
Amicon, Witten  
Nunc, Denmark  
American Can Co, Chicago  
Becton Dickinson, New Jersey  
Whatman, Maidstone, USA  
Nalge, Hereford (UK)  
Merck, Mannheim  
  
Brand, Wertheim  
  
Dynex, Denkendorf  
Shimadzu, Duisburg

## 2.2 Media, Buffers and Solutions

### 2.2.1 Cultivation and isolation of Trypanosomes

#### Basic medium, pH 7.4

(According to Eagle, 1959, modified by Duszenko *et al*, 1985, 1992)

|   | [mg/l] |
|---|--------|
| CaCl <sub>2</sub> x H <sub>2</sub> O                | 265    |
| KCl   | 400    |
| MgSO <sub>4</sub> x 7H <sub>2</sub> O               | 200    |
| NaH <sub>2</sub> PO <sub>4</sub> x H <sub>2</sub> O | 140    |
| NaCl  | 6800   |
| HEPES   | 7140   |
| Phenol Red  | 10     |

These chemicals were dissolved in water and the pH was adjusted to 7.4

|                                    |     |
|------------------------------------|-----|
| L-Arginine/HCl                     | 126 |
| L-Cysteine                         | 24  |
| L-Histidine/HCl x H <sub>2</sub> O | 42  |
| L-Isoleucine                       | 52  |
| L-Leucine                          | 52  |
| L-Lysine                           | 73  |
| L-Methionine                       | 15  |
| L-Phenylalanine                    | 100 |
| L-Threonine                        | 48  |
| L-Tryptophan                       | 10  |
| L-Tyrosine                         | 100 |
| L-Valine                           | 46  |
| L-Ornithine/HCl                    | 10  |
| L-Adenosine                        | 12  |

These amino acids and adenosine were dissolved separately, mixed together and then added to following stock solutions:

|                                       | [ml/l] |
|---------------------------------------|--------|
| MEM nonessential amino acids solution | 10     |
| MEM vitamine solution                 | 10     |

This so called basic medium was sterilized and could be stored at 4°C for up to three months

#### Citrat-Glucose-Anticoagulant (CGA) pH 7.7

|   |       |
|---|-------|
| Na <sub>3</sub> -citrat x 2H <sub>2</sub> O | 10 mM |
| Glucose                                     | 40 mM |

## Materials and methods

### Separation buffer, pH 8.0 (Lanham & godfrey, 1970)

|  |       |
|--|-------|
| Na <sub>2</sub> HP0 <sub>4</sub> x 2H <sub>2</sub> O | 57 mM |
| KH <sub>2</sub> P0 <sub>4</sub>                      | 3 mM  |
| NaCl   | 44 mM |
| Glucose  | 55 mM |

### Complete culture medium

For preparation of a complete culture medium the following stock solutions were added to the basic medium.

|  |                                 | [ml/l] |
|--|---------------------------------|--------|
| NaHC03                                     | (750mg/10ml distilled water)    | 30     |
| L-Cysteine                                 | (30.3mg/10ml distilled water)   | 10     |
| Bathocuproindi-Sulfonate (BCS)             | (5.6mg/10ml distilled water)    | 1      |
| Hypoxanthine                               | (13.6mg/10ml 0.1N NaOH)         | 10     |
| 2`-Desoxythymidine                         | (3.9mg/10ml distilled water)    | 10     |
| L-Glutamine                                | (292mg/10ml distilled water)    | 10     |
| Sodiumpyruvate                             | (220mg/ 10ml distilled water)   | 10     |
| Myristic acid linked on defatted BSA (50x) |                                 | 20     |
| Fetal calf serum                           | (heat inactivated: 30min: 56°C) | 100    |
| Penicillin-Streptomycin-solution           |                                 | 5      |

Preparation of the myristic acid linked to defatted BSA (Ferguson & Cross 1984): 24 mg myristic acid was dissolved in 100µl ethanol (95%). This myristic acid was then gradually added to a BSA solution (1 g defatted BSA/20ml distilled water). The medium was sterilized and stored at 4°C for about 4 weeks.

### Culture medium for Procyclics pH 7.4

The following stock solutions were added to the basic medium to cultivate of procyclic parasites:

|                                  |                                 | [ml/l] |
|----------------------------------|---------------------------------|--------|
| Glutmine                         | (5.6mg/10ml distilled water)    | 10     |
| Haemin                           | (25mg/10ml distilled water)     | 3      |
| Pyruvate                         | (220mg/10ml distilled water)    | 10     |
| Proline                          | (600mg/10ml distilled water)    | 10     |
| Fetal calf serum                 | (heat inactivated. 30min. 56°C) | 100    |
| Penicillin-Streptomycin-solution |                                 | 5      |

The culture medium was sterilized and stored at 4°C for up to 4 weeks

### 2.2.2 Lysate preparation from *T. brucei*

Cytosolic protein fraction and Membrane protein fraction.

#### Cytosolic protein lysis buffer

|                              |                    |
|------------------------------|--------------------|
| ZnCl <sub>2</sub>            | 200 µM             |
| Tris-HCl (pH 7.2)            | 10 mM              |
| Protease Inhibitor(Complete) | 1 tablet in 25 ml. |

#### Membrane protein lysis buffer

|                              |                   |
|------------------------------|-------------------|
| Tris-HCl (pH 7.2)            | 10 mM             |
| Trinton X 100                | 0.25% (v/v)       |
| Protease Inhibitor(Complete) | 1 tablet in 25 ml |

### 2.2.3 Prostaglandin D Synthase assay.

Assay Solution, 2.5µl phenol in 1ml Tris-HCl (pH 7.4, 100 mM)

### 2.2.4. SDS-PAGE According to Laemmli.

| <u>Running gel (2.6% C)</u> | (10% T) | (12% T)  |
|-----------------------------|---------|----------|
| Rotiphoresis Gel 30         | 15.2 ml | 18.4 ml  |
| 1M Tris-HCl pH 8.8          | 17.2 ml | 17.2 ml  |
| Distilled water             | 13.2 ml | 10.16 ml |
| 10% SDS                     | 456 µl  | 456 µl   |
| 105 Ammonium peroxodisulfat | 156 µl  | 156 µl   |
| TEMED                       | 48 µl   | 48 µl    |

#### Stacking gel

|                             |         |
|-----------------------------|---------|
| Rotiphoresis Gel 30         | 4.0 ml  |
| 1M Tris-HCl pH 8.8          | 3.0 ml  |
| Distilled water             | 16.8 ml |
| 10% SDS                     | 24 0µl  |
| 10% Ammonium peroxodisulfat | 12 0µl  |
| TEMED                       | 24 µl   |

#### Sample buffer 2x

|                 |        |
|-----------------|--------|
| Tris-HCl pH 6.8 | 180 mM |
| SDS             | 4.4%   |
| Glycerol        | 19.4%  |

## Materials and methods

### DTT/BPB (20x)

|                 |      |
|-----------------|------|
| DTT             | 1 M  |
| Bromophenolblue | 0.2% |

### Running buffer

|          |        |
|----------|--------|
| Tris-HCl | 25 mM  |
| Glycine  | 192 mM |
| SDS      | 0.1%   |

### **2.2.4.1 Coomassie Staining**

#### Staining Solution

|                            |           |
|----------------------------|-----------|
| Coomassie Brilliant Blue R | 0.1%      |
| Ethanol                    | 40% (v/v) |
| Acetic acid                | 10% (v/v) |

#### Destaining Solution

|             |           |
|-------------|-----------|
| Acetic acid | 10% (v/v) |
| Ethanol     | 40% (v/v) |

### **2.2.4.2 Silver Staining**

#### Fixative

|                    |                 |
|--------------------|-----------------|
| Acetic acid        | 24ml            |
| Ethanol            | 80ml            |
| Formaldehyde (37%) | 100µl           |
| Distilled water    | <i>ad</i> 200ml |

#### Wash Solution

|         |           |
|---------|-----------|
| Ethanol | 50% (v/v) |
|---------|-----------|

#### Incubation Solution

|  |                  |
|--|------------------|
| 10% Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> ·5H <sub>2</sub> O | 400 µl           |
| Distilled water  | <i>ad</i> 200 ml |

#### Silver Staining

|                    |                  |
|--------------------|------------------|
| Silver nitrate     | 0.4 g            |
| Formaldehyde (37%) | 150 µl           |
| Distilled water    | <i>ad</i> 200 ml |

#### Developing Solution

## Materials and methods

|  |                  |
|--|------------------|
| Na <sub>2</sub> CO <sub>3</sub>                    | 18 g             |
| 10% Na <sub>2</sub> S <sub>2</sub> CO <sub>3</sub> | 12 µl            |
| Formaldehyde (37%)                                 | 150 µl           |
| Distilled water                                    | <i>ad</i> 300 ml |

### Stop Solution

|                 |                  |
|-----------------|------------------|
| Acetic acid     | 24 ml            |
| Ethanol         | 80ml             |
| Distilled water | <i>ad</i> 200 ml |

### Gel storage Solution

|          |           |
|----------|-----------|
| Ethanol  | 25% (v/v) |
| Glycerol | 2% (v/v)  |

## 2.2.5 Western- Blotting

### Transfer buffer, pH 9.2 (Bjerruma Schafer-Nielsen, 1986)

|          |          |
|----------|----------|
| Tris/HCl | 48 mM    |
| Glycine  | 39 mM    |
| SDS      | 0.00375% |
| Methanol | 20%      |

### Blocking buffer

|                             |     |
|-----------------------------|-----|
| Defatted milk power in PBST | 10% |
|-----------------------------|-----|

### PBS

|                                  |        |
|----------------------------------|--------|
| Na <sub>2</sub> HP0 <sub>4</sub> | 80 mM  |
| NaH <sub>2</sub> P0 <sub>4</sub> | 20 mM  |
| NaCl                             | 100 mM |

### PBS-Tween

|       |      |
|-------|------|
| Tween | 0.1% |
|-------|------|

### Stripping buffer, pH 6.7

|                   |         |
|-------------------|---------|
| 2-mercaptoethanol | 100 mM  |
| SDS               | 2%      |
| Tris-HCl          | 62.5 mM |

## 2.2.6 Thin Layer Chromatography

### Developing Solvent

|               |       |
|---------------|-------|
| Diethyl-ether | 90 ml |
| Methanol      | 2 ml  |
| Acetic acid   | 1 ml  |

### Stop Solvent

## Materials and methods

|               |             |
|---------------|-------------|
| Diethyl-ether | 30 ml       |
| Methanol      | 4 ml        |
| Citric acid   | 1ml (100mM) |

### 2.2.7 2-Dimensional Gel Electrophoresis

#### Lysis Buffer

|                               |                         |
|-------------------------------|-------------------------|
| Urea                          | 9 M                     |
| CHAPS                         | 4% (w/v)                |
| DDT                           | 1% (w/v)                |
| Complete (protease-inhibitor) | 4mM (1 tablet in 25 ml) |
| Pharmalyte (3-10)             | 2% (v/v)                |
| Trinton X- 100                | 0.1% ( v/v)             |

#### Rehydration Solution (450µl on 24 cm strip)

|                   |                   |
|-------------------|-------------------|
| Urea              | 8 M               |
| CHAPS             | 1%                |
| DDT               | 50 mM             |
| Pharmalyte        | 0.5% (v/v)        |
| IPG buffer (3-10) | 2.25 µl(0.2% w/v) |
| Brilliant Blue.   | 0,002%            |

#### Equilibration buffer

|                 |                 |
|-----------------|-----------------|
| Urea            | 6 M             |
| SDS             | 2%              |
| Tris-HCl pH 8.8 | 50mM (3,5% v/v) |
| Glycerol        | 30% (v/v)       |

#### Equilibration buffer 1

|                                    |        |
|------------------------------------|--------|
| DDT (Equilibration buffer in10 ml) | 100 mg |
|------------------------------------|--------|

#### Equilibration buffer 2

|   |        |
|---|--------|
| Iodoacetamide (Equilibration buffer in 10 ml) | 250 mg |
|---|--------|

## Materials and methods

### Agarose sealing gel

|                |            |
|----------------|------------|
| Tris           | 25 mM      |
| Glycine        | 192 mM     |
| SDS            | 0.1% (w/v) |
| Agarose        | 0.5%       |
| Brilliant-blue | 0.002%     |

### Preparation of Homogeneous Gel (10%).

|                                  |            |
|----------------------------------|------------|
| Acrylamide (Rotiphoresis Gel 30) | 150 ml     |
| Tris-HCl.(1.5 M pH 8.8).         | 113 ml     |
| Distilled water                  | 178 ml     |
| 10% SDS                          | 4.5 ml     |
| 10% APS                          | 4.5 ml     |
| TEMED                            | 82 $\mu$ l |

### Water saturated butanol

|                                  |       |
|----------------------------------|-------|
| Butanol ( <i>n</i> or <i>t</i> ) | 50 ml |
| Distilled water                  | 10 ml |

### Gel storage solution

|                   |         |
|-------------------|---------|
| Tris-HCl (pH 8.8) | 0.375 M |
| SDS               | 0.1%    |

### SDS electrophoresis running buffer

|                   |        |
|-------------------|--------|
| Tris-HCl (pH 8.8) | 250 mM |
| Glycine           | 1.92 M |
| SDS               | 1%     |

### 2.2.8 In-gel trypsin digestion.

#### Solution A

|                      |       |
|----------------------|-------|
| Ammonium Bicarbonate | 50 mM |
|----------------------|-------|

#### Solution B

|                      |       |
|----------------------|-------|
| Acetonitrile         | 50%   |
| Ammonium Bicarbonate | 25 mM |



## Materials and methods

### Solution C

|                      |       |
|----------------------|-------|
| DTT                  | 10 mM |
| Ammonium Bicarbonate | 50 mM |

### Solution D

|                      |        |
|----------------------|--------|
| Iodoacetamide        | 100 mM |
| Ammonium Bicarbonate | 50 mM  |

### Solution E

|                      |       |
|----------------------|-------|
| Ammonium Bicarbonate | 25 mM |
|----------------------|-------|

### Solution F

|              |     |
|--------------|-----|
| Formic acid  | 5%  |
| Acetonitrile | 50% |

## **2.2.9 Desalting and determination of protein concentration**

### Wetting solution

|                    |      |
|--------------------|------|
| Acetonitrile (ACN) | 100% |
|--------------------|------|

### Equilibration Solution and Wash Solution

|                              |      |
|------------------------------|------|
| TFA (in Milli-Q grade water) | 0.1% |
|------------------------------|------|

### Elution Solution

|                    |      |
|--------------------|------|
| Acetonitrile (ACN) | 50%  |
| TFA                | 0.1% |

## **2.2.10 Immunoprecipitation**

TSA (tyramide signal amplification)

## Materials and methods

|                     |             |
|---------------------|-------------|
| Tris- HCl (pH 8.09) | 10mM        |
| NaCl                | 0.14 M      |
| NaN <sub>3</sub>    | 0.025 (w/v) |

### Dilution buffer

|              |            |
|--------------|------------|
| Triton X-100 | 0.1% (v/v) |
| BSA          | 0.1% (w/v) |

## 2.3 Methods

### General Methods

#### 2.3.1. Protein quantitation assay

The BCA working reagent, was prepared by mixing 50 parts of BCA protein assay reagent A (contains BCA) with one part of reagent B (contains CuSO<sub>4</sub>). Then 25 µl of each standard sample or diluent (as empty control) were pipetted into wells of a microwell-plate. 400 µl working reagent were added into each well sequentially. The plate was then covered and incubated at 37°C for 30 mins in a water bath. After incubation, the plate was cooled to room temperature before final measurement. The protein concentration was measured colorimetrically at  $\lambda=570$  nm with a spectrophotometer. The program “Revelation Version 2.0” provided by the manufacturer was used for data processing

#### Bicinchoninic acid (BCA) protein assay and preparation of diluted BSA serial standards

The BSA standards were prepared by diluting a 2.0 mg/ml BSA stock solution serially diluted with the same diluent as a list of standard dilutions with a working range from 20 µg/ml to 2000 µg/ml is shown below:

## Materials and methods

| <b>Volume of BSA</b>                | <b>Volume of diluent</b> | <b>Final BSA concentration</b> |
|-------------------------------------|--------------------------|--------------------------------|
| 300 $\mu$ l of Albumin Standard     | 0 $\mu$ l                | 2000 $\mu$ g /ml               |
| (A) 375 $\mu$ l of albumin standard | 125 $\mu$ l              | 1500 $\mu$ g /ml (A)           |
| (B) 325 $\mu$ l of albumin standard | 325 $\mu$ l              | 1000 $\mu$ g /ml (B)           |
| (C) 175 $\mu$ l of (A)              | 175 $\mu$ l              | 750 $\mu$ g /ml (C)            |
| (D) 325 $\mu$ l of (B)              | 325 $\mu$ l              | 500 $\mu$ g /ml (D)            |
| (E) 325 $\mu$ l of (D)              | 325 $\mu$ l              | 250 $\mu$ g /ml (E)            |
| (F) 325 $\mu$ l of (E)              | 325 $\mu$ l              | 125 $\mu$ g /ml (F)            |
| (G) 100 $\mu$ l of (F)              | 400 $\mu$ l              | 25 $\mu$ g /ml (G)             |

### 2.3.1.2. SDS-PAGE according to Laemmli.

SDS-PAGE was accomplished in Mighty Small II SE 250 gel chambers according to the method of Laemmli (Laemmli, 1970) with 4% stacking gel and 10% or 12% running gel. For determination of the molecular mass scale suitable molecular markers were used.

### 2.3.1.3 Commassie Staining

Following electrophoresis, the gel was stained in 1 mg/ml Commassie Blue R for at least 4 h. The gel was destained in destaining solution for about 2h.

### 2.3.1.4. Silver staining

Silver staining is a very sensitive method with a detection limit between 1-10 ng. It is based on the high reducibility of silver ions. The silver ions form complexes with proteins much stronger than with the polyacrylamide gel. Complexed silver ions can be reduced much faster than free silver ions. During the whole procedure of silver staining, the gels were shaken continuously. The gel was fixed in fixative for 10 min and washed with ethanol (50%) 3 times for 10 min each, and then incubated 1 min in incubation buffer. During this period, the sodium thiosulfate in the solution acts as complexing agents and links the proteins by forming covalent bonds. The gel was washed with water 3 times for 20 sec. The gel was then

## Materials and methods

incubated in silver stain solution for 20 min and washed 2 min with water. Thereafter the gel was soaked in developing solution until bands or protein spots appeared. The gels were treated with stop solution and washed with 50% for 20 min.

### 2.3.1.5 Gel drying and preserving

After staining the results had to be stored and the gels had to be preserved. Still wet, the gels were scanned on a transilluminating scanner and stored as TIFF-files with a resolution of 200-300 dpi. Later the gels were preserved. The gels were “sandwiched” between two sheets of wet cellophane in gel storage solution (25% ethanol and 2% glycerol). Excess solution as well as air bubbles between the cellophane sheets and the gel was expelled with a ruler.

### 2.3.1.6 In-gel digestion

Protein spots were excised from the gel with a skin-biopsy punch or blue tip cut at the and placed into the washed solution in 25 mM ammonium bicarbonate/50% acetonitrile solution. After shaking 2h at room temperature, remove excess liquid, wash for 5 min with 200ul 50% acetonitrile / 50% 50 mM  $\text{NH}_4\text{HCO}_3$ , then 5 minutes with 100% ACN to dehydrate gel piece. Vortex during incubations. Remove ACN, and either let air-dry on bench or in fume hood for 10 mins, or evaporate briefly in speedvac, without heat for about 15 mins.

Remove excess liquid, wash for 5 min with 200ul 50% acetonitrile / 50% 50 mM  $\text{NH}_4\text{HCO}_3$ , then 5 min with 100% ACN to dehydrate gel pieces. Vortex during incubations, Remove ACN, and either let air-dry on bench or in fume hood for 10 min, or evaporate briefly in speed-vac, without heat. The gel pieces should be noticeably shrunken and probably white.

Cover gel pieces with 50 ul 10 mM DTT in 100 mM  $\text{NH}_4\text{HCO}_3$ . Vortex, spin briefly, reduce proteins for 45-60 mins at 56°C. Cool to room temp (20°C), remove DTT solution and add 50 ul of 55 mM iodoacetamide in 100 mM  $\text{NH}_4\text{HCO}_3$ . Vortex and spin briefly, incubate for 45 min in dark place at room temp. Remove iodoacetamide, discard. Wash gel pieces with 100 ul of 100 mM  $\text{NH}_4\text{HCO}_3$  for 5 minutes with vortexing, then twice with 50% ACN / 50% 50 mM  $\text{NH}_4\text{HCO}_3$  for 5 minutes with vortexing. Dehydrate with 100ul acetonitrile as above.

## Materials and methods

Remove remaining liquid and speedvac dry if necessary. Add enough 12.5 ng/ul trypsin solution to just cover the gel pieces. This volume will vary but is usually around 20ul. Rehydrate the gel pieces at 4 °C for 30 min in buffer containing 50 mM NH<sub>4</sub>HCO<sub>3</sub> and trypsin. The reason for keeping it cold is to get as much active trypsin sucked into the gel as possible before it starts autodigesting itself. Spin briefly, add more NH<sub>4</sub>HCO<sub>3</sub> to cover gel pieces, typically another 25ul. Digest overnight at 37 °C (or at least for 4h). Transfer the digest solution supernatant (if any) into a clean 0.65 mL tube.

### 2.3.1.7 Sample purification Zip Tip (C<sub>18</sub>)

Sample purification was performed according to a protocol by Millipore (2001). A long, narrow pipette tip packed with 0.3 ml (C<sub>18</sub>) reversed-phase medium served as a chromatography column. Sample liquid was driven through the column by using 10µl pipette. Prior to use, the column was washed with 15 µl of acetonitrile-0.1 % TFA (8:2 v/v) followed by an equilibration step with 10 µl of 0.1% (v/v) TFA. The peptide sample was acidified with 1 µl 2% (v/v) TFA to obtain a final concentration of about 0.1-0.52% (v/v). Then the sample was loaded onto the column and was slowly pumped over the reversed-phase medium. A washing step was performed with 10 µl 0.1% TFA, and the column was emptied completely by pressing air through it for a few seconds. Finally, 3µl matrix as an eluent was evaporated with pressurised air. 1µl was loaded directly onto the target of the MALDI-QTOF mass spectrometer.

### 2.3.1.8 Western blotting analyses.

Western blotting was performed using a *Semi-Dry-Apparatus*. Three filter papers were laid on the anode plate. Then nitrocellulose membrane, gel and three filter papers were added one after another. All the materials were soaked with transfer buffer before use. The electro-transfer was carried out by a constant electric current of 5mA/cm<sup>2</sup> for 30 mins.

Cell lysate from bloodstream form cells were separated through SDS-PAGE. Affinity purified sheep anti-PGDS were used as primary antibody and peroxidase conjugated with goat anti-rabbit IgG was used as second antibody.

## Materials and methods

### 2.3.1.9 Fluorography.

After gel electrophoresis and gel blotted, the membrane gel was incubated in Amplify™ for 15-30 mins. The wet membrane was exposed to Biomax MR (Kodak) film. The film then was developed and fixed in Konica SRX 101A. The exposure time varied according to the intensity of protein bands.

### 2.3.2 Two-dimensional Gel Electrophoresis

#### 2.3.2.1. First dimension-isoelectric focussing (IEF)

Isoelectric focusing—ReadyStrip IPG strips 3-10, (24cm) were used for isoelectric focusing separation. Samples were concentrated by 2D protein clean up kit and prepared in rehydration sample buffer (9 M urea, 4% CHAPS, 50 mM dithiothreitol, and 0.2% w/v Pharmalyte 3-10 Amersham) and loaded into the rehydration/equilibration tray. Strips were placed gel side down onto the sample and overlaid with 4 ml of drystrip cover fluid (Amersham) to prevent evaporation during the rehydration process. The tray was covered and placed on a stable place at room temperature for twelve hours to rehydrate the IPG strips and load the protein sample. After rehydration, a wet paper wick was placed at both ends of the channels covering the wire electrodes, and the IPG strips were transferred into the focusing tray (gel side up) and covered with 4 ml of drystrip cover fluid. The focusing tray was then placed into the Ettan IPGphor for focusing (500 V for 1 h, linear ramping to 1000 V for 2 h, and 8,000V for 11 h).

#### 2.3.2.2.. Preparation of second-dimension gel.

Add 1% DDT (w/v) to freshly prepare to equilibration buffer (10 ml) in a tube containing IPG stripes for 15 minutes with constant shaking. Remove first equilibration solution and add 2.5% IAA (w/v) and equilibrate the IPG stripe for 10 minutes with constant agitating. Lay the prepared gel flat on a clean surface. Using a forceps, remove the equilibrated IPG strip from the equilibration tube and rinse with SDS electrophoresis buffer. Carefully lay the IPG strip in front of the gel. Apply molecular marker on a piece of whatmann paper (25ng) and seal with

## Materials and methods

agarose sealing gel. Place the marker next to one end of the IPG strip. Seal the IPG strip and the marker with agarose sealing gel.

Second dimension electrophoresis. Prior to running the second dimension, the IPG strips were equilibrated in SDS-PAGE equilibration buffer (6 M Urea, 0.375 M Tris- pH 8.8, 2% SDS, 30% glycerol, 1% dithiothreitol (w/v) and 2.5% Iodoacetamide (w/v)) for 15 min each and then loaded onto an SDS-PAGE gel, then seal the gel with 25mM Tris, 192mM glycine, 0.1% (w/v) SDS, Bromophenol Blue, 0.5% (w/v) Agarose for 5 mins for the second dimension.

Gels were electrophoresed for the first 30 min at 5 mA/gel and 100 W, following a 600 V 375 mA and 100 W for 7-8 hours. The temperature of the lower buffer chamber solution was kept at 10°C by a tube fixed to a circular cooling pump from Mult Temp III.

Electrophoresis was finished when the bromophenol blue line in the gels reached a line that has been etched 2 cm from the lower edge of the frontal gel plate. After electrophoresis, the gels were transferred into 400ml/gel 2D-fixation solution. After shaking for 2 hours, the gels were left standing overnight in the same solution at room temperature.

### 2.3.2.3. Peptide mass fingerprinting by MALDI-TOF mass spectrometry

Mass spectra of the peptide mixture were obtained using the Bruker Reflex II mass spectrometer operated in the reflector mode. The instrument functions in the “delayed extraction” mode that ensures a mass resolution up to at least 6000 Dalton over the entire mass range and a mass accuracy of better than 0.1 Dalton with internal calibration. A total of 100-140 single-shot spectra were accumulated from each sample. They were calibrated using the monoisotopic peak from a known auto-digestion product of bovine trypsin (residues 50-69,  $M+H^+ = 2163.06$  Da) and the matrix trimer ion ( $3M+H^+ = 568.14$  Da) as internal standards. The XMASS 5.0 software packages provided by the manufacturer were used for data processing.

### 2.3.2.4 Protein ladder sequencing of peptide fragments

## Materials and methods

These experiments were performed within the selection cell ( $Q_1$ ) and the collision cell ( $Q_2$ ). All ions were transmitted resulting in the measurement of the entire mass range. The ion of interest was selected at first in cell  $Q_1$ , then, this precursor ion was split in the collision cell  $Q_2$  using argon as a collision gas. The ensuing fragments were analyzed in the TOF section of the instrument. The instrument was calibrated externally with peptides of known masses. The data processing was done with the “ANALYST” software packages provided by the manufacturer



**Computer aided analysis of protein mass fingerprints.**

The identification of proteins by their peptide mass fingerprints was mainly performed with the Mascot Software (Matrix Science Ltd), and additionally with Profound or PeptideSearch as search engines. The parameters were chosen as shown in below.

**Mascot: Peptide Mass Fingerprint**

Your name: JINGXIE      Email: jing.xie@charite.de

Search title: spot 3

Database: NCBI nr

Taxonomy: Homo sapiens (human)

Enzyme: Trypsin      Allow up to: 1 missed cleavages

**Fixed modifications:** Acetyl (K), Acetyl (N-term), Amide (C-term), Biotinylated (K), Biotinylated (N-term)

**Variable modifications:** Oxidation (HW), Oxidation (M), PEO Biotin (C), Phospho (ST), Phospho (Y)

Protein mass:      kDa      Peptide tol.: 0.1 Da

Mass values:  MH+     M<sub>r</sub>      Monoisotopic  Average

Data file: E:\JING\MALDI-TOP\MALDI-peaklist.txt      Durchsuchen...

Query: NE Contents of this field are ignored if a data file is specified.

Overview:       Report top: 5 hits

Start Search...      Reset Form

Copyright © 2000 Matrix Science Ltd. All Rights Reserved. Call Number 3575

**Mascot Search Results**

User: JINGXIE  
 Email: jing.xie@charite.de  
 Search title: spot 3  
 MS data file: E:\JING\MALDI-TOP\MALDI-peaklist\0611.txt  
 Database: NCBI nr 20020718 (1034279 sequences; 327146032 residues)  
 Taxonomy: Homo sapiens (human) (116231 sequences)  
 Timestamp: 1 Aug 2002 at 13:52:49 GMT  
 Top Score: 215 for gi1114549, ATP synthase beta chain, mitochondrial precursor

**Probability Based Mouse Score**

Score is  $-10 \cdot \log(P)$ , where P is the probability that the observed match is a random event. Protein scores greater than 63 are significant (p<0.05).

**Concise Protein Summary Report**

[Switch to full Protein Summary Report](#)

To create a bookmark for this report, right click this link: [Concise Summary Report \(spot 3\)](#)

Re-Search All      Search Unmatched

1. **gi1114549**      Mass: 56525      Total score: **215**      Peptides matched: 28  
 ATP synthase beta chain, mitochondrial precursor

**gi1129235**      Mass: 57919      Total score: **196**      Peptides matched: 26  
 (M001686) ATP synthase, F1 transporting, mitochondrial F1 complex, beta polypeptide; ATP synthase, F1 transporting

**gi1129232**      Mass: 55861      Total score: **167**      Peptides matched: 24  
 (M1943) ATP synthase beta subunit [Homo sapiens]

**gi128431**      Mass: 34026      Total score: **120**      Peptides matched: 17  
 (U05686) beta-subunit (AA 1-312) [Homo sapiens]

**gi120152961**      Mass: 16792      Total score: **42**      Peptides matched: 6  
 (M006710) ATP synthase, F1 transporting, mitochondrial F1 complex, beta polypeptide [Homo sapiens]

The first step for protein search in database, in Mascot search engine is to select the parameters such as taxonomy, which would allow missed cleavages, variable modifications, and peptide masses.

**{MATRIX} Mascot Search Results**  
**SCIENCE**

**Protein View**

Match to: [gil114549](#); Score: **215**  
**ATP synthase beta chain, mitochondrial precursor**  
 Found in search of E:\JING\MALDI-TOF\MALDI-peaklist\c0611.txt

Nominal mass (M<sub>0</sub>): **56525**; Calculated pI value: **5.26**  
 NCBI BLAST search of [gil114549](#) against nr  
 Unformatted [sequence string](#) for pasting into other applications

Taxonomy: [Homo sapiens](#)  
 Links to retrieve other entries containing this sequence from NCBI Entrez:  
[gil106207](#) from [Homo sapiens](#)  
[gil179201](#) from [Homo sapiens](#)  
[gil14603307](#) from [Homo sapiens](#)  
[gil16741373](#) from [Homo sapiens](#)

Variable modifications: Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Number of mass values searched: **60**  
 Number of mass values matched: **28**  
 Sequence Coverage: **50%**

Matched peptides shown in **Bold Red**

```

1  MLGFVGRVAA APASGALRRL TPSASLPPAQ LLLRAAPTAV HPVRDYAAQT
51  SPSFKAGAAAT GRIVAVIGAV VDVQFDEGLP PILNALEVQG RETRLVLEVA
101 ONLGESTVRT IANDGTEGLV RGQKVLDSGA PIKIPVGPET LGRIMNFIGE
151 PIDERGPIKT KQFAPINAEA PEFWMSVEQ EILVIGIEVV DLLAPIAKGG
201 KIGLFGGACV GKTVLINELI MNVAKANGCY SWFAGVCERT REGNDLYIEM
251 IESGVINLID ATSKVALVYG QMNEPPGARA RVALTGLTVA EYFRDQEGQD
301 YLLFDINIFK FIQAGSEYSA LLGKIPSAVG IQPILAIQMG INQERITTK
351 KGSITSVQAI YWPADDLTDP APATTFANLD ATTVLSRAIA ELGITPAVDP
401 LDSTSRINDP NIVGSENYDV ARGVQKILQD YKSLQDI IAI LGMDELSEED
451 ELTVSRARKI QRFLSQPFQV AEVFTGIMGE LVPLKETIKG FQILAGEYD
501 HLPEQAFYMV GPIEEAVAKA DKLAEENSS
    
```

The first protein is usually the best fit. The full name of the of the candidate protein together with its *gi*-number, the theoretical molecular weight, the probability based Mascot-score and the number of matched peptides are all listed as seen above. Normally, there are several peptides or proteins listed in one suggestion. They generally are various fragments of the same protein.

## Materials and methods

After protein has been identified, the fingerprint data were compared with the theoretical digestion product of the protein. If no clear relation of the molecular weights between the “experiment” and the “theoretical” fragments could be found, I tried using less stringent criteria to improve the matching rates. This was specially the case for large peptide fragments with molecular weights above 2,000 Da. The less stringent criteria comprised the larger tolerance of mass deviation of  $\pm 0.5$  Da.

### **2.3.3 Cultivation and Isolation of trypanosomes from rat.**

Long slender blood stream forms of *T. b. brucei* were grown in rats. Rats were injected with  $3.0 \times 10^7$  trypanosomes intraperitoneally in a volume of 500 $\mu$ l. After 2.5 days, 5 $\mu$ l blood from infected rat was added to 995 $\mu$ l of CGA for control of the infection. Neubauer cell counter (0.02mm) was used for counting the number of trypanosomes in the infected rat. The trypanosomes grew to about 0.5-1.0  $\times 10^9$  cells/ml. The trypanosomes have 6h generation time. If the cell concentration was above  $5 \times 10^8$ /ml, the rats were killed with CO<sub>2</sub>. The blood was collected in the thorax by cutting the *Vena cava inferior*. Some CGA (~1 ml) was given to the thoracic cavity and the centrifuged at 1,300g for 10 min at 4°C and the *buffy coat*, which contained the trypanosomes, was resuspend in separation buffer. The buffy coat was applied to a column with 70 ml DEAE-cellulose and washed with separation buffer immediately. The flow-through containing the trypanosomes was collected and spun again as before.

#### 2.3.3.1 Lysis of trypanosomes.

The freshly isolated trypanosomes were lysed in cold lysis buffer at a cell density of  $1 \times 10^9$ /ml and immediately homogenized with a Dounce-Homogenisator. The cell lysis was controlled by phase microscopy. After cell lysis 500 $\mu$ l (10 mM Tris-HCl + protease inhibitor) was added to the lysate. The lysate was centrifuge at 12,000g for 10 min at 4°C to remove cell debris and the supernant was immediately stored at -20°C.

## Materials and methods

### 2.3.3.2. Isolation of cytosolic and membrane protein

The supernatant fraction from Trypanosomes lysis was used as Cytosolic protein. The supernatant was speed vac for 30min and used for protein precipitation. After precipitation, BCA kit was used for protein quantification.

For the isolation of membrane protein fraction, the supernatant from Trypanosomes lysis was centrifuged at 60,000g for 1h to remove most of the VSG (variant surface glyco-protein). The pellet was washed three times in 10 mM Tris-HCl with protease inhibitor. The pellet was incubated in for 12 h, in lysis buffer containing (9 M Urea 4% CHAPS 1% DDT 4 mM Complete protease-inhibitor) 2% v/v pharmalyte 3-10 (0,8 % w/v). The pellet was finally centrifuged for 10 min at 100,000g for 10 min. The supernatant contain the membrane protein fraction. The 2-D gel clean kit was used for protein precipitation.

### 2.3.3.4 *In vitro* cultivation of trypanosomes

#### Cultivation of Bloodstream Form Trypanosomes *in vitro*

The freshly isolated trypanosomes under sterile condition were cultivated at 37°C in water saturated atmosphere containing 5% CO<sub>2</sub>. The medium was incubated at 37°C before used. Cells were harvested at a density of about 1-2 x 10<sup>6</sup>/ml. For induction of trypanosomes with 25 µM Arachidonic acid for 18 h, 0.1µg/ml penicillin was added to the culture medium. The pellet was lysed in cold lysis buffer containing (10 mM Tris HCl, 0.2mM ZnCl<sub>2</sub> and protease inhibitor) at a cell density of 2 x10<sup>8</sup> in Dounced Homogenisator. The cell lysis was controlled by phase microscope. After cell lysis, the lysate was centrifuged at 12,000g for 10 min at 4°C to remove cell debris and the supernatant was stored for cytosolic protein precipitation using 2-D protein clean kit.

### 2.3.4 Prostaglandin D Synthase assay.

#### 2.3.4.1 Prostaglandin D Synthase assay.

Add 5µl (5 Units) of PGHS to 95µl assay solution, incubate for 60 sec at 37°C. Add 2.5µl of Hematin to assay Solution. Finally add 1.7µl of [<sup>14</sup>C] AA (0.1m Ci/ml 3.7MBq/ml). The

## Materials and methods

reaction was carried out at 37°C for 2 min. Add 1mM Glutathione, 20µl (20µg) of protein lysate to the reaction mixture and incubate for 90 sec. The reaction was terminated by the addition of (100µl) of a stop solution (30:4:1 vol/vol/vol diethyl ether/methanol/2 M citric acid. Add 20mg of Sodium sulphate to remove most of the water from the reaction mixture. The organic phase (50 µl) was applied to 20 x 20-cm silica gel plates (Merck) at 4°C, and the plates were developed with a solvent system of 90:2:1 vol/vol/vol diethyl ether/methanol/acetic acid at -20°C. The radioactivity on the plates was monitored and analyzed after 2 days by Fluorescent Imaging Analyzer FLA 3000 and Ad-aware software 6.0 (Fuji Photo Film Co.)

### 2.3.4.2. Protein Purification (Gel filtration and Ion-exchange chromatography).

Gel filtration is an important preparative technique since it is often a chromatographic step in the purification of proteins, polysaccharides and nucleic acids. Filling the chromatography column with matrix is called “packing.” Matrix that is suspended in the buffer and ready to be poured is referred to as the “slurry.” Gel beads were equilibrated at 4°C with 10 volumes of the column buffer prior to the loading. The slurry is carefully poured into the closed column to minimize bubbling and turbulence. A reservoir of buffer is connected to the column and the column is opened. The flow of buffer forces the matrix down to form an even, homogeneous pack. The packed matrix is called the “bed” and the volume it occupies is termed the “bed volume.” It is very important not to allow the bed to run dry. Otherwise, cracks and fissures develop and the matrix has to be removed and repacked. The gel filtration matrix consists of microscopic beads that contain pores and internal channels. Larger molecules tend to flow around and in between the beads. Smaller molecules tend to spend more time in the maze of channels and pores in the bed. The total volume of buffer between the beads is the “void volume”. Consequently, the larger, higher molecular weight molecules are eluted from the column before smaller molecules. Larger molecules take the faster, more direct path that involves less time in the. The column was then eluted with elution buffer at a flow rate of 2ml fraction in 10min. Fractions were then used for protein quantification and enzyme assay.

## Materials and methods

### 2.3.4.3. Immunoprecipitation

Add 20 µg of antibody to the eppendorf tube containing the cold precleared lysate. Incubate at 4°C for 1 hour. Add 50 µl of washed 1: 1 Protein A/G (sepharore) slurry in pre-chilled Lysis Buffer. Incubate over night at 4°C on a rocking platform or a rotator. Spin the eppendorf tube at 10000xg for 30 seconds at 4°C.

Carefully remove supernatant completely and wash the beads 3-5 times with 500 µl of Lysis Buffer. Care should be given to remove the supernatant completely in these washes.

After the last wash, aspirate supernatant and add 5µl of Rot-loading buffer (1X) to bead pellet. Vortex and heat to 100°C for 10 minutes. Spin at 10000xg for 5 minutes, collect supernatant and load onto the gel.

### 2.3.4.4. Western Blotting and Analysis of Prostaglandin D Synthase in Trypanosomal Lysate.

Protein from isolated (Cytosolic and Membrane.Cells lysate) bloodstream form cells were separated through SDS-PAGE. Western blot was performed using Semi-Dry-Apparatus. Three filter papers were laid on the anode plate. Then nitrocellulose membrane, gel and 3 filter papers were added one after another. All the materials were soaked with transfer buffer before use. The electro transfer was carried out by a constant electric current of 5.5 mA/cm<sup>2</sup> for 30 min. Purified Bovine Prostaglandin D synthase was used as first antibody. Wash 3x in washing buffer. Conjugated goat anti-rabbit IgG was used as second antibody. Wash 3x in washing buffer. Then use ECL western blotting detection reagent for 2min. Place Biomarx film on the Nitrocellulose membrane for 10 mins and develop the film in a Konica SRX Developer.

## 2.4 Sample preparation for MALDI analysis.

The peptide were solved in 10µl 0.1% TFA. 1µl of the sample was spotted onto the MALDI target plate and mixed with 1µl of 2% TFA and 1µl matrix. After the sample spots had ben air-dried completely, they were rinsed twice with 5-10 µl 0.1% TFA, and the liquid was evaporated with speed vaccum or pressured air.

## Materials and methods

### 2.4.1 Sample purification Zip Tip (C<sub>18</sub>)

Sample purification was performed according (Gobon 2001 and Millipore). A long narrow pipette tip packed with 0.3 ml (C<sub>18</sub>) reversed-phase medium served as a chromatography column. Sample liquid was driven through the column by using 10µl pipette. Prior to use, the column was washed with 15 µl of acetonitrile-0.1 % TFA (8:2 v/v) followed by an equilibration step with 10 µl of 0.1% (v/v) TFA. The peptide sample was acidified with 1 µl 2% (v/v) TFA to obtain a final concentration of about 0.1-0.52% (v/v). Then the sample was loaded onto the column and was slowly pumped over the reversed-phase medium. A washing step was performed with 10 µl 0.1% TFA, and the column was emptied completely by pressing air through it for a few seconds. Finally, 3µl matrix as an eluent was evaporated with pressurised air. 1µl was loaded directly onto the target of the MALDI-QTOF mass spectrometer.

These experiments were performed within the selection cell (Q<sub>1</sub>) and the collisions cell (Q<sub>2</sub>). All ions were transmitted resulting in the measurement of the entire mass range. The ion of interest was selected at first in a cell Q<sub>1</sub>, the precursor ion was split in the collision cell Q<sub>2</sub> using argon as a collision Gas. The ensuring fragments were analyzed in the TOF section of the instrument. The instrument was calibrated with peptides of known masses. The data processing was done with the “ANALYST” software packages by manufacturer.

## 2.5 Special Equipment.

MALDI-TOF mass spectrometer (Reflex II from Brunker-Daltonik, Bremen).

### Procedure

Mass spectra of the peptide mixture were obtained using the Brunker Reflex II mass spectrometer operated in the reflectory mode. The instrument functions in the “delayed extraction” mode that ensures a mass resolution up to at least 6000 Da over the entire mass range and a mass accuracy of a better than 0.1Da with internal calibration. A total of 100-140 single-shot spectra were accumulated from each sample. They were calibrated using the monoisotopic peak from a known auto-digestion product of bovine trypsin (residues 50-69,

## Materials and methods

M+H<sup>+</sup>-2163.06 Da) and the matrix trimer ion (3M+H<sup>+</sup> - 568.14 Da) as internal standards. The XMASS 5.0 software packages provided by the manufacturer were used for data processing.

These experiments were performed within the selection cell (Q<sub>1</sub>) and the collisions cell (Q<sub>2</sub>). All ions were transmitted resulting in the measurement of the entire mass range. The ion of interest was selected at first in a cell Q<sub>1</sub>, then this precursor ion was split in the collision cell Q<sub>2</sub> using argon as a collision gas. The ensuring fragments were analyzed in the TOF section of the instrument. The instrument was calibrated extremely with peptides of known masses. The data processing was done with the “ANALYST” software packages by manufacturer.

### **2.5.1 Computer aided analysis of protein mass fingerprints.**

The identification of proteins by their peptide mass fingerprints was mainly performed with the Mascot Software (Matrix Science Ltd), and additionally with Profound or PeptideSearch as search engines. The parameters were chosen as shown in database search based on peptide mass fingerprint spectra (page 25 and 26).



## Results

### 3.1 Production of Prostaglandins from arachidonic acid by *T. brucei*.

Preliminary attempts to isolate the prostaglandin synthases from *T. brucei* (a parasite of the *Trypanosomatidae* family) by conventional enzyme purification methods or immunoprecipitation under low stringency conditions to the sheep enzymes were unsuccessful.

Since 2000, *de novo* synthesis of PGD<sub>2</sub> and PGF<sub>2α</sub> from PGH<sub>2</sub> in lysate of *T. brucei* from bloodstream forms have been investigated in our laboratory. We grew trypanosomes in the presence of exogenously added arachidonic acid (AA) up to 25μM which had no obvious effect on cell growth. Prostaglandin accumulation in the media increased (Kubata *et al.*, 2000), suggesting that *in vivo* *T. brucei* would rely on arachidonic acid uptake from its environment (host tissue) to release significant amounts of PGs. PGF<sub>2α</sub> have been shown to be the major prostanoid synthesized upon AA addition to the growth medium during the cultivation of trypanosomes. Other PGs like PGD<sub>2</sub> and PGE<sub>2</sub> increased more than 5-fold.

Bloodstream form trypanosomes (1x10<sup>10</sup> cells) were isolated from infected rats and lysed in 10 mM Tris-HCl buffer containing the protease inhibitor complete. Protein fractions were obtained by differential centrifugation at 13,000xg for 10 min and 100,000xg for 1 h at 4°C. The cytosolic protein fraction was concentrated under vacuum (in a speed vacuum) under a N<sub>2</sub> gas flow. Protein concentration was determined by using bicinchoninic acid reagent (BCA) with BSA as standard. The concentrated fractions were used for PGs assays.

#### 3.1.2 Production of prostaglandins from the cytosolic protein fraction of the *T. brucei* bloodstream form.

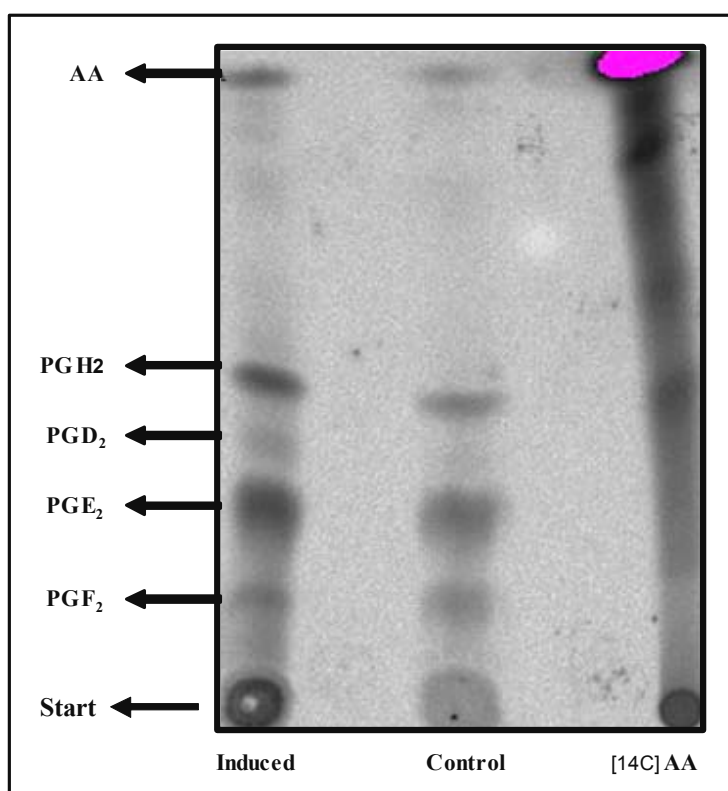
For the production of PG from AA, the reaction mixture described by Kubata *et al.*, 2000 was used with few modifications. 5μl of PGHS (EIA-assay kits, Cayman Chemicals) was added to 95μl assay solution containing 2.5μl of hematin and 1mM glutathione in 20μl (25μg) protein lysate. 1.7μl of [<sup>14</sup>C] AA (0.1mCi/ml 3.7 MBq/ml) was used as substrate. The reaction was carried out at 37°C for 2 min and terminated by the addition of 100μl stop solution 30:4:1 (vol/vol/vol) diethyl ether/methanol/citric acid (2M). 20 mg of sodium sulphate was added to bind most of the water from the reaction mixture. The organic phase (10 μl) was applied onto 20 x 20-cm silica gel plates (Merck) at 4°C, and the plates were developed with a solvent system of

## Results

90:2:1 (vol/vol/vol) diethylether/methanol/acetic acid at  $-20^{\circ}\text{C}$ . The radioactivity on the plates was monitored and analyzed after 2 days using the Fluorescent Imaging Analyzer (FLA 3000) and Ad-aware software 6.0 (Fuji Photo Film Co.) (Fig. 3.1).

Obviously, different prostaglandin bands are the result of PG synthase activities within the *T. brucei* lysates. The fluorography results showed also an increase in the production of PGs after the parasites were induced with arachidonic acid for more than 18 h.

The presence of phospholipase  $A_2$  (Nok *et al.*, 1993; Eintracht *et al.*, 1998), an enzyme that catalyzes the release of AA from phospholipids in *T. brucei* indicates the existence of a free intracellular AA source in this parasitic protozoan. Thus, the small amounts of PGs secreted by *T. brucei* in the absence of exogenously added AA in control in Fig. 3.1 were most likely produced from the intracellular AA. Alternatively, it could also be produced from trace amount of AA present in the serum used for medium preparation.



**Fig. 3.1: Fluorography from induced Cytosolic protein fraction ( $1 \times 10^{10}$  cells) of Prostaglandin assay.**

Cytosolic fractions from long slender bloodstream form parasites ( $25 \mu\text{g}$  protein) were incubated with  $1.7 \mu\text{l}$  of  $[^{14}\text{C}]$  Arachidonic acid at  $37^{\circ}\text{C}$  for 2 min. Arrows indicate the positions of  $\text{PGH}_2$ ,  $\text{PGD}_2$ ,  $\text{PGE}_2$ , and  $\text{PGF}_2\alpha$ . Note that there is no  $\text{PGD}_2$  in the control. *T. brucei* lysate activity (lane 1) induced cytosolic protein fraction; (lane 2), non-induced protein fraction.

### 3.2 Two dimension gel electrophoresis of *T. brucei* proteins.

The aim of this study was the identification of proteins, especially those related to PG metabolism, from an established reference 2D-gel electrophoresis map (Proteomics) of *T. brucei*. Slender bloodstream form trypanosomes were used since high cell numbers of this form could be isolated from infected rats. The whole project started with the observation that *T. brucei* produce high amounts of different prostaglandins when arachidonic acid was exogenous arachidonic acid added to the growth medium (Kubata. *et al.*, 2000). Cells from induced and non-induced trypanosomes were lysed and separated by differential centrifugation into cytosolic and membrane fractions. These fractions were separated on a 2D-gel electrophoresis and stained with silver nitrate. Protein identification was performed with MALDI-TOF mass spectrometry in collaboration with the group of Dr. Kammerer (Pharmacy department of Tübingen University) and subsequent database searching. The identified proteins were marked on the 2D-gel map to obtain a final reference map (Fig. 3.17, page 77).

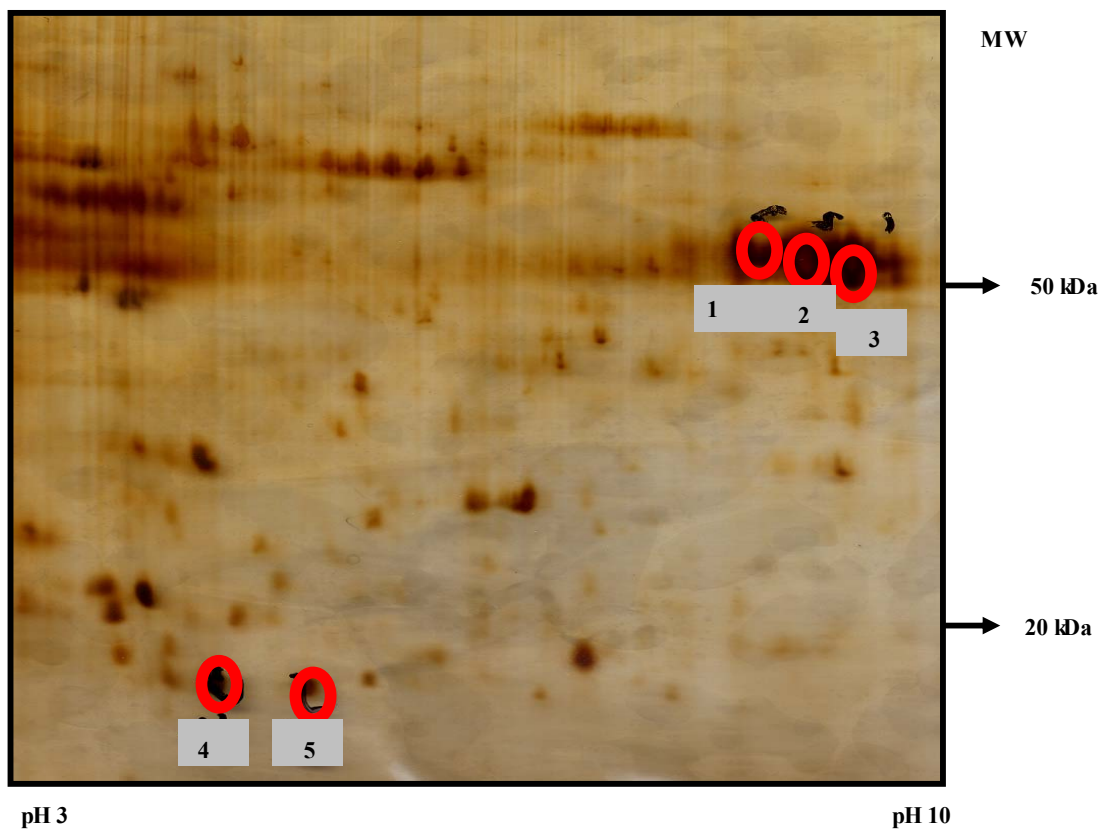
#### 3.2.1 Preparation of protein samples

There was a modification of the usual lysis protocol by using 0.2 mM ZnCl<sub>2</sub> in Tris-HCl hypotonic buffer. ZnCl<sub>2</sub> inhibits phospholipase C and prevents VSG to be released from the plasma membrane into the cytosolic fraction. Subsequent resuspension of the separated membrane fraction with the ZnCl<sub>2</sub> free Tris-HCl buffer then activated the endogenous phospholipase C leading to cleavage of the GPI anchor of VSG. The membrane fraction was then centrifuged again to remove the soluble VSG from the membrane proteins. Hypotonic lysis was used, because mechanical lysis of cells could lead to denaturation of proteins through the mechanical process and the use of a less aggressive method was an advantage in cell lysis of trypanosomes. We also modified the protocols of Klose (1999a) to isolate membrane proteins leading to an increase in the purity and amount of protein. The membrane fraction was treated with high concentrations of urea and with detergent (CHAPS) to solubilize membrane proteins. The protein concentration of the final sample was measured by using the BCA protein assay method. The protein concentration used for each 2D-gel electrophoresis was about 200 and 400 µg per gel.

## Results

### 3.2.2 Two-dimensional electrophoresis

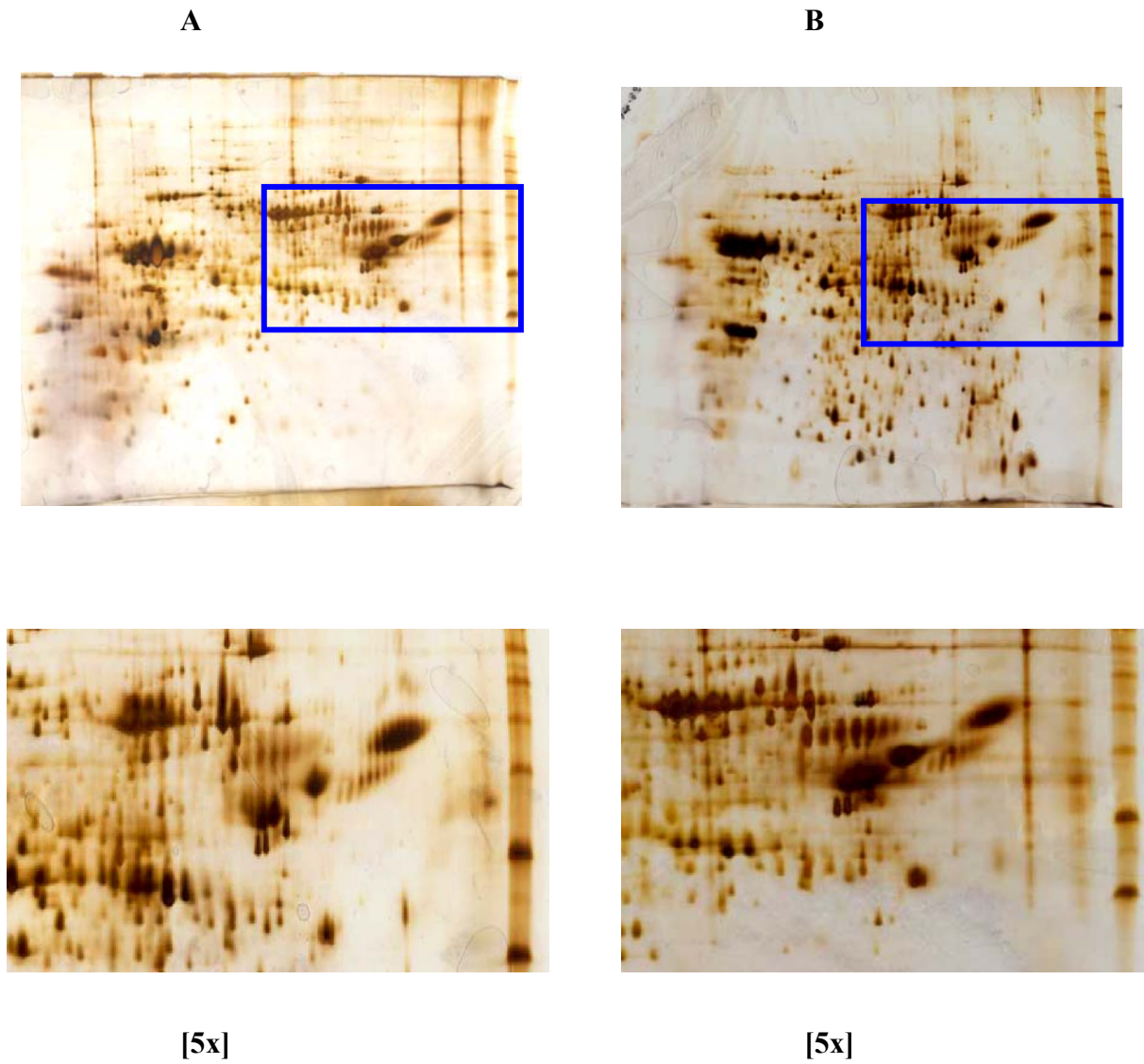
200  $\mu\text{g}$  protein sample was loaded on the dry strip for isoelectric focussing. A broad range of pH 3-10 was used for isoelectric focussing in order to obtain a better resolution of more proteins on silver nitrate stained gels. The isoelectric point of most protein spots detectable on the gel was between pH 4 – pH 8.



**Fig. 3.2: 2D-gel electrophoresis showing VSG protein spots.**

Cells were lysed in 10mM Tris- HCl. 100  $\mu\text{g}$  protein from the crude lysate was used for 2D-gel electrophoresis. As judge from MALDI-TOF data, Spot 1 to 5 shows that different VSG forms (mfVSG, sVSG, VSG with different glycosylation patterns and degradation products etc.) were located at different IP values and different molecular weight.

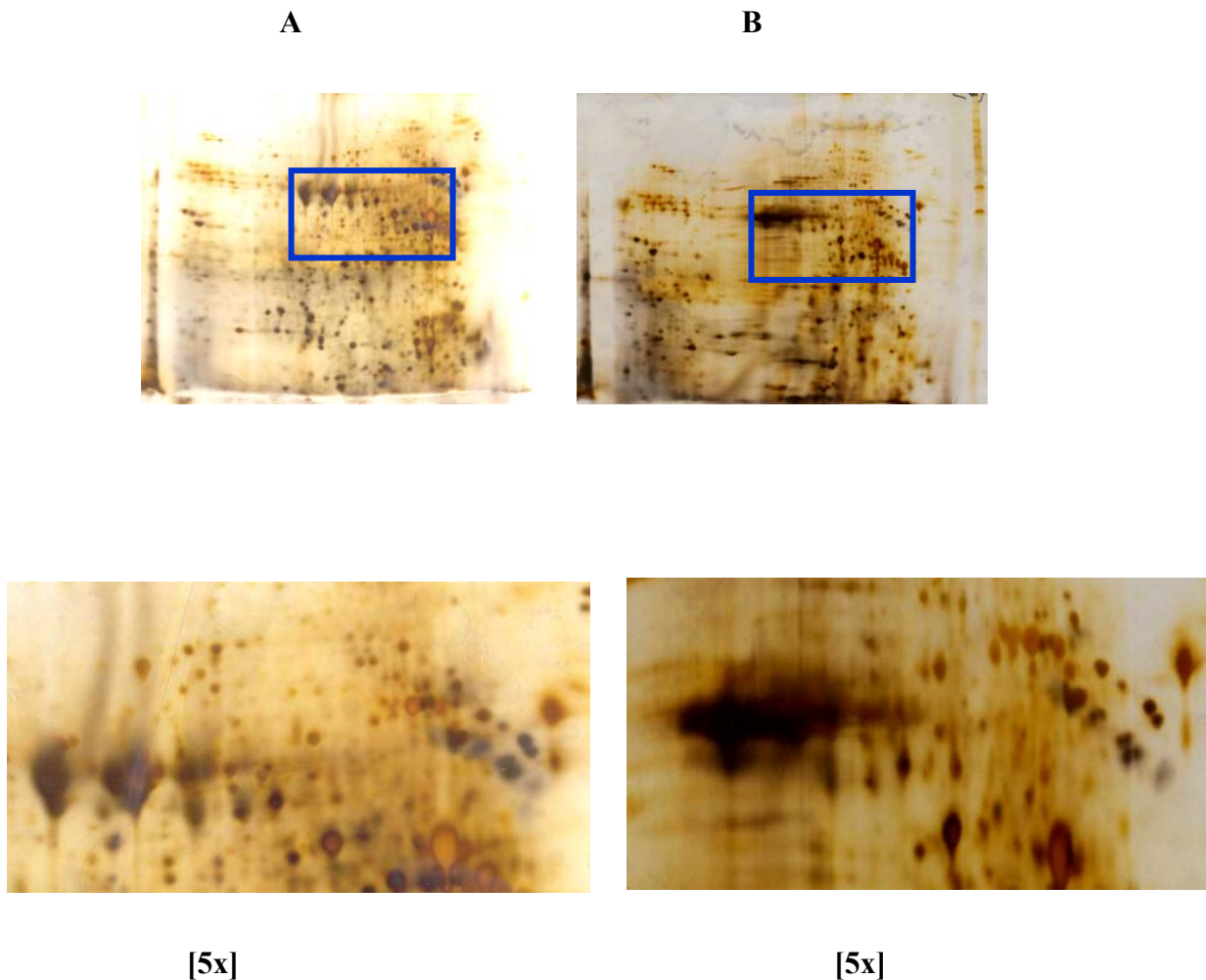
## Results



**Fig. 3.3: Comparison of the same region of *T. brucei* cytosolic protein gels.**

Gels A (100 µg protein) and B (200 µg protein) are corresponding gel-sections which demonstrate the reproducibility of 2D-gel electrophoresis. Both gels were stained with silver nitrate. Note the different magnification scale used

## Results

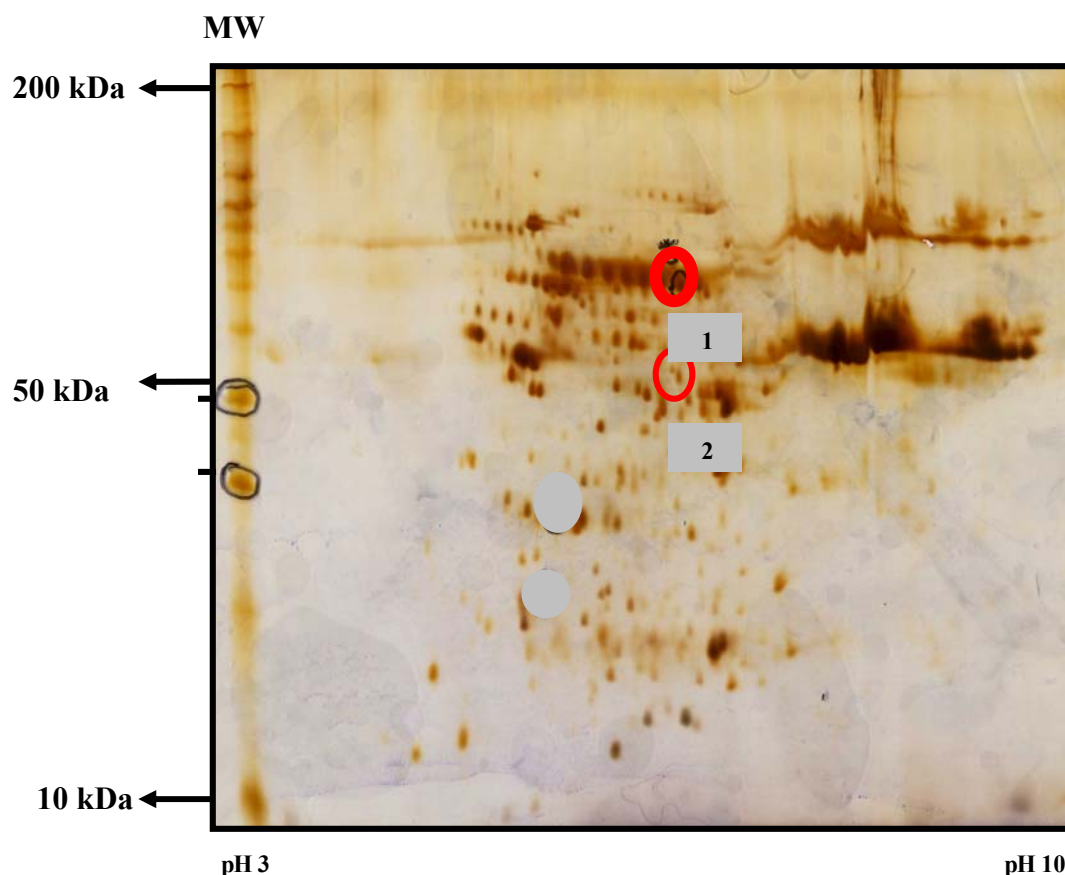


**Fig. 3.4: Comparison of the same region from membrane protein gels.**

Gels A (100  $\mu\text{g}$  protein) and B (200  $\mu\text{g}$  protein) are corresponding gel-sections from membrane fraction. These gels demonstrate the reproducibility of 2D-gel electrophoresis. All gels were stained with silver nitrate. Note the different magnification scale used

The second dimension (SDS-PAGE) was run using molecular weight standards. The range of the standard marker was between 10 and 200 kDa. The reproducibility of the 2D-gel electrophoresis runs was demonstrated by analyzing a defined section from four different gels. Most protein spots run to very similar locations on each gel except in the case of the extreme basic proteins which were difficult to separate during rehydration of the strip after the first dimension run (Fig. 3.4).

## Results



**Fig. 3.5: 2-D gel electrophoresis with 100 µg cytosolic protein.**

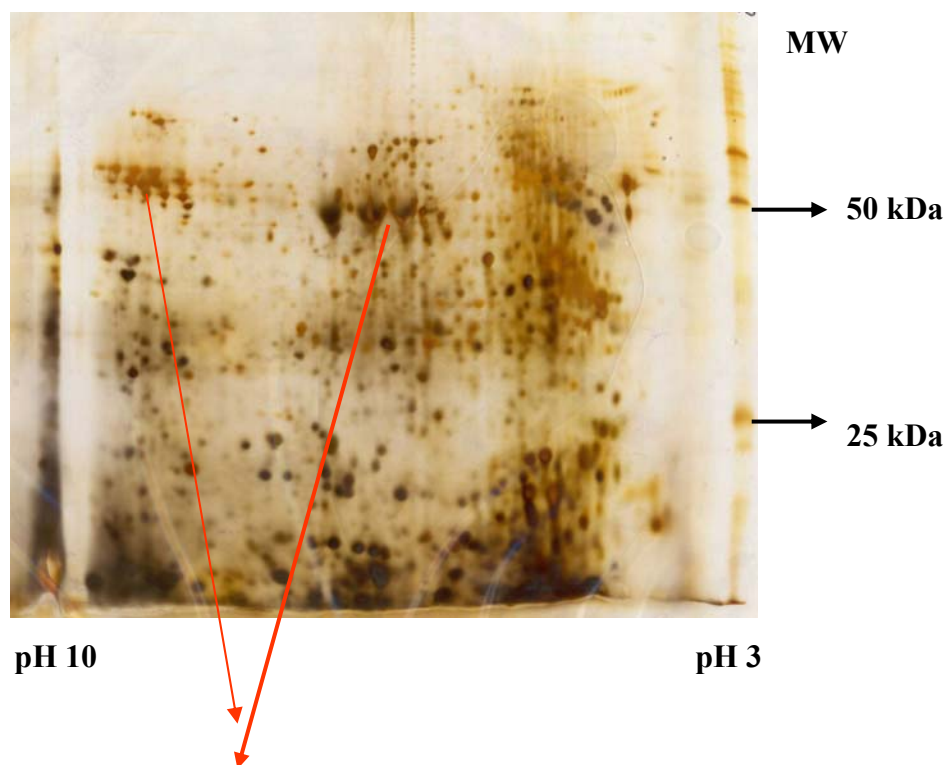
MALDI-TOF analysis of two highly expressed protein spots from the cytosolic protein fraction revealed 95% sequence identity from VSG (spot-1) and 86% sequence identity from Enolase (spot-2).

*T. brucei* bloodstream form were lysed in 10mM Tris-HCl containing protease inhibitor complex. The lysate was centrifuged at 13000xg for 10 min. The supernant was loaded on IPG stripes pH 3-10. Rehydration of the stripes was carried out for twelve hours. The stripes were focused for eleven hours on an increasing voltage gradient. The second dimension gel electrophoresis was run for six hours and followed by silver staining. Spots were excised from gel digested with trypsin and finally desalted with Zip-tip<sub>18</sub>. MALDI-TOF analysis of defined major spot reveals VSG, a highly expressed protein in *T. brucei* (Fig. 3.5).

Enolase, (spot 2, Fig. 3.5) has been shown (Michels *et al.*, 2005) to play a specific role in regulating certain enzymes in both the glycosome (hexokinase, phosphofructokinase and glycerolkinase) and the cytosol (pyruvate kinase), but not others (glucose-6-phosphate isomerase

## Results

and triose-phosphate isomerase) in *T. brucei*. This enzyme is highly expressed in bloodstream forms which depend exclusively on glycolysis as energy source.



**Fig. 3.6: 2D-gel electrophoresis analysis of membrane protein fraction from *T. brucei*.**

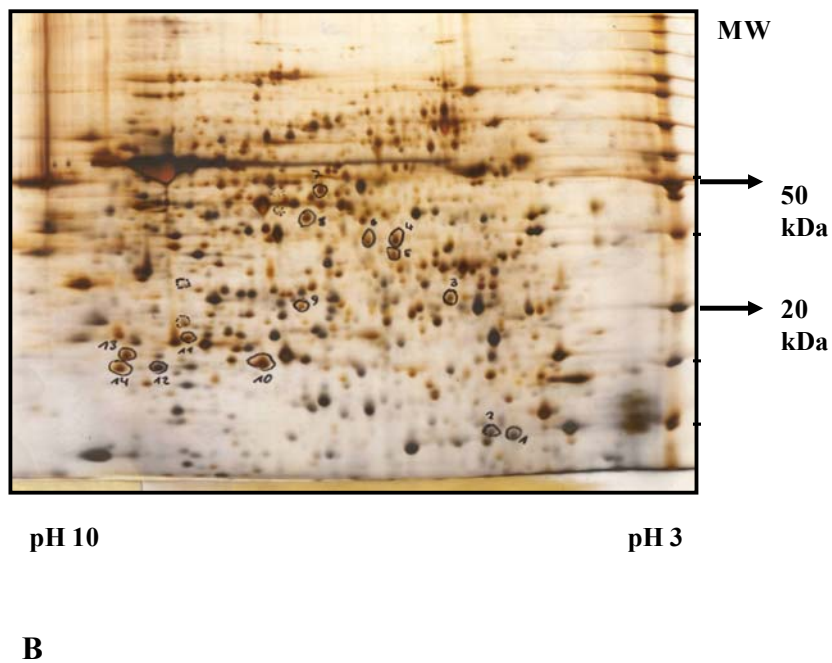
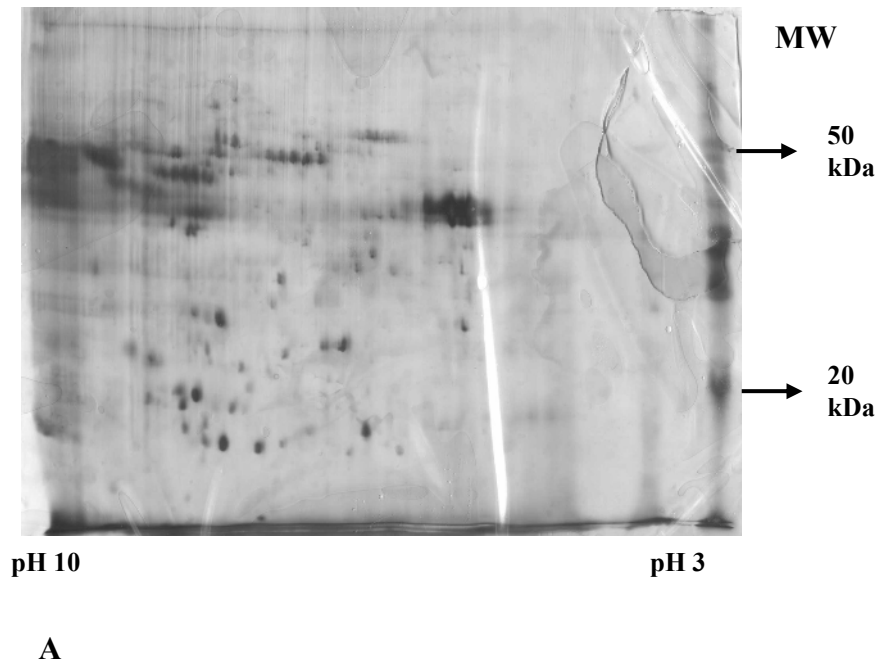
*T. brucei* cells ( $1 \times 10^{10}$ ) were lysed in 10mM Tris-HCl containing 0.2mM  $ZnCl_2$  and membrane fraction was solubilized in 10mM Tris-HCl containing 0.1% Triton X 100 for membrane protein. Red arrows indicate VSG in membrane fraction

During VSG synthesis, mfVSG (membrane form VSG) should be the first product, during is then transformed into sVSG (soluble VSG). To identify the process in which VSG could be easily reduced from the membrane protein fraction, 0.2 mM  $ZnCl_2$ , a very efficient inhibitor of GPI-specific phospholipase (GPI-PLC) was added to lysis buffer. In this experiment the GPI-PLC activity was successfully inhibited. Following cell lysis samples were centrifuged at (60,000xg, 1 h. 4°C) to separate membrane-bound proteins from VSG in the soluble protein fraction. As shown in (Fig. 3.6) VSG was found membrane fraction. This represent the membrane bound form of internal membranes (rER, Golgi vesicles). A total elimination of VSG was not achieved, since 0.2 mM  $ZnCl_2$  is the  $IC_{50}$  for the GPI-PLC enzyme (Cross, 1984).



## Results

### 3.3 Gel staining



**Fig. 3.7: Difference between Coomassie and silver stained gels**

Gels above demonstrate the difference between a Coomassie Brilliant Blue stained gel (A) and a silver stained gel (B). Both gels have been run under the same conditions. 100  $\mu\text{g}$  cytosolic protein was loaded on gel B. In contrast, 200  $\mu\text{g}$  cytosolic protein was loaded on gel A. As expected, there was a higher resolution of protein spots in gel (B) than in gel (A).

## Results

The difference between silver staining and Coomassie staining is that the silver staining is more sensitive but the Coomassie brilliant blue stain has less influence on proteins. The silver stained gel may show more spots, but for the purpose of protein identification, staining with Coomassie stain is more suitable since it does not modify the proteins covalently. The silver staining is ten times more sensitive and requires less amount of protein sample as compared to the Coomassie staining.

Although more protein is needed for Coomassie staining, the detection of more protein spots could be seen on a silver stained gel from only 100 µg protein sample. In comparison, less than 30 spots were detected on gel loaded with 200 µg protein and stained with Coomassie stain. However, the subsequent protein analysis by MALDI-TOF mass spectrometry showed some drawback in the silver stained gels. For protein identification a total of five spots were excised from gel stained with Coomassie brilliant blue. The Coomassie gel was then destained and subsequently restained with silver. The protein spots appeared to be much darker than in the preceding Coomassie stained gel. Additional protein spots that had not been detected before from the Coomassie-gels were visible. After reduction of silver with iodoacetamide and DTT and subsequent trypsin digestion some of these spots were also subjected to MALDI-TOF mass spectrometry. In contrast to the spots from the Coomassie gels, we could only identify few spots from the silver stained gel (Fig. 3.7).

### **3.4 Protein (peptide mass fingerprints) analysis.**

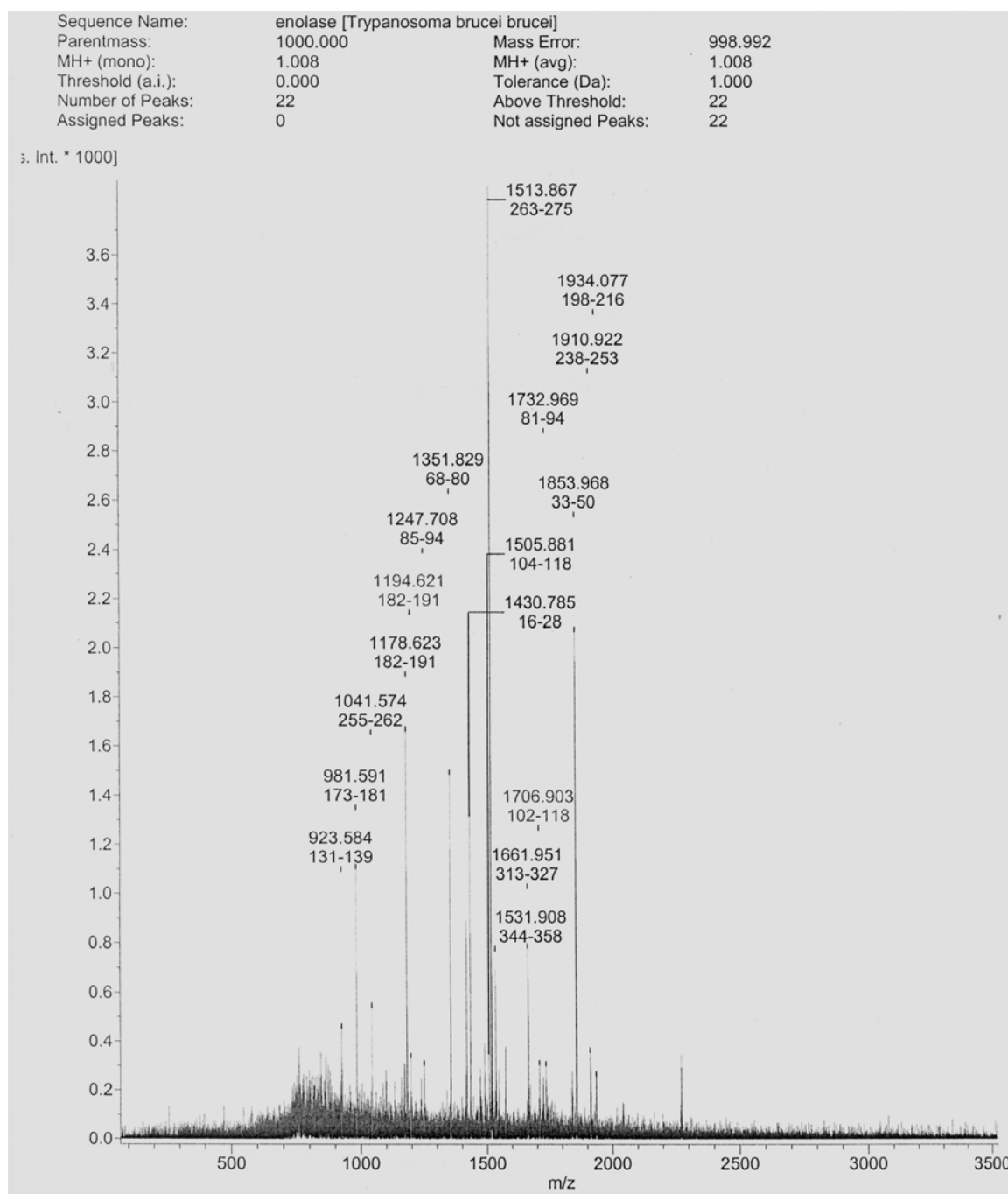
#### **3.4.1. MALDI-TOF and MALDI-QTOF tandem mass spectrometry**

The proteins were identified by MALDI-TOF mass spectrometry on the basis of their peptide mass fingerprints, and by MALDI-QTOF tandem mass spectrometry on the basis of their peptide fragment ladders. A total of 50 spots were excised from two large silver-stained 2D- gels. Following in-gel digestion with trypsin, the peptide mixture of each protein was analyzed by MALDI-TOF mass spectrometry as shown in (Fig. 3.8).

Out of the 27 peptides were detected by MALDI-TOF analysis. Using the Mascot search theoretically, all the measured peptide masses should match the corresponding “in silico” digested fragments of a certain protein in the database. However, in the experimental setting the highest percentage of matched peptide masses in this study was more than 90%. The average percentage of matched peptide masses was about 70% and that of the covered sequence was 25-

## Results

98%. In order to confirm these results, verification of each protein spot was obtained with JVirGel software (Münch *et al.*, 2003).



**Fig. 3.8: Mass spectrum of *T. brucei* Enolase.**

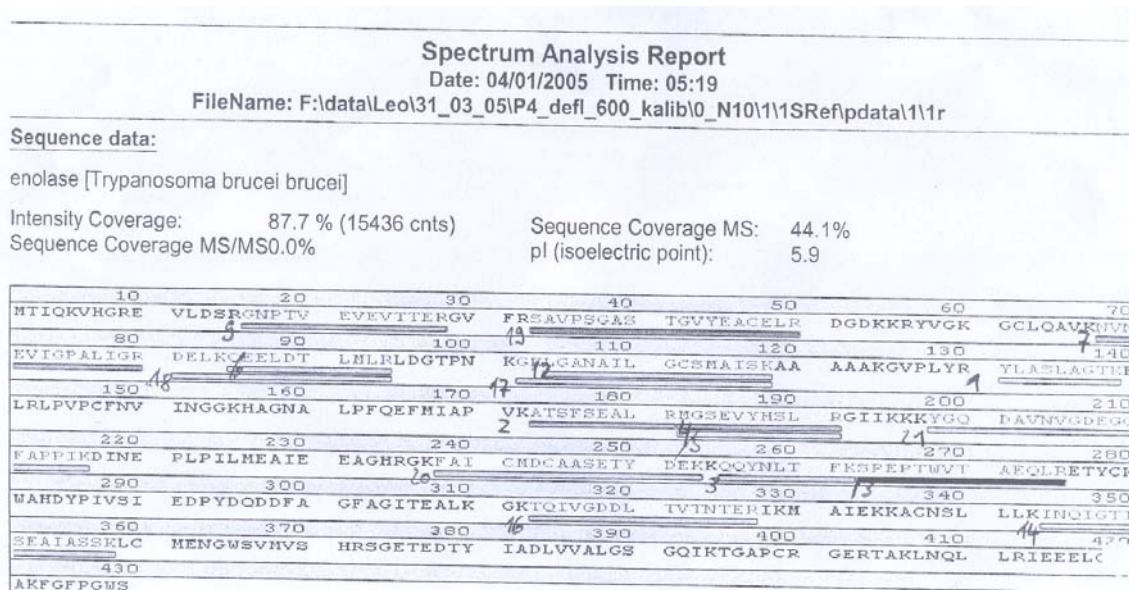
Mass spectra showing the determination of a partial peptide sequence of enolase

### 3.4.2 Enolase

Enolase mass spectrum (Fig. 3.6) shows an example using the mass spectrum information of protein. Each measured peptide mass was compared to the “in silico” digested ones. The matched peptides were highlighted and marked. The peptide masses of the self-digested trypsin fragments were marked. In order to match more peptides, less stringent criteria were used. The peptides (e.g. with one or two missed cleavages) were also highlighted. If the mass spectrum was not good enough to secure a protein identity, the amino acid sequence of a single fragment was determined by MALDI-QTOF tandem mass spectrometry. The sequence information could then be used as sequence tag to find the protein in the database.

Only few peptides were detected including 3 peptides of the self-digested trypsin. Sometimes salts from the buffer interfere with the MALDI-TOF mass spectrum. Therefore the peptides were first desalted by reversed-phase chromatography using ZIP-TIP<sub>18</sub>. After desalting, a total of 22 peptides of spot 20 could be detected with MALDI-TOF mass spectrometry. However, since the spectrum of 17 peptide fragments was still not good enough to identify the protein, MALDI-QTOF analysis had to be carried out on one isolated peptide. The sequence result of the isolated polypeptide spot (enolase) led to the identification of the 47,358 kDa. The sequencing of 40 protein spots, with corresponding 27 different proteins was identified. Most of these proteins were identified by MALDI-TOF mass spectrometry, only five spots needed to be analyzed with MALDI-TOF/LC /MS spectrometry

## Results



**Fig. 3.9: Mass spectra showing the determination of a partial peptide sequence of enolase.**

Report from mass spectrum analysis of enolase measured with MALDI-TOF mass spectrometry.

Enolase (ENO), one of the rate limiting enzymes in the glycolytic pathway in *T. brucei* was identified as spot (20) as shown (Fig 3.8 and Fig. 3.9), with intensity coverage of 87.7% and sequence coverage of 44.1%. The observations made by Albert *et al.*, (2005) suggest that enolase seems to down-regulate enzyme activities rather than enzyme level. This regulation is specific for only certain enzymes within glycosomes. Cells which were transfected with a plasmid producing double-stranded RNA decrease specific enzyme levels in the presence of the RNAi inducer tetracycline that suggests that this enzyme also participates in the regulatory mechanism. There is no information available as yet regarding how this regulation occurs. It may involve either a key metabolite (for example, phosphoenolpyruvate, the cytosolic concentration of which is expected to decrease when ENO becomes rate-limiting) and/or mechanisms of signaling to the glycosome (for example, coupled to covalent modification of organellar HXK, PFK, and glycerol kinase). No significant effects on the activities or concentrations of other proteins were seen when HXK and PYK were targeted by RNAi, at least not during the initial period (Moyersoen *et al.*, 2003). All results obtained in the RNAi experiments with HXK, PFK, PYK, PGAM, and ENO indicate the existence of 50% glycolytic flux threshold for sustaining normal growth (at least for periods of 24 h). The levels of several proteins were affected, and cell numbers declined when glycolytic flux was less than 50%.

## Results

This finding that trypanosome growth was affected when the glycolytic flux was decreased below a 50% threshold is in agreement with earlier observation that trypanosomes cannot survive for an extended period of time (12 h) using a completely anaerobic metabolism with the yield of one (instead of two) ATP molecule/glucose molecule (Helfert *et al.*, 2001)). These findings have interesting implication for drug targeting. The corollary is that only partial inhibition of enzymes may be sufficient to kill trypanosomes.

### **3.5 The strategy of protein identification.**

#### **3.5.1 Database search for protein identification**

Several search engines on the internet including Mascot and MS-Fit were used to identify the separated protein (Fig. 3.10). These programs match the peptide masses from a protein spot with the “in silico” digested peptide masses of all known *T. brucei* proteins in the National Centre for Biotechnology Information non-redundant (NCBIInr) protein database (Sanger Institute Genome Sequencing Site [http://www.sanger.ac.uk/Projects/T\\_brucei/](http://www.sanger.ac.uk/Projects/T_brucei/) TIGR or Genome Sequencing Site <http://www.tigr.org/>). At least five or more matching peptides were required for a secure identity assignment. With the Mascot search engine, most samples could be identified satisfactorily with a significant probability score ( $p < 0.05$ ). However, several spectra had to be handled with other search engines like MS-Fit. A few spots could be identified additionally that way. As mentioned above, there were some spots that could only be identified by their peptide sequence information gained from MALDI-TOF/LC mass spectrometry. Some of these protein spots are listed in (Tab. 3.4. on pages 74 and 76). The theoretical and the experimental molecular weights (MW) and isoelectric points (pI) of the identified proteins, and their corresponding SWISS-PROT accession numbers are also listed, along with the scores from the mass spectrometry analysis, and in some spots with the peptides, and the sequence coverage as shown in (Table 3.2, 3.3, 3.4, and Pages 74 - 76).

#### **3.6 Protein identification and functions of the identified *T. brucei* protein**

According to the annotation in SWISS-PROT, most of the proteins identified are either members of central metabolic pathways (including glycolysis) or cellular cycle and transport proteins. Other proteins include those that have a role in cell protection, apoptosis and protein biosynthesis.

## Results

[gi|66360261](#)      **Mass: 31718**    **Score: 212**    **Queries**  
**matched: 5**

Chain B, The Crystal Structure Of Prostaglandin F  
 Synthase From Trypanosoma Brucei

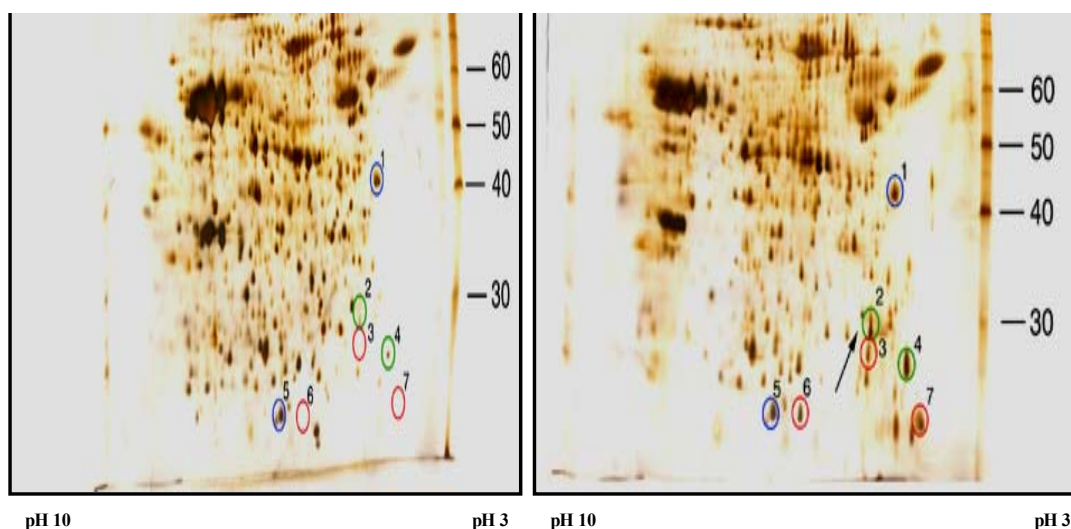
| Query              | Observed     | Mr(expt)      | Mr(calc)      | Delta      | Mis | Score | Expect     | Rank | Peptide            |
|--------------------|--------------|---------------|---------------|------------|-----|-------|------------|------|--------------------|
| <a href="#">9</a>  | 405.23<br>46 | 808.454<br>7  | 808.411<br>9  | 0.04<br>28 | 0   | 36    | 0.03<br>5  | 1    | K.FIDTWK.<br>A     |
| <a href="#">19</a> | 445.27<br>02 | 888.525<br>9  | 888.485<br>1  | 0.04<br>08 | 0   | 49    | 0.00<br>25 | 1    | K.TAAQVM<br>LR.W   |
| <a href="#">27</a> | 465.76<br>42 | 929.513<br>8  | 929.475<br>2  | 0.03<br>86 | 0   | 46    | 0.00<br>44 | 1    | R.AIASC GV<br>PR.E |
| <a href="#">31</a> | 483.78<br>42 | 965.553<br>8  | 965.506<br>9  | 0.04<br>69 | 0   | 45    | 0.00<br>45 | 1    | R.EELFVTT<br>K.L   |
| <a href="#">32</a> | 516.31<br>38 | 1030.61<br>30 | 1030.54<br>47 | 0.06<br>83 | 0   | 35    | 0.06<br>7  | 1    | R.HIDTA AI<br>YK.N |

**Fig. 3.10: Mascot peptide sequence result from Prostaglandin F<sub>2</sub>α synthase.**

Result of peptide query from mascot data bank for prostaglandin F synthase.

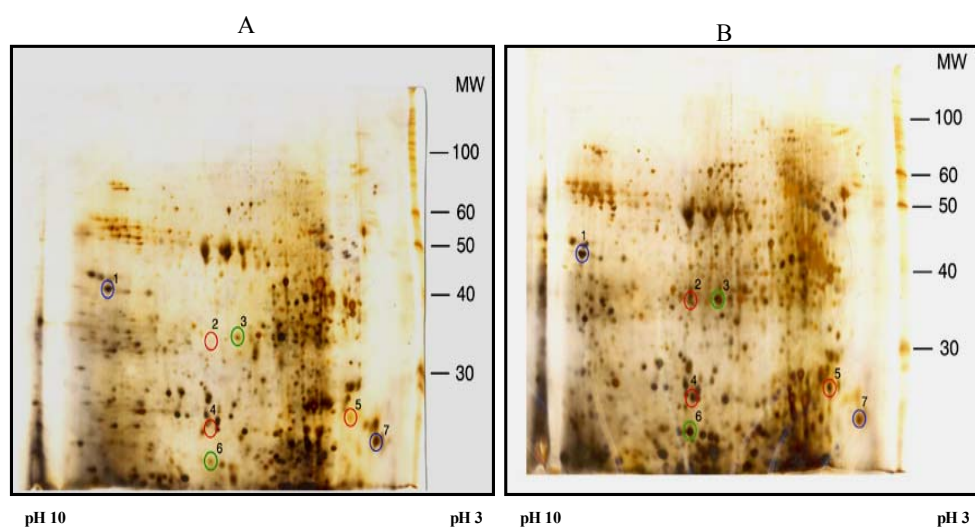
Table 3.4 (Fig. 3.16, page 77-78) shows a *T. brucei* partial proteome map. All of the identified spots are highlighted with white numbers near to the corresponding spot. The respective pIs and MWs are shown as well. The detailed information of the proteins is listed in the reference map (Table. 3.4). Out of the more than 300 visible protein spots on the silver gel, only about 10% could be identified. Among the 32 identified proteins, 85% were annotated according to NCBI and SWISS-PROT databases as *T. brucei* protein. Although there was separation of *T. brucei* cytosolic and membrane protein fraction, both fractions still contained some proteins from each other compartment. From these proteins, 20 spots corresponds to 11 different gene-products from subcellular components of either cytosolic or membrane protein fractions. The location of these proteins is on Table 3.5 below. There were few proteins remaining whose functions and localization are unknown. All of the identified spots are grouped in Table 3.5 according to their location and function. The spots that did not give a result on MALDI-TOF mass spectrometry were very faint spots that contained too little protein to produce a satisfactory spectrum.

## Results



**Fig. 3.11: 2-D gel electrophoresis and analysis of non-induced and induced *T. brucei* cytosolic protein fraction.**

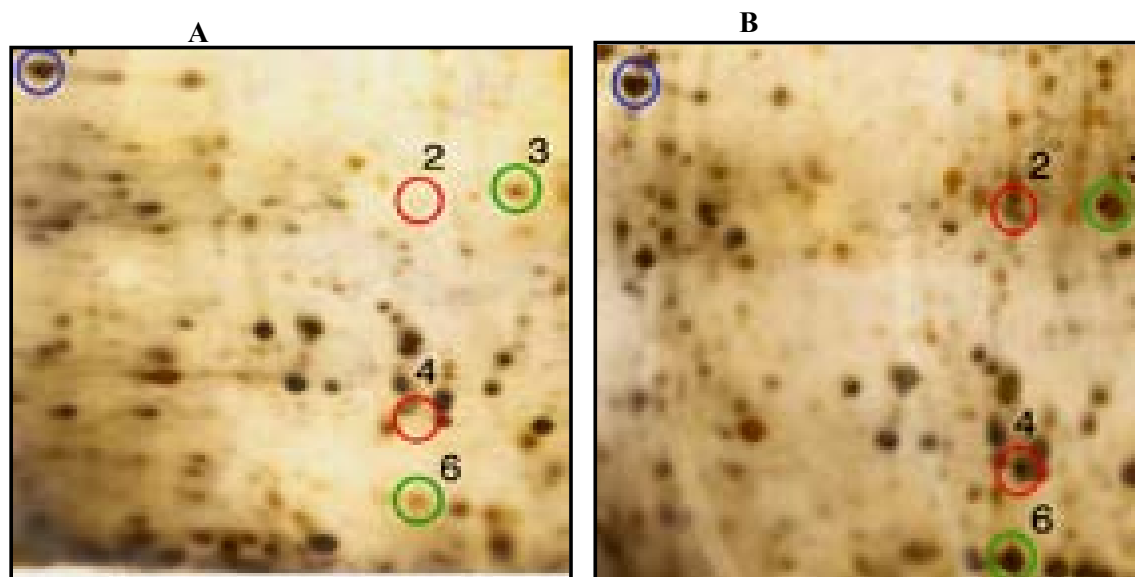
Cells were induced with 25 $\mu$ M AA for 18 hrs.  $2 \times 10^8$  cells were lysed in 10mM Tris-HCl containing 0.2mM ZnCl<sub>2</sub> and centrifuged for 13,000xg for 10min. 200 $\mu$ g protein supernatant was loaded on the IPG stripes. The result show differential expression of few proteins. Red cycles show new spots in induced cells, green shows highly expressed proteins in induced cells and blue cycle show similar protein expression in both samples.



**Fig. 3.12: 2-D gel electrophoresis and analysis of non-induced (A) and induced (B) *T. brucei* membrane protein fraction.**

*T. brucei* cells were induced with 25 $\mu$ M AA for 18 hrs.  $1 \times 10^{10}$  cells were lysed in 10mM Tris-HCl containing 0.2mM ZnCl<sub>2</sub> and centrifuged for 13,000xg for 10mins. Soluble fraction from 13,000xg was further centrifuged for 1hr at 60,000xg, to remove sVSG. The pellet was treated with (9M Urea and 4% w/v CHAPS). The supernatant was used as membrane fraction. 200  $\mu$ g protein from the membrane fraction was loaded on the IPG stripes. Highly expressed proteins were excised and identified with LC/MS spectrometry. The result show differential expression of few proteins. Red cycle indicate new spots in induced cells (Gel B), green cycle were highly expressed protein in induced cells and blue cycle are similar protein expression





**Fig. 3.13: 2D-gel electrophoresis and analysis of a section of induced and non-induced membrane protein.** Cells ( $1 \times 10^{10}$ ) membrane fraction was solubilized in 10 mM Tris-HCl containing 0.1% Triton X-100 and incubated over night at  $4^{\circ}\text{C}$ . Supernatant from this fraction was used for the 10% 2D-gel electrophoresis.

### 3.6.1 Prostaglandin $F_{2\alpha}$ synthase, VSGs, and glycolytic enzymes in *T. brucei*.

Fig 3.16 shows the differential protein expression from induced and non induced *T. brucei* cells in the presence of  $25\mu\text{M}$  AA for 18 hrs. Gel (A), non-induced membrane fraction and Gel (B) induced membrane fraction. Red circle shows new spots in the induced cell. Green cycle shows highly expressed protein in induced cells and blue shows similar protein expression.

The composition of proteins and their total content in cells depend to a large extent upon the isolation procedure used. This investigation showed that the number of protein found in the intact cells treated with 10 mM Tris-HCL containing Triton X 100 or urea and CHAPS buffer differ from those in the cells determined after homogenization. The differences, however were predictable (Horrobin, 1978), as it is well known that each factor that damages a cell (chemical, mechanical or thermal) provokes a chain of reaction that leads to denaturation of protein and to *de novo* biosynthesis.

## Results

**Table 3.1 Result from *T. brucei* sequences of differentially expressed induced cytosolic proteins. Matrix desorption/ionisation-time-of-flight (MALDI-TOF) mass spectrometry of sequenced protein.**

|    | Protein name  | mass  | score | accession number           |
|----|---|-------|-------|----------------------------|
| 1. | Prostaglandin F synthase from [ <i>T. bruceii</i> ]                             | 31718 | 212   | <a href="#">gi66360261</a> |
| 2  | ADP-ribosylation factor-like protein 3A, putative [ <i>Trypanosoma brucei</i> ] | 20096 | 114   | <a href="#">gi62175532</a> |
| 3  | Hypothetical protein, conserved [ <i>Trypanosoma brucei</i> ]                   | 24452 | 73    | <a href="#">gi70835126</a> |
| 4  | Hypothetical protein, conserved [ <i>Trypanosoma cruzi</i> ]                    | 20117 | 39    | <a href="#">gi70831317</a> |
| 5  | Trans-sialidase, putative [ <i>Trypanosoma cruzi</i> ]                          | 29677 | 30    | <a href="#">gi71403257</a> |
| 5b | Polygalacturonase 3 [ <i>Phytophthora cinnamomi</i> ]                           | 20117 | 39    | <a href="#">gi70831317</a> |
| 6  | Trans-sialidase, putative [ <i>Trypanosoma cruzi</i> ]                          | 31622 | 46    | <a href="#">gi70834304</a> |
| 7  | Eukaryotic translation initiation factor 5a [ <i>Trypanosoma brucei</i> ]       | 17923 | 122   | <a href="#">gi70833553</a> |

Infection of mammals by African trypanosomes results in the release of high levels of PGs (Gombe *et al.*, 1989; Doua *et al.*, 1990) that may, in part be involved in the pathogenesis of the disease. The up-regulation of PG production during African trypanosomiasis in *T. brucei* that exhibits a PG synthase activity capable of specifically converting PGH<sub>2</sub> to PGF<sub>2</sub>α has been reported (Alafiatayo *et al.*, 1994). In addition, despite the obvious importance of PGs in the pathogenesis of parasitic infections and of PGs production in parasitic protozoa little is known about the molecular mechanisms of PGs production in these organisms.

We have shown (Kubata *et al.*, 2000) that the parasite itself produces PGs and may contribute directly to the production of these mediators in mammals. However, the physiological relevance

## Results

of PGs production in *T. brucei* remains unknown. This opens up ways to investigate the role of TbPGFS in *T. brucei* replication, development and may provide a new tool to study the role of parasite derived PGs in the pathogenesis of African trypanosomiasis. This protein belongs to the flavin-dependent oxidoreductase family and can reduce either trypanocidal agents (nifurtimox and others) or hydroperoxides. It has been suggested that this enzyme is responsible for most of the antioxidant activity in *T. cruzi* (Kubata *et al.*, 2002). The presence of this protein in high abundance, as shown by 2DE, suggests a need to study this protein in more detail as it is a good candidate as target for drug design



**Fig. 3.14: *T. brucei* induced with AA for 18h. 400 µg membrane protein.**

*T. brucei* cells ( $1 \times 10^{10}$ ) were lysed in 10mM Tris-HCl, containing 0.2mM ZnCl<sub>2</sub> supernatant from 13,000xg centrifugation were further spun for 1hr at 60,000xg, to remove sVSG. The pellet was washed in 10mM Tris-HCl treated with (9M Urea and 4% w/v CHAPS) for membrane fraction. Highly expressed proteins were excised, digested and identified by MALDI-TOF/LC/MS spectrometry.

The poor identification of membrane proteins are due to their low absolute peptide content despite the fact that they might be present in the same molar range as high molecular weight proteins. Even if they can be detected on Coomassie stained gels they might be difficult to

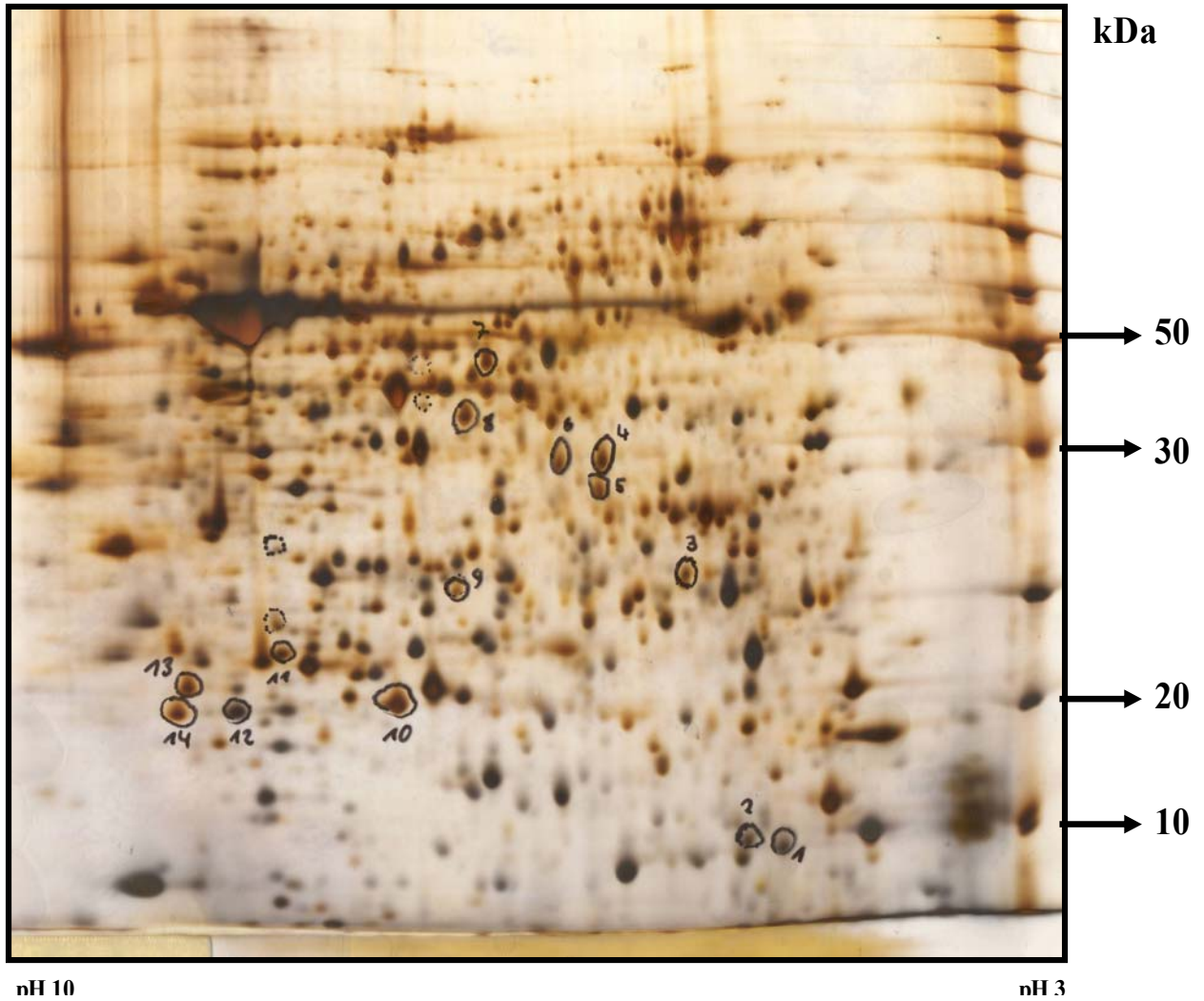
## Results

identify via MALDI-TOF mass spectrometry since there are only few peptide fragments available for a peptide fingerprint analysis. In this case one has to resort to the sequence determination of a peptide fragment by MALDI-TOF/LC/MS mass spectrometry.

**Table 3.2: Result from *T. brucei* highly expressed protein from membrane fraction.**

| Spot ID and protein name   | mass   | score | accession number            |
|--|--------|-------|-----------------------------|
| A Void.  |        |       |                             |
| B beta tubulin [ <i>Trypanosoma brucei</i> TREU927]                    | 50413  | 293   | <a href="#">gil72386160</a> |
| C pentamidine resistance protein [ <i>Leishmania major</i> ]           | 195721 | 21    | <a href="#">gil32330114</a> |
| D beta tubulin [ <i>Trypanosoma brucei</i> TREU927]                    | 50413  | 251   | <a href="#">gil72386160</a> |
| F fructose-bisphosphate aldolase (ald) (EC 4.1.2.13)                   | 41643  | 208   | <a href="#">gil161968</a>   |
| G variant surface glycoprotein MITat 1.8 [ <i>Trypanosoma brucei</i> ] | 50790  | 214   | <a href="#">gil60739050</a> |
| H variant surface glycoprotein [ <i>Trypanosoma brucei</i> ]           | 44492  | 102   | <a href="#">gil6492217</a>  |
| I Variant surface glycoprotein MITAT 1.2 precursor (VSG 221)           | 51466  | 348   | <a href="#">gil139611</a>   |
| J Variant surface glycoprotein MITAT 1.2 precursor (VSG 221)           | 51466  | 629   | <a href="#">gil139611</a>   |
| K Variant surface glycoprotein MITAT 1.2 precursor (VSG 221)           | 51466  | 506   | <a href="#">gil139611</a>   |
| L Variant surface glycoprotein MITAT 1.2 precursor (VSG 221)           | 51466  | 588   | <a href="#">gil139611</a>   |

## Results



**Fig. 3.15: 2D-Gel of the *T. brucei* cytosolic protein fraction (without ZnCl<sub>2</sub>) reference map.**

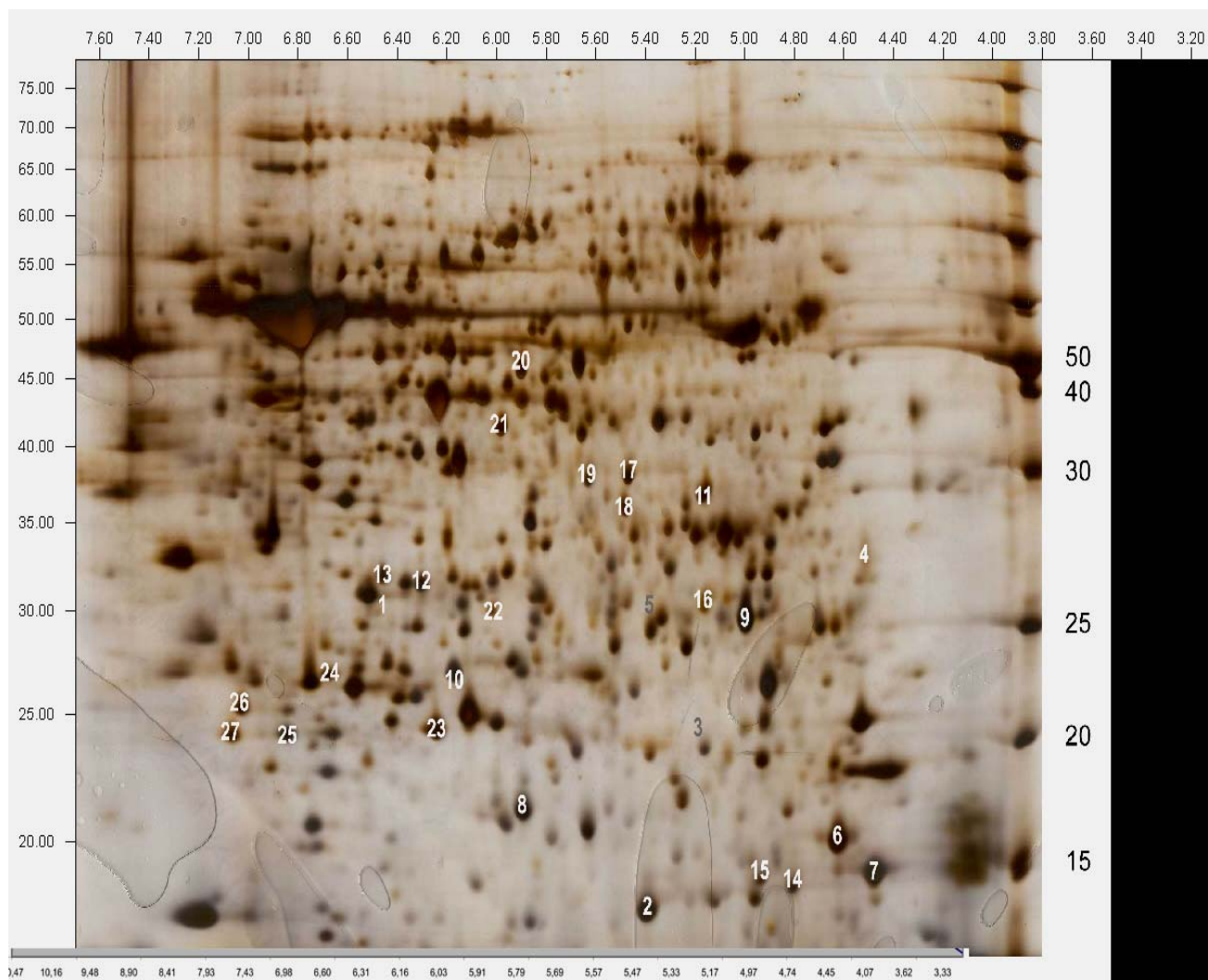
200 µg of cytosolic proteins was separated by IEF using the IPGphor system (Amersham). After first dimension, strips were equilibrated and run in 10% SDS-PAGE gel. 14 protein spots were excised for MALDI-TOF sequencing as shown in Table 3.3.

## Results

**Table 3.3: Result from *T. brucei* sequenced highly expressed cytosolic protein fraction.**

| Spot ID and Protein name                           | Mass  | Score. | Accession number            |
|--|-------|--------|-----------------------------|
| 1 40S ribosomal protein S12, putative.             | 16279 | 134    | <a href="#">gil70832745</a> |
| 2 Ran-binding protein 1, putative                  | 17692 | 87     | <a href="#">gil70833806</a> |
| 3 Ran-binding protein 1                            | 17692 | 56     | <a href="#">gil70833806</a> |
| 4 Adenosine kinase, putative                       | 38447 | 389    | <a href="#">gil62360454</a> |
| 5 Hypothetical protein, conserved                  | 36796 | 49     | <a href="#">gil62176207</a> |
| 6 Adenosine kinase, putative                       | 38397 | 94     | <a href="#">gil62360449</a> |
| 7 Chain A, Structure Of Trypanosoma Brucei Enolase | 47358 | 68     | <a href="#">gil30749888</a> |
| 8 Hypothetical protein, conserved                  | 42444 | 92     | <a href="#">gil62359432</a> |
| 9 Pteridine reductase                              | 28852 | 78     | <a href="#">gil3220185</a>  |
| 10 Hypoxanthine-guanine phosphoribosyltransferase  | 23573 | 235    | <a href="#">gil70832066</a> |
| 11 Proteasome alpha 7 subunit, putative            | 28019 | 382    | <a href="#">gil70834160</a> |
| 12 Iron superoxide dismutase                       | 23380 | 113    | <a href="#">gil70834946</a> |
| 13 Proteasome beta 2 subunit                       | 27613 | 127    | <a href="#">gil70831568</a> |
| 14 Guanylate kinase, putative                      | 20264 | 201    | <a href="#">gil70833045</a> |

## Results



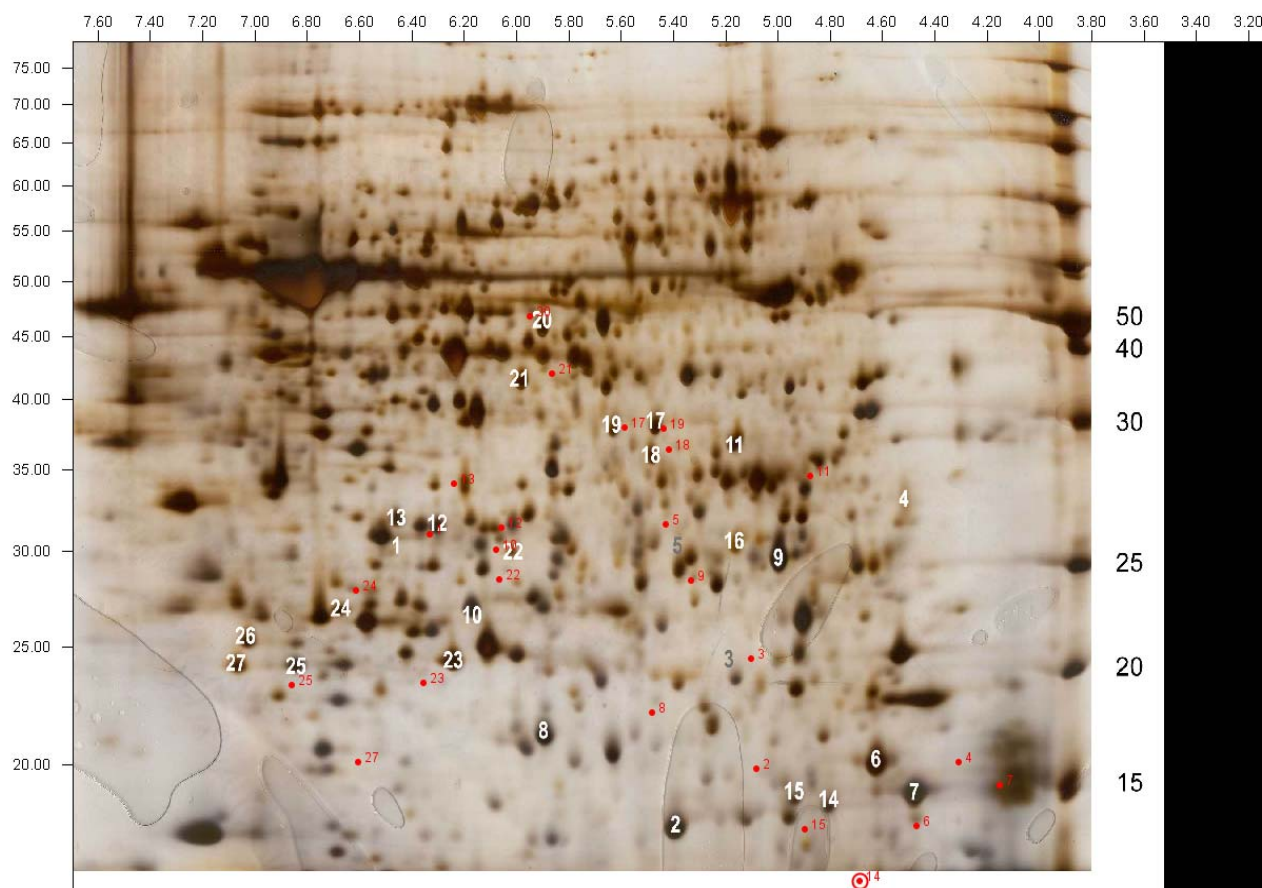
**Fig. 3.16: *T. brucei* protein reference gel map**

2D-gel electrophoresis reference map of *T. brucei* proteins.

### 3.6.2 Comparison of theoretical and the experimentally observed pI and MW

The theoretical pI and MW of proteins shown in the protein list were calculated with the “JVirgel soft ware” tool of the SWISS-PROT database. This tool calculates the pI of a protein by calculating the mean of the pK values of its amino acids as described by (Münch *et al.*, 1993). In (Fig. 3.16 and 3.17), a total of five proteins on the reference map could be detected in more than one spot. Most of the multiple spot proteins are probably isoforms of the same proteins. However, spots (B, D, I, J) in Fig 3.14, and spot (15 and 16) in Fig 3.16 correspond to the same gene but show a marked difference in pI and MW. Similar to these proteins are (spots 8 and 25) on Fig 3.16 that were identified by sequence information corresponds to the same gene (Table 3.4).

## Results



**Fig. 3.17: 2D-gel electrophoresis of soluble proteins from *T. brucei* reference map.**

The white numbers indicate the sequenced spots and red figures indicate the predicted protein spots using JVirGel program (Münch *et al.*, 2003)

**Table 3.4: Result from *T. brucei* sequenced protein reference map.**

| spots tab   |  |                             |       |               |                |               |                    |                     |
|-------------|--|-----------------------------|-------|---------------|----------------|---------------|--------------------|---------------------|
| Spot Number | Proteinname  | NCBI-AccessionNR            | MW    | calculated pi | MA SCO T-Score | induced by AA | cytosolic fraction | cut from gel        |
| 1           | prostaglandin F synthase                                 | <a href="#">gi 70833932</a> | 31201 | 6.32          | 212            | +             | yes                | 140905_cyto_induced |
| 2           | ADP-ribosylation factor-like protein 3A, putative        | <a href="#">gi 62175532</a> | 20096 | 5.34          | 114            | =             | yes                | 140905_cyto_induced |
| 3           | hypothetical protein, conserved                          | <a href="#">gi 70835126</a> | 24452 | 5.31          | 73             | +             | yes                | 140905_cyto_induced |
| 4           | nascent polypeptide associated complex subunit, putative | <a href="#">gi 70831317</a> | 20117 | 4.57          | 39             | +             | yes                | 140905_cyto_induced |



Results

| spots tab   |   |                             |       |               |                |               |                     |                               |
|-------------|---|-----------------------------|-------|---------------|----------------|---------------|---------------------|-------------------------------|
| Spot Number | Proteinname   | NCBI-AccessionNR            | MW    | calculated pi | MA SCO T-Score | induced by AA | cyto solic fraction | cut from gel                  |
| 5           | haloacid dehalogenase-like hydrolase, putative            | <a href="#">gi 70834304</a> | 31622 | 5.54          | 46             | +             | yes                 | 140905_cyto_induced           |
| 6           | eukaryotic translation initiation factor 5a, putative     | <a href="#">gi 70833553</a> | 17923 | 4.75          | 122            | =             | yes                 | 140905_cyto_induced, _control |
| 7           | translationally controlled tumor protein (TCTP), putative | <a href="#">gi 62359603</a> | 19367 | 4.45          | 57             | =             | yes                 | 140905_cyto_control           |
| 8           | iron-containing superoxide dismutase                      | <a href="#">gi 14029752</a> | 22235 | 5.56          | 115            | =             | yes                 | 140905_cyto_control           |
| 9           | translation elongation factor 1-beta, putative            | <a href="#">gi 62358758</a> | 28614 | 5.52          | 262            | =             | yes                 | 140905_cyto_control           |
| 10          | lysophospholipase, putative                               | <a href="#">gi 62175145</a> | 30245 | 6.09          | 248            | =             | yes                 | 140905_cyto_control           |
| 11          | 60S acidic ribosomal subunit protein, putative            | <a href="#">gi 70833676</a> | 34891 | 5.12          | 230            | =             | yes                 | 140905_cyto_control           |
| 12          | protein kinase, putative                                  | <a href="#">gi 7083334</a>  | 31526 | 6.07          | 66             | =             | yes                 | 140905_cyto_control           |
| 13          | dihydroorotate dehydrogenase, putative                    | <a href="#">gi 62176736</a> | 34432 | 6.24          | 31             | =             | yes                 | 140905_cyto_control           |
| 14          | 40S ribosomal protein S12, putative                       | <a href="#">gi 70832745</a> | 16279 | 4.94          | 134            | =             | yes                 | 211105_cyto_-ZnCl2            |
| 15          | Ran-binding protein 1, putative                           | <a href="#">gi 70833806</a> | 17692 | 5.12          | 87             | =             | yes                 | 211105_cyto_-ZnCl2            |
| 16          | Ran-binding protein 1, putative                           | <a href="#">gi 70833806</a> | 17692 | 5.12          | 56             | =             | yes                 | 211105_cyto_-ZnCl2            |
| 17          | adenosine kinase, putative                                | <a href="#">gi 62360454</a> | 38447 | 5.69          | 389            | =             | yes                 | 211105_cyto_-ZnCl2            |
| 18          | hypothetical protein, conserved                           | <a href="#">gi 62176207</a> | 36796 | 5.55          | 49             | =             | yes                 | 211105_cyto_-ZnCl2            |
| 19          | adenosine kinase, putative                                | <a href="#">gi 62360449</a> | 38397 | 5.57          | 94             | =             | yes                 | 211105_cyto_-ZnCl2            |
| 20          | enolase   | <a href="#">gi 30749888</a> | 47358 | 6.01          | 214            | =             | yes                 | 211105_cyto_-ZnCl2            |
| 21          | hypothetical protein                                      | <a href="#">gi 62359432</a> | 42444 | 5.92          | 92             | =             | yes                 | 211105_cyto_-ZnCl2            |
| 22          | pteridine reductase                                       | <a href="#">gi 3220185</a>  |       |               | 78             | =             | yes                 | 211105_cyto_-ZnCl2            |
| 23          | hypoxanthine-guanine phosphoribosyltransferase            | <a href="#">gi 70832066</a> |       |               | 235            | =             | yes                 | 211105_cyto_-ZnCl2            |
| 24          | proteasome alpha 7 subunit, putative                      | <a href="#">gi 70834160</a> |       |               | 382            | =             | yes                 | 211105_cyto_-ZnCl2            |
| 25          | iron superoxide dismutase                                 | <a href="#">gi 70834946</a> |       |               | 113            | =             | yes                 | 211105_cyto_-ZnCl2            |
| 26          | proteasome beta 2 subunit, putative                       | <a href="#">gi 70831568</a> | 27613 | 8.30          | 127            | =             | yes                 | 211105_cyto_-ZnCl2            |
| 27          | guanylate kinase, putative                                | <a href="#">gi 70833045</a> | 20264 | 6.53          | 201            | =             | yes                 | 28852                         |

**Table 3.5: *T. brucei* proteins identified by MALDI-TOF with known functions grouped into functional categories**

| <b>Functional destination</b>                     | <b>Protein</b>  |
|---|---|
| <b>Metabolism</b>                                 | Prostaglandin F synthase<br>Haloacid dehalogenase-like hydrolase<br>Enolase<br>Hypoxanthine-guanine Phosphoribosyltransferase<br>Pteridine reductase<br>Adenosine kinase,<br>Guanylate kinase, putative<br>Iron superoxide dismutase<br>Lysophospholipase,<br><br>Proteasome beta 2 sub unit<br>Proteasome alpha 7 sub unit |
| <b>Protein destination</b>                        | Ran-binding protein 1,<br>Translationally controlled tumor protein (TCTP),  |
| <b>Cell cycle growth and protein biosynthesis</b> | ADP-ribosylation factor-like protein 3A<br>Nascent polypeptide associated complex subunit<br>Nascent polypeptide associated complex subunit<br>Eukaryotic translation initiation factor 5a<br>Translation elongation factor 1-beta.<br>40S ribosomal protein S12,<br>60S acidic ribosomal subunit protein                   |

The JVirGel program was used for a further analysis of protein spots after the separation of the cytosolic and the membrane protein fraction from the whole proteome. The program offers three different analytical modes: a serial calculation of MW, pI, pH-dependent charge curves and hydrophobicity profiles, a virtual 2D gel as Java™ applet; and a virtual 2D gel as clickable HTML document; and a serial calculation of MW, pI, pH-dependent charge curves and hydrophobicity profiles. Using this program which separates proteins according to the MW, pI, pH-dependent charge curves and hydrophobicity profiles, it was possible to identify several proteins from experimental data as compared to the theoretical approach which does not take into account any protein-specific differences in the pI of charged amino acid residues, resulting from the interaction with neighbouring residues as shown in (Ffig. 3.17). Protein modifications resulting as additional charge, such as a bound phosphate group, which usually changes the overall pI of the protein, are not included. Usually, the deviation in pIs for these reason do not exceed a value of one pH unit (Skoog, and Wichman, 1986; Patrickios. and Yamasaki, 1995).

## Results

There are four proteins highly expressed from the sequencing results that are highly expressed which have attracted many studies in recent years. They are prostaglandin, enolase, (Ernest *et al.*, 1998), ADP-ribosylation factor like protein and hypoxanthine-guaine phosphoribosyltransferase. These proteins have been cloned and their molecular functions have been elucidated. Prostaglandin F Synthase (Kubata *et al.*, 2000) has been shown to be highly expressed in *T. cruzi* proteome map (Robello *et al.*, 2004).

### 3.6.3 ADP-ribosylation-factor like protein (ARL1).

Identification of nine uncharacterized ADP-ribosylation factors (Arf/Arls) in the *T. brucei* and *T. cruzi* genomes and ten in *L. major* members of the Arf family of proteins in three trypanosomatid species has been characterized (Smith *et al.*, 2005). Fig 3.20 spot (2) is one of the identified ARL Protein in cytosolic bloodstream form *T. brucei*.

ADP-ribosylation factor are a family of GTP binding proteins. ARL3A is believed to have a role in flagellum biosynthesis as overexpression of a constitutively active Q70L mutant form of the *L. donovani* protein leads to flagellar disappearance in promastigotes (Cuvillier *et al.*, 2000). Parasites expressing the mutant protein have no defects in cell division or differentiation, can infect and survive within macrophages but are unable to survive in the insect vector (Cuvillier *et al.*, 2003). As the known trypanosomatid ARL3A homologues share a high degree of identity at the amino acid level (79-98%), it is likely that they have similar roles in flagellum development in these parasite species. Characterization of the *T. brucei* ARL1 homologue has revealed that the protein is localized in the Golgi apparatus and is expressed only in the mammalian bloodstream form of the parasite and not in the insect procyclic stage. RNA interference has been used to demonstrate that ARL1 is essential for viability in *T. brucei* bloodstream parasites ((Cuvillier *et al.*, 2003). Prior to cell death, depletion of ARL1 protein in bloodstream parasites results in abnormal morphology, including disintegration of the Golgi structure, multiple flagella and nuclei, and the presence of large numbers of vesicles. The cells have only a minor apparent defect in endocytosis but exocytosis of variant surface glycoprotein to the parasite surface is significantly delayed (Cuvillier *et al.*, 2003). In procyclic cells, RNA interference of ARL1 has no effect on parasite growth or morphology. There may be different pathways regulating the Golgi structure and function in the two major life cycle stages of *T. brucei*. The only other studies on Arf proteins in these organisms to date have identified ARF1 in *T. cruzi* (de Sa-Freire *et al.*, 2003) and characterized ARL3A in *Leishmania* spp. (Sturm *et al.*, 1998; Cuvillier *et al.*, 2000).

## Results

The most striking difference between insect and host stages of *T. brucei* to date is in the rate of cellular protein trafficking, including transport through the flagellar pocket, a deep invagination of the plasma membrane which acts as the sole site of exocytosis and endocytosis in the cell (Overath and Engstler 2004). As extracellular pathogens, bloodstream forms *T. brucei* are under constant pressure from the host's immune system. The dominant surface protein VSG has a major role in host's immune evasion, undergoing antigenic variation to allow persistent infection (McCulloch 2004). The protein is also rapidly endocytosed, clearing surface-bound immunoglobulins, before recycling back on to the plasma membrane. The entire surface VSG of the parasite has a short turnover time, more than double the membrane turnover rates reported in mammalian cells. The relevant internal machinery of the cell needs to be channelled towards the maintenance of this vital process. In contrast, protein transport is much slower in the procyclic stage, consistent with a significant down-regulation of proteins functioning in endocytosis, such as clathrin. Another major difference between the two life-cycle stages is in their metabolic requirements. Bloodstream cells are entirely dependent on glycolysis for their ATP supply, metabolizing glucose into pyruvate in specialized peroxisome like organelles, the glycosomes. Energy metabolism is considerably more complex in insect stage cells, characterized by aerobic fermentation of glucose when available, and the additional utilization of lipids and amino acids, particularly proline, which is used by the tsetse fly as its main energy source during flight (Lamour *et al.*, 2005). It is feasible that the need to prioritize sorting and trafficking of very different sets of enzymes, receptors, effectors and metabolites in the two life-cycle stages of *T. brucei* has exerted sufficient evolutionary pressure for the specialization of golgi function in these cell types.

### **3.6.4 *T. brucei*, Hypoxanthine-guanine phosphoribosyltransferase (HPRT).**

Hypoxanthine-guanine phosphoribosyltransferase (HGPR) spot (23) on Fig 3.20 is another highly expressed protein. Biochemical and metabolic studies have substantiated the existence of a plethora of purine salvage enzymes in *T. brucei*, including phosphoribosyltransferases (PRTs), nucleoside kinases, and nucleoside cleavage activities. The unique features of the purine salvage system of *T. brucei* and the related parasites *Trypanosoma cruzi* and *Leishmania* cause these pathogenic hemoflagellates to be selectively susceptible to the cytotoxic effects of several pyrazolopyrimidine analogs of hypoxanthine and inosine (Ullman 1984, Allen and Ullman

## Results

1993). For instance, *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodiensense* bloodstream forms are all sensitive to allopurinol 4-hydroxypyrazolo 3, 4-d pyrimidine; (HPP) (Balber *et al.*, 1985), a pyrazolopyrimidine isomer of hypoxanthine that is nontoxic to humans and used extensively in the treatment of hyperuricemia and gout (Palella and Fox 1989)

Metabolism of HPP in *T. brucei*, as well as in *Leishmania* and *T. cruzi*, is initiated by the enzyme hypoxanthine- guanine phosphoribosyltransferase (HGPRT): IMP:pyrophosphate phosphoribosyltransferase, the unique substrate specificity of the HGPRT enzyme of these parasites suggests that the enzyme may serve as a rational target for antiparasitic drugs.

The isolation and purification of the molecular clone encoding the *T. brucei* hypoxanthine-guanine phosphoribosyltransferase (HGPRT) in *E. coli* has been shown by Allen and Ullman 1993. In *E. coli* which is genetically deficient in both HPRT and XGPRT, it has been shown that the enzyme specific for hypoxanthine and guanine does not recognize either xanthine or adenine. Moreover, as hypoxanthine is the salvageable purine present in the highest concentrations in human blood and within the cerebrospinal fluid (Eells and Spector, 1983) the milieu inhabited by the parasite, GPRT are likely to provide a critical nutritional function in *T. brucei*.

The expression of the recombinant *T. brucei* HGPRT in the S4606 *E. coli* establishes that the enzyme can recognize HPP as a substrate. The procyclic forms of *T. brucei*, *T. b. gambiense*, and *T. b. rhodesiense* can further convert HPP to aminopyrazolpyrimidine nucleotides and incorporate the triphosphate of the latter into RNA. This metabolic sequence does not exist in humans or mammals, and the procyclic forms of the parasite are selectively sensitive to HPP. The curative effects of HPP against African trypanosomiasis in humans have not been established, but in the year 1992, Martinez and Marr showed that the pyrazolopyrimidine exhibits demonstrable therapeutic efficacy in clinical trials against both cutaneous leishmaniasis and Chagas disease.

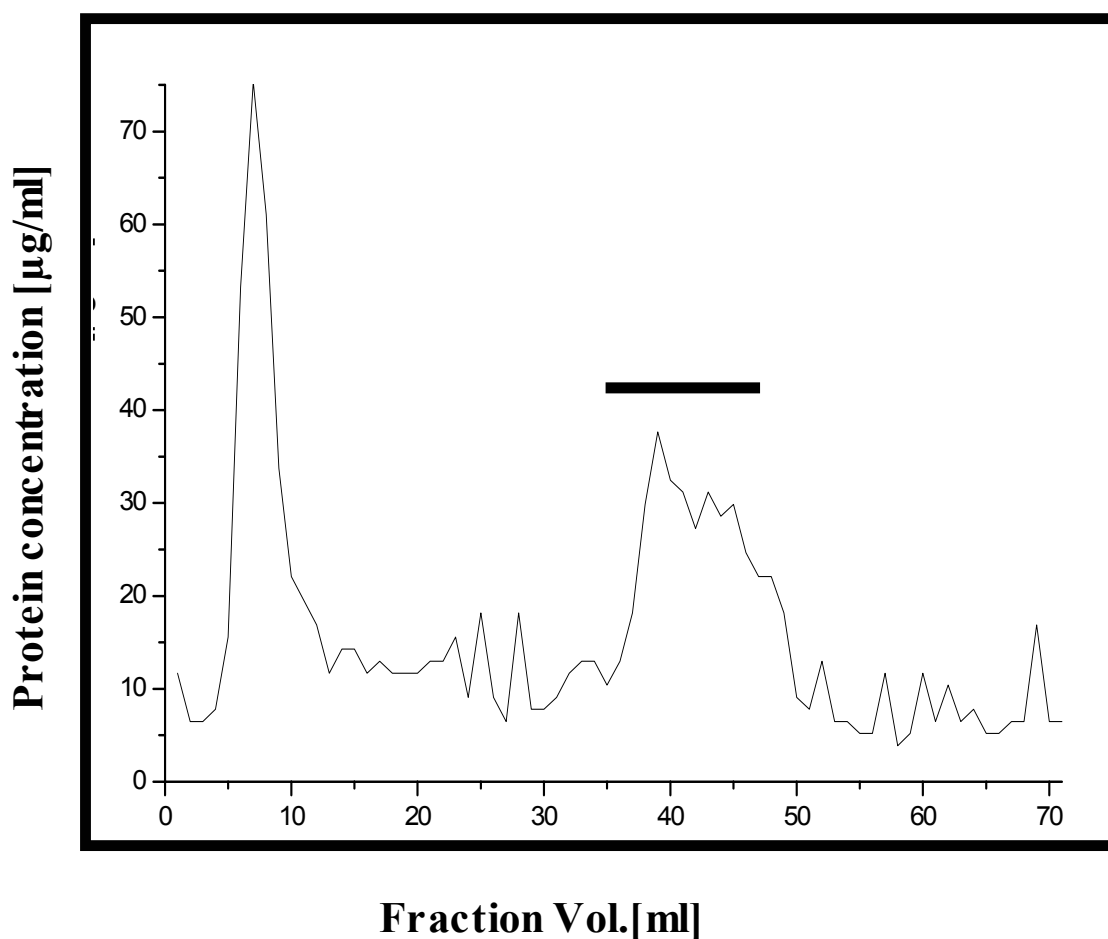
The selective toxicity of HPP toward *T. brucei* and the potentially central role of HGPRT in purine salvage imply that the parasite HGPRT may provide a biochemical rational target for the therapeutic manipulation or improve therapies for the treatment of African trypanosomiasis.

### 3.7 Ion-exchange chromatography and immunoprecipitation.

Further attempts were undertaken through convention methods of ion-exchange chromatography and immunoprecipitation of protein for the isolation of prostaglandin D synthase. Membrane protein was separated into different fractions using sephadex G-75. Fractions 38-48 were pooled and used for anion exchange chromatography (Fig. 3.18 and 3.19).

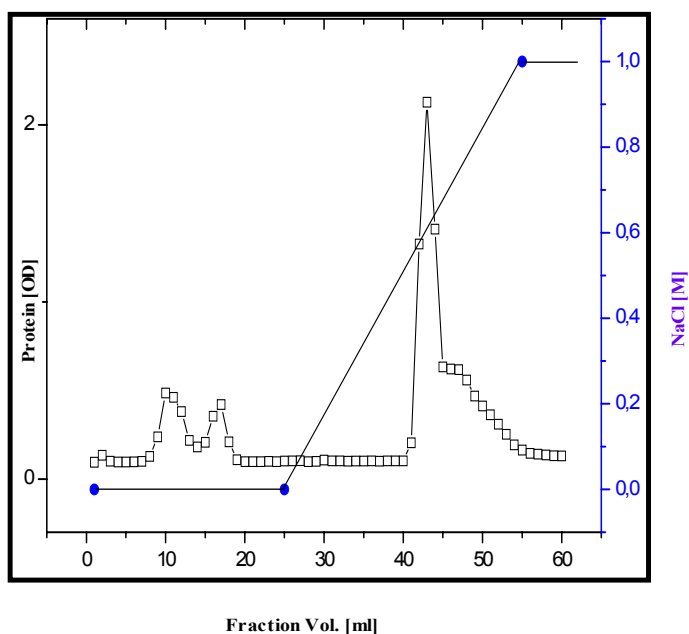
#### 3.7.1 Production of PG from AA with Membrane protein fraction.

## Gel filtration Sephadex-75



**Fig. 3.18 : *T. brucei* membrane protein fraction elution profile.**  
Elution profile of membrane protein fraction from Gel filtration (Superdex 75).

## Results



**Fig. 3.19: Anion-exchange; diethylaminoethyl sephadex (DEAE) chromatography elution profile of membrane protein.**

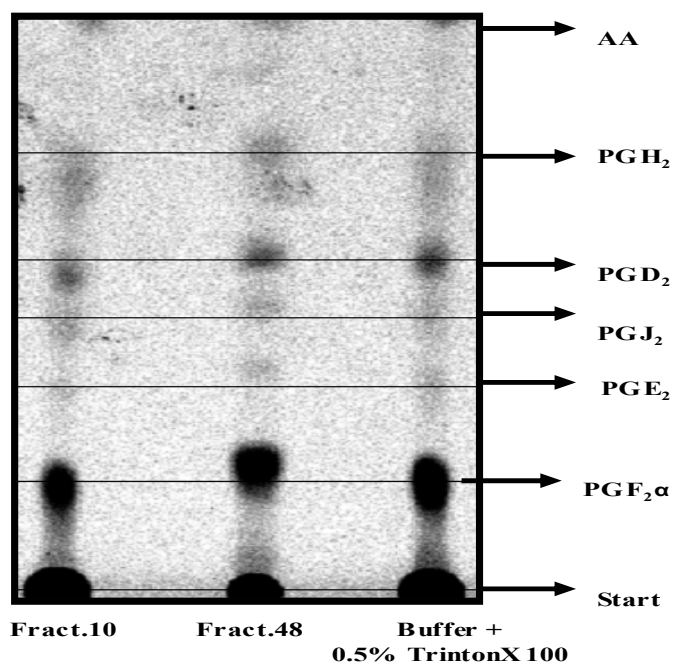
0-1M NaCl in 1mM Tris-HCl was used to elute protein from the anion-exchange material

To isolate the membrane protein fraction the lysate was centrifuged for 60 min (60,000xg, 4°C). The pellet was washed three times in 10 mM Tris-HCl buffer containing the protease inhibitor complete. The pellet was incubated in 10 mM Tris-HCl containing 0.1% (v/v) Triton X 100 overnight at 4°C. The membrane fraction was separated from insoluble debris by centrifugation for (20 min at 100,000xg 4°C). This membrane fraction from long slender bloodstream form parasites were loaded onto Superdex 75 gel filtration column which has been equilibrated with 1mM Tris-HCl containing 0.01% Triton X 100. Then, 1mM Tris-HCl was used for protein elution. Fraction 38-50 (Fig. 3.18) were pooled and used for anion-exchange chromatography. This column was eluted using a flow rate of 0.1ml/min and a linear gradient 0-1 M NaCl in 1mM Tris-HCl. Two protein fractions were pooled and concentrated with vacuum under a N<sub>2</sub> gas flow. 25µg protein from each fraction was used for the in vitro synthesis of PGs (Fig. 3.20).

The in-vitro PG assay of the membrane protein fraction showed increase activity of the PGD synthase and PGF synthase as compared to cytosolic (Fig 3.20 and Fig. 3. 1, page 56). These result as seen in the fluorography result, showed an increase in the production of prostaglandin D and F<sub>2</sub>α. This result may be due to transformation of PGH<sub>2</sub> to PGF<sub>2</sub>α which is achieved in cells through endoperoxide reductase and transformation of PGH<sub>2</sub> to PGF<sub>2</sub>α through endoperoxide isomerase (Decker 1985).The result seen in control Fig 3. 20, (buffer and Triton X 100) may be

## Results

due to the presence of Triton X 100 in the buffer and it has been shown to have effect in enzyme assays (Baker 1990).



**Fig. 3.20: Fluorography from induced *T. brucei* membrane protein fraction ( $1 \times 10^{10}$  cells) of Prostaglandin in vitro synthesis**

Membrane fractions from long slender bloodstream form parasites. The high expression of PGF<sub>2</sub>α and PGD<sub>2</sub> in *T. brucei* may result from transformation of PGH<sub>2</sub> to PGF<sub>2</sub>α which is achieved in cells through endoperoxide reductase

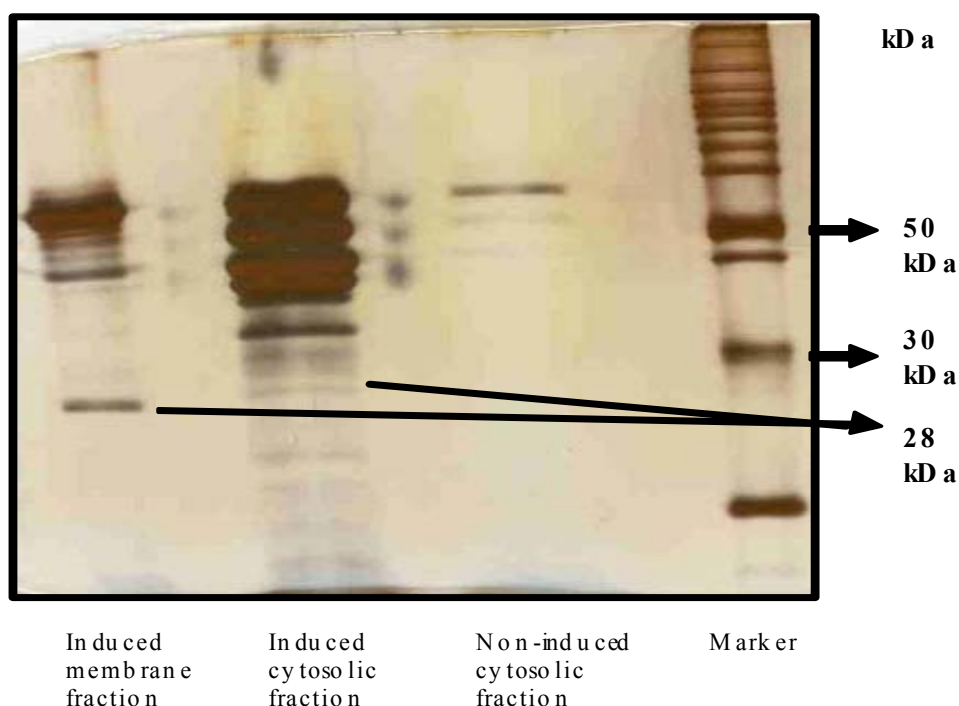
### 3.7.2 Immunoprecipitation of *T. brucei* protein fractions using anti-PGDS from sheep.

Immunoprecipitation (IP) is one of the most widely used methods for antigen detection and purification. The characteristic of IP reactions is their potential to deliver not only the target protein but also other macromolecules that interact with the target (Adams *et al.*, 2002). The principle of an IP is simple. An antibody (monoclonal or polyclonal) against a specific target antigen is allowed to form an immune complex with that target in a sample, such as a cell lysate or membrane protein fraction. The immune complex, Protein A sepharose binds to the antibody, which is bound to its antigen. Finally, components of the bound immune complex (both antigen and antibody) were eluted from the column and analyzed by SDS-PAGE gel electrophoresis (Fig. 3. 21) followed by Western blot detection to verify the identity of the antigen (Fig. 3 .22 page 88). The assumption that is usually made when associated proteins are co-precipitated is that these proteins are related to the function of the target antigen at the cellular level (Liebler 2002).



## Results

Using antibody from peptide sequence of porcine PGDS raised in rabbits (Dacheux *et al.*, 1999), for the immuno-precipitation of induced membrane and cytosolic protein lysate. Protein A sepharose was washed twice with PBS. 100  $\mu$ l protein A sepharose bead slurry (50%) per 1 ml was added to various protein fractions and incubated at 4°C for 10 min on a Shaker. The protein A beads were removed by centrifugation at 14,000 rpm at 4°C for 10 minutes and the supernatant was transferred to a fresh centrifuge tube. 500  $\mu$ g antibody against PGDS (Sheep) was added to 250 $\mu$ g of each protein fraction. The protein/antibody mixture was gently rotated overnight at 4°C by end over end rotation. The immunocomplex was captured by adding 100  $\mu$ l protein A bead slurry and gently shaken on a shaker overnight at 4°C. The Sepharose beads were collected by pulse centrifugation (i.e., 5 seconds in the microcentrifuge at 14,000 rpm). The supernatant was washed 3 times with 800  $\mu$ l ice-cold PBS buffer. The sepharose beads were resuspended in 40  $\mu$ l 2x sample buffer and gently mixed. The sepharose beads were boiled for 5 min to dissociate the immunocomplexes from the beads. The beads were collected by centrifugation and SDS-PAGE was performed with the supernatant.



**Fig. 3.21: Immuno-precipitation of anti PGDS (sheep) with induced membrane and cytosolic protein fractions from *T. brucei*.**

200 $\mu$ g of induced membrane protein fraction was precipitated with 100 $\mu$ g anti-PGDS (Dacheux *et al.*, 1999) in protein A sepharose. The silver stained gel shows a specific band of about 28 kDa. This protein band was excised from gel and sequenced. MALDI-TOF analysis showed 38% sequence identity with VSG.

## Results

The result showed (Fig. 3.21) that the PGDS could be detected after the immuno-precipitation except that co-elution of antibody fragments with antigen which might lead to results in bands interfering with detection of the co-precipitated proteins on SDS-PAGE. The protein detected was in the pI and MW range of the PGDS isolated in Sheep (Dacheux *et al.*, 1999 and 2003).

The protein band was excised and digested with trypsin. The result of MALDI-TOF was a 38% sequence identity to VSG from *T. brucei* (Fig 3.22). This result indicates that the PGDS and many similar proteins separate in the same range as the VSG which is highly expressed in *T. brucei*, and this protein could it overweight the PGDS in the excised protein band. The ideal situation would be to conduct the co-IP without contamination of the eluted antigen with antibody. When this potential interference is eliminated, only the co-precipitated proteins will be present and detected on a gel.

### **MASCOT** Mascot Search Results

#### Protein View

Match to: **VSM2\_TRYBB**; Score: **868**  
VARIANT SURFACE GLYCOPROTEIN MITAT 1.2 PRECURSOR (VSG 221). - ...  
Found in search of DATA.TXT

Nominal mass ( $M_r$ ): **51850**; Calculated pI value: **6.73**  
NCBI BLAST search of **VSM2\_TRYBB** against nr  
Unformatted [sequence string](#) for pasting into other applications

Taxonomy: [Trypanosoma brucei brucei](#)

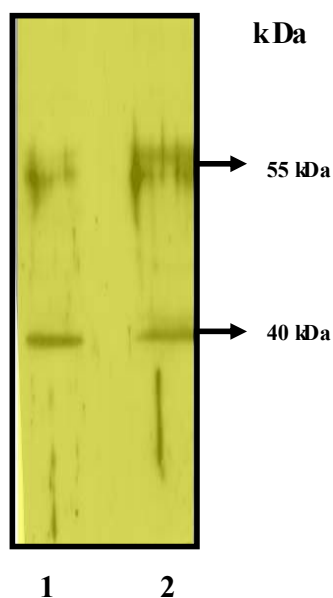
Fixed modifications: S-pyridylethyl (C)  
Variable modifications: Oxidation (M)  
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
Sequence Coverage: **38%**

Matched peptides shown in **Bold Red**

```
1  MPSNQEARLF  LAVLVLAQVL  PILVDSAAEK  GFKQAFWQPL  CQVSEELDDQ
51  PKGALFTLQA  AASKIQKMRD  AALRASIYAE  INHGTNRACA  AVIVANHYAM
101 KADSGLEALK  QTLSSQEVTA  TATASYLKGR  IDEYLNLLLQ  TKESGTSGCM
151  MDTSGTNTVT  KAGGTIGGVP  CKLQLSPIQP  KRPAATYLGK  AGYVGLTRQA
201  DAANNFHDND  AECRLASGHN  TNLGKSGQL  SAAVTMAAGY  VTVANSQTAV
251  TVQALDALQE  ASGAAHQPI  DAWKAKKALT  GAETAEFRNE  TAGIAGKTGV
301  TKLVEEALLK  KKDSEASEIQ  TELKKYFSGH  ENEQWTAIEK  LISEQPVAQN
351  LVGDNQPTKL  GELEGNAKLT  TILAYRMET  AGKFEVLTQK  HKPAESQQQA
401  AETEGSCNKK  DQNECKSPCK  WHNDAENKKC  TLDKEEAKKV  ADETAKDGT
451  GNTNTTGSSN  SFVISKTPW  LAVLLF
```

**Fig. 3.22: MALDI-TOF sequence of VSG from co-precipitated protein**  
The sequence result of the excised protein band from immuno-precipitation.

### 3.2.2 Western Blotting with anti-PGDS from sheep.



**Fig. 3.23: Western blotting and fluorography analysis of the anti-PGDS with *T. brucei* protein**

100  $\mu$ g cytosolic (lane 1) and membrane (lane 2) fraction were run on 12% SDS-PAGE. The proteins were transferred onto Hybond<sup>TM</sup> ECL<sup>TM</sup> a Nitrocellulose membrane and detected with anti-PGDS. The primary antibody was diluted at 1:2000 in PBS-T. The second antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, was used at 1:10,000 dilution in PBS-T.

There was increase in the secretion of the PGDS after 18 hours in the presence of arachidonic acid in growth medium as shown in Fig. 3.1 prostaglandin assay. The protein from cytosolic and membrane fractions were detected by Western blotting following SDS gel electrophoresis at a range of 40-55 kDa (Fig. 3.23). There are some draw backs in the traditional immunoprecipitation protocol (IP). The co-IP has equally certain deficiencies relating to the fundamental format of the assay, the antibody and associated chemistry. One of the most commonly encountered problems with co-IP approach is interference from antibody bands in gel analysis. In some case several proteins may be co-precipitated with the target protein in the presence of the co-eluted antibody heavy and light chains (fragments 25 and 50 kDa bands in reducing SDS-PAGE gel) in which results may be misleading.

#### 4. Discussion

The aim of this study was to establish a reference proteome map of *Trypanosoma brucei* and to identify new proteins synthesized when *T. brucei* bloodstream form are grown in growth media supplied with exogenously arachidonic acid.

Robello and his group has (2004) recently shown a partial proteome map of the causative agent of Chagas disease: *Trypanosoma cruzi*. The use of a JVirGel program which offers a virtual 2D gel and separates protein according to the MW, pI, pH-dependent charge curves could be used to identify several proteins from experimental data in addition to the theoretical approach. However, for diagnostic purposes most proteins are generally not available. In contrast, the *T. brucei* proteome would allow the study of proteins at different stages of infection and presence of additional substrates in growth media would show expression of new proteins or inhibition of specific protein biosynthesis. Additionally it would also allow investigation of the genetic variability within *T. brucei* in a larger control of trypanosomiasis.

##### 4.1 Protein isolation

Isolation of proteins is the first step for the identification of protein in a proteome map followed by the investigation of the protein function in vitro studies. Subfractionation of cellular components can accumulate low-abundance proteins and make their detection easier. The effect of enrichment of membrane proteins is illustrated in Fig. 3.14. The number of spots in the membrane fraction gel is distinctly reduced as compared to that of the gel from cytosolic protein fraction. The background noise and the complexity of the sample are thus reduced. The introduction of several modifications to standard protocols in this study is to solve some basic problems of protein identification. First of all, in order to isolate the membrane protein fraction more easily, the use of CHAPS, Triton X-100 or digitonin are necessary. However, this membrane destabilizing agent disrupts membranes indiscriminately, i.e. the membrane of subcellular organelles will be attacked as well causing micelles. Bronfman (*et al.*, 1998) found out that subcellular organelles are only affected if the concentration of digitonin is higher than 0.5-1.0 mg/ml. I therefore used only low concentrations of Triton X-100 (0.1 %/ v/v). The *T. brucei* were lysed in hypotonic buffer containing (10 mM Tris-HCl, HCl). Lysis of cell will occur due to the osmotic pressure of the used buffer. Secondly, the mechanical homogenizing cycle three times for 10 seconds each was to ensure disruption of

## Discussion.

all cells. After the third round of homogenization about 95% cells were disrupted. The number of repetitive homogenizing cycles depends not only on the type of biological material but also on the type of pestle and on the rotating speed of the homogenizer. Thirdly, separation of the cytosolic fraction from subcellular organelles by centrifugation is equally important for the separation of proteins to respective fractions. For this task standard protocols suggest the use of a continuous centrifugation. However, this method did not lead to a satisfactory result. Therefore, it was difficult to assure the purity of the protein, especially when the membrane amount of the protein fraction is very limited. So I opted for the use of detergent and Zinc chloride to reduce the sVSG in the lysate.

## 4.2 Two-dimensional gel electrophoresis

### 4.2.1 The choice of carrier ampholytes for isoelectric focussing.

Commercially available immobilized pH-gradients stripes for isoelectric focussing was used instead of carrier ampholytes. The advantages of immobilized pH-gradients stripes to run IEF-separations are as follow

#### Immobilized pH-gradients

- A Good protein resolution
- B A stable pH-gradient
- C Easier to handle
- D Excellent reproducibility
- E No batch-to-batch differences
- F Minor lot-to-lot differences in the pre-cast gels
- G Higher concentration of protein has little effect on the pH-gradient and the pI - resolution which is about 0.016 pH unit

The variability and stability of the pH gradient stripes are easy to handle. In addition, the excellent reproducibility of the result and easy equilibration method after the isoelectric focussing were advantages in my choice of immobilien stripes. In contrast, a variable batch-to-batch reproducibility, the effect of protein concentration on the shape of the pH-gradient, and the cathodic drift of the pH-gradient with time are draw backs in Carrier ampholytes.

## Discussion.

Since the aim of this study was to detect as many *T. brucei* protein spots as possible, optimum resolution was paramount. I thus chose to use 0.005% (v/v) carrier ampholytes buffer to run my IEF-separations. On the other hand, the disadvantages of carrier ampholytes were minimized with the choice of the same batch of ampholyte mixture. The effect of the protein sample on the pH-gradient was also investigated and is discussed below.

### 4.2.2 Reproducibility

The reproducibility of 2D-gel electrophoresis and protein separations can be assessed in three aspects:

- A. The presence/absence of spots : i. e. the reproducibility of protein resolution,
- B. The position of spots : i. e. the reproducibility of protein spot location on the gel.
- C. The size of spots: i. e. the reproducibility of protein abundance.

In order to test the reproducibility of the gel-runs, cytosolic proteins from *T. brucei* was carried out on four different gels. Fig 3.3

**Immobilien stripes.** The gel-runs achieved a satisfactory reproducibility of protein resolution since each spot on one gel was reproduced on the other gels.

A. The reproducibility of protein spots was only satisfactory when spots were compared to one another in a small perimeter from a certain anchor-point, i.e. the corresponding spots were only congruent when the gels were compared subsection by subsection. These running differences between two large gels might be due to minor inconsistencies of running conditions (temperature, buffer-composition), gel quality or protein-concentration.

B Most of the protein spots achieved satisfactory quantitative reproducibility. However, the varying reproducibility of certain protein spots might be due to additional interfering factors from the VSG protein which is highly expressed. The tight binding or affiliation of the mVSG with the outer membrane of the *T. brucei* makes its removal difficult. The degree of VSG removal most probably depends on the cell fractionation process, which is the most difficult step during protein isolation.

Several other factors can also impair the reproducibility. For example if the protein samples are thawed more than once not only the resolution of the protein spots but also their quantity is reduced (Fig. 3.18). This phenomenon is most probably due to the denaturation of proteins

## Discussion.

and subsequent precipitation during the freezing and thawing process. As expected the effect is most pronounced in the case of low abundance proteins.

### **The number of the visualized proteins on the gel**

In order to resolve as many protein spots as possible, 24 cm large stripes were used during 2D-gel electrophoresis (Lehner *et al.*, 2003). More protein spots were separated on a silver stained 2D-electrophoresis gel. The actual number of proteins from *T. brucei* protein content is unknown. Until now, a total of more than 500 different proteins have been registered in a special database of *T. brucei* proteins (TIGER Proteins Database). This database comprises information from “SwissProt”, “GenBank”, and “Genome Database” (GDB). Additionally, the existence of protein-isoforms increases the spot-number considerably. The number of isoforms of a certain protein varies between two to ten (Keith *et al.*, 2005). Although not all spots of putative isoforms were analyzed in this study, one can assume that a horizontal array of spots represent isoforms of the same protein which were less than 10%.

Loss of proteins is doubtless taking place during the whole process from protein preparation until staining of the gel. In order to minimize this loss a highly standardized sample preparation protocol introduced from (Lehner *et al.*, 2003) was used. One key point of this protocol is to solubilize hydrophobic proteins and keep them soluble. This is achieved by using CHAPS and high concentrations of Urea to break up non-covalent interactions and by using DTT to break up disulfide-bridges. Another key point is to reduce protease activities as good as possible. This is achieved by adding a cocktail of protease inhibitors and by performing all preparations at 4°C.

### **4.2.3 Staining of the gel**

In this study, silver staining was much more sensitive than the Coomassie staining. On the other hand, for the purpose of protein identification by MALDI-TOF mass spectrometry the Coomassie stain seemed to be superior. Some of the silver stained spots could not be identified. However, this disappointing result might not be due to the silver stain itself but due to the method of excising the protein spots out the gel. Therefore, the reason for the low identification rate of proteins in silver-stained gels was more likely caused by the low abundance of certain proteins than by a strong influence of silver ions on these proteins. Other authors like Shevchenko (1996a) and Rabilloud (1998) have demonstrated that silverstained gels can indeed be used for satisfactory mass spectrometric identification of proteins.

#### 4.2.4 Protein identification

The proteins were identified mainly by MALDI-TOF mass spectrometry on the basis of their peptide mass fingerprints and in some cases by sequencing a peptide tag with MALDI-TOF/LC/MS tandem mass spectrometry.

According to the annotations in SWISS-PROT and NCBI, more than 60% of the identified proteins were *T. brucei* proteins. The other 20% identified proteins included 10% cytoplasmic proteins in membrane protein fraction and 10% unknown proteins (most of them were only present as expressed sequence tags (EST)). The majority of the spectra were of good quality which easily allowed protein identification. The high accuracy in mass determination of the peptide fragments is made possible by MALDI-TOF mass spectrometry with “delayed extraction”. The delayed extraction method allows peptide fragments of the same weight to be better focused. This leads to sharp and accurate spectra which allow the reliable identification of proteins even with limited sequence coverage (Jensen *et al.*, 1996). Therefore, the results were reliable even with sequence coverage of some of the protein ranging from 14- 86%. The array of identified proteins contained some proteins from other cell compartments. This “contamination” cannot simply be attributed to the purification method of the protein. Such proteins as the VSGs or glycosome associated protein aldolase (Fig. 3.16) are very abundant. These proteins are difficult to remove completely from the membrane protein fraction and are absent when cytosolic protein fraction was investigated. However, one cannot rule out that these proteins might interact with membranes as well, since some other non-membrane proteins are known to have very tight links to the glycosomes. They either bind to the outer membrane of glycosomes or transport material to the glycosome.

There are 27 spots Fig 3.16; on the list, which could be identified. This might be due to the fact that the spectrum was not good enough or that the spectrum could not be matched with proteins listed within the databases. Besides the low abundance of certain proteins in the sample, several other reasons could also account for this. If two spots are very close to each other on the gel or overlapping each other, the proteins are mixed during excision of the spot. The spectra from those overlapping spots may confuse the database search. Peptide loss during the tryptic digest or the failure to extract the peptides from the gel pieces for MALDI-TOF mass spectrometry may be another reason for low amplitude mass spectra.

Notably, a large part of unidentified spots comprises small and very large proteins whose molecular weights are either below 15 kDa as well as above 60 kDa. Some of these proteins



## Discussion.

are fragments of larger proteins. Fig 3.17 (spot 15 and 16) is an example for these kind of proteins. These spot belong to the smallest protein among the identified ones. Its molecular weight is around 15-25 kDa.

### 4.3 The *Trypanosoma brucei* proteome reference map

A partial *T. brucei* reference proteome map has been established in this work. This map will be used as a tool for further investigations of *T. brucei* proteins. A total of 27 proteins from *T. brucei* origin, have been identified on this reference map (Fig. 3.17). The sub-cellular location and the function of each identified protein were looked up in various databases and original publications.

#### 4.3.1 The identified proteins

Similar to previous analyses of the *Trypanosoma cruzi* proteome (Robello, 2004), most of the identified proteins are hydrophilic or easy to solubilize proteins. Although 10 proteins of the list were annotated to be membrane-associated proteins, only four of them were clearly transmembrane proteins with at least one transmembrane domain. Unfortunately, most of the membrane-encoded proteins could not be identified in my study. The difficulty of the detection of membrane and basic proteins seems to be connected to the principle of 2D-electrophoresis. This method has certain limitations to detect four kinds of proteins (Gygi *et al.*, 2000; Nordhoff *et al.*, 2001):

- A. hydrophobic proteins,
- B. proteins of multi-protein-complexes,
- C. proteins with a very basic pI and
- D. very low molecular weight proteins

The critical point for a sufficient membrane-protein separation is the isoelectric focussing in the first dimension:

- A. Since membrane proteins are mostly hydrophobic proteins, they need to be solubilized to make them migrate in an electric field. This can be achieved by detergents (e.g. SDS or CHAPS). Detergents, however, affect the isoelectric point of proteins (Rabilloud, 1996). Therefore, only urea as a protein denaturant can be added to the isoelectric focussing gel to keep the proteins in solution. CHAPS a zwitterionic detergent, was added to the sample buffer to facilitate the solubility of hydrophobic

## Discussion.

proteins during sample preparation. However, its effect subsides as soon as the proteins enter the IEF-gel. It seems that urea in the IEF-gel can hardly counteract the tendency of the hydrophobic proteins to aggregate. Only few membrane-proteins do maintain their solubility and migrate towards their isoelectric points. The rest of the membrane proteins seem to aggregate and do not even enter the IEF-gel.

- B The proteins that are part of multi-protein-complexes are also denatured and solubilized by the use of CHAPS and urea during sample preparation. When the proteins enter the IEF-gel the effect of CHAPS begins to subside and some of the proteins start to refold and partially reconstitute themselves into multi-protein aggregates that cannot migrate through the pores of the 3.5% Polyacrylamide IEF-gel.
- C If the proteins have a very basic pI (>10) which is often the case for membrane proteins, they do not focus properly in the IEF-gel. At the basic end of the IEF-gel the pH gradient does not reach its equilibrium (Klose, 1995). This would cause the loss of very basic proteins unless they are captured in a higher percentage in cup loading of sample gel.
- D Small proteins with a molecular weight below 10 kDa are difficult to detect (Klose, 1995). This is due to the fact that the pores of the 10% second dimension SDS-PAGE-gel are too large to focus the small proteins properly (Carroll *et al.*, 2002). For proteins of this size a polyacrylamide-percentage of 20-22% would be appropriate. This could be achieved by pouring a 5-20% gradient-gel. This procedure is very laborious and difficult to reproduce exactly and would make different second dimension separations difficult to compare. Beyond that, the small proteins fail to stain with Coomassie blue are very faint in silver stained gels.
- The under-representation of transmembrane-proteins on a regular 2D-gel is a severe drawback of the method. Further work has to be done to make these proteins more soluble and accessible for isoelectric focussing. (Rabilloud *et al.*, 1996, 1999; Henningsen *et al.*, 2002; Navarre *et al.*, 2002)

### 4.3.2 Multiple spots proteins

Six proteins on my list are represented by multiple spots (Fig 3.14 and table 3.4). Most of them are present in pairs and are probably isoforms of the same protein.

The isoforms could be subdivided into:

- Naturally occurring isoforms that are formed by any kind of *in vivo* post-translational modification of proteins, such as phosphorylation, glycosylation or acetylation
- Isoforms caused by artifacts during sample preparation, which might be generated by the reaction of unpolymerized acrylamide with SH-groups or by oxidation of Methionine residues during sample preparation.

These modifications do not change the molecular weight of a protein so much that a difference in molecular weight might be detected in the second dimension run. However, they might well cause a shift of the isoelectric points either to the acidic or to the basic side.

There were proteins that were present in multiple spots and differed considerably in molecular weight and/or isoelectric point: VSG (Fig. 3.6), beta-tubulin (Fig. 3.14) and Ran-binding protein (Fig. 3.17). The large differences in molecular weight could be caused by differential splicing of the same precursor-mRNA. Proteolysis during the whole procedure might be another possible reason.

### 4.3.3 Comparison of my results with other *trypanosomal* proteomic projects

Identified proteins were grouped into functional categories (Table 5). Some proteins expected to be expressed in different subcellular compartments (cytoplasm, glycosomes and membrane) accomplishing different cellular functions in the parasite were identified showing, that this methodology could be used to identify a range of proteins expressed by the bloodstream form *T. brucei* VSG is the major protein in the soluble protein fraction as seen in Fig. 3.11. This result is similar to beta-tubulin, the major protein expressed in *T. cruzi* (Robello *et al.*, 2004).

The other major proteins identified were Prostaglandin F synthase, Enolase, and ADP-ribosylation factor.

Biochemical and metabolic studies have substantiated the existence of a plethora of purine salvage enzymes in *T. brucei*, including hypoxanthine-guanine phosphoribosyltransferase

## Discussion.

(HGPRT), phosphoribosyltransferases, (PRTs), nucleoside kinases, and nucleoside cleavage activities (Ullman, 1984 and Allen and Ullman 1993).

Prostaglandin F synthase has been shown to be highly upregulated in *T. brucei* when grown in the presence of arachidonic acid (Kubata *et al.*, 2000). This protein belongs to the flavin-dependent oxidoreductase family and can reduce either trypanocidal agents (nifurtimox and others) or hydroperoxides. It has been suggested that this enzyme is responsible for most of the antioxidant activity in *T. cruzi* (Kubata *et al.*, 2002). The presence of this protein in high abundance, as shown by 2D- gel electrophoresis, suggests a need to study this protein in more detail as a possible target for drug design

Enolase one of the rate limiting enzymes, in the glycolytic pathway in bloodform *T. brucei* was equally identified with a very high sequence intensity and coverage (Michels *et al.*, 2005).

Similar to my experience, the results published by Robello do not include many transmembrane proteins either. I searched for most of the proteins and only two proteins in this database carry transmembrane domains. A similar control was done with these proteins in other databases, which carried a SWISS-PROT accession number. None of these proteins had a transmembrane domain. Since Robello`s group experiments were carried out with similar methods, the limitation and drawbacks of the 2D-electrophoresis become clear.

### **4.3.4 Comparison of the theoretical and the experimentally determined pIs and molecular weights**

#### **4.3.4.1 Comparison of the experimentally determined pI and the theoretical pI**

The correlation between experimentally and theoretical pI values in this study is not as tight as described by other authors (Bjellqvist *et al.*, 1993; Perrot *et al.*, 1999). However, the correlation still tends to be linear. The proteins with a large difference between experimental and theoretical pI values were separately into the following categories:

- Some proteins on my reference map are only ESTs in available public databases. Since ESTs might be incomplete or encode a pre-protein, the theoretical MW and pI of the EST derived proteins were unreliable.

## Discussion.

- Variation in protein expression patterns might be due to alternative splicing events on the mRNA-level or due to post-translational modification. These modifications may be due to glycolysation, partial hydrolysis or phosphorylation and might cause a substantial difference between the experimentally pI value and the theoretical one. This was verified by the fact that some of those obviously deviating spots were found to be isoforms of respective proteins.
- By direct measurement, the linearity of the pH-gradient in the IEF-gel could only be verified between pH 3-8. The pH-gradient of the IEF-gel is not linear at its basic end. Therefore, experimentally shown pI values of those proteins focussing at the basic end were unreliable. This results in a pI-difference larger than 1.00 pH unit for almost all the basic proteins whose theoretical pI is larger than pH 9.00.
- Preparation artefacts (e.g. partial hydrolysis by proteases) might cause protein degradation. The ensuing fragments run differently on the 2D-gel than the intact proteins and might be as well a reason for larger deviations between the theoretical and the experimental observed pI-values.

### **4.3.4.2 Comparison of the experimental MW and the theoretical MW**

The correlation of experimental and theoretical molecular weights is better than that of the isoelectric points. In my study I used a mass reference marker set which only spanned the range between 10 kDa and 200 kDa. Therefore, the MW of the proteins could not be determined with certainty when they run below 15 kDa or above 120 kDa.

### Summary

African trypanosomiasis is caused by extra cellular parasitic protozoa that can be transmitted by the bite of a tsetse fly. The disease is caused by sub-species of *Trypanosoma brucei*, an extra-cellular eukaryotic flagellated parasite. There is no prophylactic chemotherapy or prospect of a vaccine and current treatment is inadequate. Drugs for late-stage disease are highly toxic. Livestock trypanosomiasis is caused by closely related *Trypanosoma* species. It has the greatest impact in sub-Saharan Africa, where the tsetse fly vector is common (WHO, 2002).

The complication of genomics results and analysis caused a big shift of interest from genomics to proteomics. The main analytical tool used for protein separation in proteomics is two-dimensional gel electrophoresis (2DGE) (O'Farrell, 1975 and Fey and Larsen, 2001).. This technique separates proteins according to their masses and charges. These independent attributes enables separation of thousands of proteins in a single run.

Since numerous proteins of a whole cell (its "proteome") connect the genotype with the phenotype, we set out to study the proteome of the bloodstream form in *T. brucei* which are infectious and known to cause diseases in human and animals. Deviating protein patterns between the different stages could direct the attention to disease-specific proteins and genes, which might be involved in the expression of infection and cause of disease.

After separation, most proteins can be identified by mass spectroscopy. In studies performed to identify specific proteins related to a given metabolic process or disease it is, however, much more efficient to detect and identify only differentiated protein groups of samples.

In order to understand the molecular basis of the parasites differentiation, we were interested in characterization of specific proteins expressed in bloodstream forms. However, the vast evolutionary distance between *trypanosomes* and the higher eukaryotes presents significant problems with functional assignment based on sequence similarities, and frequently homologues cannot be identified with sufficient confidence to be informative. Direct identification of proteins in isolated organelles has the potential of providing robust functional insight and is a powerful approach for initial assignment. The cytoplasm protein fraction and membrane protein fraction of *T. brucei* were used in this work to analyse the protozoan proteomics.

## Summary

Proteomics is a rapidly developing technique, which allows the efficient isolation of multiple protein families and it is a valuable tool for global patterns of gene expression. It allows the studies of membrane as well as cytosolic proteins.

The rapidly growing development of bioinformatics, for example the use of software's like The JVirGel will also transform the handling of the multitude of data accumulating in proteomic experiments. The JVirGel software creates and visualizes virtual two-dimensional (2D) protein gels based on the migration behaviour of proteins in dependence of their theoretical molecular weights in combination with their calculated isoelectric points. Although, 9068 protein-coding genes and many pseudo genes have been identified, (science 2005) a number of potential new proteins are yet to be characterized. Analysis of *T. brucei* proteins using two-dimension gel electrophoresis, may offer prognostic analysis and information on disease mechanism.

These results suggest the first step towards the generation of proteome profiles for use in future studies on protein expression, especially those accompanying the differentiation of the parasite. Comparison of the different stage-specific protein profiles will allow us to identify candidates as targets for drug action. Protein data using JVirGel software together with the provision of the complete genome sequence for *T. brucei*, the application of the genomic and post-genomic technologies should provide advances in the understanding of the biology of this parasite and the identification of key factors for virulence, drug resistance and infectivity. In this study the slender bloodstream form of *T. brucei* 221 were used because they are easy to grow in rats. These parasites can equally be cultivated at 37°C to grow to a cell density of about  $1 \times 10^6$ /ml. This approach allows the cultivation of high cell numbers without excessive expenditure of work and cost.

I first had to establish a protocol for separating trypanosomal proteins by iso-electric focusing to produce a reference map for the first time. In this project, it was possible to identify many cytoplasmic and membrane proteins on the proteome-map of *T. brucei*.

In proteomics the combination of the high-resolution two-dimensional electrophoresis and matrix assisted laser desorption/ionization–time–of–flight–mass spectrometry (MALDI-TOF-MS) is currently the method of choice for protein identification. More than 300 protein spots were detected on a silver-stained two-dimensional gel. Analysis of 50 spots among them those highly expressed when *T. brucei* when grown in the presence of arachidonic acid was carried out. The protein spots from gel were digested with trypsin in-gel digestion followed by subsequent MALDI-TOF mass spectrometry in a process termed “peptide mass fingerprinting”. Following a database search, 27 protein spots were identified (belonging to

## Summary

different functional groups of proteins). 20 of the identified proteins are components of the main biological and cell regulation pathways located in *T. brucei*

With this study, I established a partial data base and reference proteome map of the bloodstream form *T. brucei*. These data will facilitate further addition of information to *T. brucei* proteome, which may aid drug design in the treatment of patients with and control of trypanosomiasis and the control of infection. Future research based on this dissertation should mainly focus in the identification of different proteins between the bloodstream forms, the procyclic forms and the short stumpy forms of *T. brucei*.



## Analyse des Proteoms von *Trypanosoma brucei* unter besonderer Berücksichtigung des Stoffwechsels von Prostaglandinen

*Trypanosoma brucei* ist die Ursache für die afrikanische Schlafkrankheit (Trypanosomiasis), welche hauptsächlich die ärmsten Teile der Bevölkerung in den Entwicklungsländern in Zentralafrika und der Subsahara-Region bedroht. *Trypanosoma brucei* hat einen Mechanismus entwickelt, um der Immunabwehr des Wirts zu entkommen, der auf der Antigenvariation der über Glycosylphosphatidylinositol-Anker verbundenen Oberflächenproteine beruht. Die Genexpression ist hauptsächlich auf post-transkriptioneller Ebene reguliert.

Die vollständige Aufklärung dieses Organismus wurde immer wieder erschwert durch Herausforderungen technischer Art. Dennoch ermöglicht der Fortschritt verschiedener Disziplinen wie Genomik, Proteomik und der Erstellung und Analyse von 2D-Gelen verbunden mit Methoden wie der *matrix-assisted laser desorption/ionisation-time-of-flight* (MALDI-TOF) *mass spectrometry* (MS) einen neuen Ansatz um schnell funktionelle Daten über Proteine zu bekommen.

Im Gegensatz zu konventionellen biochemischen Ansätzen, die auf die Beobachtung einiger weniger Proteine abzielen, lässt sich durch proteomische Methoden die globale Proteinexpression im großen Maßstab untersuchen. Aus der direkten Messung der verschiedenen Proteinexpressionslevel lassen sich Rückschlüsse auf die Aktivität relevanter Proteine unter verschiedenen Bedingungen ziehen.

Die Methoden der Proteomik bieten ein wichtiges Werkzeug um differentielle Expression von Proteinen während der verschiedenen Lebenszyklus-Phasen und verschiedenen Kulturbedingungen des Parasiten zu beobachten.

**Literature**

- Abbott, (1999). A post-genomic challenge: learning to read patterns of protein synthesis. *Nature*. Dec 16;402(6763):715-20.
- Acosta-Serrano A, Vassella E, Liniger M, Kunz Renggli C, Brun R, Roditi I, Englund PT. (2001) The surface coat of procyclic *Trypanosoma brucei*: programmed expression and proteolytic cleavage of procyclin in the tsetse fly *Proc Natl Acad Sci U S A*. 2001 Feb 13;98(4):1513-8. Epub 2001 Feb 6.
- Adams, P.D., *et al.* (2002). Identification of Associated Proteins by Coimmunoprecipitation, *In Protein-Protein Interactions – A Molecular Cloning Manual*. Golemis, E., Ed., Cold Spring Harbor Laboratory Press, pp 59-74
- Alafiatayo RA, Cookson MR, Pentreath VW. (1994). Production of prostaglandins D2 and E2 by mouse fibroblasts and astrocytes in culture caused by *Trypanosoma brucei brucei* products and endotoxin. *Parasitol Res*. 80(3):223-9.
- Albert MA, Haanstra JR, Hannaert V, Van Roy J, Opperdoes FR, Bakker BM, Michels PA. (2005). Related Articles, Links Experimental and in silico analyses of glycolytic flux control in bloodstream form *Trypanosoma brucei*. *J Biol Chem*. Aug 5;280(31):28306-15.
- Allen CL, Goulding D, Field MC. (2003). Clathrin-mediated endocytosis is essential in *Trypanosoma brucei*. *EMBO J*. Oct 1;22(19):4991-5002
- Allen TE, Ullman B. (1993). Cloning and expression of the hypoxanthine-guanine phosphoribosyltransferase gene from *Trypanosoma brucei*. *Nucleic Acids Res*. Nov 25;21(23):5431-8.
- Ameisen JC. (1996). The origin of programmed cell death. *Science*. May 31;272(5266):1278-9.
- Ameisen. JC. (1995). From cell activation to cell depletion. The programmed cell death hypothesis of AIDS pathogenesis. *Adv Exp Med Biol*.;374:139-63.
- Arnoult D, Parone P, Martinou JC, Antonsson B, Estaquier J, Ameisen JC. (2002). Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome c release in response to several proapoptotic stimuli. *J Cell Biol*. Dec 23;159(6):923-9
- Acosta-Serrano A, Vassella E, Liniger M, Kunz Renggli C, Brun R, Roditi I, Englund PT. (2001). The surface coat of procyclic *Trypanosoma brucei*: programmed expression and proteolytic cleavage of procyclin in the tsetse fly *Proc Natl Acad Sci U S A*. 2001 Feb 13;98(4):1513-8. Epub 2001 Feb 6.
- Bacchi CJ, Nathan HC, Yarett N, Goldberg B, McCann PP, Sjoerdsma A, Saric M, Clarkson AB Jr. (1994). Combination chemotherapy of drug-resistant *Trypanosoma brucei rhodesiense* infections in mice using DL-alpha-difluoromethylornithine and standard trypanocides. *Antimicrob Agents Chemother*. Mar;38(3):563-9.

## Literature

- Baetz AL, Leiting SE, Bryner J, Barnett D. (1981). Parturition changes of plasma concentrations of prostaglandin F and 13,14-dihydro-15-ketoprostaglandin metabolites in pregnant animals exposed to *Sarcocystis cruzi* or *Campylobacter fetus*. Am J Vet Res. Jan;42(1):22-4.
- Balber AE, Gonias SL, Pizzo SV. (1985). *Trypanosoma brucei gambiense*: growth in vitro of bloodstream forms inhibited by dichlorodiammineplatinum (II) compounds and hypolipidemic drugs. Exp Parasitol. Feb;59(1):74-80.
- Baker RR. (1990). The eicosanoids: a historical overview. Clin Biochem. Oct;23(5):455-8. Review.
- Barrett MP. (1999). The fall and rise of sleeping sickness. Lancet. Apr 3;353(9159):1113-4
- Bastin P, Sherwin T, Gull K. (1998) Paraflagellar rod is vital for *trypanosome* motility. Nature. Feb 5;391(6667):548.
- Baudet C, Perret E, Delpech B, Kaghad M, Brachet P, Wion D, Caput D. (1998). Differentially expressed genes in C6.9 glioma cells during vitamin D-induced cell death program. Cell Death Differ. Jan;5(1):116-25.
- Belley A, Chadee K. (1995). Eicosanoid production by parasites: from pathogenesis to immunomodulation? Parasitol Today. Sep;11(9):327-34.
- Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, Lennard NJ, Caler E, Hamlin NE, Haas B, Bohme U, Hannick L, Aslett MA, Shallom J, Marcello L, Hou L, Wickstead B, Alsmark UC, Arrowsmith C, Atkin RJ, Barron AJ, Bringaud F, Brooks K, Carrington M, Cherevach I, Chillingworth TJ, Churcher C, Clark LN, Corton CH, Cronin A, Davies RM, Doggett J, Djikeng A, Feldblyum T, Field MC, Fraser A, Goodhead I, Hance Z, Harper D, Harris BR, Hauser H, Hostetler J, Ivens A, Jagels K, Johnson D, Johnson J, Jones K, Kerhornou AX, Koo H, Larke N, Landfear S, Larkin C, Leech V, Line A, Lord A, Macleod A, Mooney PJ, Moule S, Martin DM, Morgan GW, Mungall K, Norbertczak H, Ormond D, Pai G, Peacock CS, Peterson J, Quail MA, Rabbinowitsch E, Rajandream MA, Reitter C, Salzberg SL, Sanders M, Schobel S, Sharp S, Simmonds M, Simpson AJ, Tallon L, Turner CM, Tait A, Tivey AR, Van Aken S, Walker D, Wanless D, Wang S, White B, White O, Whitehead S, Woodward J, Wortman J, Adams MD, Embley TM, Gull K, Ullu E, Barry JD, Fairlamb AH, Opperdoes F, Barrell BG, Donelson JE, Hall N, Fraser CM, Melville SE, El-Sayed NM. (2005). The genome of the African trypanosome *Trypanosoma brucei*. Science 2005 Jul 15;309(5733):416-22.
- Bjellqvist B, Pasquali C, Ravier F, Sanchez JC, Hochstrasser D. A. (1993). Nonlinear wide-range immobilized pH gradient for two-dimensional electrophoresis and its definition in a relevant pH scale. Electrophoresis. Dec;14(12):1357-65.
- Bochud-Allemann N, Schneider A (2002). Mitochondrial substrate level phosphorylation is essential for growth of procyclic *Trypanosoma brucei*. J Biol Chem. Sep.; 277 (36) : 32849-54.
- Besteiro S, Barrett MP, Riviere L, Bringaud F. (2005). Energy generation in insect stages of *Trypanosoma brucei*: metabolism in flux. Trends Parasitol. Apr;21(4):185-91

## Literature

- Bronfman M, Loyola G, Koenig CS. (1998). Isolation of intact organelles by differential centrifugation of digitonin-treated hepatocytes using a table Eppendorf centrifuge. *Anal Biochem.* Jan 15;255(2):252-6.
- Brun R, Schumacher R, Schmid C, Kunz C, Burri C. (2001). The phenomenon of treatment failures in Human African Trypanosomiasis. *Trop Med Int Health.* Nov;6(11):906-14.
- Briggs LJ, McKean PG, Baines A, Moreira-Leite F, Davidge J, Vaughan S, Gull K. (2004). The flagella connector of *Trypanosoma brucei*: an unusual mobile transmembrane junction. *J Cell Sci.* Apr 1;117(Pt 9):1641-51.
- Carroll KM, Carey EM, Helm BA. (2002). Protein mapping in rat basophilic leukaemia cells. *J Chromatogr B Analyt Technol Biomed Life Sci.* May 5;771(1-2):289-301
- Chamrad DC, Koerting G, Gobom J, Thiele H, Klose J, Meyer HE, Blueggel M. (2003). Interpretation of mass spectrometry data for high-throughput proteomics. *Anal Bioanal Chem.* Aug;376(7):1014-22. Epub 2003 Jul 5.
- Chose O, Noel C, Gerbod D, Brenner C, Viscogliosi E, Roseto A. (2002) A form of cell death with some features resembling apoptosis in the amitochondrial unicellular organism *Trichomonas vaginalis*. *Exp Cell Res.* May 15;276(1):32-9.
- Cross GA (1984). Structure of the variant glycoproteins and surface coat of *Trypanosoma brucei*. *Philos Trans R Soc Lond B Biol Sci.* Nov 13;307(1131):3-12.
- Cross GA. (1996) Antigenic variation in trypanosomes: secrets surface slowly. *Bioessays.* Apr;18(4):283-91.
- Cuvillier A, Redon F, Antoine JC, Chardin P, DeVos T, Merlin G. (2000). LdARL-3A, a *Leishmania promastigote*-specific ADP-ribosylation factor-like protein, is essential for flagellum integrity. *J Cell Sci.* Jun;113 ( Pt 11):2065-74.
- Cuvillier A, Miranda JC, Ambit A, Barral A, Merlin G. (2003) Abortive infection of *Lutzomyia longipalpis* insect vectors by aflagellated LdARL-3A-Q70L overexpressing *Leishmania amazonensis* parasites. *Cell Microbiol.* Oct;5(10):717-28.
- Daniel LW, Grab LT, Kearns MW, Morris AJ. (2004) Differential role for phospholipase D1 and phospholipase D2 in 12-O-tetradecanoyl-13-phorbol acetate-stimulated MAPK activation, Cox-2 and IL-8 expression. *Biochim Biophys Acta.* Feb 27;1636(1):29-39.
- Dacheux JL, Fouchecourt S, Castella S, Dacheux F, Dacheux JL. (2003). Prostaglandin D2 synthase secreted in the caput epididymidis displays spatial and temporal delay between messenger RNA and protein expression during postnatal development. *Biol Reprod.* Jan;68(1):174-9
- Dacheux JL, Fouchecourt S, Dacheux F. (1999). Glutathione-independent prostaglandin D2 synthase in ram and stallion epididymal fluids: origin and regulation. *Biol Reprod.* Mar;60(3):558-66

## Literature

- Dauguschies A and Joachim A. (2000). Eicosanoids in parasites and parasitic infections. *Adv Parasitol.*;46:181-240
- Decker K.(1985). Eicosanoids, signal molecules of liver cells. *Semin Liver Dis.* 1985 May;5(2):175-90
- De Maria E. *et al.*, 1998 (De Maria R, Rippo MR, Schuchman EH, Testi R. Acidic sphingomyelinase (ASM) is necessary for fas-induced GD3 ganglioside accumulation and efficient apoptosis of lymphoid cells. *J Exp Med.* 1998 Mar 16;187(6):897-902
- De Petrocellis L, Di Marzo V. (1994). Aquatic invertebrates open up new perspectives in eicosanoid research: biosynthesis and bioactivity. *Prostaglandins Leukot Essent Fatty Acids.* Oct;51(4):215-29
- De Sa-Freire A, Nepomuceno-Silva JL, da Paixao JC, de Mendonca SM, de Melo LD, Lopes UG. (2003). TcArf1: a *Trypanosoma cruzi* ADP-ribosylation factor. *Parasitol Res.* Sep;91(2):166-70
- Dey I, Keller K, Belley A, Chadee K. (2003). Identification and characterization of a cyclooxygenase-like enzyme from *Entamoeba histolytica*. *Proc Natl Acad Sci U S A.* Nov 11;100(23):13561-6.
- Doua F Pentreath VW, Rees K, Owolabi OA, Philip KA. (1990). The somnogenic T lymphocyte suppressor prostaglandin D<sub>2</sub> is selectively elevated in cerebrospinal fluid of advanced sleeping sickness patients. *Trans R Soc Trop Med Hyg.* Nov-Dec;84(6):795-9
- Dutton, J E. (1902) Preliminary note upon a trypanosomes occurring in the blood of a man. Thomson Yates Laboratory Report 4, 455-467
- Donges J, Gotzelmann M. (1988). Digenetic trematodes: multiplication of the intramolluscan stages in some species is potentially unlimited. *J Parasitol.* Oct;74(5):884-5.
- Duszenko M, Figarella K (2006). Trends in parasitology (In press)
- Hesse F, Selzer PM, Muhlstadt K. Duszenko.M, (1995). A novel cultivation technique for long-term maintenance of bloodstream form *trypanosomes* in vitro. *Mol Biochem Parasitol.* Mar;70(1-2):157-66.
- Eells JT, Spector R. (1983). Purine and pyrimidine base and nucleoside concentrations in human cerebrospinal fluid and plasma. *Neurochem Res.* Nov;8(11):1451-7.
- Eintracht J, Maathai R, Mellors A, Ruben L. (1998). Calcium entry in *Trypanosoma brucei* is regulated by phospholipase A2 and arachidonic acid. *Biochem J.* Dec 15;336 ( Pt 3):659-66
- Ernest I, Callens M, Uttaro AD, Chevalier N, Opperdoes FR, Muirhead H, Michels PA. 1998 Pyruvate kinase of *Trypanosoma brucei*: overexpression, purification, and functional

## Literature

- characterization of wild-type and mutated enzyme. *Protein Expr Purif.* Aug;13(3):373-82.
- Ersfeld K and Gull K. (1997). Partitioning of large and minichromosomes in *Trypanosoma brucei*. *Science.* Apr 25;276(5312):611-4.
- Evan GI, Waters CM, Penn LZ, Hancock DC, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M. (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell.* Apr 3;69(1):119-28.
- Fernandez J, Gharahdaghi F, Mische SM. (1998). Routine identification of proteins from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels or polyvinyl difluoride membranes using matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). *Electrophoresis.* May;19(6):1036-45.
- Ferrante A and Allison A.C. (1983). Alternative pathway activation of complement by African *trypanosomes* lacking a glycoprotein coat. *Parasite Immunol.* Sep;5(5):491-8.
- Fevre EM, Picozzi K, Jannin J, Welburn SC, Maudlin I. (2006). Human african trypanosomiasis: epidemiology and control. *Adv Parasitol.* 61:167-221
- Fey S.J, Larsen MR, Sorensen GL, Larsen PM, Roepstorff P. (2001). Phospho-proteomics: evaluation of the use of enzymatic de-phosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. *Proteomics.* Feb;1(2):223-38.
- Figarella K, Uzcategui NL, Beck A, Schoenfeld C, Kubata BK, Lang F, Duszenko M. (2006). Prostaglandin-induced programmed cell death in *Trypanosoma brucei* involves oxidative stress. *Cell Death Differ.* 2006 Feb 3;
- Figarella K, Rawer M, Uzcategui NL, Kubata BK, Lauber K, Madeo F, Wesselborg S, Duszenko M (2005). Prostaglandin D2 induces programmed cell death in *Trypanosoma brucei* bloodstream form. *Cell Death Differ.* Apr;12(4):335-46.
- Fukushima T, Isobe A, Hojo N, Shiwaku K, Yamane Y, Torii M. (1993). The metabolism of arachidonic acid to prostaglandin E2 in plerocercoids of *Spirometra erinacei*. *Parasitol Res.* 79(8):634-8.
- Garcia-Salcedo JA, Perez-Morga D, Gijon P, Dilbeck V, Pays E, Nolan DP. (2004). A differential role for actin during the life cycle of *Trypanosoma brucei*. *EMBO J.* Feb 25;23(4):780-9
- Garrels JJ, McLaughlin CS, Warner JR, Futcher B, Latter GI, Kobayashi R, Schwender B, Volpe T, Anderson DS, Mesquita-Fuentes R, Payne WE. (1997). Proteome studies of *Saccharomyces cerevisiae*: identification and characterization of abundant proteins. *Electrophoresis.* Aug;18(8):1347-60
- Giavalisco P, Nordhoff E, Lehrach H, Gobom J, Klose J. Extraction of proteins from plant tissues for two-dimensional electrophoresis analysis. *Electrophoresis.* (2003) Jan;24(1-

## Literature

2):207-16.

- Gibson W, Backhouse T, Griffiths A. (2002). The human serum resistance associated gene is ubiquitous and conserved in *Trypanosoma brucei rhodesiense* throughout East Africa. *Infect Genet Evol.* May;1(3):207-14
- Gombe S Mutayoba BM, Meyer HH, Osaso J. (1989). *Trypanosome*-induced increase in prostaglandin F(2alpha) and its relationship with corpus luteum function in the goat. *Theriogenology.* Oct;32(4):545-55.
- Grunfelder CG, Engstler M, Weise F, Schwarz H, Stierhof YD, Boshart M, Overath P. (2002). Accumulation of a GPI-anchored protein at the cell surface requires sorting at multiple intracellular levels. *Traffic.* Aug;3(8):547-59
- Gull K Tyler KM, Higgs PG, Matthews. (2001). Limitation of *Trypanosoma brucei* parasitaemia results from density-dependent parasite differentiation and parasite killing by the host immune response. *Proc Biol Sci.* Nov 7;268(1482):2235-43
- Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R. (2000). Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc Natl Acad Sci U S A.* Aug 15;97(17):9390-5
- Hajduk SL and Priest JW. (1994). SL. Developmental regulation of *Trypanosoma brucei* cytochrome c reductase during bloodstream to procyclic differentiation. *Mol Biochem Parasitol.* Jun;65(2):291-304.
- Hamberg M, Samuelsson B. (1973). Detection and isolation of an endoperoxide intermediate in prostaglandin biosynthesis. *Proc Natl Acad Sci U S A.* Mar;70(3):899-903.
- Hamberg M. (1974). Quantitative studies on prostaglandin synthesis in man. 3. Excretion of the major urinary metabolite of prostaglandins F 1 alpha and F 2 alpha during pregnancy. *Life Sci.* Jan 16;14(2):247-52.
- Hamm B, Schindler A, Mecke D Duszenko, M.. (1990). Differentiation of *Trypanosoma brucei* bloodstream trypomastigotes from long slender to short stumpy-like forms in axenic culture. *Mol Biochem Parasitol.* Apr;40(1):13-22.
- He CY, Pypaert M, Warren G. (2005). Golgi duplication in *Trypanosoma brucei* requires Centrin2. *Science.* Nov 18;310(5751):1196-8
- Helfert S, Estevez AM, Bakker B, Michels P, Clayton C. (2001). Roles of triosephosphate isomerase and aerobic metabolism in *Trypanosoma brucei*. *Biochem J.* Jul 1;357(Pt 1):117-25
- Hemler M and Lands WE. (1976). Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *J Biol Chem.* Sep 25;251(18):5575-9.

## Literature

- Hengartner MO. (1997). Genetic control of programmed cell death and aging in the nematode *Caenorhabditis elegans*. *Exp Gerontol.* Jul-Oct;32(4-5):363-74.
- Henningsen R, Gale BL, Straub KM, DeNagel DC. (2002). Application of zwitterionic detergents to the solubilization of integral membrane proteins for two-dimensional gel electrophoresis and mass spectrometry. *Proteomics.* Nov;2(11):1479-88.
- Hesse F, Selzer PM, Muhlstadt K, Duszenko M. (1995 ). A novel cultivation technique for long-term maintenance of bloodstream form trypanosomes in vitro. *Mol Biochem Parasitol.* Mar;70(1-2):157-66
- Horrobin F, Manku M, Glen I, McGoldrick S, Duffy F, Boal D, Murck H, Green A. (2003). Prostaglandins Leukot Essent Fatty Acids. Jul;69(1):1-26
- Hung CH, Qiao X, Lee PT, Lee MG. (1994). Clathrin-dependent targeting of receptors to the flagellar pocket of procyclic-form *Trypanosoma brucei*. *Eukaryot Cell.* Aug;3(4):1004-14.
- Hutchings NR, Donelson JE, Hill KL. (2002). Trypanin is a cytoskeletal linker protein and is required for cell motility in African trypanosomes. *J Cell Biol.* Mar 4;156(5):867-77
- Jannin J and Cattand P.(2004). *Curr Opin Infect Dis.* 2004 Dec;17(6):565-71
- Jarving R, Jarving I, Kurg R, Brash AR, Samel N. (2004). On the evolutionary origin of cyclooxygenase (COX) isozymes: characterization of marine invertebrate COX genes points to independent duplication events in vertebrate and invertebrate lineages. *J Biol Chem.* Apr 2;279(14):13624-33
- Jensen ON, Podtelejnikov A, Mann M. (1996). Delayed extraction improves specificity in database searches by matrix-assisted laser desorption/ionization peptide maps. *Rapid Commun Mass Spectrom.* 10(11):1371-8.
- Jensen ON, Kulkarni S, Aldrich JV, Barofsky DF. (1996). Characterization of peptide-oligonucleotide heteroconjugates by mass spectrometry. *Nucleic Acids Res.* Oct 1;24(19):3866-72
- Karas M, Hillenkamp F. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal Chem.* Oct 15;60(20):2299-301.
- Klose J, Nock C, Herrmann M, Stuhler K, Marcus K, Bluggel M, Krause E, Schalkwyk LC, Rastan S, Brown SD, Bussow K, Himmelbauer H, Lehrach H. (2002). Genetic analysis of the mouse brain proteome. *Nat Genet.* Apr;30(4):385-93.
- Klose J. (1999a). Large-gel 2-D electrophoresis. *Methods Mol Biol.* 112:147-72
- Klose J and Kobalz U. (1995). Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. *Electrophoresis.* Jun;16(6):1034-59



## Literature

- Klose J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals *Humangenetik.* ;26(3):231-43.
- Kubata BK Kabututu Z, Martin SK, Nozaki T, Kawazu S, Okada T, Munday CJ, Duszenko M, Lazarus M, Thuita LW, Urade Y. (2002). Prostaglandin production from arachidonic acid and evidence for a 9,11-endoperoxide prostaglandin H2 reductase in *Leishmania*. *Int J Parasitol.* Dec 19;32(14):1693-700
- Kubata BK, Duszenko M, Kabututu Z, Rawer M, Szallies A, Fujimori K, Inui T, Nozaki T, Yamashita K, Horii T, Urade Y, Hayaishi O. (2000). Identification of a novel prostaglandin f(2alpha) synthase in *Trypanosoma brucei*. *J Exp Med.* Nov 6;192(9):1327-38.
- Kubata BK, Kabututu Z, Nozaki T, Munday CJ, Fukuzumi S, Ohkubo K, Lazarus M, Maruyama T, Martin SK, Duszenko M, Urade Y. (2002). A key role for old yellow enzyme in the metabolism of drugs by *Trypanosoma cruzi*. *J Exp Med.* Nov 4;196(9):1241-51
- Kuhn H and Borngraber S. (1999). Mammalian 15-lipoxygenases. Enzymatic properties and biological implications. *Adv Exp Med Biol.* ;447:5-28.
- Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* Aug 15;227(5259):680-5.
- Lamour N, Riviere L, Coustou V, Coombs GH, Barrett MP, Bringaud F. (2005). Proline metabolism in procyclic *Trypanosoma brucei* is down-regulated in the presence of glucose. *J Biol Chem.* 2005 Mar 25;280(12):11902-10.
- Lee KY, Park JS, Jee YK, Rosen GD. (2002). Triptolide sensitizes lung cancer cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by inhibition of NF-kappaB activation. *Exp Mol Med.* Dec 31;34(6):462-8.
- Lehner I, Niehof M, Borlak J. (2003). An optimized method for the isolation and identification of membrane proteins. *Electrophoresis.* Jun;24(11):1795-808.
- Levine ND. (1980). Some corrections of coccidian (Apicomplexa: Protozoa) nomenclature. *J Parasitol.* Oct;66(5):830-4
- Liebler, D.C. (2002). Identifying protein-protein interactions and protein complexes. *In* Introduction to Proteomics, Tools for the New Biology, Humana Press, pp.151-165.
- Lin HY, Rocher LL, McQuillan MA, Schmaltz S, Palella TD, Fox IH. (1989). Cyclosporine-induced hyperuricemia and gout. *N Engl J Med.* 1989 Aug 3;321(5):287-92.
- Liu LX, Serhan CN, Weller PF. (1990). Intravascular filarial parasites elaborate cyclooxygenase-derived eicosanoids. *J Exp Med.* Sep 1;172(3):993-6.

## Literature

- Lopez MF. (2000). Better approaches to finding the needle in a haystack: optimizing proteome analysis through automation. *Electrophoresis*. Apr;21(6):1082-93
- Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*. Apr 29;362(6423):847-9.
- MacLean L, Chisi JE, Odiit M, Gibson WC, Ferris V, Picozzi K, Sternberg JM.(2004) *Infect Immun*. Dec;72(12):7040-4.
- Madeo, F. Frohlich K.U. (1999). Apoptosis in yeast--a monocellular organism exhibits altruistic behaviour. *FEBS Lett*. May 4;473(1):6-9.
- Madeo F, Frohlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Frohlich KU.(1999) Oxygen stress: a regulator of apoptosis in yeast.*J Cell Biol*. May 17;145(4):757-67
- Maillet I, Lagniel G, Perrot M, Boucherie H, Labarre J. (1996). Rapid identification of yeast proteins on two-dimensional gels. *J Biol Chem*. Apr 26;271(17):10263-70
- Mariante RM, Guimaraes CA, Linden R, Benchimol M. (2003). Hydrogen peroxide induces caspase activation and programmed cell death in the *amitochondrial Tritrichomonas foetus*. *Histochem Cell Biol*. Aug;120(2):129-41
- Martinez, S. and Marr, J.J. (1992). Allopurinol in the treatment of American cutaneous *leishmaniasis*.*N Engl J Med*. Mar 12;326(11):741-4.
- Maslov DA.and Simpson L. (1994). Ancient origin of RNA editing in *kinetoplastid protozoa*. *Curr Opin Genet Dev*. Dec;4(6):887-94.
- Matthews KR. (2005). The developmental cell biology of *Trypanosoma brucei*. *J Cell Cell Sci*. May 1;118(Pt 9):2078.
- Matthews. KR and Hendriks EF. (2005). Disruption of the developmental programme of *Trypanosoma brucei* by genetic ablation of TbZFP1, a differentiation-enriched CCCH protein. *Mol Microbiol*. Aug;57(3):706-16
- Matthews KR, Ellis JR, Paterou A. (2004). Molecular regulation of the life cycle of African *trypanosomes*. *Trends Parasitol*. Jan;20(1):
- Maxey KM, Westcott JY, MacDonald J, Wenzel SE. (1998). Immunoaffinity resin for purification of urinary leukotriene E4.Prostaglandins Other Lipid Mediat. Apr;55(5-6):301-21
- McCulloch R. (2004). Antigenic variation in African *trypanosomes*: monitoring progress. *Trends Parasitol*. Mar;20(3):117-21.
- Meyer DJ, Muimo R, Thomas M, Coates D, Isaac RE. (1996). Purification and characterization of prostaglandin-H E-isomerase, a sigma-class glutathione S-transferase, from *Ascaridia galli*. *Biochem J*. Jan 1;313 ( Pt 1):223-7.

## Literature

- Mhlanga JD. (1996). Sleeping sickness: perspectives in African trypanosomiasis. *Sci Prog.* ;79
- Michels PA, Moyersoen J, Krazy H, Galland N, Herman M, Hannaert V. (2005). Peroxisomes, glyoxysomes and glycosomes (review) *Mol Membr Biol.* Jan-Apr;22(1-2):133-45.
- Miyamoto T, Ogino N, Yamamoto S, Hayaishi O. (1976). Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J Biol Chem.* May 10;251(9):2629-36.
- Miyares CM and Hollands I. (1976). Identification of substances with biological activity similar to prostaglandins in *Cestoda* of *Moniezia* genus *Rev Cubana Med Trop.* Jan-Apr;28(1):21-7
- Moreira-Leite FF, Sherwin T, Kohl L, Gull K. (2001). A *trypanosome* structure involved in transmitting cytoplasmic information during cell division. *Science.* Oct 19;294(5542):610-2.
- Moreira ME, Del Portillo HA, Milder RV, Balanco JM, Barcinski MA. (1996). Heat shock induction of apoptosis in promastigotes of the unicellular organism *Leishmania (Leishmania) amazonensis*. *J Cell Physiol.* May;167(2):305-13.
- Morgan GW, Goulding D, Field MC. (2004). The single dynamin-like protein of *Trypanosoma brucei* regulates mitochondrial division and is not required for endocytosis. *J Biol Chem.* Mar 12;279(11):10692-701.
- Moyersoen J, Choe J, Kumar A, Voncken FG, Hol WG, Michels PA. (2003) Characterization of *Trypanosoma brucei* PEX14 and its role in the import of glycosomal matrix proteins. *Eur J Biochem.* May;270(9):2059-67.
- Münch R, Hiller K, Schobert M, Hundertmark C, Jahn D. (2003). JVirGel: Calculation of virtual two-dimensional protein gels. *Nucleic Acids Res.* Jul 1;31(13):3862-5.
- Murphy NB and Welburn SC. (1997). Programmed cell death in procyclic *Trypanosoma brucei rhodesiense* is associated with differential expression of mRNAs. *Cell Death Differ.* Jul;4(5):365-70
- Nakhasi HL, Lee N, Bertholet S, Debrabant A, Muller J, Duncan R. (2002). Programmed cell death in the unicellular protozoan parasite *Leishmania*. *Cell Death Differ.* Jan;9(1):53-64
- Navarre C, Degand H, Bennett KL, Crawford JS, Mortz E, Boutry M. (2002). Subproteomics: identification of plasma membrane proteins from the yeast *Saccharomyces cerevisiae*. *Proteomics.* Dec;2(12):1706-14.
- Nielsen ML, Bennett KL, Larsen B, Moniatte M, Mann M..(2002). Peptide end sequencing by orthogonal MALDI tandem mass spectrometry. *J Proteome Res.* Jan-Feb;1(1):63-71.

## Literature

- Nok AJ, Esievo KA, Ibrahim S, Ukoha AI, Ikediobi CO. (1993). Phospholipase A2 from *Trypanosoma congolense*: characterization and haematological properties. *Cell Biochem Funct. Jun*;11(2):125-30.
- Nordhoff E, Egelhofer V, Giavalisco P, Eickhoff H, Horn M, Przewieslik T, Theiss D, Schneider U, Lehrach H, Gobom J. (2001). Large-gel two-dimensional electrophoresis-matrix assisted laser desorption/ionization-time of flight-mass spectrometry: an analytical challenge for studying complex protein mixtures. *Electrophoresis. Aug*;22(14):2844-55
- O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem. May* 25;250(10):4007-21
- Ogbadoyi EO, Robinson DR, Gull K. (2003). A high-order trans-membrane structural linkage is responsible for mitochondrial genome positioning and segregation by flagellar basal bodies in *trypanosomes*. *Mol Biol Cell. May*;14(5):1769-79.
- Ogino N, Ohki S, Yamamoto S, Hayaishi O. (1978). Prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. Inactivation and activation by heme and other metalloporphyrins. *J Biol Chem. Jul* 25;253(14):5061-8.
- Overath P and Engstler. M. (2004). Endocytosis, membrane recycling and sorting of GPI-anchored proteins: *Trypanosoma brucei* as a model system. *Mol Microbiol. Aug*;53(3):735-44.
- Overath P, Haag J, Lischke A, O'hUigin C. (2001). The surface structure of *trypanosomes* in relation to their molecular phylogeny. *Int J Parasitol. May* 1;31(5-6):468-71.
- Palella TD, Davidson BL, Pashmforoush M, Kelley WN. (1989). Human hypoxanthine-guanine phosphoribosyltransferase deficiency. The molecular defect in a patient with gout (HPRTAshville). *J Biol Chem. Jan* 5;264(1):520-5.
- Parsons M. (2004). Glycosomes: parasites and the divergence of peroxisomal purpose. *Mol Microbiol. Aug*;53(3):717-24.
- Pays E, Vanhamme L, Perez-Morga D., (2004). Antigenic variation in *Trypanosoma brucei*: facts, challenges and mysteries. *Curr Opin Microbiol. Aug*;7(4):369-74
- Pennisi. E. (1998). Taking a structured approach to understanding proteins. *Science. 1998 Feb* 13;279(5353):978-9 Perrot *et al.*, 1999
- Pentreath VW, Rees K, Owolabi OA, Philip KA, Doua F. (1990). The somnogenic T lymphocyte suppressor prostaglandin D2 is selectively elevated in cerebrospinal fluid of advanced sleeping sickness patients. *Trans R Soc Trop Med Hyg. Nov-Dec*;84(6):795-9
- Pepin J and Milord F. (1994). The treatment of human African *trypanosomiasis*. *Adv Parasitol. ;33*:1-47.
- Perrot M, Sagliocco F, Mini T, Monribot C, Schneider U, Shevchenko A, Mann M, Jenö P, Boucherie H. (1999). Two-dimensional gel protein database of *Saccharomyces*

## Literature

- cerevisiae* Electrophoresis. Aug;20(11):2280-98
- Reiner NE and Malemud CJ. (1985). Arachidonic acid metabolism by murine peritoneal macrophages infected with *Leishmania donovani*: in vitro evidence for parasite-induced alterations in cyclooxygenase and lipoxygenase pathways. J Immunol. Jan;134(1):556-63.
- Reiner NE and Malemud CJ. (1984). Arachidonic acid metabolism in murine *leishmaniasis* (*Donovani*): ex-vivo evidence for increased cyclooxygenase and 5-lipoxygenase activity in spleen cells. Cell Immunol. Oct 15;88(2):501-10.
- Ridgley EL, Xiong ZH, Ruben L. (1999). Reactive oxygen species activate a Ca<sup>2+</sup>-dependent cell death pathway in the unicellular organism *Trypanosoma brucei brucei*. Biochem J. May 15;340 ( Pt 1):33-40.
- Rabilloud T. (1999). Solubilization of proteins in 2-D electrophoresis. An outline. Methods Mol Biol. ;112:9-19.
- Rabilloud T. (1998). Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. Electrophoresis. May;19(5):758-60.
- Rabilloud T. (1996). Solubilization of proteins for electrophoretic analyses. Electrophoresis. May;17(5):813-29.
- Rabilloud T, Girardot V, Lawrence JJ. (1996). One- and two-dimensional histone separations in acidic gels: usefulness of methylene blue-driven photopolymerization. Electrophoresis. Jan;17(1):67-73.
- Rabilloud T, Brodard V, Peltre G, Righetti PG, Etori C. (1992). Modified silver staining for immobilized pH gradients. Electrophoresis. Apr;13(4):264-6.
- Rabilloud T. (1990). Mechanisms of protein silver staining in polyacrylamide gels: a 10-year synthesis. Electrophoresis. Oct;11(10):785-94.
- Robello C, Parodi-Talice A, Duran R, Arrambide N, Prieto V, Pineyro MD, Pritsch O, Cayota A, Cervenansky C, Robello C. (2004). Proteome analysis of the causative agent of Chagas disease: *Trypanosoma cruzi*. Int J Parasitol. Jul;34(8):881-6.
- Roditi I, Liniger M., (2002). Dressed for success: the surface coats of insect-borne protozoan parasites. Trends Microbiol. Mar;10(3):128-34. Review.
- Rupp G and Porter. M.E. (2003) A subunit of the dynein regulatory complex in *Chlamydomonas* is a homologue of a growth arrest-specific gene product. J Cell Biol. Jul 7;162(1):47-57
- Sherwin T and Gull K. (1989b). Visualization of detyrosination along single microtubules reveals novel mechanisms of assembly during cytoskeletal duplication in *trypanosomes*. Cell. Apr 21;57(2):211-21

## Literature

- Shevchenko A.. (1996b). Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proc Natl Acad Sci U S A. Dec 10;93(25):14440-5.
- Shevchenko A, Wilm M, Vorm O, Mann M. (1996a). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem. Mar 1;68(5):850-8.
- Simmons DL, Xie WL, Chipman JG, Robertson DL, Erikson RL. (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci U S A. Apr 1;88(7):2692-6.
- SMITHIES O and POULIK MD. (1956). Two-dimensional electrophoresis of serum proteins. Nature. Jun 2;177(4518):1033
- Smith DF, Price HP, Panethymitaki C, Goulding D. (2005). Functional analysis of TbARL1, an N-myristoylated Golgi protein essential for viability in bloodstream *trypanosomes*. J Cell Sci. Feb 15;118(Pt 4):831-41.
- Smith WL and Lands WE. (1972). Oxygenation of polyunsaturated fatty acids during prostaglandin biosynthesis by sheep vesicular gland. Biochemistry. Aug 15;11(17):3276-85.
- Sperling K., (2001). From proteomics to genomics. Electrophoresis. Aug;22(14):2835-7.
- Stephens and Fantham. (1910) Huisenga J. (1899). Measuring differences between *Trypanosoma brucei*, Plimmer and Bradford and *Trypanosoma rhodesiense*. Preliminary communication. Trop Geogr Med. Jun;22(2):221-6
- Stevens JR and Gibson WC. (1999). The molecular evolution of *trypanosomes*. Parasitol Today. Nov;15(11):432-7
- Stevens JR, Noyes HA, Dover GA, Gibson WC. (1999). The ancient and divergent origins of the human pathogenic trypanosomes, *Trypanosoma brucei* and *T. cruzi*. Parasitology. Jan;118 ( Pt 1):107-16.
- Sturm NR, Van Valkenburgh H, Kahn RA, Campbell DA. (1998). Characterization of a GTP-binding protein in the ADP-ribosylation factor subfamily from *Leishmania tarentolae*. Biochim Biophys Acta. Nov 8;1442(2-3):347-52.
- Swain M and Ross NW. (1995). Silver stain protocol for proteins yielding high resolution and transparent background in sodium dodecyl sulfate-polyacrylamide gels. Electrophoresis. Jun;16(6):948-51
- Switzer RC 3rd, Merril CR, Shifrin S. (1979). A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal Biochem. Sep 15;98(1):231-7.
- Szallies A, Kubata BK, Duszenko M. (2002). A metacaspase of *Trypanosoma brucei* causes loss of respiration competence and clonal death in the yeast *Saccharomyces cerevisiae*. FEBS Lett. Apr 24;517(1-3):144-50.

## Literature

- Trauth BC, Klas C, Peters AM, Matzku S, Moller P, Falk W, Debatin KM, Krammer PH. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science*. Jul 21;245(4915):301-5.
- Turner CM, McLellan S, Lindergard LA, Bisoni L, Tait A, MacLeod (2004). *A. Parasitology*. Oct;129(Pt 4):445-54.
- Ullman,B. (1984). IMP dehydrogenase mutants: cell culture model for hyperuricemia. *Adv Exp Med Biol.*;165 Pt A:373-9
- Valmsen K, Jarving I, Boeglin WE, Varvas K, Koljak R, Pehk T, Brash AR, Samel N. (2001). The origin of 15R-prostaglandins in the Caribbean coral *Plexaura homomalla*: molecular cloning and expression of a novel cyclooxygenase. *Proc Natl Acad Sci U S A*. Jul 3;98(14):7700-5
- Van der Ouderaa FJ, Buytenhek M, Nugteren DH, Van Dorp DA. (1977). Purification and characterisation of prostaglandin endoperoxide synthetase from sheep vesicular glands.*Biochim Biophys Acta*. May 25;487(2):315-31
- Van Weelden SW, Fast B, Vogt A, van der Meer P, Saas J, van Hellemond JJ, Tielens AG, Boshart M. (2003). Procyclic *Trypanosoma brucei* do not use Krebs cycle activity for energy generation. *J Biol Chem*. Apr 11;278(15):12854-63.
- Vaughan S. and Gull K. (2003). The *trypanosome* flagellum. *J Cell Sci*.Mar 1;116(Pt 5):757-9
- Vaux DL. (2002). Apoptosis timeline.*Cell Death Differ*. Apr;9(4):349-54.
- Vaux DL and Strasser A. (1996). The molecular biology of apoptosis. *Proc Natl Acad Sci U S A*. Mar 19;93(6):2239-44
- Vickerman K. (1994). The evolutionary expansion of the *trypanosomatid* flagellates. *Int J Parasitol*. Dec;24(8):1317-31
- Vickerman K. (1985). Developmental cycles and biology of pathogenic *trypanosomes*.*Br Med Bull*. Apr;41(2):105-14.
- Weber K and Osborn M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J Biol Chem*.Aug 25;244(16):4406-12
- Webster P, Russo DC, Black SJ. (1990).The interaction of *Trypanosoma brucei* with antibodies to variant surface glycoproteins. *J Cell Sci*. Jun;96 ( Pt 2):249-55.
- Welburn SC, Barcinski MA, Williams GT. (1997). Programmed cell death in *trypanosomatids*. *Parasitol Today*. Jan;13(1):22-6.
- Welburn SC, Hide G, Tait A, Maudlin I. (1996). The origins, dynamics and generation of *Trypanosoma brucei rhodesiense* epidemics in East Africa. *Parasitol Today*.Feb;12(2):50-5.

## Literature

- Wickstead B, Ersfeld K, Gull K. (2004). The small chromosomes of *Trypanosoma brucei* involved in antigenic variation are constructed around repetitive palindromes. *Genome Res.* Jun;14(6):1014-24
- Wickstead B, Ersfeld K, Gull K. (2003). The frequency of gene targeting in *Trypanosoma brucei* is independent of target site copy number. *Nucleic Acids Res.* 2003 Jul 5;31(14) :3993-4000.
- Wilkins MR, Hochstrasser DF, Sanchez JC, Bairoch A, Appel RD. (1996). Integrating two-dimensional gel databases using the Melanie II software. *Trends Biochem Sci.* Dec;21(12):496-7
- WHO, 2005. Tropical diseases and control
- WHO, 2004. Tropical diseases and control
- WHO, 2002. Tropical diseases and control
- Zangger H, Mottram JC, Fasel N. (2002). Cell death in *Leishmania* induced by stress and differentiation: programmed cell death or necrosis? *Cell Death Differ.* Oct;9(10):1126-39



## Appendix

My Teachers.

Victor Adeniyi, O. Bakare, H. Bagci, M. Duszenko, B. Frank, U. Hesseman, S. Ingce, A Jung,  
R. Lucius, R Mattes, D. Mecke, B. Oputa, C. Osagie, A.J. Pfitzner, P. Renz und D Wolf.

## Appendix

### Lebenslauf

|                           |  |
|---------------------------|--|
| Name                      | Leonard Ehigie   |
| Geburtstag                | 11. 02. 59.  |
| Geburtsort                | Benin-City, Nigeria  |
| Eltern                    | Lawrence Ehigie<br>Dorah Ehigie  |
| <b>Schule und Studium</b> | Besuch der Baptist Schule, Benin-City.   |
| 1982-1986                 | Studium der Biologie an der Middle East<br>Tecnical University (Turkey).   |
| 1986                      | Abschluss Bachelor of Science  |
| 1992-1993.                | Besuch der deutschen Sprachschule  |
| 1993-1997                 | Studium der Biologie an der Universität<br>Hohenheim<br>Abschluss als Diplom-Biologe   |
| 1998-2000                 | Forschungstätigkeit an der Universität<br>Hohenheim als Wissenschaftlicher Mitarbeiter   |
| 2000-2005                 | Promotion am Physiologisch-Chemischen Institut<br>der Universität Tübingen, Molekulare<br>Parasitologie, Prof. Dr M. Duszenko.<br><i>Prostaglandin Synthase</i> Isolierung und <i>T. brucei</i><br>Proteome Analysis |
| Seit 2005                 | Wissenschaftlicher Mitarbeiter am Universität<br>klinikum Tuebingen (AG-Gawaz), Platelets<br>Proteome.   |