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Characterization of Glutamate Carboxypeptidase II (GCPII) Ortholog of *Schistosoma mansoni*

Diploma Thesis

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DECLARATION

I, Enoch Mensah Boateng, hereby declare that the work herein presented is the result of my own investigation under the supervision of Dr. Jan Dvorak, and that except for other people's work which has been duly acknowledged, this thesis has not been presented to this university or elsewhere for any degree. I also declare, I did not infringe the copyright of third parties in connection of creation this diploma thesis.

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DATE.....	DATE.....

ACKNOWLEDGEMENT

I would like to express my sincerest appreciation to my supervisor, Dr. Jan Dvorak for believing in me and giving the opportunity to be part of his research team. I know it wasn't easy for him. I did mess up bigly several times and he got disappointed in me many times, yet he did not give up on me. Sometimes he went wild and was hard on me but I knew he wanted me to be the best. I also never forgot how he commends me for a good work done anytime I got something right. I thank you so much Jan, for your patient endurance in supervision, enthusiastic encouragement and useful comments as well as advices on this research work .For the first time I felt I had a 'supervisor'

I will like to say **THANK YOU** to Lenka Ulrychova of IOCB for her massive support and directions towards successful completion of my thesis. I know I was a source of burden to her but she was patient with me and contained me. Thank you for the worm sectioning and microscopic work. I can't thank you enough but deep down, I will always remember you. To Dr. Martin Horn, I say be blessed for opening your lab to me and to all the members of IOCB lab, I appreciate your kind gestures and help in diverse ways.

Again I'll like to show my heartfelt gratefulness to Dr. Cyril Barinka for letting me use his lab, IBT, CAS, Biocev for a number of experiments. To Bara, Petra Jana and all the team at IBT, a big shout out to you.

Finally, it is pleasure to thanks my family, friends and team members of our newly established Molecular Helminthology lab, CULS for any form of support I received from you.

ABSTRACT

Blood flukes of the genus Schistosoma (*Schistosoma mansoni),* among other species cause schistosomiasis, a parasitic disease that infects over 250 million people across the world, with adverse social, economic and health impact and for which there is a need to investigate for alternative chemotherapeutic interventions. This thesis focused on biological characterization of glutamate carboxypeptidase of *S. mansoni* (SmGCPII), a protease in *S. mansoni,* to identify the expression sites of the protein in *S. mansoni* and subsequently identify the functions of the protein in the model organism comparative to humans and other mammals. This objective was performed by RNA *in situ* hybridization and expression of recombinant protein in two expression systems (*Escherichia coli* and *Kluyveromyces lactis*)

By *in situ* hybridization, it was detected that GCPII is localized in the esophagus, gastrodermis, oral and ventral suckers, testis and some cells of the parenchyma of the adult male worm and also in the ovary, oviduct, vitellaria and some parenchyma cells of the Adult female worm. These findings suggested a possible reproduction, digestion and other unknown functions of GCPII in the model organism. Recombinant SmGCPII was successfully expressed *in E. coli*, purified and used as antigen to immunize rabbit. The expression of recombinant SmGCPII was unsuccessful in *K. lactis* for a number of unconfirmed reasons. It is expected that antibodies will be generated from the immunization to facilitate further biochemical studies through a number of approaches in the near future.

Keywords: Glutamate, carboxypeptidase, ortholog, *Schistosoma mansoni,* characterization, recombinant, i*n situ* hybridization

LIST OF ABBREVIATIONS

YPD: Yeast extract peptone dextrose

DAPI: 4',6-diamidino-2-phenylindole

DEPC: Diethyl pyrocarbonate

RT: Room Temperature

TNT: Tris-NaCl-Tween buffer

MAB: Maleic acid buffer

AA: Amino acid

PSMA: Prostate specific membrane antigen

SOC: Super optimal broth

IPTG: Isopropyl β-D-1-thiogalactopyranoside

PCR: Polymerase chain reaction

LB: Lysogeny broth

dNTP: Deoxynucleotide

EDTA: Ethylenediaminetetraacetic acid

MBP: Maltose binding protein

HRP: Horseradish peroxidase

DTT: Dithiothreitol

SDS –PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

20x SSC: 20 times concentration of saline-sodium citrate

GCPII: Glutamate carboxypeptidase

SmGCPII: *Schistosoma mansoni* glutamate carboxypeptidase

rSmGCPII: recombinant *Schistosoma mansoni* glutamate carboxypeptidase

RNA: Ribonucleic acid

mRNA: Messenger ribonucleic acid

PBS: Phosphate-buffered saline

CDC: Center for disease control and prevention

NCBI: National Center for Biotechnology Information

RCSB: Research Collaboratory for Structural Bioinformatics

EU: European union

Cy5 TSA: Cyanine 5 Tyramide signal amplification assay

O/N: Overnight

PIC: Protein inhibitory cocktail

THP: Tetrahydropyran

TBST: Tris-buffered saline, 0.1% Tween 20

PVDF: Polyvinylidene difluoride

DNA: Deoxyribonucleic acid

cDNA: complementary deoxyribonucleic acid

BLAST: Basic Local Alignment Search Tool

YPG: Yeast extract peptones galactose

MALDI -TOF: Matrix assisted laser desorption/ionization time of flight

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Introduction

Parasitic organisms affect millions of people and animals globally resulting in serious diseases and often to deaths. Among causative agents, parasitic worms, usually referred to as helminths (despite of their different evolutionary relationship or phylogeny) are prominently associated with the many harmful health and economic consequences. In this research, one paramount parasitic worm from the class Trematoda, blood fluke from the family Schistosomatidae is studied. The infection caused by *Schistosoma. mansoni* and other significant parasitic species *Schistosoma haematobium,* and *Schistosoma japonicum* of the same genus, (WHO, 2010) affects hundreds of millions of people and it is not limited to only humans but livestock and other permissive mammals (Ravindran and Kumar, 2012). There has been an account of the initial emergence of re-occurrence of schistosomiasis in Europe (Boissier *et al*., 2016).

The severity of schistosomiasis is exemplified in the chronic state of the disease where infections persist for years resulting in serious signs and symptoms from the interaction between parasites egg and that of the host immune responses. Neglected or improper treatment may consequently lead to death (McManus *et al*., 2010). Currently, only drug, praziquantel is the most effective treatment for schistosomiasis, while researchers are yet to make any discovery of potent vaccine or new drug to avert possible future resistance of the parasite to praziquantel.

Proteases (aka proteolytic enzymes, proteinases or peptidases) are enzymes involved in protein catabolism or modifications by hydrolyzing peptide bonds. These enzymes are present in all living organisms and even for viruses and are significant for their physiological processes and ultimately, survival (Barrett *et al.*, 2001). The essence of proteases in living organisms is invaluable because of their roles in physiological processes, such as comprehensive protein synthesis and digestion, blood coagulation, fertilization, cell differentiation, hormone processing and immune system responses (*van der Hoorn,* 2008; Sims, 2006). It is anticipated that the absence of proteases will have a detrimental consequence on the hydrolysis of a peptide bond in that, it would take hundreds of years at 25°C to hydrolyse peptide bond whereas its presence will amount to hydrolysing up to one million peptide bonds per second (Sajid and McKerrow, 2002).

Trematodes in general and schistosomes in specific, like all other organisms have a number of proteases that help them in regulating physiological processes within and outside their host

during various stages of their life cycle. (Cesari *et al*., 2000; Dvorak *et al*., 2005; Sajid *et al*., 2003). For instance, *S. mansoni* cathepsin B1 (SmCB1) is a cysteine protease which was the first *S. mansoni* sequenced proteolytic enzyme and is the most abundant cysteine protease in gastrointestinal system which is thought to be necessary for hemoglobin digestion (Baig *et al*., 2002). Glutamate carboxypeptidase II (GCPII) is also an example of the numerous proteases found in parasitic worms but with unspecified localization sites and functions (Barinka *et al*., 2004).

Human (GCPII E.C. 3.4.17.21), also known as N-acetyl-L-aspartyl-L-glutamate peptidase I (NAALADase I), NAAG peptidase, or prostate-specific membrane antigen (PSMA) is an enzyme encoded by the *FOLH1* (folate hydrolase 1) gene (O'Keefe *et al*., 2004). There are 750 amino acids in the Human GCPII and it weighs approximately 84 kDa (Barinka *et al*., 2004). GCPII is a di-zinc membrane metalloproteases. Most of the enzyme resides in the extracellular space. GCPII is a class II membrane glycoprotein. It catalyzes the hydrolysis of N-acetylaspartylglutamate (NAAG) to glutamate and N-acetylaspartate (NAA) according to the reaction scheme to the right (Mesters *et al*., 2006).

In this study, my research was focused on initial biological characterization of GCPII orthologs from *S. mansoni* because; *S. mansoni* stands for a suitable primary model organism with relatively simple body organization. *S. mansoni*, apart from being influential model for basic and translated biomedical research, also doubles as exceptional direct laboratory model for schistosomiasis of humans and animals importance (Howe *et al*., 2016). The investigation to characterize GCPII of *S. mansoni* with potential impact on human biomedical and parasitological research in view of the fact that GCPII is found in all prokaryotic and eukaryotic organisms both organisms including plants, helminths and vertebrates, thus the essentiality of GCPII appears to be universal in all living organisms (Barrett *et al.*, 2001).

1.1 Objectives of the Study

The general objective of the study is to biologically characterize GCP II ortholog in blood fluke *S. mansoni* (here called SmGCPII) in order to reveal the primary function(s) of GCPII in *S. mansoni*.

Specific Objectives

In order to achieve the general objective of the study, the following specific objectives were formulated.

1. Localization of mRNA coding SmGCPII protease using RNA *in-situ* hybridization technique in *S. mansoni*.
2. Expression of recombinant SmGCPII in a selected expression system (*Escherichia coli* and *Kluyveromyces lactis*) in order to produce polyclonal antibodies.

1.2 Hypothesis

The characterization of SmGCP II should help us to identify common (basal) functions of GCPII molecules in relatively primitive and distant organisms with potential impact on human biomedical and parasitological research. The reason for such research is that, (A) GCPII is present in all phyla from unicellular yeasts to multicellular organisms including plants, helminths and vertebrates, thus the essentiality seems to be universal (Barrett , 2001). (B) *S. mansoni*, is a direct laboratory model to several flukes that are of humans and animals importance with potential impact for vaccine discovery and it is influential model for basic and translated biomedical research as an effective tool to help understand the roles of helminths parasites in biomedicine (Howe *et al*., 2016).

CHAPTER 2

LITERATURE REVIEW

2.1 Background and Taxonomy of Schistosomiasis

Schistosomes are trematodes ('flukes') from the phylum Platyhelminthes. The adult worm occupies the vascular system of birds and mammals ('blood flukes'). The genus *Schistosoma* contains 19 species (WHO, 2010) five of which (*Schistosoma haematobium, S. mansoni, Schistosoma. japonicum, Schistosoma. mekongi* and *Schistosoma. intercalatum*) are of major pathological importance. All schistosomes that infect humans belong to the genus *Schistosoma* of the family Schistosomatidae, which contains 11 other genera, some of which cause human cercarial dermatitis despite not able to establish patent infection (Kasny *et al*., 2011. The geographical distribution and etiology of schistosomiasis reveal the exceptional life cycle of *Schistosoma* species. Schistosomes infect susceptible freshwater snails in endemic areas, usually with specific species of schistosomes infecting specific species of snails. The infected snails release cercariae 4-6 weeks after infection. The cercariae are fork-tailed, free-swimming larvae approximately 1mm in length. They can survive in fresh water up to 72 hours, during which time they must attach to human skin or to that of another susceptible host mammal or die (Ahmed, 2017).

The genesis of schistosomiasis, also referred to as bilharziasis, or snail fever, can be traced to Theodore Bilharz, a German surgeon who worked in Cairo, and first identified the etiological agent *S. hematobium* in 1851 (Nawal, 2010). Most human schistosomiasis is caused by *S. mansoni, S. haematobium,* and *S. japonicum*. It has been revealed that, less prevalent species, such as *Schistosoma Mekong, Schistosoma sinensium, Schistosoma malayensis, Schistosoma hippopotami, Orienabilharzia (Schistosoma) turkestanicum, Schistosoma incognitum, Schistosoma rodhaini, Schistosoma nasale, Schistosoma spindale, Schistosoma indicum, Schistosoma margrebowiei, Schistosoma leiperi, Schistosoma mattheei, Schistosoma curassoni, Schistosoma bovis* and *Schistosoma intercalatum*, may also cause systemic human disease. More than 250 million people, majority of who live in Africa, are infected with schistosomiasis, (WHO, 2010) and an estimated 700 million people are at risk of infection in 76 countries where the disease is considered endemic, as their agricultural work, domestic routines, and recreational

activities expose them to infested water (WHO, 2016). Estimated annual deaths of 200,000 are ascribed to schistosomiasis (Chistulo *et al*., 2004). Probably more than 95% of human schistosomiasis is due to *S. mansoni* and *S. haematobium*. Several of the 'non-human' species, including *Schistosoma mattheei* and *Schistosoma bovis*, are of veterinary importance, and both domestic and feral animals are major reservoirs of *S. japonicum* infection as well (WHO, 2010)

About 120 million people were symptomatic with schistosomiasis, with 20 million having severe clinical disease (WHO, 2010). Especially, women and children performing their daily activities, washing clothes in infested water and men using infected water for agricultural purposes are at higher risk (WHO, 2016). Forty million women of childbearing age are infected (Friedman *et al*., 2007). Approximately 10 million women in Africa have schistosomiasis during pregnancy (Friedman *et al*., 2007) and in endemic areas; the infection is usually acquired at childhood through contact with infected water or source (WHO, 2010). As well, refugee movements, migration to urban areas and increasing population size results in need for power and water and consequent building of large water dams which disperses agents of schistosomiasis (Kabatereine *et al*., 2004). The intensity and prevalence of infection has been found to rise with age and peaks usually between ages 15 and 20 years (Leder and Weller, 2009; Hagan and Gryeels, 1994; King *et al*., 1988) with older adults exhibiting no significant change in the prevalence of disease.

2.2 Biology, Life Cycle and Morphology of Schistosomes

Unlike other pathologically significant hermaphroditic trematodes, schistosomes are dioecious). The adult worms are about 1 cm long, and the male has a deep ventral groove or schist (hence the term 'schistosome') called canalis gynaecophorus in which the female worm resides in so called permanently in copula. Worms of each sex have an oral sucker at the anterior end used for blood meal intake and regurgitation since there is only one gut opening and doubles as a means of attachment to the venous epithelium of the host and for locomotion of the paired worms (Walker, 2011).

Life cycle

Mature free-swimming larvae called cercariae are shed from the infected snail into the surrounding fresh water and begin a new cycle. After attachment to the host, cercariae then move

around through intact skin to dermal veins and, over the next several days, to the pulmonary vasculature. During this movement, the cercariae transform into schistosomula; shed tail and outer glycocalyx layer while mounting double-lipid-bilayer teguments that are highly resistant to host immune responses (Ahmed, 2017). The worms then migrate through the pulmonary capillaries to the systemic circulation, which carries them to the portal veins, where they mature sexually (Ahmed, 2017). Schistosomula may circulate randomly through the pulmonary systemic blood vessels before getting entrapped in the liver (Basch, 1991). Adult male and female pair within the portal vasculature and together, they migrate along the endothelium, against portal blood flow, to the mesenteric veins or in one case of *S. haematobium* to the veins of urine bladder where they begin egg production (Ahmed, 2017). The eggs, which are highly antigenic and can induce an intense granulomatous response, migrate through the bowel or bladder wall to be shed via feces or urine. During this time (approximately 10 days), the eggs begin to mature and larvae called miracidia are formed inside (Ahmed, 2017).

Eggs that are not shed successfully may remain in the tissues or be swept back to the portal circulation (from the mesenteric vessels and be entrapped in the liver of the definite host (Corachan, 2002; Houston *et al*., 2004) or to the pulmonary circulation (from the vesicular vessels via the inferior vena cava).

The free-swimming miracidia that are released from the eggs into fresh water survive for a short time during which time they must infect a susceptible snail host to complete the life cycle. Within the infected snail, 2 generations of asexual stages - sporocysts multiply (Ahmed, 2017), there is division of germ cells in the mother sporocyst to produce daughter sporocysts, which move around to the snail's digestive gland called hepatopancreas. Inside the digestive gland, germ cells within the daughter sporocyst divide again, producing thousands of infective larvae, known as cercariae).

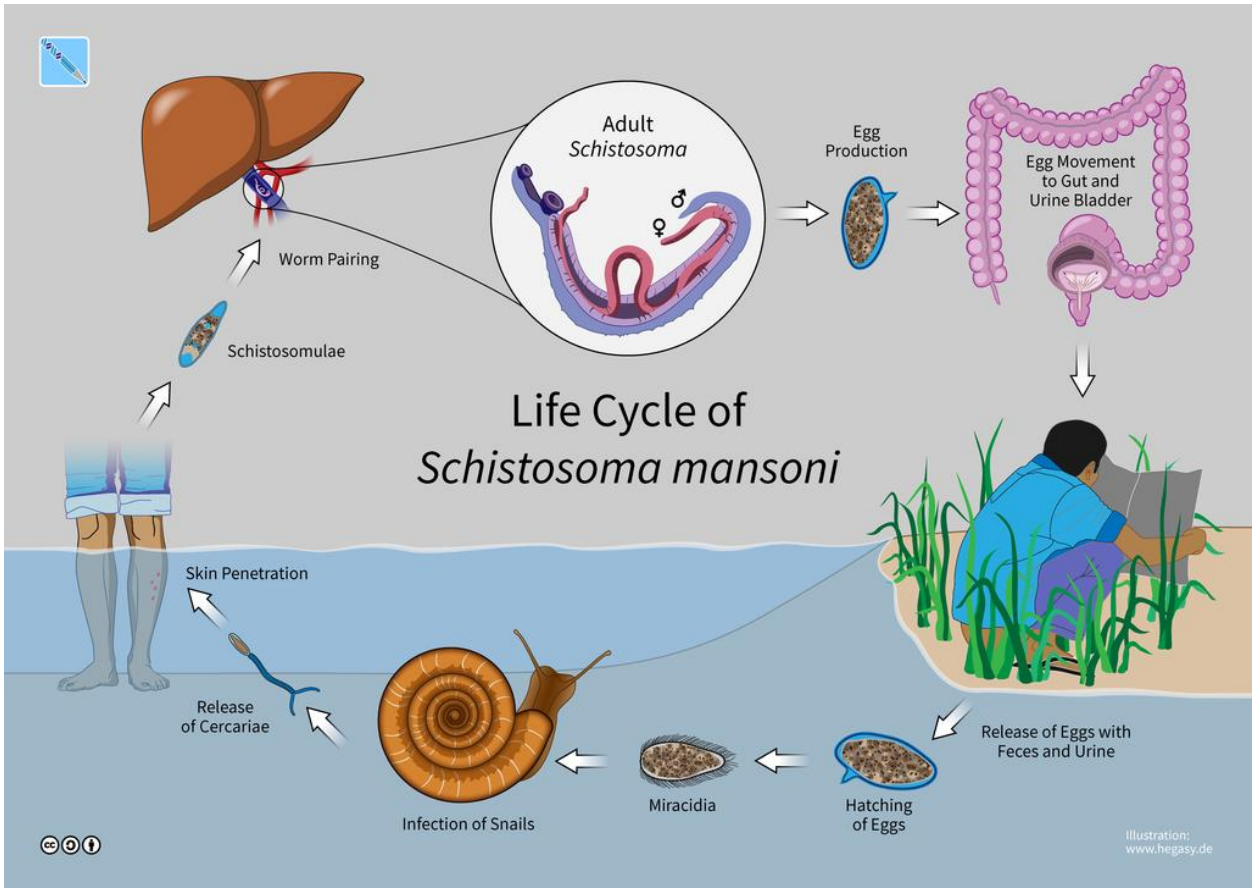

Figure 1 The life-cycle of Schistosoma species adopted from online CDC resource https://www.cdc.gov/parasites/schistosomiasis/biology.html. White arrows indicate stages to which the human host, free-living aquatic stages and those affecting the intermediate snail host are affected.

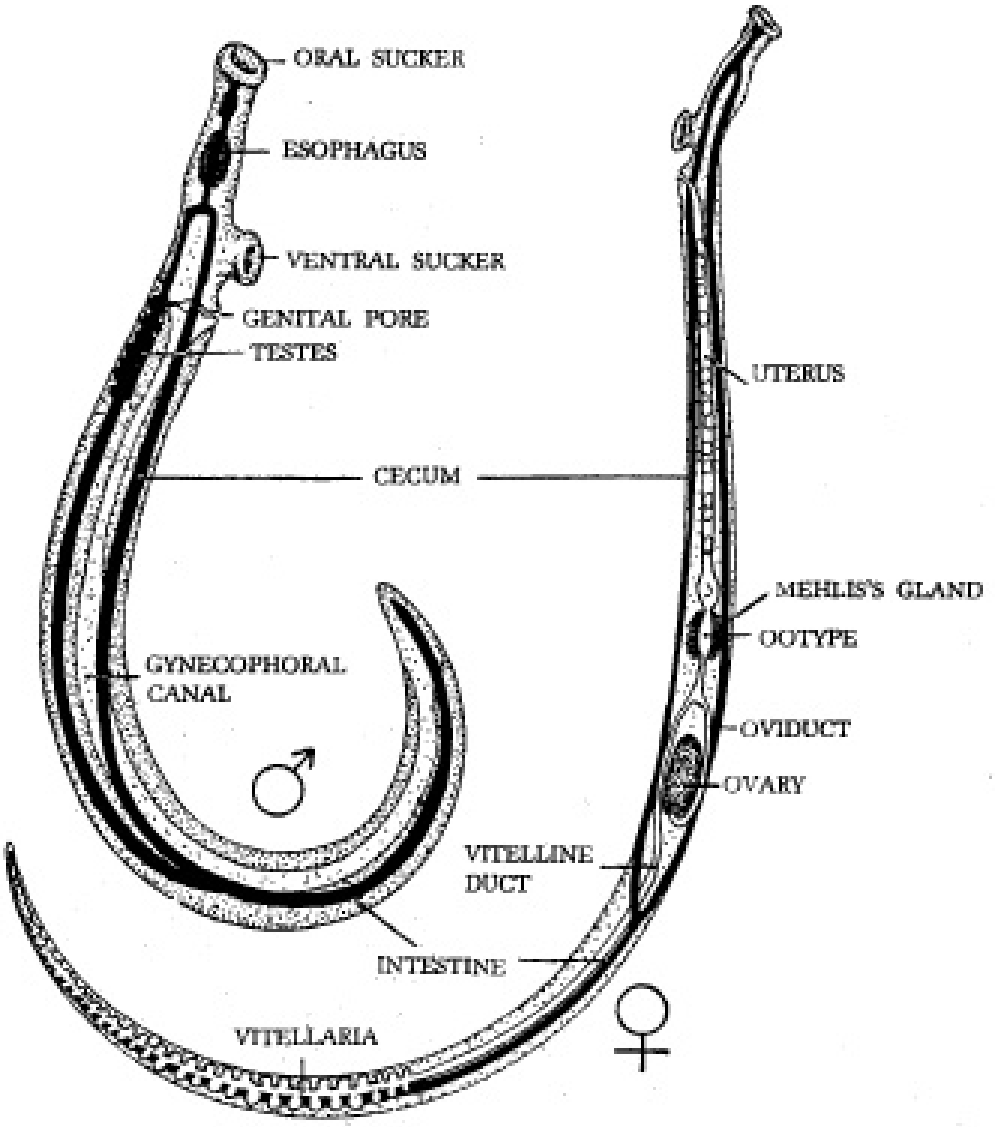

Figure 2. A labeled morphology of adult male and female *S. mansoni* adopted from https://www.slideshare.net/rajud521/schistosomes, downloaded 21/03/2018 11:18 am

There is not one general snail intermediate host for all species of schistosoma. Different species of the genus *Schistosoma* have different types of snails serving as their intermediate hosts; these hosts are *Biomphalaria spp. for S. mansoni, Oncomelania spp*. for *S. japonicum, Tricula spp.* (*Neotricula aperta*) for *S. mekongi* and *Bulinus spp.* for *S haematobium* and *S. intercalatum* (Sturrock, 2001; Leder and Weller, 2009)

2.3 Pathophysiology

Acute schistosomiasis (Katayama syndrome) is a systemic, serum sickness-like illness that develops after several weeks in some, but not all (Ahmed, 2017), individuals with new schistosomal infections. Acute schistosomiasis may match up to the first cycle of egg deposition and is associated with marked peripheral eosinophilia and circulating immune complexes. It is most common with *S. japonicum* and *S. mansoni* infections and is most likely occurring in heavily infected individuals after primary infection (Ross *et al*., 2007). Symptoms may include fever, malaise, myalgia, fatigue, non-productive cough, diarrhea (with or without the presence of blood), hematuria (*S. hematobium*), and right upper quadrant pain. Acute schistosomiasis is seen in people who are infected for the first time who traveled to endemic areas. In the case of *S. japonicum* it is also associated with either a super-infection or a hypersensitivity reaction in previously infected people (Ross *et al*., 2007).

The pathology of chronic schistosomiasis, which is far more common than the acute form of the infection, results from egg-induced immune response, (Ahmed, 2017) granuloma formation, and associated fibrotic changes. Although cercarial and adult worms are minimally immunogenic, schistosomal eggs are highly immunogenic and induce vigorous circulating and local immune responses. As the eggs penetrate the urinary system, they can find their way to the female genital region and form granulomas in the uterus, fallopian tube, and ovaries. CNS involvement occurs because of embolization of eggs from the portal mesenteric system to the brain and spinal cord through the paravertebral venous plexus (Corachan, 2002; Houston *et al*., 2004). Most of schistosomiasis symptoms are due to immunologic reactions to *Schistosoma* eggs trapped in tissues and the liver organ. Antigens released from the egg stimulate a granulomatous reaction involving T cells, macrophages, and eosinophils that results in clinical disease. Disease symptoms and signs are dependent on the number and location of eggs trapped in the tissues of the host. At the preliminary stages, the inflammatory reaction is readily reversible. In the latter stages of the disease, the pathology is associated with collagen deposition and fibrosis, resulting in organ damage that may be only partially reversible (Corachan, 2002; Houston *et al*., 2004; Mohammed *et al*., 2007).

Adult worms can absorb host proteins. If not attacked by the immune system, they can live for years in the blood stream as they are coated with host antigens **(**Ahmed, 2017**)**.

Immunopathological studies have shown that schistosomiasis results from the host's immune response to schistosome eggs and the granulomatous reaction evoked by the antigens they secrete (Ross *et al*., 2002; Gryseels *et al*., 2006; Wynn *et al*., 2004; Burke *et al*., 2009). Granulomas, which develop at the sites of maximal accumulation of eggs, destroy the eggs but result in fibrosis in host tissues (Ross *et al*., 2002; Gryseels *et al*., 2006). Granuloma formation and the local inflammatory response mediated by CD4+ T-helper-2 lymphocytes help facilitate the passage of eggs into the lumen of the gut or urinary tract. Chronic intestinal schistosomiasis can present with acute complications of appendicitis, (Badmos *et al*., 2006)

2.4 Epidemiology

A restricted study of enhanced community intervention performed in rural Chinese villages demonstrated significant improvements in human, snail, and wild mouse schistosomiasis infection rates. Along with the preexisting programs for the annual treatment of farmers and cattle, efforts were made to optimize animal grazing sites, sewage management, drinking water supplies, and health education with regard to schistosomiasis (Wang *et al*., 2009).

According to the World Health Organization (WHO) (2010), the global distribution of schistosomiasis has changed in that it has been eradicated from Japan and the Lesser Antilles Islands; transmission has been stopped in Tunisia; and transmission is very low in Morocco, Saudi Arabia, Venezuela, and Puerto Rico. Nevertheless, the human cost of schistosomal infections remains high, and the disease contributes to comorbidity with other infections, including hepatitis, human immunodeficiency virus (HIV), and malaria, in endemic regions (Kallestrup *et al*., 2006).

2.5 Control of Schistosomiasis

The World Health Organisation (W.H.O.) advocates 3 main interventions to control parasitic helminthiases: 1) chemotherapy to reduce morbidity due to high worm burdens, particularly in children, 2) improved sanitation as a means to reduce parasite transmission and 3) health education to encourage populations to modify their water contact behaviors and thus reduce both

transmission and the risk of re-infection post-treatment (Montresor *et al*., 2002). There is no effective vaccine for schistosomiasis, although this remains an attractive prospect for long-term control of infections, particularly where behavioral modifications are unrealistic (e.g. in very young children and communities without access to sanitation) and access to chemotherapy is sporadic (Chitsulo *et al*., 2000).

2.6 Role of Proteases in Survival of Parasitic Worms

The enzymatic activities of proteases is pivotal in host-parasite interactions such as invasion, migration through the tissues, hatching (reproduction), excystment (McKerrow *et al*., 2009) nutrients acquisition through haemoglobin digestion, immune evasion, activation and modulation of inflammation which are all central to their survival and their ability to cause diseases (Caffrey *et al*., 2004; Delcroix *et al*., 2007; Horak *et al*., 2008; McKerrow *et al*., 2006). Different classes and types of proteases are found in parasites with specific functions usually towards population multiplication, creating disease condition (morbidity) and virulence. For this reason, proteases have been intensively studied as targets for vaccine and drug therapies in recent biomedical research (Bos *et al*., 2009).

For decades, proteases have become the center of attention in many branches of science owing to their fundamental functions in cellular biology and their potential veterinary, medical and industrial application. In biomedical science research in general and parasitology (trematodology) in specific, peptidases have become striking molecules since their roles in both acute and chronic stages of many parasitic diseases including schistosomiasis cannot be undermined (McKerrow *et al*., 2006; WHO, 2006).

2.7 Classification of Proteases

Proteases can be classified into three main groups based on their on their catalytic residue, evolutionary phylogeny and optimal pH within which they function. The seven broad groups under catalytic type residue classification are: serine proteases cysteine proteases, threonine proteases, aspartic proteases, glutamic proteases , asparagine and metalloproteases (Rawlings *et al*., 2009). The classification based on evolutionary phylogeny takes in to consideration clans or superfamilies of the protease and this emanates from the structure, mechanism and catalytic

residue order of peptidases. Within each 'clan', proteases are classified into families based on similarity of sequence. Many hundreds of related proteases may be found within each clan. Trypsin, elastase, thrombin and streptogrisin are examples of related proteases within the S1 family. Currently more than 50 clans are known, each indicating an independent evolutionary origin of proteolysis and these are based on the MEROP database. (Oda, 2012; Rawlings *et al*., 2009; Rawlings and Barrett, 1995). On the other hand, proteases may be classified by the optimal pH in which they perform best. Acid proteases, neutral proteases and basic proteases or alkaline proteases are the main types (Mitchell *et al*., 2007).

Of all the classification systems, the use of functional, homology and molecular structure as a mean of classifying proteases is presently considered the most relevant approach to distinguish and categorize peptidases. This database of classification was promoted by Rawlings and Barrett (1995) and it is based strongly on primary and tertiary structures of proteases. More information about protease classification system is available to the general public and biomedical researchers on MEROPS (Rawlings and Morton, 2008).

2.7.1 Basic Classification of Proteases Based on Action Sites

Endopeptidases usually cleave internal peptide alpha-bonds of polypeptide chain away from N-terminus or C-terminus. Oligo-peptidases which fall under endopeptidases cleave shorter peptides and no proteins.

Exopeptidases generally cleave peptide alpha-bonds adjacent to N-terminus or C-terminus of polypeptide chain. Examples of exopeptidases are; aminopeptidases which cleave a single amino acid residue from N-terminus, carboxypeptidases also cleaving a single AA residue from C-terminus, dipeptidylpeptidases cleave a dipeptide from N-terminus and tripeptidylpeptidases cleave a tripeptide from N-terminus. Other exopeptidases are peptidyldipeptidases that cleave a dipeptide from C-terminus and dipeptidases cleave dipeptides. Both termini are usually expected to be free.

Omegapeptidases are the third group of proteases based on site of action. They cleave peptide alpha-bonds with no preference for N-terminus or C-terminus and can cleave also isopeptide bonds. (Kasny *et al*., 2009).

2.7.2 Numerical Classification

Enzyme Commission number (EC) is a numerical classification which groups enzymes according to the chemical reaction they catalyze. Proteases (E.C. 3.4) is group of hydrolases which catalyze hydrolyses of peptide bonds. Proteases (E.C. 3.4.2) are group of proteases which are classified according to their functional amino acids in active site. This classification provides five mechanistic classes of proteases: the serine proteases (E.C. 3.4.21), cysteine proteases (E.C. 3.4.22), aspartic acid proteases (E.C. 3.4.23), metalloproteases (E.C. 3.4.24) and threonine protease (E.C. 3.4.25).

2.8 Proteases of *S. mansoni*

Most studied and characterized proteases are gut proteases. Both male and female of *S. mansoni* adults rely on blood meal, predominantly containing red cells and serum proteins. Red cells are hemolyzed in esophagus and gained hemoglobin digested in the gut. This digestion is performed by a various number of proteolytic enzymes, mainly cysteine and aspartic proteases. These gut proteases are expressed as zymogens with low pH optimum typical forschistosoma gut. Several of them were expressed in various laboratory expression systems, biochemically characterized and immunohistochemically localized. Study carried out by Delcroix *et al*., (2006) investigated the proteolytic network to find out their common cooperation in the digestive process. They suggested that cathepsin B1 (SmCB1), cathepsin L1 (SmCL1), cathepsin D (SmCD), cathepsin C (SmCC), asparaginyl endopeptidase (SmAE) and aminopeptidase possibly cooperate to initiate host protein breakdown (Delcroix *et al*., 2006). SmCB1 was first sequenced protease of *S. mansoni* and is the most abundant cysteine protease in gastrointestinal region (Baig *et al*., 2002) and another cathepsin B termed, SmCB2, was located on the surface of *S.mansoni* tegument.

Serine proteases are an indispensable part of the proteolytic network in schistosomes due to their substantial activities in the developmental stages of the worms and its infection of the human host (Dvorak *et al*., 2016)., proteases involved in the invasion process are termed cercarial elastases (CEs) is the best characterized and most studied group of serine proteases from *S. mansoni*. (Salter *et al*., 2000; Dvorak *et al*., 2008; Ingram *et al*., 2012; Horn *et al*., 2014).The CEs have basic features of S1 family proteases, but possess several distinctive trait including ; missing disulfides between Cys130–Cys194 and Cys184–Cys212 of chymotrypsin. This protease

possesses typical serine peptidase catalytic triad in the active site (His68/Asp126/Ser218; numbering for SmCE; AN: MER03620, P12546; Rawlings *et al*., 2008). On the Other hand, *S. mansoni* serine proteases SmSP1, SmSP2, SmSP3 and SmSP4 may play a modulation role in the host parasite interactions (Dvorak and Horn, 2018)

GCPII is one of the numerous proteases found in organisms with yet unknown function and localization in worms. It has been found in certain organs of rodents, mammals and humans. It is a membrane peptidase produced in the prostate, central and peripheral nervous system, kidney, small intestine, and tumor-associated neovasculature (Barinka *et al*., 2004)

2.9 Mammalian GCPII Proteases and Their Orthologs (History and Nomenclature)

First suggestion of GCPII in scientific writings can be traced to 1987 when it happened concurrently from two completely unrelated research areas. Robinson *et al*. offered a study identifying a protein, denominated N-acetylated alpha-linked acidic dipeptidase (NAALADase), responsible for hydrolysis of neurotransmitter N-acetyl-L-aspartyl-L-glutamate (NAAG) in the brain of rat (Robinson *et al*., 1987). At the same time, Horoszewicz *et al* (1987), produced monoclonal antibody 7E11-C5.3 against a new marker of epithelial prostatic cancer cell line LNCaP (lymph node carcinoma of the prostate) which was entitled prostate specific membrane antigen (PSMA). To make things even more complicated, Halsted identified a new enzyme in 1991, designated folate hydrolase (FOLH), in jejunal brush-border which hydrolyses and as a result enables successive assimilation of dietary folates.

In humans, the prime sites of GCPII expression under normal physiological situations are small intestine (jejunal brush border membranes), nervous system (astrocytes and Schwann cells) and kidney (proximal tubules). Research have indicated a broader GCPII expression outline in humans and that there has been a positive but much lower expression (compared to the above mentioned organs) results in the heart, lacrimal glands, pancreas, bladder, skin, liver, lung, colon, breast, testis, (Rovenska *et al*., 2008; Silver *et al*., 1997)

Probably due to the very distant research fields, for the those five years, neuroscientists studied NAALADase, urologists PSMA and dieticians FOLH without realizing that all these names refer to a single protein entity. In 1996, two publications emerged showing that the PSMA possesses

folate hydrolase activity (Pinto *et al*., 1996) and that it also embodies the characteristics of a neuropeptidase (Carter *et al*., 1996). Shortly after, Coyle laboratory of made an attempt to unify the nomenclature by performing a thorough analysis of NAALADase, PSMA and FOLH (ref). They demonstrated the equal properties of these proteins and suggested the usage of the name "glutamate carboxypeptidase II" (Luthi-Carter *et al*., 1998; Halsted *et al*., 1998). This designation was subsequently adopted by International Union of Biochemistry and Molecular Biology (IUBMB).

However, even after many years of discovery, the original names for GCPII, mainly PSMA, are still frequently used. Unfortunately, this makes the orientation in the GCPII research field quite convoluted. All through this thesis the name GCPII will be used preferentially.

2.10 Human GCPII on RNA Level

Alternative Splicing: An mRNA coding for full-length GCPII protein (ENTREZ code NM_004476.1) goes through various alternative splicing. Several different splice variants such as PSM', PSM-C, PSM-D, PSM-E and PSM-F have been identified (Bzdega *et al*., 2002; Cao *et al*., 2007). Additionally, many other alternatively spliced transcripts of FOLH1 gene can be found among the ESTs (expressed sequence tags) at the NCBI database UniGene (http://www.ncbi.nlm.nih.gov/unigene), which indicates that the list of described splice variants is probably incomplete.

The PSM' was the first identified and the most studied GCPII splice variant. PSM' lacks the nucleotides 114-379 at the 3' end of the first exon compared to the "wild-type" mRNA. In reality, a protein, which cleaved NAAG and started with alanine at location 60, was identified in the LNCaP cells and in the prostate carcinoma. This protein was named PSM' since it was considered to be a translational product of PSM' mRNA (Grauer *et al*., 1998; Lapidus *et al*., 2000). However, the true origin of PSM' protein is probably more complex. GCPII protein was shown to be N-glycosylated and this post-translational modification was proven to be essential for its enzymatic activity (Barinka *et al*., 2004; Ghosh and Heston, 2003).

2.11 Expression of mRNA in Human Tissues

Almost all studies determining the expression profile of mRNA coding for GCPII showed a significantly increased mRNA level in the prostate compared to other tissues (Cunha *et al*., 2006; Pangalos *et al*., 1999; Renneberg *et al*., 1999). Chang *et al*. (1999) posited that GCPII mRNA expression is induced in malignant non-prostatic tissues such as the bladder, lung, or pancreas. However, a subsequent study by Pangalos *et al* (1999), detected mRNA expression in variety of human tissues including brain, colon, heart, kidneys, lungs, ovary, pancreas, spleen, small intestine and testis. These data suggest that the GCPII mRNA expression is widespread in human but it may be further induced by the malignant processes within the cell.

2.12 GCPII-like Proteases; Their Protein Levels, Structure and Domain Organization.

GCPII is classified as a type II transmembrane di-zinc metallopeptidase which, based on the sequence homology with the aminopeptidases from *Streptomyces griseus* and *Vibrio proteolyticus*, belongs to the MEROPS clan MH; subfamily M28B (Rawlings *et al*., 2014; Rawlings and Barrett, 1997). It consists of short intracellular N-terminal part (AAs 1-18), one transmembrane helix (AAs 19-43) and large extracellular C-terminal part (AAs 44-750) (Mesters 2006). GCPII contains 10 N-glycosylation motifs (Asn-X-Ser/Thr) within its primary sequence and these post translational modifications were shown to be necessary for GCPII enzymatic activity (Barinka *et al*., 2004; Ghosh and Heston, 2003). Properly modified GCPII molecule embodies a molecular weight of approximately 100 kDa while the protein without posttranslational modifications has a molecular weight of 84 kDa (Israeli *et al*., 1994).

Structural information about GCPII has been primarily gained from the X-ray crystallography experiments. The first X-ray structure of GCPII refined at 3.5 Å was published in 2005 (Davis *et al*., 2005). However, the detailed description of GCPII active site and its mechanism of substrate recognition were not known until 2006 when a series of high resolution structures (refined at up to 2.0 Å) was published (Mesters *et al*., 2006). A homodimeric structure of GCPII extracellular part is shown in Figure 3. The GCPII monomer can be divided into three distinct domains: protease-like domain (AAs 59116 and 352-590), apical domain (AAs 117-351) and C-terminal domain (AAs 591-750). Additionally, four inorganic ions (two Zn^{2+}, one Cl^{-} and one Ca^{2+}) and seven N-glycosylation sites have been identified within the crystal structure model of GCPII.

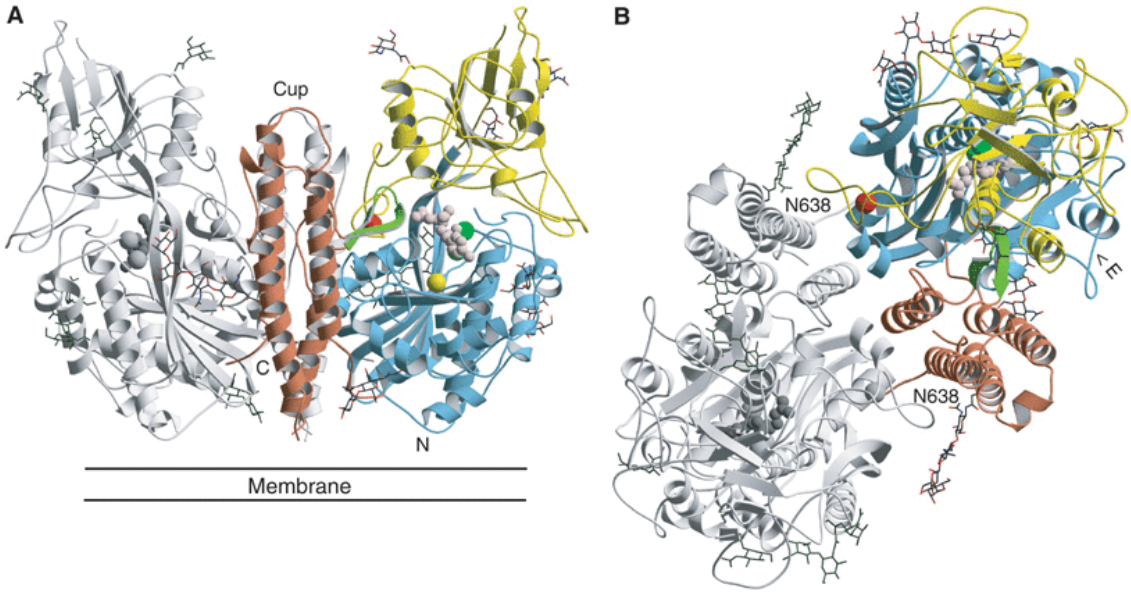

Figure 3: X-ray structure model of extracellular portion of GCPII. One monomer is shown in wheat and the other is colored according to the domain organization; protease-like domain in red (AAs 57-116 and 352-590), apical domain in yellow (AAs 117-351) and C-terminal domain in orange (AAs 591-750). Carbon atoms of carbohydrate moieties of colored monomer are shown in cyan while in other monomer are shown in wheat. All carbohydrates are shown as stick and their oxygen atoms are shown in red and nitrogen atoms in blue. Protein backbone is shown in ribbon representation while zinc ions are depicted as blue, chloride ion as green and calcium ion as magenta spheres. N- and C-termini of the GCPII extracellular domain are labeled as N and C, respectively. The orientation of GCPII homodimer towards the cell is schematically depicted by the illustration of the plasma membrane. (adopted from Mesters *et al*., 2006)

A major breakthrough in the field came a year later, when a new crystallization conditions for GCPII, which led to the structures refined to as low as 1.65 Å, were identified (Barinka *et al*., 2007). Since then, the X-ray crystallography has become a method routinely applied for investigation of GCPII substrate specificity and enabled to perform several structure-activity relationship (SAR) studies which aimed to design potent and specific inhibitors of GCPII (Barinka *et al*., 2007; Pavlicek *et al*., 2014). Currently, more than 40 different X-ray structures of GCPII have been deposited in RCSB Protein Data Bank.

2.13 GCPII as an Enzyme

GCPII substrates: As the name suggests, GCPII is a hydrolase which cleaves off C-terminal glutamate from its substrate. In a human body this enzymatic activity executes two different but important physiological functions. Firstly, GCPII is responsible for the cleavage of N-acetyl-L-aspartyl-L-glutamate (NAAG), the most abundant peptide neurotransmitter in human brain (Neale *et al*., 2000), yielding N-acetyl-L-aspartate and L-glutamate (Robinson *et al*., 1987).

Secondly, GCPII enzymatic activity is crucial for the metabolism of dietary folates. Human body accepts folate (known also as pteroyl-γ-L-glutamate or vitamin B9) in a polyglutamylated form. GCPII located in the brush border membrane of the small intestine is responsible for the consecutive cleavage of these glutamates and thus enables the absorption of the folates by epithelial cells (Horoszewicz *et al*., 1987; Halsted *et al*., 1987).

Beside these two endogenous substrates, GCPII is able to process other peptide substrates. Study by Barinka *et al*., showed that GCPII efficiently processes N-acetylated dipeptides which contain acidic residues (Glu/Asp) in P1 position and either acidic residues (Glu/Asp) or methionine in P1' position (e.g., N-acetyl-L-glutamyl-L-glutamate or N-acetyl-L-aspartyl-L-methionine) (Barinka *et al*., 2002).

2.14 GCPII Expression in Human Body

GCPII protein expression in human healthy and malignant tissues has been intensively studied throughout the past two decades. Unfortunately, except for a high protein expression in both benign and malignant prostate tissue, the data on GCPII expression in other human tissues are somewhat inconsistent (Kinoshita *et al*., 2006; Troyer *et al*., 1995)

The explanation for such big discrepancy may lie in a variety of detection methods used in these studies. Different methods have variable detection limits and their results also often rely on the experience of the researcher. Moreover, different mAbs recognize distinct epitopes (e.g. within intracellular or extracellular portion of GCPII) and may bind to its antigen in different forms (e.g., native or denatured form of GCPII). (Gala *et al*., 2000; Lopes *et al*., 1990; Silver *et al*., 1997)

Unfortunately, these basic mAbs characteristics are often not even known or specified for majority of the commonly used mAbs and additionally many researchers do not provide sufficient information (e.g., concentration of used mAb) to enable reliable assessment and/or reproducibility of their experiments.

Nevertheless, the data from healthy tissue samples indicate that GCPII expression is not exclusive for the prostate, but can also be found in other tissues such as brain, small intestine, colon and kidney. In malignant tissues, the only clear consistency of the data relates to the strong GCPII expression in prostate carcinoma. On the other hand, considering histological localization, a majority of the studies demonstrated that GCPII is primarily expressed in tumor-associated neovasculature of various types of solid carcinomas (Chang *et al*., 1999; Haffner *et al*., 2012; Liu *et al*., 1998; Huang *et al*., 2004).

Prior to this research, a preliminary analysis conducted by Dvorak *et al* (personal communication) on *S. mansoni* proposes that only a single GCPII orthologs, SmGCPII for *S. mansoni*, exist as proteins, thus creating a more convenient study system in *S. mansoni* compared to higher animals. Research has shown that only genes coding GCPII are present in trematodes and nematodes, allowing for simple study of their GCPII ancestors before latter diversification (Lambert and Mitchell, 2007).

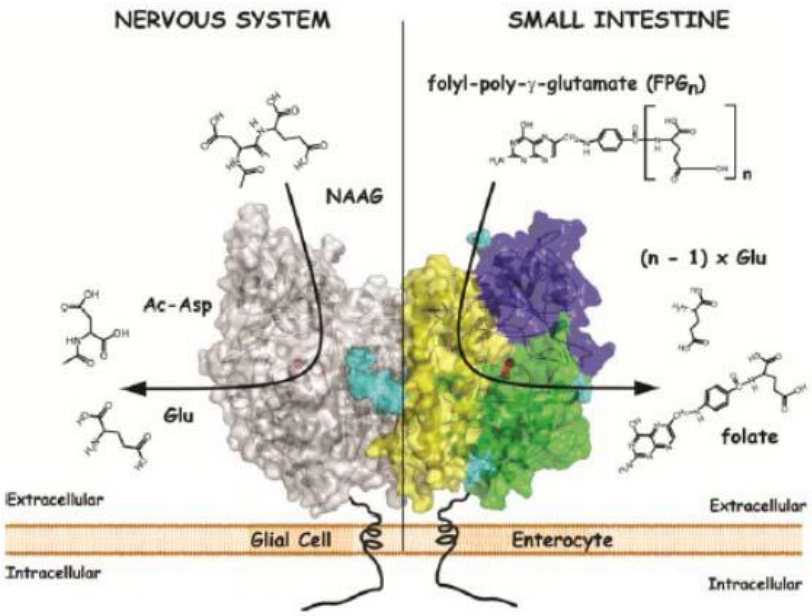

Figure 4. presents a pictorial view of the two identified physiological functions of GCPII found in mammals GCP2. On the left of Figure 2 shows the GCP2 that hydrolyzes NAAG, the most abundant neuropeptide in mammalian brains in the nervous system, enouraging thus cross-talk between neurons and surrounding glial cells. The right scheme is located in the jejunum; the enzyme found on enterocytes lining intestinal lumen encourages the absorption of folate-monoglutamate into the blood stream by chopping off polyglutamylated tails of dietary folates. **(**Adopted and adjusted from Barinka *et al*., 2007**)**

***S. mansoni* GCPII protein ortholog sequence**

MWQEISQNLCKNVSDTFLLEYMHKFCGGNPHCSGSEGNYEIANFIERSWIEWGWPVVD
RQEFYVTLPLGPPENGPWNEVLLTNSDGTEVIHGAQNSVTVPSKSEICSQNVTVDNSDSK
QLPVYQAYSCSGVSFGYLVFVNYARRKDLLLFDKLQGRKKGEPSHICNKNLIVIARLGN
GTRQSKLKNLMEHCTCGQNNTSLPDHHPGALILYPDPQDFAADGLVYPNGKGLPGDAP
VFGHMNMKYAGGGDSTCTGFPSLPHIYRTDTLVQGDALTQIPVQPVGYDDAKIFLSSLE
GPTIPNDWDTRLATHLGPSTKTCLKVVVHNQVSKNPVKLCNIVGIMPGEITPTSTESDQY
IIMGNHHDSWVQGACDPGSGMVILQEIARILGEAYRNGFKPRRTIILGSWDGEEFSVLGS
THFVHKSEYELLSRCVVYINSDCPVKGHKNFSARTDSLLIDSLINAAKLVPVDPPINMQS
FYDEWLNNKMSDRNEPVITSLGGGSDHIPFAYRLGIPSTYPEFLPDDGLYNTPVYHTAYD
IIDVVERFTDPASFTGHLPRHRLITRLILTLIIQFACTPRLPLSILRCSQCLLDDWLKFMELV
THQIPNISEYGVNLDWVLEEIQKFKKSSQDFEDFANTVERNCTSFPSYLNRILVGVSKHF
VAAGQCEKSSLKNVIQGTTGYKSVYFPHVKSSFKNLKMLYDKCKTKTLNSTELYDFKL
ELSNVLNCLQQLTNWLRNSWIGLTDFSLAL.

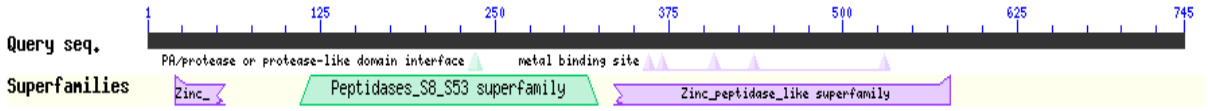

Figure 5. Protein sequence of SmGCPII as analyzed by BLAST on NCBI website. The first domain indicated in violet bar is a zinc peptidase of M28 family. Zinc peptidases play vital roles in metabolic and signaling pathways throughout all kingdoms of life. Peptidase family M28 contains aminopeptidases and carboxypeptidases, and has co-catalytic zinc ions. Each zinc ion is tetrahedrally co-ordinated, with three amino acid ligands plus activated water; one aspartate residue binds both metal ions. Protease-associated (PA) domain is an insert domain in a diverse fraction of proteases. The significance of the PA domain to many of the proteins in which it is inserted is undetermined. It may be a protein-protein interaction domain. At peptidase active sites, the PA domain may participate in substrate binding and/or promoting conformational changes, which influence the stability and accessibility of the site to substrate.There is not TFR-dimer domain prediction at the C-terminus of the protein, in this sequence of SmGCPII. H. https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 06/03/2018

CHAPTER 3

MATERIALS AND METHODS

3.1 Background Materials and Methods

In the process of investigation, a numbers of standard protocols were followed, however in some cases, based on the experiences of the supervisor and collaborating lab; some of the protocols were modified. Work involved three laboratories involved in this study are; Laboratory of Structural Biology at the Institute of Biotechnology (BIOCEV), Institute of Organic Chemistry and Biochemistry (IOCB) and Laboratory of Molecular Helminthology, Czech University of Life Sciences, headed by my supervisor.

3.2 Chemicals Used

Acetic acid	Penta, Czech Republic
Agarose	Thermo Fisher
Dextran Sulphate	Sigma-Aldrich, Czech Republic
Xylen	Penta, Czech Republic
Ethanol (absolute and 96%)	Lach-Ner, Czech Republic
Tyrmide Signal Amplification System (TSA)	PerkinElmer, Czech Republic
Hydrochloric acid (HCl)	Penta, Czech Republic
Formamide	Thermo Fisher, Germany
Glycine	USB, USA
Glycerol	Penta, Czech Republic
Tween 20	Sigma-Aldrich, Czech Republic
Methanol	Penta, Czech Republic

Sodium Chloride	Penta, Czech Republic
YPD agar	Sigma-Aldrich, USA
LB medium	Sigma-Aldrich, USA
Sodium hydroxide	Penta, Czech Republic
Glucose	Sigma-Aldrich, USA
Ampicillin	Sigma-Aldrich, USA
Immidazole	Sigma-Aldrich, USA
Guanidine hydrochloride	Sigma-Aldrich, USA
Saline-Sodium Citrate (20x SSC)	Thermo Fisher,Germany
Maleic acid	Sigma-Aldrich, USA
Sodium citrate	enta, Czech Republic
Diethylpyrocarbonate Water (DEPC H_2O)	Sigma-Aldrich, Czech Republic
2-Amino-2-hydroxymethyl-propan-1,3-diol (Tris)	Promega, USA
Urea	Sigma-Aldrich, Czech Republic
Coomassie brilliant blue	Fluka, Germany
$NiSO_4$	Sigma-Aldrich, Czech Republic
Precast SDS PAGE gels	GenScript
EDTA	Sigma-Aldrich Czech Republic
YPG	Sigma-Aldrich, USA

3.3 Buffers used

Phosphate-buffered saline (PBS)

Tris-buffered saline-Tween buffer (TBS-T)

Maleic acid buffer

TAE buffer

SDS PAGE buffer

3.4 Acquisition and Preparation of *S. mansoni*

3.4.1 Ethics Statement

All the animals used in the study were housed in accordance with European regulations for transport, housing and care of laboratory animals (Directive 2010/63/EU on the protection of animals used for scientific purposes) in the Institute of Immunology and Microbiology of the First Faculty of Medicine, Charles University and the General University Hospital in Prague, Czech Republic. The animals were maintained by certified persons (certificate number CZ 02627) in the laboratories specifically accredited by the Ministry of Agriculture of the Czech Republic in accordance with the animal welfare laws of the Czech Republic and EU.

3.4.2 Acquisition and Maintenance of *S. mansoni*

Acquisition and maintenance of a Puerto Rican isolate of *S. mansoni* was ensured in our laboratory through the intermediate snail host, *Biomphalaria glabrata* and the laboratory mouse, (infected at the age of 5– 6 weeks). The cycle was maintained according to previously used protocols (Horn *et al*, 2014).

3.5 Part 1(*In situ* Hybridization)

3.5.1Cloning of PCR DNA with pGEM-T Easy Vector (Promega)

PCR Screening

Messenger RNA was isolated from adult *S. mansoni* and transcribed into single stranded cDNA by reverse transcriptase (done by supervisor). AishGCPII forward and reverse as well as BishGCPII forward and reverse primers were used with cDNA for PCR amplification. The amplification reaction consisted of 35 cycles at 94°C for 2 minutes (initial denaturation), 60°C for 30 seconds (annealing) and 72°C for 40 seconds (elongation) using *Taq* polymerase . Gel electrophoresis was run using agarose and TAE running buffer GCPII A and B fragments were excised, purified and subsequently develop RNA *in situ* hybridization probes by first cloning into pGEM-T-Easy vector.

The sequences for primers used are (Sigma-Aldrich):

AishSmGCPIIFRD – 5'- GTGGCAACCCTCACTGTTCT

AishSmGCPIIREV – 5'- GAGTTCCGTTGCCCAGTCTA

BishSmGCPIIFRD – 5' - TCCTGGCGCTCTTATTCTGT

BishSmGCPIIREV – 5' - CAAGCACCTTGTACCCATGA

Ligation into pGEM-T- Easy (Promega)

Separate ligation reactions for GCPII A and GCPII B were set up by mixing appropriate concentrations and volumes of Ligation Buffer, pGEM-T Easy Vector (50ng), amplified GCPII A (in one reaction) and GCPII B (in another reaction), T4 DNA Ligase (3 Weiss units/µl) and deionized water to a defined final volume (see protocol in appendix..). The reactions were mixed in two different 0.5ml tubes (with low DNA binding capacity) and incubated for 1 hour at room temperature.

Transformation of JM109 High Efficiency Competent Cells

50µl of thawed JM109 High Efficiency Competent Cells was added to 2µl of a briefly centrifuged ligation reaction in a sterile 1.5ml tube on ice, mixed and incubated for 20 minutes. The cells were heat-shocked for 45–50 seconds in a water bath at exactly 42°C and was immediately returned to ice for 2 minutes. 950µl of cold (4°C) SOC medium was added to each transformation reaction followed by 60 minutes incubation at 37°C with shaking.

100µl of each transformation culture was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates. A control culture was also set up according to manufacturer's protocol with overnight incubation of plates at 37°C and selection of white colonies (transformed cells) afterwards. Plasmids were isolated for probe preparation.

3.5.2 Linearization of DNA and Preparation of Antisense Probe for RNA ISH

Single-stranded RNA probes for GCPIIA and GCPII B antisense were prepared *in vitro* using commercial DIG RNA Labeling KIT (SP6/T7) (Roche) and DNA construct. Hence this procedure, digoxigenin (DIG) labeled RNA probes were produced and later used for RNA *in situ* hybridization experiment (chapter 3.5.4).

Five µg of DNA plasmid containing GCPII A sequence was digested by NdeI restrictase (NEB) while 5µg of DNA plasmid containing GCPII B was digested by SacII (NEB) at 37 °C, O/N and subsequently purified by PCR clean up kit (Sigma) according to the manufacturer's protocol. The linearized plasmids were then used as a template for single-stranded antisense RNA probe production. Antisense GCPIIA probe was transcribed under SP6 RNA polymerase while antisense GCPII B was transcribed under T7 RNA polymerase according to the manufacturer instruction (Roche).

Preliminary experiment and analysis showed that, both GCPII A and GCPII B antisense probes had similar effect on tissues therefore GCPII A was used for subsequent experiments.

Below are the sequences of DNA fragments used:

GTGGCAACCCTCACTGTTCTGGTAGCGAAGGAAATTACGAAATTGCGAACTTCATAGAAAGGAGCTGG
ATAGAATGGGGTTGGCCTGTTGTCGACAGACAAGAATTTTATGTGACTCTACCTTTGGGCCCTCCTGAA
AATGGTCCATGGAATGAGGTTCTTCTTACCAACTCGGACGGTACCGAAGTCATACACGGAGCTCAAAA
TAGTGTTACTGTTCCATCGAAGTCTGAAATATGCTCTCAAAATGTAACTGTTGACAACTCCGACAGTAA
GCAGTTACCTGTTTATCAGGCTTATTCGTGCTCTGGTGTGTCGTTTGGATATTTGGTATTTGTGAACTAT
GCAAGACGTAAGGATTTGCTGTTGTTTGATAAATTGCAAGGCCGAAAAAAGGGTGAACCTAGTCATAT
ATGTAACAAGAATTTGATTGTTATTGCTAGACTGGGCAACGGAACTCTCCTGGCGCTCTTATTCTGTAT
CCTGATCCACAAGATTTTGCTGCAGATGGTCTAGTTTATCCTAATGGCAAAGGGCTACCAGGGGATGC
TCCTGTATTTGGTCATATGAATATGAAATATGCTGGCGGTGGTGACTCGACCTGCACTGGATTTCCATC
GCTTCCCCATATTTATCGAACTGATACACTTGTACAAGGTGATGCCCTTACACAGATTCCTGTTCAACC
TGTTGGTTATGATGATGCCAAAATATTTTTATCGAGCTTAGAGGGACCGACTATCCCTAATGATTGGGA
TACTCGTTTGGCGACTCATTTGGGACCATCAACTAAAACCTGCTTAAAAGTTGTAGTACATAATCAAGT
CTCTAAAAACCCAGTGAAGTTATGTAATATAGTGGGTATAATGCCGGGTGAAATAACACCCACTTCAA
CTGAATCTGATCAATATATAATTATGGGTAATCATCATGATTCATGGGTACAAGGTGCTTG

AishSmGCP2FRD	80	20	60.16	55.00	4.00	0.00	GTGGCAACCCTCACTGTTCT
AishSmGCP2REV	538	20	60.25	55.00	3.00	2.00	GAGTTCCGTTGCCCAGTCTA
BishSmGCP2FRD	612	20	59.98	50.00	4.00	0.00	TCCTGGCGCTCTTATTCTGT
BishSmGCP2REV	1106	20	59.57	50.00	5.00	2.00	CAAGCACCTTGTACCCATGA

3.5.3 *S. mansoni* Adult Worm Fixation and Sectioning

These steps were followed for the preparation of worm tissues for *in situ* hybridization (chapter 3.5.4). Worms (males and females) were washed for 90-120 minutes in 4% formaldehyde at room temperature (RT) and subsequently incubated in 30%, 50%, 70%, 96% ethanol solution for 45 minutes at RT each. After those incubations, the worms were immersed in a mixture of absolute ethanol containing Chromotrop 2R, and then absolute alcohol for 45 minutes at RT both. Subsequently, sample was incubated in methyl benzoate at RT, males for 45 minutes and females for 20 minutes, followed with two times of 5 minutes washing in benzene. Worms were then embedded into paraffin blocks according to the standard methodology. Section (8 µm) were prepared on microtome and transferred on microscopic glasses.

3.5.4 Detection of GCPII mRNA within *S. mansoni* Adult Worm Sections using RNA ISH

RNA *in situ* hybridization experiment was performed under RNA-free conditions and that all buffers and dilutions were made using RNA-free water. First, tissues on slides were de-paraffinized in xylen for 10 minutes at RT and then transferred to absolute ethanol under the same conditions. Tissue re-hydration was performed by 5 minutes of incubation in 96%, 70%, 50%, 25% ethanol (in decreasing order of ethanol concentration) with final wash in H_2O. Then, sections were incubated for 20 minutes in 0.2N HCl at RT and subsequently incubated in 10mM sodium-citrate buffer (pH 6.0) for 15minutes in boiling water bath and then cooled down to RT for 30 minutes. Later washes were performed in 0.2% glycine for 5 minutes at RT and then in 20% acetic acid at 4°C for 15 seconds. Then, slides were incubated in 1x phosphate-buffered saline (PBS) for 5 minutes at RT, in 20% glycerol for 15 minutes at RT. After this, sections were rinsed shortly in 2x SSC (two times concentrated) and heated to 70°C for 10minutes and subsequently were shortly cooled down on ice-cold metal plate. Then, probe-hybridization mixture (preparation of probe mixture is explained in next chapter) was immediately applied on the sections. Slides were covered with cover-glass, placed in a humid chamber and hybridized at 42 °C, O/N.

After hybridization were performed stringency washes in 2x SSC with 0.1% Tween20 heated to hybridization temperature, then in 1x SSC and in 0.5x SSC at RT for 15 minutes each. The stringency washes were followed by incubation in maleic acid buffer (MAB) for 5 minutes at RT and in 4 % blocking solution for 30 minutes. To label the hybridized probes, anti-*digoxigenin horseradish peroxidase labeled* antibodies (anti-DIG-HRP) diluted in 2% blocking solution in the proportion 1 : 500 were applied onto the hybridized worm tissue and incubated for 2 hours at 37 °C. Washing of slides for 20 minutes in MAB with agitation at RT and 2x 5 minutes each in TNT also at RT were performed immediately after incubation. Finally, cyanine 5 tyramide signal amplification assay (Cy5-TSA) was used to give and amplify signal for mRNA GCPII according to the manufactured protocol which was followed by 2 times washes in TNT buffer for 8 minutes each at RT. In the last step, sections were embedded in prolong diamond antifade mountant with 4',6-diamidino-2 -phenylindole **(**DAPI).

The fluorescent signal was visualized using Olympus IX83 microsocope equipped with PCO edge 5.5. camera and CoolLED pE-4000 LED illumination system. DAPI signal was detected by excitation diode 365 nm and emission filter 440/40 nm, Cy5 using diode 635 nm and emission filter 700/75. Appropriate lightning settings were determined using control slides underwent the whole in situ hybridization procedure however without any probe to define the background signal threshold. Image stacks of optical sections were processed using the Fiji software.

3.5.5 Preparation of Probe - Hybridization Mixture

For a total of 2.5mL hybridization mixture, 0.625 ml of 5x SSC, 2.5 mg of torula yeast RNA of concentration 10mg/mL, 0.35 mL of 1x PBS, 1.25 mL 50% deionised formamide, 1% of tween 20 and 0.25 g of 10% dextran sulphate were mixed together. The mixture was divided into two parts. The first part containing 5x SSC, torula yeast RNA and 1x PBS was mixed with 2µL of SmGCPII probe in sterile tube and heated at 70°C for 12 minutes after which the second part of the of the hybridization buffer containing deionized formamide, 1% tween 20 and 10% dextran sulfate was added. The whole experiment was conducted in 5 replications to authenticate the consistency in result obtained.

Part 2

3.6 Expression of Recombinant Protein (GCPII ortholog) in *E. coli*

3.6.1 Cloning of SmGCPII into Gateway pEC527 Expression Plasmid and Transformation of *E. coli* cells (Genscript)

Plasmid clone was made in the laboratory of Dr. Cyril Barinka (IBT, CAS, Biocev) using Gateway Cloning Technology. The Gateway cloning technology is based on the site-specific recombination system used by phageλ to integrate its DNA in the *E. coli* chromosome. Both organisms have specific recombination sites called *att*P in phage λ site and *att*B in *E. coli*. The integration process (lysogeny) is catalyzed by 2 enzymes: the phage λ encoded protein Int (Integrase) and the *E. coli* protein IHF (Integration Host Factor). Upon integration, the recombination between *att*B (25 nt) and *att*P (243 nt) sites generate *att*L (100 nt) and *att*R (168 nt) sites that flank the integrated phage λ DNA (see Figure 6A and B).

The process is reversible and the excision is again catalyzed Int and IHF in combination with the phageλ protein Xis. The *att*L and *att*R sites surrounding the inserted phage DNA recombine site-specifically during the excision event to reform the *att*P site in phage λ and the *att*B site in the *E. coli* chromosome (Genscript).

Plasmid pEC527 (approximately 10 ng) was added to a thawed vial of BL21 *E. coli* competent cells (Genscript) and incubated on ice for 30 minutes. Mixture was heat-shocked for 30 seconds at 42^{o}C and then transferred immediately onto ice. 250µl of SOC with RT was added to mixture which was incubated for 30 minutes with shaking at 37^{o}C. The transformed cells was added to 10ml Lysogeny broth (LB) with ampicillin and cultured overnight at 37^{o}C.

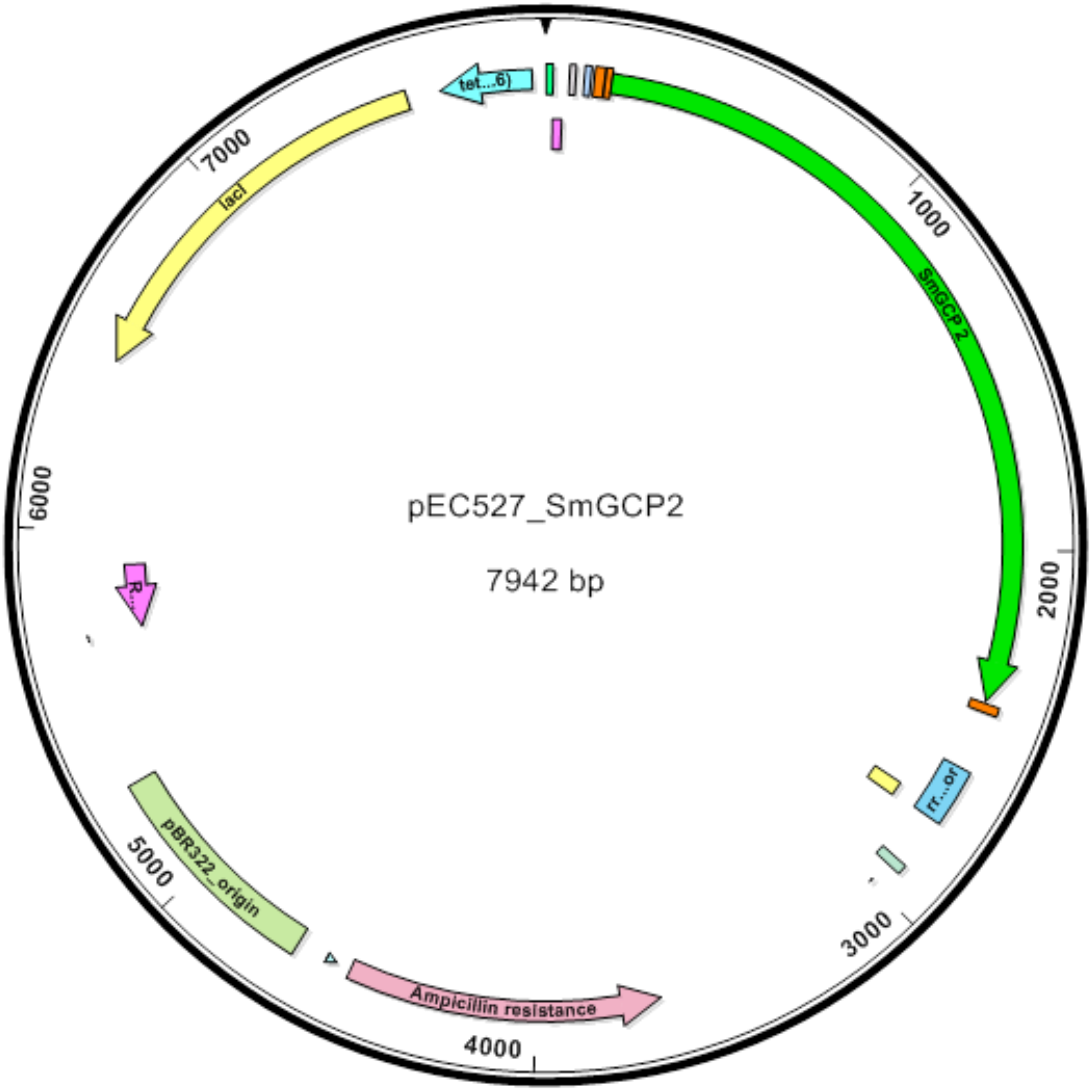

Figure 6A. Schematic map of the Gateway pEC527 expression plasmid containing SmGCPII (green) with *att* rich sites

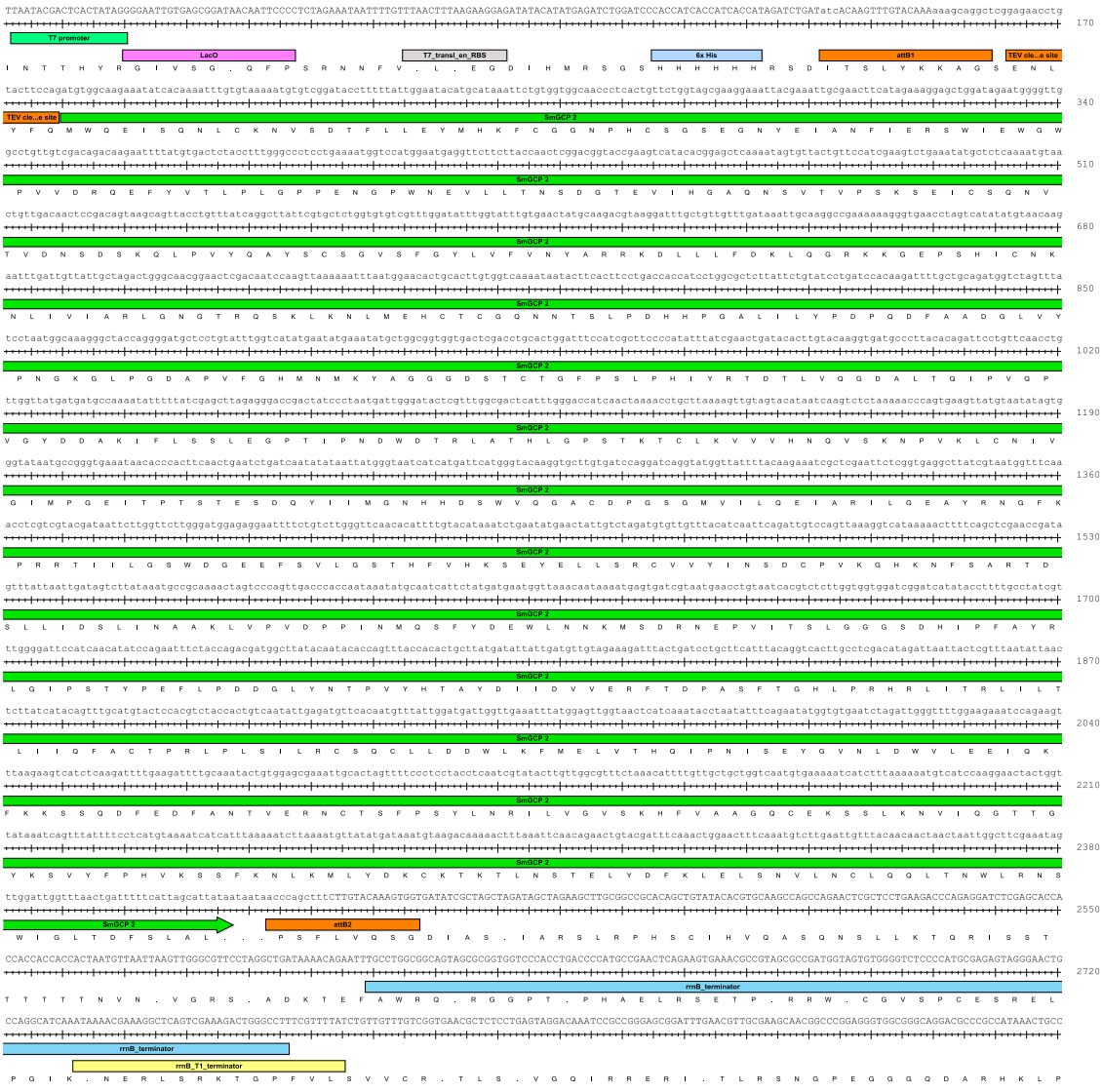

Figure 6B: Schematic representation of cloning of protein of interest (SmGCPII) to *E. coli* bacteria. Green color indicates protein of interest SmGCP2 with *att* recombination sites and T7 promoter

3.6.2 Expression of SmGCPII

Overnight inoculum was poured into the 200ml LB media (100µg/ml ampicillin, 20mM glucose) and grew at 37°C for 5 hours until it reached OD600= 0.7.Grown bacteria culture was spun down by centrifugation at 3000g for 10 min. Pellet was resuspended in LB medium (100µg/ml ampicillin, 1mM IPTG) and grown overnight at 37°C. The culture was centrifuged at 3000g for 10 min and resuspended in lysis buffer (20mM tris/HCl, pH=8). Resuspended culture was sonicated and centrifuged at12 000g for 10 min at 4°C. Resultant pellet was sonicated two times

in isolation buffer (8M Urea, 20mM Tris/HCl, pH=8) and centrifuged at 12 000g for 10 min at 4°C.

3.6.3 Purification by FPLC (Fast protein liquid chromatography)

A chelating sepharose column was charged with 50mM $CoSO_4$ with wash buffer composed of (20 mM Na-phosphate + 0.5 M NaCl + 8 M urea with pH of 8.0, and equilibrated with the isolation buffer. Samples were consequently washed by the wash buffer (8M urea, 20mM Tris,

Prepurification was done using 20 mM Tris/HCl buffer of pH 6.0 for sonication. 10mM immidazole, pH=8) and eluted by the elution buffer (8M urea, 20mM Tris, 0,5M immidazole, pH=8). Protein isolation was authenticated by SDS-PAGE.

Due do failure to obtain a desired result (insoluble and impure recombinant SmGCPII) (Figure 13), a second transformation and expression of SmGCPII in *E. coli* was conducted but in high volume. SmGCPII was cultured in 6L LB + ampicillin + glucose medium. Isolation and purification was done as described in (chapter 3.6.4). This approach gave a dissolved but impure recombinant SmGCPII (14A). In a third attempt to purify rSmGCPII, sample was washed several times in 8M urea buffer and then dissolved in 10M urea without FPLC (Figure 14B).

3.6.4 Isolation and Purification of Inclusion Bodies from *E. coli* (6L culture)

The isolation was done by using lysis buffer of composition; 200 ml of 1x PBS, 200mg of lysozyme, 2 tablets of protease inhibitor cocktail, 60 µl of 1x benzyonase and 80 µl of 1mM PMSF inhibitor (phenylmethyl sulfonyl fluoride) for 30 minutes and sonicated on ice for 10 minutes. A number of centrifugations and re-suspensions were done to obtain pure pellet. Re-suspension of pellet in buffer made up of (180 ml of ice-cold 1x PBS + 1.8 ml of 5mM EDTA + 900 µl of 5 mM DTT + 16.4 ml of 1M urea) was done followed by sonication on ice for 3 minutes. Content was again centrifuged at 5000 g for 10 minutes and supernatant discarded. Pellet was further dissolved in guanidine based buffer composed of (30 ml of ice-cold 50mM phosphate, pH 8.0 + 6 M guanidine + 1mM DTT) then sonication. Centrifugation of the mixture at 30000 g, 10 minutes was done successively. The supernatant was then collected for further purification. Purification and elution were done in 6M guanidine, elution with 6M guanidine + 5% acetic acid respectively using Ni-NTA purification system for FPLC.

3.6.5 Gel Running, Staining and Imaging

In the first part of the experiment containing 200ml of culture with 8M urea as wash and elution buffer, 5µl each of our samples from injection (load), flow through, washes and elutions were mixed with 5µl of sample buffer and 10uL of PBS to make up loading samples. 10% SDS-PAGE gel was then loaded with samples and a molecular weight marker (standard) and run at 150v for 90 minutes. Gel was washed and boiled twice for 1 minute each in distilled water. Gel was then stained with Coomasie brilliant blue and then scanned to obtain image using hp digital scanner (Figure 13).

The above procedure was followed for the first part of the second experiment containing 6L of culture (with 6M guanidine as wash and elution buffers). However, images were then taken using LAS 4000 camera (Figure 14A). From the second part of the second experiment where cytoplasmic fractions were washed with 8M urea and dissolved with 10M urea, three different loads from the dissolved samples + loading buffer were loaded on to 10% SDS-Page alongside a molecular weight marker and run at 150v for 90 minutes. Boiling, staining and removal of stain from gel were done followed by imaging with LAS 4000 digital camera (Figure 14B).

3.7 Expression of Recombinant Protein (SmGCPII) in *K. lactis* Cells

3.7.1 Cell Preparation

Already prepared and portioned *K. lactis* cells from IBT laboratory were used. These aliquot of cells were spun at a revolution of 4000 g for 5 minutes at 4^{o}C after which supernatant was discarded and pellet suspended in 0.8ml of ice cold water for another spinning at 4000g for 5 minutes at 4^{o}C. Cells (pellets) were re-suspended second time in 0.4ml of ice cold water and were portioned into 40µl each for the electroporation.

3.7.2 DNA Preparation

Plasmid was obtained through the use of Gateway cloning technology as described above in the (chapter 3.6.1). SacII was used to linearized 5µg of DNA GCPII (20µl of DNA contained 5µg of DNA). A reaction volume of 50µl, containing 20µl of DNA (5g), 5µl of 10x cutmart, 1µl of

SacII restrictase and 24µl of deionized water was incubated for 2 hours at 37^{o}C. Agarose gel electrophoresis was run to to verify restriction digest of plasmid.

3.7.3 Electroporation

10µL of linearized DNA (plasmid) was added to 40µl of cells. The mixture was homogenized and transferred to a pre-chilled cuvette. Cuvette was dried and electroporated at the following parameters (exponential decay, 2500V, 25µF, 200 Ω). 1ml of a mixture of YPD medium with 2M sorbitol was added immediately after electroporation and incubated for 1 hour at 30^{o}C without shaking. Content was transferred into 1.5 ml Eppendorf tube for centrifugation at 7000rpm for 30 seconds at RT. Pellet was collected and suspended in 1 ml of sterile water with another spinning at 7000rpm for 30 seconds at RT. The pellet was re-suspended in 80µl of sterile water for cultivation on a plate with YCB agar containing 5mM acetamide. For negative control, only 80µl of mixture of YPD and sorbitol (without cell) was spread on plate. For the transformed cells, two plates with YCB media were used. One plate had low concentration (10%) of the cells while the other had high concentration (90%). This was followed by incubation at 30°C, bottom up until Colonies appeared after 3 days.

3.7.4 Monocolonies Selection

1) After 3 days of cultivation, with visible colonies appearing(A). A Selection of 10-12 big monocolonies were tranferred onto new YCB acetamide plate for each of the plates with transformed cells using a sterile microbial loop.

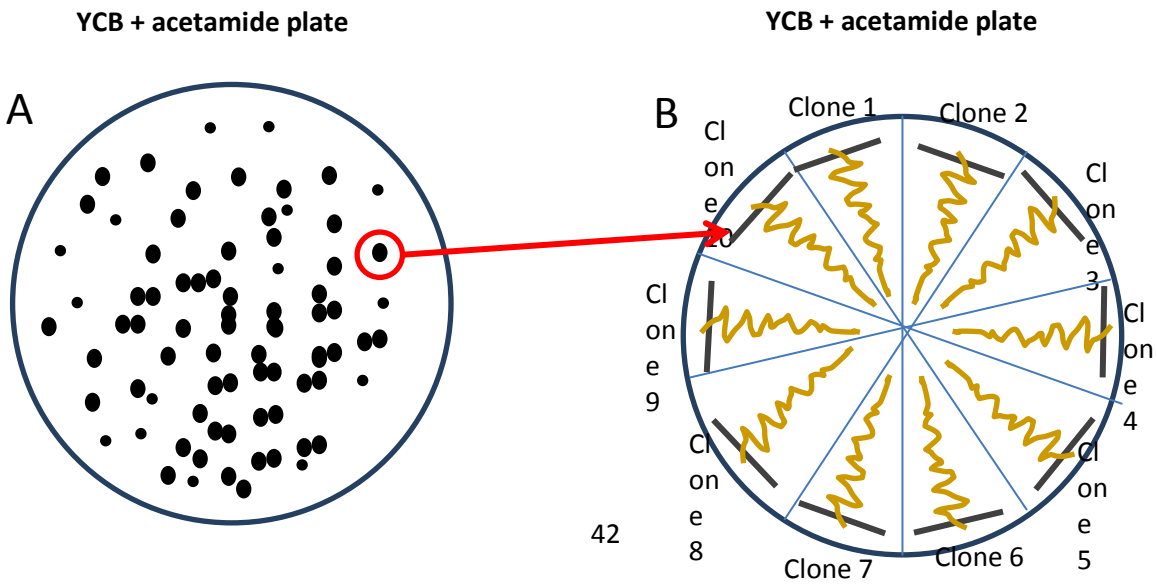

Figure 7. Monoclonal selection of transformed cells from electroporation (Plate A to Plate B)

Cultivation was again ensured for 2-3 days at 30°C in an incubator with bottom up orientation of plates. After day 3, colonies in the central part of plate (B) streaked onto new YCB

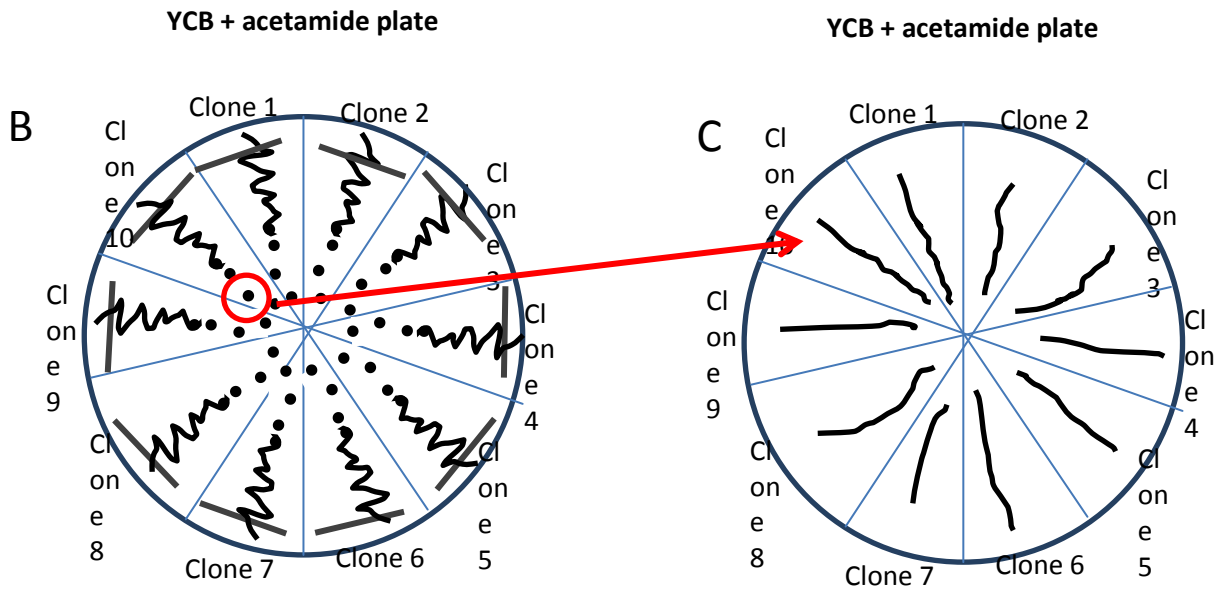

Figure 8. Monoclonal selection of transformed cells from electroporation (Plate B to PlateC)

acetamide plate (C) and incubated for 3 days. Biomass from plate C was used to inoculate YPD for expression test.

3.7.5 Expression Test of *K. lactis* Colonies (Abcam)

Biomass from plate "C" from *K. lactis* electroporation was used to inoculate 400 µL of YPD medium for the subsequent cultivation in 96 wells plate (one well per clone for a total of 16 clones). This was then cultivated at 30 °C, 300 rpm, overnight.

The overnight culture was used to prepare glycerol stock in which a mixture containing 200µL of culture and 200 µl of sterile 50% glycerol was prepared and frozen in – 80 °C freezer for future use. 5 µl of overnight culture was used to inoculate 400 µL of YPG (yeast extract (1 % w/v). 20 g/l peptone (2 % w/v). 20 g/l galactose) and incubated at 30°C, 300 rpm for 3 days. 200 µl of culture from each well was stored at – 80°C. Cells were spun in plate for 20 min at 2250 rpm at 4°C. A mixture containing 960µl yeast buster, 9.6µl 100x THP, 19.2 µl 50x PIC and 2.4 µl benzonaze was prepared for a total of 16 colonies and added to the pelleted yeast and incubated 60 minutes at RT then subsequent spinning at 20000 rpm for 20 minutes at RT and the supernatant was analyzed using western blot analysis protocol (Chapter 3.7.6) .

3.7.6 Loading and Running of Gel

Equal amounts of protein (50µl) were loaded into the wells of the SDS-PAGE gel, along with molecular weight marker. Running of the gel was done for 2 hours at 150V. The Polyvinylidene difluoride (*PVDF*) membrane was activated in methanol for 1 minute and rinse with transfer buffer before preparing the stack.

Figure 9, Example of prepared stack adopted from Abcam website.

(http://www.abcam.com/protocols/general-western-blot-protocol accessed on 01/23/2018)

3.7.7 Antibody Staining and Image Acquisition

Blocking of the membrane was done for 1 hour at RT using blocking buffer with His tag antibodies. The membrane was incubated with His tag antibodies in blocking buffer for an hour at RT. Three times washes of membrane in TBST (Tris-buffered saline, 0.1% Tween 20)

(TBST), 10 minutes each was performed. Blot was probed against anti-MBP antibodies in blocking buffer at RT for 1 hour. Membrane was washed in three washes of TBST, 5 minutes each. Excess reagent was removed and the membrane covered in transparent plastic wrap. Images were acquired image using darkroom development techniques for both chemiluminescence, and normal image scanning methods for colorimetric detection. The expression in *K. lactis* was repeated thrice but with no success.

CHAPTER 4

RESULTS

4.1. RNA *in situ* Hybridization

Transcript (mRNA) coding GCPII in *S. mansoni* adult males and females were localized via RNA *in situ* hybridization followed by Cy5-TSA microscopy detection assay. Antisense probe prepared *in vitro* was hybridized with mRNA coding SmGCPII which is present in the adult worm tissue. The labeled tissues were observed by fluorescence microscopy. Strong signal of mRNA specific for GCPII was localized in esophagus, gastrodermis, oral and ventral sucker, testes, and in some specific cells in parenchyma of the males (Figure. 11). Females showed strong signal in ovaria, oviduct, vitellaria and in some specific parenchyma cells (Figure. 12). No signal was observed in tegument and muscles of both genders and surprisingly, no signal in female gastrodermis comparing to males.

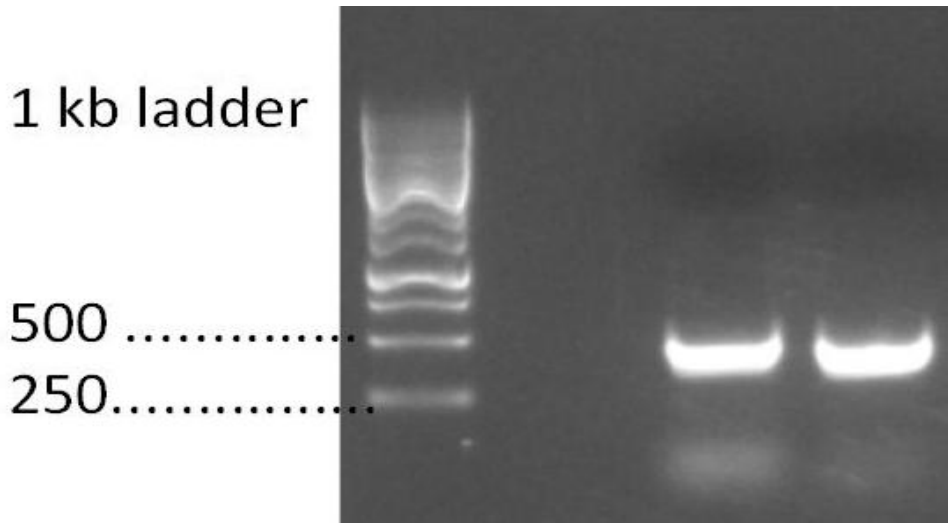

Figure 10. Result of PCR to get probes for GCPII A and GCPII B for *in situ* hybridization. On the first column is a ladder standard and label sizes. The sizes of both fragments of DNA are approximately 500 base pairs.

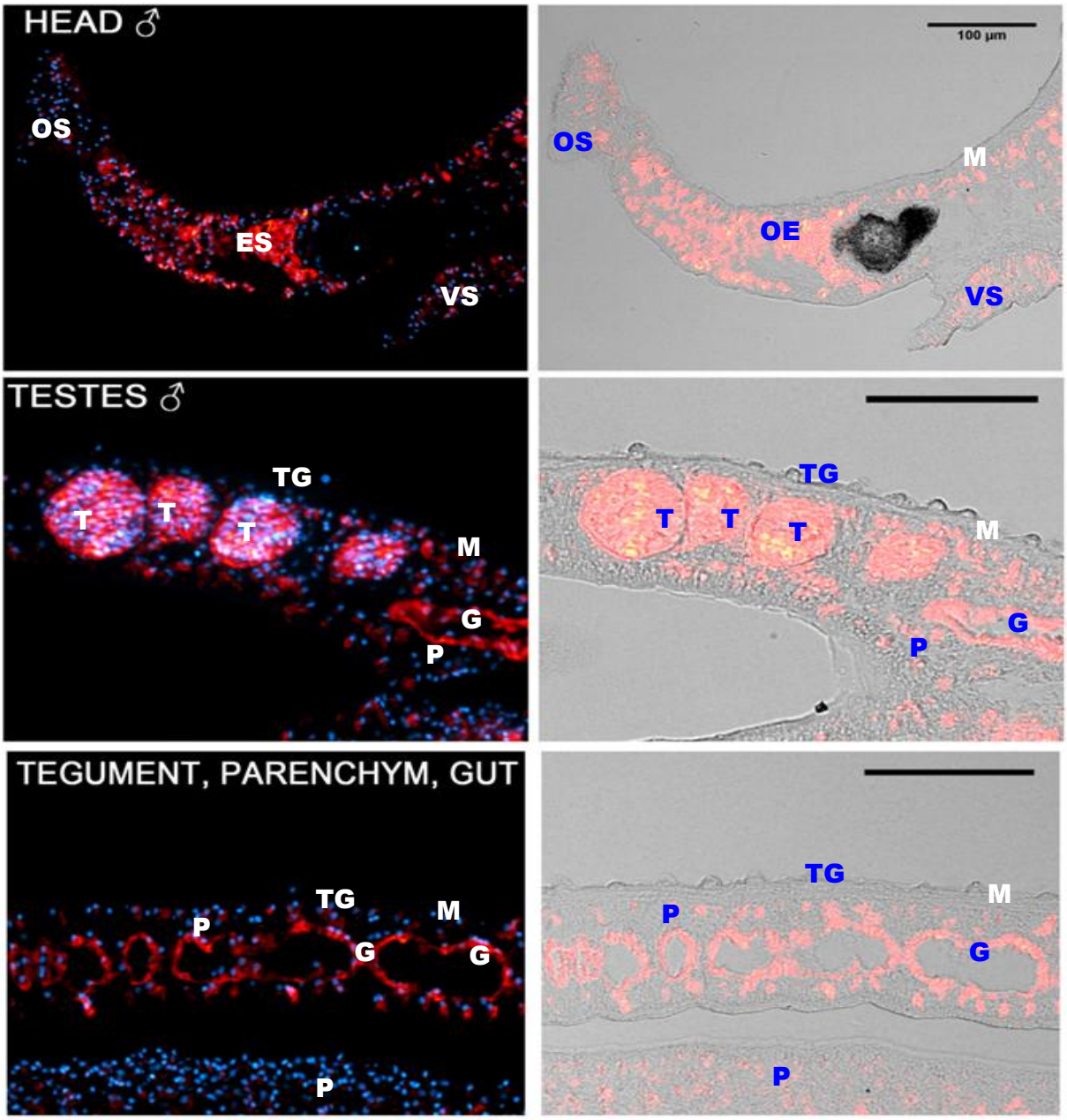

Figure 11. Localization of mRNA coding GCPII in *S. mansoni* adult males. Sections of *S. mansoni* adult males were hybridized with an antisense GCPII probe (red). Strong signal was detected in esophagus (ES), gastrodermis (G), oral sucker (OS), in specialized types of parenchyma cells (P) within the body, testis (T), and in the area of ventral sucker (VS). The muscle tissue (M) and tegument (TG) appear to have no signal at all. DAPI was used to label nuclear DNA (blue). The left columns show merged fluorescent channels; in the right columns is red fluorescent signal merged with differential interference contrast. The scale bar represents 100µm.

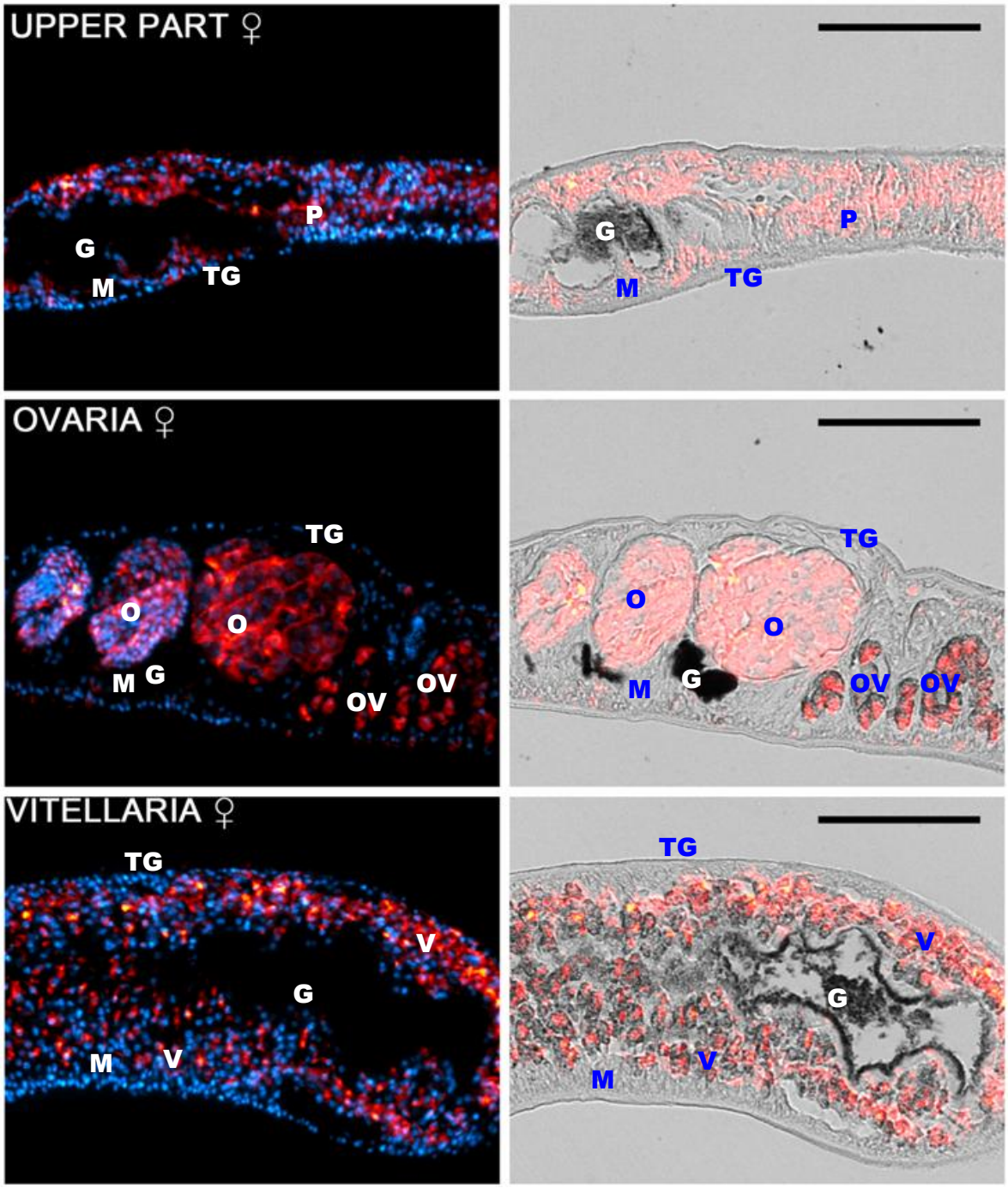

Figure 12. Localization of mRNA coding GCPII in *S. mansoni* adult females. Sections of *S. mansoni* adult females were hybridized with an antisense GCPII probe (red). Fluorescent signal was detected in ovaria (O), oviduct (OV), in specific parenchyma cells (P) and vitellaria (V) while the gastrodermis (G), muscle tissue (M) and tegument (TG) appear to have no signal at all. DAPI was used to label nuclear DNA (blue). The left columns show merged fluorescent channels; in the right columns is red fluorescent signal merged with differential interference contrast. The scale bar represents 100µm.

4.2. Recombinant Expression of SmGCPII in *E. coli*

Three different purification approaches were tested as recombinant protein appeared to be problematic in terms of solubility and consequent purification All the three results show successful expression of recombinant SmGCPII in *E. coli* as analysis of recombinant protein size (molecular weight) corresponds with the theoretical mass of GCPII discussed in literature (84kDa) Bands were excised from SDS-PAGE and submitted for mass spectrometry at Biocev. Samples were trypsinized (broken to small parts by bovine trypsine) and analyzed by MALDI - TOF. Lower big band was sequenced, and the result shows it was bacterial protein. Sequence of Upper band from first results was our SmGCPII ortholog (Figure 13A and B). However, there was issue with solubility with respect to Figure 13. Inclusion bodies isolated from 200mL culture were washed and resuspended in 8M urea buffer and subjected to FPLC and analyzed with 10% SDS-PAGE. Result from protein analysis (Figure 13) shows the presence of recombinant with poor solubility SmGCPII in loads as well as flow through.

In a second experiment, inclusion bodies from 6L culture were after washes solubilized in 6M guanidine buffer and purified with Ni-NTA purification system (Themo Fisher). 10% SDS-PAGE analysis of the purified sample (Figure 14A) showed that recombinant SmGCPII was soluble in 6M guanidine buffer but did not bind to column.

During a repeated purification process, portion of the inclusion bodies from 6L bacteria culture was washed several times in 8M urea buffer and dissolved in 10M urea buffer with no FPLC. Samples from 10M urea buffer were analysed with 10% SDS-PAGE (Figure 14B) and as well presence of the protein was verified by mass-spectrometry. Result shows a relatively clean and soluble recombinant SmGCPII (Figure 14B) and lower molecular bands represent in majority degradation products of SmGCPII.

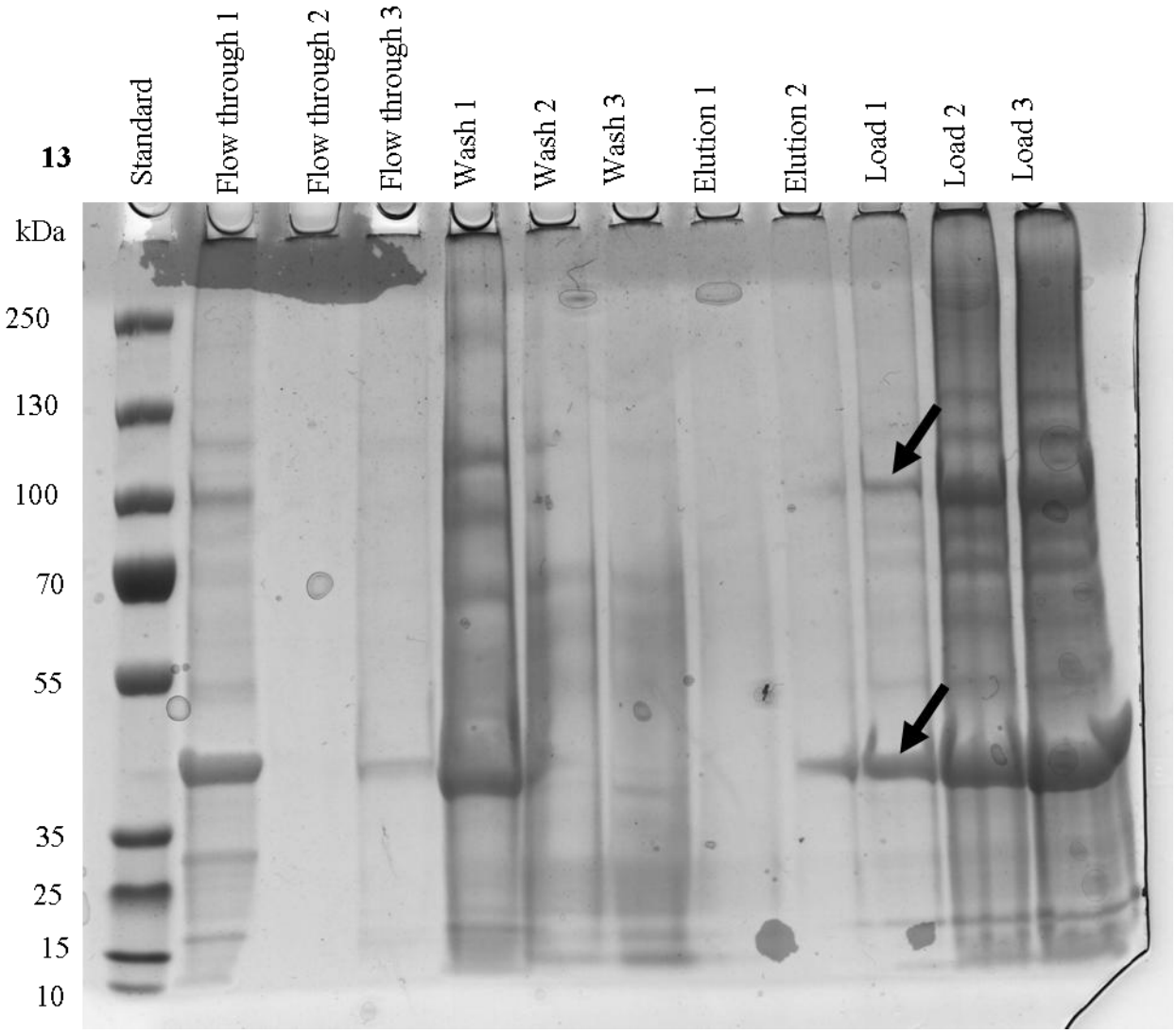

Figure 13. **SDS-PAGE of FPLC recombinant SmGCPII from *E. coli.*** On the vertical left column are sizes of protein bands according to standard used (PageRuler Plus Prestained Protein Ladder). On the top upper horizontal row are labels of samples loaded onto SDS-PAGE. In this, a soluble (with minor solubility problem with inclusion body) but unclean recombinant SmGCPII PROTEIN was found in loads. Wash buffer made from (8M urea + 0.5M NaCl + 20mM sodium phosphate 10mM immidazole) and elution buffer made of (wash buffer + 0.5M immidazole) were used for washes and elution respectively. The arrows point to the two bands which were excised for mass spectrometer analysis (see Figure 13A and 13B)

Concise Summary Repo | Mascot Search Results: XP_(

matrixscience.com/cgi/master_results.pl?file=../data/20171214/FTmAoiSOL.dat

13A

MATRIX SCIENCE Mascot Search Results

```
User           : petr
Email          : ppompach@yahoo.com
Search title   : BarA
Database       : NCBIprot 20171024 (135744157 sequences; 49805139192 residues)
Timestamp      : 14 Dec 2017 at 15:35:10 GMT
Top Score      : 172 for XP_018651911.1, NAALADASE L peptidase (M28 family) [Schistosoma mansoni]
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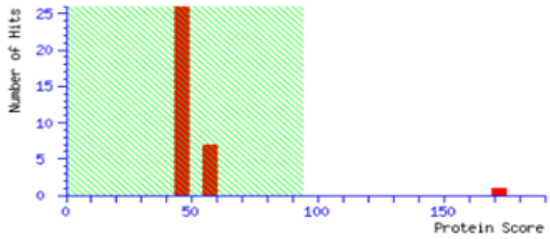
Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 94 are significant (p<0.05).



Concise Protein Summary Report

Format As: Concise Protein Summary | Help

Significance threshold p< 0.05 | Max. number of hits 5

Preferred taxonomy: All entries

Re-Search All | Search Unmatched

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1.    XP_018651911.1    Mass: 84552    Score: 172    Expect: 8.6e-10  Matches: 23
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      XP_012791899.1    Mass: 84904    Score: 71     Expect: 10  Matches: 16
      N-acetylated-alpha-linked acidic dipeptidase 2 [Schistosoma haematobium]
      WP_065444372.1    Mass: 56530    Score: 47     Expect: 2.7e+03  Matches: 6
      NAD(P)/FAD-dependent oxidoreductase [Mycobacterium malmoense]
      WP_065495025.1    Mass: 56502    Score: 47     Expect: 2.7e+03  Matches: 6
      NAD(P)/FAD-dependent oxidoreductase [Mycobacterium malmoense]
      XP_010629033.1    Mass: 24307    Score: 46     Expect: 3e+03  Matches: 4
      PREDICTED: ras-related protein Rab-37 isoform X2 [Fukomys damarensis]
      WP_026738891.1    Mass: 24728    Score: 46     Expect: 3.2e+03  Matches: 4
      arylesterase [Lonsdalea quercina]
      WP_026742808.1    Mass: 24714    Score: 46     Expect: 3.2e+03  Matches: 4
      arylesterase [Lonsdalea quercina]
      EHL19516.1    Mass: 7738    Score: 46    Expect: 3.7e+03  Matches: 3
      hypothetical protein HMPREF9628_00237 [Peptoanaerobacter stomatis]
```

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2.    KRH19272.1    Mass: 7851    Score: 59    Expect: 1.7e+02  Matches: 7
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13B

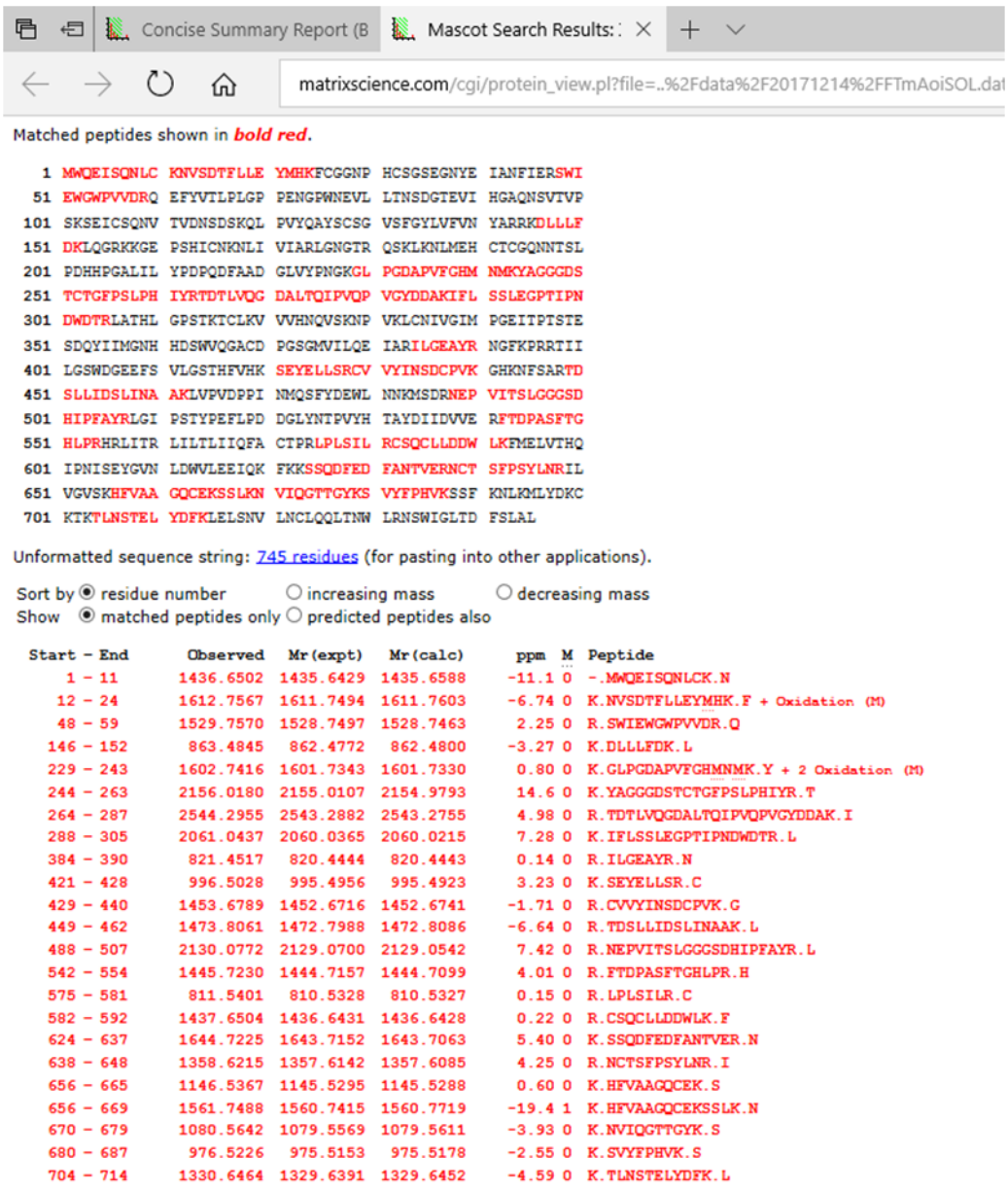

Figure 13A and B show the results of two excised band from protein purification shown by arrows in (Figure 13) in a mass spectrometer analysis and sequencing. Samples (excised bands) were trypsinized and analyzed by MALDI – TOF (Mass spectrometry facility Charles University Prague, Biocev). Sequence of lower band, corresponds to bacterial protein while upper band from (Figure 13) was recombinant SmGCPII.

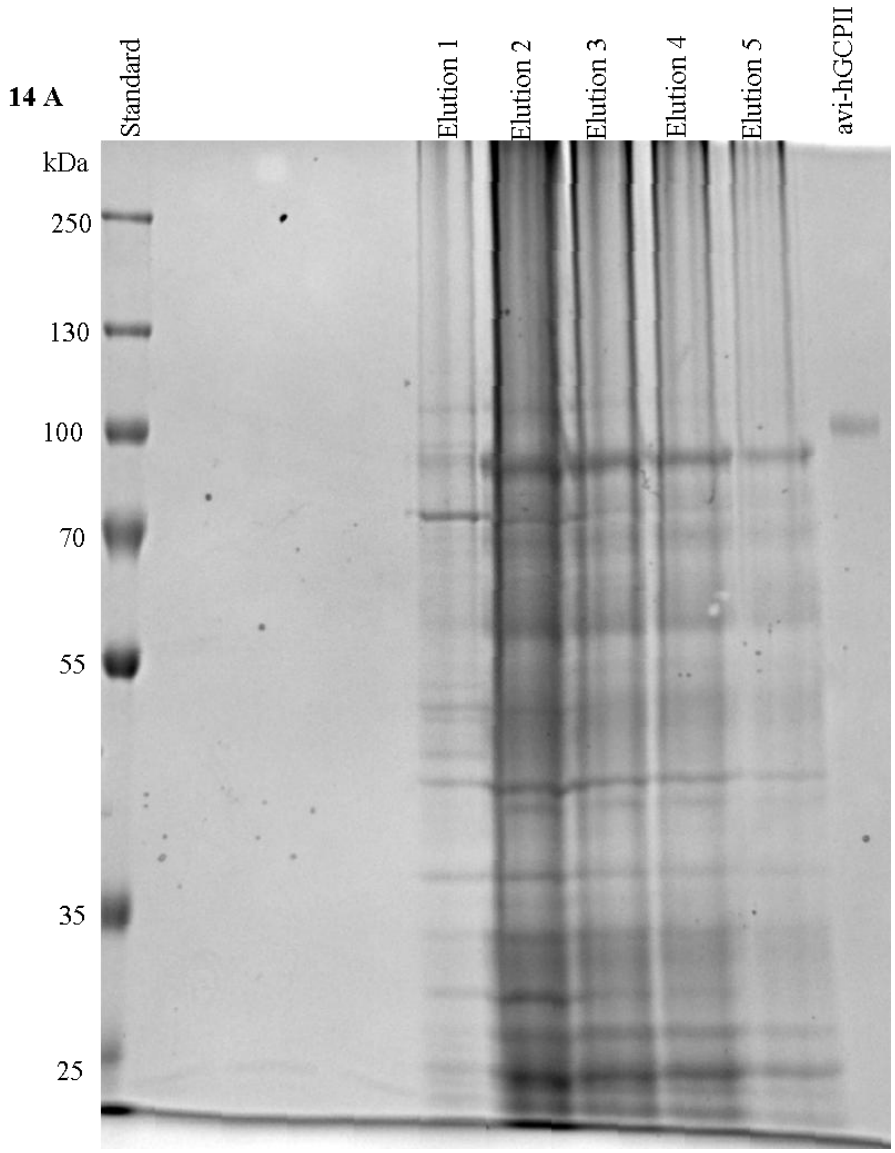

Figure 14A. SDS-PAGE of FPLC solubilized recombinant SmGCPII from *E. coli* by 6M guanidine hydrochloride. Left column and top rows are labels of protein sizes (PageRuler Plus Prestained Protein Ladder) and loaded samples (5 elutions which were precipitated in ethanol) respectively. Dissolution was done in a buffer of (50mM phosphate + 6M guanidine + 1mM DTT) with Ni-NTA purification in 6M guanidine, elution with 6M guanidine + 5% acetic acid and further precipitation in ethanol. Recombinant SmGCPII was found in all fractions. Although soluble, expressed recombinant SmGCPII did not bind strongly to the affinity column. The last line is labeled avi-hGCPII (recombinant extracellular human GCPII) protein used as a control.

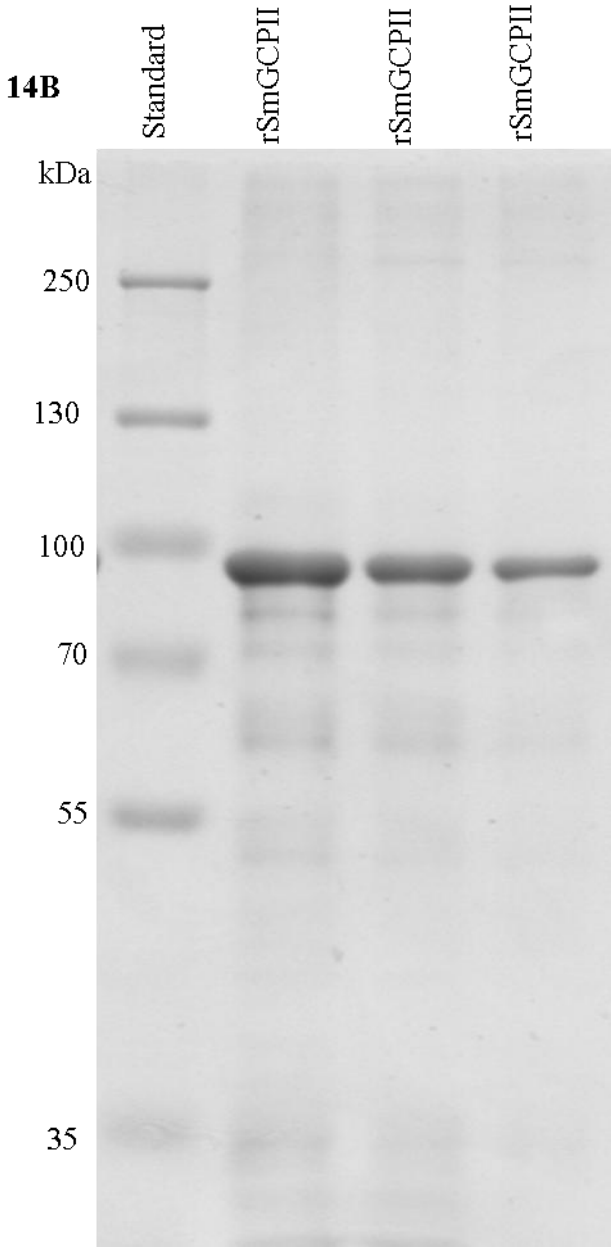

Figure 14B. SDS-PAGE of soluble and relatively pure recombinant SmGCPII from *E. coli.* Left column and top rows are labels of protein sizes (Page Ruler Plus Prestained Protein Ladder) and loaded samples respectively. Sample was washed in 8M urea buffer and dissolved in 10M urea buffer. Soluble recombinant SmGCPII was found in all 3 loaded samples with sizes of the expected protein and conforms to mass spectrometry analysis.

4.3 Recombinant Expression of SmGCPII in *K.lactis*

Cultures from 16 monocolonies from *K. lactis* cells electroporated with SmGCPII were ressolved by western blot analysis (Abcam). Equal amounts of prepared samples were loaded onto SDS-PAGE gel, along with molecular weight marker (PageRuler Plus Prestained Protein Ladder). Content from the SDS-PAGE was blotted unto *PVDF membrane*, blot was probed against anti-MBP antibodies and images were taken with LAS 4000 camera. Unfortunately, the blot analysis shows no success with the expression of recombinant SmGCPII in *K. lactis* (Figure 15) Experiment with the yeast transformation and consequent colony screens was repeated three times.

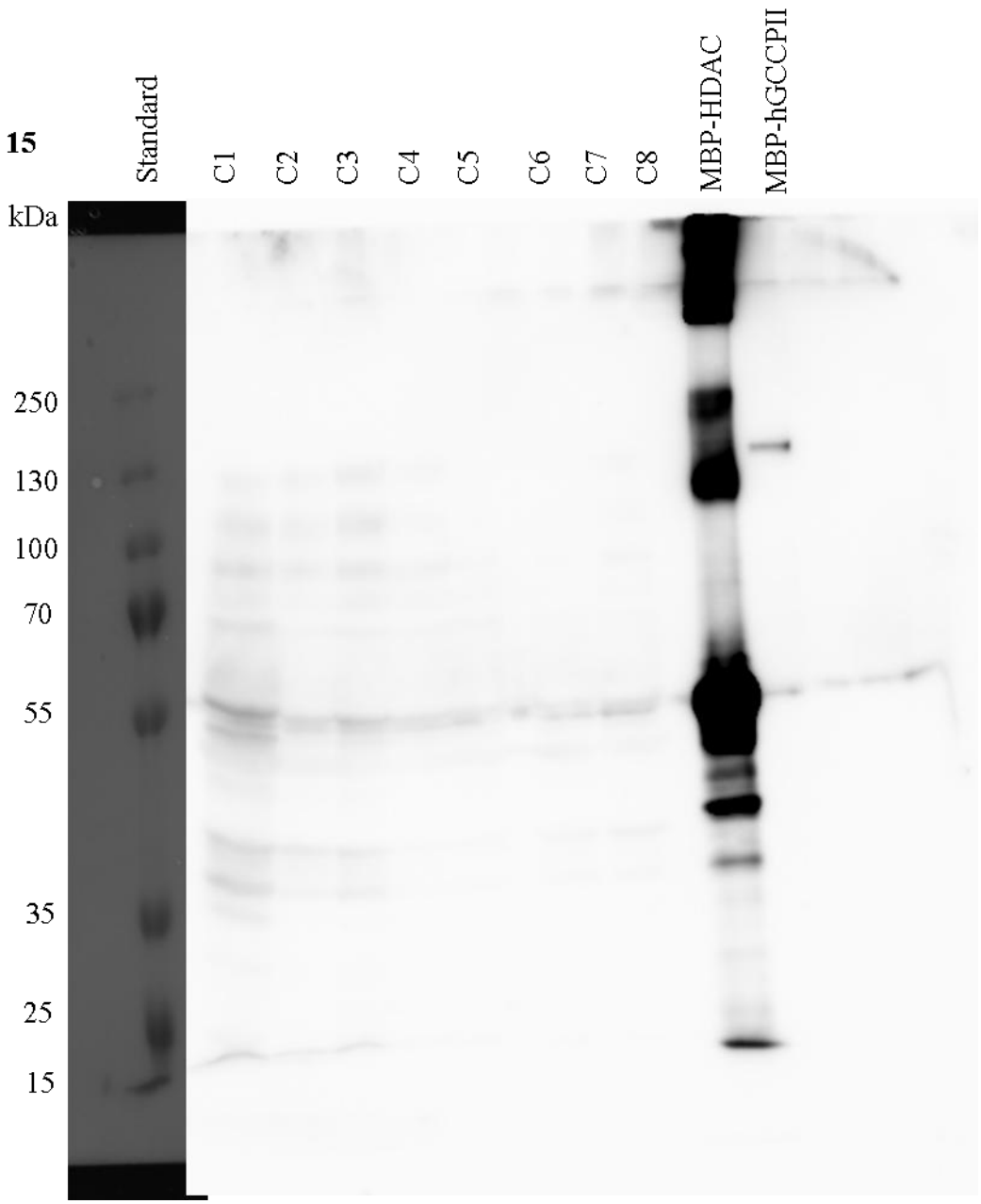

Figure 15. Example of the Western blot screening putative presence of recombinant SmGCPII in *K. lactis* colonies. On the extreme left is found labels of protein sizes in kDa. On the top row is found labels of samples loaded, a standard, positive controls and C1-C8 (colony 1 to colony 8). The image shows no band from C1-C8 that corresponds with the expected recombinant SmGCPII size. There is only background or unspecific bands depicted in the result. Positive control is a human GCPII with MBP tag.

CHAPTER 5

DISCUSSION AND CONCLUSION

This thesis is a part of bigger research project "Orthologs of glutamate carboxypeptidase II in model organisms – search for physiological roles and therapeutic potential of the enigmatic enzyme" which aims at identifying the function of GCPII ancestral protein found in studied organisms (*S. mansoni* and *Caenorhabditis elegans*) and the biological roles and physiological function in the organism. This thesis was geared towards a characterization of GCPII ortholog of *S. mansoni* through localization using mRNA *in situ* hybridization and expression of recombinant protein in *E. coli* and *K. lactis*.

This chapter will discuss the observations from the methodology in chapter 3 as well as detail explanation of the results as summarized in chapter 4.

5.1 RNA *in situ* Hybridization

In situ hybridization is one of the many ways of revealing DNA, RNA or nucleic acid sequence in a portion or section of tissue. It uses a labeled complementary DNA, RNA or modified nucleic acids strand (probe, usually antisense) to localize a specific DNA or RNA sequence in a portion or section of tissue. The antisense probe and mRNA binds and visualizing of probe is in the tissue slice becomes possible (Corthell, 2014). The procedures followed in Part I of Chapter 3 were to achieve a successful *in situ* hybridization.

PCR product was generated and used for the preparation of probes for in situ hybridization. The gel analysis of PCR product shows a size of approximately 500 base pairs for both DNA fragments (GCPII A and GCPII B) (Figure 10). Through mRNA *in situ* hybridization and tissue analysis, mRNA coding GCPII is expressed in certain organs or tissues of both male and female adult *S. mansoni*. Through immunolocalization with a horseradish peroxidase (HRP) antibody, GCPII is distributed in the testis, oral and ventral suckers, esophagus and parenchyma of adult male *S. mansoni* while there was no localization signal in the muscle cells and tegument of the analyzed sections of male worms (Figure 11). In humans, various investigations have revealed that GCPII was expressed in sites including central nervous system, testis, small intestines and salivary glands (Kinoshita *et al*., 2006; Mhawech-Fauceglia *et al*., 2007; Rovenska *et al*., 2008;

Sacha *et al*., 2007) which is an indication of the role of human GCPII in reproduction and protein digestion among others in humans. The presence of mRNA coding GCPII in the testis of the adult male *S. mansoni* may suggest a reproductive function concomitant to that of humans GCPII. Although there is variation between the gastrodermis of the adult *S. mansoni* and the small intestines of humans in terms of morphology, both structures are mainly for digestion of a number of proteins in both organisms, hence the likelihood that the localization of GCPII in the gastrodermis of male *S. mansoni* imposes digestive function of absorbed host blood protein similar to digestion of protein in the small intestine of humans. The presence of GCPII in the esophagus, which may act as a salivary gland in the male *S. mansoni* may have a role that corresponds with the localization of GCPII in the salivary glands of both mouse and humans (Knedlík *et al*., 2017) however its specific funtion is unknown.

In sections of the adult female *S. mansoni*, there were strong signals of mRNA of GCPII in the ovary, oviduct, vitellaria and some cells in the parenchyma (refer to Figure 12). Literature on human GCPIII, the closest othorlog of human GCPII (with about 70% sequence identity) reveals the localization of GCPIII in the ovary, testis, brain etc (Bzdega *et al*., 2004; Pangalos *et al*., 1999) of humans. The localization of GCPII in ovary, oviduct and vetillaria all of which are reproductive structures of the adult female *S. mansoni* is in indicative of the reproduction function of GCPII in the studied organism synonymous to that of GCPIII in humans. The parenchyma is known to perform a number of functions in flatworms. Depending on the type of cell, the prenchyma may perform functions such as transport of materials, structural interactions with other tissues, modifiable tissue for morphogenesis, oxygen storage, and perhaps other functions that are yet to be discovered (Conn, 1993). Depending on which cells of the parenchyma contain GCPII, GCPII may be assisting in any of the above mentioned functions performed by the parenchyma

Again, there were absences of signals in the tegument and muscle cells. The absence of the mRNA for GCPII could mean that GCPII it does not form part of the proteins that constitute the tegument and muscle cells of S. mansoni (van Balkom *et al*., 2005). The absence of GCPII in the tegument could also mean that, GCPPII does not play any significant role in the absorption of nutrients (Bogitsh *et al*., 2013) from the host cells however, these assumptions are inconclusive,

specific antibodies may be required for further localization and biochemical analysis to ascertain the current results.

Beguilingly, GCPII is found in the male gastrodermis but is apparently absent from the female gastrodermis (Figures 11 and 12). This suggests that GCPII may have male-specific proteolytic function at the male-female interface.

Even though desirable observations and results were obtained, however, there are few challenges associated with ISH. A disadvantage of applying *in situ* hybridization techniques is the difficulty in identifying targets that have low DNA and RNA copies. Also the presence of mRNA in a tissue does not necessarily mean the presence of the protein (Qian and Llyod, 2003) hence the need for production of antibodies for further biochemical studies.

5.2 Recombinant Expression of SmGCPII in *E. coli*

Second part of my work was focused on protein expression. Expression of SmGCPII was carried out to obtain recombinant protein for immunization to produce antibodies for further biochemical studies. SmGCPII was successfully expressed in *E. coli*, as SDS-PAGE analysis and sequence of recombinant protein show that the molecular weight and sequence of recombinant SmGCPII correspond to theoretical molecular weight and sequence of GCPII (Figures 13, 13A and B). Recombinant SmGCPII was purified, dissolved and used for immunization in rabbit for generation of sera for further investigations. Refolding process which led to dissolution of recombinant was carried out in three buffers but successful was in the last one which contained 10M urea buffer. High amount of urea salt with increased protein denaturation properties of the buffer and probably caused at dissolution of misfolded recombinant SmGCPII (Figure 14B). The evidence of poor solubility and poor purification is shown by the outcome of SDS-PAGE analysis of the content washed and eluted with 8M urea buffer (Figure 13). The poor solubility and inability of the recombinant SmGCPII to bind to column during FPLC purification process can be attributed to a number of assumed reasons. In the case of poor solubility, it can be said that 8M urea buffer used for the assay was not optimal for this recombinant SmGCPII. For poor purification and inability for recombinant SmGCPII to bind to columns possible reasons could be that, SmGCPII possesses complex cysteine bridges near each other. This fact could have led to a misfolding of recombinant SmGCPII which was not completely affected by presence of urea.

Alternatively, recombinant GCPII did not bind column due to absence of His- taq which might have been cleaved off in *E. coli* during the expression processes. Similar reason can be assigned to why recombinant will not bind column even in the cases where 6M guanidine hydrochloride and 10M urea were used to dissolve recombinant.

Comparatively, high 10M urea concentration is relatively the best among the three buffers for purifying recombinant SmGCPII. Also, 6Mguanidine and 10M urea buffers are better suited for dissolving the recombinant SmGCPII compared to the 8M urea counterpart (refer to Figures 13 14A and 14B).

5.3 Recombinant Expression of SmGCPII in *K. lactis*

Another approach to get recombinant SmGCPII expressed was the use of *K. lactis* as expression medium under low temperature. These conditions result in slow and gradual protein production which should provide enough time for correct protein folding. The expression was carried out under low temperature for few days but SDS-PAGE and activity assay revealed that protein expression was not successful (Figure 15). There are several possible explanations for failure of these expressions.

The first is that produced protein was not stable in *K. lactis*. After folded protein is created, it could be degraded by *K. lactis* due to lack of translation. Secondly, the reason could be that, expressed protein was immediately misfolded and degraded. Third reason could be related to age of cells. According to information I gathered from colleagues from researchgate (https://www.researchgate.net/), a very common problem in recombinant protein expression is that, the expression efficiency of host cells diminished or even become null in older stocks and since the *K. lactis* cells used were from stock that had been prepared for a long time, it might have contributed towards unsuccessful expression.

5.4 Conclusion

The aim of this thesis was to biologically characterize GCPII ortholog of *S. mansoni*. This characterization was performed through the use of RNA *in situ* and initial attempt was done to express t SmGCPII in *E.coli* and *K. lactis* expression systems in order to produce antibodies. By PCR, probes for preparation of RNA in situ hybridization were generated. The RNA *in situ*

hybridization revealed the major site of GCPII mRNA location in adult male and female *S. mansoni* such as gastrodermis, esophagus, oral and ventral suckers, testis and some parenchyma cells of unknown function in the male and ovaries, oviduct, vitellaria and some parenchyma cells in the female. Based on the localization sites, suggested functions of GCPII in the model organism were compared to the functions of GCPII in humans and mouse. However valid these comparisons are, there is still the need for further biochemical characterization and precise localization studies to confirm the certainty of the discussed functions in this thesis.

The expression of recombinant protein was successful in *E.coli* but not in *K. lactis*. Expressed recombinant SmGCPII was trypsinized and analzyed by MALDI – TOF mass spectrometry. Result of the analysis confirms successful expression of SmGCPII in *E.coli*. The successful expression of rSmGCPII is actually a step towards antibodies production.The expressed recombinant SmGCPII was used recemtly as an antigen for immunization in rabbit.

The objective of this thesis is a meager part of a bigger research with the aim of identifying and comparing the functions of GCPII orthologs in two model organisms, *S. mansoni* and *C. elegans.*

Several approaches such immunohistochemistry, RNA interference in *S. mansoni* and *C. elegans* gene knockout mutants, functional biochemical characterization (substrate specificity profiling), endopeptidase profiling, exopeptidase profiling and The Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) will be adopted to arrive at a desired conclusion. Structural studies involving molecular modeling and X-ray crystallography will also be a major component of future studies. (Dvorak *et al*. 2016; Barinka *et al*., 2002; Tykvart *et al*., 2015; O'Donoghue *et al*., 2012).

REFERENCES

Ahmed, H.S, (2017). Schistosomiasis (Bilharzia). Accessed online at: *https://emedicine.medscape.com/article/228392-overview*

Badmos, K. B., Komolafe, A. O., & Rotimi, O. (2006). Schistosomiasis presenting as acute appendicitis. *East African Medical Journal*, *83*(10), 528–532.

Baig, S., Damian, R. T., & Peterson, D. S. (2002). A novel cathepsin B active site motif is shared by helminth bloodfeeders. *Experimental Parasitology*, *101*(2–3), 83–89. https://doi.org/10.1016/S0014-4894(02)00105-4

Barinka, C., Byun, Y., Dusich, C. L., Banerjee, S. R., Chen, Y., Castanares, M., … Lubkowski, J. (2008). Interactions between human glutamate carboxypeptidase II and urea-based inhibitors: Structural characterization. *Journal of Medicinal Chemistry*, *51*(24), 7737–7743. https://doi.org/10.1021/jm800765e

Barinka, C., Hlouchova, K., Rovenska, M., Majer, P., Dauter, M., Hin, N., … Lubkowski, J. (2008). Structural Basis of Interactions between Human Glutamate Carboxypeptidase II and Its Substrate Analogs. *Journal of Molecular Biology*, *376*(5), 1438–1450. https://doi.org/10.1016/j.jmb.2007.12.066

Barinka, C., Rinnová, M., Šácha, P., Rojas, C., Majer, P., Slusher, B. S., & Konvalinka, J. (2002). Substrate specificity, inhibition and enzymological analysis of recombinant human glutamate carboxypeptidase II. *Journal of Neurochemistry*, *80*(3), 477–487. https://doi.org/10.1046/j.0022-3042.2001.00715.x

Bařinka, C., Rovenská, M., Mlčochová, P., Hlouchová, K., Plechanovová, A., Majer, P., … Lubkowski, J. (2007). Structural insight into the pharmacophore pocket of human glutamate carboxypeptidase II. *Journal of Medicinal Chemistry*, *50*(14), 3267–3273. https://doi.org/10.1021/jm070133w

Barinka, C., Sácha, P., Sklenár, J., Man, P., Bezouska, K., Slusher, B. S., & Konvalinka, J. (2004). Identification of the N-glycosylation sites on glutamate carboxypeptidase II necessary for proteolytic activity. *Protein Science : A Publication of the Protein Society*, *13*(6), 1627–1635. https://doi.org/10.1110/ps.04622104

Barinka, C., Starkova, J., Konvalinka, J., & Lubkowski, J. (2007). A high-resolution structure of ligand-free human glutamate carboxypeptidase II. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*, *63*(3), 150–153. https://doi.org/10.1107/S174430910700379X

Barrett, A J. (2001). Proteases. *Current Protocols in Protein Science / Editorial Board, John E. Coligan ... [et Al.]*, *Chapter 21*(1989), Unit 21.1. https://doi.org/10.1002/0471140864.ps2101s21

Basch, P. F. (1981). Cultivation of Schistosoma mansoni In vitro. I. Establishment of Cultures from Cercariae and Development until Pairing. *Source: The Journal of Parasitology*, *67*(2), 179–185. https://doi.org/10.2307/3280632

Bogitsh, B. B., Carter, C. E., & Oeltmann, T. T. (2013). *Human Parasitology*. *Human Parasitology*. https://doi.org/10.1016/C2010-0-65681-1

Boissier, J., Grech-Angelini, S., Webster, B. L., Allienne, J. F., Huyse, T., Mas-Coma, S., … Mitta, G. (2016). Outbreak of urogenital schistosomiasis in Corsica (France): an epidemiological case study. *The Lancet Infectious Diseases*, *16*(8), 971–979. https://doi.org/10.1016/S1473-3099(16)00175-4

Bos, D. H., Mayfield, C., & Minchella, D. J. (2009). Analysis of regulatory protease sequences identified through bioinformatic data mining of the Schistosoma mansoni genome. *BMC Genomics*, *10*, 488. https://doi.org/10.1186/1471-2164-10-488

Burke, M. L., Jones, M. K., Gobert, G. N., Li, Y. S., Ellis, M. K., & McManus, D. P. (2009). Immunopathogenesis of human schistosomiasis. *Parasite Immunology*. https://doi.org/10.1111/j.1365-3024.2009.01098.x

Bzdega, T., Crowe, S. L., Ramadan, E. R., Sciarretta, K. H., Olszewski, R. T., Ojeifo, O. A., … Neale, J. H. (2004). The cloning and characterization of a second brain enzyme with NAAG peptidase activity. *Journal of Neurochemistry*, *89*(3), 627–635. https://doi.org/10.1111/j.1471-4159.2004.02361.x

Bzdega, T., Turi, T., Wroblewska, B., She, D., Chung, H. S., Kim, H., & Neale, J. H. (2002). Molecular Cloning of a Peptidase Against N-Acetylaspartylglutamate from a Rat Hippocampal cDNA Library. *Journal of Neurochemistry*, *69*(6), 2270–2277. https://doi.org/10.1046/j.1471-4159.1997.69062270.x

Caffrey, C. R., McKerrow, J. H., Salter, J. P., & Sajid, M. (2004). Blood "n" guts: An update on schistosome digestive peptidases. *Trends in Parasitology*. https://doi.org/10.1016/j.pt.2004.03.004

Cao, K. Y., Mao, X. P., Wang, D. H., Xu, L., Yuan, G. Q., Dai, S. Q., … Qiu, S. P. (2007). High expression of PSM-E correlated with tumor grade in prostate cancer: A new alternatively spliced variant of prostate-specific membrane antigen. *Prostate*, *67*(16), 1791–1800. https://doi.org/10.1002/pros.20664

Carter, R. E., Feldman, A. R., & Coyle, J. T. (1996). Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase.

Proceedings of the National Academy of Sciences, *93*(2), 749–753. https://doi.org/10.1073/pnas.93.2.749

Cesari, I. M., Ballen, D. E., Perrone, T., Oriol, O., Hoebeke, J., & Bout, D. (2000). Enzyme activities in *Schistosoma mansoni* soluble egg antigen. *The Journal of Parasitology*, *86*(5), 1137–1140. https://doi.org/10.1645/0022-3395(2000)086[1137:EAISMS]2.0.CO;2

Cesari, I. M., Valdivieso, E., & Schrével, J. (1998). Biochemical characterization of cathepsin D from adult Schistosoma mansoni worms. *Memórias Do Instituto Oswaldo Cruz*, *93 Suppl 1*, 165–168. https://doi.org/10.1590/S0074-02761998000700024

Chang, S. S., O'Keefe, D. S., Bacich, D. J., Reuter, V. E., Heston, W. D. W., & Gaudin, P. B. (1999). Prostate-specific membrane antigen is produced in tumor-associated neovasculature. *Clinical Cancer Research*, *5*(10), 2674–2681. https://doi.org/10537328

Chitsulo, L., Engels, D., Montresor, A., & Savioli, L. (2000). The global status of schistosomiasis and its control. *Acta Tropica*, *77*(1), 41–51. https://doi.org/10.1016/S0001-706X(00)00122-4

Chitsulo, L., Loverde, P., & Engels, D. (2004). Schistosomiasis. *Nature Reviews Microbiology*. https://doi.org/10.1038/nrmicro801

Conn B.D., (1993): The Biology of Flatworms (Platyhelminthes): Parenchyma cells and extracellular matrices. *Transactions of the American Microscopical Society*, 112 (4). 241-261.

Corachan, M. (2002). Schistosomiasis and international travel. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *35*, 446–450. https://doi.org/10.1086/341895

Corthell, J. T. (2014). *Basic Molecular Protocols in Neuroscience: Tips, Tricks, and Pitfalls*. *Basic Molecular Protocols in Neuroscience: Tips, Tricks, and Pitfalls*. https://doi.org/10.1016/C2014-0-00083-4

Cunha, A. C., Weigle, B., Kiessling, A., Bachmann, M., & Rieber, E. P. (2006). Tissue-specificity of prostate specific antigens: Comparative analysis of transcript levels in prostate and non-prostatic tissues. *Cancer Letters*, *236*(2), 229–238. https://doi.org/10.1016/j.canlet.2005.05.021

Davis, M. I., Bennett, M. J., Thomas, L. M., & Bjorkman, P. J. (2005). Crystal structure of prostate-specific membrane antigen, a tumor marker and peptidase. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(17), 5981–5986. https://doi.org/10.1073/pnas.0502101102

Delcroix, M., Medzihradsky, K., Caffrey, C. R., Fetter, R. D., & McKerrow, J. H. (2007). Proteomic analysis of adult *S. mansoni* gut contents. *Molecular and Biochemical Parasitology*, *154*(1), 95–97. https://doi.org/10.1016/j.molbiopara.2007.03.008

Delcroix, M., Sajid, M., Caffrey, C. R., Lim, K. C., Dvořák, J., Hsieh, I., … McKerrow, J. H. (2006). A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. *Journal of Biological Chemistry*, *281*(51), 39316–39329. https://doi.org/10.1074/jbc.M607128200

Dvorak, J., & Horn, M. (2018). Serine proteases in schistosomes and other trematodes. *International Journal for Parasitology*. https://doi.org/10.1016/j.ijpara.2018.01.001

Dvořák, J., Delcroix, M., Rossi, A., Vopálenský, V., Pospíšek, M., Šedinová, M., … Caffrey, C. R. (2005). Multiple cathepsin B isoforms in schistosomula of Trichobilharzia regenti: Identification, characterisation and putative role in migration and nutrition. *International Journal for Parasitology*, *35*(8), 895–910. https://doi.org/10.1016/j.ijpara.2005.02.018

Dvořák, J., Fajtová, P., Ulrychová, L., Leontovyč, A., Rojo-Arreola, L., Suzuki, B. M., … O'Donoghue, A. J. (2016). Excretion/secretion products from Schistosoma mansoni adults, eggs and schistosomula have unique peptidase specificity profiles. *Biochimie*, *122*, 99–109. https://doi.org/10.1016/j.biochi.2015.09.025

Dvořák, J., Mashiyama, S. T., Braschi, S., Sajid, M., Knudsen, G. M., Hansell, E., … McKerrow, J. H. (2008). Differential use of protease families for invasion by schistosome cercariae. *Biochimie*, *90*(2), 345–358. https://doi.org/10.1016/j.biochi.2007.08.013

Friedman, J. F., Mital, P., Kanzaria, H. K., Olds, G. R., & Kurtis, J. D. (2007). Schistosomiasis and pregnancy. *Trends in Parasitology*. https://doi.org/10.1016/j.pt.2007.02.006

Gala, J. L., Loric, S., Guiot, Y., Denmeade, S. R., Gady, a, Brasseur, F., … Tombal, B. (2000). Expression of prostate-specific membrane antigen in transitional cell carcinoma of the bladder: prognostic value? *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *6*(October), 4049–4054.

Ghosh, A., & Heston, W. D. W. (2003). Effect of carbohydrate moieties on the folate hydrolysis activity of the prostate specific membrane antigen. *Prostate*, *57*(2), 140–151. https://doi.org/10.1002/pros.10289

Grauer, L. S., Lawler, K. D., Marignac, J. L., Kumar, A., Goel, A. S., & Wolfert, R. L. (1998). Identification, purification, and subcellular localization of prostate-specific membrane antigen PSM' protein in the LNCaP prostatic carcinoma cell line. *Cancer Research*, *58*(21), 4787–4789. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9809977

Gryseels, B., Polman, K., Clerinx, J., & Kestens, L. (2006). Human schistosomiasis. *Lancet*. https://doi.org/10.1016/S0140-6736(06)69440-3

Haffner, M. C., Laimer, J., Chaux, A., Schäfer, G., Obrist, P., Brunner, A., … Bander, N. H. (2012). High expression of prostate-specific membrane antigen in the tumor-associated neovasculature is associated with worse prognosis in squamous cell carcinoma of the oral cavity. *Modern Pathology*, *25*(8). https://doi.org/10.1038/modpathol.2012.66

Hagan, P., & Gryseels, B. (1994). Schistosomiasis research and the European Community. *Tropical and Geographical Medicine*, *46*(4 Spec No), 259–268.

Halsted, C. H. (1991). Jejunal brush-border folate hydrolase. A novel enzyme. *The Western Journal of Medicine*, *155*, 605–609.

Halsted, C. H., Ling, E. H., Luthi-Carter, R., Villanueva, J. A., Gardner, J. M., & Coyle, J. T. (1998). Folylpoly-γ-glutamate carboxypeptidase from pig jejunum: Molecular characterization and relation to glutamate carboxypeptidase II. *Journal of Biological Chemistry*, *273*(32), 20417–20424. https://doi.org/10.1074/jbc.273.32.20417

Horák, P., Mikeš, L., Rudolfová, J., & Kolářová, L. (2008). Penetration of Trichobilharzia cercariae into mammals: dangerous or negligible event? *Parasite (Paris, France)*, *15*(3), 299–303. https://doi.org/10.1051/parasite/2008153299

Horn, M., Fajtová, P., Rojo Arreola, L., Ulrychová, L., Bartošová-Sojková, P., Franta, Z., … Dvořák, J. (2014). Trypsin- and Chymotrypsin-Like Serine Proteases in Schistosoma mansoni - "The Undiscovered Country." *PLoS Neglected Tropical Diseases*, *8*(3). https://doi.org/10.1371/journal.pntd.0002766

Horoszewicz, J. S., Kawinski, E., & Murphy, G. P. (1987). Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. *Anticancer Research*, *7*(5 B), 927–935. https://doi.org/10.1016/0734-9750(93)90273-P

Houston, S., Kowalewska-Grochowska, K., Naik, S., McKean, J., Johnson, E. S., & Warren, K. (2004). First report of Schistosoma mekongi infection with brain involvement. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *38*(1), e1–e6. https://doi.org/10.1086/379826

Howe, K. L., Bolt, B. J., Cain, S., Chan, J., Chen, W. J., Davis, P., … Sternberg, P. W. (2016). WormBase 2016: Expanding to enable helminth genomic research. *Nucleic Acids Research*, *44*(D1), D774–D780. https://doi.org/10.1093/nar/gkv1217

http://www.who.int/tdr. Accessed: 02/5/2018.

Huang, X., Bennett, M., & Thorpe, P. E. (2004). Anti-tumor effects and lack of side effects in mice of an immunotoxin directed against human and mouse prostate-specific membrane antigen. *Prostate*, *61*(1), 1–11. https://doi.org/10.1002/pros.20074

Ingram, J. R., Rafi, S. B., Eroy-Reveles, A. A., Ray, M., Lambeth, L., Hsieh, I., … McKerrow, J. H. (2012). Investigation of the proteolytic functions of an expanded cercarial elastase

gene family in Schistosoma mansoni. *PLoS Neglected Tropical Diseases*, *6*(4). https://doi.org/10.1371/journal.pntd.0001589

Israeli, R. S., Powell, C. T., Corr, J. G., Fair, W. R., & Heston, W. D. (1994). Expression of the prostate-specific membrane antigen. *Cancer Research*, *54*(7), 1807–1811. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7511053

Kabatereine, N. B., Brooker, S., Tukahebwa, E. M., Kazibwe, F., & Onapa, A. W. (2004). Epidemiology and geography of Schistosoma mansoni in Uganda: implications for planning control. *Tropical Medicine and International Health*, *9*(3), 372–380. https://doi.org/10.1046/j.1365-3156.2003.01176.x

Kallestrup, P., Zinyama, R., Gomo, E., Butterworth, A. E., van Dam, G. J., Gerstoft, J., … Ullum, H. (2006). Schistosomiasis and HIV in rural Zimbabwe: efficacy of treatment of schistosomiasis in individuals with HIV coinfection. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *42*(12), 1781–1789. https://doi.org/10.1086/504380

Kašný, M., Mikeš, L., Dolečková, K., Hampl, V., Dvořák, J., Novotný, M., & Horák, P. (2011). Cathepsins B1 and B2 of trichobilharzia SPP., bird schistosomes causing cercarial dermatitis. *Advances in Experimental Medicine and Biology*, *712*, 136–154. https://doi.org/10.1007/978-1-4419-8414-2_9

Kašný, M., Mikeš, L., Hampl, V., Dvořák, J., Caffrey, C. R., Dalton, J. P., & Horák, P. (2009). Chapter 4 Peptidases of Trematodes. *Advances in Parasitology*. https://doi.org/10.1016/S0065-308X(09)69004-7

King, C. H., Keating, C. E., Muruka, J. F., Ouma, J. H., Houser, H., Arap Siongok, T. K., & Mahmoud, A. A. F. (1988). Urinary tract morbidity in schistosomiasis haematobia: Associations with age and intensity of infection in an endemic area of Coast Province, Kenya. *American Journal of Tropical Medicine and Hygiene*, *39*(4), 361–368. https://doi.org/10.4269/ajtmh.1988.39.361

Kinoshita, Y., Kuratsukuri, K., Landas, S., Imaida, K., Rovito, P. M., Wang, C. Y., & Haas, G. P. (2006). Expression of prostate-specific membrane antigen in normal and malignant human tissues. *World Journal of Surgery*, *30*(4), 628–636. https://doi.org/10.1007/s00268-005-0544-5

Knedlík, T., Vorlová, B., Navrátil, V., Tykvart, J., Sedlák, F., Vaculín, Š., … Konvalinka, J. (2017). Mouse glutamate carboxypeptidase II (GCPII) has a similar enzyme activity and inhibition profile but a different tissue distribution to human GCPII. *FEBS Open Bio*, *7*(9), 1362–1378. https://doi.org/10.1002/2211-5463.12276

Lambert, L. A., & Mitchell, S. L. (2007). Molecular evolution of the transferrin receptor/glutamate carboxypeptidase II family. *Journal of Molecular Evolution*, *64*(1), 113–128. https://doi.org/10.1007/s00239-006-0137-4

Lapa, M., Dias, B., Jardim, C., Fernandes, C. J. C., Dourado, P. M. M., Figueiredo, M., … Souza, R. (2009). Cardiopulmonary manifestations of hepatosplenic schistosomiasis. *Circulation*, *119*(11), 1518–1523. https://doi.org/10.1161/CIRCULATIONAHA.108.803221

Lapidus, R. G., Tiffany, C. W., Isaacs, J. T., & Slusher, B. S. (2000). Prostate-specific membrane antigen (PSMA) enzyme activity is elevated in prostate cancer cells. *The Prostate*, *45*(4), 350–354. https://doi.org/10.1002/1097-0045(20001201)45:4<350::AID-PROS10>3.0.CO;2-U [pii]

Leder K., Weller P. (2009). Epidemiology; pathogenesis; and clinical features of schistosomiasis. *UpToDate*. 1-9. Accessed online at www.uptodate.com/ [Full Text].

Liu, H., Rajasekaran, A. K., Moy, P., Xia, Y., Kim, S., Navarro, V., … Bander, N. H. (1998). Constitutive and antibody-induced internalization of prostate-specific membrane antigen. *Cancer Research*, *58*(18), 4055–4060.

Lopes, A. D., Davis, W. L., Rosenstraus, M. J., Uveges, A. J., & Gilman, S. C. (1990). Immunohistochemical and Pharmacokinetic Characterization of the Site-specific Immunoconjugate CYT-356 Derived from Antiprostate Monoclonal Antibody 7E11-C5. *Cancer Research*, *50*(19), 6423–6429.

Luthi-Carter, R., Barczak, A. K., Speno, H., & Coyle, J. T. (1998). Hydrolysis of the neuropeptide N-acetylaspartylglutamate (NAAG) by cloned human glutamate carboxypeptidase II. *Brain Research*, *795*(1–2), 341–348. https://doi.org/10.1016/S0006-8993(98)00244-3

McKerrow, J. H., Caffrey, C., Kelly, B., Loke, P., & Sajid, M. (2006). PROTEASES IN PARASITIC DISEASES. *Annual Review of Pathology: Mechanisms of Disease*, *1*(1), 497–536. https://doi.org/10.1146/annurev.pathol.1.110304.100151

McManus, D. P., Gray, D. J., Li, Y., Feng, Z., Williams, G. M., Stewart, D., … Ross, A. G. (2010). Schistosomiasis in the People's Republic of China: The era of the three Gorges Dam. *Clinical Microbiology Reviews*. https://doi.org/10.1128/CMR.00044-09

Mesters, J. R., Barinka, C., Li, W., Tsukamoto, T., Majer, P., Slusher, B. S., … Hilgenfeld, R. (2006). Structure of glutamate carboxypeptidase II, a drug target in neuronal damage and prostate cancer. *EMBO Journal*, *25*(6), 1375–1384. https://doi.org/10.1038/sj.emboj.7600969

Mhawech-Faucegli, P., Zhang, S., Terraccian, L., Sauter, G., Chadhuri, A.; Herrmann, F.R., Penetrante, R. (2007). Prostate-specific membrane antigen (PSMA) protein expression in normal and neoplastic tissues and its sensitivity and specificity in prostate adenocarcinoma: an immunohistochemical study using mutiple tumour tissue microarray technique. *Histopathology*, *50*, 472-483. https://www.ncbi.nlm.nih.gov/pubmed/17448023

Mitchell, R.S., Kumar, V., Abbas, A.K., Fausto, N. (2007). Robbins Basic Pathology. *Philadelphia: Saunders*. (8) 122.

Mohammed, A. Z., Edino, S. T., & Samaila, A. a. (2007). Surgical pathology of schistosomiasis. *Journal of the National Medical Association*, *99*(5), 570–574.

Montresor, A., Crompton, D. W. T., Gyorkos, T. W. and Savioli, L. (2002**)**. Helminth control in school age children: a guide for managers of control programmes. World Health Organization Document, World Health Organisation.

Neale, J. H., Bzdega, T., & Wroblewska, B. (2000). N-acetylaspartylglutamate: The most abundant peptide neurotransmitter in the mammalian central nervous system. *Journal of Neurochemistry*. https://doi.org/10.1046/j.1471-4159.2000.0750443.x

Nour, N. M. (2010). Schistosomiasis: health effects on women. *Reviews in Obstetrics & Gynecology*, *3*(1), 28–32. https://doi.org/10.3909/riog0109

O'Donoghue, A. J., Alegra Eroy-Reveles, A. A., Knudsen, G. M., Ingram, J., Zhou, M., Statnekov, J. B., … Craik, C. S. (2012). Global identification of peptidase specificity by multiplex substrate profiling. *Nature Methods*, *9*(11), 1095–1100. https://doi.org/10.1038/nmeth.2182

O'Keefe, D. S., Bacich, D. J., & Heston, W. D. W. (2004). Comparative Analysis of Prostate-Specific Membrane Antigen (PSMA) Versus a Prostate-Specific Membrane Antigen-Like Gene. *Prostate*, *58*(2), 200–210. https://doi.org/10.1002/pros.10319

Oda, K. (2012). New families of carboxyl peptidases: Serine-carboxyl peptidases and glutamic peptidases. *Journal of Biochemistry*. https://doi.org/10.1093/jb/mvr129

Pangalos, M. N., Neefs, J. M., Somers, M., Verhasselt, P., Bekkers, M., Van Der Helm, L., … Gordon, R. D. (1999). Isolation and expression of novel human glutamate carboxypeptidases with N-acetylated α-linked acidic dipeptidase and dipeptidyl peptidase IV activity. *Journal of Biological Chemistry*, *274*(13), 8470–8483. https://doi.org/10.1074/jbc.274.13.8470

Pavlicek, J., Ptacek, J., Cerny, J., Byun, Y., Skultetyova, L., Pomper, M. G., … Barinka, C. (2014). Structural characterization of P1′-diversified urea-based inhibitors of glutamate carboxypeptidase II. *Bioorganic and Medicinal Chemistry Letters*, *24*(10), 2340–2345. https://doi.org/10.1016/j.bmcl.2014.03.066

Pinto, J. T., Suffoletto, B. P., Berzin, T. M., Qiao, C. H., Lin, S., Tong, W. P., … Heston, W. D. (1996). Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *2*(September), 1445–1451.

Qian, X., & Lloyd, R. V. (2003). Recent developments in signal amplification methods for *in situ* hybridization. *Diagnostic Molecular Pathology*, *12*(1), 1–13. https://doi.org/10.1097/00019606-200303000-00001

Ravindran, R., & Kumar, A. (2012). Nasal schistosomiasis among large ruminants in Wayanad, India. *Southeast Asian Journal of Tropical Medicine and Public Health*, *43*(3), 586–588.

Rawlings N.D., Barrett A.J., Bateman A. (2009). "MEROPS: the peptidase database". *Nucleic Acids Research. 38 (Database issue): 227–233.* https://doi.org/10.1093/nar/gkp971

Rawlings, N. D., & Barrett, A. J. (1995). Evolutionary families of metallopeptidases. *Methods in Enzymology*, *248*(C), 183–228. https://doi.org/10.1016/0076-6879(95)48015-3

Rawlings, N. D., & Barrett, A. J. (1997). Structure of membrane glutamate carboxypeptidase. *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*, *1339*(2), 247–252. https://doi.org/10.1016/S0167-4838(97)00008-3

Rawlings, N. D., & Morton, F. R. (2008). The MEROPS batch BLAST: A tool to detect peptidases and their non-peptidase homologues in a genome. *Biochimie*, *90*(2), 243–259. https://doi.org/10.1016/j.biochi.2007.09.014

Rawlings, N. D., Waller, M., Barrett, A. J., & Bateman, A. (2014). *MEROPS* : the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Research*, *42*(D1), D503–D509. https://doi.org/10.1093/nar/gkt953

Renneberg, H., Friedetzky, A., Konrad, L., Kurek, R., Weingärtner, K., Wennemuth, G., … Aumüller, G. (1999). Prostate specific membrane antigen (PSM) is expressed in various human tissues: Implication for the use of PSM reverse transcription polymerase chain reaction to detect hematogenous prostate cancer spread. *Urological Research*, *27*(1), 23–27. https://doi.org/10.1007/s002400050085

Robinson, M. B., Blakely, R. D., Couto, R., & Coyle, J. T. (1987). Hydrolysis of the brain dipeptide N-acetyl-L-aspartyl-L-glutamate. Identification and characterization of a novel N-acetylated α-linked acidic dipeptidase activity from rat brain. *Journal of Biological Chemistry*, *262*(30), 14498–14506.

Ross, A. G. P., Bartley, P. B., Sleigh, A. C., Olds, G. R., Li, Y., Williams, G. M., & McManus, D. P. (2002). Schistosomiasis. *New England Journal of Medicine*, *346*(16), 1212–1220. https://doi.org/10.1056/NEJMra012396

Ross, A. G., Vickers, D., Olds, G. R., Shah, S. M., & McManus, D. P. (2007). Katayama syndrome. *Lancet Infectious Diseases*. https://doi.org/10.1016/S1473-3099(07)70053-1

Rovenska, M., Hlouchova, K., Sacha, P., Mlcochova, P., Horak, V., Zamecnik, J., … Konvalinka, J. (2008). Tissue expression and enzymologic characterization of human

prostate specific membrane antigen and its rat and pig orthologs. *Prostate*, *68*(2), 171–182. https://doi.org/10.1002/pros.20676

Sácha, P., Zámecník, J., Barinka, C., Hlouchová, K., Vícha, a, Mlcochová, P., … Konvalinka, J. (2007). Expression of glutamate carboxypeptidase II in human brain. *Neuroscience*, *144*(4), 1361–1372. https://doi.org/10.1016/j.neuroscience.2006.10.022

Sajid, M., & McKerrow, J. H. (2002). Cysteine proteases of parasitic organisms. *Molecular and Biochemical Parasitology*. https://doi.org/10.1016/S0166-6851(01)00438-8

Sajid, M., McKerrow, J. H., Hansell, E., Mathieu, M. A., Lucas, K. D., Hsieh, I., … Caffrey, C. R. (2003). Functional expression and characterization of Schistosoma mansoni cathepsin B and its trans-activation by an endogenous asparaginyl endopeptidase. *Molecular and Biochemical Parasitology*, *131*(1), 65–75. https://doi.org/10.1016/S0166-6851(03)00194-4

Salter, J. P., Lim, K. C., Hansell, E., Hsieh, I., & McKerrow, J. H. (2000). Schistosome invasion of human skin and degradation of dermal elastin are mediated by a single serine protease. *Journal of Biological Chemistry*, *275*(49), 38667–38673. https://doi.org/10.1074/jbc.M006997200

Silver, D. A., Pellicer, I., Fair, W. R., Heston, W. D., & Cordon-Cardo, C. (1997). Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *3*(1), 81–85. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9815541

Sims, G. K. (2006). Nitrogen starvation promotes biodegradation of N-heterocyclic compounds in soil. *Soil Biology and Biochemistry*, *38*(8), 2478–2480. https://doi.org/10.1016/j.soilbio.2006.01.006.

Sturrock, R. F., Diaw, O. T., Talla, I., Niang, M., Piau, J. P., & Capron, A. (2001). Seasonality in the transmission of schistosomiasis and in populations of its snail intermediate hosts in and around a sugar irrigation scheme at Richard Toll, Senegal. *Parasitology*, *123 Suppl*(2001), S77–S89. https://doi.org/10.1017/S0031182001008125

Su, S. L., Huang, I. P., Fair, W. R., Thomas Powell, C., & Heston, W. D. W. (1995). Alternatively Spliced Variants of Prostate-specific Membrane Antigen RNA: Ratio of Expression as a Potential Measurement of Progression. *Cancer Research*, *55*(7), 1441–1443.

Troyer, J. K., Beckett, M. L., & Wright, G. L. (1995). Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *International Journal of Cancer. Journal International Du Cancer*, *62*(5), 552–558. https://doi.org/10.1002/ijc.2910620511

Tykvart, J., Bařinka, C., Svoboda, M., Navrátil, V., Souček, R., Hubálek, M., … Konvalinka, J. (2015). Structural and biochemical characterization of a novel aminopeptidase from human

intestine. *The Journal of Biological Chemistry*, *290*(18), 11321–11336. https://doi.org/10.1074/jbc.M114.628149

Van Balkom, B. W. M., Van Gestel, R. A., Brouwers, J. F. H. M., Krijgsveld, J., Tielens, A. G. M., Heck, A. J. R., & Van Hellemond, J. J. (2005). Mass spectrometric analysis of the Schistosoma mansoni tegumental sub-proteome. *Journal of Proteome Research*, *4*(3), 958–966. https://doi.org/10.1021/pr050036w

van der Hoorn, R. A. L. (2008). Plant Proteases: From Phenotypes to Molecular Mechanisms. *Annual Review of Plant Biology*, *59*(1), 191–223. https://doi.org/10.1146/annurev.arplant.59.032607.092835.

Walker, A. (2011). Insights into the functional biology of schistosomes. *Parasites and Vectors*, *4*(1). https://doi.org/10.1186/1756-3305-4-203

Wang, L.-D., Chen, H., Guo, J., Zeng, X., Hong, X., Xiong, J., … Zhou, X. (2009). A strategy to control transmission of Schistosoma japonicum in China. *The New England Journal of Medicine*, *360*(2), 121–128. https://doi.org/10.1056/NEJMoa0800135

World Health Organization (2006). Preventive chemotherapy in human helminthiasis. *… Use of Anthelminthic Drugs in Control …*, 62. Retrieved from http://whqlibdoc.who.int/publications/2006/9241547103_eng.pdf%5Cnhttp://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Preventive+chemotherapy+in+human+helminthiasis#8

World Health Organization (WHO). (2016). WHO: Weekly epidemiological record Relevé épidémiologique hebdomadaire. *World Health Organization Geneva*, *21*(83), 421–428. https://doi.org/10.1186/1750-9378-2-15.Voir

World Health Organization. (2010). Fact sheet on Schistosomiasis. *Health Topics*. Retrieved from http://www.who.int/mediacentre/factsheets/fs115/en/index.html

World Health Organization. (2010). *Weekly epidemiological record: relevé épidémiologique hebdomadaire; position paper on pertussis vaccines*. *The Weekly Epidemiological Record* (Vol. 90). https://doi.org/10.1371/jour

Wynn, T. A., Thompson, R. W., Cheever, A. W., & Mentink-Kane, M. M. (2004). Immunopathogenesis of schistosomiasis. *Immunological Reviews*. https://doi.org/10.1111/j.0105-2896.2004.00176.x