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Characterization of thioredoxin and glutathione reductase activities of *Mesocestoides vogae*, a flatworm parasite useful as a laboratory model for the screening of drugs.

Inaugural - Dissertation
zur Erlangung der Doktorwürde der
Medizinischen Fakultät
der
Julius-Maximilians-Universität Würzburg
vorgelegt von

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Würzburg, April 2014

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Tag der mündlichen Prüfung: 24.11.2014

Die Promovendin ist Ärztin

Für Opa, weil du eben doch immer dabei bist.

Abbreviations

Ab	Antibody
Abs	absorbance
APS	ammonium persulfate
aura	auranofin
DMSO	dimethyl sulfoxide
DTNB	5,5’-dithiobis (2-dinitrobenzoic acid)
EMEM	Eagle's minimal essential medium
Gpx	glutathione peroxidase
GR	glutathione reductase
Grx	glutaredoxin
GSH	glutathione
GSSG	glutathione disulfide
IgG	Immunoglobulin G
iNOS	inducible nitric oxide synthase
MHC II	major histocompatibility complex class II)
min	minute
MSR	methionine sulfoxide reductase
NTD	neglected tropical diseases
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide ph osphate
nox	NADPH oxidase
OPZ	oltipraz
PAMPs	pathogen-associated molecular patterns
PAT	potassium antimonyl tartrate

PBS	phosphate buffered saline
PRR	pattern recognition receptors
Prx	peroxiredoxin
rEg TGR	recombinant TGR of *E.granulosus*
RNAi	RNA interference
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute
sec	second
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
sp.	species (singular)
spp.	species (plural)
TEMED	tetramethylethylenediamine
TGR	thioredoxin glutathione reductase
TNB	thionitrobenzoic acid
TR	thioredoxin reductase
Trx	thioredoxin

Content

1.Introduction

1.1 Flatworms as a health problem

Helminth parasites cause some of the most common infections in developing regions of the world and are the causative agents of so-called “neglected tropical diseases” (NTD) (Hotez et al., 2007). Helminths are metazoan organisms with complex multicelullar organization which undergo complex metamorphoses and migrations within the host. They are divided in two phyla: The nematodes (roundworms) and the platyhelminths (flatworms). The parasitic platyhelminths are divided in flukes (trematodes), tapeworms (cestodes) and monogenea, of which the first two include the agents of serious human diseases (Otero et al., 2010).

1.1.1 Trematodes

Trematodes have an unsegmented body, an incomplete digestive tract and two suckers. Most have a complex life cycle that involves one or two intermediate hosts. They are divided in blood flukes (which inhabit the blood in some stage of their life cycle) and tissue flukes which infect lungs, bile ducts and biological tissues.

The most prevalent and devastating trematode infection is schistosomiasis, caused by trematodes of the genus *Schistosoma spp. (*blood flukes) which is widespread in Africa, Central America, Middle East and Southamerica (see figure 1.1). In total more than 200 million people are affected and an estimated 200 000 people die annually only in Sub-Saharan Africa, where the prevalence of infection reaches 90% (Hotez et al., 2008). Schistosomias can be intestinal (*S.mansoni, S.mekongi, S.japonicum, S.intercalatum*) or urogenital (*S.haematobium)* depending on the species (Ross and Salinas 2010). In his intestinal form the worms occupy mesenteric veins, and their eggs pass into the lumen of the intestine and reach the faeces. The urogenital form resides in veins and are draining the urinary tract (First WHO report on neglected tropical diseases 2010, available at http://whqlibdoc.who.int/publications/2010

/9789241564090_eng.pdf). For this work *F.hepatica* should also be mentioned as relevant trematode (tissue fluke) which is the agent of fasciolosis.

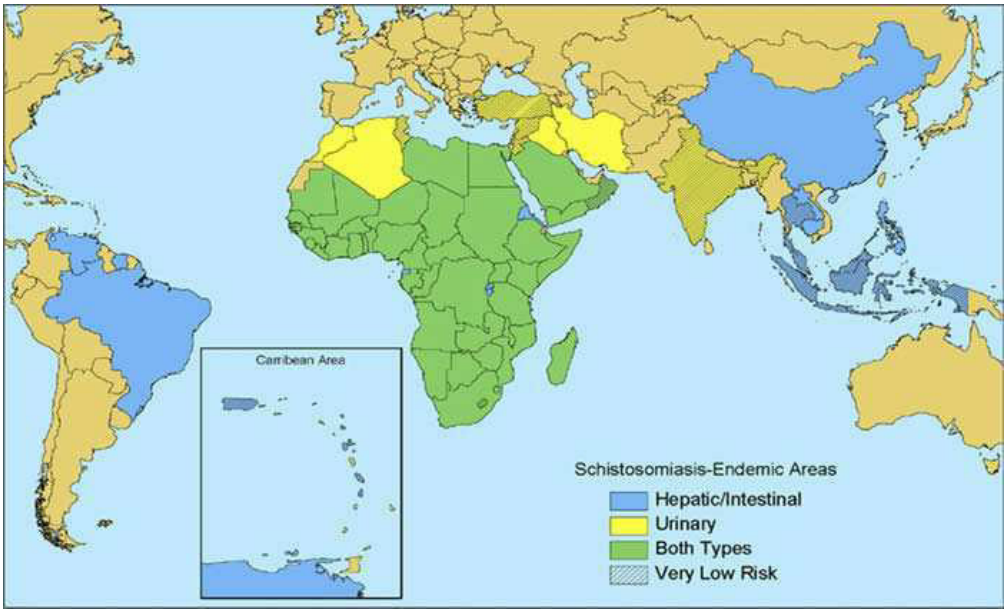


Figure 1.1: Global distribution of schistosomiasis (US Center for Disease Control and Prevention)

1.1.2 Cestodes

Morphological features of adult cestodes are an elongated tape-like body and the absence of an alimentary canal, in both the adult and the larval stage (Smyth and McManus, 1989). Furhermore the body is composed of successive segments (proglottids). In general, cestodes are hermaphroditic and need one or two intermediary hosts. The adult cestodes vary in size from 3 to 7 mm (*Echinococus spp*.) to more than 10 meters (*Taenia spp*.). Cestode infections are less prevalent than those due to trematodes, but include serious human diseases such as cysticerosis and hydatid disease. The former is caused by the larval stage (cysticerci) of *T. solium*. Cysticerci that develop in the central nervous system cause neurocysticercosis which is the most frequent preventable cause of epilepsy in the developing world (WHO). Cystic and alveolar echinococcosis are caused by *E.granulosus* and *E. multilocularis*. The former is prevalent in livestock farming areas (and therefore prevalent in Uruguay where the experiments for this work were conducted). It is characterized by the

development of the larval stage of *E.granulosus* in humans and ruminant livestock which are the intermediate hosts (Ross and Salinas, 2010) and mainly dogs are the definitive hosts of the adult tapeworm (definitive host: sexual reproduction. Intermediate host: asexual reproduction); this infection is maintained by pastoralism, whereas *E.multilocularis* is maintained by a sylvatic life cycle. Because this work deals mostly with drugs against *E.granulosus* a short overview over its lifecycle is given in figure 1.2 below and finally compared to that of *E.multilocularis*.

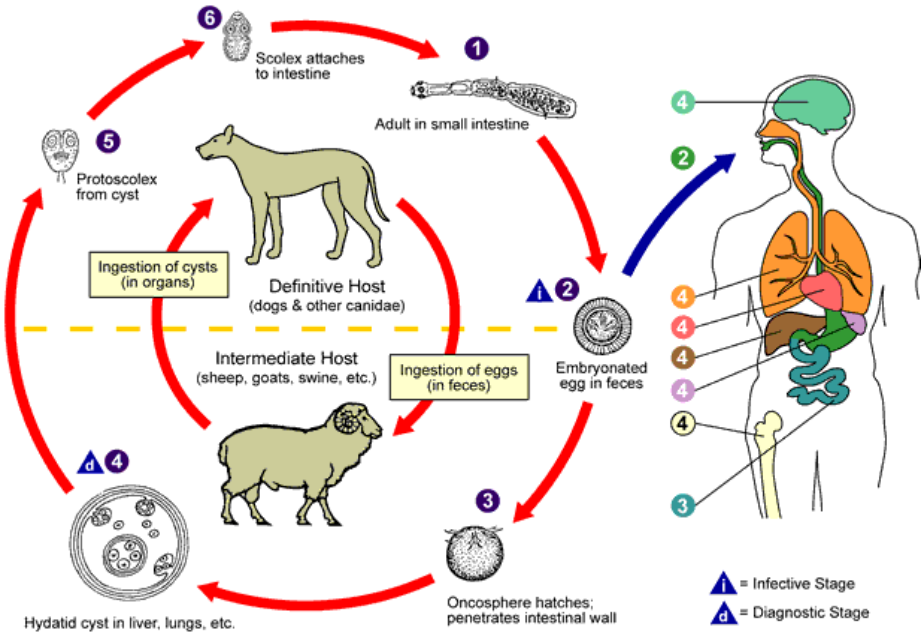


Figure 1.2: Overview over the lifecycle of *E.granulosus*

Lifecycle

1 The adult *Echinococcus granulosus*, which is about 3-6 mm in length, resides in the bowel of its definite host

2 Gravid proglottids release eggs that are passed in the feces.

3 These eggs are then ingested by a suitable intermediate host, including sheep, goat, swine, cattle, horses and camels. The eggs then hatch in the bowels and release **oncospheres** that penetrate the intestinal wall. These oncospheres then migrate through the circulatory system to various organs of the host.

4 At the organ site, the oncosphere develops into a **hydatid cyst**. This cyst enlarges gradually, producing **protoscolices** and **daughter cysts** that fill the cyst interior.

5 These cyst-containing organs are then ingested by the definite host, causing infection. After ingestion, the protoscolices evaginate, producing **protoscolexes**.

6 The **scolexes** of the organisms attach to the intestine of the definite host and develop into adults in 32-80 days.

Human infections

2 Humans can become infected if they ingest substances infected with *Echinococcus* eggs.

3 The eggs then release **oncospheres** in the small intestine.

4 4 4 4 4 4 At these places, oncospheres migrate through the circulatory system and produce **hydatid cysts**.

Note: The same life cycle occurs with E. multilocularis (1.2 to 3.7 mm) except for these differences:
-Definite hosts = usually foxes and canines
-Intermediate hosts = small rodents
-Larval growth remains indefinitely in the proliferative stage, resulting in invasion of the surrounding tissues
(Graphic and text copied from http://www.cdc.gov/parasites/echinococcosis/biology.html)

1.2 The need for new drugs against platyhelminth infections

Until today only a few drugs are available for the treatment of platyhelminth infections. Of the 1556 new chemical entities marketed between 1975 and 2004, only four drugs were developed to treat helminthiases (Hotez et al., 2008) and only three of these drugs are also effective against platyhelminth infections: Oxamiquine, praziquantel and benzimidazoles (e.g. albendazole and mebendazole). Oxamiquine is of particular importance against infections caused by *S.mansoni* but it is not active against the other human-parasitic schistosomes (Cioli and Pica-Mattoccia, 2003). On the contrary, praziquantel is the drug of choice against infections with all species of *Schistosoma* (Coles, 2006) and it is also highly potent against cestode adult stages and taeniasis (Olson et al., 2012). In contrast it is important to highlight that praziquantel is not effective against the metacestode (larval) stages of platyhelminths (Bygott and Chiodini, 2009). Hence, platyhelminth infections which are caused by a larval stage (e.g. echinococcosis) cannot be cured with praziquantel on itself. Therefore the benzimidazoles, are also of particular importance to treat this kind of infections and here albendazole and mebendazole are used for chemotherapeutic treatment of cystic and alveolar echinoccosis. Nevertheless it has been shown that a combination of albendazole plus praziquantel is more effective than only benzimidazole alone (Bekhti and Pirotte, 1987; Wen et al., 1994). Furthermore chemotherapy mostly has to be combined with surgical removal of (e.g.) the hydatic cyst caused by *E.granulosus*.
Unfortunately, there are more limitations regarding the mentioned therapy of flatworm infections with praziquantel and benzimidazoles (from now on BZ):

1) Especially against *E.multilocularis*, BZ treatment is very limited because BZ seems only to be parasitostatic rather than parasitocidal and therefore has to be given lifelong (Olsen et al., 2012) This could be due to a limited access of BZ to *E.multilocularis* when applied *in vivo* (Brehm et al., 2000).

2) Severe side effects were reported within BZ chemotherapeutic treatment of *E. granulosus, E.multiloclaris* and *T.solium* because of the similarity of the drug target in the parasite and its host. (Olsen et al., 2012).
3) The occurrence of drug resistance against many anthelminthic drugs has been reported:

a) *S.mansoni* isolates have shown to be resistant to oxamniquine (Cioli et al., 1993).

b) There are reported several cases where BZ treatment was not effective in reducing the *Echinoccocus* larvae growth in patients and therefore an infection with a resistant strain was supposed (Amman and Eckert, 1995) This is underlined by the report of resistance to BZs of *E.granulosus* (Coles, 2006). More BZ resistances are described e.g. for *Fasciola spp.* that showed resistance to the previously effective drug triclabendazole (which also belongs to the BZs) (Brennan et al., 2007). The reason for BZ resistance has already been studied in detail for some nematodes such as *Haemonchus contortus* and *Caenorhabditis elegans* (Driscoll et al., 1989) but additional data are still pending.

c) Finally and most important praziquantel-resistant isolates of *Schistosoma spp*. have already been identified (Doenhoff, 2002) which is alarming since praziquantel is the single drug useful against Schistosoma infections.

There are reported successes with vaccineation to protect sheep, goats, and bovines against hydatid disease caused by the cysts of *E.granulosus* (Heath et al., 2003).
Nevertheless, economically, there is no market to produce vaccines for mass prevention of platyhelminth infection in humans (Otero et al., 2010).
Summing up, the absence of vaccines, the scarcity of effective drugs and prospect of drug resistance could have dire consequences for the people affected of NTD as well as

for the economic sector of the affected countries. Therefore there exists an urgent need for new and effective drugs for plathyhelminth infections.

1.3 Flatworms and their hosts: a delicate balance

In the following, the mechanism of host defense against parasites and the way how parasites deal with the host effector arms are described.

1) Host defenses

Host-parasite relation can be seen as a co-evolutionary process, in which the host aims to eliminate the parasite, or at least to limit their damage and the parasite aims to avoid recognition and killing by the host and to perpetuate its lifecycle. Host defenses involve physical and chemical barriers to avoid infection, but once infected, the immune systems activates the defense mechanisms. For the purpose of this work, focus will be laid on the host defenses against parasites and in particular in the oxidative stress imposed on the parasite by the host effector mechanisms. In this context two groups of actors are of high importance: macrophages and phagocytic leucocytes (mostly neutrophils, but also monocytes and eosonophils) which are both part of the innate immune response (Nelson and Cox, 2005). Although they are able to recognize pathogens directly, their binding improves dramatically when microbes are opsonized by complements or antibodies (Kindt et al., 2007) for which they possess receptors for ImmunoglobulinG (IgG) and complement factors. In the case of bacteria, binding leads to phagocytosis by effector cells (Klebanoff, 2005). Although phagocytosis of flatworms which are metazoan (multicellular) organism is not possible because of their large size, these cells play also an important role in killing worms or their larval stages. The macrophages have a membrane bound oxidase system whose core enzyme is a complex called NADPH oxidase (nox) (figure 1.3.a). Nox has the capacity to release large amounts of reactive oxygen species (ROS). Besides superoxide anion ($\bullet O_2^-$), hydroxyl radical (OH•), hydrogen peroxide (H_2O_2) and hypochlorite anion (ClO^-) belong to the ROS (figure 1.3.b). In the course of this, $\bullet O_2^-$ is the initial point for the formation of other ROS. The oxygen needed by the macrophages to support ROS

production is provided by a metabolic process, known as the respiratory burst in which the oxygen uptake rate of the cell increases rapidly (Kindt et al., 2007). Nox is activated by the recognition of the parasite by pattern recognition receptors (PRR) and antibody (Ab) receptors that recognize pathogen-associated molecular patterns (PAMPs) and Ab, respectively. The activation itself takes place through phosphorylation of the enzyme (Iles and Forman, 2002). Upon activation the macrophage releases $\bullet O_2^-$ towards the surface of the extracellular parasite (figure 1.3.a). It should be highlighted that the extracellular space in the case of non-phagocytosable parasites (e.g. worms) is topologically equivalent to the lumen of the phagosome in the case of intracellular parasites (Salinas et al., 2006). Therefore the release of $\bullet O_2^-$ towards to extracellular space for non-phagosytosable parasites is similar to the release of $\bullet O_2^-$ towards inside of the phagosome for bacteria. In addition, activated neutrophils and eosinophils release myeloperoxidase and eosinophil peroxidase, respectively. Myeloperoxidase and eosinophil peroxidase themselves catalyse the conversion of H_2O_2 into hypohalous acids that are powerful oxidants and can give rise to further ROS (Halliwell and Gutteridge, 2007; cited by Bonilla and Otero et al., 2011). Apart from the ability to kill foreign organisms, macrophages also present peptide-fragments of the antigen through MHCII (major histocompatibility complex class II) to activate T- helper cells and therefore link innate to adaptive immunity.

The superoxide dismutase (SOD)-catalyzed and spontaneous dismutation of $\bullet O_2^-$ generates H_2O_2 (figure 1.3.b). H_2O_2 is not highly reactive but can react with metal ions via Fenton reaction in which OH• is generated. OH• itself is a powerful oxidant that can abstract an electron from almost any organic molecule (Bonilla and Otero et al 2011). Furthermore (figure 1.3.b) the reaction of nitric oxide (•NO), a gaseous radical produced by inducible nitric oxide synthase (iNOS), reacts with $\bullet O_2^-$ and form reactive nitrogen species (RNS) like peroxynitrite ($ONOO^-$) (Knight, 2000). This is a potent oxidant nitrating agent that further decomposes into additional reactive species (Radi et al., 2001). Large amounts of nitrogen dioxide radical ($\bullet NO_2$) are also produced by hemolytic cleavage of the peroxo bond of the protonated form of peroxynitrite (ONOOH) (Bonilla and Otero et al, 2011).

Ultimately ROS and RNS generated through the oxidase system of macrophages and neutrophil granulocytes are able to cause the death of ingested microorganism or extracellular parasite (flatworm in our case) by disabling protein functions (e.g. reacting with tyrosine residues, metal centers, cystein and methionine), causing lipid peroxidation and DNA damage (Bonilla and Otero et al., 2011) as displayed in figure 1).

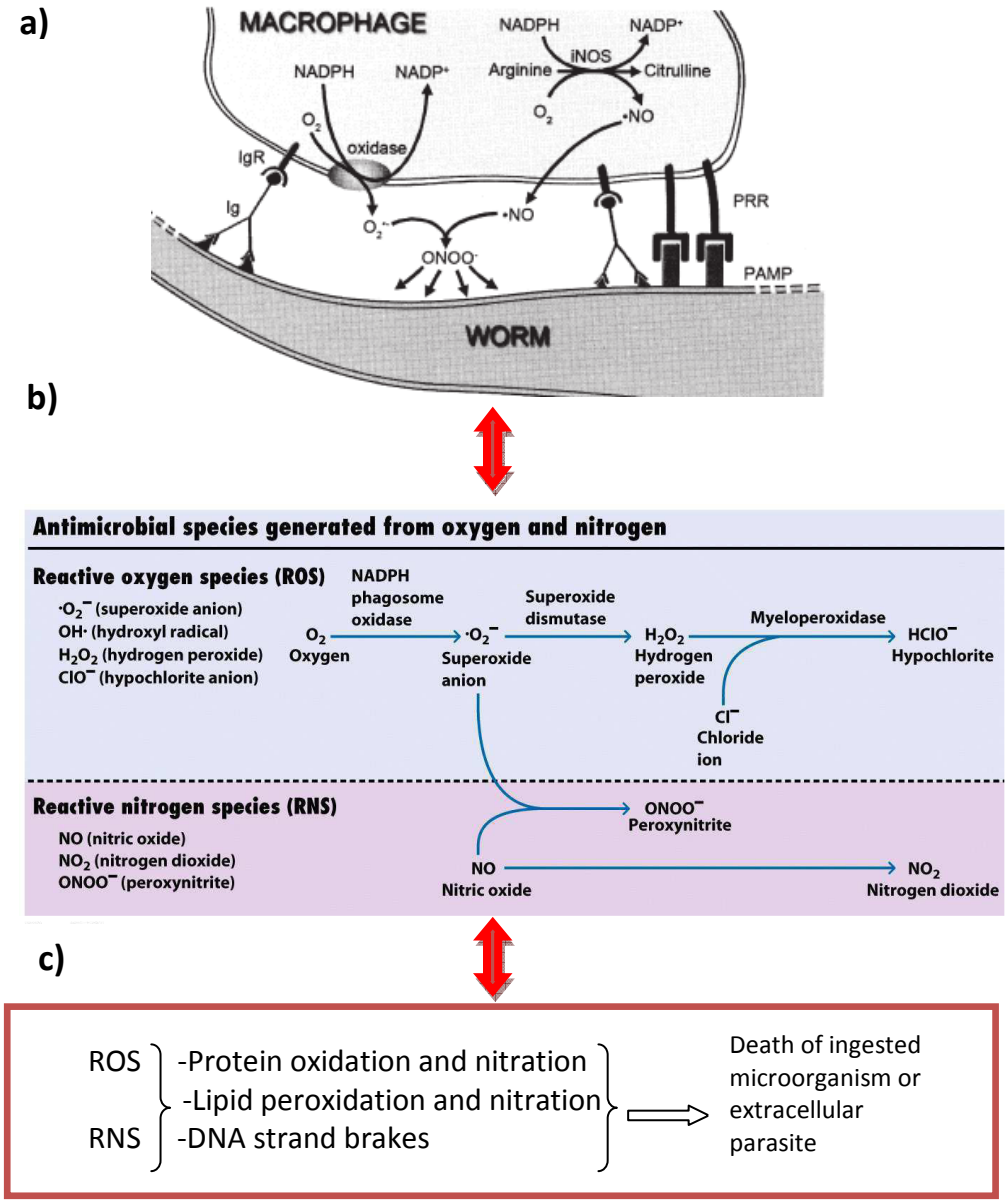


Figure 1.3: (Text see next page)

Figure 1.3.: Reactive oxygen and nitrogen species generated by the host immune response and antioxidant defense:
a) Recognition of parasites by host leukocytes occurs by PRR that bind PAMPs, or through antibodies and leads to activation of host immune cells. Upon activation, these cells produce superoxide ($\cdot O2^-$) and nitric oxide (·NO) radicals. ·NO is produced in the cytosol (but can cross membranes) by inducible nitric oxide synthase (iNOS); ·O2– is produced by a multi-component, membrane-associated NADPH oxidase (nox). $\cdot O2^-$ is released towards the extracellular space in the case of non-phagocytosable parasites *(here:* worms), or towards the phagosome (topologically equivalent to the extracellular space) in the case of intracellular parasites *(*e.g.*,* protozoans).
b) The oxygen needed to support ROS production is provided by a respiratory burst and $\cdot O2^-$ is the initial point for the formation of other ROS such as H_2O_2 and ClO^-. Here the removal of $\cdot O2^-$ through the superoxide dismutase limits the formation of $ONOO^-$ which is the result of the reaction of •NO with $\cdot O2^-$ and which belongs to the reactive nitrogen species (RNS). Large amounts of $\bullet NO_2$ are also produced.
c) Ultimately ROS and RNS generated through the oxidase system of macrophages and neutrophil granulocytes are able to cause the death of ingested microorganism or extracellular parasite

(References: a) Salinas et al., 2006 (graphic and text) b) Kindt, et al., 2006 (graphic)).

2) How the parasite deals with the host effector arms

To understand the way in which flatworms deal with oxidative stress imposed by the immune system, it is important to know how eukaryotic do so in general. Therefore the general mechanism of eukaryotic organism to deal with oxidative stress is described and afterwards will be compared to those of flatworms.

a) Mechanism of eukaryotic organism to deal with oxidative stress

$\bullet O_2^-$ is not very harmful itself but can lead to other highly toxic species (Salinas and Cardozo, 2000), in particular to OH• and $ONOO^-$ (see 1.3). To minimize attacks through oxidative stress, eukaryotic organisms possess adequate mechanisms of detoxification. To eliminate $\bullet O_2^-$ they possess the above mentioned superoxide dismutases (SODs) which catalyze the reaction $2\,^{\bullet}O_2 + 2H^+ \rightarrow H_2O_2 + O_2$. In this way the removal of $\bullet O_2^-$ limits the formation of $ONOO^-$ (which belongs to the RNS, see above section and figure 1.3.b). There are three major families of SODs, depending on the metal cofactor: Cu/Zn (which bind both copper and zinc), Fe and Mn types (which bind either iron or manganese), and the Ni type which binds nickel. In eukaryotic organism two types of SODs have been described and are discriminated: MnSOD which is localized in the mitochondria, and CuZnSOD which is found in the cytosol, peroxisomes and extracellular fluids (Fridovich, 1995). H_2O_2 which is produced by SODs should also be

eliminated to avoid oxidative damage. Therefore most aerobic organism have the enzyme catalase (flatworms lack this enzyme, see below) which catalyzes the decomposition of H_2O_2 to H_2O and O_2 (Chelikani et al., 2004). Peroxidases also catalyze the decomposition of H_2O_2 through the reduction of lipid and H_2O_2 to alcohols and water, respectively:

ROOR' + electron donor (2 e^-) + 2H^+ → ROH + R´OH.

In animals such peroxidases are selenol- or thiol-dependent peroxidases: glutathione peroxidase (Gpx) which uses glutathione as an electron donor, and peroxiredoxin (Prx, formerly known as thioredoxin peroxidase) which uses electrons provided by the dithiol-disulfide oxidoreductase thioredoxin (Trx). Gpxs and Prxs contain a conserved cystein (Cys) or selenocyseine (Sec) residue that undergoes a cycle of peroxide oxidation and thiol-dependent reduction during catalysis (Rhee et al., 2005).

While Gpx reduces hydrogen and organic peroxides to water and alcohol, respectively, this process is accompanied by the oxidation of two moles of glutathione (GSH) (which is the co-substrate) to GSSG (which is the dimeric oxidized form). Furthermore, this involves an additional electron transfer step from GSH to glutaredoxin (Grx) , a small thiol-disulfide oxidoreductase belonging to the thioredoxin (Trx) family which is able to reduce several different targets (see bottom left on the figure 1.3.); in turn glutathione is reduced by glutathione reductase (GR) (Cioli et al., 2008).

Prx, the other thiol peroxidase, besides its ability to reduce peroxides, is also able to reduce peroxinitrite to nitrite (NO_2^-). To reduce oxidized Prx, thioredoxin (Trx) is needed, which in turn is reduced by thioredoxin reductase (TR). The whole described redox cycle can be seen in figure 1.4.

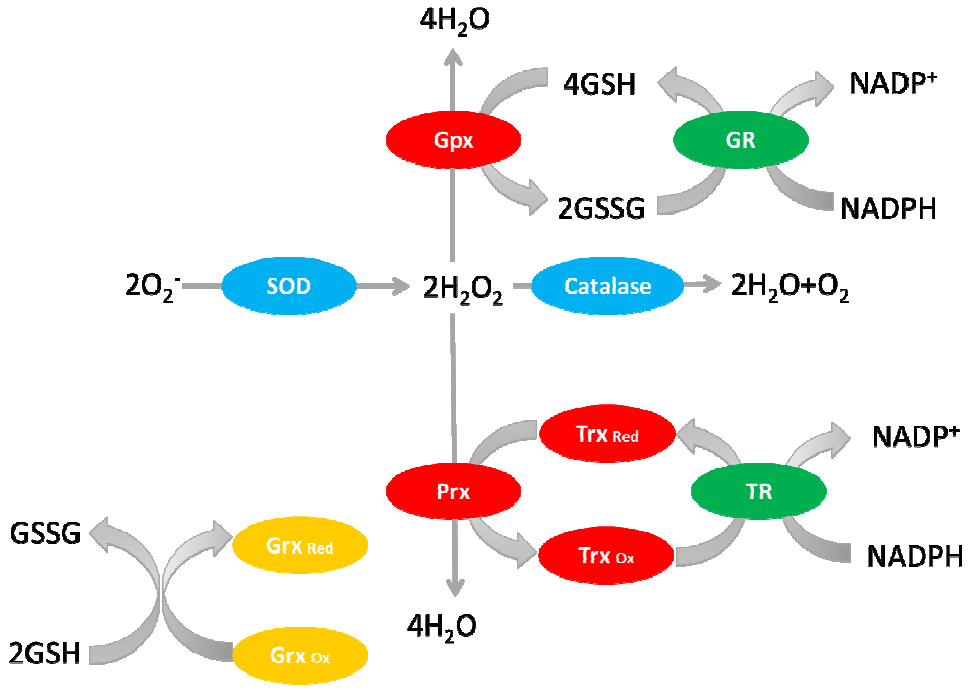


Figure 1.4.: Redox cycles in the Trx and GSH systems:
Most eukaryotic organism possess the enzyme catalase to eliminate the hydrogen peroxide produced by the SODs. Peroxidases (Gpx and Prx) also catalyze the decomposition of hydrogen peroxide though the reduction of lipid and hydrogen peroxides to alcohols and water, respectively. In the case of Gpx this process is accompanied by the oxidation of GSH to GSSG. Furthermore, this involves an additional electron transfer step from GSH to Grx (see bottom left); in turn GSH is reduced by GR. On the other, to reduce oxidized Prx, Trx is needed, which in turn is reduced by TR. (Modified from http://smu.edu/biology/faculty/radyuk.asp).

In summary, apart from catalase that converts H_2O_2 to H_2O and O_2, there are two systems that are involved in the detoxification of ROS and RNS: the first one depends on GSH and the second one on Trx. In both systems the reducing equivalents are provided by NADPH (Kuntz et al., 2007). Both systems are dependent on class I pyridine-nucleotide thiol-disulfide oxidoreductases:

- Thioredoxin reductase (TR) which reduces the oxidized (disulfide) form of Trx (Trx-S_2) to Trx-$(SH)_2$ by NADPH (Holmgren, 1985).

- Glutathione reductase (GR) that reduces GSSG to 2GSH by NADPH (Meister and Anderson, 1983).

All in all Trx, TR and NADPH is called the thioredoxin system (Holmgren and Bjornstedt, 1995). In turn, the glutathione system composed of GR, GSH, GRx and NADPH. In addition to providing electrons to Gpx and Prx, the thioredoxin and glutathione systems also provide electrons to other antioxidants such as methionine sulfoxide reductase (MSR) and operate as protein disulfide reductase system, and therefore not only avoid oxidative damage by metabolizing peroxides, but also participate in oxidative damage repair mechanisms.

b) The redox homeostasis in flatworms:

A balance characterized by the absence of catalase and the reliance on thiol-dependent peroxidases

There are similarities but also differences in the manner in which platyhelminth parasites deal with oxidative stress in comparison to the eukaryotic species. The survival of parasites within their hosts is directly related to their compendium of antioxidant enzymes (McGonigle et al., 1998). As mentioned above, flatworms also provide SODs to eliminate $\bullet O_2^-$, resulting in the production of H_2O_2.

The existence of MnSOD and different isoforms of CuZnSOD in flatworms was described in previous biochemical studies and CuZnSOD activity was found *in vivo* and *in vitro* flatworm secretions (Hong et al., 1992; Salinas and Cardozo, 2000). As already described, Gpx, Prx or catalase are needed to avoid oxidative damage through H_2O_2. It is important to emphasize that platyhelminth parasites such as *Schistosoma spp*. (Berriman M. et al., 2009) or *Echinococcus spp*. (G. Salinas, personal communication) lack any catalase-encoding gene in their genomes (Tsai et al., 2013). So the detoxification of H_2O_2 depends only on Gpx and Prx. The presence of selenocysteine-(Sec-) containing Gpx in flatworms was initially demonstrated in *S.mansoni* (Williams et al., 1992). With regard to Prx, initial studies with this enzyme were carried out in *F. hepatica* (Sayed and Williams, 2004) and has also been found in *E. granulosus*

(McGonigle et al., 1998). Furthermore Prxs seems to be higher expressed than Gpxs in these organisms.

Flatworms possess a linked Thioredoxin-Glutathione System

As seen above, the regeneration of Gpx and Prxs depends on the presence of GSH and Trx. These glutathione and thioredoxin systems are essential for redox homeostasis in eukaryotic organisms where they are present in the cytosol and the mitochondria. Instead of having thioredoxin and glutathione systems, platyhelminth parasites possess linked Trx-GSH systems in both mitochondrial and the cytosolic compartment, and overall redox homeostasis is controlled by the selenocysteine-containing enzyme thioredoxin-glutathione reductase (TGR) (Otero et al., 2010). So far, studies carried out with several platyhelminth parasites, e.g. *S.mansoni* (Alger and Williams, 2002), *E.granulosus* (Agorio et al., 2003; Bonilla et al., 2008), *F. hepatica* (Guevara-Flores et al., 2011) and *T.crassiceps* (Rendón et al., 2004) have shown that all of them possess TGR and lack conventional TR and GR (as seen in figure 1.5.), a biochemical scenario different to that of their mammalian hosts (which possess TR, GR and TGR) (Sun et al., 2005) and to the one present in free-living platyhelminths (which also possess TR, GR and TGR) (Otero et al., 2010).

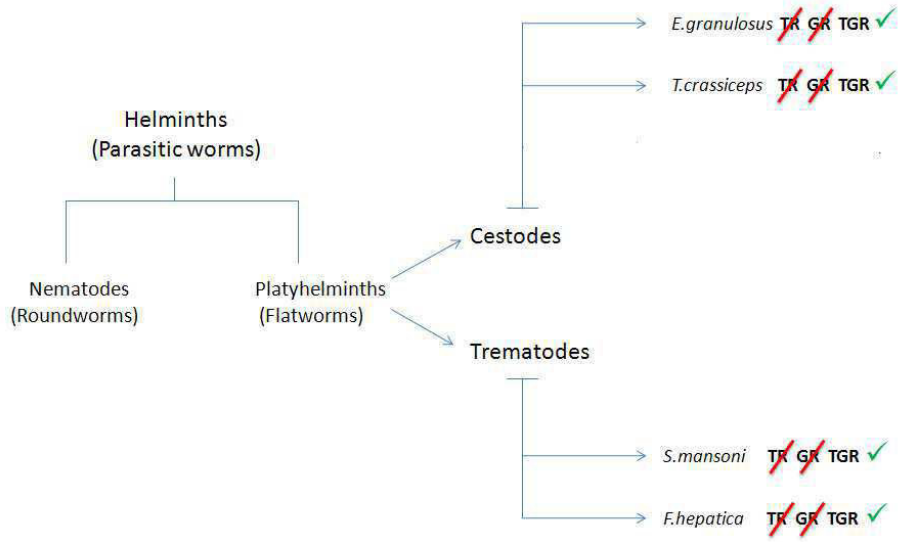


Figure 1.5.: Instead of having thioredoxin and glutathione systems the platyhelminth parasites displayed in this figure possess linked Trx-GSH systems through which overall redox homeostasis is controlled by the selenocysteine-containing enzyme thioredoxin-glutathione reductase (TGR).

TGR is an isoform of the TR which contains an N-terminal glutaredoxin-like domain. Like TR, TGR is dependent on selenocysteine (Sec) (Guevara-Flores et al., 2010).. Through this fusion of the TR and the glutaredoxin domain it achieves a broad substrate specificity and provides reducing equivalents for Trx, GSH and Grx-dependent reaction (figure 1.6a) (Sun et al., 2001).

This fusion was first described in mouse testis (Guevara-Flores et al., 2010). TGR is a selenoprotein with a carboxyl (C) -terminal GCUG active site motif, where “U” is Sec. (Kuntz et al., 2007). Sec is a Cys-analog with a selenium-containing selenol group in place of the sulfur-containing thiol-group in Cys. This yields Sec a stronger nucleophile than Cys with a lower pK_a than the latter (Johansson et al., 2005) (pK_a is the negative base-10 logarithm of the acid dissociation constant. The acid dissociation constant is the equilibrium constant of the dissociation reaction of an acid of a solution. Thus, a smaller pK_a means to be more acid). The mode of the reaction mechanism of TGR indicates that electrons flow from NADPH to FAD to the CX_4C redox center, then to the C-terminal GCUG redox center (TR domain) of the second subunit and finally to the

CX_2C redox center of the Grx domain of the first subunit. The fully reduced enzyme can reduce either oxidized Trx using the C-terminal active site GCUG, or GSSG through the CX_2 redox center of the Grx domain (Sun et al., 2001). The hypothesis that the C-terminal redox center donates electrons to the fused Grx domain is supported by the fact that the Grx domain is linked to the TR domain by a flexible hinge to support the reduction of both oxidized Trx and Grx (figure 1.6.b).

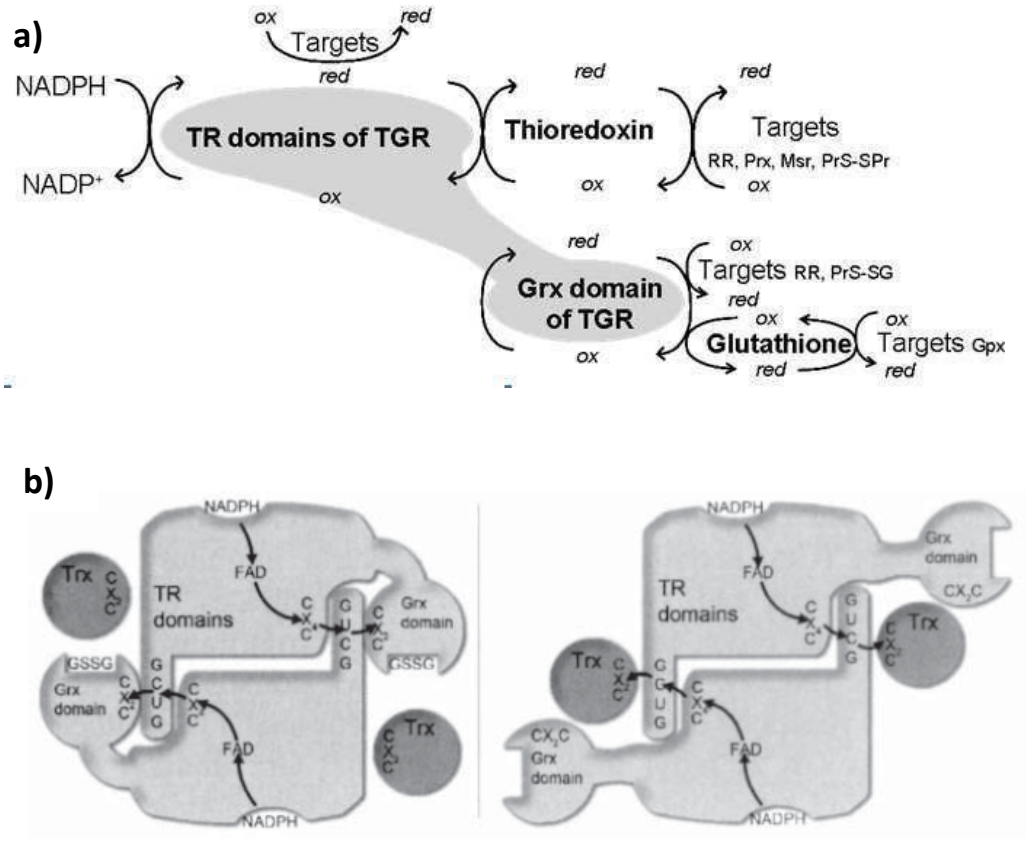


Figure 1.6.: TGR is an oxidoreductase shown to possess TR, GR and GRx activities.
a) Trx and GR are replaced with TGR which provides reducing equivalents for Trx-, GSH- and Grx-dependent reactions.
b) The hypothesis that the C-terminal redox center donates electrons to the fused Grx domain implies that the Grx domain of TGR would be linked to the TR domains by a flexible hinge to allow reduction of the oxidized Trx. (Salinas et al., 2006 (graphic and text)).

1.4 TGR as drug target

Due to the differences in the ROS detoxification systems of flatworms and their eukaryotic hosts, TGR was considered as a potential target for drug design. The notion is to target the TGR pharmacologically. This would affect the overall redox homeostasis of the flatworm, but not that of the host, and therefore would shift this delicate balance in favor of the host.

Recent studies strongly support this idea: inhibition of TGR expression by RNA interference (RNAi) caused death of *S. mansoni*, demonstrating that it is an essential enzyme (Kuntz et al., 2007). TGR also fulfills other requirements as a drug target: it is expressed constitutively, can be easily produced in a recombinant form, there is a low cost and simple biochemical assay to test its activity, and lastly and very important, it is a "druggable enzyme": the Sec residue contains a highly nucleophilic lateral chain, susceptible to be targeted by electrophiles. The fact that both GR and TR activities of TGR are dependent on Sec is important when considering TGR as a chemotherapeutic target since targeting this active site residue would compromise the overall redox homeostasis of the parasites (Bonilla and Otero et al., 2011).

Identified inhibitors of TGR and TR that contain Sec are organic gold compounds which are widely used in the treatment of rheumatoid arthritis (Gromer et al., 1998). Best studied is the gold compound auranofin which is a potent inhibitor of flatworm TGRs and human TR, but not human GR (Gromer et al., 1998). Its inhibition effect has been studied in *Schistosoma spp*. where auranofin led to partial healing by reducing the worm burden in mice in an experimental infection (Kuntz et al., 2007). Furthermore, auranofin was capable to kill *Schistosoma* in various ages *in vitro* which confirmed the essential nature of the target (Cioli et al., 2008). The inhibition of *Schistosoma* TGR by auranofin was also studied by X-ray crystalography in which a catalytic role of the C-terminal Sec was suggested as a possible mechanism of auranofin to inhibit TGRs and TRs (Angelucci et al., 2009).

The effect of auranofin on Sec-containing TGR and TR has also been studied in *Taenia crassiceps* (Rendón et al., 2004), *F. hepatica* (C. Carmona, Universidad de la República, Montevideo, Uruguay, personal conversation) and *E.granulosus* in which the larval

worms of *E.granulosus* died after 12 hours at a 10 µM concentration of auranofin (Bonilla et al., 2008).

Moreover, additional gold and platinum phosphole compounds which do not inhibit TR but human GR have been described. These compounds might be also useful as studying objects for new antiparasitig drugs (Deponte, M. et al., 2005).

Finally, a chemical library of 71028 potential inhibitors of *S.mansoni* TGR and Prx activity was screened in a quantitative high-throughput essay (Simeonov et al., 2008). Subsequently, Sayed et al. (2008) investigated new compounds of the series of oxadiazole 2-oxides (also known as furoxanes) and phosphinic amides and found that one furoxane was effective against all *S. mansoni* developmental stages and showed low toxicity against mammalian cell lines.

In the work of Kuntz et al. (Kuntz et al., 2007), a number of the identified TGR inhibitors of *S.mansoni* like auranofin, potassium antimonyl tartrate (PAT) or oltipraz (OPZ) were screened against recombinant TGR of *S.mansoni*. Here TGR inhibitors that kill the parasite and cure infection were found. Salinas and Ross (2010) recently identified potent inhibitors of *E.granulosus* TGR that killed protoscolex stage (larvae of *E.granulosus*) *in vitro* by screening a library of electrophilic compounds. Here, the successful inhibition with the furoxanes VL16E, VL63 and VL should be mentioned as well as the inhibition with WPQ75 which belongs to the thiadiazole-family. A small overview over the inhibitors which are relevant in this work can be seen in figure 1.7.

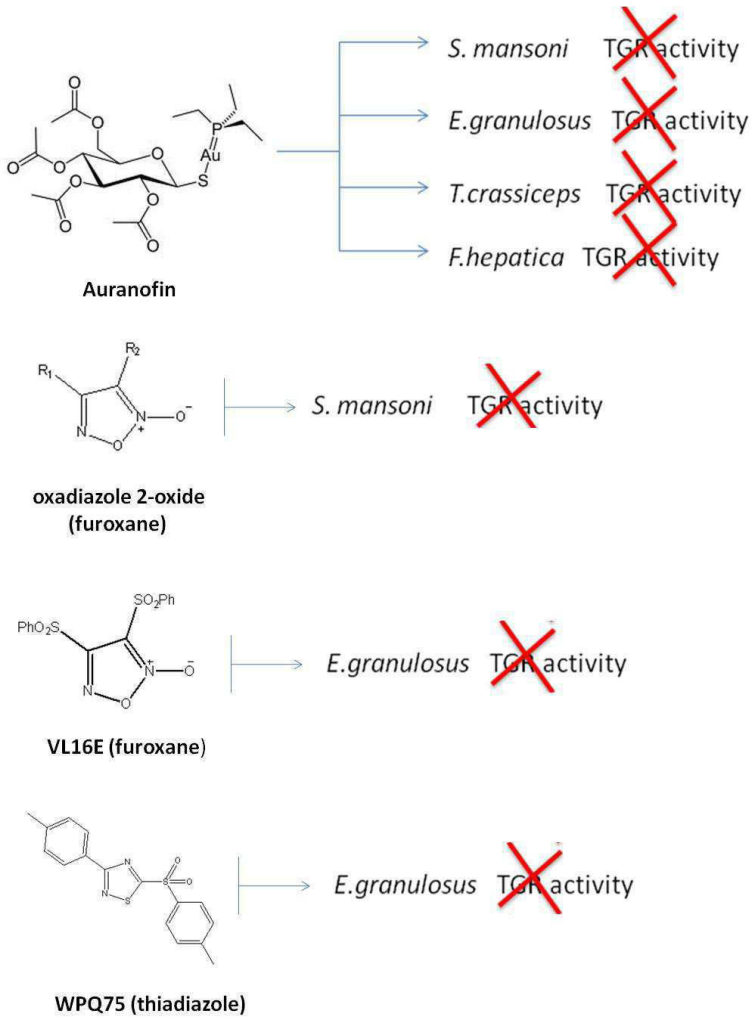


Figure 1.7.: Identified inhibitors of TGR activity in different platyhelminth parasites: Auranofin is the best studied TGR inhibitor that has an effect on the TGR of several flatworms. Compounds of the furoxane-family have an effect both on *S.mansoni* and *E.granulosus* (VL16E in this case). WPQ75 is the latest identified inhibitor of flatworm TGR proven to inhibit TGR activity in *E.granulosus*..

1.5 *Mesocestoides vogae*: a potential model useful for testing flatworm TGR inhibitors?

To test *in vivo* the identified inhibitors of TGR (this work only refers to the recently found TGR inhibitors of *E.granulosus*) requires an accessible infection model. *E.granulosus* is not a useful cestode parasite for *in vivo* assessment of drugs. Indeed, *E. granulosus* experimental infection in mice takes about 10 month to develop into cysts. As an alternative, *Mesocestoides vogae* (syn. *corti*) provides an excellent infection

model to study flatworm infections: it is very rare in humans, can be easily manipulated both *in vivo* and *in vitro* and grows extremely fast in mice being possible to analyze larval growth in two weeks (Hemphill et al., 2010).

It was firstly isolated by Specht and Voga (1965) and its tetrathyridia stage of the strain was described as "*M. vogae*" (Saldaña J et al., 2001). *M.vogae* belongs to the genus *Mesocestoides sp.,* the adult stage of which dwells in the intestine of carnivores. Basically a three-host cycle is assumed although the first intermediate host(s) has (have) not yet been identified (ants were recently suggested). The second intermediate hosts are vertebrates like amphibian, reptile, birds and mammalians (Eckert et al., 2008).

This flatworm multiplies asexually and extremely fast in the peritoneal cavity and liver of mice (Specht and Widmer, 1972) and has been proposed as a useful system for the studies on cestode biology (Hart, 1968; Mitchell et al., 1977). Its suitability as a model for the screening of drugs against cestodes has also been reported (Saldaña et al., 2003).

2.Objectives

Since *M.vogae* is a very good infection model for screening drugs, and with the aim to assess TGR inhibitors as possible drugs to treat flatworm infections, this study addressed the thioredoxin and glutathione pathways of *M.vogae*. To this end it was first invesigated whether the biochemical pathways that maintain redox homesotasis in *M. vogae* conform to the general biochemical scenario proposed for other platyhelminth parasites. The central question was if *M.vogae* possesses TR and GR activity and if these activities derive from TGR or other thiol-disulfide pyridine nucleotide oxidoreductases (like separately conventional TR and GR). After this it was investigated if some recently identified TGR-inhibitors of *E.granulosus* are also be able to inhibit thioredoxin- and glutathione reductase activities in *M.vogae* and whether they are able to inactivate the worm both *in vitro* and *in vivo*.

3. Material and Methods

3.1 Used equipment and chemicals

a) Used equipment

- Centrifuge: Sigma 3-18 K
- Ion exchange chromatography: ÄKTApurifier, GE Healtcare, results were evaluated with the program Unicorn™ 5.2., GE Healthcare
- Spectrophotometer: spectrophotometer T70+, PG Instruments, results were evaluated with the program UVWin 5 software v 5.1.1
- Nikon stereoscope

b) Used chemicals

Unless stated otherwise stated all chemicals were bought from Sigma-Aldrich.

3.2 Principals of the used purification techniques

Different techniques to purify recombinant TGR of *E.granulosus* (rEg TGR) and native TGR of *M.vogae* were used. The basic principles are described in the following sections.

a) Affinity chromatography

Affinity chromatography is based on a reversible interaction between a protein and a specific ligand coupled to a chromatography matrix. In the case of this work a 2´5´ADP-Agarose column (GE Healthcare) was used. 2'5' ADP is a NADP structural analog immobilized on Sepharose™ 4B. It binds and immobilizes enzymes requiring this cofactor. The idea was, that the TGR will bind to the 2'5' ADP matrix and can be eluted with NADPH (as the real substrate) which will detach TGR from the matrix.

b) Ammonium sulfate precipitation

The solubility of proteins depends (among others variables) on the salt concentration in the solution. Ammonium sulfate precipitation or salt fractionation is also known as "salting in, salting out". This name comes from the functionality of this method: "Salting in" means that salt stabilize the charged groups on a protein molecule at low concentration by attracting the protein into the solution and increase the solubility of the protein. When the salt concentration is increased, less water is available to dissolve the protein and finally a point of maximum of the protein solubility is reached. Consequently the protein starts to precipitate. This phenomenon of protein precipitation in the presence of excess salt is known as *salting-out* (experiment description by Wang NS, Department of Chemical and Biomolecular Engineering, University of Maryland, USA. Available at: http://www.eng.umd.edu/~nsw/ench485 /lab6a.htm). Different proteins precipitate with different maximums of salt concentration. Accordingly, it should be figured out with which amount of salt (in our case ammonium sulfate) the rEg TGR and native TGR of *M.vogae* will precipitate completely.

c) PD-10 Desalting column

The used column PD-10 (GE Healthcare) has a Sephadex G25 Matrix which exhibits a molecular sieve effect. Purification is based on the principle of gel filtration. High molecular weight substances (proteins in our case) can pass the matrix more rapidly (within or just after the void volume of the column) than smaller molecules (salts in our case). The void volume of the used column was 3,5 ml, column volume was 2,5 ml.

d) Ion exchange chromatography

Ion exchange chromatography is based on a competitive interaction of charged molecules. By using Ion exchange chromatography, proteins can be separated according to their net charge. The net charge of the protein depends on the pH. If a pH lower than the isoelectric point is used, the protein should be positively charged and if a pH higher than the isoelectric point is used, the protein should be negatively

charged. An Ion exchange matrix consists of charged functional groups to which the sample can bind. The samples are retained on the stationary phase and can be eluted by changing the ionic strength of the mobile phase (in our case through the increase of the salt concentration of the elution buffer). The used column was connected with an UV-detector so that eluted proteins can be detected (absorption at 280 nM). TGR is supposed to have an isoelectric point of 6,7 (which is the theoretical isoelectric point of recombinant TGR of *E.granulosus* (M. Bonilla, Cátedra de Inmunología, Institut of Hygiene, Montevideo, Uruguay, personal communication)) and therefore TGR should be positively charged at a pH lower than 6,7 and negatively charged at a pH higher than 6,7.

e) Hydroxyapatite chromatography

The formula of hydroxyapatite is $Ca_{10}(PO_4)_6(OH)_2$. The functional groups are positive charge pairs of crystal calcium ions and negative charge groups oxygen groups which are associated with triplets of crystal phosphates (see instruction manual Bio Rad Laboratories,http://wolfson.huji.ac.il/purification/PDF/Hydroxyapatite/BIORADCeramic.pdf) .The separation mechanism of hydroxyapatite chromatography is very complex. It could be described as a mix of anion and cation exchange by which the hydroxyapatite can react with the negative charged carboxyl group or the positive charged amino groups of the protein respectively. The phosphate groups of the hydroxyapatite interact more strongly with the carboxyl groups of the proteins therefor a buffer with increasing phosphor concentration is normally used.

3.3 Enzymatic Assays

To detect TR and GR activities two different enzymatic assays were used:

a) DTNB reduction assay for TR activity

The reduction of 5,5'-dithiobis (2-dinitrobenzoic acid) (DTNB) with concomitant NADPH (reduced nicotinamide adenine dinucleotide phosphate**)** oxidation was determined by the increase in absorbance (abs) at 412 nm due to formation of 5'-thionitrobenzoic

acid (TNB) at 25 °C (Arner et al., 1999; Holmgren and Björstedt, 1995). The enzyme (TR) catalyses the following reaction:

$$DTNB^{2-} + NADPH + H^{+} \xrightleftharpoons{TR} 2TNB^{-} + NADP^{+}$$

The assays were performed using:

- 100 µl dipotassium phosphate (K_2HPO_4) 0,5 M, EDTA 10 mM, pH 7 (final concentration: dipotassium phosphate 500 mM,, EDTA 1 mM)
- 100 µl DTNB 50 mM (final concentration 5 mM)
- 5 µl NADPH 20 mM (final concentration 0,1 mM)
- Extract to be tested (amount see each experiment)
- H_2O milliQ to achieve a total volume of the mixture of 1000 µl

The Δ[Abs/min] was calculated for the first and second minute (min) prior to measurement of absorbance was started. The change of absorbance during 3 minutes was observed.

The consumption of NADPH was calculated using the law of Beer-Lambert: ε×l×c=abs. (ε =molar extinction coefficent for the absorbing material at wavelength in units of 1/mol×cm; l= light path in the absorbing material; c=concentration of the absorbing solution; abs=measured Δ[Abs/min]).

With a light path of 1 cm in our case, a ε for DTNB of 13600 1/mol×cm and bearing in mind that 2 molecules of DTNB are comsumed per molecule of NADPH the formula Δ[NADPH/min]= Δ[Abs/min] / 2 × 13600 1/ 1/mol×cm was established.

b) GR assay

GR activity was assayed as a NADPH-dependent reduction of oxidized gluatahione (GSSG) to glutathione (GSH), which is followed as the decrease in absorbance at 340 nm (Carlberg and Mannervik, 1985).

The enzyme (GR) catalyses the following reaction:

$$GSSG + NADPH + H^{+} \xrightleftharpoons{GR} 2GSH + NADP^{+}$$

The conventional GR-assay is performed using:

- 100 µl dipotassium phosphate (K_2HPO_4) 0,5 M, EDTA 10 mM, pH7 (final concentration: dipotassium phosphate 500 mM,, EDTA 1 mM
- 15 µl GSSG 20 nM (final concentration: 300 µM)
- 5 µl NADPH 20 mM (final concentration 0,1 mM)
- Extract to be tested (amount see each experiment)
- H_2O milliQ to achieve a total volume of the mixture of 1000 µl

Mainly, a modified GR assay was used which includes 15 µl GSH 20 nM (final concentration 300 µM) besides the above mentioned ingredients.
The Δ[Abs/min] was calculated among the first and second minute when measurement of absorbance was started. The change of absorbance during 3 minutes and (even longer within the hysteresis experiments) was observed.
The corresponding consumption of NADPH was calculated using the law of Beer-Lambert again (see TR assay) with a ε for GSSG of 6200 1/mol×cm and consequently establishing the formula Δ[NADPH/min]= Δ[Abs/min] / 6200 1/ 1/mol×cm.

3.4 SDS-Page gels

The following solutions for preparing resolving gels for Tris-glycine SDS-Polyacrylamide Gel -Electrophoresis were used (Schagger und Jagow 1987):

To prepare 10 ml of 12% gel:

- 3,3 ml H_2O
- 4,0 ml 30% acrylamide mix
- 2,5 ml Tris 1,5 M, pH 8,8
- 0,1 ml 10% sodium dodecyl sulfate (SDS)
- 0,1 ml 10% ammonium persulfate (APS)
- 0,0004 ml tetramethylethylenediamine (TEMED)

For 10 ml of 10% gel 4,0 ml of H_2O and 3,3 ml 30% acrylamide mix were used. The other ingredients were the same as with 12 % gel.

To prepare 5% Stacking gel

- H_2O 2,1 ml
- 0,5 ml 30% acrylamide mix
- 0,38 ml Tris 1 M, pH 6,8
- 0,03 ml 10% SDS
- 0,03 ml 10% APS
- 0,003 TEMED

Coomassie Staining and Destaining

	Coomasie Stain solution	Coomasie Destain solution
methanol	40%	20%
acetic acid	10%	10%
Coomassie R250	0,1%	--

3.5 Origin of TR and GR activities in *M.vogae*

In the following, the way to reveal the origin of TR and GR activities in *M.vogae* is described.

3.5.1 Preparation *of M.vogae* extracts

Extracts of *M.vogae* were obtained by disruption of *M. vogae* larval worms (*M.vogae* larval worms were provided by Jenny Saldaña, Laboratorio de Experimentación Animal, Departamento de Ciencias Farmacéuticas, Facultad de Química, Universidad de la República, Montevideo, Uruguay) with a mortar and a pestle under liquid nitrogen The resulting suspension was further homogenized by ultrasound, and the suspension centrifuged at 18,000 rpm during 30 minutes. The resulting extract was diluted 1:1 using Hanks Balanced Salts as medium (modified from Guevara-Flores et al., 2010; Kuntz et al., 2007).

3.5.2 Measurement of TR and GR activities in *M.vogae*

To detect potential TR activity, the DTNB Reduction Assay for TR Activity was utilized (see 3.3 from now on "TR assay") using 20 µl of *M.vogae* extracts. GR activity was firstly measured with the conventional GR assay (see 3.3 using 40 µl of the extracts.

3.5.3 Verification of hysteretic behavior of *M.vogae* unpurified extracts

To test if *M.vogae* exhibits hysteretic behavior (definition and meaning of hysteretic behavior see results 4.1.2), the GR assay in a modified form was used. In doing so, the composition of the buffer of the assay was changed by adding GSH to a final concentration of 300 µM.

3.5.4 Optimization of TGR purification

Recombinant *E. granulosus* TGR (rEg TGR; Bonilla et al., 2008) was kindly provided by M. Bonilla (Cátedra de Inmunología at the Institut of Hygiene, Montevideo, Uruguay). In all experiments, wild-type rEg TGR_{GCUG} (where U is Sec and GCUG is the C-terminal tetrapeptide) was used at 10 µM concentration.

a) Purification of rEg TGR by affinity chromatography

The principle of this method is described in 3.2

rEg TGR (10 µM) was applied onto a 2'5'-ADP-Agarose column (GE Healthcare) and washed with Tris 50 mM, EDTA 1 mM, pH 7,5. It was eluted with NADPH (2 ml steps of 50 µM, 100 µM, 200 µM, 400 µl, 800 µM). 15 µl aliquots of each eluted fraction were run on an electrophoresis gel (SDS-Page 12% gel) under denaturing conditions.

The experiment was repeated under identical conditions but with pH 5 and pH 8 of the binding buffer instead of pH 7,5. As readout for successful isolation, the characteristic yellow color of rEgTGR in aqueous solution (G.Salinas, personal conversation) was measured.

b) Purification of rEg TGR by ammonium sulfate precipitation

The principle of this technique is described in 3.2. The used solution of saturated ammonium sulfate ($(NH_4)_2SO_4$) was prepared by dissolving 761 g of $(NH_4)_2SO_4$ in 1000

ml H_2O. The pH was adjusted to 7 and the solution filtered (Millipore, pore size: 0,22 µM).

For each precipitation 80 µl of rEg TGR (10 µM) was utilized. The precipitation of the extract was carried out in five sequential steps of 20, 25, 30, 40 and 50% of ammonium sulfate saturation. The used amounts of $(NH_4)_2SO_4$, rEg TGR and the resulting percentages of precipitation can be seen in table 3.1 below.

Amount of recombinant TGR of *E.granulosus*	Amount of $(NH_4)_2SO_4$, pH 7	Percentage of precipitation
80 µl	20 µl	20%
80 µl	26,6 µl	25%
80 µl	34,3 µl	30%
80 µl	53,3 µl	40%
80 µl	80 µl	50%

Table 3.1: Used amounts for the precipitation of rEg TGR with $(NH_4)_2SO_4$.

In every precipitation step, after adding $(NH_4)_2SO_4$ slowly drop by drop, the mix was left to precipitating for 3 hours (room temperature). Afterwards the sample was centrifuged at 4°C (18,000 rpm) for 10 minutes. The supernatant was separated from the pellet and the pellet was dissolved in 1 ml Tris 20 mM, NaCl 10 mM, pH 8. All samples were analyzed in SDS-PAGE 10% gel according to standard techniques (see 3.4) using 5 µl aliquots of each fraction.

c) Purification of rEg TGR by anion exchange chromatography

The principle of ion exchange chromatography is 3.2 2 different columns for anion exchange chromatography were used:

Column HiTrap Mono Q™ 5/50 GL (Mono Q)

This column (5×50 mm) has a polystyrene matrix and is classified as a strong anion exchanger. The charged group is $-CH_2-N^+(CH_3)_3$. Its column volume (CV) is 1 ml.

The flow rate was always 1 ml/min. Binding buffer was Tris 20 mM, NaCl 10 mM, pH 8. Elution was performed with Tris 20 mM, NaCl 400 mM, pH 8. The column was equilibrated with 10 CV elution buffer. Then 10 CV of binding buffer were passed through column. 0,2 ml of rEg TGR (10 µM) was dissolved at the ratio of 1:10 with binding buffer (thus 2 ml in total) and injected in the column. Afterwards the column was washed with 2 CV of binding buffer. Subsequently a gradient-elution was carried out in which the salt concentration was increased step by step using a linear gradient and achieved 100% within 15 CV.

Column HiTrap DEAE Sepharose Fast Flow (DEAE)

This column (1,6×2,5 cm) has a 6% highly cross-linked agarose matrix and is classified as a weak anion exchanger. The charged group is $-CH_2CH_2CH_2SO_4^{2-}$. Its CV is 5 ml.

The treatment was essentially the same as with the column Mono Q (see above); the same binding and elution buffers were used at a flow rate of 1 ml/min, but the gradient was performed within 10 CV instead of 15 CV.

d) Purification of rEG TGR by cation exchange

2 different columns for cation exchange chromatography were used:

Column HiTrap Resource™ 15S (Resource S)

This column (6,4×30 mm) depends on a divinylbenzene matrix and is known as a strong anion exchanger. Its ligand is methyl sulfonate. The CV of the column used was 1 ml. Flow rate was always 1 ml/min.

As binding buffer sodium phosphate (NaH_2PO_4) 20 mM, pH 3,5 was used. Elution buffer was NaH_2PO_4 20 mM, NaCl 1 M, ph 3,5. The column was equilibrated with 10 CV of elution buffer and afterwards washed with 10 CV of binding buffer. The pH of rEg TGR (10 µM) was adjusted to 3,5. Subsequently 0,2 ml of the rEg TGR (10 µM) was

dissolved in 1,8 ml binding buffer and then injected. The column was washed with 2 CV of binding buffer and bound proteins eluted with a linear gradient with increasing salt concentration which achieved 1 M of NaCl within 10 CV.

Column HiTrap Heparin HP (Heparin HP)

This column (0,7 × 2,5 cm) exhibits a highly cross-linked spherical agarose matrix, the ligand is heparin. Heparin operates usually as an affinity ligand. In this experiment its attribute as a high capacity cation exchanger due to its anionic sulfate groups (see instruction manual: http://www.gelifesciences.com/aptrix/upp01077.nsf/content /Products?OpenDocument&parentid=570&moduleid=14811) was used.

CV was 1 ml. The course of action was the same as in the case of the column Resource S (see above). 0,2 ml of rEg TGR (10 µM) dissolved in 1,8 ml of binding buffer was used. It is worth noting that first intents to dissolve the TGR in a pH of 5,5 binding buffer failed because TGR precipitated at this pH. Finally binding buffer was NaH_2PO_4 10 mM, pH 3,5 and elution was performed with an increasing salt concentration of NaH_2PO_4 10 mM, NaCl 1M, pH 3,5 within 10 CV.

e) Purification of rEg TGR by hydroxyapatite chromatography

100 µl rEg TGR (10 µM) were dissolved in 1 ml of Tris 20 mM NaCl 10 mM, pH 8 (thus total volume of the mixture 1,1 ml) and applied on a hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)-column. The column was prepared by mixing 1 g of hydroxyapatite (Bio Gel HTP, BioRAD Laboratories) with 7 ml Tris 20 mM NaCl 10 mM, pH 8. 3 ml of monosodium phosphate (NaH_2PO_4) 10 mM, pH 8 was used as binding buffer and it was eluted with increasing concentrations of NaH_2PO_4: 20 mM, 40 mM, 80 mM, 160 mM and 360 mM – all of them at pH 8. 15 µl aliquots of each fraction were analyzed on a SDS-PAGE 12 % according to the standard techniques.

3.5.5 Purification of native TGR in *M.vogae*

The methods that seemed to be successful within the purification of rEG TGR were tested using *M.vogae* extracts.

a) Extraction of native TR/GR activities of *M.vogae* by affinity chromatography

1 ml of *M.vogaes* extract was dissolved in 3 ml Tris 20 mM, NaCl 100 mM, pH 7,5 and applied on a 2'5'-ADP-Agarose column (GE Healthcare). The flow through was collected and the column was washed with 5 ml Tris 20 mM, NaCl 100 mM, pH 7,5. Elution was performed with 0,2 ml Tris 20 mM, NaCl 200 mM, NADPH 1 mM, pH 7,5. 50 µl aliquots of the flow through- and eluted fractions were tested for TR activity using the TR assay.

b) Purification of native TR/GR activities of *M.vogae* by ammonium sulfate precipitation

The salt precipitation with $(NH_4)_2SO_4$ was first carried out following two schemes of three steps: 20, 40 and 85% saturation and 25, 50 and 85% saturation. This change in the salt fractionation scheme was performed to optimize purification. Below the procedure with 25, 50 and 85% is described in details (see Figure 3.1) while the results of both attempts are listed in the results (IV.1.3.2.B).

$(NH_4)_2SO_4$ was added drop by drop (procedure was the same as with rEg TGR of *M.vogae* (see 3.5.4). To achieve a salt-saturation of 25%, 1,38 ml of a $(NH_4)_2SO_4$ was added to the start extract volume (5,5 ml) . The suspension was led three hours precipitating and afterwards it was centrifuged at 4 °C (15,000 rpm). The supernatant fraction was separated from the pellet fraction. While the pellet fraction was dissolved in 1 ml Tris 20 mM, NaCl 10 mM, pH 8 (to measure TR and GR activity, see below), the supernatant fraction was brought to 50% salt-saturation by adding 3,67 ml $(NH_4)_2SO_4$ (altogether the mixture contained now 5,5 ml extract of *M.vogae* and 5,5 ml $(NH_4)_2SO_4$. The previous procedure was repeated the supernatant fraction was again separated from the pellet fraction. The pellet fraction (named as 25-50% pellet fraction) was dissolved in 2,5 ml Tris 20 mM, NaCl 10 mM, pH 8 and TR and GR activities were measured. To the supernatant fraction 25,6 ml of $(NH_4)_2SO_4$ was added to achieve a salt saturation of 85% (named as 50-85% pellet fraction). After centrifugation, the pellet was dissolved in 3,5 ml Tris 20 mM, NaCl 10 mM, pH 8 and TR and GR activities were measured again. To measure the TR and GR activities 20 µl respectively 40 µl of the samples where used.

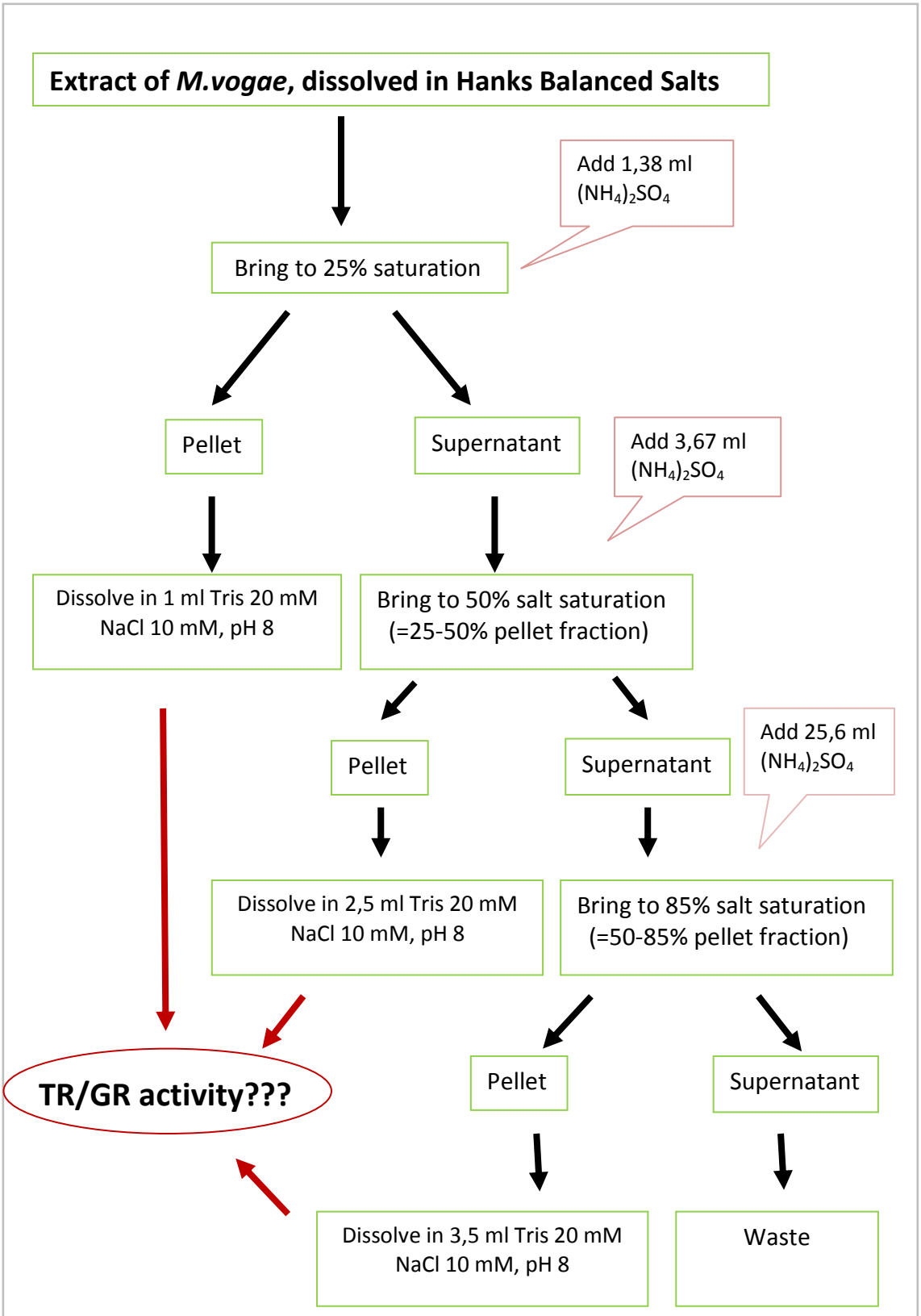


Figure 3.1: Steps of *M.vogae* extract ammonium sulfate precipitation with $NH_4)_2SO_4$

The column was equilibrated with 25 ml (=25 CV) equilibration buffer (Tris 20 mM NaCl 10 mM, pH 8). The 25-50% pellet fraction (with a volume of 2,5 ml) was applied on the PD-10 column. Elution was performed with 3,5 ml equilibration buffer. The TR and GR activities of the eluted fraction were measured as previously described (using 20 µl and 40 µl of the sample, respectively).

c) Purification of native TGR of *M.vogae* via ion exchange chromatography

The volume of the extract purified by the use of $(NH_4)_2SO_4$ and desalted using a PD10 column (see 3.3) accounted 3,5 ml. This volume was brought to 5ml by adding 1,5 ml Tris 20 mM NaCl 10 mM, pH 8 (binding buffer).

The procedure was the same as using within r*Eg TGR* (see 3.5.4) Elution buffer was Tris 20 mM, NaCl 400 mM, pH 8. The gradient-elution was carried out in which the salt concentration was increased step by step using a linear gradient and achieved 100% within 10 CV.

The whole volume of 5 ml was injected. Flow rate was again 1 ml/min. The flow through (unbound proteins) was collected, and the eluted fractions were collected in 1 ml fractions. Collection of eluted fractions began when absorbance was detected at 280 nm (protein absorb at this wavelength) - in other words, fractions containing no proteins were not collected. Subsequently TR and GR activities were tested in each fraction (using 20 µl and 40 µl of the samples, respectively). 5 µl aliquots of the fractions were also analyzed on a SDS-Page 12% gel. The experiment was repeated several times and proved to be very reproducible.

d) Purification of native TGR of *M.vogae* by hydroxyapatite chromatography

The fractions of ion exchange chromatography possessing the highest TR and GR activity (fractions 6, 7, 8, see results 4.1.4) were pooled and desalted with a PD10-column using NaH_2PO_4 10 mM, pH 8. The mixture was applied on a hydroxyapatite-column and washed with 3,5 ml NaH_2PO_4 10 mM, pH 8. The flow through was collected. Subsequently elution was performed with increasing concentration of NaH_2PO_4: 20 mM, 40 mM, 80 mM and 160 mM and 360 mM. The total volume of the flow through was 3,5 ml, the 20mM, 40mM and 80 mM fractions had a total volume of

0,5 ml, the 160 mM fraction had a total volume of 0,4 ml and the 360 mM fraction had a total volume of 1 ml. TR activity was detected using 50 µl of each sample. 20 µl aliquots of each fraction were electrophoresed on a SDS-Page 12% gel.

3.5.6 Verification of hysteretic behavior of *M.vogae*

Semi-purified extracts of *M.vogae* (this is the fraction eluted from Mono Q, i.e. after salt fractionation and anion exchange) were used.

To check if this extracts of *M.vogae* exhibit hysteretic behavior the modified GR Assay was used. On this occasion the concentration of GSH in the buffer was increased step by step to see if, and under what conditions hysteresis disappears. Furthermore, it was tested if the hysteretic behavior can be influenced using no GSH and a high concentration of GSSG instead ("high" in comparison with the conventional GR assay, in this case 600 µM).

The used concentrations of GSSG and GSH can be seen in table 3.2 below. The other compounds of the buffer (1ml in total) were the same as included in the convention GR assay (see 3.3) In all cases 30 µl of the semi-purified *M.vogae* extracts were used.

Concentration GSSG	Concentration GSH
600 µM	0 µM
300 µM (=conventional GR assay)	0 µM (=conventional GR assay)
300 µM	20 µM
300 µM	50 µM
300 µM	100 µM
300 µM	300 µM

Table 3.2: Concentrations of GSSG and GSH to change the [GSSG]/[GSH] ratio.

3.5.7 Inhibition of TR and GR activities of *M.vogae* with the gold compound auranofin

The anion exchange semi-purified extracts of *M.vogae* were used again. The start concentration of auranofin was 4 µM dissolved in dimethyl sulfoxide (DMSO). Grade of inhibition was tested using the TR and GR assay. The concentrations for TR and GR,

respectively and the composition of the essays are named in the tables 3.3.a and 3.3.b below.

The reaction in the case of the TR assay was started after a 3 minutes preincubation by adding DTNB. The reaction in the GR assay was started by adding GSSG after 3 minutes preincubation. To avoid hysteresis during the GR assay a chose very small concentration of GSSG (60 µM) was used while omitting GSH. These conditions were chosen based on previous results observed for the hysteretic behavior with recombinant TGR of *E.granulosus*.

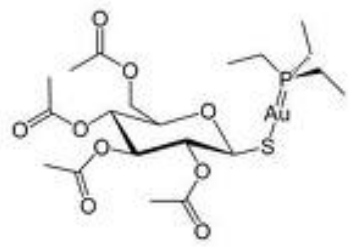


Figure 3.3: Chemical structure auranofin

Concentration Aura	Buffer	H_2O	DTNB (50 mM)	NADPH (20 mM)	Aura/DMSO	Extract
Extract without inhibition	100 µl	685 µl	100 µl	5 µl	0 µl /10 µl	100 µl
Aura 20 nM	100 µl	685 µl	100 µl	5 µl	2 µl of aura 10 nM/ 8 µl DMSO	100 µl
Aura 1 nM	100 µl	685 µl	100 µl	5 µl	2 µl of aura 500 nM/ 8 µl DMSO	100 µl
Aura 0,5 nM	100 µl	685 µl	100 µl	5 µl	4 µl aura 50 nM/ 6 µl DMSO	100 µl
Aura 0,2 nM	100 µl	685 µl	100 µl	5 µl	10 µl aura 0,5 nM/ 0 µl DMSO	100 µl
Aura 0,75 nM	100 µl	685 µl	100 µl	5 µl	1,5 µl aura 500 nM/ 8,5 µl DMSO	100 µl

Table 3.3.a: Used amounts of the ingredients within the TR assay under different concentrations of auranofin.

Concentration Aura	Buffer	H_2O	GSSG (800 µM)	NADPH (20 mM)	Aura/DMSO	Extract
Extract without inhibition	100 µl	770 µl	75 µl	5 µl	0 µl /10 µl	40 µl
Aura 10 nM	100 µl	770 µl	75 µl	5 µl	10 µl aura 1 µM/ 0 µl DMSO	40 µl
Aura 8 nM	100 µl	770 µl	75 µl	5 µl	8 µl of aura 1 µM / 2 µl DMSO	40 µl
Aura 6 nM	100 µl	770 µl	75 µl	5 µl	6 µl aura 1 µM/ 4 µl DMSO	40 µl
Aura 4 nM	100 µl	770 µl	75 µl	5 µl	4 µl aura 1 µM/ 6 µl DMSO	40 µl
Aura 2 nM	100 µl	770 µl	75 µl	5 µl	2 µl aura 1 µM/ 8 µl DMSO	40 µl
Aura 1 nM	100 µl	770 µl	75 µl	5 µl	10 µl aura 100 nM/ 0 µl DMSO	40 µl
Aura 0,5 nM	100 µl	770 µl	75 µl	5 µl	5 µl aura 100 nM/ 5 µl DMSO	40 µl
Aura 0,25 nM	100 µl	770 µl	75 µl	5 µl	2,5 µl aura 100 nM/ 7,5 µl DMSO	40 µl

Table 3.3.b: Used amounts of the ingredients within the GR assay under different concentrations of auranofin.

3.6 Inhibition of TGR activity of *M.vogae* by recently identified inhibitors of flatworm TGR

3.6.1 Inhibition of TGR activity of *M.vogae* with VL16E

Inhibition of TGR activity of *M.vogae* were performed with different concentrations of the N-oxide VL16E (provided by the Laboratory of Medicinal Organic Chemistry form the Faculty of Sciences, Universidad de la República, Montevideo, Uruguay). Its chemical structure can be seen in figure 3.4 below. Mainly the TR activity was measured. Only with two concentrations the GR activity (0,1µM and 10 µM VL16E) was also measured. This course of action was justified by the results of the previous experiments which showed that *M.vogae* possesses, in all likelihood, TGR and not conventional TR or GR (see results and discussion). The stock of VL16E was 10 mM and was diluted to achieve the needed concentrations. The reaction of the TR-assay was started after an incubation time of 3 minutes by adjoining DTNB. All used amounts can be seen in table 3.4 below.

Figure 3.4: Chemical structure of VL16E

Concentration VL 63	Buffer	H_2O	DTNB (50 mM)	NADPH (20 mM)	VL63/DMSO	Extract
Extract without inhibition	100 µl	765 µl	100 µl	5 µl	0 µl /10 µl	20 µl
VL16E 100 µM	100 µl	765 µl	100 µl	5 µl	10 µl VL63 100 mM/ 0 µl DMSO	20 µl
VL16E 10 µM	100 µl	765 µl	100 µl	5 µl	10 µl VL63 1 mM/ 0 µl DMSO	20 µl
VL16E 1 µM	100 µl	765 µl	100 µl	5 µl	10 µl VL63 0,1 mM/ 0 µl DMSO	20 µl
VL16E 0,5 µM	100 µl	765 µl	100 µl	5 µl	5 µl VL63 0,1 mM/ 5 µl DMSO	20 µl
VL16E 0,3 µM	100 µl	765 µl	100 µl	5 µl	3 µl VL63 0,1 mM/ 7 µl DMSO	20 µl
VL16E 0,1 µM	100 µl	765 µl	100 µl	5 µl	10 µl VL63 0,01 mM/ 0 µl DMSO	20 µl
VL16E 0,05 µM	100 µl	765 µl	100 µl	5 µl	5 µl VL63 0,01 mM/ 5 µl DMSO	20 µl
VL16E 0,01 µM	100 µl	765 µl	100 µl	5 µl	1 µl VL63 0,01 mM/ 9 µl DMSO	20 µl

Table 3.4: Used amounts of the ingredients within the TR assay under different concentrations of VL63.

3.6.2 Inhibition of TGR activity of *M.vogae* with WPQ 75

The course of action was the same as with VL63. TGR was inhibited with different concentrations of the Thiadiazole WPQ75 (also provided by the Laboratory of Medicinal Organic Chemistry form the Faculty of Sciences, Universidad de la República, Montevideo, Uruguay). The chemical structure can be seen in figure 3.5. Again, the grade of inhibition was measured mainly with the TR assay and for two concentrations with the GR assay (10 µM and 0,1 µM). The stock of WPQ 75 was 10 mM and was diluted to achieve the needed concentrations. The reaction of the TR-assay was started

after 3 minutes preincubation by adding DTNB. All used amounts can be seen in table 3.5.

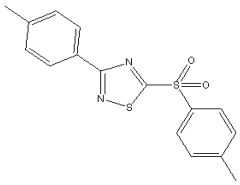


Figure 3.5: Chemical structure of WPQ75

Concentration WPQ75	Buffer	H_2O	DTNB (50 mM)	NADPH (20 mM)	WPQ75/DMSO	Extract
Extract without inhibition	100 µl	765 µl	100 µl	5 µl	0 µl /10 µl	20 µl
WPQ75 10 µM	100 µl	765 µl	100 µl	5 µl	10 µl WPQ75 1 mM/ 0 µl DMSO	20 µl
WPQ75 0,5 µM	100 µl	765 µl	100 µl	5 µl	5 µl WPQ75 0,1 mM/ 5 µl DMSO	20 µl
WPQ75 0,25 µM	100 µl	765 µl	100 µl	5 µl	2,5 µl WPQ75 0,1 mM/ 7,5 µl DMSO	20 µl
WPQ75 0,1 µM	100 µl	765 µl	100 µl	5 µl	1 µl WPQ75 0,1mM/ 9 µl DMSO	20 µl
WPQ75 0,05 µM	100 µl	765 µl	100 µl	5 µl	5 µl WPQ75 0,01 mM/ 5 µl DMSO	20 µl
WPQ75 0,01 µM	100 µl	765 µl	100 µl	5 µl	1 µl WPQ75 0,01 mM/ 9 µl DMSO	20 µl

Table 3.5: Used amounts of the ingredients within the TR assay under different concentrations WPQ75.

3.7 Testing of TGR inhibitors of *M.vogae in vitro*

To see if auranofin, VL16E and WPQ75 are also able to kill *M.vogae in vitro,* 0,5 ml *M.vogae* larval worms were incubated with 20 mM of each drug in Eagle's minimal essential medium (EMEM) which contained ampicillin and kanamycine, pH 7,4 . As control sample the behavior of *M.vogae* was tested with praziquantel. After an incubation time of 3 hours (37°C) the different treated fractions of *M.vogae* were observed under a steroskope and were filmed with a 75-magnification From then on

the different fractions were observed every 3 hours during 24 hours. All the time the same medium was used.

3.8 Testing of TGR inhibitors of *M.vogae in vivo*

This was performed by an external laboratory instructed by us (see results 4.4).

4. Results

4.1 TR and GR activities of *M.vogae* derive from TGR

To investigate wheter that TR and GR activities in *M.vogae* derive from TGR it was tried to purify native TGR of *M.vogae* (with methods firstly optimized with recombinant rEG TGR) and to test if *M.vogaes* TGR exhibits properties that are already proven to be typical for the existence of TGR in flatworms.

4.1.1 Extracts of *M.vogae* exhibit TR and GR activities

A first set of experiments addressed the question whether *M. vogae* exhibits TR and/or GR activities. To this end, parasite extracts were generated and analyzed in the DTNB enzyme assay, for TR activity, and in GR-assay for GR-activity (as outlined in M&M 3.3). As depicted in Fig. 4.1., the extracts clearly displayed TR activity with a consumption of 45,7 uM of NADPH per minute (the measured Δ[Abs]/min was 0,113). The total volume of the extract was 11 ml (see 3.5.1), hence it resulted a total Δ[Abs]/min of 1,24 and consequently a consumption of 45,70 µM NADPH per minute (way of calculation see 3.3).

In the standard GR activity assay, on the other hand, no activity was measured (data not shown). As previously shown (Rendón et al., 2004), this could be due the fact that flatworm TGRs usually exhibit hysteretic behaviour in the presence of high concentrations of GSSG. In a next set of experiments, it was therefore analyzed *M. vogae* extracts for this characteristic.

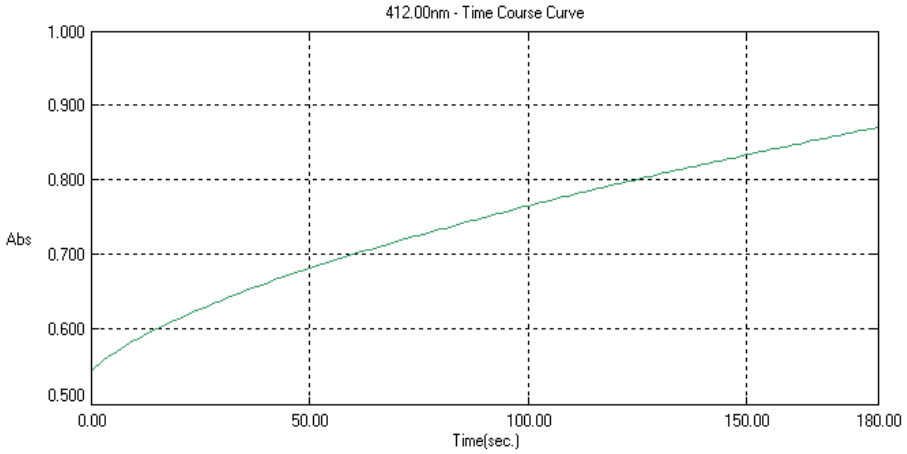


Figure 4.1: Extracts of *M.vogae* possess TR activity.
The assay was carried at concentrations of NADPH of 0,1 mM and DTNB of 5 mM . Reaction was started by the addition of *M.vogae* extracts (20µl).
Abs=Absorbance, Time (sec)=time in seconds

4.1.2 Unpurified extracts of *M.vogae* exhibit hysteretic behavior

The hysteresis is characterized by a lag time before the steady state velocity is reached (Rendón et al., 2004). The time lag depends on the conditions (concentration of substrate, product and enzyme); in the case of flatworm TGRs, the GR activity is temporarily hysteretic at high GSSG concentrations, low GSH concentration and low enzyme concentration. Hysteretic behavior is a typical indication for the existence of TGR in flatworms (Rendón et al., 2004) Thus, this hypothesis was tested by increasing the concentration of GSH and using a higher concentration of GSSG while omitting GSH in the assay.

As figure 4.2 displays, GR activity appeared in the extract after changing the composition of the buffer of the assay by adding GSH to the final concentration of 300 µM. The measured Δ[Abs]/min was 0,08. This results in a total Δ[Abs]/min of 0,88 and consequently a consumption of 141,94 µM NADPH per minute. Thus, it was decided to work in all the following experiments only with this modified GR assay to avoid the hysteretic behavior.

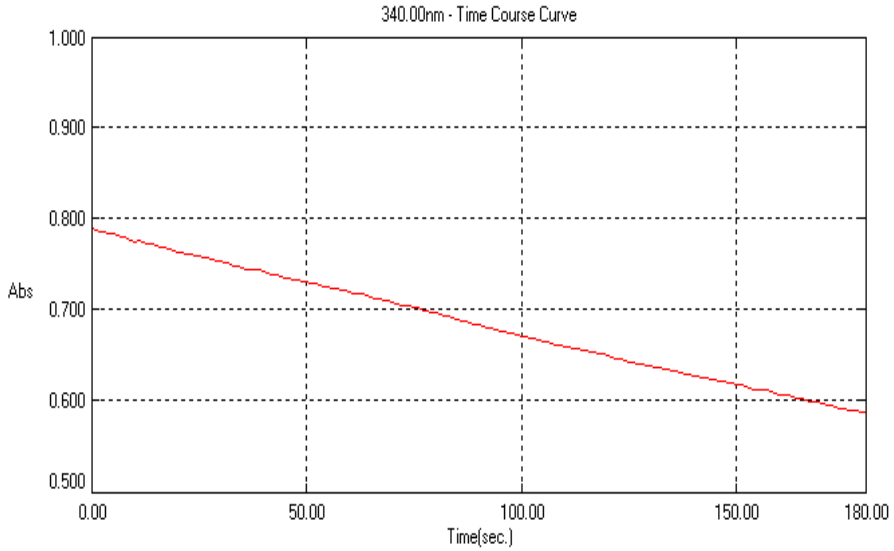


Figure 4.2: Extracts of *M.vogae* possess GR activity using a modified form of the conventional GR assay.
The assay was carried out at concentrations of GSSG of 300 µM, GSH 300 µM and NADPH 100 µM. Reaction was started by the addition of *M.vogae* extracts (40 µl).
Abs=Absorbance, Time(sec)=time in seconds

4.1.3 Optimization of TGR purification

In order to establish protocols for the purification *of M. vogae* protein extracts with GR/TR activity, use was first made of rEg TGR that should exhibit similar biochemical properties as *M. vogae* TGR, including parameters that are relevant for chromatography.

a) Purification of rEg TGR by affinity chromatography

Affinity chromatography with 2´5´ADP agarose was initially attempted with Tris 50 mM, EDTA 1 mM, pH 7,5 as binding buffer. The elution was performed with different NADPH concentrations. The analysis with SDS-Page 12% gel shows that rEg TGR was present in nearly all eluted fractions (figure 4.3.a). The flow through fraction also contained TGR, suggesting that the column was overload. The experiment was repeated under identical conditions using binding buffers with pH 5 an pH 8 instead of pH 7,5. Similar results were obtained in either case: Benefiting of the characteristically yellow color of the TGR, it was demonstrated that the results after changing the pH of

the buffer corresponded to those with the initial buffer (exemplary shown for pH 5 in figure 4.3.b).

Because 2'5'ADP agarose binds other nucleotide-binding proteins and rEg TGR did not appear in a single fraction, the purification with 2'5'-ADP-agarose may not be as useful as initially thought for crude extracts. In any case, the binding of rEg TGR to the matrix indicates that it could be used as one step in a purification procedure, eluting with the highest NADPH concentration.

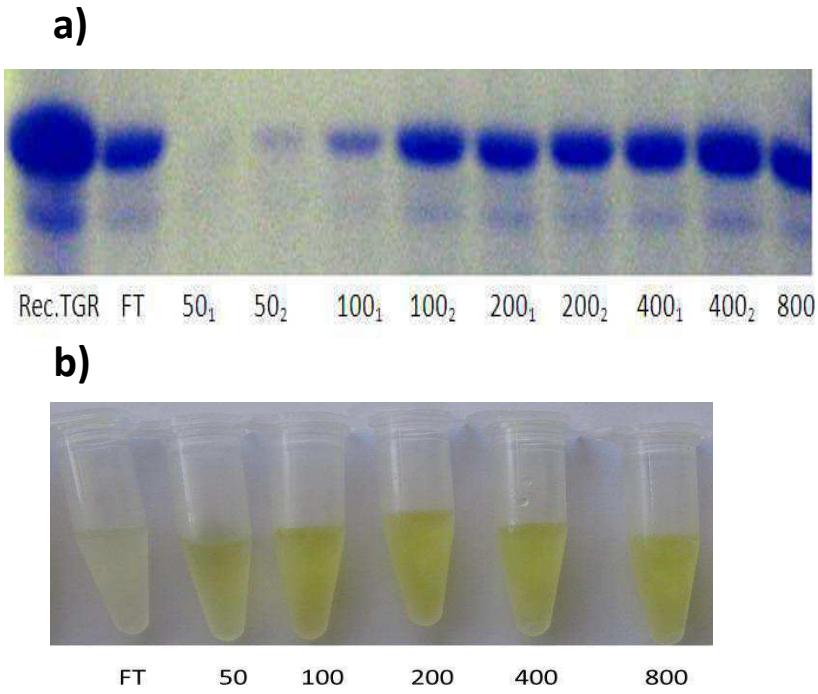


Figure 4.3: Using affinity chromatography, rEg TGR was present in nearly all eluted fractions.
rEg TGR was applied onto a 2'5'-ADP agarose matrix and eluted with different concentrations of NADPH (50-800 µM, named 50-800 in the graphic).

a) Binding buffer used was Tris 50 mM EDTA 1 mM, pH 7,5. The eluted fractions (each in 2 fractions with a volume of 100 µl) and the flow through (FT) were analyzed in SDS-Page 10% gel. Unpurified rEg TGR ran as reference in the gel (left track). It should be noted that the major band corresponds to the full length TGR (67 kDa), the smaller band corresponds to the TR domains of TGR. A small fraction of TGR is sometimes cleaved between the TR domains (56 kDa) and the Grx domain (11 kDa).

b) Binding buffer used was Tris 50 mM, EDTA 1 mM, pH 5. It was benefited of the characteristically yellow color of the TGR. As readout for successful isolation, the characteristic yellow color of rEgTGR in aqueous solution was measured.

b) Purification of rEg TGR by ammonium sulfate precipitation

In a next step, the influence of $(NH_4)_2SO_4$ concentrations on the precipitation of TGR from aqueous solution was tested. To this end, rEg TGR (in Tris 20 mM, NaCl 10 mM, pH 8)was subjected to precipitation with various concentrations of $(NH_4)_2SO_4$.

As shown in Fig 4.4, rEg TGR began to precipitate with 25% of a saturated solution of $(NH_4)_2SO_4$ (i.e. there was TGR in the pellet and in the supernatant). At 30% most of the TGR was in the pellet (precipitated) and a small fraction in the supernatant; and with 40% of a saturated solution it was ascertained that the whole TGR was completely precipitated (besides the analysis with SDS-Page 10% gel, visual inspection was a good indicator of whether TGR was either in the pellet-, the supernatant- or in both fractions since TGR possesses the already mentioned characteristic yellow color). Referring to these results and hypothesizing that the native TGR of *M.vogae* could react in a similar way as rEg TGR it was decided to precipitate the *M.vogae* extract with 20% and 40% of $(NH_4)_2SO_4$.

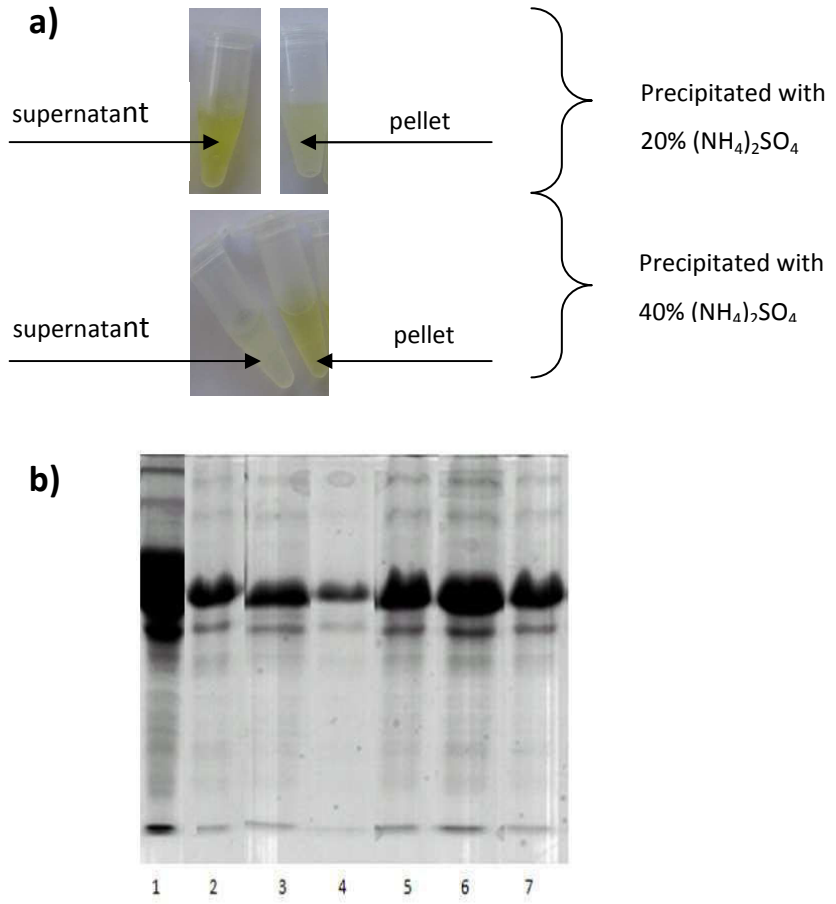


Figure 4.4: The analysis by visual inspection (a) and by SDS-Page 10% gel (b) showed that no rEg precipitated with 20% $(NH_4)_2SO_4$ and all the rEg precipitated with 40% of $(NH_4)_2SO_4$

Lane 1: rEg before precipitation	
Lane 2: Supernatant fraction	precipitated with 25% $(NH_4)_2SO_4$
Lane 3: Pellet fraction	precipitated with 25% $(NH_4)_2SO_4$
Lane 4: Supernatant fraction	precipitated with 30% $(NH_4)_2SO_4$
Lane 5: Pellet fraction	precipitated with 30% $(NH_4)_2SO_4$
Lane 6: Pellet fraction	precipitated with 40% $(NH_4)_2SO_4$
Lane 7: Pellet fraction	precipitated with 50% $(NH_4)_2SO_4$

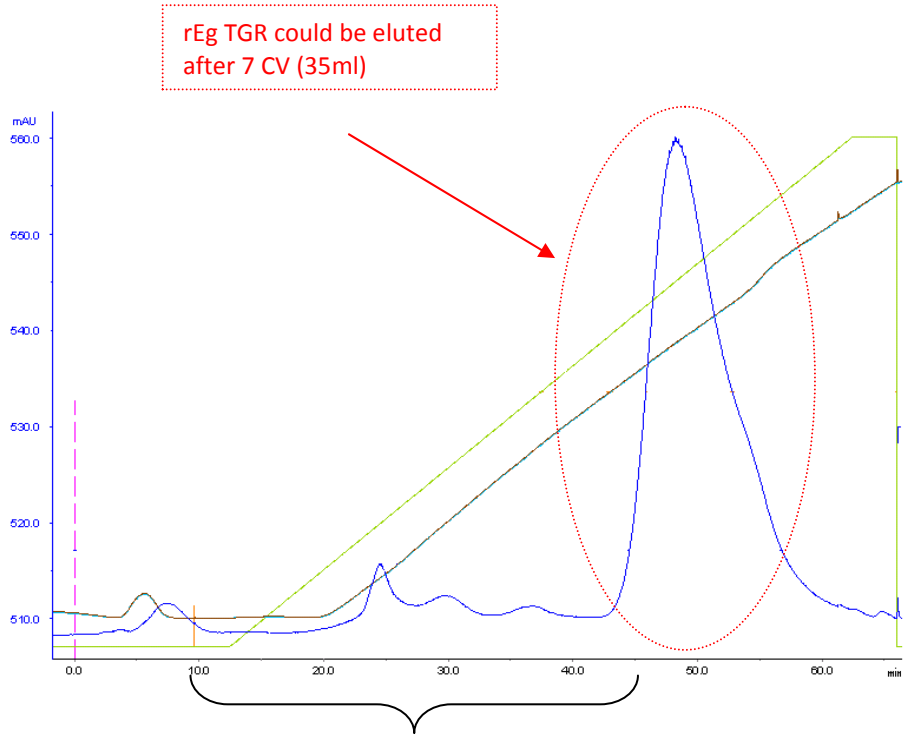
c) Purification of rEg TGR by Ion exchange chromatography

To optimize the conditions, experiments with Ion exchange chromatography were made. Here, two different columns (anion exchange columns as well as cation exchangers) were used to verify which column could be useful for the next step of purification of rEg TGR.

Anion exchange chromatography

Two different anion exchange columns were used: a Mono Q column (with a column volume (CV) of 1 ml) and a DEAE column (with a CV of 5ml see M&M 3.2). In the case of the column Mono Q, the whole rEg TGR bound to the matrix. Furthermore, it appeared in the gradient-elution-fraction after 13 CV (13 ml) which corresponds to approximately 350 mM salt concentration of the passing elution buffer. Similar results were obtained when using the DEAE column: the rEg TGR also bound to the matrix and appeared in the gradient-elution-fraction after 7 CV (35 ml) which corresponds approximately to a salt concentration of 300 mM – to explain this result: eluted was with Tris 20 mM NaCl 400 mM, pH 8 using an increasing salt gradient where concentration of NaCl achieved its maximum within 10 CV (50 ml), thus the salt concentration after 35 ml is approximately 300mM. The results of one run with column DEAE are shown in figure 4.5.

All in all anion exchange seems to be a useful technique to purify rEg TGR and therefore should also useful to purify the TGR of *M.vogae*.



35 min=35 ml (flow rate 1ml/min)
=7 CV (1CV=5ml)

Figure 4.5: Purification of rEg TGR using an anion exchange column (column DEAE)

rose: moment of injection
green: salt concentration (linear gradient from 10 mM to 400 mM NaCl)
blue: absorption at 280 nm (protein concentration)
brown: conductance
orange: starting point of passing elution buffer
min=minutes, mAU=milli absorption unit

Binding buffer was Tris 20 mM NaCl 10 mM, pH 8; eluted was with Tris 20 mM NaCl 400 mM, pH 8 using an increasing salt gradient where concentration of NaCl achieved its maximum within 10 CV (50 ml). The graphic shows that rEg TGR could be eluted after 7 CV (35 ml).

Cation exchange chomatography

Two different cation exchange columns were used: a column Heparin HP and a Resource S column. Both with the column Heparin HP and with the column Resource S, rEg TGR did not appear in the eluted fractions but in the flow through where the activity was found (exemplary shown for the column Resource S in figure 4.6). Therefore the rEg TGR is not considered to bind to these columns and consequently cation exchange was not used for the purification of the native TGR of *M.vogae* afterwards.

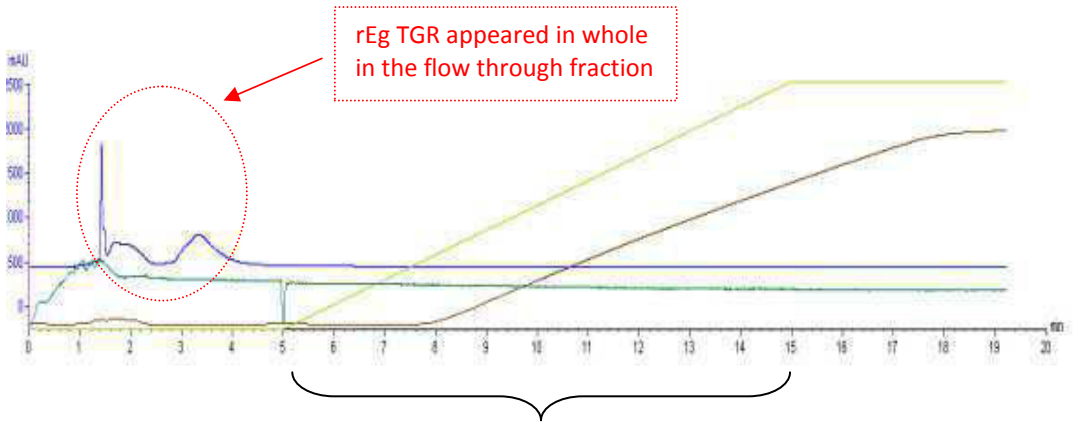


Figure 4.6: Purification of rEg TGR with cation exchange (column Resource S)

rose: moment of injection
light green: salt concentration (linear gradient from 10 to 400 mM NaCl)
blue: absorption at 280 nm (protein concentration)
brown: conductance
dark green: pressure
min=minutes, mAU=milli absorption unit

Binding buffer was Tris 20mM NaCl 10 mM, pH 8; elution buffer was performed with Tris 20mM NaCl 400mM, pH8 using an increasing salt gradient where concentration of NaCl achieved its maximum within 10 CV (10 minutes).

d) Purification of rEg TGR by hydroxyapatite chromatography

In a next step it was tested if hydroxyapatite chromatography could be an useful instrument to purify rEg TGR. As the results of the SDS-Page 12% gel (figure 4.7) show, the whole rEg TGR bound to the column (because there is no visible bound in the flow through fraction). By eluting with NaH_2PO_4 20 mM nearly nothing of the TGR appeared in the gel and only a few after eluting with NaH_2PO_4 40 mM. The biggest part of TGR was found using an elution buffer of NaH_2PO_4 80 mM and NaH_2PO_4 160 mM. Thus, rEg TGR appeared concentrated in the fractions $80_{1\text{-}2}$-$160_{1\text{-}2}$ and not distributed in all fractions. This result shows that hydroxyapatite chromatography can be used for purification of the rEg TGR and was considered encouraging. For this reason it was decided to use this method of purification also with native TGR of *M.vogae* (in combination with the other successful methods already described).

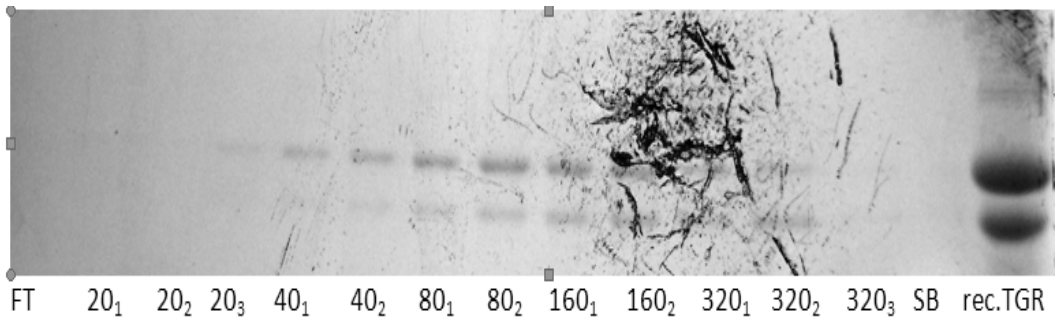


Figure 4.7: SDS-Page 12% gel after eluting rEG TGR from a hydroxyapatite column
Increasing gradients of NaH_2PO_4 wer used. rEg TGR appeared mostly in the fractions 80_1-160_2.

Marking:
FT= Flow through, rec.TGR= rEg TGR as a reference
20_1-20_3 = NaH_2PO_4 20 mM, pH 8 (each fraction quantified 0,33 ml)
40_1-40_2 = NaH_2PO_4 40 mM, pH 8 (each fraction quantified 0,5 ml)
80_1-80_2 = NaH_2PO_4 80 mM, pH 8 (each fraction quantified 0,5 ml)
160_1-160_2 =NaH_2PO_4 160 mM, pH 8 (each fraction quantified 0,5 ml)
320_1-320_2 =NaH_2PO_4 320 mM, pH 8 (each fraction quantified 0,33 ml)

4.1.4 Purification of native TGR of *M.vogae*

Taking into account the results of the optimization of TGR purification it was decided to purify the native TGR of *M.vogae* with a combination of affinity chromatography (2'5' ADP-agarose), ammonium sulfate-precipitation, anion exchange and hydroxyapatite chromatography.

a) Extraction of TR and GR activities of M.vogae by affinity chromatography

Because rEg TGR bound to the 2'5' ADP-Agarose column (see 4.1.4) it was decided to use this method as an initial step to isolate *M. vogaes* TR and GR activities: In this way it would be possible to separate nucleotide-binding proteins from other proteins present in the extract. However, when passing the *M. vogae* extract to the column, the TR activity did not bind efficiently to the matrix: instead most of it remained in the flow through (see figure 4.8). It should be mentioned that GR activity was not found in the eluted fractions either.

Concluding it was figured out that affinity chromatography using 2'5'-ADP-Agarose did not bind TR and GR activities of *M.vogae*. This was an unexpected result as discussed 5.1.6.

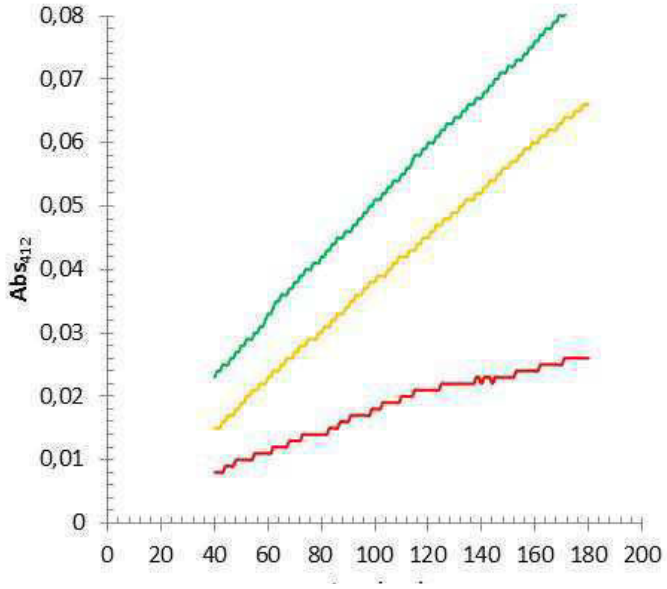


Figure 4.8: Purification of native TGR of *M.vogae* by affinity chromatography
TR activity of *M.vogae* was mostly found in the flow through fractions.

Green: Extract *M.vogae* before passing the columns
Yellow: Flow through ADP-agarose
Red: Eluted fraction ADP-agarose
Time (sec)= time in seconds, Abs_{412}=absorbance at 412nm

A 2'5' ADP agarose column was used. The assays were carried out at constant concentrations of DTNB and NADPH (5mM and 0,1mM, respectively). Reaction was started by the addition of each sample (20µ). The green and the yellow marked fractions had the same total volume (4 ml) the red fraction had a total volume of 0,2 ml. It should be mentioned that in order to avoid overlapping of the date, all (also future) curves were displaced vertically.

b) Extraction of TR and GR activities of *M.vogae* by ammonium sulfate precipitation

Referring to the results of the experiments with rEg TGR it was decided to precipitate the extract of *M.vogae* incorporating 3 steps of 20% and 40% and 85% of $(NH_4)_2SO_4$. The detection of TR with the TR assay for each fraction is shown in figure 4.9.

Table 4.1 below sums up the activity recovered in each step of the salt fractionation, and compares those values with the activity of the initial extract. All in all the results show that some TR activity was found in the 20%-pellet fraction, whereas more activity was found in the 20-40% pellet-fraction. Interestingly, TR activity was also found in the 40-85% pellet-fraction. Thus, it can be said that not all TR and GR activities precipitated

with 40% of $(NH_4)_2SO_4$ as expected from the results with rEg TGR. Therefore, the salt fractionation scheme was changed to avoid leaving TR activity in the 20 % pellet fraction and to precipitate all the activity in the second step. Thus, a new extract was precipitated sequentially at 25%, 50% and 85%.

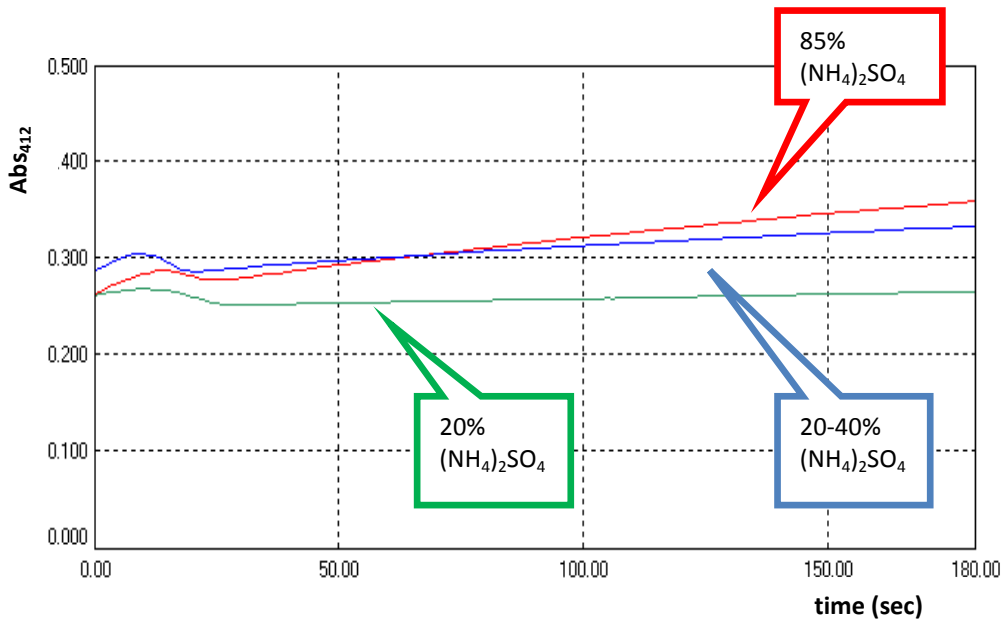


Figure 4.9: TR activities of *M.vogae* extracts after salt precipitation
TR activity was mostly found in the 20-40%- and 85% -pellet fraction.

The assays were carried out at constant concentrations of DTNB and NADPH (5 mM and 0,1 mM, respectively). Reaction was started by the addition of the re-dissolved precipitated fractions (10 µl). All fractions had the same total volume of 5 ml.
time (sec)=time in seconds Abs_{412}*=absorbance at 412 nm*

Measured sample (10µl)	Total Volume	Measured Δ[Abs]/min and total Δ[Abs]/min	Δ[NADPH]/min
Extract	5 ml	0,072×5=0,36	13,24 µM
Pellet 20%	5 ml	0,006×5=0,03	1,10 µM
Pellet 20-40%	5 ml	0,018×5=0,09	3,30 µM
Pellet 85%	5 ml	0,030×5=0,15	5,52 µM

Table 4.1: Measured TR activity and resulting values referring to figure 4.9.

TR activities for all steps of precipitation with $(NH_4)_2SO_4$ carried out with 25%, 50% and 85% are shown in Fig 4.10. The table 4.2 below sums up the activity recovered in each step of the salt fractionation, and compares those values with the activity of the initial extract. To be able to carry on with the purification of *M.vogae* TR and GR activities from the extract and to measure the TR and GR activity again with a lower concentration of salt, a PD-10 Desalting column was used to desalt the 25-50% pellet fraction which is also performed in Fig 4.10.

The results indicate that negligible activity was present in the pellet of the 25% fraction and in the pellet of the 50-85% fraction. Virtually all the activity was recovered in the 25-50% pellet-fraction.

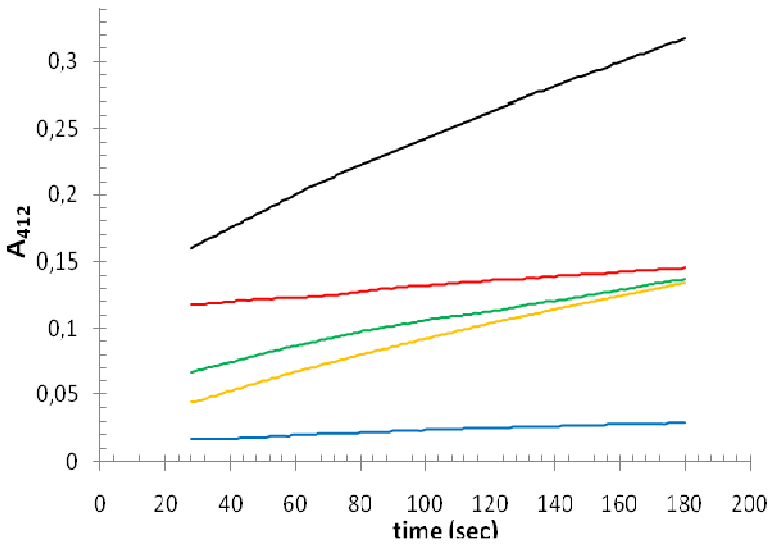


Figure 4.10:TR activities of *M.vogae* extracts after salt precipitation
TR activity was mostly found in the 25-50% pellet-fractions

Green: Extracts *M.vogae* before the precipitation
Red: Pellet 25% $(NH_4)_2SO_4$
Black: Pellet 25-50% $(NH_4)_2SO_4$ before PD10
Yellow: Pellet 25-50% $(NH_4)_2SO_4$ after PD10
Blue: Pellet 50-85% $(NH_4)_2SO_4$
Time (sec)= time in seconds A_{412}=absorbance at 412 nm

The assays were carried out at constant concentrations of DTNB and NADPH (5mM and 0,1mM respectively) Reaction was started by the addition of the re-dissolved precipitated compounds (20 µl). The total volume of each fraction can be seen in the table 4.2 below.

Measured sample (20 µl)	Total Volume	Measured Δ[Abs]/min and total Δ[Abs]/min	Δ[NADPH]/min
Extract before the purification	5,5 ml	0,027×5,5 = 0,149	5,48 µM
Pellet 25%	1 ml	0,013×1 = 0,013	0,48 µM
Pellet 25-50% before PD 10	2,5 ml	0,062×2,5 =0,155	5,57 µM
Pellet 25-50% after PD10	3,5 ml	0,037×3,5 =0,130	4,78 µM
Pellet 50-85%	3,5 ml	0,005×3,5 =0,018	0,64 µM

Table 4.2: Measured TR activities and resulting values referring to figure 4.10 above.

To evaluate if the increase of purity of *M. vogae* TR was accompanied by comparatively the same increase of purity GR activity, the GR activity of each precipitation step was measured with the GR assay. As shown in figure 4.11, the extracts of *M.vogae* had GR-activity just as the 25-50% pellet fraction whereas only marginal activity was found in the 25%-pellet fraction and the 50-85%-pellet fraction. The corresponding measured Δ[Abs]/min and the calculated Δ[NADPH]/min are shown in table 4.3 which is performed in the same manner as table 4.2 to enable the comparison of the data.

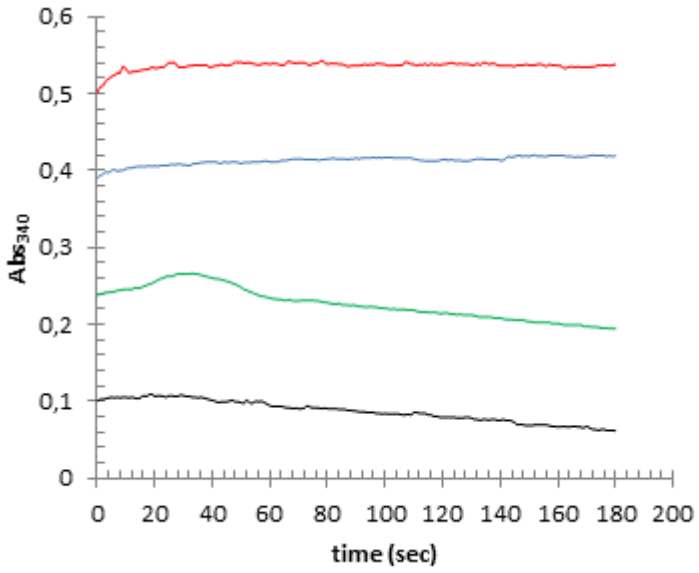


Figure 4.11: GR activities of *M.vogae* extracts after salt precipitation
GR activity was mostly found in the 25-50% pellet fraction.

Green: *M.vogae extracts*
Red: Pellet 25% $(NH_4)_2SO_4$
Black: pellet 25-50% $(NH_4)_2SO_4$
Blue: pellet 50-85% $(NH_4)_2SO_4$
Time (sec)= time in seconds A_{412}=absorbance at 412 nm

The assays were carried out at constant concentrations of GSSG (0,3 mM), GSH (0,3 mM) and NADPH (0,1 mM). Reaction was started by the addition of the precipitated compounds (40 µl).
The total volume of each fraction can be seen in the table 4.3 below.

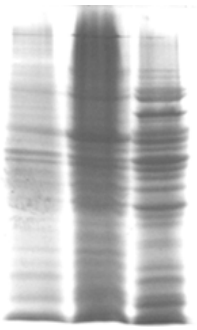
Measured sample (40µl)	Total Volume	Measured Δ[Abs]/min and total Δ[Abs]/min	Δ[NADPH]/min
Extract before the purification	5,5 ml	0,018×5,5 = 0,099	15,97 µM
Pellet 25%	1 ml	0	0µM
Pellet 25-50% after PD10	3,5 ml	0,024×3,5 =0,084	13,55 µM
Pellet 50-85%	3,5 ml	0	0 µM

Table 4.3: Measured GR activity and resulting values referring to figure 4.11.

Drawing a comparison of the TR/GR coefficient (referring to the NADPH consumption in each case) before and after the precipitation with $(NH_4)_2SO_4$ it is clear that the TR and GR activity measured within the purification changed in the same ratio:

$$coeff.\frac{TR}{GR} = \frac{TR}{GR} \leftrightarrow \underbrace{\frac{5,48}{15,97} = 0,34}_{\text{Unpurified } M.vogae \text{ extracts}} \leftrightarrow \underbrace{\frac{4,78}{13,55} = 0,35}_{\text{25-50\% pellet fraction}}$$

To get an idea of the grade of pureness of *M.vogae* extracts (and to be able to compare the pureness after more steps of purification) the results of SDS-Page 12% gel is shown below (figure 4.12). As it can be appreciated more proteins precipitate in the 25-50 % fraction than in the 25% or 50-85% fractions. Although there are many proteins in the 25-50% fractions, there was enrichment in TR and GR activities, since they are absent in the 25% or 50-85% fractions.



1 2 3

Figure 4.12: SDS-Page 12% gel after salt precipitation of *M.vogae* extracts
Many proteins still remain in the 25-50% pellet fraction

Lane 1: 25% pellet fraction
Lane 2: 25-50% pellet fraction
Lane 3: 50-85% pellet fraction

c) Extraction of TR and GR activities of *M.vogae* by anion exchange chromatography

Taking into account the results of the optimization of the purification of rEg TGR (see 4.1.3) the next step of purification was performed by anion exchange chromatography using a Mono Q column.

M.vogae TGR was purified using the column Mono Q although the tests with the column DEAE (with rEg) were successful, too. This was justified because the segregation of proteins using Mono Q or DEAE is based on the same principles (both are anion exchangers) so that the extract would not be cleaner by using both columns consecutively. As figure 4.13.a below shows, the column Mono Q is capable to separate TR and GR activities from *M.vogae* extracts. The TR and GR activity bound to the Mono Q and elute with a salt concentration of 400 mM (the salt concentration was increased step by step using a linear gradient which achieved 100% (=400 nm) within 10 CV).

The results of the DTNB Reduction Assay and one corresponding value of GR detected by the GR assay can be seen in figure 4.13.b. Furthermore the corresponding values of

all eluted fractions are illustrated in table 4.4 which is performed in the same way as the tables shown before.

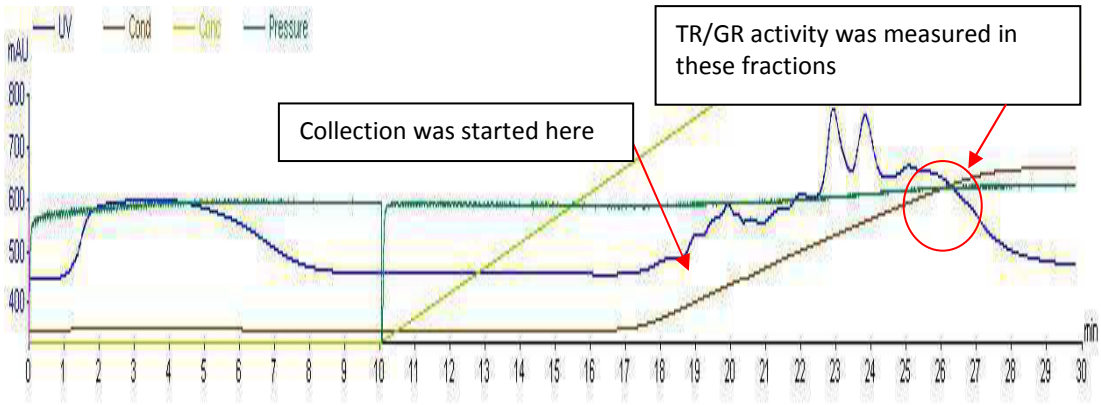


Figure 4.13.a: Purification of native TGR of *M.vogae* by anion exchange (column Mono Q).
rose: moment of injection
dark green: pressure
light green: salt concentration (linear gradient from 10 to 400 mM NaCl)
blue: absorption at 280 nm (protein detection)
brown: conductance
min=minutes, mAU=milli absorption unit

Binding buffer was Tris 20 mM NaCl 10 mM, pH 8; eluted was with Tris 20 mM NaCl 400 mM, pH 8 using an increasing salt gradient where concentration of NaCl achieved its maximum within 10 CV (10 ml).

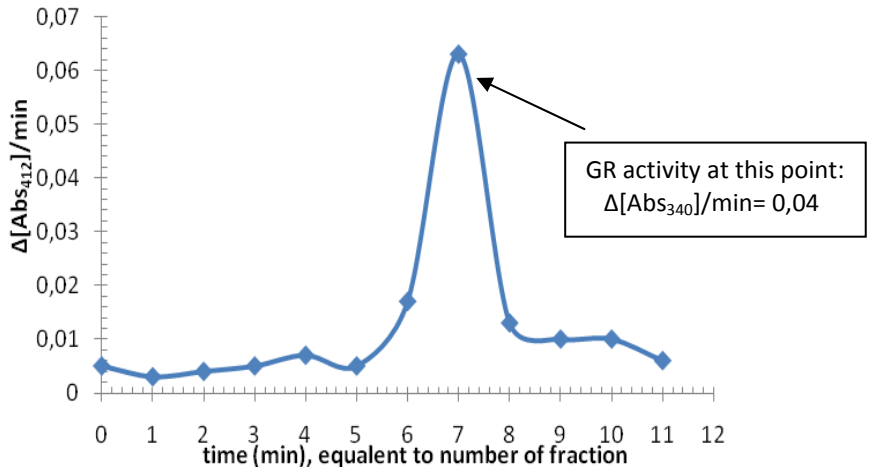


Figure 4.13.b: TR activity denoted in $\Delta[Abs_{412}]$/min after purification by anion exchange (column Mono Q).
The assays were carried out at constant concentrations of DTNB and NADPH (5 mM and 0,1mM, respectively). Reaction was started by the addition of each sample (20 µl). The flow through fraction is named as 0. Furthermore the measured GR activity for fraction 7 is also named. In this case the assay was carried out at a concentration of GSSG of 0,3 mM, GSH 0,3 mM and NADPH 0,1 mM. Reaction was started by the addition of the sample (40 µl). The flow through had a total volume of 3,5 ml, all the other fractions had a total volume of 1 ml. All corresponding values are listed in the table 4.4 below.

Measured sample (20 µl)	Total Volume	Measured Δ[Abs]/min and total Δ[Abs]/min	Δ[NADPH]/min
semi-purified extract before passing Mono Q	3,5 ml	0,037×3,5 =0,130	4,78 µM
Flow through	5 ml	0,001×5 =0,005	0,18 µM
1st eluted fraction	1 ml	0,003×1 =0,003	0,11 µM
2nd eluted fraction	1 ml	0,004×1 =0,004	0,15 µM
3rd eluted fraction	1 ml	0,005×1 =0,005	0,18 µM
4th eluted fraction	1 ml	0,007×1 =0,007	0,64 µM
5th eluted fraction	1 ml	0,005×1 =0,005	0,18 µM
6th eluted fraction	1 ml	0,017×1 =0,017	0,63 µM
7th eluted fraction	1 ml	0,063×1 =0,063	2,32 µM
8th eluted fraction	1 ml	0,013×1 =0,013	0,48 µM
9th eluted fraction	1 ml	0,010×1 =0,010	0,37 µM
10th eluted fraction	1 ml	0,010×1 =0,010	0,37 µM
11th eluted fraction	1 ml	0,006×1 =0,006	0,22 µM

Table 4.4: Measured TR activities and resulting values referring to figure 4.13.b.

The 7th fraction contains the major part of TR- and GR activity The TR/GR coefficient before and after this step of purification (referring to the NADPH consumption in each case) was calculated again.

Furthermore the coefficient of the unpurified extract of *M.vogae* is listed again to get a general idea of its value during the whole purification:

$$coeff.\frac{TR}{GR} = \frac{TR}{GR} \leftrightarrow \underbrace{\frac{5,48}{15,97} = 0,34}_{\text{Unpurified } M.vogae \text{ extracts}} \leftrightarrow \underbrace{\frac{4,78}{13,55} = 0,35}_{\text{25-50\% pellet fraction}} \leftrightarrow \underbrace{\frac{2,32}{6,45} = 0,35}_{\text{Fraction 7 of anion exchange}}$$

As the calculation shows, the coefficient TR/GR was always nearly the same in all steps of purification respectively that TR and GR co-purified in all steps of purification. Furthermore the analysis in SDS-Page 12% gel (see figure 4.14) shows that the extract of *M.vogae* was highly cleaner after the purification by anion exchange in comparison to its grade of pureness before passing the column Mono Q (see figure 4.12).

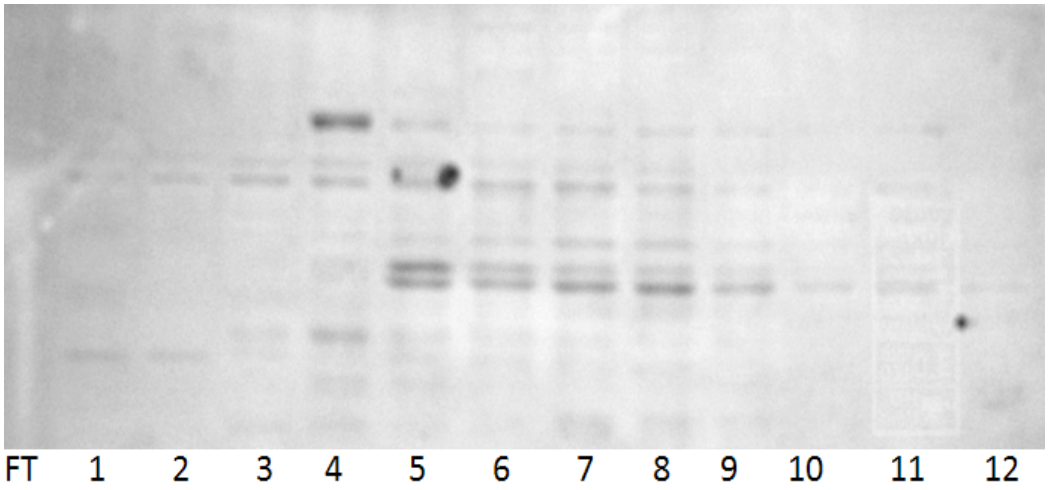


Figure 4.14: Grade of pureness of semi-purified extracts of *M.vogae* is displayed in a SDS-Page gel 12%
After applying on the Mono Q matrix the eluted fractions (named 1-12) and the flow through (FT) were analyzed in SDS-Page gel 12%

d) Extraction of TR and GR activities of *M.vogae* by hydroxyapatite chromatography

As a next purification step, hydroxyapatite chromatography was intended. Therefor the fractions of ion exchange chromatography possessing the highest TR and GR activity (fractions 6, 7, 8) were pooled and desalted with a PD10-column.

Although there was activity in nearly all eluted fractions, most activity was found in the flow through fraction which strongly points out that the TR activities of *M.vogae* did not bind efficiently to the hydroxyapatite-column. Indeed, the part of the TR activity which bound to the column represents 35% of the total recovered TR activity. The values can be seen in table 4.5.

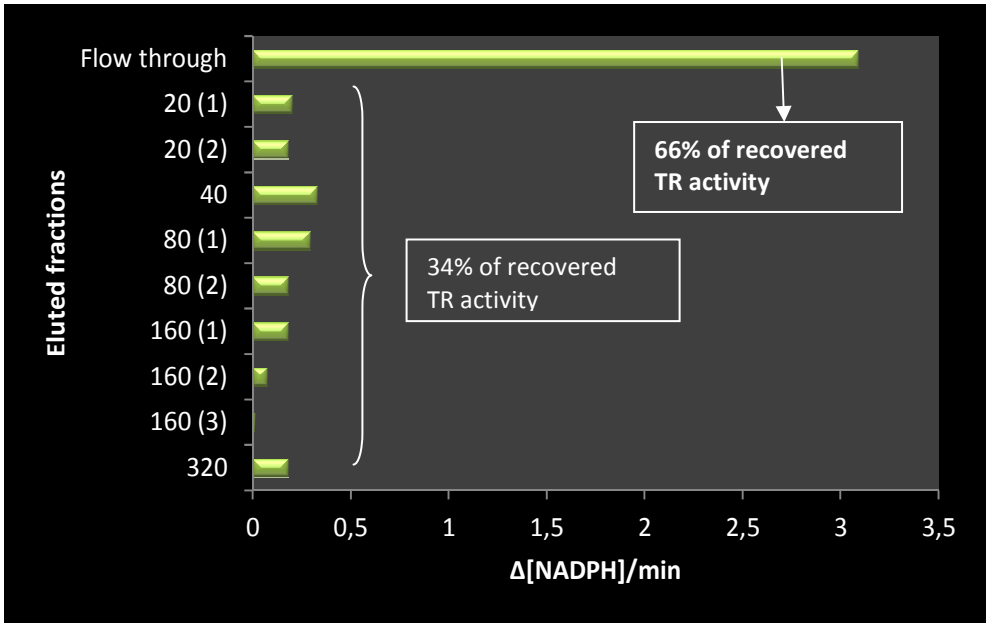


Figure 4.15: TR activities of *M.vogae* extracts after passing a column of hydroxyapatite. Eluted was with increasing concentrations of (NaH_2PO_4): 20 nM -320 nM which are named as fraction 20 -320 in the graphic. The corresponding volumes of each fraction and the exact data can be found in the table 4.5 below.
The assays were carried out at constant concentrations of DTNB and NADPH (5 mM and 0,1 mM respectively) Reaction was started by the addition of each sample (50 µl).

Measured sample (50 µl)	Total Volume	Measured Δ[Abs]/min and total Δ[Abs]/min	Δ[NADPH]/min
Flow through fraction	3,5 ml	0,024×3,5=0,084	3,09 µM
(NaH_2PO_4) 20 mM first fraction	0,5 ml	0,014×0,5=0,007	0,2 µM
(NaH_2PO_4) 20 mM second fraction	0,5 ml	0,01×0,5=0,005	0,18 µM
(NaH_2PO_4) 40 mM first fraction	0,5 ml	0,018×0,5=0,009	0,33 µM
(NaH2PO4) 80 mM first fraction	0,5 ml	0,016×0,5=0,008	0,29 µM
(NaH2PO4) 80 mM second fraction	0,5 ml	0,010×0,5=0,005	0,18 µM
(NaH2PO4) 160 mM first fraction	0,4 ml	0,012×0,4=0,005	0,18 µM
(NaH2PO4) 160 mM second fraction	0,4 ml	0,004×0,5=0,002	0,07 µM
(NaH2PO4) 160 mM third fraction	0,4 ml	0,008×0,4=0,003	0,01 µM
(NaH2PO4) 320 mM	1 ml	0,005×1=0,005	0,18 µM
			4,71
			(Total sum of Δ[NADPH]/min)

Table 4.5: Measured TR activities and resulting values referring to figure 4.15.

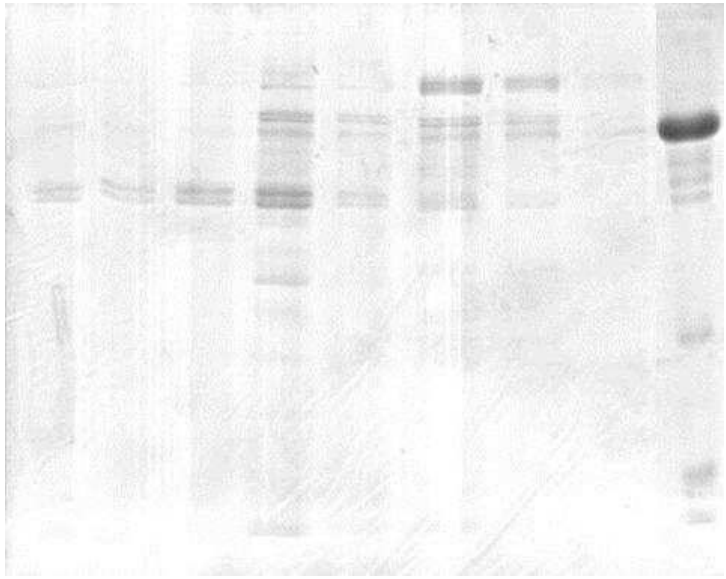
Furthermore, another observation should be noted: As described in Material and Methods (see 3.5.4), the pooled and desalted fractions of ion exchange chromatography which possessed the highest TR and GR activity were used. The measured total Δ[NADPH]/min of these fractions was 3,43 (0,63+2,32+0,48 see table). Comparing this value with the sum of the in table 4.5 above named fractions (4,71 ×

2/5[1] = 1,88) it is getting clear that even most of the activity was not recovered from the column.

Nevertheless a SDS-Page 12% gel (see figure 4.16) was carried out. This gel shows that a high grade of pureness was achieved in this purification step compared to the gel after ion exchange chromatography (see figure 4.14)

Altogether because the TR activity appeared in such a unexpected manner (a bigger part didn't bind to the column and most of the bound activity was not recovered from the column) it was decided not to measure the GR activity and for further experiments only the partially purified extract of *M.vogae* was used: the fraction obtained after a combination of salt precipitation and anion exchange.

[1] It is important to highlight that the TR assays were carried out with 50 µl of each eluted fraction (instead of 20 µl as it was done the experiments before) consequently the resulted sum has to be multiplied by 2/5.)



FT 20_1 20_2 40 80_1 80_2 160_1 160_2 rEG TGR

Figure 4.16: Grade of pureness of semi-purified extracts of *M.vogae* is displayed in a SDS-Page gel 12%
After applying *M.vogae* extract on a hydroxyapatite column, elution was performed with increasing concentrations of NaH_2PO_4 and each eluted fraction was analyzed in SDS-Page 12% gel.

FT= flow through (total volume 3,5 ml)
20_1= eluted was with (NaH_2PO_4) 20 mM, Ph 8 (total volume 0,5 ml)
20_2= eluted was with (NaH_2PO_4) 20 mM, Ph 8 (total volume 0,5 ml)
40= eluted was with (NaH_2PO_4) 40 mM, Ph 8 (total volume 0,5 ml)
80_1= eluted was with (NaH_2PO_4) 80 mM, Ph 8 (total volume 0,5 ml)
80_2= eluted was with (NaH_2PO_4) 80 mM, Ph 8 (total volume 0,5 ml)
160_1= eluted was with (NaH_2PO_4) 80 mM, Ph 8 (total volume 0,4 ml)
160_2= eluted was with (NaH_2PO_4) 80 mM, Ph 8 (total volume 0,4 ml)
In the right line rEg TGR is shown to have an idea which band is the TGR of *M.vogae*. It should be notice though, that a smaller band than rEg TGR would be expected for native *M.vogae*, since the former encodes for a 4 kDa leader absent in the native enzyme.

4.1.5 Semi-purified extracts of *M.vogae* exhibit hysteretic behavior

To complete the observations of hysteresis existence in the extracts of (unpurified) *M.vogae* (4.1.2) it was figured out if this behavior also recurs in the semi-purified extracts of *M.vogae* (this is the fraction eluted from Mono Q, i.e. after salt fractionation and anion exchange).

Therefore the GR Assay in a modified form was used. On this occasion the concentration of GSH in the buffer was increased step by step (10 µm, 20 µm, 50 µm, 100 µm, 300 µm) to see if, and under what conditions hysteresis disappears. Furthermore, it was tested if the hysteretic behavior can be influenced using no GSH and a high concentration of GSSG instead ("high" in comparison with the conventional GR assay, in this case 600 µM).

Figure 4.17 shows the result of the modified GR assays: It was verified that the semi-purified extracts also exhibited hysteretic behavior. The hysteretic activity became evident at a concentration of GSH lower than 20 µM (and with a concentration of GSSG 300 µM) or with a high concentration (600 µM) of GSSG (and absence of GSH). These results confirm the observation of the hysteresis existence in unpurified extracts (4.1.2) and reinforce the hypothesis that *M.vogae* possesses TGR.

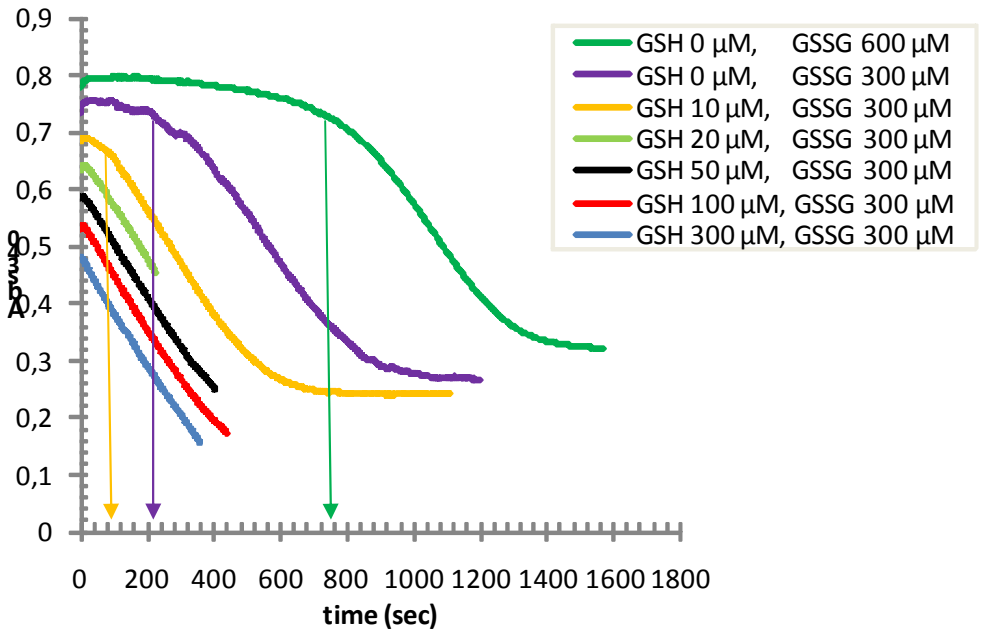


Figure 4.17: Hysteretic behavior of GR activity of semi-purified extracts of *M.vogae*
In all cases the reaction was started by the addition of 30 µl of semi-purified *M.vogae*s extracts at the indicated final concentrations of GSSG and GSH. All assays were carried out at a concentration of NADPH of 100 µM.
The arrows marc approximately the length of the hysteresis.
Abs_{340}=absorbance at 340nm, time (sec) = time in seconds

4.1.6 TR and GR activities of *M.vogae* can be inhibited with the gold compound auranofin

To verify the existence of TGR in *M.vogae*, inhibition experiments using the organogold compound auranofin (aura) were performed. This drug is known as Sec-containing TR and -TGR inhibitor (Gromer et al., 1998). Its chemical structure can be seen in figure figure 3.3. It was tested if TR and GR activities of *M.vogae* extracts could be inhibited by auranofin. If doing so it would be a strong indication of Sec-containing TGR like in *E.granulosus* (see 1.4). The anion exchanged semi-purified extracts of *M.vogae* were used again. The grade of inhibition was tested using the TR and GR assay.

As result, it was possible to inhibit both TR and GR activity of the semi-purified extract of *M.vogae* with different concentration of the gold compound. TR activity of the extract was inhibited completely under a concentration of auranofin 1 nM, whereas 75% remaining activity was observed at a 0,5 nM concentration of auranofin.

The results of the inhibition of the GR activity (which is crucial to differ if *M.vogae* possesses particular TR or TGR,see discussion 5.1.5) are shown in table 4.6 and the corresponding figure 4.18 where the corresponding values for TR activity can also be found. 58,3% remaining GR activity was detected within a concentration of 0,5 nM auranofin. However it requires a concentration of 10 nM auranofin to achieve nearly a complete inhibition of GR activity (2,7 % remaining GR activity). Conventional GRs are not inhibited at a nM range with auranofin, but at µM range – usually between three to four order of magnitude higher concentration are required to inhibit conventional GR when compared with TR (Gromer, 1998). Furthermore the fact that similar concentrations of auranofin were required to inhibit TR and GR activity strongly suggest that both activities are dependent on Sec, and therefore derive from a TGR.

Concentration auranofin	Δ[Abs/min]	ΔNADPH/min	Remaining activity
0 nM	0,036	5,80 µM	100%
0,5 nM	0,021	3,39 µM	58,3%
1 nM	0,018	2,90 µM	50%
2 nM	0,014	2,26 µM	38,8%
6 nM	0,012	1,94 µM	33,3%
8 nM	0,007	1,13 µM	19,4%
10 nM	0,001	0,16 µM	2,7%

Table 4.6: Δ[Abs/min], resulting ΔNADPH/min and remaining GR activity after inhibition of GR activity with different concentrations of auranofin. The table corresponds to figure 4.18 below.

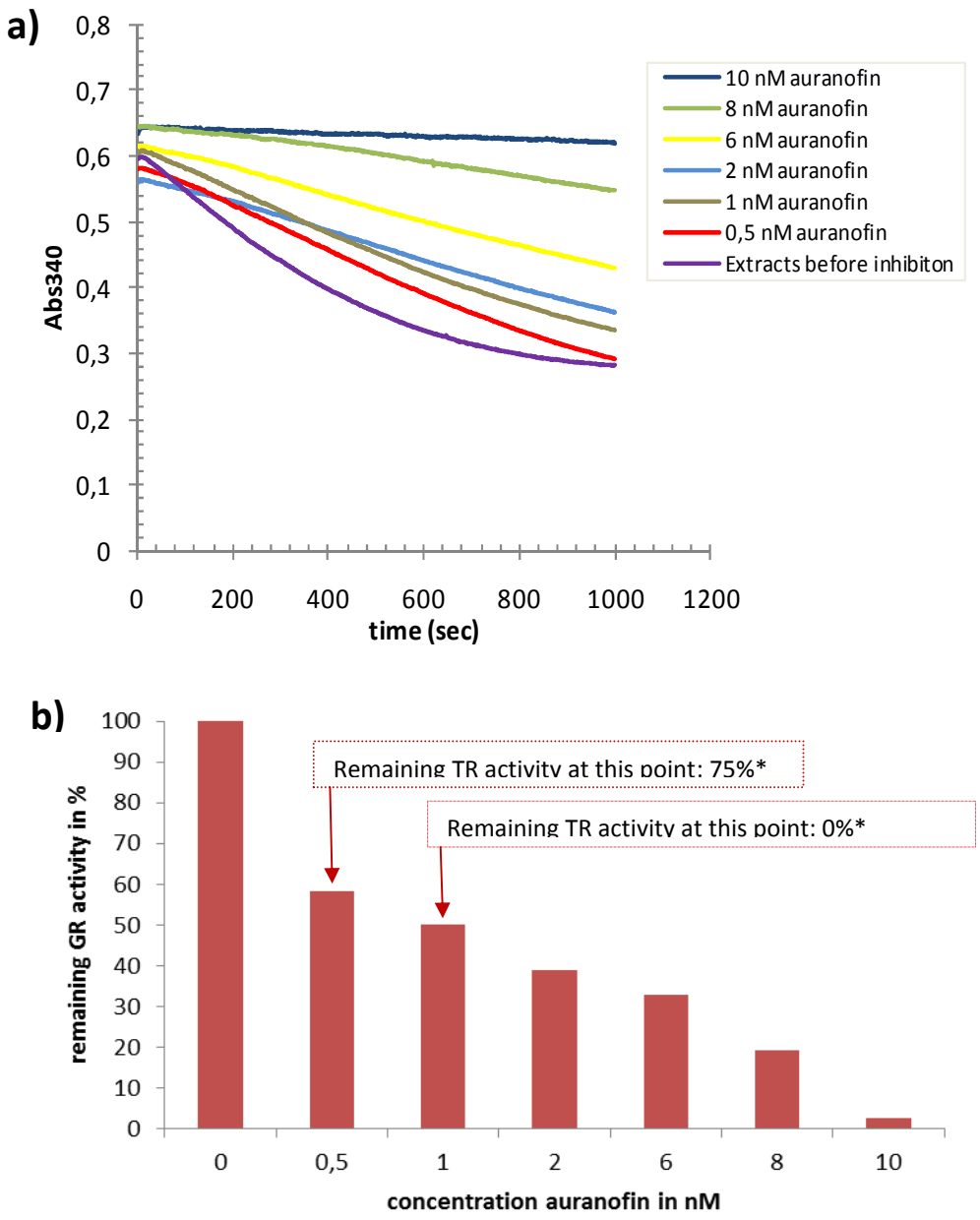


Figure 4.18: Inhibition of GR activity with different concentrations of auranofin and corresponding values of remaining TR activity (arrows).

a) GR assay was carried out at constant concentrations of GSSG (0,06 mM), NADPH (0,1 mM) and without GSH. Reaction was started by addition of GSSG (after a preincubation time of 3 minutes)

b) There was only 2,7% remaining GR activity measured under a concentration of 10 nM auranofin whereby there was still 58,3% remaining GR activity at a 0,5 nM concentration of auranofin.

* TR assay was carried out at constant concentration of DTNB (0,1 mM) and NADPH (0,1 mM). Reaction was started by the addition of DTNB after a preincubation time of 3 minutes.

4.2 TGR activity of *M.vogae* can be inhibited by recently identified inhibitors of flatworm TGR.

After performing the experiment with auranofin, it was tested if recently identified TGR inhibitors of *E.granulosus* are able to inhibit TR and GR activities in the semi-purified extracts. Therefore VL16E and WPQ75 were chosen as these compounds were already identified as potent inhibitors of *E.granulosus* TGR that killed protoscolex stage (larvae of *E.granulosus*) *in vitro* (see introduction1.4).

Because all results in 4.1 strongly pointed out that *M.vogae* possesses TGR instead of separate TR and GR, it was decided to test the inhibition with VL16E and WPQ75 mainly with the TR activity to be sure that no hysteretic behavior could disturb the experiment. Nevertheless a few control samples with GR were also carried out. It should be mentioned that the inhibition effect of different concentrations of VL16E and WPQ75 to *M.vogaes'* TGR was only tested one time. As seen in the figures 4.19 (corresponding to table 4.7) and figure 4.20 (corresponding to table 4.8) a total inhibition of the TR activity was achieved within a concentration of 10 µM of each tested inhibitor. These results are consistent with the needed concentration of WPQ75 and VL16E to achieve 100% inhibition of the TGR activity of *E.granulosus*. (Ross and Salinas, 2010).

4.2.1 TGR activity of *M.vogae* can be inhibited with VL16E

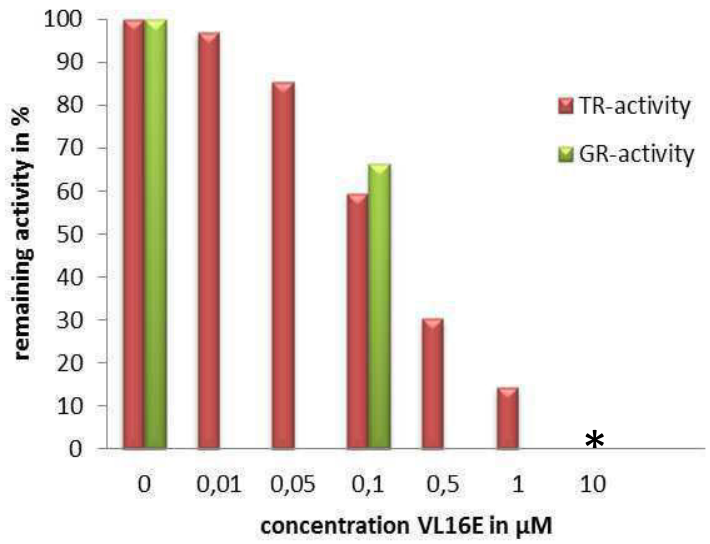


Figure 4.19: Inhibition of *M. vogae* TR activities by VL16E
The graphic displays the remaining TR-activity under different concentrations of VL16E. Assays were carried out at constant concentrations of NADPH (0,1 mM) and DTNB (0,1 mM). Reaction was started by addition of DTNB (after 3 minutes preincubation)
Green balk: A control sample using the GR assay was also carried out at constant concentrations of GSSG (0,06 mM), NADPH (0,1 mM) and without GSH. Reaction was started by addition of GSSG (after 3 minutes preincubation).
Note: This experiment was only carried out one time.

*There was neither found TR nor GR activity under a concentration of 10 µM VL16E.

Concentration VL16E	Δ[Abs/min]	ΔNADPH/min	Remaining activity
0	0,069	2,54 µM	100%
0,01	0,067	2,46 µM	97,1 %
0,05	0,059	2,17 µM	85,6 %
0,1	0,010	0,37 µM	59,4%
0,5	0,021	0,77 µM	30,4%
1	0,010	0,37 µM	14,5%
10	0	0 µM	0 %

Table 4.7: Measured Δ[Abs/min], resulting ΔNADPH/min and remaining TR activity after inhibition of TR activity with different concentrations of VL16E. Table corresponds to Figure 4.19 above.

4.2.2 TGR activity of *M.vogae* can be inhibited with WPQ75

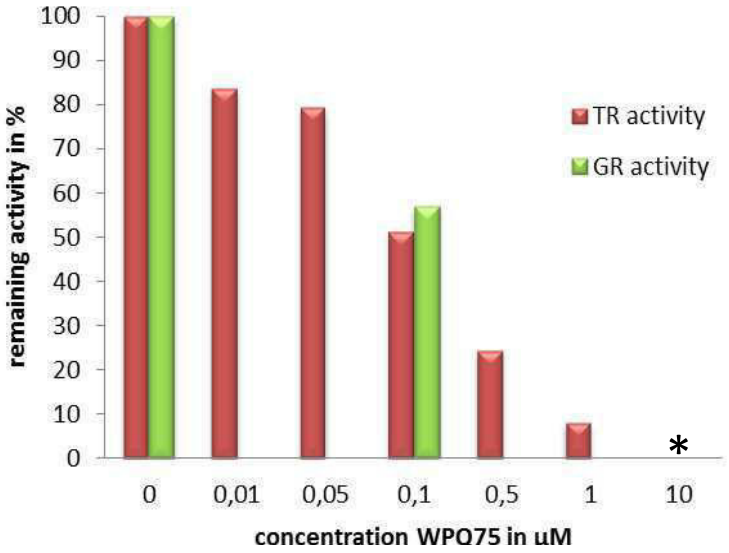


Figure 4.20: Inhibition of *M. vogae* TR activities by WPQ75
The graphic displays the remaining TR-activity under different concentrations of WPQ75
Assays were carried out at constant concentrations of NADPH (0,1 mM) and DTNB (0,1 mM). Reaction was started by addition of DTNB (after 3 minutes preincubation)
Green balk: A control sample using the GR assay was also carried out at constant concentrations of GSSG (0,06 mM), NADPH (0,1 mM) and without GSH. Reaction was started by addition of GSSG (after 3 minutes preincubation).
Note: This experiment was only carried out one time.

*There was neither found TR nor GR activity under a concentration of 10 µM WPQ75

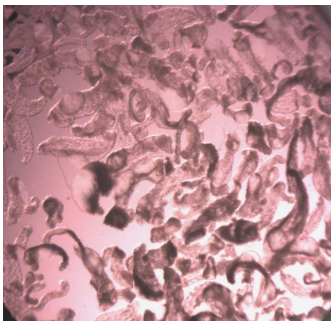
Concentration WPQ 75	Δ[Abs/min]	ΔNADPH/min	Remaining activity
0 µM	0,074	2,721 µM	100%
0,01 µM	0,062	2,279 µM	83,8 %
0,05 µM	0,059	2,169 µM	79,7 %
0,1 µM	0,038	1,397 µM	51,3%
0,5 µM	0,018	0,662 µM	24,3%
1 µM	0,006	0,221 µM	8,1%
10 µM	0	0 µM	0 %

Table 4.8: Measured Δ[Abs/min], resulting ΔNADPH/min and remaining TR activity after inhibition of TR activity with different concentrations of WPQ75 Table corresponds to Figure 4.20 above.

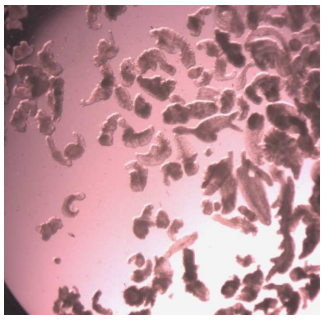
4.3 Recently identified TGR inhibitors are able to kill *M.vogae* larval worms *in vitro*

Since the previous experiments clearly indicated that *M. vogae* TGR can be inhibited by recently identified inhibitors such as VL16E and WPQ75, the effect of these compounds on in vitro cultivated parasite larvae was tested. An overview over the tested inhibitors is seen in figure 4.21:

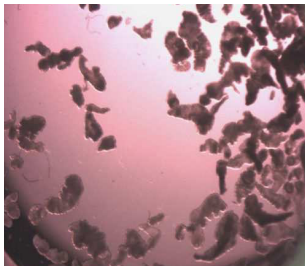
Praziquantel (a) as reference drug for adult flatworms, auranofin (b) as reference for the inhibition of Sec-containing TGR, VL16E (c) and WPQ75 (d) as two of the recently identified inhibitors of rEg TGR (Ross and Salinas, 2010) and *M. vogae* TGR (this work) were tested as drugs at 20 µM against *in vitro* culture of *M.vogae* larval worms. Viability was measured by visual inspection of their mobility. All groups of worms showed reduced mobility after 6 hours of culture. After 12 hours there was no movement at all observed in group a), b) and c), and *M.vogae* larval worms died which was assumed because there wasn't observed movement after 24 hours neither. The worms of the group treated with WPQ75 moved less after 12 hours but instead of dying they recuperate and showed full mobility again after 24 hours (the medium was not changed which means that the worms were all the time incubated with the start-medium of the respective drug). Hence, the used concentration of WPQ75 seems to be too low as discussed in 5.3. The change of mobility of each group can be seen in attachment 1 (CD-Rom).



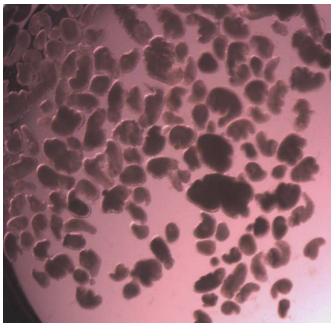
a) M.Vogae with praziquantel



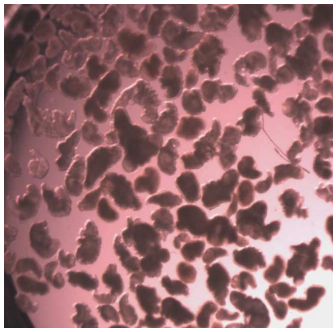
b) M.Vogae with auranofin



M.vogae without any drug treatment



c) M.Vogae with VL16E



d) M.Vogae with WPQ75

Figure 4.21: *In vitro* assessment of recently identified TGR inhibitors on *M.vogae* larval worms
Movement can be seen in attached CD-Rom. All drugs were used at a 20 µM concentration. The pictures show *M.vogae* after 12 hours incubation**.**
The worms in a), b) and c) lost all mobility during this time, only the worms in d) stayed alive and could recuperate after 24 hours. The picture at the right shows *M.vogae* without any drug treatment.

4.4 TGR inhibitors are also able to kill *M.vogae* in experimental infection in mice.

As already mentioned in Material and Methods (3.8) this experiment was performed by an external laboratory (Laboratorio de Experimentación Animal, Departamento de Ciencias Farmacéuticas, Facultad de Química, Universidad de la República, Montevideo, Uruguay) instructed by the author of this work. These series of experiments were carried out both with praziquantel (to have a well explored reference with this effective drug against flatworms (Saldaña et al., 2003) and VL16E.

The *in vivo* experiments were carried out with three groups of mice (each group consisted of 8 mice and were named group 1, 2 and 3). Each group was infected with the tetrathyridia stage of *M.vogae*. On day 14, 15 and 16 post-infection, group 1 received 0,25 ml of vehicle (the solvent in which praziquantel and VL16E were dissolved: 5% dimethyl sulfoxide in phosphate buffered saline (PBS)); group 2 received praziquantel (20mg/kg); group 3 received VL16E (20 mg/kg, equimolar dosis to that of praziquantel because both compounds have similar molecular weight). The mice were killed on day 17 post-infection and the increase of the worm burden was determined. Here it was figured out that the percentage of reduction (which means the value of reduction of worm burden in each group in comparison with the group of control which was treated with the vehicle) of VL16E corresponded to the group treated with praziquantel (27% and 28% respectively). This strongly indicates that the effectiveness of VL16E is equal to that of praziquantel.

This exemplify run with VL16E shows, that this recently identified inhibitor is also be able to kill *M.vogae in vivo*.

5. Discussion

As previous work showed, the TGR-System is a promising target for the development of new drugs against flatworm infection. This was stated due to the differences in the ROS detoxification systems of flatworms and their eukaryotic host. As this work referred to the recently found TGR inhibitors of *E.granulosus*, it had to be dealt with the problem, that *E.granulosus* is not a useful cestode parasite for *in vivo* assessment of drugs. Indeed, *E. granulosus* experimental infection in mice takes about 10 month to develop into cysts. Therefore this work dealt with the question if *M.vogae* could be used as infection model to test recently identified inhibitors of *E.granulosus'* TGR.

Therefor three main questions were addressed in this work:

1) Does *M.vogae* possess TGR as the single enzyme that controls the pathways of thiol-dependent redox homeostasis and in doing so does it conform to the biochemical scenario proposed for other platyhelminth parasites? This question will be discussed in section 5.1.

2) If *M.vogae* redox homeostasis is fully dependent on TGR, is it possible to inhibit TGR activity of *M.vogae* with recently identified TGR-inhibitors of *E.granulosus*? This question will be discussed in section 5.2.

3) And finally can *M.vogae* be killed with these inhibitors *in vitro* (5.3) as well as *in vivo* (5.4)?

5.1 *M.vogae* possesses TGR

Several evidences in this work strongly indicate that *M.vogae* possesses a TGR system which is similar to these of other flatworm parasites:

5.1.1 *M.vogae* extracts possess TR and GR activity

It was assured that *M.vogae* exhibits TR and GR activity. Important to say is that in the first experiments using the conventional GR assay (Carlberg and Mannervik, 1985) no GR activity was detected. It was assumed that the absence of GR activity has derived

from the hysteretic behavior typical of the GR activity of flatworm TGRs at the standard conditions of the assay (Guevara-Flores et al., 2011). Hysteresis means literally to lag behind, and therefore there is a lag time before the steady state velocity is reached (Rendón et al., 2004). The time lag depends on the conditions (concentration of substrate, product and enzyme); in the case of flatworm TGRs, the GR activity is temporarily hysteretic at high GSSG concentrations, low GSH concentration and low enzyme concentration. Until now the biochemical reason for this phenomenon is unknown. Hysteretic behavior has been associated with changes in the conformation and/or oligomerization in response to substrates, products, or modifiers (Frieden, 1970). Bonilla et al. (2008) did not detect changes in the oligomerization state of TGR while incubating the enzyme with high concentrations of GSSG. Based on glutathionylation of Cys_{88} they postulated that the hysteresis correlates to the glutahionylation state of TGR. Recently, results of explorations of the hysteretic behavior of *F.hepatica* TGR deny this hypothesis arguing that a sequence comparison revealed that in the enzyme of *F.hepatica* Cys_{88} is absent (Guavera-Flores et al., 2011). More recent results of Bonilla et al. (2011) showed that a mutant in the resolving Cys of the Grx domain drastically reduces the hysteresic behavior, implicating this residue in the phenomenon, most likely by glutathionylation. This residue is also present in *F.hepatica*.

To explore if the observed TR and GR activity really derived from TGR it was acted in a couple of steps build on each other:

5.1.2 The GR activity of *M.vogae* extracts has hysteresis at high [GSSG]/[GSH] ratio typical of TGR existence

Apart from the reasons for hysteresis it was highlighted that hysteretic behavior is supposed to be a typical indication for the existence of flatworm TGRs. This peculiar kinetic behavior was already proven in *T.crassiceps* (Rendón et al., 2004; Guevera-Flores et al., 2010), *E.granulosus* (Bonilla et al., 2008), *F.hepatica* (Guevara-Flores et al., 2011) and in *S. mansoni* (M.Bonilla, personal communication). In this context it is also important to mention that there is only one case described where hysteresis was also

found in individually presented GR namely in the GR of human erythrocytes (Worthington and Rosemeyer, 1975). Therefore, the existence of hysteretic behavior can be considered a “TGR signature”.

In this work hysteretic behavior could be established in the extracts of *M.vogae*. This statement is justified by the fact that GR activity abruptly occurred by changing the [GSSG]/[GSH] ratio in the used GR assay. In other words, GR activity could be detected by adding equal concentration of GSH (in refer to GSSG) to the assay.

Regarding to this observation it was decided to continue the following experiments with this modified form of the GR assay to avoid hysteresis.

5.1.3 TR and GR activities co-purified in all steps of purification

The purification with ammonium sulfate saturation (with saturation steps of 25 and 50% of ammonium sulfate) followed by ion exchange chromatography using the column Mono Q (anion exchange) achieved a higher purity grade of *M.vogae* TGR. Although it could not be purified to homogeneity the awareness was gained that TR and GR activities co-purified in all steps of purification. This strongly indicates that it was worked with TGR and not with single existing TR or GR. Furthermore the ratio of GR to TR activity of 4:1 was maintained during all purification steps (this is an indication that GSSG is a preferred substrate than DTNB; this latter is a non-physiological substrate).

5.1.4 Semi-purified extracts of *M.vogae* also exhibit hysteretic behavior

To complete the observations of hysteresis existence in the extracts of *M.vogae*, this behavior was also be demonstrated in the semi-purified extracts of *M.vogae* (the fraction eluted from Mono Q, i.e. after salt fractionation and anion exchange).Here it was shown again that the hysteresis is strongly dependent of the [GSSG]/[GSH] ratio. These experiments emphasized the already made argumentation for the existence of TGR in *M.vogae*.

5.1.5 TR and GR activities of semi-purified *M.vogae* extracts can be inhibited with the gold compound auranofin

In this step it was verified that the TR and GR activities of the semi-purified extract can be inhibited by auranofin, a gold compound known specifically to inhibit seleno-dependent enzymes (Gromer et al., 1998).Here, the concentration of auranofin required was in the nanomolar range. This is consistent with earlier observations with the TR activity of human placenta (Gromer et al., 1998), TGR of *S.mansoni* (Alger et al., 2002), TGR of *E.granulosus* (Bonilla et al., 2008), TGR of *T.crassiceps* (Rendón et al., 2004) and TGR of *F.hepatica* (C. Carmona, personal communication). Here it should be emphasized that the observation that TR activity is inhibited by auranofin itself is not sufficient to postulate TGR existence (it could be Sec-containing TGR or Sec-containing TR). Rather, it is important that the GR activity could also be inhibited in the nanomolar range (typical for Sec-containing enzymes as real target of the drug) because conventional GRs are not inhibited at a nM range with auranofin, but at µM range (usually between three to four order of magnitude higher concentration are required to inhibit conventional GR when compared with TR) (Gromer et al., 1998). So, the fact that concentrations of auranofin in the nM range were required to inhibit TR and GR activity strongly suggest that both activities are dependent on Sec, which implies a selenocysteine residue and therefore TGR existence in *M.vogae*. Here it should be noted that a reason for the non-linear inhibition of GR activity with auranofin could not be dissected (58,3% remaining GR activity was detected within a concentration of 0,5 nM auranofin – however it required a concentration of 10 nM auranofin to achieve nearly a complete inhibition of GR activity). Further experiments are required to shed light on this issue.

5.1.6 First conclusions

Up to now, all results and observations strongly indicate that the observed TR and GR activities in *M.vogae* are most likely derived from TGR possession.

However, also differences between *M.vogae* and rEg TGR could be observed.

Here, especially the failed attempts regarding to the purification of *M.vogae* abstracts with affinity chromatography (2’5’-ADP agarose) and hydroxyapatite chromatography, respectively, should be mentioned. In both cases the attempts with rEg TGR seemed to

be promising: The rEg TGR bound to a 2'5' ADP agarose matrix and could be eluted with different concentrations of NADPH. In the case of the hydroxyapatite column the experiment worked even better and the rEg TGR appeared nearly on its whole in a SDS-PAGE 12% gel. However, in both cases, with affinity- and hydroxyapatite chromatography, the native TGR of *M.vogae* did not bind in a satisfactory manner to the particular column. The 2'5' ADP agarose column worked even worse than the hydroxyapatite column. Although there might be differences between rEg TGR and native *M. vogae* TGR (e.g. that the rEg TGR encodes for a 4 kDa leader absent in the native enzyme and they could differ in their sequences, and therefore in physicochemical properties) the fact that TR and GR activity did not bind to the affinity column is a totally unexpected result. Further work is needed to determine whether different binding buffers (with a different pH) are required. Further considerations should also be made in the case of the unsuccessful attempt to purify rEg TGR with the help of cation exchange (which was not tried with *M.vogae* extract for the failure with rEg TGR). A pH below the theoretical isoelectric point of rEg TGR which is 6,7 (M. Bonilla, personal communication) was used and therefore rEg TGR should be positively charged at the pH used (3,5) and bind to the column. It cannot be explained why rEg TGR did not bind to the column. It seems that there are small differences between the biochemistry of *M.vogae* and the *E.granulosus* system. Additional experiments would be required to address this issue. This could be performed by the cloning and characterization of the TGR-gen of *M.vogae.* Here also an establishment of a recombinant form of *M.vogae's* TGR should be considered.

5.2 TGR activity of *M.vogae* can be inhibited with recently identified flatworm TGR-inhibitors

In recently studies, new interesting inhibitors of flatworm TGR could be established. As fundamental basis the works of Simeonov et al. where a library of 71028 potential inhibitors were established (Simeonov et al., 2008), Sayed et al. about inhibitors of the TGR of *S. mansoni* (Sayed et al., 2008) and finally a work of Andricopulo et al. about TR inhibitors of *Plasmodium falciparum* (Andricopulo et al., 2006) should be highlighted.

A crucial role within the choice of the compounds in this work played the work of Ross and Salinas (2010) who showed that WPQ75 and VL16E are able to kill larvae of *E.granulosus*) *in vitro* .These compounds were identified as a result of a screening of 85 selected electrophilic compounds, being the rationales behind to target the nucleophilic Sec residue of TGR. Here, WPQ75 and VL16E were among the most effective inhibitors of TGR activity of *E.granulosus*

Hence in this work it was tested if these two inhibitors of *E.granulosus* are able to inhibit also *M.vogae* TGR activity

It was demonstrated that the found TR and GR activities can be inhibited with the thiadiazole WPQ75 and the N-oxide VL16E (which is a furoxan derivate).

The TR and GR activities of *M.vogae* could be totally (100%) inhibited at a 10 µM concentration of WPQ63 whereas the remaining activity was only 5,8% at a 10 µM concentration of VL16E.

These results strongly support the above discussed dates in refer to the similarity of *M.vogae* and *E.granulosus* systems and justified the further studies, which included the corresponding experiments *in vitro* and *in vivo*.

5.3 The identified TGR inhibitors are also able to kill *M.vogae in vitro*

The ability of the inhibitors (WPQ75 and VL16E) to kill the larval stage of *M.vogae in vitro* was tested at a concentration of 20 µM within 12 hours. Praziquantel and auranofin were used as controls. VL16E, as well as the control, but not WPQ75, kill all larval worms. In the case of WPQ75, larval worms showed reduced movements at 12 hours, but they recovered after 24 hours. The lower efficacy of WPQ75 *in vitro* highlights differences in absorption or metabolization of this compound by the larval worm. In any case, higher concentrations of WPQ75 should be tested before assaying its antiflatworm activity in experimental infection. In summary the experiments *in vitro* encouraged again the similarity of the redox systems of *M.vogae* with other TGR-dependent systems.

5.4 The identified TGR inhibitors are also able to kill *M.vogae in vivo*

From all that has been already discussed, it can be concluded that it was very worthwhile to test TGR inhibitors of *E.granulosus* in *M.vogae* as model *in vivo.* Therefor VL16E was chosen because inhibition experiments *in vitro* worked better out than with WPQ75 As described in 4.4 this was performed by an external laboratory instructed by the author of this work.

The mice which were infected with the tetrathyridia stage of *M.vogae* and received both praziquantel (to have a well explored reference with this effective drug against flatworms (Saldaña et al., 2003)) and VL16E. After killing the mice the worm burden in each mouse was measured. Here it was figured out that the percentage of reduction (which means the value of reduction of worm burden in each group in comparison with the group of control which was treated with the vehicle) of VL16E corresponded to the group treated with praziquantel (27% and 28% respectively). This strongly indicates that the effectiveness of VL16E is equal to that of praziquantel.

This exemplify run with VL16E showed, that this recently identified inhibitor is also be able to kill *M.vogae in vivo*.

5.5 Final Conclusion and perspectives

As seen in this discussion the overall results strongly indicate that *M.vogae* possess TGR and lacks conventional GR and TR.

In the future other methods to purify TGR from *M.vogae* extracts to homogeneity should be envisaged. Here it should be paid attention on the purification with 2’5’-ADP Agarose and hydroxyapatite which led to good results with rEg TGR. If enough material is obtained gel filtration (size fractionation) could also be useful, since it separates proteins on a different principle (size). Furthermore cation exchange chromatography could be attempted again using different binding buffers (with different pH).

With regard to the proven possibility to inhibit TR and GR activities of *M.vogae* by TGR inhibitors of *E.granulosus* it could be interesting to establish more precisely curves of inhibition. Furthermore the inhibition experiment should be repeated several times to prove the reproducibility.

In this work, the effects of TGR Inhibitors *in vitro* were only tested by the observation of the mobility of *M.vogae* larval worms. Likewise other methods to test the viability in *in vitro* experiment should be established because observation of mobility can only be used as a first clue to assess the effectiveness of vitality. Therefore the *in vitro* experiment could be repeated with vital dye staining (e.g. the trypan blue exclusion test for cell viability) for a precise evaluation of the viability.

Finally it should be discussed if all data of drug development against *M.vogae* in future studies can be directly applied to *E.granulosus*. The suitability *of M.vogae* as a useful system for the studies on cestode biology (Hart, 1968; Mitchell et al., 1977) and as model for the screening of drugs against cestodes (Saldaña et al., 2003) has already been proven in some cases. As this work showed, the redox system of *M.vogae* and *E.granulosus* are both TGR- dependent. Furthermore, recently identified inhibitors of *E.granulosus'* TGR were revealed as also potent against *M.vogaes* TGR. Nevertheless there seems to be some differences in absorption or metabolization of the inhibitors (at least of WPQ75) by the larval worm. Furthermore, a note of caution should be taking into account when considering that *M. vogae* results can be directly extrapolated to *Echinococcus*. For instance, praziquantel which was able to kill *M.vogae* larvae *in vitro* and *in vivo,* was not effective against the metacestode (larval) stage of *E.granulosus* (Bygott and Chiodini, 2009, Olsen et al., 2011) which is the agent of human echinococcosis.

With the knowledge that *M.vogae* possesses TGR it could be considered to start an automated high throughput screening (HTS). This could be peformed on 96-well microplates. Larval worms could be incubated with 96 different inhibitors and the viability could be measured using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay which is a colorimetric assay for measurement of *in vitro* cytoxity (Mosmann, 1983) The results could be read on a multiwell scanning spectrophotometer (ELISA reader). In parallel the 96 compounds could be tested for TR activity assay using *M.vogae* extracts, or even better purified TGR, using the DTNB assay to prove if TR activity inhibition correlates with lethality. Such an assay was already established for testing inhibitors of *S. mansoni* TGR (Lea et

al., 2008) where the development of a 1,536 well-plate based assay for TGR (that utilizes the DTNB assay as a quantitative measure).

Furthermore with the knowledge of the lethal effect of the TGR inhibitor VL16E *in vivo,* it can be started to test all identified inhibitors of TGR activity of *E.granulosus* and other flatworms (e.g. *S.mansoni*) *in vivo* It could be also interestingly to know if the identified inhibitors suppress also human TR or GR which could interfere with the development of the corresponding future drug against flatworm infection.

6. Abstract

6.1. Abstract (English version)

Flatworm parasites (platyhelminths) cause serious infection diseases in humans, such as schistosomiasis and hydatid disease, mainly prevalent in developing countries. However, the current repertoire of drug armamentarium used to combat flatworm infections is limited. For instance, praziquantel is the only drug available for mass treatment of *Schistosoma* infections. In contrast to their hosts, flatworm parasites possess a distinct redox arrangement of redox pathways in which the selenoenzyme thioredoxin glutathione reductase (TGR) controls the overall redox homeostasis. Interference with this enzyme leads to parasite death. Hence, this key redox enzyme seems to be a new promising drug target against flatworm infections.

Because most flatworms are difficult to cultivate in the laboratory (e.g. *Echinococcus granulosus* experimental infection in mice takes about 10 month to develop into cysts), this work was focused on *Mesocestoides vogae* (syn. *corti*), a non-human flatworm parasite which is an interesting laboratory model to study other flatworm infections: it is very rare in humans, can be easily manipulated both *in vivo* and *in vitro* and grows extremely fast in mice. With the aim to assess TGR inhibitors as possible drugs to treat flatworm infections, the thioredoxin and glutathione pathways of *M.vogae* were studied. Here, the objectives were to study whether the biochemical pathways that maintain the redox homeostasis in *M. vogae* conform to the general biochemical scenario proposed for other platyhelminth parasites.

Here, it was proven that *M. vogae* extracts possess both thioredoxin and glutathione reductase activities. The thioredoxin and glutathione reductase activities were partially purified from total extracts by a combination of ammonium sulfate precipitation, anion exchange and hydroxyapatite chromatography. Both activities co-purified in all steps which strongly indicates the existence of TGR rather than a single TR and GR. Furthermore partially purified activities could be inhibited by the organogold compound auranofin, a known TGR inhibitor. Moreover, the glutathione reductase activity displays hysteresis (a peculiar kinetic behavior) at high

concentrations of oxidised glutathione, a feature typical of flatworm TGRs, but not of conventional GR. Although *M. vogae* activities could not be purified to homogeneity, the overall results strongly indicate that this flatworm possesses TGR and lacks conventional GR and TR.

Furthermore the thiadiazole WPQ75 and the N-oxide VL16E (a furoxan derivate) were identified as inhibitors of TGR activity of *M.vogae* at a 10 µM concentration. These inhibitors were able to kill *M.vogae* larval worms *in vitro* as well as in experimental infection in mice.

Due to the existence of TGR activity in *M.vogae*, the possibility to inhibit this activity with recently discovered inhibitors of flatworm TGR and the successes achieved by testing these inhibitors both *in vitro* and in *vivo,* it is strongly evident that *M. vogae* would be an excellent model to assess TGR inhibitors in flatworm infections.

6.2. Abstract/Zusammenfassung (German version)

Infektionen mit Plattwürmern (Platyhelminthen) führen zu schwerwiegenden Infektionskrankheiten wie zum Beispiel Schistosomiasis und Echinokokkose. Diese Krankheiten sind vor allem in Entwicklungsländern weit verbreitet. Dennoch gibt es bisher nur wenige Medikamente, um solche Wurminfektionen zu therapieren. Beispielsweise ist Praziquantel der einzig verfügbare Wirkstoff zur Behandlung von Schistosoma-Infektionen.

Im Gegensatz zur ihrem Wirt besitzen Plattwürmer ein eigenes Redox-System bei dem das Selenoenzym Thioredoxin Glutathion Reduktase (TGR) als Schlüsselenzym für die gesamte Redoxhomöostase fungiert. Die Hemmung dieses Enzyms führt deshalb direkt zum Tod des Parasiten. Aus diesem Grund scheint die TGR ein vielversprechender Angriffspunkt für gezielte Pharmakotherapie zu sein.

Da die Gewinnung der meisten Plattwürmer als Material für Forschungszwecke sehr schwierig ist (beispielsweise braucht *E.granulosus* in experimentellen Infektionen von Mäusen über 10 Monate um sich in Zysten zu entwickeln), konzentriert sich diese Doktorarbeit auf *Mesocestoides vogae (*Syn.corti), einen nicht-humanpathogenen Parasiten, der ein sehr interessantes Modell für die Erforschung aller anderer Infektionen mit Plattwürmern darstellt: Er infiziert nur sehr selten Menschen, kann sowohl *in vitro* als auch *in vivo* einfach manipuliert werden und wächst sehr schnell in infizierten Mäusen heran.

Mit dem Ziel, mögliche Wirkstoffe auf ihre Wirksamkeit zur Behandlung von Plattwurminfektionen zu testen, wurden also die Signalwege von Glutathion- und Thioredoxinreduktase (GR und TR) von *M.vogae* untersucht. Das Ziel dabei war es, zu untersuchen, ob die biochemischen Wege zur Aufrechterhaltung der Redoxhomöostase von *M.vogae* denen von anderen Plattwürmern entsprechen. Die Frage war, ob auch *M.vogae* TGR als Schlüsselenzym besitzt, oder zum Beispiel einzeln vorliegende GR und TR (was *M.vogae* als Modell für TGR Inhibitoren unbrauchbar machen würde).

Es wurde gezeigt, dass *M.vogae* sowohl TR, als auch GR Aktivitäten besitzt. Diese Aktivitäten wurden aus *M.vogae* Extrakten unter Zuhilfenahme von Anionen-Austauschern, Amoniumsalzpräzipitation und Hydroxyapatit-Chromatographie isoliert. Während dieser Reinigung veränderten sich die TR und GR Aktivitäten immer im gleichen Verhältnis, was ein starker Hinweis darauf ist, dass man es mit TGR und nicht mit einzeln vorliegenden TR und GR zu tun hatte.

Darüber hinaus konnten TGR-Aktivitäten von teilgereinigten Extrakten mit Auranofin (einer organischen Goldverbindung) gehemmt werden. Auranofin ist ein bekannter Inhibitor der TGR. Außerdem zeigten die gemessenen GR Aktivitäten unter hohen Konzentrationen von oxidiertem Glutathion Hysterese (eine besondere kinetische Eigenschaft). Diese Eigenschaft ist ebenfalls typisch für die TGR von Plattwürmern, dagegen aber nicht für herkömmliche (einzeln vorliegende) GR. Obwohl die Extrakte von *M.vogae* nicht bis zu vollkommener Homogenität gebracht werden konnten, geben die Ergebnisse einen starken Hinweis darauf, dass *M.vogae* anstelle von einzeln vorliegender TR und GR die kombinierte Form dieser Enzyme, also TGR, besitzt.

In einem weiteren Schritt wurde gezeigt, dass Thiadiazol WPQ75 und das N-Oxid VL16E in einer Konzentration von 10 µM als Inhibitoren der TGR-Aktivitäten von *M.vogae* wirksam sind. Diese Stoffe konnten *M.vogae* außerdem sowohl *in vitro* als auch *in vivo* abtöten.

Dadurch, dass gezeigt wurde, dass *M.vogae* TGR Aktivitäten aufweist, dass es möglich ist, diese Aktivität mit kürzlich entdeckten Inhibitoren von Plattwurm TGR zu inhibieren und dass diese Inhibitoren sowohl *in vitro* als auch *in vivo* aktiv gegen *M.vogae* wirken, ist der Beweis erbracht worden, dass *M.vogae* ein exzellentes Modell darstellt um die Wirksamkeit von TGR Inhibitoren auf andere Plattwürmer zu testen.

7.References

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7.2. List of figures

Figure 1.1: http://www.nathnac.org/pro/factsheets/pdfs/schistosomiasis.pdf (Juli 2012)

Figure 1.3.a: Salinas G, Lobanov A, Gladyshev VN. 2006. Selenoproteins in parasites. In: Hatfield DL, Berry MJ, Gladyshev VH (Ed.), Selenium: Its molecular biology and role in human health. Springer, New York, 358.

Figure 1.3.b: Kindt TJ, Goldsby RA, Osborne BA. 2007. Kuby Immunology. W.H. Freeman and Company, New York and Houndmills, 68.

Figure 1.4: Modified from: http://smu.edu/biology/faculty/radyuk.asp (Juli 2012)

Figure 1.6: Salinas G, Lobanov A, Gladyshev VN. 2006. Selenoproteins in parasites. In: Hatfield DL, Berry MJ, Gladyshev VH (Ed.), Selenium: Its molecular biology and role in human health. Springer, New York, 361f.

Gran parte de las dificultades por las que atraviesa el mundo se deben a que los ignorantes están completamente seguros y los inteligentes llenos de dudas.

(Bertrand Russel)

Thanks to:

All the Uruguayan new friends, namely Gustavo, Cecilia, Mariana, Lucía, Laura, Leo, y Fernanda for one of the best times of my life. Muchas gracias, nunca olvidaré el tiempo que pasé en Uruguay!

Klaus Brehm who gave me the chance to go to Uruguay and to make this exceptional experience.

My family and friends who always accept my absence.

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