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# Identification and characterization of immunomodulating proteins in *Giardia lamblia*

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Degree project in biology, Bachelor of science, 2008

Examensarbete i biologi 15 hp till kandidatexamen, 2008

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## Summary

Many eukaryotic parasites infect their hosts by attaching to the intestinal surface without invading the mucosa. *Giardia lamblia* is such a parasite and it infects the upper small intestine. *G. lamblia* is a binucleated, flagellated protozoan and is a major cause of diarrhea (giardiasis) in mammals world-wide but not much is known about its interaction with the host. Intestinal parasites must modulate the intestinal immune system to resist clearance by the immune system. It is believed that *Giardia lamblia*, like some nematodes and other parasites, has developed several mechanisms that interfere with the intestinal immune system, e.g. secretion of cytokine-like molecules and protease inhibitors. Very little inflammation is seen during acute giardiasis and the infection often becomes chronic. This suggests that the parasite can immunomodulate the host actively.

In this degree project I searched the newly sequenced *Giardia* genome for the presence of genes homologous to immunosuppressive genes already identified in intestinal parasitic worms. I identified a gene encoding a serine-protease inhibitor (serpin). Serpins have been shown to be secreted by intestinal nematodes and have immunomodulatory functions. The giardial serpin has a predicted 47 amino-acid signal sequence indicating that it is a secreted protein. Sequence comparisons showed a close relationship of the *Giardia* serpin gene to those of serpin genes in *Chlamydomonas* species among others. The giardial serpin was over-produced as a recombinant protein in *E. coli* and the recombinant protein showed trypsin-inhibiting activities. Epitope tagging showed localization to lysosome-like structures in *Giardia* trophozoites. Thus, I conclude that I have identified a protein in *Giardia* with potential immunomodulatory functions.

## **Abbreviations**

Abs – absorbance

BLAST – basic local alignment search tool

DTT – dithiothreitol

GST *fusion* – glutathione-S-transferase fusion

IEC – intestinal epithelial cell

IPTG –  $\beta$ -D-thiogalactopyranoside

LB – Luria Bertini medium

OD – optical density

ORF – open reading frame

Pfam – protein family

PBS – phosphate buffered saline

PCR – polymerase chain reaction

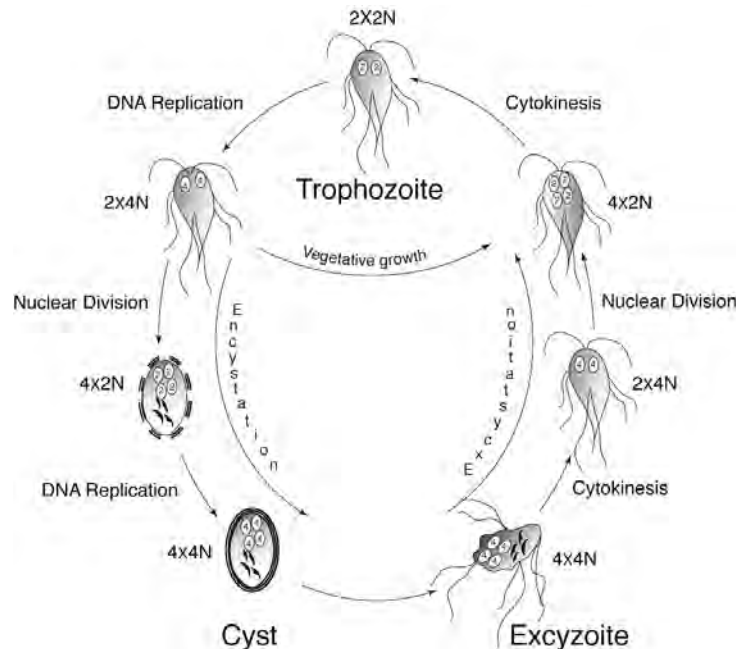
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

## Introduction

### *History and Biology*

*Giardia lamblia* is a eukaryotic microbe that commonly causes diarrheal diseases in all kinds of vertebrates, including reptiles, birds, mammals and amphibians worldwide [8]. The earliest record of the existence of the parasite was made by the Dutch scientist van Leeuwenhoek in 1681 [8] when examining his own, probably diarrhoeal, stools in his microscope. But it took almost 200 years til the parasite was examined more greatly, this time by the Czech physician Lambl in 1859 [8]. The human form of the microbe, *G. lamblia*, in fact has its name from him and that name was officially accepted in the 70's after some wide discussions of different names.

*G. lamblia* is a binucleated, flagellated protozoan with two major stages in its lifecycle [8], a cyst-stage and an excysted trophozoite stage (fig 1). The cyst stage has a fecal-oral route of infection and is most often ingested by a host via infected water, occasionally via food, while the excysted trophozoite colonizes the host's small intestine, predominantly in the mid-jejunum [9]. When the cyst enters the stomach and is exposed to its acidic environment it excysts to become a trophozoite [8].



**Fig. 1.** The *G. lamblia* lifecycle. Shows the development of the parasite, from a cyst surviving in unfortunate environments to the trophozoite and back again to a new infectable cyst (with permission from the copyright holder S. Svård).

The vegetative trophozoite undergoes asexual reproduction; it divides by binary fission and has two nuclei with 5 chromosomes each, resulting in a haploid genome size of ~12 Mb [8]. The trophozoite has 8 flagella suited in 4 pairs; anterior, ventral, lateral and caudal flagella. In addition to their role in the motility of the parasite, flagella have been suggested to be important in food uptake, attachment and freeing of the parasite in the intestines epithelial cells [8]. The parasite subsequently undergoes dramatic biological changes to survive outside the intestine of their host, by differentiating into the resistant cyst (encystation) again [10]. The cyst has an outer cell wall approximately 0.3-0.5  $\mu\text{m}$

thick and a inner layer of two membranes that give a good protection to the parasite. [8] The cyst must be able to resist different harms like disinfectants, acidic fluids from the stomach and fresh water among others to be able to infect a new host.

The pathogenesis of *Giardia* varies from asymptomatic carriers to chronic giardiasis (a severe kind of diarrhea causing malabsorption syndrome) [8]. If symptomatic, symptoms usually occur in 6-15 days after infection and lasts 2-4 days. There is evidence of self limitation of the disease within 2-4 weeks, indicating an effective host immune response. Both the innate and adaptive immune responses have been shown to play important roles in the control of *G. lamblia* [9, 8].

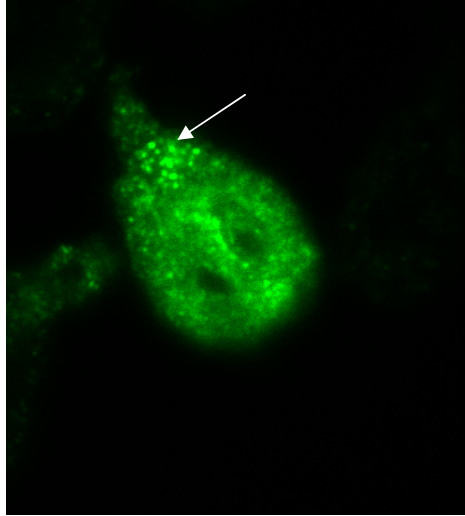
#### *Serine protease inhibitors*

The protolytic activity found in the small intestine of humans and animals is due to serine proteases such as trypsin and chymotrypsin. These proteases cleave peptide substrates on the carboxyl side of lysine and arginine and the lateral chains of aromatic amino acids [2]. Trypsin is secreted into the pancreatic duct from which it enters the intestine after suitable stimulus. All vertebrates studied so far, including man, secrete two different trypsinogens, an anionic and a cationic trypsinogen, that differ in their isoelectric points [1].

To achieve control of activation of serine proteases most organisms use very potent *serine protease inhibitors* (serpins) in the circulation or intestinal fluid. Serpins are secreted with the trypsinogen into the duodenal juice, but, due to their relatively low amount and sensitivity to trypsin digestion, they do not interfere to a significant extent with the trypsin activity in the intestinal lumen. Probably the inhibitor prevents premature trypsinogen activation within the gland or in the pancreatic duct.

Serpins have also been found in several infectious organisms, such as helminths (e.g. *Trichinella spiralis*) and other parasites (e.g. *Toxoplasma gondii*) [3 & 8]. These parasites like *G. lamblia* have several life stages, and infections are initiated in the small intestine and have shown expression of serpins. In most parasites a number of biological processes are important, such as digestion, complement activation, apoptosis, cellular and extracellular remodelling, and the proteolytic processing of proteins [4]. According to Mangan *et al.* there are more than 1000 different serpins and they are represented in all domains of life. The large number found of serpins probably reflects the large variety and range of proteases in life that need to be regulated.

Recently the entire genome of *G. lamblia* has been sequenced and a gene sequence similar to that encoding serpins in helminths and other parasites was identified [5]. Thus, it is possible that *Giardia* produces and secretes serpin. The Svärd group has found that cell lysates of *Giardia* inhibit trypsin and that acutely infected giardiasis patients show reduced protein breakdown [6]. Furthermore, recent data suggest that *Giardia* can immunosuppress its host actively [9]. The serpin protein gene has recently been epitope tagged with the AU-1 tag, transfected into *Giardia* and localized. The serpin protein was found localized close to the plasma membrane in vesicles (Fig. 2, S. Svärd, personal communication). These vesicles are lysosome-like and known to be involved in protein degradation/secretion. Furthermore, the expression of serpin is high in trophozoites and it is slightly up-regulated upon contact with human intestinal epithelial cells (S. Svärd, personal communication).



**Fig 2.** Image of *G. lamblia* transfected with a serpin-AU-1 construct. Arrow show possible serpin secreting vacuoles, activated during stress (with permission from the copyright holder S. Svård).

### *Aims*

This study aimed to investigate whether there are any serpin-like genes in the *Giardia* genome and if their encoded proteins have trypsin-inhibiting activities. I identified a sequence in the *Giardia* genome verified coding a serpin, carrying a secretory signal peptide. Overproduction of recombinant giardial serpin constructs showed that it had trypsin-inhibiting activities.

## Results

### Comparing genome sequences

The *Giardia* database was searched for the presence of a serpin gene in the genome of *G. lamblia* type A-WB-06 using the BLAST P algorithm in comparing the already known serpin of the pathogenic nematode *Trichinella spiralis* to the *Giardia* database. One open reading frame (ORF) was detected, ORF 4653. This possible serpin gene encodes a signal peptide (in the N-terminal), 47 amino acids long. Also recognized in the protein was a Pfam motif found in all serpins (see fig 3 c). Interestingly there are a couple of amino acids not equal to the general Pfam sequences.

a)

Trich	13	AYSLYQLEBAGNVFFSPVSIPLALAMVFFGSNGTNTQLLNVMFKAGWKKNRKTKAMRSF	72
Giard	15	AYFTEERLQSKGVVYSSWSLPHLFIPLAAVTGSHDDLLTAM-----QLSEKEASPPA	68
Trich	73	VSSLTIDEYYDASLKLANRLYANDQYPILHPFLKDVKRYLSSDLVSVNFADTEAARLQIN	132
Giard	69	VSQIASLLREDSALALAANIYAKSVSPTVE-FVALAKRYFDITIEPLKSA-----AQVN	121
Trich	133	KWVSDQTNHKINDLLQSGTVEANTRLIAVNAIYFKASWDEVFDEAHTKPKKFFYPHPSSI	192
Giard	122	NWCSAQTKGLINSIVDD---VKDTPDAILISAIYFKANWALKFSDKDDTKMLPFTSLDGRTQ	178
Trich	193	KIPMMTQTNGYSYYETEDYQFLGMDYYPEYLKMFILLPK-SGKTLSELQOKF-NGETLLN	250
Giard	179	DRELMYLKAKLQYASSDSAQMVKLNYNDSNVSAIIVLPKETGKASLKAALRYDNFFPTDG	238
Trich	251	LVSKVSGAEVVKVTIPKMKFEKQMNLEALKKLGIEDLFIPGKADLSGICVKEKLYVSDIV	310
Giard	239	FLTK----EVLKLPKFRLESSHDLKDHLVLRGAKSMF--GVCDNCG-SLGAPMSVTKVI	291
Trich	311	HKAYLEFNXXXXXXXXXXXXVRIVPMGVM-YEDSPEFVADHPFLFFI	356
Giard	292	QKAVIEVDEEGTTAAAVTAVRMLRCAMMAPPEIYHVICDRPFWFML	338

b)

SVHDDLLTAMQLSEKEASPPAVSQIASLLREDSALALAANIYAKSVSPTVEFVALAKRYF  
DITIEPLKSAQAQVNNWCSAQTKGLINSIVDDVKDTPDAILISAIYFKANWALKFSDKDDTK  
MLPFTSLDGRTQDRELMYLKAKLQYASSDSAQMVKLNYNDSNVSAIIVLPKETGKASL  
KAALRYDNFFPTDGFLTKEVVLKLPKFRLESSHDLKDHLVLRGAKSMFGVCDNCGSL  
GAPMSVTKVIQKAVIEVDEEGTTAAAVTAVRMLRCAMMAPPEIYHVICDRPFWFML  
ERDGMPLFLTALL

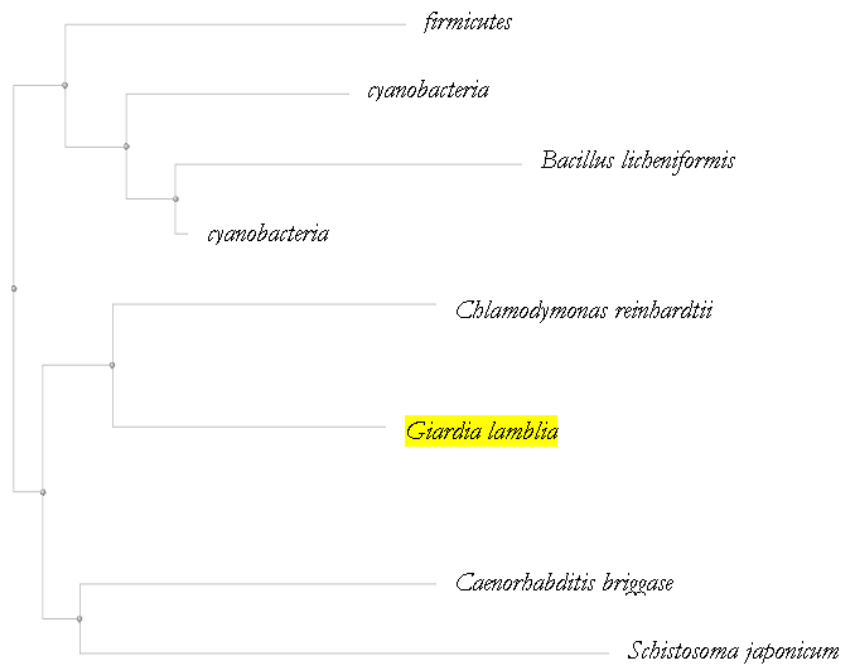
c)

Score = 65.2 bits (1.8e-16), Expect = Identities = 0/59 (0%), Gaps = 53/59 (89%)

Giardia: 2	ATALPQALFSDYVA-----*	YFTEERLQSKGVVYSSWSLPHLFIPLAAVTGSHDDLL	54
Serpin: 1	ldsralkLasantdFafsLYkeLveskspdkNiffSPvSIssALAmLsLGAKgnTrtQil	+++++ L s +++ ++e + +k +++S S++ +L + g+ ++ +l	60

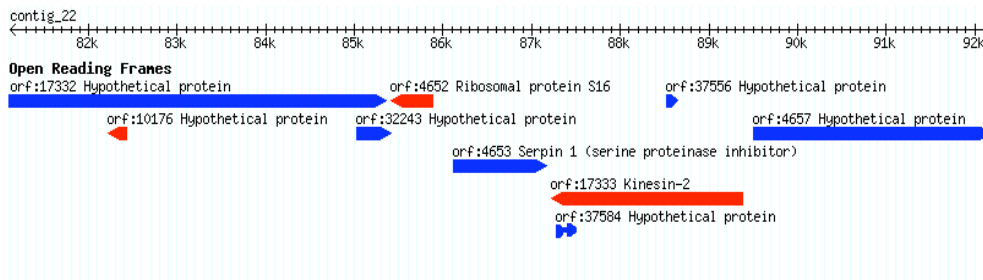
**Fig 3.** Alignment of amino acid sequences of serpins from *G. lamblia* and *T. spiralis*, using BLAST P. **b).** Amino acid sequence of *G. lamblia* genotype A-WB-06 possible serpin from gene ORF 4653. This gene encodes a 47 amino acid long signal protein in the beginning which is not included here. **c)** The protein family (Pfam) motif in *Giardia* compared with the general sequence of serpins, taken from the *Giardia* data base. Slightly differences between this amino acid sequence and the general Pfam, marked with an asterisk.

BLAST P was also used to compare the serpin of *G. lamblia* with other organisms. This analysis showed that this gene is similar to the serpin genes in the green alga *Chlamydomonas reinhardtii*, the pathogenic nematode *Caenorhabditis briggsae* and the pathogenic fluke *Schistosoma japonicum*. A phylogenetic tree were constructed for these genes (fig. 4)



**Fig 4.** Phylogenetic tree showing the relationship of serpin gene sequences from different organisms. The yellow marked is the *Giardia* serpin (ORF: 4653).

The localization of the serpin gene in the *Giardia* genome was studied using the *Giardia* data base. The gene is localized close to the genes encoding the ribosomal protein S16 and kinesin-2 (fig. 5).

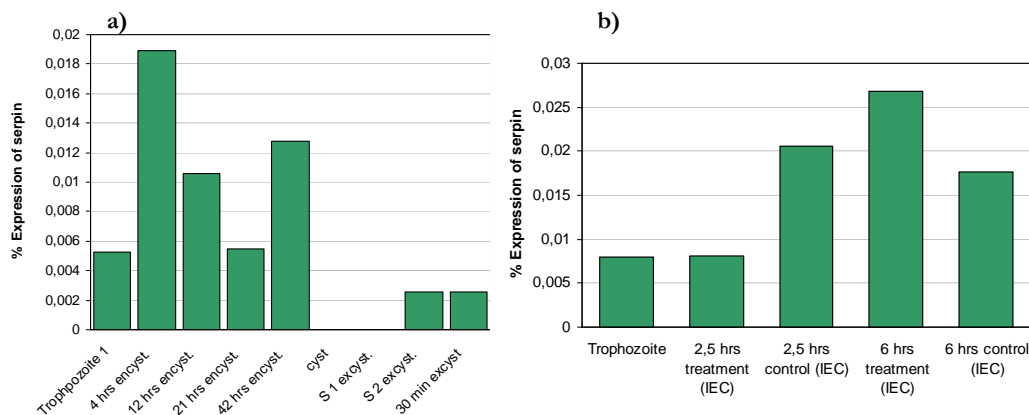


**Fig 5.** Part of the genome of *G. lamblia* genotype A-WB-06 showing genes encoding serpin (ORF: 4653), ribosomal protein S16 (ORF: 4652) and kinesin downstream (ORF: 17333).

I was also interested in the expression profile of the giardial serpin gene. In the *Giardia* data base there are expression data from the life cycle of free living parasite and parasite interaction with human intestinal epithelial cells (IECs). Total RNA had been isolated from *Giardia* at various stages of differentiation (trophozoites, encystation, cyst and excystation) and from trophozoites after co-incubation with intestinal epithelial cells, IECs. This RNA was used in Serial Analysis of Gene Expression (SAGE), a powerful experimental tool that does not depend upon construction of gene specific probes or primers, but instead simply samples any polyadenylated RNA transcript that includes an *Nla*III restriction site (5'-CATG-3') [5]. SAGE provides both the identity of expressed genes and levels of their expression. Figure 6 shows that the serpin gene was expressed in



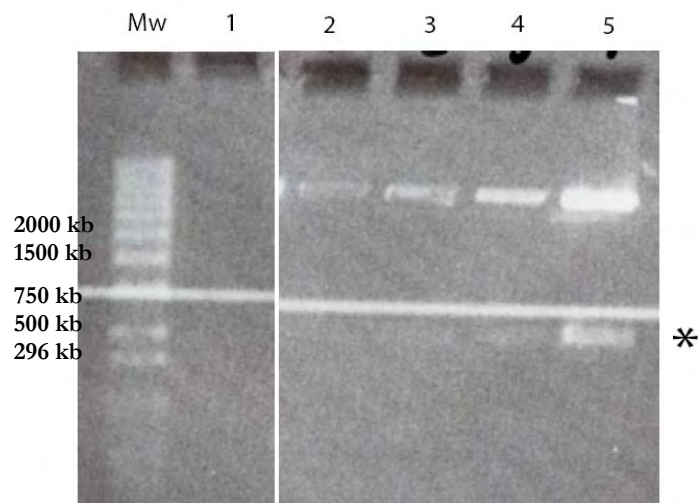
trophozoites and upregulated early in encystation (fig. 6a). Interestingly, the expression was also upregulated after 6 hrs interaction with IECs (fig. 6b).



**Fig 6,** Expression of serpin in *G. lamblia* as described in the *Giardia* data base, [www.giardiadb.org](http://www.giardiadb.org). **a)** Expression of serpin in the *Giardia* life cycle, from trophozoite (free living parasite) through encystation stages (4 hrs to 42 hrs) to the encapsulated cyst. S1, stage 1, treatment of cysts with acid. S2, stage 2, treatment of cysts with trypsin. 30 min excyst is growth in growth medium for 30 min after S2 treatment. **b)** Expression of serpin of *G. lamblia* during interaction with human intestinal cells (IEC). Trophozoite is the free living parasite in ordinary growth medium. Treatment 2.5 and 6 hrs means co-incubation of trophozoites and IECs in DMEM medium. Control means trophozoites in DMEM, no IECs [ref. 5 &7]. The percentage means the percentage of serpin expression tags compared to the total number of sequenced tags (40000) from each stage.

#### *Making and growth of competent cells and pure proteins*

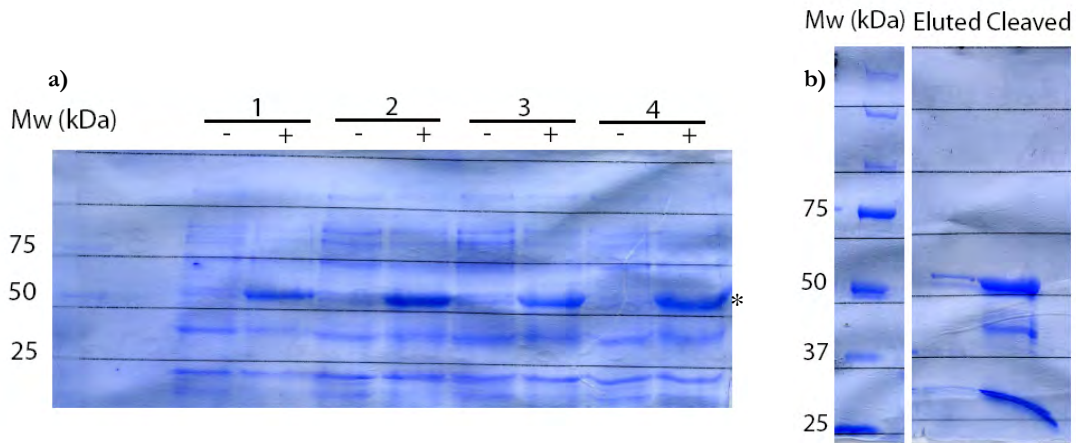
For biochemical investigations of the possible serpin encoded by the *Giardia* genome this gene had to be cloned and over-expressed as a recombinant protein in *E. coli*. The gene was polymerase chain reaction (PCR) amplified, cleaved and inserted into the plasmid pGEX-6P3 as a GST fusion. Restriction enzymes, BamH1 and EcoR1 were used during cleavage. Agarose gel analysis of the serpin construct showed a band at the correct size, 900 base pairs (fig. 7). Several serpin constructs were made and colony #5 showed best results out of the five tested colonies.



**Fig 7.** Serpin constructs cleaved by the restriction enzymes Bam H1 and Eco R1 and analyzed by agarose gel electrophoresis. The wells were loaded with plasmids from different colonies. Colony 5 (marked with a star) of the serpin had the clearest result and therefore was chosen for further use.

Plasmids from clone number 5 were transformed into *E. coli* BL-21 and serpin expression induced. Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It was clear that induction was needed to get expression of recombinant proteins (fig. 8a).

Culture #4 was chosen for large scale expression. After purification of protein from these cells both a cleavage and an elution to remove the GST of the protein from the gel beads were done. An SDS-PAGE gel showed that cleavage of this serpin GST fusion was inefficient, and no proteins were eluted with glutathione (fig. 8b). Band 1 is GST+serpin, band 2 serpin and band 3 GST. This shows that the serpin interfered with cleavage by the serine protease, PreScission Protease (GE-Healthcare, Uppsala, Sweden). The non-cleaved serpin GST-fusion was chosen for all the inhibition tests, since too little cleaved product was produced when eluting the protein. However the GST-serpin fusion seemed to be degraded quite fast, even when stored in a cold room (+4 °C), and got useless after being stored over the weekend. Therefore a new stock of pure serpin had to be made all over again, from the same colony as the first time, to be able to do some further testing.

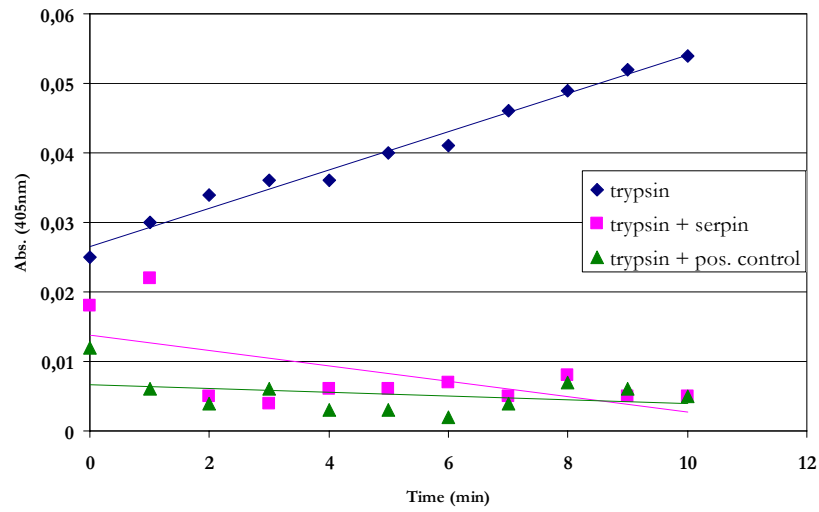


**Fig 8.** Analysis of serpin expression in *E. coli*. **a)** Sodium dodecyl sulfate polyacrylamide gel loaded with either protein extracts with IPTG or not, + indicates IPTG added and – indicates no IPTG added, in four different colonies (1-4). Protein extract from colony number four was chosen for further use. \* is serpin-GST fusion. **b)** An SDS-PAGE analysis with the results of cleaned and purified proteins by elution and cleavage of GST in the last step of purification. Band 1 is GST+serpin, band 2 serpin and band 3 GST. This shows that the serpin interfered with cleavage by the serine protease, PreScission Protease (GE-Healthcare, Uppsala, Sweden) The size of serpin is ~38 kb (2). The horizontal lines in the gels are just marks from the paper they were pictured from.

#### *Inhibitory activity of G. lamblia serpin*

Protease inhibition by the purified serpin was analyzed by incubating the recombinant protein with trypsin and a trypsin substrate, Bz-L-Arg-4-NA. After about 10 minutes the trypsin catalytic activity ceased. Therefore measurements were only made for 10 minutes in every test. Three different samples were made and tested; trypsin, trypsin + serpin and trypsin + a positive control. The positive control was a serpin from soy bean, having a proved inhibitory effect on trypsin.

The analysis of data (fig 9) clearly showed that this serpin of *G. lamblia* in fact was a protease inhibitor. The catalytic activity of trypsin, measured by absorbance from 4-nitroaniline released was inhibited by the serpin, just as in the positive control.



**Fig 9.** Serpin activity of trypsin. Protease inhibition by the purified serpin was analyzed by incubating the recombinant protein with trypsin and a trypsin substrate, Bz-L-Arg-4-NA. Used samples was trypsin, trypsin + serpin and trypsin + positive control (a soy bean protein having a proved trypsin inhibitory effect).

## Discussion

The *G. lamblia* serpin gene sequence showed a high degree of similarity to the *T. spiralis* serpin gene, which made further data base investigation interesting. The *G. lamblia* sequence encoded a signal protein. This makes sense since many secreted proteins have a signal protein sequence. PCR primers were designed so that the signal peptide was not included in the PCR product, since it can cause problems during over-expression and insertion into the plasmid. The *Giardia* serpin also contains a Pfam sequence. A Pfam domain is found in pretty much all trypsin inhibitory proteins investigated so far, which is a good indicator that this serpin really is an trypsin inhibitory protein (S. Svärd, personal communication).

A phylogenetic tree for the possible serpin gene in *G. lamblia* showed its closest neighbor to be *Chlamydomonas reinhardtii*. It's also related to *Schistosoma japonicum*. The second is a nematode, causing schistosomiasis in mammals and *C. reinhardtii* is a eukaryotic alga living in water. These organisms also encode proved protease inhibitors. Another relative is *Caenorhabditis briggsae*, a very close relative to *C. elegans*, a nematode often used in laboratory research and whose whole genome has been sequenced. *C. elegans* and *C. briggsae* also encode serine protease inhibitors. This is also good evidence that the serpin in *G. lamblia* also might be a protease inhibitor, since it is a relatives encode inhibitory proteins.

Over the years scientists have made more experiments on the possible serpin gene. During differentiation of the parasite expression of the serpin is the highest during encystation, probably because this is a stressful state of the parasite. During interaction with human cells the highest expression of serpin occurs after a couple of hours. This is most likely because it takes a while for the human cells to react and start immunoregulating the parasite. When the human cells do, the expression of serpin increases which makes sense since it's possibly a protein protecting the parasite in some way (S. Svärd, personal communication).

The Svärd group at ICM is currently producing specific polyclonal antibodies against serpin with the recombinant protein produced here. These antibodies will be used for further localization studies under different conditions and to attempts to identify serpin in culture supernatants. This can show if the serpin is secreted by the parasite, which would aid in understanding the role of serpin during *Giardia*-host interactions.

## Material and Methods

### *Biological materials*

All biological materials used are listed in Table 1. This includes the strain of *Giardia* I used as “template” for cloning of my protein.

**Table 1.** Strains used in lab.

Strains	Genotype	Source and references
<i>E.coli</i> Top 10	F- <i>ompT gal dcm lon</i> <i>bsdS<sub>B</sub>(<math>\tau_B</math> <math>m_B</math>)</i> $\lambda$ (DE3 [ <i>lacI</i> <i>lacUV5-T7 gene 1 ind1</i> <i>sam7 nin5</i> ])	Invitrogen, Amsterdam Holland
<i>E.coli</i> BL-21	F- <i>mcrA</i> $\Delta$ ( <i>mrr-bsdRMS-</i> <i>mcrBC</i> ) $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74 nupG recA1</i> <i>araD139</i> $\Delta$ ( <i>ara-leu</i> )7697 <i>galE15 galK16 rpsL(Str<sup>R</sup>)</i> <i>endA1</i> $\lambda$ -	Invitrogen, Amsterdam Holland
Plasmid pGEX-6P3	<i>Amp<sup>R</sup>, lacZ, gst</i>	GE Healthcare, Uppsala Sweden
<i>Giardia</i> -WB	Assemblage A	ATCC3957, S. Svärd Group, ICM Uppsala University

### *Identification of the serpin sequence in G. lamblia*

The *Giardia* database GiardiaDB ([www.gmod.mbl.edu/gb](http://www.gmod.mbl.edu/gb)) was used to search for genes in *G. lamblia* genotype A-WB-06, homologous to serpin genes from intestinal nematodes and protozoa. The sequences of the nematode *Trichinella spiralis* serpin gene (accession number AAF63473) was used as bait in the BLAST search of *Giardia* data base. The BLOSUM 62 algorithm was used for comparisons. The protein encoded by *Giardia*, ORF 4653, was BLASTed against all sequences at NCBI and among the first 100 hits serpin genes from a variety of organisms were identified. The most similar sequence was a serpin protein from *Chlamydomonas reinhardtii* according to the distance tree of the BLAST results.

A phylogenetic tree was also constructed showing an overview of the other closest neighboring species using tree-mapping neighbor joining in the data base BLAST P, with setting maximum sequence distance of 0.65. If the fraction of mismatched bases for any pair of sequences is larger than this value, both sequences are excluded from tree generation. The distance model used was the Grishin model. It can be computed for fraction of mismatched amino acids larger than 0.75.

### *Serial Analysis of Gene Expression*

Serial Analysis of Gene Expression (SAGE) has been used to study expression throughout the *Giardia* life cycle and during interaction with human Caco-2 cells. Data are published at the *Giardia* data base homepage [www.giardiadb.org](http://www.giardiadb.org) and also described by Morrison [5] and Ringquist [7].

### *Gel electrophoresis*

#### Agarose gels:

1.5 % agarose gels was prepared by mixing 0.75 g agarose with 50 ml 1x TAE buffer (2 ml 0.5 M EDTA, 4.84 g Tris base and 1.142 ml glacial acetic acid dissolved in dH<sub>2</sub>O to a final volume of 1000 ml) and heating til the agarose was dissolved. When the agarose mix was cooled to approx. 50°C 0.8 µl ethidiumbromide 10 mg/ml was added. The liquid was poured onto a gel tray with a well comb. A marker was made by mixing 80 µl H<sub>2</sub>O 10 µl 1 kb DNA ladder (Invitrogen, Amsterdam, Holland) and 10 µl loading dye. The samples were run at 100V until the required separation had been obtained. The gel was examined under UV-light and an image was recorded and copied.

#### Sodium dodecyl sulfate polyacrylamide gel electrophoresis:

An SDS-polyacrylamide gel consists of a resolving and a stacking gel.

10 ml 10 % resolving gel was prepared by mixing 4 ml dH<sub>2</sub>O, 3.3 ml 30 % acrylamide/bis 37.5:1 (Bio-Rad, Stockholm Sweden), 2.5 ml 1.5 M Tris-HCl pH 8.8, 0.1 ml 10 % sodium dodecyl sulfate (SDS), 0.1 ml 10 % ammonium persulfate and 4 µl TEMED (Bio-Rad, Stockholm Sweden). 5 ml 5 % stacking gel was prepared by mixing 3.4 ml dH<sub>2</sub>O, 0.83 ml 30 % acrylamide/bis 37.5:1 (Bio-Rad, Stockholm Sweden), 0.63 ml 1.5 M Tris-HCl pH 6.8 , 0.05 ml 10 % SDS, 0.05 ml 10 % ammonium persulfate and 5 µl TEMED (Bio-Rad, Stockholm, Sweden). Protein samples were mixed with 6×SDS loading buffer containing 50 mM Tris-HCl pH 6.8, 30 % glycerol, 10 % SDS, 0.6 M dithiothreitol (DTT) and 0.012 % bromophenol blue (BPB). Protein samples were boiled for 5 minutes and loaded on the 10 % one-dimensional gels for analysis. Precision Plus Protein Kaleidoscope Standard (Bio-Rad, Stockholm, Sweden) was used as a molecular marker in gels to be stained. Gels were run at 100 V through the stacking gel and 150 V through the separation gel. After electrophoresis the gel was soaked in Coomassie Brilliant Blue solution (40 % methanol, 10 % HAc, 0.1 % Coomassie Brilliant Blue R250), heated in microwave (1000 W) for 1 minute and incubated with shaking for 15 minutes at room temperature. After staining the gel was fixed by soaking it in 10 % ethanol followed by heating in microwave (1000 W) for 1 minute and incubated with shaking at room temperature til the background was low enough. The gel was dried on a vacuum-drier for 1 hour.

### *Sequencing and cloning of the serpin-gene.*

Polymerase chain reaction (PCR) primers were designed for the ORF 4653 of *G. lamblia* genotype A-WB-06. These were (Forw): 5'-CCGATCCTCTGTTTCACGACGACCTCCTCACG-3' and (Rev): 5'-CCGAATTCCTTAAAGAAGAGCCTGCAGGAA-3' (Sigma Genosys, Stockholm Sweden). PCR was carried out with PCR-beads (GE-Healthcare, Uppsala Sweden), 0.8 µM primers and 1 µl template (genomic *Giardia*-WB DNA, 1 ng) for 30 cycles of 30 sec. denaturation at 96 °C 30 sec annealing at 55 °C and extension for 1 min at 72 °C with a final extension at 72 °C for 10 min. The PCR products were subjected to agarose gel

electrophoresis and extracted from the gel using a gel extraction kit (NucleoSpin® Extrakt II, Machery-Nagel, Düren, Germany). The extracted PCR fragments (0.2 µg) were cleaved with restriction enzymes, BamH1 and EcoR1, and ligated between BamH1 and EcoR1 sites of a plasmid pGEX-6P3 (table 1) with 1.5 µl ligation buffer (Invitrogen, Amsterdam, Holland) and 1.0 µl DNA ligase (Invitrogen, Amsterdam, Holland). The ligated plasmid was transformed into competent cells (*E.coli* – Top 10) using a heat shock method (cells and plasmids on ice 30 min followed by 42°C for 1 minute), grown in 1 ml LB medium (10 g/L Bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5) 1 hr at 37 °C and then spread on LA+ 100 ug/ml ampicillin plates. 4 colonies were inoculated in 2.5 ml LB medium + 2.5 µl ampicillin (50 mg/ml) and incubated in a shaker over night at 37°C. Plasmid DNA was prepared using a Nucleospin plasmid kit (Machery-Nagel, Düren, Germany). Ligation was confirmed by another cleavage of the PCR product in the vector between the BamH1 and EcoR1 sites. Sequencing was formed by the Rudbeck laboratory on the purified plasmids using the forward primer described above.

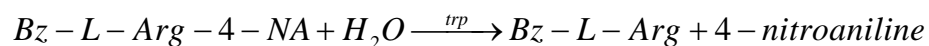
#### *Expression and purification of recombinant protein*

The confirmed serpin construct was transformed into chemically competent *E. coli* BL-21 gold cells (Invitrogen, Amsterdam, Holland). The cells were grown in LB medium to an optical density (OD) of ~0.6. Expression was induced by adding β-D-thiogalactopyranoside (IPTG) to a concentration of 1 mmol (10 µl IPTG/ml bacteria culture). The serpin was expressed at 37 °C for 3 hours. Protein extracts were then confirmed by SDS-PAGE gel from bacterial pellets from 100 µl of each culture. Large scale expression was performed in 1 liter cultures of LB medium in 2 liter flasks at 37 °C for 6 hrs. The culture was centrifuged (3000 x g) and the pellet resuspended in 120 ml STE-buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl and 1 mM EDTA) with lysozyme (Sigma, Stockholm, Sweden) (100 µg/ml) and incubated on ice for 15 min. Dithiothreitol (DTT) was added to a final conc. of 5 mM, and bacteria were lysed by addition of N-laurylsarcosine to 1.5 %. The lysate was sonicated in MSE 100 Watt Ultrasonic Disintegrator for 4 x 30 s at amplitude 5 and then centrifuged at 10 000 x g for 15 min at 4 °C. The supernatant was mixed with Triton X 100 (final conc. 1.7 %) and 1 ml 50 % Glutathione Sepharose slurry (GE-Healthcare, Uppsala, Sweden) was added. The solution was left over night at 4 °C. The samples were then washed in phosphate buffered saline (PBS) (20 mM K-phosphate, 14 mM NaCl, pH 7.0) and centrifuged at 500 x g for 10 min at 4 °C four times. The sample was divided in two sub-samples, one washed three times in cleavage buffer (50 mM HCl-Tris pH 7.0, 0.15 M NaCl and 1 mM EDTA) to remove unbound proteins, whereafter the proteins were cleaved with PreScission Protease (GE-Healthcare, Uppsala, Sweden). The other sub-sample was washed three times in 50 mM Tris-HCl pH 8.0 before elution with glutathione elution buffer (3.07 mg glutathione/ml Tris-HCl-buffer pH 8.0). The samples were incubated at 4 °C overnight on a shake-board. The proteins were then analyzed by SDS-PAGE gel.

#### *Proteinase inhibitory activity of recombinant protein*

Purified serpin was incubated with trypsin (final concentration of 3.3 µmol/L) and a trypsin substrate, Bz-L-Arg-4-NA (final concentration 4 mM). The amount of 4-nitroaniline produced was measured as 405 OD. This is a measurement of the catalytic activity of trypsin according to the following reaction;





All solutions used for testing the inhibitory activity of serpin were made according to the manufacturer's manual (Roche Molecular Biochemical's, Stockholm, Sweden). The substrate, Bz-L-Arg-4-NA, was difficult to dissolve in TEA buffer (4.84 g Tris base, 1.142 ml glacial acetic acid and 2 ml 0.5 M EDTA dissolved in dH<sub>2</sub>O to a final volume of 1000 ml), even at high temperature. Thus it was first dissolved in 1 ml DMSO and this solution was then mixed with TEA. The positive control used was a protein from soy bean that has an inhibitory effect on trypsin. The absorbance at 405 nm was measured directly after the substrate was added and every minute for 10 minutes, after which time the catalytic activity of trypsin ceased. All steps were performed according to the manufacturer's manual (Roche Molecular Biochemical's, Stockholm, Sweden) at 25°C.

### **Acknowledgments**

I'd like to thank my supervisor Staffan Svärd for all the inspiration and support throughout the project. I also want to thank Hanna Skarin for always helping out when ever I needed it. Without those I could not have made this bachelor degree project.

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