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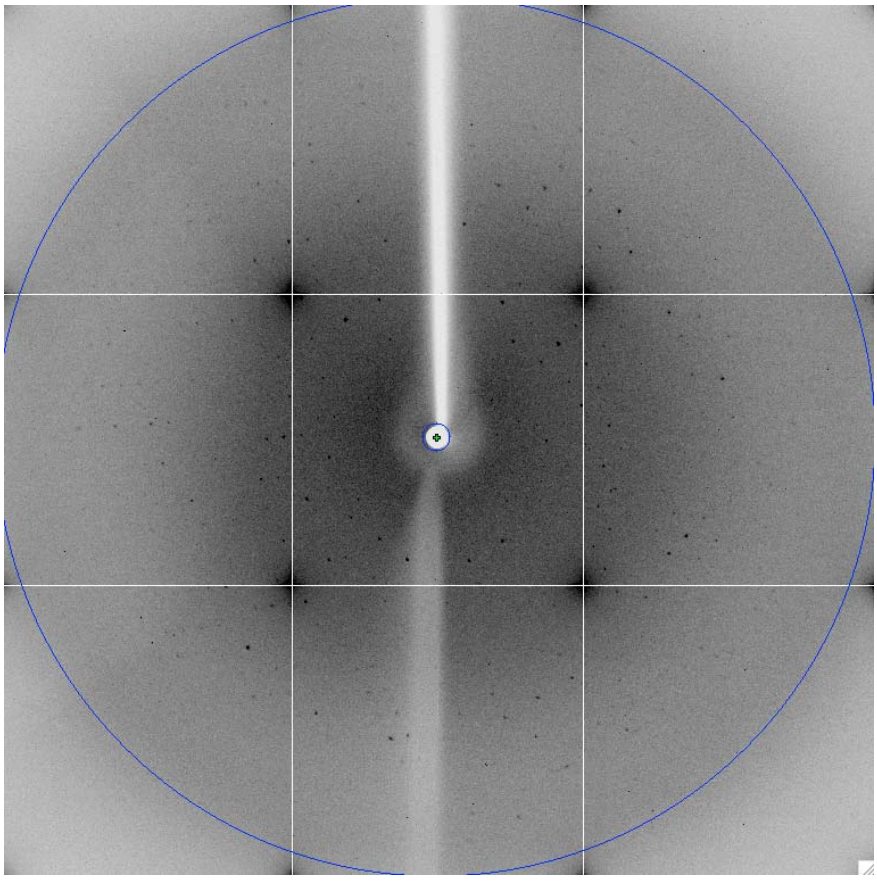
How to Kill a Parasite: A Report

An inhibitor study of ribose-5-phosphate isomerase B from *Giardia lamblia* as a possible drug target

Daniel Steinhauf



Hur man dödar en parasit: En Rapport

How to Kill a Parasite: A Report

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Abstract:

The parasite *Giardia lamblia* is known to cause the disease giardiasis, which is a major threat against the public health, especially in developing countries. A recent study has shown that over 200 million people have giardiasis now, and about 500 000 more people are infected every year. A *G. lamblia* infection is usually treated with the drugs metronidazole, albendazole, tinidazole and furazolidone. These drugs are in some cases toxic and have severe side effects. Therefore new methods of killing the parasite are of great importance. Ribose-5-phosphate isomerase (Rpi) is an enzyme active in the pentose phosphate pathway, and it catalyses the conversion of ribose 5-phosphate to ribulose 5-phosphate and *vice versa*. Some enzymes in this class can also act on 6-carbon substrates, interconverting allose 6-phosphate and allulose 6-phosphate. To find which inhibitor works best on the enzyme from *G. lamblia* (*Gl*RpiB), the enzyme was expressed as a His-tagged construct in E. coli was purified then tested to check its purity. When it was confirmed to be the right protein, the protein was used for crystallization and kinetic assays. Allose-6-phosphate was added before crystallization. Crystals formed in the presence of allose-6-phosphate. The K_m, V_{max} and k_{cat} were determined and inhibitory tests were performed. It was determined that the Km for ribose-5-phosphate is about 10 mM, and the k_{cat} is around 260sec^{-1}. The Km of for allose-6-phosphate is 0.41 mM, and k_{cat} 0.008sec^{-1}. Among 6 substrate-like compounds tested, only 4PEH (4-phospho-D-erythronohydroxamic acid) showed significant inhibition of *Gl*RpiB.

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Introduction

Giardia lamblia is a pear-shaped eukaryotic unicellular flagellated parasite. The first time *G. lamblia* was described was in 1681 by van Leeuwenhoek. The parasite was characterized in greater detail in 1859 by Lambl. The parasite differs from other eukaryotes by not having any classical mitochondria or cytochrome-mediated oxidative phosphorylation. Instead, the parasite has a mitosome, which has homologous proteins to mitochondria, suggesting a mitochondrial ancestry (S. Svard *et al* 2010). These facts indicate that the parasites use fermentative metabolism. *G. lamblia* is the most commonly detected parasite in human and mammalian intestinal tracts in the world (Adam 2001). It is known to cause the disease giardiasis, which is a major threat against the public health in developing countries (Tian 2010). It has also been proved that the *Giardia* parasite can jump between dogs and humans, if both live in the same location (Traube *et a.* 2004). A recent study has shown that over 200 million people now suffer from giardiasis, and about 500 000 more people are infected every year.

The *Giardia* life cycle is made up of an infective cyst and a vegetative trophozoite. The vegetative trophozoite is pear-shaped and about 12-15 μm long. The trophozoite has two nuclei that are similar in appearance and function, as they are replicated at the same time and transcriptionally active. The infective cyst has an inner double membrane layer and an outer filamentous layer that is made up from *N*-acetylgalactosamine. In contrast to the trophozoite, the cyst has four nuclei. *G. lamblia* has like most eukaryotes, linear chromosomes with telomeres on the edges. Sequencing of the genome has shown a very high G-C content (Adam 2001).

One is infected by ingesting the infective cyst, while the trophozoite causes the ~~a~~symptomatic infection. When a cyst comes into contact with the hydrochloric acid in the stomach, the outer filamentous layer breaks down and the cyst forms the trophozoite, which then moves into the small intestine. The trophozoite attaches to the intestinal wall by a ventral adhesive disk. This disk is a unique feature for *G. lamblia,* and is very important for the survival of the parasite, as no cellular invasion or receptor mediated adhesion has been reported. The disk has a concave shape and spans the whole ventral surface of the parasite. In the disk the proteins actin, myosin and topomyosin are found, which are needed for contraction. After the parasite has attached itself to the intestinal wall, it starts to take nutrients from the host and replicate. When the body flushes the small intestine with biliary fluid, some of the trophozoites form cysts which move into the jejunum with the biliary fluid and exit the host with the feces. The cysts can now infect a new host, thus completing the transmission cycle (Adam 2001). Symptoms start to appear two weeks after infection, and last for two to five days. *Giardia* infection usually does not cause a long lasting infection in people without immunodeficiency. Persons who have an immunodeficiency are more likely to suffer from a chronic infection, and in combination with starvation it is highly lethal. There have been cases of people without immunodeficiency who have suffered from a chronic infection with *Giardia* (Rivero et al.2010). A *G. lamblia* infection is usually treated with the drugs metronidazole, albendazole, tinidazole and furazolidone. These drugs are in some cases toxic and may have severe side effects (Tian 2010). Therefore a new method of killing the parasite would be of great importance.

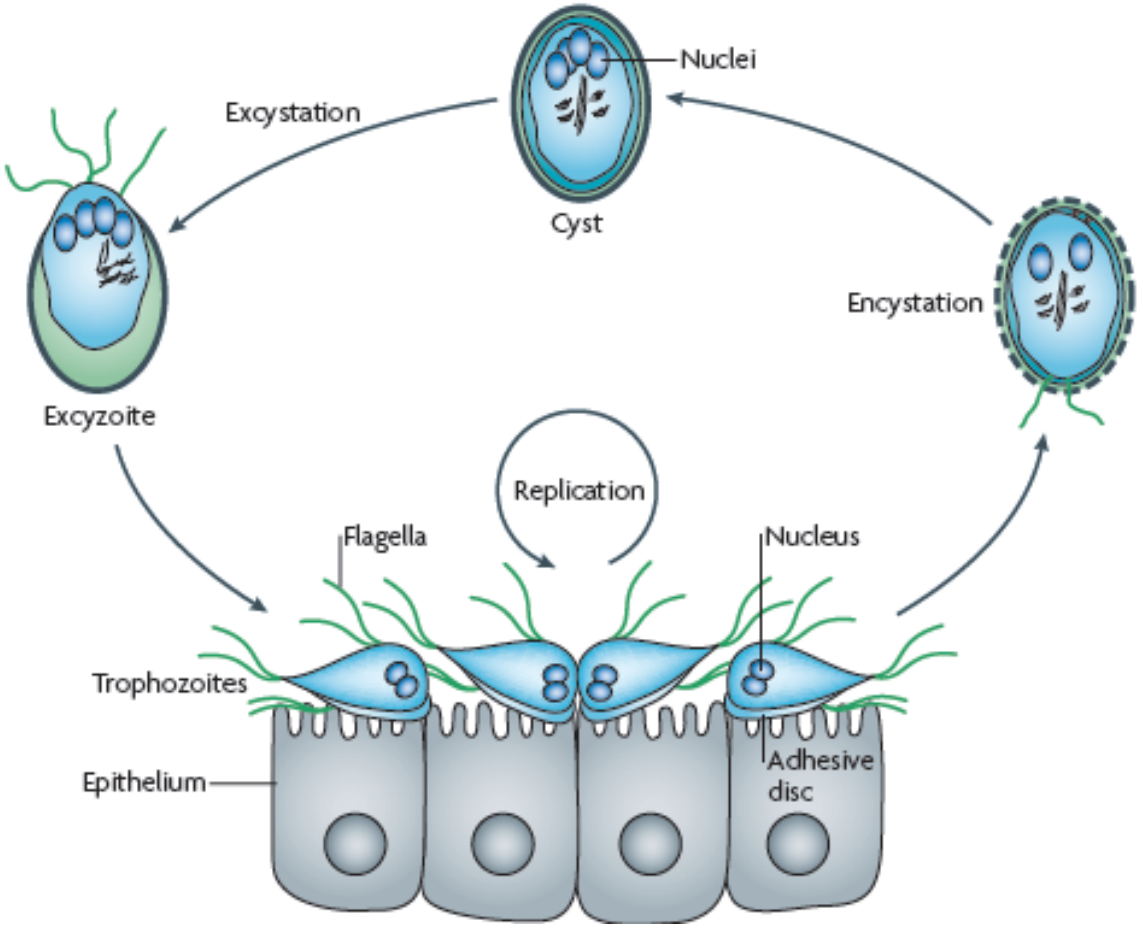

Figure 1. Picture showing the life cycle of *G. lamblia* with trophozoites attached to epithelial cells by the adhesive disk at the bottom of the picture and the infective cyst at the top of the picture (S. Svard *et al* 2010).

Ribose-5-phosphate isomerase (Rpi) is an enzyme active in the pentose phosphate pathway and it catalyses the conversion of ribose-5-phosphate to ribulose-5-phosphate and *vice versa* (Rose *et al* 2005). One important product of the pentose phosphate pathway is NADPH, which is used as a reductive agent in biological reactions. The pathway is also used to synthesize nucleic acids and other important biochemical compounds (Berg *et al* 2006). There are two completely unrelated types of Rpi: A and B. The two types of proteins have evolved convergently which means that they have different evolutionary ancestors but perform the same biochemical reaction. In agreement with the sequence information, the two types of Rpi have completely different structures, and their active sites show very little similarity. Humans have the A type of Rpi, while *Giardia* has the B type, which makes *Gl*RpiB a perfect candidate for a drug target, as a drug could be designed to inhibit the B type but not the A type, thus killing the parasite but not the patient (Stern *et al* 2006). *Gl*RpiB has a molecular weight 17804 Da in the construct with a His-tag and an isoeletric point of 6.5.

Materials and Methods

All calculations and buffer recipes can be found in the Appendix.

Expression of protein

Protein was induced and expressed as a His-tagged construct (A. Stern, unpublished data) by using homemade chemically competent BL21-AI *E. coli* cells (Invitrogen). A cell culture was started from a single colony in LB medium containing 100μg/ml of ampicilin. After overnight incubation the culture was diluted 1/50 in 800ml of fresh LB medium containing 100μg/ml of ampicilin. Three hours later the protein expression was induced using 0.02g/L of arabinose in the first expression and 0.2g/L of arabinose in the second expression for three hours. Cells were collected by centrifugation and stored at -20^{o}C.

Protein purification

To the bacterial pellet from the 800 ml of culture, 20 ml of lysis buffer was added to break up the cells which enable extraction of the expressed protein. The culture was run through a cell disruptor to make sure every cell was fully destroyed. The solution of disrupted cells was then centrifuged at maximum speed until the supernatant was clear. A nickel column was used to extract *Gl*RpiB from the solution of disrupted cells. The gene incorporated into the expression vector used had a His-tag sequence, which made it stick to the column while other proteins without a His-tag pass through the column. When the column is washed with imidazole-containing elution buffer, the interaction between the metal and the His-tag is broken and the protein is eluted. The nickel column used was a Ni Sepharose High Performance column sold by GE Healthcare. The column was first washed with distilled water and then equilibrated with lysis buffer. The supernatant was poured into the top of the column. When the supernatant has passed through the column and collected at the bottom, non-bound proteins were eluted with lysis buffer and collected. The column was then washed with wash buffer 1 and the eluate was collected. The same process was repeated with wash buffer 2. Finally, *Gl*RpiB was eluted with elution buffer and the eluate was collected.

Concentration of protein

All fractions containing protein were analyzed by SDS-PAGE, and fractions containing the protein with the predicted molecular weight of *Gl*RpiB were concentrated by a Vivaspin kit with a 5000 Da cut-off.

Measuring protein concentration

To know accurate protein concentration is important for kinetics, where accurate values are needed to get accurate kinetic values. The protein concentration was measured using a spectrophotometer and the Bradford method, calibrated with BSA (bovine serum albumin) provided with the Bradford reagents. In the Bradford method the absorbance (at 595nm) is divided with the Bradford factor which in this case is 0.04 μg/ml (obtained from the calibration with BSA). The Bradford method is linear till the region 10 μg -20 μg and measurements outside the linear face may not be accurate.

Gel filtration experiments

To verify that the product is of the right size and to investigate it aggregation state as well to purify the sample further, a HiLoad Superdex 75 preparative grade gel filtration column was used and followed according to the manufacturer's instructions. The column had a flow rate of 1ml/min and a column volume of 120.6 ml. The column was calibrated with the proteins ribonuclease A (15600 Da), Chymotrypsinogen A (20400 Da), Ovoalbumin (48100 Da) and Albumin (63500 Da). The buffer used was the gel filtration buffer. All fractions eluted from the column were collected.

Measurement of protein size and purity

To follow the purification process and make sure the expressed protein is of the right size, a SDS-PAGE was performed. The Phast system was used and followed according to the manufacturer's instructions. For the fractions from the gel filtration chromatography, 1 µl of fraction was mixed with 5 µl of 6x SDS buffer. From the eluted fractions collected when the nickel column was washed with wash buffer 1 and wash buffer 2 2.5 µl of sample was taken and mixed with 1 µl 6x SDS buffer. From the soluble and insoluble crude extracts, and the flowthrough from the nickel column, 1µl was mixed with 4 µl of distilled water and 1 µl of 6x SDS buffer. All the samples were heated at 95^{o}C for 20 seconds and then applied to the SDS-PAGE gel. A native gel was also performed to get an understanding of the protein's behavior when it is not denatured by SDS, and to see if this differs with substrate bound. 1.5 µl of 4 mg/ml protein in cacodylate buffer in pH 7.5, 6.5 and 5 were mixed with 1 µl of 5mM of ribose-5-phosphate, 1 µl of loading buffer and 2.5 µl of distilled water.

Crystallization

Crystallization of protein is essential when solving the structure of any protein by x-ray crystallography. In the crystal, all the protein molecules are aligned in the same direction so that when the crystal is hit by x-rays, the scattered x-rays will form a specific pattern on an x-ray detection plate. The spot pattern is used to solve the structure of the protein provided that some method of obtaining the correct phases is available. The crystallization plates used were MRC-2 plates from Hampton Research with different commercially available screens. The first screen set up was an optimum solubility screen, to see under what conditions the protein would precipitate. Those conditions are important to avoid because crystals are formed from initially clear drops. The screens after the optimum solubility screen had different buffers in which the protein would crystallize, as well as different precipitants. The plates and screens were set up using a Douglas Instruments Oryx 8 protein crystallization robot.

Kinetics

To be able to test potential inhibitors, one must be confident in the protein's K_m, V_{max} and k_{cat} values. In a kinetic test, a known concentration of substrate is added to the protein and the amount of product is measured as a function of time. Two different types of substrates were used:

Ribose 5-phosphate

The protein was diluted 1:41500 which gave a concentration of 0.002 mg/ml ($1.14 * 10^{-4}$mM) from the original 83 mg/ml. 80 µl Tris buffer was mixed with 10 µl of substrate in a cuvette and incubated for 5 minutes. After the incubation, 10 µl enzyme solution was added to the

cuvette which gave a final enzyme concentration of 0.0002mg/ml, and the increase in absorbance at 290 nm was measured for 180 seconds.

Allose 6-phosphate

The assay is based on the kinetic assay of Laurent Salmon *et. al* (2004). Incubation buffer and allose 6-phosphate were placed in 1.5 ml tubes, and 10 µl of enzyme was added to the tube to initialize the reaction. The tubes were placed at 26°C for 10 and 20 minutes.100 µl of HCl was added to each test tube to stop the reaction, after which 100 µl of TBA (thiobarbituric acid)-HCl solution (20 mM) was added to each test tube to bind to the product (allulose 6-phosphate). All test tubes were heated at 80°C for 6 minutes to convert the product and the TBA to a second product, which absorbs light. The tubes were placed on ice for 20 seconds to stop all activity in the test tubes. The tubes were incubated at room temperature for one hour. The absorbance at 438 nm was measured. The absorbance was considered as a change over time where the initial time and absorbance is assumed to be the moment the substrate and enzyme were mixed in the cuvette.

Inhibitor tests

70 µl Tris buffer pH 7.5 was mixed with 10 µl of ribose-5-phosphate with a final concentration of 5mM and 10 µl of inhibitor with a final concentration of 10mM in a cuvette and incubated for 5 minutes. After the incubation, 10 µl enzyme was added to the cuvette. The final concentration of enzyme in the cuvette was 1.8 µM. The change in absorbance at 290 nm for 180 seconds was measured at room temperature. All samples with inhibitor were repeated in triplicate. Samples containing 80 µl Tris buffer at pH 7.5 was mixed with 10 µl of substrate in a cuvette and incubated for 5 minutes. After the incubation, 10 µl enzyme was added to the cuvette. This was done to see what the change in absorbance was when there was no inhibitor present. If the triplets with inhibitor have a lower change in absorbance than the triplets without the inhibitor, the inhibitor works.

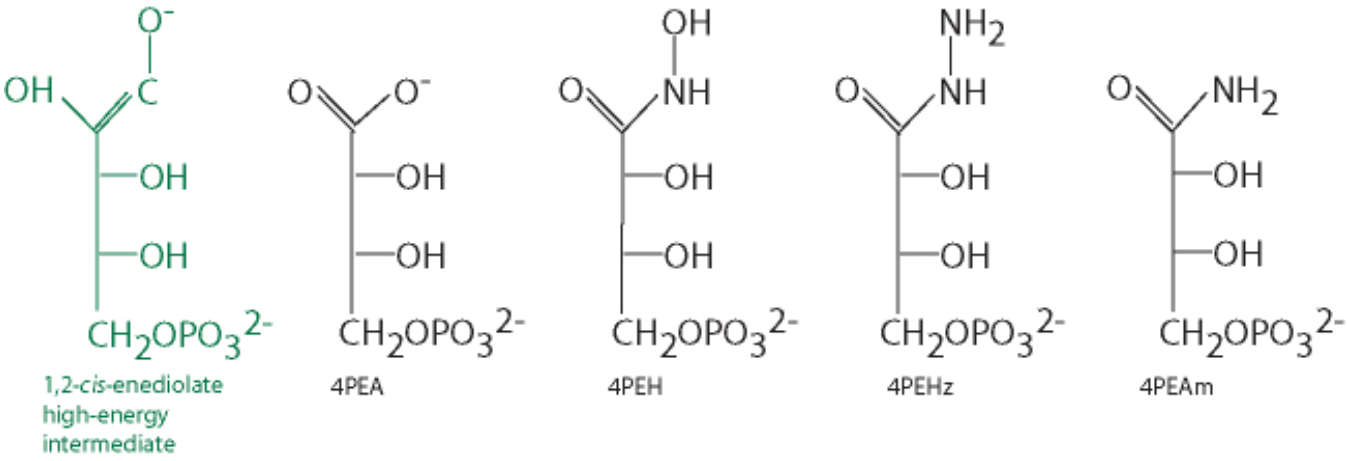

Figure 2. Picture showing the name and the structure of the four-carbon inhibitors.

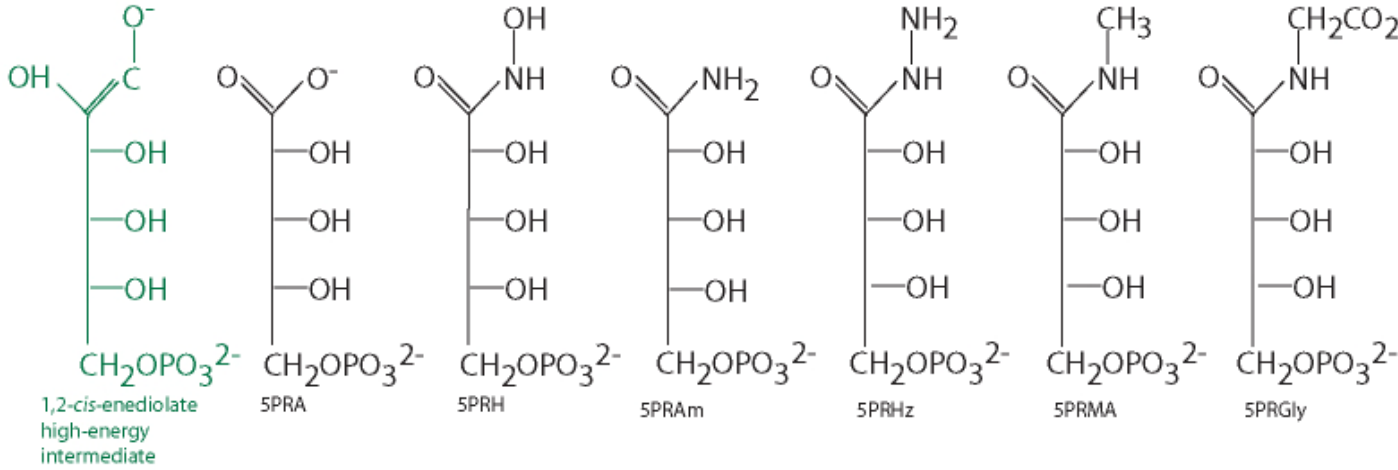

Figure 3. Picture showing the name and the structure of the five-carbon inhibitors.

Results

All calculations and buffer recipes can be found in the appendix.

Final concentration of protein and estimation of yield

First purification

For concentration of the protein with a Vivaspin column, fractions 22-25 (see Figure 2) from the gel filtration chromatography were pooled, as they most likely had the highest amount of the protein of interest amongst the fractions, this because of the height of the peak and the molecular weight of the protein that represented the peak on the gel chromatogram. To measure the concentration of protein the Bradford method was used, calibrated with BSA. The solution gave an absorbance of 0.733, which corresponds to an initial concentration of 83 mg/ml. This gives a yield of 20mg protein/L of cell culture.

Second purification

For concentration of the protein with a Vivaspin column, fractions B12-B14 (see Figure 3) from the gel filtration chromatography were used as they most likely had the highest amount of the protein of interest amongst the fractions because of the height of the peak and the molecular weight of the protein that represented the peak on the gel chromatogram. After the purification and during the concentration of protein, the protein solution was placed in two separate 20ml Falcon tubes one with cacodylate buffer in pH 7.0 and the other tube with cacodylate buffer in pH 6. The absorbance of the sample at pH 7.0 was 0.342 using the Bradford method, corresponding to 86 mg/ml protein in the concentrated sample and a yield of 42.5mg of protein/L of cell culture. The absorbance of the sample at pH 6.0 was 0.114 corresponding to 29 mg/ml after concentration and a yield of 14 mg of protein/L of culture.

Gel filtration chromatography

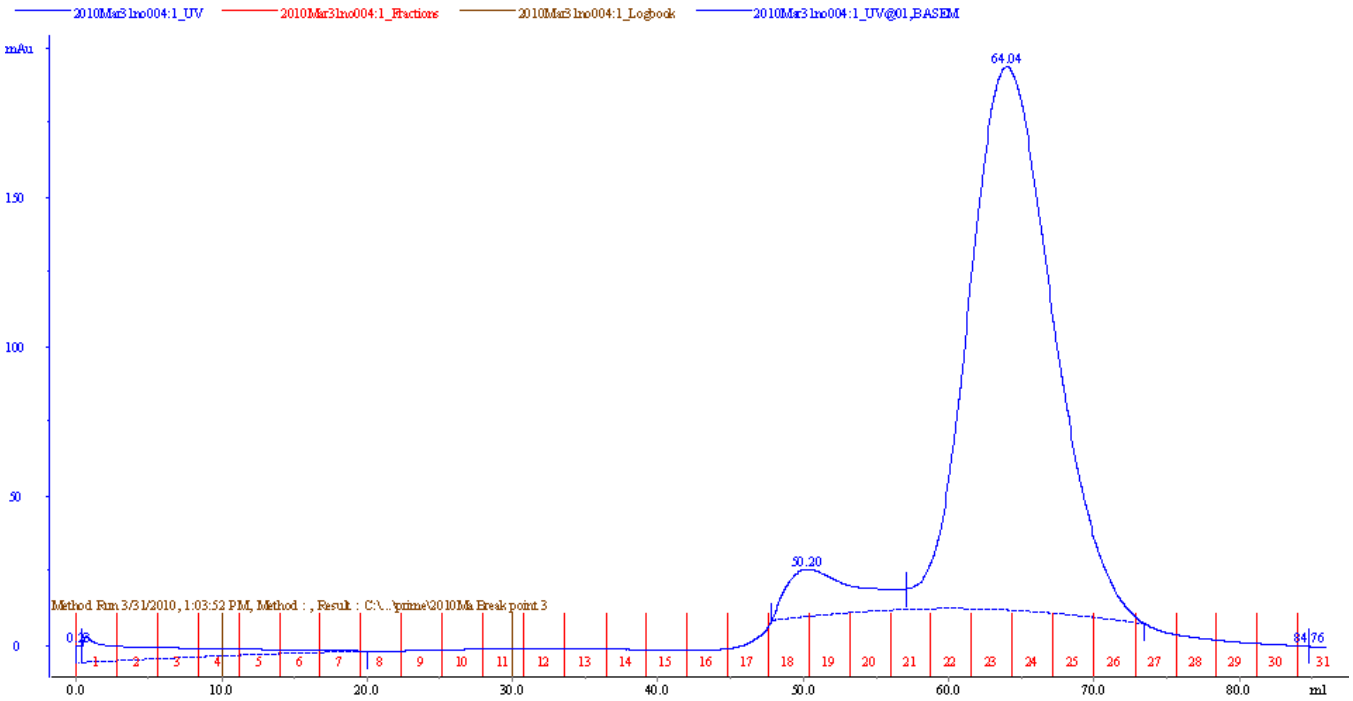

Figure 4. Gel chromatography graph from the second purification showing two peaks. The x-axis displays the elution volumes and the fraction numbers which are show in red numbers. The y-axis displays the mAu scale.

In Figure 4, two peaks are seen with different heights. The larger peak has an elution volume of 63.9 ml while the smaller peak has an elution volume of 50.0 ml. The K_{av} for the large peak was calculated to be 0.2636. By using a standard curve for the gel filtration column, the log of the molecular weight of the large peak was calculated to be 4.5 which gives a molecular weight of 33,8 kDa. The K_{av} for the small peak was calculated to be 0.0831, which is outside the calibration range and therefore the small peak represents a protein that is larger then the biggest protein used to calibrate the column (Albumin, 63500 Da).

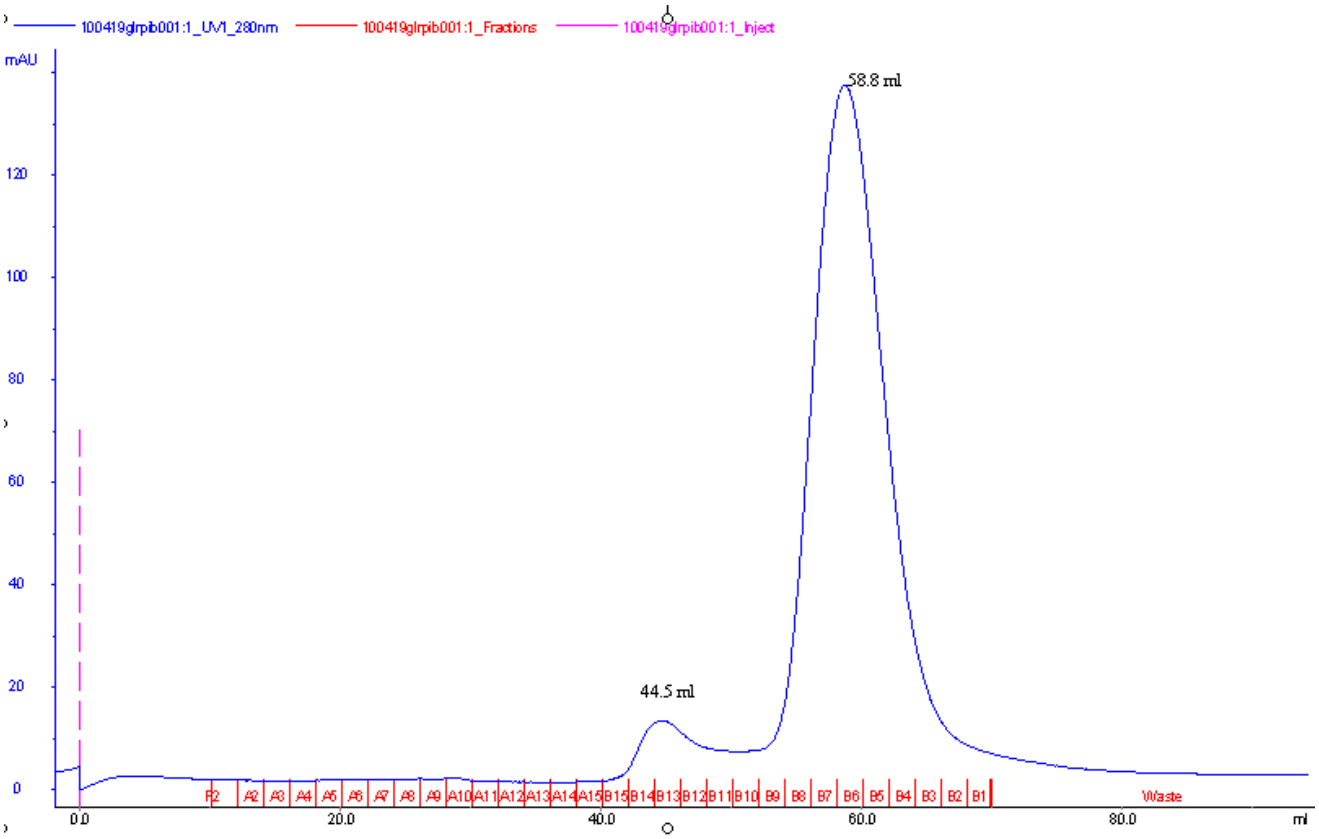

Figure 5. Gel chromatogram from the second purification showing two peaks. The x-axis displays the elution volumes and the fraction numbers, which are shown in red. The y-axis displays the absorbance scale in units of mAu.

In Figure 5, two peaks are seen with different mAu values. There is a large difference in the mAu values between the two peaks. The smaller peak has an elution volume of 44.5ml while the large peak has an elution volume of 58.8ml. The smaller peak has a K_{av} value of 0.0116, which is outside the calibration range and therefore the small peak represents a protein that is larger then the biggest protein used to calibrate the column (Albumin, 63500 Da).
The larger peak has a Kav value of 0.191. By using a standard curve for the gel filtration column the log of the molecular weight was calculated to be 4.66 which give a molecular weight of 45.7 kDa.

Verification of protein size by SDS-PAGE

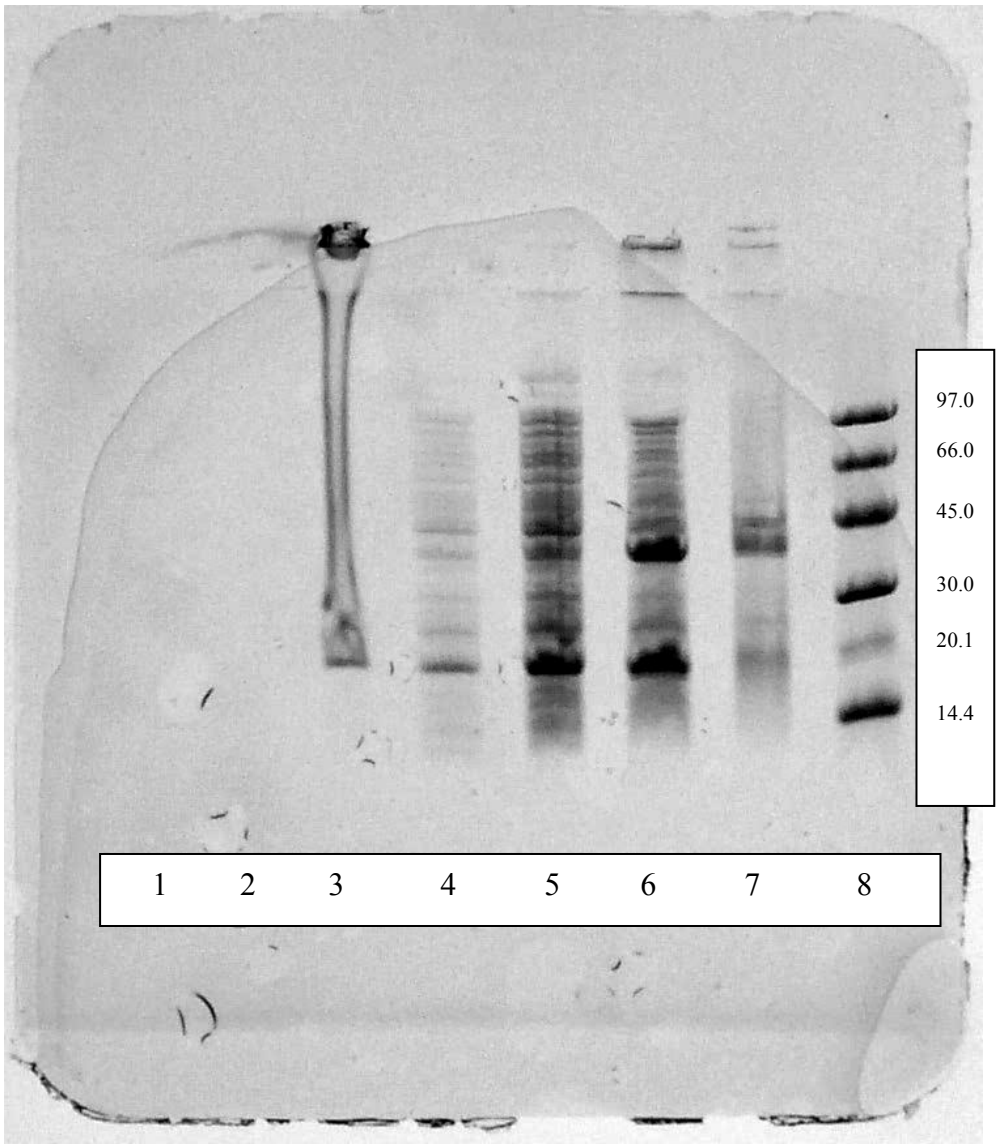

Figure 6. SDS-PAGE gel showing different fractions and eluates from the first protein purification. Lane 1 contains fraction 18 from the first gel filtration chromatography (see Figure 4). Lane 2 contains fraction 17 from the first gel filtration chromatography (see Figure 4). Lane 3 contains the insoluble crude extract. Lane 4 contains the second wash of the nickel column. Lane 5 contains the flowthrough from the nickel column. Lane 6 contains the crude soluble extract. Lane 7 contains the first wash of the nickel column. Lane 8 contains a Low Molecular Weight marker in kDa. The gel gradient is 8-25% acrylamide

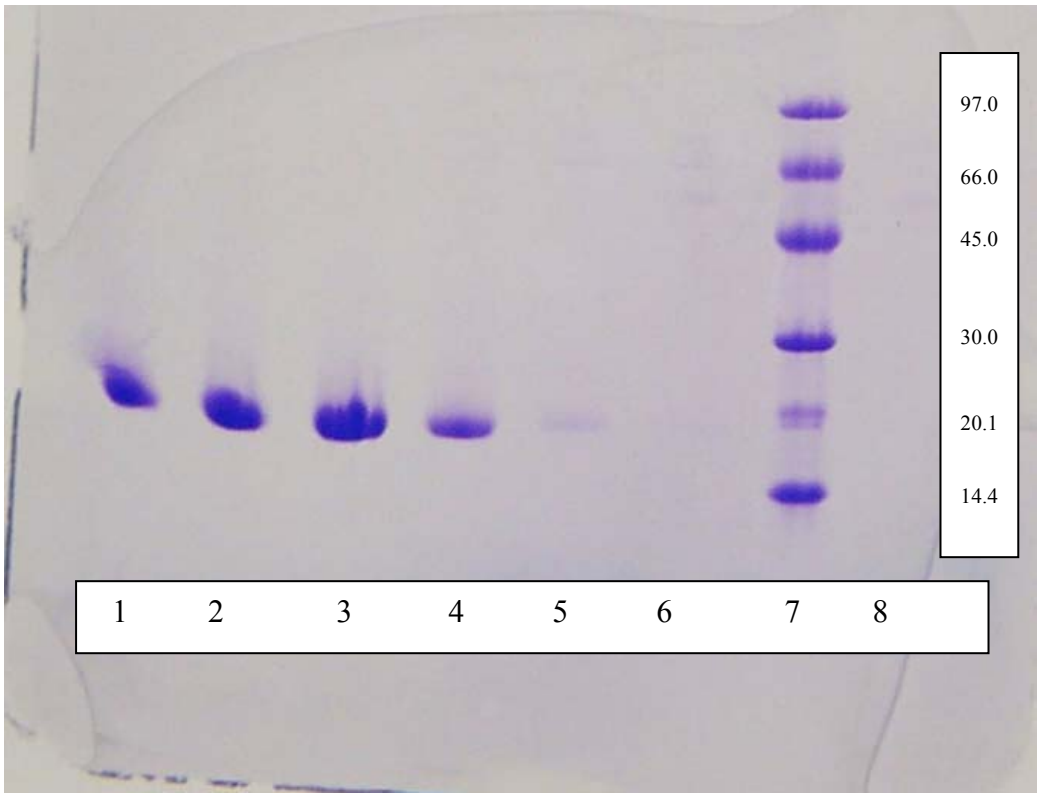

Figure 7. SDS-PAGE gel showing different fractions from the first gel filtration chromatography experiment (see Figure 4). Lanes 1 to 6 contain fractions in the order 25 to 20. Lane 7 contains a Low Molecular Weight marker in kDa. Lane 8 contains fraction 19. The gel gradient is 8-25% acrylamide.

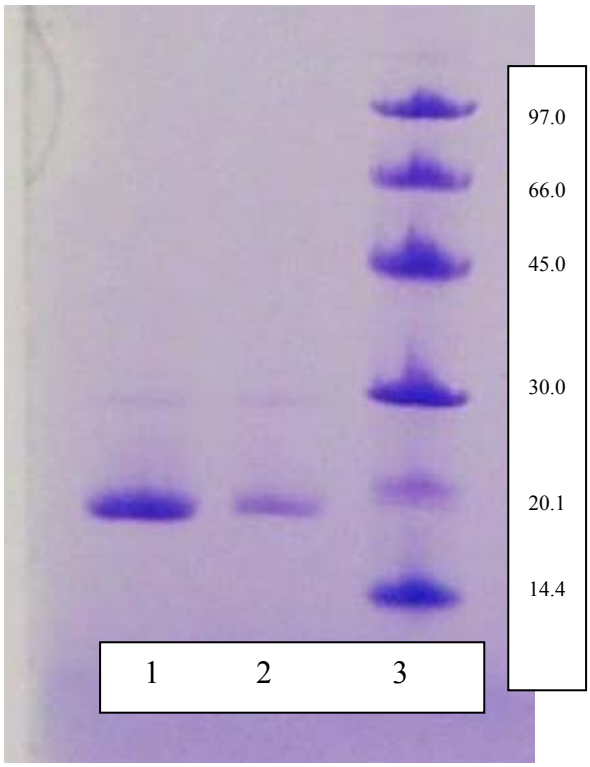

Figure 8. SDS-PAGE gel showing different fractions from the first gel filtration chromatography (see Figure 4). Lane 1 contains fraction 26. Lane 2 contains fraction 27. Lane three contains a Low Molecular Weight marker, with sizes marked in kDa. The gel gradient is 8-25% acrylamide.

As seen in Figure 6, there are clear bands at the right size for the monomer of *G. lamblia* ribose-5-phosphate isomerase B in lanes the following fractions: the second wash of the nickel column, the flowthrough from the nickel column and in the crude soluble extract. The fraction from the first wash of the nickel column shows a weak band at the right size for the protein. Fractions 18 and 17 from the gel filtration chromatography experiment (see figure 4) show no visible product. The SDS-PAGE gel in Figure 7 has strong bands at the right size in the fractions 25, 24, 23 and 22 from the gel filtration chromatography experiment (see figure 4). Fraction 21 has a weak band at the right size while fraction 20 and 19 do not have any visible product. In Figure 8 there are bands at the right size in lanes with fraction 26 and 27 from the gel filtration experiment (see figure 4) but the band in the lane containing fraction 26 is much more intense than the lane containing fraction 27.

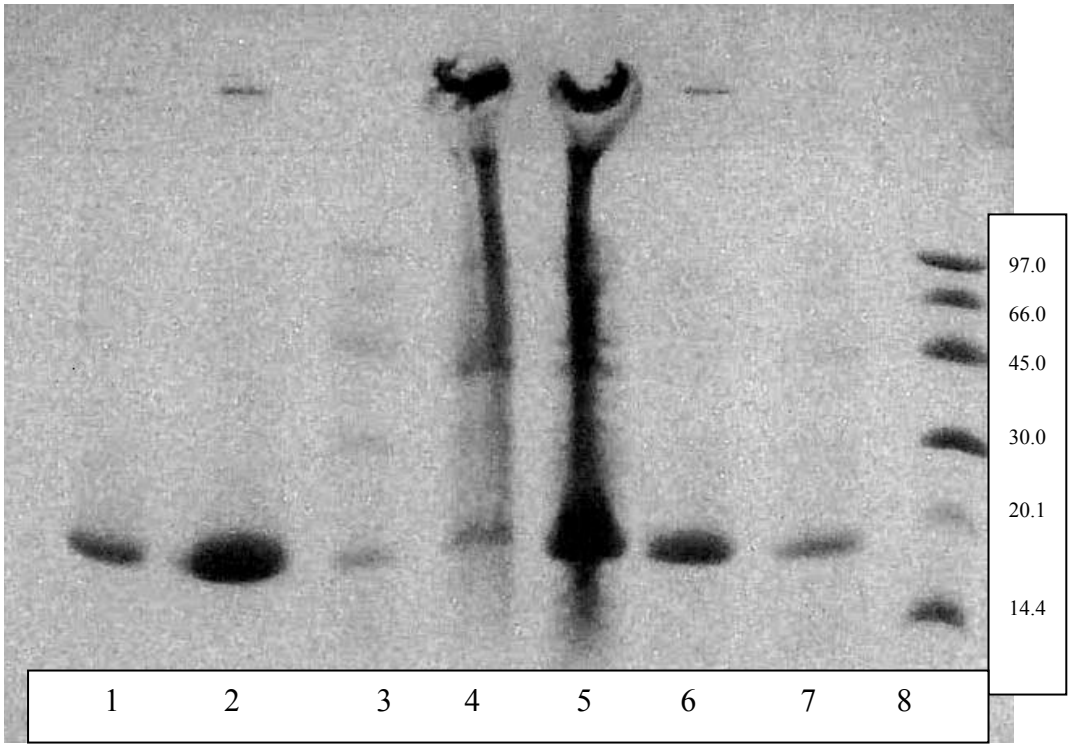

Figure 9. SDS-PAGE gel showing the different eluates from the second purification. Lane 1 contains the eluate from the nickel column collected when the column was washed with the elution buffer. Lane 2 contains 4µg/µl of protein kept in a buffer with a pH of 7.5. Lane 3 contains the flowthrough from the nickel column. Lane 4 contains the insoluble crude extract from the protein purification. Lane 5 contains the soluble crude extract from the protein purification. Lane 6 contains the eluate from the nickel column when it was washed with the second wash buffer. Lane 7 contains the eluate collected from the nickel column when it was washed with the first wash buffer. Lane eight contains a Low Molecular Weight marker, with sizes marked in kDa. The gel was a homogenous 20% acrylamide gel.

As seen in Figure 9, there are strong bands at the right size for *G. lamblia* ribose-5-phosphate isomerase B in lanes with product from eluate from the nickel column collected when the column was washed with elution buffer, 4µg/µl of protein kept in a buffer with a pH of 7.5, soluble crude extract from the protein purification, and the eluate collected from the nickel column when it was washed with the second wash buffer. There are weaker bands at the right size in lanes with the flowthrough from the nickel column, the insoluble crude extract from the protein purification and the eluate collected from the nickel column when it was washed with the first wash buffer.

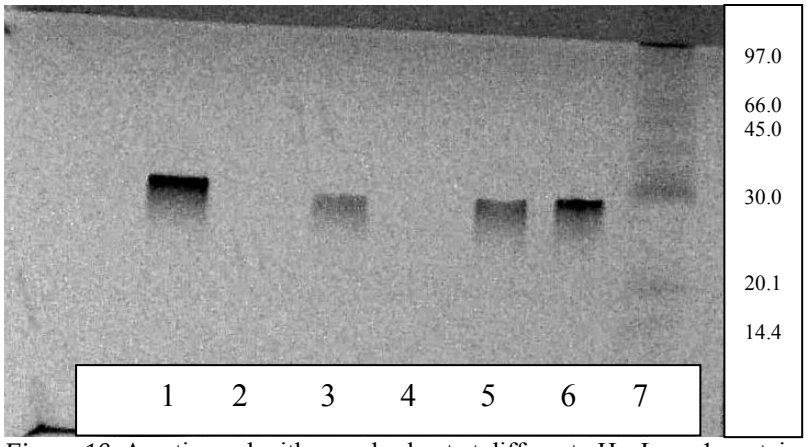

Figure 10. A native gel with samples kept at different pHs. Lane 1 contains RpiB kept in cacodylate buffer of pH 5.0 with ribose-5-phosphate. Lane 2 contains RpiB kept in cacodylate buffer of pH 6.5 with ribose-5-phosphate. Lane 3 contains RpiB kept in cacodylate buffer of pH 7.5 with ribose-5-phosphate. Lane 4 contains RpiB kept in cacodylate buffer of pH 5.0. Lane 5 contains RpiB kept in cacodylate buffer of pH 7.5. Lane 6 contains RpiB kept in cacodylate buffer of pH 6.5. Lane 7 contains a Low Molecular Weight marker, with sizes marked in kDa.

As seen in Figure 10, there are strong bands in lanes containing RpiB kept in cacodylate buffer of pH 5.0 with ribose-5-phosphate and RpiB kept in cacodylate buffer of pH 6.5. There are weaker bands in lanes containing RpiB kept in cacodylate buffer of pH 7.5 with ribose-5-phosphate and RpiB kept in cacodylate buffer of pH 7.5. There are no bands in lanes containing RpiB kept in cacodylate buffer of pH 6.5 with ribose-5-phosphate and RpiB kept in cacodylate buffer of pH 5.0. All bands are just under the 30.0 kDa marker.

Crystallization

Table 1. The table shows the screens run and their tendency to form clear drops or crystals.

Run #	Screen	Buffer	Protein concentration (mg/ml)	Ligand	Precipitant/clear drop ratio	Other notes
#001	Optimum solubility screen	Gel filtration	20	No ligand	63/192 precipitated. 97/192 clear	
#002	PACT	Cacodylate pH6.0	10	Ribose-5-phosphate	179/192 precipitated 13/192 clear.	
#003	JSCG+	Cacodylate pH 7.0	5	Ribose-5-phosphate	121/192 precipitated 70/192 clear	Crystals formed after 16 days. In cacodylate buffer pH7.0
#004	PACT	Cacodylate pH 7.0	5	Allose-6-phosphate	176/192 precipitated 16/192 clear	
#005	PEG/ION	Cacodylate pH 7.0/pH 5.0	5	Ribose-5-phosphate/ Allose-6-phosphate	126/192 precipitated 66/192 clear	
#006	Structure screen I	Cacodylate pH 7.5	4	Ribose-5-phosphate/	177/192 precipitated	

				Allose-6-phosphate	22/192 clear	
#007	Structure Screen I	Hepes pH 8.0/ cacodylate pH 7.0	2	Ribose-5-phosphate/ Allose-6-phosphate	87/192 precipitated 106/192 clear	Stored at 28°C, crystals formed after 48 hours in Hepes buffer pH 8.0.
#008	Structure Screen I	Hepes pH 8.0/ cacodylate pH 7.0	2	Ribose-5-phosphate/ Allose-6-phosphate	105/192 precipitated 86/192 clear	

The first column shows the run number of the plate, where #001 was the first plate run and #008 was the last plate run.. The sixth column shows the precipitant and clear drop ratio. Column seven shows any other notes important for the results.

The two last crystallization plates were both Structure Screens I, one stored in room temperature while the other was stored at 28°C. Both had proteins in buffers of pH 8.0 and pH 7.0 with either ribose-5-phosphate or allose-6-phosphate added. After 16 hours, long heavily twinned needle-shaped crystals had formed in droplets with allose-6-phosphate added, and conditions of 0.2 M magnesium acetate tetrahydrate, 0.1M cacodylate, pH 6.5 and 20% PEG 8000. These poor crystals were used to seed crystals in conditions 0.2 M magnesium acetate tetrahydrate, 0.1M Cacodylate pH 6.5 and 20% PEG 8000 but this only gave drops with precipitation. The other plate stored in 28°C gave crystals after 48 hours in droplets with allose-6-phosphate added, and conditions of 0.2 M calcium acetate hydrate, 0.1 M Na cacodylate pH 6.5 and 18% (w/v) PEG 8000. The crystals were taken out of the drop and stored in liquid nitrogen.

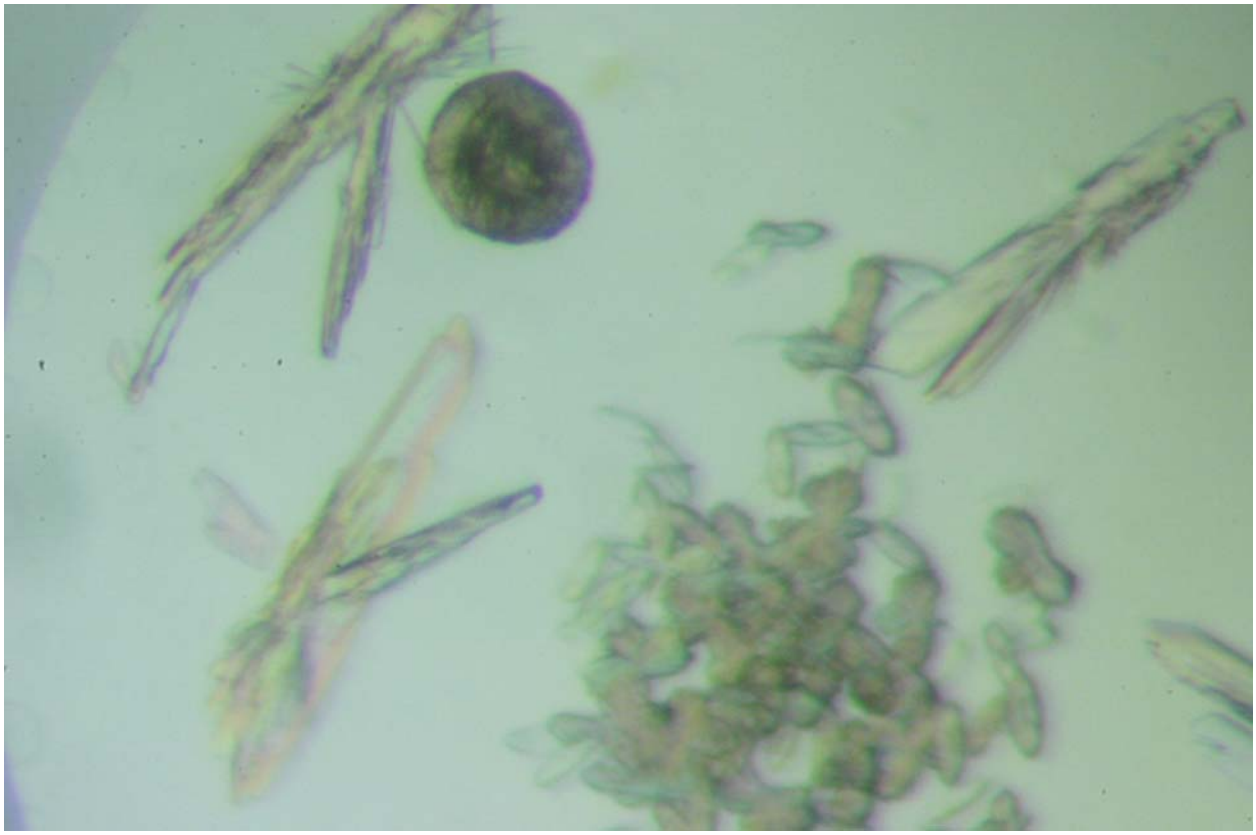

Figure 11. A picture of the needle shaped crystals that appeared in the Structure Screen I at room temperature. The circular crystals are suspected to be salt.

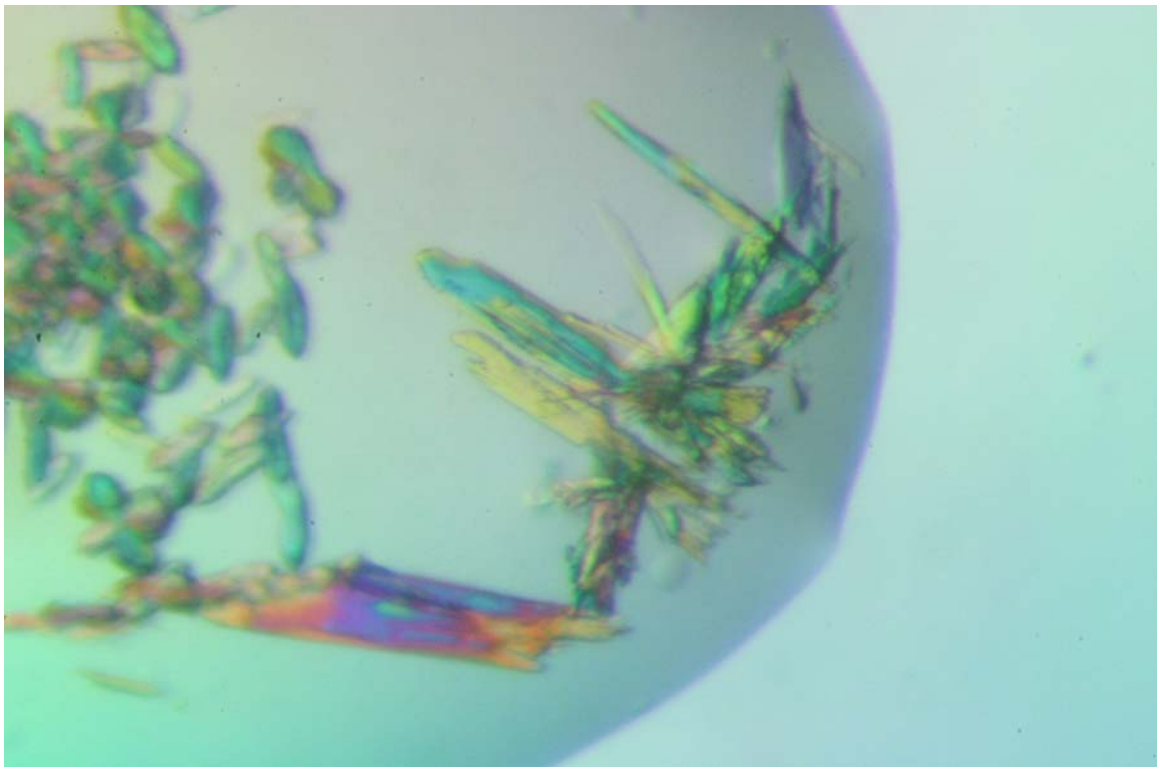

Figure 12. Here are needle shaped crystals from the same drop as in Figure 8 but they are viewed with polarized light.

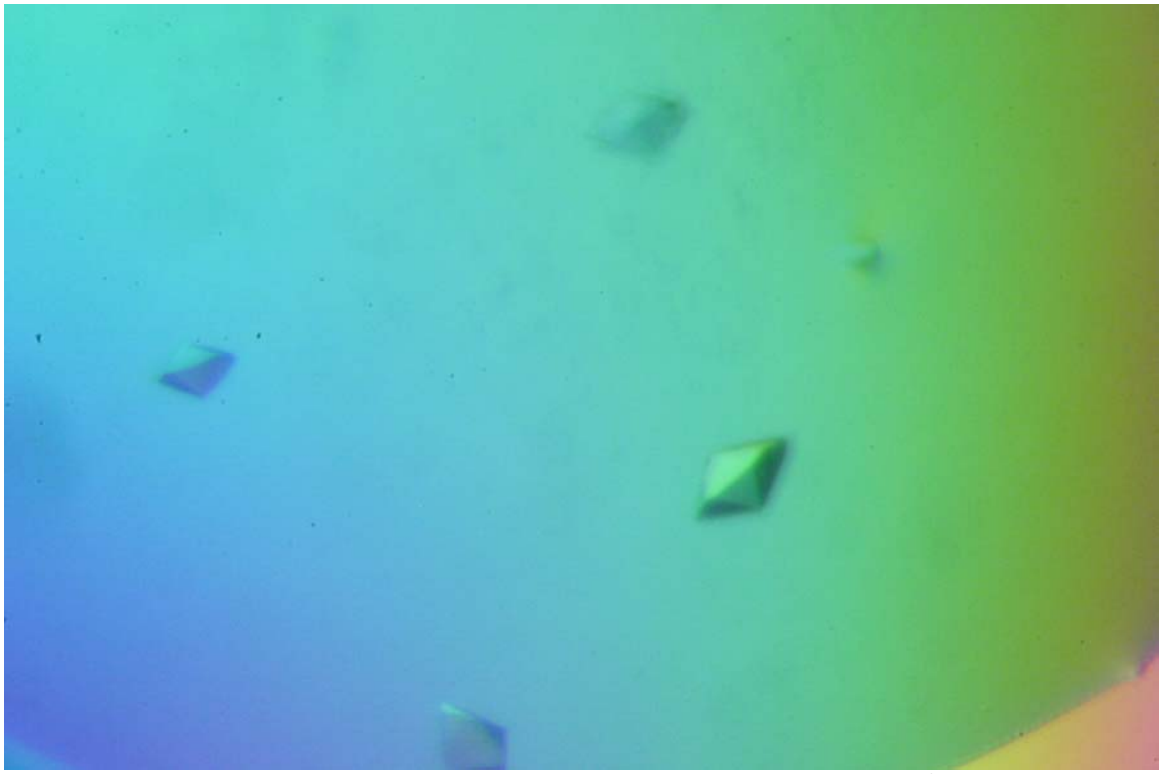

Figure 13. This is a picture of the crystals obtained in the Structure Screen I kept at 28°C. The droplet is viewed with polarized light.

Kinetics

Ribose-5-phosphate isomerase activity

Table 2. Results from the first kinetic assay of *Gl*RpiB activity.

Substrate concentration (mM)	Change in absorbance over 3 minutes	Velocity (mM/3 min)	1/v	Average 1/v	1/[S]	Average 1/[S]	[S]/v	Average [S]/v
5	0,0114	0,158333	6,315789	5,693106	0,2	0,2	31,57901	28,465575
5	0,0142	0,197222	5,070423		0,2		25,35214	
6	0,0195	0,270833	3,692308	4,726154	0,166667	0,166667	22,15387	28,356945
6	0,0125	0,173611	5,76		0,166667		34,56002	
7,5	0,018	0,25	4	4,384106	0,133333	0,133333	30	32,880815
7,5	0,0151	0,209722	4,768212		0,133333		35,76163	
10	0,0215	0,298611	3,348837	3,9385695	0,1	0,1	33,48838	39,385735
10	0,0159	0,220833	4,528302		0,1		45,28309	
13,3	0,0195	0,270833	3,692308	3,903297	0,075188	0,075188	49,10775	51,913825
13,3	0,0175	0,243056	4,114286		0,075188		54,7199	
30	0,0268	0,372222	2,686567	2,4179105	0,033333	0,033333	80,59706	72,53732
30	0,0335	0,465278	2,149254		0,033333		64,47758	
40	0,0346	0,480556	2,080925	1,885533	0,025	0,025	83,23692	75,42126
40	0,0426	0,591667	1,690141		0,025		67,6056	
50	0,0266	0,369444	2,706767	2,216693	0,02	0,02	135,3385	110,8347
50	0,0417	0,579167	1,726619		0,02		86,33089	

The table shows the values obtained from the kinetic assay of ribose-5-phosphate isomerase activity. The fifth column shows the 1/v values for the Lineweaver-Burke plot. The sixth column shows the 1/[S] values for the Lineweaver-Burke plot. The seventh column shows the substrate concentration divided by the velocity. The final enzyme concentration was 0.0002 mg/ml. The blank measurement was zero.

To convert the change in absorbance over 3 minutes to velocity (mM/3 min). The change in absorbance is divided by the extinction coefficient for ribulose-5-phosphate which is 72. For example:

Change in absorbance over 3 minutes 0.0114/72 = 0.00015 M => 0.00015 M * 1000 = 0.15 mM/3 min.

The extinction coefficient for ribose-5-phosphate is very low which gives a small change in absorbance over time.

As seen in Table 2 and Figure 12, there is an increase in the velocity of the reaction as the substrate concentration increases.

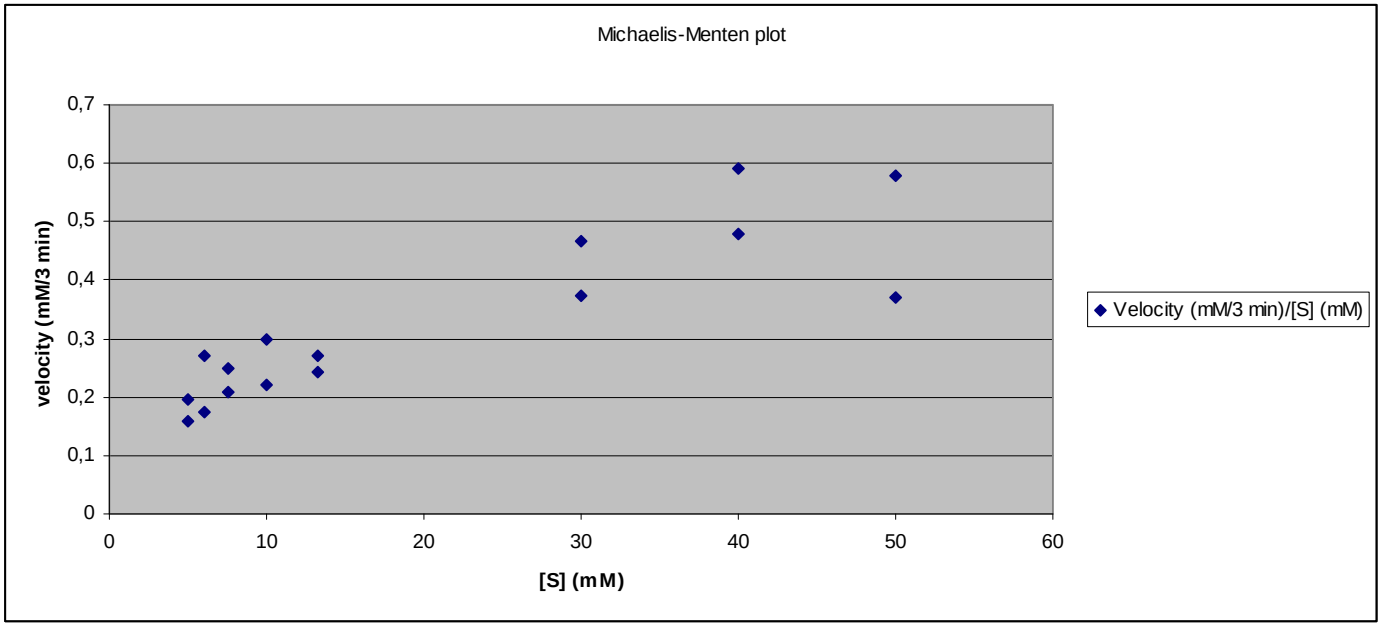

Figure 14. Michaelis-Menten plot with the velocity on the y-axis and the substrate concentration on the x-axis.

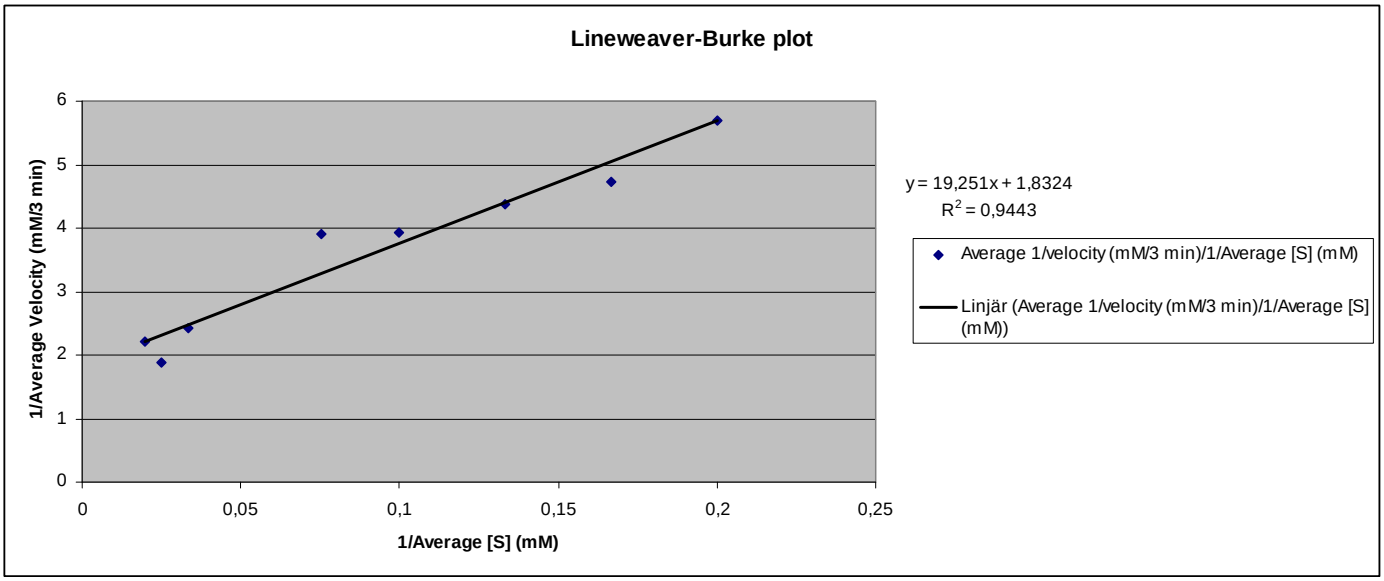

Figure 15. Lineweaver-Burke plot created with the values obtained in the kinetic assay presented in Table 2. The x-axis shows 1/Average [S] values and the y-axis shows the 1/Average velocity values.

From the Lineweaver-Burke plot the following kinetic constants were calculated:
K_m = 8.19 mM
V_{max} = 0.18mM/min
k_{cat} = 262sec^{-1}

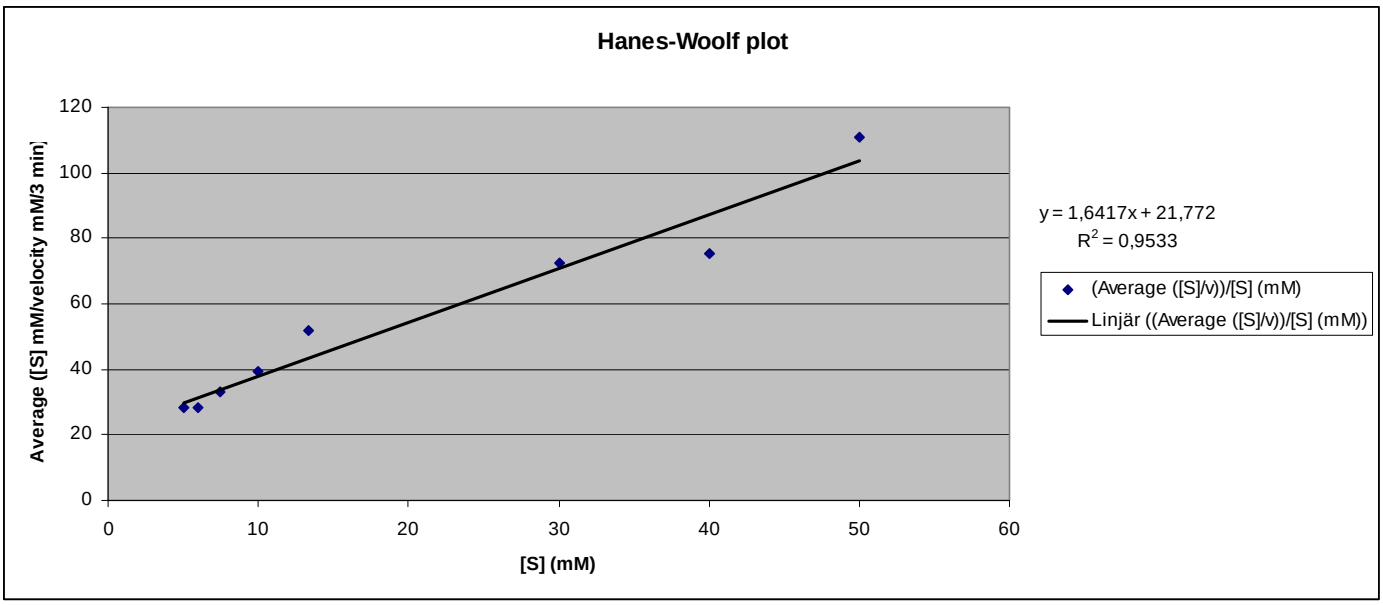

Figure 16. A Hanes-Woolf plot made from the data presented in Table 2. On the x-axis average ([S]/v) is displayed and on the y-axis the [S] is displayed.

From the Hanes-Woolf plot, the following kinetic constants were calculated:
K_m = 13.3 mM
V_{max} = 0.20 mM/min
k_{cat} = 291sec^{-1}

Allose-6-phosphate isomerase activity

Table 3. Results from the second kinetic assay of *GlRpiB* activity.

Substrate concentration mM	Absorbance at 20 min	Absorbance at 20 min with blank subtracted	Velocity (mM/min)	1/[S]	1/v	[S]/v
0	0,35	0	0	0	0	0
0,1	0,46	0,11	0,00019	10	5054,545	505,4545
0,2	0,68	0,14	0,00025	5	3971,429	794,2857
0,3	0,78	0,23	0,00041	3,333333333	2417,391	725,2174
0,5	0,97	0,41	0,00073	2	1356,098	678,0488
1	1,49	0,36	0,00064	1	1544,444	1544,444
2	1,53	0,45	0,00080	0,5	1235,556	2471,111

The table shows the results from the kinetic assay with allose 6-phosphate. The fifth column shows 1/[S]. The sixth column shows 1/v. The seventh column shows the substrate concentration divided by the velocity. The enzyme concentration was 1.8µM. All the values were obtained from the samples which were left on the heating block for 20 minutes.

To convert the absorbance at 20 minutes to velocity (mM/ min), the change in absorbance is divided by the extinction coefficient for the Allulose-6-phosphate + TBA product which is 27800 M and by the time (20 minutes). For example:

Absorbance at 20 min 0.11/27800 = 0.0000039 M => 0.0000039 M * 1000 = 0,0039 mM => 0,0039 mM/20 min = 0,00019 mM/min

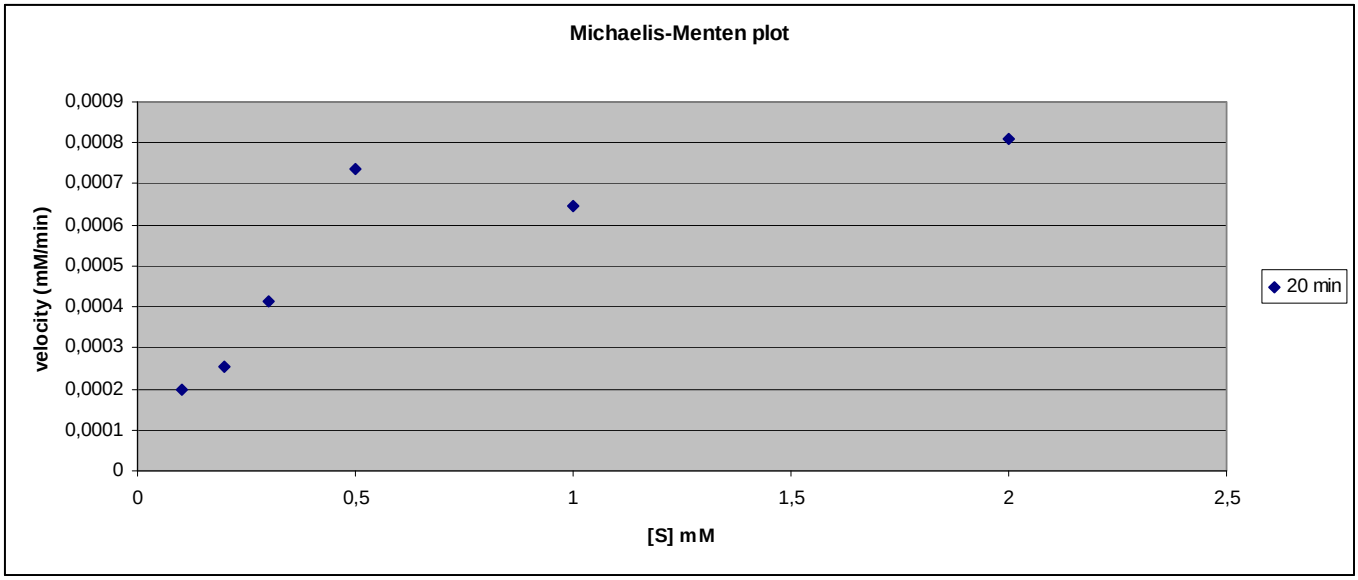

Figure 17. Michaelis-Menten plot with the velocity on the y-axis and the substrate concentration on the x-axis.

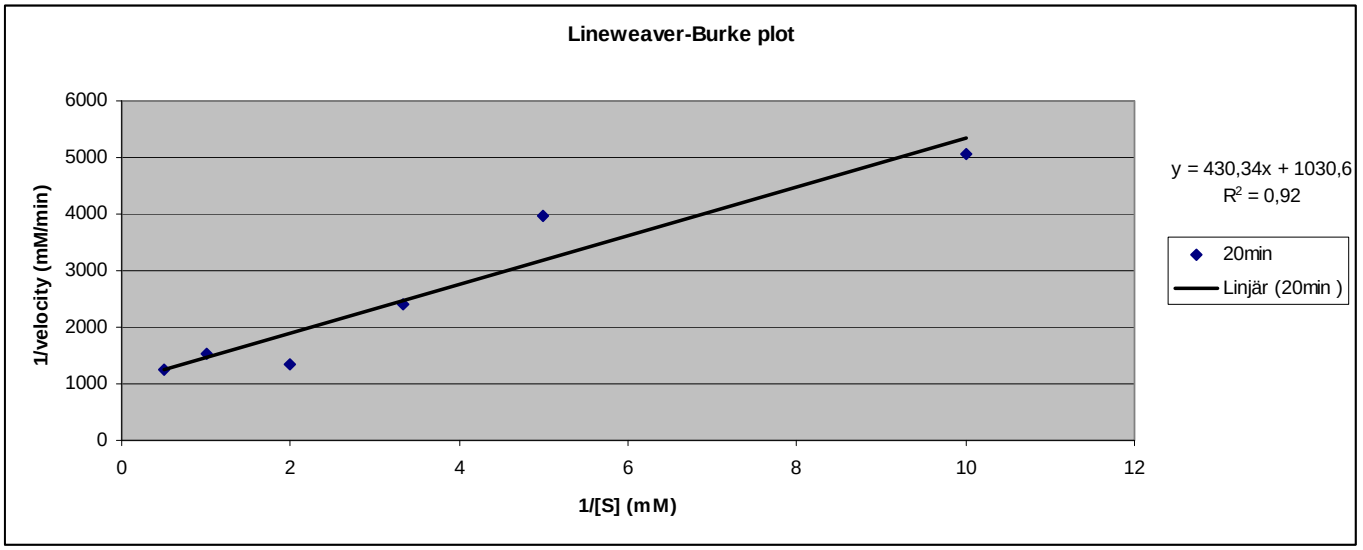

Figure 18. Lineweaver-Burke plot created with the values obtained in the kinetic assay presented in Table 3. The x-axis shows 1/[S] values and the y-axis shows the 1/v values.

From the Lineweaver-Burke plot the following kinetic constants were calculated:
K_m = 0.41mM
V_{max} = 0.0009 mM/min
k_{cat} = 0.008sec^{-1}

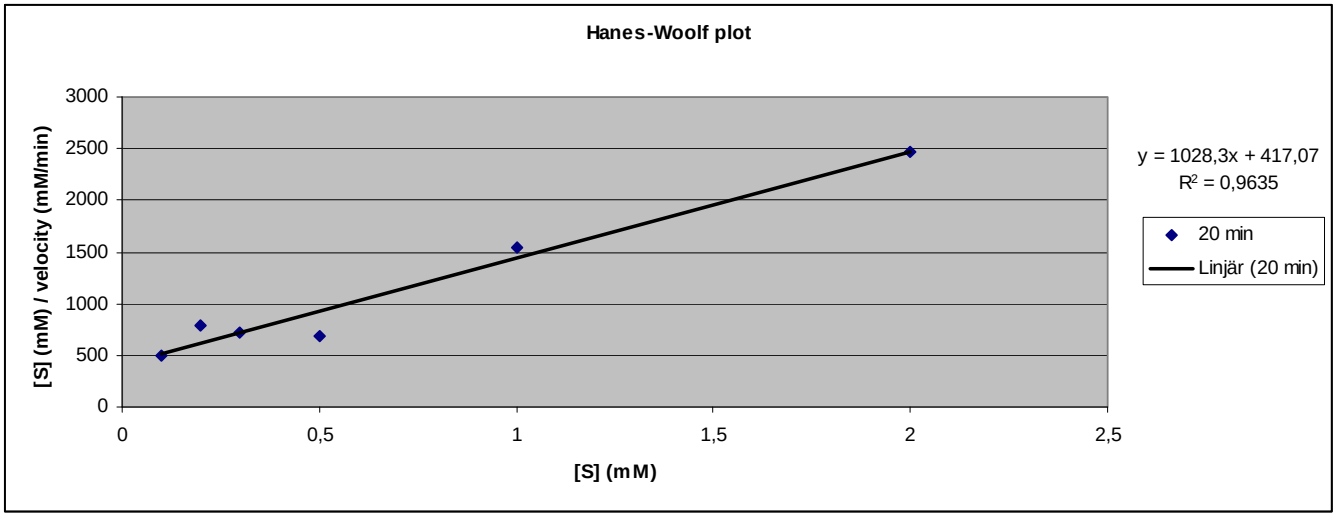

Figure 19. A Hanes-Woolf plot made from the data obtained in Table 2. On the x-axis, [S]/v is shown and on the y-axis the [S] is shown.

K_m = 0.41mM
V_{max} = 0.0009 mM/min
k_{cat} = 0.008sec^{-1}

Inhibitor tests

Table 4. Results from inhibitor test.

Tube content Final [I]: 10mM, Final [S]: 5mM	Change in absorbance during a period of 180 seconds	Average Change in absorbance during a period of 180 seconds
Enzyme blank	0	
4PEH	0.00084	0.0021
4PEH	0	
4PEH	0.0056	
Without inhibitor	0.0112	0.0106
Without inhibitor	0.0058	
Without inhibitor	0.0148	
5PRGly	0.0092	
5PRGly	0.0093	
Without inhibitor	0.0100	0.0099
Without inhibitor	0.0100	
Without inhibitor	0.0098	
4PEHz	0.0130	0.0133
4PEHz	0.0100	
4PEHz	0.0170	
Without inhibitor	0.0083	0.0097
Without inhibitor	0.0100	
Without inhibitor	0.0110	
5PRA/5PRH	0.0110	0.0101
5PRA/5PRH	0.0097	
Without inhibitor	0.0096	0.0110
Without inhibitor	0.0104	

5PRAm	0.0132	0,0115
5PRAm	0.0112	
Without inhibitor	0.0102	0,0076
Without inhibitor	0.0105	
5PRMA	0.0111	
5PRMA	0.0104	
Without inhibitor	0.0105	0,0106
Without inhibitor	0.0103	
Without inhibitor	0.0112	

The table displays the change in absorbance over 180 seconds to it correspondent inhibitor. The first column contains the test tube content and the second column contains the change in absorbance during a period of 180 seconds. 5PRA and 5PRH amounts were limiting so only duplicates are shown for that measurement and both inhibitors were run at the same time.

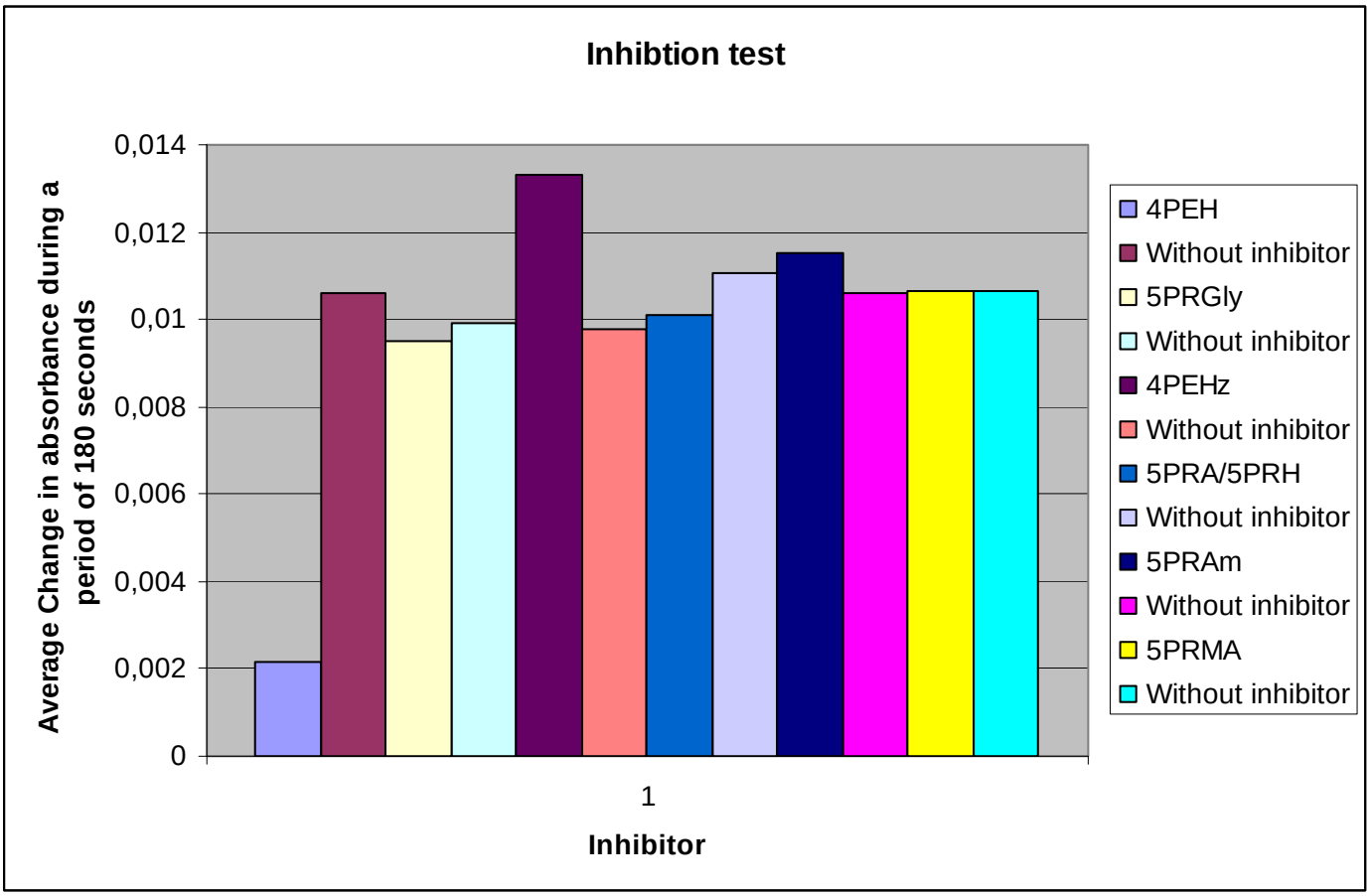

Figure 20. A bar diagram displaying the inhibitors' effects. The y-axis shows the average change in absorbance over 180 seconds. The x-axis shows the values for the inhibitor and a corresponding measurement without inhibitor. The box to the right shows which staple corresponds to what inhibitor. The staple to the right of the inhibitor staple is the blank without inhibitor which is needed to see if the inhibitor works.

As seen in Table 4, the change in absorbance without inhibitor is about 0.0100 during a period of 180 seconds. The final concentration of inhibitor was 10mM. The final concentration of enzyme was 0.0002 mg/ml (0.0114 μM). The first six samples had a final substrate concentration of 5 mM while the other samples had a final concentration of 10 mM. It was decided to increase the substrate concentration because if the change in absorbance would become smaller it could be too little to measure. There are only duplicates of SPRAM because the amount of inhibitor available was limited. The only inhibitor that seems to have a great affect on the change in absorbance was 4PEH.

Discussion

During the concentration of protein at pH 6.0 during the second purification, the protein precipitated in the Vivaspin tube and therefore not all of the protein could be recovered which gave a smaller yield. It thus seems that the protein is more soluble in higher pHs.

As seen in Figure 4, there were two peaks eluted from the gel filtration column. The higher peak had a calculated size of 33.7 kDa which is about the size of the dimer of *Gl*RpiB which is 35 kDa. In the second purification as seen in Figure 5, using the same column the higher peak had an estimated molecular weight of 45.7 kDa, which is much larger than the dimer. The smaller peak has a molecular weight of 110.2 kDa. In both purifications there was an small peak which was at a larger size than the dimer. The small peaks with the large molecular weights could be explained by inclusion bodies which would not be soluble but sill have a His-tag and thus get stuck to the nickel column and eluted with *Gl*RpiB.

The gel seen in Figure 7 shows *Gl*Rpi B in fractions 25 to 21. Figure 8 the protein is present in fractions 26 and 27 which corresponds to the largest (dimer) peak in the gel filtration peak in Figure 2. In Figure 9, lane 1 has a band at the right for *Gl*Rpi B which indicates that the protein in the second purification is also of the right size. The native gel (see Figure 10) indicates that the protein is a homogeneous dimer as all of the bands are ≈ 30.0 kDa (GlRpiB = 17804 Da with His-tag construct).

By looking at Table 1 it is very clear that the protein crystallizes in higher pH and that crystals form earlier in higher temperature than room temperature. Cacodylate seems to be needed to form crystals as in every condition crystals formed cacodylate was present. The crystals in Figure 11 were sent to the synchrotron in Grenoble but the resolution of the data obtained (≈3Å) was too low to solve the structure of the protein.

The plot in Figure 14 for ribose-5-phosphate isomerase activity dose not have the ideal shape of a Michaelis-Menten plot but dose generally follow the expected pattern. The Lineweaver-Burke plot does not look strange but the K_m is 8.2 mM which is high compared to *Trypanosoma cruzi* (which also is a parasitic protozoa) Rpi B which in the study made by Ana L. Stern *et al* has a K_m of 4mM. Compared to the RpiB from the slow growing parasite *Mycobacterium tuberculosis* (K_m = 3.7mM) the *Gl*RpiB K_m is also high (Roos *et al* 2004). When the values were placed in a Hanes-Woolf plot all values increased. The k_{cat} values in both the Hanes-Woolf and the Lineweaver-Burke are very large. It is about twice as large as the k_{cat} for *Mt*RpiB ($k_{cat} = 120sec^{-1}$) with ribose-5-phosphate (Roos *et al* 2004). Because no structure has been solved for *Gl*RpiB it is hard to speculate if the structure might affect the high K_m and k_{cat} values.

The plot for the kinetic assay with allose-6-phosphate is seen in Figure 17, is a better fit to the Michaelis-Menten equation. The values obtained from the Hanes-Woolf plot are identical to the values obtained from the Lineweaver-Burke plot which strengthens one's trust in the kinetic values. Because of the high K_m obtained in the kinetic assay were ribose-5-phosphate is used as a substrate indicates that the protein does not prefer to work with ribose-5-phosphate. In fact, in the parasite could use RpiB to produce ribulose-5-phosphate from ribose-5-phosphate.

As seen in Table 4 and Figure 20, 4PEH (4-phospho-D-erythronohydroxamic acid) is the only inhibitor that differs significantly in the test with and without inhibitor. As seen in figure 2 4PEH is very similar to ribose-5-phosphate except that it has a four carbon back bone while

ribose has a five carbon and 4PEH has a NH-OH group bound to it. The inhibitor 5PRH has the same group bound but it has a five carbon back bone (as seen in figure 3) which would inhibit the reaction allose-6-phosphate/allulose-6-phosphate but when looking at the numbers in Table 4 there is only a small change in the absorbance between the samples with inhibitor and without inhibitor. Because the small change in 5PRH it can not be concluded that the NH-OH group is the single factor that determines whether the compound inhibits the protein. The reason for 5PRH low change in absorbance between samples with and without inhibitor could be because it is a six carbon sugar and is placed differently in the protein and therefore the NH-OH group is placed wrong in the active site.

What can be concluded from the results is that the protein precipitates in lower pHs as seen in table 1 were screens with lower pHs had more precipitated drops than clear. The tests showed that the protein has a higher K_m and k_{cat} compared to other parasites that infect humans. The inhibitory test showed that the compound that inhibited the enzyme reaction with the greatest effect from the compounds tested was 4PEH as it lowered the average change in absorbance compared to the control without inhibitor (as seen in table 4 and figure 20). Therefore 4PEH is the best compound to develop as an agent against *Giardia lamblia* infection from the compounds tested.

Further work

For further work calculating the K_i for 4PEH would be of great importance as it will tell one if the inhibitor is competitive, non-competitive or uncompetitive. After the K_i of the inhibitor has been determined the chemical structure of 4PEH has to be changed as it will not be taken up by the parasite because of the phosphate group bound to it. When one knows that the compound will taken up by the parasite initial drug test can take place.

Acknowledgments

I would like to thank Sherry Mowbray for being a great supervisor who let me do my bachelor thesis at her department and for giving me such an interesting and exciting subject to work with. I also want to thank Sherry for all the feedback during my time in the lab and with my report. I want to give a special thanks to Ana Laura Stern for being my co-supervisor and helping me with problems in the lab and with my report. I would also like to thank Ana for having such a positive attitude to my (sometimes stupid) questions and ideas, and for being such a great person to work with. Finally I would like to thank all the people at x-ray for being the best colleagues one could have and for taking me in. To you all I wish you the best!

Thank you!

Appendix

Buffers:

Lysis buffer:

- 50 mM NaH_2PO_4 (pH8)
- 300 mM NaCl

- 10 mM Imidazole
- 1% (v/v) Glycerol
- 400 mM phenylmethylsulfonyl fluoride (Serine protease inhibitor)
- 1 mM β-mercaptoethanol

Wash buffer 1:

- 50 mM NaH_2PO_4 (pH8)
- 300 mM NaCl
- 50 mM Imidazole
- 1% (v/v) Glycerol

Wash buffer 2:

- 50 mM NaH_2PO_4 (pH 8)
- 300 mM NaCl
- 100 mM Imidazole
- 1% (v/v) Glycerol

Elution buffer:

- 50 mM NaH_2PO_4 (pH 8)
- 300 mM NaCl
- 300 mM Imidazole
- 1% (v/v) Glycerol
- 10 mM β-mercaptoethanol

Gel filtration buffer:

- 20 mM Tris HCl (pH 7.5)
- 150 mM NaCl
- 1% v/v Glycerol
- 10 mM β-mercaptoethanol
- 1 mM EDTA

Cacodylate buffer:

- 20 mM Cacodylate, pHs: 5.0, 6.0, 6.5, 7.0, 7.5
- 150 mM NaCl
- 1% v/v Glycerol
- 10 mM β-mercaptoethanol

Hepes buffer:

- 5 mM β-mercaptoethanol
- 150 mM NaCl
- 20 mM Hepes pH 8.0

Incubation buffer

- 50 mM TrisHCl pH 7.6; 5 mM
- MESNA

Raw data

Gel filtration chromatography

Table 5. Gel filtration chromatography calibration and results.

Protein	Molecular Weight (Da)	Ve (ml)	LogMW	Kav
Ribonuclease A	15600	76,2	4,1931	0,4233
ChymotrypsinogenA	20400	68,6	4,3096	0,3246
Ovoalbumin	48100	57,7	4,6821	0,1831
Albumin	63500	53,5	4,8028	0,1285
Large peak in Figure 1	31600	63,9	4.4997	0,2636
Small peak in Figure 1	77326	50	4,8883	0,0831
Large peak in Figure 2	43876	58.8	4.6422	0,1974
Small peak in Figure 2	110235	44,5	5,0423	0,0116

The table shows proteins used to calibrate the HiLoad Superdex 75 preparative grade column and the peaks with corresponding molecular weights, elution volumes, Log molecular weight and K_{av} values.

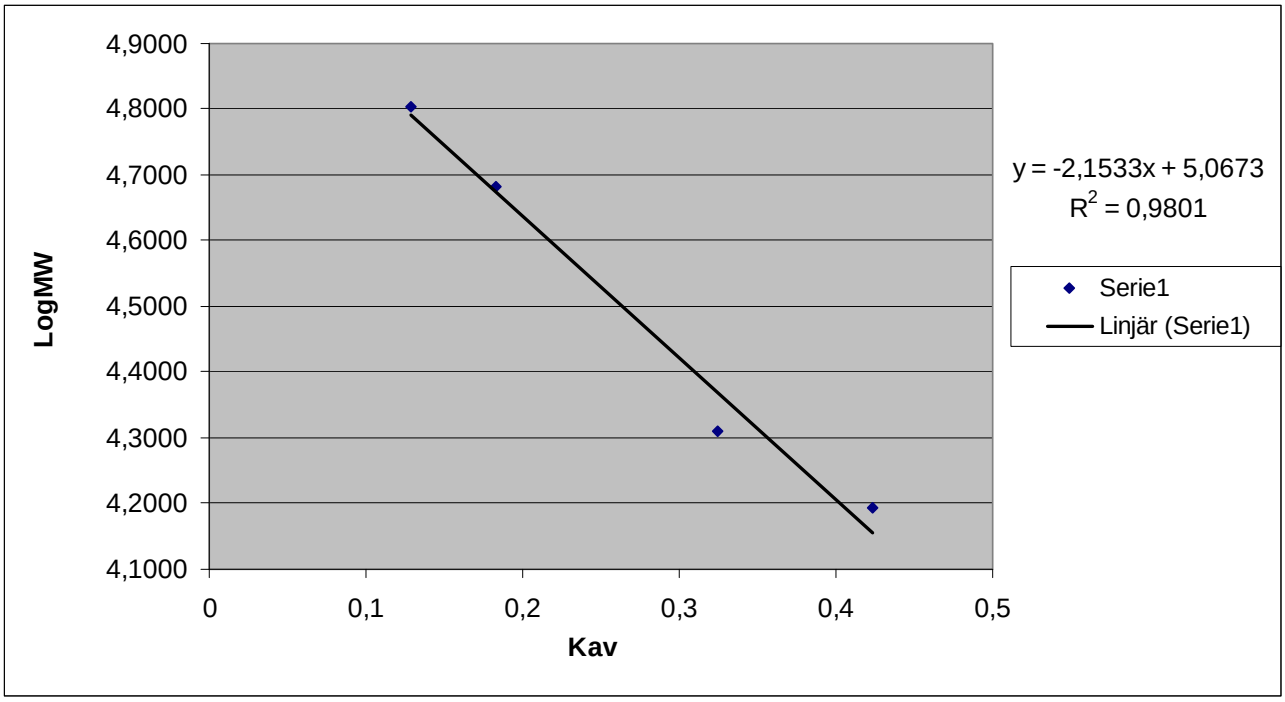

Figure 19. The figure is a graph used for calculating the Log molecular weight from the K_{av} by using the equation of the trendline. Series 1 is made up by the proteins ribonuclease A, Chymotrypsinogen A, ovalbumin and albumin.

$K_{av} = V_e\text{-}V_o/V_t\text{-}V_o$

$V_o = 43.6$ ml
$V_t = 120.6$ ml

V_t-V_o = 77 ml

Large peak in Figure 1
K_{av} = 63.9 ml - 43.6 ml/120.6 ml – 43.6 ml
K_{av} = 0.2636

y = -2.1533x + 5.0673
y = -2.1533 * 0.2636 + 5.0673
y = 4.4997
Log MW = 4.4997 => $10^{4.4997}$ = 31.6 kDa

Small peak in Figure 1
Outside calibration range

Large peak in Figure 2
K_{av} = 58.8 ml - 43.6 ml/120.6 ml – 43.6 ml
K_{av} = 0,1974

y = -2.1533x + 5.0673
y = -2.1533 * 0,1974 + 5.0673
y = 4.6422
Log MW = 4.6422 => $10^{4.6422}$ = 43.8 kDa

Small peak in Figure 2
Outside calibration range

Concentration and yield of protein

First purification:
Absorbance of 0.733/0.04 µg/ml = 18.325 µg => 18.325 µg/0.22 µl = 83µg/µl = 83mg/ml

The absorbance is divided with the Bradford constant (0.04 µg/ml) which gives the amount of protein in the 1ml of Bradford dye solution (18.325 µg). The amount of protein is divided by the real volume of protein (0.22 µl) which gives the initial protein concentration.

Yield = 83mg/ml * 0.2 ml = 16.6mg => 16.6mg/0.8l L = 20mg protein/L of cell culture

To get the yield the concentration is multiplied by the amount of purified sample (0.2 ml) and then divided by the amount of cell culture (0.81 L). This gives the amount of protein from one liter of cell culture. Because the sample in the second purification was divided into two batches one with cacodylate buffer at pH 7.0 and one with cacodylate buffer at pH 6.0 the amount of purified sample has to be multiplied by two.

Second purification:
pH 7.0: absorbance of 0.342/0.04 µg/ml = 8.55 µg => 8.55 µg/0.1 µl = 86 µg/µl = 86 mg/ml

Yield = 86mg/ml * 0.2 ml = 17.2 mg => 17.2 mg * 2 = 34.4 mg/0.81 L = 42.5mg of protein/L of cell culture

pH 6.0: absorbance of 0.114/0.04 µg/ml = 2.85µg => 2.85µg/0.1µl = 29µg/µl = 29mg/ml

Yield = 28.5mg/ml * 0.2 ml = 5.7mg => 5.7mg * 2 = 11.4 mg/0.81 L = 14 mg of protein/L of culture

Kinetics:

K_m and V_{max} can be calculated from the lineweaver burk plot. If 1/[S] is plotted on the x-axis and 1/velocity is plotted on the y-axis, $-1/K_m$ is were the line crosses the x-axis. Which means that if y = 0 in the line equation of the plot then $x = -1/K_m$. $1/V_{max}$ is were the line crosses the y-axis. Which means that if x = 0 in the line equation of the plot then $y = 1/V_{max}$.

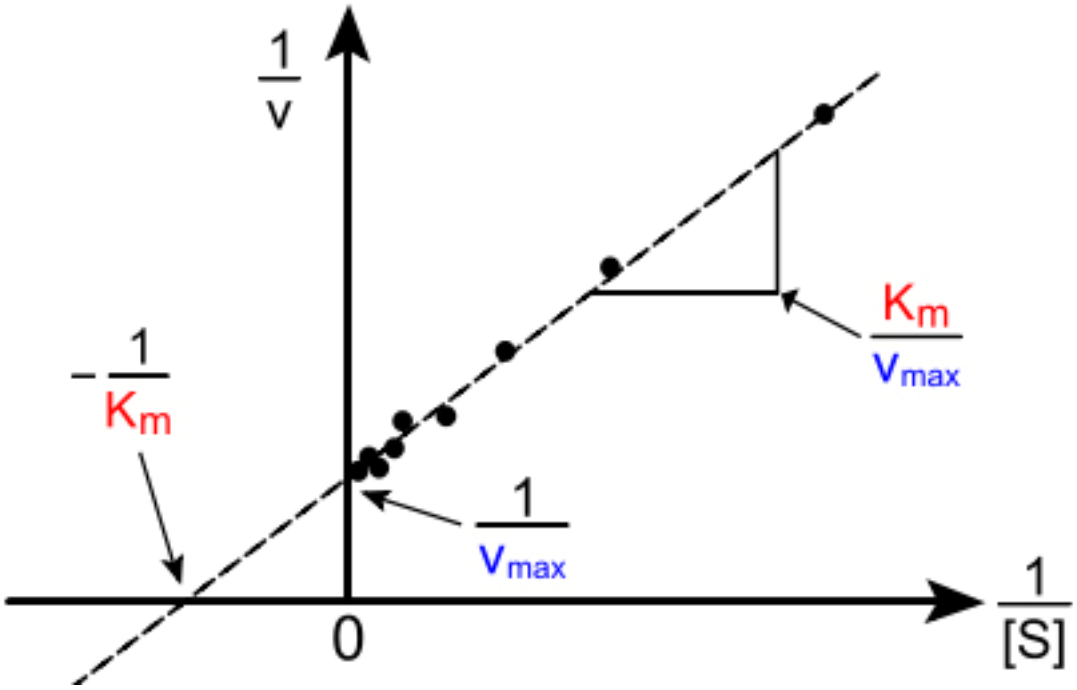

Figure 20. A Limeweaver plot showing were the $-1/K_m$ and $1/V_{max}$ can be calculated from with 1/velocity on the y-axis and 1/[S] on the x-axis (Wikipedia1).

K_m and V_{max} can also be calculated by using a Hanes-Woolf plot. If the [S] is plotted on the x-axis and [S]/v is plotted on the y-axis, $-K_m$ is were the line crosses the x-axis and $1/V_{max}$ is the angel of the slope. If y = 0 in the line equation of the plot then $x = -K_m$. Hanes-Woolf plots are often considered less sensitive as the substrate concentration is present on both axis.

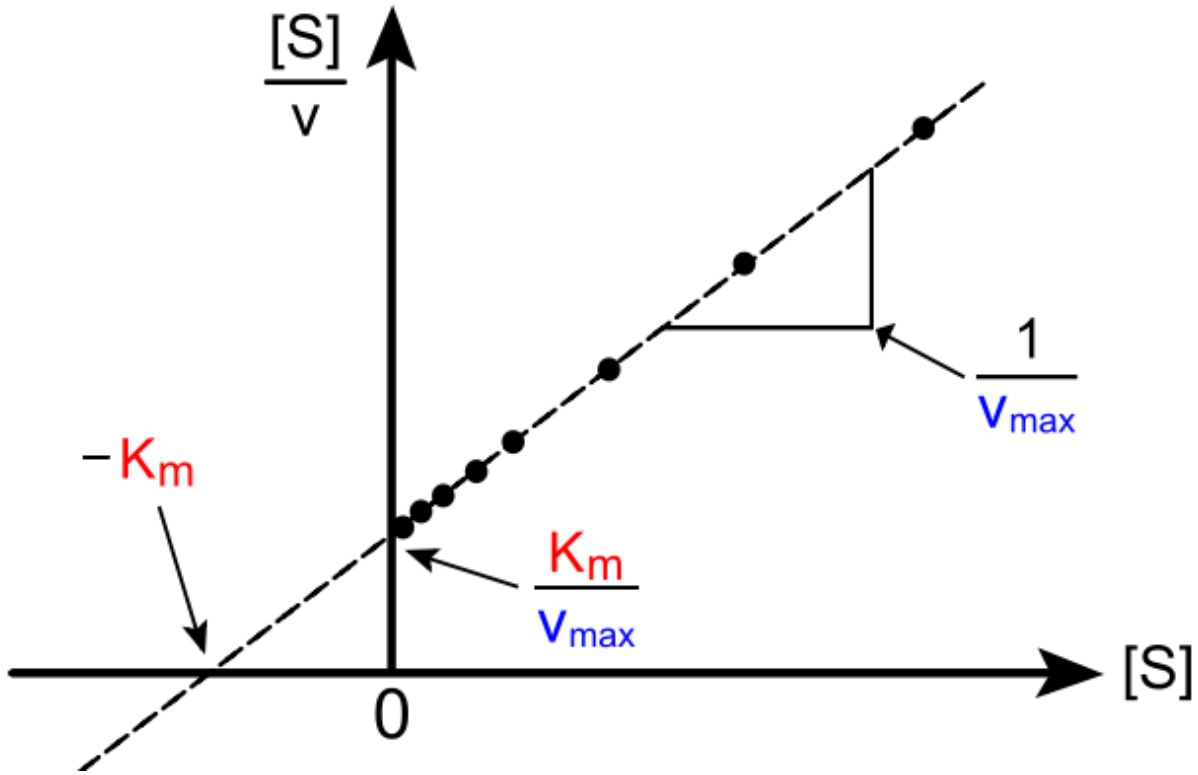

Figure 21. A Hanes-Woolf plot showing were $-K_m$ and $1/V_{max}$ can be calculated from with the substrate concentration on the x-axis and the substrate concentration divided by the velocity on the y-axis (wikipedia 2).

Ribose-5-phosphate:

K_m, V_{max} and k_{cat} using the Lineweaver-Burke plot (Figure 15)
K_m: y = 19.251x + 1.8324 => 0 = 19.251x + 1.8324 => x = -0.0951 => -1/-0.0951 = 10.5 mM

V_{max}: y = 19.251x + 1.8324 => y = 19.251 * 0 + 1.8324 => y = 1.8324 => 1/1.8324 = 0.54 mM/3 min = 0.18 mM/min

The final concentration of enzyme in the cuvette was: 0.0002mg/ml.
[E]: 0.0002 mg/ml /17500 Da = $1.142 * 10^{-8}$ M => $1.142 * 10^{-8}$ M * 1000 = 0.00001142 mM

$k_{cat} = V_{max}/[E]$
k_{cat}: 0.18 mM/min /0.00001142 mM = 15761 min^{-1} => 15761 min^{-1}/60 = 262sec^{-1}

K_m, V_{max} and k_{cat} using the Hanes-Woolf plot (Figure 16)
K_m: y = 1.6417x + 21.771 => 0 = 1.6417x + 21.771 => x = -13.2612 => -1*-0.1221 = 13.3 mM

V_{max}: y = 1.6417x + 21.771 => 1/1.6417 = 0.61 mM/3 min => 0.20 mM/min

$k_{cat} = V_{max}/[E]$
k_{cat}: 0.20 mM/min/0.00001142 mM = 17513 min^{-1} => 17513 min^{-1}/60 = 291sec^{-1}

Allose-6-phosphate:

K_m, V_{max} and k_{cat} using the Lineweaver-Burke plot (Figure 17)
K_m: y = 430.34x + 1030.6 => 0 = 430.34x + 1030.6 => x = -2.3948 => -1/-2.3948 = 0.41 mM

V_{max}: y = 430.34x + 1030.6 => y = 430.34 * 0 + 1030.6 => y = 1030.6 => 1/1030.6 = 0.0009 mM/min

The final concentration of enzyme in the cuvette was: 1.8 μM
[E]: 1.8 μM/1000 = 0.0018 mM

$k_{cat} = V_{max}/[E]$
k_{cat}: 0.0009 mM/min/0.0018 mM = $0.5min^{-1}$ => $0.5min^{-1}/60 = 0.008sec^{-1}$

K_m, V_{max} and k_{cat} using the Hanes-Woolf plot (Figure 18)
K_m: 0 = 1028.3x + 417.07 => x = -0.4055 => -1*-0.4055 = 0.41

V_{max}: 1/417.07 = 0.0009

The final concentration of enzyme in the cuvette was: 1.8 μM
[E]: 1.8 μM/1000 = 0.0018 mM

$k_{cat} = V_{max}/[E]$
k_{cat}: 0.0009 mM/min/0.0018 mM = $0.5min^{-1}$ => $0.5min^{-1}/60 = 0.008sec^{-1}$

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Picture references

S. Svard, Ankarklev J, Jerlström-Hultqvist J, Ringqvist E, Troell K (2010) *Behind the smile: cell biology and disease mechanisms of Giardia species*. **Nat Rev Microbiol**: 413-22

Wikipedia 1: http://upload.wikimedia.org/wikipedia/commons/7/70/Lineweaver-Burke_plot.svg

Wikipedia 2: http://upload.wikimedia.org/wikipedia/commons/9/9e/Hanes-Woolf_plot.svg