UPPSALA
UNIVERSITET

UPTEC X 13 021
Examensarbete 30 hp
Juni 2013

Site directed mutagenesis on a recombinant lectin domain with the aim for increased solubility

Anton Linde

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 13 021	**Date of issue 2013-06**
Author **Anton Linde**	
Title (English) **Site directed mutagenesis on a recombinant lectin domain with the aim for increased solubility**	
Title (Swedish)	
Abstract A series of mutations were performed on a recombinant fucose binding lectin domain from the fungi *Aleuria aurantia* in order to break oligomer formation. The mutated variants showed lowered solubility than the wild type, indicating that the oligomer formation was inhibited and is important to solubilize the protein domain.	
Keywords Lectin, site directed mutagenesis, fucosylation	
Supervisors **Bengt-Harald Johnsson, Linköpings universitet**	
Scientific reviewer **Per Hammarström, Linköpings universitet**	
Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **32**

Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 471 4687

Site directed mutagenesis on a recombinant lectin domain with the aim for increased solubility

Anton Linde

Populärvetenskaplig sammanfattning

För att i detalj förstå molekylärbiologiska förlopp har man på senare år börjat lägga allt större vikt på att studera proteiners ytkolhydrater och dess funktioner. Denna nya gren inom life science, som på engelska kallas ”Functional Glycomics” blir allt hetare inom forskningsvärlden. Glykosylering av proteiner anses vara inblandat i en mängd cellulära funktioner och har kunnat knytas till en rad olika sjukdomsförlopp, däribland olika former av cancer. Plasmaproteinet orosomucoid har visat sig få en förändring i sina kolhydratkedjor vid vissa leversjukdomar, särskilt leverchiros. Förändringen ligger i en ökad mängd av monosackariden fukos. För att studera kolhydrater kan man använda särskilda kolhydratbindande proteiner ”lektiner” som specifikt binder olika kolhydratstrukturer utan att modifiera dem. Ett lektin ifrån svampen *Aleuria aurantia* har specificitet mot just fukos och kan användas för att selektera och binda fukosinnehållande oligosackarider. Ur teknisk och diagnostisk synpunkt är det viktigt att kunna mäta fukos på ett enkelt och okomplicerat sätt. Därför har vi med gentekniska metoder isolerat ett genfragment, från *Aleuria aurantia*, som ger upphov till ett mindre protein som endast binder en fukosenhet per protein istället för 5 som hos det nativa proteinet. Detta examensarbete har som fokus att vidareutveckla denna proteinkonstruktion för att prova möjliga vägar för att öka löslighet och samtidigt förbättra stabilitet och integritet i de oxidativa miljöer som är vanliga i detektionsammanhang.

Svampen Mönjeskål (*Aleuria aurantia*)

Examensarbete 30 hp
Civilingenjörsprogrammet Molekylär bioteknik
Uppsala universitet, maj 2013

Table of contents

Abbreviations

AAL	*Aleuria aurantia* lectin
AGP	alpha(1)-acid glycoprotein
CV	Column volume
D-man	D-Mannose
DMSO	Dimethylsulfoxide
dsDNA	Double stranded deoxyribonucleic acid
ELISA	Enzyme Linked ImmunoSorbent Assay
ELLA	Enzyme Linked Lectin Assay
Fuc	Fucose
GalNAc	*N*-Acetylgalactosamine
IDA	iminodiacetic acid
IMAC	Immobilized Metal ion Affinity Chromatography
LB	Lysogeny broth
MAAL	Monomeric *Aleuria aurantia* lectin
NeuNAc	N-acetylneuraminic acid
NTA	nitrilotriacetic acid
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PNPP	p-Nitrophenyl Phosphate
rAAL	His-tagged recombinant *Aleuria aurantia* lectin
S2-AAL	His-tagged recombinant site 2 of *Aleuria aurantia* lectin
TMB	3, 3', 5, 5'-Tetramethylbenzidine
WT	Wild type
α-glc	α-Glucose
α-gal	α-Galactose
α-man	α-Mannose
β-gal	β-Galactose

Aim

To produce a suitable tool for detection of liver cirrhosis, it is of importance to have a monospecific binder directed towards a specific target. It has been shown that fucosylation of the plasma glycoprotein orosomucoid is linked to liver cirrhosis, and this may be used as a marker for detection. The carbohydrate binding protein (i.e. lectin) from *Aleuria aurantia* (AAL) is a homo dimer which has five binding sites on each of its two domains, which all have slight different specificity and affinity towards the monosaccharide fucose, and especially for fucose containing oligosaccharides. If the lectin is used in its native form, the difference in specificity of the sugar binding sites would obviously cause low selectivity in the assay. Further, the fact of having multiple binding sites will lead to multi site attachment of target glycoprotein and possible crosslinking could occur. The binding site which is called AAL site 2 (S2-AAL) has shown signs of having the highest affinity of the 5 sites and was therefore chosen as a candidate tool for fucose binding. The DNA sequence of this site was cloned in *E. coli* and the protein fragment was successfully expressed. However, it has been found that this protein construct forms large portions of insoluble inclusion bodies during expression, and the minor soluble fraction is unstable in solution and aggregates forming different types of complexes. The aim of this work was to investigate if the solubility of S2-AAL could be increased by replacing some chosen hydrophobic amino acids with hydrophilic ones in order to break the proposed interactions that could contribute to aggregation. Another goal was to increase the stability towards oxidation of a cysteine residue in the lectin, for use in oxidative environments, which are common in most detection assays.

Introduction

Liver cirrhosis and a quest for a molecular marker

Cirrhosis is the name for pathological degradation of liver tissue. The underlying cause is mainly chronic liver inflammation, caused by high alcohol consumption or hepatitis virus infection. Cirrhosis means that large sections of the liver tissue has died and been replaced by fibrosis and scar tissue, which lowers the total liver function. A significant proportion of patients had liver cirrhosis due to inflammatory liver disease, whose causes are not due to the victim's lifestyle (Farrell *et al.,* 2006). In table 1, some causes of cirrhosis is listed. Cirrhosis is generally irreversible and treatment is generally focused on alleviating symptoms and preventing further liver damage. The only reliable resort in late stage of cirrhosis is liver transplantation and currently the only analytical method used for diagnosis is biopsy (Farrell *et al.,* 2006).

Table 1. A list of some known causes of cirrhosis with references.

Cause	Reference
Alcohol abuse	Sørensen *et al.,* 1984
Chronic viral hepatitis infection	Farrell *et al.,* 2006
Autoimmunity	Farrell *et al.,* 2006
Hemochromatosis	Farrell *et al.,* 2006
Deficiency of Alpha1-antitrypsin	Mahadevaa *et al.,* 1998
Wilson's disease	Strand *et al.,* 1998
Prolonged cholestasis	Jansen *et al.,* 2003
Cystic fibrosis	Feigelson *et al.,* 1993
Overdose of certain drugs	Zimmerman *et al.,* 2000

Fucosylation

Nearly half of all eukaryotic proteins are glycosylated, making it one of the most important post-translational modifications. The function of glycosylation is far from fully explored, but changes in glycosylation patterns are associated with many biological events such as cell growth migration, and prolifieration, pathogen attachment and entrance (Buskas *et al.,* 2006), and inflammatory response (Urien *et al.,* 1991). Fucose is a monosaccharide and one of the essential sugars needed for cell to cell communication (Zhao *et al.,* 1999). Fucose occurs in two enantiomeric forms: L-fucose and D-fucose, and the L-form is the common type in nature, which is rare for sugars (figure 1.). In humans it is included in extracellular muco-and glycoproteins in breast milk (bifidus factor) and in blood group substances (A, B and H, Lea). In bacteria, it occurs in an antigenic polysaccharide in the bacterial cell wall (Osborn *et al.,* 1969).
Alpha(1)-acid glycoprotein (AGP), also known as orosomucoid, is a highly glycosylated and normally occurring plasma protein in humans, that acts as a carrier for basic drugs, steroids and protease inhibitors (Colombo *et al.,* 2006), (Urien *et al.,* 1991). It also acts as a natural anti-inflammatory agent (Williams *et al.,* 1997), but the biological function is far from fully understood. Lars Rydén et al., has shown that the fucosylation of AGP appears in different inflammatory diseases (Rydén *et al.,* Dec 2002) (figure 2) and could be used as a marker for liver cirrhosis (Rydén *et al.,* Jan 2002).

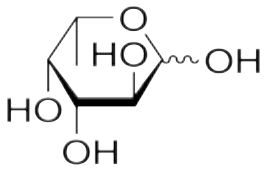

Figure 1. Structure of L-Fucose

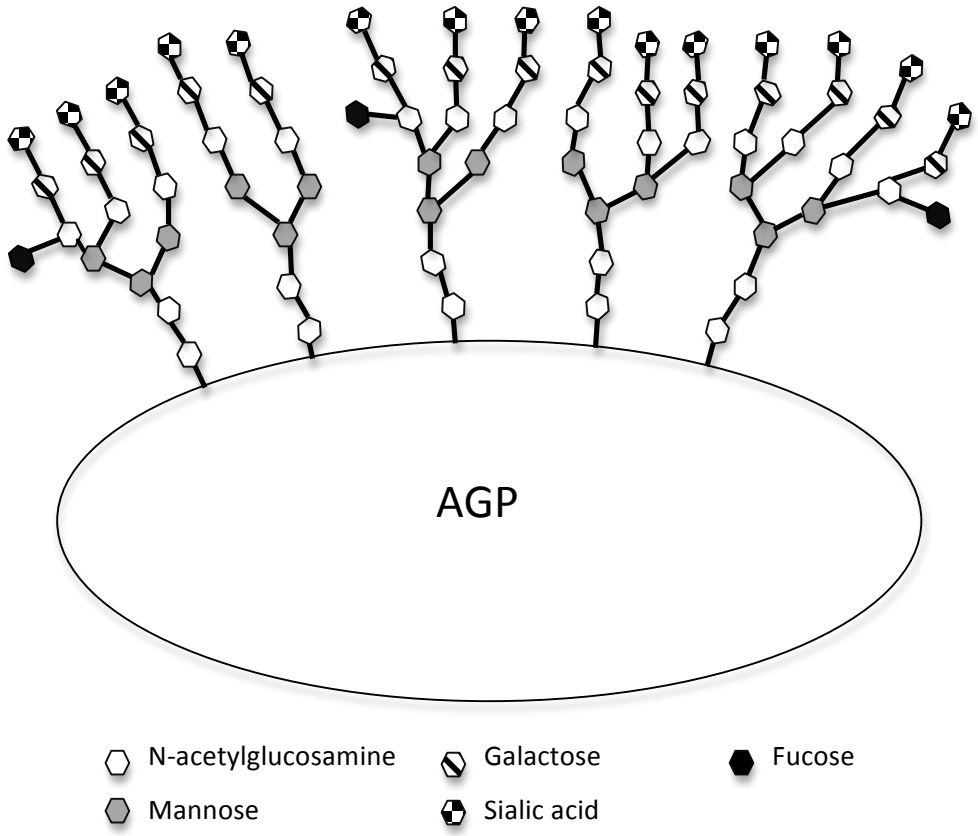

Figure 2. Shematic layout of AGP, showing fucosylation on branched oligosacharides. The illustration only represents an example of several possible combinations of fucosylation of oligosacharides.

Lectins

Lectins are non-enzymatic proteins of non-immunoglobulin origin that binds carbohydrates reversibly without modyfing their glycosyl linkage. The name "Lectin" is derived from the latin word lectus meaning "to select" (Beuth *et al.,* 1995). They are present in prokaryotes and all eukaryotic kingdoms, but they are especially abundant in legume plants. Lectins were first described in 1888 by Peter Hermann Stillmark in his doctor thesis where he noted that seed extracts of Castor bean, *Ricinus cumminensis* could cause clumping of erythrocytes (agglutination) (Beuth *et al.,* 1995). An alternative older name for those lectins was therefore "hemaagglutatin". They play an important role in molecular recognition in biological systems such as cell adhesion; aid the immune system with the recognition of glycosylation patterns typical for pathogens (Springer *et al.,* 1990). In plants, they are for example involved in germination of pollen (Southworth *et al.,* 1975). The ability to specifically bind different carbohydrate motifs has been used in many applications in biotechnology and diagnostics such as: blood typing (Schertz *et al.,* 1960), as well as lectin-glycoprotein assays, lectin blotting, lectin conjugate precipitation and affinity chromatography of glycoproteins, glycolipids, polysaccharides, viruses and cells (Nilsson *et al.,* 2007). Lectins from various origin presents different sugar specificity as seen in table 2.

Table 2. Examples of lectins and their sugar specificity (Medicago AB, lectin selection table, 2010)

Lectin	Sugar specificity
Arachis hypogaea	β-gal(1->3)galNAc
Artocarpus integrifolia	α-gal->OMe
Concanavalin A	α-man, α-glc
Crotalaria juncea	gal > galNAc
Galanthus nivalis	Non-reduc. D-man
Glycine Max	galNac
Lens Culinaris	α-man > α-glc
Narcissus pseudonarcissus	A-D-man
Phaseolus vulgaris	oligosaccharide
Pisum sativum	α-man > α-glc
Triticum vulgaris	(glcNAc)2, NeuNAc
Viccia ervilia	α-man > α-glc

Aleuria aurantia

AAL is a lectin present in the fruiting bodies of the mushroom *Aleuria aurantia*. It is natively a double barrel dimer, which has 5 binding sites for fucose on each domain (figure 3). Each site binds free fucose and fucose containing oligosaccharides with a slight difference in affinity. Traditionally this lectin is purified from the fruiting bodies of *Aleuria aurantia* by affinity chromatography on an immobilized-fucose column, followed by elution with fucose. (Fujihashi *et al*., 2003) However, it has been shown that one of the five sites binds fucose with so high affinity that it will not be removed even after extensive dialysis (Olausson *et al.,* 2008). This binding site seemed promising as an analytic tool for detection of fucosylation. Therefore, a recombinant HIS-tagged version of the lectin was produced, which could be purified without involving free fucose. AAL-site2 was considered the most probable candidate for being the high affinity binding site. S2 has been cloned and successfully produced in *E. coli*. (Olausson *et al.,* 2011). However, this little fragment has poor solubility and tends to form insoluble aggregates.

As mentioned in the introduction the aim of this project was to investigate if the solubility of the domain "S2" could be increased by site directed mutagenesis aimed to exchange a number of

amino acids which side chains are believed to contribute to hydrophobic interactions leading to aggregation. Another aim was to increase the oxidation stability by exchanging a cysteine to a serine.

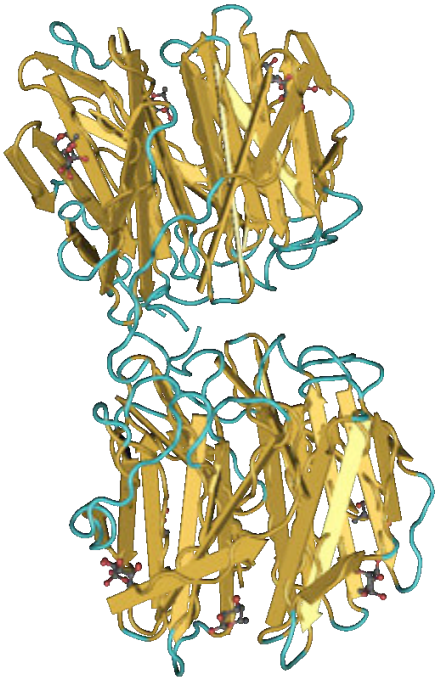

Figure 3. Crystall structure of AAL double barrel with five fucose ligands (shown with sticks and balls) attached to each monomer.

Inclusion bodies

Inclusion bodies are insoluble cytoplasmic or nuclear protein aggregates. They are typically formed when recombinant eukaryotic proteins are over expressed in *E. coli* or other hosts. The reason behind the formation is due to differences in protein production and folding mechanisms in eukaryotic and prokaryotic systems. Differences are for example the presence of chaperons and other folding proteins in eukaryotes, and the protein production rate, which is higher in engineered prokaryotic systems thereby affecting the formation of these aggregates. The formation of insoluble protein aggregates can be minimized by controlling the cultivation parameters such as temperature, pH, induction time and the concentration and type of inducer used. A reduction in temperature and inducer concentration often increases the amount of soluble protein. This can be explained by the overall reduced protein expression rate. The change in temperature can also have an effect in the physical folding process, as higher temperatures increases hydrophobic effects that can lead to exposure of hydrophobic side chains that otherwise would be trapped inside the folded polypeptide. A decrease in media pH during expression (which will be the case if the cultivation is left uncontrolled) has also been shown to decrease the portion of soluble protein (Strandberg *et al.,* 1991). Engineering of the construct and strain genome can also be performed with the aim to lower the recombinant gene expression rate. Such modifications can include type of promoter and codon usage. In some cases co-expression with chaperons may also aid in correct folding (Villaverde *et al.,* 2003).

Metods

PCR site directed mutagenesis

The technique used to achieve the desired mutations in S2-AAL is called PCR Site directed mutagenesis. This technique was first described by Michael Smith (Hutschison *et al.,* 1978) who in 1993 shared the Nobel price in chemistry with Kary B. Mullis, who invented the polymerase chain reaction used to amplify DNA (Mullis *et al*., 1986). The process starts with a denaturing step, were heating causes the double stranded DNA template to disrupt its base pairing, yielding single stranded DNA molecules. The denaturation step is followed by an annealing step where the temperature allows the primers to anneal to the single stranded DNA template, and the DNA polymerase binds and starts to polymerize. In the following step, where the elongation takes place, the temperature is risen to the optimum for the Taq-polymerase used. When the whole sequence has been synthesized the process starts over. When performing PCR site directed mutagenesis, primers with an introduced mutation is used. The newly formed copies are produced with staggered nicks and must be repaired by super competent cells. Before transformation, the parental, non mutated DNA is digested by an endonuclease (Dpn1) that is specific towards hemimethylated and methylated DNA. The nicked DNA that is left is then transformed by a heat pulsing procedure. (Stratagene, 2009) (figure 4)

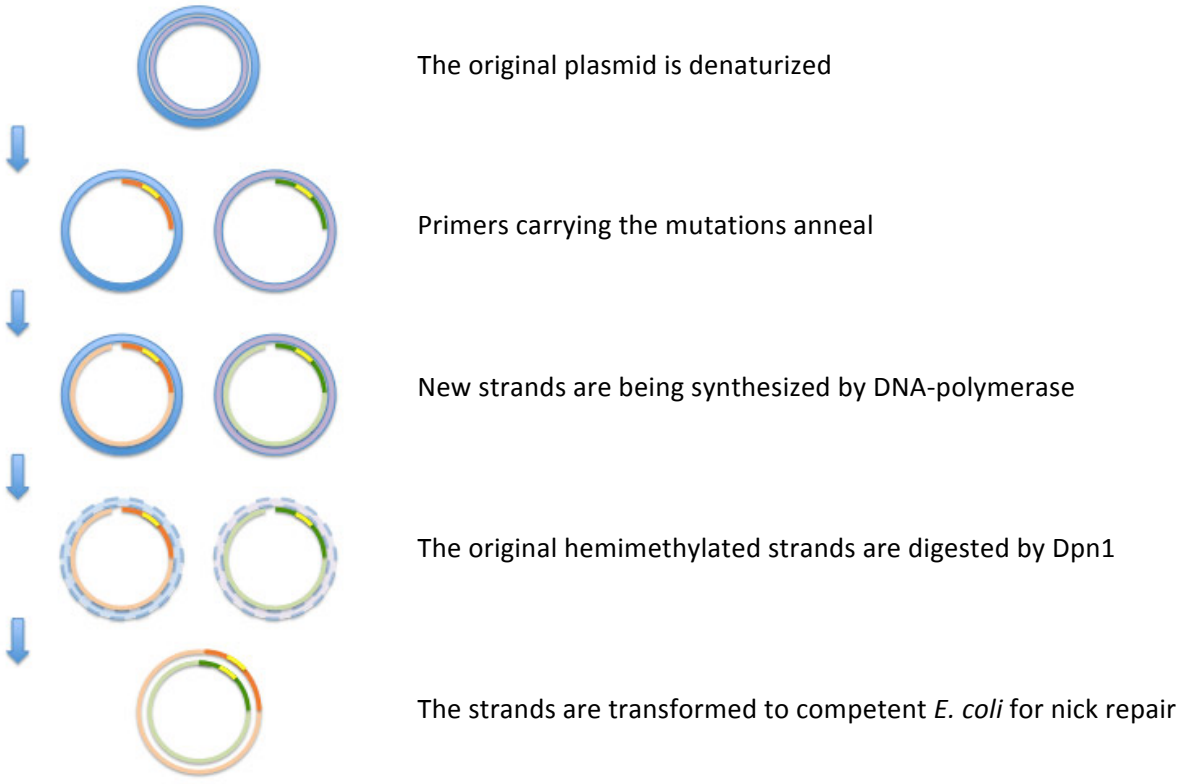

Figure 4. PCR amplification. First of all the DNA is denatured and the resulting strands are separated. Then the primers are annealed and the polymerase begins to synthesize the new strands. Dpn1 is added to digest the original DNA, and the resulting DNA is transformed into competent cells for nick repair.

Primer design

When designing primers for mutagenesis, there are many things that must be taken into account. First of all, the primer needs to cover the location to be mutated with an overlap on both sides. The length of the primers is also of importance. Too short primers may bind unspecifically to different areas of the template and would make it difficult to bind to the desired area due to large proportions of mismatch compared to matched pairs. On the other hand, too long primers often fail to anneal properly because they tend to form hair pins (stem-loops) and other secondary structures due to base pairing to complementary regions within the same strand. Something to strive for is to let the primers end with at least one cytosine or guanine, which increases the bond strength at the edges without letting the melting point increase to excessive levels. The melting point of the primers decides the annealing temperature and can be calculated by the simple formula T_m= (4[G+C]) + (2[A+T]) °C. Where [G+C] is the number of guanine and cytosine bases and [A+T] is the number of adenine and thymine bases (Stratagene, 2009). DMSO can be used in the reaction mixture to "lower" the melting point of the strands.

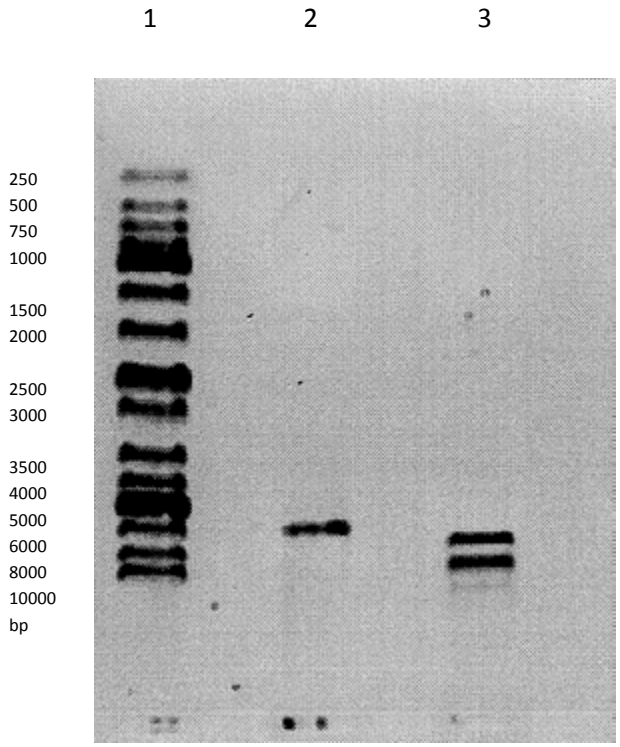

Figure 5. Agarose gel electrophoresis of the pET-28 vector containing the S2-AAL insert. From left: 1. Ladder "gene ruler 1kb, Fermentas", 2. pET-28 plasmid linearized with NdeI-restriction enzyme, 3. Uncut pET-28 plasmid.

Protein expression in *E. coli*

The *E. coli* strand XL1Blue (Agilent, Santa Clara, California, USA), which is good for plasmid production is not especially suitable for protein expression, so a good protein producing strain had to be selected. The pET-28 plasmid used (Merck KGaA, Darmstadt, Germany)(figure 5) carries the T7-promoter, which is not recognized by the *E. coli* RNA polymerase, so a strain with the λ prophage T7 RNA polymerase gene had to be chosen. BL21-DE3 fulfills these criteria. The technique used to transform the plasmids between those strains is called electroporation. The cells are subjected to a short electric pulse that widens pores in the cellular membrane, allowing DNA to migrate in to the cell. The transformed bacteria are then grown on agar plates containing antibiotic, for selection of the plasmid containing clones.

IMAC purification

The 6xHIS-tagged protein was purified by Ni^{2+}-NTA IMAC. The technique (Immobilized Metal Affinity Chromatography) is based on the principle that free histidine and to a lesser degree cysteine, tryptophan and arginine residues coordinates divalent metal ions (typical Ni^{2+}, Co^{2+}, Zn^{2+}, or Cu^{2+}) with their side chains (Abe *et al*., 2009). The metal ion is typicaly immobilized on agarose beads with the use of a linker and a chelator (typical IDA or NTA). When the HIS-tagged protein is bound, it may be eluted with an increasing amount of imidazole (side chain of histidine), which competes with the binding sites. Alternative elution with low pH, ammonium chloride or histidine is also possible. Excess of imidazole in the elution fractions may afterwards be removed by dialysis or gelfiltration.

Experimental procedures

Plasmid preparation

One colony of Top10 *E. coli* cells containing the PET-28a plasmid with an S2-AAL insert was transferred to 40 ml of LB medium with kanamycin (10 g NaCl, 10 g Peptone, 5 g Yeast extract and 30 mg kanamycin per liter) and grown at 37 ˚C over night with vigorous shaking. The bacterial pellet was then collected by centrifugation (10 min, 4500 x g) and the plasmids were prepared using a QIAprep® miniprep kit according to their protocol (Qiagen, Hilden, Germany).

Design of primers for mutagenesis

The primers were designed so each mutation could be performed independently from each other, without overlapping, so all seven possible combinations could be produced successively (See figure 6, 7, and 8). The length of the primers were adjusted so that the melting point would be high enough, see table 3. The primers were also checked for potential secondary structures.

```
  1  mpteflytsk iaaiswaatg grqqrvyfqd lngkireaqr ggdnpwtggs sqnvigeakl
 61  fsplaavtwk saqgiqirvy cvnkdnilse fvydgskwit gqlgsvgvkv gsnsklaalq
121 wggsesappn irvyyqksng sgssiheyvw sgkwtagasf gstvpgtgig ataigpgrlr
181 iyyqatdnki rehcwdsnsw yvggfsasas agvsiaaisw gstpnirvyw qkgreelyea
241 ayggswntpg qikdasrptp slpdtfiaan ssgnidisvf fqasgvslqq wqwisgkgws
301 igavvptgtp agw
```

Figure 6. The amino acid sequence of AAL in one letter code. The sequence which corresponds to S2-AAL is highlighted in grey.

```
1   atgcctaccgaattcctctacacctcgaaaattgcagccatctcttgggctgccaccggc
61  ggccgccagcaacgcgtctacttccaagaccttaatggcaagatccgcgaggctcagcgc
121 gggggagacaatccatggaccggcgggtcgagccagaatgtaatcggcgaagcaaagctt
181 ttttcgccactggctgctgtcacgtggaaaagtgctcagggcatacagatccgtgtttac
241 tgcgtcaataaggataacatcctctccgaatttgtgtatgacggttcgaagtggatcacc
301 ggacagctgggcagtgtcggcgtcaaggtgggctccaattcgaagcttgctgcgcttcag
361 tggggcggatctgagagcgccccccaaacatccgagtttactaccagaagagcaacggt
421 agtgggagctcaatccacgagtatgtctggtcgggcaaatggacggctggcgcaagcttt
481 gggtcaacggtgccaggaacgggtatcggagccaccgccatcgggccaggtcgcctgagg
541 atctactaccaggctactgacaacaagatccgtgagcactgttgggactccaacagttgg
601 tacgtgggggggttctcggccagcgcttccgccggcgtctccatcgcggcgatttcttgg
661 ggcagtacacccaacatccgggtctactggcagaaaggtagggaggaattgtacgaggct
721 gcctatggcggttcatggaacactcctggtcagatcaaggacgcatccaggcctacgccc
781 tcgttgccagacacctttattgctgcgaactcctcggggaacatcgacatctctgtgttc
841 ttccaagctagcggcgtctccttgcagcagtggcaatggatctccggcaagggctggtcc
901 atcggcgcggttgttcccactggcactcccgcgggatgg
```

Figure 7. The DNA sequence of AAL. PCR primers marked in grey and mutations marked in red.

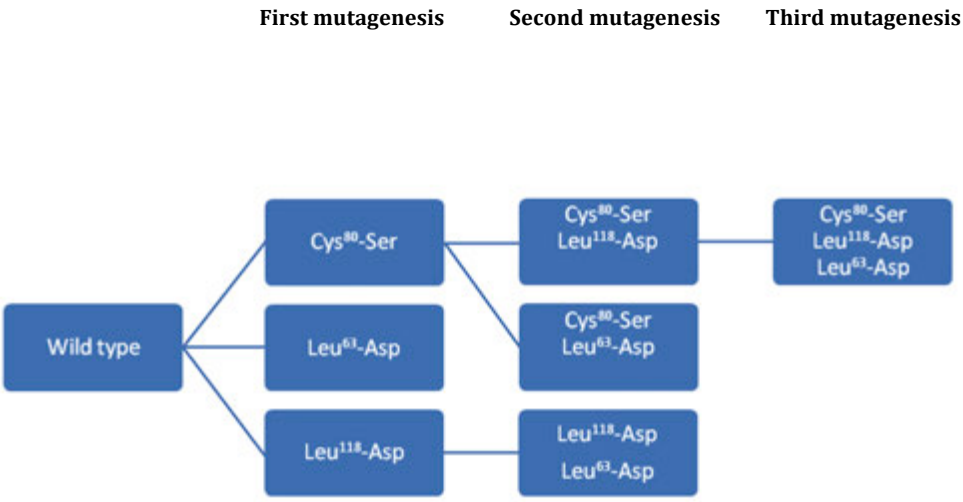

Figure 8. Mutagenesis strategy. All primers were designed so each single mutation could be performed independently from each other without overlapping. The schematic picture shows the arrangement used in this work to produce the wanted variants but it is just one of the possible arrangements that lead to the same goal.

Table 3. Primers used to create the desired mutations.

Primer	Sequence	Mp °C
Cys^{80}-ser sense	5'ggcatacagatccgtgtttactccgtcaataaggataac	66
Cys^{80}-ser antisense	3'ccgtatgtctaggcacaaatgaggcagttattcctattg	66
Leu^{63}-asp sense	5'cgaagcaaagctttttttcgccagacgctgctgtcacgtgg	71
Leu^{63}-asp Antisense	3'gcttcgtttcgaaaaaagcggtctgcgacgacagtgcacc	71
Leu^{118}-asp sense	5'cgaagcttgctgcggatcagtggggcgatctg	70
Leu^{118}-asp antisense	3'gcttcgaacgacgcctagtcaccccgcctagac	70

First mutagenesis (single amino acid replaced variants)

To generate site directed mutations in order to increase the solubility of the protein a QuickChange® II Site Directed Mutagenesis Kit (Agilent technologies, Santa Clara, USA) was used. The standard protocol recommended in the manual was modified and optimized with the addition of DMSO to the PCR-mixture (table 4), and with a lengthening of the elongation step (table 5). The amount of polymerase and dNTP was halved compared to the recommendations. As template, a pET-28a plasmid with an S2-AAL insert was used at the concentration of 20 ng/µl. Autoclaved Milli-Q water was used to dilute all the DNA samples, and a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) was used to measure the DNA concentrations. The primers were constructed using Agilent Technologies QuikChange Primer Design Program, with slight manual modifications. Primers were supplied by DNA Technologies A/S and diluted to 4.0 pmol/µl before use. The PCR-mixture was prepared and kept on ice until temperature cycling started. A Robocycler gradient 96 (Stratagene, La Jolla, USA) was used for rapid thermocycling. Note that the residues to be replaced are numbered with the assumption that the N-terminal methionine is removed by methionine aminopeptidase (Sherman F, 1985).

Table 4. PCR mixture used for the first mutagenesis to produce the single amino acid replaced variants.

	Resulting variant		
	Cys^{80}-Ser	*Leu^{63}-Asp*	*Leu^{118}-Asp*
Plasmid template (µl)	1	1	1
Primer (µl)	2.43	2.37	2.87
Primer, antisense (µl)	2.43	2.37	2.87
dNTP mix (µl)	0.5	0.5	0.5
10 x Reaction buffer (µl)	2.5	2.5	2.5
DMSO (µl)	0.5	0.5	0.5
H_20 (µl)	15.76	15.64	14.76
	Then add		
PfuUltra HF DNA polymersase (µl)	0.5	0.5	0.5

Following the temperature cycling, 1 µl of Dpn1 restriction enzyme was added to each tube. The reaction mixture was then thoroughly mixed by pipetting up and down several times. The mixture was spun down for one minute using a bench top centrifuge, and incubated at 37 °C for 1 hour on a heating block, to digest unmutated dsDNA. 1 µl of the Dpn1 treated DNA was transferred to separate aliquots (50 µl) of gently thawed XL1-Blue super competent cells. The mixture was gently mixed and left on ice for 30 minutes before it was heat pulsed for 45 seconds at 42 °C. The mixture was again put on ice for 2 minutes and then 0.5 ml of NZY^+ broth (5 g NaCl, 2 g $MgSO_4$ x $7H_20$, 5 g yeast extract and 10 g casein hydrolysate per liter), preheated to 42 °C, was added. The cells were then incubated in growth medium for 1 hour at 37 °C with vigorous shaking. For each aliquot, 500 µl was divided and streaked on two LB-agar plates with kanamycin, and then incubated over night at 37 °C. The colonies were re-streaked once, and two colonies of each mutation from the new plates were incubated in 10 ml LB over night at 37 °C with vigorous shaking. The cell pellet was collected by centrifugation and the plasmids were purified with the use of a QIAprep® miniprep kit, and MilliQ was used in the elution step instead of the supplied tris-HCl buffer. Before further use as template, the purified plasmids were sent to GATC Biotech in Germany for confirmation of the desired mutations, and to verify that no other mutations had occurred.

Table 5. PCR parameters.

Segment	Cycles	Temperature	Time
1	1	95 °C	30 seconds
2	17	95 °C	30 seconds
		55 °C	1 minute
		68 °C	7 minutes
3	1	4 °C	∞

Second mutagenesis (double amino acid replaced variants)

To generate the 3 possible variants with two different amino acids replaced (*Cys^{80}-Ser, Leu^{63}-Asp; Leu^{63}-Asp , Leu^{118}-Asp; and Leu^{118}-Asp, Cys^{80}-Ser*) a second mutagenesis was carried out. As template the previous single mutated plasmids were used at the concentration of 20 ng/µl. The same thermo cycling parameters were used as in the first mutagenesis. Layout presented in table 6.

Table 6. PCR mixture used for the second mutagenesis to produce the double amino acid replaced variants.

	Resulting variant		
	Cys^{80}-Ser, Leu^{63}-Asp	*Leu^{63}-Asp ,Leu^{118}-Asp*	*Leu^{118}-Asp, Cys^{80}-Ser*
Plasmid template (µl)	1(Cys^{80}-Ser)	1 (Leu^{118}-Asp)	1 (Cys^{80}-Ser)
Primer (µl)	2.37	2.37	2.87
Primer, antisense (µl)	2.37	2.37	2.87
dNTP mix (µl)	0.5	0.5	0.5
10 x Reaction buffer (µl)	2.5	2.5	2.5
DMSO (µl)	0.5	0.5	0.5
H_20 (µl)	15.76	15.76	14.76
	Then add		
PfuUltra HF DNA polymersase (µl)	0.5	0.5	0.5

Third mutagenesis (triple amino acid replaced variants)

To generate the variant with three different amino acids replaced (*Cys^{80}-Ser,Leu^{63}-Asp,Leu^{118}-Asp*) a third mutagenesis was carried out. As template the previous (Cys^{80}-Ser, Leu^{118}-Asp) was used at the concentration of 20 ng/µl. The same thermo cycling parameters were used as in the first mutagenesis. Layout presented in table 7.

Table 7. PCR mixture used for the third mutagenesis to produce the triple amino acid replaced variant.

	Resulting variant
	Cys^{80}-Ser, Leu^{63}-Asp, Leu^{118}-Asp
Plasmid template (µl)	1(Cys^{80}-Ser, Leu^{118}-Asp)
Primer (µl)	2.37
Primer, antisense (µl)	2.37
dNTP mix (µl)	0.5
10 x Reaction buffer (µl)	2.5
DMSO (µl)	0.5
H_20 (µl)	15.76
	Then add
PfuUltra HF DNA polymersase (µl)	0.5

Preparation of electrocompetent cells

For protein production, the *E. coli* strain BL21-DE3 was selected. 0.5 L LB medium was inoculated with 1/100 overnight culture. The cells were incubated at 37 °C until OD_{600} was 0.5-0.7, and then chilled on ice for 20 minutes. The cells were collected by centrifugation (4000 x g, 15 min, 4 °C) and the supernatant was discarded. The cells were resuspended in 500 ml 10% glycerol followed by centrifugation (4000 x g, 15 min, 4 °C) and the supernatant was discarded again. This purification step was repeated four times in total, with decreased volume of the solution with glycerol (250 ml, 20 ml, 1 ml). The resulting glycerol preparation was then aliquoted in 40 µl portions, freezed with liquid nitrogen, and stored at -70 °C.

Protein production – Test induction

Growth at 37 °C

The three plasmids with mutated inserts were transformed into BL21-DE3 with the use of electroporation. For each variant, one colony was transferred to 10 ml of LB medium with kanamycin and incubated at 37 °C over night with vigorous shaking. 50 ml LB medium was then inoculated with 1/20 overnight culture and grown until OD_{600} = 0.5-0.7. A sample of 1 ml of each was collected (Non-induced control). IPTG was added to a final concentration of 1 mM, and the flasks were incubated at 37 °C, with vigorous shaking for 4 hours. A sample of 1 ml of each was then collected (induced control) and the rest were centrifuged (4000 x g, 20 min, 4 °C), and the pellet was resuspended in 5 ml lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM Imidazole, pH 8.0). The cells were lysed by sonication (3 x (30 s pulse / 30 s rest)) on ice. The lysate was then centrifuged (10 000 x g, 30 min) and a sample of the supernatant was collected (Soluble protein extract). The resulting pellet was resuspended in lysis buffer and kept on ice (Insoluble protein extract). All samples were analyzed by SDS-PAGE.

Growth at 20 °C and 16 °C

Exactly the same procedure was repeated but with a temperature lowered to 20 °C and 16 °C respectively, 1 hour before induction. 37 °C was still used during the growth phase. This was done in order to investigate if the proportion of soluble protein could be increased with lowered temperatures. To achieve this temperature, the heating incubator was placed in room temperature for 20 °C, and in a cold room to achieve an induction temperature of 16 °C. The temperature was verified with an external thermometer.

Large scale protein production in *E. coli* BL21-DE3

For each of the 8 variants, one colony was transferred to 20 ml of LB medium with kanamycin and incubated at 37 °C over night with vigorous shaking. 2 L LB medium was then inoculated with 1/100 overnight culture and grown until OD_{600} = 0.5-0.7. IPTG was added to a final concentration of 0.5 mM and the flasks were incubated at 20 °C with vigorous shaking over night (16h). The bacterial slurry was centrifuged (3000 x g, 30 min, 4 °C) and the pellet was then resuspended in 5 ml lysis buffer. The cells were lysed by sonication (3 x (30 s pulse / 30 s rest)) on ice. The lysate was centrifuged (10 000 x g, 30 min) and a sample of the supernatant was collected (Soluble protein extract). The resulting pellet was resuspended in lysis buffer and kept on ice (Insoluble protein extract).

Purification of S2-AAL variants

For each variant, 3 ml NTA-Superflow agarose (Qiagen, Hilden, Germany) was equilibrated with 5 column volumes (CV) of lysis buffer. The crude lysate was then mixed with the gel and left to incubate (45 min, 4 °C, end-over-end). The slurry was packed in PD10 gravity flow columns (GE Healthcare Life Sciences, Uppsala, Sweden) and washed with 10 CV of lysis buffer followed by 10 CV of wash buffer (50 mM NaH_2PO_4, 500 mM NaCl, 20 mM Imidazole) before eluting with elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 300 mM Imidazole). The elution was collected in 3 ml samples and as no UV-detector was used, the fractions were visualized by Bradford assay, and the protein containing fractions were pooled and dialysed against 1 L PBS prepared from PBS-tablets (Medicago, Uppsala, Sweden).

Purification optimization

Because the original purification protocol failed to give pure protein samples, and the difficulty to follow the chromatography without an UV-detector, the protein production and purification was redone. This time, a smaller scale (200 ml medium) was used, and the purification protocol was modified by doubling the volumes used in the wash steps and by modifying the wash buffers (doubled amount of imidazole and with the addition of 0.05% of the detergent tween™ 20).

Results

Mutagenesis

The mutagenesis was successful in all cases and sequencing of the entire coding region verified the desired mutations, and showed that no external mutations had arisen in the 8 variants.

Production of S2-AAL-variants under different conditions

All variants were shown to grow well and produce large amounts of the wanted protein upon induction. All proteins showed different solubility characteristics and repeated protein production and solubility experiments showed consistency between runs.

In order to find the optimal conditions for productions of a soluble and stable variant of S2-AAL, all variants were produced by incubating *E. coli* cultures at 37, 20 and 16 ˚C respectively. 20 ˚C was later chosen for the large scale production for practical reasons. After induction and incubation, cells were harvested by centrifugation and lysed by sonication as described in the experimental section. After lysis the soluble and insoluble material was separated by centrifugation, and both supernatants and pellets was analyzed on a 15% homogenous SDS-PAGE gel with Tris-Glycine-SDS (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) as running buffer and stained with Coomassie brilliant blue R-250 (see figure 9-12).

Production at 37 °C

As seen in figure 9 and 10, the variants Leu_{118}-Asp, Cys_{80}-Ser, Leu_{63}-Asp and wt-S2-AAL were expressed with high yield at this temperature. However, the proteins were almost completely located in the insoluble fraction, indicating formation of aggregates during protein synthesis.

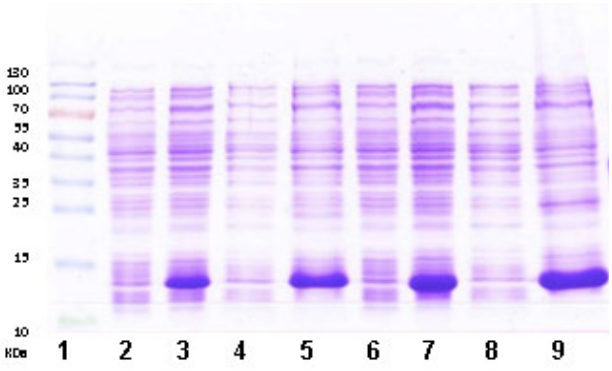

Figure 9.
Variants grown and induced at 37 °C.

1 MW ladder
2 Leu_{118}-asp non induced control
3 Leu_{118}-asp induced control
4 Leu_{118}-asp soluble protein
5 Leu_{118}-asp insoluble protein
6 Wild type non induced
7 Wild type induced control
8 Wild type soluble protein
9 Wild type insoluble protein

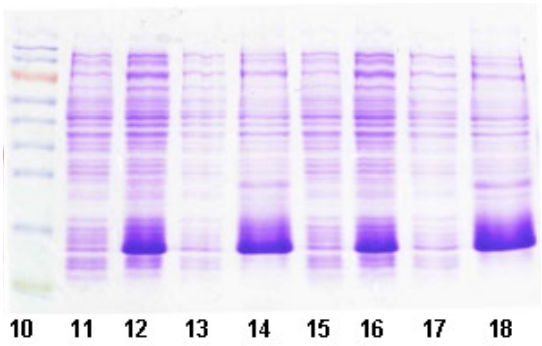

Figure 10.
Variants grown and induced at 37 °C.

10 MW ladder
11 Cys_{80}-ser non induced control
12 Cys_{80}-ser induced control
13 Cys_{80}-ser soluble protein
14 Cys_{80}-ser insoluble protein
15 Leu_{63}-asp non induced
16 Leu_{63}-asp induced control
17 Leu_{63}-asp soluble protein
18 Leu_{63}-asp insoluble protein

Production at 20 °C

As seen in figure 11, when cells were induced at 20 °C, the total recombinant protein yield was greatly reduced, but the fraction of soluble protein was increased. This is most notably in the wild type protein where the band representing the soluble fraction has about the same intensity as the insoluble fraction.

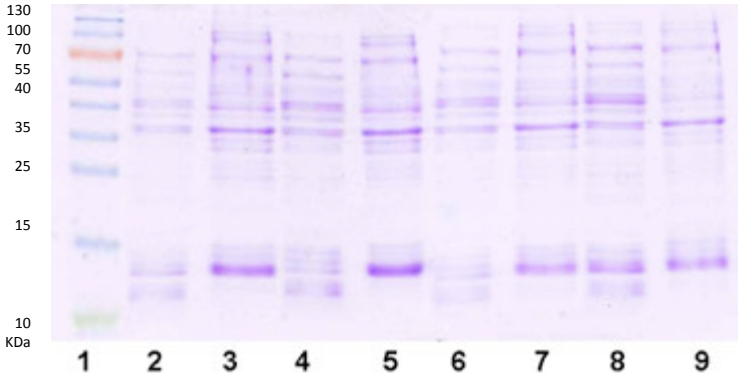

Figure 11. Variants grown and induced at 20 °C.

1 MW ladder
2 Cys-Ser soluble protein
3 Cys-Ser insoluble protein
4 Leu63-Asp soluble protein
5 Leu63-Asp insoluble protein
6 Leu118-Asp soluble protein
7 Leu118-Asp insoluble protein
8 Wild type soluble protein
9 Wild type insoluble protein

Production at 16 °C

As seen in figure 12, the trend continues with lower total protein yield, but higher solubility of the target protein when induced at 16 °C. The wild type even produced greater amount of soluble than insoluble protein, and the Cys-Ser variant produced about equal amounts of soluble and insoluble protein.

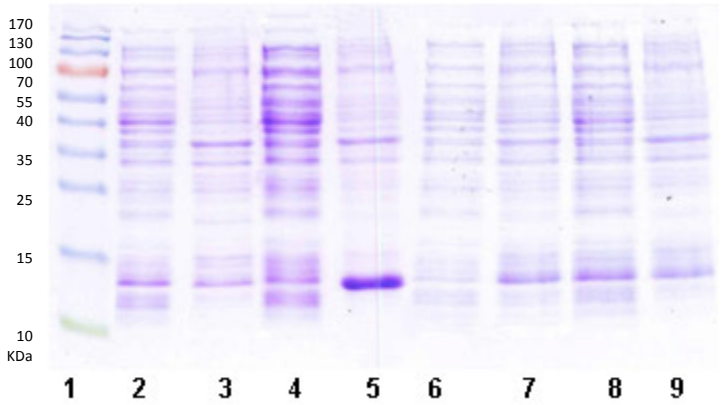

Figure 12.
Variants grown and induced at 16 °C.

1 MW ladder
2 Cys-Ser soluble protein
3 Cys-Ser insoluble protein
4 Leu63-Asp soluble protein
5 Leu63-Asp insoluble protein
6 Leu118-Asp soluble protein
7 Leu118-Asp insoluble protein
8 Wild type soluble protein
9 Wild type insoluble protein

Large scale protein production of all variants at 20 °C

All variants were grown and induced at 20 °C and compared by SDS-PAGE. As seen in figure 13 and 14, the solubility is drastically reduced as more residues are exchanged. This confirms earlier comparisons and shows that the double and triple exchanged variants lower the solubility drastically.

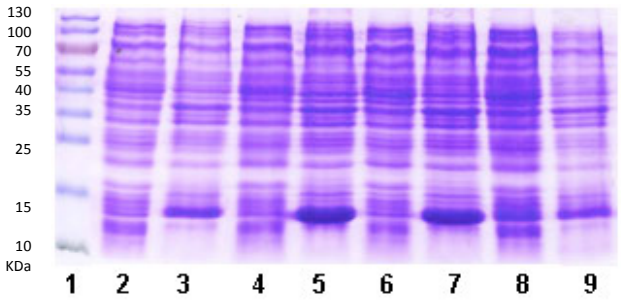

Figure 13.
Variants grown and induced at 20 °C.

1 MW ladder
2 Cys-Ser soluble protein
3 Cys-Ser in soluble protein
4 Leu63-Asp soluble protein
5 Leu63-Asp insoluble protein
6 Leu118-Asp soluble protein
7 Leu118-Asp insoluble protein
8 Wild type soluble protein
9 Wild type insoluble protein

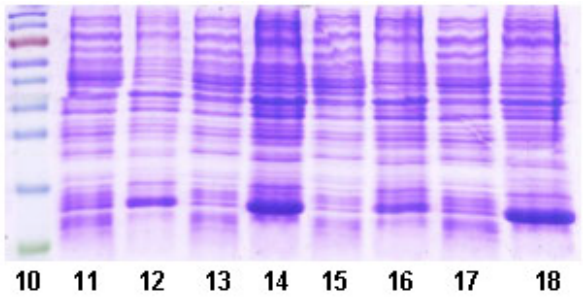

Figure 14.
Variants grown and induced at 20 °C.

10 MW ladder
11 Leu63-Asp, Cys-Ser soluble protein
12 Leu63-Asp, Cys-Ser insoluble protein
13 Leu118-Asp, Cys-Ser soluble protein
14 Leu118-Asp, Cys-Ser insoluble protein
15 Leu118-Asp, Leu63-Asp soluble protein
16 Leu118-Asp, Leu63-Asp insoluble protein
17 Tripple variant, soluble protein
18 Tripple variant, insoluble protein

Native protein purification

All variants were grown and induced at 20 °C and after harvesting, the clarified cell homogenates were incubated with IMAC resin, and packed in gravity flow columns. As no UV-detector was used, and the protocol used was not optimized, the elution fractions were not composed of pure S2 protein. However, the purification runs confirmed that the wild type and Cys-Ser yielded soluble protein that could be purified using IMAC. The purifications also showed weak bands, indicating that the Cys-ser,Leu_{63}-Asp and Cys-Ser,Leu_{118}-Asp variants are somewhat soluble. However, the other types are lacking bands of the same size. All data is shown in figure 15.

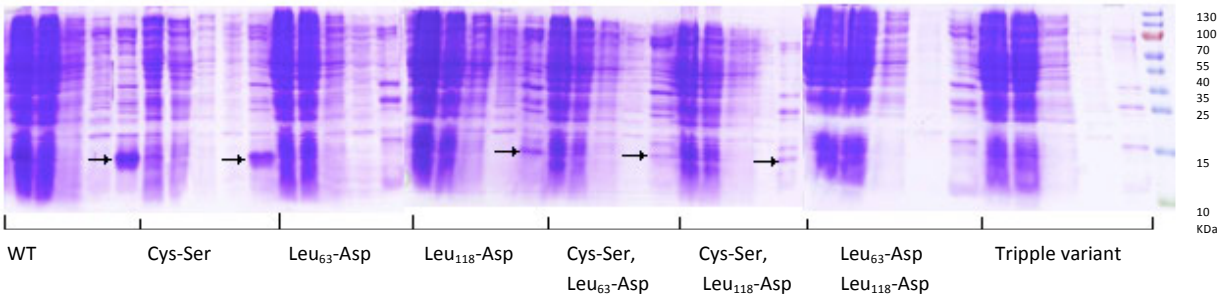

Figure 15. Standard purification protocol.

Gel: 3ml Ni-NTA-Superflow.
LC = load control, Cellslurry
FT = flow through,
W1 = wash1, 50 mM NaH_2PO_4, 500 mM NaCl, 10 mM Imidazole, 10 CV
W2 = wash2 50 mM NaH_2PO_4, 500 mM NaCl, 20 mM Imidazole, 10 CV
E = Elution 50 mM NaH_2PO_4, 500 mM NaCl, 300 mM Imidazole

1 WT: LC,FT,W1,W2,E
2 Cys-Ser: LC,FT,W1,W2,E
3 Leu_{63}-Asp: LC,FT,W1,W2,E
4 Leu_{118}-Asp LC,FT,W1,W2,E
5 Cys-Ser, Leu_{63}-Asp LC,FT,W1,W2,E
6 Cys-Ser, Leu_{118}-Asp LC,FT,W1,W2,E
7 Leu_{63}-Asp, Leu_{118}-Asp LC,FT,W1,W2,E
8 Tripple variant LC,FT,W1,W2,E

In the second purification run, an extended wash step was used. The purity obtained was much higher; however the protein load to column volume was lower this time, resulting in more diluted samples (data not shown). After dialysis the purified proteins were this time stored at +5 °C instead of at -20 °C. However, they still formed insoluble aggregates after a while, indicating instability of this small lectin fragment. Because of the instability and low solubility of the S2 variants, it was decided that no further characterization was to be made.

Discussion and conclusion

Figure 16. Crystall structure of monomeric AAL. S2 marked in yellow.

It has been shown previously that S2 forms different types of soluble oligomers, and recent studies indicate that this construct probably consists of a mixture of monomers, dimers and trimers (Figure 16-18) but that the most abundant oligomer is a trimer (Olausson *et al*., 2011). In this study, both protein solubility tests and protein purifications showed reduced solubility for the modified variants (Figure 9- 15). However, one variant, namely Cys-Ser, showed only a slight decrease in solubility. This indicates that this cysteine is not involved to any great extent in the formation of oligomers. One aim of this project is thereby achieved by creating a more oxidation stable variant, that otherwise could form disulfide bonds leading to dimerization with itself, or other proteins with exposed thiol groups.

The replacement of the hydrophobic leucine residues did lower the solubility drastically. This indicates that those residues are in fact involved in hydrophobic interactions that contribute to the creation of soluble oligomers. By replacing the leucine with hydrophilic aspartic acid residues, the created protein variant fails to form soluble oligomers, and because it is not stable by its own in solution, it precipitates after a while, forming aggregates. Another possibility is that the indicated leucine residues are important for the stability of the monomer, and that other residues may be involved in the formation of oligomers. Future work may be focused on finding a way to make S2 soluble by its own, and eliminating the interactions that leads to aggregations. One possible approach is to design a construct with three identical S2 units linked together. This may produce a stable and reliable trimer. However, what we really want is a protein with only one binding site, to avoid crosslinking. So another way to go, may be to fuse this fragment with a larger inert protein, which may stabilize it. An additional advantage is that the fusion protein can be used as an affinity anchor aiding in purification and detection. A third way to go, is to replace all residues involved in carbohydrate binding, in all sites except the S2 site, in the native five site protein. This would hopefully create a stable protein, with just one functional binding site.

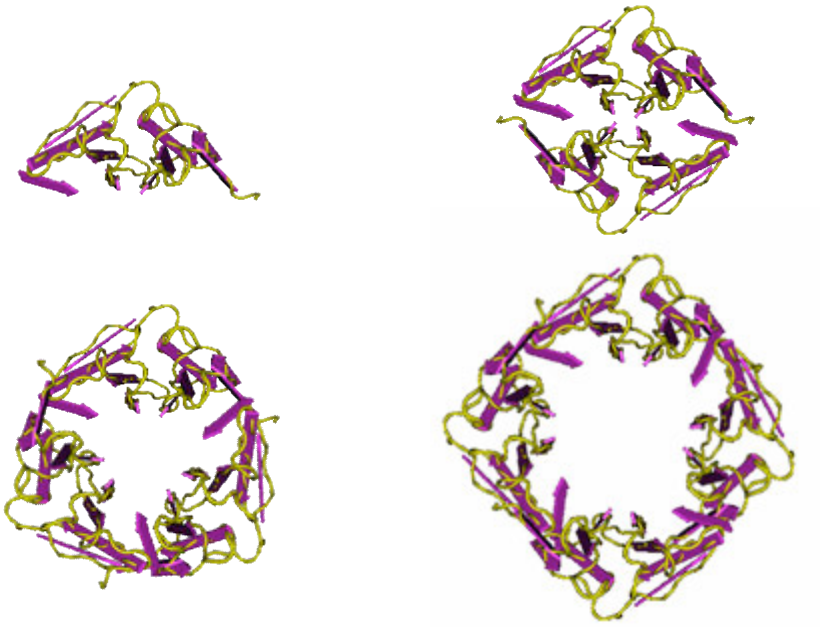

Figure 17. proposed structure of monomeric, dimeric, trimeric and tetrameric forms of S2

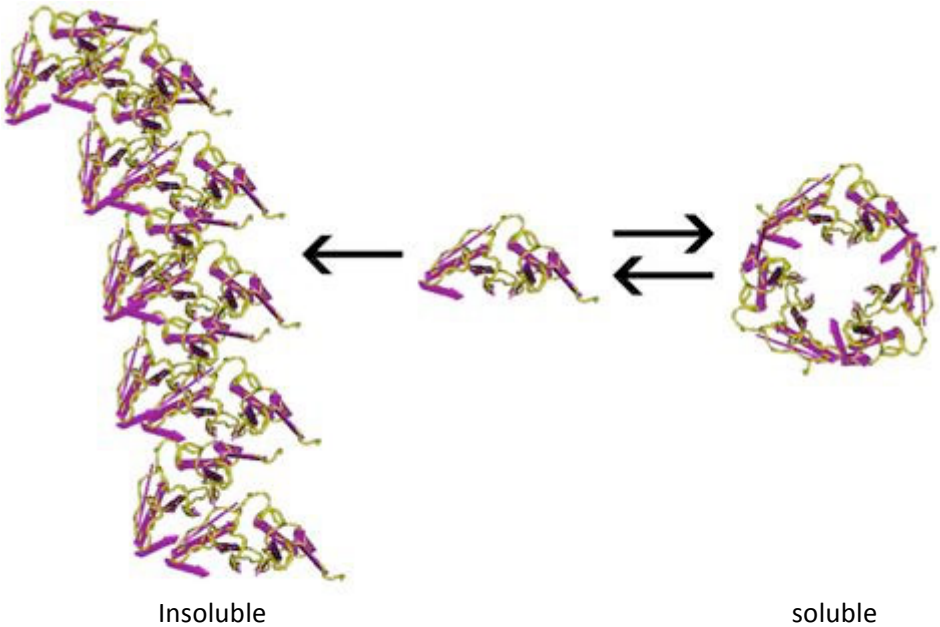

Figure 18. Proposed equilibrium of the formation of soluble oligo-structures and insoluble aggregates. By breaking the hydrophobic interactions involved in the formation of soluble oligomers, the equilibrium is thought to be shifted to the left, and thus insoluble aggregates precipitates after storage.

Acknowledgments

I would like to thank Professor Bengt-Harald Jonsson, my supervisor at the Department of Physics, Biology and Chemistry (IFM) at Linköping University for my opportunity to work on this exciting project, and for his highly valued guidance and advice regarding protein chemistry and DNA technology during the project. I also want to thank him for his patience during proofreading and correspondence regarding the report. At the Faculty of Health Sciences (FHS) I would like to thank Professor Peter Påhlsson for his big knowledge regarding clinical chemistry and diagnostics, and also Doctor Eva Åström, who worked on a different parallel ptoject on the same protein, and gave me an introduction and advice in the beginning of the project. I also want to thank Doctor Daniel Kanmert, my lab instructor, who helped me a lot with practical biochemical methodology, and furthermore, I would like to thank all personnel in the biotechnology and organic chemistry corridor for their kindness.

References

R. Abe , M. Kudou, Y. Tanaka, T. Arakawa, K. Tsumoto*."Immobilized metal affinity chromatography in the presence of arginine"*. Biochem. Biophys. Res. Commun. Vol. 381, No. 3, (Apr. 2009) pp. 306-310.

J. Beuth, H. L. Ko, G. Pulverer, G. Uhlenbruck and H. Pichlmaier. "*Importance of lectins for the prevention of bacterial infections and cancer metastases*" Glycoconjugate Journal Vol. 12, No. 1, (Feb. 1995) pp. 1-6.

T. Buskas, S. Ingale, and G. Boons. *"Glycopeptides as versatile tools for glycobiology"* Glycobiology Vol. 16, No. 8, (May. 2006) pp. 113-136.

S. Colombo ,T. Buclin, L.A. Décosterd, A. Telenti, H. Furrer, B.L Lee, J. Biollaz and C.B Eap *"Orosomucoid (alpha1-acid glycoprotein) plasma concentration and genetic variants: effects on human immunodeficiency virus protease inhibitor clearance and cellular accumulation"*. Clin. Pharmacol. Vol. 80, No. 4, (Jun. 2006) pp. 307–18.

G.C. Farrell, C.Z. Larter *"Nonalcoholic fatty liver disease: From steatosis to cirrhosis"* Hepatology, Vol. 43, No. 3, (Feb. 2006) pp. 99-112.

J. Feigelson, C. Anagnostopoulos, M. Poquet, Y. Pecau, A. Munck, J. Navarro *"Liver cirrhosis in cystic fibrosis--therapeutic implications and long term follow up"* Arch. Dis. Child., Vol. 68, No. 5, (May. 1993) pp. 653-657.

M. Fujihashi, D. Peapus, N. Kamiya, Y. Nagata, and K. Mik. *"Crystal Structure of Fucose-Specific Lectin from Aleuria aurantia Binding Ligands at Three of Its Five Sugar Recognition Sites"* Biochemistry. Vol. 42, No. 38, (Sep. 2003) pp. 11093-11099.

C.A. Hutschison, C.A. Philipps, M.H. Edgell, S. Gillham, P. Jahnke, M. Smith *"Mutagenesis at a Specific Position in a DNA Sequence"* J. Biol. Chem. Vol. 253, No. 18, (Sep. 1978) pp. 6551-6560.

P. L. M. Jansen, E. Sturm *"Genetic cholestasis, causes and consequences for hepatobiliary transport"* Liver international Vol. 23, No. 5, (Oct. 2003) pp. 315-322.

R. Mahadevaa, D. A. Lomasa, *"Alpha1-antitrypsin deficiency, cirrhosis and emphysema"* Thorax, Vol. 53, No. 6, (Jun. 1998) pp. 501-505.

"Lectin selection table" Medicago AB, Accessed Maj 24, 2011, http://www.medicago.se/absolute-lectins-application-table

K. Mullis, F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich, *"Specific Enzymatic Amplification of DNA In Vitro: The Polymerase Chain Reaction"* Quant Biol., Vol. 51, (Jan. 1986) pp. 263-273.

C.L. Nilsson *"Lectins analytic technologies"* (2007) Elsevier B.V pp. 195.

J. Olausson, L. Tibell, B. Jonsson and P. Påhlsson, *"Detection of a high affinity binding site in recombinant Aleuria aurantia lectin"* Glycoconjugate journal, Vol. 25, No. 8, (May. 2008) pp. 753-762.

J. Olausson, E. Åström, B. Jonsson, L. A. E. Tibell and P. Påhlsson. "*Production and characterization of a monomeric form and a single-site form of* Aleuria aurantia *lectin"* Glycobiology Vol. 21, No. 1, (Aug. 2011) pp. 34-44.

M.J. Osborn, *"Structure and Biosynthesis of the Bacterial Cell Wall"* Annual Review of Biochemistry, Vol. 38, (Jul. 1969) pp. 501-538.

L. Rydén, P. Påhlsson, A. Lundbland, T. Skogh "*Fucosylation of a1-acid glycoprotein (orosomucoid) compared with traditional biochemical markers of inflammation in recent onset rheumatoid arthritis"* Clin Chim Acta. Vol. 317, No. 1-2, (Jan. 2002) pp. 221-229.

L. Rydén, P. Påhlsson, S. Lindgren. *"Diagnostic accuracy of alpha(1)-acid glycoprotein fucosylation for liver cirrhosis in patients undergoing hepatic biopsy"* Clin. Chem. Vol. 48, No. 12, (Dec. 2002) pp. 2195-2201.

K. F. Schertz, W. C. Boyd, W. Jurgelsky, J.R., and E Cabanillas *"Seed Extracts with Agglutinating activity for Human Blood"* Economic Botany, Vol. 14, No. 3, (Jul. 1960), pp. 232-240.

F. Sherman, J.W. Stewart, S. Tsunasawa. *"Methionine or not methionine at the beginning of a protein"* BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology Vol. 3, No.1, (July. 1985) pp. 27–31.

D. Southworth. "*Lectins stimulate pollen germination"* Nature, Vol. 258, (Dec. 1975) pp. 600-602.

T. A. Springer, *"Adhesion receptors of the immune system"* Nature, Vol. 346, (Aug. 1990) pp. 425-434.

L. Strandberg and S.O. Enfors *"Factors influencing inclusion body formation in the production of a fused protein in Escherichia coli"* Appl Environ Microbiol, Vol. 57 (June. 1991) No. 6, 1669–1674.

S. Strand, W. J. Hofmann, A. Grambihler, H. Hug, M. Volkmann, G. Otto, H. Wesch, S. M. Mariani, V. Hack, W. Stremmel, P. H. Krammer and P. R. Galle. *"Hepatic failure and liver cell damage in acute Wilson's disease involve CD95 (APO-1 /Fas) Mediated apoptosis"* Nature Medicine Vol. 4, No. 5, (May. 1998) pp. 588-93.

"QuikChange II Site-Directed Mutagenesis Kit" manual, Stratagene, 2009.

T. A. Sørensen, K. D. Bentsen, K. Eghøje, M. Orholm, G. Høybye, P. Offersen *"Prospective evaluation of alcohol abuse and alcoholic liver injury in men as predictors of development of cirrhosis"* The Lancet, Vol. 324, No. 8397, (Aug. 1984) pp. 241 – 244.

S. Urien. F. Brée, B. Testa, J.P. Tillement . *"pH-dependency of basic ligand binding to alpha 1-acid glycoprotein (orosomucoid)"*. Biochem. J. Vol. 280, No. 1, (Nov. 1991) pp. 277–80.

A. Villaverde, M. Mar Carrió *"Protein aggregation in recombinant bacteria: biological role of inclusion Bodies"* Biotechnology letters, Vol. 25, No. 1, (May. 203) pp. 1385-1395.

J.P. Williams, M.R. Weiser, T.T.V. Pechet, L. Kobzik, F.D. Moore Jr., and H.B. Hechtman *"α_1-Acid glycoprotein reduces local and remote injuries after intestinal ischemia in the rat "* Am. J. Physiol. Gastrointest. Liver Physiol. Vol. 273, No. 5, (Nov. 1997) pp. 1031-1035.

Y. Zhao, M. Takahashi, J. Gu, E. Miyoshi, A. Matsumoto, S. Kitazume, N.Taniguchi *"Functional roles of* N*-glycans in cell signaling and cell adhesion in cancer"* Cancer Sci. Vol. 99, No. 7, (Jul. 1999), pp. 1304-1310.

H. J. Zimmerman, *"Drug-induced liver disease"* Hepatology Vol. 4, No. 1, (Feb. 2000) pp. 73-96.

Appendix

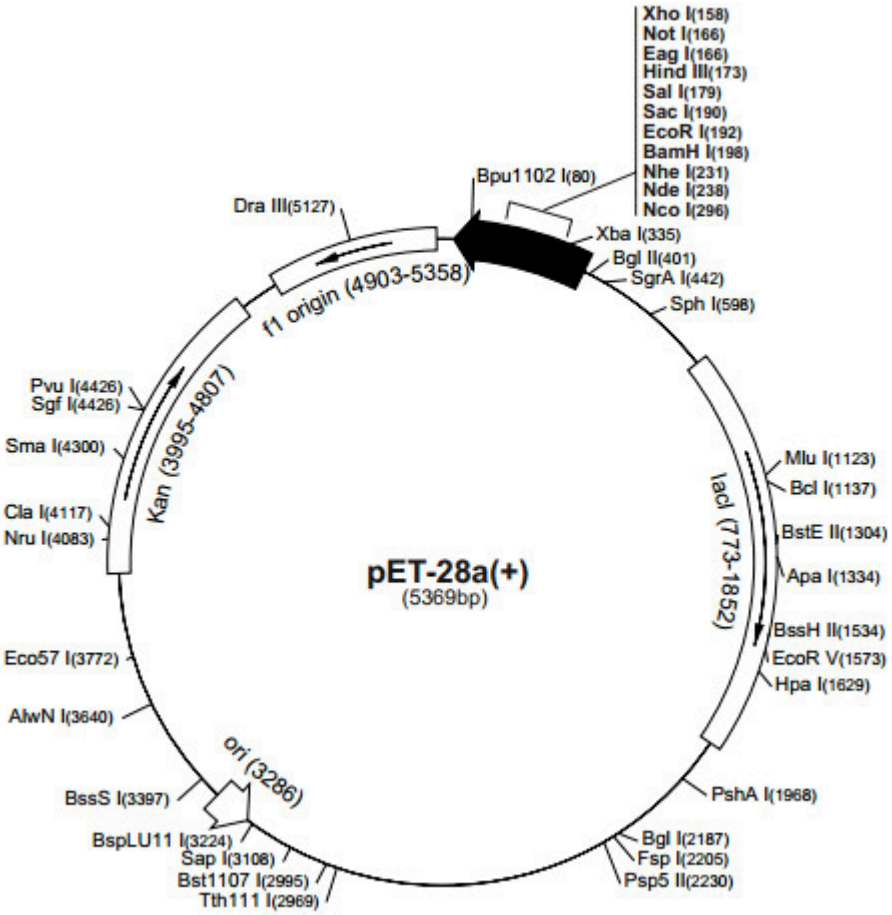

Figure 19. pEt-28 vector

ELLA

Enzyme-linked lectin assay is a technique similar to ELISA, used to quantify and detect specific carbohydrate motifs. It was first described by McCoy *et al.* in 1983, and the principle is that a sample which typically consists of diluted serum is immobilized to the walls and bottom of the wells of a standard 96 well micro titer plate. To the wells, a lectin directed against the carbohydrate motif to be determined, is added. The Lectin could be directly conjugated with the enzyme (Horse radish peroxidase, alkaline phosphatase etc) or coupled via a linker such as biotin-avidin, after it has bound to the target, and excess lectin has been washed off. A chromogenic substrate (TMB, pnpp etc) specific to the enzyme used, is added, and the signal can then be detected with a standard ELISA plate reader.