This is to certify that the
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OVER-EXPRESSION, PURIFICATION AND CRYSTALLIZATION
OF THE Rmt1/ACETYLATED H4 PEPTIDE AND SNAP43

presented by

Blanka Jelencic

has been accepted towards fulfillment
of the requirements for the

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Major Professor's Signature

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OVER-EXPRESSION, PURIFICATION AND CRYSTALLIZATION OF THE Rmt1/ACETYLATED H4 PEPTIDE AND SNAP43

By

Blanka Jelencic

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ABSTRACT

OVER-EXPRESSION, PURIFICATION AND CRYSTALLIZATION OF Rmt1/ACETYLATED H4 PEPTIDE AND SNAP43

SNAPc (Small Nuclear RNA Activating Protein complex) is a basal transcription factor responsible for recruitment of RNA pol II or RNA pol III machinery to snRNA promoters. SNAPc is a five subunit complex but we focused on a "mini-SNAPc" complex composed of SNAP190(1-505), SNAP50, SNAP43 and SNAP19. Because it is the minimal complex required for transcription initiation and DNA binding. A novel approach was used to over-express all the required subunits of the complex resulting in increased expression levels, higher purity and better stability. Using this recombinant complex, several promising crystallization conditions were identified. Furthermore, the co-expressed complex is active for DNA binding.

Arginine methyl transferase-1 (Rmt1) was shown to methylate histone tails, which in turn changes chromatin packing and can lead to increased gene expression or gene silencing. It was recently shown that Rmt1 exhibits preferential affinity for an acetylated H4 histone tail, compared to a non-acetylated histone tail. In order to investigate the mechanism for preferential binding of Rmt1 to H4 histone tails containing acetylated lysines, Rmt1 was over-expressed and purified to homogeneity, mixed with the acetylated H4 peptide, and crystallized. Several promising crystallization conditions were identified for both wild type and mutant (Δ22) Rmt1 and in the absence and presence of the acetylated H4 peptide.

To my family
for believing in me
and to Andrej for taking me with him

ACKNOWLEDGMENTS

I sincerely thank to my advisor, Prof. James H. Geiger for his encouragement and guidance throughout the research. I would like to thank my second reader, Prof. Bill Henry for providing plasmids for SNAP subunits, and more importantly for being available for frequent discussions of results and suggestions for new experiments to try. I also want to thank our collaborator, Prof. Min-Hao Kuo for providing plasmids for wild type Rmt1 and Rmt1(Δ22) and for suggestions regarding the Rmt1 project. I would also like to thank Dr. Kathleen Foley for introducing me to the world of cloning. I would like to thank Andrej Hanzlowsky for his help on the Rmt1 and SNAPc projects. His never ending suggestions on how to solve what often appeared to be unsolvable problems are the reason for the success of this work. I am also appreciative of my coworkers Stacy, Suzy, Lei, Soheila, Sara, Alfy, Dorothy, and Justin as well as all other present and past members of my group for their help, discussions, and for being around and all the good times we shared.

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LIST OF ABBREVIATIONS

A	alanine
A/ADE	adenine
Amp	ampicillin
Ac	acetyl
bp	base pair
C	cysteine
Chlor	chloramphenicol
C/CYT	cytosine
C terminal	carboxy terminal
D	aspartic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonucleosidase
DTT	dithiothreitol
DSE	distal sequence element
E	glutamic acid
F	phenylalanine

G	glycine
G/GUA	Guanine
GST	Glutathione S-Transferase
GSH	glutathione
H	histidine
HTH	helix turn helix
Hepes	N-[2-hydroxyethyl] piperazine-N'-[ethane sulfonic acid]
I	isoleucine
IPTG	Isopropyl-B-D-Thiogalactopyranoside
K	lysine
Kan	kanamycin
kDa	kiloDalton
Da	Dalton
L	leucine
M	methionine
MCS	multiple cloning site
µg	microgram
mL	milliliter

mm	millimeter
MW	molecular weight
mRNA	messenger RNA
N	asparagine
ng	nanogram
N-terminal	amino terminal
P	proline
PCR	polymerase chain reaction
pol	polymerase
PDB	Protein Data Bank
PEG	polyethylene glycerol
PMSF	phenylmethylsulfonylfluoride
PSE	proximal sequence element
Q	glutamine
R	arginine
RNA	Ribonucleic Acid
RNAP	Ribonucleic Acid Polymerase
rRNA	Ribosomal RNA

S	serine
SNAP	small nuclear RNA activating protein
SNAPc	small nuclear RNA activating protein complex
mSNAPc	mini SNAP complex
snRNA	small nuclear RNA
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
Str	Streptomycin
T	threonine
T/THY	Thymine
TBP	TATA Binding Protein
TFII(A)	Transcription Factor II (X)
Tris	2-Amino-2-(hydroxymethyl)-1,3-propendiol
tRNA	transfer RNA
U	uracil
V	valine
W	tryptophan
Y	tyrosine

1. Introduction

1.1. Transcription

Transcription is the synthesis of RNA from a DNA template. Transcription has four main events: pre-initiation, initiation, elongation and termination. Pre-initiation in which RNA polymerase with the aid of additional general transcription factors (GTFs) recognizes a promoter is the first step of transcription. Several factors influence binding of polymerase to the promoter region of the DNA and subsequently regulate gene expression. Accurate control of gene expression is necessary for normal cell operation and is achieved by transcription factors that bind to specific promoter regions and recruit the RNA polymerase machinery. Failure of transcription factors to specifically recruit the RNA polymerase and accurately regulate gene expression can cause abnormal cell function, apoptosis and cancer[1,2,3].

In prokaryotes, RNA is synthesized by a single kind of polymerase. By contrast, the nucleus of eukaryotes contains three types of RNA polymerases differing in template specificity, localization and susceptibility to inhibitors. RNA polymerase I is located in nucleoli, where it transcribes the tandem array of genes for 18S, 5.8S and 28S ribosomal RNA[4]. The other ribosomal RNA molecule (5S rRNA) and all the transfer RNA molecules (tRNA) are synthesized by RNA polymerase III[5], which is located in the nucleoplasm. The precursors of messenger RNA (mRNA) are synthesized by RNA polymerase II, which is also located in nucleoplasm. Several small RNA molecules, such as U1 small nuclear RNA (snRNA) are also synthesized by RNA polymerase II[6].

The human small nuclear RNA genes encode snRNAs which are involved in RNA processing reactions such as mRNA splicing. The genes are characterized by the presence of a proximal sequence element (PSE) and a distal sequence element (DSE). In the human genes, the U1 and U2 snRNA promoters serve as the prototypic pol II snRNA promoters, and the U6 snRNA serves as the prototypic pol III snRNA promoter. The human pol II snRNA core promoters contain only one essential element, the PSE. The pol III snRNA core promoters consist of two elements, the PSE and the TATA box located at a fixed distance downstream (Figure 1)[7,8].

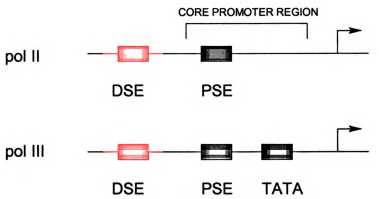


Figure 1: Structure of *H. sapiens* snRNA promoters

1.2. Basal transcription factors

Basal transcription factors are required for accurate recruitment of the RNA polymerase machinery. RNA polymerase alone can initiate transcription but the process happens randomly and without control. RNA polymerase III transcription factors include both general factors and gene specific factors. General factors include TFIIIB, TFIIIC, and SNAPc. In yeast TFIIIB is the central initiation factor and it alone can recruit RNA polymerase III (RNAP III) and define the transcription start site. TFIIIA, TFIIIC and other transcription factors bind DNA and serve as assembly factors for TFIIIB. TFIIIB is composed of three subunits: TBP, Brf1 and Bdp1[9]. TATA box binding protein (TBP) is an essential component of the transcription initiation machinery and is required for the expression of most nuclear genes[1].

Small nuclear RNA activating protein complex (SNAPc) is a basal transcription factor required for specific transcription by RNA polymerase II (RNAP II) and RNAPIII of small nuclear RNA (snRNA) genes. SNAPc-dependant promoters that are recognized by RNAPIII have both a PSE[10] and a TATA box while promoters recognized by RNAPII have only a PSE. TBP on the other hand is required for transcription initiation of both classes of genes (Figure 2).

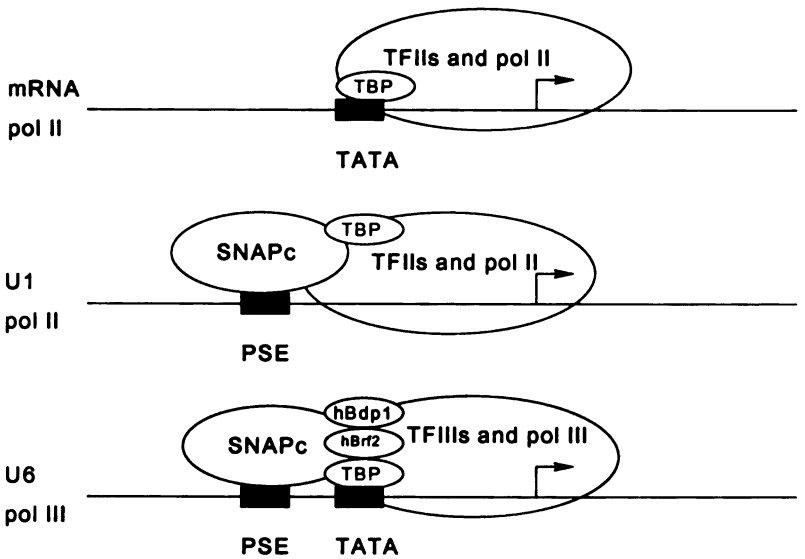


Figure 2: Composition of pol II and pol III transcription initiation complexes

1.3. Small nuclear RNA activating protein complex (SNAPc)

SNAPc binds to the proximal sequence element (PSE) and recruits TBP, and after that several other transcription factors and RNA polymerase can be recruited[11]. SNAPc is composed of five subunits SNAP190[12,13], SNAP50[14], SNAP43[15], SNAP45[16] and SNAP19 (Figure 3)[17]. The N-terminus of SNAP190, the largest subunit of SNAPc, interacts with the SNAP50, SNAP43 and SNAP19 subunits of SNAPc, TBP and DNA (Figure 4). The Myb domain[12] of SNAP190 responsible for DNA binding is composed of four and a half Myb homology repeats. Oct-1 interacts directly with a small region of SNAP190 (800-930). SNAP50 interacts with SNAP190, SNAP43, TBP and DNA. SNAP43 interacts strongly with the N-terminal region of SNAP190, SNAP50, SNAP19 and TBP. SNAP45 interacts with the C-terminus of SNAP190 and TBP[12]. SNAP19 interacts with SNAP190 and SNAP43 and stabilizes the interaction between the two.

SNAP19 is interestingly not required for the assembly of the SNAP190, SNAP43 and SNAP50 complex, but the assembly of the complex is strengthened in the presence of SNAP19 (Figure 4).

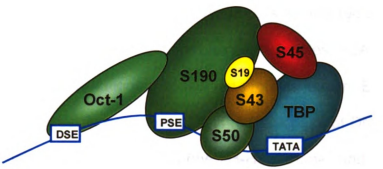


Figure 3: SNAPc dependant pol III transcription

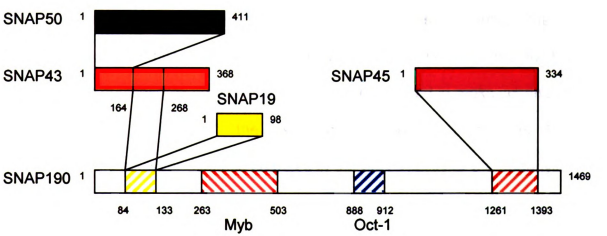


Figure 4: Schematic representation of the SNAP190 amino acid sequence showing relevant domains (adopted from Hernandez et al[11]).

1.4. Chromatin architecture

Histone proteins, assembled with DNA to form nucleosomes, are the basic building blocks of chromatin. The nearly invariant way in which DNA can wrap around the histone proteins in eukaryotic cells is reflected by the conservation of the histone proteins. The four core histone molecules, H2A, H2B, H3 and H4 are among the most evolutionary conserved proteins known. Each nucleosomal unit is formed by wrapping approximately 146 base pairs of DNA around the histone octamer core particle containing one H3-H4 tetramer and two H2A-H2B dimers (Figure 5, 6). The accuracy of this model was confirmed by crystal structure determination of the histone octamer. The crystal structure[18] revealed that the conserved C-terminal domains of core histone proteins have similar conformations that are crucial for the assembly of histones. In contrast the N-terminal tails of core histones are less structured and are not essential for the integrity of the nucleosome particle. Histone tails are believed to make secondary and more flexible contacts with the DNA and neighboring nucleosomes. This interaction would allow for dynamic changes in the accessibility of the underlying genome.

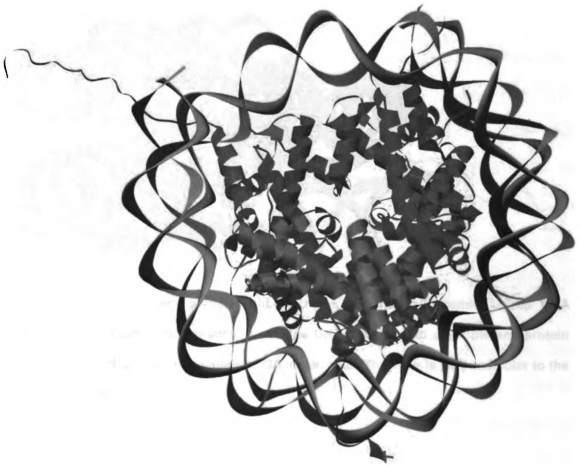


Figure 5: Nucleosome core particle: ribbon traces for the palindromic 146-bp DNA Fragment from Human X-chromosome α-satellite DNA (yellow) and eight histone protein main chains (red: H3, blue: H4, magenta: H2A, cyan: H2B. The view is down the DNA superhelix axis[18].

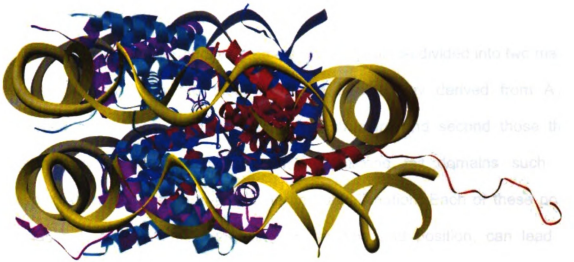


Figure 6: Nucleosome core particle: ribbon traces for the palindromic 146-bp DNA Fragment from Human X-chromosome α-satellite DNA (yellow) and eight histone protein main chains (red: H3, blue: H4, magenta: H2A, cyan: H2B. The view is perpendicular to the DNA superhelix axis[18].

1.5. Chromatin remodeling

The enzymes that modify chromatin packing can be divided into two major groups[19,20]. First there are those that use free energy derived from ATP hydrolysis and actively disrupt nucleosomal structure and second those that cause covalent modifications[21,22,23] on the histone tail domains such as phosporylation, acetylation, methylation and ubiquitination. Each of these post-translational modifications, depending on its type and position, can lead to different changes in chromatin packing. This change is due to charge changes of the histone tails which can stabilize or destabilize the chromatin[24,25].

At present the function of histone phosphorylation is not well known. One possibility is that the addition of negatively charged phosphate groups to the N-terminal H3 tails may disrupt the electrostatic interaction between the basic H3 tail and the negatively charged DNA backbone, thus resulting in higher accessibility of the genome to nuclear factors. The other possibility is that the secondary modification may serve as a recognition site for recruitment of transcription factors and regulatory complexes. Similarly histone acetylation[26] has been shown to change the affinity of some regulatory proteins towards the histone tail. A structure known as the bromodomain (Figure 7)[27,28] is an example of such acetylation dependant binding to histone tails. It has been shown that the bromodomain, found in many nuclear histone acetyl transferases (HATs) is responsible for recognition of acetyl-lysine sites. In vitro peptide binding assays showed that multiply acetylated H4 peptides show much greater binding to the double bromodomain than the unacetylated peptides[29,30].

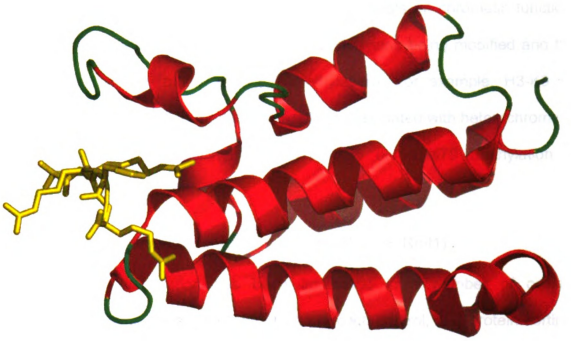


Figure 7: Ribbon diagram of Gcn5p showing the bundle of four main α-helixes (Red) with the Nζ-acetyl lysine side chain of the H4 peptide (yellow) bound in a deep slot at the top of the helix bundle[27].

Histone tail methylation can have multiple effects on chromatin function, including transcription, depending on the specific lysine being modified and the level of methylation (mono-, di- or tri-methylation). For example, H3-K9 di-methylation and H3-K27 tri-methylation are both associated with heterochromatin formation and gene silencing. H3-K4, H3-K36 or H3-K79 methylation is associated with active chromatin.

1.6. *S. cerevisiae* protein-arginine methyltransferase (Rmt1)

Arginine methylation has been implicated in a number of cellular processes including transcription regulation, growth control, and protein sorting. The most studied arginine methyltransferase is the type I enzyme, which catalyzes the S-adenosyl-L-methionine (SAM) dependant formation of monomethyl arginine and asymmetric dimethylarginine. Many substrates have been identified for the type I enzyme, which often methylates mRNA binding proteins at Arg-Gly-Gly, Arg-X-Arg (X represents any amino-acid) or Gly-Arg-Gly. Rmt1 and its mammalian homolog PRMT1 are able to methylate a number of yeast and mammalian polypeptides including histones, recombinant heterogeneous ribonucleoprotein A1, cytochrome c and myoglobin. PRMT1 can interact with the immediate-early gene products BTG1 and TIS21, the interferon α/β receptor and interleukin enhancer binding factor 3 (ILF3)[31,32].

There are two major types of protein arginine methyltransferases that transfer the methyl group from S-adenosyl-L-methionine (SAM) to the guanidino group of arginines in protein substrates resulting in homocystein (AdoHcy) and methylated proteins. Both types catalyze the formation of monomethyl arginine

but differ in dimethylarginine products (Figure 8). Type I PRMTs form asymmetric dimethylarginine and type II form symmetric dimethylarginine. Type I PRMTs have numerous substrates while type II PRMTs have only one known substrate the myelin basic protein.

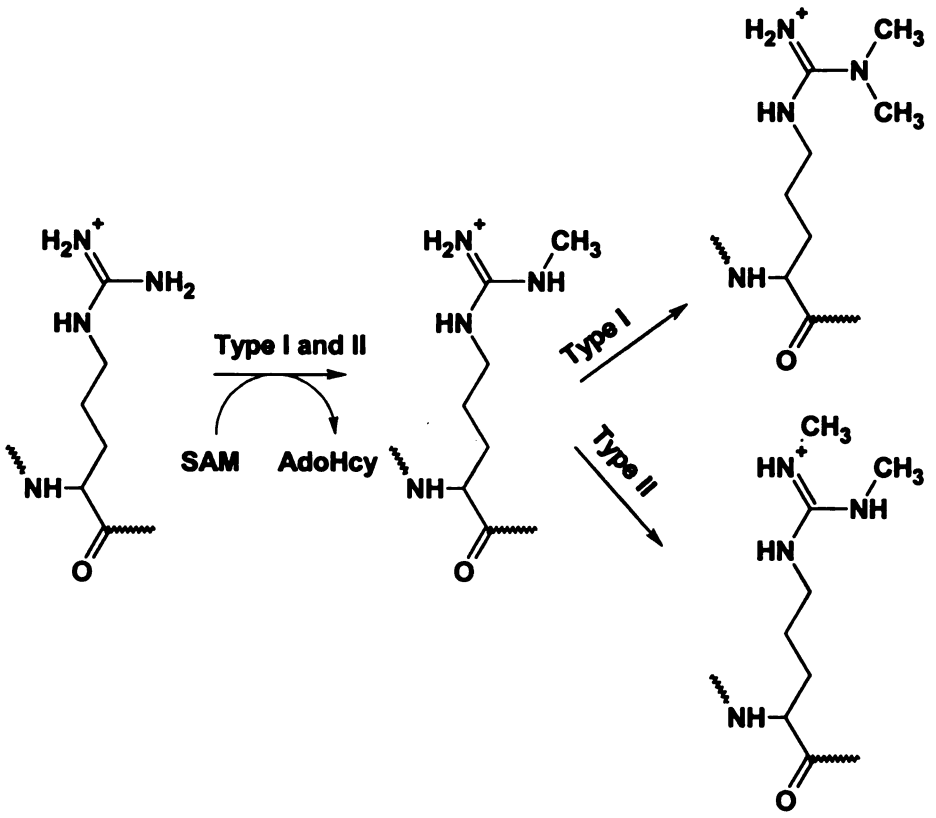


Figure 8: Two major types of protein arginine methylation

1.7. Structure and function of methyl transferase

Recently crystal structures for three methyl transferases; SET7/9, LSRT and DIM-5 in complex with their substrate were determined. All three structures have a narrow, doughnut-like hole at the catalytic site where methyl transfer occurs. The peptide substrate binds on one side of the channel and the methyl donor occupies the opposite face. Once the lysine is docked in the active site,

one hydrogen from the ε-NH_3 protrudes through the hole to the opposite side where the transfer reaction occurs. It was shown that changing one of the amino acids that controls the environment of the hydrogen-bonding of the ε-NH_3 group will result in transformation of a mono- or tri-methyl transferase to a di-methyl transferase.

In the case of arginine methyl transferase several crystal structures were recently determined[31,33,34]. All the proteins have high sequence similarity and all the proteins form a dimer that has the shape of a donut. The Rmt-1 dimer is formed by hydrophobic interactions between a dimerization arm or antenna of one monomer and the head of the other (Figure 9). Furthermore the crystal structure of the yeast Rmt1[33] revealed an even more complex ordering of the dimers. Rmt1 forms a hexamer; a trimer of dimers and collectively all six monomers fill a large flattened spheroid space about 70 Å high and 110Å in diameter (Figure 10). Dimerization of yeast Rmt1, rat PRMT1 and rat PRMT3 appears to be essential for methylation activity. Several other PRMT genes were recently identified through genome sequencing projects. In addition several sequences were identified that share strong similarity with PRMT1 in *Drosophila*, *Xenopus*, zebrafish, sea urchin, rice and tomato, indicating that PRMTs are a highly conserved family of proteins in eukaryotes. The proteins vary in length from 348 (*S. cerevisiae* Rmt1) to 608 (CARM1) amino acids, but they all contain a conserved core region of about 310 amino acids.

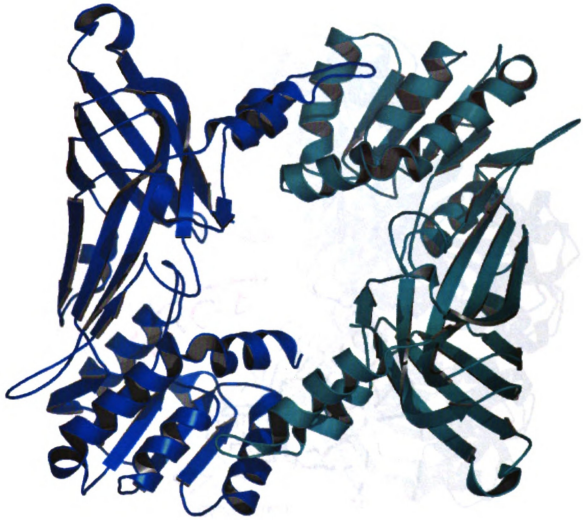


Figure 9: Ribbon representation of the Rmt1 dimer, viewed from inside the hexamer. Each monomer is in different color[33].

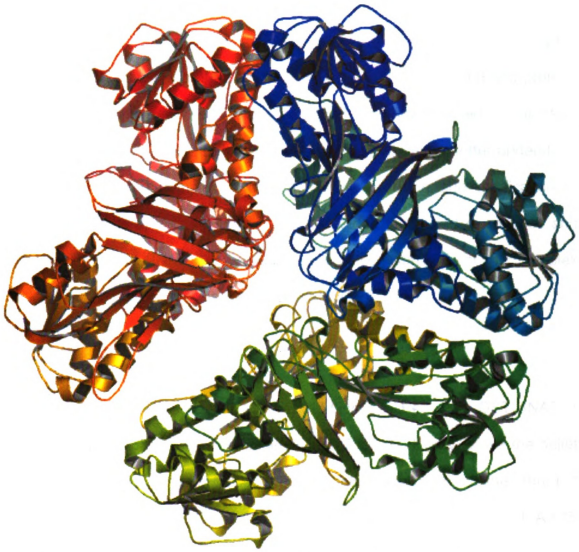


Figure 10: Ribbon representation of the Rmt1 hexamer. Each monomer is in different color[33].

2. Results: over-expression and purification of SNAP43

The SNAPc complex in an important basal transcription factor responsible for transcription initiation of small nuclear RNA genes. The function and protein-protein interactions have been well researched. We first focused on structure determination of separate subunits of the complex in order to better understand the behavior of the complex. Since the over-expression and purification of separate subunits is not feasible we finally decided to try and co-express and crystallize the mini SNAPc. If successful the solved structure of this complex would yield a wealth of information about the SNAPc.

2.1. Cloning of SNAP43

The plasmid DNA coding for the wild-type SNAP43 as a GST-SNAP43 fusion peptide (p43ExXB-1) was obtained from Prof. Bill Henry. Since the belief was that GST-SNAP43 is expressed in the insoluble fraction and is therefore not folded, we decided that a HIS tagged version of SNAP43 should be tried. A HIS tagged protein can be purified from the crude cell extract under denaturing conditions with urea and then refolded. We decided to insert the DNA sequence for SNAP43 into a pET-14b vector, which has an N-terminal HIS tag sequence followed by a thrombin site.

To achieve this goal the DNA sequence was first incorporated into a pBSKS bluescript cloning plasmid and then into the pET vector. A PCR reaction was performed using TURBO PFU and the p43ExXB-1 plasmid as a template. After successful amplification the samples were analyzed on an agarose gel,

selected bands were excised, and the PCR product was purified using a QIAGEN gel extraction kit. The purified PCR products and the pBSKS vector were digested with *EcoRI* and *BamHI* restriction enzymes, followed by gel purification of the selected bands. A ligation reaction was performed, followed by a transformation into *E. coli* JM109 competent cells. Several colonies were selected and grown to 5 mL cultures. DNA was isolated from the cultures using the QIAGEN Plasmid Mini kit. The validity of the prepared plasmids was verified first by test cleavage of the DNA with *EcoRI* and *BamHI* restriction enzymes and by analyzing the reactions on the agarose gel. Fortunately every selected colony appeared to be correct.

The prepared SNAP43 cloning plasmid (pBSKS43-1) and the pET14b expression plasmid were cleaved with *NdeI* and *BamHI* and gel purified. A ligation reaction was performed with the cleaved insert and pET14b vector and a transformation into *E. coli* JM109 was performed. Selected colonies were grown in 5 mL cultures and the plasmid DNA was isolated using the QIAGEN Plasmid Mini kit. The validity of the resulting DNA (pETH43-1) was verified by *NdeI* digest followed by *BamHI* digest. Again all selected colonies were correct.

2.2. Over-expression of HIS-SNAP43

The plasmid DNA coding for HIS-SNAP43 was transformed into *E. coli* BL21(DE3) competent cells and the cultures were grown at 37°C until an OD_{600} of 0.6, induced with 1mM IPTG, and grown at 16°C for 16 h. Several attempts were made to purify the protein from the soluble cell extract using Ni-NTA resin. Since no evidence was obtained that the protein is at all present in the soluble

fraction, the insoluble part of the cells was resuspended in 6M urea and the solubilized cell extract was again loaded to Ni-NTA beads. Again no clear evidence of SNAP43 presence was detected. Several different buffers with differences in pH, salt concentrations and additives were tested for binding of HIS-SNAP43 but in no case did we clearly detect the protein of interest. After extensive screening of binding conditions and the inability to detect reasonable quantities of SNAP43 we decided to change our strategy. We were however able to detect small quantities of SNAP43 in the purified fractions by Western Blot (data not shown).

2.3. Over-expression, refolding and purification of GST-SNAP43

Since the HIS-SNAP43 expression failed, we decided to refold the insoluble GST-SNAP43. The GST-SNAP43 fusion peptide was over-expressed in BL21(DE3) Codon Plus strain of *E. coli*. The cultures were grown at 16°C and the cell extract was prepared by sonication of the cells in 6M urea and removal of insoluble cell debris by centrifugation. The solubilized cell extract was dialyzed against a buffer containing lower concentrations of urea. After urea was completely removed the insoluble material was removed by centrifugation and the supernatant was loaded on to GST resin. After binding, the resin was washed and analyzed by SDS-PAGE. A single band was visible that runs at about 70 kDa and could therefore be GST-SNAP43. After thrombin cleavage and elution, the fractions and the beads were analyzed. The GST-SNAP43 band disappeared but cleaved SNAP43 was not visible in any fraction. Most likely, the thrombin

cleavage was allowed to proceed for too long and the protein was destroyed (data not shown).

Using this method we were able to over-express and partially purify reasonable amounts of SNAP43, but the time needed for complete cleavage of the linker between the GST tag and the protein had yet to be determined.

The refolding experiment of the GST-SNAP43 used the whole cell solubilized extract. After discussing the matter with Prof. Bill Henry it appeared that GST-SNAP43 is in fact at least in part soluble. Armed with this new information a new attempt to purify the protein from the soluble fraction of the cell extract was made. After binding and several washes, the beads were analyzed by SDS-PAGE and a large quantity of GST-SNAP43 appeared to be immobilized on the beads. Time controlled reactions with thrombin revealed that 20 u of thrombin and 4 h of cutting time at 4°C are sufficient for complete cleavage of the tag without protein degradation. The protein was eluted off the beads but the majority of the protein remained on the beads. If buffers with increasing amounts of salt are used in elution, more protein is successfully eluted. It is interesting that eluting the protein with high volumes of high salt buffers does not elute the protein but every time the salt concentration is increased, more SNAP43 is eluted. For maximal recovery, the protein beads were washed with the buffers while shaking for 30 min at 4°C. After every elution the salt concentration was increased and elution repeated (Figure 11). After elution with 2 M salt much protein was still immobilized on the resin. This result is surprising but it can be used to explain the failure we had with the His tagged protein.

Another problem in the purification of SNAP43 was soon identified. After elution of the protein an attempt was made to buffer exchange and concentrate the protein using the Centricon-30 concentrators. Although the volume of the solution containing SNAP43 was reduced drastically during centrifugation, the concentration of the protein did not change. Even after 50 fold reduction of the volume the concentration of the protein was almost equal to the starting concentration. Interestingly no precipitation was observed. The protein sample after the concentration was analyzed and compared with the starting material. The SDS-PAGE revealed that the two samples are almost identical, with an increase of the background bands in the concentrated sample but a decrease of the SNAP43 suggesting that SNAP43 sticks to the membrane (data not shown).

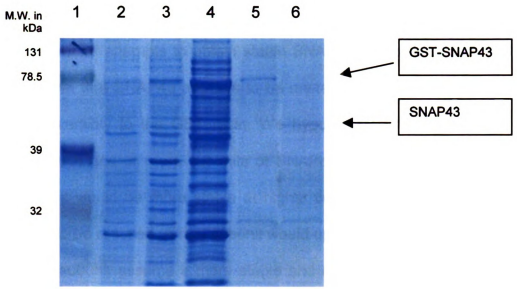


Figure 11: Expression and purification of GST-SNAP43

1. M.W. marker
2. Cells before induction
3. Cells after induction
4. Cell extract
5. Proteins bound to GST resin
6. Cleaved protein on the GST resin

Several changes to the condition for concentration were made by changing salt, glycerol and additive concentrations but the changes did not improve the recovery of the protein during concentration. Furthermore the concentration of SNAP43 decreased even when left at 4°C in only 2 h. No precipitation was observed but the concentration of the protein dropped from 0.2 mg/mL to 0.06 mg/mL. The protein is either being degraded or the protein sticks to the flask. Knowing the difficulties in keeping SNAP43 in solution, it became clear that getting the concentration high enough for crystallization trials and maintaining a stable homogenous sample of crystallographic quality is not feasible.

2.4. Co-expression of SNAP43 with other SNAPc subunits

Previous attempts to obtain stable SNAP43 samples of sufficient quality have shown that SNAP43 appears to be unstable at higher concentrations and binds irreversibly to the GST resin. We hypothesized that if SNAP43 is in a complex with one or more subunits of SNAPc such as SNAP190, SNAP50 or SNAP19, it would be more stable at higher concentrations and sticking to the resin would be reduced. A free subunit would upon formation of the complex with the other subunit assume a more stable and more compact conformation. The unspecific interactions with the resin might be minimized by shielding an exposed surface of SNAP43 due to protein-protein contacts.

Other members of our research group have been working on other subunits of the mSNAPc. And several conclusions can be made from their experience with the mSNAPc subunits. SNAP190(1-505) expresses in reasonable quantities, can be easily purified and concentrated. However crystallization trials yielded no protein crystals. It was observed that SNAP190(1-505) has low solubility, when precipitate was observed even with low precipitant concentrations.

The smallest subunit SNAP19 can be easily expressed and purified, but the crystals that were obtained were of extremely poor quality. Recent data obtained by my coworker shows that SNAP19 as a free subunit has no or very little secondary structure. Using NMR techniques it was shown that a free SNAP19 is most likely a random coil in solution. It is very unlikely that an unordered peptide with almost 100 residues will crystallize.

SNAP50 can be expressed in soluble form, but it is extremely hard to obtain pure material. After thrombin cleavage and even before that several bands of equal intensity can be seen on SDS-PAGE. It is most likely that SNAP50 undergoes proteolitic degradation in the cell. Attempts to remove the proteolitic products have failed. Wild-type SNAP50 must be extremely susceptible to degradation probably due to unordered regions. It was also observed that SNAP50, like SNAP43, sticks to the GST resin, suggesting that SNAP50 also has some exposed surfaces that unspecificly interact with the resin.

From this data one can conclude that the separate subunits of the mSNAPc are either weakly ordered, have some exposed surfaces which unspecificly interact with the resin used in purification or are expressed in degraded form.

To test our hypothesis, a transformation of SNAP43 (pSBET43-1; HIS tag, kanamycin resistance) was made into *E. coli* BL21(DE3) Codon Plus RIL. A single colony was selected and competent cells were prepared by $CaCl_2$ treatment. Plasmids coding for SNAP50, SNAP190(1-505) and SNAP19 with GST-tags and ampicilin resistance were transformed into SNAP43 competent cells. Cells were grown on AMP/KAN/CHLOR plates and in AMP/KAN/CHLOR media (see Methods and materials 4.1).

2.5. Co-expression of HIS-SNAP43 with GST-SNAP50

To purify the proteins using the GST beads the cell extract was manipulated following the GST purification method. To purify the proteins using the Ni-NTA beads the cell extract was manipulated following the Ni-NTA

purification method. When Ni-NTA agarose was used to purify the binary complex thru HIS-SNAP43 no apparent band on the gel was visible that corresponds to SNAP43 or GST-SNAP50. On the other hand we were able to observe small quantities of SNAP43 and GST-SNAP50 when GST resin was used (Figure 12). We were unable to elute the proteins in detectable levels neither with the glutathione nor with thrombin cleavage. We can conclude that co-expression of SNAP43 with SNAP50 did not improve the yield or the stability of SNAP43. Interestingly GST-SNAP50 appears to be of higher purity (only one band can be observed) compared to the situation where SNAP50 is expressed and purified alone.

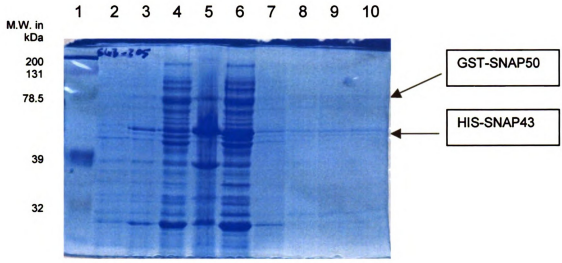


Figure 12: Co-expression of SNAP43 and SNAP50, GST purification

1. M.W. marker
2. Cells before induction
3. Cells after induction
4. Cell extract
5. Insoluble part of cells
6. Flow thru
7. First wash of the resin

8-10. GST resin with bound proteins

2.6. Co-expression of HIS-SNAP43 with GST-SNAP190(1-505)

The GST and Ni-NTA purifications were performed as described before. When the binary complex was purified thru GST-SNAP190(1-505) we were able to observe a strong band corresponding to GST-SNAP190 and a weaker band corresponding to HIS-SNAP43 (Figure 13).

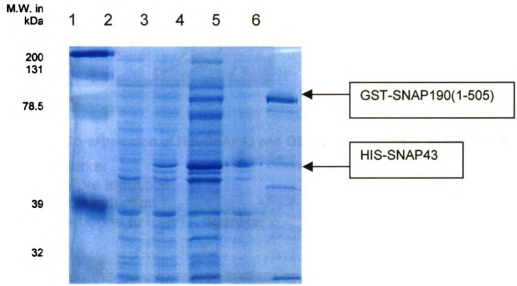


Figure 13: Co-expression of HIS-SNAP43 and GST-SNAP190(1-505), GST purification

1. M.W. marker
2. Cells before induction
3. Cells after induction
4. Cell extract
5. Insoluble part of cells
6. Proteins bound to GST resin

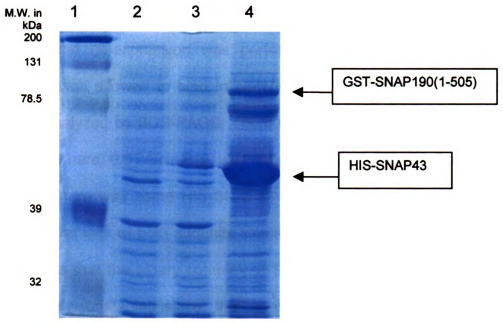


Figure 14: Co-expression of HIS-SNAP43 and GST-SNAP190(1-505), Ni-NTA purification

1. M.W. marker
2. Cells before induction
3. Cells after induction
4. Proteins bound to Ni-NTA agarose

Using this co-expression system and two different purification approaches it can be concluded that both SNAP43 and SNAP190(1-505) are expressed in significant amounts. From the fact that the bands for the two proteins are substochiometric on both GST, where the GST-SNAP190(1-505) band is stronger and Ni-NTA beads, where the HIS-SNAP43 band is stronger (Figure 14), one can conclude that the binary complex does form, since both subunits can be purified using one type of beads, but it is not very stable, since one subunit can be gradually washed away.

2.7. Co-expression of HIS-SNAP43 with GST-SNAP19

The co-expression was performed as described previously. The cell extract was allowed to bind to Ni-NTA resin and to GST resin and the beads were analyzed by SDS-PAGE. There was no HIS-SNAP43 present on the GST beads where GST-SNAP19 was present in large quantities. On the Ni-NTA beads there was some HIS-SNAP43 observed but no GST-SNAP19 could be seen. The lack of both subunits simultaneously present on either type of beads suggests that the binary complex of SNAP43 and SNAP19 does not form or is extremely unstable, since the second subunit was washed away under purification conditions (data not shown).

2.8. Co-expression of SNAP190, SNAP50, SNAP43 and SNAP19

Using the binary co-expression systems of SNAP43 with other SNAPc subunits that interact with SNAP43 we have observed that although the expression levels, purity or stability of SNAP43 are not significantly increased, the proteins in most cases co-purify to some extent. One other binary co-expression system was tested by other members of my group. The case where GST-SNAP190(1-505) was co-expressed with GST-SNAP50 yielded significant levels of soluble and pure material after the first step of purification. This is a purification of wild-type SNAP50 expressed in bacteria, where SNAP50 is represented as a single band according to SDS-PAGE and is soluble. Both SNAP190(1-505) and SNAP50 were cleaved with thrombin and eluted off the

GST resin. The proteins could also be concentrated and crystallization trials were set up although the homogeneity of the sample was not achieved.

Interestingly the co-expression of SNAP190(1-505) and SNAP50 had a great effect on the purity of SNAP50 although the proteins are thought not to interact strongly. The co-expression of SNAP43 with any other subunit of SNAPc did not drastically improve the situation although SNAP43 is thought to interact strongly with SNAP190(1-505), SNAP50 and SNAP19. SNAP190(1-505) and SNAP50 co-purified with SNAP43 to some degree suggesting a strong interaction between the two subunits.

Since the binary co-expression system did not work for SNAP43, a ternary system was designed as a cooperative project between several members of our group employing GST-SNAP190(1-505), SNAP43 and SNAP50. The DNA sequence for each subunit was on a plasmid with unique antibiotic resistance. GST-SNAP190(1-505) was on a pGST-190(1-505) plasmid with ampicilin resistance and SNAP43 and SNAP50 were placed on pCDFDuet-1 (pCDF43-1, streptomycin resistance) and pRSFDuet-1 (pRSF50-1, kanamycin resistance) respectively. Using the three plasmids all three proteins can be expressed in the same cell and at the same time in the BL21(DE3) Codon Plus cells (see Methods and materials 4.1 and 4.2). The result was striking. All three proteins expressed in significant levels (2 mg/L) and the purity was high. The GST tag was removed by thrombin cleavage and none of the subunits were degraded during the cleavage process, although the cleavage was performed for 16 h, which is usually enough to completely over digest the separate subunits (Figure 15). All

three subunits co-purified on the size exclusion and ion exchange chromatographic columns. When ion exchange chromatography was used, some leaking of SNAP43 was observed in some fractions, suggesting that SNAP43 was not tightly bound in the complex. Nevertheless this expression system yielded more soluble and stable SNAP43 than any previously tested expression system. The complex can even be concentrated up to 5 mg/mL and crystallization trials were set up.

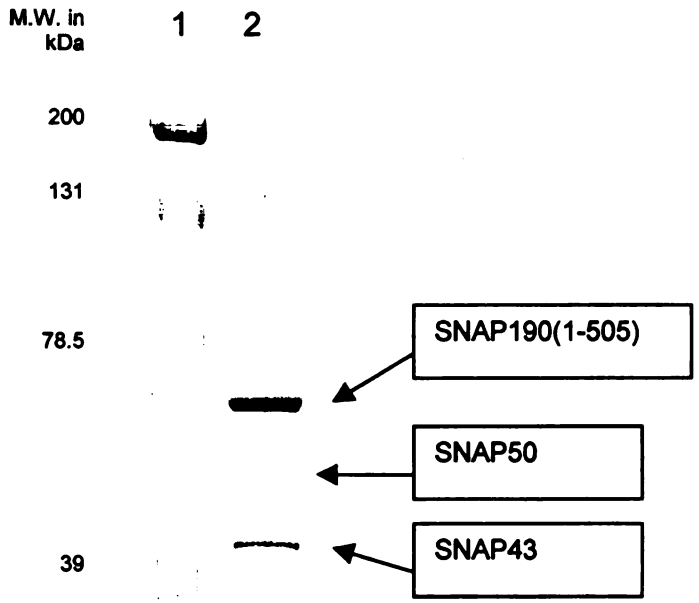


Figure 15: Co-expression of SNAP190(1-505), SNAP43 and SNAP50

1. M.W. marker
2. Purified SNAP190(1-505), SNAP43, SNAP50 complex

SNAP19 was added to the above system in order to enhance the stability of the mSNAPc. DNA sequences for SNAP43 and SNAP50 were cloned into pCDFDuet-1 (pCDF43/50-1) and SNAP19 was cloned into pRSFDuet-1 (pRSF19-1). The expression levels of SNAP50, SNAP43, SNAP19 and SNAP190(1-505) were higher (4 mg/L) than in the above system and SNAP19 was present in super-stoichiometric levels and co-purified with the complex. This system of four proteins behaved similar to the system with the three proteins, but there was no loss of subunits observed during purification, and the recovery of material that was submitted to purification was improved drastically (Figure 16).

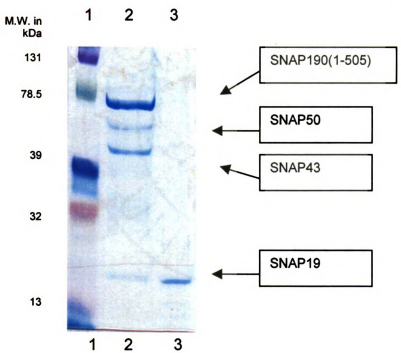


Figure 16: Co-expression of SNAP190(1-505), SNAP50, SNAP43 and SNAP19

1. M.W. marker (Blue 200.000, Red 131.000, Green 78.500, Violet 39.000, Orange 32.000, Magenta 13.000)
2. Purified SNAP190(1-505), SNAP43, SNAP50, SNAP19 complex
3. Purified SNAP19

The optimization steps for purification of the ternary and quaternary complex are currently in progress and crystallization trials are being set up. DNA binding assays and transcription initiation assays are to be performed in the near future. A crystal form was obtained (2.5 M 1,6-hexanediol, 0.1 M sodium citrate pH 5.4) where the crystals are 0.1 mm long and 0.02 mm wide (Figure 17). The crystals appeared in 3 days and grew to its maximum size in one weak. No diffraction was obtained since the crystals are small, but the crystals grew in the original sparse matrix screen and fine screening is in progress.

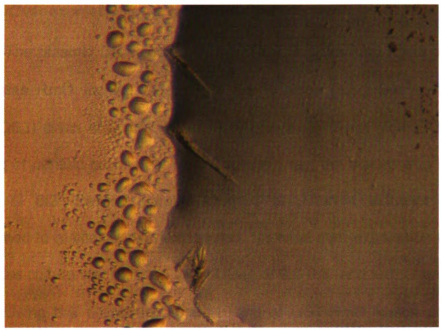


Figure 17: Crystals of SNAP190(1-505)/SNAP50/SNAP43/SNAP19 complex

3. Results: over-expression, purification and crystallization of wild type and mutant S. cerevisiae Rmt1/H4 peptide complex

Rmt1 was shown to preferentially bind to the H4 histone tails with acetylated lysine (K8). Interestingly the solved crystal structure and the amino-acid sequence do not show the presence of a bromodomain, which is responsible for recognition of the acetylated lysines in HATs. The crystal structure of Rmt1 in complex with the H4 acetylated peptide would yield a detailed picture of how the Rmt1 recognizes the acetylated lysine of the H4 tail.

3.1. Over-expression of *S. cerevisiae* Rmt1

The plasmid DNA coding for wild type *S. cerevisiae* Rmt1 and mutant *S. cerevisiae* Rmt1 lacking the first 22 amino acids (Δ22) was transformed into BL21(DE3) strain of *E. coli*. Preliminary growths of Rmt1 yielded approximately 2-3 mg of purified protein per liter of culture when the culture was induced at an OD_{600} of 0.3. Several parallel growths at different induction points were performed to maximize over-expression. Maximal over-expression was achieved when the culture was induced at an OD_{600} of 0.6-0.8 and further grown for 4 h at 37°C. Using this procedure about 10 mg of GST-Rmt1 fusion protein can be grown in 1 L culture.

3.2. Purification of Rmt1

Rmt1 is expressed as a GST fusion peptide and can therefore be purified using the glutathione resin. After cell disruption with the sonicator and removal of

solid cell debris the cleared cell extract was mixed with glutathione resin and allowed to bind. After several washes with increasing salt concentration immobilized protein was analyzed with SDS-PAGE.

The general protocol for eluting the bound protein off the resin calls for use of thrombin, which cleaves the linker between the GST and the protein and releases the protein. The second method for eluting the protein is elution with glutathione. Glutathione dissolved in the elution buffer will compete with the glutathione that is immobilized on the beads for the GST. If the concentration of dissolved glutathione is sufficient the protein will be eluted off the beads. However this way the fusion protein has to be cleaved and free GST separated from the protein of interest.

When we tried to employ the thrombin cleavage method on GST-Rmt1 we were unable to elute the protein. SDS-PAGE analysis showed that the cleavage of the linker was complete; however the protein remained on the beads. We suspected that the protein might be nonspecifically but strongly interacting with the resin. To disrupt this interaction we tried eluting with buffers containing higher amounts of salt. A range of buffers containing 0.1 to 1 M KCl was tested without success.

The glutathione elution method was tried next. We were able to completely remove the fusion peptide off the resin. Thrombin was added to the eluted fractions of the GST-Rmt1 fusion peptide to cleave the linker and only after a few hours precipitate was formed. After centrifugation the supernatant and

the pellet were analyzed with SDS-PAGE and the majority of the protein was precipitated. Only a fraction of total protein was present in solution.

From this data we concluded that the protein does not unspecificly bind to the resin but rather forms large aggregates and precipitates. A range of buffers containing 0.1-1 M KCl was again tested. The protein precipitated in every case but only the aliquot containing 0.1 M KCl had low amounts of soluble protein. Given this result a new range of salt was tested and when the concentration of KCl is 50 mM or less the cleavage is complete without precipitation and the protein is completely soluble. Interestingly if salt is increased even up to 1 M the protein does not precipitate once it has been cleaved.

Since the separation of GST and Rmt1 involves additional purification steps, we decided to try the thrombin cleavage method but in a buffer with low salt concentrations. Cleavage and elution was complete without precipitation and large quantities of reasonably pure Rmt1 were obtained. Again if salt is added to Rmt1 the protein does not precipitate once it has been cleaved but if the cleavage is done even in 0.1 M KCl the protein precipitates immediately during cleavage. This phenomenon can be explained by the fact that the active form of the Rmt1 is a dimer. The activity assays performed by Prof. Min Hao Kuo showed that the GST-Rmt1 is not very active when the cleaved Rmt1 is. This suggests that the GST-Rmt1 can not form an active dimer due to GST interference. Once the GST is removed a surface area on the Rmt1 becomes exposed and a dimer must be formed fast or the protein will precipitate due to unspecific interactions. When the cleavage is done in high salt the unspecific

interactions are stronger than in lower salt and the protein precipitates faster than it is able to form a stable and active dimer. Once sufficient time is allowed for the Rmt1 to form a dimer it can be exposed to high salt conditions but will not precipitate.

Rmt1 was further purified using the Pharmacia FPLC chromatography system equipped with a Source Q ion exchange column. The protein was loaded in the same buffer that was used in the GST purification but diluted twofold with water (25 mM KCl), and a salt gradient ranging from 15-30% KCl was used to elute the protein. The protein was eluted in a range from 18-25 % KCl but its purity was not much higher and the yield was only about 20%. The result was surprising since the protein was relatively pure according to SDS-PAGE before the ion exchange chromatography. An increase in the backflow pressure was detected during two consecutive loads to the ion exchange column suggesting that the protein might be sticking to the ion exchange beads. This can explain the low yield of this purification. When a column with smaller bed volume (1.5 mL) was used, the recovery of the protein was increased to 60% and the purity was estimated to be >95% (Figure 18).

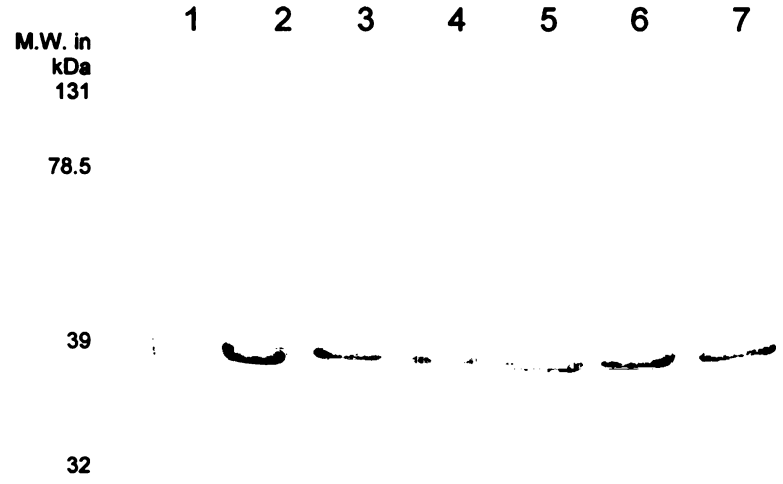


Figure 18: Ion-exchange chromatography of wild type Rmt1.

1. M.W. marker
2. Rmt1 after GST purification step (input)
3. Fraction 9
4. Fraction 10
5. Fraction 11
6. Fraction 12
7. Fraction 13

Ion-exchange chromatography was performed on a Biotech Pharmacia FPLC system. 1.5 mL of Source® 15Q ion-exchange beads were used in Omnifit 5/10 column. The buffer flow was 5 mL/min and 5 mL fractions were collected. The protein was loaded in low salt (50 mM NaCl) and eluted with salt gradient. Low salt buffer (10 mM Tris pH 7.9, 10 mM Tris pH 7.9, 50 mM NaCl, 3mM DTT) and high salt buffer (10 mM Tris pH 7.9, 10 mM Tris pH 7.9, 1 M NaCl, 3mM DTT) were used. The protein eluted in 150-200 mM NaCl.

Size exclusion gel filtration was used to further purify the protein. Resulting fractions were analyzed by Bradford assay and SDS-PAGE. Two apparent peaks appear on the chromatogram (Figure 19). The first peak elutes in a volume that corresponds to more than 600 kDa and the second peak corresponds to approximately 90 kDa. The SDS-PAGE shows that Rmt1 is the only component of the second peak. The protein purified by gel filtration corresponding to the Rmt1 dimer is of superior purity and was also used for crystallization trials (Figure 20).

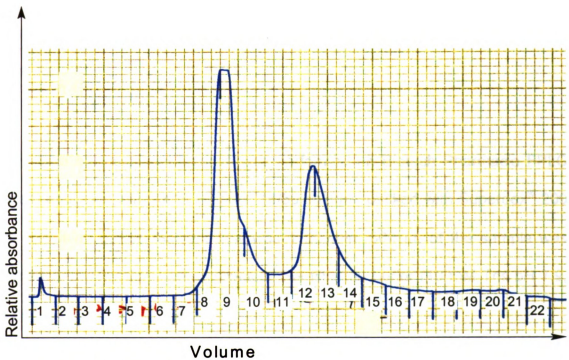


Figure 19: Plot of wild type Rmt1 gel filtration

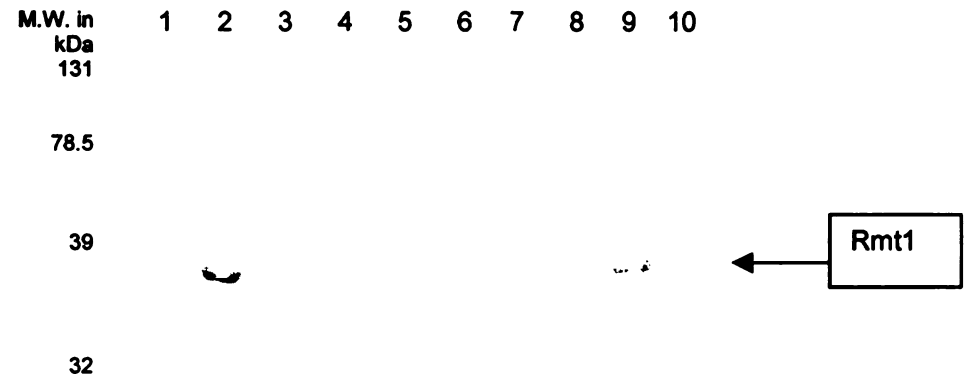


Figure 20: SDS-PAGE of wild type Rmt1 gel filtration

1. M.W. marker
2. Rmt1 after GST purification step (input)
3. Fraction 7
4. Fraction 8
5. Fraction 9
6. Fraction 10
7. Fraction 11
8. Fraction 12
9. Fraction 13
10. Fraction 14

Size exclusion gel filtration was performed on Biotech Pharmacia FPLC system equipped with XK19 HiLoad® 16/60 column, packed with Superdex® 200 size exclusion beads. Solvent flow was 1 mL/min. 500 μL of the sample was injected. 1 mL fractions were collected. Gel filtration buffer (10 mM Tris pH 7.9, 50 mM NaCl, 3 mM DTT) was used.

The protein was concentrated using Centriprep-30 (Amicon) by centrifugation at 4000 rpm. The final concentration of the protein was 6 mg/mL as determined by the Bradford assay.

3.3. Crystallization of Rmt1 and Rmt1/H4 peptide complex

Rmt1 and a mutant Rmt1(Δ22) were previously crystallized and the structure was solved for the mutant Rmt1. The crystals of Rmt1 were not of sufficient quality for crystallization purposes. Limited digest experiments and N-terminal sequencing identified Rmt1 lacking the first 22 amino acids. Well diffracting crystals were obtained for Rmt1(Δ22) [33]. It appears that the first 22 amino acids were unordered and as a result the diffraction of the wild type Rmt1 was poor.

We have attempted to crystallize the wild type and mutant Rmt1 with the acetylated and nonacetylated H4 peptide.

The purified and concentrated Rmt1 was mixed with the acetylated H4 peptide in 1:2 ratios. Immediately after the addition of the peptide the solution turned cloudy. The voluminous precipitate was removed with centrifugation and the concentration of the mixture was determined to be close to zero. The addition of the peptide to the concentrated sample of either wild type or mutant Rmt1 causes a structural change that result in fast aggregation. If the peptides are added to the protein, when the concentration of the protein is below 0.5 mg/mL, the aggregation is not observed.

The purified wild type or mutant Rmt1 was mixed with the peptides at a 0.5 mg/mL or lower concentration of the protein and 10 fold excess of the

peptide. The mixture was buffer exchanged and concentrated using the Centriprep-10 by centrifugation at 4000 rpm. The concentrated solution of the protein and peptide was analyzed with SDS-PAGE and the presence of the peptide was confirmed (Figure 21). The final concentration of the Rmt1 with the peptide was 5 mg/mL.

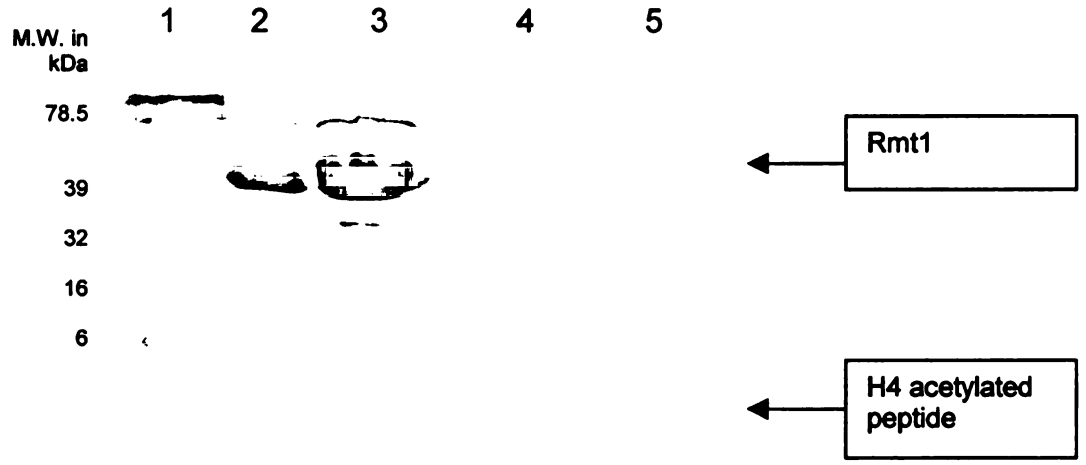


Figure 21: Wild type Rmt1 with H4 acetylated peptide

1. M.W. marker
2. Rmt1 after GST purification step, mixed with the H4 acetylated peptide
3. Rmt1 after GST purification step, mixed with the H4 acetylated peptide, buffer exchanged and concentrated
4, 5. H4 acetylated peptide

Hanging drop vapor diffusion was used to crystallize the proteins. After extensive screening, crystals of wild type Rmt1 and Rmt1(Δ22)/Acetylated peptide were obtained. The crystals appear in only 30 min, grow to its maximum size in 12 h and have a form of long needles (Figure 22). The previously reported crystallization condition surprisingly did not yield any crystals for our material.

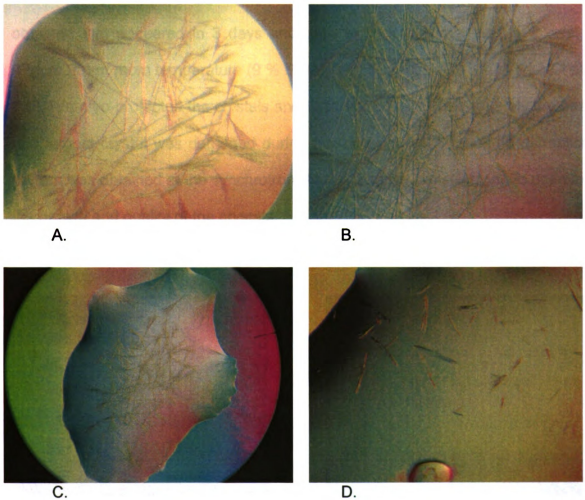
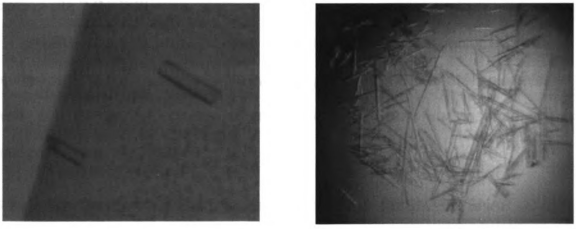


Figure 22: Wild type Rmt1 crystals.

A., B., C. Crystals grown in 9% w/v PEG 4000, 0.1 M Hepes pH 7.5.

D. Crystals grown in 9% w/v PEG5000MME, 0.1 M Hepes pH 7.5

Systematically changing the crystallization condition yielded better crystals of wild type Rmt1 that gave diffraction up to 15 Å at home (Figure 23) but the crystals were small, not single and hard to repeat. After several optimization steps we found that adding 50-200 mM NaCl to the crystallization condition greatly improves the repeatability and the quality of the crystals. Several single but small crystals (the biggest crystal was 0.1 mm in the long direction) were obtained that appeared in 3 days and grew to their maximal size in 2 weeks (Figure 23) at room temperature (9 % PEG 4000, 50 mM NaCl, 0.1 M TrisHCl pH 7.5). We cryo-protected the crystals and flash froze them. Unfortunately the best and largest crystal was damaged during freezing and as a result no diffraction pattern was obtained at the synchrotron. At this time we are still trying to optimize the growth and quality of the crystals.



A B

Figure 23: Crystals of wild type Rmt1 (A) and Rmt1Δ22/Acetylated peptide complex (B)

4. Methods and materials

4.1. Preparation of expression plasmids for SNAP proteins

pCDF43-1 was prepared from pCDFDuet-1 (Novagen) vector by insertion of the SNAP43 coding region, which was amplified from a p43ExXB-1 plasmid, via *NdeI* and *BamHI/BglII* cloning sites. The coding region was inserted in the multiple cloning site 2 (MCS2).

pRSF50-1 and pCDF50-1 were prepared from pRSFDuet-1 and pCDFDuet-1 (Novagen) vector by insertion of the SNAP50 coding region, which was amplified from a pGST-50-2 plasmid, via *NcoI* and *SacI* cloning sites. The coding region was inserted in the multiple cloning site 1 (MCS1).

pCDF43/50-1 was prepared from pRSF50-1 vector by insertion of the SNAP43 coding region, which was amplified from a p43ExXB-1 plasmid, via *NdeI* and *BamHI/BglII* cloning sites. The coding region was inserted in the multiple cloning site 2 (MCS2).

pRSF19-1 was prepared from pRSFDuet-1 (Novagen) vector by insertion of the SNAP19 coding region, which was amplified from a pGST19R plasmid, via *NdeI* and *BamHI/BglII* cloning sites. The coding region was inserted in the multiple cloning site 2 (MCS2).

4.2. Preparation of an *E. coli* strain for expression of mSNAPc (SNAP 190/50/43)

Plasmid (pGST-190(1-505)) coding for SNAP190(1-505) was transformed in to *E. Coli* BL21(DE3) Codon Plus™ RIL (Stratagene) competent cells (see 4.7) and the colonies were allowed to grow for 16 h on the Amp/Chlor agar plates.

Competent cells were prepared (see 4.8) and pRSF50-1 was transformed in to this cell line and the colonies were allowed to grow for 16 h on the Amp/Kan/Chlor agar plates. Competent cells were prepared and pCDF43-1 was transformed into this cell line and the colonies were allowed to grow for 16 h on the Amp/Kan/Chlor/Strep agar plates.

The concentrations of antibiotics used were: ampicillin 50 μg/mL, chloramphenicol 50 μg/mL, kanamycin 10 μg/mL and streptomycin 10 μg/mL.

4.3. Preparation of an *E. coli* strain for expression of mrSNAPc (SNAP 190/50/43/19)

Plasmid (pGST-190(1-505)) coding for SNAP190(1-505) was transformed in to *E. Coli* BL21(DE3) Codon Plus™ RIL (Stratagene) competent cells (see 4.7) and the colonies were allowed to grow for 16 h on the Amp/Chlor agar plates. Competent cells were prepared (see 4.8) and pRSF19-1 was transformed in to this cell line and the colonies were allowed to grow for 16 h on the Amp/Kan/Chlor agar plates. Competent cells were prepared and pCDF43/53-1 was transformed into this cell line and the colonies were allowed to grow for 16 h on the Amp/Kan/Chlor/Strep agar plates.

The concentrations of antibiotics used were: ampicillin 50 μg/mL, chloramphenicol 50 μg/mL, kanamycin 10 μg/mL and streptomycin 10 μg/mL.

4.4. Preparation of culture medium

10 g of Bacto™ Tryptone, 5 g of Bacto™ Yeast Extract and 5 g of NaCl was mixed in 1 L of DI water. 5 mL of culture medium was transferred to test tubes, 50 mL of culture medium was transferred to 250 mL Erlenmeyer flask and 1 L was transferred to 4 L flasks. The flasks were covered with aluminum foil and autoclaved.

4.5. Preparation of agar plates

Agar plates were prepared by mixing 15 g of Bacto™ Agar in one liter of culture medium and autoclaved. The medium was allowed to cool to about 50°C. Antibiotics were added and the medium was poured into Petri dishes, closed immediately, allowed to cool down and harden and stored at 4°C.

4.6. Transformation into BL21(DE3) and DH5α

E. coli BL21(DE3) or DH5α (Stratagene) competent cells were slowly unfrozen on ice and 50 μL aliquots were prepared. 1.5 μL of DNA (0.05-0.2 μg/μL) was added to the cells and incubated on ice for 30 minutes. The cells were immersed in a water bath set at 42°C for 45 seconds. The cells were then placed on ice for 2 minutes. 900 μL of cold LB medium was added to the cells and the cells were shaken for 1 h at 37°C. The cells were then plated on agarose plates containing the appropriate antibiotics and incubated at 37°C for 16 h.

4.7. Transformation into BL21(DE3) Codon Plus

E. coli BL21(DE3) Codon Plus™ RIL competent cells (Stratagene) were slowly unfrozen on ice and 50 μL aliquots were prepared. 1.5 μL of DNA (0.05-0.2 μg/μL) was added to the cells and incubated on ice for 30 minutes. Then the cells were immersed in a water bath set at 42°C for 20 seconds. The cells were then placed on ice for 2 minutes, 900 μL of preheated (42°C) LB medium was added to the cells and the cells were shaken for 1 h at 37°C. The cells were plated on agarose plates containing the appropriate antibiotics and incubated at 37°C for 16 h.

4.8. Preparation of competent cells

A single colony was used to inoculate 50 mL of LB medium containing the appropriate antibiotics. The culture was allowed to grow at 37°C until OD_{600} was 0.3-0.4. The culture was placed on ice for 5 minutes and the cell pellet was collected by centrifugation. The pellet was resuspended in 4 mL of frozen storage buffer (FSB) and incubated on ice for 20 minutes. Cell pellet was collected by centrifugation and resuspended in 3 mL of FSB. 100 μL aliquots were prepared and frozen on dry ice. Competent cells were stored at -80°C.

FSB (frozen storage buffer)

10 mM potassium acetate pH 6.2

100 mM KCl

50 mM $CaCl_2$

10% v/v glycerol

4.9. Preparation of glycerol stocks

50 mL of LB medium containing the appropriate antibiotic was inoculated with a single colony from a freshly grown plate. The cells were grown at 37°C for 12 h, then 12 mL of glycerol was added and the growth continued for 1 h. The cells were then aliquoted in 1 mL volumes and stored at -80°C. A single 1 mL aliquot can be slowly thawed and used to inoculate up to ten 50 mL cultures.

4.10. Over-expression of Rmt1 and Rmt1(Δ22)

50 mL of LB medium in a 250 mL flask containing 40 μg/mL of ampicilin was inoculated with 100 μL of the glycerol stock. The flasks were then grown for 6 h at 37°C for 6 h while shaking at 250 rpm. After the 6 h growth the cells were transferred to 1 L LB medium containing 40 μg/mL of ampicilin. The cells were shaken at 37°C at 250 rpm until the OD_{600} was 0.7. At his point 1 mL of 1M IPTG was added to induce the over expression of Rmt1. The cells were allowed to grow for an additional 4 h at 37°C while shaking at 250 rpm. The cells were harvested using centrifugation and stored at -20°C for future use.

4.11. Preparation of cell extract for GST purification

The cells were allowed to thaw on ice. 40 mL of HEMGT-250 buffer for every liter of cell growth and a protease inhibitor tablet were added. Cells were

disrupted with sonication. The resulting mixture was pelleted using centrifugation. The supernatant was stored and the pellet was resuspended in equal volume as in the first step and sonicated again. The mixture was pelleted using centrifugation. The supernatants were combined and the pellet was discarded. The supernatant was frozen in liquid nitrogen and stored at -80°C or used immediately.

4.12. Preparation of cell extract for Ni-NTA purification

The cell extract for Ni-NTA purification was prepared as described above, but with HMG buffer, where EDTA and Tween-20 were omitted.

4.13. GST purification (thrombin cleavage) of Rmt1

40 mL of cell extract was mixed with 3 mL of GST resin purchased from Sigma and allowed to shake for 2 h at 4°C. The beads were removed with centrifugation and washed with 40 mL of HEMGT-250. This washing procedure was repeated twice with HEMGT-250, once with HEMGT-500, once with HEMGT-1000 and twice with HEMGT-50. After the final wash 10 mL of HEMGT-50 and 20 units of thrombin were added to the beads. The mixture was incubated for 16 h at 4°C while shaking. After complete cleavage PMSF was added to inhibit thrombin. The cleaved protein was eluted off the beads and the beads were washed twice with 10 mL of HEMGT-50 for complete recovery of the protein. The resulting fractions were analyzed with SDS-PAGE and the Bradford assay. Using this procedure 4.5 mg of cleaved Rmt1 can be isolated from 1 L of cell growth.

BUFFERS:

HEMGT-250

25 mM Hepes pH 7.9
2 mM EDTA
12.5 mM $MgCl_2$
10% Glycerol
0.1 % v/v Tween-20
3 mM DTT
250 mM KCl

HEMGT-100

Same as the above but 100 mM KCl

HEMGT-1000

Same as the above but 1 M KCl

HEMGT-50

12.5 mM Hepes pH 7.9
1 mM EDTA
6.25 mM $MgCl_2$
5% Glycerol
0.05 % v/v Tween-20
1.5 mM DTT
50 mM KCl

4.14. GST purification (glutathione elution) of Rmt1

The GST-Rmt1 fusion peptide was bound to the GST resin and washed as described above. After the wash with HEMGT-50, the glutathione elution buffer (HEMGT-50, 10 mM glutathione pH 7.9) was added and the mixture was incubated for 30 min while shaking. The fractions were eluted and beads washed with glutathione elution buffer for complete elution of the protein. Using this procedure approximately 10 mg of GST-Rmt1 fusion peptide can be isolated.

4.15. GST purification of mSNAPc

40 mL of cell extract was mixed with 5 mL of GST resin purchased from Sigma and allowed to shake for 12 h at 4°C. The beads were removed and washed by centrifugation with 40 mL of HEMGT-250. This washing procedure was repeated twice with HEMGT-250, once with HEMGT-500 and again once with HEMGT-250. The beads were then transferred to a column equipped with a frit and washed with HEMGT-250 until the concentration of the protein material in the wash was below 0.005 as measured with the Bradford assay. After the final wash 10 mL of HEMGT-250 and 20 units of thrombin were added to the beads. The mixture was incubated for 16 h at 4°C while shaking. After complete cleavage PMSF was added to inhibit thrombin. The cleaved protein was eluted off the beads and the beads were washed twice with 10 mL of HEMGT-250 for complete recovery of the protein. The resulting fractions were analyzed with SDS-PAGE and the Bradford assay.

4.16. FPLC ion exchange chromatography

Ion-exchange chromatography was performed on a Biotech Pharmacia FPLC system. Source® 15Q or Source 15S ion-exchange beads were used in Omnifit 5/10 column.

Buffer A (low salt buffer)

10 mM Tris pH 7.9

50 mM NaCl

3 mM DTT

Buffer B (high salt)

10 mM Tris pH 7.9

1 M NaCl

3 mM DTT

4.17. Size exclusion gel filtration

Size exclusion gel filtration was performed on Biotech Pharmacia FPLC system equipped with XK19 HiLoad® 16/60 column, packed with Superdex® 200 size exclusion beads.

Gel filtration buffer

10 mM Tris pH 7.9

50 mM NaCl

3 mM DTT

4.18. Bradford protein assay

The Bradford Solution® was diluted with water to prepare a 20 % solution. For simple concentration measurements 20 μL of sample solution was added to 1 mL of prepared Bradford mixture, mixed and the absorbance at 600 nm was measured. The concentration of all protein material is the value of OD_{600} in mg/mL. For more concentrated samples, smaller amounts of sample solution were added so that the OD_{600} does not exceed 0.6. The concentration of the total protein material present in the sample can be calculated by the following formula obtained from a standard curve.

$$c(mg/ml) = \frac{V(\mu L) \bullet OD_{600}}{20\mu L}$$

4.19. SDS-PAGE

SDS-PAGE analysis was performed as described in the Biorad Protean Mini instruction manual. The separating (resolving) gel was prepared by mixing the required reagents (Table 1) adding 50μL of 10% ammonium persulfate and 5μL of TEMED and the mixture was wortexed for 30 s and poured into the gel cassette to about 1 cm from the top of the cassette. The gel solution was overlaid with t-butyl alcohol and allowed to polymerize (5-15 min). The overlaying alcohol was removed and the gel surface on the top of the cassette was wiped dry with filter paper. The stacking gel solution (6% gel) was prepared and the gel cassette was filled to the top with this solution. The well comb was inserted in the top portion of the cassette and the gel was allowed to polymerase (10-15 min).

Gel percentage	ddH_2O (mL)	30% Degassed Acrylamide (mL)	Gel Buffer (mL)	10% w/v SDS (mL)
6%	6.2	1.2	2.5	0.1
9%	4.4	3.0	2.5	0.1
12%	3.4	4.0	2.5	0.1
17%	1.7	5.7	2.5	0.1
20%	0	7.4	2.5	0.1

Table 1: SDS-PAGE gel formulation

After complete polymerization of the gel the gel cassette was installed into the electrophoresis apparatus. The electrode chamber was filled with electrophoresis running buffer. The wells were loaded with 1-15 μL of sample and the gel was allowed to run at a constant voltage of 220 V for 40-60 min. After

the tracing line reached the bottom of the gel the gel was removed from the cassette and placed in the Commassie staining solution. This was then microwaved for 30 s and allowed to shake for one additional minute. The gel was removed from the staining solution and placed into destaining solution. This was microwaved for 30 s and allowed to shake until the gel was destained (20 min – 2 h). After the gels were destained, they were rinsed with water and soaked in 5% v/v glycerol for 1 h. Gels were then placed between two cellophane sheets and allowed to dry for 24 h.

5. References

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