universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Recombinant expression, purification and quaternary structure of human RNase Z^S, RNase Z^L and tRNA ribonucleotidyl transferase

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer. nat.)

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List of abbreviations

APS:	ammonium persulfate
ATP:	adenosine 5'-triphosphate
BS^3:	suberic acid bis(3-sulfo-N-hydroxysuccinimide ester)
BSA:	bovine serum albumin
CTP:	cytidine 5’-triphosphate
DTT:	dithiothreitol (threo-1,4-dimercaptobutane-2,3-diol)
E. coli:	Escherichia coli
EDC:	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide
EDTA:	ethylenedinitrilotetraacetic acid
GMP:	guanosine 5’-monophosphate
GTP:	guanosine 5’-triphosphate
HEPES:	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IMAC:	immobilized metal ion affinity chromatography
IPTG:	isopropyl β-D-1-thiogalactopyranoside
LB:	Luria-Bertani broth
LPA:	linear polyacrylamide
MCS:	multiple cloning site
mRNA:	messenger RNA
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PEG:	polyethylene glycol
RNA:	ribonucleic acid
rbs:	ribosome binding site
RSB:	reticulocyte solubilization buffer
SDS:	sodium dodecyl sulfate
SEC:	size exclusion chromatography
Sulfo-NHS:	N-hydroxysulfosuccinimide
TBE:	Tris-Borate-EDTA buffer
TEMED:	N,N,N′,N′-Tetramethylethylenediamine
Tricine:	N-[Tris(hydroxymethyl)methyl]glycine

Tris: 2-amino-2-(hydroxymethyl)propane-1,3-diol

TritonX-100: octylphenol ethylene oxide condensate

tRNA: transfer RNA

Tween-20: polyoxyethylenesorbitan monolaurate

UTP: uridine 5’-triphosphate

Objectives

The primary objective of this diploma thesis was the heterologous, recombinant expression of two human enzymes and their purification. Recombinant RNase Z^S and RNase Z^L had to be expressed in Escherichia coli and purified to near homogeneity in order to use them as parts of an in vitro tRNA-processing system for the study of pathogenic mutations of mitochondrial tRNAs. Furthermore, the human enzyme tRNA ribonucleotidyl transferase had to be produced in the same way and for the same purpose.

A secondary objective was the analysis of the quaternary structure of the RNase Z isoforms. Experiments on purified RNase Z^L should show, if this enzyme is active as monomer or homodimer.

Ziele

Primäres Ziel dieser Diplomarbeit war die heterologe Expression zweier rekombinanter, humaner Enzyme und deren Reinigung. Rekombinante RNase Z^S und RNase Z^L sollten in Escherichia coli exprimiert und fast bis zur Homogenität gereinigt werden, um sie als Teile eines in vitro tRNA-Prozessierungssystems zu verwenden, mit welchem pathogene Mutationen mitochondrialer tRNAs erforscht werden sollen. Darüber hinaus sollte das humane Enzym tRNA-Ribonukleotidyltransferase auf dieselbe Weise und zum selben Zweck produziert werden.

Sekundäres Ziel war die Analyse der Quartärstruktur der beiden RNase Z-Isoformen. Experimente an gereinigter RNase Z^L sollten zeigen, ob dieses Enzym als Monomer oder Homodimer aktiv ist.

Introduction to tRNA-processing

Structure and Function of tRNAs

In all organisms the process of ribosomal protein synthesis requires transfer RNA (tRNA) molecules as adaptors between messenger RNAs (mRNA) and peptide bond formation. A tRNA is specific for one type of amino acid, which is covalently linked to its 3' end. Within a loop on the opposite side the tRNA contains the anticodon: three nucleotides, whose sequences are specific for the amino acid. The anticodon enables the tRNA to recognize the complementary codon on the mRNA by means of base pairing within the ribosome. As a consequence the amino acid is able to perform a nucleophilic attack on the activated carboxyl, which connects the growing polypeptide chain with the previous tRNA, thus forming a peptide bond through the action of the ribosome. Then the previous tRNA is released from the ribosome and can be reloaded with an amino acid by its aminoacyl tRNA synthetase.

Transfer RNAs are small RNA molecules, whose length is between 50 and 105 bp (usually about 80 bp). Intramolecular base pairing results in the typical secondary structure, as shown below for an aminoacylated phenylalanine tRNA (Figure 1A). The secondary structure comprises the 5' end, the D loop, the anticodon loop, the variable V loop, the T loop, also called "TΨC loop", and the 3' end or "acceptor end" with the terminal, invariant CCA sequence following the so called "discriminator nucleotide", in a typical clover leaf structure. The tertiary structure shows a compact, L-shaped tRNA in two different orientations (Figure 1B and 1C). The anticodon loop is on the long end of the L, whereas the acceptor stem is on the short end of the L. In contrast to the secondary structure the T and D loop are rather close to each other in the tertiary structure. Below the outlines of the secondary and tertiary structure the primary structure is shown for this phenylalanyl tRNA (Figure 1D). The primary structure also gives a good survey of the many modified nucleotides in tRNAs.

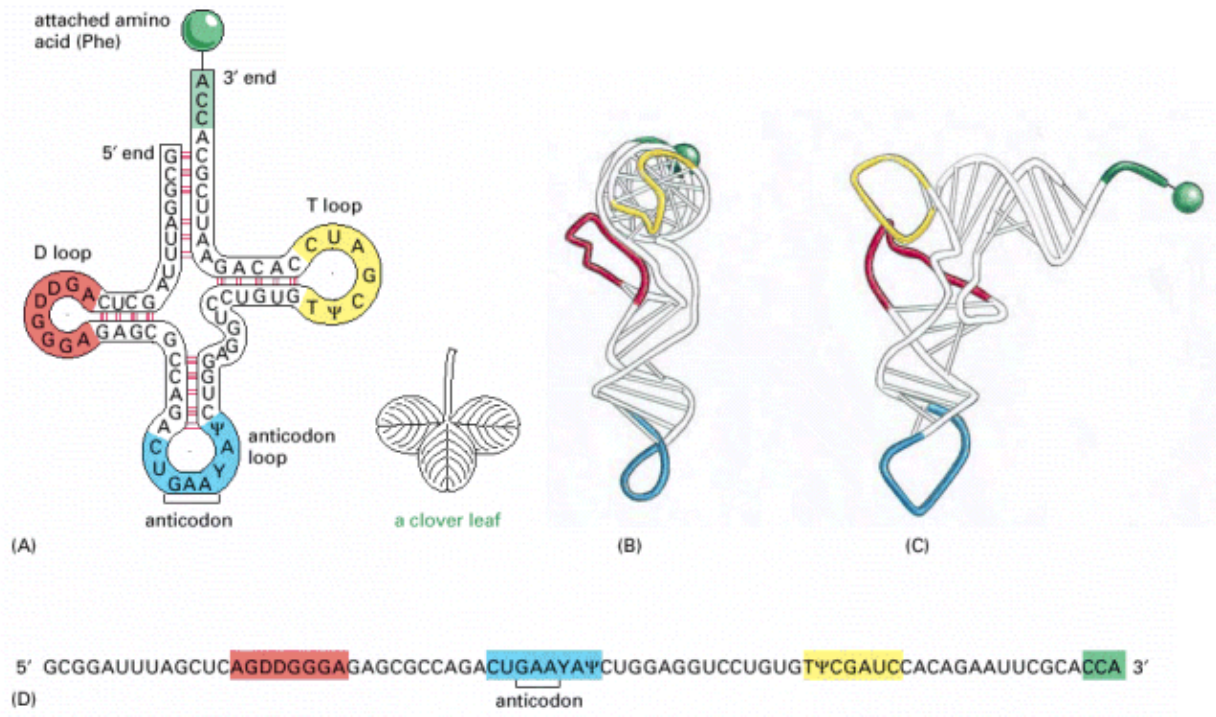

Figure 1. Primary (D), secondary (A) and tertiary (B and C) structure of a tRNA. Posttranscriptionally modified nucleotides: pseudouridine ("Ψ"), dihydrouridine ("D"), wyosine ("Y") and ribothymidine ("T"). See text for further details and comments.
(taken from http://www.ncbi.nlm.nih.gov/books/bookres.fcgi/mboc4/ch6f52.gif)

Biosynthesis of tRNAs

Transfer RNAs are transcribed as precursors: they contain 5' leader and 3' trailer sequences. In order to get functional tRNAs these leaders and trailers have to be removed and the 3' terminal CCA has to be added in those organisms, which lack a genetically encoded CCA (all eukaryotes, many bacteria and some archaea). First of all the 5' leader sequence is removed by an endonuclease followed by the removal of the 3' trailer by an endonuclease or by endo- and exonucleases. Next the terminal CCA sequence is added by another enzyme in organisms, whose tRNA genes do not genetically encode the CCA. All these enzymes recognize tRNAs by their tertiary structure and by interacting only with their sugar-phosphate backbones. Since these enzymes do not bind tRNAs in a sequence-specific way, they work on every tRNA in one organism. Then introns are spliced, which occurs in some tRNAs of most eukaryotes and some archaea. Finally many nucleotides are chemically modified ("posttranscriptionally modified nucleotides") (Hopper and Phizicky 2003).

Generation of mature 5' ends

After transcription all tRNAs contain 5' leader and 3' trailer sequences, which have to be removed in order to obtain functional tRNAs. First the 5' leader sequence is

removed by an enzyme called RNase P, which has been found in organisms from all three domains of life. This enzyme consists of one to ten protein subunits (1 in bacteria, 4 in archaea, 9 or 10 in eukarya) and an RNA. Therefore RNase P is a ribonucleoprotein and the RNA component shows - at least in vitro - basic catalytic activity (Willkomm and Hartmann 2007) (Kikovska, Svärd et al. 2007). However, it has been demonstrated recently that at least human mitochondrial RNase P does not need a trans-acting RNA in order to perform its catalysis and that this enzyme is composed of protein only (Holzmann, Frank et al. 2008).

RNase P is an endonuclease dependent on divalent metal ions. Its catalytic activity is the hydrolysis of phosphodiester bonds in precursor tRNAs leaving a phosphate on the tRNA, thus creating mature 5' ends by removing the 5' leader sequence (Figure 2) (Kazantsev and Pace 2006).

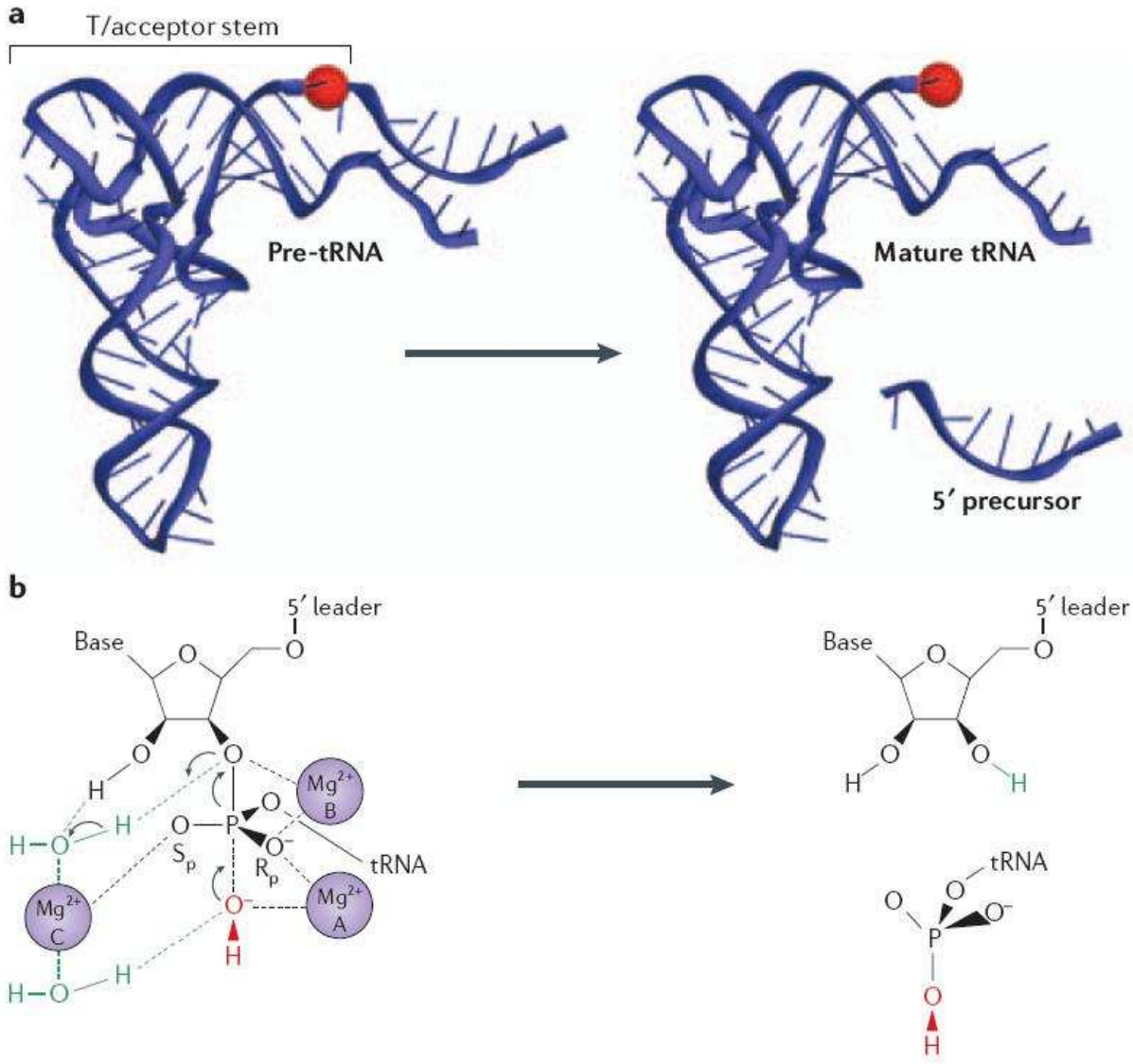

Figure 2. Catalytic activity of RNase P. Removal of 5' leader sequence from pre-tRNA (a) and mechanism of hydrolytic cleavage reaction (b).
(taken from (Kazantsev and Pace 2006))

Generation of mature 3' ends

The next step to gain functional tRNA is the removal of the 3' trailer. Among the organisms studied so far there are two pathways to achieve this: in the "exonucleolytic" pathway the precursor-tRNA is endonucleolytically cut downstream and trimmed to its mature 3' end by exonucleases. Only a single endonucleolytic cut is needed in the "endonucleolytic" pathway, which is then performed by an enzyme called RNase Z (Figure 3). Both pathways sometimes coexist in organisms: apparently they serve as backup systems for each other and/or involved enzymes have other functions as well (Redko, Li de la Sierra-Gallay et al. 2007).

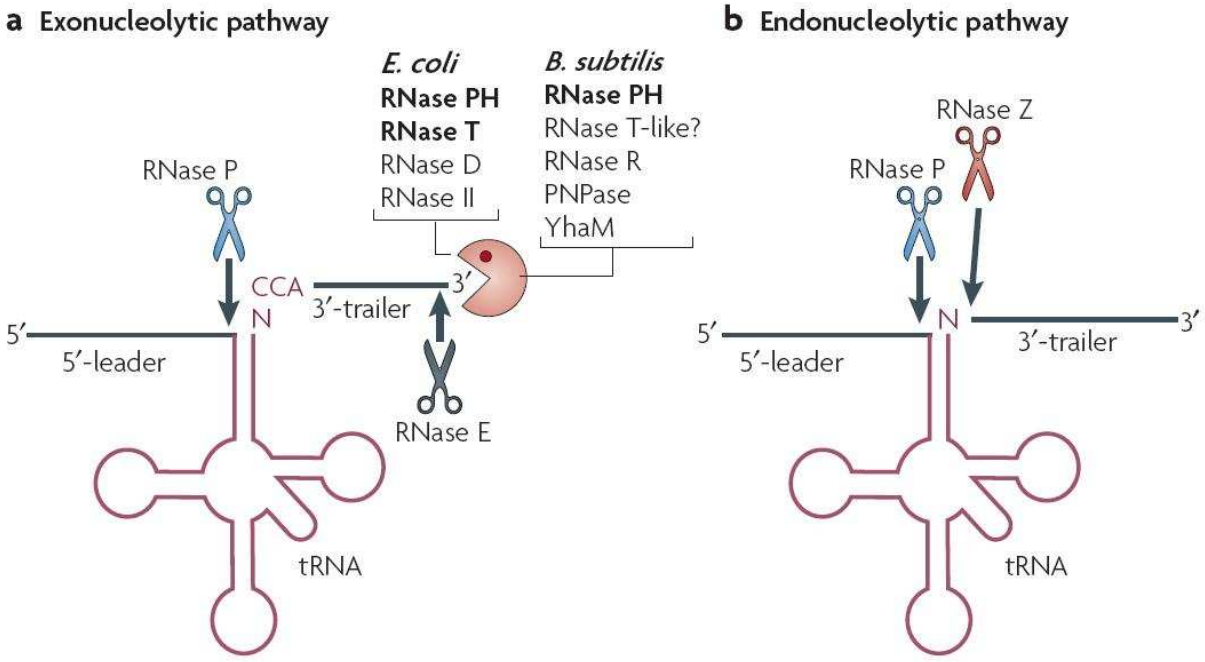

Figure 3. Exonucleolytic (a) and endonucleolytic (b) pathway for creating mature 3' ends of tRNAs. Endonucleases are shown as scissors, whereas exonucleases are depicted by a "pacman" symbol. (taken from (Redko, Li de la Sierra-Gallay et al. 2007))

Many bacteria as well as all archaea and eukaryotes sequenced so far contain at least one copy of an orthologous RNase Z gene (Späth, Canino et al. 2007). This enzyme belongs to the family of zinc-dependent metallo-hydrolases, which is part of the β-lactamase superfamily (Redko, Li de la Sierra-Gallay et al. 2007). There are two isoforms of RNase Z: a long one of 750 to 930 amino acids depending on the organism, called RNase Z^L, which can only be found in eukaryotes, and a short one between 280 and 360 amino acids, called RNase Z^S, which exists in organisms of all three domains of life. Based on structural studies it is assumed that the long form originated from the short form by gene duplication, hence that RNase Z^S and RNase Z^L are paralogues (Späth, Canino et al. 2007).

Typical structural features of RNase Z are (Figure 4): the “His motif” or “HxHxDH motif” is a conserved sequence motif serving for metal coordination in the active site. The “flexible arm” or “exosite” participates in binding of substrate but is outside the active site. Other features are targeting signals for mitochondria or chloroplasts and the “pseudo His-motif” or “Ψ-His-motif”, a degenerated His-motif without the ability to coordinate metal ions, present only in eukaryal RNase Z^L (Vogel, Schilling et al. 2005).

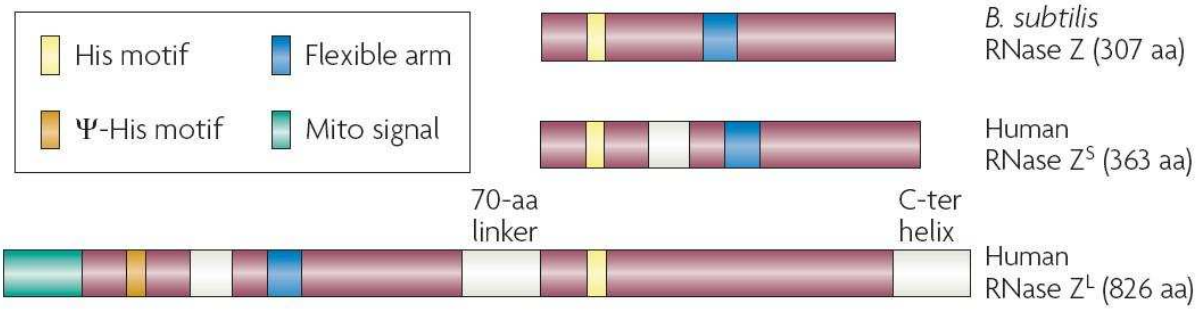

Figure 4. Domains of RNase Z. Abbrevations explained above except for “Mito signal”, which is a mitochondrial targeting signal.
(taken from (Redko, Li de la Sierra-Gallay et al. 2007))

The crystal structures of RNase Z^S enzymes from Bacillus subtilis, Escherichia coli and Thermotoga maritima form homodimers, which is therefore assumed to be true for all other orthologous RNase Z^S enzymes (Ceballos and Vioque 2007). RNase Z^L, on the other hand, is generally assumed to be a monomer due to its domain structure, which seems to resemble a covalently linked dimer (Figure 4). However, no experimental data have been published so far concerning the quaternary structure of RNase Z^L.

RNase Z is an endonuclease, which catalyses cleavage of the 3’ trailer from the precursor tRNA by hydrolysis of a phosphodiester bond. A 5’ phosphate is left on the trailer and a 3’-hydroxyl group remains on the tRNA (Vogel, Schilling et al. 2005). The proposed mechanism for this hydrolysis comprises two initial steps (Figure 5): firstly, the phosphate group, which connects the last nucleotide of the tRNA with the first nucleotide of the 3’ trailer, is polarized by two zinc ions (as for RNase Z^S from Bacillus subtilis). Secondly, a water molecule (“water147” in Figure 5c) is deprotonated by an aspartic acid residue (“D67”). As a result, this hydroxyl ion performs a nucleophilic attack on the polarized phosphate, whereupon a proton is regained from a nearby located water molecule (“wat5”) (Li de la Sierra-Gallay, Pellegrini et al. 2005).

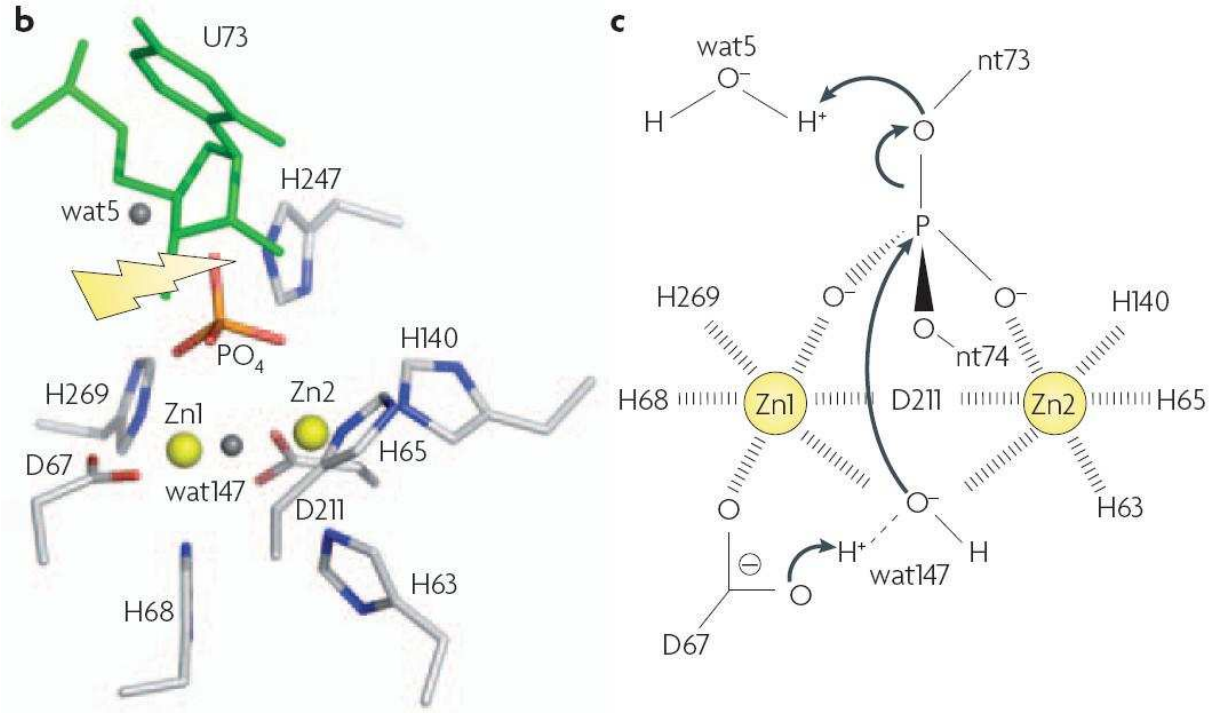

Figure 5. Active site (b) and mechanism of hydrolytic cleavage reaction (c) of RNase Z^S (B. subtilis). Nitrogen atoms of histidine residues in blue, oxygen atoms of aspartate in red. Zn ions depicted as yellow spheres, water molecules as grey spheres. tRNA shown in green.
(taken from (Redko, Li de la Sierra-Gallay et al. 2007))

If a tRNA is processed by RNase Z (endonucleolytic pathway) and if its terminal CCA is not genetically encoded, cleavage will usually occur 3' to the discriminator nucleotide and the CCA will have to be added by an enzyme called tRNA nucleotidyl transferase. In Homo sapiens and many other organisms maturation of 3' ends of tRNAs is believed to work that way (RNase Z + tRNA ribonucleotidyl transferase).

Aside from processing tRNAs there are other functions assumed for RNase Z, some of which have been discovered in several organisms. There are three reasons for this assumption: firstly, RNase Z is present in Escherichia coli, whose tRNAs are processed by the exonucleolytic pathway. Secondly, RNase Z is essential in Saccharomyces cerevisiae, although its tRNA maturation can be backed up by the exonucleolytic pathway. Thirdly, RNase Z has been shown to cleave other substrates as well in vitro (Vogel, Schilling et al. 2005).

Mutations in the gene for human RNase Z^L (called "*ELAC2*", whereas the gene for RNase Z^S is called "*ELAC1*") located at chromosome 17p are suspected to be linked to prostate cancer in humans. Therefore, *ELAC2* is known as "candidate prostate cancer susceptibility gene" (Takaku, Minagawa et al. 2003).

Addition of terminal CCA

All tRNAs have the same invariant sequence at their 3' end on the acceptor stem: CCA. These three nucleotides can either be encoded genetically or added post-transcriptionally by an enzyme called tRNA ribonucleotidyl transferase (name of corresponding human gene: "*TRNT1*"). This enzyme is also responsible for repair of the CCA-sequence and can therefore be found in all organisms (Augustin, Reichert et al. 2003).

In contrast to other polymerases tRNA ribonucleotidyl transferases, also called CCA-adding enzymes or CCases, do not use nucleic acid templates to incorporate the correct nucleotides, but mimic the normal Watson-Crick hydrogen bonding in two different ways: Archaeal class I CCA-adding enzymes use a combination of hydrogen bonds from amino acid sidechains and backbone phosphate groups of the tRNA to select the right nucleotide. Bacterial and eukaryal class II CCA-adding enzymes, on the other hand, perform this task solely by protein templating, which means hydrogen bonding with amino acid residues of the enzyme (Xiong and Steitz 2006).

Human tRNA ribonucleotidyl transferase has a remarkable quaternary structure: it seems to form homodimers covalently linked by an intermolecular disulfide bond (Augustin, Reichert et al. 2003).

Technical introduction

Expression via pET-System

T7 RNA polymerase

The pET-System (Novagen) is used for heterologous expression of recombinant proteins in Escherichia coli. This system is a so called “T7 expression system”, which means that the actual transcription of recombinant proteins is performed by T7 RNA polymerase. This enzyme originates from bacteriophage T7 and its advantages include high activity (~200 nucleotides per second at 37°C), selectivity for its own promoter (“T7 promoter”) without recognition of other promoters and having only one subunit of about 99 kDa, hence one gene.

Regulation of target gene expression

In order to start transcription and, as a result, translation of the recombinant protein, a source of T7 RNA polymerase and an appropriate T7 promoter in front of the actual target gene on the expression vector are necessary. The gene for T7 RNA polymerase is usually integrated in the chromosome of an appropriate expression host in a region derived from DE3 (Figure 6). This bacteriophage is a lambda derivative comprising the gene for the lac-repressor (“lac I”) and the gene for T7 RNA polymerase under the control of lacUV5 promoter. Therefore, such hosts are referred to as “DE3 lysogens”, abbreviated as “(DE3)”. The lacUV5 promoter contains the lac-operator sequence, which serves as binding site for the lac-repressor. Quantitative transcription of T7 RNA polymerase is prevented by binding of repressor to operator, thus hampering bacterial RNA polymerase. Due to the ability of IPTG to bind to the lac-repressor, thereby inducing a conformational change, which keeps the repressor from binding to the lac-operator, transcription of T7 RNA polymerase is enabled by simply adding IPTG (Figure 6).

As mentioned before, the target gene is under the control of a “T7lac promoter”, which is not only recognized by the T7 RNA polymerase but also contains a lac-operator sequence to prevent transcription by basal levels of T7 RNA polymerase in the absence of IPTG.

In addition to these control mechanisms there are some vectors in the pET series, which offer more stringent control of expression and some, which do not even have a

lac-operator in front of the target gene (“plain” T7 promoter). While a less tight control promotes protein production, more stringent regulation will protect Escherichia coli from possible toxic effects of recombinant proteins during growth. Therefore, the toxicity of recombinant protein has to be considered before choosing a pET expression vector on the basis of its control mechanism.

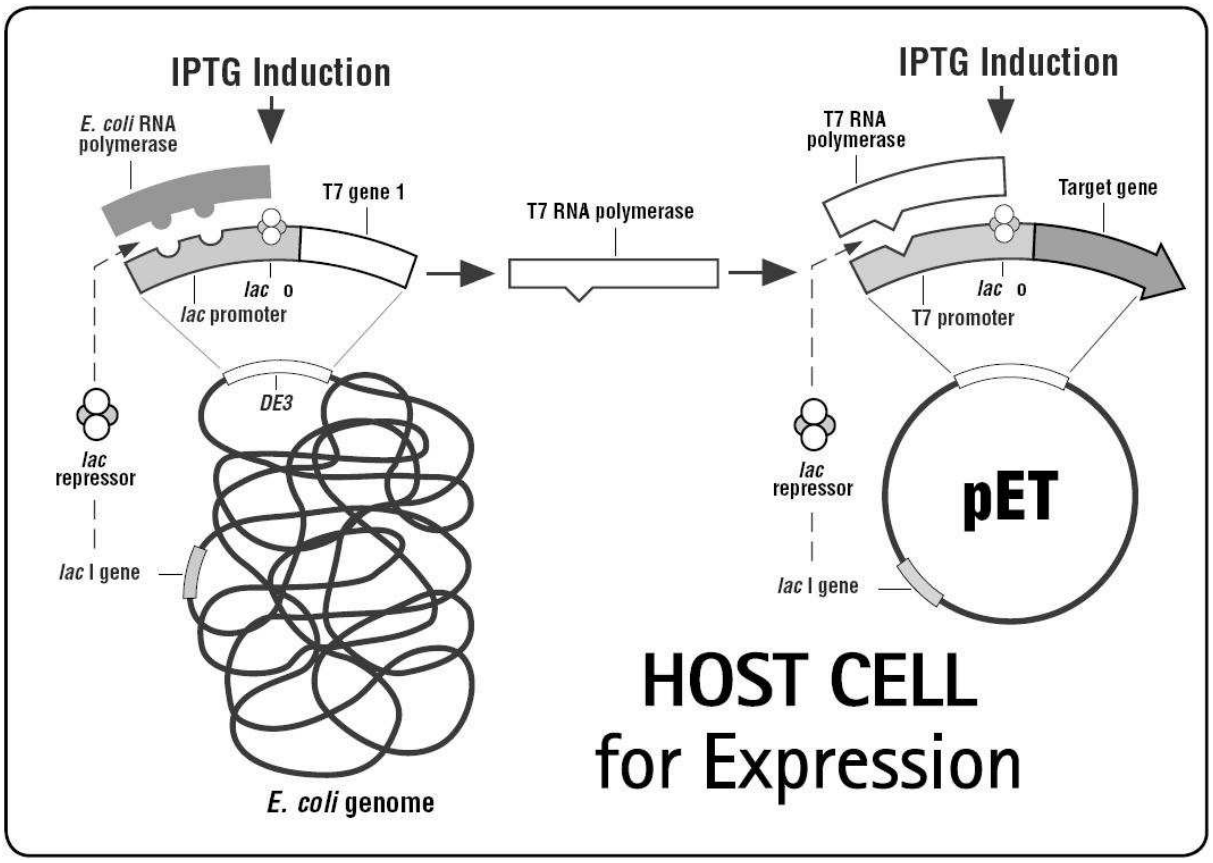

Figure 6. IPTG inducible T7 expression system using a vector with T7lac promoter in front of the target gene. Lac-operator abbreviated as “lac o”. See text for details and comments.
(taken from pET System manual (Novagen) with small modifications)

Fusion tags

In general, fusion tags are artificial polypeptides or natural proteins fused to proteins of interest in order to facilitate purification of these recombinant proteins by affinity chromatography, to enhance solubility, promote disulfide bond formation, allow detection via Western blot or even enable the export into the periplasm. Normally their sequences are fused to 5’ or 3’ ends of target genes resulting in N- or C-terminal tags, but internal tags are possible as well. After purification many tags can be removed from the recombinant protein by special proteases. Since all pET expression vectors encode one or more N- or C-terminal fusion tags, the need for one or the other tag should be considered carefully, when choosing a vector.

pET expression vector

The Novagen pET System Manual distinguishes between transcription and translation vectors. Transcription vectors lack the sequence of a ribosome binding site in contrast to translation vectors, which encode the ribosome binding site from a gene of phage T7.

Other general features of an expression vector are: origin of replication, selective marker (usually an antibiotic resistance gene) and multiple cloning site (contains multiple restriction sites), as can be seen in Figure 7. As mentioned below in the section “Materials and Methods”, pET21b(+) was used as expression vector and therefore the vector map of the pET21 series is shown. This vector includes, apart from all the basic features as described above, an f1 origin for generation of single stranded plasmid DNA upon infection with a helper phage and the gene lac I, which codes for the lac-repressor.

The solid black arrow in Figure 7 represents the part of the vector, where the target gene should be inserted and which is transcribed by T7 RNA polymerase upon induction by addition of IPTG. This region, called “cloning/expression region”, is shown in more detail in Figure 8 with all the features necessary for cloning (MCS), regulation (lac-operator as part of the “T7lac promoter”), transcription (T7 promoter, T7 terminator) and translation (ribosome binding site) as well as the tags (T7-tag, His-tag). It should be considered that the T7-tag can be removed from any pET21 with the aid of Nde I (or Nhe I) and a second restriction endonuclease cutting in the MCS for cloning a gene into the vector.

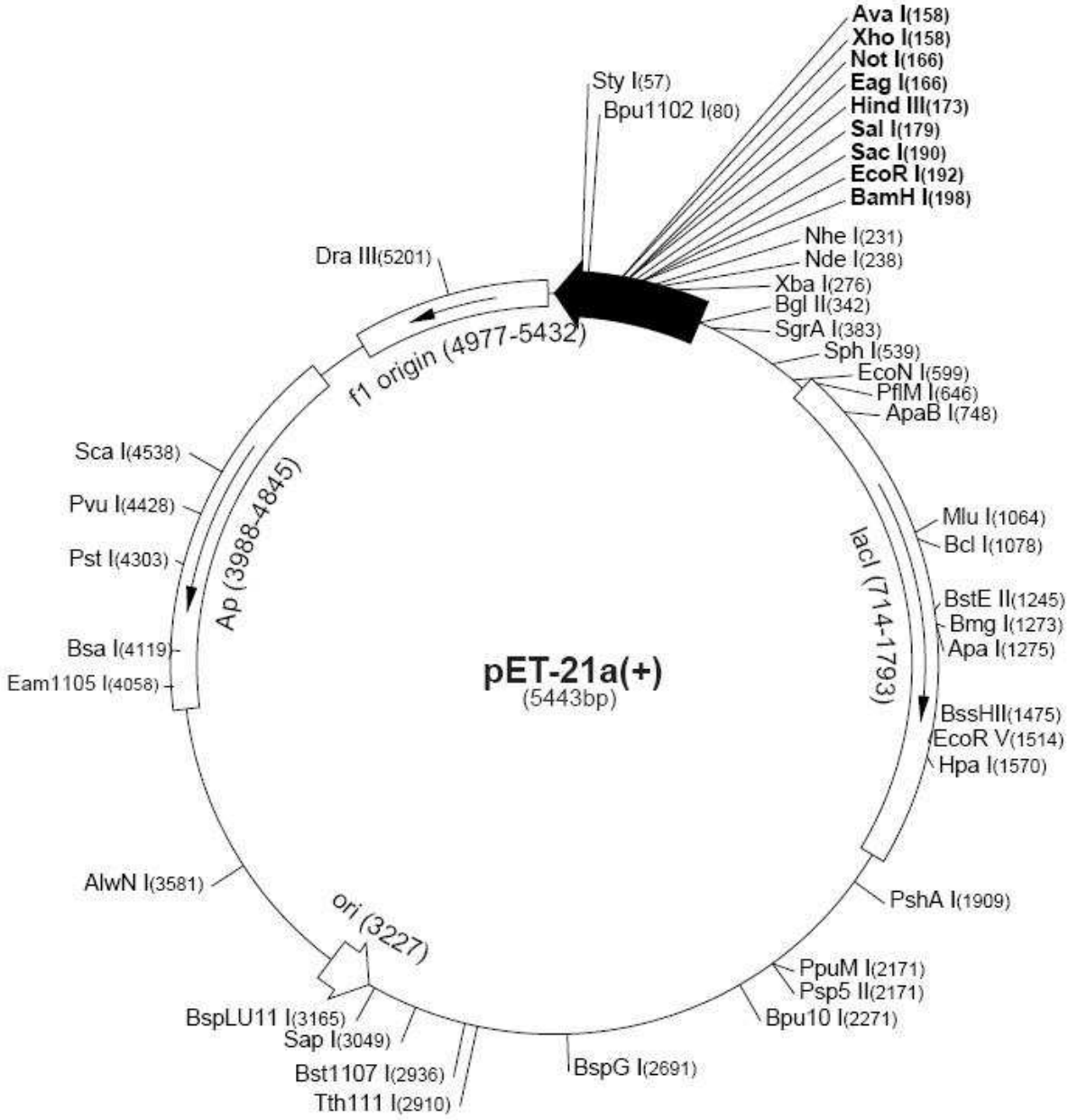

Figure 7. Vector map of pET21a(+). According to Novagen 1bp needs to be subtracted from each site beyond BamHI at 198 to get the correct map of pET21b(+), which is 5442bp in length. See text for details and comments.
(taken from http://www.emdbiosciences.com/html/NVG/home.html)

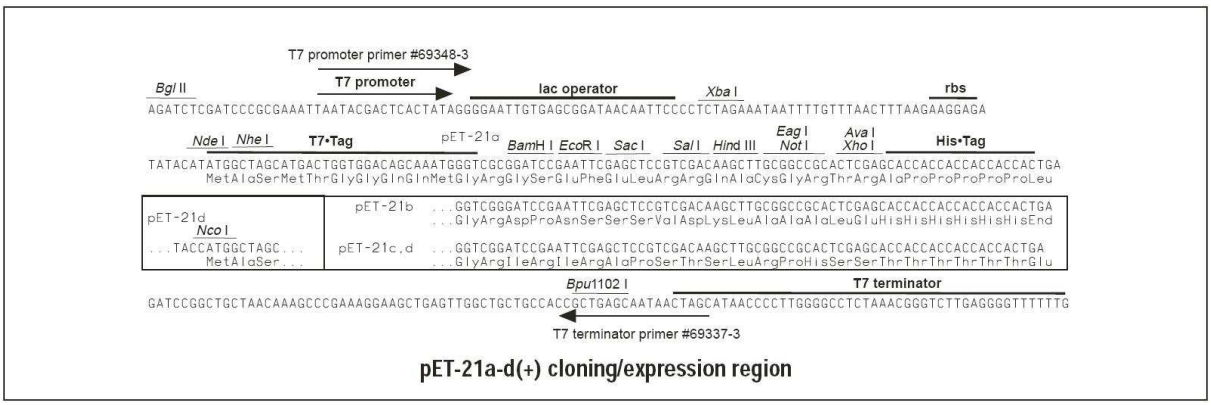

Figure 8. Cloning/expression region of pET21a-d(+).
(taken from http://www.emdbiosciences.com/html/NVG/home.html)

Expression via IMPACT system

IMPACT (Intein mediated purification with an affinity chitin-binding tag, New England Biolabs) is a T7 expression system (see above), which utilizes a special tag. This tag consists of a chitin binding domain, which is used for affinity purification on a chitin column, and an intein domain (from Saccharomyces cerevisiae), whose self-cleavage activity upon induction by addition of thiols is used to elute the purified target protein from the column (Figure 9).

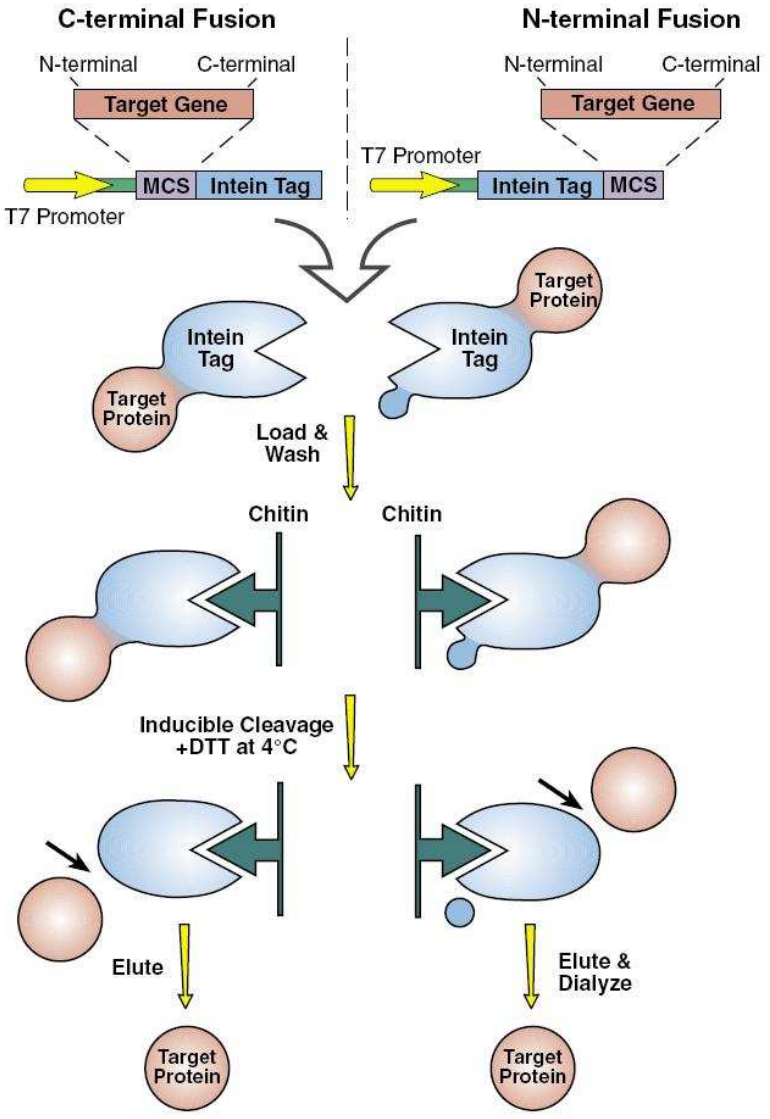

Figure 9. IMPACT system. Affinity chromatography and differences between C- and N-terminal fusion tags therein are shown. See text for details and comments.
(taken from IMPACT™-CN instruction manual, version 2.1, New England Biolabs)

There are four vectors available: two coding for C- and two coding for N-terminal fusion tags. One of each kind gives rise to recombinant proteins, which contain additional amino acids between protein and intein tag for more efficient cleavage, whereas the others lack these additional nucleotides. If an N-terminal fusion tag is

chosen, a small (~1.6 kDa) secondary cleavage product will be eluted in the course of affinity chromatography. The lack of additional amino acids without subsequent treatment with proteases combined with a one-step purification procedure is unique and beneficial, if purified native recombinant protein is required.

Purification via IMAC

Immobilized metal ion affinity chromatography ("IMAC") is a kind of affinity chromatography, which uses the affinity of amino acids to metal ions for separation purposes. Since histidines have a high affinity towards divalent metal cations, immobilized Ni^{2+} (or other divalent cations) can be used to bind histidine-tagged recombinant proteins, thus retaining them (Figure 10). These divalent metal cations are immobilized by chelating groups, which are coupled to the matrix (e.g. sepharose). Beads of this material are used as stationary phase in the course of affinity chromatography and chelated divalent cations are able to bind histidine-tagged recombinant proteins (in the liquid mobile phase) by coordinate binding. Usually imidazole is used in excessive amounts for competitive elution: This molecule is able to coordinate the divalent metal cation just like the histidines, therefore replacing them, hence the whole protein.

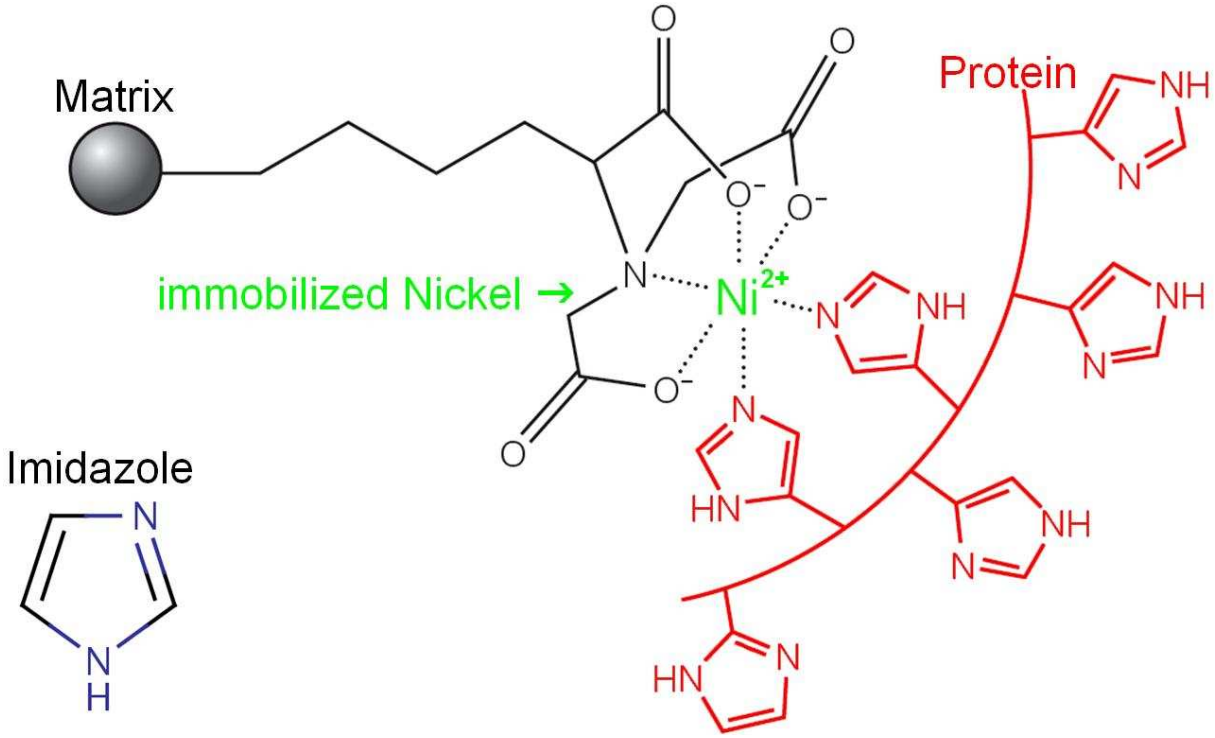

Figure 10. Immobilized metal ion affinity chromatography with hexahistidine-tagged protein. The eluent imidazole displaces the histidines in a competitive manner.
(imidazole taken from http://www.bmrb.wisc.edu/metabolomics/standards/imidazole/lit/4744.png)

Analysis of quarternary structure

Size exclusion chromatography

Size exclusion chromatography is a chromatographic method, which separates molecules according to their size. When an aqueous solution is used as mobile phase, it is sometimes called “gel filtration chromatography”.

The stationary phase consists of small polymer beads with many pores of different sizes and the molecules to be separated are dissolved in the liquid mobile phase. Whereas larger molecules can enter only large pores or none at all, if they are too big, smaller molecules are able to enter more pores or all, if they are too small. Therefore, small molecules have a longer distance to cover, thus elute later than larger ones (Figure 11).

If gel filtration chromatography is performed with proteins under physiological conditions, they will elute according to their oligomeric state, whereupon monomers can be distinguished from dimers or oligomers.

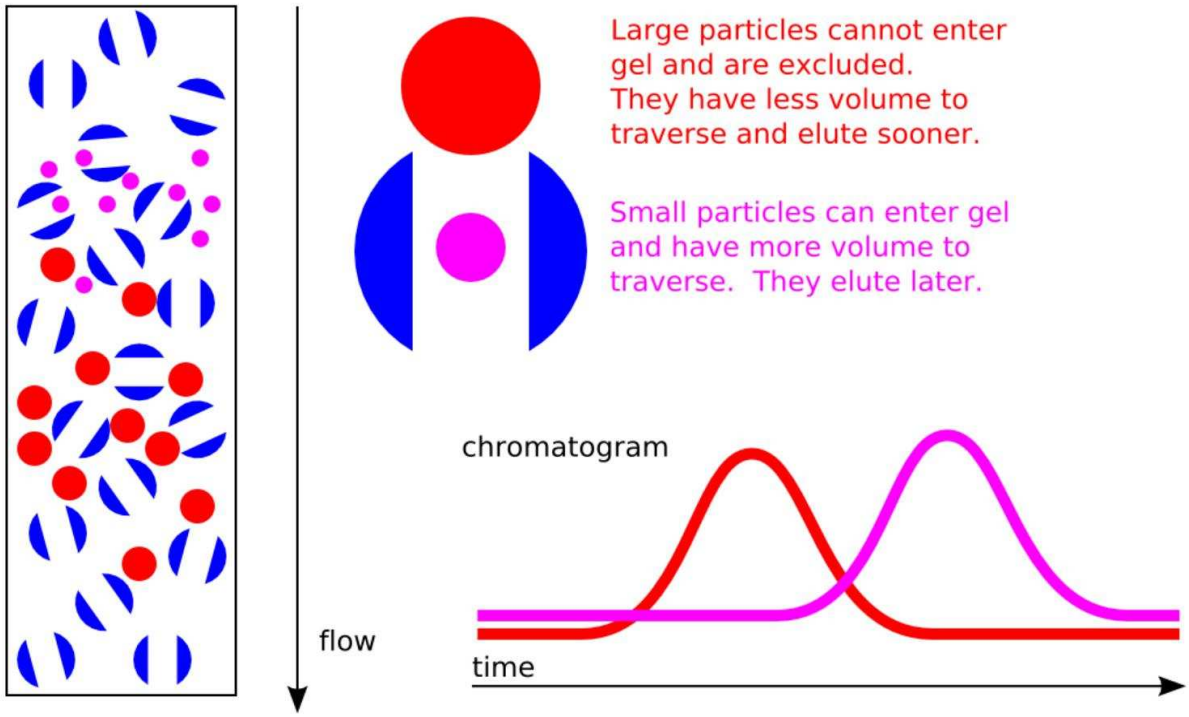

Figure 11. Separation mechanism of size exclusion chromatography. (taken from http://en.wikipedia.org/wiki/Image:SizeExChrom.png)

Glycerol gradient centrifugation

Glycerol gradient centrifugation (a kind of “rate zonal centrifugation”) is a method for determining the molecular mass or “size” of a molecule. A glycerol gradient is set up in a centrifugation tube and the sample (mixture of molecules with different molecular

masses) is added on top. After centrifugation molecules will be separated according to their molecular mass and density (Figure 12).

If glycerol gradient centrifugation is performed with proteins under physiological conditions, they will sediment according to their oligomeric state, whereupon monomers can be distinguished from dimers or oligomers.

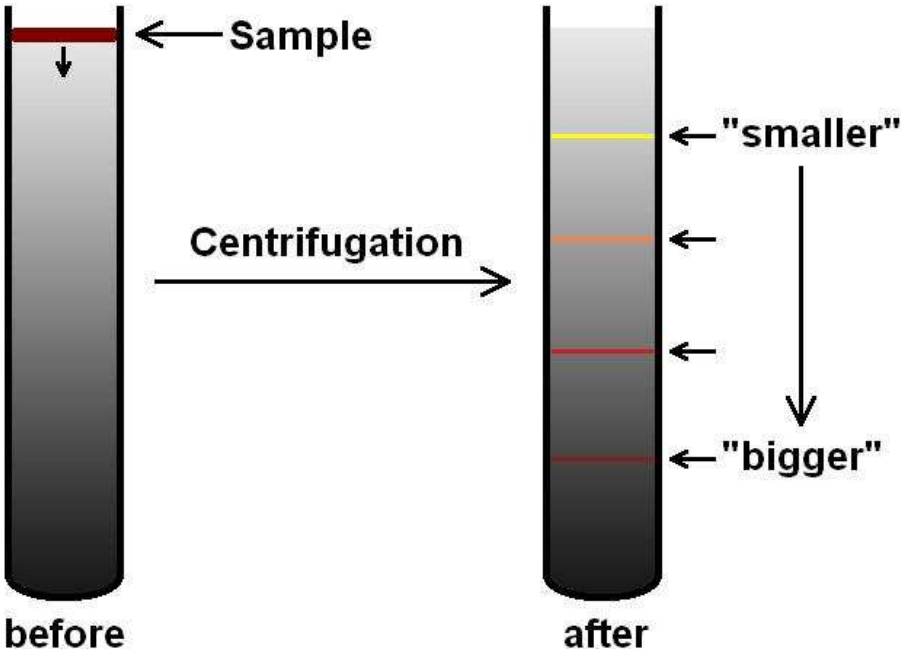

Figure 12. Separation by rate zonal centrifugation (e. g. glycerol gradient centrifugation) according to molecular mass ("size").

Chemical cross-linking

Chemical cross-linking is a method that chemically links two adjacent molecules (mostly polymers as well as biopolymers, e. g. proteins) by a covalent bond. Since cross-linking can only occur between adjacent molecules, this reaction is interpreted as evidence that the participating molecules are in close physical neighbourhood and/or interact with each other. If proteins under physiological conditions can be cross-linked, it will usually be assumed that they form dimers or oligomers depending on the size of the products. The impossibility of getting cross-linked, however, cannot be taken as reference that proteins are active as monomers. Cross-linked proteins can be analyzed by conventional SDS-PAGE, although gradient gels might be useful, if bands are not focused well enough.

The reagents catalyzing this reaction are often classified according to the different functional groups they react with and according to the spacer they introduce. EDC, for example, is a zero-length (no spacer introduced between the cross-linked molecules), heterobifunctional (reacts with two different functional groups) cross-

linking reagent coupling carboxyl groups to primary amines, whereas BS^3 is a homobifunctional (reacts with two functional groups of the same kind), amine-reactive (reacts with primary amines) cross-linking reagent introducing an 8-carbon spacer.

Blue native PAGE

Blue native PAGE is a kind of polyacrylamide gel electrophoresis, in which proteins can be separated under native, not denaturing conditions (without SDS). Coomassie Brilliant Blue G250 is used for providing a uniform negative charge to proteins necessary for separation according to mass or “size” instead of isoelectric point. Therefore, dimers or oligomers should be visible as bands after staining of the gel. These bands correspond to masses approximately two or more times as high as their monomeric forms. Proteins of known molecular mass and quarternary structure are used as mass standards.

As in chemical cross-linking, gradient gels might be useful, if bands are not focused well enough.

Materials and Methods

Materials

Buffers and solutions

- 0.5x TBE: 44.5 mM Tris, 44.5 mM H_3BO_3, 1 mM EDTA
- 5x Laemmli buffer: 300 mM Tris-Cl (pH 6.8), 60% glycerol, 10% SDS, 0.025% bromophenol blue, 7% 2-mercaptoethanol
- LB: 0.5% yeast extract, 1% tryptone, 1% NaCl, pH 7
- LB agar plates: 1.5% agar, 0.5% yeast extract, 1% tryptone, 1% NaCl, pH 7
- M3: 210 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl (pH 7.6), 10 mM KCl, 6 mM EDTA
- MS: 420 mM mannitol, 140 mM sucrose, 10 mM Tris-Cl (pH 7.6), 5 mM EDTA
- PBS: 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4
- PBST: 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, 0.3% Tween-20, pH 7.4
- RSB: 10 mM Tris-Cl (pH 7.6), 10 mM NaCl, 1.5 mM $CaCl_2$
- SOB: 0.5% yeast extract, 2% tryptone, 0.5% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, pH 7
- SOC: 0.5% yeast extract, 2% tryptone, 0.5% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7

Bacterial strains (E. coli)

DH5 served as host for cloning of newly ligated, recombinant plasmids. Its genotype includes, among others, knockouts in recA1 and endA1. Furthermore it has a high transformation efficiency and achieves a good plasmid output.

Beside the general expression host BL21(DE3) some other expression hosts were used as well, all of which are λDE3 lysogens: Rosetta2(DE3) is basically a BL21(DE3), which contains a plasmid carrying additional genes for tRNAs. Since E. coli uses some codons less often compared to eukaryotes, the corresponding tRNAs are called “rare tRNAs”. By expressing higher levels of seven rare tRNAs Rosetta2(DE3) is adjusted to the eukaryotic codon usage, thus expression of eukaryotic genes is improved. The additional plasmid, called “pRARE2”, which

provides chloramphenicol restistance for selection, is compatible to all pET vector plasmids.

A second derivative of BL21(DE3), ArcticExpress™(DE3), constitutively expresses two cold-adapted chaperonins (Cpn10 and Cpn60) from the psychrophilic bacterium Oleispira antarctica. This should decrease the formation of inclusion bodies and therefore enhance solubility of recombinant proteins in addition to expression at low temperatures (<15℃), which is generally thought to improve solubility due to better folding properties. The genes for the chaperonins are located on an additional plasmid (provides gentamycin resistance for selection), which is compatible with all pET vector plasmids but not compatible with pRARE2, because they have the same origin of replication (P15a of pACYC).

A new strain of ArcticExpress™(DE3) was created by transforming it with pRARE2 isolated from Rosetta2(DE3) by Miniprep and amplified by DH5. This strain, called "AE-RARE(DE3)", was only stable under permanent balancing selection with gentamycin and chloramphenicol, since the plasmid providing the additional chaperonins was not compatible with pRARE2.

Vectors (plasmids)

Among the vectors of the pET-System (Novagen) the translation vector pET21b(+) was chosen. It provides all the features necessary for replication (origin of replication), cloning (MCS), selection (ampicillin resistance gene), regulation (lac-operator and lac-repressor gene), transcription (T7 promoter, T7 terminator) and translation (ribosome binding site) as well as two tags: an N-terminal T7-tag and a C-terminal hexahistidine-tag (+ stop codon). The sequence of the T7-tag can be removed from the vector, if Nde I (or Nhe I) and a second restriction endonuclease cutting in the MCS are used for cloning a gene into the vector. Since there are no sequences for protease cleavage sites in the cloning/expression region of pET21b(+), both tags cannot be removed posttranslationally from the recombinant protein.

In addition all four vectors of the IMPACT system (pTYB1, pTYB2, pTYB11 and pTYB12) were used for some basic experiments. They provide the same features as described above for pET21b(+), but have an intein-tag instead of a T7-tag or His-tag. This tag is either C-terminal (pTYB1 and pTYB2) or N-terminal (pTYB11 and pTYB12). Two vectors (pTYB2 and pTYB12) code for additional amino acids

between target protein and tag causing a more efficient intein-cleavage and two vectors (pTYB1 and pTYB11) do not code for additional amino acids resulting in native recombinant proteins without any extra residues.

Substrates for enzyme assay

Three different radiolabelled human pre-tRNAs were used for enzyme assays. These pre-tRNAs were endonucleolytically cleaved by RNase Z^S or RNase Z^L or elongated by tRNA ribonucleotidyl transferase in the course of the enzyme assays. Nuclear pre-$tRNA^{Arg}$ ("Arg2": 3'trailer, no 5' leader sequence) was used for enzyme assays with RNase Z^S, mitochondrial pre-$tRNA^{Tyr}$ ("Y2": 3'trailer, no 5' leader sequence) was used for enzyme assays with RNase Z^L and mitochondrial pre-$tRNA^{Tyr}$ ("Y6": no 5' leader and no 3' trailer sequence, but no terminal CCA either) was used for enzyme assays with tRNA ribonucleotidyl transferase. The nucleotides ATP and CTP were added to the assays with tRNA ribonucleotidyl transferase.

Methods

Polymerase chain reaction for amplification of inserts

PCRs were performed in 100 µl total volumes each and contained 0.2 mM dAGCT nucleotide mix, 10 ng template DNA, 0.5 µM oligonucleotide primers and 1.25 units Pfu Ultra™ DNA polymerase (Stratagene) in the reaction buffer supplied with the enzyme. 5% dimethyl sulfoxide was added to the reaction for amplification of *ELAC2* only.

The initiation step was performed at 95°C for 60 seconds, cycling was repeated 35 times and included: denaturation at 95°C for 15 seconds, annealing at 58°C (*ELAC1*) and 61°C (*ELAC2*) respectively for 30 seconds, elongation at 72°C for 150 seconds. Final elongation was done at 72°C for 10 minutes.

Polymerase chain reaction for analyzing colonies

Bacterial colonies were picked up and resuspended in 20 µl double distilled water. Half of each suspension was heated up to 95°C for 5 minutes and used as template for PCR, whereas the other half was stored at 4°C for further use.

PCRs were performed in 15 µl total volumes each and contained 0.2 mM dAGCU nucleotide mix, 1.5 µl of boiled E. coli as template, 0.5 µM oligonucleotide primers

and 0.19 units Taq DNA polymerase (LC) in the reaction buffer supplied with the enzyme.

The initiation step was performed at 95℃ for 60 seconds, cycling was repeated 35 times and included: denaturation at 95℃ for 15 seconds, annealing at 58℃ (*ELAC1*) and 61℃ (*ELAC2*) respectively for 30 seconds, elongation at 72℃ for 50 seconds. Final elongation was done at 72℃ for 200 seconds.

Restriction control of DNA

In order to control, whether purified plasmids met the expectations, they were restricted using 5 units of each restriction enzyme, mostly about 500 ng of DNA in a total volume of 10 µl. Digestion was performed at 37℃ for 90 minutes followed by agarose gel electrophoresis. Restriction buffers were used depending on the enzymes.

Restriction of inserts and vector

Approximately 7 µg of plasmid pET21b(+) were digested with 40 units Nde I and Xho I each in a total volume of 200 µl, whereas 2 µg *ELAC1* (PCR product) and 4.8 µg *ELAC2* (PCR product) were digested with 30 Units Nde I and 20 Units Xho I respectively in total volumes of 60 µl each. R+ (Fermentas) served as restriction buffer for all 3 reactions which were allowed to proceed at 37℃ over night.

Dephosphorylation of vector

Digested plasmids (200 µl reaction volume) were supplemented with 2 µl of alkaline phosphatase (1 Unit/µl) and dephosphorylated at 37°C for 30 minutes.

Phenol-chloroform extraction and concentration of nucleic acids

Purification of nucleic acids was done by phenol extraction: samples were mixed by vortexing at full speed for one minute with equal volumes of a mixture of phenol, chloroform and isoamyl alcohol (25:24:1). As phases had been separated using a bench top microcentrifuge at full speed for two minutes, the upper phases were mixed by vortexing with chloroform/isoamyl alcohol to get rid of the phenol, followed by centrifugation in order to separate phases. To increase yield double distilled water was used for back-extraction.

Precipitation was performed by adding 0.1 volumes of 10 M ammonium acetate and 2.5 volumes of pure ethanol. A small amount of LPA (40 µg) served as carrier. Times

and temperatures of precipitation varied between 40 minutes at 4℃ and over night at 4℃ or -20℃. After centrifugation with a bench top microcentrifuge at full speed for 15 minutes the pellets were washed with 70% ethanol, dried at room temperature and dissolved in double distilled water.
Concentrations of DNA were estimated by measuring the absorption at 260 nm assuming that 50 ng DNA per µl correspond to an absorption of 1.

Agarose gel electrophoresis

Before loading samples onto gel, 0.1 volume of buffer (0.2% bromphenol blue, 0.2% xylene cyanol, 10 mM EDTA, 50% sucrose, 20% glycerol) was added. As an appropriate gel (0.7 to 1% standard electrophoresis Agarose in 0.5x TBE, 0.5 µg ethidium bromide per ml) had been prepared, it was submerged in 0.5x TBE and run at 5 V/cm constant voltage. Transillumination with longwavelength ultraviolet light was used for visualization of nucleic acids.

Gel-purification of DNA

QIAquick Gel Extraction Kit was used for purification of DNA after agarose gel electrophoresis. Bands containing nucleic acids of interest were cut out of the gel, dissolved in the same volume of QG buffer, heated to 50℃ for 10 minutes while shaking and trapped on spin columns supplied with the kit using a bench top microcentrifuge at full speed for 1 minute. When washing with buffer PE was completed, 50 µl of buffer EB was used to elute nucleic acids.
Concentrations of DNA were estimated by measuring absorption at 260 nm assuming that 50 ng DNA per µl correspond to an absorption of 1.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Prior to loading samples onto gel, they were supplemented with 0.25 volumes 5x Laemmli buffer and heated up to 95℃ for 5 minutes. Only minigels were used for SDS-PAGE. Resolving gels contained 8% acrylamide/bisacrylamide (29:1), 375 mM Tris-Cl (pH 8.8) and 0.1% SDS, whereas stacking gels contained 5% acrylamide/bisacrylamide (29:1), 125 mM Tris-Cl (pH 6.8) and 0.1% SDS. Polymerisation was caused by addition of 0.01 volumes of 10% APS and 0.0006 (resolving gel) or 0.001 (stacking gel) volumes of TEMED. Gels were run at constant voltages: 60 V were used for the entry of samples into gel, whereas 120 V were

applied during separation. Electrophoresis was terminated when the bromophenol blue had left the bottom of the gel.

Coomassie staining of SDS-polyacrylamide gels

In order to visualize proteins gels were usually stained with Coomassie solution (0.1% Coomassie Brilliant Blue R-250 (CBR-250), 50% methanol, 10% acetic acid) for 30 to 60 minutes and destained afterwards using a solution consisting of 12% methanol and 7% acetic acid.
Alternatively silver staining was performed (see below).

Silver staining of SDS-polyacrylamide gels

Gels were fixed in the first solution (50% methanol, 12% acetic acid) for 30 minutes, washed twice in the second solution (5% methanol, 6% acetic acid) for 15 minutes each, reduced with 1% glutaraldehyde for 30 minutes and washed thrice with double distilled water for 10 minutes each. After a final washing step with 32.4 µM DTT for 30 minutes the gels were stained with 0.1% silver nitrate for 30 minutes, washed again with double distilled water and twice with 283 mM sodium carbonate prior to developing with 0.0185% formaldehyde for about 3 minutes. When bands were visible, the reaction was stopped by replacing the formaldehyde solution with 3% acetic acid.
Alternative the following protocol was used: Gels were fixed in the first solution (50% methanol, 5% acetic acid) for 30 minutes, washed in 50% methanol and twice in double distilled water for 10 minutes each, sensitized in 0.02% precooled sodium thiosulfate for 1 minute and washed twice with double distilled water. As gels had been stained with 0.1% silver nitrate for 20 minutes, they were washed twice with double distilled water and once with 283 mM sodium carbonate prior to developing with 0.037% formaldehyde for about 1 minute. When bands were visible, the reaction was stopped by replacing the formaldehyde solution with 3% acetic acid.

Preparation of competent Escherichia coli

Bacteria were taken from deep frozen samples and streaked out on LB agar plates with appropriate antibiotics and grown at 37℃ over night. As single colonies were resuspended in 55 ml SOB each, bacteria were allowed to grow on a shaker at 23℃ over night. When absorption at 600 nm reached 0.6, 50 ml were transferred into one falcon tube each, placed on ice for 10 minutes and centrifuged with 2500g at 4℃ for

15 minutes. After decantation pellets were resuspended in 16 ml cold TB medium and incubated on ice for 10 minutes followed by centrifugation with 2500g at 4℃ for 10 minutes. After decantation pellets were resuspended in 4 ml cold TB medium and dimethyl sulfoxide was added to a final concentration of 7% followed by incubation on ice for 10 minutes. Aliquots of 200 µl of this suspension were transferred to microcentrifuge tubes, shock-frozen in liquid nitrogen and stored at -80℃ for further use.

Ligation

Ligation was performed in 10 µl total volumes using 1 unit of Ligase in the appropriate ligation buffer, 52 ng of restricted, dephosphorylated and purified pET21b(+), 40 ng *ELAC1* (PCR product, restricted and purified) and 98 ng *ELAC2* (PCR product, restricted and purified) respectively, which corresponds to a molar ratio of 4:1 of insert to vector. The reactions were allowed to proceed at 16℃ over night.

Transformation of competent Escherichia coli

Usually 50 µl of competent E. coli were transformed with 1 to 10 ng of DNA (25 to 30 ng if following ligation) and put on ice for 30 minutes. After heat shock at 42℃ for 45 seconds 4 volumes (mostly 200 µl) of SOC were added and the bacteria were incubated on a shaker at 37℃ for 1 hour. Between 5 0 and 200 µl of that suspension were streaked out on LB agar plates supplemented with the appropriate antibiotics and grown at 37℃ over night.

Miniprep

Wizard®Plus SV Minipreps DNAPurification System was used for Miniprep. Usually 10 ml of bacterial cell suspension were harvested in a microcentrifugation tube using 5 cycles of centrifugation at full speed for 2 minutes followed by decantation. Pellets were resuspended in 250 µl cell resuspension solution, then 250 µl of cell lysis solution were added and tubes were inverted 4 times. As 10 µl alkaline protease solution were added, tubes were inverted 4 times and reactions allowed to proceed at room temperature for 5 minutes followed by addition of 350 µl neutralisation solution and inversion of tubes (4 times). In order to maximize yield, tubes were centrifuged repeatedly for 10 minutes and supernatants collected each time by pipetting them onto spin columns.

As DNA was trapped in the stationary phase by centrifugation at full speed for 1 minute, it was washed twice with 750 µl and 250 µl column wash solution respectively. Columns were put on new microcentrifugation tubes and DNA was eluted twice using 50 µl nuclease-free water each time resulting in 100 µl purified DNA. Concentrations were estimated by measuring the absorption at 260 nm assuming that 50 ng DNA per µl correspond to an apsorption of 1.

Escherichia coli glycerol stock

Bacterial cultures were grown in LB medium on a shaker at 37°C. During exponential phase or sometimes during stationary phase 400 µl were taken and added to 40 µl 80% glycerol, mixed briefly, shock-frozen in liquid nitrogen and stored at -80°C.

In vitro transcription and purification of labelled pre-tRNA

The following components were used for a single in vitro transcription in a total volume of 10 µl: 5x in vitro transcription buffer (Fermentas), 1 µl of the restricted plasmid phY2/BamHI and phArg2/SacI respectively or hY6 PCR product as template, 0.5 mM ACGMP-Mix (0.5 mM ATP, 0.5 mM CTP, 0.2 mM GTP, 1 mM GMP), 0.1 mM UTP, 0.1 mM (α^{32}P)UTP (10 µCi/µl; 800 Ci/mMol), 0.5 µl RNasin and 20 units of T7 or 17 units of T3 RNA polymerase (Promega) according to the template used. Reactions were allowed to proceed at 40°C for 1 hour and for another 30 minutes after a second addition of 7.5 units of corresponding polymerase. In order to terminate the reaction 50 µl “stop-solution” (4 M guanidine thiocyanate, 1 M ammonium acetate, 10 mM EDTA) and 2 µl of LPA (20 mg/ml) were added. Nucleic acids were precipitated by addition of 150 µl 100% ethanol at 4°C over night.

After centrifugation with a bench top microcentrifuge at full speed for 15 minutes pellets were washed with 450 µl 70% ethanol and allowed to dry at room temperature followed by dissolvation in 8 µl buffer F2 (85% formamide, 5% glycerol, 220 mM sucrose, 10 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol). Prior to loading onto a denaturing, 0.5 mm thick polyacrylamide gel (8% acrylamide/bisacrylamide (19:1), 7 M urea in 1x TBE; polymerized by addition of 0.01 volumes of 10% APS and 0.001 volumes of TEMED) samples were heated up to 95°C for 5 minutes. Gels were run at 20-25 V/cm constant voltage in 0.5x TBE.

When gel electrophoresis was complete, the glass plates were separated and the gel exposed to a phosphoimager screen for 1 minute. The bands corresponding to the pre-tRNAs were cut out and incubated with 200 µl elution buffer (0.5 M ammonium

acetate, 0.1% SDS, 1 mM EDTA) over night. As buffer had been withdrawn, gel slices were incubated with 100 µl elution buffer for a second time on a shaker at 35℃ for a couple of hours (for maximization of yield). Extracts were pooled and phenol extraction was performed. 0.01 volumes of LPA (20 mg/ml) and 2.5 volumes of 100% ethanol were added in order to precipitate RNA at -20℃ over night.

After centrifugation with a bench top microcentrifuge at full speed for 15 minutes pellets were washed with 1 volume of 70% ethanol and allowed to dry at room temperature followed by dissolvation in 1 mM EDTA. The amount of EDTA was chosen in relation to the emitted radiation, which was estimated by a handheld radiation monitor. Pre-tRNAs were stored at -20℃.

Enzyme assay

Assays were performed in total volumes of 20 µl each in 30 mM Tris-Cl (pH 7.6), 4.5 mM MgCl, 30 mM KCl, 20 µg BSA, 5 mM DTT, 0.01 volumes of RNasin, an appropriate amount of radiolabelled pre-tRNA and 1 to 3 µl of purified enzyme. Additionally 1 mM ATP and 0.1 mM CTP were added to enzyme assays with tRNA nucleotidyl transferase. Reactions were allowed to proceed at 25℃ for 10 to 30 minutes. Reaction time and amount of enzyme solution were chosen to match the estimated concentrations of purified enzyme in the fractions. In order to terminate reactions 50 µl “stop-solution” (4 M guanidine thiocyanate, 1 M ammonium acetate, 10 mM EDTA) and 2 µl LPA (20 mg/ml) were added. Nucleic acids were precipitated with addition of 175 µl 100% ethanol at -20℃ over night.

Denaturing polyacrylamide gel electrophoresis

After centrifugation with a bench top microcentrifuge at full speed for 15 minutes pellets were washed with 300 µl 70% ethanol and allowed to dry at room temperature followed by dissolvation in 7 µl of buffer F2 (85% formamide, 5% glycerol, 220 mM sucrose, 10 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol).

Prior to loading onto a denaturing, 0.5 mm thick polyacrylamide gel (8% acrylamide/bisacrylamide (19:1), 7 M urea in 1x TBE; polymerized by addition of 0.01 volumes of 10% APS and 0.001 volumes of TEMED) samples were heated up to 95℃ for 5 minutes. Gels were run at 20-25 V/cm con stant voltage in 0.5x TBE.

When gel electrophoresis was complete, glass plates were separated and gel exposed to a phosphoimager screen at least over night.

Expression of recombinant protein

In order to obtain single colonies transformed E. coli were streaked out on LB agar plates supplemented with the appropriate antibiotics and grown at 37℃ over night. For each expression one colony was resuspended in 6 ml LB supplemented with the appropriate antibiotics and grown on a shaker at 37℃ over night. These fluid cultures were used to inoculate 300 ml LB (+ appropriate antibiotics) and grown on a shaker at 37℃ for about 2.5 hours. When the absorption at 600 nm reached 0.7, fluid cultures were cooled down to 16℃, supplemented with 10 µM $ZnCl_2$ and 1 mM IPTG and incubated on a shaker at 16℃ over night.

The next day liquid cultures were placed on ice and exact volumes and absorptions at 600 nm were determined. After transfer into centrifuge tubes and subsequent centrifugation with 3000g at 4℃ for 20 minutes supernatants were decanted and pellets washed with 150 ml buffer (20 mM Tris-Cl (pH 7.5), 100 mM NaCl). 15 ml each were transferred into smaller centrifuge tubes and spun down with 3600 rpm at 4℃ for 15 minutes. As supernatants had been decanted, pellets were shock-frozen in liquid nitrogen and stored at -80℃ for further use.

Lysis of bacteria

Pellets of deep-frozen E. coli were quickly thawed and resuspended in an amount of lysis buffer (20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 15% Glycerin, 0.002 volumes protease inhibitor, 0.1 mM DTT) corresponding to a calculated absorption of 10. As 20 µl had been withdrawn as samples for SDS-PAGE analysis, lysis was performed in sodium chloride ice baths using a Bandelin Sonopuls HD 70 with standard horn SH 70 G, MS 73 titanium probe and high frequency generator GM 70. Samples were treated with 100% power and 50% cycle thrice for 5 minutes each and allowed to cool down for 5 minutes in between. After centrifugation with 20000g at 4℃ for 20 minutes bacterial extracts were transferred with syringes into chilled centrifuge tubes on ice by passing through syringe filters (0.22 µm). For SDS-PAGE analysis 20 µl samples of extracts and resuspended pellets were taken after lysis.

Small scale expression

In order to optimize expression parameters many expression experiments were performed with smaller volumes (i. e. < 10 ml) as described above. Usually a single colony was resuspended in 1 to 2 ml LB with appropriate antibiotics and grown on a

shaker at 37℃ over night. A small amount thereof was used to inoculate < 10 ml LB (+ appropriate antibiotics) in sterile culture tubes and grown on a shaker at 37℃ for about 2 to 3 hours. When the absorption at 600 nm reached 0.5 to 0.8, fluid cultures were induced by addition of IPTG (usually 1 mM) and incubated on a shaker at different temperatures for various times.

Afterwards liquid cultures were placed on ice and exact volumes and absorptions at 600 nm were determined. After transfer into centrifuge tubes and subsequent centrifugation with a microcentrifuge at full speed supernatants were decanted. Pellets were shock-frozen in liquid nitrogen and stored at -80℃ for further use or were immediately resuspended in lysis buffer and lysis performed with sonication (100% power and 50% cycle, nine times for 10 seconds each with interruptions of 20 seconds for cooling down) in an ice bath. Alternatively lysis was performed using lysozyme (0.5 µg/µl) on a shaker at 4℃ for 30 minutes, 0.5% TritonX-100 and 10 µg/ml DNase I were added subsequently and allowed to react at 4℃ for another 15 minutes. Centrifugation at 20000g at 4℃ for 10 minutes was used for sedimentation of cell debris.

Aliquots of unbroken bacteria, bacterial extract and resuspended cell debris (all normalized to cell density and volume) were analysed via SDS-PAGE in order to gain information on the optimal parameters for expression of recombinant proteins.

Immobilized metal ion affinity chromatography

Buffer A (20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 15% Glycerol, 0.1% Tween-20, 0.1 mM DTT, 0.001 volumes protease inhibitor) and B (20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 15% Glycerol, 0.1% Tween-20, 0.1 mM DTT, 0.001 volumes protease inhibitor, 500 mM imidazole) were prepared, filtrated (0.22 µm) and degassed. A prepacked HisTrap HP 1 ml column (GE Healthcare) was connected to an ÄKTA explorer chromatography system and equilibrated with a mixture of 92% buffer A and 8% buffer B corresponding to 40 mM imidazole. Meanwhile 2 M imidazole was added to the bacterial extract to a final concentration of 40 mM.

When extract was injected into the chromatography system at a flow rate of 0.25 ml per minute using a superloop, collection of 1 ml fractions was started. As injection had been completed, the column was washed with a mixture of 80% buffer A and 20% buffer B (100 mM imidazole, RNase Z^S and tRNA ribonucleotidyl transferase) or 84% A and 16% B (80 mM imidazole, RNase Z^L) at a flow rate of 0.7 ml per minute

for about 8 column volumes. Elution was caused by switching to a mixture of 40% buffer A and 60% buffer B corresponding to 300 mM imidazole at a flow rate of 0.25 ml per minute for approximately 4 column volumes. As a last step the column was washed with 100% buffer B (500 mM imidazole) for 5 column volumes and, as collection of fractions had been ceased, with 20% ethanol.
Selected fractions were analyzed by SDS-PAGE and those containing recombinant protein by enzyme assay.

Storage of recombinant proteins

Fractions containing recombinant RNase Z were pooled, concentrated using a Vivaspin 2 centrifugal device (Vivascience) and diluted by addition of buffer to these final concentrations: 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 50% glycerol, 5 mM DTT, 1 mM EDTA, 0.1% Tween-20, 0.1% protease inhibitor and 30 mM imidazole (artefact of purification procedure). This enzyme solution was split into 100 µl aliquots, some of which were shock-frozen in liquid nitrogen and stored at -80°C, whereas others were stored at -20°C.

Size exclusion chromatography

After immobilized metal affinity chromatography three fractions of purified RNase Z^L or RNase Z^S were pooled and concentrated using a Vivaspin 2 centrifugal device (Vivascience). The resulting concentrate was supplemented with proteins of known molecular weight and quarternary structure, which served as molecular weight markers: 200 µg chymotrypsinogen, 100 µg ovalbumin, 100 or 200 µg BSA, 200 or 600 µg aldolase, 200 µg catalase and 100 or 200 µg ferritin.
A prepacked Superdex 200 10/300 GL chromatography column (GE Healthcare) with approximately 24 ml bed volume was connected to an ÄKTA explorer chromatography system and equilibrated with buffer (20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 15% Glycerol, 0.1% Tween-20, 0.1 mM DTT, 0.001 volumes protease inhibitor). 100 µl of protein sample were applied at a flow rate of 0.25 ml per minute (RNase Z^L) or 0.45 ml per minute (RNase Z^S). When the absorption at 260 nm began to rise, collection of 0.5 ml (RNase Z^S) or 0.25 ml (RNase Z^L) fractions was started. As one bed volume of buffer had passed through the column after injection of sample, chromatography was ceased and column washed with 20% ethanol.
Fractions were analyzed via SDS-PAGE and enzyme assay.

Glycerol gradient centrifugation

Glycerol gradient centrifugations were performed using different salt concentrations (100, 150 or 300 mM NaCl) but always the same linear glycerol density gradient (15-30% glycerol). Further components of the gradients were 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.1 mM DTT and 0.001 volumes of protease inhibitor. 4 ml gradients were generated in Beckman Polyallomer centrifuge tubes (11x60 mm) using a gradient mixer connected to a peristaltic pump.

Mitochondrial extracts or purified recombinant proteins were mixed with equal volumes of buffer (20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.1 mM DTT, 0.001 volumes of protease inhibitor) containing no glycerol but NaCl in such concentrations that the resulting concentration was the same as in the glycerol gradient. Microcon centrifugal filter devices (50 kD cut-off) were used to concentrate samples to 100 µl. Samples were supplemented with four proteins, which served as molecular weight markers: 20 µg ovalbumin, 20 µg BSA, 40 µg catalase and 120 µg aldolase. As samples were layered over glycerol gradients, centrifugation was performed with 485000g at 4℃ for 14 hours using a swing-out rotor (SW60, Beckman).

Glycerol gradients were fractionized and fractions (approximately 160 µl each) analyzed via SDS-PAGE and enzyme assay.

Chemical cross-linking

Buffers of stored enzyme solutions were displaced by repeated dilution with buffer (20 mM HEPES-Na (pH 7.6), 150 mM NaCl) and concentration with Microcon centrifugal filter devices (50 kD cut-off).

Chemical cross-linking reactions were performed in 20 µl total volumes using approximately 10 µg of purified enzyme and either 0.05% glutaraldehyde, 1 mM BS^3 or 50 mM EDC with 5 mM Sulfo-NHS. Reactions were allowed to proceed for 30 minutes at room temperature and terminated by addition of 2 µl 1 M Tris-Cl (pH 7.5). As 0.25 volumes of 5x Laemmli buffer had been added, samples were heated up to 70℃ for 10 minutes. SDS-PAGE was performed as desc ribed above with the exceptions that gradient gels (4-12% acrylamide/bisacrylamide (29:1), polymerized by only 0.0075 volumes of APS and 0.0006 volumes of TEMED) were used as resolving gels and that the stacking gels contained only 3.5% acrylamide/bisacrylamide (29:1).

Coomassie staining was used for visualization of proteins, as described above.

Blue native polyacrylamide gel electrophoresis

Buffers of stored enzyme solutions were displaced by dilution with 10 mM imidazole (pH 7.0) and concentration with Microcon centrifugal filter devices (50 kD cut-off). Samples were supplemented with 0.25 volumes of 5x sample buffer (25 mM imidazole, 250 mM 6-aminohexanoic acid, 25% Glycerin, 0.5% Coomassie Brilliant Blue G250) and approximately 6 µg of protein were loaded onto the gel. Additionally 3 µg catalase, 5 µg BSA, 5 µg ovalbumin, 10 µg thyroglobulin, 10 µg ferritin and 24 µg aldolase were loaded as molecular weight markers.

Gradient gels (8-18% acrylamide/bisacrylamide (29:1)) were used as resolving gels containing 25 mM imidazole, 500 mM 6-aminohexanoic acid and polymerization was started by addition of 0.008 volumes of APS and 0.0004 volumes of TEMED. Stacking gels contained 4% acrylamide/bisacrylamide (29:1), 25 mM imidazole, 500 mM 6-aminohexanoic acid and were polymerized by addition of 0.01 volumes of APS and 0.001 volumes of TEMED.

Electrophoresis was performed with cathode buffer containing 50 mM tricine, 7.5 mM imidazole, 0.02% Coomassie Brilliant Blue G250, pH 7.0, whereas 25 mM imidazole (pH 7.0) served as anode buffer. Gels were run at constant voltages: 100 V were used for the entry of samples into gel (45 minutes), whereas 200 V were applied during separation (90 minutes). Electrophoresis was terminated when Coomassie Brilliant Blue had left the bottom of the gel.

Coomassie staining was used for visualization of proteins, as described above, but with a shorter incubation time (10 minutes).

Results

Cloning and Transformation

The first steps towards expression of recombinant human RNase Z^S, RNase Z^L and tRNA ribonucleotidyl transferase were amplification of genes (“inserts”) and vector, the integration of inserts into vector by restriction and ligation and subsequent transformation of expression hosts.

Inserts were amplified by PCR. Agarose gel electrophoresis was performed with a small amount thereof to confirm correct base pair length of amplicons. The expression vector was amplified by DH5 and recovered via Miniprep. A small amount thereof was restricted in order to confirm the correct identity of this plasmid. Bands of inserts and digested vector met all expectations (data not shown).

Vector and inserts (*ELAC1*, *ELAC2* and *TRNT1*) were restricted by Nde I / Xho I. When the vector had been dephosphorylated, proteins were removed by phenol-chloroform extraction with subsequent concentration by precipitation and resolvation. Vector and inserts were purified by agarose gel electrophoresis, gel slices with DNA of correct length cut out and DNA was extracted subsequently. Agarose gel electrophresis with a small amount thereof confirmed purity of vector and inserts (data not shown).

Vector-insert constructs were ligated and competent DH5 were transformed with them. Several colonies of each transformation experiment were selected and tested via PCR for successful transformation. Products were visualized by agarose gel electrophoresis and successfully transformed DH5 were used to inoculate a liquid medium (data not shown).

Vector-insert constructs were retrieved from DH5 via Miniprep, restrictions of small amounts thereof were performed and agarose gel electrophoresis confirmed identity and verified correct insertion (data not shown). One clone of each vector-insert construct was chosen and sequences of target genes were verified by sequencing (data not shown). Thereupon competent host strains for expression were transformed with these vector-insert constructs and small scale expression experiments confirmed expression of recombinant proteins (data not shown).

Optimization of Expression

At first, only pET21b(+) was used as expression vector and only two genes (*ELAC1* for RNase Z^S and *ELAC2* for RNase Z^L) were inserted into this vector. Vector-insert constructs were named pET21-*ELAC1* and pET21-*ELAC2*, respectively.

BL21(DE3) or Rosetta2(DE3)

Since human genes had to be expressed in Escherichia coli, the differences between bacterial and eukaryal codon usage were expected to be a major problem for expression. Therefore two different strains of expression hosts were transformed with vector-insert constructs at the beginning: the general expression host BL21(DE3) and Rosetta2(DE3). Due to increased expression of seven rare tRNA genes, Rosetta2(DE3) is adjusted to the eukaryotic codon usage.

In order to decide, which host (BL21(DE3) or Rosetta2(DE3)) was better suited for expression of each RNase Z, this experiment was performed: As both hosts had been transformed with either plasmid (pET21-*ELAC1* and pET21-*ELAC2*), small scale expressions were performed throughout different periods of time (0, 1, 2, 3 and 4 hours) at 30℃ using 1 mM IPTG for induction. When bacteria had been harvested and lysed, crude lysates were analyzed via SDS-PAGE. Subsequent Coomassie staining was used for visualisation.

It turned out that RNase Z^S could be produced by both strains (Figure 13), whereas RNase Z^L could only be expressed in Rosetta2(DE3) most likely due to increased expression of seven rare tRNA genes (Figure 14).

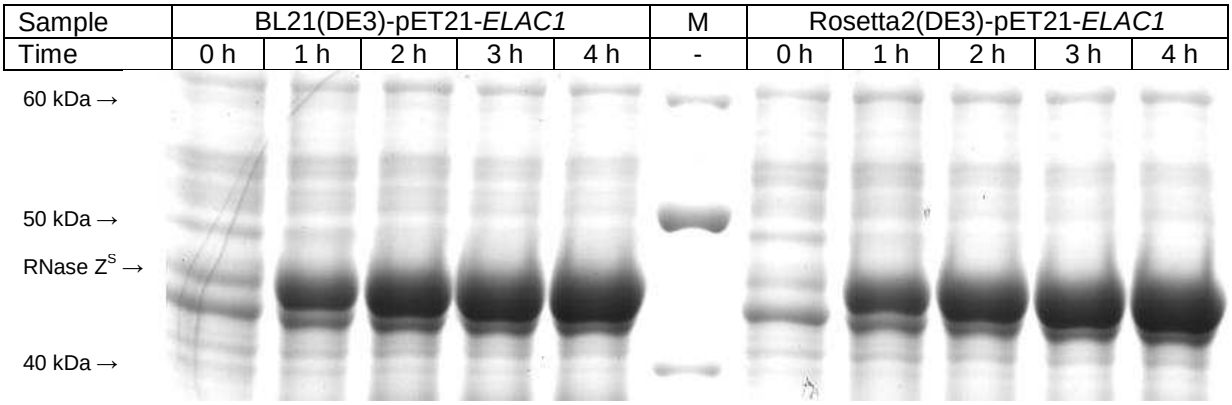

Figure 13. Suitability of BL21(DE3) or Rosetta2(DE3) for expression of RNase Z^S via pET21b+. SDS-PAGE of proteomes of BL21(DE3) and Rosetta2(DE3) having expressed RNase Z^S at 30℃ for different periods of time. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

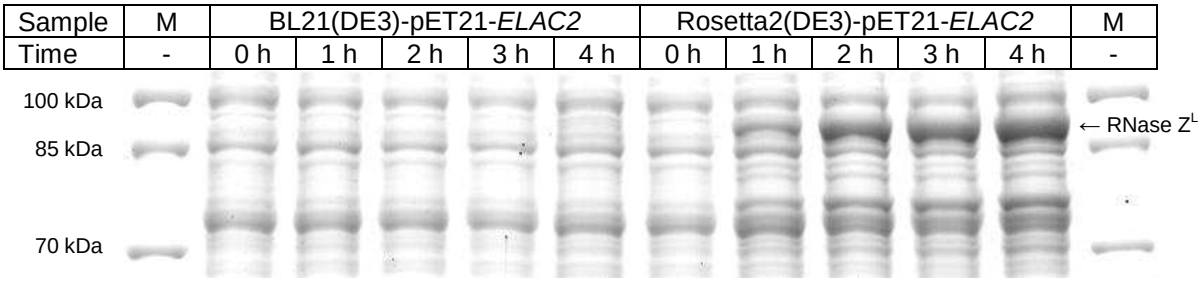

Figure 14. Suitability of BL21(DE3) or Rosetta2(DE3) for expression of RNase Z^L via pET21b+. SDS-PAGE of proteomes of BL21(DE3) and Rosetta2(DE3) having expressed RNase Z^L at 30℃ for different periods of time. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

Expression time and temperature

Expression of both RNases Z had been demonstrated to be possible. The next question was, if they could be expressed in a soluble manner, which was not yet clear. Enzymes, which are normally in an aqueous environment, need to be folded correctly in order to be soluble and enzymatically active. A recommended method to increase production of correctly folded, thus soluble recombinant protein is to lower expression temperature. This was tested by small scale expression experiments at different temperatures for different times: 15℃ for 5h, 20℃ for 4h, 25℃ for 3h and 30℃ for 2h. When bacteria had been harvested and lysed, cell debris were sedimented by centrifugation. Extract and resuspended cell debris were analyzed via SDS-PAGE. Subsequent Coomassie staining was used for visualisation of expression.

Although the total amount of soluble RNase Z^S was only slightly higher at lower expression temperatures than at higher expression temperatures, the ratio of soluble to insoluble protein was highly increased (Figure 15). Therefore, expression of

recombinant proteins at 15℃ for a longer period of time (over night) was chosen. Soluble RNase Z^L is difficult to recognize on photos, because amounts were often very low. Nevertheless, the increase of insoluble RNase Z^L at higher temperatures is visible (Figure 16). Therefore, it was assumed that expression at 15℃ for a longer period of time (over night) was favourable for this protein, too.

Fraction	M	soluble					insoluble				
Temp. [℃]	-	15	15	20	25	30	15	15	20	25	30
Induced	-	-	+	+	+	+	-	+	+	+	+

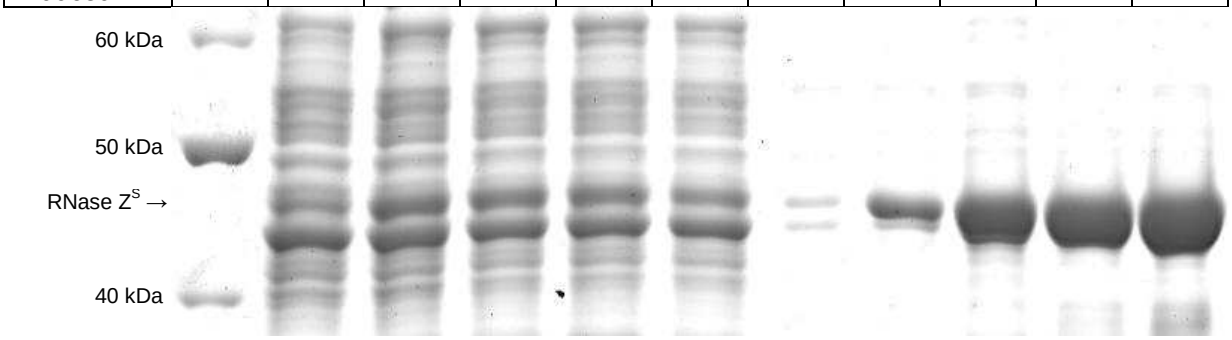

Figure 15. Expression and solubility of RNase Z^S by BL21(DE3) via pET21b+ at different times and temperatures. SDS-PAGE of soluble and insoluble fractions after lysis of BL21(DE3) having grown at 15℃ for 5h (not induced) or having expressed RNase Z^S at 15℃ for 5h, at 20℃ for 4h, at 25℃ for 3h or at 30℃ for 2h. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

Fraction	M	soluble					insoluble				
Temp. [℃]	-	15	15	20	25	30	15	15	20	25	30
Induced	-	-	+	+	+	+	-	+	+	+	+

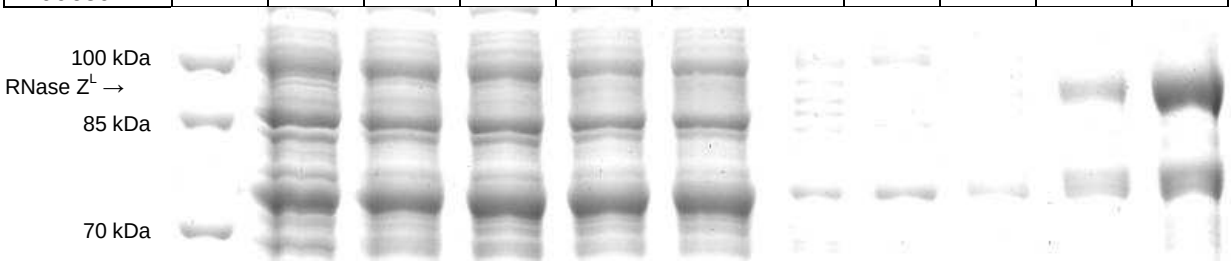

Figure 16. Expression and solubility of RNase Z^L by Rosetta2(DE3) via pET21b+ at different times and temperatures. SDS-PAGE of soluble and insoluble fractions after lysis of Rosetta2(DE3) having grown at 15℃ for 5h (not induced) or having expressed RNase Z^L at 15℃ for 5h, at 20℃ for 4h, at 25℃ for 3h or at 30℃ for 2h. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

Concentration of IPTG

In order to find out, which effect, if any, the alteration of IPTG concentrations had on expression of recombinant proteins, this experiment was performed: small scale expressions of RNase Z^S and RNase Z^L induced by addition of 0.2 mM or 1 mM IPTG at 15℃ for 5h. When bacteria had been harvested and lysed, SDS-PAGE and subsequent Coomassie staining were used for visualisation of expression.

Because RNase Z^L was produced in rather low amounts, it was hard to recognize small differences in expression of this recombinant protein, especially on photos. Therefore, only the result for the experiment with RNase Z^S is shown below (Figure 17). Addition of 1 mM IPTG resulted in production of more insoluble protein, but production of soluble protein was not increased. Therefore, induction by addition of 0.2 mM IPTG might have been sufficient. Nevertheless, 1 mM IPTG was used later on, since it seems to be a standard concentration for expression experiments. Moreover, it was considered unreasonable to further hamper expression at 15℃ by using less IPTG than 1 mM.

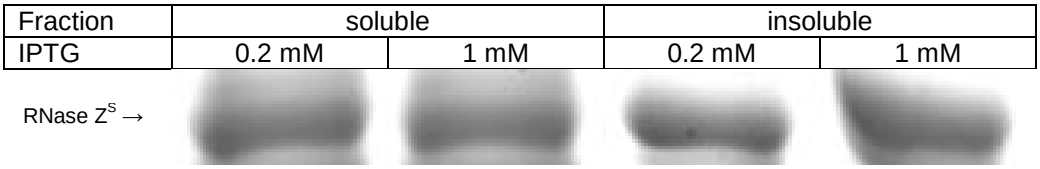

Figure 17. Effect of IPTG concentration on expression of RNase Z^S via pET21b+. SDS-PAGE of soluble and insoluble fractions after lysis of BL21(DE3) having expressed RNase Z^S at 15℃ for 5h. Induction of expression by addition of 0.2 mM or 1 mM IPTG. All amounts have been normalized to cell density and volume at the end of expression experiments.

Addition of cofactor

Addition of cofactor can lead to better solubility of recombinant proteins due to better folding properties. Since Zn^{2+} is assumed to be a cofactor for both RNases Z, it was tested, whether addition of micromolar amounts can enhance solubility. RNase Z^L was produced in the lowest amounts of all three recombinant proteins. Furthermore, it had a rather unfavourable ratio of soluble to insoluble protein. Therefore, this experiment was performed only with this enzyme: a small scale expression induced by addition of 1 mM IPTG at 15℃ over night with ad dition of 0 µM, 10 µM, 100 µM and 1 mM $ZnCl_2$. When bacteria had been harvested and lysed, SDS-PAGE and subsequent Coomassie staining were used for visualisation of expression.

There was no visible positive effect of additional $ZnCl_2$ on solubility of RNase Z^L and Zn^{2+} provided by LB medium might have been sufficient (Figure 18). Nevertheless, it was considered reasonable to add 10 µM $ZnCl_2$ in further expression experiments of both RNases Z, since there was no negative effect on expression until concentrations reach 1 mM.

Fraction	M	soluble					insoluble				
Induced	-	-	+	+	+	+	-	+	+	+	+
$ZnCl_2$ [µM]	-	0	0	10	100	1000	0	0	10	100	1000

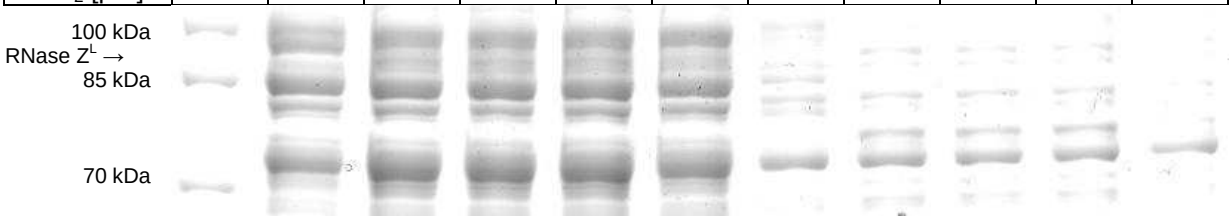

Figure 18. Effect of additional $ZnCl_2$ on expression of RNase Z^L via pET21b+. SDS-PAGE of soluble and insoluble fractions after lysis of Rosetta2(DE3) having grown at 15℃ over night (not induced control) or having expressed RNase Z^L at 15℃ over night in presence of increasing amoun ts of $ZnCl_2$ (0 µM, 10 µM, 100 µM or 1 mM). Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

ArcticExpress™(DE3) and AE-RARE(DE3)

Because both RNases Z tended to form inclusion bodies during expression to a rather large extent (see Figures 15 and 16), ArcticExpress™(DE3) was tested for its ability to produce more soluble protein. RNase Z^L could only be expressed in E. coli strains with increased expression of seven rare tRNA genes as Rosetta2(DE3). Therefore, “AE-RARE(DE3)” was made (ArcticExpress™(DE3) transformed with plasmid pRARE2 of Rosetta2(DE3)). These cells were made competent again and thereafter transformed with pET21-*ELAC2*. In order to decide, whether ArcticExpress™(DE3) and AE-RARE(DE3) were superior to BL21(DE3) and Rosetta2(DE3), small scale expressions induced by addition of 1 mM IPTG were performed at 15℃ over night. When bacteria had bee n harvested and lysed, SDS-PAGE and subsequent Coomassie staining were used for visualisation of expression. It turned out that ArcticExpress™(DE3) and AE-RARE(DE3) did not produce more soluble recombinant protein (only less insoluble protein) but large amounts of chaperonins (only Cpn60 is visible in Figures 19 and 20). Moreover, both strains needed an additional antibiotic (gentamycin) and grew more slowly. Therefore, they were judged inferior to BL21(DE3) or Rosetta2(DE3) and not used for expression of recombinant proteins.

Fraction	M	proteome				soluble		insoluble	
Strain	-	AE	BL	AE	BL	AE	BL	AE	BL
Induced	-	-	-	+	+	+	+	+	+

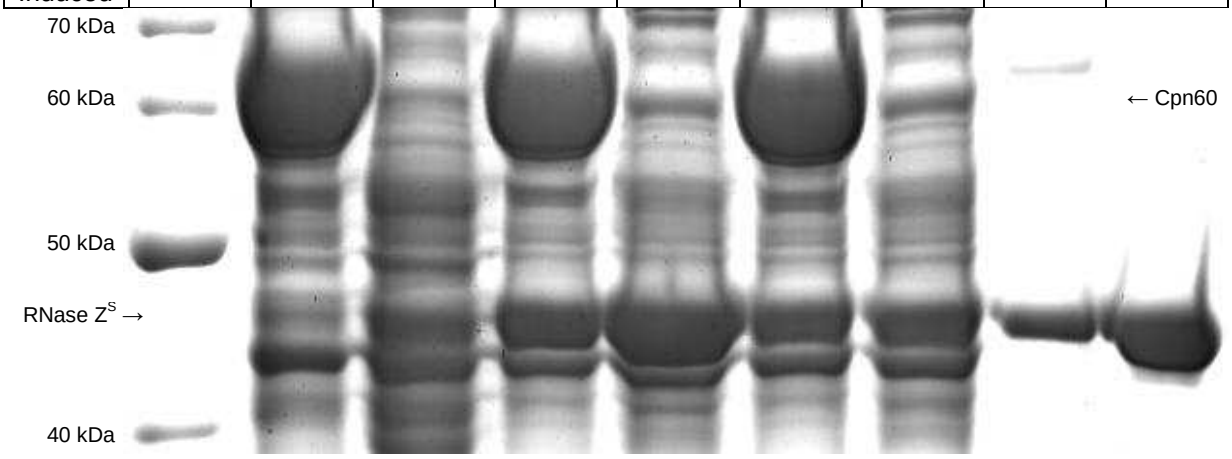

Figure 19. Suitability of BL21(DE3) ("BL") or ArcticExpress™(DE3) ("AE") for expression of RNase Z^S via pET21b+. SDS-PAGE of proteomes, soluble and insoluble fractions after lysis of BL21(DE3) or ArcticExpress™(DE3) having grown at 15℃ over night (not induced controls) or having expressed RNase Z^S at 15℃ over night. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

Fraction	M	proteome				soluble		insoluble	
Strain	-	AE-R	Ros2	AE-R	Ros2	AE-R	Ros2	AE-R	Ros2
Induced	-	-	-	+	+	+	+	+	+

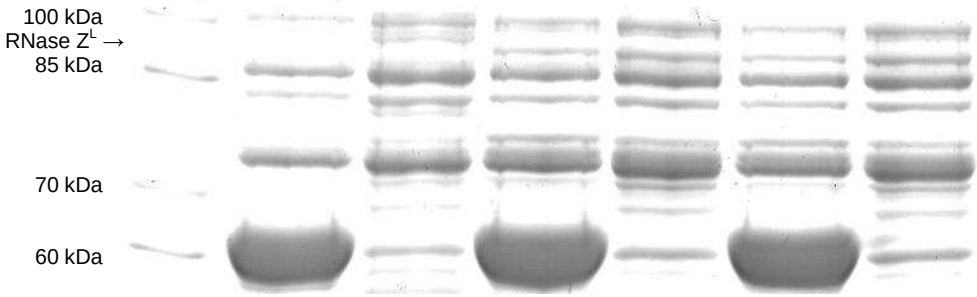

Figure 20. Suitability of Rosetta2(DE3) ("Ros2") or AE-RARE(DE3) ("AE-R") for expression of RNase Z^L via pET21b+. SDS-PAGE of proteomes, soluble and insoluble fractions after lysis of Rosetta2(DE3) or AE-RARE(DE3) having grown at 15℃ over night (not induced controls) or having expressed RNase Z^L at 15℃ over night. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

IMPACT system

Since expression of RNase Z^L by the pET-System yielded the least total protein production of all three recombinant proteins and usually a low ratio of soluble to insoluble protein, another T7 expression system was tested: the IMPACT system.

After cloning and transformation procedures of pTYB2-*ELAC2* (C-terminal fusion) and pTYB12-*ELAC2* (N-terminal fusion) small scale expression experiments were performed at different temperatures for different times (30℃ for 4h, 25℃ for 6h, 20 and 15℃ over night) using 1 mM IPTG for induction. When bacteria had been

harvested and lysed, SDS-PAGE and subsequent Coomassie staining were used for visualisation of expression.

RNase Z^L expressed via pTYB2 (Figure 21) as well as pTYB12 (Figure 22) seemed to form inclusion bodies to an even larger extent compared to RNase Z^L expressed by pET21b+. Furthermore, the purification was quite disappointing, as tested in a small scale experiment (data not shown), and the IMPACT system was therefore considered inferior to the pET-system.

Fraction	proteome								M	soluble				insoluble			
Temp. [℃]	15	20	25	30	15	20	25	30	-	15	20	25	30	15	20	25	30
Induced	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+

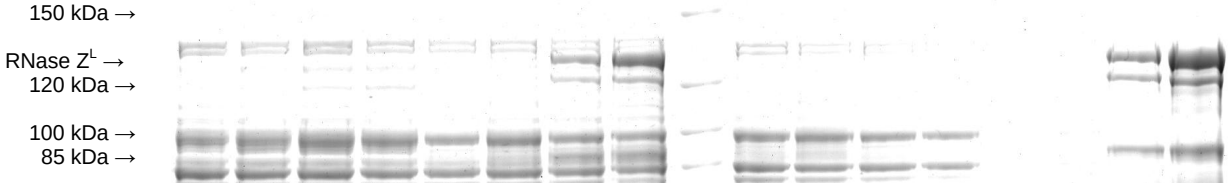

Figure 21. IMPACT system: Expression of RNase Z^L via pTYB2. SDS-PAGE of proteomes, soluble and insoluble fractions after lysis of Rosetta2(DE3) having grown (not induced controls) or having expressed RNase Z^L at 15℃ over night, at 20℃ over night, at 25℃ fo r 6h or at 30℃ for 4h. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

Fraction	M	proteome								soluble				insoluble			
Temp. [℃]	-	15	20	25	30	15	20	25	30	15	20	25	30	15	20	25	30
Induced	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+

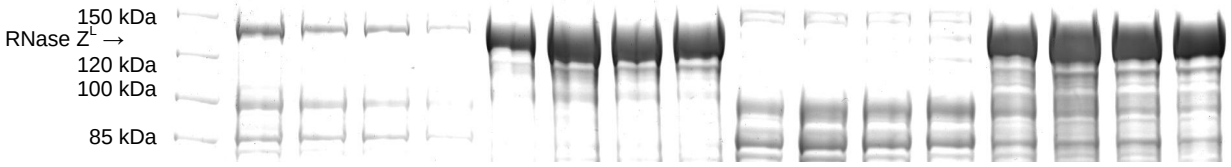

Figure 22. IMPACT system: Expression of RNase Z^L via pTYB12. SDS-PAGE of proteomes, soluble and insoluble fractions after lysis of Rosetta2(DE3) having grown (not induced controls) or having expressed RNase Z^L at 15℃ over night, at 20℃ over night, at 25℃ fo r 6h or at 30℃ for 4h. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

Optimization of lysis

Although optimization of lysis was actually no optimization of expression, the effect on the appearance of proteins (soluble/insoluble) after SDS-PAGE and staining procedures seemed to be comparable, because imperfect lysis mimicked bad solubility of recombinant proteins.

Sonication

Since aqueous solutions of lysozyme seemed to lose activity quite fast when stored at 4℃, they had to be prepared freshly for each se t of expression experiments and

subsequent lysis of bacteria. In order to overcome this disadvantage lysis by sonication was tested and successfully optimized for small scale expression experiments (data not shown). Since this method seemed to be more promising for expression experiments with larger volumes, it was optimized for this purpose, too.

As described in “Materials and Methods”, RNase Z^S was expressed by BL21(DE3) in 300 ml LB medium, cells were washed, divided into 1/10 volumes, centrifuged and pellets were shock-frozen in liquid nitrogen and stored at -80℃ for further use. Having thawed one aliquot of this expression experiment, cells were resuspended in lysis buffer and lysed by sonication (100% power and 50% cycle) on sodium chloride ice bath for as long as 18 minutes. Small samples were taken prior to lysis and every three minutes during lysis, which offered enough time for cooling down. SDS-PAGE was performed with these samples and Coomassie staining was used for visualisation of proteins.

Although sonication for nine minutes seemed to be enough judged by the photo below (Figure 23), 15 minutes (five minutes of sonication for three times alternating with five minutes of cooling in between) were used later on.

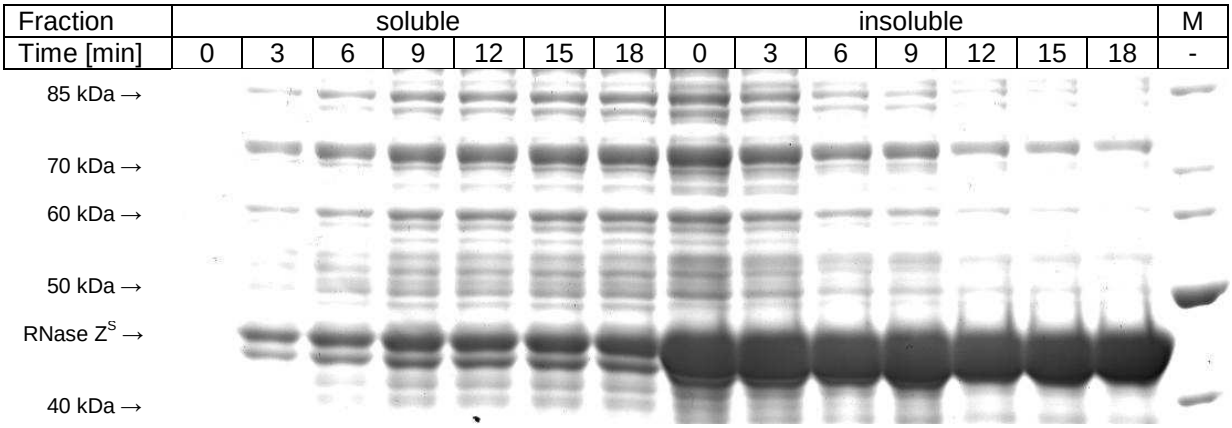

Figure 23. Optimization of sonication. SDS-PAGE of soluble and insoluble fractions after lysis of BL21(DE3) having expressed RNase Z^S. Samples were taken after 0, 3, 6, 9, 12, 15 and 18 minutes of sonication. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

Composition of lysis buffer

In order to test, whether recombinant proteins were better soluble in lysis buffer containing different amounts of NaCl, this experiment was performed: small scale expression of RNase Z^S by BL21(DE3) via pET21b+ at 15℃ over night using 1 mM IPTG for induction. Lysis was performed in buffers containing, among other chemicals, different amounts of NaCl (100 mM or 500 mM). SDS-PAGE and

subsequent Coomassie staining were used for visualisation of expression and solubility of RNase Z^S.

It seemed that RNase Z^S was more soluble in the presence of 100 mM NaCl compared to 500 mM NaCl (Figure 24). Therefore, 100 mM NaCl was used in further lysis buffers for all three recombinant proteins.

Fraction	M	proteome		soluble		insoluble	
Induced	-	-	+	+	+	+	+
NaCl [mM]	-	-	-	100	500	100	500

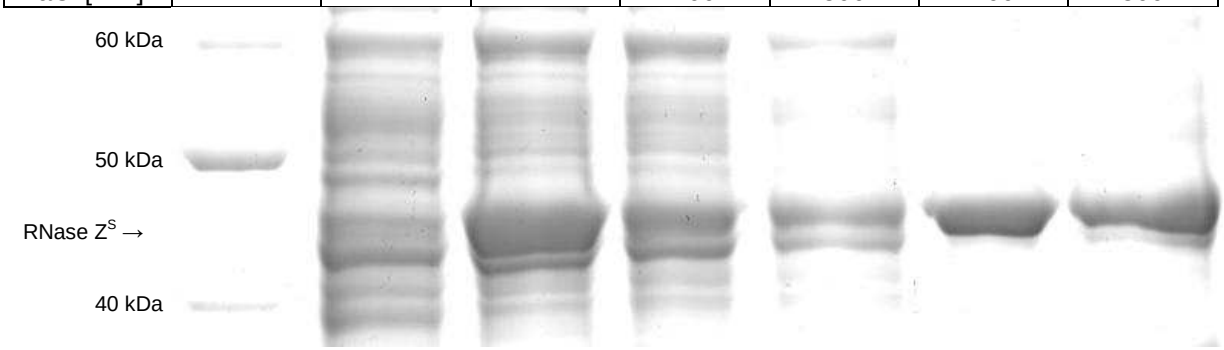

Figure 24. Effect of different NaCl concentrations on solubility of RNase Z^S in lysis buffer. SDS-PAGE of proteome, soluble and insoluble fractions after lysis of BL21(DE3) having grown at 15℃ over night (not induced control) or having expressed RNase Z^S. Lysis was performed in buffer containing 100 mM NaCl or 500 mM NaCl. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

Addition of anionic detergents after lysis

Since anionic detergents such as Tween-20 and TritonX-100 are used for lysis of bacteria after treatment with lysozyme due to destabilization of cytoplasmic membranes, it was assumed that they may have a positive effect on lysis after sonication as well. Therefore, this experiment was performed: small scale expression of RNase Z^S by BL21(DE3) via pET21b+ at 37℃ for 2h using 1 mM IPTG for induction followed by lysis in buffer and subsequent addition of anionic detergents (TritonX-100 and Tween-20) in different amounts (0%, 0.2% and 0.5%). SDS-PAGE and subsequent Coomassie staining were used for visualisation of expression of RNase Z^S and for control of lysis.

Since there was no positive effect of either detergent on lysis after sonication (Figure 25), both were omitted in further experiments.

Fraction	M	soluble			prot	insoluble			soluble			insoluble		
Detergent	-	TritonX-100			-	TritonX-100			Tween-20			Tween-20		
Conc. [%]	-	0.0	0.2	0.5	-	0.0	0.2	0.5	0.0	0.2	0.5	0.0	0.2	0.5

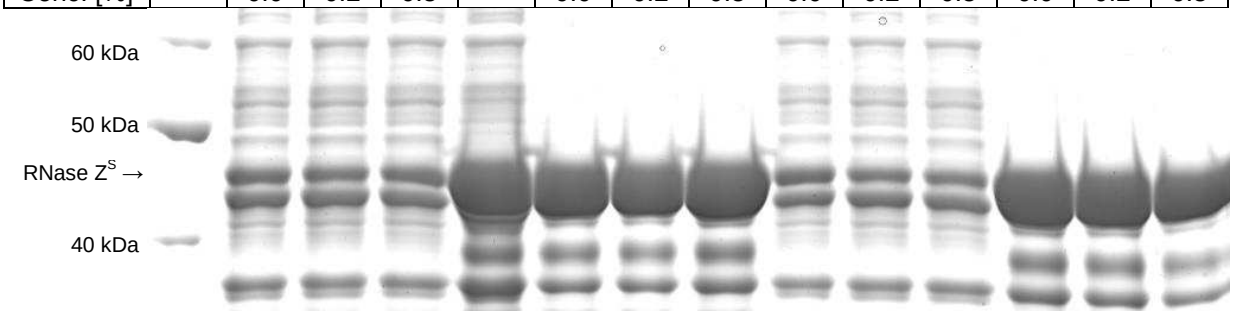

Figure 25. Effect of addition of anionic detergents after sonication of BL21(DE3). SDS-PAGE of proteome ("prot"), soluble and insoluble fractions after lysis of BL21(DE3) having expressed RNase Z^S. TritonX-100 or Tween-20 was added in different amounts after sonication. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard. All amounts have been normalized to cell density and volume at the end of expression experiments.

Summary of optimization

Having performed many experiments concerning optimization of expression of recombinant proteins, these parameters were chosen finally:

1. host: BL21(DE3) for expression of tRNA ribonucleotidyltransferase and RNase Z^S, Rosetta2(DE3) for expression of RNase Z^L
2. system: T7 expression system, vector pET21b(+)
3. temperature: 15℃ during expression
4. time: expression over night
5. cofactor: addition of 10 µM $ZnCl_2$ prior to induction
6. induction: addition of 1 mM IPTG
7. lysis buffer composition: 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 15% Glycerin, 0.002 volumes protease inhibitor, 0.1 mM DTT
8. lysis: five minutes of sonication on sodium chloride ice bath for three times alternating with five minutes of cooling in between
9. anionic detergents: no addition of Tween-20 or TritonX-100 after lysis

When the expression of RNase Z^S and RNase Z^L had been optimized, these parameters were adopted for the expression of tRNA ribonucleotidyl transferase as well. RNase Z^S, RNase Z^L and tRNA ribonucleotidyltransferase were subsequently expressed as described in "Materials and Methods" in larger amounts. Cells were washed, divided into 1/10 volumes, centrifuged and pellets were shock-frozen in liquid nitrogen and stored at -80℃ for further use. Lysis was performed with sonication, usually immediately prior to purification.

Purification of recombinant proteins

Having completed expression experiments, the next step was to purify the recombinant proteins. Since all three recombinant proteins were expressed with C-terminal hexahistidine-tags, purification was done by IMAC using columns prepacked with “Ni Sepharose High Performance” connected to an ÄKTA explorer chromatography system.

Optimal conditions for IMAC (primarily concentration of imidazole in chromatography buffer during equilibration, loading, washing and elution) are variable depending on the protein to be purified and have therefore to be determined individually prior to purification. For this purpose an IMAC was done using linear increasing concentrations of imidazole for elution after equilibration, loading and washing without imidazole. Comparison of SDS-PAGE analysis of fractions and absorption at 280 nm to corresponding concentrations of imidazole during IMAC allowed determination of imidazole concentrations needed for washing and elution of recombinant proteins. Imidazole concentrations for equilibration and loading were determined independently in subsequent experiments.

Purifications using IMAC were analyzed by SDS-PAGE of fractions and activities were checked by enzyme assays of fractions and subsequent denaturing polyacrylamide gel electrophoresis of substrate and processing products. Three different radiolabelled human pre-tRNAs were used for enzyme assays. These pre-tRNAs were endonucleolytically cleaved by RNase Z^S or RNase Z^L or elongated by tRNA ribonucleotidyl transferase in the course of the enzyme assays.

Purification of RNase Z^S

In order to determine concentrations needed for washing and elution, this IMAC was performed: after equilibration, loading and washing without imidazole, linear increasing concentrations of imidazole ranging from 0 mM to 500 mM were applied during IMAC of RNase Z^S. Absorption at 280 nm, conductivity and pressure were recorded during chromatography (Figure 26).

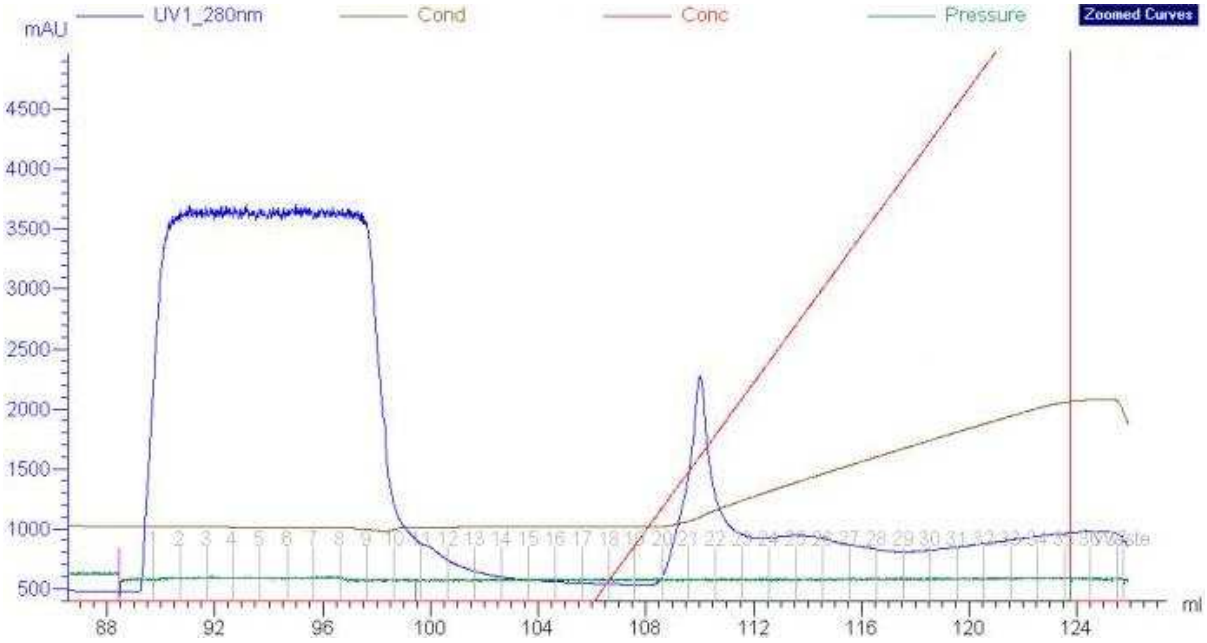

Figure 26. Determination of imidazole concentration required for washing and elution of RNase Z^S. Chromatogram of IMAC showing absorption at 280 nm (blue), conductivity (brown), pressure (green) and concentration of imidazole (red) ranging from 0 mM to 500 mM imidazole.

Fractions (1 ml each) were collected during IMAC and subsequently analyzed via SDS-PAGE. This facilitated the decision, which imidazole concentrations were apt for washing and elution (Figure 27).

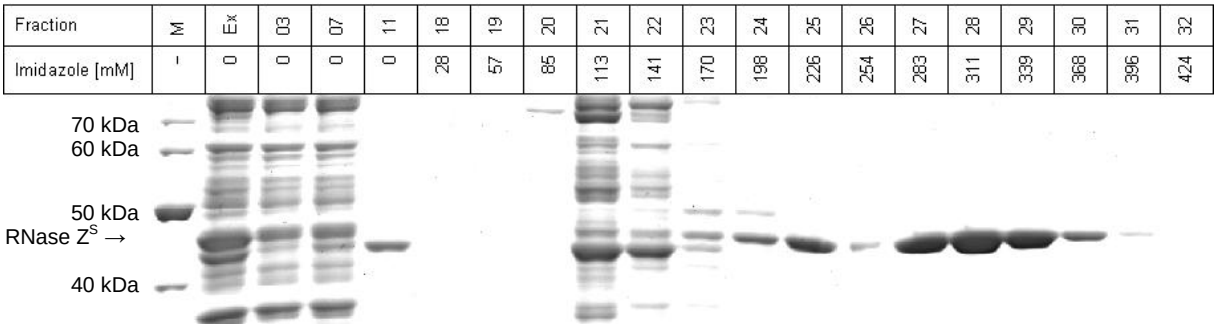

Figure 27. Analysis of IMAC of RNase Z^S having used a linear imidazole gradient. SDS-PAGE of bacterial extract ("Ex") and selected fractions taken during IMAC. Imidazole concentrations were calculated (linear progression). Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard.

Judged by this results 100 mM seemed to be the optimal concentration of imidazole for washing, while 300 mM imidazole were enough for complete elution of RNase Z^S. Additional chromatography experiments helped to determine imidazole concentrations needed for equilibration and during loading of bacterial extract. As it turned out a concentration of 40 mM imidazole helped to prevent unspecific binding of bacterial proteins during loading and was low enough to enable RNase Z^S to bind to the chromatography material in the column. Optimized IMACs of RNase Z^S were

performed using step gradients of imidazole concentrations and yielded highly concentrated and satisfactorily purified protein (Figures 28 and 29).

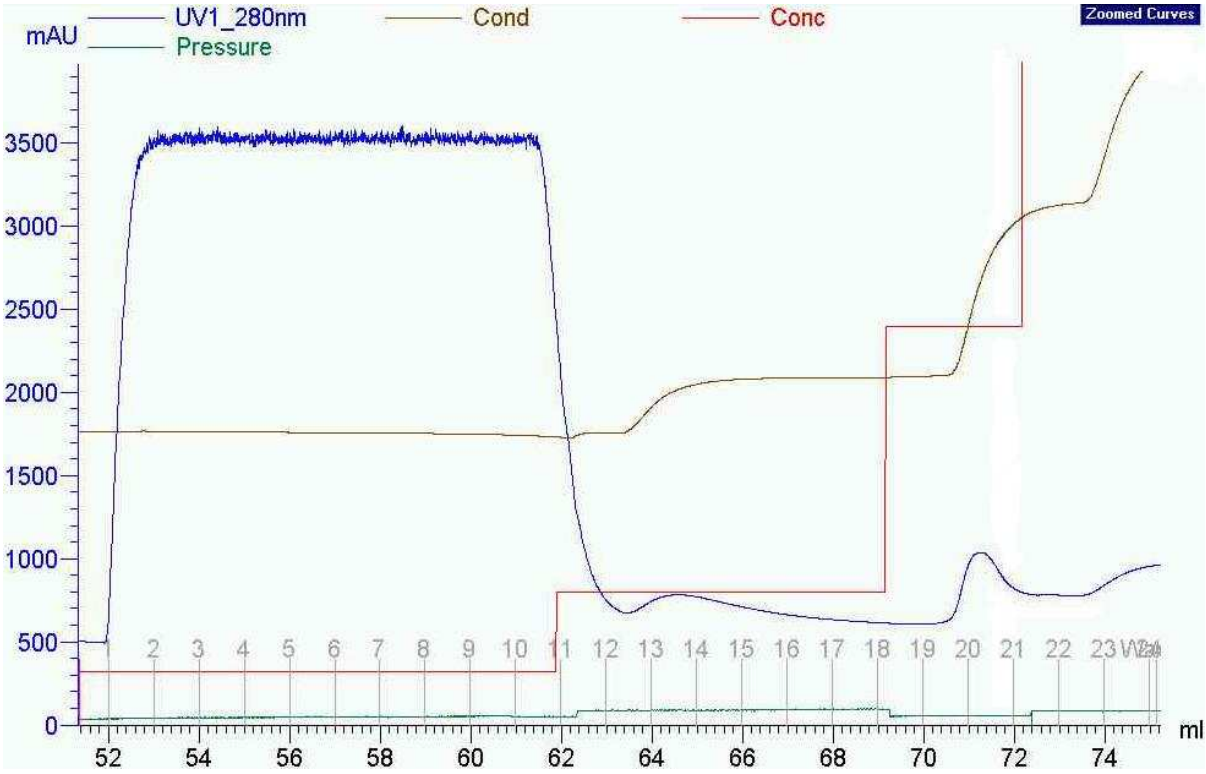

Figure 28. Optimized IMAC of RNase Z^S. Chromatogram showing absorption at 280 nm (blue), conductivity (brown), pressure (green) and concentration of imidazole (red): a concentration of 40 mM imidazole was used for equilibration of column and loading of extract, 100 mM imidazole for washing and 300 mM imidazole for elution. In order to ensure that the column was free of proteins after IMAC, a concentration of 500 mM imidazole was used after elution.

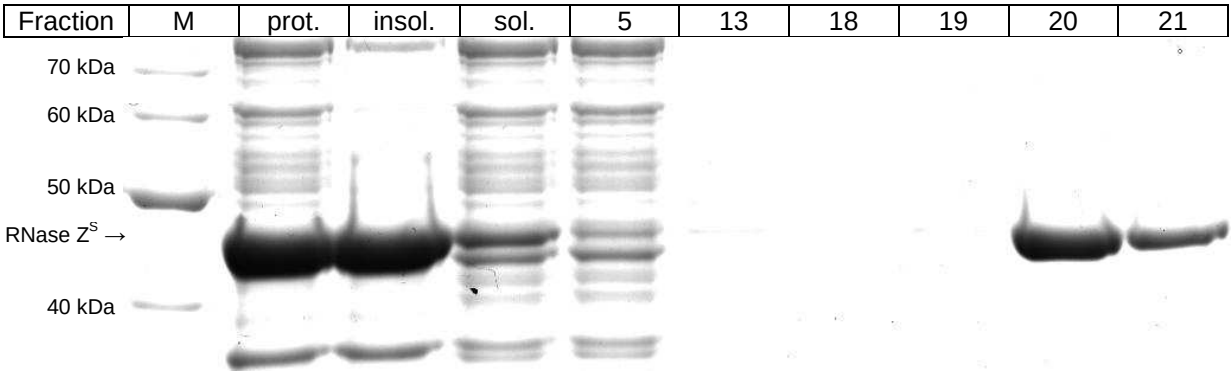

Figure 29. Analysis of optimized IMAC of RNase Z^S. SDS-PAGE of proteome ("prot."), insoluble ("insol.") and soluble ("sol." = bacterial extract) fractions after lysis and fractions collected during IMAC. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard.

When purification of RNase Z^S had been completed, enzyme assays were made with selected fractions thereof in order to test, whether purified protein showed activity. After electrophoresis of assays the gel was incubated with a phosphoimager screen. Analysis of the phosphoimager screen confirmed that the 3' trailer was removed from the substrate (radiolabelled nuclear pre-tRNA^{Arg}), hence that purified RNase Z^S was active (Figure 30).

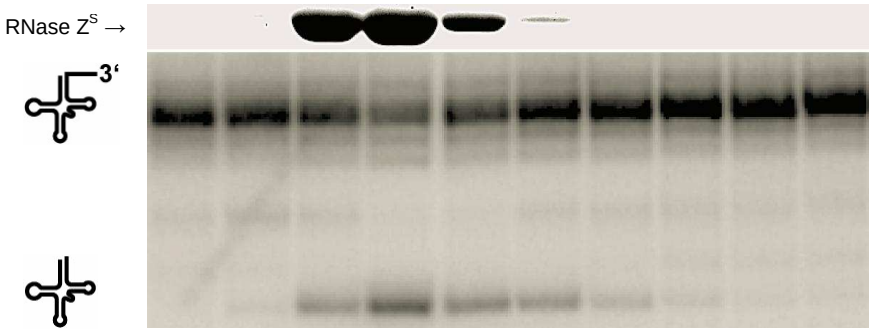

Figure 30. Activity of RNase Z^S. SDS-PAGE (above) and autoradiogram of denaturing polyacrylamide gel electrophoresis (below) of ten consecutive fractions collected during elution of RNase Z^S in the course of affinity chromatography. Radiolabelled nuclear pre-$tRNA^{Arg}$ served as substrate for enzyme assays.

Purification of RNase Z^L

In order to determine concentrations needed for washing and elution, this IMAC was performed: after equilibration, loading and washing without imidazole, linear increasing concentrations of imidazole ranging from 0 mM to 300 mM were applied during IMAC of RNase Z^L. Absorption at 280 nm, conductivity and pressure were recorded during chromatography (Figure 31).

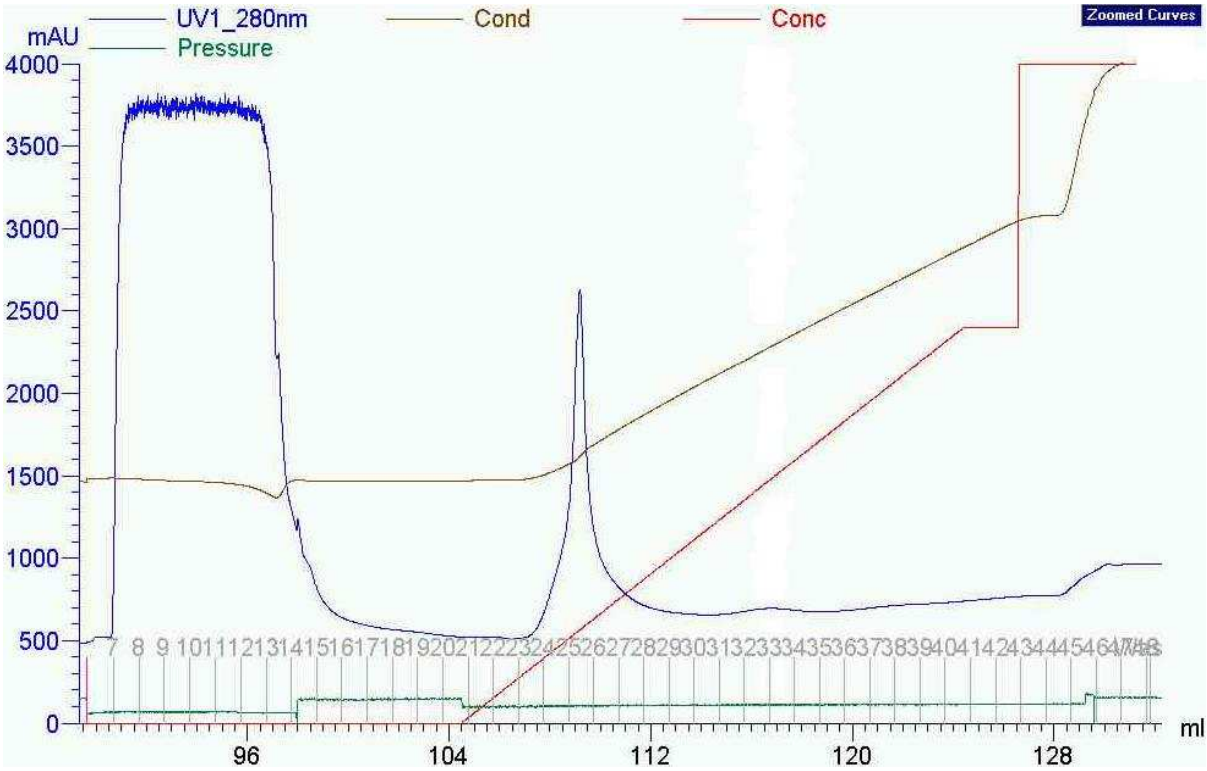

Figure 31. Determination of imidazole concentration required for washing and elution of RNase Z^L. Chromatogram of IMAC showing absorption at 280 nm (blue), conductivity (brown), pressure (green) and concentration of imidazole (red) ranging from 0 mM to 300 mM imidazole. In order to ensure that the column was free of proteins after IMAC, a concentration of 500 mM imidazole was used after elution.

Fractions (1 ml each) were collected during IMAC and subsequently analyzed via SDS-PAGE. This facilitated the decision, which imidazole concentrations were apt for washing and elution (Figure 32).

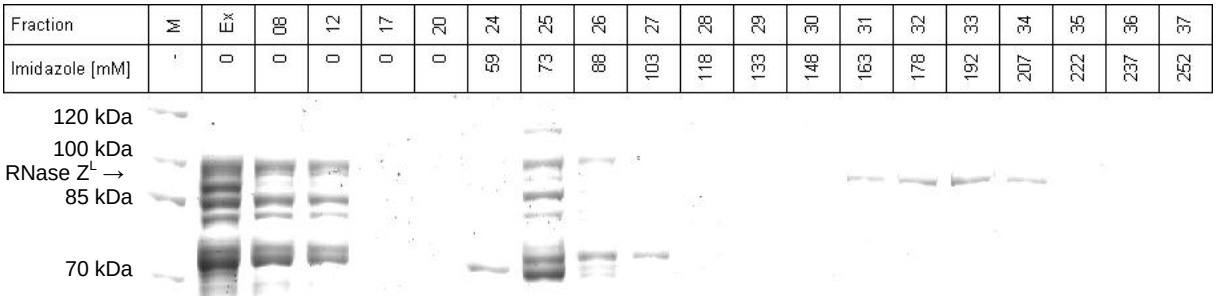

Figure 32. Analysis of IMAC of RNase Z^L having used a linear imidazole gradient. SDS-PAGE of bacterial extract ("Ex") and selected fractions taken during IMAC. Imidazole concentrations were calculated (linear progression). Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard.

Judged by this results 80 mM seemed to be the optimal concentration of imidazole for washing, while 300 mM imidazole were enough for complete elution of RNase Z^L. Additional chromatography experiments helped to determine imidazole concentrations needed for equilibration and during loading of bacterial extract. As it turned out a concentration of 40 mM imidazole helped to prevent unspecific binding of bacterial proteins during loading and was low enough to enable RNase Z^L to bind to the chromatography material in the column. Optimized IMACs of RNase Z^L were performed using step gradients of imidazole concentrations and yielded highly concentrated and satisfactorily purified protein (Figures 33 and 34).

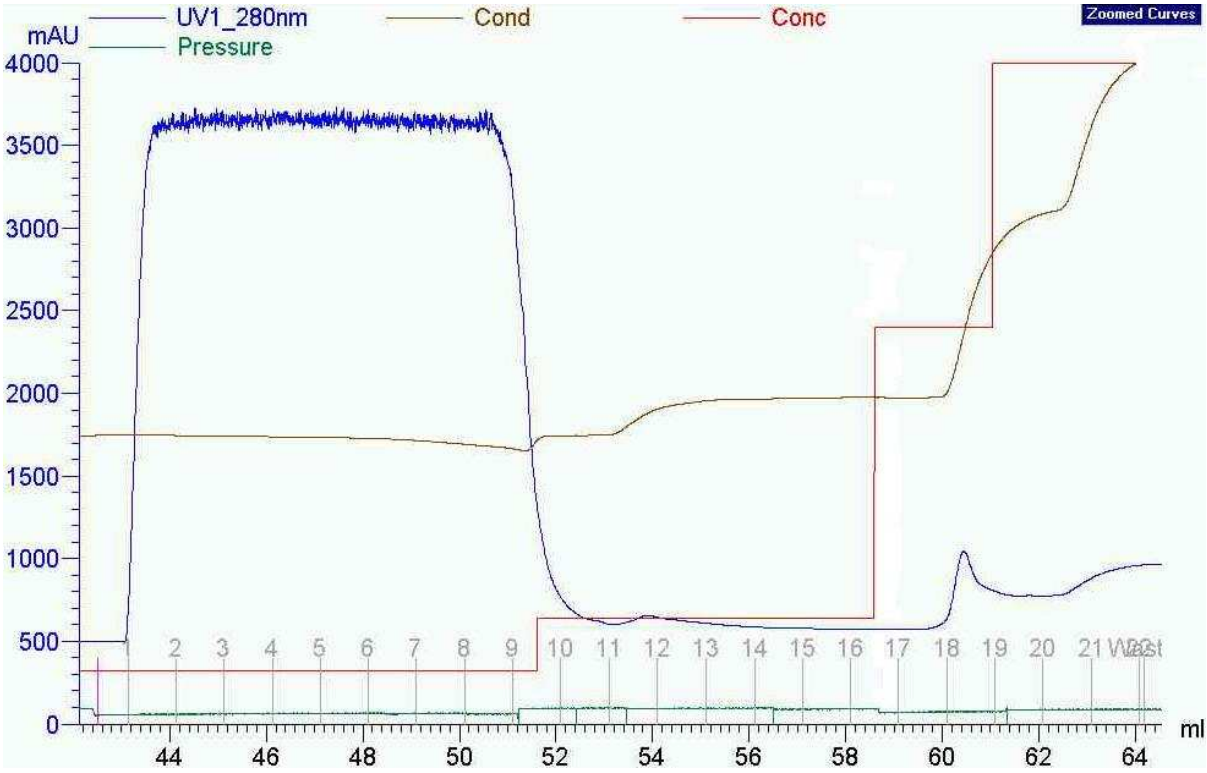

Figure 33. Optimized IMAC of RNase Z^L. Chromatogram showing absorption at 280 nm (blue), conductivity (brown), pressure (green) and concentration of imidazole (red): a concentration of 40 mM imidazole was used for equilibration of column and loading of extract, 80 mM imidazole for washing and 300 mM imidazole for elution. In order to ensure that the column was free of proteins after IMAC, a concentration of 500 mM imidazole was used after elution.

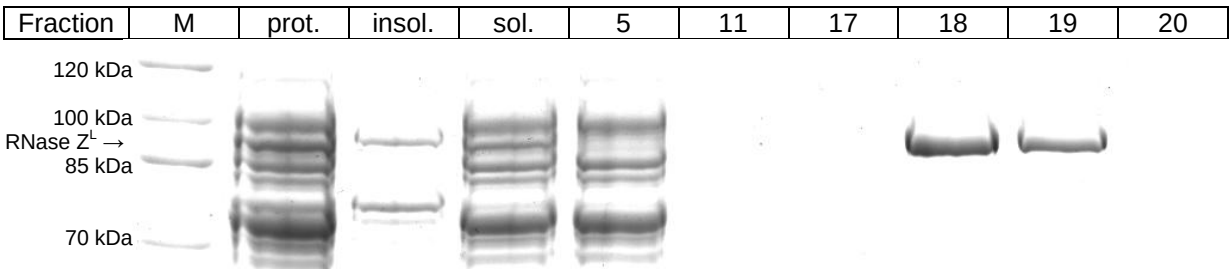

Figure 34. Analysis of optimized IMAC of RNase Z^L. SDS-PAGE of proteome ("prot."), insoluble ("insol.") and soluble ("sol." = bacterial extract) fractions after lysis and fractions collected during IMAC. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard.

When purification of RNase Z^L had been completed, enzyme assays were made with selected fractions thereof in order to test, whether purified protein showed activity. After electrophoresis of assays the gel was incubated with a phosphoimager screen. Analysis of the phosphoimager screen confirmed that the 3' trailer was removed from the substrate (radiolabelled mitochondrial pre-$tRNA^{Tyr}$), hence that purified RNase Z^L was active (Figure 35).

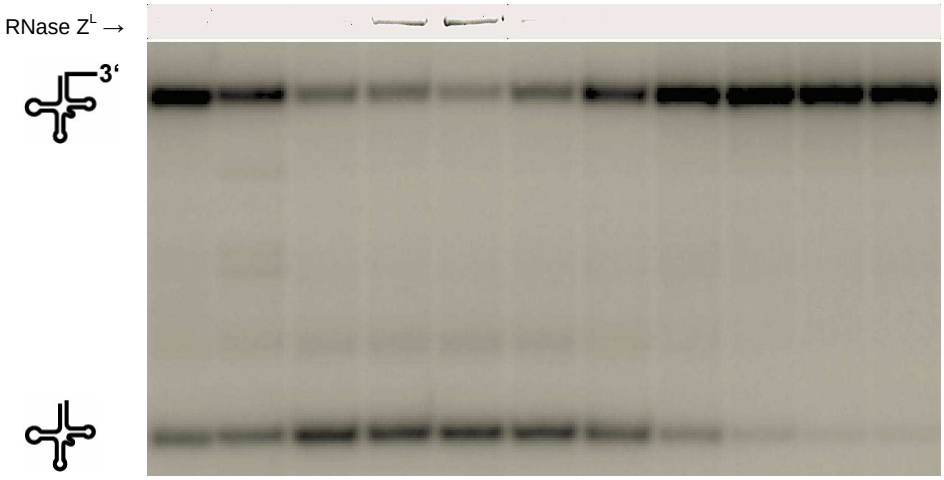

Figure 35. Activity of RNase Z^L. SDS-PAGE (above) and autoradiogram of denaturing polyacrylamide gel electrophoresis (below) of eleven consecutive fractions collected during elution of RNase Z^L in the course of affinity chromatography. Radiolabelled mitochondrial pre-tRNA^{Tyr} served as substrate for enzyme assays.

Purification of tRNA ribonucleotidyl transferase

In order to determine concentrations needed for washing and elution, this IMAC was performed: after equilibration, loading and washing without imidazole, linear increasing concentrations of imidazole ranging from 0 mM to 300 mM were applied during IMAC of tRNA ribonucleotidyl transferase. Absorption at 280 nm, conductivity and pressure were recorded during chromatography (Figure 36).

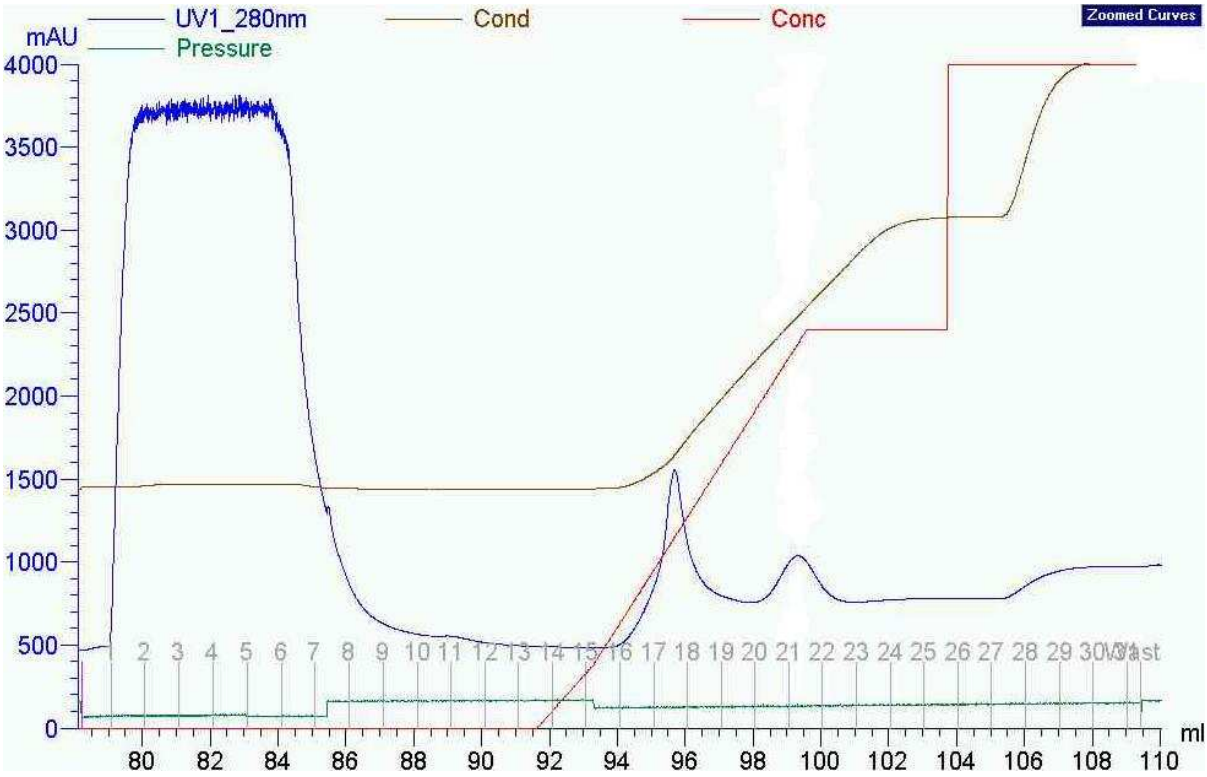

Figure 36. Determination of imidazole concentration required for washing and elution of tRNA ribonucleotidyl transferase. Chromatogram of IMAC showing absorption at 280 nm (blue), conductivity (brown), pressure (green) and concentration of imidazole (red) ranging from 0 mM to 300 mM imidazole. In order to ensure that the column was free of proteins after IMAC, a concentration of 500 mM imidazole was used after elution.

Fractions (1 ml each) were collected during IMAC and subsequently analyzed via SDS-PAGE. This facilitated the decision, which imidazole concentrations were apt for washing and elution (Figure 37).

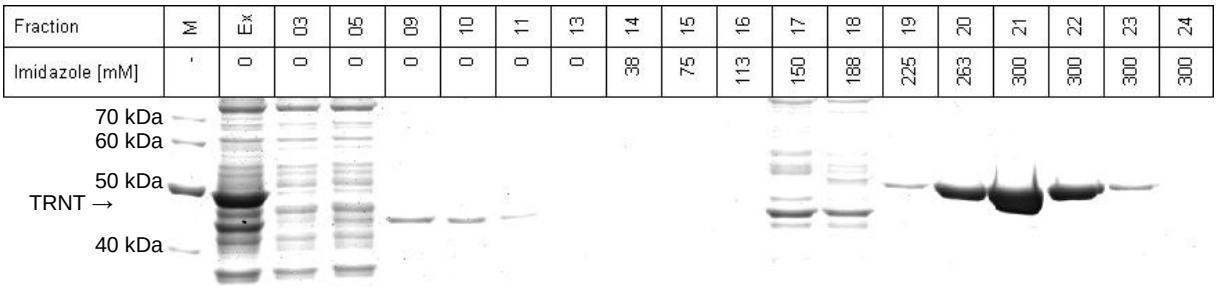

Figure 37. Analysis of IMAC of tRNA ribonucleotidyl transferase ("TRNT") having used a linear imidazole gradient. SDS-PAGE of bacterial extract ("Ex") and selected fractions taken during IMAC. Imidazole concentrations were calculated (linear progression). Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard.

Since the linear imidazole gradient for IMAC of tRNA ribonucleotidyl transferase was obviously to steep, conditions for IMAC of this enzyme were chosen based on the experiences with RNase Z^S and RNase Z^L. Therefore a concentration of 100 mM imidazole was chosen for washing, while 300 mM imidazole were chosen for elution of tRNA ribonucleotidyl transferase.

Additional chromatography experiments helped to determine imidazole concentrations needed for equilibration and during loading of bacterial extract. As it turned out a concentration of 40 mM imidazole helped to prevent unspecific binding of bacterial proteins during loading and was low enough to enable tRNA ribonucleotidyl transferase to bind to the chromatography material in the column. Optimized IMACs of tRNA ribonucleotidyl transferase were performed using step gradients of imidazole concentrations and yielded highly concentrated and satisfactorily purified protein (Figures 38 and 39).

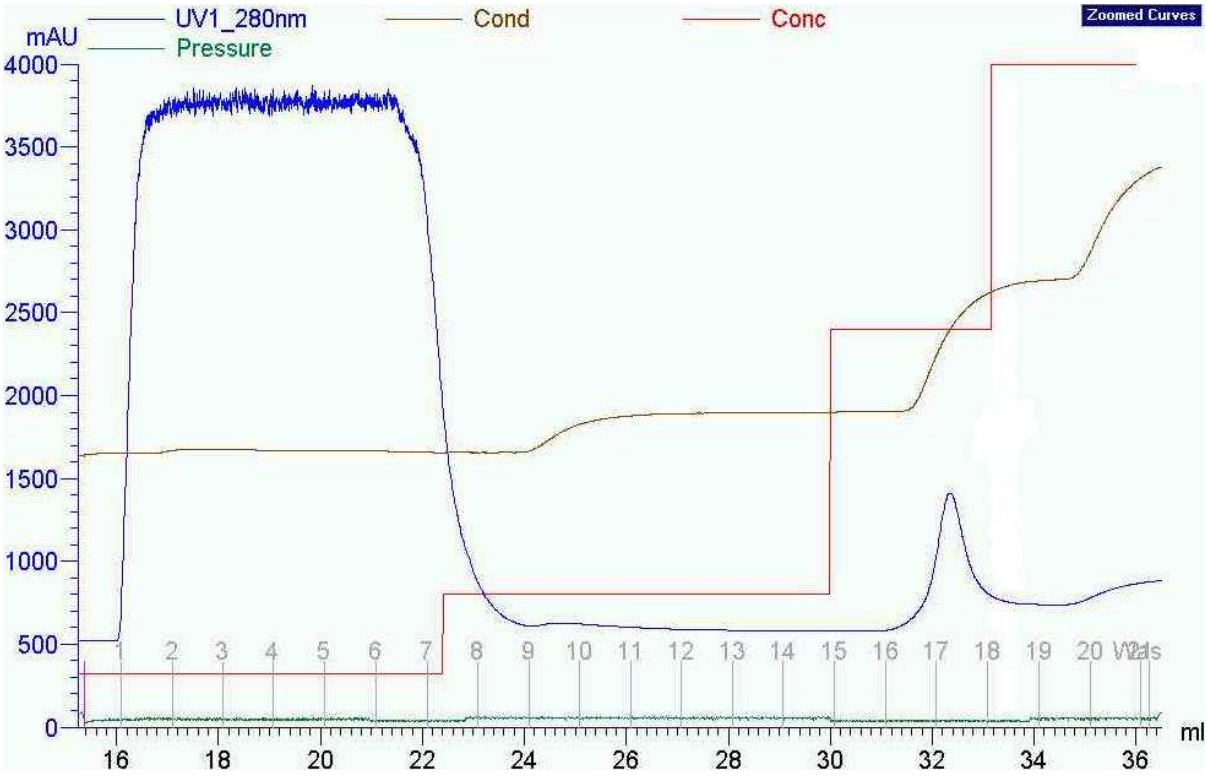

Figure 38. Optimized IMAC of tRNA ribonucleotidyl transferase. Chromatogram showing absorption at 280 nm (blue), conductivity (brown), pressure (green) and concentration of imidazole (red): a concentration of 40 mM imidazole was used for equilibration of column and loading of extract, 100 mM imidazole for washing and 300 mM imidazole for elution. In order to ensure that the column was free of proteins after IMAC, a concentration of 500 mM imidazole was used after elution.

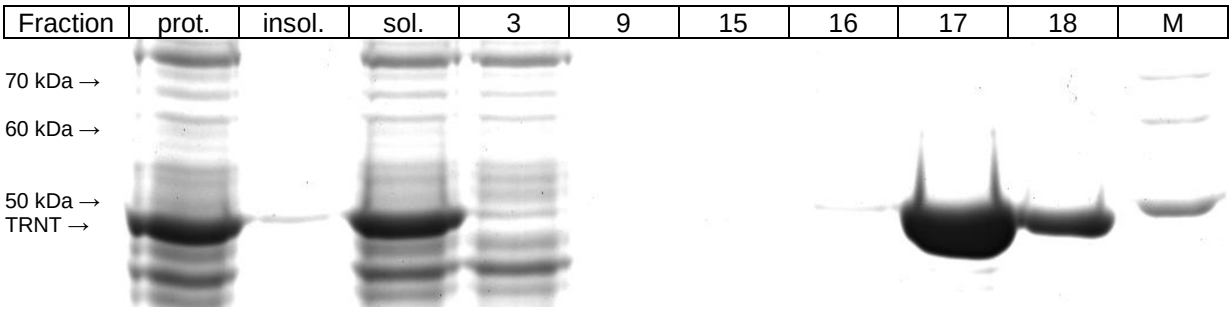

Figure 39. Analysis of optimized IMAC of tRNA ribonucleotidyl transferase ("TRNT"). SDS-PAGE of proteome ("prot."), insoluble ("insol.") and soluble ("sol." = bacterial extract) fractions after lysis and fractions collected during IMAC. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard.

When purification of tRNA ribonucleotidyl transferase had been completed, enzyme assays were made with the purified protein in order to test, whether it showed activity. After electrophoresis of assays the gel was incubated with a phosphoimager screen. Analysis of the phosphoimager screen confirmed that the 3' terminal nucleotides (CCA) were added to the substrate (radiolabelled mitochondrial pre-tRNA^{Tyr} without a 3' trailer sequence), hence that purified tRNA ribonucleotidyl transferase was active (Figure 40). Adenine, however, was not added to the pre-tRNA quantitatively, if concentration of enzyme was too low.

TRNT [µg/ml]	0	10	10	1	0.1
ATP (1 mM)	+	-	+	+	+

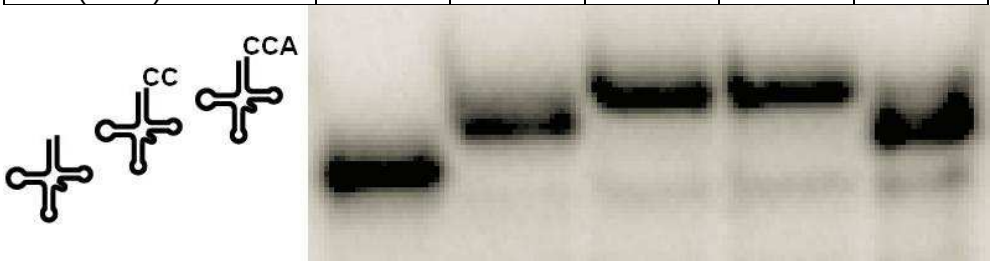

Figure 40. Activity of tRNA ribonucleotidyl transferase. Autoradiogram of denaturing polyacrylamide gel electrophoresis having used purified tRNA ribonucleotidyl transferase ("TRNT") for enzyme assays. Radiolabelled mitochondrial pre-tRNA^{Tyr} served as substrate. Note that 0.1 mM CTP was present in every reaction buffer.

Storage of purified proteins

Having optimized IMACs of all three recombinant proteins storage conditions were tested. Therefore, fractions of purified RNase Z^{S} and RNase Z^{L} were concentrated by filtration (as described in "Materials and Methods") and tenfold diluted by addition of six different storage buffers. Final concentrations are shown below.

- buffer 1: 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 50% glycerol, 5 mM DTT, 0.1% Tween-20, 0.1% protease inhibitor and 30 mM imidazole (remaining from the purification procedure)
- buffer 2: as buffer 1, except for 100 mM $(NH_4)_2SO_4$ instead of NaCl, which was reduced to a final concentration of 10 mM remaining from the preceding procedures
- buffer 3: as buffer 1, except for addition of 20 mM $MgCl_2$
- buffer 4: as buffer 1, except for addition of 1 mg/ml BSA
- buffer 5: as buffer 1, except for addition of 1 mM EDTA
- buffer 6: as buffer 1, except for addition of 5% PEG-6000

Enzymes were stored at -20℃ (liquidly) and at -80° C after being shock-frozen in liquid nitrogen. Enzyme assays thereof were made immediately after dilution by buffer 1 to 6 and after 5 days of storage (as described in “Materials and Methods”). After electrophoresis of assays the gel was incubated with a phosphoimager screen. Analysis of the phosphoimager screen showed that addition of 1 mM EDTA (buffer 5) was favourable, most probably due to its inhibition of metalloproteases. Ammonium sulfate (buffer 2) seemed to conserve a high level of activity, too, if used as salt in storage buffers (Figure 41).

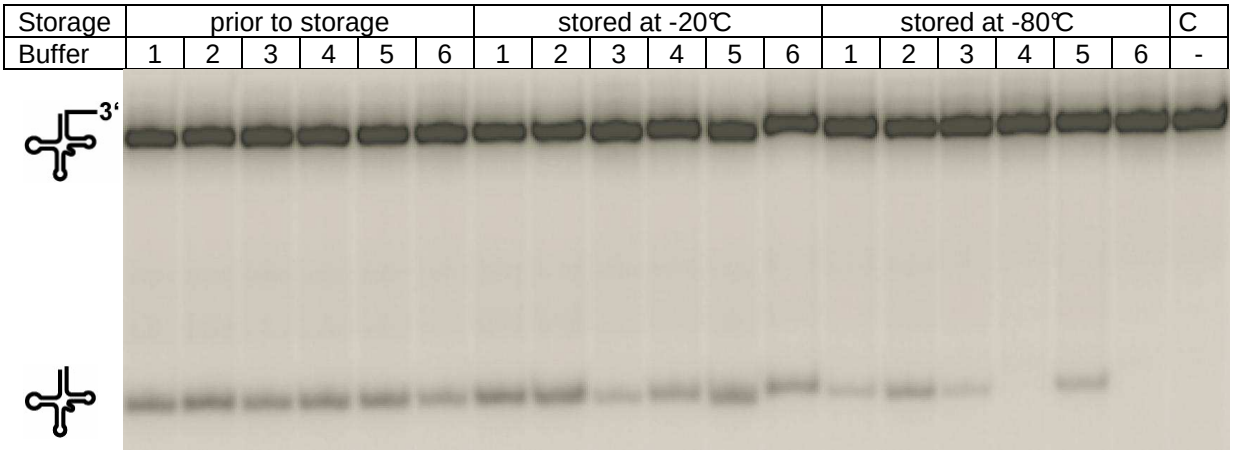

Figure 41. Activity of differently stored RNase Z^L. Autoradiogram of denaturing polyacrylamide gel electrophoresis having used purified and differently stored RNase Z^L for enzyme assays (“C” is the negative control without enzyme). Radiolabelled mitochondrial pre-$tRNA^{Tyr}$ served as substrate. See text for information on buffers 1 to 6.

Nevertheless, it was still unclear, whether $(NH_4)_2SO_4$ surpassed NaCl as salt in storage buffers or not. Therefore, another experiment was set up with buffer 2 (+ addition of 1 mM EDTA) and buffer 5. After electrophoresis of assays the gel was incubated with a phosphoimager screen. Since the analysis of the phosphoimager screen showed no significant difference between both salts, NaCl was chosen (data not shown). Therefore, the final storage buffer was composed of 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 50% glycerol, 5 mM DTT, 1 mM EDTA, 0.1% Tween-20, 0.1% protease inhibitor and 30 mM imidazole (remaining from the purification procedure).

Quarternary structure

The primary objective (expression and purification of three enzymes) was achieved. Since these enzymes were active (Figures 30, 35 and 40) and could be stored liquidly at -20℃ and -80℃ retaining most of their activity, all goals of expression and purification had been met. A secondary objective was the analysis of the quaternary structure of the RNase Z isoforms.

In order to determine, if recombinant proteins RNase Z^S and RNase Z^L are active as mono- or dimers, four different experiments were performed: gel filtration chromatography (size exclusion chromatography with aqueous mobile phase), glycerol gradient centrifugation, blue native PAGE and chemical cross-linking. The latter two were done with recombinant tRNA ribonucleotidyl transferase as well. Proteins as chymotrypsinogen (25 kDa), ovalbumin (43 kDa), BSA (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) served as molecular weight standards.

Size exclusion chromatography

Size exclusion chromatographies were performed and selected fractions thereof were analyzed via SDS-PAGE and enzyme assays. Four marker proteins were used for size exclusion chromatography of purified RNase Z^S: chymotrypsinogen, BSA, aldolase and ferritin. RNase Z^S (monomer: ~41 kDa) seemed to elute prior to BSA (67 kDa) but after aldolase (158 kDa), which can be seen as monomer of ~40 kDa in SDS-PAGE analysis (Figure 42). This indicated that RNase Z^S had a native molecular weight between BSA and aldolase and was therefore believed to form homodimers. Ferritin (440 kDa), an oligomer consisting of 24 subunits, cannot be seen in the SDS-PAGE below (Figure 42), whereas chymotrypsinogen (25 kDa) is visible at the lower end of the gel eluting at last.

Since all marker proteins were stored frozen in aqueous solution and had to be thawed prior to use each time, chymotrypsinogen may have been activated by unspecific proteolysis forming chymotrypsin. Protease chmyotrypsin could be responsible for degradation products of proteins, which can be seen in SDS-PAGE analysis as undefined bands.

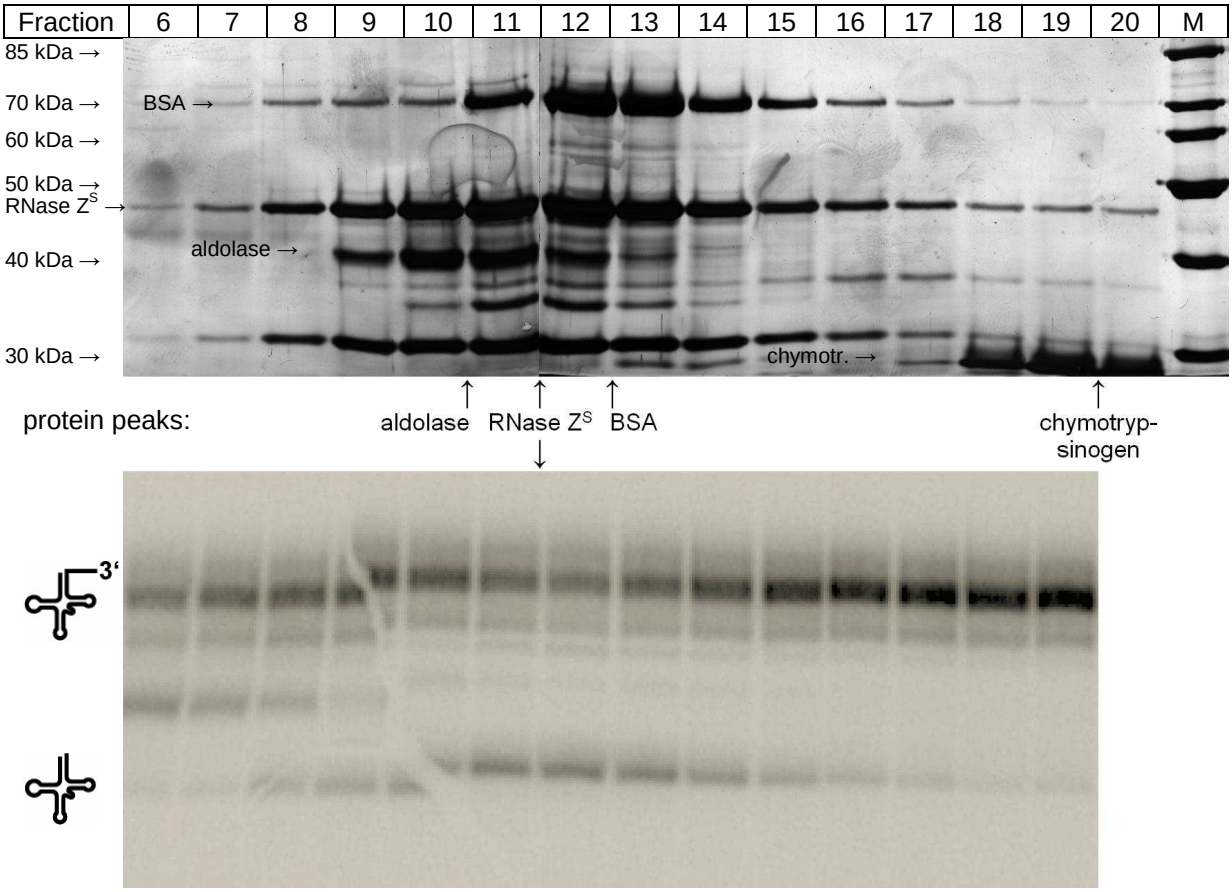

Figure 42. Size exclusion chromatography of RNase Z^S. SDS-PAGE (above) and autoradiogram of denaturing polyacrylamide gel electrophoresis (below) of 15/14 consecutive fractions collected during SEC. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard for SDS-PAGE. Radiolabelled nuclear pre-$tRNA^{Arg}$ served as substrate for enzyme assays. Note that there are many protein bands in SDS-PAGE visible corresponding to proteolytical fragments, which may be caused by chymotrypsin, which in turns may have originated from chymotrypsinogen.

Since resolution of size exclusion chromatography with RNase Z^S was rather unsatisfying, parameters were changed for SEC with RNase Z^L: smaller fractions (0.25 ml) were taken and the flow rate was decreased to 0.25 ml per minute. Furthermore chymotrypsinogen and ferritin were omitted as marker proteins and ovalbumin (43 kDa) and catalase (232 kDa) were used instead.

Surprisingly recombinant RNase Z^L seemed to elute at the same time or even slightly earlier as aldolase (158 kDa, can be seen as ~40 kDa monomer in SDS-PAGE analysis). This indicated that RNase Z^L (monomer: ~92 kDa) had a native molecular weight as aldolase or even higher and could therefore be believed to form homodimers judged solely by this experiment. Catalase, which can be seen in SDS-PAGE analysis as monomer of ~58 kDa, peaked at first (approximately at fraction 20 to 21), followed by RNase Z^L (fractions ~22 to 23), whereas aldolase seemed to peak at fraction ~23. BSA reached its maximum not until fractions ~26 to 27 and ovalbumin eluted at last (maximum at fraction ~31).

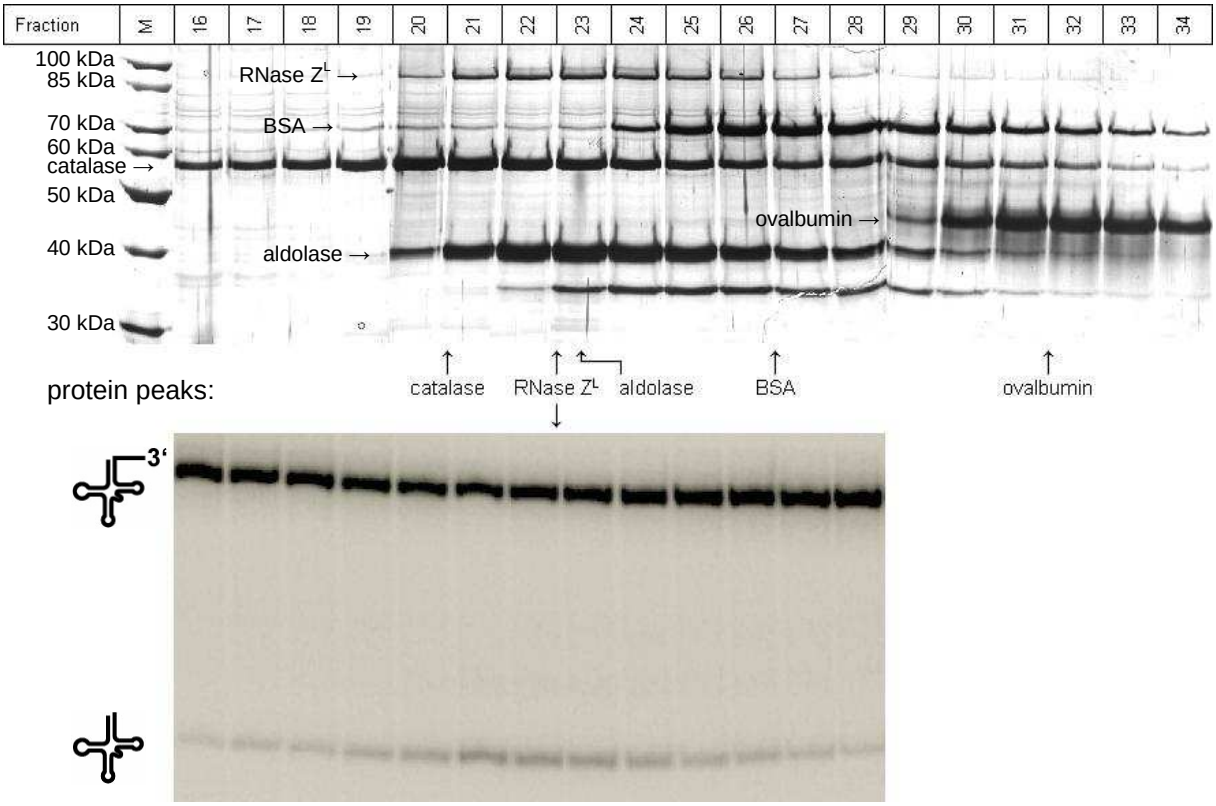

Figure 43. Size exclusion chromatography of RNase Z^L. SDS-PAGE (above) and autoradiogram of denaturing polyacrylamide gel electrophoresis (below) of 19/13 consecutive fractions collected during SEC. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard for SDS-PAGE. Radiolabelled mitochondrial pre-$tRNA^{Tyr}$ served as substrate for enzyme assays.

Glycerol gradient centrifugation

Glycerol gradient centrifugations were performed and selected fractions thereof analyzed via SDS-PAGE and enzyme assays. These experiments were performed with recombinant RNase Z^L and RNase Z^S as well as with mitochondrial extracts from HeLa cells (native RNase Z^L) in different concentrations of NaCl (100, 150 and 300 mM).

SDS-PAGE and enzyme assays of glycerol gradient centrifugation of recombinant RNase Z^L in 100 mM NaCl are shown below (Figure 44). Fractions were taken from top to bottom, therefore ovalbumin (43 kDa) can be seen first (mostly fraction ~8) in SDS-PAGE analysis followed by BSA (67 kDa), which peaked at fraction 10. Recombinant RNase Z^L (monomer: ~92 kDa) can be seen in fraction 11 in SDS-PAGE analysis and mostly in fractions 10 to 12 in enzyme assay analysis. Aldolase (158 kDa) can be seen as ~40 kDa monomer in fractions 15 to 16 and catalase (232 kDa) as ~58 kDa monomer in fractions 20 to 21. Recombinant RNase Z^L seemed to have a native molecular weight between BSA and aldolase and hence seemed to be monomeric judged by glycerol gradient centrifugation.

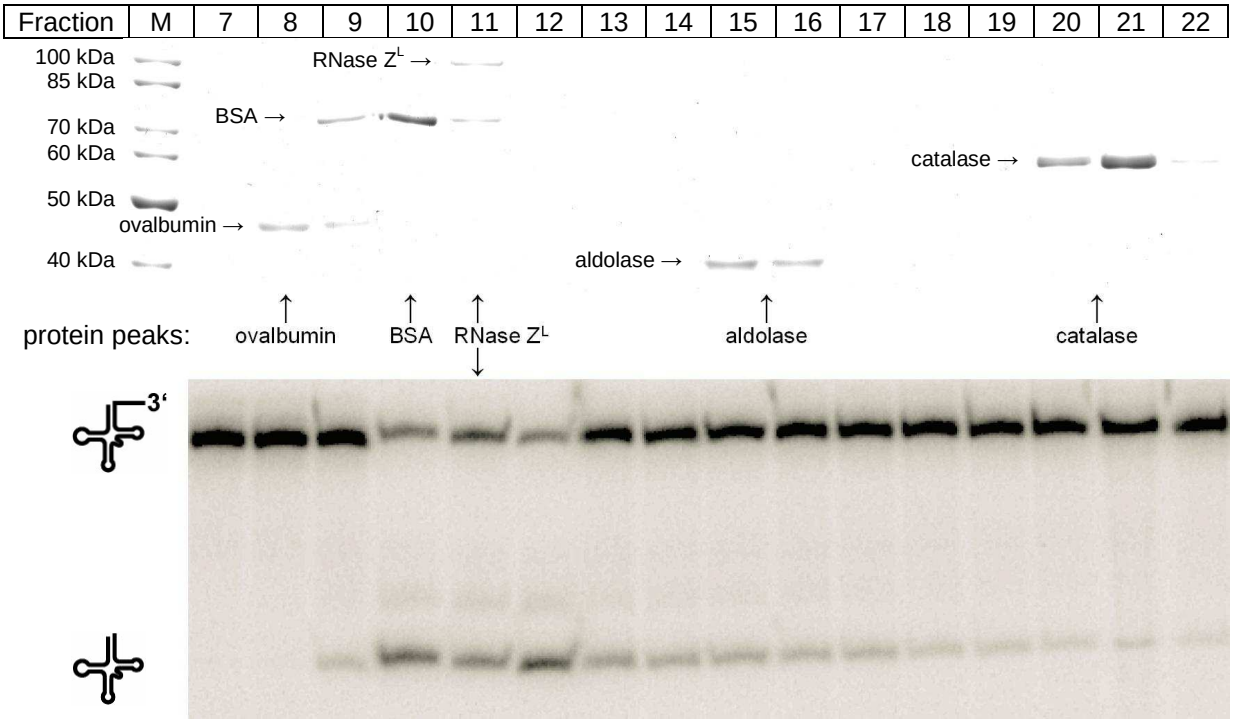

Figure 44. Glycerol gradient centrifugation of RNase Z^L. SDS-PAGE (above) and autoradiogram of denaturing polyacrylamide gel electrophoresis (below) of 16 consecutive fractions collected after glycerol gradient centrifugation. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard for SDS-PAGE. Radiolabelled mitochondrial pre-$tRNA^{Tyr}$ served as substrate for enzyme assays.

In glycerol gradient centrifugation experiments with lower salt concentrations (100 mM and 150 mM NaCl) recombinant RNase Z^L tended to associate forming very unstable "dimers": SDS-PAGE analysis of glycerol gradient centrifugations under these conditions showed clearly visible bands of recombinant RNase Z^L (as shown above) and very thin bands. Clearly visible bands corresponded to a molecular weight of ~85 kDa judged by comparison with marker proteins and thin bands corresponded to ~140 kDa. These thin bands, however, cannot be seen on the photos. In addition, enzyme activities seemed to decrease more slowly with increasing number of fraction and hence higher molecular weight compared to glycerol gradients with 300 mM NaCl.

Recombinant RNase Z^S (monomer: ~41 kDa) showed a molecular weight corresponding to ~80 kDa judged by comparison with marker proteins. In contrast to recombinant RNase Z^L this result was independent of NaCl concentrations (150 or 300 mM) and RNase Z^S was therefore believed to form homodimers.

The results of glycerol gradient centrifugations of mitochondrial extracts from HeLa cells (native RNase Z^L) matched those of recombinant RNase Z^L to some extent: increasing salt concentrations decreased molecular weight. But native RNase Z^L seemed to be even heavier than recombinant RNase Z^L probably due to other

proteins sticking to it. This was in accordance with the results of Rossmanith and Karwan: they performed glycerol gradient centrifugations with the same glycerol gradients (15-30% glycerol) as presented within this thesis, but with 150 mM KCl instead of NaCl as the main difference. Mitochondrial RNase Z^L extracted from HeLa cells (called "(mt)3' pre-tRNase") seemed to have a molecular weight of ~225 kDa in those experiments (Rossmanith and Karwan 1998).

	native RNase Z^L	recomb. RNase Z^L	recomb. RNase Z^S
100 mM NaCl	200 kDa	85 kDa (+ 140 kDa)	-
150 mM NaCl	160 kDa	85 kDa (+ 140 kDa)	80 kDa
300 mM NaCl	110 kDa	85 kDa	80 kDa
calculated monomer	92 kDa	92 kDa	41 kDa

Figure 45. Glycerol gradient centrifugations with different salt concentrations. Information in brackets indicate molecular weights corresponding to thin bands of RNase Z^L visible in SDS-PAGE (see text for further information).

Blue native PAGE

Size exclusion chromatography and glycerol gradient centrifugation suggested contradictory results for the quarternary structure of recombinant RNase Z^L (monomer or dimer). Therefore, other methods were used to confirm one result or the other. Blue native PAGE was performed using ovalbumin (43 kDa), BSA (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) as molecular weight standards. Recombinant RNase Z^S and RNase Z^L as well as tRNA ribonucleotidyl transferase were tested, whether they showed formation of mono- or dimers in blue native PAGE.

Whereas RNase Z^S could not be resolved at all, RNase Z^L seemed to have a molecular weight similar to catalase (232 kDa). This indicated formation of homodimers, but there was a vague band in the range of the monomer (~92 kDa),

too. Recombinant tRNA ribonucleotidyl transferase, on the other hand, migrated as a monomer (~51 kDa) between ovalbumin and BSA.

Protein	ovalb.	BSA	aldol.	catal.	ferritin	thyro.	Z^S	Z^L	TRNT
kDa	43	67	158	232	440	669			

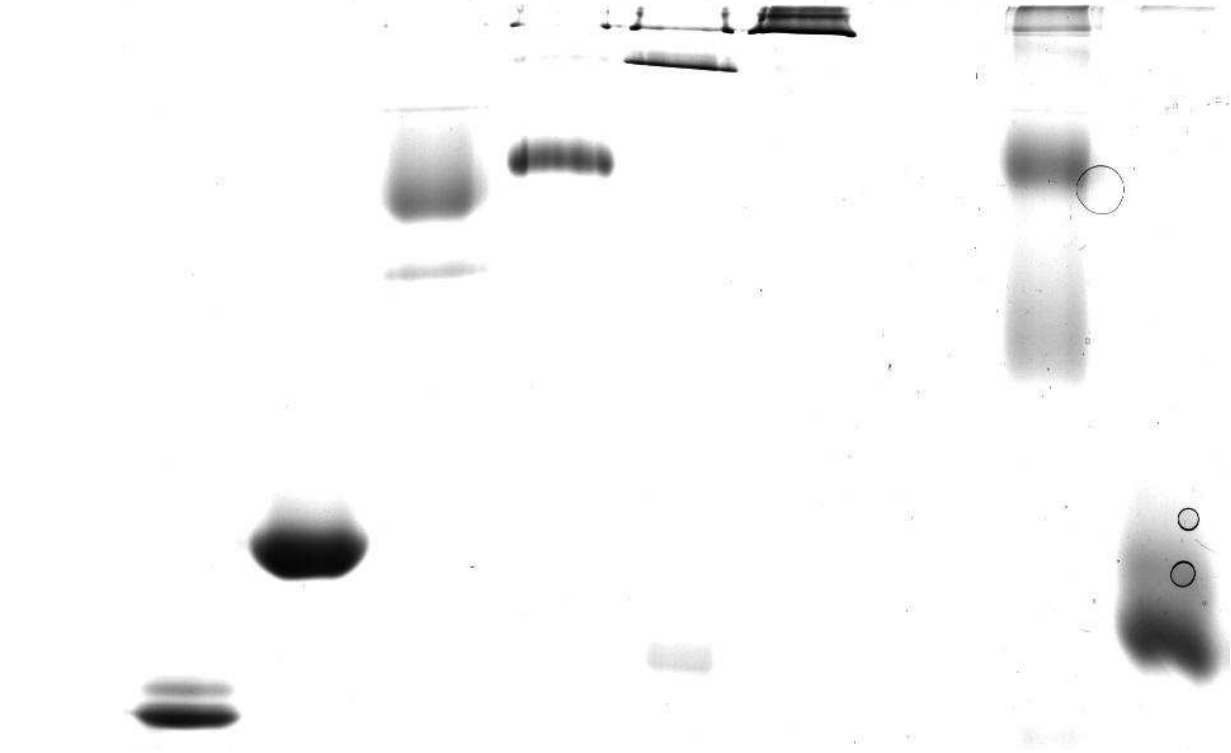

Figure 46. Blue native PAGE of recombinant proteins. Ovalbumin, BSA, aldolase, catalase, ferritin and thyroglobulin were used as molecular weight standards. Recombinant proteins: RNase Z^S ("Z^S"), RNase Z^L ("Z^L") and tRNA ribonucleotidyl transferase ("TNRT"). Note that RNase Z^S could not be displayed in blue native PAGE.

Chemical cross-linking

Another experiment to confirm formation of di- or oligomers is chemical cross-linking. Glutaraldehyde, BS^3 and EDC were used for this purpose on recombinant RNase Z^S and RNase Z^L. These proteins were subsequently analyzed via SDS-PAGE.

Dimers of RNase Z^S could clearly be cross-linked by EDC and BS^3 and to a lesser extent by glutaraldehyde. RNase Z^L, on the other hand, showed no formation of dimers upon treatment with cross-linkers. Treatment of RNase Z^L with glutaraldehyde produced a vague smear over a distinct monomer-band, but glutaraldehyde is not a specific cross-linker.

Protein	M	RNase Z^S				RNase Z^L			
Cross-linker	-	-	glutar.	EDC	BS^3	-	glutar.	EDC	BS^3

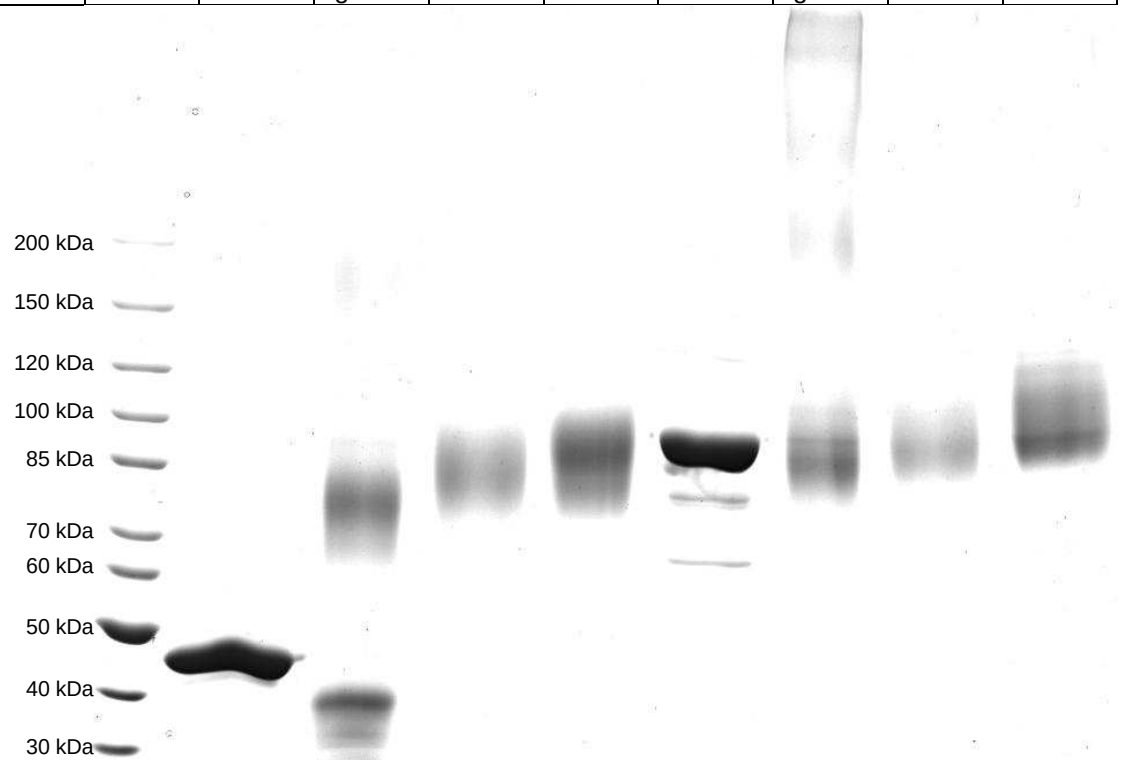

Figure 47. Chemical cross-linking of recombinant RNases Z. SDS-PAGE of RNase Z^S and RNase Z^L treated with cross-linkers glutaraldehyde, EDC and BS^3. Marker (PageRuler™ unstained protein ladder, Fermentas) served as molecular weight standard.

Discussion

Codon usage was crucial for expression of RNase Z^L

The main goal of this thesis was the recombinant expression of three human enzymes: RNase Z^S, RNase Z^L and tRNA ribonucleotidyl transferase. It is well known that many eukaryotic genes can be expressed in Escherichia coli to a different extent, whereas others cannot be expressed at all. Therefore it was assumed prior to cloning and transformation procedures that it might be useful to consider two different strains of expression hosts: BL21(DE3), the standard expression host for T7 expression systems, and Rosetta2(DE3), basically a BL21(DE3) with improved ability of expressing eukaryotic genes due to an additional set of rare tRNAs.

As it turned out, the initial transformation of two different hosts was chosen wisely, since RNase Z^L could not be expressed in BL21(DE3), but nonetheless in Rosetta2(DE3). Most probably this was due to Rosetta's additional rare tRNAs, which aided the expression of eukaryotic genes. Nevertheless, optimization of expression of RNase Z^L in Rosetta2(DE3) was necessary, since the ratio of soluble to insoluble protein was rather low. Recombinant RNase Z^S and tRNA ribonucleotidyl transferase, on the other hand, could be expressed in the standard expression host BL21(DE3), maybe due to a less human-specific codon usage and shorter length of the protein.

Decrease in the expression rate of RNase Z reduced the formation of inclusion bodies

Formation of inclusion bodies reached rather high levels (50% and higher) during expression of RNase Z^S and RNase Z^L. Since inclusion bodies are insoluble aggregates of misfolded proteins, overproduction of recombinant protein seemed to be responsible. Recombinant protein can make up half of total cell protein after a few hours of expression. This overproduction may lead to formation of inclusion bodies in two different ways: high concentrations of folding intermediates might accumulate inside the cell, if recombinant proteins are normally folding spontaneously. If they need help from the cellular folding machinery, i. e. chaperonins, the bacterial folding machinery might become overloaded, which leads to misfolded recombinant proteins. Or, in other words, if transcription and thus translation of recombinant proteins, which need chaperonins for proper folding, exceed maximal reaction rate (V_{max}) of these

chaperonins, they will become saturated and, as a consequence, spare proteins will become misfolded. These misfolded proteins form inclusion bodies inside the cell due to their bad solubility. Therefore the ratio of soluble to insoluble recombinant protein can be increased by lowering its transcription and/or translation (Sørensen and Mortensen 2005). Recombinant tRNA ribonucleotidyl transferase, on the other hand, was almost completely soluble and thus formed almost no inclusion bodies upon expression in BL21(DE3). This could be due to a different mode of folding in comparison to RNase Z^S and RNase Z^L.

Strategies for slowing down expression and thus avoiding formation of inclusion bodies include lowering expression temperature and using less IPTG for induction. In general, lowering temperature leads to lower reaction rates: proteins are expressed more slowly, proteases are less active and growth is reduced significantly. Therefore unfolded proteins are less likely to accumulate or overload chaperonins and active proteins become less likely degraded by proteases, but expression times have to be extended. Additionally, higher temperatures seem to favour aggregation reactions over proper folding of proteins, wherefore lowering of expression temperature is believed to support folding reactions (Sørensen and Mortensen 2005). The results of expression experiments with different temperatures presented within this thesis were as expected: lowering expression temperature increased ratio of soluble to insoluble recombinant protein, thus minimizing formation of inclusion bodies.

Using lower concentrations of IPTG for induction is believed to reduce formation of inclusion bodies due to slowing down expression of recombinant protein, thereby preventing accumulation of unfolded protein and/or keeping chaperonins from becoming overloaded. However, the effect of lower concentrations of IPTG for induction was not as dramatic as changing expression temperature. There are two possible reasons: firstly, lower concentrations of IPTG do not affect aggregation and folding reactions directly. Secondly, Escherichia coli are able to take up IPTG actively via beta-galactoside permease, wherefore the concentration inside the cell does not correlate directly with the concentration outside the cell. Since expression at low temperatures seemed very promising in order to minimize formation of inclusion bodies and since the effect of lower IPTG concentrations was not very convincing, further hampering of expression seemed not reasonable. Therefore 1 mM IPTG was used for expression in almost all experiments.

Alternative strategies for decreasing inclusion body formation during expression of RNase Z were less effective

Additionally, formation of inclusion bodies may be decreased by adding N- or C-terminal extensions (i. e. solubility promoting tags), co-expressing chaperonins (e. g. GroEL + GroES) or adding cofactor to growth medium. The latter aids proteins, which cannot fold properly without their cofactor.

Co-expression of cold adapted chaperonins

Since another strain of E. coli (Rosetta2(DE3)) had such a great effect on the expression of RNase Z^L, it was reasonable that a different strain might reduce formation of inclusion bodies as well. Therefore ArcticExpress™(DE3) and AE-RARE(DE3) were transformed and their expression of recombinant proteins compared to BL21(DE3) and Rosetta2(DE3) respectively. ArcticExpress™(DE3) is basically a BL21(DE3) expressing additional chaperonins: Cpn10 and Cpn60 of Oleispira antarctica (homologues of GroEL and GroES of Escherichia coli). Since chaperonin complex GroEL/GroES has reduced activity at low temperatures, their cold adapted homologues from a psychrophilic bacterium were expected to overcome this problem. But ArcticExpress™(DE3) and AE-RARE(DE3) had several disadvantages: firstly, they did not produce more soluble protein, only less insoluble compared to BL21(DE3) and Rosetta2(DE3) respectively. Secondly, they expressed Cpn10 and Cpn60 in such large amounts that recombinant proteins made up even less of total protein. Thirdly, this massive overproduction of chaperonins seemed to consume a lot of the cells' energy, which led to slower growth, to which the challenge of an additional antibiotic (gentamycin) might have probably contributed to as well. Forthly, pRARE2 (additional tRNA genes) and the plasmid providing chaperonins Cpn10 and Cpn60 were not compatible, since they had the same origin of replication (P15a of pACYC). Therefore AE-RARE(DE3) was unstable and needed permanent selection using chloramphenicol and gentamycin. For all those reasons BL21(DE3) and Rosetta2(DE3) were preferred for expression.

Addition of cofactor

Some proteins will not fold properly, if they lack their cofactor. All RNase Z are believed to need Zn^{2+} as cofactor for these reasons: Firstly, RNase Z^S from Bacillus subtilis contains two Zn^{2+} per dimer judged by crystal structure analysis (Li de la

Sierra-Gallay, Pellegrini et al. 2005). Secondly, the crystal structure of RNase Z^S from Escherichia coli shows two zinc ions in the active site of each monomer and, if metal-depleted, is not active until Zn^{2+} is supplemented (Vogel, Schilling et al. 2002) (Kostelecky, Pohl et al. 2006). Thirdly, RNase Z^S from Arabidopsis thaliana shows very high affinity for Zn^{2+}. If it is depleted of metals, nevertheless, its activity can be restored by addition of Mg^{2+}, Mn^{2+}, Ca^{2+} and, of course, Zn^{2+} (Späth, Canino et al. 2007). Taken together, this leads to the assumption that all RNase Z^S need zinc ions and all RNase Z^L do probably, too. SDS-PAGE analysis of recombinant RNase Z^S and RNase Z^L expressed via Escherichia coli showed that addition of cofactor Zn^{2+} did not improve folding and hence decrease formation of inclusion bodies significantly. Nevertheless, $ZnCl_2$ was added in micromolar amounts (10 µM) to Escherichia coli expressing recombinant RNase Z^S or RNase Z^L, although concentration of Zn^{2+} present in LB medium might have been sufficient.

Alternative expression system

Since the IMPACT system was available, it was tested, whether it might provide better expression or solubility of RNase Z^L. Expression of intein-tagged RNase Z^L was surprisingly high judged by SDS-PAGE, although these tags are rather big. RNase Z^L tagged at the C-terminus was only expressed at 25°C and above, but N-terminally tagged RNase Z^L was expressed at all temperatures (15 to 30°C) in rather high amounts. Unfortunately, all intein-tagged RNase Z^L variants seemed to be insoluble, i. e. they formed inclusion bodies. Nevertheless, it was tried to purify intein-tagged RNase Z^L, because even small amounts of soluble protein (undetectable by SDS-PAGE) might have gotten concentrated in the course of affinity chromatography. But the results of some small scale expression and purification experiments showed that RNase Z^L remained undetectable via SDS-PAGE, even after affinity chromatography procedures. Furthermore, self-cleavage of fusion protein (intein-RNase Z^L) was quite inefficient judged by a small experiment and reached satisfying levels not under 16°C. Leaving a protein, which is otherwise handled with extreme care, at 16°C for a couple of hours seemed quite unreasonable, moreover.

Using alternative tags might improve solubility of recombinant proteins, as reported for tags as GST, Trx or NusA. Since these tags are rather big, they need to be removed from the protein by proteases after purification procedures. Faced with the

unsatisfying efficiency of self-cleavage by intein it was considered unreasonable to use other proteases on recombinant proteins, although they might have been more efficient. Hexahistidine-tags, on the other hand, offer some advantages: firstly, purification procedures will be rather simple, if removal of tag is not necessary. Secondly, purification by IMAC is very efficient and leads to well concentrated recombinant proteins. Thirdly, expression of hexahistidine-tagged RNase Z^S, RNase Z^L and tRNA ribonucleotidyl transferase yielded enough soluble protein, which could be enriched in the course of affinity chromatography. For all those reasons expression with pET-system using the hexahistidine tags was preferred over the IMPACT system.

Imperfect lysis mimicked bad solubility of recombinant proteins

As first expression experiments were done, lysis of bacteria was performed using lysozyme. Therefore, an aqueous solution of this enzyme was made, kept on ice and stored at 4℃ after first use. As it turned out, this enzyme lost its activity quite fast under these conditions.

As described in “Materials and Methods” crude lysates were centrifuged for separating cell debris from extract. This procedure made imperfect lysis mimic formation of inclusion bodies, since unbroken bacteria sedimented as well taking enclosed recombinant proteins with them. Therefore, solubility of recombinant proteins seemed to get worse each time, when the same aqueous solution of lysozyme was used after storage at 4℃ for a couple of days. Soon it was realized that bad lysis was to blame for this effect and alternatives were sought to preparing lysozyme freshly each time. Since all devices needed for sonication were available, sonication was tested and optimized for small bacterial suspensions (0.5 to 1 ml) as well as larger ones (6 to 11 ml).

Because formation of inclusion bodies was judged by SDS-PAGE, it could not be excluded that remaining insoluble recombinant protein was in reality soluble but still enclosed by its host, when the crude lysate was centrifuged. Therefore, experiments concerning composition of lysis buffer and addition of anionic detergents after lysis were done. Anionic detergents seemed to have no effect on lysis after sonication, which is quite reasonable considering the difference between lysis by lysozyme and sonication: while lysozyme destroys the cell wall leaving the cell membrane

vulnerable to attack by detergents, sonication causes microscopic bubbles inside the cell “exploding” it from the inside. Therefore, anionic detergents are necessary for lysis with lysozyme, while they have no (or little) effect after lysis by sonication. That is why no anionic detergents were added after lysis by sonication in further experiments.

Composition of lysis buffer was mainly based on conventional wisdom (20 mM Tris-Cl (pH 7.5), 15% Glycerin, 0.002 volumes protease inhibitor, 0.1 mM DTT) and only concentration of NaCl was subjected to further investigation. A concentration of 100 mM NaCl seemed to get more RNase Z^S dissolved in lysis buffer than 500 mM. Therefore a concentration of 100 mM sodium chloride was chosen for all lysis buffers in further experiments.

IMAC: Step elution causes concentration of recombinant protein

Immobilized metal ion affinity chromatography (IMAC) was used for single-step purification of histidine-tagged proteins. Furthermore, it had a pleasant side effect: concentration of recombinant protein.

Usually, imidazole is used for elution, which displaces the His-tag and hence the whole protein from an immobilized nickel cation in a competitive manner. This means that different concentrations of imidazole have different effects: while high concentrations cause instant elution of recombinant proteins, low concentrations just remove unspecifically bound proteins and all graduations are possible in between. Therefore step gradients of imidazole concentration can be applied as well as linear gradients in the course of affinity chromatography.

Linear gradients offer the possibility of determining the imidazole concentrations necessary for washing and elution of recombinant protein. This is especially useful, since different histidine-tagged proteins need different imidazole concentrations for these steps. Therefore linear gradients of imidazole concentrations are usually applied during first affinity chromatography of a newly expressed recombinant protein in order to gain information on concentrations needed for washing and elution. When these parameters for affinity chromatography of a recombinant protein are known, step gradients can be used instead. Nevertheless, these parameters should be balanced between purity (higher imidazole concentration during loading and washing) and yield (lower imidazole concentration during loading and washing)

depending on further use of purified proteins. Since recombinant RNase Z^S, RNase Z^L and tRNA ribonucleotidyl transferase were intended for use as parts of an in vitro tRNA-processing system in order to study pathogenic mutations of mitochondrial tRNAs, it was assumed that small impurities of bacterial proteins would not hamper their enzymatic activities or alter reaction products in significant amounts. Therefore, yield seemed to be more important than absolute purity, when concentrations for washing and elution were determined for each protein after first purifications using linear gradients of imidazole.

Step gradients offer two advantages: firstly, imidazole concentrations at any point of affinity chromatography are clearly defined, since they are not changing anytime. Therefore the purified protein will be in a buffer of well known composition. Secondly, increasing the imidazole concentration at once for elution ("step elution") causes (almost) all recombinant proteins to elute at once, thus concentrating them in very few fractions. This was impressively demonstrated by the optimized affinity chromatography of tRNA ribonucleotidyl transferase, which was concentrated in almost one single fraction (1 ml) and with an astonishing purity. Step elution was also used in the course of affinity chromatography of RNase Z^S and RNase Z^L.

In conclusion, immobilized metal ion affinity chromatography using a chromatography system (ÄKTA explorer) proved to be fast, easy to handle and provided surprisingly good results for a single-step purification procedure.

Recombinant proteins were enzymatically active

Another main goal of the primary objective of this diploma thesis was that the recombinant proteins RNase Z^S, RNase Z^L and tRNA ribonucleotidyl transferase had to be enzymatically active. Therefore enzyme assays were performed using radiolabelled pre-tRNAs as substrates. These experiments were performed immediately after purification procedures (IMAC) and after some time of storage. The results of this assays showed that the recombinant proteins were enzymatically active immediately after IMAC as well as after some time of storage under different conditions (fluid at -20°C or frozen at -80°C). It has to be mentioned, however, that RNase Z^L seemed to be more active than RNase Z^S in all these experiments, but activity depends on the substrate used for assays. Since no special experiments concerning enzyme kinetics were performed, no clear statement can be made on the differences in enzyme activity between RNase Z^S and RNase Z^L.

However, after all preceding procedures (heterologous expression, lysis, purification and storage) all recombinant proteins performed their intended enzymatic reactions well. Therefore, they could be used as parts of an in vitro tRNA-processing system so as to study pathogenic mutations of mitochondrial tRNAs. This meant that all goals of expression and purification had been met.

Human RNase Z^S is a homodimer

Crystal structures of RNase Z^S from Bacillus subtilis, Escherichia coli and Thermotoga maritima suggest homodimeric proteins. Therefore all other orthologues of RNase Z^S are believed to form homodimers as well (Ceballos and Vioque 2007).

This assumption was supported by various experiments with human recombinant RNase Z^S, at least for this particular protein. In the course of this thesis four different methods were used to address the quaternary structure of human RNase Z^S: size exclusion chromatography, glycerol gradient centrifugation, blue native PAGE and chemical cross-linking. With the exception of blue native PAGE, in which recombinant RNase Z^S could not be displayed, all other three experiments indicated a dimeric quaternary structure for this protein.

In size exclusion chromatography recombinant RNase Z^S (monomer: ~41 kDa) had a molecular weight between aldolase (158 kDa) and BSA (67 kDa). Since recombinant RNase Z^S was purified and remaining impurities (bacterial proteins) were negligible and therefore not reasonable as interaction partners, this means that recombinant RNase Z^S could be assumed to form homodimers judged by this experiment.

Molecular weight of recombinant RNase Z^S was calculated based on results of glycerol gradient centrifugation: approximately 80 kDa. In contrast to RNase Z^L this result was independent of salt concentration used in buffer (150 or 300 mM NaCl). This finding clearly supported the assumption that recombinant RNase Z^S forms stable homodimers.

Chemical cross-linking was performed using three different cross-linkers: glutaraldehyde, EDC and BS^3. All three of them were able to covalently cross-link recombinant RNase Z^S as shown via SDS-PAGE. This was another evidence for recombinant RNase Z^S being a homodimer, because only adjacent proteins can be cross-linked by these chemicals.

Summed up, all results indicated the formation of homodimers for recombinant RNase Z^S. Since it was reported that hexahistidine-tags can cause dimerization at

least for one DNA binding protein called $\pi^{30.5}$, the results for recombinant RNase Z^S can only be assumed to be true for native RNase Z^S with some caution (Wu and Filutowicz 1999). To eliminate this problem, the same experiments could be made with purified native RNase Z^S, a two-hybrid screening could be performed or the crystal structure of the native protein could be determined.

Quaternary structure of RNase Z^L is still unclear

Although it is widely assumed that all RNases Z^L are active as monomers, there seems to be no experimental evidence to support this assumption. Basically their domain structure, which resembles a covalently linked dimer, is taken as a hint for being a monomer. This domain structure is believed to have arisen by gene duplication of RNase Z^S and hence is taken as reference of its origin assuming that RNase Z^S and RNase Z^L are paralogues. The structure of RNase Z^L was predicted by Redko et al. using data of crystal structures of RNase Z^S from Bacillus subtilis, Escherichia coli and Thermotoga maritima. This structure shows a monomer capable of processing one tRNA at a time (Redko, Li de la Sierra-Gallay et al. 2007).

In the course of this thesis some experiments were performed to contribute to this ongoing debate: size exclusion chromatography, glycerol gradient centrifugation, blue native PAGE and chemical cross-linking. Most interestingly, two experiments confirmed a monomeric form of RNase Z^L, whereas the other two contradicted this current opinion and showed formation of dimers.

In size exclusion chromatography recombinant RNase Z^L (monomer: ~92 kDa) seemed to have a molecular weight as aldolase (158 kDa) or even slightly higher. Since recombinant RNase Z^L was purified and remaining impurities (bacterial proteins) were negligible and therefore not reasonable as interaction partners, this means that recombinant RNase Z^L could be assumed to form homodimers judged by this experiment.

Molecular weight of recombinant RNase Z^L was also calculated based on results of glycerol gradient centrifugation: approximately 85 kDa. In contrast to recombinant RNase Z^S this result was dependent on salt concentration used in buffer (100, 150 or 300 mM NaCl): if low salt concentrations were used (100 or 150 mM NaCl), a slight second peak appeared correlating to a molecular weight of ~140 kDa. This peak disappeared, if 300 mM NaCl were used in the buffer. Since dimers should normally be stable in 300 mM NaCl, this result supported the current opinion on RNase Z^L

being a monomer. Glycerol gradient centrifugations were also performed using mitochondrial extracts containing native human RNase Z^L. Calculated molecular weight at low salt concentration seemed even higher, but decreased with increasing salt concentrations: 200 kDa (100 mM NaCl), 160 kDa (150 mM NaCl) and 110 kDa (300 mM NaCl). This suggested one or more interaction partners in the mitochondrial extract rather than formation of homodimers.

Blue native PAGE, on the other hand, showed formation of homodimers. Recombinant RNase Z^L seemed to have a molecular weight as catalase (232 kDa) judged by this experiment, although there was a slight band correlating to a monomeric form. Strangely recombinant RNase Z^S could not be displayed in blue native PAGE and tRNA ribonucleotidyl transferase seemed to be a monomer contradicting current opinion of being a covalently linked homodimer. Maybe its linkage (a disulfide bond) became reduced in the course of the gel electrophoresis.

Chemical cross-linking was performed using three different cross-linkers: glutaraldehyde, EDC and BS^3. All three of them were not able to cross-link recombinant RNase Z^L covalently as shown via SDS-PAGE, although there seemed to be a light smear in the range of a dimeric RNase Z^L if treated with glutaraldehyde. But since glutaraldehyde is no specific cross-linker and side reactions are likely, it can be claimed that recombinant RNase Z^L could not be cross-linked by these three chemicals. Nevertheless, it has to be mentioned that failure of cross-linking is no evidence for a monomeric protein.

Taken together, no clear statement could be made, whether recombinant RNase Z^L was active as mono- or dimer. Maybe it just aggregated and formed loose “dimers” in the absence of any other interaction partner depending on the surrounding conditions. Furthermore, reported effects of hexahistidine-tags being capable of conveying dimerization complicate any statement on native RNase Z^L derived from recombinant RNase Z^L (Wu and Filutowicz 1999). To eliminate this problem, the same experiments could be made with purified native RNase Z^L, a two-hybrid screening could be performed or the crystal structure of the native protein could be determined.

Abstract

All tRNAs are transcribed as immature precursors, which have to be processed at the 3' and at the 5' end. This diploma thesis is about the enzymes maturating the 3' end in humans: RNase Z (two isozymes: RNase Z^S and RNase Z^L) removes extra nucleotides from the 3' end and tRNA ribonucleotidyl transferase adds and/or repairs the sequence CCA subsequently. Primary objective of this thesis was the recombinant expression of these proteins in Escherichia coli and their purification in order to use them as parts of an in vitro tRNA-processing system so as to study pathogenic mutations of mitochondrial tRNAs.

When bacterial expression was achieved, it was optimized to minimize formation of inclusion bodies. As a result, the pET-system (Novagen) was used for expression of C-terminally hexahistidine-tagged versions of all three proteins. RNase Z^S and tRNA ribonucleotidyl transferase were expressed via BL21(DE3) at low temperatures (~15℃) over night using 1 mM IPTG for induction and RNase Z^L was expressed via Rosetta2(DE3) under the same conditions.

Purification of recombinant proteins was done via affinity chromatography using an ÄKTA explorer chromatography system. The procedure was optimized using step gradients of imidazole concentration for loading, washing and elution. Proteins were checked for activity and subsequently stored in buffer containing 50% glycerol at -20℃ or frozen at -80℃.

Secondary objective was gaining some information on quaternary structure of both RNases Z. Four different methods were used: size exclusion chromatography, glycerol gradient centrifugation, blue native PAGE and chemical cross-linking. RNase Z^S seemes to be a homodimer judged by size exclusion chromatography, glycerol gradient centrifugation and chemical cross-linking. The quaternary structure of RNase Z^L, on the other hand, is still unclear. Whereas size exclusion chromatography and blue native PAGE suggested a homodimer, glycerol gradient centrifugation appeared to indicate a monomer or a salt-labile homodimer depending on the buffer conditions used. Chemical cross-linking failed.

Zusammenfassung

Alle tRNAs werden als unreife Vorläufer transkribiert, die an ihren 3'- und 5'-Enden prozessiert werden müssen. Diese Diplomarbeit beschäftigt sich mit den Enzymen, die für die Reifung der 3'-Enden beim Menschen zuständig sind: RNase Z (zwei Isozyme: RNase Z^S und RNase Z^L) entfernt überzählige Nukleotide am 3'-Ende und tRNA-Ribonukleotidyltransferase addiert und/oder repariert anschließend die Sequenz CCA. Primäres Ziel dieser Arbeit war die rekombinante Expression dieser Proteine in Escherichia coli und deren Reinigung, um sie als Teile eines in vitro Prozessierungssystems zur Erforschung pathogener Mutationen von tRNAs zu benutzen.

Nach erfolgreicher bakterieller Expression wurde diese optimiert, um die Einschlusskörperbildung zu minimieren. In der Folge wurde das pET-System (Novagen) für die Expression von C-terminal mit einem Hexahistidintag versehenen Versionen aller drei Proteine benutzt. RNase Z^S und tRNA-Ribonukleotidyltransferase wurden mittels BL21(DE3) bei niedrigen Temperaturen (~15℃) über Nacht unter Verwendung von 1 mM IPTG zur Induktion exprimiert und RNase Z^L wurde mittels Rosetta2(DE3) unter den selben Bedingungen exprimiert.

Die Reinigung der rekombinanten Proteine erfolgte per Affinitätschromatographie, wobei ein „ÄKTA explorer" Chromatographiesystem benutzt wurde. Die Prozedur wurde optimiert mittels Stufengradienten der Imidazolkonzentration für Laden, Waschen und Eluieren. Die Proteine wurden auf Aktivität getestet and anschließend in einem Buffer mit 50% Glycerin auf -20℃ oder gefroren auf -80℃ gelagert.

Sekundäres Ziel war die Erlangung von Information bezüglich der Quartärstruktur beider RNase Z. Vier verschiedene Methoden wurden angewandt: Gelfiltration, Glyceringradientenzentrifugation, blue native PAGE und chemisches Cross-linking. RNase Z^S schien ein Homodimer zu sein, wofür Gelfiltration, Glyceringradientenzentrifugation und chemisches Cross-linking sprachen. Im Gegensatz dazu ist die Quartärstruktur von RNase Z^L weiterhin unklar. Während Gelfiltration und blue native PAGE ein Homodimer suggerierten, schien die Glyceringradientenzentrifugation ein Monomer oder ein salzlabiles Homodimer zu zeigen abhängig von den verwendeten Bufferbedingungen. Chemisches Cross-linking schlug fehl.

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