UNIVERSITY OF THESSALY

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE HUMAN PLACENTAL RIBONUCLEASE 8

Final Year Project Thesis – Ferezi Emmanouela

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«Structural and functional characterization of the human placental ribonuclease 8»

«Δομικός και λειτουργικός χαρακτηρισμός της ανθρώπινης ριβονουκλεάσης 8 του πλακούντα»

THE ADVISORY COMMITTEE

Ester Boix

Assistant Professor of Biochemistry at the Department of Biochemistry and Molecular Biology, University Autonoma of Barcelona

Demetres D. Leonidas

Associate Professor of Biochemistry at the Department of Biochemistry and Biotechnology, University of Thessaly

Nicolaos Balatsos

Assistant Professor of Biochemistry at the Department of Biochemistry and Biotechnology, University of Thessaly

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CONTENTS

Summary

In the present work human Ribonuclease 8, a prominent member of the pancreatic ribonuclease A superfamily was produced by heterologous expression in *E. coli*, purified by chromatography and characterized by determining its enzymatic activity. The enzyme was expressed in *E. coli* BL21 cells, transformed with the pET11c vector that had an insertion of a synthetic gene encoding Ribonuclease 8. Ribonuclease was purified from inclusion bodies using a denaturation/refolding protocol and liquid chromatography using a cation exchange column and a reverse phase C18 column. Purity of the isolated protein was evaluated using SDS page electrophoresis, and quantified by the Bradford method. Ribonuclease activity of the purified protein was determined using poly(U) as a substrate by SDS electrophoresis.

Περίληψη

Στη παρούσα εργασία, η ανθρώπινη ριβονουκλεάση 8, ένα διακεκριμένο μέλος της παγκρεατικής υπεροικογένειας Α ριβονουκλεασών, παράχθηκε με ετερόλογη έκφραση σε Ε. coli, καθαρίστηκε με χρωματογραφία και χαρακτηρίστηκε με προσδιορισμό της ενζυματικής της δράσης. Το ένζυμο εκφράστηκε σε Ε. coli BL21 κύτταρα, μετασχηματίσθηκε με το φορέα pET11c που περιέχει μια ένθεση ενός συνθετικού γονιδίου που κωδικοποιεί τη ριβονουκλεάση 8. Η ριβονουκλεάση καθαρίστηκε από έγκλειστα σωμάτια χρησιμοποιώντας ένα πρωτόκολλο μετουσίωσης/αναδίπλωσης και υγρή χρωματογραφία χρησιμοποιώντας μια στήλη ανταλλαγής κατιόντων και μια στήλη αντίστροφης φάσης C18. Η καθαρότητα της απομονωμένης πρωτεΐνης εκτιμήθηκε χρησιμοποιώντας ηλεκτροφόρηση SDS – PAGE και υπολογίστηκε με τη μέθοδο Bradford. Η ενζυμική δραστικότητα της καθαρισμένης πρωτεΐνης προσδιορίστηκε χρησιμοποιώντας πολύ (U) ως υπόστρωμα σε SDS ηλεκτροφόρηση.

THEORETICAL PART

1. Ribonucleases

Ribonuclease (commonly abbreviated RNase) is a type of nuclease that catalyzes the degradation of RNA into smaller components. Ribonucleases can be divided into endoribonucleases and exoribonucleases. Endoribonucleases are ribonuclease endonucleases, which are enzymes that separate phosphodiester bonds that occur within molecular chains. Phosphodiester bonds occur between phosphates and carbohydrates, and are very important to the structure of RNA. Exoribonucleases are ribonuclease exonucleases that are responsible for degrading RNA through the removal of nucleotides towards the ends of the molecule strands. Nucleotides are molecules that, when joined together, form RNA or DNA. Exoribonuclease degrades all forms of RNA — transfer RNA, messenger RNA, ribosomal RNA, and MiRNA.

All organisms studied contain many RNases of many different classes, showing that RNA degradation is a very ancient and important process. As well as cleaning of cellular RNA that is no longer required, RNases play key roles in the maturation of all RNA molecules, both messenger RNAs that carry genetic material for making proteins, and non-coding RNAs that function in varied cellular processes. In addition, active RNA degradation systems are a first defense against RNA viruses, and provide the underlying machinery for more advanced cellular immune strategies such as RNAi.

Some cells also secrete copious quantities of non-specific RNases such as A and T1. RNases are, therefore, extremely common, resulting in very short lifespans for any RNA that is not in a protected environment. It is worth noting that all intracellular RNAs are protected from RNase activity by a number of strategies including 5' end capping, 3' end polyadenylation, and folding within an RNA protein complex (ribonucleoprotein particle or RNP).

Another mechanism of protection is ribonuclease inhibitor (RI), which comprises a relatively large fraction of cellular protein (~0.1%) in some cell types, and which binds to certain ribonucleases with the highest affinity of any protein-protein interaction; the dissociation constant for the RI-RNase A complex is ~20 fM under physiological conditions. RI is used in most laboratories that study RNA to protect their samples against degradation from environmental RNases.

Similar to restriction enzymes, which cleave highly specific sequences of double-stranded DNA, a variety of endoribonucleases that recognize and cleave specific sequences of single-stranded RNA have been recently classified.

RNases play a critical role in many biological processes, including angiogenesis and self-incompatibility in flowering plants (angiosperms). Also, RNases

in prokaryotic toxin-antitoxin systems are proposed to function as plasmid stability loci, and as stress-response elements when present on the chromosome.

2. Ribonuclease A

Pancreatic ribonuclease A (RNase A) is an endonuclease. It catalyzes the cleavage of the phosphodiester bond between the 5'-ribose of a nucleotide and the phosphate group attached to the 3'-ribose of an adjacent pyrimidine nucleotide. This cleavage forms a 2',3'-cyclic phosphate, which is then hydrolyzed to the corresponding 3'-nucleoside phosphate (Figure 1).

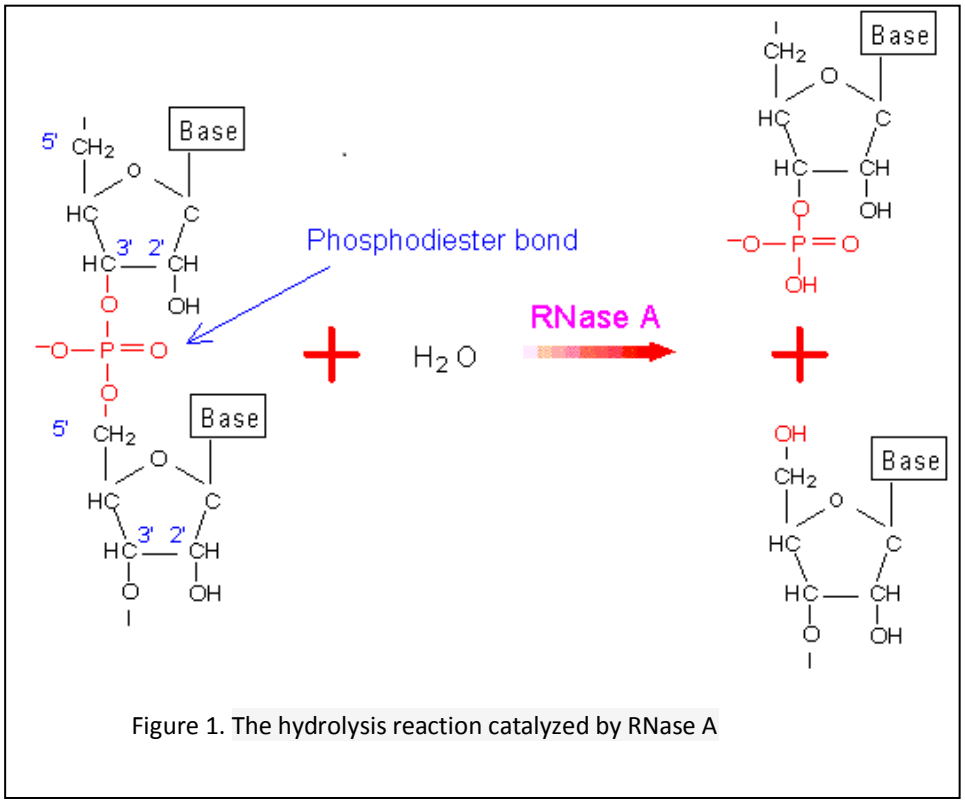


Figure 1. The hydrolysis reaction catalyzed by RNase A

RNase is found in greatest quantity in ruminant pancreases. The major component of the crystalline enzyme is RNase A; a minor component is RNase B. RNase B is the glycosylated form of RNase A.

2.1 History: The work of Jones in 1920 is usually cited as the "beginning" of pancreatic ribonucleases scientific field. RNase A was isolated by Dubos and Thompson in 1938 and crystallized by Kunitz in 1940. In 1947 Worthington was the first company to manufacture highly purified crystalline RNase A. In the early 1950s, the company Armour prepared crude crystalline enzyme, and offered it at a very affordable price. Through the 1960s and 1970s, RNase A was a favorite to study primarily because it is remarkably thermostable and present at high concentration in an accessible source, bovine pancreas. These studies led to the elucidation of its crystal structure, determination of the amino acid sequence, identification of the

catalytic mechanism, and clarification of folding pathways. RNase A was the first enzyme and third protein for which a correct amino acid sequence was determined.

Four Nobel prizes have been awarded for work associated with studies of RNase. The vast literature and numerous studies have made RNase A the most extensively studied enzyme of the 20th century.

Recent work continues to investigate the synthesis and maturation of RNase A in the endoplasmic reticulum of live cells. Much work is also still being dedicated to studying the folding and aggregation of RNase A. The enzyme's role in cancer development and gene regulation is being studied, and it is being developed into cancer chemotherapeutic agents.

2.2 Structure: RNase A is made up of a single polypeptide chain of 124 residues. Of the 20 natural amino acids, RNase A possesses 19 of them, excluding tryptophan. It can be characterized as a two-layer α + β protein that is folded in half to resemble a taco, with a deep cleft for binding the RNA substrate. The first layer is composed of three alpha helices (residues 3-13, 24-34 and 50-60) from the N-terminal half of the protein. The second layer consist of three β-hairpins (residues 61-74, 79-104 and 105-124 from the C-terminal half) arranged in two β-sheets. The hairpins 61-74 and 105-124 form a four-stranded, antiparallel β-sheet that lies on helix 3 (residues 50-60). The longest β-hairpin 79-104 mates with a short β-strand (residues 42-45) to form a three-stranded, antiparallel β-sheet that lies on helix 2 (residues 24-34).

RNase A has four disulfide bonds in its native state: Cys26-Cys84, Cys58-110, Cys40-95 and Cys65-72. The first two (26-84 and 58-110) are essential for conformational folding. Each joins an alpha helix of the first layer to a beta sheet of the second layer, forming a small hydrophobic core in its vicinity. The latter two disulfide bonds (40-95 and 65-72) are less essential for folding. Either one can be reduced (but not both) without affecting the native structure under physiological conditions. These disulfide bonds connect loop segments and are relatively exposed to solvent. Interestingly, the 65-72 disulfide bond has an extraordinarily high propensity to form, significantly more than would be expected from its loop entropy, both as a peptide and in the full-length protein. This suggests that the 61-74 β-hairpin has a high propensity to fold conformationally. The structure of RNase A is shown in figure 2.

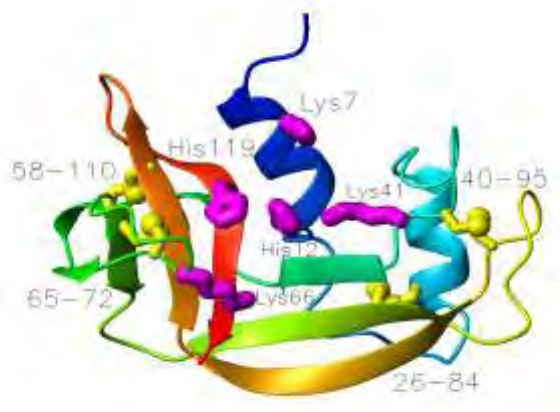


Figure 2. The backbone ribbon is colored from blue (N-terminus) to red (C-terminus). The side chains of the four disulfide-bonded cysteines are shown in yellow, with their sulfur atoms highlighted as small spheres. Residues important for catalysis are shown in figure.

2.3 Specificity: RNase A catalyzes the cleavage of the phosphodiester bonds in two steps: (i) formation of the pentavalent phosphate transition state and (ii) degradation of the 2’3’ cyclic phosphate intermediate, using three main catalytic residues (His12, Lys41, and His119). An important part of the reaction is the ability of histidine (His 12 and His119) to both accept and donate electrons, allowing these histidine to be an acid or a base, making the reaction pH dependent.

RNA hydrolysis begins when His12 abstracts a proton from the 2’ OH group on RNA, assisting thus in the nucleophilic attack of the 2’ oxygen on the electrophilic phosphorus atom. A transition state is then formed, having a pentavalent phosphate, which is stabilized by the positively charged amino group of Lys41 and the main chain amide nitrogen of Phe120. His119 then protonates the 5' oxygen on the ribose ring and the transition state falls to form a 2’3’cyclic phosphate intermediate.

In a secondary and separate reaction, the 2’,3’ cyclic phosphate is hydrolyzed to a mixture of 2'phosphate and 3' hydroxyl. His12 donates a proton to the leaving group of this reaction, the 3’ oxygen of the cyclic intermediate. Simultaneously, His-119 abstracts the proton from a water molecule, activating it for nucleophilic attack. The activated water molecule attacks the cyclic phosphate causing the cleavage of the 2'3’ cyclic phosphate intermediate. The truncated nucleotide is then released with a 3’ phosphate group

The enzyme cleaves at cytidine residues twice as fast as at uridyl residues. Thr45 has been found to be most important for mediating the pyrimidine specificity, both by forming hydrogen bonds with pyrimidine bases and sterically excluding purine bases. The side chain of Asp83 is important for stabilizing the transition state during the cleavage of uridine-containing substrates; this residue has no effect on the kinetics of cytidine cleavage. (Figure 3).

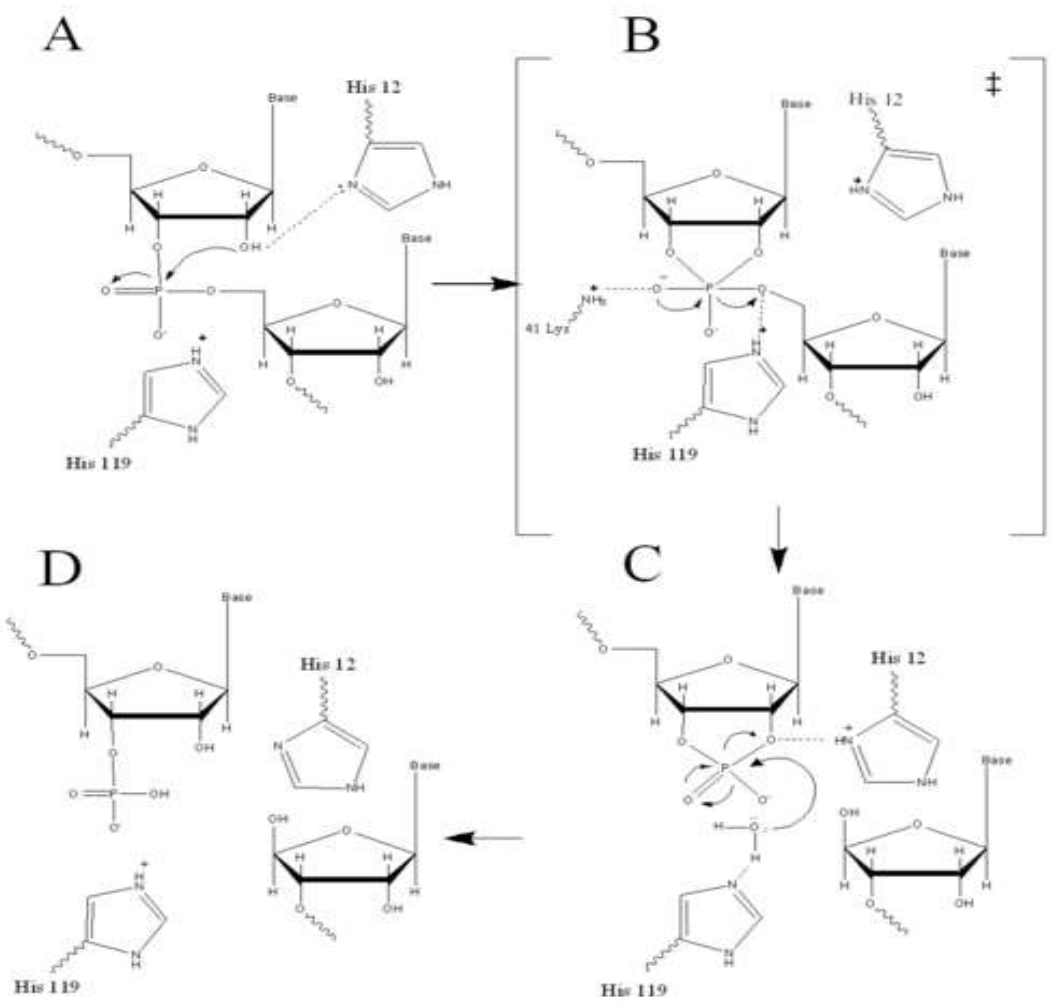


Figure 3. RNase A Catalysis. (A) Initial attack of 2'hydroxyl stabilized by His12. (B) Pentavalent phosphorous intermediate. (C) 2'3' cyclic intermediate degradation. (D) Finished products: Two distinctive nucleotide sequences.

3. The RNase A superfamily

The initial analysis of the draft human genome sequence reveals several gene families that are unique to vertebrate species. Among them, the ribonuclease (RNase A) gene superfamily is the only one that encodes proteins with enzymatic activity. The evolution of this vertebrate-specific enzyme family has received increasing attention because it serves as an excellent model for understanding the origin of new genes and novel gene function.

For over a half century since RNase A was first purified, the RNase A superfamily, of which the RNase A is the prototype, has been the one of the most intensively studied protein superfamilies in biochemistry, structural biology, enzymology and molecular evolution.

This super-family constitutes a group of homologous proteins isolated from many vertebrates, but it has not been found outside vertebrates. The ribonucleolytic activity originated multiple times during evolution and non-vertebrate organisms possess ribonucleases that are non-homologous to vertebrate RNase A. In fact, the RNase A superfamily is believed to be the sole enzyme family that is vertebrate specific. RNase proteins are typically composed of a signal peptide of about 25 amino acids and a mature peptide of about 130 amino acids. They have 3 catalytic residues (1 lysine and 2 histidine residues) at proper positions and 6 to 8 cysteine residues that form three to four disulfide bonds. Except for these conserved residues, RNases are quite divergent with sequence identities varying from 20 to nearly 100%. They also exhibit diverse expression patterns and possess various catalytic activities against specific RNA substrates. A wide variety of physiological functions are known for RNases, including degradation of dietary RNAs in the digestive gut, angiogenesis, and innate immunity. The RNase A superfamily is extremely dynamic, with high rates of gene duplication and gene loss, resulting in variable numbers of genes in different species.

The complete identification of human RNase genes was obtained a few years ago when human chromosome 14q.11.2 could be read and analyzed. The chromosome was found to contain all human RNases. However, only eight of them (RNases 1 – 8) are true RNases, called ‘’canonical RNases’’; the remaining ‘’noncanonical’’ five, numbered 9 – 13. Most differences between noncanonical and canonical RNases are found at the C – terminal region of the proteins, but RNases 9 and 10 contain an insertion of 40 – 50 residues in their signals peptides. As for their function, RNases 9 and 10 appear to have a role in the re productive tract, at least in some species, as they are expressed in mouse and porcine epididymis. The eight canonical RNases are: RNase 1 (pancreatic RNase), RNase 2 (eosinophil-derived neurotoxin, EDN), RNase 3 (eosinophil cationic protein, ECP), RNase 4, RNase 5 (also

known as angiogenin, another rapidly-evolving ribonuclease known to promote blood vessel growth with recently-discovered antibacterial activity), RNase 6 (k6),RNase 7 (an anti-pathogen ribonuclease identified in human skin) and RNase 8 .

RNases exhibit diverse expression patterns and have varying catalytic activities against specific RNA substrates. They also exhibit a variety of physiological functions, including digestion of RNA released from foregut bacteria of herbivorous mammals (RNase 1), angiogenesis (RNase 5) and inhibition of viral infection (RNases 2 and 3). The biochemical nature of RNase A ribonucleases and the emergence of the RNase A gene superfamily have been reviewed extensively. A phylogenetic tree documenting the relationships among representative RNase A ribonucleases is shown in Figure 3. Upon completion of the human genome, eight fully, catalytically active RNase A ribonucleases (numbered 1–8) were identified together with five divergent sequences that encode proteins that are structurally incapable of degrading polymeric RNA. Each RNase A RNase displays some degree of nucleotide preference within RNA substrates, but they do not have rigidly selective recognition or cleavage sites. Among the signature features of this gene family, all active RNase A ribonuclease coding sequences are initiated with a signal sequence, all mature proteins include six to eight appropriately spaced cysteines that form distinct disulfide bonds, and all include two catalytic histidine residues and a single lysine residue, the latter within an invariant sequence motif (CKXXNTF). As a rule, RNase A ribonuclease coding sequences are found on a single exon and in the human genome, are found on chromosome 14.

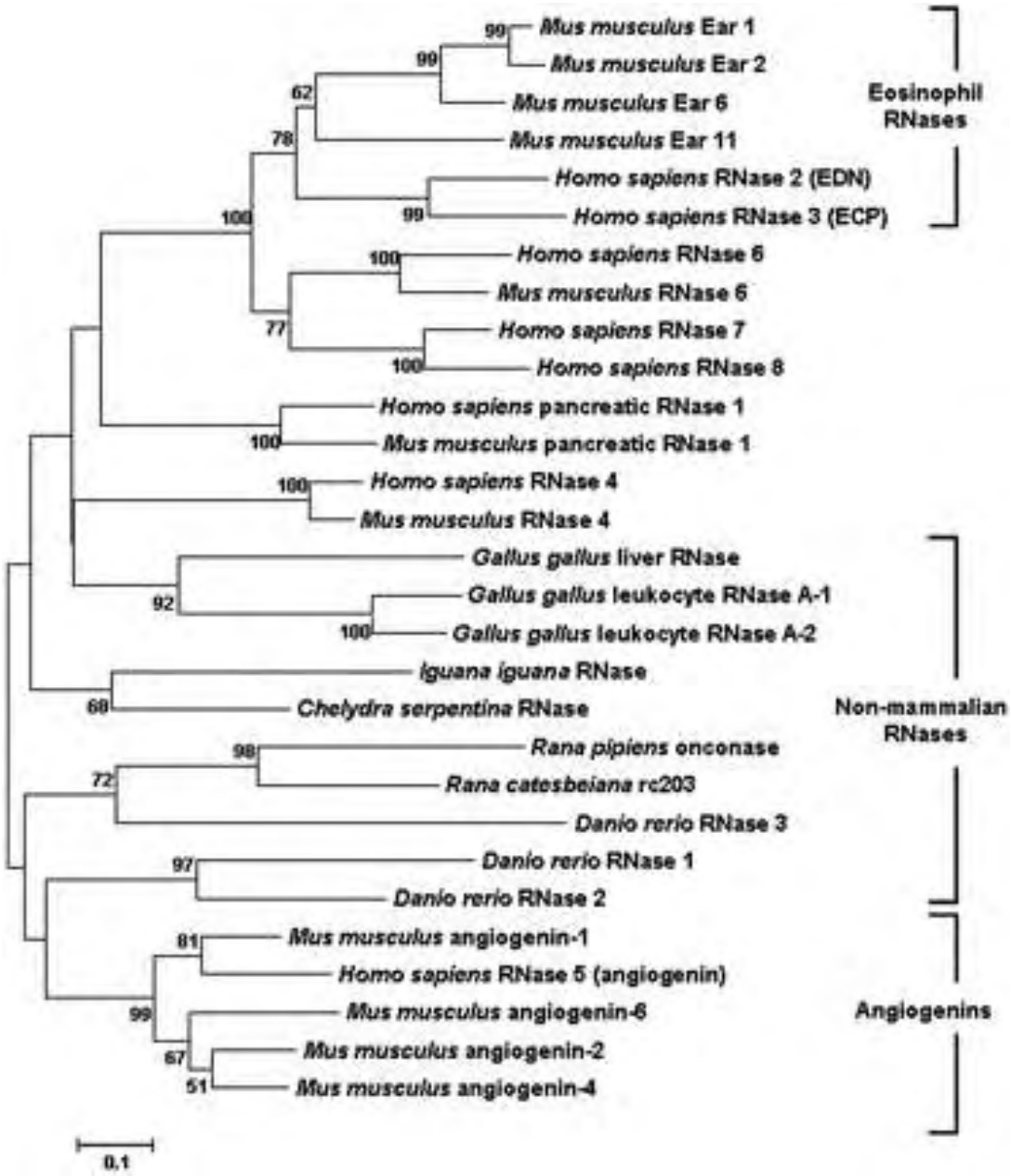


Figure 4. The relationships among representative RNase A ribonucleases

4. Antimicrobial RNases

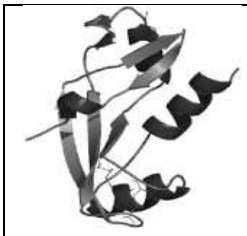
Antimicrobial proteins comprise various groups of small gene-encoded endogenous proteins exhibiting a broad spectrum of microbicidal activity against bacteria, fungi, and viruses. Antimicrobial proteins offer a fast response against invading potentially pathogenic microorganisms, thus playing an important role in innate immunity. The widespread occurrence of antimicrobial proteins in the plant and animal kingdoms reflects the significance of these evolutionarily ancient host defense molecules. In addition, humans produce various classes of antimicrobial proteins such as alpha- and beta-defensins, the cathelicidin LL-37, histatins, lysozyme, and dermcidin.

Another class of human antimicrobial proteins is represented by members of the RNase A superfamily. Recent evolutionary studies suggest that the RNase A family started off in vertebrates as a host-defence protein. The comparative analysis in mammals and birds indicates that this family was probably originated from an RNase 5-like gene. The hypothesis is supported by the fact that only RNase 5-like RNases have been reported outside the mammalian class. The RNase 5 group would be the most ancient form of this family and all the other members would have arisen during mammalian evolution. Cho and coworkers (Cho, S., Beintema, J.J., and Zhang, J. The ribonuclease A superfamily of mammals and birds: Identifying new members and tracing evolutionary histories) suggest an ancestral host defence function, considering that multiple lineages of this family, which includes EDN, ECP, angiogenin and RNase 7, have antipathogenic activities (figure 4). Besides, many members of the family exhibit high rates of amino acid substitution, as frequently observed in immunity genes. Additionally, the so-called antimicrobial RNases have a high pI, a property that is often associated with an antibacterial capacity. In fact, a high isoelectric point is characteristic for all the family members (Table 1) and is considered to be important for conferring them affinity to its negatively charged RNA substrate. The positive net charge has also been reported to be critical, but not sufficient, for the antibacterial activity of the family members with reported antimicrobial properties (Table 1). The ribonuclease activity is conserved within the family members, but so far, the described RNases antimicrobial capacities do not depend on the RNase catalytic activity.

Recently, a novel member of the RNase A superfamiliy, termed RNase 8, has been discovered by searching the human genome databases. Interestingly, RNase 8 and RNase 7 have an amino acid sequence similarity of 78% and a genomic distance of only 15,000 bp, suggesting that their genes may have evolved from a common ancestor gene by a duplication event. RNase 7 exhibits potent antimicrobial activity against gram-negative and gram-positive bacteria. The high similarity of RNase 8 to the antimicrobially active RNase 7 suggests that RNase 8 might also act as an antimicrobial protein. Also, is known that the pI of RNase 8 is 8,6.

Table 1. Antimicrobial properties and proposed function of RNase A family members involved in the host defence immunity

RNases		UniProt number	pI	Related antimicrobial properties	Expression and purification source	Proposed role
RNase 2a 	Human RNase 2 (EDN)	P10153	10.4	Antiviral activity Selective chemoattractant for dendritic cells Induction of proinflammatory mediators	Purified from eosinophil granules and liverExpressed in liver, spleen, neutrophils and activated monocytes and macrophages	Innate immunity Antiviral host defence Induction and regulation of innate and adaptive antimicrobial immunity
	Mouse eosinophil associated RNase 2 (mEAR2)	P97425	9.75	Selective chemoattractant for dendritic cells	Purified from eosinophil granules Expressed in lung, liver and spleen	
	Rat eosinophil associated RNase (EAR-2; R15)	Q5WN11	8.65	Activity against both Gram-negative and Gram-positive strains Activity against E. coli	Purified from eosinophil granules	Antimicrobial host defence
RNase 3b 	Human RNase 3 (ECP)	P12724	11.4	Lysis of bacterial membranes and synthetic lipid bilayers Bactericidal activity against Gram-negative and Gram-positive strains Immunomodulator capacity	Purified from eosinophil granules Expression in eosinophils	Antipathogen role associated to immune defence response mediated by eosinophils
	Rat eosinophil associated RNase (Ear-1; EAR11)	P70709	10.03	Activity against E.coli Activity against both Gram-negative and Gram-positive strains	Purified from eosinophil granules	Antimicrobial host defence
RNase 5	Human RNase 5 (angiogenin, ANG)	P03950	10.5	Antimicrobial activity against systemic bacteria Bactericidal and fungicidal activity	Expression induced during inflammationIncrease of protein levels after acute phase response	Systemic innate immunity response

						
	Mouse angiogenin (Ang 1)	(Ang 1) P21570	10.1	Antimicrobial activity against systemic bacteria Bactericidal and fungicidal activity	Expressed in liver, lung and pancreas	Systemic innate immunity response
	Mouse angiogenin (Ang 4)	Q80Z85	9.6	Antimicrobial activity against intestinal microbes	Produced by Paneth cells Expression induced by bacteria infection	Innate immunity of intestinal epithelium and regulation of intestinal flora
RNase 7	Human RNase 7 (Skin derived RNase)	Q9H1E1	10.3	Broad spectrum antimicrobial activity against both Gram-negative and Gram-positive strains Activity against yeast (Candida albicans) Bacteria membr ane permeabilization	Purified from skin Stratum corneum, cultured primary keratinocytes, psoriatic-scale material and articular joints Expressed in skin and respiratory, urogenital and intestinal tracts, in keratinocytes induced by bacterial challenge and interferon and in somatic tissues (liver, kidney, skeletal muscle and heart)	Innate immunity defence of human epithelia (skin and respiratory, gastrointestinal and genitourinary tracts) Host skin barrier against cutaneous infections

5. RNase 8

The human RNase 8 has been identified as a member of the ribonuclease A superfamily and as antimicrobial protein which may contribute to host defense. The physiological function of RNase 8 is still unclear, although it has a ribonucleolytic activity and exclusive expression in the placenta. RNase 8 is most similar to RNase 7 (78% sequence identity) and least similar to RNase 5 (29%). Is a divergent paralog of RNase 7, which is lysine-enriched, highly conserved, has prominent antimicrobial activity, and is expressed in both normal and diseased skin. It has 154 amino acids, including a signal peptide of 27 amino acids. The isoelectric point (pI) of the mature peptide is 8.6. RNase 8 has eight cysteine residues, one catalytic lysine residue and two catalytic histidine residues that are all among the characteristic features of all mammalian RNase A ribonucleases (except for RNase 5, with only six cysteine residues). The RNase 8 gene also encodes the RNase A superfamily invariant 'signature' motif, CKXXNTF, which includes the catalytic lysine residue. One unusual feature of RNase 8 is that a cysteine residue (at position 81 of the human RNase 8 mature protein) that is conserved among all other RNases has changed to glycine, while a new cysteine residue has appeared at position 66.

RNase 8 is active as an antibacterial agent against several Gram – positive and Gram – negative bacteria; its apparent function is to protect placenta from infections.

EXPERIMENTAL PART

1. OBJECTIVES

The objective of the presented work was the optimization of the heterologous expression and purification protocol of RNase 8 so as to produce enough quantities of pure protein that will allow comprehensive structural and functional studies.

2. MATERIALS AND METHODS

2.1 Plasmid vectors

A plasmid is a small DNA molecule that is physically separate from, and can replicate independently of, chromosomal DNA within a cell. Most commonly found as small circular, double-stranded DNA molecules in bacteria, plasmids are sometimes present in archaea and eukaryotic organisms. In nature, plasmids carry genes that may benefit survival of the organism (e.g. antibiotic resistance), and can frequently be transmitted from one bacterium to another (even of another species) via horizontal gene transfer. Artificial plasmids are widely used as vectors in molecular cloning, serving to drive the replication of recombinant DNA sequences within host organisms.

Plasmids can be recombinant DNA vectors, if they have some basic properties:

- Small size (plasmid sizes vary from 1 to over 1,000 kbp).
- High copy number (number of plasmids maintained per cell.
- Marker gene(s) for easy selection in bacteria or other hosts.
- A multiple cloning site/region (MCS or MCR) – a short section of DNA containing several unique restriction enzyme recognition sequences.
- Visual marker for selecting bacteria carrying plasmids with inserts (blue white screening).
- Promoters for in vitro transcription.

In the present work the plasmid vector pET11c was selected for cloning RNase 8. This vector consists of a lacl gene, which encodes the repressor protein lac, the T7 promoter, the lac operator that may prevent the transcription, the poly-linker, the factor f1 and an ampicillin resistance gene. The gene encoding the RNase 8 protein is embedded in the poly-linker.

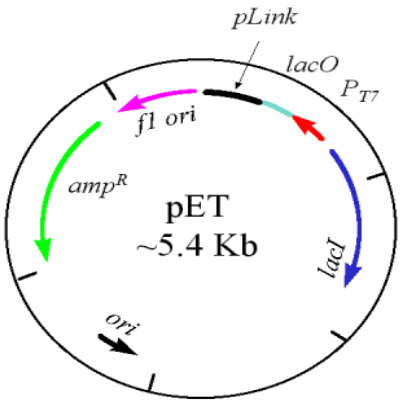


Figure 1. The pET plasmid vector

2.2 E. Coli as host cell for the expression of RNase 8

E. coli is a Gram – negative, facultative anaerobic, rod – shaped bacterium that belongs at the normal flora of the gut. Optimal growth of E. Coli occurs at 37°C but some laboratory strains can multiply at temperatures of up to 49°C. Development environment requires the presence of organic compounds as a source of carbon and energy, nitrogen, sulfur and salts.

E. Coli is the most common bacterial specie that used for expression of proteins. Commercially available are many different bacterial strains of *E. Coli* which differ from each other in terms of properties. In this work we used the strain BL21, which has the genotype:

F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])

2.3 Protein expression and induction

In the course of expression and isolation of a protein, the main points that must be determined before the production of the protein on a large scale are:

- The growth conditions of cultivation (temperature, ventilation, medium, incubation time).
- The IPTG concentration that is required during induction, and the temperature at which the induction will happen
- Determine whether the target protein is expressed in a soluble form or in inclusion bodies

Firstly, a preliminary analysis of the expression levels in various conditions in small scale cultures is performed. Based on the results, a protein purification protocol is then designed by controlling the effectiveness of different methods. The purpose of the protein purification usually dictates the design of a purification protocol since different purity levels are required for various experiments. In addition, since multiple purification steps are usually required to achieve purity, and in each step there is a loss of the protein, a purification protocol should have as few steps as possible.

2.3.1 Induction

IPTG (isopropylthiogalactoside) was selected as the inducer compound since it shows structural similarities with allolactose (Figure2), while it is not a substrate of the enzyme nor it is catabolized, thus ensuring continues induction.

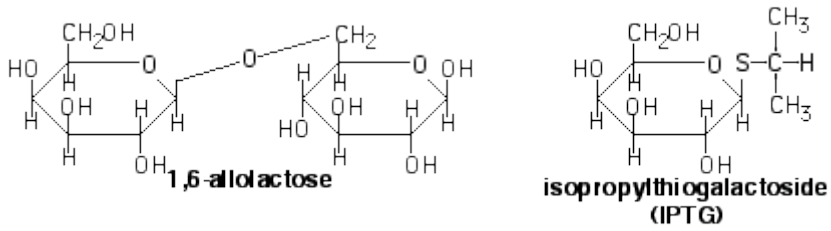


Figure 2. Chemical structure of 1,6 – allolactose and IPTG

Experimental procedure

- A small quantity of the desired cells (*E. Coli* strain BL21) stored at -80^{o}C are inoculated in an adequate amount of LB growth medium/ antibiotic Amp (c = 100mg/ml)
- Incubation in a 37^{o}C orbital shaker overnight (start later in the day so cultures do not grow too long)
- 40ml of the overnight culture were used to inoculate 2l of LB medium containing ampicillin (400mg/ml) and grow at 37^{o}C until an OD600 of approximately 0,6 was reached.

- The culture was cooled to room temperature and the protein expression was induced by adding IPTG to a final concentration of 1mM.
- The culture is incubated at a suitable temperature, under stirring at 250rpm for a further period of 3 – 4h.

2.4 Cell collection

The produced proteins can be detected either in the sediment or the supernatant fraction after centrifugation of the culture. The extracellular soluble proteins can be detected in the supernatant fraction while the insoluble or the intracellular proteins are in the pellet.

RNase 8 is an insoluble intracellular protein and therefore, when the induction finishes, the pellet is collected.

The cultures are transferred into sterile centrifuge tubes and then centrifuged at 12.000 rpm for 10 min at 4^{o}C. After centrifugation, the supernatant is discarded and the pellet may be used for further processing, or can be stored at –20^{o}C.

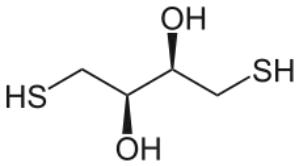
2.5 Cell lysis

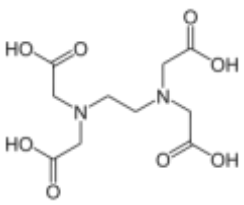
Cell lysis is a process in which a cell is broken down or destroyed as a result of some external force or condition. Lysis can happen through natural means, such as viral infections, or through artificial means (mechanically) for research purposes.

In this experimental work a mechanic cell lysis method was selected using ultrasound (sonication). In this method high-frequency sound waves are used to disrupt and destroy cells.

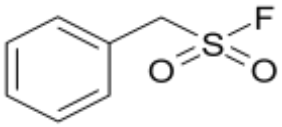
The pellet is resuspended in lysis solution (160ml; solution A) composed of:

- 15 mM (pH 7) Na_2HPO_4
- 5mM DTT → is frequently used to reduce disulfide bonds of a protein and prevent intramolecular and intermolecular disulfide bonds from forming between cysteine residues. Also, it prevents the formation of agglomerates.





- 2mM EDTA → chelator that binds ions necessary for the action of proteases.
- Protease inhibitor (PMSF) → serine protease inhibitor



The cellular suspension is sonicated for 5 min, twice in order to obtain a homogenous solution. The cellular suspension is maintained on ice during sonication to avoid overheating which can lead to the destruction of proteins.

The solution is then centrifuged at 4^{o}C for 30 min at 16.000 rpm and the pH of the supernatant solution is checked (it should have a value of 7.0).

2.6 Chromatographic purification of RNase 8

2.6.1 Ion exchanged chromatography (cationic)

The method of Ion Exchange Chromatography (IEX) for the separation of biomolecules was introduced in the 1960s and continues to play a major role in the separation and purification of biomolecules. Today, IEX is one of the most frequently used techniques for purification of proteins, peptides, nucleic acids and other charged biomolecules, offering high resolution and group separations with high loading capacity. The technique is capable of separating molecular species that have only minor differences in their charge properties. The solution to be injected is usually called a sample, and the individually separated components are called analytes.

Ion-exchange chromatography retains analyte molecules on the column based on coulombic (ionic) interactions. The stationary phase surface displays ionic functional groups (R-X) that interact with analyte ions of opposite charge. This type of chromatography is further subdivided into cation exchange chromatography and anion exchange chromatography. The ionic compound consisting of the cationic species M+ and the anionic species B- can be retained by the stationary phase.

Cation exchange chromatography retains positively charged cations because the stationary phase displays a negatively charged functional group:

$$\mathrm{R\text{-}X^-C^+ + M^+B^- \rightleftarrows R\text{-}X^-M^+ + C^+ + B^-}$$

Anion exchange chromatography retains anions using positively charged functional group:

$$\mathrm{R\text{-}X^+A^- + M^+B^- \rightleftarrows R\text{-}X^+B^- + M^+ + A^-}$$

In the present work two cation exchange columns (DEAE – sepharose and CM – sepharose, Figure 3) were used with an eluting solution (solution A) of a pH value of 7.0 so that RNase which has a pI value of 8.6 was positively charged.

Figure 3. Cationic chromatography

The chromatographical purification of RNase 8 is composed by the following steps:

- Equilibration with solution A, to allow the binding with RNase 8.
- Sample injection (V = 160ml). The flow rate is 1ml/min so the process will take about 2 – 3 hours. The chromatography system consists of two columns. The first is a DEAE – sepharose, which is positively charged because of $NH3^{+}$. So, the first column will commit the negatively charged proteins (no RNase 8). The second is a CM – sepharose, which is negatively charged because of COO^{-}, so it commits the positively charged proteins.
- Wash column with solution A. This ensures the removal of all proteins, which did not bind at the column.
- NaCl linear gradient elution of RNase 8 from the second column by using (solution A + NaCl 0,5M, V = 200ml) (Figure 4). With the gradual increase of

ionic strength, the proteins are removed from the column depending on the strength of their binding to it. Initially, the weakly bound proteins will elute while the strongly bound proteins will be retained and elute in the last fractions with the highest concentration of salt.

Figure 4. Elution of RNase 8 using NaCl gradient

- Protein concentration in fractions is monitored by measuring the optical absorbance at a wavelength of 280nm.

2.6.2 Gel filtration chromatography

For further purification, the protein sample obtained from the affinity chromatography was applied to a Hiload 26/20 superdex 200 gel filtration column.

Gel filtration separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. Unlike ion exchanged chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution. Consequently, a significant advantage of gel filtration is that conditions can be varied to suit the type of sample

or the requirements for further purification, analysis or storage without altering the separation.

Gel filtration is well suited for biomolecules that may be sensitive to changes in pH, concentration of metal ions or cofactors and harsh environmental conditions. Separations can be performed in the presence of essential ions or cofactors, detergents, urea, guanidine hydrochloride, at high or low ionic strength at 37^{o}C. Purified proteins can be collected in any chosen buffer.

To perform a separation, gel filtration medium is packed into a column to form a packed bed. The medium is a porous matrix in the form of spherical particles that have been chosen for their chemical and physical stability, and inertness. The packed bed is equilibrated with buffer which fills the pores of the matrix and the space between the particles. The liquid inside the pores is sometimes referred to as the stationary phase and this liquid is in the equilibrium with the liquid outside the particles, referred to as the mobile phase. It should be noted that samples are eluted isocratically. A wash step using the running buffer is usually included at the end of separation to facilitate the removal of any molecules that may have been retained on the column and to prepare the column for a new run.

The buffer which was used in this case is composed by:

- 50mM Na_2HPO_4
- 200mM NaCl
- 2mM EDTA
- 5mM DTT

The peak fractions corresponding to the protein were concentrated up to a protein concentration of 1 mg/ml and loaded on a reversed – phase chromatography column.

2.6.3 Reversed – phase chromatography

Besides being a polishing step, reversed – phase chromatography was used mainly to desalt the protein solution in order to store it dry without salts after lyophilization. Up to 1 ml of the protein solution obtained in the previous chromatography step was injected on a reversed – phase HPLC column (Symmetry C18) equilibrated with a mixture of 90% solvent A (H_2O + 0,1% TFA) and 10% solvent B (acetonitrile + 0,1% TFA). The elution was carried out at a flow rate of 1 ml/min with an initial 10 min wash and a 10 min linear gradient from the initial conditions to 75% solution A plus 25% solution B followed by a second 30 min linear gradient from 75% solution A plus 25% solution B to 50% solution A plus 50% solution B. The purity

of the protein fraction was checked by SDS – PAGE. The peak fractions corresponding to the RNase 8 were mixed and immediately frozen and lyophilized. Protein was stored at – 20 ^{o}C.

Reversed-phase chromatography (also called RPC, reverse-phase chromatography, or hydrophobic chromatography) includes any chromatographic method that uses a hydrophobic stationary phase. RPC refers to liquid (rather than gas) chromatography.

The term "reversed-phase" has a historical background. In the 1970s, most liquid chromatography was performed using a solid support stationary phase (also called a "column") containing unmodified silica or alumina resins. This method is now called "normal phase chromatography". In normal phase chromatography, the stationary phase is hydrophilic and therefore has a strong affinity for hydrophilic molecules in the mobile phase. Thus, the hydrophilic molecules in the mobile phase tend to bind (or "adsorb") to the column, while the hydrophobic molecules pass through the column and are eluted first. In normal phase chromatography, hydrophilic molecules can be eluted from the column by increasing the polarity of the solution in the mobile phase.

The introduction of a technique using alkyl chains covalently bonded to the solid support created a hydrophobic stationary phase, which has a stronger affinity for hydrophobic compounds. The use of a hydrophobic stationary phase can be considered the opposite, or "reverse", of normal phase chromatography - hence the term "reversed-phase chromatography". Reversed-phase chromatography employs a polar (aqueous) mobile phase. As a result, hydrophobic molecules in the polar mobile phase tend to adsorb to the hydrophobic stationary phase, and hydrophilic molecules in the mobile phase will pass through the column and are eluted first. Hydrophobic molecules can be eluted from the column by decreasing the polarity of the mobile phase using an organic (non-polar) solvent, which reduces hydrophobic interactions. The more hydrophobic the molecule, the more strongly it will bind to the stationary phase, and the higher the concentration of organic solvent that will be required to elute the molecule.

2.7 SDS – polyacrylamide gel electrophoresis

Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field.

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. It is used in clinical chemistry to separate proteins by charge and/or size

(IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving. Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins.

Gel electrophoresis uses a gel as an anticonvective medium and/or sieving medium during electrophoresis, the movement of a charged particle in an electrical field. Gels suppress the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of molecules; gels can also simply serve to maintain the finished separation, so that a post electrophoresis stain can be applied.

Electrophoresis is a process which enables the sorting of molecules based on size. Using an electric field, molecules (such as DNA) can be made to move through a gel made of agar or polyacrylamide. The electric field consists of a negative charge at one end which pushes the molecules through the gel, and a positive charge at the other end that pulls the molecules through the gel. The molecules being sorted are dispensed into a well in the gel material. The gel is placed in an electrophoresis chamber, which is then connected to a power source. When the electric current is applied, the larger molecules move more slowly through the gel while the smaller molecules move faster. The different sized molecules form distinct bands on the gel.

In most cases, the gel is a cross-linked polymer whose composition and porosity is chosen based on the specific weight and composition of the target to be analyzed. When separating proteins or small nucleic acids (DNA, RNA, or oligonucleotides) the gel is usually composed of different concentrations of acrylamide and a cross-linker, producing different sized mesh networks of polyacrylamide. When separating larger nucleic acids (greater than a few hundred bases), the preferred matrix is purified agarose. In both cases, the gel forms a solid, yet porous matrix. Acrylamide, in contrast to polyacrylamide, is a neurotoxin and must be handled using appropriate safety precautions to avoid poisoning. Agarose is composed of long unbranched chains of uncharged carbohydrate without cross links resulting in a gel with large pores allowing for the separation of macromolecules and macromolecular complexes

The types of gel most typically used are agarose and polyacrylamide gels. Each type of gel is well-suited to different types and sizes of analyte. Polyacrylamide gels are usually used for proteins, and have very high resolving power for small

fragments of DNA (5-500 bp). Agarose gels on the other hand have lower resolving power for DNA but have greater range of separation, and are therefore used for DNA fragments of usually 50-20,000 bp in size, but resolution of over 6 Mb is possible with pulsed field gel electrophoresis.

In this experimental work is used SDS – PAGE (polyacrilamide gel electrophoresis). The purpose of SDS-PAGE is to separate proteins according to their size, and no other physical feature.

SDS (sodium dodecyl sulfate) is a detergent (soap) that can dissolve hydrophobic molecules but also has a negative charge (sulfate) attached to it. Therefore, if a cell is incubated with SDS, the membranes will be dissolved, all the proteins will be solubilized by the detergent, plus all the proteins will be covered with many negative charges (denaturation).

If the proteins are denatured and put into an electric field, they will all move towards the positive pole at the same rate, with no separation by size. So we need to put the proteins into an environment that will allow different sized proteins to move at different rates. The environment of choice is polyacrylamide, which is a polymer of acrylamide monomers. When this polymer is formed, it turns into a gel and we will use electricity to pull the proteins through the gel so the entire process is called polyacrylamide gel electrophoresis (PAGE).

Further denaturation of the proteins before electrophoresis is usually performed by using reagents which elevate the disulfide bonds, such as 2-mercaptoethanol and by heating at 100 º C. In this system, two sequential gels are actually used; the top gel, called the stacking gel, is slightly acidic (pH 6.8) and has a low (5.5%) acrylamide concentration to make a porous gel. Under these conditions proteins separate poorly but form thin, sharply defined bands. The lower gel, called the separating, or resolving gel, is more basic (pH 8.8), and has a higher polyacrylamide content (in our case, 12%), which causes the gel to have narrower channels or pores. As a protein, concentrated into sharp bands by the stacking gel, travels through the separating gel, the narrower pores have a sieving effect, allowing smaller proteins to travel more easily and hence rapidly, than larger proteins.

Following electrophoresis, the gel may be stained (for proteins, most commonly with Coomassie Brilliant Blue), allowing visualization of the separated proteins, or processed further. After staining, different species biomolecules appear as distinct bands within the gel. It is common to run molecular weight size markers of known molecular weight in a separate lane in the gel to calibrate the gel and determine the approximate molecular mass of unknown biomolecules by comparing the distance traveled relative to the marker.

2.7.1 Experimental procedure

- The stacking gels (stacking gel-5%) and separating (running gel-5% -15%) are usually prepared in flat, vertical form, thickness 0,8-2 mm and varied dimensions. To prepare the used glass plates and special separator films (spacers) in a suitable pattern.
- The stacking and separating gels were prepared according to table 1, which is showing the quantities of used reagents.
- The separating gel is prepared first and is transferred to the space between the plates, filling 75% of the space.
- The gel is covered with isopropanol to protect the gel from the oxygen. The isopropanol is removed when the polymerization is over and the two phases are separated (30 – 60 minutes).
- Stacking gel is prepared and transmitted to the space between the plates forming a layer on top of the separating gel, up to the surface.
- APS and TEMED are added at the end, just before transfer the solutions in the plates in order not to start the polymerization too early.
- Suitable plastic comb is placed in order to form a specified number of wells.
- Mixing the samples with loading buffer (30% glycerol, 9% SDS, 15% β – mecraptoethanol, 0.05% bromophenol blue dissolve in Tris/HCl 180mM, pH 6.8) in the ratio sample/buffer 2 to 1, which can allow viewing the proteins after separation from electrophoresis. Also, is meant to help weigh down the proteins, so that they can sink into the bottom of the wells and not float in the buffer solution.
- Samples are heated for 2 – 3 min at 100^{o}C.

Separating gel	
For 2 gels (15%)	**For 1 gel (15%)**
2,5 ml H_2O	1,25 ml H_2O
2,5 ml solution B (1,5 M Tris/HCl, pH 8.8, 0.4% SDS)	1,25 ml solution B
5 ml solution A (30.4% acrylamide, 0.8% bis – acrylamide)	2,5 ml solution A
40µl APS (10%)	20µl APS(10%)
10µl TEMED	5µl TEME
Stacking gel	
1,9 ml H_2O	
0,775 ml solution C	
0,5 ml solution A	
40µl APS	
5µl TEMED	

Table 1. Quantities of reagents for the preparation of separating and stacking gels.

2.7.2 Samples placing and gel staining

- The comb is removed and the electrophoresis device is assembled.
- A large volume of running buffer (250mM Tris/HCl, pH 8.3, 1.9M glycin, 1% SDS and distilled water up to 1 lt) is placed in the device.
- The samples are loaded in the wells, using a microsyringe.
- The device is connected with the power supply and a constant intensity power is applied.
- The electrophoresis stops when the dye reaches about 1cm from the edge of the gel.
- Gel(s) are laid down in Coomasie Brilliant Blue staining solution (30% methanol, 8% acetic acid, 0.15% Coomasie blue) for 10 min.
- Destaining is performed with a solution of 30% ethanol and 7% acetic acid until the excess of dye is washed out.

2.8 Ribonuclease activity by zymogram

Zymography is an electrophoretic technique that includes a substrate copolymerised with the polyacrylamide gel for the detection of enzymes and their activity. Samples are prepared without denaturing the active enzymes present in the samples. Following electrophoresis, the gel is placed in an enzyme activation buffer which allows the enzymes present in the sample to become active and digest the substrates copolymerised in the gel. The zymogram is subsequently stained and the areas of enzyme activity and digestion become visible.

Gelatin is the most commonly used substrate, and is useful for demonstrating the activity of gelatin-degrading proteases, but zymography has been applied to a variety of enzymes, including xylanases, proteases, lipases, etc.

In the presented work, the zymogram method is employed with polyurydylic acid (poly(U)) as a substrate, in order to determine the bands which correspond to the protein fractions with ribonuclease activity. After the separations of the proteins by electrophoresis, SDS is eliminated and gel is incubated. Proteins with ribonuclease activity in contact with poly(U) locally degrade the polynucleotide. RNase 8, which displays ribonuclease activity, is visualized by negative staining. Substrate which is not degraded is stained with toluidine blue, and bands of degraded polynucleotide remain without dye.

2.8.1 Experimental procedure

In principle the method is the same as SDS – 15% PAGE for SDS polyacrylamide gel for electrophoresis with few modifications:

- Dilute 3 mg of poly(U) (potassium salt of poly(U): SIGMA) in 2ml of distilled water.
- Leave 10 minutes at room temperature and then 5 minutes at 55 ^{o}C.
- Prepare solution for the separating gel (6 ml of solution A and 2 ml of solution B) and keep it for 1 – 2 min at 55oC.
- Mix quickly poly(U) with polyacrylamide solution and add 10 µL TEMED and 40 µL of APS.
- Pour with pipette 5ml of separating gel between glasses in electrophoretic kit without forming bubbles.
- Over the separating gel place certain quantity of isopropanol.
- Leave the gel to polymerize completely for 30 min.

- Preparation of stacking gel (the same with SDS – PAGE electrophoresis) and transmitted to the space between the plates forming a layer on top of the separating gel, up to the surface.
- Suitable plastic comb is placed in order to form a specified number of wells.
- Mix the samples with loading buffer (without β - mercaptoethanol) in the ratio sample/buffer 2 to 1.
- The samples are loaded in the wells, using a microsyringe.
- Connect the device with the power supply device and apply constant intensity power.
- Stop the electrophoresis when the dye reaches about 1cm from the edge of the gel.
- The gel is washed with isopropanol and Tris/HCl 50mM (3 times for 5 – 10 min)
- To eliminate isopropanol, the gel is washed with cooled buffer 50mM Tris/HCl (e times for 5 – 10 min)
- The gel is incubated at room in the buffer 100mM Tris/HCl for 1 hour.
- Stain the gel for 10 min with toluidine blue and destain excess of color with distilled water.

2.9 Antimicrobial activity

To determine if RNase 8 shows an antimicrobial activity, the following experiment was performed:

- Preparation of medium (12,5 gr of L.B and 7,5 gr of agar dissolved in 0,5lt of water).
- Sterilization for 20 minutes at 20^{o}C (dry heat).
- The medium is shared in 10 plates and left in room temperature until is solidified.
- Bacteria dilution (10 μL of E. Coli is added to 990 μL of buffer) and protein dilution (there are 10 Eppendorf type vials). All contain 50 μL of the buffer except the first one, which contains 78,5 μL. In the first one 21,5 μL of protein are added. At the second vial 50 μL of the content of the first one is added and so on).
- Mix protein with bacteria (to each vial 50 μL of diluted bacteria are added).
- Vials are placed to 37^{o}C for 4 hours (cabin).
- 25 μL of each vial is added to each plate and the plates are placed for 12 hours at 37^{o}C (cabin).
- Colonies measurement.
- Finding the c/50 using a computer.

The plate wells with no bacteria colonies present the antimicrobial activity of the protein.

2.10 Crystallization

Crystallization of proteins is a biochemical technique, which is originally used to isolate and control the purity of the proteins, as the small crystallinity of molecules is indication of purity. In recent years the use of crystallization for this purpose has been abandoned because the crystals may contain up to 10 % other impurities. Like many other types of molecules, proteins can be prompted to form crystals, when the solution in which they are dissolved becomes supersaturated. Under these conditions, individual protein molecules can pack in a repeating array, held together by noncovalent interactions. These crystals can then be used in structural biology to study the molecular structure of the protein, for various industrial or biotechnological purposes.

Crystallization of protein molecules has been known for over 150 years. In 1934, John Desmond Bernal and his student Dorothy Hodgkin discovered that protein crystals surrounded by their mother liquor gave better diffraction patterns than dried crystals. Using pepsin, they were the first to discern the diffraction pattern of a wet, globular protein. Prior to Bernal and Hodgkin, protein crystallography had only been performed in dry conditions with inconsistent and unreliable results. The selection of the proteins was based on the availability and the ease with which it could be crystallized and not on their biological importance, as the interest of structural biology those first years, was the development of methods of X-ray for the solution of structures than the approach of crystallization processes. Today, with the help of tools from biotechnology, it is probably easier for the researcher to acquire the necessary amounts of protein for crystallization (even, in some cases quite mg), as the goal is not only crystal growth but also achieving the appropriate size for these.

Proteins are biological macromolecules and function in an aqueous environment, so protein crystallization is predominantly carried out in water. Protein crystallization is traditionally considered challenging due to the restrictions of the aqueous environment, difficulties in obtaining high-quality protein samples, as well as sensitivity of protein samples to temperature, pH, ionic strength, and other factors. Proteins vary greatly in their physicochemical characteristics, and so crystallization of a particular protein is rarely predictable. Determination of appropriate crystallization conditions for a given protein often requires empirical testing of many conditions before a successful crystallization condition is found.

Specifically, the factors, which affect crystallization, are:

A. Endogenous physicochemical parameters

1. Supersaturation (concentration of protein and precipitating agent)
2. Temperature, pH (changes)
3. Time (balancing and growth rates)
4. Ionic strength and the chemical purity (nature of precipitating factors, buffers, additional chemicals)
5. Diffusion and transport (gels, microgravity)
6. Volume and sample geometry and experimental devices (surface of crystallization devices)
7. Solid particles, interactions with the walls and with the median surfaces (homogeneous, heterogeneous nucleation)
8. Phenomena dependent on the density or viscosity (differences between crystal and mother liquor)
9. Pressure, electric and magnetic fields
10. Vibration and sound (acoustic waves)
11. Range of events, repeatability (researcher or robot)

B. Biochemical and biophysical parameters

1. Sensitivity of the protein structure in physical parameters (temperature, pH, ionic strength, solvents)
2. Binding of other ligands (substrates, cofactors, metal ions, other ions)
3. Special additives (agonists, nonionic detergents, polyamines)
4. Properties of proteins (oxidation, hydrophobicity, hydrophilicity)
5. Aging of the sample (redox changes, denaturation, decomposition)

C. Biological parameters

1. Very small amounts of most proteins in nature
2. Biological and physiological state of organisms or cells, which are containing the proteins (thermophiles, psychrophiles, allofiloi, mesophilic organisms, static or growing phase cells)
3. Bacterial infections

D. Purity of macromolecules

1. Macromolecular transfections (with other macromolecules, or other small molecules)
2. (Micro) sequence heterogeneity (cuts by proteases or nucleases -fractions of macromolecules can be crystallized easier - some or heterogeneous translational modifications)
3. Structural (micro) heterogeneity (degree and method of polymerization, aggregates, denaturation)

2.10.1 Solubility diagrams

The solubility curve (S) defines the sub saturating and super saturation zones and determines the balance state between the saturated and crystallized protein in solution. Below the saturation curve, the protein will never be crystallized (sub saturating), and above the solubility curve the protein concentration is higher than the balancing concentration for a given electrolyte concentration (super saturation) and is divided in three other areas:

1. Precipitation Zone: protein turns into blob.
2. Nucleation Zone (crystallization nuclei): Crystallization nuclei are the smallest organized forms of protein aggregates and are formed in the region where the protein excess gets crystalline form. Close to the precipitation zone there is a plurality of microcrystals which can be mixed with the protein's amorphous precipitate. Finding the nucleation zone is a primary purpose of crystallization experiments.
3. Metastable zone (crystal growth): a supersaturated solution of protein cannot create crystallization nuclei for a long time, unless it is assisted mechanically. This zone corresponds to the ideal crystal growth of crystallization nuclei which is preexisting without creating new ones.

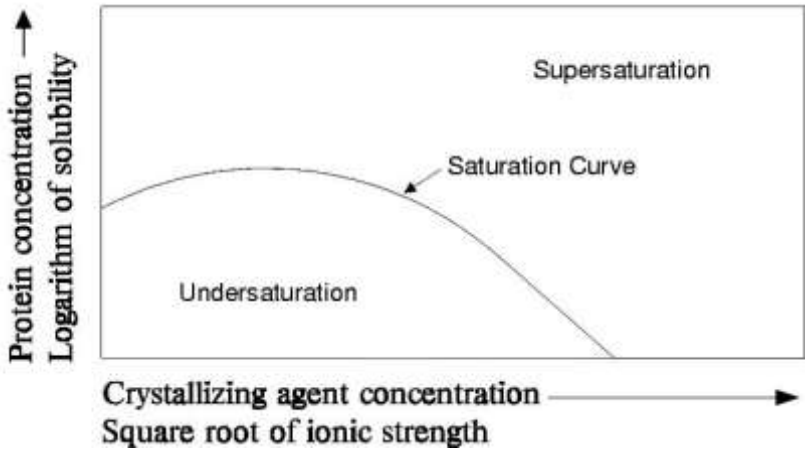


Figure 5. The phase diagram plots the solubility curve of a protein. The horizonal axis shows the parameter being varied (usually precipitant concentration)and the vertical axis shows the protein concentration.

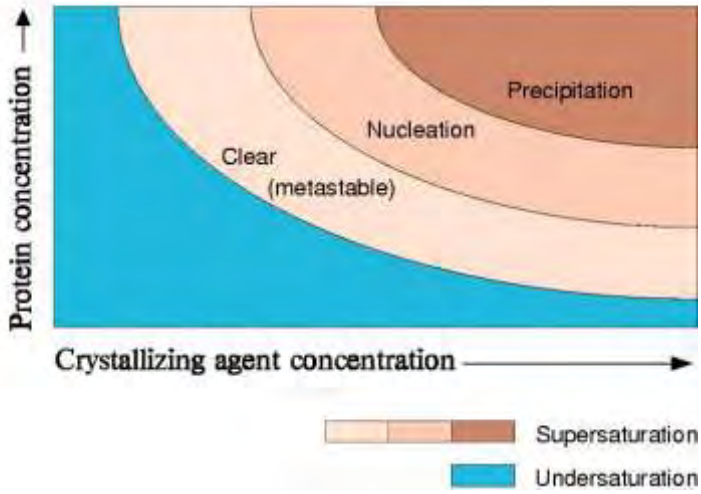


Figure 6. The Diagram showing zones for crystal nucleation, growth and precipitation.

2.10.2 Crystal-Crystalline grid

Crystal is the normal, repetitive arrangement of atoms or molecules in three dimensions. It consists of similar cuvettes, which have the same orientation and are repeated in the three dimensions. This helps create a regular three dimensional grid. The volume unit of the matrix with sides a, b, c, is called elementary cell and can be selected in various ways. They are limited to having shapes, which do not leave gaps, when repeated by shifting. In the simplest case, the unit cell contains a single molecule, but usually consists of two or more molecules associated with some kind of symmetrical arrangement. This helps fill the space more effectively when It Is compared to having a single molecule.

The interactions that hold proteins in the crystal lattice are called crystal contacts.

The highest possible purity of the proteins is required for crystallization experiments, as the unevenness, because of biochemical modifications (proteolysis, phosphorylation, glycosylation) or other factors, stops the proteins from crystallization.

Large and well-formed crystals are required for the crystallographic study of a protein. Therefore it needs initially to create crystallization nuclei which are allowed to grow in the metastable zone. The protein must exceed an energy barrier in order to be able to crystallize, similar to that of typical chemical reactions, as shown in the figure below.

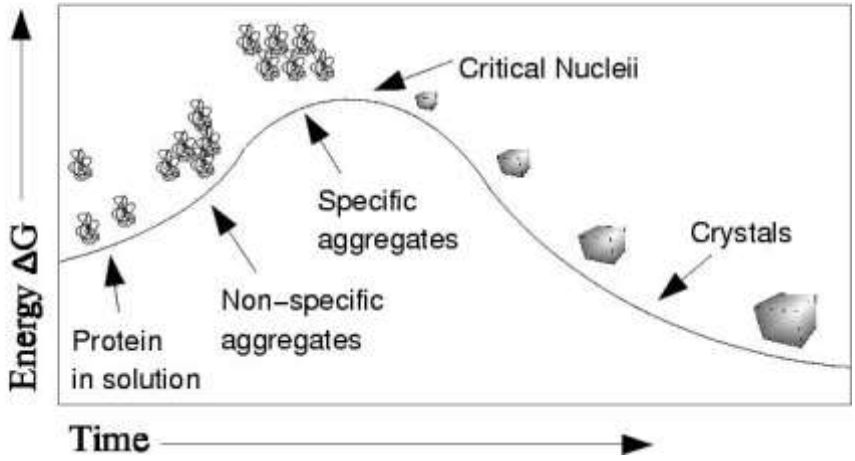


Figure 7. Energy diagram for crystallization

The protein solution must pass from the soluble phase in the nucleation zone and stay there for a short time, so as to not create many crystallization nuclei which are leading to many smaller-sized crystals. (The increase in the volume of crystals in solution with many crystallization nuclei leads to duplication crystal lattice, which in turn leads to badly formed crystals). Then, the cores of the protein solution, when dropped back to the metastable zone, will continue to grow and give well-formed crystals. As the crystals grow, the concentration of the dissolved protein diminishes; resulting in the displacement of the metastable zone. It is evident that a major concern in crystallization experiments is to create conditions of continuous monitoring of the metastable zone.

The data above suggests that we must control the way of reducing the solubility of the protein in solution. Rapid decrease in solubility leads to amorphous precipitates. Therefore, the approach of super saturation point is performed slowly, by gradually changing factors such as concentration, ionic strength, pH, or the dielectric constant of the protein solution.

Protein concentration: many proteins have been crystallized from solutions which are containing one to hundreds of mg / ml protein. However, for experimental testing the desired concentration is 10-20 mg / ml, if it is possible.

Ionic strength: the ionic atmosphere changes the solubility of proteins through the phenomena:

1. Salting in: proteins are more soluble in the presence of a small amount of electrolyte than in pure water as the electrolyte ions bound to the surface of the protein and increase hydrophilicity. In this case, the protein can be crystallized by lowering the concentration of the electrolyte (salt).
2. Salting out: if a strong electrolyte (e.g ammonium sulfate) is added to the protein solution (where the solubility is greater than that of proteins), then the competition of ions for water molecules among themselves is increased, thereby removing water molecules from the protein solution. The result of this is the decrease of its solubility.

pH: the net charge of the protein can be changed by varying the protons, changing the pH or by the ion binding to polar groups of the protein. The higher the net charge of the protein is, the more soluble it has. When it has a pure charge of zero, it is located at the point of minimum solubility. This happens when the protein is in the isoelectric point (pI). The pH is a very important factor for the crystallization and requires the use of appropriate buffers.

Temperature: the solubility of the protein is directly dependent on the temperature, but varies widely from protein to protein in combination with the ionic strength, the presence of organic solvents and the specific experimental conditions. Assays are done at refrigerator temperature (4-6 ^{o}C) or at a steady room temperature (17-24 ^{o}C).

Organic – non polar solvents: solutions which are used are ethanol or pentanediol in admixture with an aqueous solution, to lower the solubility of the protein. Bounds to polar groups of the protein surface, so the proteins appear less polar, or simply reduce the effective number of polar groups of the solvent.

2.10.3 Crystallization techniques

Sometimes the same approach has been implemented in a slightly different form in several crystallization methods, leading to a rather large number of techniques. More than 20 techniques have been reported and these are listed in the table 2 below, together with the number of macromolecules and crystal forms successfully crystallized by each of these methods.

The most common protein crystallization methods are batch crystallization, vapor diffusion and dialysis.

Batch crystallization

It is the classical technique which is used for crystallization of enzymes as well. (Successful production of large crystals of lysozyme, and ribonuclease enzymes of the family of trypsin). All components are directly combined into a single, supersaturated protein solution, which is then left undisturbed. The protein is dissolved in a low ionic strength to give a solution of high concentration. The precipitating factor (salt or organic solvent) is then added in order to bring the solution in a state of super saturation. After standing for hours or days, the crystals appear. When super saturation conditions are not known, the precipitation factor is added in small quantities over the day.

An advantage of this method is that the samples are protected from airborne contamination, as they are never exposed to the air during the experiment. A disadvantage of the method is that there is little possibility in controlling the growth of crystals and the requirement of large amounts of protein. Nevertheless it is useful for proteins whose rates of nucleation and crystal growth are low when super saturation is achieved, or for those that give well-formed crystals even at high growth rates.

Crystallization method	No. of macromolecules	Total crystals
Bulk dialysis	63	71
Batch method	245	385
Vapor diffusion	163	197
Microdialysis	119	145
Vapor diffusion on plates or slides	244	332
Vapor diffusion in hanging drops	534	635
Free interface diffusion	45	58
Dialysis against distilled water	16	23
Dialysis	44	55
Microbatch	5	5
Vapor diffusion in Lagerkvist cells	11	11
Concentration by evaporation	96	104
Temperature crystallization	18	36
Dialysis against low ionic strength	2	2
Electrodialysis against tap water	1	1
Liquid bridge	1	2
pH induced crystallization	1	1
Diffusion in capillaries	2	2
Direct addition of precipitant	7	8

Vapor diffusion with microscopic seeding	17	19
Concentration by ultracentrifuge	2	2
Macroseeding	43	44
Microcapillary batch method	2	2

Table 2. Total number of crystal forms reported grown by various techniques (Data from the NIST/CARB/NASA Biological Crystallization Database)

Vapor diffusion

The method of vapor diffusion is the most widely used one. There are two common procedures for conducting vapor diffusion crystallization: they are either based on a drop, which is supported by some surface, in which case it is called a sitting drop, or based on an arrangement, where the drop is suspended from some surface and it is called a hanging drop (it is the one that is used in this experimental work). A third approach in which the protein sample droplet is simultaneously in contact with both an upper and lower surface, called a sandwich drop, is sometimes used, but for special purposes only such as making optical measurements.

In both vapor diffusion experiments a protein droplet containing purified protein, buffer and precipitant, is closed in a microwell with a reservoir of similar buffers and precipitants. Nevertheless their concentrations are higher than in the protein droplet. The reservoir does not contain the protein. At the very beginning of the experiment the protein and precipitant in the protein droplet are in too low of a concentration to cause the protein to crystallize, therefore the system is undersaturated. During the course of the experiment water vaporizes from the protein droplet and is collected in the reservoir. The decrease in water in the protein droplet moves the protein droplet, from a state of undersaturation to a state, where the protein and precipitant concentration in the protein droplet are sufficiently high, so that the protein crystallization occurs in the protein droplet. The net transfer of water from the protein droplet to the reservoir is equilibrium and that is the reason why optimum temperature conditions in the microwell should be maintaine,d until crystallization is complete.

A major advantage of the vapor diffusion technique is the possibility of affecting the equilibration rate and thus approaching supersaturation more slowly, by varying the distance between the reservoir and the crystallization drop. The equilibration process can also be slowed down by inserting an oil barrier over the reservoir. A further advantage is the ability to alter the composition and/or the concentration of the components in the crystallizing solution during the experiment without having to touch the drop. This can be achieved by either concentration or dilution of the reservoir.

However, this method has some disadvantages too: once nucleation conditions are achieved, the solution remains highly supersaturated, so that both nucleation and rapid crystal growth can occur simultaneously. Moreover, during the experiment the conditions are changing continuously, thus leaving the investigator guessing at which conditions crystallization takes place.

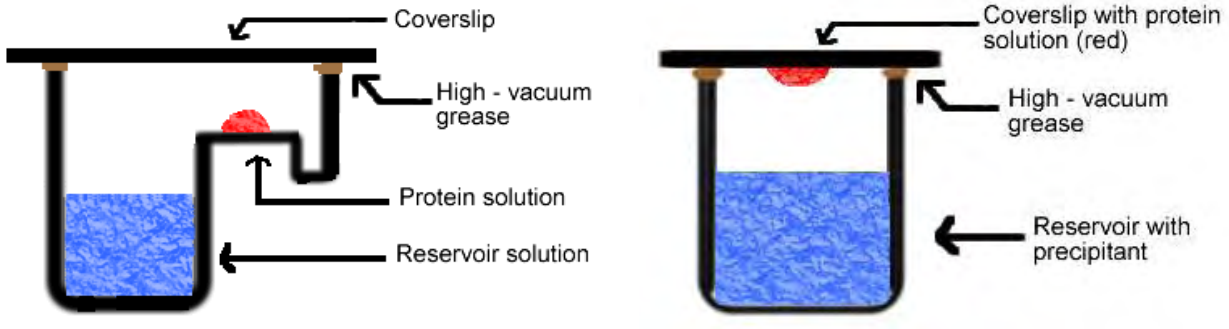


Figure 8. Sitting drop and hanging drop vapor diffusion

Dialysis

Just as with batch methods, the protein concentration remains constant during crystallization by dialysis. However, in this method the solution composition is altered by diffusion of low molecular weight components through a semipermeable membrane (which is produced by cellophane or cellulose and have pores of different sizes and each time are chosen for the size of the protein which are going to be separated.). In this way a protein may be brought slowly towards its precipitation point by dialysis against a solution of concentrated salt or organic solvent. But also dialysis against distilled water is a very productive method. It relies on the limited solubility of many proteins at very low ionic strength, because proteins need to be surrounded by a cloud of positively and negatively charged ions to be soluble. However, dialysis against distilled water is useful only when it does not induce aggregation or denaturation of the protein.

Dialysis has several advantages, including the ability to change the protein solution composition accurately an infinite number of times and the fact is that, as the differential between concentration inside and outside the membrane decreases, the rate of equilibration decreases. Moreover, the protein concentration remains constant, so that only one parameter varies at a time. This offers a better insight in the roles of different parameters.

2.10.4 Crystallisation plate preparation protocol

- Technique used: hanging drop method
- Pre-greased LINBRO™ 24-well crystallisation plate
- Crystallisation mix (reservoir volume): 800 µL
- Crystallisation condition we have worked with – composition:
  - Ribonouclease 8, 10mg/ml in sodium acetate 50mM pH 3.5
  - Initial crystallisation mixture: 12% PEG (polyethelene glycol) 20000g/mol + MES (2-(*N*-morpholino)ethanesulfonic acid) 100mM pH 6.5
- Prepare reservoir mixtures deciding which conditions and concentrations we will be working with.
- Calculate the corresponding crystallisation mixture component volume out of the initial stock concentration ($V_{stock} = C_{reservoir} \cdot V_{reservoir} / C_{stock}$).
- Once all well's reservoir volumes have been calculated, pour gently each component amount (e.g. 80 µL buffer) into the well. A concrete addition order is preferred in order.
- As we will be using the hanging drop method the protein will be hanging from a glass coverslip (should be clean enough to avoid any dust interfering with the final drop + reservoir crystallization mixture drop) placed over the well (already filled with the reservoir mixture). In order to prepare the final protein+reservoir drop, simply take 1µL of the protein sample, put it on the coverslip and then add 1µL of the reservoir composition (crystallisation condition) very gently. Avoid making any bubbles or strong mixing, as the protein could be damaged. To avoid eventual bad mixing or precipitation problems, we will start adding mQ (twice distillated) water and then go on with the buffer and finally salt.
- Turn over the coverslip rapidly and put it directly over the pre greased well border. Once all wells have been covered, cover the whole plate itself and keep it at 16^{o}C.

RESULTS – DISCUSSION

1. Protein expression and purification

For the production of RNase 8, it was used the plasmid vector pET11c, which as already mentioned (p. 15) allows the expression of the gene. Then, this plasmid vector was used for the transformation of E. Coli BL21 cells, according to the transformation protocol, which was described above in presence of antibiotic, in a final concentration of 400 mg/ml.

1.1 Protein production

- Transformation into receptive cells E. Coli BL21 by the use of antibiotic Amp (100μl/ml)
- 2lt culture of bacterial cells at a temperature of 37^{o}C under stirring using antibiotic Amp for 3 hours until the OD_{600} = 0,6.
- Induction in a temperature of 37oC for 4 hours using IPTG to a final concentration of 1mM.
- Collection of cells by centrifugation (12.000 rpm for 10 min at 4^{o}C). Then, the supernatant is discarded and the pellet may be used for further processing, or can be stored at – 20^{o}C.
- resuspending of the cells in lysis buffer (160 ml). it follows the lysis of the cells by sonication and the fractionation of the cellular components by centrifugation. The supernatant is received, which is used for the isolation and purification of protein.

1.2 Isolation and purification of RNase 8

For the purification of RNase 8 three types of chromatography were used:

- Ion exchanged
- Gel filtration
- Reverse – phase

The solution of the protein which results from the condensation was loaded in a DEAE – sepharose and CM – sepharose column, which was first equilibrated with the solution A with a flow of 1 ml/min. The washout of columns by the same solution follows (solution A). Then, the protein was eluted from the CM – sepharose column (which is negatively charged) with the use of NaCl linear gradient (solution A + NaCl 0,5M, V = 200ml). The result of the chromatography it is shown in the figure 1.

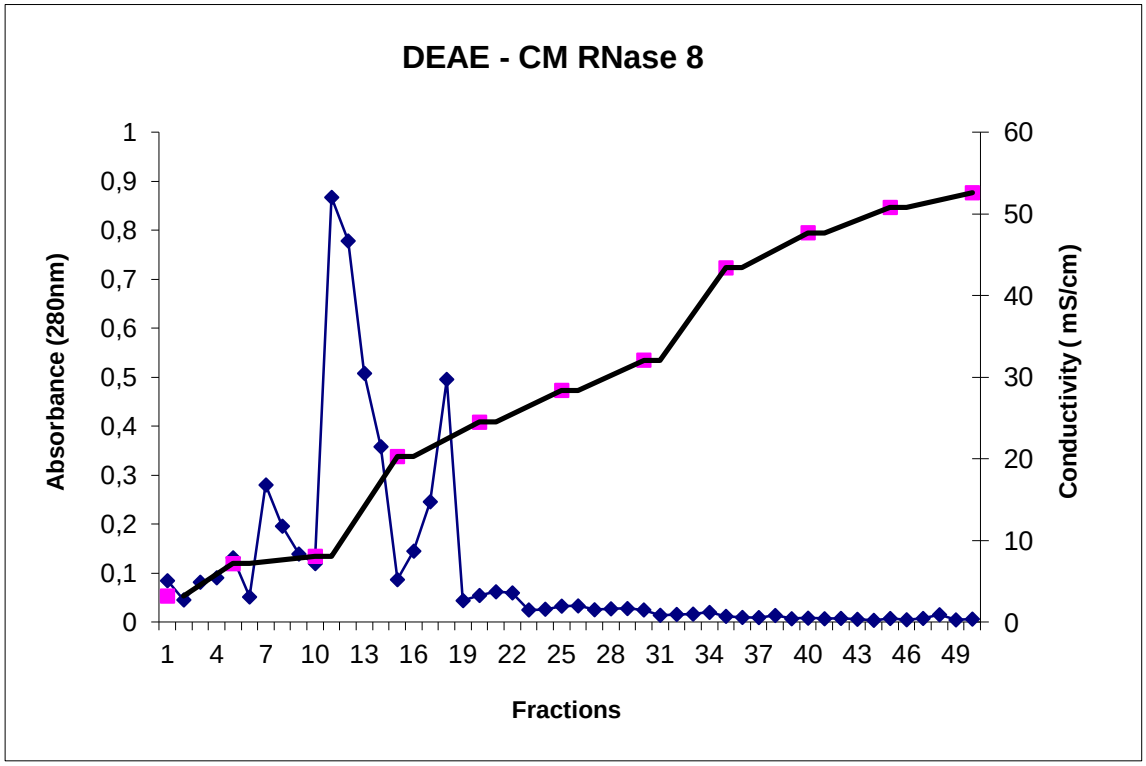


Figure 1. The chromatographic purification of RNase 8 by using an ion exchanged chromatography (DEAE – sepharose and CM – sepharose column). Conductivity is marked with pink and elution profile with blue.

As it is seen in the figure, a peak is given by four fractions (11 - 14), which are collected and mixed. Then, this mixture is used in the gel filtration chromatography, for further purification. The results from this chromatography are appearing in figure 2. In this case, only one peak is given, by the fractions 105 – 117.

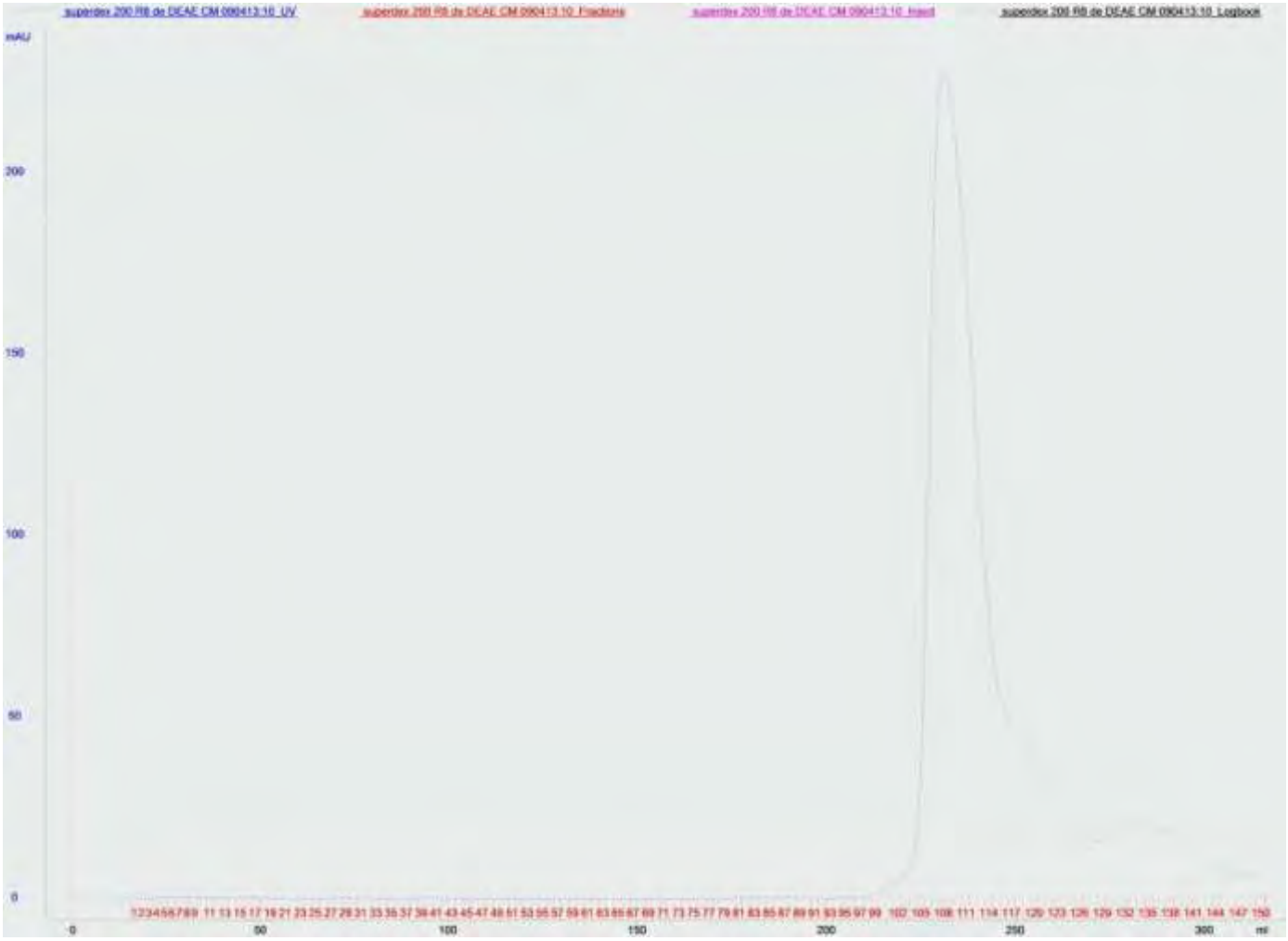


Figure 2. The chromatographic purification of RNase 8 by using a gel filtration chromatography (superdex 200 column)

Subsequently, the fractions which give peak in the previous chromatography are mixed and used in a reverse – phase chromatography in order to desalt the protein solution and to store it dry without salts after lyophilization. In the figure below (figure 3) the results of this last chromatography are shown.

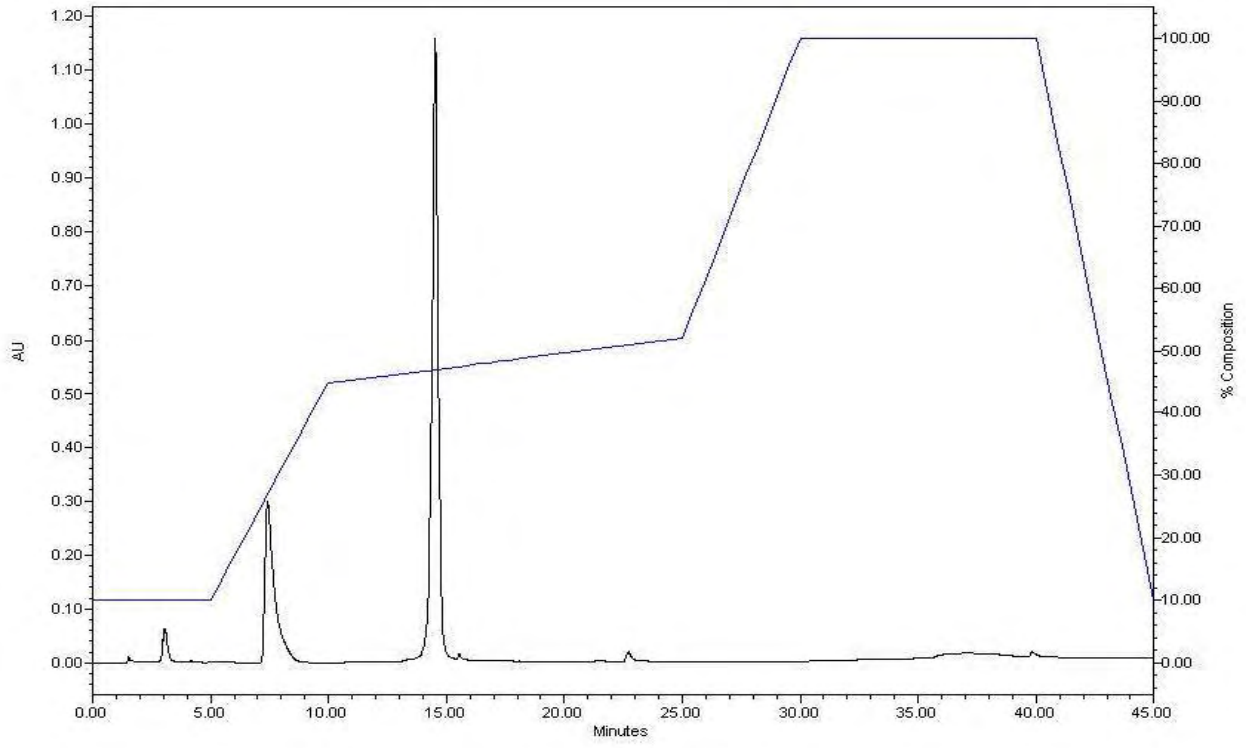


Figure 3. Reverse – phase chromatography

The purification of the protein had as result the production of 6 – 7 mg of pure protein per liter of bacterial culture.

2. SDS – polyacrylamide gel electrophoresis

The purity of the protein fraction was checked by SDS – PAGE. A sample (20μl) of the peak fractions corresponding to the RNase 8 in each chromatography was collected and uploaded in the gel with the process which is mentioned above (p.26). More specific:

- As a marker an RNaseA is used in which 5μl of loading buffer was added. A total amount of 7,5μl of RNaseA is also added in the first well of the gel.
- During the process of induction (p.17), a sample (B.I) was taken before the injection of IPTG from the culture and another one (A.I) after adding the IPTG. These samples were resuspended in 500μl Na_2HPO_4 and then were centrifuged for 15 min. From the supernatant 20μl of each sample are taken and are mixed with 10μl of loading buffer. A total amount of 15μl of each sample is uploaded in the wells.
- A sample (20μl) is taken from the mixture of fractions which give a peak in the ion exchanged chromatography (116 – 119) and another sample from the fractions of the gel filtration chromatography (105 – 117). These samples are mixed with 10μl of loading buffer and 15μl of each are uploaded in the wells of the gel.

All the samples were heated before the uploading in the gel. The figure 4 presents the results of the SDS – polyacrylamide gel electrophoresis.

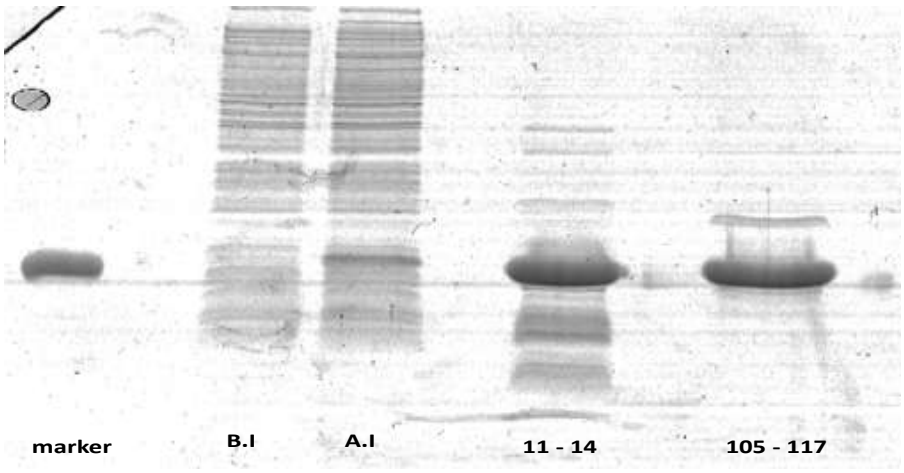


Figure 4. SDS - PAGE

3. Ribonuclease activity by zymogram

Zymography is used in order to detect if there is any enzyme activity inside the protein. RNase 8, which displays ribonuclease activity, is visualized by negative staining. The exact process is described above (p. 28).

In this case, a sample of 10µl is taken from the mixture of fractions (105 – 117) from the gel filtration chromatography. The figure 5 shows the results of this zymography, which mean that the RNase 8 presents activity.

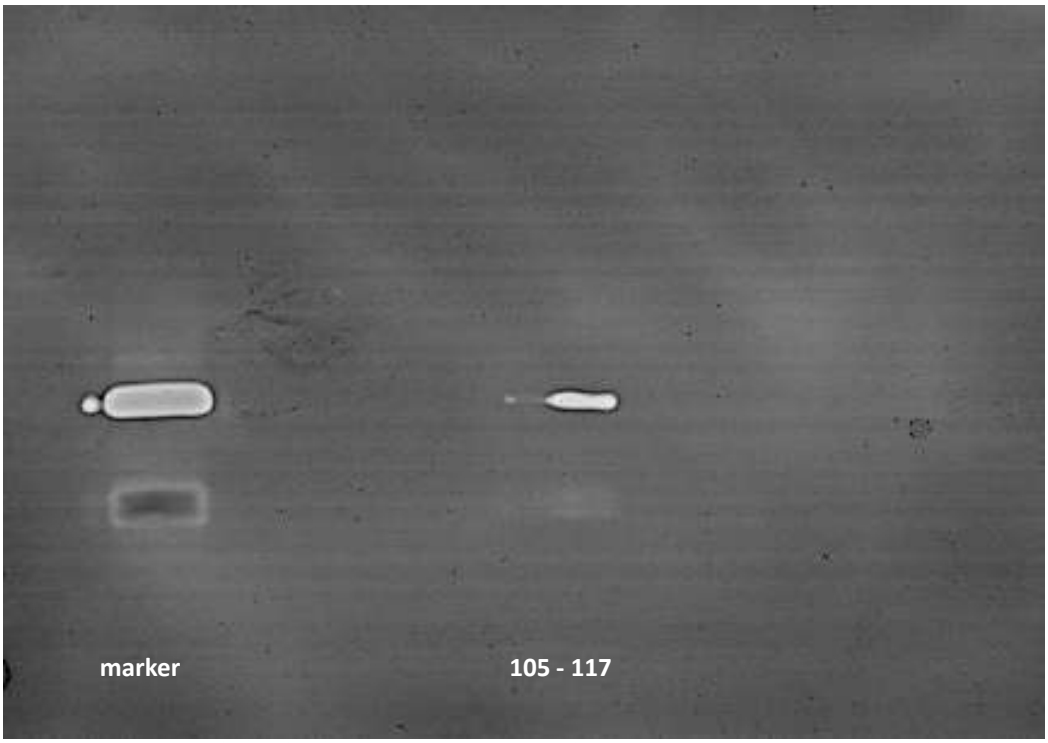


Figure 5. Zymography of RNase 8

4. Antibacterial activity

To determine if RNase 8 shows an antibacterial activity is applied the procedure wich is described above (p. 33). Each plate presents colonies, which are measured by dividing each plate into 4 quadrants. The results are the following:

1. (0,0195μM) → 424x4 = 1696
2. (0,039 μM) → 340x4 = 1360
3. (0,078 μM) → 277x4 = 1108
4. (0,156 μM) → 264x4 = 1056
5. (0,3125 μM) → 253x4 = 1012
6. (0,625 μM) → 140x4 = 560
7. (1,25 μM) → 139x4 = 556
8. (2,5 μM) → 54x4 = 216
9. (5 μM) → 25x4 = 100
10. (10 μM) → 21x4 = 84

Then, by using the computer it is possible to find the c/50 (x_0).

$$x_0 = 0.92 \pm 0.1$$

5. Crystallization

The experimental procedure of crystallization is described above (page 43). Several tests took place under different conditions. The table below shows the conditions for each test of the experiment:

	1	2	3	4	5	6
A	12% PEG 20000 MES 125mM pH 6.5	12% PEG 20000 MES 125mM pH 6.5	12% PEG 20000 MES 125mM pH 6.5	12% PEG 20000 MES 125mM pH 6.5	5% PEG 20000 MES 125mM pH 6.5	20% PEG 20000 MES 125mM pH 6.5
B	12% PEG 20000 MES 0.1M pH 5	12% PEG 20000 MES 0.1M pH 6	12% PEG 20000 MES 0.1M pH 6.5	12% PEG 20000 MES 0.1M pH 7	12% PEG 20000 MES 0.1M pH 7.5	12% PEG 20000 MES 0.1M pH 8
C	12% PEG 20000 MES 0.1M pH 5	12% PEG 20000 MES 0.1M pH 6	12% PEG 20000 MES 0.1M pH 6.5	12% PEG 20000 MES 0.1M pH 7	12% PEG 20000 MES 0.1M pH 7.5	12% PEG 20000 MES 0.1M pH 8
D	4% PEG 20000 MES 0.1M pH 6.5	6% PEG 20000 MES 0.1M pH 6.5	8% PEG 20000 MES 0.1M pH 6.5	10% PEG 20000 MES 0.1M pH 6.5	12% PEG 20000 MES 0.1M pH 6.5	14% PEG 20000 MES 0.1M pH 6.5

E	6% PEG 20000 MES 0.1M pH 6.5	7% PEG 20000 MES 0.1M pH 6.5	8% PEG 20000 MES 0.1M pH 6.5	9% PEG 20000 MES 0.1M pH 6.5	10% PEG 20000 MES 0.1M pH 6.5	11% PEG 20000 MES 0.1M pH 6.5
F	12% PEG 20000 MES 0.1M pH 6.5	13% PEG 20000 MES 0.1M pH 6.5	14% PEG 20000 MES 0.1M pH 6.5			

From the tests and the visual results, the following conclusions came out:

1. **12%** PEG 20.000 should be used (from test A)
2. the **pH** should be **6.5** (from test B and C)
3. **11%** PEG 20.000 is the best choice (from tests D and E)

However, it was not possible to take crystals. That means that more tests should take place under a wider and also different variety of conditions.

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