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STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF AN RNASE 7 VARIANT

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«Structural and functional characterization of an RNase 7 variant»

«Δομικός και λειτουργικός χαρακτηρισμός μίας μεταλλαγμένης μορφής της Ριβονουκλεάσης 7»

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CONTENTS

Summary

In the present work human Ribonuclease 7, 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 with zymography. The enzyme was expressed in *E. coli* BL21 cells, transformed with the pET11c vector that had an insertion of a synthetic gene encoding Ribonuclease 7. Ribonuclease 7 was purified from inclusion bodies using a denaturation/refolding protocol and cation exchange chromatography using a DEAE – sepharose and a CM – sepharose column. Purity of the isolated protein was evaluated using SDS page electrophoresis, and quantification by the Beer-Lambert principle. Ribonuclease activity of the purified protein was determined using poly(U) as a substrate by SDS electrophoresis. Evaluation of the antimicrobial activity was performed by counting colonies in agar plates after incubating bacteria with the proteins. Finally, attempts for crystallization of the Ribonuclease 7 were performed using the method of the hanging drop.

Περίληψη

Στην παρούσα εργασία, η ανθρώπινη ριβονουκλεάση 7, ένα διακεκριμένο μέλος της παγκρεατικής υπεροικογένειας της ριβονουκλεάσης Α, παράχθηκε με ετερόλογη έκφραση σε *E. coli*, καθαρίστηκε με χρωματογραφικές μεθόδους και χαρακτηρίστηκε με προσδιορισμό της ενζυματικής της δράσης με ζυμογραφία. Το ένζυμο εκφράστηκε στα βακτηριακά στελέχη της E. coli BL21, τα οποία μετασχηματίσθηκαν με το φορέα pET11c που περιέχει μια ένθεση ενός συνθετικού γονιδίου που κωδικοποιεί τη ριβονουκλεάση 7. Η ριβονουκλεάση καθαρίστηκε από έγκλειστα σωμάτια χρησιμοποιώντας ένα πρωτόκολλο μετουσίωσης/αναδίπλωσης και χρωματογραφία κατιονικής ανταλλαγής, χρησιμοποιώντας μια στήλη DEAE – σεφαρόζης και μία στήλη CM – σεφαρόζης. Η καθαρότητα της απομονωμένης πρωτεΐνης εκτιμήθηκε χρησιμοποιώντας ηλεκτροφόρηση SDS – PAGE και η ποσότητα της υπολογίστηκε με τη μέθοδο Beer-Lambert. Η ενζυμική δραστικότητα της καθαρής πρωτεΐνης προσδιορίστηκε χρησιμοποιώντας ως υπόστρωμα πολύ(U) σε ηλεκτροφόρηση SDS και η εκτίμηση της αντιμικροβιακής της δράσης έγινε μετρώντας τις αποικίες βακτηρίων που σχηματίστηκαν σε τρυβλία άγαρ, μετά από επώαση των πρωτεϊνών με τα βακτήρια. Τέλος, καταβλήθηκαν προσπάθειες για κρυστάλλωση της Ριβονουκλεάσης 7, χρησιμοποιώντας τη μέθοδο της κρεμάμενης σταγόνας.

THEORETICAL PART

1. INTRODUCTION

1.1 Ribonucleases

Ribonuclease, which is commonly abbreviated as RNase, is a type of nuclease that catalyzes the degradation of RNA into smaller components. Ribonucleases can be divided into endoribonucleases and exoribonucleases and comprise several sub-classes.

1.2 Function

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, so that cells do not become cluttered with waste, 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. RNASE genes are active in many cells and tissues, including different immune system cells. The variety of tissue activity likely supports the ability of RNase enzymes to help fight infection throughout the body.

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 (ribonucloprotein particle or RNP).

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. Genes in the RNASE family

The HUGO Gene Nomenclature Committee (HGNC) provides a list of genes in the RNASE family.

Approved Symbol	Approved Name	Previous Symbols	Synonyms	Chromosome
RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	RNS1		14q11.1
RNASE2	ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)	RNS2	EDN	14q24-q31
RNASE3	ribonuclease, RNase A family, 3	RNS3	ECP	14q24-q31
RNASE4	ribonuclease, RNase A family, 4			14q11
ANG	angiogenin, ribonuclease, RNase A family, 5		RNASE5	14q11.1-q11.2
RNASE6	ribonuclease, RNase A family, k6	RNS6		14q11
RNASE7	ribonuclease, RNase A family, 7			14q11.1
RNASE8	ribonuclease, RNase A family, 8			14q11.1
RNASE9	ribonuclease, RNase A family, 9 (non-active)		h461	14q11.2
RNASE10	ribonuclease, RNase A family, 10 (non-active)		RNASE9	14q11.1
RNASE11	ribonuclease, RNase A family, 11 (non-active)	C14orf6		14q11.1
RNASE12	ribonuclease, RNase A family, 12 (non-active)			14q11.1
RNASE13	ribonuclease, RNase A family, 13 (non-active)			14q11.1

Table 1. Genes in the RNASE family.

Proteins encoded by members of the RNASE gene family all share a similar structure and enzyme activity. All the genes in the RNASE gene family are found on chromosome 14. Thirteen genes have been identified in the RNASE gene family, but only eight of the genes in this family are designated by the letters RNASE followed by a unique number identifier, starting with the number one. For example, the first identified member of this gene family is the gene RNASE1.

3. Ribonuclease A (RNase A)

Ribonuclease A (RNase A) is a pancreatic endoribonucleases that cleaves single-stranded RNA. Bovine pancreatic RNase A is one of the classic model systems of protein science.

3.1 History

The importance of bovine pancreatic RNase A was secured when the Armour & Co purified a kilogram of it, and gave 10 mg samples away free to any interested scientists. The ability to have a single lot of purified enzyme instantly made RNase A a predominant model system for protein studies.

RNase A was the model protein used to work out many spectroscopic methods for assaying protein structure, including absorbance, circular dichroism/optical rotary dispersion, Raman, EPR and NMR spectroscopy. RNase A was also the first model protein for the development of several chemical and structural methods, such as limited proteolysis of disordered segments, chemical modification of exposed side chains, and antigenic recognition.

RNase A was the first enzyme for which a correct catalytic mechanism was proposed, even before its structure was known. RNase A was also the first protein showing the effects of non-native isomers of X-Pro peptide bonds in protein folding. RNase A was the first protein to be studied by multiple sequence alignment and by comparing the properties of evolutionarily related proteins.

3.2 Structure

RNase A is a relatively small protein (~13.7 kDa), the mature enzyme has 124 amino acid residues, with no carbohydrate attached. RNase A contains 19 of the 20 amino acids, lacking only tryptophan .The RNase A superfamily has been the subject of intense biochemical study for over half a century. The prototype, RNase A (bovine pancreatic ribonuclease) was the first enzyme to be sequenced and chemically synthesized, and the classic experiments on the denaturation and renaturation of this protein appear in virtually every biochemistry textbook. During the past two decades, dozens of novel ribonucleases of this superfamily have been identified in multiple vertebrate species. As a group, all RNase A superfamily members are secretory proteins, and include a classic hydrophobic signal peptide followed by a mature peptide with molecular mass of ∼12–16 kDa in unglycosylated state. All RNase A ribonucleases maintain three specific catalytic residues—one lysine and two histidines—that comprise a catalytic crevice, and six to eight appropriately-spaced cysteines that form three to four disulfide bonds. Except for these conserved residues, the RNases are otherwise quite divergent, with sequence identities varying from 30 to nearly 100%. Although all RNase A ribonucleases are enzymatically active, catalytic efficiency and substrate preference vary considerably.

Figure 1. RNase A: 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 magenta.

RNase A is a basic protein (p*I* = 9.63); its many positive charges are consistent with its binding to RNA (a poly-anion). More generally, RNase A is unusually polar or, rather, unusually lacking in hydrophobic groups, especially aliphatic ones. This may account for its need of four disulfide bonds to stabilize its structure. The low hydrophobic content may also serve to reduce the physical repulsion between highly charged groups (its own and those of its substrate RNA) and regions of low dielectric constant (the nonpolar residues).

3.3 RNase A specificity

RNase A is specific for pyrimidine nucleoside linkages. The reaction is believed to take place in two steps. In the first step, the 3',5'-phosphodiester bond is cleaved, while generating a 2',3'-cyclic phosphodiester intermediate. In the second step, the cyclic phosphodiester is hydrolyzed to a 3'-monophosphate group. The first step is nonspecific with respect to the nitrogenous base of the substrate; however, the second step is absolutely specific for pyrimidine nucleotides with terminal 2',3'-cyclic phosphates. It can be inhibited by ribonuclease inhibitor protein, by heavy metal ions, and by uridine-vanadate complexes and it can be activated by Sodium chloride and Sulfate.

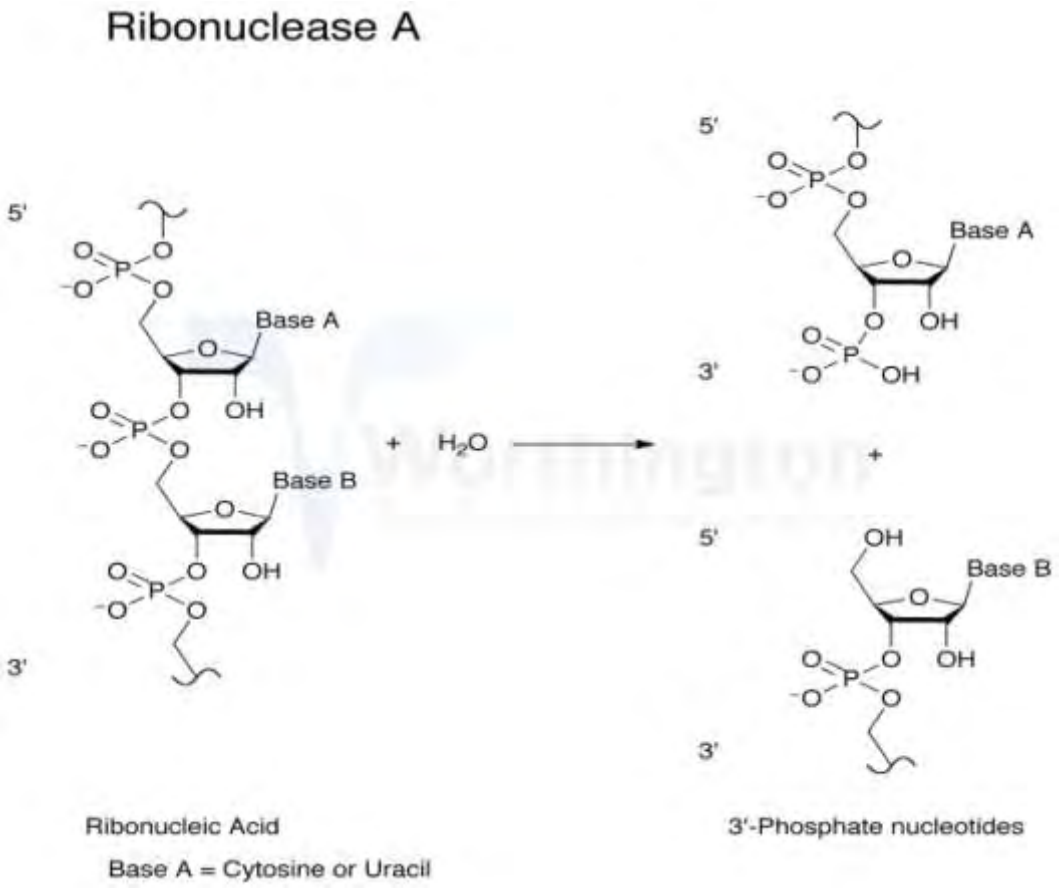

Figure 2. RNase A catalysis

3.4 Anti-cancer effects

RNase A, and to a greater extent its oligomers and some homologs (such as onconase from frogs), have cytotoxic and cytostatic effects, particularly on cancer cells. This attribute has led to the development of onconase as a cancer therapeutic. As with many protein drugs from a non-human source, the internal use of non-human ribonucleases such as onconase is limited by the patient's immune response

Antitumor ribonucleases are small (10–28 kDa) basic proteins. They were found among members of both, ribonuclease A and T1 superfamilies. Their cytotoxic properties are conferred by enzymatic activity, i.e., the ability to catalyze cleavages of phosphodiester bonds in RNA. They bind to negatively charged cell membrane, enter cells by endocytosis and translocate to cytosol where they evade mammalian protein ribonuclease inhibitor and degrade RNA.

Onconase, is the only enzyme of cytotoxic ribonucleases this class that reached clinical trials. Onconase is the smallest, very stable, less catalytically efficient and more cytotoxic than most RNase A homologues. Its cytostatic, cytotoxic and anticancer effects were extensively studied. It targets tRNA, rRNA, mRNA as well as the non-coding RNA (microRNAs). Numerous cancer lines are sensitive to Onconase; their treatment with 10 – 100 nM enzyme leads to suppression of cell cycle progression, predominantly through G_1, followed by apoptosis or cell senescence. Onconase also has anticancer properties in animal models. Many effects of this enzyme are consistent with the microRNAs, one of its critical targets. Onconase sensitizes cells to a variety of anticancer modalities and this property is of particular interest, suggesting its application as an adjunct to chemotherapy or radiotherapy in treatment of different tumors. Cytotoxic RNases as exemplified by Onconase represent a new class of antitumor agents, with an entirely different mechanism of action than the drugs currently used in the clinic. Further studies on animal models including human tumors grafted on severe combined immunodefficient (SCID) mice and clinical trials are needed to explore clinical potential of cytotoxic RNases.

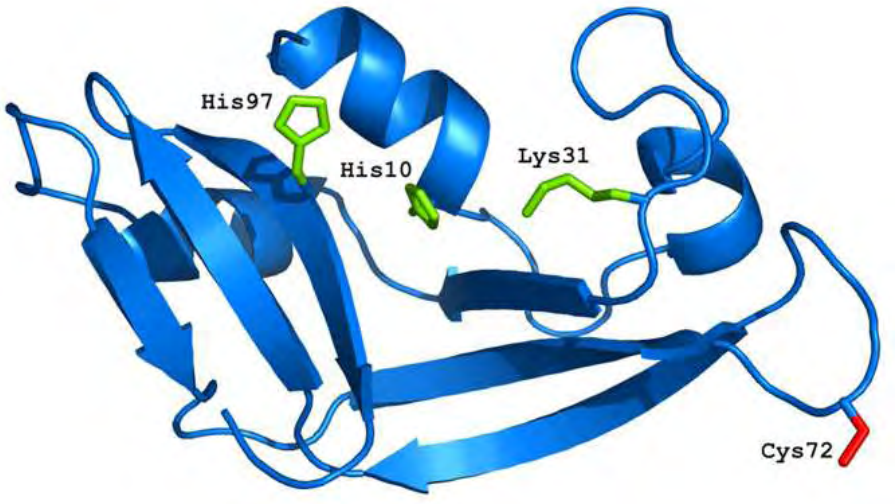

Figure 3. 3-D structure of onconase

4. RNase 7

RNase 7 belongs in the RNase A superfamily of ribonucleases identified in the human genome sequence. Members of this family, as it has been already mentioned, share a conserved structure of six or eight cysteines linked by disulfide bonds and two histidines and one lysine that form the catalytic site. RNase 7 is fairly typical for a member of the RNase A family. Its open reading frame encodes a 28-amino acid signal sequence and the mature protein sequence includes 8 cysteines and appropriately localized histidines and lysines, the latter within the family signature motif. The human RNase 7 gene is expressed in various somatic tissues including the liver, kidney, skeletal muscle and heart and in various epithelial tissues including skin, respiratory tract, genito-urinary tract and, at a low level, in the gut.

RNase 7 is an intriguing secretary and cationic ribonuclease with a calculated isoelectric point (pI) of 9,8 and has unique structure and antimicrobial activity, particularly powerful against several pathogenic microorganisms, both Gram negative and Gram positive.

4.1 RNase 7 as an AMP

Antimicrobial peptides (AMPs), a fundamental component of the innate immune system, are small cationic molecules expressed by phagocytic white cells and epithelial cells. AMPs typically have broad-spectrum antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, enveloped viruses, fungi, and some protozoa.

Ribonuclease 7 (RNase 7) is a potent AMP that was first identified in the skin, while searching for antimicrobial proteins of human skin, during screening the human genome. Molecular cloning from skin-derived primary keratinocytes and purification of RNase 7 from supernatants of cultured primary keratinocytes indicate that keratinocytes represent the major cellular source in skin and that RNase 7 is secreted. It is also demonstrated that RNase 7 protects healthy skin against infection and colonization, respectively with *Staphylococcus aureus*. RNase 7 is expressed widely and is induced in keratinocyte culture by prominent proinflammatory stimuli including TNF, IFNγ, IL-1β and IL-1α, as well as UV-irradiation and bacterial components.

Currently, the mechanisms for RNase 7's antimicrobial properties are not completely understood. Its bactericidal activity has been linked to its capacity to permeate and disrupt the bacterial cell membrane, independent of its ribonuclease activity. As it is already mentioned, RNase 7, like many human AMPs, is highly cationic protein and clusters of flexible cationic lysine residues on the surface of RNase 7 are thought to be critical for its antimicrobial activity. It is supposed that RNase 7 binds to negatively-charged bacterial membranes through these cationic residues. Upon binding to the bacterial membrane, RNase 7 incorporates itself within the membrane, disrupting its physical and functional characteristics, killing the microbe. The toxicity of RNase 7 is directed mainly towards bacteria, as studies show that it is not cytotoxic to human uroepithelial cells, red blood cells and human keratinocytes.

4.2 RNase 7 in the urinary tract

Rnase7 is a potent epithelial-derived AMP that plays a crucial role in the urinary tract, which is constantly challenged by microbial invasion, but remains free from colonization. Although little is known about how the urinary tract maintains sterility, the presence of antimicrobial peptides (AMPs) in the urine suggests that they may play a role in its protection from infection. Ribonuclease 7 (RNase 7) is a potent AMP. As far as the expression and relevance of RNase 7 in the human kidney and urinary tract are concerned, basal RNase 7 expression in kidney and bladder tissue was found by using RNA isolated from healthy human tissue and performing quantitative real-time PCR. Immunohistochemical and immunofluorescent analysis localized RNase 7 to the urothelium of the bladder, ureter, and the intercalated cells of the collecting tubules. RNase 7 is present in the urine at levels sufficient to kill bacteria. It has been stated that, on a per molar basis, RNase 7 is the most potent human AMP. Antibacterial neutralization assays showed that urinary RNase 7 has potent antimicrobial properties against Gram-negative and Gram-positive uropathogenic bacteria.

Thus, RNase 7 is expressed in the human kidney and urinary tract and it may have an important antimicrobial role in maintaining tract sterility, as it is shown by quantitative real-time PCR analysis, which demonstrates that RNase 7 expression increases with acute pyelonephritis. These results suggest that RNase 7 may be an important AMP for the defense and prevention of early infection in the upper urinary tract. However, at chronic pyelonephritis and other chronic bacterial infections, there is a lack of RNase7 induction, supporting the notion that RNase 7 may be critical to mounting an innate immune response that favors rapid microbial clearance. Thus, RNase 7 may be more important during early states of infection than in later stages of disease. The observed decrease in RNase 7 expression with chronic infection may represent a pathogenic mechanism aimed at overwhelming the innate immune response, inhibiting RNase 7 synthesis, and allowing pathogens to evade innate defenses to cause disease.

4.3 RNase 7 in cutaneous defense

The role of RNase 7 in cutaneous defense is important, as well. A detailed analysis of its *in vitro* as well as *in vivo* expression together with functional antimicrobial studies suggest that RNase 7 may play a major role in skin defense and contributes to the high resistance of human skin against colonization with the gram-positive gut bacterium *E. faecium*.

The recent isolation of the antimicrobial ribonuclease RNase 7 from *stratum corneum* of healthy skin revealed its critical role in cutaneous defense. A quantitative analysis revealed that primary keratinocytes express high levels of RNase 7 mRNA when compared to other skin-derived antimicrobial proteins such as psoriasin, hBD-2, and LL-37. This observation further strengthened the hypothesis that RNase 7 may be an important defense molecule in cutaneous innate immunity. Immunohistochemistry using RNase 7 specific antibodies revealed expression of RNase 7 in all layers of human epidermis with higher intensity in the more outer

differentiated epithelial layers. Hair follicles also stained positively which is in concordance with a recent study demonstrating RNase 7 expression in hair follicle epithelium. The expression of RNase 7 in the uppermost epidermal layers and its expression in hair follicles are in concordance with its proposed antimicrobial function, because it is expressed at areas where the first contact with bacteria takes place. Higher RNase 7 protein levels were detected in the supernatants of the primary keratinocyte cultures compared with keratinocyte extracts. These data indicate that RNase 7 is secreted from viable cells and acts primarily outside the cell, which is consistent with the proposed role of RNase 7 in antimicrobial defense.
Based on these *in vitro* data, it was speculated that RNase 7 is secreted *in vivo* on the body surface, so it was determined whether RNase 7 is present at various skin surface sites. Various amounts of RNase 7 in skin washing fluids from different body sites were recovered, which confirmed that RNase 7 is secreted *in vivo*. High variability in RNase 7 secretion levels was detected in different persons and skin locations. This suggests that levels of RNase 7 secretion may depend on environmental factors such as microbial colonization.

The high expression of RNase 7 in skin and its very potent activity against *E. faecium* suggest that the skin is protected against colonization with *E. faecium*. This would explain the low infection rate of skin areas exposed to *E. faecium* such as the perianal region. Skin extracts derived from the stratum corneum efficiently killed *E. faecium* indicating that the skin harbors defense mechanisms which inhibit cutaneous colonization with *E. faecium*.

4.4 Ribonuclease and antimicrobial activity

The high antimicrobial activity of RNase 7 against *E. faecium* raises the question of the responsible molecular killing mechanisms and of the role of the ribonuclease activity. Recombinant RNase 7 that lacks enzymatic activity, by making point mutations of the catalytic residues lys-38 and his-123, was still potent for bactericidal activity against *E. faecium*. This is consistent with other reports showing that members of the RNase A superfamily exhibit ribonuclease-independent antibacterial activity, but raises the question of the role of the enzymatic activity. It has been shown that the antiviral activity of the RNases ECP and EDN against respiratory syncytial virus (RSV) requires functional ribonuclease activity. These data suggest that the ribonuclease activity of RNase 7 may be necessary for a potential antiviral rather than antibacterial activity. However, this remains to be proven as it is not yet known whether RNase 7 exhibits antiviral activity.

The ribonuclease inhibitor interacts with RNase 7 and blocks its ribonuclease as well as antimicrobial activity. It is possible that the ribonuclease inhibitor masks the amino acid residues responsible for the antimicrobial action of RNase 7. Another explanation is that the interaction of the ribonuclease inhibitor RI induces a conformational change of RNase 7 as recently reported for the interaction of RNase 1 and the ribonuclease inhibitor.

4.5 Functional domains of RNase 7 that influence its antibacterial activity

The functional domains of RNase 7 influence its activity against bacteria. The generation of a series of RNase 7 fragments shows that contain different components of its secondary motifs starting from both the N-terminus and C-terminus of RNase 7. Each fragment is determined to have antimicrobial properties against both Gram-positive *S. saprophyticus* and Gram-negative *E. coli* and *P. mirabilis*. However, RNase 7 fragments display significant differences in their antimicrobial activity profiles. Compared to N-terminal fragments, C-terminal fragments show uniformly decreased activity against Gram-negative *E. coli*, *P. mirabilis*, and Gram-positive *S. saprophyticus*. Fragments that lack β-sheet 1, 3 and 4 also demonstrate significantly decreased activities. One fragment is found to have at least four-fold increased potency against both *E.coli* and *Staphylococcus* compared to full-length peptide. There are also identified distinct regions of the peptide that are independently responsible for Gram-negative and Gram-positive activity. These studies suggest that distinct mechanisms are responsible for RNase 7's antimicrobial activity against various pathogens.

4.6 Recombinant RNase 7 and comparison with RNase 3

Recombinant RNase 7 is ribonucleolytically active against yeast tRNA, as expected from the presence of eight conserved cysteines and the catalytic histidine–lysine– histidine triad which are signature motifs of this superfamily. The protein is atypically cationic with an isoelectric point (pI) of 10.5. Expression of recombinant RNase 7 in *Escherichia coli* completely inhibits the growth of the host bacteria, similar to what has been observed for the cationic RNase, eosinophil cationic protein (ECP/RNase 3, pI 11.4). An *in vitro* assay demonstrates dose-dependent cytotoxicity of RNase 7 against bacteria *E.coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. While RNase 7 and ECP/RNase 3 are both cationic and share this particular aspect of functional similarity, their protein sequence identity is only 40%. Of particular interest, ECP/RNase 3's cationicity is based on an (over)abundance of arginine residues, whereas RNase 7 includes an excess of lysine. This difference, in conjunction with the independent origins and different expression patterns, suggests that RNase 7 and ECP/RNase 3 may have been recruited to target different pathogens *in vivo*, if their physiological functions are indeed host defenses.

4.7 RNase 7 compared to RNase 8

RNase 7 and RNase 8 share an amino acid sequence similarity of 78% and a genomic distance of only 15,000 bp, suggesting that they may have evolved from a common ancestor gene by a duplication event. However, although these proteins show a very high similarity in their gene and protein sequence, their physiological roles seem to be completely different. RNase 8 mRNA has been shown to be expressed uniquely in the placenta, and no antibacterial/antiviral activity could be detected with recombinant material. In contrast, RNase 7 is expressed in many epithelial tissues and exhibits a broad spectrum of efficient antimicrobial activity at low micromolar concentrations against various pathogenic microorganisms, including*S. aureus*, *P. aeruginosa*, *P. acnes*, and *C. albicans* (lethal doses that achieve a CFU reduction of 90% (LD_{90}) = 0.75–1.5 μM).

4.8. Conclusion

In conclusion, the isolation of a novel epithelial-derived antimicrobial RNase identifies RNases as a novel class of endogenous epithelial antimicrobial proteins that may play an important role in the innate immunity of human epithelia and offer an immediate host response against infectious agents. It is interesting to speculate that in patients suffering from recurrent epithelial infections the production of epithelial-derived antimicrobial proteins like RNase 7 might be disturbed.

Finally, the discovery of human epithelial antimicrobial proteins like RNase 7 may further inspire the development of new strategies for the treatment of infectious diseases in which conventional antibiotics fail because of the emergence of resistant bacteria.

EXPERIMENTAL PART

1.OBJECTIVES

The work of this Thesis is part of a research project focused on the study of the structure-function relationship of RNase 7. As it is already mentioned, this protein is an epithelial-derived antimicrobial RNase that exhibits ribonuclease activity and belongs to the ribonuclease A superfamily. It is expressed in various somatic and epithelial tissues and its main source of secretion is the keratinocyte cells of the skin.

The aims of the presented work were:

1) Expression of the epithelial- derived RNase7 using *E. Coli* strains BL21, transformed with vector pET11c with insertion of synthetic gene.
2) Isolation and purification of the protein from insoluble fraction (inclusion bodies). Purification was made through denaturation and renaturation steps, using FPLC (RESOURCE S column) or HPLC (MONO S column) as the final step of purification.
3) Characterization of expressed and purified proteins with SDS polyacrilamide gel electrophoresis.
4) Determination of the ribonuclease activity of purified proteins using electrophoretic method of SDS gel by zymogram, using poly(U) as a substrate.
5) Evaluation of the antimicrobial activity by counting colonies in agar plates after mixing of diluted proteins with diluted bacteria and equilibration.
6) Crystallization of RNase7 using the hanging drop crystallization method.

2. BIOLOGICAL MATERIAL AND CULTURE MEDIA

2.1 *Esherichia Coli* as a host for pET11c for the expression of RNase7

E. coli is Gram-negative, facultative anaerobic and non-sporulating. Cells are typically rod-shaped, and can live on a wide variety of substrates. Optimal growth of *E. coli* occurs at 37 °C, but some laboratory strains can multiply at temperatures of up to 49 °C. *E. coli* and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population.

Escherichia coli encompass an enormous population of bacteria that exhibit a very high degree of both genetic and phenotypic diversity. *E. coli* is one of the most diverse bacterial species: only 20% of the genome is common to all strains.

A strain is a sub-group within the species that has unique characteristics that distinguish it from other strains. These differences are often detectable only at the molecular level; however, they may result in changes to the physiology or lifecycle of the bacterium.

In this experiment, the strain used as general host for Pt11c vector is the BL21 which has the genotype: F^- omp t $hsdS_B(r_B^-m_B^-)$ gal dcm (DE3).

2.2 Plasmids

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, such as 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.

2.3 Plasmids as vectors

Plasmids used in genetic engineering are called vectors. Plasmids serve as important tools in genetics and biotechnology labs, where they're commonly used to multiply or express particular genes. Many plasmids are commercially available for such uses. The gene to be replicated is normally inserted in copies, into a plasmid that typically contains a number of features. The plasmid should include a gene that makes the bacterial cells resistant to particular antibiotics such as Kanamycin or Ampicillin. It should also contain at least one DNA sequence that serves as an origin of replication, or ori (a starting point for DNA replication), which enables the plasmid DNA to be duplicated independently from the chromosomal DNA and to be distributed to the daughter cells, and a multiple cloning site (MCS, or polylinker). A

multiple cloning site is a short region containing several commonly used restriction sites allowing the easy insertion of DNA fragments at this location. An ideal vector should have a small size, a large number of restriction sites, marker genes for easy seletion of the clones containing the insert and promoters for in vitro transcription.

After the gene of interest is inserted, the plasmids are inserted into bacteria by a process called transformation. Then, the bacteria are exposed to the particular antibiotics. Only bacteria that take up copies of the plasmid survive, since the plasmid makes them resistant. In particular, the protecting genes are expressed and the expressed protein breaks down the antibiotics. In this way, the antibiotics act as a filter to select only the modified bacteria. Now these bacteria can be grown in large amounts, harvested, and lysed to isolate the plasmid of interest.

Another major use of plasmids is to make large amounts of proteins. In this case, researchers grow bacteria containing a plasmid harboring the gene of interest. Just as the bacterium produces proteins to confer its antibiotic resistance, it can also be induced to produce large amounts of proteins from the inserted gene.

A plasmid can contain inserts of up to 30-40 kbp. To clone longer lengths of DNA, lambda phage with lysogeny genes deleted, cosmids, bacterial artificial chromosomes, or yeast artificial chromosomes are used.

In this experiment pET11c vector is used for cloning in *E. coli*. pET11c plasmid is a 5677 bp plasmid with T7 lac promoter and a BamH I cloning site.

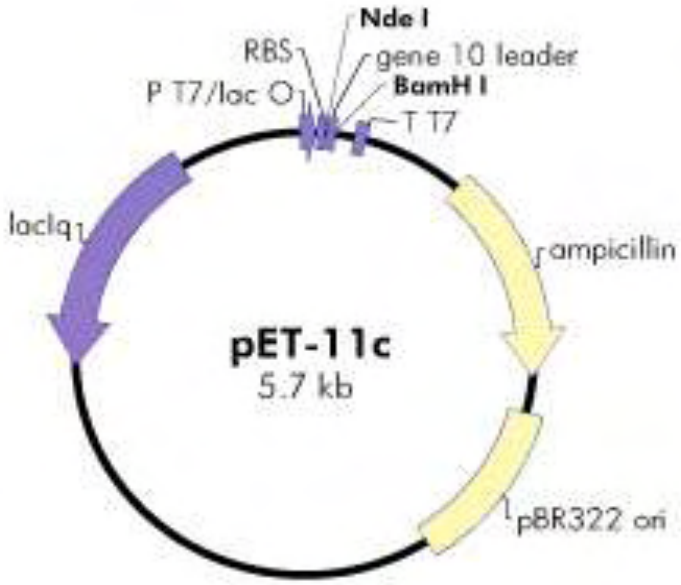

Figure 1. The pET Vector. This plasmid contains a drug resistant marker for ampicillin resistance, the T7 transcription promoter, the lac operator region to the T7 promoter, a lacI repressor ORF and a polylinker region with a Nde I cloning site,a BamH I cloning site and a ribosome binding site . There are two origins of replication - one is the f1 origin which enables the production of a single stranded vector under appropriate conditions, and the other is the conventional origin of replication.

2.4 Luria-Bertani (LB) medium (per 1 l)

Luria-Bertani (LB) broth is the most widely used medium for the growth of bacteria. Life Technologies offers LB media in both powder and ready-to-use liquid formats for convenience and ease of use.

PREPARATION : 10 g of bactotryptone, 5 g of bactoyeast extract and 10 g of NaCl are dissolved in 950 mL of deionised MQ water. Volume is adjusted in 1 L and sterilization is succeeded by autoclaving. Concentration of LB: 25 g/L.

2.5 Terrific Broth (TB) medium (per 1 l)

Terrific broth (TB) is a nutritionally rich medium for the growth of bacteria. Actually, it is a medium allowing the overexpression of the proteins and the formation of inclusion bodies. Life Technologies offers TB media in both powder and ready-to-use liquid formats for convenience and ease of use.

PREPARATION : In 900 mL of deionised MQ water 12g of bactotryptone, 24g of bacto-yeast extract and 4 mL of glycerol are added followed by autocliving for sterilization. (glycerol is added last after solubilization is succeded). After solution is cooled down to 60 ^{o}C, 100 mL of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 are added because if the TB medium is autoclived in the presence of phosphate buffer, a precipitate will occur.

TB salts (10x) : 23.1g KH2PO4 and 125.4 g K2HPO4 are dissolved in water to a final volume of 1 L and are sterilized by autoclaving.

Tryptone; Tryptone is commonly used to produce LB for the growth of *E. coli* and other microorganisms. It provides a source of amino acids for the growing bacteria**.**

Bacto-yeast extract; BD Yeast Extracts are animal-free products suitable for use as multi-functional nutritional supplements in cell culture, microbial fermentation and insect cell culture applications.

Glycerol; Glycerol has three hydroxyl groups that are responsible for its solubility in water and its hygroscopic nature and is used as a carbon source.

Buffer solution; An appropriate buffer solution added to a protein mixture during the extraction process can help improve the stability of protein molecules as these molecules are subjected to various forces designed to isolate them for study. A buffer solution can protect the integrity of the proteins while separating them from other integrated cell components.

A buffer solution should be compatible with the protein in question and able to recreate an ionic environment similar to the ionic environment of the cell. The pH balance of the buffer must correspond with that of the cell *in vivo*, while still allowing separation of the cell's component parts.

A buffer solution that can maintain protein stability during every stage of the procedure is necessary in order to avoid the need to switch buffer solutions during the process, including chromatography or electrophoresis. It's also wise to account for shifts in temperature, since the pH of some buffer solutions can change in the presence of heat.

2.6 IPTG induction

Isopropyl β-D-1-thiogalactopyranoside (IPTG) is a molecular biology reagent. This compound is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon and it is therefore used to induce protein expression where the gene is under the control of the lac operator. However, IPTG, unlike allolactose, is not hydrolyzable by β-galactosidase, its concentration therefore remains constant in an experiment.

Like allolactose, IPTG binds to the lac repressor and releases the tetrameric repressor from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon, such as the gene coding for beta-galactosidase, a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides. But unlike allolactose, the sulfur (S) atom creates a chemical bond which is non-hydrolyzable by the cell, preventing the cell from metabolizing or degrading the inducer. The concentration of IPTG therefore remains constant and the expression of lac p/o-controlled genes would not be inhibited during the experiment.

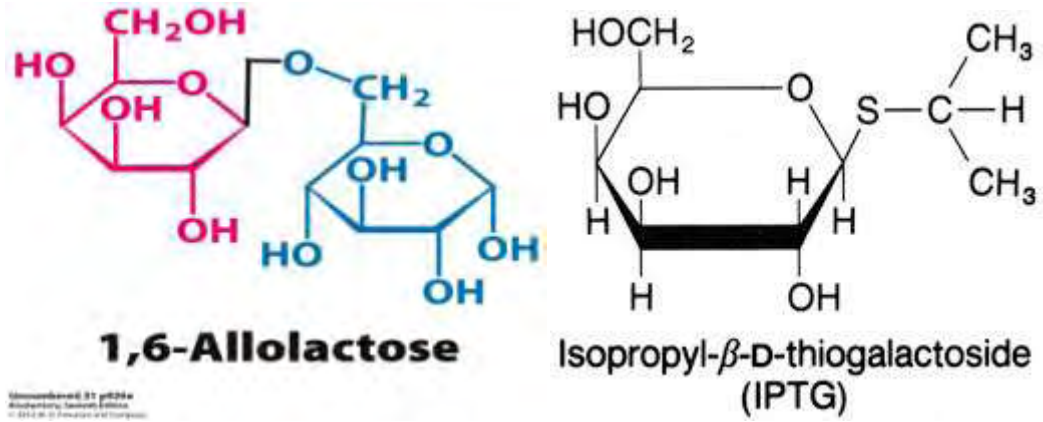

Figure 2. Chemical structure of 1,6-allolactose and its molecular mimic, IPTG

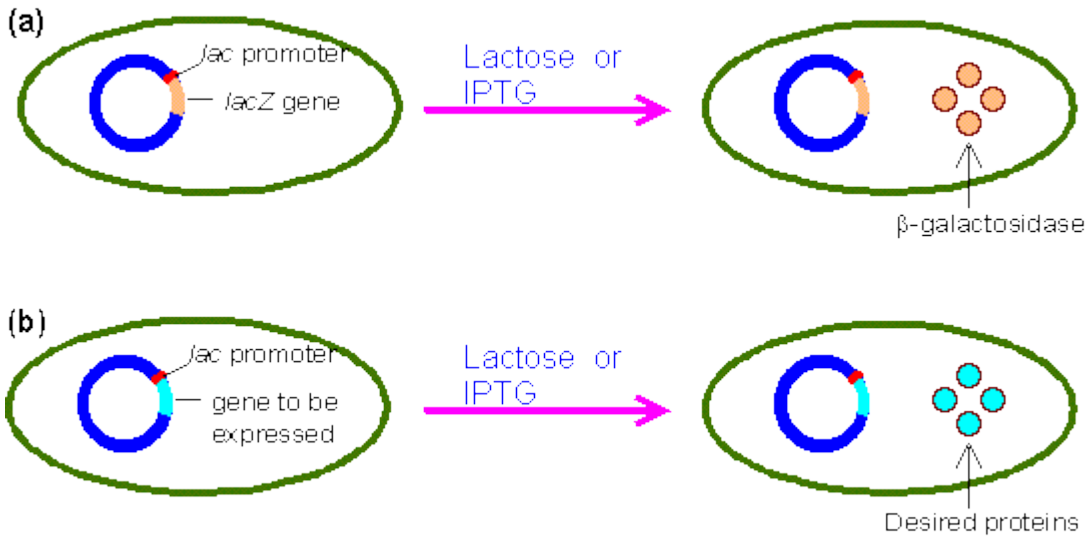

Figure 3. Production of recombinant proteins. (a) The expression vector contains the *lac* promoter and its neighboring *lacZ* gene encoding β-galactosidase. Lactose or its analog IPTG can stimulate the expression of β-galactosidase. (b) If *lacZ* is replaced by the gene encoding the protein of interest, lactose or IPTG will stimulate the expression of desired proteins

2.7 Centrifugation for cell collection

After the protein expression is achieved by induction with IPTG, the next goal is to start the purification of the protein. The first step in this long procedure is its separation from non-protein components such as nucleic acids, lipids or unbroken cells, through centrifugation, according to their size, shape, density, viscosity of the medium and rotor speed. In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it float to the top. The greater the difference in density, the faster they move.

After centrifugation, the supernatant containing the soluble hydrophilic proteins is separated from the pellet containing the hydrophobic/insoluble proteins, such as the inclusion bodies. Inclusion bodies have a relatively high density and, therefore, can be pelleted by centrifugation.

2.8 Inclusion bodies

Overproduction of heterologous proteins in the cytoplasm of E. coli is often accompanied by their misfolding and segregation into insoluble aggregates known as inclusion bodies.

It has been possible to develop improved solubilization and refolding procedures for higher recovery of bioactive protein. Proteins inside inclusion body aggregates have native-like secondary structures, but remain inactive and denatured. It is assumed that restoration of this native-like secondary structure using mild solubilization conditions will help in improved recovery of bioactive protein in comparison to solubilization using a high concentration of chaotropic agent.

ADVANTAGES	DISADVANTAGES
the recombinant protein deposited in inclusion bodies can be 50% or more of the total cellular protein.	refolding is often cumbersome and optimal condition cannot be predicted
the inclusion bodies often contain almost exclusively the overexpressed protein.	
in inclusion bodies the protein is protected from proteolytic degradation.	
expression in inclusion bodies will protect the cell against the toxicity of the recombinant protein	

Table 1. Advantages and disadvantages of inclusion bodies for protein purification.

Experimental procedure

1) A small quantity of the desired cells (E. Coli BL21) which are stored at -80^{o}C are inoculated in 500 mL of LB medium for the overnight culture of bacteria, along with ampiciline (amp) (100 μg/mL).
2) Overnight incubation in 37^{o}C shaker.
3) 200 mL of KH_2PO_4/ K_2HPO_4 through a sterilized with water tube are added, along with 8 mL amp and 40 mL bacteria of the overnight culture to

inoculate 2l of TB and are grown at 37^{o}C until an OD_{600} of approximately 0.6 is reached.

4) 1.250 μL IPTG at a final concentration of 1 mM are added to induce the protein expression.
5) The culture is incubated at a suitable temperature, under stirring for a period of 3 – 4h.
6) Before and after the IPTG induction aliquots are taken to confirm the recombinant protein expression by SDS-PAGE analysis and are centrifuged at 14.800 rpm for 1 minute.
7) The culture is centrifuged for 10 minutes at 12.000 rpm.
8) The supernatant is discarded and the pellet is kept in fridge at -20 ^{o}C to be used for purification of the recombinant RNase 7 from the inclusion bodies.

2.9 Purification

2.9.1 Sonication

In order to start the purification of our protein which is obtained from the inclusion bodies that are formed in the sediment, it is necessary to move on to cell disintegration or lysis. The goal of lysis is to disrupt parts of the cell wall or the complete cell to release biological molecules, such as organelles, proteins, DNA, mRNA. It is important that cell lysis is complete, because intact cells sediment together with the inclusion bodies, thus contaminating the preparation.

In the case of sonication for cell lysis, ultrasound high-frequency sound waves are applied to samples to agitate and disrupt the cell membranes. Whether the disruption is very gentle or very abrupt depends on the cell structure and on the purpose of lysis (e.g. DNA extraction requires softer sonication, complete protein extraction of bacteria requires a more intense ultrasound treatment), so the duration of sonication is regulated according to the purpose served. Localized heating due to the high-frequency waves can occur leading to protein denaturation and aggregation. To avoid this problem it is essential to keep samples on ice.

The buffer in which the pellet is resuspended before the sonication, is used to remove as much bacterial protein as possible from the aggregated foreign protein.

Experimental procedure

1) The pellet is resuspended with the following buffer, so the total volume will be around 80 mL, while the initial volume of the culture was 2l and then is mixed with vortex.

   Buffer:

   - 10 mM Tris HCl pH 8.0

   (for providing an ionic environment similar to that of the cell in vivo so as the stability of the protein is maintained)

   - 2 mM EDTA (for prevention of proteases)

2) The sample is inserted in tubes.
3) Each tube is sonicated 3 times for 6 minutes while kept on ice to avoid destruction of the proteins.
4) The sample is then centrifuged for 30 minutes at 16.000 rpm at 4 ^{o}C.
5) The supernatant is discarded and the pellet is kept in fridge.

2.9.2 Purification from inclusion bodies through denaturating and refolding steps

To obtain soluble active proteins from inclusion bodies, the insoluble inclusion bodies are first washed. This wash step is necessary to remove contaminants, especially proteases that may have absorbed onto the hydrophobic inclusion bodies during processing. Then, they are solubilized in denaturant form and are centrifuged to remove remaining aggregates which could act as nuclei to trigger aggregation during refolding. Then the denaturant is reduced and removed from the solubilized protein by dilution in a huge volume of refolding buffer to recover soluble and active proteins.

For this reason, denaturing buffer containing Guanidine hydrochloride denaturant is most commonly used to solubilize the inclusion bodies in denature form. Guanidine hydrochloride can unfold the misfolded secondary structures present in inclusion bodies completely into random coiled structures. Then, glutathione, which acts as a reducing agent, is added in the denaturing buffer, with which the inclusion bodies are incubated.

Glutathione (GSH) is an important antioxidant in plants, animals, fungi and some bacteria and archaea, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides. It is a tripeptide of L-cysteine, L-glutamic acid, and glycine with a gamma peptide linkage

between the carboxyl group of the glutamate side-chain and the amine group of cysteine (which is attached by normal peptide linkage to a glycine).

Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. In the reduced state, the sulfhydryl (thiol) group (SH) group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules, reducing disulfide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor.In donating an electron, glutathione is converted to its oxidized form, becoming itself reactive, so it reacts with another reactive glutathione to form glutathione disulfide (GSSG), also called L – glutathione.

So, in our experiment the reduced glutathione in the denaturing buffer donates its hydrogen in the proteins, cleaving the disulfide bonds formed between them, thus helping in the unfolding process. However, in the refolding buffer the oxidised form of glutathione, has no hydrogens, so it takes them back from the proteins, letting the formation of disulfide bonds between the proteins, meaning that they are refolded again. The best yield was obtained when the GSH / GSSH ratio was 4. The refolding buffer also contains Tris-HCl and L-arginine, which impedes aggregate formation by enhancing the solubility of folding intermediates, presumably by shielding hydrophobic regions of partially folded chains.

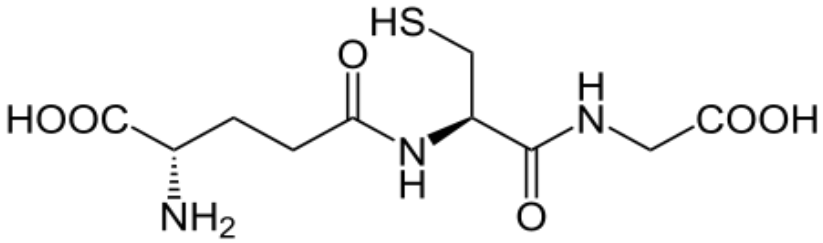

Figure 4. glutathione

Experimental procedure

1) The pellet is resuspended in 20 mL of the following buffer and then mixed with vortex for 30 minutes

Buffer:

- 50 mM Tris HCl pH 8.0
- 300 mM NaCl
- 2 mM EDTA

2) The sample is then centrifuged for 30 minutes at 12.000 rpm at 4 ^{o}C.
3) The supernatant is discarded and the pellet is kept in fridge.
4) 25 mL of the following buffer is added gradually in the pellet in order to be resuspended.

Denaturing buffer:

- 6 M Guanidine – HCl
- 100 mM Tris - acetate pH 8.0
- 2 mM EDTA

5) After the pH is checked to be ensured that is still 8.0 reduced glutathion is added to the final concentration of 80 mM. Since after the glutathion the pH starts decreasing, at the same time solid Tris (hydroxymethyl) aminomethane is added in order to be kept stable (8.0).
6) The solution is covered with parafilm and is left being sterilized for 2 h in nitrous atmosphere.
7) Then, centrifugation follows in 12.000 rpm for 30 minutes.
8) This time the pellet is discarded and the supernatant is kept.
9) The supernatant is incubated gradually (5 mL / min) in 2 l of the following buffer, which is kept in the fridge. (sample A)

Refolding buffer:

- 100 mM Tris - acetate HCl pH 7,5
- 0,5 M L - arginine
- 0,3 mM L – glutathione

2.9.3 Chromatographic purification of RNase 7 with ion exchanged chromatography (cationic)

Purification and characterization of target proteins were then carried out by using high-resolution ion-exchange chromatography and SDS-PAGE gel, respectively.

Ion-exchange chromatography (IEX) is a process that allows the separation of ions and polar molecules based on their affinity to the ion exchanger. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids, 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 this experiment, two cation exchange columns are used : DEAE – sepharose and CM – sepharose (figure 5). DEAE-Sepharose consists of the anion-exchange reactive group , diethylaminoethanol (DEAE) covalently linked to Sepharose (a polysaccharide polymer). DEAE is positively charged due to the presence of the amino-group (NH_3^+). So, it binds negatively charged proteins and allows positively charged proteins to pass through the column, such as RNase 7. Then, CM – sepharose is used, which is negatively charged due to the presence of the carboxyl group (COO^-), thus letting the binding of the positively charged proteins. The eluting solution passing through the columns has a p.H of 7.5, so RNase 7 with a p.I of 9.8 is positively charged.

- The column is flushed with deionised MQ water, so that the storage solution (usually 20% ethanol) is washed out.
- Equilibration with buffer A (150 mM sodium acetate pH 5.0) , to allow the binding with RNase 7.
- Filtration of sample A so as to be condensed and prevent loading of misfolded proteins in the column. After filtration, it is loaded in the column.
- The washout of columns by the same buffer follows (buffer A) for 5h for the removal of all proteins, which did not bind at the column.
- Elution of the protein from the CM – sepharose column (which is negatively charged) with the use of NaCl linear gradient. More specifically, the column is connected with 2 solutions. The one contains buffer A , 5 mM EDTA , 20 mM DTT →prevents aggregation (buffer B) and the other buffer B and NaCl pH 5, 2M, V=200 mL (figure 6). With this way, sodium chloride is transferred gradually in the column, so with the gradual increase of ionic strength, the proteins are removed from the column depending on the strength of their

binding to it. The proteins which are weakly bound in the column will elute first, while the ones that are bound will be retained and eluted in the last fractions, which contain the highest concentration of salt.

- After the column has been used, it is washed with distilled deionised water, kept in 20% ethanol to prevent microbial growth and is stored at 4 ^{o}C. The flow rate of the sample was 2 mL/min and the fraction size was 4 mL.
- The protein concentration of the fractions is calculated by measuring their optical absorbance at a wavelength of 280 nm.

Figure 5. Cation exchange chromatography

Figure 6. Elution of RNase 7 with NaCl.

2.10 Characterization by SDS – polyacrylamide gel electrophoresis

The term electrophoresis refers to the movement of charged molecules in response to an electric field, resulting in their separation.

In an electric field, proteins move toward the electrode of opposite charge. The rate at which they move (migration rate,) is governed by a complex relationship between the physical characteristics of both the electrophoresis system and the proteins. Factors affecting protein electrophoresis include the strength of the electric field, the temperature of the system, the pH, ion type, and concentration of the buffer as well as the size, shape, and charge of the proteins. Proteins come in a wide range of sizes and shapes and have charges imparted to them by the dissociation constants of their constituent amino acids. As a result, proteins have characteristic migration rates that can be exploited for the purpose of separation. Protein electrophoresis can be performed in either liquid or gel-based media and can also be used to move proteins from one medium to another.

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 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 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.

If several samples have been loaded into adjacent wells in the gel, they will run parallel in individual lanes. Depending on the number of different molecules, each lane shows separation of the components from the original mixture as one or more distinct bands, one band per component. Incomplete separation of the components can lead to overlapping bands, or to indistinguishable smears representing multiple unresolved components. Bands in different lanes that end up at the same distance from the top contain molecules that passed through the gel with the same speed, which usually means they are approximately the same size. There are molecular weight size markers available that contain a mixture of molecules of known sizes. If such a marker was run on one lane in the gel parallel to the unknown samples, the bands observed can be compared to those of the unknown in order to determine their size. The distance a band travels is approximately inversely proportional to the logarithm of the size of the molecule.

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.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which is used in this experiment, is a technique for separating proteins based on

their ability to move within an electrical current, which is a function of the length of their polypeptide chains or of their molecular weight. It uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins.

SDS is an anionic detergent, meaning that when dissolved, its molecules have a negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field.

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).

In this system, two sequential gels are actually used; a stacking gel component, which has a low (5.5%) acrylamide concentration to make a porous gel. Under these conditions proteins separate poorly but are focused as thin, sharply defined bands at the beginning of the electrophoretic run. The lower gel, called separating or resolving gel has a higher polyacrylamide content, which causes the gel to have narrower channels or pores. So, it separates the proteins based on their mass weight, allowing smaller proteins to travel more easily and hence rapidly, than larger proteins. This classic system uses a discontinuous buffer system where the pH and ionic strength of the buffer used for running the gel (Tris pH 8.3) is different from the buffers used in the stacking gel (Tris, pH 6.8) and resolving gel (Tris, pH 8.8).

After the electrophoresis is complete, the molecules in the gel can be stained to make them visible. DNA may be visualized using ethidium bromide which, when intercalated into DNA, fluoresce under ultraviolet light, while protein may be visualised using silver stain or Coomassie Brilliant Blue dye.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed.

Experimental procedure

1) Two glass plates are washed and then are put together in the electrophoresis chamber.
2) Then, the separating gel is prepared and fills the gap between the glass plates (75% of the space), with the help of a pipette.
3) The gel is covered with isopropanol until its polymerization (30 – 60 minutes).
4) Isoporopanol is removed and the stacking gel is then added above the separating gel, filling the space up to the surface of the plates.
5) A plastic comb is used to squize the gel in order to form a specified number of wells.
6) After polymerization of the second gel, the plastic comb is removed and the gel is placed in the electrophoresis device.
7) In the meantime, 20 μL of each sample are mixed with 10 μL loading buffer (30% glycerol, 9% SDS, 15% β – mecraptoethanol, 0.05% bromophenol blue dissolved in Tris/HCl 180mM, pH 6.8). For the marker, 10 μL are mixed with 5 μL loading buffer.
8) The samples are heated for 2 – 3 min at 100^{o}C.
9) Running buffer 1x (250 mM Tris/HCl, pH 8.3, 1.9 M glycin, 1% SDS and distilled water up to 1 L) is poured in the device but not until the top.
10) The samples are loaded in the wells, using a microsyringe and then the rest of the running buffer is poured.
11) The device is connected with the power supply and the electrophoresis stops when the dye of the protein wells reaches about 1cm from the edge of the gel.
12) After the device is removed from the power supply the plates are removed and are laid down in paper.
13) Then they are carefully removed and the gel is placed in in Coomasie Brilliant Blue staining solution (30% methanol, 8% acetic acid, 0.15% Coomasie blue) for 10 min, while left shaking mildly.
14) After the removal of the coomasie, destaining is performed with a solution of 30% ethanol and 7% acetic acid until the excess of dye is washed out.

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
40 µL APS (10%)	20 µL APS(10%)
10µl TEMED	5µl TEMED
Stacking gel	
1,9 mL H_2O	
0,775 mL solution C	
0,5 mL solution A	
40 µL APS	
5 µL TEMED	

Table 2. Reagents for the preparation of the separating and stacking gel.

- **Bisacrylamide** (N,N'-Methylenebisacrylamide) ($C_7H_{10}N_2O_2$) ; Bisacrylamide is the most frequently used cross linking agent for polyacrylamide gels, and is present in much smaller quantities than acrylamide. Chemically it can be thought of as two acrylamide molecules coupled head to head at their non-reactive ends. Bisacrylamide can crosslink two polyacrylamide chains to one another, thereby resulting in a gel.
- **Sodium Dodecyl Sulfate (SDS**) ($C_{12}H_{25}NaO_4S$)**;** as it is already mentioned, SDS is a strong anionic detergent agent used to denature native proteins to unfolded, individual polypeptides. It binds around the polypeptide backbone, so, in this process, the intrinsic charges of polypeptides becomes negligible when compared to the negative charges contributed by SDS. Thus polypeptides after treatment become rod-like structures possessing a uniform charge density. The electrophoretic mobility of these proteins is a linear function of the logarithms of their molecular weights. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass-charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.

- **Ammonium persulfate** (APS) ($N_2H_8S_2O_8$)**;** APS is a source of free radicals and is often used as an initiator for gel formation.
- **TEMED** (N, N, N', N'-tetramethylethylenediamine) ($C_6H_{16}N_2$); TEMED stabilizes free radicals and improves polymerization. The lowest catalytic concentrations that allow polymerization in a reasonable period of time should be used. Since the combination of APS and TEMED is essential for the polymerization of the gel, these reagents are added just before the transfer of the solutions in the plates.
- **Isopropanol;** Right after the seperating gel is placed among the glass plates, isopropanol is added, so as to exclude air and to obtain an even interface between the gels.
- **Loading buffer;** All protein samples before electrophoresis are mixed with loading buffer, which helps weigh down the solution, so that it can sink into the bottom of the wells and not float in the buffer solution. Bromphenol blue does not stain the samples, but helps their visualisation while loading them. The loading buffer, also contains a dense substance, such as glycerol. It is the glycerol which due to its density will make the sample more dense than the buffer which the gel is run in, and will prevent it floating out of the well. β-mercaptoethanol, which is also present n the loading buffer, is used to break disulphide bonds.
- **Running buffer;** The gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value.
- **Coomassie Brilliant Blue R-250 (CBB) (**$C_{45}H_{44}N_3NaO_7S_2$). CBB is the most popular protein stain. It is an anionic dye, which non-specifically binds to proteins. The structure of CBB is predominantly non-polar, and it is usually used in methanolic solution acidified with acetic acid. Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated into the gel can be removed by destaining with the same solution without the dye. The proteins are detected as blue bands on a clear background. As SDS is also anionic, it may interfere with staining process. Therefore, large volume of staining solution is recommended, at least ten times the volume of the gel.

2.11 Ribonuclease activity by zymogram

Zymography is an electrophoretic method, based on SDS-PAGE, used for measuring proteolytic activity. It includes an enzyme substrate copolymerized with the polyacrylamide gel. Samples are prepared without boiling to preserve the structure and activity of the enzyme. 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 areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme.

In this experimental work, the separating gel consists of certain amount of polyurydylic acid (poly(U)) as a substrate, in order to determine bands which correspond to the protein fractions with ribonuclease activity. Since it is mixed with other reagents, it is applied everywhere in the gel, meaning that enzymes with ribonuclease activity in contact with poly(U), locally degrade the polynynucleotide and are represented as clear bands in the gel. After the separations of the proteins by electrophoresis, the gel is washed with three different solutions, playing different roles each. The first solution (solution 1) contains isopropanol and is used to remove SDS, while the second (solution 2) is used to remove the isopropanol. The third solution (solution 3), containing an increased concentration of Tris, compared to the other two, increases the ionic charge of the protein, thus providing the best conditions for the expression of the ribonuclease activity. The gel is then stained with toluidine blue, allowing the visualization of the bands representing the ribonuclease activity, which remain without dye.

Experimental procedure (quantities for 2 gels)

1) 6 mg of poly(U) (potassium salt of poly(U): SIGMA) are diluted in 2,5 mL of distilled water.
2) The soution is left for 10 minutes at room temperature and then 5 minutes at 55 ^{o}C.
3) 5 mL of solution A (same as SDS-PAGE) and 2,5 mL of solution B (same as SDS-PAGE) are heated for 1 – 2 min at 55oC, so as to have the same temperature with the poly (U) substrate, with which are mixed together.
4) 10 μL TEMED and 40 μL of APS are added.
5) 5 mL of separating gel is poured with a pipette between glasses in electrophoretic kit without forming bubbles.
6) The separating gel is covered with certain quantity of isopropanol and is left for approximately 30 min. to be polymerized.

7) The stacking gel (the same with SDS – PAGE electrophoresis) is prepared and after the removal of the isopropanol, is placed between the plates forming the upper layer above the separating gel, reaching up to the surface of the plates.
8) A plastic comb is used to squize the gel in order to form a specified number of wells.
9) After polymerization of the second gel, the plastic comb is removed and the gel is placed in the electrophoresis device.
10) The samples are mixed with loading buffer (without β - mercaptoethanol) in the ratio sample/buffer 2 to 1 and are loaded in the wells (no more than 15 μL), using a microsyringe.
11) The device is connected with the power supply and the electrophoresis stops when the dye of the protein wells reaches about 1cm from the edge of the gel.
12) The gel is washed with solution 1; cooled buffer Tris/HCl 10 mM, pH 7.5 which has 20% isopropanol (3 times for 5 – 10 min)
13) The gel is washed with solution 2; cooled buffer containing 10 mM Tris/HCl, pH 7.5 (3 times for 5 – 10 min)
14) The gel is incubated at room temperature with solution 3; buffer containing 100 mM Tris/HCl for 1 hour.
15) The gel is stained for 10 min with toluidine blue (0.2% toluidine blue in 10mM Tris/HCl, pH 7.5) and the excess of color is distained with distilled water.

2.12 Antimicrobial activity

In order to determine, if RNase 7 shows antimicrobial activity, the Diffusion or agar plate method is performed. The proteins are mixed with the bacteria, after both of them are diluted and then, after incubation they are spread in agar plates to be incubated. Then, the bacterial colonies are measured in each plate and the minimum inhibitory concentration (MIC) is calculated. MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of an organism after overnight incubation. MIC is important to confirm resistance of microorganisms to an antimicrobial agent, such as RNase 7 in our case, and also to monitor the activity of new antimicrobial agents. A lower MIC is an indication of a better antimicrobial agent.

Experimental procedure

1) Initially, the agar plates are prepared; 12,5 gr of L.B and 7,5 gr of agar are dissolved in 0,5 L of water. Then, they are sterilized for 20 minutes at 120^{o}C (dry heat). Last, after the medium is cooled, it is shared in 10 plates and left in room temperature until it is solidified.
2) Bacterial dilution
   10 μL of *E. Coli* is added in 990 μL of buffer in an eppendorf type tube. Then, 10 μL are added in 990 μL of buffer and the same procedure follows one more time so the final dilution is 1/10.000.
3) Protein dilution
   The initial concentration of RNase 7 is 62 mM, while the final concentration should be 20 mM in 100 μL. So, according to the type $C_1XV_1 = C_2V_2$, 32 μL of RNase 7 are added in 68 μL of buffer in the first eppendorf type tube. Then, 50 μL of the dilution of the first eppendorf type tube are added in a second eppendorf type tube which already contains 50 μL buffer. The same procedure goes on for n=10 eppendorf type tubes and 50 μL of the last eppendorf type tube are discarded, so each eppendorf type tube finally contains 50 μL. In each step, the concentration of the protein is reduced by half.
4) Mixing of RNase 7 with diluted bacteria
   In every eppendorf type tube containing 50 μL (protein+buffer), 50 μL of diluted bacteria are added.
5) The eppendorf type tubes are incubated at 37 ^{o}C for 4 h.
6) The incubated bacteria solution is spread in agar plates (25 μL per plate) and is left at 37 ^{o}C for 12 h.
7) The colonies are measured and the c/50 is calculated through a computer programme.

The plates, in which no bacteria growth is found, represent the antimicrobial activity of RNase 7. Actually, the first plate with no bacterial growth represents the MIC of the protein.

2.13 Crystallization

Crystallization is the process for the formation of solid crystals from an homogenous solution**.** More than 80% of the substances used in pharmaceuticals, fine chemicals, agrochemicals, food and cosmetics are isolated or formulated in

their solid form**.** Crystallization is in general the last chemical purification step in the production of ingredients.

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. Proteins, like many other molecules, 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. The goal of crystallization is usually to produce a well-ordered crystal that is lacking in contaminants and large enough to provide a diffraction pattern when hit with x-ray. This diffraction pattern can then be analyzed to discern the protein's three-dimensional structure. Solving the three-dimensional (3D) structure of a protein represents a prerequisite and critical step towards complete understanding of its biological function. In addition, knowledge of the 3D structure is useful for research areas that rely on protein structure, such as rational protein design, bioinformatics, biodiversity, and studies on mechanisms of human health and disease.

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. In 1958, the structure of myoglobin, determined by X-ray crystallography, was first reported by John Kendrew, who also won a Nobel Prize in Chemistry in 1962 for this discovery. The interest of X-ray diffractionists was influential in promoting efforts to reproducibly grow high quality protein crystals, but also led to efforts to increase success rates and to automate the crystallization process. The background for the latter is formed by the fact that with the extraordinary advances in data collection and computing techniques and with the revolution in pharmacology and biotechnology, the need for new macromolecular crystals very soon greatly surpassed their supply. Clearly, at present, the challenge is to obtain enough knowledge about the ideal conditions for protein crystallization, initially through the 'trial-and-error' principle and then, based on this knowledge, to develop new, more directed methods in order to be able to readily crystallize any protein.

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 and the fragile nature of protein crystals. Proteins have

irregularly shaped surfaces, which results in the formation of large channels within any protein crystal. Therefore, the noncovalent bonds that hold together the lattice must often be formed through several layers of solvent molecules. In addition to overcoming the inherent fragility of protein crystals, the successful production of X-ray worthy crystals is dependent upon a number of environmental factors because so much variation exists among proteins, with each individual requiring unique conditions for successful crystallization. Therefore, attempting to crystallize a protein without a proven protocol can be very tedious. Some factors that require consideration are protein purity, pH, and concentration of protein, temperature, and precipitants. In order for sufficient homogeneity, the protein should usually be at least 97% pure. pH conditions are also very important, as different pH's can result in different packing orientations. Buffers, such as Tris-HCl, are often necessary for the maintenance of a particular pH. Precipitants, such as ammonium sulfate or polyethylene glycol, are compounds that cause the protein to precipitate out of solution. Specifically, the factors which affect crystallization, are presented in the following table.

Physical factors	Chemical factors	Biochemical factors
Temperature	Precipitant type	Sample purity
Methodology	Precipitant concentration	Macromolecular impurities
Time	pH and buffer	Aggregation
Pressure	Ionic strength	Posttranslational modifications
Gravity, convection and sedimentation	Reducing/oxidizing environment	Sample source
Vibrations and sound	Sample concentration	Sample storage
Magnetic fields	Metal ions	Proteolysis
Electric fields	Detergents	Chemical modifications
Dielectric properties	Small molecule impurities	Sequence modifications
Viscosity	Polyions	Sample symmetry
Equilibration rate	Crosslinkers	Sample pI
Nucleants	Heavy metals	Sample history
Volume	Reagent source	Ligands,co-factors, inhibitors
Particulate/amorphous material	Reagent purity	Microbial contamination
Surface of crystallization device	Reagent formulation	Purification method
Sample handling		

Table 3. Factors that affect crystallization

2.13.1 The thermodynamics of crystal growth

Crystallization is a complex process, involving multiple equilibria between different states of the crystallizing species. The three stages of crystallization common to all molecules are nucleation, crystal growth and cessation of growth. During nucleation enough molecules associate in three dimensions to form a thermodynamically stable aggregate, the so called critical nucleus. These nuclei provide surfaces suitable for crystal growth, which can occur by a couple of different mechanisms. Crystal growth ceases when the solution is sufficiently depleted of protein molecules, deformation-induced strain destabilizes the lattice, or the growing crystal faces become poisoned by impurities.

The solubility of a solute is the maximum amount of solute that can be dissolved in a given amount of solvent at a given temperature.

- A saturated solution is a solution in which no more solute can be dissolved at a given temperature.
- An unsaturated solution is a solution into which more solute can be dissolved.
- A supersaturated solution is an unstable solution as it contains more dissolved solute than a saturated solution.

A classical explanation of crystal nuclei formation and growth is given by the two-dimensional solubility diagram shown in figure 7. The solubility curve divides the concentration space into two areas - the undersaturated and supersaturated zones. Each point on this curve corresponds to a concentration at which the solution is in equilibrium with the precipitating agent. These correspond to the situation either at the end of the crystal growth process from a supersaturated solution or to a situation when crystal dissolution occurs in an undersaturated solution. In the area under the solubility curve, the solution is undersaturated and the crystallisation will never take place. Above the solubility curve lies the supersaturation zone; here, for a given concentration of precipitating agent, the protein concentration is higher than that at equilibrium. Depending on the kinetics to reach equilibrium and the level of supersaturation, this region may itself be subdivided into three zones:

1. The precipitation zone is where the excess of protein molecules immediately separates from the solution to form amorphous aggregates.
2. The nucleation zone is where the excess of protein molecules aggregates in a crystalline form. Near the precipitation zone, crystallisation may occur as a shower of microcrystals, which can be confused with precipitate.
3. A metastable zone; a supersaturated solution may not nucleate for a long period, unless the solution is mechanically shocked or a seed crystal introduced. To grow well-ordered crystals of large size, the optimal

conditions would have to begin with the formation of a preferably single nucleus in the nucleation zone just beyond the metastable zone. As the crystals grow, the solution would return to the metastable region and no more nuclei could occur. The remaining nuclei would grow, at a decreasing rate that would help to avoid defect formation, until equilibrium is reached.

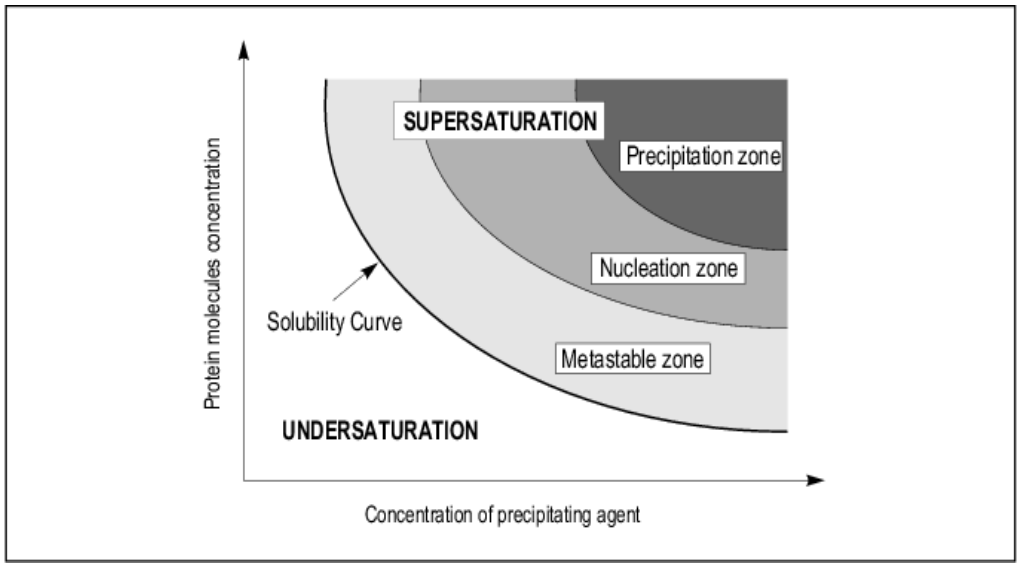

Figure 7. Two-dimensional solubility diagram.

2.13.2 Physical techniques of protein crystallization

A number of techniques have been developed for bringing a protein solution into a supersaturation state. Among them, the following three methods are frequently used: (1) micro-batch, (2) vapour-diffusion and (3) dialysis. Although supersaturation of a protein solution could be achieved by means of each of these techniques, the underlying principles of these methods vary.

The **micro-batch method** is a variation of the simple batch crystallisation technique, where the concentrated protein solution is mixed with concentrated precipitant in a closed vessel to produce a final supersaturated concentration, which may eventually lead to crystallisation. This can be done with large amounts of solutions, and typically results in larger crystals owing to the larger volumes of solute present and the lower chance of impurities diffusing onto the face of the crystal. In the micro-batch technique, smaller volumes, as little as 0.5 mL, can be used. The protein sample and precipitant solution are dispensed into the well of a plate and the well is covered with paraffin oil to prevent evaporation (figure 8a). During the incubation period, the concentration of a precipitant agent remains constant since

evaporation is limited and, therefore, the volume of the drop remains the same during the experiment. On the other hand, the concentration of the protein changes on formation of either crystals or amorphous precipitant. If the concentration of precipitant agent is chosen in such a way that the solution is in an undersaturated state, crystallisation will never occur.

In terms of phase diagram, as shown in figure 9a, this condition is indicated by point A. The protein will immediately precipitate if the starting point is located in the precipitation zone of the solubility diagram, point C. Therefore, only those points in the phase diagram (point B in figure 9a) which lie between the solubility and precipitation curves represent starting conditions for a successful crystallisation experiment.

The main disadvantage of this method is that the equilibration occurs very rapidly, thus affecting the rate of crystal growth and consequently the quality of the obtained crystals. The second disadvantage is that the manipulation of the crystals from the drop covered by oil is very difficult. However, since the use of very small volumes of protein solution can be made, the micro-batch technique is quite useful as an initial screening method. Although the evaporation of water from the drop covered by oil is negligible, it does occur, and therefore the 'life-time' of micro-batch trials is usually about 2 to 3 weeks.

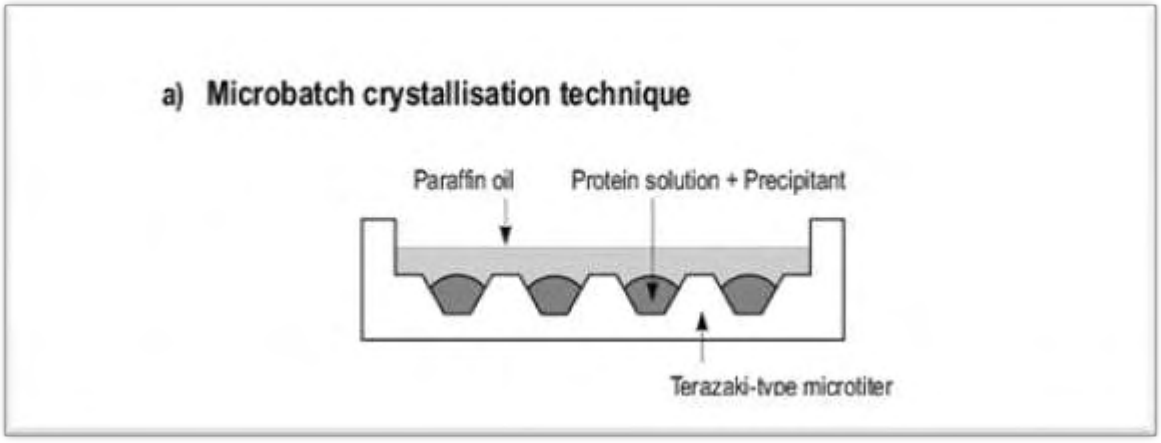

Figure 8a: Protein crystallisation techniques. Schematic representation of microbatch crystallisation technique.

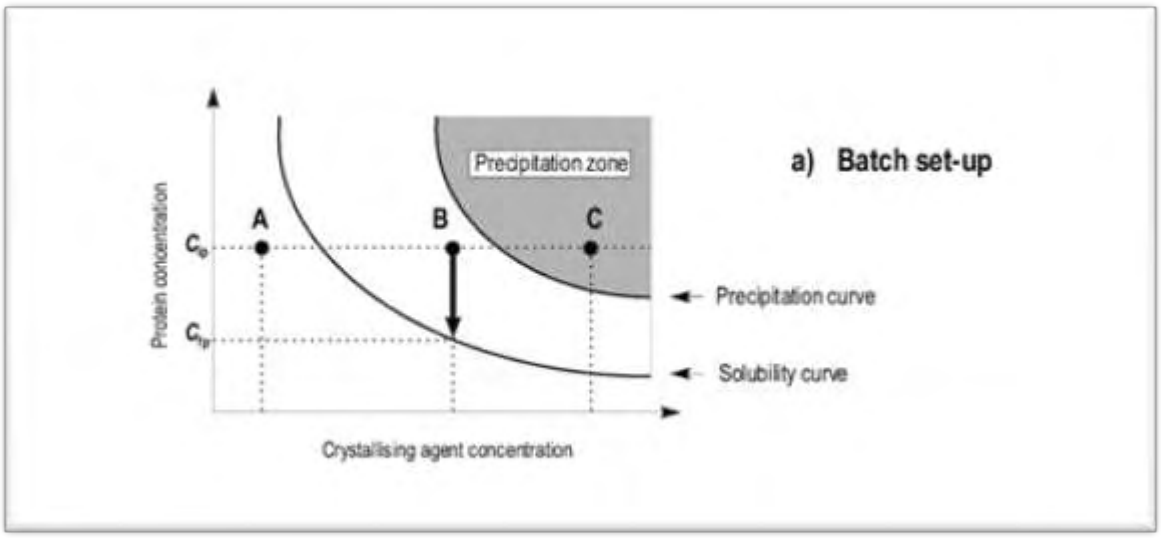

Figure 9a : Various crystallisation set-ups explained in terms of phase diagrams. Schematic representation of solubility phase diagram and correlation between protein and crystallising agent concentrations in batch crystallisation experiments. C_{ip} and C_i are the initial concentrations of protein and crystallising agent respectively, C_{fp} and C_f are their final concentrations.

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 (figure 8b), or based on an arrangement, where the drop is suspended from some surface and it is called a hanging drop (figure 8b), which is also the technique 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 (figure 8b), 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 maintained until crystallization is complete.

During a vapour-diffusion experiment, the protein will start to concentrate from an unsaturated state (point A, concentration C_{pi}) to reach a supersaturated state (point B). As the first crystals appear the concentration of protein will decrease (figure 9b). The crystal will then grow until the concentration of the protein in the drop reaches the solubility curve (point C, at concentration C_{fp}).

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.

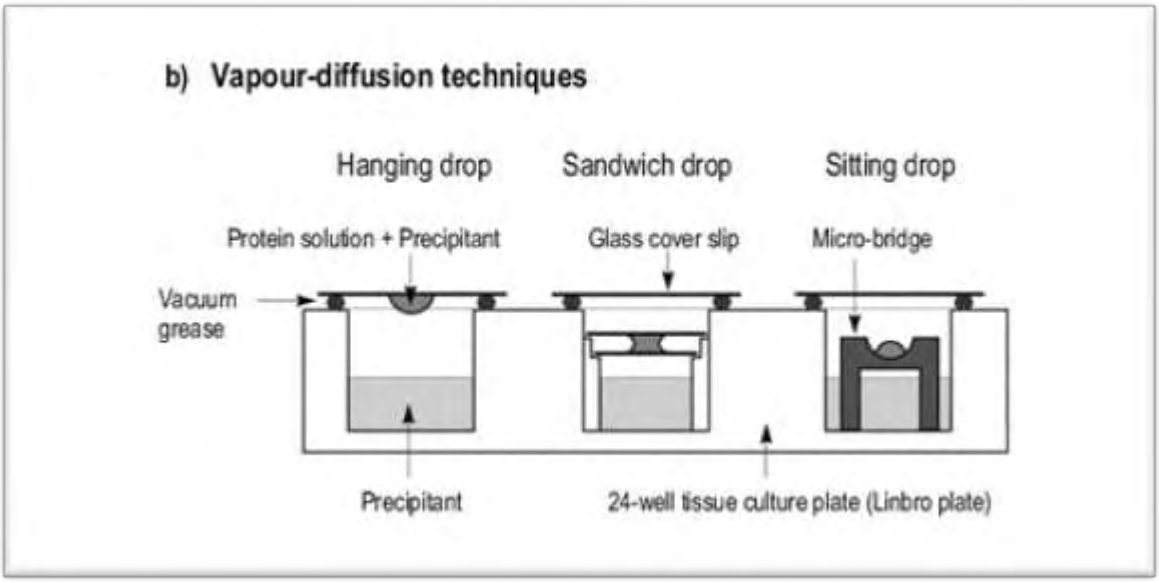

Figure 8b : Protein crystallisation techniques. Schematic representation of vapour-diffusion crystallisation technique.

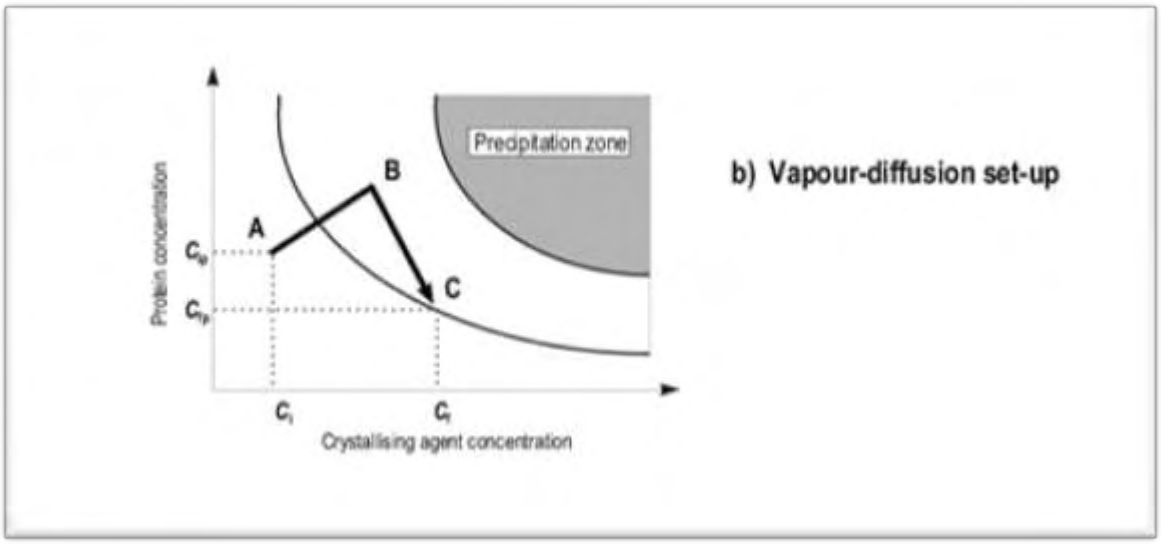

Figure 9b: Various crystallisation set-ups explained in terms of phase diagrams. Schematic representation of solubility phase diagram and correlation between protein and crystallising agent concentrations in vapour-diffusion. C_{ip} and C_i are the initial concentrations of protein and crystallising agent respectively, C_{fp} and C_f are their final concentrations.

Dialysis techniques utilize diffusion and equilibration of small precipitant molecules through a semipermeable membrane as a means of slowly approaching the concentration at which the macromolecule solute crystallizes. Initially, the protein solution is contained within the dialysis membrane, which is then equilibrated against a precipitant solution. Equilibration against the precipitant in the surrounding solvent slowly achieves supersaturation for the solute within the dialysis membrane, eventually resulting in crystallization. A dialysis membrane can be used to cover the opening of a dialysis button, allowing diffusion of the surrounding solvent into the solute through the dialysis membrane (figure 8c). Dialysis buttons themselves come in a variety of sizes from 7 mL to 200 mL.

The protein solution at the start of the dialysis experiment is in an undersaturated state (figure 9c). The concentration of the precipitant agent slowly increases as its diffusion through the membrane takes place. Thus, the system goes from an undersaturated state into the metastable region through the point S on the solubility curve.

The advantage of dialysis over other methods is in the ease with which the precipitating solution can be varied, simply by moving the entire dialysis button or sack from one condition to another. Thus, the protein solution can be continuously recycled until the correct conditions for crystallization are found.

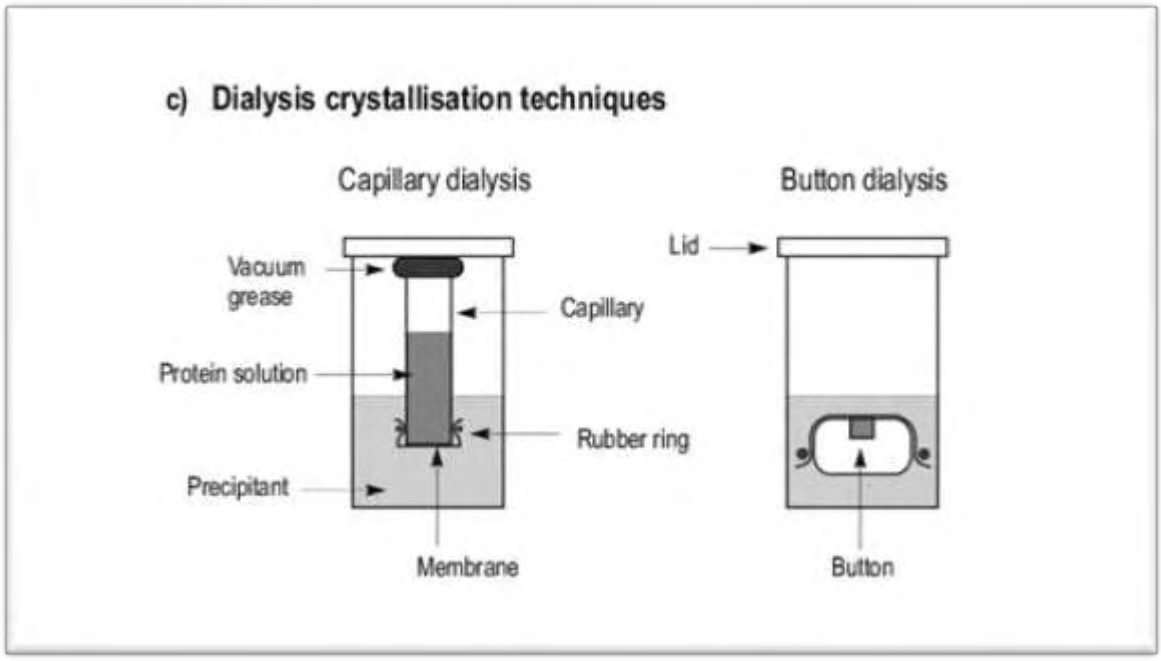

Figure 8c : Protein crystallization techniques. Schematic representation of dialysis crystallization technique.

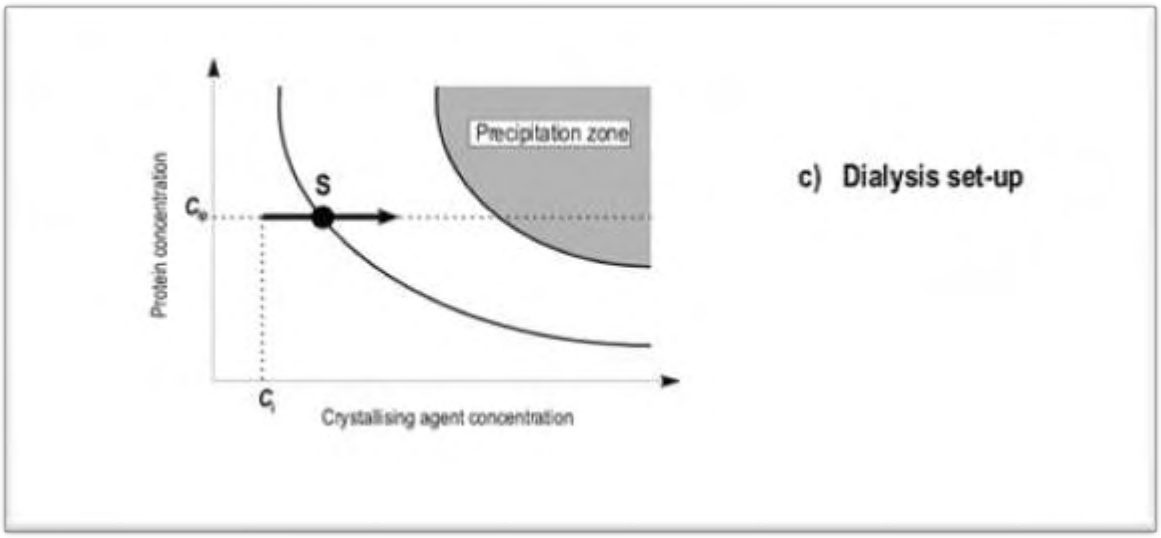

Figure 9c. Various crystallization set-ups explained in terms of phase diagrams. Schematic representation of solubility phase diagram and correlation between protein and crystallizing agent concentrations in dialysis crystallization experiments. C_{ip} and C_i are the initial concentrations of protein and crystallizing agent respectively, C_{fp} and C_f are their final concentrations.

2.13.3 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:
  - Ribonuclease 7, "Rosenberg" variant, 30 mg/mL in sodium cacodylate buffer 20mM pH5
  - Initial crystallisation mixture: (NH4)2SO4 2,4M + Bicine buffer 0,1M pH9
- Reservoir mixtures are prepared, deciding which conditions and concentrations we will be working with i. e. We study what varying crystallisation components' concentrations (ammonium sulphate, buffer, pH values, etc.) may result in
- The corresponding crystallization mixture component volume is calculated out of the initial stock concentration ($V_{stock} = C_{reservoir} \cdot V_{reservoir} / C_{stock}$).

  Stock concentrations**:** $(NH_4)_2SO_4$ 3,5 M
  Different buffers (TRIS, CHES), pH values (8,5; 9; 9,5; 10)

- Once all well's reservoir volumes have been calculated, we pour gently each component amount (e.g. 80 µL buffer) into the well. A concrete addition order is preferred in order to avoid eventual bad mixing or precipitation problems. Thus, first mQ (twice distillated) water is added, then the buffer and finally the salt.
- Since 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, we simply take 1µL of the protein sample, put it on the coverslip and then add 1µL of the reservoir composition (crystallisation condition) very gently. We 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.
- We turn over the coverslip rapidly and put it directly over the pre greased well border. Once all wells have been covered, we cover the whole plate itself and keep it at 16^{o}C.

RESULTS – DISCUSSION

1. Protein expression and purification

The recombinant RNase 7 was expressed using *Escherichia Coli* BL21 cells transformed with vector Pet11C with insertion of RNase 7 synthetic gene. Protein is obtained and purified from the inclusion bodies.

1.1 Protein production

- Transformation into receptive cells *E. Coli* BL21 by the use of antibiotic Amp (100 μg/mL)
- 2 L culture of bacterial cells are inoculated at a temperature of 37^{o}C under stirring along with the antibiotic Amp until an OD_{600}=0.6 is reached (3h).
- Induction in a temperature of 37^{o}C for 4 hours with IPTG to a final concentration of 1 mM.
- Aliquots before and after induction are kept to be used for SDS-PAGE electrophoresis.
- 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. The pellet contains the inclusion bodies, which are insoluble aggregates.

1.2 Purification

Sonication is necessary for the purification of our recombinant protein from inclusion bodies. It is important that cell lysis is complete, because intact cells sediment together with the inclusion bodies, thus contaminating the preparation.

- Resuspension of the pellet in 80 mL of lysis buffer to remove as much bacterial protein as possible from the aggregated foreign protein and then sonication for the lysis of cells and centrifugation (16.000 rpm for 30 min at 4^{o}C). The supernatant is scattered and the pellet is kept in fridge.

The inclusion bodies are still insoluble, so in order to obtain soluble active proteins from inclusion bodies denaturing and refolding steps are necessary. First, they are washed to remove contaminants, and then they are solubilised in denaturing buffer which contains a reducing agent. After centrifugation, the supernatant which contains the unfolded proteins is gradually incubated in refolding buffer to recover soluble and active proteins.

- Resuspension of the pellet in 20 mL of washing buffer.
- Centrifugation for 30 minutes at 12.000 rpm at 4 ^{o}C.

- Resuspension of the pellet in 25 mL of denaturing buffer, which is reduced with glutathion (80 mM).
- Centrifugation in 12.000 rpm for 30 minutes and gradual incubation (5 mL / min) of the supernatant this time to 2 L of refolding buffer. (sample A)

The final step for the purification of the recombinant RNase 7 is achieved by high-resolution ion-exchange chromatography.

- DEAE – sepharose and CM – sepharose column is first equilibrated with the solution A with a flow of 1 mL/min.
- Filtration of sample A so as to be condensed and prevent loading of misfolded proteins in the column. After filtration, it is loaded in the column.
- The washout of columns by the same solution follows (solution A) for 5h.
- Elution of the protein from the CM – sepharose column (which is negatively charged) with the use of NaCl linear gradient (solution A + NaCl).

The results are shown in figure 1. The peaks represent proteins and the most characteristic peak is an indication of our protein.

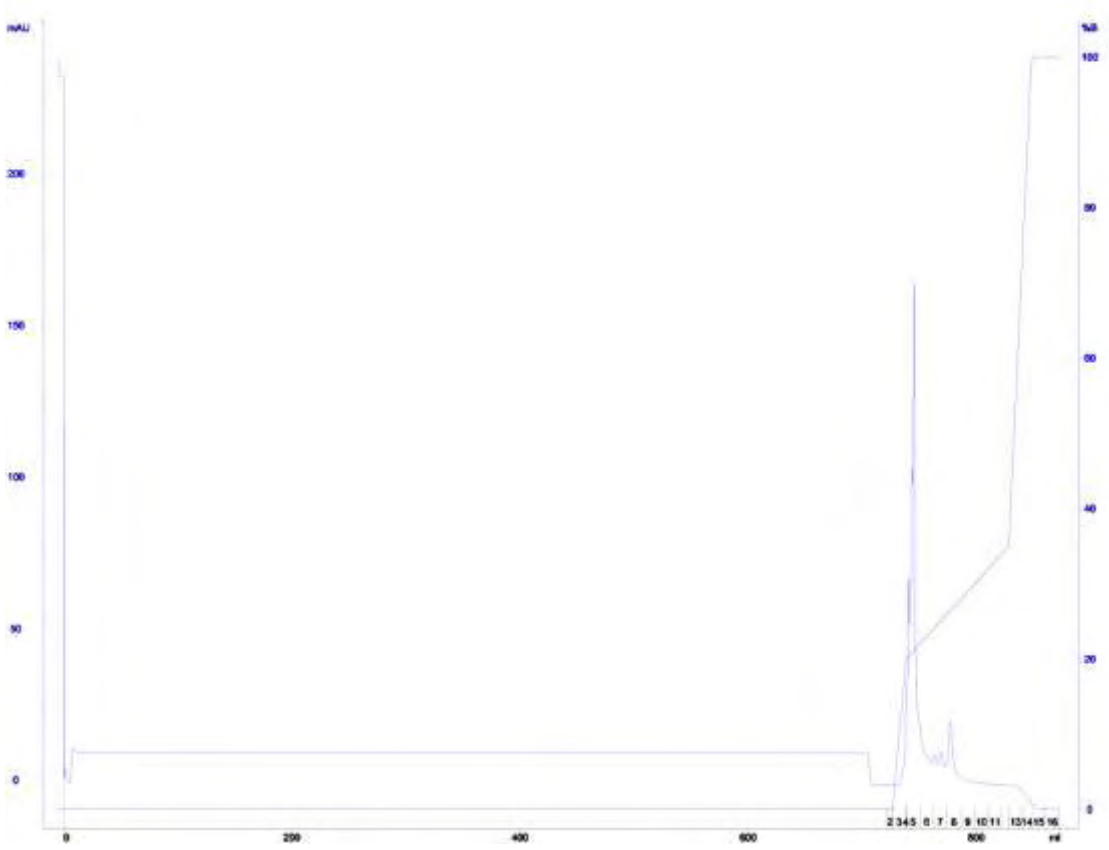

Figure 1. Ion exchange chromatography with Resource S column using HPLC.

The fraction 5 shows a peak. So after centrifugation, 1mL of the protein has absorbance A=0.4 in 280nm. As a reference we used water. The quantity of the protein obtained is 38,2 mM calculated by the Beer-Lambert law, A=ebc where:

- ε is the molar absorption coefficient with units of L mol^{-1} cm^{-1}
- b is the path length that the incident light cross expressed in cm
- c is the concentration of the compound in solution, expressed in mol L^{-1}

So if we calculate the total amount of the protein (converting molecular concentration in mass) we purified and divided it by the total volume (in L) of the bacterial culture used to express this protein. Then this results in the production of 8 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. From the results of the chromatography, we speculate that the fraction of the protein in the 5^{th} tube corresponds to the purified RNase 7, since it shows the highest peak. To assure this speculation, 20μl of this fraction are mixed with 10μl loading buffer and are uploaded in the gel. Samples before induction and after induction are also uploaded in the gel with the process which is mentioned previously (p.37).

- As a marker, an RNase A sample (10 μL) is used which is mixed with 5 μL of loading buffer and is placed in the first well of the gel (7,5 μL)
- During the process of induction (p.25), 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 are centrifuged for 5 min. In the pellet 20 μL TRIS/HCl are added and 10 μL loading buffer. A total amount of 20 μL of each sample is uploaded in the wells.
- We centrifuge (10 min in 10 ^{o}C) the fraction from the chromatography, which is in tube 5. We put it in a special eppendorf type tubes along with water, which holds the protein, and discards water and salts (5 centrifugations). A total amount of 20 μL of the sample is uploaded in the wells..

All the samples were heated before the uploading in the gel. The figure 2 presents the results of the SDS – polyacrylamide gel electrophoresis.

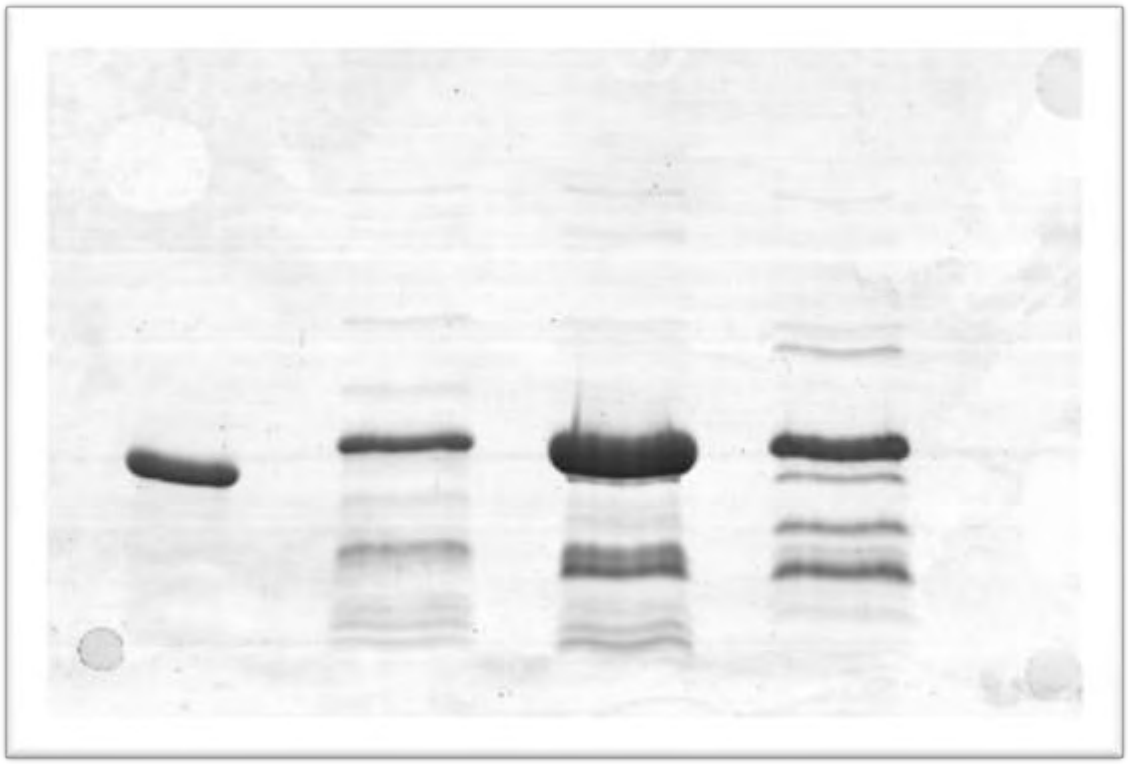

Figure 2. SDS-PAGE electrophoresis. The first band starting from the right is the marker, the second the sample B.I, the third the sample A.I and the last that from the chromatography (tube 5).

As it is seen, the expression of the protein is induced after the injection of IPTG, since it is represented with a more intense band in the gel, compared to the one before induction. The last band confirms that the purified fraction from the chromatography stands for the desired protein, RNase 7.

3. Ribonuclease activity by zymogram

Zymography is used in order to determine if our protein displays enzyme activity. The exact process is described above (p. 40). In this case, a sample of 10µl is taken from fraction 5 of the ion exchange chromatography. Samples are prepared without boiling to preserve the structure and activity of the enzyme.

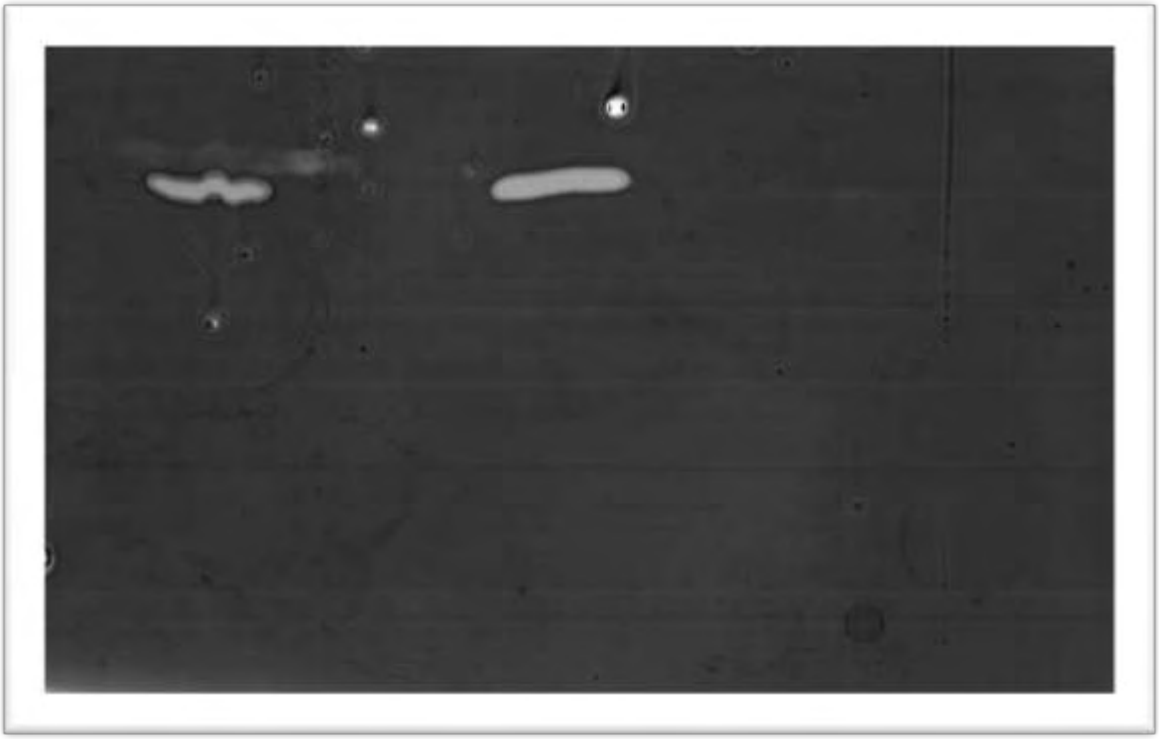

Figure 3. Zymogram.: . The first band starting from the right is the marker and the second the sample from the chromatography (tube 5).

As it is seen, RNase 7, which is visualized by negative staining, has digested the poly-U substrate, and is represented as clear band in the gel, which means that has ribonuclease activity.

4. Antibacterial activity

To determine if RNase 8 displays antibacterial activity, the diffusion or agar plate method is performed, as described previously (p.42). In each plate, we measure the colonies by dividing it into 4 quadrants. The results are the following:

1. (0,0195μM) → 274x4 = 1096
2. (0,039 μM) → 283x4 = 1132
3. (0,078 μM) → 140x4 = 160
4. (0,156 μM) → 21x4 = 84
5. (0,3125 μM) → 0
6. (0,625 μM) → 0
7. (1,25 μM) → 0
8. (2,5 μM) → 0
9. (5 μM) → 0
10. (10 μM) → 0

From these data, we can come to the conclusion that as the protein concentration increases, the bacterial growth is inhibited, until there are plates, where no bacterial growth is noticed. This inhibition, starts from the plate 5, which represents also the MIC of the protein and of course continues in the next plates, where the concentration of RNase 7 is even higher. So, RNase 7 justifies its characterization as an AMP. More specifically, its antibacterial activity is calculated with the help of a computer programme : c/50 = 0.62± 0.3

5. Crystallization

The experimental procedure of crystallization is described previously (p.52). Through the trial-and-error principle, several experiments took place took place under different conditions, in order to find the optimal ones for the formation of crystals. The table below shows the conditions for each test of the experiment:

Plate 1: **different pH values and buffers**	
$(NH_4)_2SO_4$ 2,4M TRIS 0,1M pH9	$(NH_4)_2SO_4$ 2,4M TRIS 0,1M pH9,5
$(NH_4)_2SO_4$ 2,4M CHES 0,1M pH9,5	$(NH_4)_2SO_4$ 2,4M CHES 0,1M pH10

Results: Better results are obtained with TRIS, where there was not too much precipitation. So, in the next plate, we use TRIS as buffer, investigating the values.

Plate 2: **different $(NH_4)_2SO_4$ concentration at previous plate's best pH values**				
$(NH_4)_2SO_4$ 1,5M	$(NH_4)_2SO_4$ 1,85M	$(NH_4)_2SO_4$ 2,2M	$(NH_4)_2SO_4$ 2,5M	TRIS/HCl 0,1M pH 9
				TRIS/HCl 0,1M pH 9,5

Results: Better results are obtained at the concentration of 1,85M $(NH_4)_2SO_4$, where a procrystal structure is observed. So, in the next plate we investigate a shorter $(NH_4)_2SO_4$ concentration range.

Plate 3:**shorter (NH4)2SO4 concentration range at wider TRIS buffer pH value range**					
$(NH_4)_2SO_4$ 1,7M	$(NH_4)_2SO_4$ 1,8M	$(NH_4)_2SO_4$ 1,9M	$(NH_4)_2SO_4$ 2M	$(NH_4)_2SO_4$ 2,1M	TRIS/HCl 0,1M pH 8,5
					TRIS/HCl 0,1M pH 9
					TRIS/HCl 0,1M pH 9,5

Results: No significant results (i.e. crystals) were obtained. Only crystal-like structures were observed. Therefore, more conditions should be explored to achieve crystal growth.

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