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THESIS SUMMARY

Biosinteza proteinelor modulatoare de apoptoză și investigarea interacțiuniilor dintre ele

Biosynthesis and interaction studies of apoptosis modulating proteins

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CONTENT

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INTRODUCTION

Over the past decade several peptides have been considered as potential therapeutic sources of various diseases. The interest in bioactive peptides have increased in pharmacology and medical science due to their increasing knowledge of the diverse modes of action. Therapeutic peptides generally have been derived from three sources: natural or bioactive peptides produced by plants, animal or human, peptides isolated from genetic or recombinant libraries and peptides discovered from chemical libraries. Peptide therapies are limited by chemical and enzymatic labilities of peptides [1]. Many peptides are inactive when applied orally, and even parenteral application is often not possible [2]. Application via mucous membranes (e.g., nasal) is promising. A major strategy in peptide chemistry is the desing of chemical modification in order to increase its chemical and enzymatic stability, to extend the time of action, and to increase activity and selectivity towards the receptor [3].

Classified by cell targets, peptides have two major groups [4]. The first one includes peptides which are active against microbial and cancer cells, but not being active against healthy mammalian cells. The second group contains anti cancer peptides that have activity against all three types of cells (microbial, normal and cancerous).

Industrial peptide production is commonly based on three alternative technologies: solid-phase synthesis, liquid-phase synthesis, and in vivo biotechnological recombinant technology. Chemical synthetic strategies are challenging in economic terms. Production of peptides with recombinant techniques proved to be a more effective.

Peptides have received special attention in molecular biology in recent times, because peptides could be used in the study of protein structure and function. Apoptosis is an essential process in the development of cells. The inhibitor of apoptosis proteins suppresses cell death. This suppression is caused by inhibiting the activity of caspases [5]. The mitochondrial protein SMAC (Second Mitochondria-derived Activator of Caspases) promotes apoptosis by eliminating the inhibitory effect of IAP's through physical interactions. The amino-terminal sequences in SMAC protein (AVPI) are required for this function. SMAC promotes apoptosis by activating caspases in the cytochrome c/Apaf-1/caspase-9 pathway [5]. In the case of apoptosis researchers focused on small molecule Smac mimetics [6] that target the *BIR3* domain IAP's. These IAP inhibitors are currently under investigation as anticancer drugs in clinical trials [5, 6].

In this work, I have studied biosynthesis of small peptides with 13 amino acid length. These peptides contain AVPI tetrapeptide moiety of SMAC, which are involved in the interaction with IAP's. Furthermore, C-terminal labeling of peptides was executed by fluorescent labeling reaction. An Alexa Flour dye was used for crosslinking reaction.

Interaction studies were performed by fluorescence polarization method. In addition, BIR3 domain of IAP's were also produced by recombinant techniques. During our research we investigated heterologous expression of IAP's. In this regard, solubilization from inclusion body and successful refolding of XIAP was executed using nondenaturing conditions. This research includes also in vitro investigation of protein-protein interaction by pull-down technique.

The thesis is divided in three main chapters. In the first chapter, in a literature overview, the importance of small peptides in present scientific perspective is presented. Followed by the presentation of SMAC mimetic molecules, their effect on apoptosis and present therapeutic applications. Furthermore in this chapter the method that we consider the most effective and appropriate for peptide production is discussed in detail.

In the second chapter the materials and methods used during the practical work are presented, beginning from the recombinant techniques to the investigation of peptide-protein interaction.

The third chapter contains the actual research work and the discussion of its results. At the beginning of the chapter, the design and biosynthesis of small peptides is presented. It includes cloning procedures, selection of appropriate vector and host cell line, heterologous expression and efficient purification results. Furthermore, this chapter contains our results about biosynthesis of IAP's. The above mentioned recombinant techniques were used in this phase of research as well. In addition, the solubilization from inclusion body and refolding of XIAP is presented. And finally, the interaction studies of BIR3 domain and fluorescent labeled small peptides are discussed.

CHAPTER 1.

LITERATURE OVERVIEW

1.1. PEPTIDES

1.1.2. Therapeutic peptides-Peptide drugs

A number of important biochemical functions of life are influenced by peptides. Peptides have received special attention in molecular biology in recent times for several reasons. At first, peptides could be used in the study of protein structure and function. For example, synthetic peptides can be used as probes to determine protein-peptide interactions. Inhibitory peptides are also used in clinical research to examine the effects of peptides on the inhibition of cancer proteins [4] and other diseases. Bioactive peptides have an increased interest of pharmacology and medical science due to their increasing knowledge of the diverse modes of action. The isolation and targeted application of peptides is gaining importance for the treatment of pathologic processes. New therapeutic methods based on peptides for a series of diseases give rise to the hope that diseases can be amenable to therapy. In following table we present some of these commercial bioactive peptides.

Table 1.1. Commercial bioactive peptides

Generic name (trade name)	Disease/target	Properties	Reference
Ecallantide (Kalbitor®)	Hereditary angioedema	Plasma kallikrein inhibitor	[9]
Telavancin (Vibativ®)	Skin infection	Antibacterial agent	[10]
Romidepsin (Istodax®)	Cutaneous T-cell lymphoma	HDAC inhibitor	[11]
Liraglutide (Victoza®)	Type 2 diabetes	GPL-1 receptor agonist	[12]
Boceprevir (Victrelis™)	Hepatitis C Virus genotype 1	NS3/4A protease inhibitor	[13]
Telaprevir (Incivek®)	Hepatitis C Virus genotype 2	NS3/4A protease inhibitor	[14]
Brentuximab vedotin (Adcetris™)	Hodgkin's lymphoma	CD30 directed	[15]
Icatibant (Firazyr®)	Hereditary angioedema	Bradykinin B2 receptor antagonist	[16]

Therapeutic peptides generally have been derived from three sources: (a) natural or bioactive peptides produced by plants, animal or human (derived from naturally occurring peptide hormones or from fragments of larger proteins); (b) peptides isolated from genetic or recombinant libraries and (c) peptides discovered from chemical libraries.

Peptide therapies are limited by chemical and enzymatic labilities of peptides. Many peptides are inactive when applied orally, and even parenteral application is often not possible [2]. Application via mucous membranes (e.g., nasal) is promising. A major strategy in peptide chemistry is directed towards chemical modification increase chemical and enzymatic stability.

Classified by cell targets peptides have two major groups [4]. The first one includes peptides active against microbial and cancer cells, but not being active against healthy mammalian cells. The second group contains anti-cancer peptides that have activity against all three types of cells (microbial, normal and cancerous).

According to literature, more than 50 peptide drugs were commercialized, and hundreds of peptides are in a preclinical stage or under clinical trials. The annual sales of peptide drugs is ~25 billion USD and peptide drug sales are estimated to exceed 50 billion USD in 2024 [17].

1.2. BIOSYNTHESIS

Nowadays, many therapeutic peptides and proteins are produced by recombinant techniques. Heterologous expression is defined as expression of a gene or a part of a gene in a host organism, where the gene naturally does not exist in expression system. Insertion of the target gene in the heterologous host is performed by recombinant techniques [18]. Bacteria, fungi, mammalian cell, animal cell, plant cell can be host cells. Heterologous expression systems are based on the hypothesis that the basic principles of protein expression and function are similar in all organisms. Proteins and peptides have several features that should be carefully observed when choosing a host system for their production, such as size, intracellular localization or secretion, proper folding, and glycosylation pattern.

Theoretically the steps needed for obtaining a recombinant peptide are pretty straight forward. At first the gene of interest has to be cloned into an expression vector, results the corresponding plasmid. This plasmid in the second step is transformed into the host cell. The following step is induction the host cell, which starts the peptide production. After these procedures the peptide is ready for purification and characterization. In the past, many reviews have described the heterologous expression systems with great detail [63-66].

In practice, planning an expression and carrying out it seems difficult [18]. Circumstances needed to be considered during planning are follows: we need to examine ways to isolate gene of interests, choosing the appropriate and well-functioning expression vector,

choosing the appropiate organism for expression, troubleshooting the recombinant peptide production, purification and structural analysis.

1.2.2. Host selection

Bacterial cells can be engineered to express non-native genes, resulting in the production of recombinant peptides and proteins, which have various biotechnological and pharmaceutical applications. In eukaryotes, such as mammalian and yeast cells, which have large genomes, a higher recombinant protein expression can be difficult.

The choice of the host cell whose will produce the peptide will initiate the outline of the whole process. Host systems that are available, e.g. bacteria, yeast, fungi, plant cells, animal cells and unicellular algae all have strengths and weaknesses and their choice may be depending of the peptide of interest. In recent literature review we will focus on heterologous expression in bacteria, narrowly recombinant peptide expression in *Escherichia* coli.

E. coli cells have been the most popular cells of producing recombinant proteins for over two decades. In the Escherichia coli expression system, the expression is induced with isopropyl b-D-1-thiogalactopyranoside (IPTG). Studies have shown low expression levels of peptides. The production process requires the accomplishment of three individual factors: expression, solubilization and purification [23].

E. coli expression system remain to be the preferred system for laboratory investigations and initial development in commercial activities because is a well-established host with short culturing time, and it is easy to manipulate genetically [18]. In addition we can remember that *E. coli* needs low cost media. The number of plasmid copies within the cell can range from one to several hundreds. The copy number of plasmids is a combined property of the replicons present in the plasmid, the cell, and its current metabolic state. For example the ColE1-family [24] of replicons are used in popular vector systems such as pET (pMB1, 15–60 copies/cell) and pUC (pMB1 variant, 500–700 copies/cell). Within *E. coli* expression, the T7 system is the most popular approach for producing peptides and proteins. In this system, an expression vector containing a gene of interest cloned downstream of the T7 promoter is introduced into a T7 expression host [18]. T7 expression hosts such as *DE3* strains [25] , [26] or T7 express strains carry a chromosomal copy of the phage T7 RNA polymerase gene. When inducer is added, T7 RNA polymerase is expressed and becomes dedicated to transcription of the gene of interest.

Recombinant gene expression in *E. coli* is normally under the control of an inducible promoter that requires the presence of a specific metabolite or synthetic inducer to direct transcription of the gene of interest. Several different promoters are in use with the most common being lac/tac, lacUV5, araBAD, and rhaBAD [27],[28], [29].

The advantages of using E. coli as the host organism are the following [28]:

- It has fast growth kinetics, in glucose-salts media and given the optimal environmental conditions, the doubling time is about 20 min.
- High cell density cultures are easily achieved. The theoretical density limit of an *E. coli* liquid culture is estimated to be about 200 g dry cell weight/l or roughly 1 × 1013 viable bacteria/ml, which is less than 0.1% of the theoretical limit. For this reason, high cell-density culture methods were designed for *E. coli* growth.
- Rich complex media can be made from readily available and inexpensive components.
- Transformation with exogenous DNA is fast and easy.

1.2.4. Protein purification and affinity tags

Protein and peptide affinity tags have become highly popular tools for purifying recombinant proteins and native protein complexes for excellent reason. The advantages of using a protein/peptide tag fused to the recombinant protein/peptide are mainly to facilitate its purification and detection. Simple purification is possible using affinity chromatography. Some tags allow strong binding to chromatography media in the presence of denaturants, making on-column refolding possible. Tags may also improve the stability and solubility of recombinant proteins.

The use of recombinant proteins has increased greatly in recent years. Simultaneously, techniques and products used for their expression and purification showed rapid development in past 20 years. Most of the available protein and peptide affinity tags can be categorizes into three classes. The first class of epitope affinity tags contain those peptides or proteins which are fused to small molecule ligands linked to a solid support. This group contains the hexahistidine tag, which binds to immobilized metal [30], [31] and glutathione S-transferase protein fusions which bind to glutathione attached to chromatography resign [32]. In the second class of affinity tags, are those peptide tags which bind to a protein-binding partner immobilized to chromatography resign. Accordingly, calmodulin-bindig peptide binds specifically to calmodulin allowing proteins fused to the peptide to be purified over calmodulin resin. The third class of epitope affinity tags can be considered as a type of second class. The protein-binding partner attached to the resin is an antibody which recognize specific peptide epitopes [33]. For example, the FLAG peptide can be used with one of several anti-FLAG antibody resin [93],[35].

1.3. CELL DEATH AND APOPTOSIS

In multicellular organism apoptosis is a normal and important process. Apoptosis means genetically programmed cell death. Apopoptosis has been implicated in many human diseases: cancer, disease, inflammation and neurodegradation. Mapping up critical apoptosis regulators is a possessing strategy for the development new therapies [18]. Two main apoptotic pathways (Fig.1.3.) are known: the intrinsic (mitochondrial) and the extrinsic pathways.

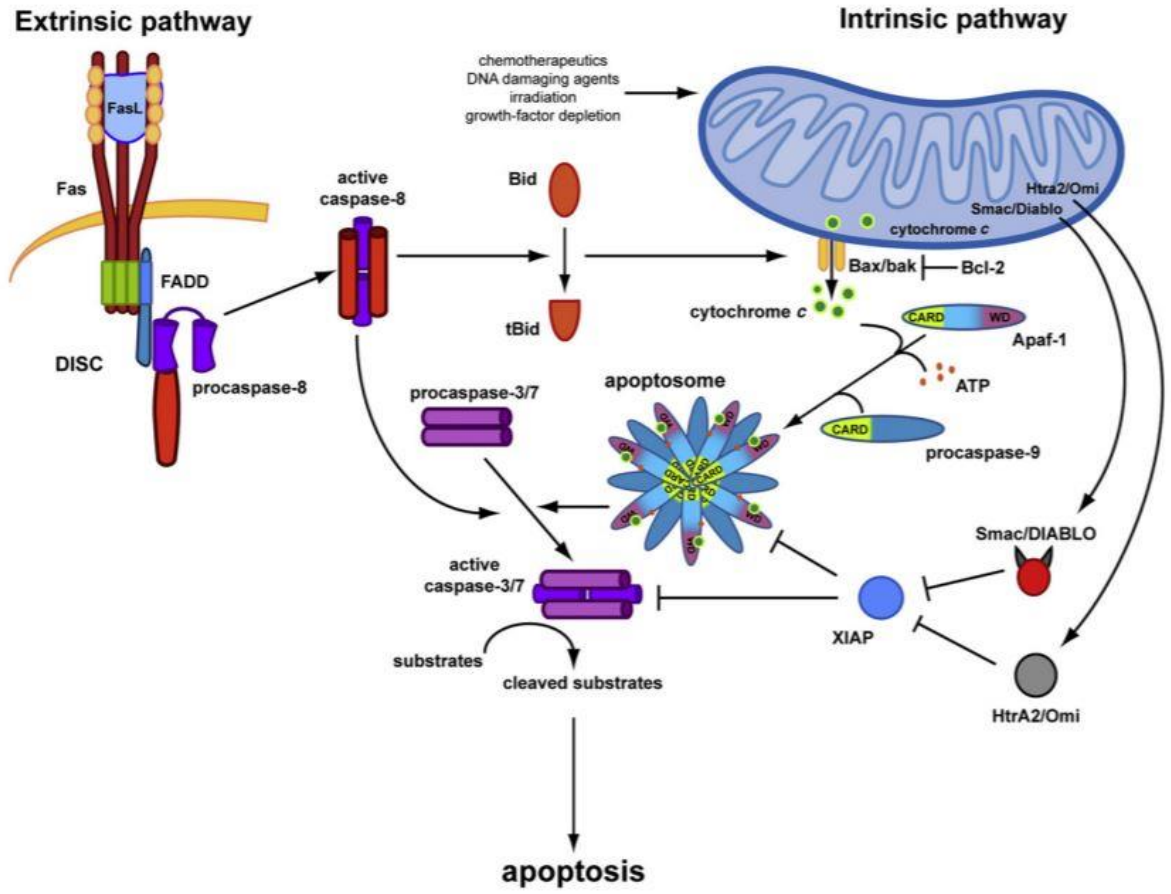


Fig.1.3. Overview of the Extrinsic and Intrinsic Apoptotic Pathways [36]

The intrinsic pathway is characterized by permeabilization of mitochondria induced by stress signals (e.g. chemotherapeutic drugs and radiation). At the molecular level the intrinsic pathway involves the translocation and oligomerization of BAX or BAC proteins, then they forms a pore in the outer mitochondrial wall leading to the release of cytochrome c and second mitochondrial-derived activator of caspases (Smac/DIABLO-direct IAP-binding protein with low pI) from mitochondria into the cytosol [37]. Apoptosome forming take place in the cytosol by cytochrome c, apoptotic protease activating factor 1 (APAF1) and procaspase-9 [38]. So the apoptosome is a multi-protein complex, which cleaves pro-caspases-9 into active caspase-9. Active caspase-9 activates effector caspases (caspase-3 and caspase-7), which cleave down-stream cell-death substrates, leading to apoptosis.

The extrinsic pathway is initiated by the binding of death ligands (Fas/Apo-1, TNF-α, Apo2L/TRAIL to their corresponding cognate death receptors (CD95/FasR, TNFR1 and DR4/DR5) on the cell surface [39]. This binding results in recruitment of multiprotein death-inducing signaling complex (DISC) at the plasma membrane. An adapter protein from DISC recruits procaspase-8 into the complex and results auto activation of caspase-8. Then cleavage of caspase-8 activates caspase-3 and caspase-7 [40]. The two pathways are linked and the molecules in one pathway can influence the other pathway [41].

Both pathways proceeds via activating caspases. Caspases are the main executioners of apoptosis and are found in inactive form, named procaspases, that activated by proteolytic cleavage. In addition, caspases can be grouped into upstream initiator caspases (as caspases 8 and 9) and downstream effector caspases (as caspases 3,6 and 7) [42].

Proteins involved is apoptotic machinery can be classified according to their role into sensors and effectors [43]. The cellular environment is monitored by sensors, which detect signals that initiate apoptosis (as DNA abnormalities). Sensors activate the effectors of apoptotic cell death.

The inhibitor of apoptosis proteins contains approximately 70 amino acid motifs [18] termed baculovirus IAP repeat domains (BIR) [44]. The most potent caspases inhibitor in IAP family is human X-linked inhibitor of apoptosis protein (XIAP), contains 3 BIR domains in the N-terminal region and a RING domain endowed with E3 ubiquitin ligase activity, in the C-terminal region. According to literature High levels of XIAP have been found in several cancer cell lines. The physiological amount of Smac-DIABLO released from the mitochondria may not be sufficient to overcome the inhibitory effect of XIAP on the caspases, thus preventing apoptosis [45]. Inactivation of overexpressed XIAP by Smac mimetic molecules may relieve caspase binding, thus promoting apoptosis in malignant cells [18].

1.4. SMAC MIMETICS

1.4.1 Protein interactions and mimetic molecules

The design of a protein mimetic molecules is based on the small peptide molecules or non-peptide molecules which are able to mimicking the properties or biological activity of a protein owing to the presence of the secondary structure and other features that are analogous to those of the original protein. Generally, protein interactions are dependents other proteins, peptides or specific organic molecules. The main purpose of drug design is to intervene specifically with these interactions. As we know protein structure dictates protein function.

The chemical and physical properties of peptides and proteins are determined by the nature of amino acid side chains and the polyamide peptide backbone. This nature of proteins

involve that the small mimetic molecules are often south to recapitulate key protein contacts (interactions). The mimetic must be capable to bind with protein natural binding partner. Nowadays various methods exist for developing a specific mimetic [46], including computational as well as experimental screening methods. In recent research we are focusing on peptide based protein mimetic molecules. Specifically, instead of target protein the active part is selected, resulting a small peptide with specific amino acid sequence.

Referring to above presented literature overview, peptides shown a great pharmaceutical potential as active drugs and diagnostics in several clinical area such as endocrinology, urology, obstetrics, oncology, etc. and as functional excipients in drug delivery systems to overcome tissue and cellular membrane barriers.

1.4.2. Structural characteristics of Smac mimetic molecules

Several strategies have been developed for the design of small-molecular inhibitors of XIAP [6]. One solution has been to block the interaction between XIAP BIR2 and caspases-3/-7 [6]. The crystal structure of Smac protein in a complex with the XIAP BIR3 domain has been developed in 2000 by Shi's group [47]. Structural information at the atomic level has been the basis for the design of Smac mimetic molecules.

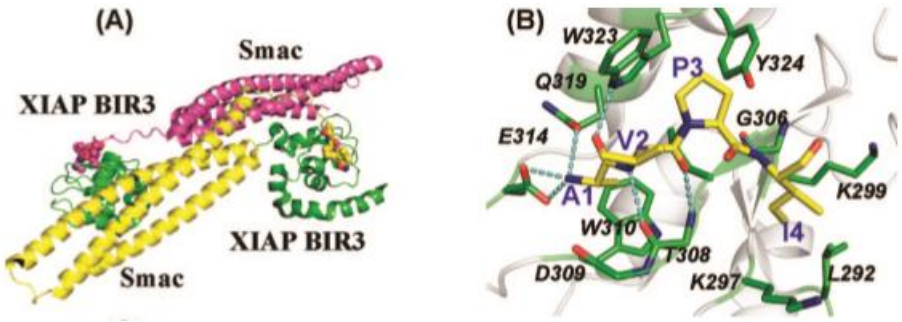


Fig.1.4. Schematic representation of the Smac/XIAP-BIR3 structures [6]

The crystal structure showed (Fig.1.4.) that Smac protein forms an elongated homidimer (A), in addition the N-terminal four residues (Ala1-Val2-Pro3-Ile4) in Smac recognize and bind to a surface groove on XIAP BIR3 (B) removing caspases-9 inhibition and bind with lower affinity to the BIR2 domain, reducing inhibition by caspases-3 and -7 . The free amino group of the Ala1 forms strong hydrogen bonds to the Glu314 and Gln319 residues on BIR3. The backbone carbonyl group forms a hydrogen bond to the indole NH group in Trp323 [6]. The above mentioned alanine's methyl group inserts into a small hydrophobic pocket. Furthermore the amino and carbonyl groups of Val2 form optimal hydrogen bonds with the carbonyl and amino groups of Thr308, while the Val2 side chain, with no interactions with protein residues is exposed to solvent [47]. Proline has Van der Waals contacts with the side chains of Trp323

an Tyr324 by five membered-ring [48]. The amino group of Ile4 residue forms a hydrogen bond with the carbonyl group in Gly306. Its hydrophobic side chain inserts into a hydrophobic pocket formed by side chains of Leu292 and Val298 and the hydrophobic portion of the side chains in Lys297 and Lys299. The IAP family member's cIAP1 and cIAP2 are similar to XIAP. As with XIAP, the BIR2 and BIR3 domains of cIAP1and cIAP2 also bind caspases and Smac. In contrast to XIAP, the dominant role of cIAP1 and cIAP2 in apoptosis regulation appears to occur in the context of TNF signaling via TNFR1, where these proteins play an essential role in NF-κB induction and suppression of TNF-induced apoptosis [49].

In the past few years researchers focused on small molecule Smac mimetics that target the BIR3 domain of XIAP despite there have been only few reports on the design of compounds that effects inhibition by binding to the BIR2 domain of XIAP. These IAP inhibitors are currently under investigation as anticancer drugs in clinical trials. Knowing in detail structure features can be concluded some structure-activity relationship specificities (Fig.1.5.). Position (a) is methyl or ethyl preferred. In case of methylation two positions exist, one position (b) where methylation is not tolerated and another position (c) where methylation is tolerated. In addition, at position (d) Gly, Asp, pro is not favorable. At the (e) part of the molecule larger hydrophobic groups are more optimal.

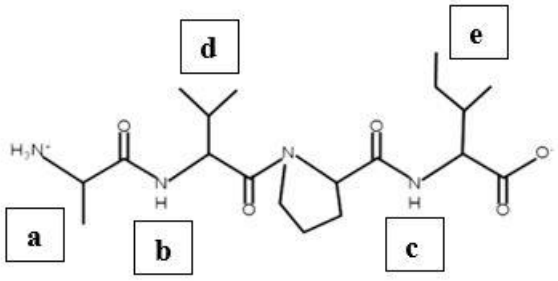


Fig.1.5. Structure specificities of AVPI peptide

In the past few years a number of researcher groups have designed and synthetized many peptidic and nonpeptidic Smac mimetics. Furthermore, a series of bivalent Smac mimetic molecules which mimic the interaction of Smac proteins with XIAP have been synthetized [50]. According to literature bivalent Smac mimetic molecules can achieve much higher affinity to XIAP that the corresponding monovalent Smac mimetic induction of apoptosis in tumor cells [51].

Currently, several Smac mimetics are under evaluation in early clinical trials. These mimetics are the followings: LCL161 [52], AT-406/Debio1143 [53], TL32711/birinapant [53], HGS1029, GDC-0917/CUDC-427 [54].

The first Smac mimetic to enter clinical trials was designed by Genentech and was one of the series monovalent compounds. Initial studies have shown that the GDC-0152 to be a potent antagonist of XIAP, cIAP1/2.

In addition, Ling's group elaborate theoretically studies on the interaction of XIAP-BIR2 domain. With the goal of to get an insight into the binding nature molecular docking and dynamics simulations were used to study the binding of XIAP-BIR3 with three groups of Smac mimetics [55].

CHAPTER 3.

RESULTS

Over the past decade peptides have received special attention in molecular biology in recent times. As we described in introduction, we focused on SMAC mimetic molecules. The aim of our research was to develop an appropriate method for biosynthesis and C-terminal labeling of small peptides. These proteomimetic molecules could be chemically modified according to their application. *E. coli* expression system, against to chemical synthesis, remain to be the preferred system for laboratory investigations and initial development in commercial activities because is a well-established host with short culturing time and easy genetic manipulation [18], [56].

3.1. BIOSYNTHESIS OF CIAP1, CIAP2 AND XIAP

For biosynthesis of cIAP1, cIAP2 and XIAP *E. coli* expression system was designed. The recombinant construct (pGEX) contained a glutathione S-transferase (GST) fusion partner, which allows the purification of the protein by affinity chromatography. As host cell *E. coli* Rosetta™(DE3)pLysS cell line was chosen. Rosetta host strains are BL21 lacZY derivatives designed to enhance the expression levels of proteins that contain codons rarely used in *E. coli*. The tRNA genes are driven by their native promoters. In Rosetta (DE3)pLysS the rare tRNA genes are present on pRARE plasmids that carry the T7 lysozyme.

In case of intracellular protein production, the amount of target protein is directly proportional to the amount of biomass. Induction of the cells results in the expression of the protein. For sensitive heterologous proteins, it is necessary to optimize expression conditions to achieve higher yields. At this stage of our research the growth of the cell culture was examined to determine optimal cell density for induction [18].

3.1.2. Optimization of XIAP protein expression [18]

In this part of my thesis, results about shake flask expression of XIAP-GST in M9 minimal medium induced with different final concentrations of IPTG are summarized. During the experiment samples taken every 3-3 hours to determine target protein concentration were examined by SDS-PAGE analysis.

XIAP is a 53 kDa sized protein. In niether case no protein expression can be observed at 37°C. It is observed that proteins were produced only at 18 °C for 12 hours (Fig.3.3.). We can observe differences at concentration of inducer. Unfortunately, the target proteins were expressed in inclusion bodies, therefore solubilization and refolding are necessary steps towards

protein purification. Frequently, human proteins expressed in prokaryotic systems form denatured inclusion bodies. To obtain the active form of protein the most common technique is denaturation of the protein aggregates followed by refolding of inclusion proteins. Conventional denaturants for solubilization are urea, guanidine hydrochloride and sodium dodecyl sulphate (SDS), while refolding can be achieved by several techniques found in the literature [57], [58].

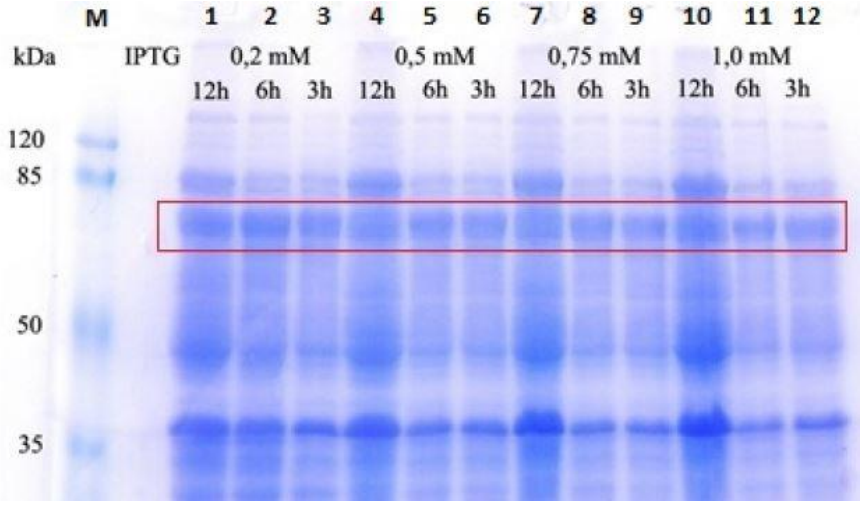


Fig.3.3. Heterologous expression of XIAP-GST at 18 °C.
Protein samples were separated by 10% SDS-PAGE and stained with CBB.
M:Prestained Protein Ladder 20-120 kDa, (molecular weight marker from Thermo Scientific™); 1: 0.2 mM IPTG induction-12h; 2: 0.2 mM IPTG induction- 6h; 3: 0.2 mM IPTG induction- 3h; 4: 0.5 mM IPTG induction-12h; 5: 0.5 mM IPTG induction-6h; 6: 0.5 mM IPTG induction-3h; 7: 0.75 mM IPTG induction-12h; 8: 0.75 mM IPTG induction-6h; 9: 0.75 mM IPTG induction-3h; 10: 1.0 mM IPTG induction-12h; 11: 1.0 mM IPTG induction-6h; 12: 1.0 mM IPTG induction-3h. [18]

3.1.3. Solubilization of XIAP-GST

The refolding of protein was executed using detergents. XIAP contains a RING domain and 20 cysteine residues, possibly GST-fusion construct is expressed in *E. coli* as inclusion bodies by standard expression protocols, which can be effectively solubilized by *N*-Lauroylsarcosine according to our results. For the refolding of the protein, we used a detergent and micelle-assisted, chromatography-based modified procedure initially presented by Massiah et. al [59] , which was further developed using different detergents, optimized for XIAP.

After cell lysis, analyzed bacterial supernatant (Fig.3.4, lane 3) and pelleted fraction (Fig.3.4, lane 4), show the target protein aggregated in inclusion bodies. Natively folded proteins can be extracted from inclusion bodies using *N-l*auroylsarcosine. In our case, in a first experiment, the solubilization step was carried out according to the protocol developed by Massiah's group. The results suggest that virtually the total amount of the insoluble GST-XIAP was solubilized successfully at 10% *N-l*auroylsarcosine added to the lysis buffer. Fig.3.4, lane

6. shows inclusion body supernatant after solubilization, while lane 7. shows the pellet after solubilization.

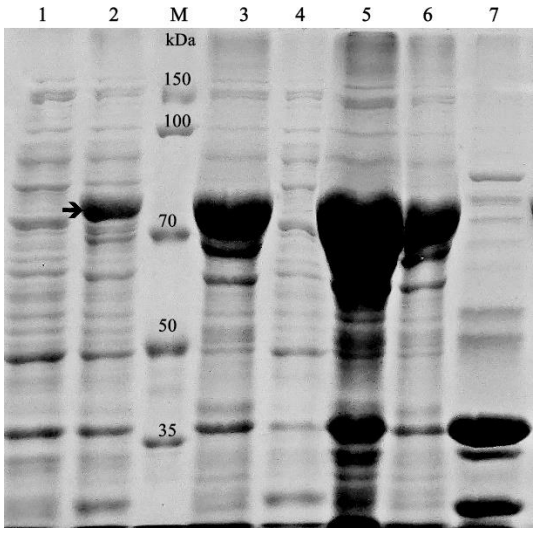


Fig.3.4. 10 % SDS-PAGE analysis of XIAP-GST expressed in *E. coli at 18 °C*.
Lane 1. uninduced total cell lysate, *lane 2.* total cell lysate after induction of 0.2 mM IPTG after 8 hours of culture, *lane M.* Protein molecular weight marker (Novagen, Sigma Aldrich), *lane 3.* cell lysate after homogenization, *lane 4.* supernatant after centrifugation, *lane 5.* pellet after centrifugation, *lane 6.* supernatant after solubilization with 10% *N-l*auroylsarcosine, *lane7.* pellet after solubilization. Proteins stained with Coomassie Brilliant Blue R250.

Knowing that mild solubilization of inclusion body aggregates is the key for improving recovery of bioactive protein, we continued our work with assessment of *N-l*auroylsarcosine concentration effect on solubiliztaion yield of our target protein. The refolding protocol, based on the solubilization of proteins without chaotropic agents was optimized and used for the XIAP-GST construct.

The exact mechanism of action of *N-l*auroylsarcosine is unknown, it is supossed that *N-l*auroylsarcosine molecules disrupt aggregation by encapsulating proteins, while higher concentrations of the solubilization agent could interfere subsequent affinity purification of the GST-tagged proteins. However some data are suggesting that during dilution of the sarkosyl-solubilized proteins (at 0.01% *N-l*auroylsarcosine concentration) the detergent can act as a folding enhancer by preventing aggregation of the partially folded conformers via hydrophobic interaction [60], [61].

In the next step solubilized protein fractions were analysed by electrophoretic microchip using a 2100 Bioanalyzer and Protein 230 kit for molecular weight determination and quantifying proteins. Based on the electropherogram, as Fig.3.6. shows, the apparent molecular weight of the target protein was about 80.4 kDa, consistent with the calculated molecular mass

of the GST-XIAP construct (78 kDa), while the solubilized protein concentration is 1.025 µg /µl. In total, we obtained 20 mg solubilized GST-XIAP.

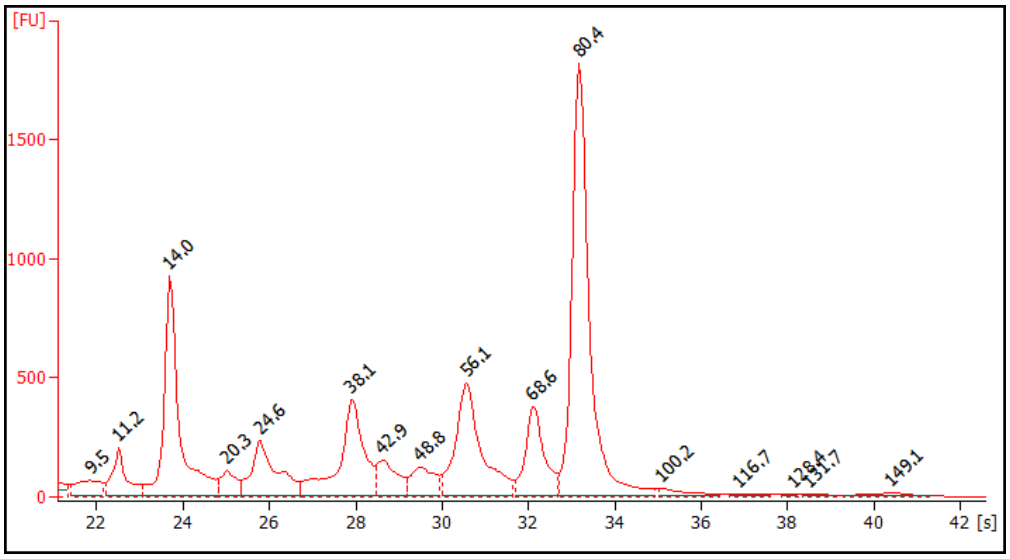


Fig.3.6. Electrophoretic microchip analysis of supernatant after solubilization with N-lauroylsarcosine.

3.1.4. Detergent-aided refolding and purification of XIAP-GST protein by affinity chromatography and gel-filtration

The CMC of *N*-lauroylsarcosine is greater than 0.5% (14 mM). Unfortunatelly the removal of detergents with CMC greater than 5 mM is not possible by dialysis. Removal of *N*-lauroylsarcosine is necessary for efficient affinity purification and refolding of the target protein. There are several studies in the literature for removal of *N*-lauroylsarcosine by dilution and dialysis [62], [63]. A low CMC indicates that the equilibrium between detergent monomers and detergent micelles is almost completely on the micelle side. In addition, the micelles are of large size, whileonly detergent monomers can diffuse easily into the surrounding buffer. *N*-lauroylsarcosine may be sequestered by adding a nonionic detergent, such as OTG.

Table 3.1. Detergent-aided refolding conditions

Number	Triton-X (%)	CHAPS (mM)	OTG (%)
1.	1	10	-
2.	2	20	-
3.	3	30	-
4.	1	-	1
5.	1	-	1.5
6.	-	10	1

Optimizing refolding conditions for the recombinant XIAP-GST was carried out adding TritonX-100, CHAPS and OTG in the appropriate ratios, testing 6 different refolding conditions, as described in the Materials and methods section. The affinity purification was performed in batch mode. All three detergents (TritonX-100, CHAPS and OTG) resulted in binding of GST to the GSH Sepharose matrix. No protein precipitation was observed in case of conditions 1, 2, 4, 5, 6, while in case of condition 3 (Fig.3.7, lane 3), using the highest concentrations of TritonX-100 and 30% CHAPS, almost the total amount of target protein precipitated. Purification of the refolded protein was followed by SDS-PAGE (Fig.3.7.). Based on our results, refolding aided by 2% Triton X-100 and 20% CHAPS (Fig.3.7, lane 2) can be considered to be most adequate for XIAP-GST in our experimental setting.

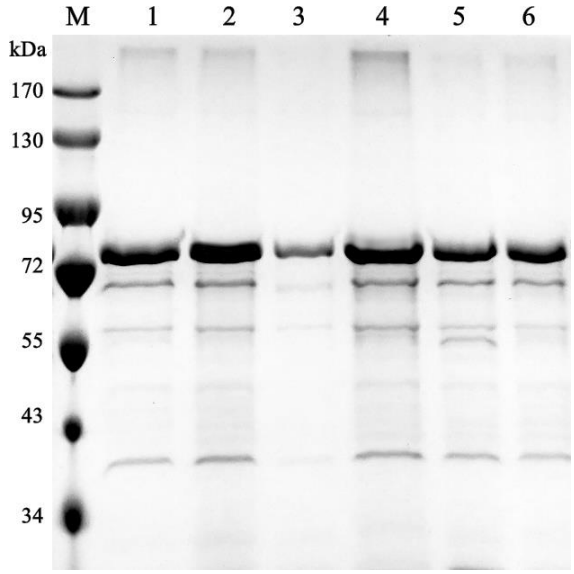


Fig.3.7. Affinity purification of refolded XIAP-GST protein
Lane M. Protein molecular weight marker (Thermo Scientific), *lane 1.* 1% TritonX-100 and 10 mM CHAPS; *lane 2.* 2% TritonX-100 and 20mM CHAPS; *lane 3* 3% TritonX-100 and 30mM CHAPS; *lane 4.* 1% TritonX-100 and 1% OTG; *lane 5.* 1% TritonX-100 and 1.5 % OTG; *lane 6.* 10 mM CHAPS and 1% OTG.

In summary, refolding and purification experiments can be considered successful. Proteins were efficiently refolded. The highest efficiency, i.e. 87.11% refolded protein, was achieved by experiment 2. (2% TritonX-100 and 20 mM CHAPS). It was followed by experiment 1. (79.5 %) and experiment 5. (73.79 %). Not much lower efficiency can be considered in case of experiment 4. (68.08 %) and experiment 6. (67.36 %). In the interest of obtaining high purity protein, for further purification was chosen experiment 2.

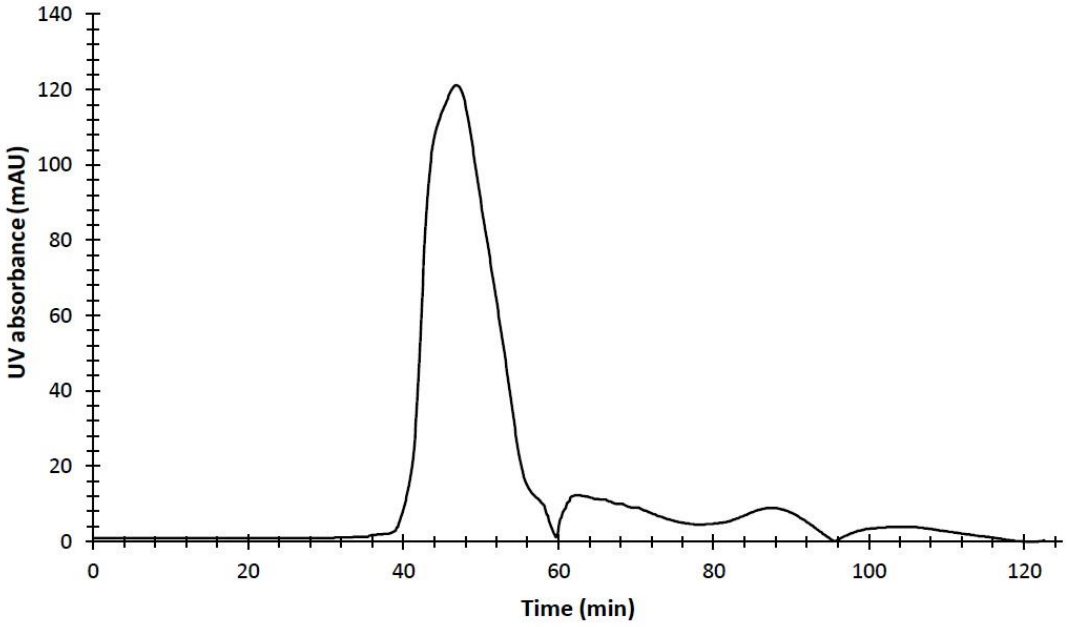


Fig.3.9. Elution profile of refolded XIAP-GST after passage through a HiLoad 16/600 Superdex 75 pg column.

For further purification of target protein we performed size-exclusion chromatography, using HiLoad 16/600 Superdex 75 pg column. The detection was set at 280 nm.The column was first loaded with Tris-HCl buffer (pH=7.5), followed by sample injection to column. In Fig.3.9. shows the elution profile of XIAP-GST. In the chromatogram one large peak and two small peaks can be observed. This purification step resulted in 97% purity of refolded protein. The overall yield of refolded protein was 15.67 mg/200 ml *E. coli* culture.

3.1.5. Identification of the refolded protein

The pull-down assay is a suitable method for examining protein–protein direct interactions between two purified proteins using affinity purification. The ability of GST-XIAP fusion protein to bind on the GST Sepharose beads was essential to perform GST pull-down experiments. This method may provide decisive data in terms of whether the study is suitable for structure determination. In our case XIAP-SMAC direct interaction indicated the authenticity of refolded and purified protein. This interaction was previously described in literature[64], [65].

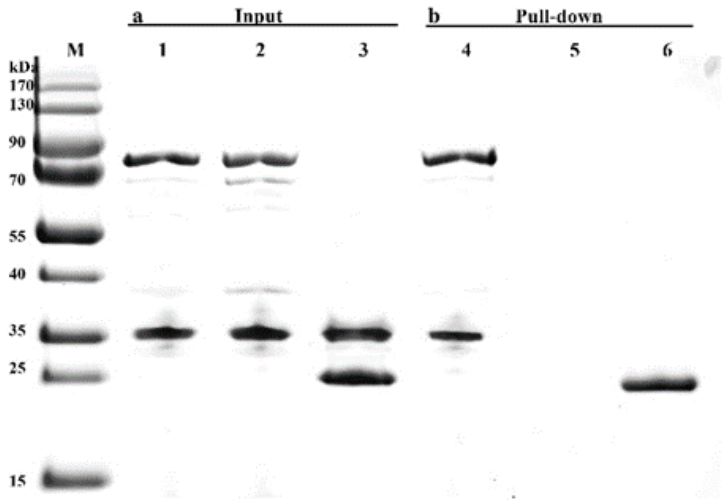


Fig.3.11. GST pull-down binding assays of the refolded GST-XIAP with recombinant SMAC
(a) shows input of protein samples in pull-down binding assays of recombinant SMAC as prey with refolded GST-XIAP and unfolded GST-XIAP as a bait.
lane 1: refolded and purified GST-XIAP and SMAC; lane 2: unfolded GST-XIAP and SMAC; lane 3: SMAC and GST control.
(b) shows pull-down binding assays of the prey protein with the bait proteins.
lane 4: interaction complex of refolded and purified GST-XIAP and SMAC; lane 5: interaction complex of unfolded GST-XIAP and SMAC; lane 6: SCMAC and GST control

During the interaction complex, first band was identified as XIAP (78 kDa), while the second band was recognized as SMAC (approximately 30 kDa). As the above-presented results show, solubilization and refolding of XIAP-GST was successful. Refolded and purified GST-XIAP show appropriate interaction against SMAC (Fig.3.11, lane 4.), while in case of unfolded protein there are no interaction protein bands detected after pull-down (Fig.3.11, lane 5). This can also be considered as a proof that only refolded proteins interact with SMAC. GST is adopted as a negative control.

Overall, our findings conclude that starting from 200 ml expression culture, 19.62mg solubilized XIAP-GST can be obtained by adding 2% N-Lauroylsarcosine in the lysis buffer, 92.34% of the protein can be refolded using 2% TritonX-100 and 20 mM CHAPS during affinity purification, while size-exclusion chromatography assures a purification of 97% of the refolded protein solution, thus obtaining an overall yield of 17.57 mg refolded, purified XIAP-GST.

3.2. BIOSYNTHESIS OF BIR3 DOMAIN OF IAP'S

Genes of BIR3 domain was cloned succesfully into pMAL-p2x expression vector. This vector system contains as fusion partner maltose binding protein (MBP) and the signal peptide on pre-MBP directs fusion proteins to the periplasm. Shake flask protein expression was achieved in *E. coli Rosetta (DE3) pLysS* cells using Rich medium. Proteins were isolated from

periplasm and purified by affinity chromatography using amylose resin. For increasing protein purity anion exchange chromatography was performed.

3.2.1. Cloning and expression of BIR3 domain of IAP's

Construction of the three recombinant plasmid were performed as described in Methods section. Amplified target genes (105 bp) were directly subcloned into pMAL-2x expression vector in all three cases, and the expression vector was constructed successfully.

The recombinant plasmids were confirmed by double-enzyme digestion and gene sequencing.

Based on our previous research we found that *E. Coli* Rosetta cell line (Fig.3.12.**)** proves to be the appropriate host for target protein expression. The protein expression was induced with 0.3 mM IPTG. We consider that expressed protein quantity in induction at 18°C for 16 h was the most significant [66]. As Fig.3.13. shows *(lane 1)* protein expression was successfully executed. Target proteins can be observed on 10% gel around 46 kDA. Comparing expression in bacterial cytoplasm to bacterial periplasm we found that in periplasmic space much less protein can be produced. In addition, the oxidizing environment of the periplasm allows for better formation of disulfide bonds and protein folding.

Successful protein isolation from bacterial periplasm can be listed among our results. Freezing cells in 5 mM $MgCl_2$ is a simple and easy method. It is important to mention that cells have to thaw gently at 4 °C. In addition following resuspension step must be executed at 4 °C, paying attention in the gentle resuspension.

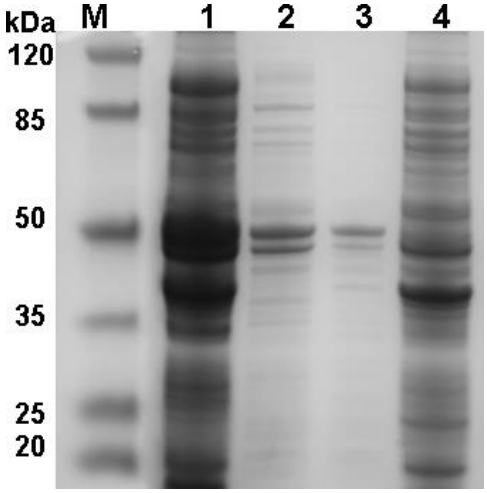


Fig.3.13.Verification of heterologous expression and protein isolation from bacterial periplasm
M: Prestained Protein Marker 20-120 kDa (Thermo Scientific); lane 1: whole cell sample; lane 2: II. periplasm extract; lane 3: I. periplasm extract; lane 4: bacterial cell sample after protein isolation from periplasm; 10% SDS-PAGE; the gel was stained with Coomassie Brilliant Blue.

The 2th and 3th lane of Fig.3.13. shows that target proteins directed to periplasm where isolated as we expected. After isolation procedure no target protein can be observed in analyzed sample.

3.2.2. Affinity purification and anion exchange chromatography

The protein purification method is based on MBP-amylose resin interaction and target protein elution using maltose. As shown Fig.3.14. proteins were eluted successfully from amylose resin with 10 mM maltose containing column buffer (lane 1, 2 and 3). Maltose binding protein is a common protein expression tag and is an approximately 42 kDa protein. BIR3 domains are composed of 35-35 amino acid residues and have a molecular mass of ~ 4 kDa.

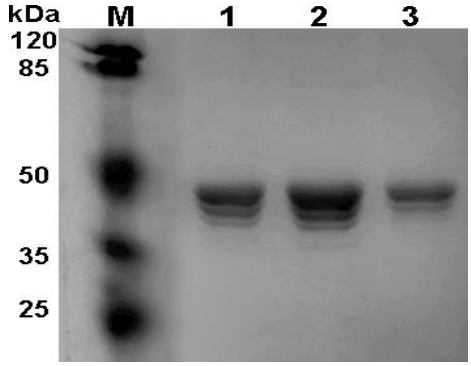


Fig.3.14. Affinity purification of XIAP-BIR3 construct
M: Prestained Protein Marker 20-120 kDa (Thermo Scientific); lane 1: first elution with 10 mM maltose; lane 2: second elution with 10 mM maltose; lane 3: third elution with 10 mM maltose;

Our purification results (Fig.3.14.) in case of MBP-XIAP/BIR3 and in the other two cases were also successful and after dialysis we achieved about 2 mg/mL protein. For increasing protein purity we performed another purification step. The isoelectric point of MBP-XIAP/BIR3 construct is around 5.01, thus anion exchange chromatography was chosen for next purification step. Protein purity was examined on 10%-SDS PAGE, presented on Fig.3.15. The results shows high protein purity was achieved.

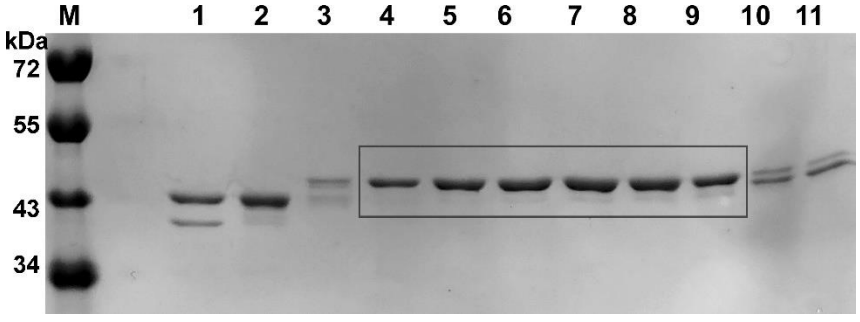


Fig.3.15. 8% SDS-PAGE analysis of eluted fractions
M: Prestained protein Marker 10-180 kDa; lane 1: flow through (10-25 min); lane 2: flow through (26-40 min); lane 3: sample 76 min; lane 4: sample 80 min; lane 5: sample 81 min; lane 6: sample 82 min; lane 7: sample 83 min; lane 8: sample 84 min; lane 9: sample 85 min; lane 10: sample 86 min; lane 11: sample 87 min.

The results shows that contaminant proteins remain in flowthrough, thus BIS-TRIS buffer system at pH=5.8 was well functioning. Negatively charged target proteins were eluted with NaCl. Based on chromatogram, presented on Fig.3.16, target proteins were eluted from column using 24 CV linear gradient elution with approximately 250-300 mM NaCl.

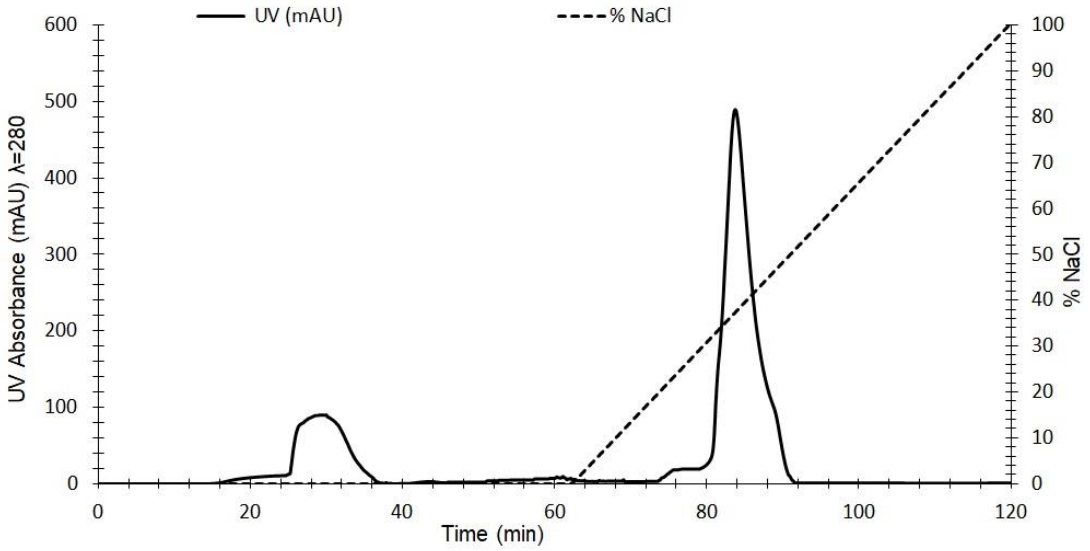


Fig.3.16. Elution profile of MBP-XIAP/BIR3 after passage through a HiTrap Q HP column. The detection was set at 280 nm;

Based on our results, it can be stated that our objective referring to biosynthesis and purification of recombinant cIAP's has been achieved. Our research extended to investigation of BIR3 domain of target proteins by biosynthesis and interaction assays. Our biologically active recombinant proteins are useful for studying the biological functions of genes in different diseases, for the development of therapeutic drugs, biomimetics and biomaterials for the biotechnology industry [18], [56].

3.3. SMAC PEPTIDE AND CONTROL PEPTIDE

3.3.1. Design and construction of SUMO fusion system for SMAC mimetic peptide expression

In the following part of the thesis I will present an expression system, developed by our group, which permits the production of sufficient quantities of recombinant peptides for protein-peptide interaction studies.

At first, hybridization of corresponding designed oligonucleotides results the target DNA templates. By examining electropherogram (Fig.3.17.) we can obviously declare a well-defined sign with 50 bp length, consistent with the calculated length of target DNA.

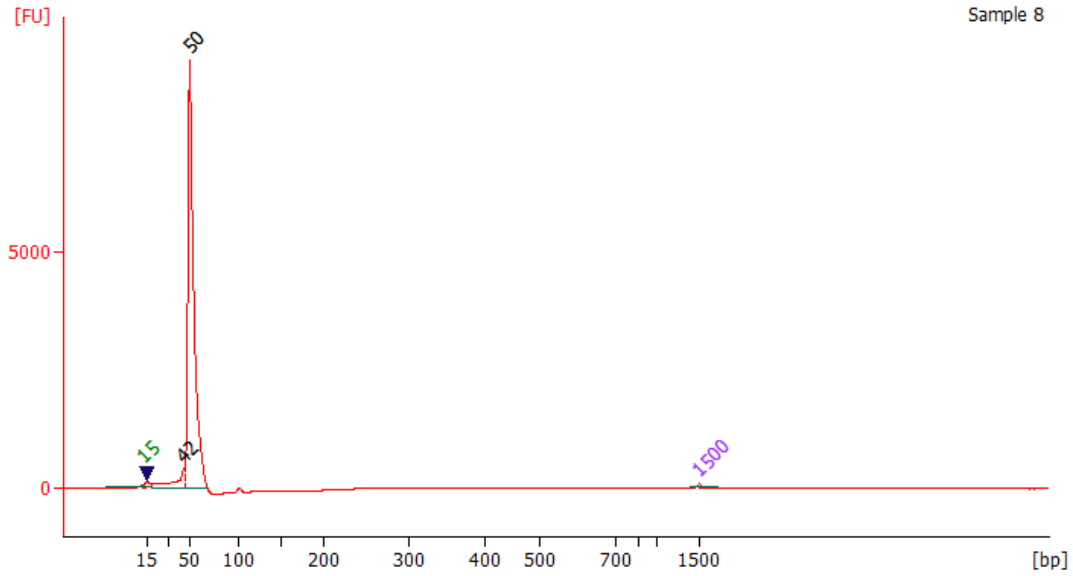


Fig.3.17. Electrophoretic microchip analysis of SMAC peptide DNA template obtained from hybridization

In addition a critical step for hybridization cooling down the reaction can be mentioned. This step must be performed slowly, in order to form double stranded DNA. The obtained DNA has blunt ends. The next step of preparing insert DNA was the addition of A-overhangs. Our designed plasmid can be formed by complementarity between the insert 3'-A overhangs and vector 3'-T overhangs. The A-tailing reaction works best when a specific amount of the DNA is used. The recommended amount is 10–100 ng (0.15–1.5 pmol) DNA for each 100 bp length of the DNA. In addition it was necessary to purify hybridized DNA.

After A-tailing reaction ligation of insert DNA to vector DNA was performed using T4 DNA ligase, then positive recombinant plasmids were selected successfully. Selection (Fig.3.18.) and examination of clones as described in Methods section were executed. The TA cloning site of pET-SUMO vector is presented in Appendix A.2, Fig.A.2.3.

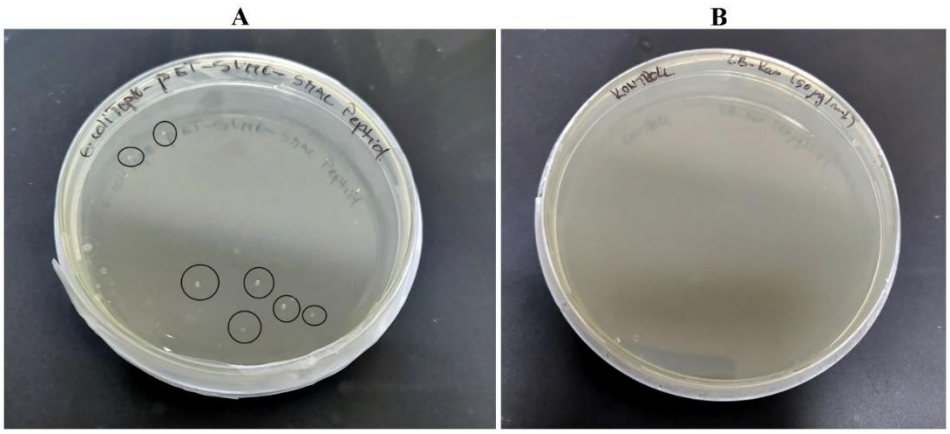


Fig.3.18. A: Colonies formed after transformation *E. coli* TOP10F' with pET-SUMO-P.SMAC recombinant plasmid
B: Controll transformation after ligation of insert and vector DNA

Amplified plasmids was around 300 bp. Amplified DNA fragments were successfully confirmed by 1% agarose gel electrophoresis presented on Fig.3.19.

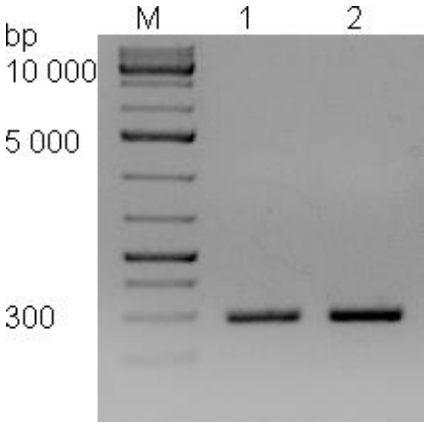


Fig. 3.19. Amplified DNA fragments;
M: GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific); lane 1: verification of pSUMO-SMAC peptide construct; lane 2: verification of pSUMO-Control peptide construct

3.3.2. Heterologous expression and purification of SUMO-Peptide constructs

Transformation of both recombinant plasmids in *E. coli* BL21(DE3) host cell line were successfully executed with high efficiency (part A, Fig.3.20.).

We can declare that expression was successful, and the selected host cell line was well-functioning. The induction with different concentration of IPTG caused difference in protein expression levels. On Fig.3.21. we can observe target proteins appearing under 20 kDa.

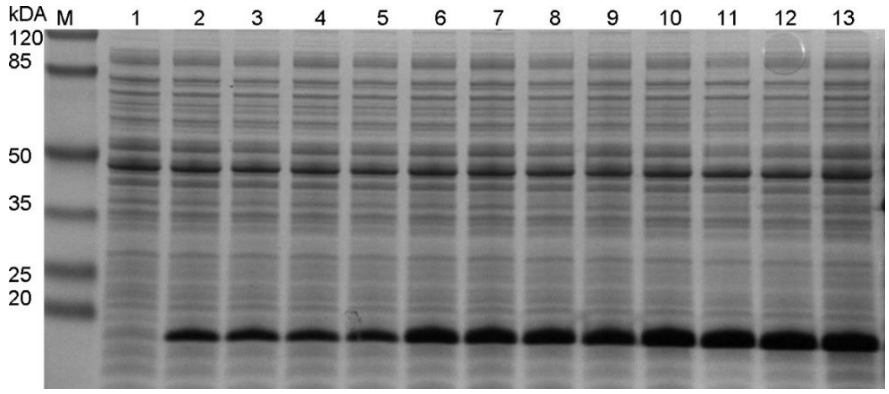


Fig.3.21. Optimization expression of SMAC+K peptide;
10% SDS-PAGE. M: Ptestained protein marker 20-120 kDa lane 1: uninduced cells; lane 2: induction with 0,3 mM IPTG-1h; lane 3: induction with 0,3 mM IPTG-2h; lane 4: induction with 0,3 mM IPTG-3h; lane 5: induction with 0,5 mM IPTG-1h; lane 6: induction with 0,5 mM IPTG-2h; lane 7: induction with 0,5 mM IPTG-3h; lane 8: induction with 0,8 mM IPTG-1h; lane 9: induction with 0,8 mM IPTG-2h; lane 10: induction with 0,8 mM IPTG-3h; lane 11: induction with 1 mM IPTG-1h; lane 12 induction with 1 mM IPTG-2h; lane 13:induction with 1 mM IPTG-3h

As we increased concentration of IPTG from 0.3 mM to 1 mM the expression level of target protein has also increased. In case of 1 mM IPTG induction the highest level of expression can be observed. If we compare the aforementioned expression in the first hour (lane 11) with the expression in the third hour of 0.3 mM (lane 4) or 0.5 mM (lane 7) IPTG induction cases, we can observe high expression level differences. In addition SUMO protein enhances protein production and solubility.

Hereinafter, heterologous expression was performed in 200-200 mL LB medium. Increasing the volume of broth higher cell count and higher target protein concentration may yield. Fig.3.22. presents heterologous expression of SUMO-SMAK-peptide construct using optimal parameters determined after optimization. Protein expression was induced by 1 mM IPTG for 3 hour at 37°C. Target proteins were expressed in bacterial cytoplasm. After cell lysis proteins were extracted in soluble form. Soluble proteins (lane 1, 2 and 3) and pellets (lane 6, 7 and 8) are presented on Fig.3.22.

For cell lysis Phosphate Buffered Saline (PBS) buffer systems was chosen by protein stabilizing properties. In addition protease inhibitor, glycerol for increasing protein stability were added. The lysis buffer contained salts (NaCl) to maintain or increase the strength of the ionic medium and detergent (Triton X-100) to dissolve the cell membranes and facilitate the release of soluble proteins and other cellular components into the solution. The experiments were conducted under pH conditions that mimic biological conditions (pH 7.4).The binding buffer contained 20mM imidazole to minimize binding of contaminating proteins and increase purity with fewer wash steps. Theoretically with increased salt (1 M NaCl) concentration we can eliminate contaminating proteins. Finally, elution of target proteins with 400 mM imidazole containing buffer were performed. Fig.3.22. presents described purification experiment. It can

be considered that this buffer system is well-functioning. Using these conditions elution fraction contains almost only target proteins (Fig.3.22, lane 4).

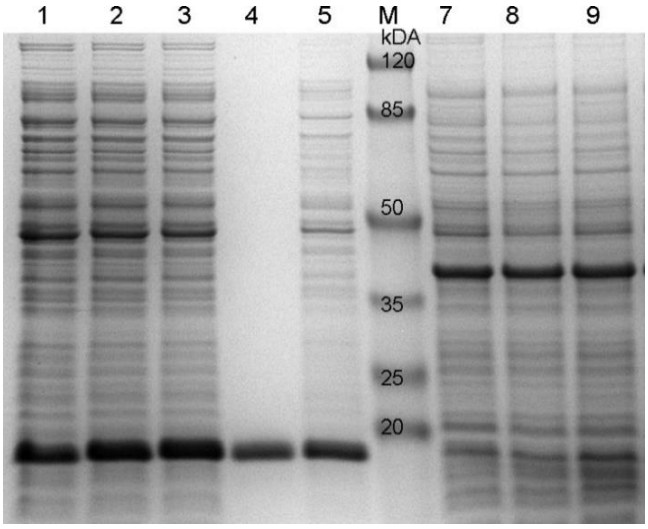
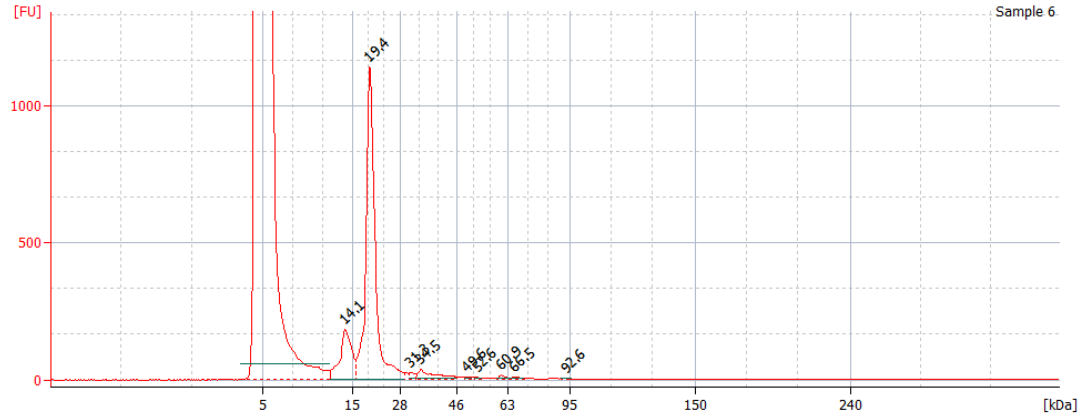


Fig.3.22. Shake flask expression of SMAC+K peptide

10% SDS-PAGE. 1: soluble protein expression in first hour, 2: soluble protein expression in second hour, 3: soluble protein expression in third hour, 4: purified SUMO-SMAC construct, 5: proteins bounded to Ni-NTA resin, M: Prestained protein Marker 20-120 7: insoluble proteins-expression first hour, 8: insoluble proteins-expression second hour, 9: insoluble proteins-expression third hour

A:



B:

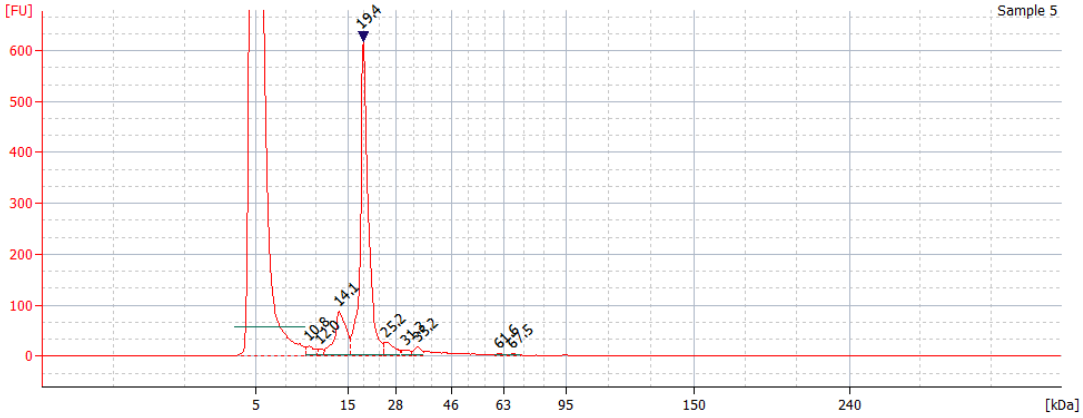


Fig.3.23. Electrophoretic microchip analysis of purified proteins
A: purified SUMO-SMAC; B: purified SUMO-Control

According to the electropherogram on figure 3.23 our target protein appears around 19 kDA. The obtained target proteins concentration is about 5 mg/mL with a relative high purity. We found that the concentration of Sumo-SMAC is higher (5.25 mg/mL) than Sumo-Control's concentration (4.02 mg/mL).

Finally, we can declare that SUMO expression system was suitable for expression of our recombinant contructs. Above presented purification system is simply practicable and high protein purity can be achieved. This heterologous expression system proved to be suitable for other recombinant peptide production.

3.3.4. Recovery of recombinant peptides

Predicted sizes for the recombinant peptides were 1.39 kDa. According to manufacturer's protocol, hydrolysis of the fusion protein was performed successfully. Peptides were separated from SUMO protein and SUMO protease using Ni-affinity purification. Recombinant peptides were confirmed by LC/MS analysis. Purity of peptides were determined

by reverse-phase high-performance liquid chromatography (HPLC) using as references, chemically synthesized SMAC peptide and Control peptide.

Designed peptides has the following properties calculated using ExPASy ProtParam and PeptideMass tools:

Table 3.3. Properties of designed peptides

Peptide/Properties	***SMAK+K***	***Control+K***
Number of amino acids	13	13
Amino acid sequence	AVPIAQKSEPHSK	AQVKSIEPAHSPK
Molecular weight (g/mol)	1391.59	1391.59
Theoretical pI	8.64	8.64
Net charge at pH 7	1.1	1.1
Estimated solubility	Good water solubility	Good water solubility
$[M - H]^-$	1389.75	1389.75
$[M+H]^+$	1391.76	1391.76
$[M - 2H]^{2-}$	694.37	694.37
$[M+3H]^{3+}$	464.59	464.59
$[M+2H]^{2+}$	696.38	696.38

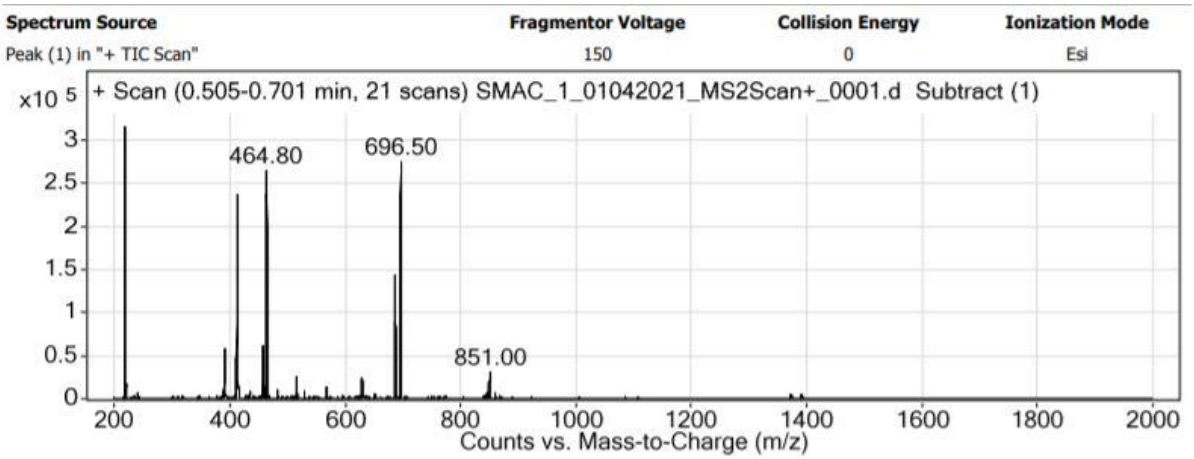


Fig.3.24. MS analysis of recombinant SMAK+K peptide

Analysis of chemically synthesized peptides in Appendix A.5 were presented.

3.3.5. C-terminal fluorescent labelling of peptides and biotinylation

To maintain the native structure of N-terminal of peptides, we performed a C-terminal fluorescent labeling reaction. Cross linking to carboxyl groups is mediated by a water-soluble carbodiimide. According to literature, carbodiimides effect conjugation of carboxyl to hydrazides result in formation of hydrazone bonds. In case of our experiment carboxyl containing amino acids (glutamine acid and C-terminal lysine) were labeled. For investigation

of labeling reaction MS analysis was executed using MALDI Biotyper. Based on the MS results, labeling reaction was successfully performed in both cases. As the following figures shows (Fig.3.25 and Fig.3.26) the laser ionization technique was suitable for investigation of appropriate ions. Efficiency of labelling reactions were acceplable. Among future plans we design optimization of labelling reaction.

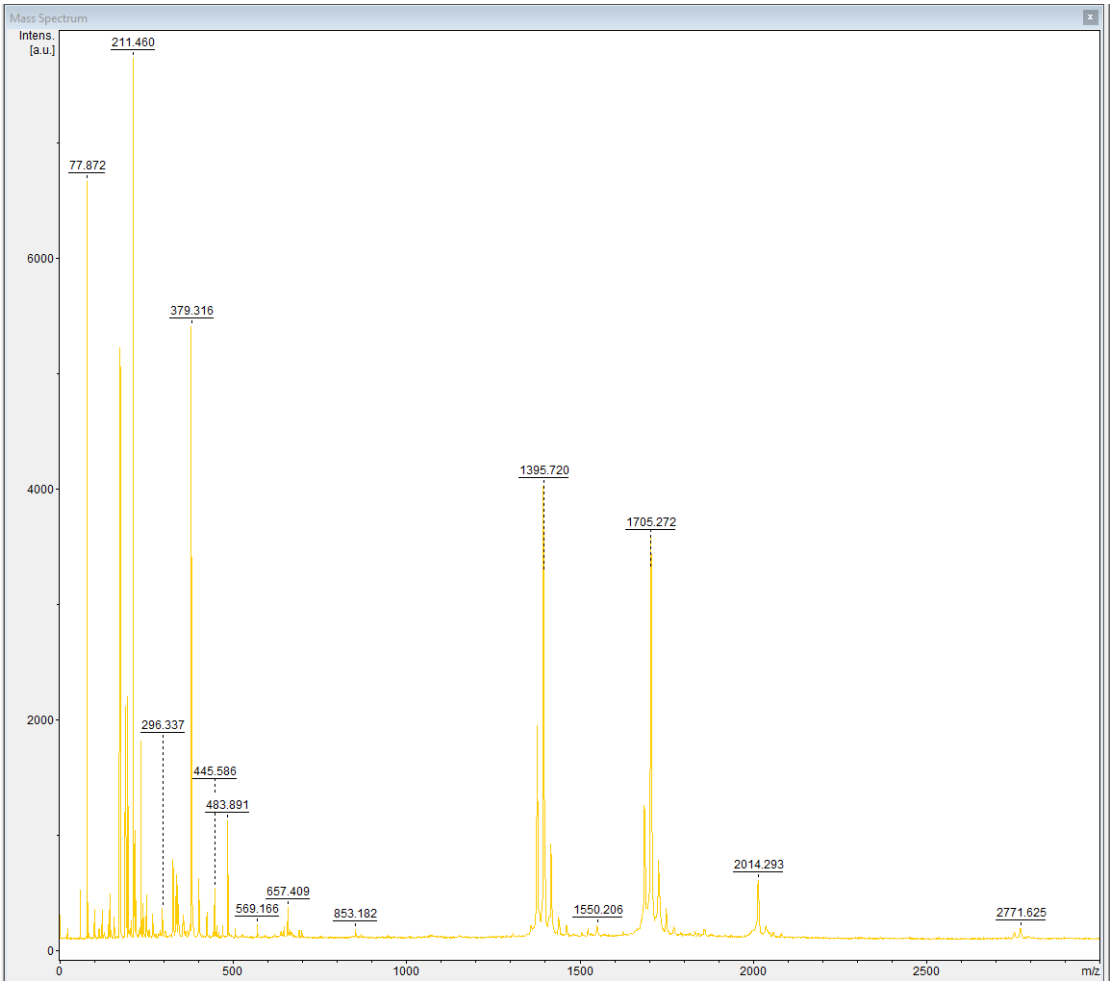


Fig.3.25. MS analysis of fluorescent labelled SMAK+K peptide

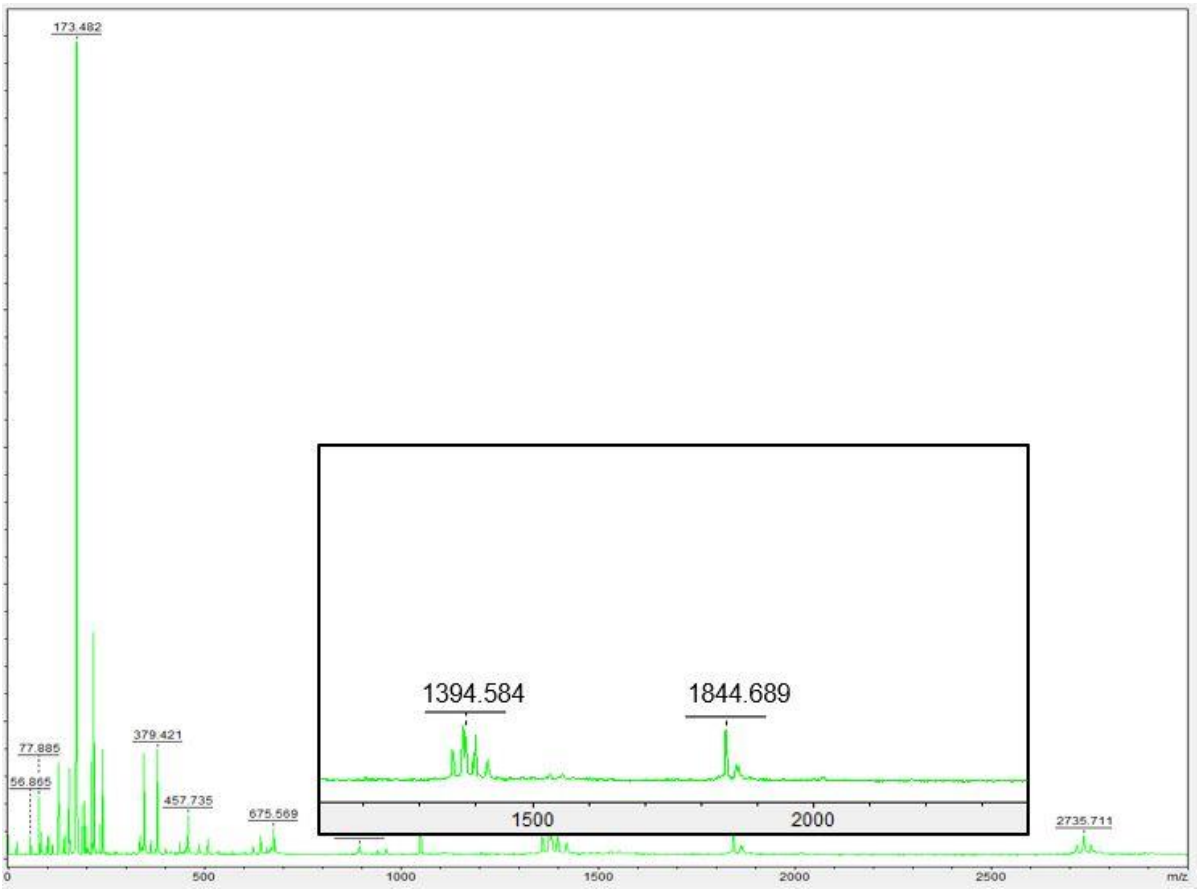


Fig.3.26. MS analysis of biotinylated SMAK+K peptide

3.3.6. Monitoring BIR3-peptide interaction

There are several examples in the literature on the classification of Smac mimetics. At first there are monovalent and bivalent Smac mimetics [51]. The monovalent compounds mimic the binding of a single AVPI motif to IAP proteins. Bivalent compounds contain two AVPI binding motifs. Monitoring BIR3-SMAC+K peptide interaction belongs major objectives of our research.

The aformentioned interaction is presented on Fig.3.24. using PyMOL molecular visualization software.

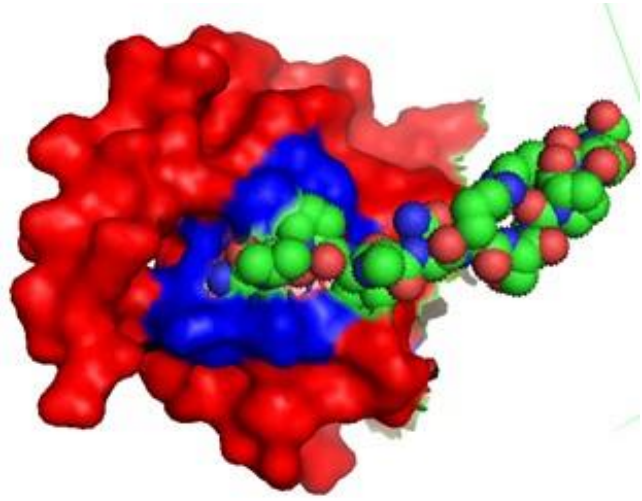


Fig.3.24. XIAP-BIR3 and SMAC+K interaction (PyMOL)

3.3.7. Pull-Down Assay

In the next part I will present pull-down assays designed for investigation BIR3 domain interactions. The pull-down assay is an *in vitro* method used to analyse protein-protein interaction using affinity purification. This is a physical interaction between two or more proteins. In our case, at first, the bait protein (namely BIR3 domain) was tagged and captured on an immobilized affinity ligand specific for the tag (amylose resin). Thereafter, unbond proteins were washed away. The next step was binding the "prey" protein to the bait protein. The "prey" proteins were Cas9 and SMAC proteins separately. After formation protein-protein interaction complex a washing step was performed. The method of protein elution depends on the affinity ligand In our case interaction complexes were eluted by 10 mM maltose. Finally, protein-protein interaction complex was analyzed by SDS-PAGE. These general steps of pull-down assay are shown on Fig.3.25.

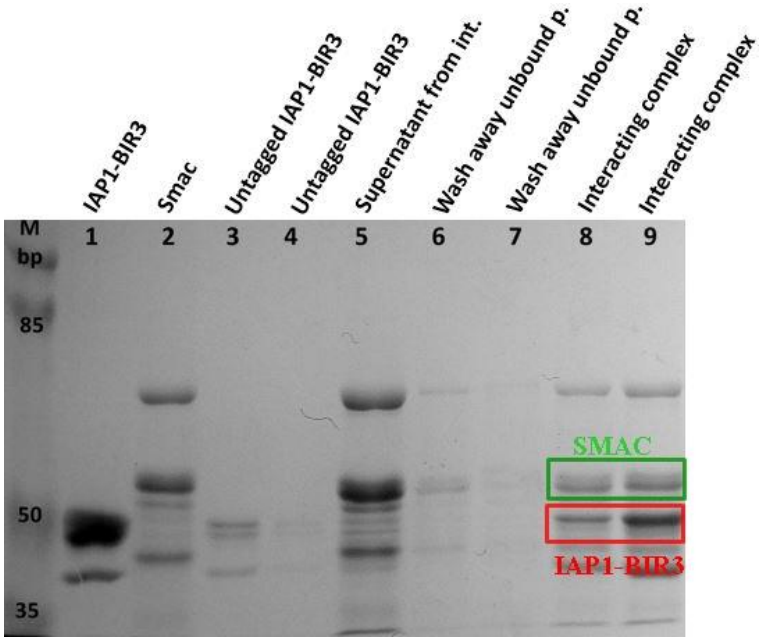


Fig.3.25. Analysis of IAP1-BIR3 and SMAC interaction by 10% SDS-PAGE

At this stage of our research we investigated intraction between IAP1-BIR3 and SMAC protein. It is important to mention that SMAC-EGFP (~ 50 kDa) protein is also recombinant protein, it belongs to our previous research [56]. For expression of the SMAC-EGFP recombinant protein, the *E. coli* expression strain Rosetta™(DE3) pLysS transformed with the recombinant vector was used successfully. A significant quantity of 20 mg/L of pure recombinant SMAC-EGFP protein was obtained in a bioreactor culture. Regarding the purification procedure, our two-step protocol proved to be successful. Protein-protein interactions were traceble thanks to the fluorescence property of the EGFP protein.

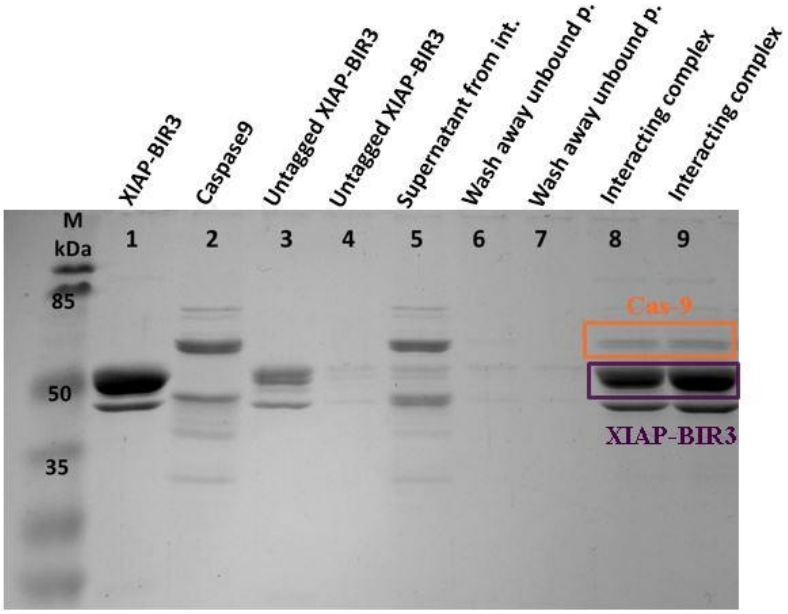


Fig.3.27. Analysis of XIAP-BIR3 and Cas9 interaction by 10% SDS-PAGE

In case of second pull down assay, ie. interaction between Cas9 and XIAP-BIR3, our results was also succesful. The bait protein (XIAP-BIR3) was successfully tagged on amylose resin, therefore untagged bait proteins were washed away from amylose resin (Fig.3.27. lane 3 and lane 4). The interaction against Cas9 protein was investigated by overnight incubation at 4 °C. As shown on figure 3.27. the amount of interacting proteins are significant. In addition, it's necessary to optimize recent protein-protein interaction assay.

CONCLUSIONS

C.1. GENERAL CONCLUSIONS

Bioactive peptides have an increased interest of pharmacology and medical science due to their increasing knowledge of the diverse modes of action. Therapeutic peptides generally have been derived from three sources: natural or bioactive peptides produced by plants, animal or human, peptides isolated from genetic or recombinant libraries and peptides discovered from chemical libraries. An important strategy in peptide chemistry is desing chemical modification in order to increase its chemical and enzymatic stability in the interest of to extend the time of action, and to increase activity and selectivity towards the receptor [3].

Industrial peptide production is commonly based on three alternative technologies: solid-phase synthesis, liquid-phase synthesis, and in vivo biotechnological recombinant technology. Chemical synthetic strategies are challenging in economic terms. The production of peptides by recombinant techniques has also contributed important progress of methodology.

Apoptosis is an essential and a general process in the development of cells. The mitochondrial protein SMAC promotes apoptosis by eliminating the inhibitory effect of IAP's through physical interactions. The amino-terminal sequences in SMAC protein are required for this function, as mutation of the first amino acid leads to loss of interaction with IAP. SMAC promotes apoptosis by activating caspases in the cytochrome c/Apaf-1/caspase-9 pathway [5]. In the case of apoptosis researchers focused on small molecule Smac mimetics that target the *BIR3* domain IAP's. These IAP inhibitors are currently under investigation as anticancer drugs in clinical trials [5, 6]. With today's knowledgewe we developed an expression system and purification procedure that allows the production of sufficient quantities of recombinant peptides for protein-peptide interaction studies. Furthermore, it can be stated that our objective referring to biosynthesis and purification of recombinant cIAP's has been achieved. It is also important to mention our successful protein refolding experiments. In addition, our research extended to investigation of BIR3 domain of target proteins by biosynthesis and interaction assays. The previously mentioned biologically active recombinant proteins are useful for studying the biological functions of genes in different diseases, for the development of therapeutic drugs, biomimetics and biomaterials for the biotechnology industry [18], [56].

C.2. ORIGINAL CONTRIBUTIONS

This thesis contributes to the study of protein and peptide chemistry, including production, purification, refolding, protein-peptide interaction assay as follows:

1. The Champion pET SUMO expression system shows to be suitable for peptide expression, notedly for small peptide biosynthesis.

2. Designed C-terminal chemical labelling of peptides using Alexa Fluor 350 hydrazide and Biotin XX hydrazide were successfully implemented. Fluorescent chemical labelling reactions takes an important role in fluorescent polarization assays.

3. The pMAL-2px expression system shows to be applicable for expression of large amount recombinant protein production.

4. The method developed for isolation of recombinant BIR3 domains of IAp's from periplasm was successfully performed.

5. We developed a quick and straightforward solubilization and detergent assisted refolding procedure using detergents for the recovery of native recombinant GST tagged XIAP protein.

C.3. FUTURE RESEARCH DEVELOPMENTS

Nowdays, peptide chemistry is an emerging field of science. The gained theoretical knowledge of peptide chemistry aspects during this study encuraged us to form a concept of a future project with the goal of engineering other recombinant small peptides. Peptides of interests based on their bilogically function will be selected. Among future plans of our research we design to elaborate in detail fuorescent polarization assays.

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APPENDINX

A.1. PUBLICATIONS AND PARTICIPATIONS AT CONFERENCES

Papers published in ISI journals

1. Pál SALAMON, Csongor K. ORBÁN, **Katalin MOLNÁR-NAGY**, Zita KOVÁCS, Klára VÁNCSA, Emese BÁLINT, Ildikó MIKLÓSSY, Beáta ALBERT, Gyöngyi TAR, Szabolcs LÁNYI, Study of native SMAC protein production in the pUbiq expression system: molecular cloning, biosynthesis and molecular modelling, *Electronic Journal of Biotechnology,* Manuscript number: EJBT-D-21-00069R2

2. **Katalin NAGY,** Csongor-Kálmán ORBÁN, Beáta ALBERT, Szabolcs LÁNYI, Biosynthesis of BIR3 domain of inhibitor of apoptosis proteins, *Scientific Bulletin Series B: Chemistry and Materials Science*, Vol. 81, Iss. 4, 2019, ISSN 1454-2331.

3. **Katalin NAGY**, Zita KOVÁCS, Pál SALAMON, Csongor-Kálmán ORBÁN, Szabolcs LÁNYI, Beáta ALBERT, Enhanced heterologous expression in *E.coli*, *Studia Universitatis Babes-Bolyai, Seria Chemia*, LXIV, 2,Tom I, 2019 (p. 101-110), ISSN (Print): 1224-7154 , 2019, Impact Factor: 0,305

4. Pál SALAMON, Ildikó MIKLÓSSY, Beáta ALBERT, Mónika KORODI, **Katalin NAGY**, Ildikó BAKOS, Szabolcs LÁNYI, Csongor ORBÁN, Heterologous expression and purification of recombinant proapoptotic human protein SMAC/diablo with EGFP as fusion partner, *Studia Universitatis Babes-Bolyai*, Seria Chemia, LXII, 2,Tom II, 2017 (p. 333-345), ISSN (Print): 1224-7154, 2017, Impact Factor: 0.305

Participation in conferences (2015-2019)

1. Krisztina KOVÁCS , **Katalin MOLNÁR-NAGY**, Zita KOVÁCS, Kálmán-Csongor ORBÁN, Beáta ALBERT, Biosynthesis and purification of apoptosis modulating BIR3 domain of IAP2, *25rd International Conference on Chemistry,* Cluj-Napoca, October 24–26, 2019

2. Pál SALAMON, Ildikó MIKLÓSSY, **Katalin NAGY**, Zita KOVÁCS, Szabolcs LÁNYI, Csongor-K. ORBÁN, Beáta ALBERT, Analysis of protein-protein interactions in cell death control, *25rd International Conference on Chemistry,* Cluj-Napoca, October 24–26, 2019

3. **Nagy Katalin** , Kovács Zita, ORBÁN Kálmán-Csongor, LÁNYI Szabolcs, ALBERT Beáta, A humán XIAP refoldálása, *XII. Szent-Györgyi Albert Konferencia*, Budapest, Április 5-6, 2019

4. SALAMON Pál, **NAGY Katalin**, KOVÁCS Zita, ALBERT Beáta, MIKLÓSSY Ildikó, Lányi Szabolcs, ORBÁN Csongor-K., A kaszpáz 9 szerepe a kaszpáz függetlennek vélt „öngyilkos" sejthalál programban, *XII. Szent-Györgyi Albert Konferencia*, Budapest, Április 5-6, 2019

5. **Katalin NAGY**, Csongor-Kálmán ORBÁN, Beáta ALBERT, Zita KOVÁCS, Pál SALAMON, Szabolcs LÁNYI, Biosynthesis, purification and protein-protein interaction studies of BIR3 domain of IAP's, *24rd International Conference on Chemistry,* Szovátafürdő, october 24-28, 2018

6. Zita KOVÁCS, Beáta ALBERT, Csongor-Kálmán ORBÁN, **Katalin NAGY**, Pál SALAMON, Szabolcs LÁNYI, Molecular cloning of human Tyrosine Kinase FER and heterologous expression, *24rd International Conference on Chemistry,* Szovátafürdő, october 24-28, 2018

7. SALAMON Pál, **NAGY Katalin**, KOVÁCS Zita, Beáta ALBERT, Ildikó MIKLÓSSY, Szabolcs LÁNYI, Csongor-Kálmán ORBÁN, Biosynthesis of Necroptosis Modulating Human Proteins, *24rd International Conference on Chemistry,* Szovátafürdő, october 24-28, 2018

8. **Katalin NAGY**, Csongor-Kálmán ORBÁN, Beáta ALBERT, Ildikó MIKLÓSSY, Szabolcs LÁNYI, Biosynthesis, purification and chemical labelling of AVPI binding motiff containing Smac mimetics, *23rd International Conference on Chemistry,* Deva, October 25-28, 2017

9. Zita KOVÁCS, Csongor-Kálmán ORBÁN, Beáta ALBERT, SALAMON Pál, Mónika KORODI, **Katalin NAGY**, Optimizing the heterologous expression of Inhibitor of Apoptosis Proteins (IAP), *23rd International Conference on Chemistry,* Deva, October 25-28, 2017

10. **Katalin NAGY**, Csongor-Kálmán ORBÁN, Beáta ALBERT, Ildikó MIKLÓSSY, Szabolcs LÁNYI, SMAC „mimicking" peptides: cloning, heterologous expression with fusion partner and purification, *22rd International Conference on Chemistry,* Temesvár, November 3-6, 2016