BIOSYNTHESIS OF BIR3 DOMAIN OF INHIBITOR OF APOPTOSIS PROTEINS

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Present work highlights cloning, heterologous expression and purification of BIR3 domain of Inhibitor of Apoptosis Protein. The BIR3 gene was cloned into pMAL-p2x expression vector which contains a maltose binding protein (MBP) fusion partner. The signal peptide on pre-MBP directs fusion proteins to the periplasm of E. Coli. Successful shake flask expression of BIR3 was achieved in E. coli Rosetta (DE3) pLysS cells at 16°C using Rich medium. The recombinant construct was isolated from periplasm and purified by affinity chromatography using amylose resin.

Keywords: cloning, heterologous expression, BIR3 domain, MBP fusion partner, periplasm, affinity chromatography

1. Introduction

In multicellular organism, the process of programmed cell death, or apoptosis, is a natural and important process. Apoptotic regulation has been implicated in many human diseases, including cancer, autoimmune disease, inflammation and neuro degradation. Mapping up critical apoptosis regulators is a possessing strategy for the development of new therapies [1, 2]. The inhibitor of apoptosis proteins (IAPs) contains 3 motifs termed baculovirus IAP repeat domains (BIR), each of them consisting of approximately 70 amino acids [2,3]. The most potent caspases inhibitor in IAP family is human X-linked inhibitor of apoptosis protein (XIAP), which contains 3 BIR domains in the N-terminal region. The IAP family member's cIAP1 and cIAP2 have an architecture similar to XIAP. As like XIAP, the BIR2 and BIR3 domains (Fig.1) of cIAP1and cIAP2 also bind caspases and Smac (Second Mitochondria-activator of Caspases) [4].

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 [5].

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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 [6, 7]. Inactivation of overexpressed XIAP by Smac mimetic molecules [1, 2, 8, 9, 10, 11, 12] may relieve caspase binding, thereby promoting apoptosis in malignant cells.

2. Materials and Methods

2.1. Chemicals and reagents

All chemicals used for experiments were purchased from commercial sources and are of analytical and molecular biology grade. Designed oligonucleotides (primers) were purchased from GeneriBiotech, Debrecen.

2.2. Designed constructions

BIR3-IAP1 construct is composed of 35 amino acid residues (~ 4,1 kDa) with the following sequence:

DDVKCFCCDGGLRCWESGDDPWVEHAKWFPRCEFL. MBP is a commonly used expression tag protein which has a molecular mass of approximately 42 kDa. MBP is encoded by the malE gene of *E. coli*. The pMAL-p2X vector encode the Factor Xa site, which allows MBP to be cleaved from the protein of interest after purification.

2.3. Target gene

Amplification of the BIR3-IAP1 gene from pGEX-4T constructions were performed by PCR using thermocycler (Agilent) with corresponding primers (*Table 1.*), designed to incorporate *EcoRI* and *HindIII* restriction sites of the PCR product. The PCR was carried out in a total volume of 50 µL (0,1 µg of template, 0,2 mM dNTP mix, 2,5 mM $MgCl_2$, 0,5 µM oligonucleotides, 5U/µL Taq DNA Polymerase) and conditions were set as follow: 1 cycle at 95°C for 5 min followed by 30 cycles each at 95°C for 45 sec, 49°C for 30 sec and 74°C for 30 sec and 1 cycle for final extension at 74°C for 5 min.

Table 1

Designed oligonucleotides

Construct	Primer	Nucleotide sequence 5'-3'	Tm (°C)
BIR3-IAP1	Fw.	CTGAATTCCCGGATGATGTCAAATGCT	
	Rev.	CGAAGCTTGGGTTACAAGAACTCACACCTT	

The amplified gene was analyzed on 1% agarose gel electrophoresis in 1X Tris-Acetate-EDTA (TAE) buffer at a constant voltage of 90 V and visualized by GelDoc System from BioRad.

2.4. Recombinant plasmid and bacterial strains

pMAL-p2x vector system was designed for cloning procedures (Fig.1). A signal sequence directs expressed proteins to periplasmic space of *E. coli*. The corresponding PCR product was digested, then directly subcloned into the vector. Ligation reaction was designed according to 5:1 ratio (insert/vector) by *Thermo Scientific* protocol. The reaction was carried out in a total volume of 20 µL, containing 50 ng vector DNA, ligase enzyme (1 U) and 10X Ligase Buffer (2 µL). After 1 hour, incubation at 22°C the ligation product was transformed into *E. coli Top10F'* cloning cell line, followed by a screening on apramycin LB plates. All plasmids were confirmed by double-enzyme digestion using NcoI (NEB,10 U/µL) and NdeI (NEB,10 U/µL).

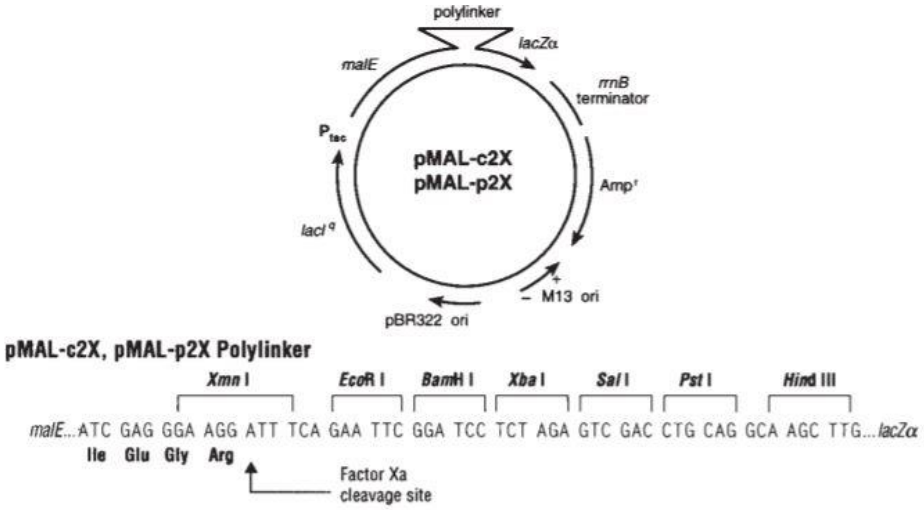

Fig.1. pMAL-2- cloning/expression region

The gene expression was realized in *E. coli Rosetta*^{Tm} (DE3)pLysS cells as host which were used for high-yield expression of recombinant proteins. Shake flask expression of proteins were carried out using *Rich medium* (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose) supplemented with the appropriate antibiotics (Ampicillin 100 µg/ml) at 37°C for 3 hour and at 18°C for

16 hour. Protein expression was induced by IPTG (0,1 mM; 0,3mM) at OD_{600}= 0,8.

2.5. Protein isolation and purification from periplasmic space

Proteins are initially isolated from periplasm by freezing cells in PBS buffer containing 5 mM $MgCl_2$ and 5 mM $MgCl_2$ for 8-72 hour. Cells were thawed at 4°C, then centrifuged at 8000 g for 10 min. The periplasmic extract contains MBP-IBM/BIR3 proteins, which were purified by affinity chromatography using amylose resin. All of the purification steps were carried out at 4°C using Tris-HCl buffer system (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT). The elution buffer consisted of 10 mM maltose added to previously mentioned Tris buffer. At the final step of protein purification, the elution fractions containing the protein of interest were dialyzed against a storage buffer (20 mM Tris, 100 mM NaCl, 1 mM DTT. 1 mM PMFS, 10% glycerol).

2. Results and Discussion

The designed recombinant plasmids were constructed successfully as described in Materials and Methods section. The map of the designed expression vector is outlined in Fig. 2. It was designed in silico using *Snap Gene* software.

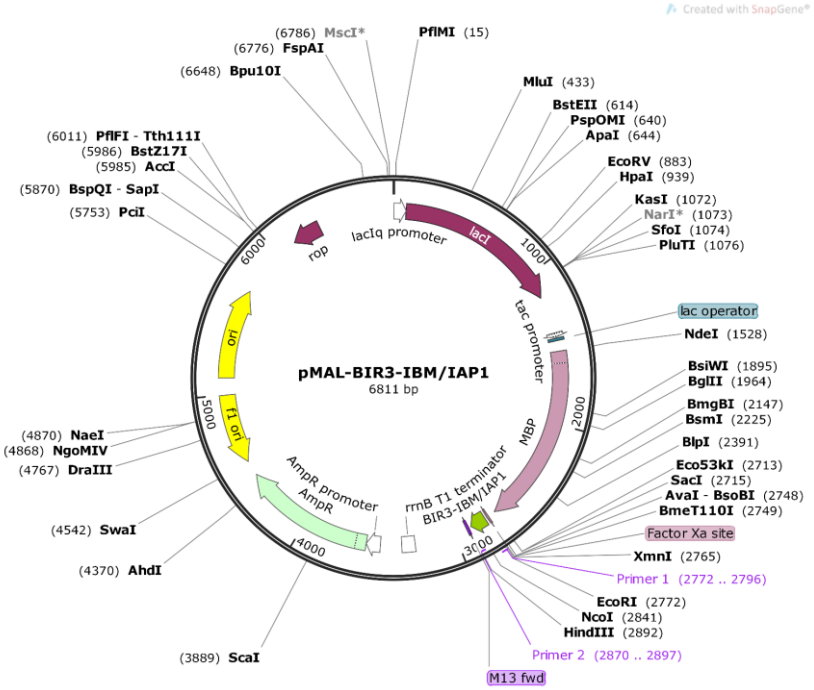

Fig.2. pMAL-p2x-BIR3-IAP1 (SnapGene™)

Amplified target genes (105 bp) were directly subcloned into pMAL-2x expression vector.

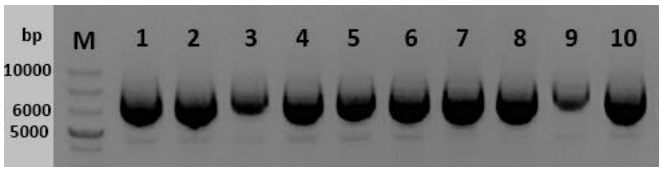

Fig.3. Selection of recombinant clones-plasmid miniprep analysis on 1% agarose gel
M: 1 kb Plus DNA Ladder; 1-10: BIR3-IBM/IAP1 clones
Selected clones: **1**, **5, 8**

Fig.3 shows realized BIR3-IAP1 clones. All plasmids were analyzed on 1% agarose gel. The recombinant plasmids were confirmed by double-enzyme digestion using NcoI-NdeI restriction enzymes (Fig.4: 1-3).

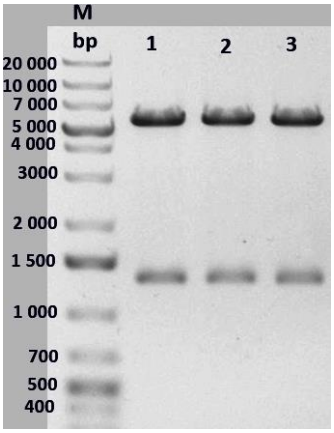

Fig. 4. Analysis of double enzyme digestion of selected clones on 1% agarose gel
M: 1 kb Plus DNA Ladder; 1: digestion of plasmid DNA from colony 1; 2: digestion of plasmid DNA from colony 5; 3: digestion of plasmid DNA from colony 8

Shake flask expression was executed successfully using Rich medium (Ampicillin 100 µg/ml). Fig.5 presents protein expression at 37°C for 3 h and 18°C for 16 h. Protein expression was induced with 0,1 mM or 0,3 mM IPTG at OD600=0,8. Using 0,3 mM IPTG induction at 18°C for 16 h, the quantity of expressed proteins was the most significant.

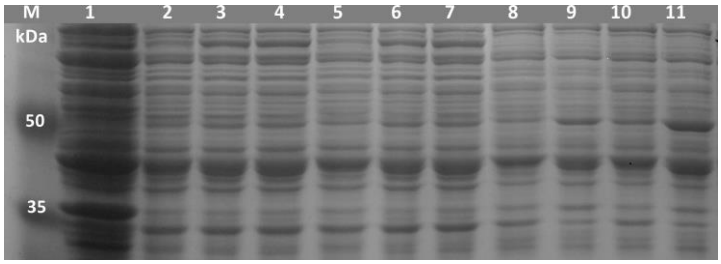

Fig. 5. BIR3-IBM/IAP1 expression at 37°C (2-7) and 18°C (8-11)
M: 120-20 kDa protein marker; 1: uninduced cells ; 2: 0,1 mM IPTG induction-1h; 3: 0,1 mM IPTG induction-2h; 4: 0,1 mM IPTG induction-3h; 5: 0,3 mM IPTG induction-1h; 6: 0,3 mM IPTG induction-2h; 7: 0,3 mM IPTG induction-3h; 8: 0,1 mM IPTG induction-4h; 9: 0,1 mM IPTG induction-16h; 10: 0,3 mM IPTG induction-4h; 11: 0,3 mM IPTG induction-16h;

During our research, several isolation methods from periplasm were tested. In conclusion freezing cells in PBS buffer containing 5 mM $MgCl_2$ for 48 h proved to be the most appropriate method (Fig.6).

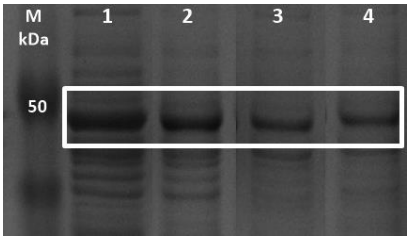

Fig. 6. BIR3-IAP1 isolation from periplasm
M: 120-20 kDa protein marker; 1: frozen cells 48 h; 2: frozen cells 24 h; 3: frozen cells 12h; 4: frozen cells 8 h

Isolated proteins from periplasmic space of E. coli were purified on amylose resin using Tris–HCl column buffer at pH= 7,4. As shown on Fig. 7 proteins were eluted successfully from amylose resin with column buffer containing 10 mM maltose. Maltose binding protein is a common protein expression tag with a molecular mass of approximately 42 kDa protein. BIR3-IBM constructions are composed of 35 amino acid residues and have a molecular mass of ~ 4 kDa.

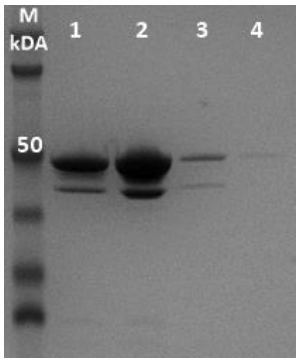

Fig. 7. BIR3-IAP1 purification
M: 120-20 kDa protein marker; 1: elution 1; 2: elution 2; 3: elution 3; 4: elution 4

4. Conclusion

Production of human BIR3 domain from Inhibitor of Apoptosis Proteins in E. coli was realized successfully. Using E. coli as expression host, a high efficiency in protein production was realized. Experimental results demonstrated that the recombinant construct was isolated from periplasm and high purity was achieved by affinity chromatography using amylose resin.

R E F E R E N C E S

[1]. *H. Sun et al.*, "Potent bivalent smac mimetics: Effect of the linker on binding to inhibitor of apoptosis proteins (IAPs) and anticancer activity", in Journal of Medicinal Chemistry, **vol. 54**, no. 9, May 2011, pp. 3306-3318

[2]. *S. Fulda*, "Smac mimetics as IAP antagonists", in Seminars in Cell & Developmental Biology, **vol. 39**, no. 3, Mar. 2015, pp. 132-138

[3]. *Cossu et al*. "Targeting the BIR Domains of Inhibitor of Apoptosis (IAP) Proteins in Cancer Treatment", in Comput Struct Biotechnol J., **vol. 17**, Jan. 2019, pp: 142–150

[4]. *Ardecky et al*., "Design, synthesis and evaluation of inhibitor of apoptosis protein (IAP) antagonists that are highly selective for the BIR2 domain of XIAP", in Bioorganic & Medicinal Chemistry Letters, **vol. 23**, no. 14, July 2013, pp. 4253-4257

[5]. *T. Grabinger et al.*, "Inhibitor of Apoptosis Protein-1 Regulates Tumor Necrosis Factor-Mediated Destruction of Intestinal Epithelial Cells", in Gastroenterology, **vol. 152**, no. 4, Mar. 2017, pp. 867-879

[6]. *X. Gao, L. Zhang, Y. Wei, Y. Yang, J. Li, H. Wu, Y. Yin*, "Prognostic Value of XIAP Level in Patients with Various Cancers: A Systematic Review and Meta-Analysis", in J Cancer, **vol. 10**, no. 6, Feb. 2019, pp. 1528-1537

[7]. *R. Saraei*, et al., "The role of XIAP in resistance to TNF-related apoptosis-inducing ligand (TRAIL) in Leukemia", in Biomedicine & Pharmacotherapy, **vol. 107**, no. 2018, May 2018, pp. 1010–1019

[8]. *A. C. West, B. P. Martin, D. A. Andrews, S.J. Hogg, A. Banerjee, G. Grigoriadis, R. W. Johnstone, J. Shortt*, "The SMAC mimetic, LCL-161, reduces survival in aggressive MYC-driven lymphoma while promoting susceptibility to endotoxic shock", in Oncogenesis, **vol. 5**, no. 4, Apr. 2016, pp. 1-6

[9]. *S. Fulda*, "Smac Mimetics to Therapeutically Target IAP Proteins in Cancer", in International Review of Cell and Molecular Biology, **vol. 330**, no. 2017, Jan. 2017, pp. 157-169

[10]. *E. Weisberg et al.*, "Smac mimetics: implications for enhancement of targeted therapies in leukemia", in Leukemia, **vol. 24**, no. 2010, Sept. 2010, pp. 2100–2109

[11]. *Gonzalez-Lopez et al*., "Design, synthesis and evaluation of monovalent Smac mimetics that bind to the BIR2 domain of the anti-apoptotic protein XIAP", in Bioorganic & Medicinal Chemistry Letters, **vol. 21**, no. 14, May. 2011, pp. 4332-4336

[12]. *Zobel et al*., "Design, Synthesis, and Biological Activity of a Potent Smac Mimetic That Sensitizes Cancer Cells to Apoptosis by Antagonizing IAPs", in ACS Chemical Biology, **vol. 8**, no.1, Sept. 2006, pp. 525-533