FACULTY OF SCIENCE

Pro Gradu

Heterologous production and DNA binding activity of *Trypanosoma cruzi* poly(ADP ribose) polymerase

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<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
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<tr>
<td>AIM</td>
<td>Auto induction media</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
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<tr>
<td>ANK</td>
<td>Ankyrin</td>
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<tr>
<td>ART</td>
<td>ADP-ribosyl transferases</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BER</td>
<td>Base excision repair</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>BRCT</td>
<td>Breast cancer susceptibility protein C-terminus</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break repair</td>
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<td>dsDNA</td>
<td>Double stranded Deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoresis mobility shift assay</td>
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<tr>
<td>H1, H2A, H2B</td>
<td>Histones</td>
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<tr>
<td>hPARP-1</td>
<td>Human poly(ADP-ribose) polymerase-1</td>
</tr>
<tr>
<td>HPS</td>
<td>Histidine, proline, and serine</td>
</tr>
<tr>
<td>HYE</td>
<td>Histidine, Tyrosine, Glutamate</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>mARTs</td>
<td>Mono-ADP-ribosyltransferases</td>
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<tr>
<td>MNNG</td>
<td>N-methyl-N-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>mtROS</td>
<td>Mitochondrial ROS</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>PAR</td>
<td>Polyanionic poly(ADP-ribose)</td>
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<tr>
<td>PARG</td>
<td>Poly-ADP-ribose-ribose-glycohydrolase</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBN</td>
<td>Phenyl-α-tert-butylnitrone</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PRD</td>
<td>PARP regulatory domain</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAM</td>
<td>Sterile Alpha Module</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SSB</td>
<td>Single-strand break</td>
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<tr>
<td>T. brucei</td>
<td>Trypanosoma brucei</td>
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<tr>
<td>T. cruzi</td>
<td>Trypanosoma cruzi</td>
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<tr>
<td>TbPARP</td>
<td>Trypanosoma brucei poly(ADP-ribose) polymerase</td>
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<tr>
<td>TcPARP</td>
<td>Trypanosoma cruzi poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco Etch Virus</td>
</tr>
<tr>
<td>TRF1</td>
<td>Telomere-repeat binding factor-1</td>
</tr>
<tr>
<td>WGR</td>
<td>Tryptophan, Glycine, Arginine</td>
</tr>
<tr>
<td>XRCC1</td>
<td>X-ray repair cross complementing1</td>
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1 REVIEW OF THE LITERATURE

1.1 Introduction.

1.1.1 Poly(ADP-ribose) polymerase (PARP)

For proper cellular functions, nuclear proteins usually undergo post-translational modifications. Poly (ADP-ribose)ylation, first identified in the 1960s, is one of these essential modifications (Chambon et al., 1963). The process is catalyzed by a family of cell-signaling enzymes known as poly(ADP-ribose) polymerases (PARPs) (Otto et al., 2005). Studies revealed that PARP enzymes are activated upon the DNA damage that occurs due to ionizing radiation, oxidative stress, chemical or biological means (Lindahl et al., 1995; D’Amours et al., 1999). These enzymes include mono-ADP-ribosyl transferases and poly-ADP-ribose polymerases (PARPs) that modify their substrates with a single ADP-ribose units and polymers of ADP-ribose, respectively (Kleine & Lüscher, 2009).

PARP genes have been found in various groups of eukaryotes and also identified in a broad variety of species including fungi (except yeast), mammals, as well as in eubacteria and archaebacteria (Hottiger et al., 2010; Ame et al., 2006; Hassa et al., 2006; Otto et al., 2005). Once activated, PARP enzymes use nicotinamide adenine dinucleotide (NAD⁺) as a substrate to transfer the ADP-ribose on acceptor proteins or to the growing chains (Schreiber et al., 2006; Spagnolo et al., 2012). Apart from repairing DNA damage, PARPs have also been shown to be involved in cell death pathways and genome modification/remodelling (Kim et al., 2005; Schreiber et al., 2006; Hassa et al., 2006, 2008).

In terms of pathological conditions that cause severe genomic stress such as ischemia-reperfusion injury, inflammation, myocardial infarction, glutamate excitotoxicity, and Parkinson disease, PARP-1 overactivation leads to cell death. Cell failure from PARP-1 overactivation has been attributed to depletion of cellular energy. That results in the release of death effector apoptosis-inducing factor (AIF) from the mitochondria, and productions of excess poly-ADP ribose (PAR) polymer, a novel death signal (David et al., 2009).

1.1.2 PARP family members

Recent studies have been focused on characterization of the structure and function of PARPs and PARP related proteins (Krishnakumar & Kraus 2010). So far, PARP enzymes have been best
characterized in humans, where seventeen family members have been found that share the PARP catalytic domain (Hottiger et al., 2010; Ame et al., 2004).

Based on their catalytic domain, PARP members have been categorized into three sub-families. The first sub-family consists of PARP-1 to PARP-5a and 5b (or Tankyrase-1 and -2), based on the presence of three conserved amino acids residues in PARP-1, Histidine (H862), Tyrosine (Y896) and Glutamate (E988). The “HYE” triad is critical for NAD⁺ binding and for synthesis of polymers (Kleine et al., 2008; Domenighini et al., 1994; Domenighini & Rappuoli, 1996; Okazaki & Moss, 1994). In the second group, which contains PARP6–PARP8, PARP10–PARP12, and PARP14–PARP16, the third amino acid, glutamate (E) of “HYE” motif is replaced by Isoleucine/Leucine/Tyrosine. Furthermore, it is also suggested that the members of this group function only as mono-ADP-ribosyltransferases (mARTs) rather than polymerases (Ruf et al., 1996; Ruf et al., 1998). The third group of ADP-ribosyltransferases contains PARP9 and PARP13. Instead of “HYE”, this group possesses “QYT” and “YYV” triads respectively. Because both members of this group contain no catalytically important glutamate and histidine residues, which are involved in NAD⁺ binding activity, it is suggested that they are catalytically inactive (Kleine et al., 2008; Aguiar et al., 2005).

1.1.3 Structure and function of PARP-1
The founding and most abundant member of the PARP family is poly(ADP-ribose)polymerase-1 PARP-1 (Amé et al., 2004). PARP-1 is highly conserved chromatin bound enzyme with a molecular weight of 116 kDa (D’Amours et al., 1999). PARP-1 has a modular structure composed of three functionally important regions (Krishnakumar & Kraus 2010), including N-terminal DNA-binding region, a central automodification region, and a C-terminal catalytic region (Hakme et al., 2008; Schreiber et al., 2006) (Figure 1).

A 46 kDa N-terminal DNA-binding region spans up to 372 amino acids in human PARP-1 (Kraus and Hottiger, 2013). The N-terminal region contains three zinc-finger domains, of which the first two motifs were referred as “nick sensors” (Shieh et al., 1998; D’Amours et al., 1999), because they are responsible for DNA binding along with other protein-protein interactions (Vira´g & Szabo´, 2002). The third zinc finger domain contributes to inter-domain interactions and is important for enzymatic activation of DNA-dependent of PARP-1 (Ba & Garg 2011; Langelier et al., 2010; Langelier et al.,
Along with zinc finger domains, DNA-binding region also contains a nuclear localization signal (NLS) that contains the caspase-cleavage site (DEVD).

The central automodification region has a molecular weight of 22 kDa, located between amino acid residues 373 and 524 in human PARP-1 and serves as a central regulatory segment of the enzyme (Kurosaki et al., 1987; Alkhatib et al., 1987; Kraus & Hottiger, 2013). It consists of a breast cancer susceptibility protein C-terminus (BRCT) motif from residues 385 to 479 (Bork et al., 1997) (Figure 1). BRCT domain is involved in protein-protein interactions and it is also found in several other DNA repairing proteins. Structure based studies on PARP-1 revealed that it contains glutamic acid residues. On activation of PARP-1 these glutamic acid residues are involved in covalent binding with poly(ADP-ribose). However, some other studies showed that, instead of glutamic acid there are lysine residues that are involved as acceptor sites for ADP ribosylation (Desmarais et al., 1991; Tao et al., 2009; Altmeyer et al., 2009). It is also reported that automodification region is important for homodimerization or heterodimerization of PARP-1 with PARP-2 (Caldecott et al., 1996; Schreiber et al., 2002; Masson et al., 1998).

The C-terminal catalytic region of human PARP-1 has a molecular weight of approximately 54 kDa and spans from residue 525 to 1014 (Kurosaki et al., 1987; Alkhatib et al., 1987; Kraus and Hottiger, 2013). There is high amino-acid sequence similarity between different species in the primary structure of the PARP-1 enzyme, with the catalytic domain showing the greatest amount of similarity. The PARP catalytic fragment contains WGR domain named after highly conserved amino acid sequence (Trp, Gly, Arg) (Simonin et al., 1993; Ruf et al., 1996). The PARP regulatory domain (PRD) might be involved in regulation of the PARP-1 branching activity and ART domain, is the catalytic core required for ART activity. It is conserved in other Diphtheria toxin-like ADP-ribosyltransferases (ARTDs) and includes the amino acids involved in catalysis and binding of NAD$^+$ (Figure 1).
1.1.4 Activation mechanism of PARP-1

PARP-1 cleaves the glycosidic bond of NAD$^+$ between nicotinamide and ribose followed by the formation of ADP-ribose from the oxidized form of nicotinamide adenine dinucleotide (NAD$^+$). The ADP-ribose is then attached to a glutamate, aspartate and carboxyterminal lysine residues of target (‘acceptor’) proteins, which are covalently modified by the addition of an ADP-ribose subunit, via formation of an ester bond between the protein and the ADP-ribose residue (Ogata et al., 1980). Structural heterogeneity of the PAR polymer is achieved by elongation and branching at the 2'-OH and 2''-OH of the ribose moiety respectively. The chemical structure of the branching site of ADP-ribose was determined by NMR and by mass spectroscopy (Miwa et al., 1981) as $O$$-$$\text{D}$-ribofuranosyl-(1''→2'')-$O$$-$$\text{D}$-ribofuranosyl-(1'' → 2')-adenosine-5',5'',5'''-tri(phosphate), commonly known as Ado(P)-Rib(P)-Rib-P. ADP-ribose is branched in an irregular manner. This generates novel ribosyl-ribosyl linkages and eventually results in the formation of polymers with the average branching frequency of approximately one branch per linear section of 20±50 units of ADP-ribose (Hayaishi et al., 1983; Miwa et al., 1979; Alvarez-Gonzalez and Jacobson 1987).

PARP-1 catalytic activity is regulated by allosteric mechanism involving several functions such as DNA insult, histones, transcription factors, nuclear enzymes, and nuclear structural proteins (D'Amours et al., 1999; Kraus & Lis, 2003; Tulin et al., 2003). PARP-1 binds to damaged DNA via second zinc finger domain in N-terminal region which leads to hydrolysis of NAD$^+$ and catalysis of the successive addition of ADP-ribose units to either acceptor proteins (heteromodification) or themselves (automodification) (D'Amours et al., 1999). If the DNA damage is repairable then the cell survives. If
DNA damage is irreparable, PARP-1 is over activated the cell may die by necrosis or parthanatos due to depletion of both NAD$^+$ and ATP (Hakmé et al., 2008; Nguewa et al., 2003).

In addition, PARP-1 enzymatic activity can also be stimulated by protein-protein interactions (Kraus & Lis, 2003). For example, during transcription, PARP-1 binds to histones, especially H1, H2A and H2B and PARsylate them, which may play a role in the regulation of chromatin structure (D'Amours et al., 1999; Kim et al., 2005; Kraus, 2008; Kraus and Lis, 2003) (Figure 2).

1.1.5 Hydrolysis of PAR

PARsylation is a dynamic process whose enzymatic activity is hydrolyzed by two different enzymes (Brochu et al., 1994). Poly-ADP-ribose phosphodiesterase/ ADP-ribose pyrophosphatase, contains pyrophosphatase activity to cleave the pyrophosphate linkages. That results to release of 5'-AMP from chain termini, phosphoribosyl-AMP from internal residues, and diphosphoribosyl-AMP from branching points (Boulikas 1992).

Another enzyme, poly-ADP-ribose-ribose-glycohydrolase (PARG), contains both endoglycosidase and exoglycosidase activities. PARG is responsible for the hydrolysis of glycosidic riboseribose bonds internally and at the ends of ADP-ribose polymers, respectively (Figure 2). The endoglycosidase activity releases free PAR chains from product which are supposed to be important signaling molecules involved in diverse cellular processes, such as cell death or cell growth. Furthermore, branched and short polymers are degraded more slowly by PARGs than long and linear poly-ADP-ribose polymers (Braun et al., 1994; Brochu et al., 1994; Ame et al., 1999). This suggests that complexity and concentration of each distinct type and structure of poly-ADP-ribose may vary not only depending on the cellular context and stimuli, but also depending on specific branching activities of different PARPs (Figure 2).
1.1.6 Trypanosoma cruzi

Trypanosomiasis is a disease found in Africa and the American continents that is caused by infection with a parasite. African trypanosomiasis, are known as sleeping sickness. The disease can affect people living on the African continent south of the Sahara Desert. American trypanosomiasis also called Chagas disease is a potentially life-threatening illness caused by the protozoan parasite, Trypanosoma cruzi (family Trypanosomatidae, order Kinetoplastida). It occurs only on the American continents, from Mexico to Argentina (Ba et al., 2010; Gupta et al., 2009; Tanowitz et al., 2009).

Among the trypanosomatids, T. cruzi represents one of the most complex life cycles involving several developmental stages (Figure 3). The cycle is initiated when insects (triatomine) of the Reduviidae family by sucking the blood of animals or human infected with the trypomastigotes. Once ingested, most of the trypomastigotes divide rapidly in the insect gut (Castro et al., 2007; Clayton, 2010). After few days the surviving trypomastigotes transform either into spherical stage (known as spheromastigotes) or into epimastigote stage. Epimastigotes migrate to the intestine where they rapidly divide and attach to the perimicrovillar membranes secreted by intestinal cells of posterior midgut (Alves et al., 2007; Nogueira et al., 2007). At the rectum, many epimastigotes detach from the intestinal surface and transform into metacyclic trypomastigote forms which are then released together with feces and urine (Garcia et al., 2007). Metacyclic trypomastigotes are highly infective for human and several other mammalian species. Usually the infection takes place through direct inoculation of these forms through the ocular mucosa or the wounded skin during insect blood meal. Once in the
vertebrate host, the metacyclic trypomastigotes invade the cells at the inoculation site like, fibroblasts, macrophages, and epithelial cells. After invasion, the cycle involves several steps like the formation of an endocytic vacuole also known as the parasitophorous vacuole, a differentiation of the long and thin trypomastigote forms in rounded with a short flagellum, characteristic of the amastigote forms (also known as intracellular spheromastigotes) and lysis of the parasitophorous vacuole membrane by enzymes secreted by the parasite (de Carvalho & de Souza, 1989) so that the amastigote forms enter direct into the bloodstream of host cell and organelles (Figure 3) (Souza et al., 2010).

![Figure 3: Generalized life cycle of the Trypanosoma cruzi](http://www.dpd.cdc.gov/dpdx/HTML/TrypanosomiasisAfrican.htm)

**1.1.7 Trypanosoma cruzi PARP (TcPARP)**

On complete genome analysis, to characterize the metabolism of trypanosomatids (*T. cruzi* and *T. brucei*), it was found that both protozoan parasites contain genes involved in DNA repair mechanism that exhibit similarities to human poly(ADP-ribose) polymerases (PARPs) (Berriman et al., 2005; El-Sayed et al., 2005). However, in contrast to higher eukaryotes having multiple PARP enzymes, there is only one PARP present in *T. cruzi* and *T. Brucet* (*TcPARP and TbPARP*). Like human hPARP-1, TcPARP shows its activation on DNA damaging agents such as H$_2$O$_2$ and UVC radiation (Fernández et al., 2008; Vilchez Larrea et al., 2011).
Recent studies on the characterization of trypanosomatid PARP (TcPARP) found out that the poly ADP-ribose polymerase is a 65 kDa protein, showing similarities to the C-terminal catalytic domain and the secondary structure with human PARP-1 to PARP-4 and TbPARP (Otto et al., 2005). Moreover, like hPARP-1, trypanosomatid PARP also contains DNA binding region, automodification region and catalytic region, but surprisingly they lack zinc finger binding domains as well as the carboxyl- terminal domain of the breast cancer gene 1 (BRCT domain) (Fernandez Villamil et al., 2008). This feature of TcPARP is homologous to human PARP-3 that also lack zinc finger DNA binding domains but contains a WGR domain.

1.2 Physiological and Pathophysiological Role of poly(ADP-ribosyl)ation

1.2.1 Physiological role of poly(ADP-ribosyl)ation
PARPs play key roles in the maintenance of genomic integrity, in the regulation of chromatin structure and transcription, and in other cellular processes. PARsylation is referred as genetic indication for many physiological pathways (Alexander 2001; Hassa & Hottiger 2008; Kim et al., 2005). In the following, I will describe pathways that are involved in physiological and pathophysiological processes.

Genome stability
The most dominated function linked to PARP-1 is DNA repair and the maintenance of genomic integrity (D'Amours et al., 1999). DNA damage can be caused by many means like alkylation, oxidation or radiation (De Murcia 2000). Several pathways have been postulated to involve PARP-1, of which three are more common. These include single-strand break repair (SSB), double strand break repair (DSB) repair and base excision repair (BER) (Bouchard et al., 2003; Woodhouse & Dianov, 2008; Bürkle 2001a; Bürkle 2001b; Masutani et al. 2003).

During DNA repair, two zinc finger domains present in amino terminal DNA-binding region play key roles for the binding of PARP-1 to single-strand breaks and double-strand breaks (DSBs) (Gradwohl et al., 1990; Hassa & Hottiger 2008). A third zinc finger was not found to be involved in DNA binding, but it is essential for coupling damage-induced changes in the DNA-binding region to modifications in PARP-1 catalytic activity (Langelier et al., 2008; Tao et al., 2008). Studies showed that after the
induction of certain types of DNA damage, including nicks and DNA DSBs, PARP-1 is rapidly recruited to the altered DNA and its catalytic activity increases 10- to 500-fold, resulting in the synthesis of protein conjugated long branched poly ADP-ribose chains (Hassa & Hottiger 2008; Haince et al., 2008). Once the polymer is formed, it could recruit hundreds of other proteins. Some of these known recruited proteins are XRCC1 (X-ray repair cross complementing1), DNA ligase III and DNA polymerase β (Caldecott et al., 1996; Masson et al., 1998; Schreiber et al., 2002). These recruited proteins are scaffolding proteins that assemble and activate the DNA base excision repair (BER) machinery (Masson et al., 1998; El-Khamisy et al., 2003). XRCC1 bind directly to PAR, whereas others are indirectly recruited because they interact with poly ADP ribose -binding proteins.

PARP-1 and PARP-2 prevent covalent complex formation of topoisomerase I with DNA and are able to remove stalled topoisomerase I from SSBs (Malanga & Althaus 2004). In addition, inhibition of PARP activity via transdominant and chemical inhibition results in the inhibition of BER in vivo and in vitro (Durkacz et al., 1980; Zwelling et al., 1982; AhnstrÔm & Ljungman 1988; Satoh & Lindahl 1992; Molinete et al., 1993). PARP-3 localizes in daughter centrioles throughout the cell cycle and interacts with PARP-1. Over expression of PARP-3 causes pause of G1/S phase (Augustin et al., 2003). These results indicate the possibility that dysfunction of PARP-2 and PARP-3 could induce mitotic defects leading to genomic instability, thus contributing to carcinogenesis.

**Transcriptional regulation & Chromatin remodeling**

Several studies reported the involvement of PARPs as transcription co-factors. PARP-1, PARP-2, PARP-9/BAL1 and PARP-14/Coast6 have been shown to regulate transcription by influencing the formation or activity of various transcription-factor complexes (Goenka et al, 2007; Juszczynski et al, 2006; Kraus, 2008; Yelamos et al, 2008). Furthermore, presence of PARP-1 on the promoters of almost all actively transcribed genes (Krishnakumar et al., 2008) suggested that it plays a role in promoting the formation of chromatin structures that are permissive to transcription. From these studies, two different mechanisms have been proposed for PARP-1. It has a histone-modifying enzymatic activity that act as a chromatin structure modulator and it can interact with gene-specific enhancer/promoter-binding complexes (Kraus & Lis 2003; Kim et al., 2005; Kraus, 2008). Independent to DNA damage, PARP-1 has been shown to play a role in remodeling chromatin structure by PARsylating histones. For both PARP-1 and PARP-2, H₁ and H₂b are the main histone targets for PARsylation (Mathis & Althaus,
According to Huletsky et al. 1989, PARP-1 could ADP-ribosylate chromatin proteins (e.g., H1 and, to a lesser extent, core histones), promoting the decondensation of chromatin and destabilization of nucleosomes. Further studies suggested that polyanionic poly(ADP-ribose) (PAR), itself acts as a core histone binding matrix. It may be either attached to proteins or present as a free polymer to further destabilize nucleosomes (Realini and Althaus, 1992). Collectively, these data suggested that PARP-1 promotes the decondensation of chromatin by causing the dissociation of nucleosomes through PARsylating H1 and core histones, as well as the generation of polyanionic, histone binding PAR (D'Amours et al. 1999, Kraus & Lis 2003, Rouleau et al. 2004; Tulin & Spradling 2003).

Another proposed mechanism for transcriptional regulation described that, PARP-1 can interact with the enhancers and promoters of genes by direct sequence-specific binding to enhancers or by recruitment via DNA binding transcription factors like NF-κB (Hassa & Hottiger, 2002; Kraus & Lis, 2003; Pavri et al., 2005).

**Cell death pathways**

In contrast to the role of PARsylation in DNA repair and cell survival after genotoxic stimuli, overactivation of PARP-1 can promote cell death pathways. Two models have been proposed to account for induction of cell death by hypersynthesis of PAR.

First mechanism includes, apoptosis, also called “programmed cell death” or “cell suicide”. Apoptosis involves DNA fragmentation by PARsylation of chromatin. Apoptosis involves PARP-1 overactivation leads to the release of the death effector apoptosis-inducing factor (AIF). The process of apoptosis can happen either through caspase-independent or caspase-dependent induction of apoptosis-inducing factor (AIF). Apoptosis-inducing factor (AIF), located in the mitochondrial intermembrane space. AIF is a flavoenzyme which possesses both death-promoting and protective functions (Klein et al., 2002).

Apoptotic process is generally divided in three main phases (initiation, effector, and execution) and requires ATP (González et al., 2001). In caspase-dependent induction of apoptosis-inducing factor (AIF), cysteine proteases called caspases digest cellular proteins. Proteins digested by caspase includes pro-caspase-3 and pro-caspase-7, Bcl-2 family proteins (Bid, BcL-XL, and Bcl2), structural proteins (actin, cytokeratin, and focal adhesion kinase), neural cell adhesion molecules, signal-transduction proteins, DNA repair and cell cycle regulatory proteins (Orrenius et al., 2003).
AIF exhibits both reactive oxygen species (ROS)-generating NAD(P)H oxidase and monodehydroascorbate reductase activities (Susin et al., 1999; Vaehsen et al., 2004; Urbano et al., 2005). Induction of cell death by high doses of genotoxic alkylating agents N-methyl-N-nitro-N-nitrosoguanidine (MNNG) or N-methyl-D-aspartate induces the opening of the mitochondrial transition pore and release of AIF from mitochondria to the cytoplasm. There, it combines with cyclophilin A to form an active DNase, which in turn translocates to the nucleus and contributes to nuclear DNA fragmentation (Ye et al., 2002; Yu et al., 2002; Wang et al., 2004).

Second mechanism of cell death by PARPs includes energy depletion or “programmed necrosis”. During necrosis, necrotic cells excrete cell content into the surrounding tissues, which may contribute to organ injury (Golstein et al., 1991). It has been suggested that ATP is an important determinant of the mode of cell death. Severe ATP depletion leads to subsequent cellular energy failure, which results in cellular dysfunction and finally in necrotic cell death (Berger et al., 1983; Berger, 1985). ATP depletion is brought about by the fall in mitochondrial permeability transition induced by cytotoxic stimuli. This causes the inhibition of mitochondrial oxidative phosphorylation that produces ATP. ATP depletion blocks caspase cleavage of PARP, leading to continued PARP activity. PARP overactivation results in NAD$^+$ depletion and further ATP depletion (Green & Reed, 1998).

Regulation of telomere length and longevity

Telomeres are essential for genome stability in all eukaryotes. They are the part of chromosome that contains tandem-repeated hexamers at the termini to form protective complexes in association with specific proteins.

One subfamily of PARPs consists of two telomere-binding enzymes called tankyrases or TRF-1 interacting ankyrin-related ADP-ribose polymerases 1 and 2 (PARP-5a/ARTD5 and PARP-5b/ARTD6, respectively). These are supposed to regulate telomere length together with telomerase. Both tankyrases 1 and 2 are multidomain proteins having on ANK domain; SAM domain; and ART domain (Smith et al., 1998). A fourth domain called HPS domain is found only in tankyrase 1. These domains are supposed to regulate telomere length together with telomerase (Cook et al., 2002). The ART domain is a catalytic domain and is common in all PARP enzymes. The ANK domain consists of 24 ankyrin
repeats and is responsible for the protein-protein interactions, and the SAM domain is required for the multimerization of the tankyrase itself. The function of HPS domain is still unknown.

Tankyrases are able to bind several proteins and PARsylate target proteins, including themselves. TRF-1 is directly and covalently PARsylated by tankyrase-1 (PARP-5a/ARTD5). TRF-1, binds and protects telomeric DNA and PARsylation leads to telomere elongation (Seimiya, 2006). Elongated telomeres are associated with immortality of cancer cells, and therefore telomerase has been a target for drug development (Folini et al., 2009).

**Cellular differentiation and proliferation**

Nuclear mono and poly-ADP-ribosylation reactions are suggested to play major roles in the regulation of cell division, cell cycle progression and or cell proliferation (Chang et al., 2004). Many fully differentiated cell types such as 3T cells, B or T lymphocytes, fibroblasts, and hepatocytes maintain the ability to proliferate, such as in the course of immune response, wound healing, or liver regeneration, respectively (Rochette-Egly et al., 1980; Butt & Sreevalsan 1983; Marini et al., 1989). It is also demonstrated that the relationship between differentiation and high levels of PARsylation activity may be dependent on the cell type (Chabert et al., 1992). Furthermore, PARsylation of histones was also proposed to facilitate the assembly and deposition of histone complexes on DNA during replication (Boulikas, 1990). Further studies will certainly elucidate the potentially distinct roles of different nuclear PARsylation activities in cellular differentiation and proliferation processes.

### 1.2.2 Pathophysiology of poly(ADP-ribosylation)

On the contrary to the "cytoprotective" functions, PARP-1’s over-expression or low expression can lead to pathological process.

**Carcinogenesis**

Inflammation reactions are considered to promote carcinogenesis by inducing genomic instability due to oxidative and nitrosative stress on cellular components (Ohshima et al., 2003). A number of lines of evidence propose the involvement of PARP-1 in human carcinogenesis. *PARP-1* gene expression in gastric cell lines, a colon cancer cell line and T-cell leukemia cell lines is reported to be significantly lower than in other cancer cell lines (Masutani et al., 2004). Similarly, a germ cell tumor contained a
possible germ line mutation, which causes amino acid substitution. (Shiokawa et al., 2005). A number of germ cell tumor cell lines show a substantial decrease in PARsylation level, suggesting a possible association between dysfunction of PARP and germ cell tumors (Masutani et al. 2003). In addition, over expression of PARP-1 is also involved in variety of cancers and its expression correlates with higher genomic instability, especially in breast cancer (Rojo et al., 2011).

**Diabetes mellitus**

During islet inflammation, destruction of insulin-producing pancreatic β-cells is responsible to cause Type-1 (juvenile) diabetes. Dysfunctioning of endothelial cells is involved in promoting retinopathy, nephropathy, neuropathy and accelerated atherosclerosis in diabetic patients. PARP-1 activation may play a role in the pathogenesis of endothelial dysfunction in diabetes (Soriano et al., 2001). Nicotinamide, a general PARP-1 inhibitor, has been reported to be protective against inflammatory insult and improve the function of residual β-cells (Kolb & Burkart 1999). Studies on PARP-1 knockout animal model described that in non-obese diabetic mice, nicotinamide protected islet cells against cytotoxic reagents and reduced the incidence of type I diabetes (Suarez-Pinzon et al., 2003).

**Ischaemia-reperfusion damage in brain, heart, kidney and bowel**

After a variety of damages, including cerebral infarct resulting from ischaemia-reperfusion damage, MPTP-induced Parkinsonism and brain trauma, over-activation of PARP-1 has been proved critical for acute neuronal cell death (De Murcia 2000).

**1.3 PARP Inhibitors**

Dramatically activation of PARP-1 by ionizing radiation and DNA methylating agents opened new ways for examining the effects of PARP inhibitors in combination with DNA-damaging agents. They are developed for multiple indications as specific, effective, and safe competitive inhibitors of PARP catalytic activity. Clinical development of PARP inhibitors follows two distinctive approaches. When PARP activity is lost, targeting the cells that are genetically predisposed to die. By combining PARP inhibition with DNA-damaging therapy to derive additional therapeutic benefit from DNA damage. Both approaches are being pursued vigorously.

The most important target is the treatment of cancer therapy, including breast, uterine, and ovarian cancers (Patel & Kaufmann, 2010). Several forms of cancer are more dependent on PARP than regular
cells, making PARP an attractive target for cancer. The focus has been on competitive inhibitors of PARP catalytic activity that may be useful as research tools, as well as clinical therapies (Rouleau et al., 2010).

**PARP inhibitors as adjuvant drugs in cancer chemotherapy**

Cytotoxic tumour therapy is one of the most frequently administered forms of cancer therapy. It involves the utilization of cytotoxic drugs or ionising radiation. The efficacy of PARP inhibitors may be due to synthetic lethality between PARP inhibition and a genetic lesion in the cancer cells. For instance, the potent PARP inhibitor AG14361 showed effectiveness against in vitro growth, when it was combined with an alkylating agent, temozolomide, a topoisomerase I inhibitor or ionizing irradiation. It was also effective in xenografts of human cancer cell lines (Calabrese et al., 2004). Another potentially useful effect of PARP inhibition was reported in acute promyelocytic leukaemia cells. Direct exposure of these cells to all-trans retinoic acid and the PARP inhibitor isoquinolinediol was noted to activate apoptosis rather than granulocytic differentiation (Berry et al., 2000).

Furthermore, studies also reported that treatment of p53-deficient breast cancer cells with a PARP inhibitor lose resistance to doxorubicin, a clinically active antitumor anthracycline antibiotic that promotes apoptosis (Munoz-Gamez et al., 2005). In the same way, germline mutations in the familial breast cancer genes *BRCA1* or *BRCA2* also reported to sensitize breast cancer cells to PARP inhibitors in a PARP-1-dependent manner (Bryant et al., 2005; Farmer et al., 2005).

**PARP inhibitors as antitumor therapy**

The development of PARP inhibitors as agents to treat tumors with certain genetic lesions is based on the concept that cells with defects in DSB repair. Recent findings revealed that poly(ADP-ribose) polymerase (PARP) inhibitors have cytotoxic effects on BRCA1- or BRCA2-deficient cells1 (Bryant et al., 2005; Farmer et al., 2005) and human tumours (Fong et al., 2009). In early clinical testing, PARP inhibitor olaparib (previously known as AZD2281) has shown significant activity against BRCA1- or BRCA2-mutant tumours (Fong et al., 2009). Because triple-negative (Oestrogen receptor (ER)-negative, progesterone receptor (PR)-negative and ERBB2-negative) breast cancer and sporadic serous ovarian cancer exhibit some of the properties of BRCA1- or BRCA2-deficient cells (Anders & Carey 2008; Turner et al., 2004), PARP inhibitors are also being tested against these tumours.
It has also been reported that the use of PARP inhibitors in combination with DNA-binding drugs may result in the enhancement of the apoptotic activity of this type of antitumor drugs. For example, use of PARP inhibitors 3-aminobenzamide or NU1025 increase apoptosis and reduce necrosis induced by the DNA minor groove binder MeOSO2(CH2)2-lexitropsin (Me-Lex) (Tentori et al., 2001).

**PARP inhibitors in Trypanosomatid infections**

Acute infection by *Trypanosoma cruzi* results in intense inflammatory reaction consisting primarily of leukocytes, including eosinophils and macrophages, together with increased expression of inflammatory mediators such as cytokines, chemokines, and nitric oxide synthase in the heart (Talvani et al., 2004; Machado et al., 2008). Most of the PARP inhibitors bind to the nicotinamide binding site and it was revealed via homology model that region is highly conserved also in *Tc*PARP.

In vitro studies showed the best inhibitors for *Tc*PARP were Olaparib and EB-47. The most common interactions sites of the inhibitors are as stacking with the tyrosine$_{907}$, and hydrogen bonds to the glycine$_{863}$ and serine$_{904}$ (Larrea et al., 2012). Structure of Olaparib in complex with human Tankyrase-2 (PARP-5b/ARTD6) has recently been solved (Narwal et al., 2012). It is find out that there are key differences in the nicotinamide binding site between Tankyrase-2 and hPARP-1, specifically in the D-loop (loop lining the donor NAD$^+$ binding site). However, no structural data of EB-47 in complex with PARPs have been discovered.

It has been studied that *T. cruzi* alters the mitochondrial membrane potential and induce a feedback cycle of mitochondrial leakage of electrons to molecular oxygen and reactive oxygen species (ROS) production in human cardiomyocytes and chagasic myocardium (Wen & Garg 2008; Gupta et al., 2009). Treatment of infected cardiomyocytes with ROS scavenger (PBN) and PARP-1 inhibitor (PJ34) disrupted the mtROS formation, blocked the nuclear translocation and activation of NF-κB and cytokine gene expression in infected cardiomyocytes. This subsequently caused DNA damage and PARP-1 activation were decreased. Furthermore, it is also reported that Phenyl-α-tert-butylnitron (PBN), a nitrone-based antioxidant, blocked the mitochondrial ROS (mtROS) formation, DNA damage, and PARP-1 activation in infected cardiomyocytes (Ba et al., 2010).
2 PURPOSE OF STUDY

*T. cruzi* is the causative agent of Chagas’ disease in Latin America and sleeping sickness in sub-Saharan Africa. Complete genome analysis of trypanosomatids (*T. cruzi* and *T. burcei*) proved the presence of genes involved in DNA repair mechanism. However, the mechanism of activation of these enzymes are likely different from that of human PARP-1, as trypanosomatid PARPs do not contain zinc finger DNA-binding domains that are responsible for the DNA nick detection and binding (Figure 4).

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**Figure 4: Structure of TcPARP**

The purpose of the research project was therefore to find out:

- Express deletion mutants in *E.coli* (Figure 4)
- Study the activity and DNA binding properties of the produced proteins.
3 MATERIALS AND METHODS

3.1 General reagents and chemicals used
Phusion high -Fidelity DNA Polymerase (Thermo scientific)
iProof DNA polymerase (Bio-Rad)
Taq DNA polymerase (Fermentas)
Benzonase (Sigma-Aldrich)
EDTA free protease inhibitor tablets (Roche)
Lysozyme from chicken egg (Fluka)
DpnI (Neb)
DNaseI (Sigma-Aldrich)
Geneaid Extraction kit
NucleoSpin Plasmid

3.2 Cell lines
Cell lines used were DH5α and Rosetta2(DE3). DH5α is a versatile strain that express transformation efficiencies of >1 x 10⁸ cfu/ µg plasmid DNA. The Rosetta2(DE3) cell line is a T7 expression host designed to enhance codon bias when expressing heterologous proteins in E. coli. Helper plasmid DE3 indicates that the host is a lysogen of λDE3, and therefore carries a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter.

3.3 Plasmids
pET-22b+: The pET-22b(+) vector carries an N-terminal signal sequence for potential periplasmic localization, plus C-terminal His-Tag sequence. For expression, the pET-22b+ vector bearing the TcPARP gene copy was transformed to E. coli BL21 Rosetta2(DE3) strain.
pNH-TrxT vector: pET expression vector with His₆-Trx (E.coli thioredoxin) in 128 aa N-terminal fusion peptide, with TEV protease cleavage site. For expression, the pNH-TrxT vector bearing the TcPARP gene copy was transformed to E. coli BL21 Rosetta2(DE3) strain.

3.4 Cloning
Five fragments of TcPARP were already cloned in pET-22b(+) vector by Salomè Vilchez Larrea. Same fragments of TcPARP were also cloned in pNH-TrxT vector (Table 1). This was made by Anton & Ichiro (2010) description of overlap extension PCR cloning. The first PCR reaction creates a linear
insert with plasmid sequences at both ends. These extensions subsequently allow the strands of the PCR product to act as a pair of oversized primers on the vector. After denaturation and annealing, the insert strands hybridize to the vector and extend to form new double-stranded plasmid.

For Primer designing, overlap extension PCR follows 3 steps. First I designed appropriate primers A and B to PCR-amplify the insert. Second, I selected the desirable insert points on the plasmid, preferably, 50 to several hundred base pairs apart. Then I selected 30–40 bp upstream of the left point of insert on the top strand of the plasmid and 30–40 bp downstream of the right point of insert on the bottom strand of the plasmid. Primer Tm was examined by using online tool (http://www.thermoscientificbio.com/webtools/tmc/). Following these three steps, primers for each mutant fragment were designed and ordered.

<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>Domains</th>
<th>Vector</th>
<th>Tag</th>
<th>Vector</th>
<th>Tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP-c003</td>
<td>Mutant 1</td>
<td>N-terminus</td>
<td>pET-22b+</td>
<td>C-His ...LEHHHHHHH</td>
<td>pNH-TrxT</td>
<td>His6- Thioredoxin - TEV</td>
</tr>
<tr>
<td>TCP-c004</td>
<td>Mutant 2</td>
<td>N-terminus + WGR</td>
<td>pET-22b+</td>
<td>C-His ...LEHHHHHHH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCP-c005</td>
<td>Mutant 3</td>
<td>N-terminus + WGR + PRD</td>
<td>pET-22b+</td>
<td>C-His ...LEHHHHHHH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCP-c006</td>
<td>Mutant 4</td>
<td>WGR + PRD+ART</td>
<td>pET-22b+</td>
<td>C-His ...LEHHHHHHH</td>
<td>pNH-TrxT</td>
<td>His6- Thioredoxin - TEV</td>
</tr>
<tr>
<td>TCP-c007</td>
<td>Mutant 6</td>
<td>ART</td>
<td>pET-22b+</td>
<td>C-His ...LEHHHHHHH</td>
<td>pNH-TrxT</td>
<td>His6- Thioredoxin - TEV</td>
</tr>
</tbody>
</table>

Table 1: Showing all constructs in pET22b+ and pNH-TrxT vectors

**Primers**

**TCP-c003**

Forward: 5’GGT ACC GAG AAC TTG TAC TTC CAA TCC ATG TCA CCA AAG AAG TTA3’
Reverse: 5’GAT CCG TAT CCA CCT TTA CTG GAG ACC GTC CTA GTA CAC GTG GCA3’

**TCP-c004**

Forward: 5’GGT ACC GAG AAC TTG TAC TTC CAA TCC ATG TCA CCA AAG AAG TTA3’
Reverse: 5’GAT CCG TAT CCA CCT TTA CTG GAG ACC GTC CTA ACG TCC CTT AAC3’
TCP-c005
Forward: 5’GGT ACC GAG AAC TTG TAC TTC CAA TCC ATG TCA CCA AAG AAG TTA3’
Reverse: 5’GAT CCG TAT CCA CCT TTA CTG GAG ACC GTC CTA GTA AAG TTT GTC3’
TCP-c006
Forward: 5’GGT ACC GAG AAC TTG TAC TTC CAA TCC ATG TAC GAA AAA GGC AGT GA3’
Reverse: 5’GAT CCG TAT CCA CCT TTA CTG GAG ACC GTC CTA ATG ATA TTT AAA3’
TCP-c007
Forward: 5’GGT ACC GAG AAC TTG TAC TTC CAA TCC ATG CTG AAC TGT GAG ATA AC3’
Reverse: 5’GAT CCG TAT CCA CCT TTA CTG GAG ACC GTC CTA ATG ATA TTT AAA3’

1st PCR (megaprimer formation)
5x Phusion buffer 10 µl
25µM Forward primer 0,5 µl
25µM Reverse primer 0,5 µl
10mM dNTPs 1 µl
Template 1 µl
Phusion polymerase 0,5 µl
dH₂O 36,5 µl
Total 50 µl

The PCR program was as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>98°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>98°C</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td>55°C</td>
<td>30 sec</td>
<td>30 cycles</td>
</tr>
<tr>
<td>72°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>hold</td>
<td></td>
</tr>
</tbody>
</table>
PCR products were purified by using Geneaid Extraction kit and their concentration was measured by using Nanodrop at absorbance 280.

**II\(^{nd}\) PCR**

In II\(^{nd}\) PCR reaction, products from I\(^{st}\) PCR used as a mega primers. The concentrations of megaprimers were 50 times more than concentration of vector i.e. 1:50 (vector: insert) ratio was taken.

5x Phusion buffer 10 µl  
Mega primer 500 ng  
10mM dNTPs 1 µl  
Template (vector) 10 ng (stock concentration: 67.7 ng/µl)  
Phusion polymerase 0.5 µl  
dH₂O  
Total 50 µl

The PCR program was as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>98°C</td>
<td>3 min</td>
<td></td>
</tr>
<tr>
<td>98°C</td>
<td>10 sec</td>
<td>18 cycles</td>
</tr>
<tr>
<td>55°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>hold</td>
<td></td>
</tr>
</tbody>
</table>

After PCR, DpnI digestion was performed by adding 2 µl of DpnI enzyme per 20 µl PCR mixture and incubated for 2.5 hours at 37°C and inactivated at 80°C for 20 minutes.

**3.5 Transformation in DH5α cells**

I thawed DH5α *E.coli* competent cells on ice until the last ice crystals disappear. Then I gently added whole DpnI digested PCR product into the DH5α tube and placed the mixture on ice for 30 minutes. Then I gave heat shock first at 42°C for 45 seconds and then on ice for 5 minutes. After heat shock, I added 1 ml LB (without antibiotics) into the mixture and incubated at 37°C for 60 minutes. After incubation, spinned down the cells and 900 µl of media was discarded and cell pellet was resuspended
in rest of the media. Then I spread the cells on selection plates (having LB, 5% sucrose (allowing negative selection) and 50 µg/ml Kanamycin as selection marker) and incubate overnight at 37°C.

### 3.6 Colony PCR

I performed colony PCR to confirm the correctly cloned colonies obtained. Colony PCR was done with sequencing primers and run on 1% agarose gel.

<table>
<thead>
<tr>
<th>10x Taq buffer</th>
<th>2 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTPs</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Template</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>13.25 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20 µl</td>
</tr>
</tbody>
</table>

The PCR program was as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>3 min</td>
<td></td>
</tr>
<tr>
<td>95°C</td>
<td>10 sec</td>
<td>34 cycles</td>
</tr>
<tr>
<td>50°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>hold</td>
<td></td>
</tr>
</tbody>
</table>

### 3.7 Plasmid extraction

Miniprep was done in LB media supplemented with 50 µg/ml kanamycin as antibiotic and inoculated colony got from DH5α transformation and incubated o/n at 37°C. Plasmid extraction was done by plasmid extraction kit (NucleoSpin Plasmid).
3.8 Transformation to Rosetta 2(DE3) cells

I thawed Rosetta 2(DE3) competent cells on ice until the last ice crystals disappeared. Then I gently added approximately 1 µl of plasmid into the cell and placed the mixture on ice for 30 minutes. After that I gave heat shock first at 42°C for 45 seconds and then on ice for 5 minutes. Then I added 1 ml LB (without antibiotics) into the mixture and incubated at 37°C for 60 minutes. After incubation, cells were spin down and 900 µl of media was discarded and cell pellet was resuspended in rest of the media. Then I spread the cells on selection LB agar plates with 50 µg/ml Kanamycin and 34 mg/ml Chloramphenicol as selection markers and incubated overnight at 37°C.

3.9 Expression test for all constructs

Small scale expression of all constructs cloned in pET-22b+ vector and pNH-TrxT vector was done in 5 ml LB media supplemented with 100 mg/ml ampicillin, and 34 mg/ml chloramphenicol or 50 µg/ml Kanamycin and 34 mg/ml Chloramphenicol respectively. Then I incubated cultures overnight at 37°C with 180 rpm. Next day, 4% of starter culture was inoculated in 15 ml of LB supplemented with antibiotics and incubated at 37°C with 180 rpm. Culture growth was measured by spectrophotometer with OD$_{600}$ until it reaches to ~ 0.4. Then I separated 5 ml culture of each construct and centrifuged them at 4200 rpm for 10 minutes and saved pellet at -20°C as non-induced sample. Then I induced rest of the cultures with different concentrations (0.5 mM, 0.2 mM, 0.1 mM) of IPTG and incubated them at 18°C with 180 rpm. Overnight culture was centrifuged and cell lysis was done by adding 200 µl lysis buffer (0.1 M HEPES pH 7.4, 500 mM NaCl, 10 mM Imidazole, 10% glycerol, 0.5 mM TCEP) along with a spoon tip of lysozyme, DNaseI and MgSO$_4$ (0.5 mg/ml) in each pellet. Cell lysis was done by using freeze thaw heat shock mechanism in liquid nitrogen (5-6 cycles). Then I centrifuged the cells and collected the supernatant. Pellet was resuspended in lysis buffer and all samples were analyzed on SDS-PAGE at 150V.

3.10 Protein expression

TcPARP fragments cloned in to pET- 22b+ and pNH-TrxT expression vectors were transformed into Rosetta 2(DE3) strain and saved as glycerol stocks at -80°C. I used a loop full of glycerol stock as starter culture in Luria broth (LB) media supplemented with 100 mg/ml ampicillin, and 34 mg/ml chloramphenicol in case of pET- 22b+ vector constructs, whereas, 50 µg/ml kanamycin and 34 mg/ml chloramphenicol for constructs in pNH-TrxT vector as antibiotics. The culture was grown overnight at
37°C, 180 rpm. The following morning 4% of bacterial culture was inoculated in 2 liters of Autoinduction media (AIM) (Formedium) supplemented with 8 g/l glycerol, 100 mg/ml ampicillin, and 34 mg/ml chloramphenicol or 50 µg /ml kanamycin and 34 mg/ml chloramphenicol for pET-22b+ and pNH-TrxT vectors, respectively. The culture was grown at 37°C until OD₆₀₀ reached 0.5 and induction was performed at 18°C overnight. Next day, I collected cells at 4200 rpm for 50 minutes at 4°C and suspended in 1.5 ml/g Lysis buffer (50 mM HEPES pH 7.4, 0.5 M NaCl, 10% glycerol, 10 mM imidazole, 0.5 mM TCEP) and stored at -20°C.

3.11 Purification of His₆-tagged protein
Cells were thawed and supplemented with benzonase (5 U/ml) and 1 tablet of EDTA free protease inhibitor along with a spoon tip of lysozyme (0.5 mg/ml). Then I incubated cells on ice for 30 minutes and sonicated the lysate 6 times on ice. Lysate was centrifuged for 30 minutes at 4°C/16000 rpm and the soluble fraction was filtered through a 0.45 mm syringe filter. The recombinant protein was purified by Ni²⁺-charged 1 ml His-Trap FF crude column (GE Healthcare), equilibrated with binding buffer (50 mM HEPES pH 7.4, 0.5 M NaCl, 10% glycerol, 10 mM imidazole, 0.5 mM TCEP). After that I loaded the lysate onto the column and washed with 30 column volumes of binding buffer and 10 column volumes of wash buffer (50 mM HEPES pH 7.4, 0.5 M NaCl, 10% glycerol, 25 mM imidazole, 0.5 mM TCEP). The protein (10 ml) was eluted with 50 mM HEPES pH 7.4, 0.5 M NaCl, 10% glycerol, 250 mM imidazole, 0.5 mM TCEP.

3.12 Heparin column purification of TCP-c004
The elute (10 ml) was diluted to 30 ml with 50 mM HEPES pH 7.0, 1 mM TCEP, 1 mM EDTA, 10 % glycerol. This resulted in NaCl concentration of 200 mM. I washed Heparin column at 1.5 ml/min with 25 ml dH₂O and after that with 25 ml Buffer A (50 mM HEPES pH 7.0, 200 mM NaCl, 0.5 mM TCEP, 1 mM EDTA, 10% glycerol). Then the protein (30 ml) was loaded in 5 ml Heparin column and washed with 30 ml Buffer A at 1 ml/min (flow through was collected). After that, heparin column was connected to BioRad Duo flow system and protein was eluted by using Buffer B (50 mM HEPES pH 7.0, 1 M NaCl, 0.5 mM TCEP, 1 mM EDTA, 10 % glycerol) with a flow rate of 2ml/min. Fractions were collected and analyzed in SDS-PAGE.
3.13 Gel Filtration
Ni-NTA eluted protein was further purified on ÄKTA purifier on Superdex 200 prep grade column at 4°C. The column was equilibrated with gel filtration buffer (20 mM HEPES pH 7.4, 0.5 M NaCl, 10% glycerol, and 0.5 mM TCEP). Fractions were collected after size exclusion chromatography and analyzed using SDS-PAGE.

3.14 SDS-PAGE
To analyze the recombinant protein produced in *E. coli* SDS-PAGE gels were used. Samples were prepared by adding 1x of the sample buffer (25 mM Tris-Cl (pH 6.8), 2% SDS, 20% glycerol and 4% β-mercaptoethanol. I boiled samples for 5 minutes at 95°C before loading on SDS gel. Electrophoresis was done at 150V for 60 minutes.

3.15 Activity assay
Activity of purified protein was measured by using a fluorometric method. The assay was described by Putt (2003) that measures the substrate utilization by quantifying the remaining NAD$^+$ after enzymatic reaction.

The final volume of reaction was 50 µl and it was carried out by using 96 well U-shaped black fluorescence microtiter plates (Greiner BioOne). Different concentrations of *Tc*PARP was incubated in the presence of 500 nM NAD$^+$ and buffer containing activated DNA (0.1 M Na-P pH 7.0, 20 µg/ml activated DNA and 15% glycerol). The reaction was carried out for 2 hours at 25°C with shaking at 300 rpm using PST-60 HL plus Thermo Shaker (Biosan). Reaction was terminated by the addition of 20 µl of 2 M KOH and 20 µl of 20% acetophenone (EtOH), and plate was incubated at room temperature for 10 minutes. Then I added 90 µl of 88% formic acid and again incubated the plate at room temperature for 20 minutes. The fluorescence was measured on a Fluoroskan Ascent FL using excitation at 355 nm and emission at 460 nm. Consumption of NAD$^+$ was detected by decrease in fluorescence.

3.16 DNA-binding assay (EMSA)
The gel electrophoresis mobility shift assay (EMSA) is used to detect the binding of dsDNA ligand with purified protein (mutant 4). Different compositions of purified protein with full length protein, activated DNA (blunt DNA) and with Tobacco Etch Virus (TEV) were made. TEV was added to newly
thioredoxin tagged protein to check the binding efficiency of the cleaved protein. TEV protease was a very useful reagent for cleaving fusion proteins.

Purified protein (2.5 µM)
Full length (2.5 µM) + Purified protein (2.5 µM)
TEV (0.14 mg/ml)
Blunt DNA (2.5 µM)
Purified protein (2.5 µM) + Blunt DNA (2.5 µM)
Full length (2.5 µM) + Blunt DNA (2.5 µM)
TEV (0.14 mg/ml) + Blunt DNA (2.5 µM)
Purified protein (2.5 µM) + TEV (0.14 mg/ml) + Blunt DNA (2.5 µM)

Total 50 µl reaction of each composition was made in small tubes. Buffer used in making mixtures contained 20 mM HEPES pH 7.4, 0.5 M NaCl, 10% glycerol, and 0.5 mM TCEP. Electrophoretic mobility shift assays (EMSAs) were carried out after incubation of all tubes on ice for 1.5 hours. After incubation, samples were run on 1% Agarose gel for 35 minutes and analyzed by using BioRad gel doc system.
4 RESULTS

4.1 Expression of all constructs in pET-22b\textsuperscript{+} vector

Small scale expression of all TcPARP fragments cloned in pET-22b\textsuperscript{+} vector in LB media at 18°C with three different IPTG concentrations was done. On SDS-PAGE analysis, it was shown that TCP-c003 and TCP-c004 (molecular weight of 13.4 kDa and 23.8 kDa, respectively), did not show band of respective size with any IPTG concentration (Figure 5a). On the other hand, TCP-c005 that has a molecular weight of 41.8 kDa, shows band of approximately correct size in all IPTG concentrations at 18°C. However, TCP-c005 protein expression showed in only insoluble fraction (Figure 5a).

Overall, SDS-PAGE analysis of soluble fractions of TCP-c003, TCP-c004 and TCP-c005 did not show bands of expected sizes (Figure 5b). This indicates that proteins might be targeted on inclusion bodies.

Figure 5a: Expression of TCP-c003, TCP-c004 and TCP-c005 at 18°C with different IPTG concentrations showed no expression of TCP-c003 and c004, while TCP-c005 showed bands of expected size in all IPTG concentrations (pellet); 5b: Soluble fraction (supernatant) showed none of the construct expressed and did not showed expected size band.

Similarly, in figure 6a, TCP-c006 that has a molecular weight of 53.9 kDa, showed bands of expected size at 18°C with all three different IPTG concentrations, but, likewise TCP-c005, protein was present only in insoluble fraction (Figure 6a). TCP-c007 has a molecular weight of 25.1 kDa, and it did not show expected size band in insoluble fraction with any IPTG concentrations (Figure 6a).
Figure 6a: Expression of TCP-c006 showed bands of expected size in all IPTG concentrations at 18°C while no expression of TCP-c007 has been shown in SDS-PAGE (pellet); 6b: Soluble fraction (supernatant) showed none of the construct expressed and did not showed expected size band.

However, SDS-PAGE analysis of soluble fractions of TCP-c006 and TCP-c007 did not showed band of expected sizes (Figure 6b). This seems that expression occurred in inclusion bodies not in soluble fraction.

4.2 Protein expression in pET 22b+ vector

Although small scale expression of all constructs with different IPTG concentrations did not show any detectable expression. Large scale expressions and purifications of few constructs were done.

Mutant 2 (TCP-c004)

Mutant 2 (TCP-c004) fragment contains N-terminus and WGR domain and has a molecular weight of 23.8 kDa. After expression and purification with Ni⁺-NTA His-Trap FF crude column followed by heparin column purification, eluted protein was analyzed on SDS-PAGE. On SDS gel, eluted protein from His-Trap FF crude did not show any purified protein as shown in lane 4 (Figure 7a); however protein eluted from heparin column showed faint bands of expected size at 23 kDa as in lane 12 and 14 of SDS gel (Figure 7a).
Figure 7: (a) Heparin column purification showed band of expected sizes in lane 12 and 14, (b) gel filtrate purification of TCP-c004 did not show any purified protein.

On the other hand, similar mutant 2 (TCP-c004) fragment was expressed and purified by gel filtration size exclusion chromatography and analyzed on SDS-PAGE where it did not showed any purified protein even in His-Trap FF crude elution as mention in lane 5 (Figure 7b).

TCP-c006
TCP-c006 fragment contains WGR+ PRD + ART domains with molecular size of 53.9 kDa. First I purified protein through Ni-NTA His-Trap FF crude column and saved a little amount for SDS-PAGE analysis and rest was concentrated and loaded in gel filtration column. After size exclusion column chromatography, multiple peaks were observed (Figure 8a). I collected six fractions at different peaks and analyzed on SDS PAGE.

Figure 8a: Gel filtrate chromatograph of TCP-c006
On SDS-PAGE analysis, lane 8, 9 and 10 showed purified protein. However, the sizes of bands were slightly higher than expected (Figure 8b).

**TCP-c007**

TCP-c007 which contains only PARP catalytic domain has a molecular mass of 25.1 kDa. After size exclusion chromatography, I collected three fractions at different peaks (Figure 9a) and analyzed on SDS PAGE.
SDS-PAGE analysis of TCP-c007 did not show any purified protein. It seems that protein was not purified properly through gel filtration because in all three collected fractions there were many bands of different sizes.

### 4.3 Cloning of new constructs

After expression and purification of three constructs of TcPARP cloned in pET-22b$^+$ vector, it seems that none of the protein were expressed and purified. To resolve this problem, same fragments were cloned in pNH-TrxT vector. The presence of thioredoxin was expected to enhance the level of expression of proteins.

Three constructs were successfully cloned in pNH-TrxT vector (Table 2).

<table>
<thead>
<tr>
<th>ID</th>
<th>Size</th>
<th>Domain</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP-c003</td>
<td>375 bp +625 bp</td>
<td>N-terminus</td>
<td>pNH-TrxT</td>
</tr>
<tr>
<td>TCP-c006</td>
<td>1401 bp +625 bp</td>
<td>WGR + PARP-reg. + PARP-cat.</td>
<td>pNH-TrxT</td>
</tr>
<tr>
<td>TCP-c007</td>
<td>633 bp +625 bp</td>
<td>PARP-cat.</td>
<td>pNH-TrxT</td>
</tr>
</tbody>
</table>

Table 2: Successfully cloned constructs in pNH-TrxT vector
Successfully cloned constructs was confirmed by PCR using sequencing primers as well as original primers and analyzed on 1% Agarose gel. Mutant 1 (TCP-c003) pNH-TrxT has an expected size of 1000 bp, mutant 4 (TCP-c006) pNH-TrxT has an expected size of 2026 bp and mutant 6 (TCP-c007) pNH-TrxT has an expected size of 1258 bp. All three constructs shown to be on expected size in agarose gel (Figure 10). Further confirmation was done by sequencing.

**4.4 Expression of TCP-c006 and TCP-c007 in pNH-TrxT vector**

Small scale expression of mutant 4 (TCP-c006) and mutant 6 (TCP-c007) was done in LB media at 18°C with 0.5mM IPTG concentration. Mutant 4 (TCP-c006) and mutant 6 (TCP-c007) have a molecular weights of approximately 68 kDa and 34 kDa respectively. Both constructs cloned in pNH-TrxT vector, having an expression as shown in lane 6 for TCP-c006 and lane 12 for TCP-c007 (Figure 11). However it seems that expressed proteins might be miss-folded and targeted to inclusion bodies as again expression is visible only in insoluble fractions (pellet) but not in soluble fractions (supernatant).
4.5 Protein purification of mutant 4 (TCP-c006) in pNH-TrxT vector

Mutant 4 (TCP-c006) fragment has no N-terminus and contains only WGR+ PRD+ ART domains. Therefore, in order to get even small amounts of this protein, a larger expression trials were carried out. After successfully cloned in pNH-TrxT vector, TCP-c006 fragment was expressed and purified with Ni⁺-NTA His-Trap FF crude column. Eluted protein was further purified by gel filtrate size exclusion column chromatography where it shows different peaks (Figure 12a). I collected fractions of eluted protein of all peaks and analyzed them on SDS-PAGE (Figure 12b).
Figure 12a: Gel filtrate chromate graph of TCP-c006pNH-TrxT vector showed different peaks.

Figure 12b: SDS-PAGE analysis showed expected size bands of purified TCP-c006 in lane 11, 12, 13 and 14. Lane 11, 12, 13 and 14 of SDS gel (Figure 12b) show expected size bands of purified protein. Further confirmation was done by activity assay and DNA binding assay.
4.6 Activity assay

A well known characteristic of PARP-1 and PARP-2 from other organisms is the activation of PARPs by nicked DNA (Villamil et al., 2007; Ame et al., 1999; Oliver et al., 1999; Podesta et al., 2004; Diefenbach and Burkle, 2005; Schreiber et al., 2006). The activity of TcPARP has previously been detected to increase in the presence of DNA strand breaks (sbDNA). A direct correlation between the rate of poly(ADP-ribosyl)ation and sbDNA concentration was obtained (Villamil et al., 2007). Therefore, the activity of TcPARP was tested for DNA dependence. The activity assay for full length TcPARP to determine the effect of pH and DNA concentrations for the enzymatic activity was already optimized by Vilchez Larrea et al., 2012. It was find out that maximum activity obtained by using 20µg/ml of DNA and the optimal buffer system was sodium phosphate at pH 7.0 (Table 3). Mutant 4 (TCP-c006) which is without N-terminus showed activity against NAD\(^+\) consumption (Figure 13).

<table>
<thead>
<tr>
<th></th>
<th>Blank Na-P</th>
<th>Control Na-P &amp; NAD(^+)</th>
<th>2.5 nM protein conc.</th>
<th>10 nM protein conc.</th>
<th>50 nM protein conc.</th>
<th>250 nM protein conc.</th>
<th>500 nM protein conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>2211.75</td>
<td>41045</td>
<td>40723.75</td>
<td>40310.75</td>
<td>40128.25</td>
<td>31903</td>
<td>26557.5</td>
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<tr>
<td>STD</td>
<td>1494.4</td>
<td>2265.19</td>
<td>1842.60</td>
<td>2376.8</td>
<td>6388.92</td>
<td>3064.85</td>
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<tr>
<td>CV</td>
<td>3.6</td>
<td>5.5</td>
<td>4.5</td>
<td>5.9</td>
<td>20.02</td>
<td>11.5%</td>
<td></td>
</tr>
<tr>
<td>Conversions</td>
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<td></td>
<td>0.7%</td>
<td>1.7%</td>
<td>2.2%</td>
<td>22.2%</td>
<td>35.2%</td>
</tr>
</tbody>
</table>

Table 3: NAD\(^+\) conversion with different concentrations

Activity assay showed that conversion of NAD\(^+\) is concentration dependent. As the concentration of protein increases conversion rate also increases. It is also noted that activity of mutant 4 (TCP-c006), which is missing the DNA binding domain, was low because 500 nM of mutant construct takes 3 hours incubation time to get 35% conversion. While full length contains only 10 nM and gets full conversion in 30 minutes.
4.7 DNA-binding assay (EMSA)

On DNA-binding assay, mutant 4 (TCP-c006) seems not to bind with ds DNA as full length TcPARP. This has been shown previously by Dr. Teemu Haikarainen with a same assay. Although the protein concentration used in assay was quiet low, it is clearly visible that binding of DNA with mutant did not happen (Figure 14). Hence it has been concluded that N-terminus is necessary for DNA binding. Lane 2 has purified protein and lane 3 has full length protein, but no band visible on these lanes. Lane 4 has TEV protease and lane 5 has ds DNA which is visible on agarose gel. Lane 6 has purified protein + DNA and it is clearly visible that protein was not bind with DNA and only single band of ds DNA was observed on gel. Lane 7 has full length protein + DNA, and it shows two bands of DNA. This preliminary experiment indicates that there is no DNA binding with the mutant, but there are two bands present with the full length. Lane 10 has TEV + DNA and only single band of DNA was visible on gel and lane 11 has Protein + TEV + DNA and single band of ds DNA shows that TEV protease cleaves protein-DNA complex.
Figure 14: DNA-binding assay (EMSA)
5 DISCUSSIONS
PARPs are a family of proteins that catalyze the conversion of nicotinamide adenine dinucleotide (NAD\(^+\)) into long poly(ADP-ribose) (PAR) chains. These highly negatively charged PAR chains can be either covalently or noncovalently attached to acceptor proteins, thereby altering their function. The most extensively studied PARP family member is PARP-1. PARP-1 has been implicated in a variety of cellular processes. Particularly, PARP1 acts as a sensor of DNA damage and initiator of the base excision repair pathway (El-Khamisy et al., 2003).

American and African trypanosomes, brucei and cruzi are responsible to cause Chagas disease and sleeping sickness in human and other mammals. These parasites are transmitted to humans either by transfusion of infected blood, from an infected mother to her child, or by its most important vector, a blood–sucking bug (Triatoma infestans, a.k.a. ‘vinchuca’), which carries the parasite in its contaminated feces (Maya et al., 2010). Complete genome studies of trypanosomatids (T. cruzi and T. brucei), showed that both protozoan parasite contain genes involved in DNA repair mechanism and exhibits similarities to human poly(ADP-ribose) polymerases (PARPs) (Berriman et al., 2005; El-Sayed et al., 2005; Atclas et al., 2008). However, the exact mechanism of binding trypanosomatid PARP with DNA is not known due to the lack of zinc finger domains which are essential elements of hPARP-1 to bind with DNA.

Current study focused on the expression, purification and activity of different TcPARP mutants to figure out the mechanism or essential components involved in DNA binding.

5.1 Cloning
Mutant TcPARP fragments were cloned in pET-22b\(^+\) vector which contains a C-terminal His-tag. Expression levels of different mutants were low in this vector. Large scale expression and purification of mutants in pET-22b\(^+\) vector did not show any purified protein. Therefore switch to another vector that contains thioredoxin in its N-terminus.

Mutant TcPARP fragments in pNH-Trx vector shows high level of expression as compared with pET-22b\(^+\) vector expression. However majority of expressions seem to be in non soluble fractions as inclusion bodies.
5.2 Protein Expression and Purification

On protein expression, I tried four different temperatures i.e. at 18°C, 25°C, 28°C, 37°C. It was find out that lower temperature gives higher expression of protein. The optimal temperature used for protein expression was 18°C. Although most of the protein targeting to inclusion bodies which might be due to protein not folded properly. Upon lysis buffer, I did not try different lysis buffer but in future it could be worthy to try different lysis buffers.

On large scale protein purification, in pET-22b\(^+\) vector, three mutant constructs (TCP-c004, TCP-c006 and TCP-c007) were purified through His\(_6\)-tag FF crude column followed by heparin and gel filtration. Only mutant 2 (TCP-c004), showed purified protein of appropriate size through heparin column.

Large scale purification in pNH-Trx vector of only one mutant construct (TCP-c006) showed protein of appropriate size. Although most of the protein seems to be in inclusion bodies but it is concluded that large scale purification was still worth to study activity assay.

5.3 Protein Activity

Protein activity test was performed to confirm whether the purified protein was active or not. It is clearly visible that TcPARP-c006 in pNH-Trx vector shows its activity in the presence of active DNA by consuming NAD\(^+\). Although the activity level of mutant 4 (TCP-c006) was quiet low as compared to full length. The activity of mutant 4 (TCP-c006), which is missing the DNA binding domain, was low to hPARP-1 because 500 nM of mutant construct takes long incubation time to get 35\% conversion. While full length contains only 10 nM and gets full conversion in 30 minutes.

5.4 DNA-binding activity

DNA-binding activity of mutant 4 (TcPARP-c006) in pNH-Trx vector did not show any binding with DNA indicates that N-terminus is responsible for binding with DNA. WGR domain not enough to activate the enzyme nor bind DNA.
6 FUTURE DIRECTIONS

From this current research studies, it has been proposed that the amount of soluble protein could be increased by using different buffers, testing salts, detergents and pH. Choose the buffer according to the stability of the target protein with respect to pH and the buffering compound. Moreover, depending on the target protein, different salt compounds like NaCl, KCl, (NH₄)₂SO₄ added to the lysis buffer to improve the stability of the target protein.

Furthermore, different vectors may also contribute in increasing protein production and stability. Like pNIC28-Bsa4 with His₆ tag in 22-aa N-terminal fusion peptide for expression of N-terminally His tagged proteins in *E.coli*. Solubility of both wild type and mutants of *Tc*PARP in different expression host system using various fusions such as MBP, T4 lysozyme etc could be tested.

In order to find out the structure of mutant construct Static light scattering (SLS) technique should be used. The intensity of the scattered light is measured as a function of angle and may be analyzed to yield the molar mass, root mean square radius, and second virial coefficient (A2). This will give a stoichiometry of the DNA binding. The results of an SLS experiments can be used as for structural studies and to determine the oligomeric state (monomer/dimer) of protein.
REFERENCES


