Buffy: A Bcl-2 like protein form *Drosophila melanogaster*

A thesis submitted for the fulfilment of the requirement of the degree of Master in Biotechnology and Bioinformatics

Submitted by:

Pradeep Penugonda Jwala Venkata Shiva

Under the supervision of Dr. Linda Ward and Dr. Marc Kvansakul

Department of Biochemistry

School of Molecular Sciences

Faculty of Science, Technology and Engineering



Bundoora, Victoria, 3086

Australia

2017

Chapter 1

Introduction

1.1 Apoptosis

Apoptosis is a self-destruction mechanism which selectively eliminates infected or unwanted cells from an organism. changes in the regulation of apoptosis can give rise to a variety of diseases. For example, neurodegenerative disease can be caused by the over enhancement of apoptosis and cancers can be caused by the inhibition of apoptosis. The disruption of apoptosis can also cause various auto-immune diseases (Reed, 2002). The principle of apoptosis was first described by Karl Vogt in 1842 (Vogt, 1842) and a further detailed description of apoptosis was given by Walther Fleming in 1885 (Flemming, 1885). In 2002, three scientists Sydney Brenner, Horvitz and John E. Sulston received the Nobel prize in medicine for their work on gene regulation of organ development and programmed cell death. They identified genes that control apoptosis in *Caenorhabditis elegans* and related this to similar genes functioning in human (Brenner, 1974, Ellis & Horvitz, 1986, Metzstein *et al*., 1998, Sulston *et al*., 1983). The term 'Apoptosis' was coined by professor Alastair R Currie to replace the term 'shrinkage necrosis' which was published in 1972 (Kerr *et al*., 1972).

1.2 History of apoptosis

The first experiment of induced cell death was performed on thymocytes where they were treated with glucocorticoid and observed that the DNA was degraded into smaller fragments because of an endonuclease activation (Wyllie, 1980). The cause of this DNA degradation is due to the apoptotic stimuli activating caspase-3 which then activates a novel heterodimeric protein and further triggers the DNA degradation (Liu *et al*., 1997).

Later, detailed research was conducted on post-embryonic cell lineages of *C.elegans* and showed that a small number of somatic cells produced during development underwent programed cell death. It was found that 131 out of 1090 produced somatic cells underwent programed cell death and this observation was highly reproducible (Sulston & Horvitz, 1977). The analysis on genes involved in programed cell death showed that more than 12 genes are involved in programed cell death in *C.elegans* (Ellis & Horvitz, 1986, Hedgecock *et al.*, 1983, Hengartner *et al.*, 1992).

Bcl-2 was the first apoptotic protein identified in *homo sapiens* and was characterized as a proto-oncogene that causes follicular lymphoma in humans (Tsujimoto & Croce, 1986). Further research showed that Bcl-2 could increase the survival of hematopoietic cells and contribute to neoplasia which makes pre-B cells immortalize with c-myc activation (Vaux *et al.*, 1988).

It has been shown that the over-expression of human Bcl-2 prevented developmental cell death in *C.elegans* (Vaux *et al.*, 1992). The processes of cell death during limb development between insects and vertebrates are similar, which implies that programmed cell death is conserved through evolution (Whitten, 1969).

1.3 Proteins involved in apoptosis

As shown in Figure 1, apoptosis is a physiological process which can occur via two different pathways. Several different proteins are involved in each of these pathways. Bcl-2 family proteins are involved in intrinsic apoptosis, and are divided into pro-apoptotic, anti-apoptotic and BH3 only proteins. The anti-apoptotic members are Bcl-2 (B cell lymphoma -2), Bcl-x_L (B cell lymphoma -x_L), Bcl-w (B cell lymphoma -w), MCL-1 (Myeloid cell leukemia 1) and A1 (Bcl-2 related gene A1). The pro-apoptotic members are BAK (Bcl-2 antagonist killer 1), BAX (Bcl-2 associated X protein) and Bok (Bcl-2 related ovarian killer). There is a class of BH3 only proteins such as BAD

(Bcl-2 antagonist of cell death), BID (Bcl-2 interacting domain death agonist), BIM (Bcl-2 interacting mediator of cell death), NOXA, PUMA (P^{53} -upregulated modulator of apoptosis), BMF (Bcl-2 modifying factor), HRK (Harakiri) and BIK (Bcl-2 interacting killer) (Gross *et al.*, 1999).

The extrinsic apoptosis is executed by the interaction between different proteins such as Fas receptor, Fas ligand, FADD – Fas associated death domain adapter protein, Disc - death-inducing signaling complex formed by Procaspase 8, pro-caspase 10, procaspase 3 (Wang *et al.*, 2010).

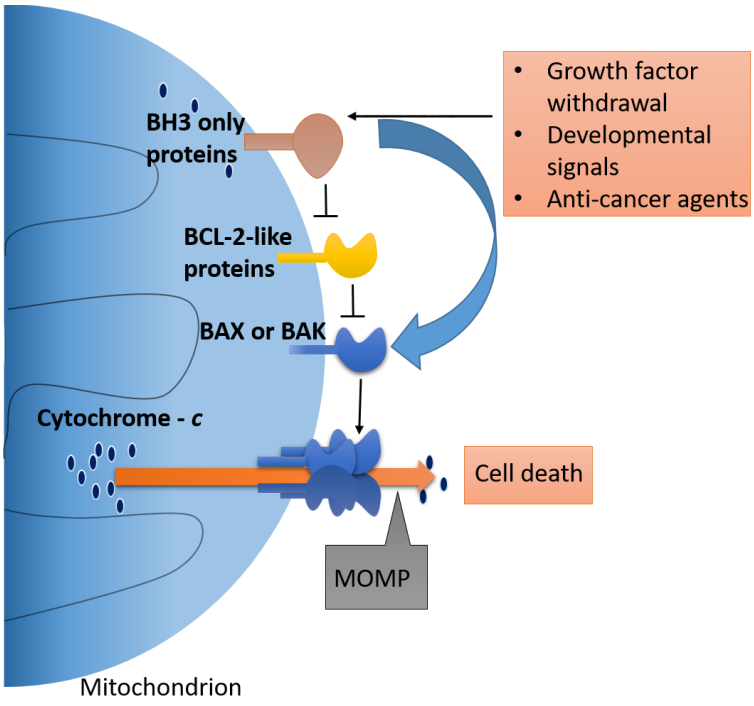


Figure 1: Intrinsic pathway. The pro-apoptotic BH3 only proteins activate pro-apoptotic proteins BAK and BAX to form pores on mitochondria which leads to MOMP - mitochondrial outer membrane permeabilization with release of cytochrome – c which triggers caspase activation cascade and results in apoptosis.

1.4 Bcl-2 family proteins

All Bcl-2 Family members are characterized by conserved regions known as BH domains or BH motifs (Sato *et al.*, 1994). There are four domains identified in Bcl-2 protein family called BH1, BH2 and BH3 (Chittenden *et al.*, 1995, Yin *et al.*, 1994).

All the anti-apoptotic Bcl-2 proteins and pro-apoptotic BAX and BAK have four similar conserved domains BH1, BH2 and BH3 which shows high structural homology. Whereas pro-apoptotic BH3 only proteins such as BIM, BAD, BMF, tBID, NOXA, BIK, PUMA and HRK have BH3 domain only (Czabotar *et al.*, 2014).

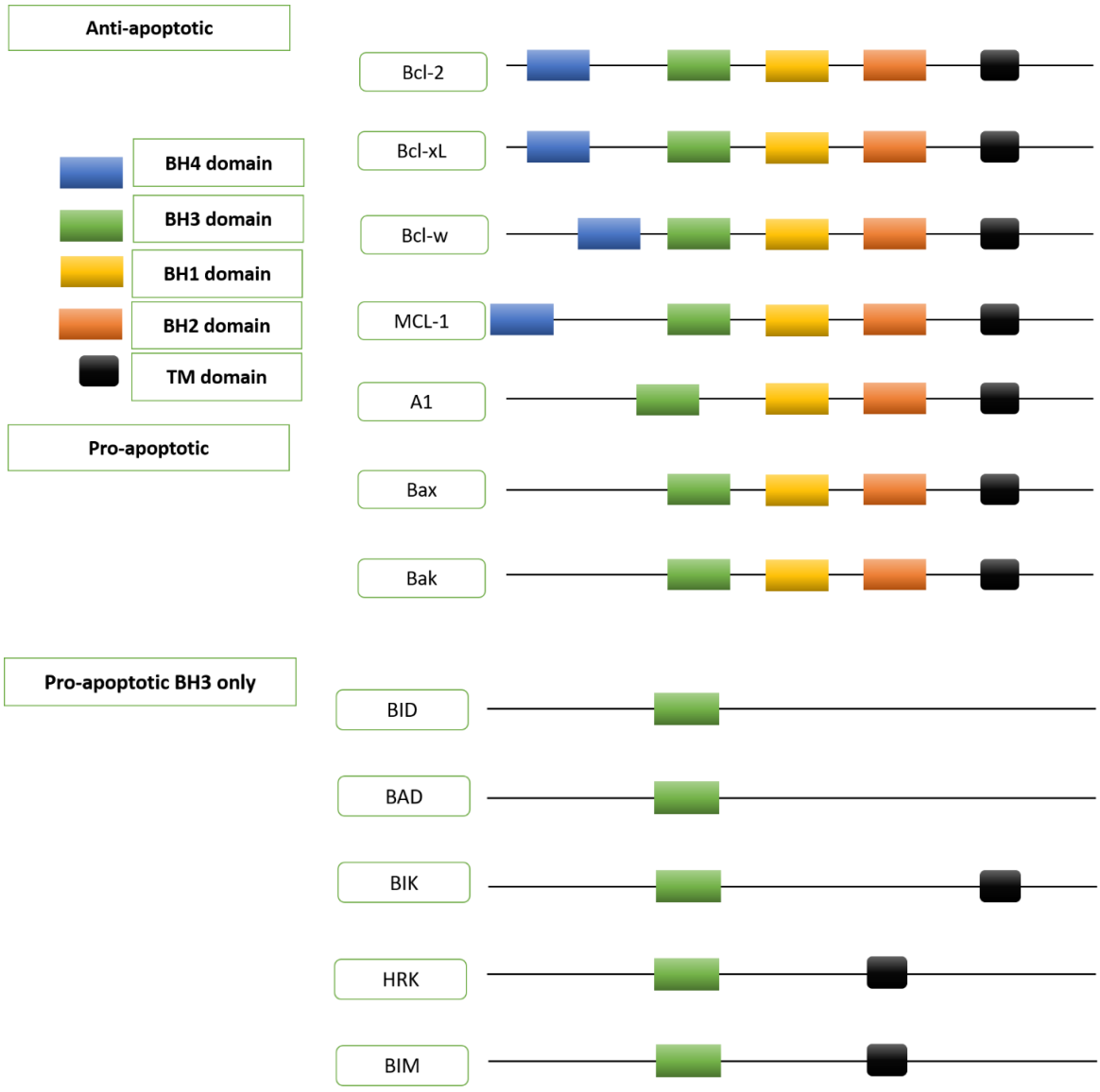


Figure 2: Homology of Bcl-2 family proteins. The pro-apoptotic and anti-apoptotic Bcl-2 family share sequence homology at four domains. The highly-conserved domain in all the Bcl-2 proteins is BH3 domain.

1.4.1 Structure

The structure of anti-apoptotic and multidomain pro-apoptotic (BAX and BAK) Bcl-2 protein family members consists predominantly of α-helices. Structure evaluation of Bcl-x_L by X-ray crystallography and NMR spectroscopy reveled that Bcl-x_L has eight α-heliceses. The α-helices 5 and α-helices 6 are bound by α-3 and α-4 on one side and α-1 and α-2 on the other side. While α-5 and α-6 form the core of the protein. α-7 and α-8 are on the opposite side. The core of the protein is mainly hydrophobic and on the surface of this hydrophobic groove the BH region is found (BH1, BH2, BH3). The bottom of the grove is enveloped by α-3 and α-4 (Muchmore *et al.*, 1996).

The structure of Bcl-2 also consists of eight α-heliceses with a hydrophobic groove on the exterior. The arrangement of all the α-heliceses is same as in Bcl-x_L. The main difference between Bcl-2 and Bcl-x_L is the presence of long loop between α-1 and α-2 in Bcl-x_L (Petros *et al.*, 2001).

BAX is a pro-apoptotic protein and its structure is similar to Bcl-2 and Bcl-x_L. BAX has nine α-heliceses out of which two α-helices form a hydrophobic central core with seven α-heliceses are packed around. The arrangement of α-1, α-2, α-3 and α-4 is similar with Bcl-x_L except that a long loop connects α-1 with α-2. All four α-heliceses form a hydrophobic groove. The arrangement of α-5, α-6, α-7, α-8 heliceses is also similar to Bcl-x_L (Suzuki *et al.*, 2000).

The α-9 is a transmembrane domain which binds to the hydrophobic groove. BAX is in the cytosol. However, when BAX is activated at the time of apoptosis due to cytokine withdrawal the intercellular pH increases and it translocates to the mitochondrial outer membrane. The α-9 comes out of the groove and helps the protein to get attached on to the mitochondrial outer membrane (Khaled *et al.*, 1999). The α-9 transmembrane domain of BAX is structurally and functionally similar to the one found in Bcl-2 and Bcl-x_L. Most of the BAK is alpha helices with α-9 always

bind to mitochondrial membrane. When BAK undergo conformational changes then it heterodimerizeses with BAX (Suzuki *et al.*, 2000).

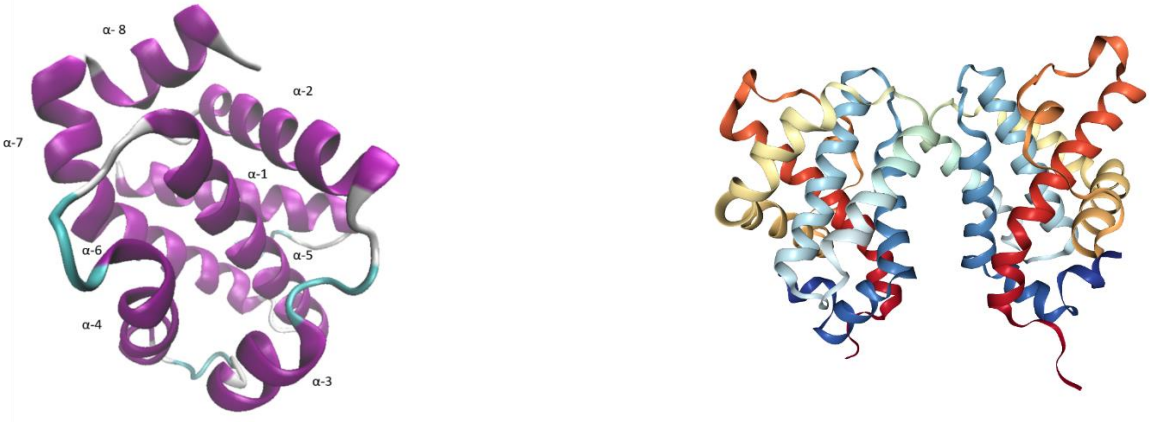


Figure 3: Structure of Bcl-x_L and BAK. The structure 1 shows the arrangement of alpha helices in Bcl-x_L (1MAZ) and structure 2 represents homodimer of BAK (2IMS).

1.4.2 Mechanism of action

There are two pathways involved in apoptosis intrinsic and extrinsic. At the time of apoptosis via intrinsic pathway different Bcl-2 family members are involved in inducing MOMP – mitochondrial outer membrane permeabilization.

At the time of cellular stress, the BH3 only molecules bind on to the free sites of anti-apoptotic molecules to inhibit them and the activators activate BAX (Suzuki *et al.*, 2000). The inactivated BAX has alpha helix 9 occupied in its active site. When it receives the stimuli the alpha helix 9 comes out of the active site and hangs out as a tail which helps BAX to form a heterodimer complex with BAK on the mitochondrial membrane to form a pore on the membrane. After activation of

BAX, not only alpha helix 9 but two other alpha helixes 5 and 6 help BAX to fix firmly on to mitochondrial membrane (Khaled *et al.*, 1999, Suzuki *et al.*, 2000).

MOMP caused by the pore formation leads to release of cytochrome *c* and triggers caspase activation by binding to ApaF1 and procaspase 9 to form an apoptosome. With an active caspase 9 in the center it activates procaspase 8. The activated caspase 8 and 9 cleave procaspase 3, 6, and 7 in to active caspase 3,6 and 7 which results in DNA fragmentation and condensation of chromatin and leads to a complete cell destruction (Boatright & Salvesen, 2003, Enari *et al.*, 1998).

BH3 only proteins play an important role in apoptosis. They are categorized into two types sensitizers and activators. The sensitizers break up the protein complex which is formed by Bcl-2 and BAK/BAX or they will bind to the anti-apoptotic proteins and inhibit them. The activators activate BAX and BAK to form pores on the mitochondrial outer membrane (Shamas-Din *et al.*, 2011).

The anti-apoptotic and pro-apoptotic proteins form heterodimers to inhibit the activity. For example, Bcl-x_L inhibits BAK by forming a heterodimer complex with BAK (Sattler *et al.*, 1997). Similarly, the heterodimerization of BAX with Bcl-2 leads to inhibition of apoptosis (Yin *et al.*, 1994). The α helix from the BH3 region of BAK inserts in to the hydrophobic groove composed of BH1, BH2 and BH3 of Bcl-x_L via hydrophobic interactions (Sattler *et al.*, 1997).

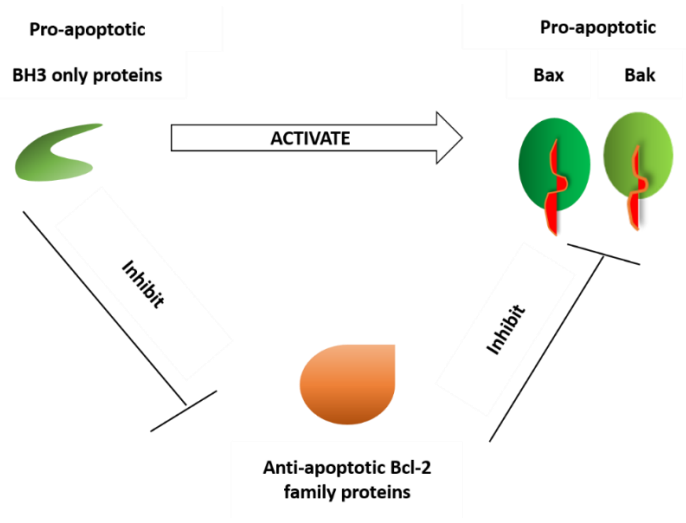


Figure 4: Activation and inactivation of Bcl-2 family members in regulation of apoptosis. The Anti-apoptotic proteins inhibits pro-apoptotic proteins, at the time of stress the BH3 only proteins activate Pro-apoptotic proteins by inhibiting Anti-apoptotic proteins.

1.5 Buffy a Bcl-2 like protein

Buffy is the second Bcl-2 homologue identified in *Drosophila melanogaster* which has an anti-apoptotic function. Debcl was the first Bcl-2 like homologue characterized in drosophila and has been shown to be pro-apoptotic (Colussi *et al.*, 2000, Quinn *et al.*, 2003).

Ectopic apoptosis was observed when Buffy levels were reduced by RNA interference and developmental cell death inhibition observed when Buffy was overexpressed. Further more, Buffy interacts with Debcl to stop cell death induced by Debcl. Buffy is found to be functionally similar to the anti-apoptotic Bcl-2 family proteins of mammals (Quinn *et al.*, 2003).

Apart from its anti-apoptotic function, Buffy was found to have an influence on nutrient stress. The study conducted on drosophila lacking the Buffy gene showed that they were more sensitive to nutrient stress. There was a twofold decrease in survival of larvae lacking the Buffy gene over

wild type larvae. This decrease in survival rate leads to autophagy initiation and mobilization of stored lipids (Monserrate *et al.*, 2012).

The localizations of Buffy and Debcl are in the endoplasmic reticulum and mitochondria respectively and both have a c-terminal membrane anchor domain (MA). The MA in Debcl is similar to Bcl-x_L which is useful in targeting mitochondria. The c-terminal positively charged residues which are present in Debcl are absent in Buffy helping to explain its endoplasmic reticulum location (Doumanis *et al.*, 2007, Tanner *et al.*, 2011).

1.6 Aim

The aim of the study is to characterise the Bcl-2 homolog present in *Drosophila melanogaster*. As a part of this study, Buffy a Bcl-2 like protein from drosophila has chosen because it may have a similar function to the anti-apoptotic Bcl-2 protein found in *Homo sapiens*. The study involves expression and purification of the protein and potentially followed by determination of secondary structure of Buffy protein using circular dichroism spectroscopy. The study also involves in knowing various interactions of Buffy protein with known Bcl-2 proteins using isothermal titration calorimetry which gives various protein interaction with known apoptotic proteins. The secondary structure predictions and binding affinities of Buffy gives a deep insight into various conserved motifs and phylogenetic relationship with other apoptotic proteins which can be used to determine potential drug target sites.

Chapter 2

Materials and Methods

2.1 Bioinformatics software

DNA and amino acid sequences for Buffy, a Bcl-2 like protein from *Drosophila melanogaster* and Human Bcl-2 family members were obtained from the National Centre of Biotechnology Information (NCBI). Protein property predictions were made using Protparam tool available through ExPASy. ApE plasmid editor program was used to construct Vector maps. The sequence alignment of amino acid sequences was processed using CLUSTAL W program.

2.2 Chemicals and reagents

Unless otherwise named all chemicals used were obtained from Sigma-Aldrich®. All plasmid constructs were obtained from GenScript® and cell lines from MBB laboratory, Latrobe University.

2.3 Cell biology and Molecular biology techniques

2.3.1 Competent cell preparation

The *Escherichia coli* XL1-Blue cells cell lines was used for plasmid propagation and *E.coli* BL-21 (DE3) cell lines for protein expression. Glycerol stock of cells was inoculated into 10 ml of Luria bertani (LB) broth (1% (w/v) NaCl, 0.5% (w/v) Bacto-Yeast extract and 1% (w/v) Bacto-Tryptone) and incubated overnight at 37°C with shaking. A 100 ml of fresh LB-broth was inoculated with 1.5 ml of overnight culture for each cell line. The subculturing of overnight culture into fresh LB-broth was executed based on the optical density at 600nm (OD_{600}) was approximately 0.1. The freshly inoculated cultures were incubated at 37°C until the OD_{600} reached between 0.5 and 0.6. Buffer 1 (100 mM $CaCl_2$) and buffer 2 (100 mM $CaCl_2$, 15% glycerol) were

put at 4°C during this incubation. Cultures were then centrifuged for 15 minutes at 600 x g in 4°C. After centrifugation, the pellet was retained and resuspended gently in 25 ml pre-chilled buffer 1 and incubated on ice for 15 minutes, followed by centrifugation at 600 x g at 4°C for 10 minutes. Again, the pellets were retained and gently resuspended in 2.5 ml of buffer 2 followed by taking 200 µL aliquots into sterile 1.5 ml tubes which were snap frozen in liquid nitrogen and stored at -80°C for future use.

2.3.2 Plasmid transformation

For transformation, each aliquot of competent cells was thawed on ice and added into a 1.5 ml tube with 50 ng of DNA for every 50 µL of competent cells and incubated for 15 minutes on ice. The incubated samples were then subjected to heat shock at 42°C for 30 seconds and incubation on ice for 1 minute. The samples were then added with 250 µL of LB-broth and incubated at 37°C for 1 hour with shaking. The culture was plated on (1.5% w/v) LB-agar plates with suitable antibiotics and incubated overnight at 37°C.

2.3.3 Glycerol stocks

The bacterial cultures were stored as glycerol stocks which were prepared by adding 1000 µL of 50% (v/v) sterile glycerol to 1000 µL of fresh transformed bacterial culture which was grown overnight in a sterile 1.5 ml tube and was snap frozen in liquid nitrogen and stored at -80°C.

2.3.4 Plasmid DNA extraction

A single colony of transformed *Escherichia coli* XL1-blue was inoculated into 5 ml of LB-broth with ampicillin (0.1 mg/ml) and incubated at 37°C. Cultures were then subjected to plasmid DNA isolation using QIAprep® Spin Miniprep kit (QIAGEN) according to manufacturer's protocol. The concentration of the plasmid was measured using Nano-drop ND-100 spectrophotometer (Thermo scientific) at 260 nm.

2.3.5 Restriction enzyme digestion

A 30 µL restriction enzyme digestion reaction was set up using 200 ng/µL DNA plasmid, high fidelity *Eco*RI and High fidelity *Bam*HI with Cutsmart buffer according to manufacturer's instructions and the DNA digest was visualised by Agarose gel electrophoresis.

2.3.6 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in 1% (w/v) agarose gel. Agarose gel was prepared by mixing of 0.5 grams of agarose with 50 ml of 1x TAE buffer (40 mM Tris, 1 mM EDTA, 0.114% (V/V) Glacial acetic acid). This was microwaved until the solution became clear and cooled down to room temperature. The GelRed (0.1% Biotium) stain of 1 µL was added and the solution was poured into a gel boat with a comb and left to set at room temperature. Once the gel was set, the samples were made in 1x DNA loading dye (5 µL of 1x DNA loading dye (5% (v/v) glycerol, 0.041% (w/v) bromophenol blue, 0.041% (w/v) xylene cyanol) is added to 15 µL sample) and then loaded into the wells with 5 µL of 1 Kb plus DNA ladder (Invitrogen). The gel was run at 100 V for 60 minutes in 1x TAE buffer. The gel was then visualised using a GelDoc™ imaging system (Bio-Rad Technologies).

2.4 Protein expression and purification

2.4.1 Small scale protein expression

Transformed *Escherichia coli* BL21 DE3 was inoculated in to fresh LB broth with ampicillin (0.1 mg/ml) and incubated overnight at 37°C. Overnight culture were sub cultured into 10 ml of fresh LB broth with ampicillin (0.1 mg/ml) such that the initial OD_{600} was 0.1. This was incubated at 37°C until the OD_{600} reached to 0.6 – 0.8. IPTG (1 mM) was added at this stage and incubated at 37°C for 4 hours. 1 ml sample before adding IPTG and a 4 hours' time course samples after

induction was kept aside. These were centrifuged at high speed for 5 minutes and the pellet was retained for SDS-PAGE analysis.

2.4.2 Large scale protein expression

A colony of transformed *Escherichia coli* Bl21 DE3 cells with pGIL Buffy was inoculated overnight into 100 ml LB broth with ampicillin (0.1 mg/ml) and incubated at 37°C shaking on incubator. The overnight culture was sub cultured into 2 L of fresh LB broth with ampicillin such that the starting OD_{600} was 0.1. This was incubated at 37°C until the OD_{600} reached to 0.6 – 0.8. At this stage, the induction is initiated with the addition of 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 1 ml of the sample was kept aside as a negative IPTG sample before addition of IPTG. The induction was carried out for 2 hours at 37°C and 1 ml sample was kept aside after each hour as a time course samples. These were centrifuged at high speed for 5 minutes and the pellet was retained for SDS-PAGE analysis. The entire 2 L culture was harvested by centrifugation at 3000 x g for 30 minutes at 4°C after the 2^{nd} hour induction, the supernatant was discarded and the cell pellet was snap frozen in liquid nitrogen and stored at -80°C.

2.4.3 Cell lysis and fractionation

The frozen bacterial cell pellet post protein induction was thawed on ice and resuspended in PBS containing protease inhibitor cocktail (Sigma-Aldrich® according to manufacturer's protocol) in a ratio of 4 ml per 1 g of cell pellet. DNase at a final concentration of 10 µg/ml was added to the cell suspension. The homogenous cell suspension was subjected to Bug Buster Liquid Homogeniser (Beijer electronics) cell lysis at 37 psi or 2.51 kbar pressure at 4°C. Soluble and insoluble fractions were separated by high speed centrifugation at 30,000 x g for 30 minutes at 4°C. These fractions were loaded on an SDS gel.

2.5 Tris-Glycine Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.5.1 Tris-Glycine (SDS-PAGE) gel and sample preparation

The Tris-Glycine SDS gel was used to perform protein analysis and the gel was prepared as per the protocol outlined in (Harlow & Lane, 1988). The gel casting apparatus was used to prepare an SDS gel with 12% (v/v) separating and 5% (v/v) stacking gel. Protein samples were prepared by resuspending the pellet from protein induction in 1x PBS (137 mM NaCL, 2.7 mM KCL, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) along with SDS loading dye (50 mM Tris-Cl pH 6.8, 2% w/v SDS, 100 mM DTT, 10% v/v glycerol, 0.1% w/v bromophenol blue) and these were boiled at 95°C for 15 minutes. The samples were loaded onto a gel along with 5 µL of seeblue® pluse2 (Invitrogen™) protein standard and it was run at 170 V for 60 minutes until the dye front reaches the bottom of the gel.

2.5.2 Coomassie blue staining and Visualisation

Following the SDS-PAGE gel electrophoresis, the gel was stained with Coomassie Brilliant Blue stain (50% v/v ethanol, 10% v/v glacial acetic acid, 0.25% w/v coomassie blue stain R250). After overnight staining, the stain is retained and gel is washed in distilled water and then distained in distaining buffer (40% v/v ethanol, 10% v/v glacial acetic acid) until the background of the gel was clear. The gel was visualized using a GelDoc™ imaging system (Bio-Rad Technologies).

2.6 Western blotting

This method was used to transfer proteins from SDS-PAGE gel on to nitrocellulose membrane (NCF) followed by probing the NCF membrane with antibodies. While the SDS-gel was running, six sheets of blotting paper and 1 sheet of NCF membrane was cut to the size of gel and soaked in 50 ml Western transfer buffer (25 mM Tris-Cl, 192 mM Glycine, 0.03% SDS, 20% Ethanol). The stacking gel was carefully removed from the separating gel. The separating gel, soaked blotting

papers and NCF membrane was taken to semi-dry Western transfer apparatus. The soaked blotting paper was laid by stacking one by one on the transfer apparatus and they were rolled flat using a clean test tube. The protective blue backing paper was removed from the NCF membrane and placed on already stacked blotting papers followed separating gel and three more blotting papers. The western transfer apparatus lid is closed and transfer was run at 150 mA for 1 hour 15 minutes. Once the transfer had completed, the SDS-gel was discarded. The NCF membrane was obtained in a clean container and stained with ponceau stain (1% v/v glacial acetic acid, 0.5% w/v ponceau stain) for 5 minutes. The NCF membrane was then washed with distilled water until the protein bands were visible. This step was necessary to confirm the transfer and a photo was taken at this stage. The NCF membrane was distained completely using distilled water followed by blocking with blotto (10% w/v skim milk powder in 0.1% v/v Tween 20-PBS). The blocking was carried out for 30 minutes with consistent shaking. The NCF membrane was then washed four times with PBS-Tween (0.1% v/v Tween 20-PBS) for 5 minutes per each wash. After the washes the NCF membrane was incubated with 10 ml of primary antibody anti-His HRP raised in mice (prepared to 1:10,000 final ratio of antibody to 0.1% v/v Tween 20-PBS with 1% w/v skim milk powder) for 1 hour on shaker. The NCF membrane was again subjected to four times washing with PBS-Tween (0.1% v/v Tween 20-PBS) followed by the incubation with 10 ml of secondary antibody anti mouse HRP raised in goat (prepared to 1:200 final ratio of antibody to 0.1% v/v Tween 20-PBS with 1% w/v skim milk powder) for 1 hour at room temperature on a shaker along with four times washing with PBS-Tween (0.1% v/v Tween 20-PBS) after incubation.

The NCF membrane was then prepared for visualisation by addition of ECL reagents (GE Healthcare) according to manufacturer's protocol. The chemiluminescence was captured using GelDOC™ imaging system.

2.7 Immobilized metal affinity chromatography (IMAC)

Purification of Histidine-tagged soluble proteins was performed using Ni-NTA beads in a column. Before the beads are packed in the column they were equilibrated in equilibration buffer (10 mM Tris-HCL pH 8.0, 300 mM NaCl, 10 mM imidazole) at a ratio of 4 ml of equilibration buffer per 200 µL of beads.

The pre-equilibrated Ni-NTA beads was mixed with the soluble fraction from the cell lysis and incubated on a rotary mixer at 4°C for 1 hour. This mixture of lysate and beads was applied to a column and the flow through was collected as the unbound fraction. The beads was washed with 5 times the volume of the beads twice, with wash buffer (20 mM Tris-HCL pH 8.0, 300 mM NaCl, 20 mM imidazole) and the wash fractions were collected. His-tagged protein was eluted in elution buffer (20 mM Tris-HCL pH 8.0, 300 mM NaCl, 250 mM imidazole) at 5 times the bed volume of beads. A 50 µL aliquot from each fraction in the purification process was taken and subjected to SDS-PAGE analysis.

2.8 TEV purification

The soluble fraction containing the TEV was purified using His-Trap fast flow column (GE Healthcare) using ÄKTA protein purification system (FPLC). The column was pre-equilibrated using SEC buffer (25 mM Tris pH 7.5, 150 mM NaCl). The lysate was pumped into the column followed by washing the column with wash buffer (25 mM Tris pH 7.5, 500 mM Nacl and 10 mM Imidazole) until the no protein is detected in washes. The bound proteins were eluted using elution buffer by following gradient 0% to 100% B in 15 column volumes. The eluted samples were run on SDS-PAGE gel to check the purity and high purity samples were pooled. The pooled sample was then concentrated using Vivaspin 20 ml concentrators and stored by adding 10% glycerol followed by flash freezing in liquid nitrogen and storing at -80°C.

2.9 Dialysis

The elutions from the His-tag purification were analysed on SDS-PAGE gel and pooled and was added into D-Tube™ Dialysers Maxi (Novagen®) with a molecular weight cut-off 6-8 kDa. The dialysis tube with protein was incubated in a beaker with 4 L dialysis buffer (50 mM Tris, pH 8.0) with continuous stirring at 4°C overnight. Following dialysis, the protein was collected from the dialysis tube into a sterile 1.5 ml tube with 10% (v/v) glycerol and snap frozen in liquid nitrogen and stored at -80°C.

2.10 Protein cleavage using TEV protease

TEV cleavage reaction was set up in a sterile Eppendorf tube by adding 1x OD_{280} of the TEV per 100 OD_{280} of the target protein to the final concentration along with 0.5 mM EDTA and 1 mM DTT. The reaction mixture was incubated overnight on a rotary mixer at 4°C with sample aliquots taken after 2 hour.

Chapter 3

Results

3.1 Buffy shares sequence homology with human Bcl-2 proteins

The Buffy protein from *Drosophila melanogaster* has been reported to be an anti-apoptotic Bcl-2 family member (Colussi *et al.*, 2000, Quinn *et al.*, 2003). As shown in Figure 5, this protein has high sequence similarity with mammalian Bcl-2. An alignment was performed with Buffy and the known Human Bcl-2 and BAX protein sequences. The alignment showed 22% similarity in BH3 and 16% in BH1 and 42% in BH2. Furthermore, the Buffy protein appears to have a C-terminal transmembrane domain which is believed to be involved in making the protein bound to membrane and these results indicate Buffy may be anti-apoptotic as there is a significant homology in BH1 and BH2.

```
Buffy       MPGTSYPTNNDNFSNGFPMATTQSERLLQAQNRRKFSFPATLHSASLLEVGGGPKETTRR
hBcl-2      ------------------MA----------------------------HAGRTGY----
hBAX        ------------------------------------------------------------


Buffy       RLSNVSDAVTRKLSYT-----IGWKAAQ----------IPAQDIISQGRCLCGHYIKRRL
hBcl-2      ---DNREIVMKYIHYKLSQRGYEWDAGDVGAAPPGAAPAPGIFSSQPGHTPHPAASRDPV
hBAX        ------------------------MDGSGEQPRGGGPTSSEQIMKTGALLLQGFIQDR-
                                                 .    . *        :
                                                   BH3 domain
Buffy       RRSGLFNKKLGLQRIRSILGSTSMGIVRD----------VFPAVQVLGDELERMHPRIYN
hBcl-2      ARTSPLQT--------PAAPGAAAGPALSPVPPVV-----HLTLRQAGDDFSRRYRRDFA
hBAX        -------A--------GRMGGEAPELALDPVPQDASTKKLSECLKRIGDELDSNME--LQ
                                 . :   . .              ::  **::.
                                          BH1 domain
Buffy       GVARQICRNPGGEFHTPDAVSLLLGAVGRELFR-VEITWSKVISLFAIAGGLSVDCVRQG
hBcl-2      EMSSQ-------LHLTPFTARGRFATVVEELFR-DGVNWGRIVAFFEFGGVMCVESVNRE
hBAX        RMI---------AAVDTDSPREVFFRVAADMFSDGNFNWGRVVALFYFASKLVLKALCTK
             :                :    :  *  ::*    ..*.::::* :.. : :..:
                               BH2 domain    Hydrophobic/transmembrane
Buffy       HPEYLPKLMESVSEVIEDELVPWINENGGWSGINTHVLPTTNSLNPLEWTTLVIGVVFGL
hBcl-2      MSPLVDNIALWMTEYLNRHLHTWIQDNGGWDAFVELYGPSMRPLFDFSWLSLKTLLSLAL
hBAX        VPELIRTIMGWTLDFLRERLLGWIQDQGGWDGLLSYFGTPTWQT-------VTIFVA-GV
                : .:     : :. .*  **:::***..:                  :   :  .:
               domain
Buffy       ILVFMILRFIFNLIVPKIYQRFTNS
hBcl-2      VGACITLGAYLGHK-----------
hBAX        LTASLTIWKKMG--------------
            : . : :   :.
```

Figure 5 Sequence alignment. the alignment of Buffy protein (Q8T8Y5) with *Homo sapiens* Bcl-2 (P10415) and BAX (Q07812) is carried out using Clustal-Omega Bioinformatics tool. The BH domain of all the three proteins are highlighted in different colours. BH3 in red, BH1 in green, BH2 in brown and transmembrane domain in blue.

3.2 Plasmid for the bacterial expression of Buffy (pGIL-*buffy*)

While previous studies have established a clear role for Buffy in the regulation of cell death mechanisms, little is known about the structure of this molecule and how it co-ordinates activities with other proteins in the cell. As one of the aims of the study is to investigate the interacting partners of Buffy, the first step in the study was to obtain purified protein for characterization studies. To do this, a bacterial expression construct was designed and purchased from the gene synthesis company Genscript. As shown in Figure 6, the pGIL vector has a T7 promotor followed by a His-Tag and MBP tag. The gene sequence encoding Buffy was inserted downstream of the

MBP tag. The gene sequence has been codon optimised and encodes from 82 – 257. This region is lacking a portion of the N-terminus which encodes the predicted BH4 domain and the C-terminal transmembrane domain. This regions were exclude to assist in obtaining soluble protein. Importantly, the construct also encodes a TEV protease cleavage site which allows the removal of the MBP tag following purification.

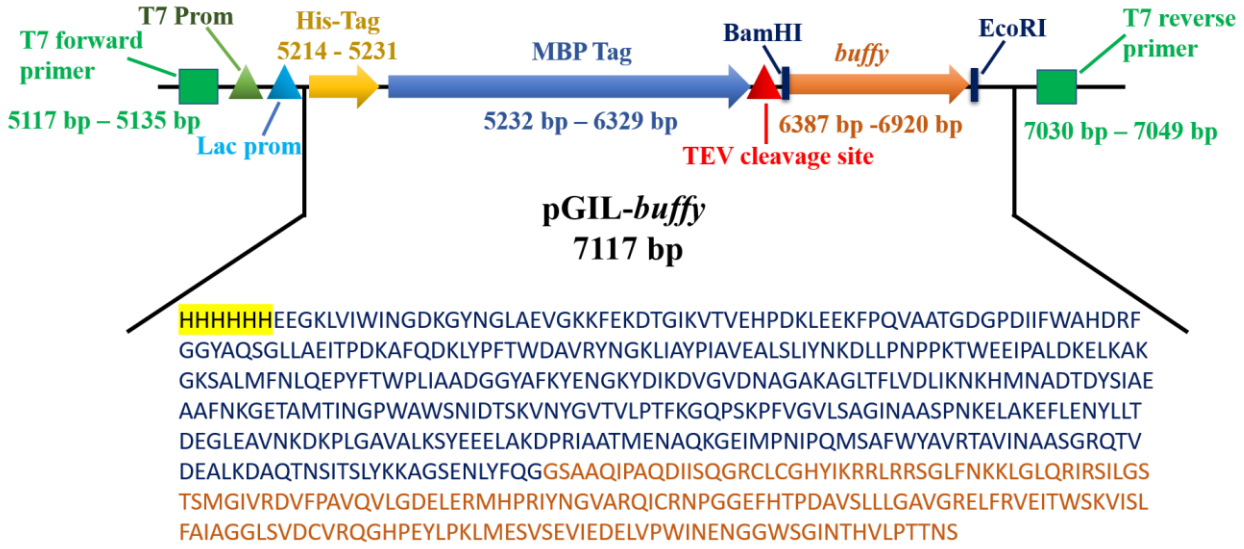


Figure 6 Vector map of pGIL-*buffy*. The vector map depicts the gene sequence encoding His-MBP-Buffy and the TEV cleavage site. The various other features including restriction enzyme sites, lac promotor, T7 promotor, T7 forward and reverse primers. The translate recombinant Buffy is shown below the vector map.

3.3 Restriction enzyme digest of pGIL-*buffy* vector

To check that the plasmid contained an insert corresponding to the expected size of *buffy*, a restriction enzyme digest was performed. The recombinant pGIL vector was transformed into XL-1 Blue *E.coli* to increase the vector copy number. The transformed *E.coli* was grown overnight and a mini-prep performed to obtain high yields of the pGIL-*buffy* vector. The plasmid DNA was

then incubated with the *EcoRI* and *BamHI* restriction enzymes for 2 hours at 37°C. As confirmed by agarose gel electrophoresis, a 540 bp insert corresponding to the expected size of the *buffy* cDNA released from the pGIL vector (Figure 7, lane3)

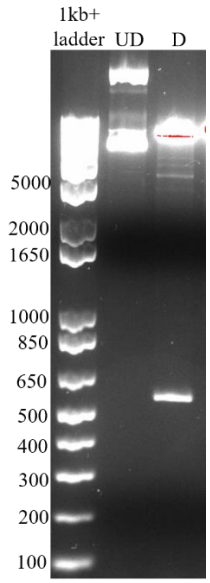


Figure 7 Restriction digestion of pGIL-*buffy*. Undigested (U) and *BamHI* and *EcoRI* digested (D) pGIL-*buffy* was analysed on a 1% agarose gel. The size of DNA fragments was determined by comparison against a 1 kb+ ladder.

3.4 Expression of His-MBP-Buffy in *E.coli* BL-21 cells

After the restriction enzyme digest, the pGIL-*buffy* vector was transformed into BL-21 *E.coli* for protein expression trials. The Buffy protein induction was carried out using 1mM IPTG at 37°C. A sample was collected prior to the addition of IPTG and further samples taken at 1 hour intervals following induction. A 12% SDS-PAGE gel was used to analyse the time course. As observed in Figure 8, a predominant band migrating at approximately 62 kDa was observed in the post-induction samples. This protein corresponds with the expected size of the His-tagged MBP Buffy fusion. Interestingly, a small drop in protein expression was observed at the later 4 hour time point.

Nonetheless the presence of enriched band at 62 kDa implies the success in the induction of the recombinant Buffy protein.

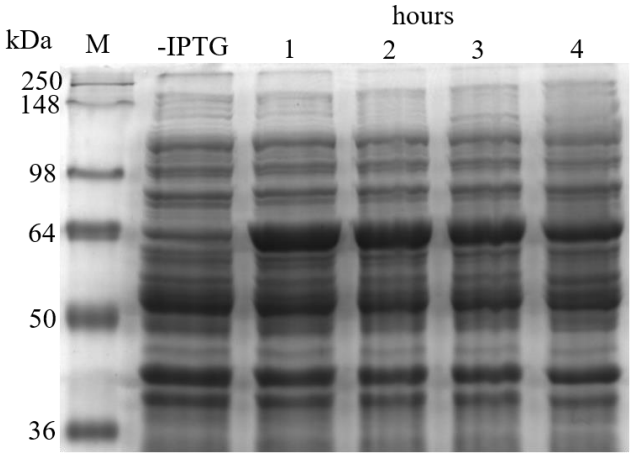


Figure 8 SDS-PAGE showing recombinant protein induction. Protein expression was performed in *E.coli* BL21 cells at 37°C using 1 mM IPTG. SDS-PAGE was used to analyse the uninduced (-IPTG) and post induction samples (1-4 hours). Gels were stained in Coomassie brilliant blue and protein sizes compared against the See blue plus 2 molecular weight markers (M).

3.5 Western transfer and immunoblot analysis of degradation product

Protein expression was further verified by Western transfer and immunoblot analysis. Both pre-induction and post-induction samples were separated by SDS-PAGE and proteins transferred to a NCF membrane using the Western blotting method. The membrane was subsequently probed with an anti-His tag antibody and chemilluminescence was recorded. As observed in the Figure 9, a band was observed 62 kDa confirming expression of the recombinant His-tagged protein. Interestingly, along with the 62 kDa species, another band at approximately 55 kDa was observed. This result suggested that a portion of the recombinant may be sensitive to degradation or cleavage. In an attempt to reduce the degree of degradation observed, expression trials at 16°C were

performed. While these result yielded reasonable levels of expression, a cleavage product was still evident.

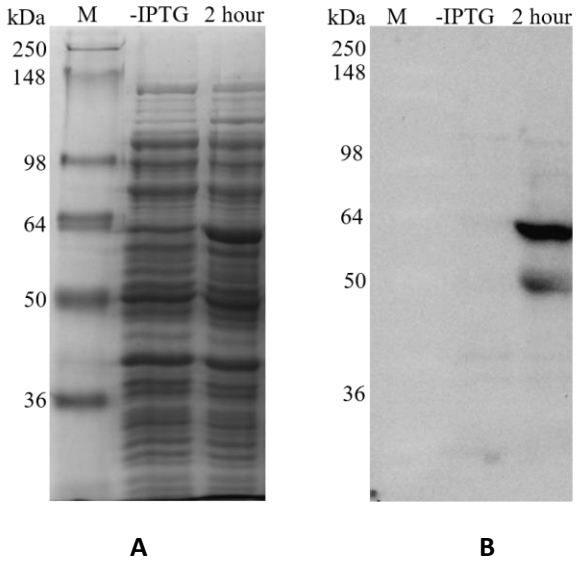


Figure 9 Western Transfer analysis of degraded product. protein expression in *E.coli* BL21 cells was analyzed using 10% SDS-PAGE where the uninduced (-IPTG) and post-induction sample (2 hour) along with molecular weight marker (M) were loaded on gel. The proteinswere transferred to a NCF membrane and chemiluminescence was captured using GelDoc™ imaging system.

3.6 Solubility testing of His-MBP-Buffy protein

A high level expression of His-MBP Buffy was observed in the *E.coli* BL-21 expression trials. The solubility of the protein was determined by lysing the cells and separating the soluble proteins by high speed centrifugation and running the fractions on SDS-PAGE. The solubility test showed that approximately 70% of the protein was soluble. Approximately 30% appeared to be in the insoluble fraction. The results also showed an apparent increase in the degradation product following processing. The total (T) fraction represents the post-expression sample following lysis

and the 55 kDa species is more prevalent. All of this degradation product appears to be fractionating to the soluble fraction.

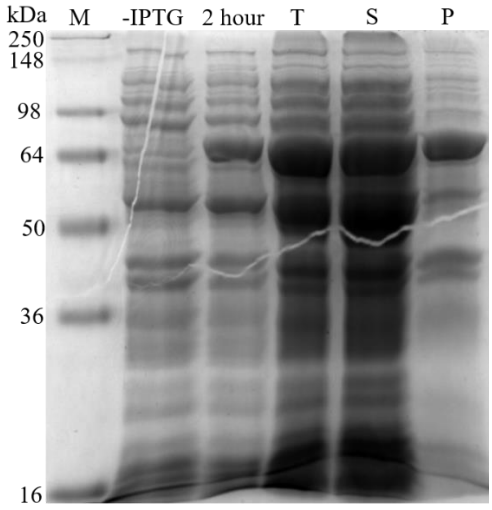


Figure 10 Solubility testing of His-MBP Buffy protein. Cells expressing the His-MBP-Buffy protein were lysed by homogenisation (T) followed by high speed centrifugation to separate the soluble (S) and pellet fractions (P). Samples were run on 12 % SDS-PAGE gels. The molecular weight marker were loaded in first lane followed by pre-induction and post-induction samples (2 hours).

3.7 Purification of His-MBP-Buffy

As many trials like adding the protease inhibitors, changing the temperature conditions, lowering the IPTG concentration and screening of other transformed colonies was employed to reduce the apparent breakdown of His-MBP-Buffy were unsuccessful, a decision to purify the protein was made with the view to try and remove the species at a later stage in the purification processes.

To purify the His-MBP-Buffy protein, large scale expression cultures were prepared and cell lysis was performed. Following isolation of the soluble fraction, a His-Tag purification was done. The

purification was carried out by incubating the lysate with nickel-agarose beads and washing the beads with low concentrations of imidazole to remove loosely bound proteins. Protein specifically bound to the beads was then eluted with higher concentrations of imidazole.

As shown by SDS-PAGE analysis, a substantial amount of His-MBP-Buffy did not bind to the beads, appearing in the unbound fraction (UB). The enriched His-tag Buffy protein (62 kDa) was eluted along with the 55 kDa protein (Figure 11). Although the IMAC purification was performed in the presence of protease inhibitor and resulted in the reduction of 55 kDa protein, this species appeared to comprise more than 50% of the total purified.

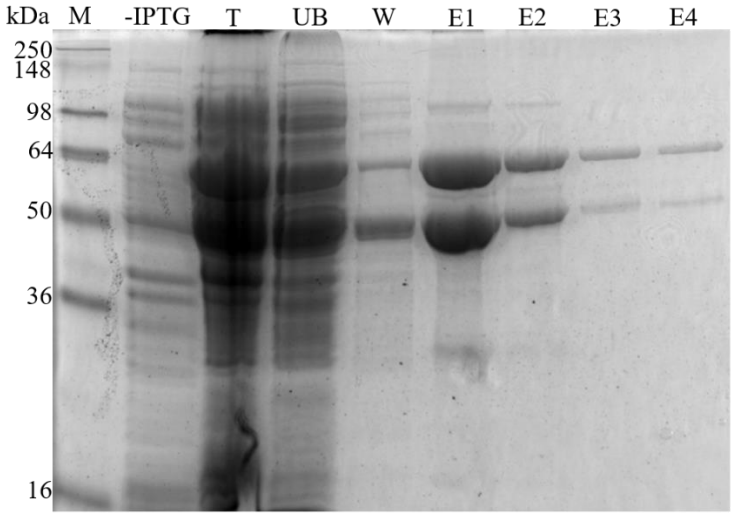


Figure 11 Purification of soluble proteins. The samples obtained at each stage of purification were subjected to SDS-PAGE. The gel shows the purification of Buffy in the presence of protease inhibitor. The gel was loaded with seeblue plus 2 molecular weight marker in the first lane followed by –IPTG (before induction), T – total sample before the separation of soluble lysate. UB – unbound, W – wash, E1 – E4 elution 1 to 4.

3.8 Mass spectrometry analysis of His-MBP-Buffy (62 kDa) and the 55 kDa proteins

The His-MBP-Buffy (62 kDa) and the 55 kDa protein band from the purification gel was excised and subjected to mass-spectrometry analysis to know where exactly the protein was getting cleaved. The mass-spectrometry result showed that the small part of Buffy was getting cleaved (532-568 residues were missing).

3.9 Expression and purification of TEV

A bacterial expression construct pRK793 with TEV (tobacco etch virus proteases) encoding gene was designed and purchased from the gene synthesis company Genscript. The Bl21-RIL cells containing pRk793-TEV were grown (large scale) at 37°C followed by IPTG induction and reduction of temperature to 30°C post-induction. Following 4-hour induction, cell lysis was performed and soluble proteins were separated by high speed centrifugation. The TEV was purified using His-Trap Fastflow column (IMAC) connected to FPLC. The eluted samples were subjected to SDS-PAGE analysis and the elutions with pure TEV was pooled. As TEV cleaves itself from the tag, their won't be a necessary cleavage process. The pure TEV and Buffy obtained was dialyzed for removing of imidazole from the buffer. The Buffy and the TEV was then concentrated down using Vivaspin 20 ml concentrators.

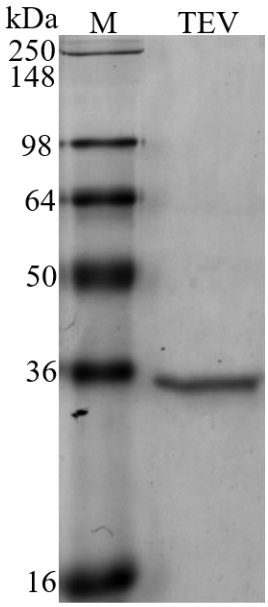


Figure 12 SDS-PAGE showing purified TEV protein. A single band was observed at approximately 35 kDa confirming the pure TEV. The gel was loaded with seeblue plus 2 marker in the first lane followed by purified TEV.

3.10 Cleavage of the His-MBP from Buffy

A trial cleavage reaction was set up by adding purified and concentrated TEV to His-MBP-Buffy in a sterile 1.5 ml tube, which was incubated at 4°C on rotatory mixer for 2 hours. The sample aliquot after two hours of incubation was run on gel which showed the cleavage reaction was a success with release of 19 kDa Buffy.

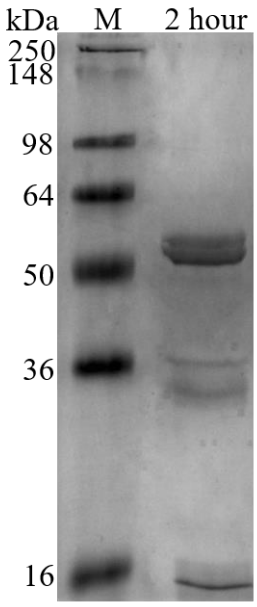


Figure 13 SDS-PAGE showing trial cleavage. The 19 kDa Buffy was obtained after a trial cleavage reaction (2hour induction) along with other species. The gel was loaded with seeblue plus 2 molecular weight marker in the first lane followed cleaved sample.

As the trial cleavage was successful with the release of 19 kDa Buffy protein, further the cleavage reaction is performed with higher amounts of Buffy which will be then subjected to reverse IMAC followed by size exclusion chromatography and further purified by ion-exchange chromatography.

Chapter 4

Discussion

Drosophila melanogaster is a model organism and study of proteins from this organism can give a deep understanding of protein function and phylogenetic relationship with other organisms. Few residues may be involved in folding of Bcl-2 proteins, in spite of having very less sequence similarity between different Bcl-2 proteins in different organisms the proteins fold in a similar fashion. This conserved sites can be found out by characterising various Bcl-2 homologs from different organisms and establishing a phylogenetic relationship.

The alignment of recombinant Buffy protein showed a significant homology in BH 1, BH 2 and BH 3 domain. The BH 1 and BH 2 domains are involved in anti-apoptotic functions, which is mainly seen in human Bcl-2 and Bcl-xL (Yin *et al*., 1994). In contrast the BH 3 domain is mainly involved in protein – protein interactions as seen in Human BAK and BAX (Kelekar & Thompson, 1998). Because there is significant similarity in all three BH domains, it is not possible to speculate whether it is a pro or antiapoptotic in nature. The literature suggests that it is a Bcl-2 like homologue and anti-apoptotic in nature, to determine this need to perform protein interaction assays (Quinn *et al*., 2003).

The recombinant Buffy was engineered to have a His-Tag and MBP tag at the N-terminus of the protein. Importantly, the hydrophobic or transmembrane domain in the recombinant protein was removed to make the protein more soluble. As codon bias may exist when synthesising a fly protein in bacteria, the gene was codon optimised during its commercial synthesis (Rosano & Ceccarelli, 2009).

While the His-tag can facilitate purification of the proteins, MBP was selected due to its known ability to enhance the solubility of the target protein to which it is fused (Kapust & Waugh, 1999). The entire recombinant DNA was engineered into the pGIL vector with a T7 promotor. Since there is a TEV recognition site in-between MBP and Buffy, after purification the His-Tag and MBP can be cleaved by the addition of TEV protease results in the release of the target Buffy protein.

The pGIL-Buffy construct was transformed into BL-21 (DE3) *E.coli* for protein expression trials. This strains is deficient in Lon and Omp T proteases (Gottesman, 1996, Grodberg & Dunn, 1988). These are the main housekeeping proteases found in *E.coli* which function to degrade proteins. This feature makes it desirable for the expression of recombinant proteins and may reduce the possibility of digestion of recombinant proteins (Grodberg & Dunn, 1988).

Induction with IPTG showed the expression of the recombinant protein at approximately 62 kDa which is the correct size of Buffy with MBP and His-tag. The induction showed a small decrease in the concentration of the expressed protein with the increase in time. This may occur when the *E.coli* cells cannot sustain the higher concentration of expressed recombinant protein. Along with the recombinant protein another secondary non-specific expression is also seen at approximately 55 kDa. A Western blot of the 2-hour induction showed both 62 kDa and 55 kDa expressions were positive for His-tag. To try and reduce this, the induction was performed at 16°C and as an alternative approach the concentration of IPTG was reduced (Baldwin, 1986, Makhatadze & Privalov, 1995, Schellman, 1997). Despite efforts to improve this, the expression result was the same at 37°C.

To monitor solubility of the protein, the soluble lysate was obtained by cell lysis and high speed centrifugation. Around 70% of the recombinant protein was found in the soluble fraction with only a minimal amount of recombinant Buffy found in the pellet. To improve the solubility in the future,

a number of measures could be trailed. Change in lysis buffer may make recombinant protein more stable and soluble, similarly using different *E.coli* strains can be employed.

As the levels of protein in the soluble fraction were sufficient, the purification of soluble His-tag proteins was carried out which resulted in elution of both the 62 kDa and 55 kDa protein bands. Mass spectrometry confirmed that the apparent secondary band was a degradation product which is a part of the target protein. To decrease the degradation, protease inhibitors were added to lysis buffer before cell-lysis and purification. The decrease in the intensity of the degraded product was seen which is now equal to the recombinant Buffy protein concentration. Other possible ways to try and remove this protein would be to trial ion-exchange. As the two proteins are very close in size, gel filtration chromatography is unlikely to be effective in separating the two species.

To perform a trial cleavage of the recombinant protein, the TEV protease had to be expressed and purified. Expressions were performed with IPTG induction of *E.coli* BL21-RIL cells containing pRk793 coding for TEV protease. The cell lysis and purification of soluble proteins were carried out and pure TEV was obtained. Since the recombinant TEV is expressed as an MBP fusion protein, that cleaves itself from MBP to yield TEV protease with an N-terminal His-tag (Kapust *et al.*, 2001), the pure TEV protease was obtained in IMAC purification. The TEV protease and the elution 1 and 2 (pooled) of the recombinant Buffy purification fractions were subjected to dialysis separately to remove the imidazole from the protein fractions. As the imidazole does not allow the cleavage of recombinant Buffy with TEV protease.

The trial cleavage showed the release of the 19 kDa protein. Since TEV protease targets a specific sequence, the shift in the size of the degradation product band indicates that some of the protein has being cleaved, however the cleavage product would be too small to appear on an SDS-PAGE gel.

4.1 Further directions

Further work could involve in upscaling and isolation of Buffy protein followed by performing of circular dichroism spectroscopy (CD) to interpret its secondary structure and subjecting to crystallization studies for resolving its quaternary structure. The ITC studies can give a deep insight in to protein interactions with other known apoptotic proteins. These studies would aid in better understanding of the mechanism of Buffy. A library of conserved motives and various protein interaction studies from a model organism can help in knowing various drug targets.

References

Baldwin RL (1986) Temperature dependence of the hydrophobic interaction in protein folding. Proc Natl Acad Sci U S A 83: 8069-72

Boatright KM, Salvesen GS (2003) Mechanisms of caspase activation. Current Opinion in Cell Biology 15: 725-31

Brenner S (1974) The genetics of Caenorhabditis elegans. Genetics 77: 71-94

Chittenden T, Flemington C, Houghton AB, Ebb RG, Gallo GJ, Elangovan B, Chinnadurai G, Lutz RJ (1995) A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. Embo j 14: 5589-96

Colussi PA, Quinn LM, Huang DC, Coombe M, Read SH, Richardson H, Kumar S (2000) Debcl, a proapoptotic Bcl-2 homologue, is a component of the Drosophila melanogaster cell death machinery. J Cell Biol 148: 703-14

Czabotar PE, Lessene G, Strasser A, Adams JM (2014) Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol 15: 49-63

Doumanis J, Dorstyn L, Kumar S (2007) Molecular determinants of the subcellular localization of the Drosophila Bcl-2 homologues DEBCL and BUFFY. Cell Death and Differentiation 14: 907-15

Ellis HM, Horvitz HR (1986) Genetic control of programmed cell death in the nematode C. elegans. Cell 44: 817-29

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391: 43-50

Flemming W (1885) Über die bildung von richtungsfiguren in säugethiereiern beim untergang graaf'scher follikel. publisher not identified,

Gottesman S (1996) Proteases and their targets in Escherichia coli. Annu Rev Genet 30: 465-506

Grodberg J, Dunn JJ (1988) ompT encodes the Escherichia coli outer membrane protease that cleaves T7 RNA polymerase during purification. J Bacteriol 170: 1245-53

Gross A, McDonnell JM, Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. Genes Dev 13: 1899-911

Harlow E, Lane D (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 139: 238

Hedgecock EM, Sulston JE, Thomson JN (1983) Mutations affecting programmed cell deaths in the nematode Caenorhabditis elegans. Science 220: 1277-9

Hengartner MO, Ellis RE, Horvitz HR (1992) Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. Nature 356: 494-9

Kapust RB, Tozser J, Fox JD, Anderson DE, Cherry S, Copeland TD, Waugh DS (2001) Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency. Protein Eng 14: 993-1000

Kapust RB, Waugh DS (1999) Escherichia coli maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Protein Sci 8: 1668-74

Kelekar A, Thompson CB (1998) Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol 8: 324-30

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British Journal of Cancer 26: 239-57

Khaled AR, Kim K, Hofmeister R, Muegge K, Durum SK (1999) Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. Proc Natl Acad Sci U S A 96: 14476-81

Liu X, Zou H, Slaughter C, Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89: 175-84

Makhatadze GI, Privalov PL (1995) Energetics of protein structure. Adv Protein Chem 47: 307-425

Metzstein MM, Stanfield GM, Horvitz HR (1998) Genetics of programmed cell death in C. elegans: past, present and future. Trends in genetics 14: 410-416

Monserrate JP, Chen MY, Brachmann CB (2012) Drosophila larvae lacking the bcl-2 gene, buffy, are sensitive to nutrient stress, maintain increased basal target of rapamycin (Tor) signaling and exhibit characteristics of altered basal energy metabolism. BMC Biol 10: 63

Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL, Ng SL, Fesik SW (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature 381: 335-41

Petros AM, Medek A, Nettesheim DG, Kim DH, Yoon HS, Swift K, Matayoshi ED, Oltersdorf T, Fesik SW (2001) Solution structure of the antiapoptotic protein bcl-2. Proc Natl Acad Sci U S A 98: 3012-7

Quinn L, Coombe M, Mills K, Daish T, Colussi P, Kumar S, Richardson H (2003) Buffy, a Drosophila Bcl-2 protein, has anti-apoptotic and cell cycle inhibitory functions. Embo j 22: 3568-79

Reed JC (2002) Apoptosis-based therapies. Nat Rev Drug Discov 1: 111-21

Rosano GL, Ceccarelli EA (2009) Rare codon content affects the solubility of recombinant proteins in a codon bias-adjusted Escherichia coli strain. Microb Cell Fact 8: 41

Sato T, Irie S, Krajewski S, Reed JC (1994) Cloning and sequencing of a cDNA encoding the rat Bcl-2 protein. Gene 140: 291-2

Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science 275: 983-6

Schellman JA (1997) Temperature, stability, and the hydrophobic interaction. Biophys J 73: 2960-4

Shamas-Din A, Brahmbhatt H, Leber B, Andrews DW (2011) BH3-only proteins: Orchestrators of apoptosis. Biochim Biophys Acta 1813: 508-20

Sulston JE, Horvitz HR (1977) Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Developmental Biology 56: 110-56

Sulston JE, Schierenberg E, White JG, Thomson J (1983) The embryonic cell lineage of the nematode Caenorhabditis elegans. Developmental Biology 100: 64-119

Suzuki M, Youle RJ, Tjandra N (2000) Structure of Bax: coregulation of dimer formation and intracellular localization. Cell 103: 645-54

Tanner EA, Blute TA, Brachmann CB, McCall K (2011) Bcl-2 proteins and autophagy regulate mitochondrial dynamics during programmed cell death in the Drosophila ovary. Development 138: 327-38

Tsujimoto Y, Croce CM (1986) Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci U S A 83: 5214-8

Vaux DL, Cory S, Adams JM (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335: 440-2

Vaux DL, Weissman IL, Kim SK (1992) Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2. Science 258: 1955-7

Vogt CI (1842) Untersuchungen über die Entwicklungsgeschichte der Geburtshelferkröte (Alytes obstetricans). Jent,

Wang L, Yang JK, Kabaleeswaran V, Rice AJ, Cruz AC, Park AY, Yin Q, Damko E, Jang SB, Raunser S, Robinson CV, Siegel RM, Walz T, Wu H (2010) The Fas-FADD death domain complex structure reveals the basis of DISC assembly and disease mutations. Nat Struct Mol Biol 17: 1324-9

Whitten JM (1969) Cell death during early morphogenesis: parallels between insect limb and vertebrate limb development. Science 163: 1456-7

Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555-6

Yin XM, Oltvai ZN, Korsmeyer SJ (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 369: 321-3