İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

RECOMBINANT ANTIGEN AND MONOCLONAL ANTIBODY PRODUCTION FOR KATANIN P60

M.Sc. Thesis by
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Department : **Advanced Technologies**

Programme : **Molecular Biology–Genetics and Biotechnology**

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

KATANİN P60 İÇİN REKOMBİNANT ANTİJEN VE MONOKLONAL ANTİKOR ÜRETİMİ

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FOREWORD

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Derya Canbaz

Medical Biologist

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ABBREVIATIONS

µg : Microgram
µl : Microliter
µM : Micromolar
µm : Micrometer
aa : Amino acid
AAA : ATPases Associated with diverse cellular Activities
ADP : Adenosine diphosphate
AP : Alkaline phosphatase
APS : Ammonium persulfate
ATP : Adenosine triphosphate
ATPase : Adenosine triphosphatease
BCIP : 5-bromo-4-chloro-3-indolyl phosphate
bp : Base pair
BSA : Bovine Serum Albumine
C : Constant region
CBB : Coomassie Brilliant Blue
cDNA : Complementary DNA
CH : Constant region of heavy chain
CL : Constant region of light chain
cm : Centimeter
cm^2 : Centimeter square
Da : Dalton
DMEM : Dulbecco's modified Eagle medium
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
dNTP : Deoxyribonucleotide
DTT : Dithiothreitol
E.coli : *Escherichia coli*
EB : Elution Buffer
EDTA : Ethylenediaminetetraacetic acid
ELISA : Enzyme-Linked Immunosorbent Assay
EtBr : Ethidium bromide
FACS : Fluorescence-Activated Cell Scanning
FBS : Fetal bovine berum
FCA : Freund's Complete Adjuvant
g : Gram
GDP : Guanosine tri-phophate
GTP : Guanosine tri-phosphate
HAT : Hypoxanthine Aminopterin Thymidine
His : Histidine
HPGRT : Hypoxanthine-guanine phosphoribosyltransferase
HRP : Horseradish peroxidise
HT : Hypoxanthine Thymidine

ICC : Immunocytochemistry
IDT : Integrated DNA Technology
IFA : Incomplete Freund's Adjuvant
IgA : Immunoglobulin A
IgD : Immunoglobulin D
IgE : Immunoglobulin E
IgG : Immunoglobulin G
IgM : Immunoglobulin M
IPTG : Isopropyl β-D-1-thiogalactopyranoside
Kb : Kilo base
kDa : Kilo dalton
L : Liter
LB : Luria-Bertani Broth
M : Molar
mA : Miliampere
Mab : Monoclonal antibody
Mabs : Monoclonal antibodies
MAPs : Microtubule-associated proteins
mg : Miligram
min : Minute
ml : Mililiter
mM : Milimolar
mm : Milimeter
mRNA : Messenger ribonucleic acid
MT : Microtubule
MTs : Microtubules
NBT : Nitroblue tetrazolium
NCBI : National Center for Biotechnology Information
Ni-NTA : Nickel-nitriloacetic acid
ng : Nanogram
nm : Nanometer
nM : Nanomolar
nrec p60 : New recombinant katanin p60
OD : Optical Density
Pab : Polyclonal antibody
Pabs : Polyclonal antibodies
PBS : Phosphate Buffered Saline
PBS-T : Phosphate Buffered Saline-Tween 20
PCR : Polymerase chain reaction
PEG : Polyethylene glycol
PET : Positron Emission Tomography
Pfu : *Pyrococcus furiosus*
pH : Power of hydrogen
PIPES : Piperazine-N,N′-bis(2-ethanesulfonic acid)
PNPP : Para-Nitrophenylphosphate
PVDF : Polyvinylidene Fluoride
rec p60 : Recombinant p60
RNA : Ribonucleic acid
rpm : Revolutions per minute
SDS : Sodium dodecyl sulphate

SDS-PAGE **:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec **:** Second
SOC **:** Super Optimal Broth with catabolite repression
SPECT **:** Single Photon Emission Computerized Tomography
TBE **:** Tris-borate-EDTA
TBS **:** Tris-Buffered Saline
TCA **:** Trichloroacetic acid
TE **:** Tris-EDTA
TEMED **:** Tetramethylethylenediamine
TTBS **:** Tween 20-Tris-Buffered Saline
T_m **:** Melting temperature
UV **:** Ultraviolet
V : Volt
γ-TuRC : γ -tubulin ring complex

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RECOMBINANT ANTIGEN AND MONOCLONAL ANTIBODY PRODUCTION FOR KATANIN p60

SUMMARY

Katanin, one of the proteins that severs microtubules, is a heterodimer composed of two subunits termed p60 and p80. The p60 subunit is an enzyme that hydrolyzes ATP to break the lattice of the microtubule where the p80 subunit regulates the activity of p60 and localizes it to the centrosomes. Severing activity of katanin p60 has critical roles in mitotic and meiotic cell divisions and in axonal growth and differentiation in postmitotic neurons. Katanin may also function in various aspects of neuronal morphology such as the length, number and branching patterns of neuritis. Although, katanin has been extensively studied, interacting proteins of katanin p60 are not well understood. Monospecific antibodies could be helpful to monitor cellular katanin and find out its interacting proteins. Therefore, in this study, it is aimed to express specific recombinant katanin p60 protein and produce monoclonal antibodies against this protein for further use in immunocytochemistry, immunoprecipitation and western blot techniques.

For this purpose, an amino acid sequence was selected from full katanin p60 protein considering specificity, solubility and inclusion of antigenic determinants of the protein and expressed efficiently. 6-8 weeks old Balb/c mice were then immunized with purified recombinant katanin p60 protein. Fusion study was carried out by using hybridoma technology with a mouse having high immune response. The main source of antibody producer B lymphocytes, spleen and lymph node cells, were fused with myeloma cells in the presence of polyethylenglycol. ELISA (Enzyme Link Immuno Sorbent Assay) cross reactivity test results showed that one hybrid clone was obtained among 295 hybrid clone as a candidate for producing a specific monoclonal antibody against katanin p60. Further ELISA tests showed that the monoclonal antibody subtype was not IgG but it could be IgA or IgM subtypes. Later, exact subtype of the monoclonal antibody was determined as IgM using mouse monoclonal antibody isotyping kit.

KATANİN P60 İÇİN REKOMBİNANT ANTİJEN VE MONOKLONAL ANTİKOR ÜRETİMİ

ÖZET

Mikrotübülleri kesen proteinlerden biri olan katanin, p60 ve p80 olarak tanımlanan iki alt üniteden oluşan bir heterodimerdir. p60 alt ünitesi mikrotübülün kafesini kırmak için ATP yi hidrolizleyen bir enzim iken, p80 alt ünitesi p60'ın aktivitesini düzenler ve sentrozoma lokalize eder. p60'ın kesim aktivitesi mitotik, miyotik hücre bölünmesinde ve postmitotik nöronların aksonal büyüme ve farklılaşmasında kritik rollere sahiptir. Katanin ayrıca, nöritlerin uzunluk, sayı ve dallanma modelleri gibi nöronal morfolojinin çeşitli durumlarında fonksiyon gösterebilmektedir. Katanin proteini şu ana kadar çok fazla çalışılmış olmasına rağmen, etkileştiği proteinler çok iyi anlaşılamamıştır. Monospesifik antikorlar, hücresel kataninin görüntülenmesi ve etkileşen proteinlerinin belirlenmesinde yardımcı olabilir. Dolayısıyla bu çalışmada, spesifik bir rekombinant katanin p60' ın ekspresyonunun yapılması ve immunositokimya, immunopresipitasyon ve western emdirimi tekniklerinde ileride kullanılmak üzere, bu proteine karşı monoklonal antikorlar üretilmesi amaçlanmaktatır.

Bu amaçla, tam katanin p60 proteininden özellikliği, çözünebilirliği ve antijenik determinant içeriği göz önünde bulundurularak bir amino asit dizisi seçildi ve verimli bir anlatım sisteminde anlatımı yapıldı. Sonra, 6-8 haftalık Balb/c türü fareler saflaştırılmış rekombinant katanin p60 proteini ile bağışıklandırıldı. Yüksek bağışık cevabı olan fare üzerinde hibridoma teknolojisi kullanılarak füzyon çalışmaları gerçekleştirildi. Antikor üreten B lenfositlerin ana kaynağı olan dalak ve lenf düğümü hücreleri, polietilenglikol varlığında miyelom hücreleri ile birleştirildi. ELISA (Enzim Bağlantılı Immuno Sorbent Ölçümü) kross reaktivite test sonuçları, 295 melez klon arasından katanin p60' a karşı spesifik monoklonal antikor üretmeye aday bir melez klon, elde edildiğini göstermiştir. Daha sonraki ELISA testleri, bu monoklonal antikorun alt tipinin IgG olmadığı ancak IgA ya da IgM alt tipi olabileceğini göstermiştir. Bununla beraber, monoklonal antikorun kesin alt tipi fare monoklonal antikor izotipleme kiti ile IgM olarak belirlenmiştir.

1. INTRODUCTION

1.1 Cytoskeleton

Cells are supported by a network of protein fibers extending throughout the cytoplasm called the cytoskeleton. This dynamic structure maintains cells' shape, enables several types of cell motility, and plays important roles in both intracellular transport (the movement of vesicles and organelles) and cellular division. The cytoskeleton is composed of three main cytoplasmic filament networks: microtubules, microfilaments, and intermediate filaments (Campbell and Reece, 2005).

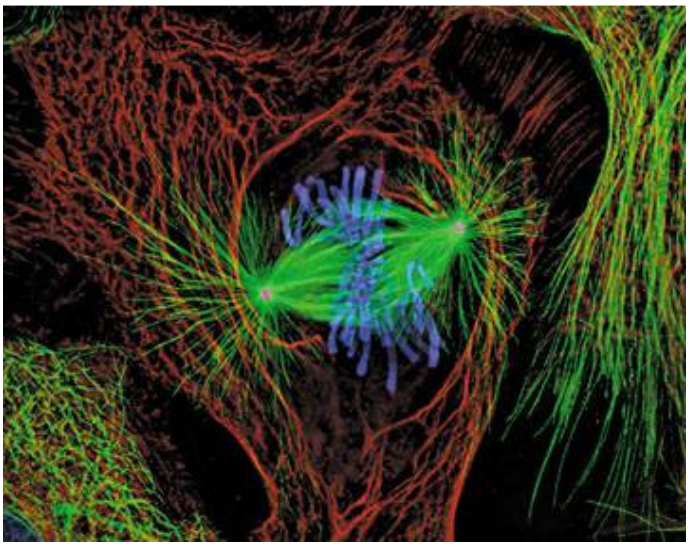

Figure 1.1: The major structural components of cytoskeleton. Centrosomes (magenta), microtubules (green), chromosomes (blue) and keratin filaments (red) are shown in the mitotic spindle that is central to the process of cell division (Dunn, 2000).

Briefly, intermediate filaments provide mechanical strength and resistance to shear stress. Microtubules determine the positions of membrane-enclosed organelles and direct intracellular transport and are involved in cell division in the course of drawing chromosomes to the poles. Microfilaments (composed of actin filaments) determine the shape of the cell's surface and are necessary for whole-cell locomotion (Alberts et al., 2002).

1.2 Microtubules

Microtubules shape and support the cell and also serve as tracks along which organelles equipped with motor proteins can move. For example, microtubules guide secretory vesicles from the Golgi apparatus to the plasma membrane by "dynein walking". Moreover, microtubules have an essential role in the separation of chromosomes during cell division (Campbell and Reece, 2005).

1.2.1 Microtubule structure and organization

Microtubules are ~25 nm diameter hollow tubes with walls made from α- tubulin and β- tubulin heterodimers stacked head to tail at 8 nm intervals to form "protofilaments" that run lengthwise along the wall (Fig 1.2). Microtubule architecture in living cells depends on the number of protofilaments, usually 13, but a very wide range is possible (Meurer-Grob et al., 2001). α- and β- tubulin monomers are proteins of about 450 amino acids each and are about 50 % identical at the amino acid level. Each monomer has a molecular mass of about 50,000 Da and there are two GTP binding sites on tubulin, a hydrolyzable site (exchangeable site) on the β-subunit and a non-hydrolyzable site on the α- subunit (Valiron et al.,2001; Meurer-Grob et al.,2001).

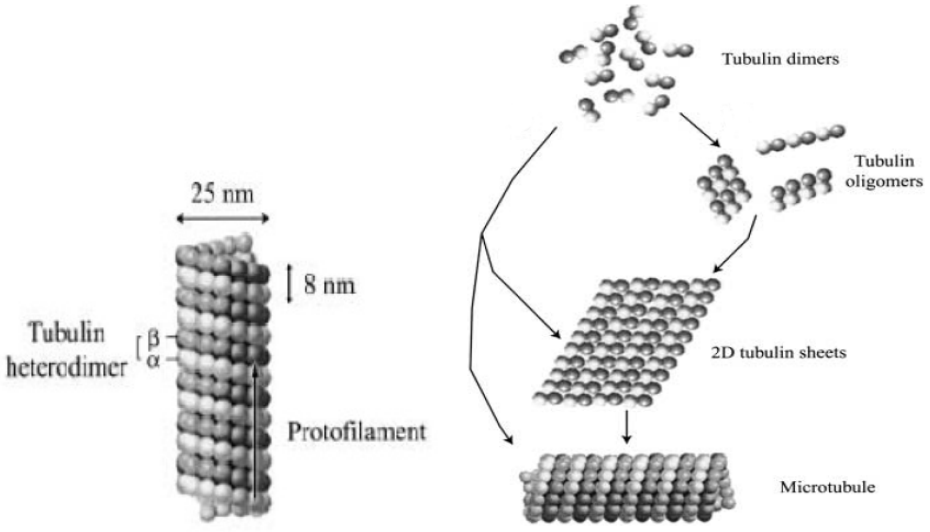

Figure 1.2: Microtubule structure (Valiron et al., 2001).

The structure of microtubule is organized in a polar manner such that the α-tubulin subunit is exposed at the minus end, while the β-tubulin subunit is exposed at the plus end (Risinger et al., 2009).

One member of the tubulin superfamily is γ- tubulin; it is localized to microtubule organizing centers, such as centrosomes and is also found in the cytoplasm as a part of a large complex called γ-tubulin ring complex (γ-TuRC) (Job et al., 2003), in which the γ-tubulin subunit serves as a template to initiate the polymerization of the α/ β- tubulin into a microtubule (Wiese and Zheng, 2006).

A full 55 % of microtubules formed in the presence of γ-TuRC have one complex at their minus end and very few have γ-TuRCs in their middles or at the plus ends (Job et al., 2003). Therefore, the faster polymerizing end was termed the 'plus' end and the more slowly polymerizing end the 'minus' end. Microtubules with free minus ends may be generated by release from a microtubule organizing center, cytoplasmic assembly, or breakage/ severing of existing microtubules (Fig. 1.3).

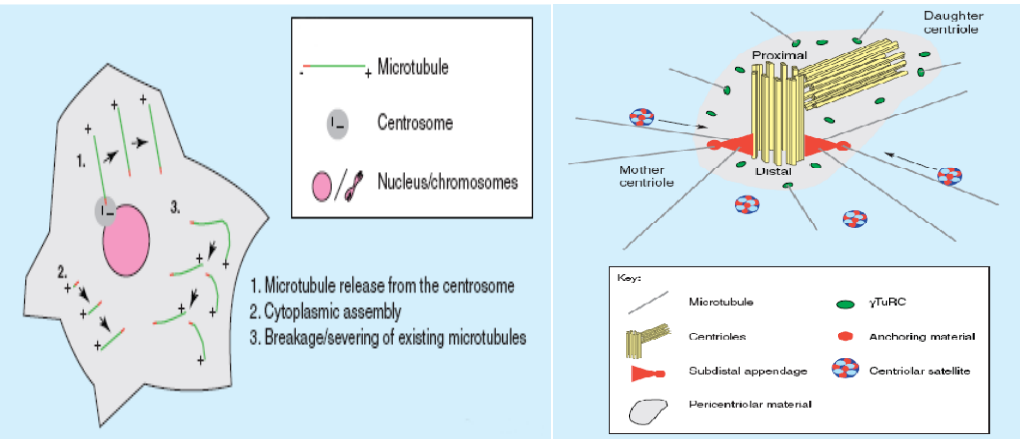

Figure 1.3: Microtubule nucleation from centrosome, adapted from Dammermann et al. (2003).

In nerve axons, the microtubules are arranged longitudinally with the plus end pointing away from the cell body, whereas in epithelial cells microtubules are organized with the plus end pointing toward the basement membrane. In most other cells, such as fibroblasts and macrophages, microtubules radiate from the cell center with the plus ends pointing toward the periphery (Hirokawa, 1998).

1.2.2 Microtubule dynamics

Microtubule dynamics are important for rapid cellular restructuring of the cytoskeleton, as well as for mediating the delivery of cellular cargos. For example,

during mitosis, dynamic MTs mediate the alignment of sister chromatids at the spindle equator during metaphase, and ultimately segregate the sister chromatids into the nascent daughter cells during anaphase (Inoue and Salmon, 1995).

Microtubule polymerization is a complex process involving a cooperative assembly of α β tubulin heterodimers followed by GTP hydrolysis. As mentioned before, the α-subunit binds to GTP in an irreversible manner, while the GTP bound to β- tubulin is exchangeable and it is hydrolyzed during polymerization. Thus, the majority of β-tubulin in the microtubule fiber is in the GDP-bound form and ‘‘capped” with GTP-bound β tubulin at the plus end. This leads to its elongation and formation of the protofilament (Singh et al., 2008).

When the GTP on β-tubulin molecule is hydrolyzed to GDP before another GTP-bound β-tubulin is added, the exposed GDP-β-tubulin leads to a conformational change that results in rapid depolymerization of the microtubule in an event known as microtubule catastrophe while a transition from a shortening phase to a growing phase is termed as a rescue. The relatively rapid lengthening and shortening at the microtubule plus end is referred to as dynamic instability (Risinger et al., 2008; Singh et al., 2008) (Fig 1.4).

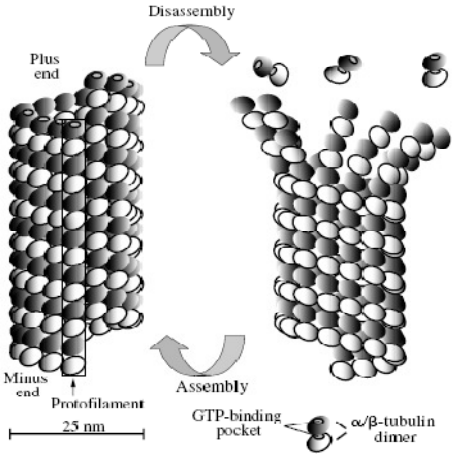

Figure 1.4: Dynamic instability (Wiese and Zheng, 2006).

Microtubule assembly and activity in the cells is considered to be precisely regulated by several proteins, called as microtubule associated proteins (MAPs) (Singh et al., 2008). MAPs bind to the tubulin subunits that make up microtubules to direct their stability (Dehmelt and Halpain, 2005). MAPs were shown to stimulate microtubule assembly. However, MAPs are not restricted only to stabilize microtubules, some

can mediate the interaction of microtubules with other cellular components and some can destabilize or severe microtubules. MAPs can act on a microtubule directly, or they can restrict access to the microtubules to other MAPs or motor proteins by binding to microtubule (Baas and Qiang, 2005). A broad range of MAPs functions suggests that it is the coordinated action of MAPs that leads to the proper microtubule functioning (Maiato et al., 2004). Coordination faults may lead to diseases, e.g. Alzheimer's (Baas and Qiang, 2005).

Both structure and the polarity of the microtubule serve as a rail on which microtubule associated motor proteins, such as kinesin and dynein superfamily proteins, convey their cargoes (Hirokawa, 1998). Transport occurs along microtubules when the appropriate motor binds to a cargo through its 'tail' and simultaneously binds to the rail through one of its 'heads'. The motor then moves along the rail in such a "walking" manner by using repeated cycles of coordinated binding and unbinding of its two heads, powered by energy derived from hydrolysis of ATP (Mallik and Gross, 2004). Impaired transport may play a role for example; impaired axonal transport of molecules by motor proteins was linked to the pathogenesis of Alzheimer's disease (Stokin and Goldstein, 2006).

1.3 Microtubule Severing

Since dynamic instability is not sufficient to explain the entirety of microtubule behaviors observed in cells, there exists an additional pathway by which microtubule dynamics can be affected: microtubules can be broken or severed along their length (Baas et al., 2005; Quarmby, 2000).

In mitotic cells, microtubule severing on the spindles is thought to contribute to spindle reorganization (Ahmad and Baas, 1995). For example, severing near the centrosome could provide the opportunity for minus-end-directed flux of the mitotic spindle microtubules during metaphase (Mitchison, 1989). Microtubule severing may also play roles in the specific activities on differentiated cells such as myocytes, epithelial cells and neurons (Quarmby and Lohret, 1999; Quarmby, 2000). In postmitotic neurons, microtubules are not used for the formation of a mitotic spindle but rather for the elongation of axons (Karabay et al., 2004). Important events for axonal differentiation such as elongation, branching, navigation, retraction, are accomplished by changes in the configuration and behavior of microtubules (Baas

and Buster, 2004). Microtubule severing in neurons is introduced by suggesting that all neuronal microtubules are nucleated at the centrosome and then relocated to populate locales such as axons and dendtires (Baas et al., 2005).

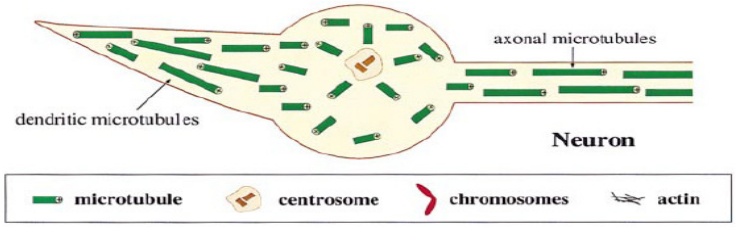

Figure 1.5: Microtubule array of the postmitotic neuron (Baas, 1999).

Microtubule is released from minus end or severed by a severing enzyme and transport of these non-centrosomal microtubules is conveyed by motor proteins (Keating et al, 1997).

1.4 Katanin

Microtubule severing activity is first identified in mitotic extracts of Xenopus laevis eggs (Vale, 1991). Microtubule severing protein is later characterized initially by purification from sea urchin eggs and named katanin that is originated the Japanese word for samurai sword "katana". This heterodimeric protein is a microtubule-stimulated ATPase and that ATP hydrolysis is performed to disassemble stable microtubules (McNally and Vale, 1993).

1.4.1 AAA superfamily ATPases

Katanin is a member of the conserved AAA family of ATPases (Confalonieri and Duguet, 1995). AAA ATPases (**A**TPases **A**ssociated with various cellular **A**ctivities) play important roles in numerous cellular activities including proteolysis, protein folding, membrane trafficking, cytoskeletal regulation, organelle biogenesis, DNA replication and intracellular motility (Vale, 2000). All members of the AAA family are Mg^{2+}dependent ATPases. The AAA motif is defined by a conserved sequence of 230 to 250 amino acids that includes the Walker signature sequences of P-loop ATPases and other regions of similarity unique to AAA proteins. The classical AAA proteins are easily recognized by their strong sequence conservation in this domain (about 30% identity) (Patel and Latterich, 1998; Vale, 2000).

AAA proteins function as an oligomeric ring complex (Hartman et al., 1998), although according to Patel and Latterich (1998) they are also monomers. Katanin exists in equilibrium between monomers and oligomers. In this case of the katanin, the oligometric state has been shown to be hexameric ring where ATP binding induces conformational changes at the interface region which increase interactions between AAA protein and its target (Hartman and Vale, 1999).

1.4.2 Katanin function and regulation

The AAA enzyme katanin breaks microtubules along the length of a microtubule by removing tubulin dimers from the wall of the microtubule. Released tubulin dimers are able to repolymerize again, so they are not proteolyzed or modified by katanin (McNally and Vale, 1993). Microtubules act as a scaffold upon which katanin oligomerizes after it has exchanged its ADP for ATP. As a consequence of ATP hydrolysis and subsequent phosphate release, the katanin undergoes a conformational change leading to destabilization of tubulin-tubulin contacts. The ADP- bound katanin has lower affinity both for other katanin molecules and for tubulin; this leads to the dissolution of the complex and recycling of the katanin (Hartman and Vale, 1999; Quarmby, 2000) (see Fig 1.6). This model suggests several possible points of regulation: a nucleotide-exchange factor could regulate loading of p60 with ATP; accessibility to microtubules could be regulated by removal of protective MAPs; oligomerization sites on p60 could be reversibly masked by regulatory factors; and other factors could stimulate or inhibit ATP hydrolysis and severing (Quarmby, 2000). However, the exact mechanism of microtubule severing is still unknown.

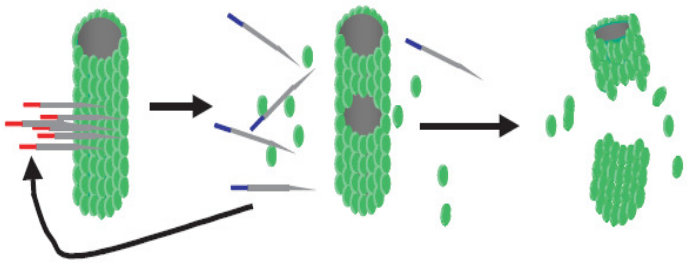

Figure 1.6: Microtubule severing by katanin (swords) (Quarmby 2000).

Katanin display an unusual microtubule- stimulated ATPase reaction in which the activity peaks at a microtubule concentration of 2 to 10 µM tubulin dimers then, decreases as the microtubule concentration is further increased (Hartman and Vale, 1999).

Early studies on katanin earmarked it as a protein that severs microtubules during mitosis. Katanin activity, assessed by the degree to which microtubules were severed, was found to be higher in mitotic extracts than interphase extracts (Vale, 1991). Then, katanin was found to be highly concentrated at centrosomes through the cell division (McNally et al., 1996). Katanin severs microtubules from their γ-TuRC caps and allows minus end depolymerization during mitosis (Buster et al., 2002). These findings support the hypothesis that katanin mediates the disassembly of microtubule minus end during poleward flux (McNally et al., 1996, Quarmby 2000; Buster et al., 2002). However, katanin's role in flux or chromosome motility has not been previously demonstrated. In addition, *Drosophila melanogaster* katanin orthologues appear to function primarily on anaphase chromosomes, where it stimulates microtubule plus-end depolymerization and Pacman-based chromatid motility (Zhang et al., 2007). In Caenorhabditis elegans, katanin is involved in meiosis, allowing the late meiotic spindle shortening (McNally et al., 2006) and increasing the MT density during spindle assembly in acentrosomal female cells from a relatively inefficient chromatin-based MT nucleation pathway (Srayko et al., 2000). Finally, katanin was also shown to participate in cilia biogenesis and in particular in the MT central pair assembly (Quarmby and Lohret, 1999; Sharma et al., 2007).

In neurons, a large number of non-centrosomal microtubules are required for growth and maintenance of neuronal processes. Therefore, microtubule severing by katanin is essential for releasing microtubules from neuronal centrosome, and also for regulating the length of the microtubules after their release (Ahmad et al., 1999). In addition, katanin is likely to be the principal means for generating the short microtubules observed in axonal growth cones and interstitial branch points (Dent et al., 1999) (see Figure 1.7).

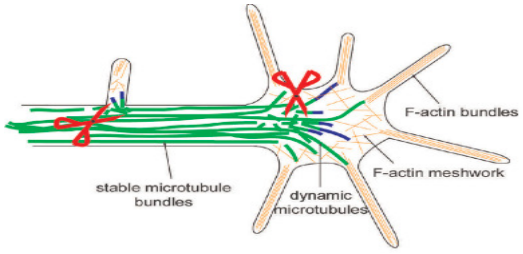

Figure 1.7: Model for microtubule severing in axon. Potential sites of action of microtubule-severing enzymes are indicated by scissors. Microtubules growing from severed ends are shown in dark blue (Roll-Mecak and Vale, 2006).

Since katanin is abundant and widely distributed in neurons, it severs microtubules elsewhere but, it is locally activated and deactivated rather than, or in addition to, being recruited to sites where microtubules need to be severed (Karabay et al., 2004). Potential mechanisms that regulate katanin-mediated microtubule severing was illustrated in vitro (McNally et al., 2002), tight regulation of the levels of katanin was observed in the neuron during axonal growth in vivo and high katanin levels was found in the rapidly growing axons but decreased rapidly once the target cells were introduced (Karabay et al., 2004).

Since there is growing evidence that microtubules can be moved into new patterns of organization by forces generated by molecular motor proteins, a model called 'cut and run' is supported in which long microtubules are stationary, but relatively short microtubules are mobile. In this model, katanin break the lattice of the microtubule polymer in order to mobilize microtubules of cell. After being reorganized, the short microtubules can once again elongate and lose their mobility (Fig. 1.8).

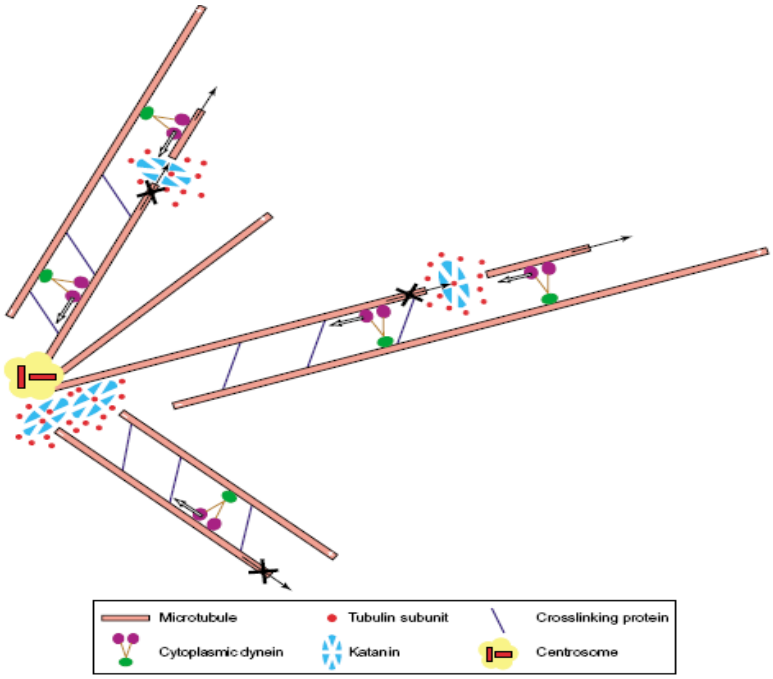

Figure 1.8: The 'cut' and 'run' model for microtubule reconfiguration (Baas et al., 2005).

According to experiments it was suggested that fibrous MAPs protect the lattice of the microtubule from being severed by katanin and phosphorylation of this MAPs results in their release from microtubule and thus enable katanin to gain access. Not all MAPs, but tau was determined that it offers strong protection against severing by

either katanin or spastin that is another microtubule severing protein. Therefore, hyperphosphorylation of tau causes it to dissociate from microtubules so microtubules become more accessible to katanin and this process is also observed in Alzheimer's disease pathology (Fig 1.9).

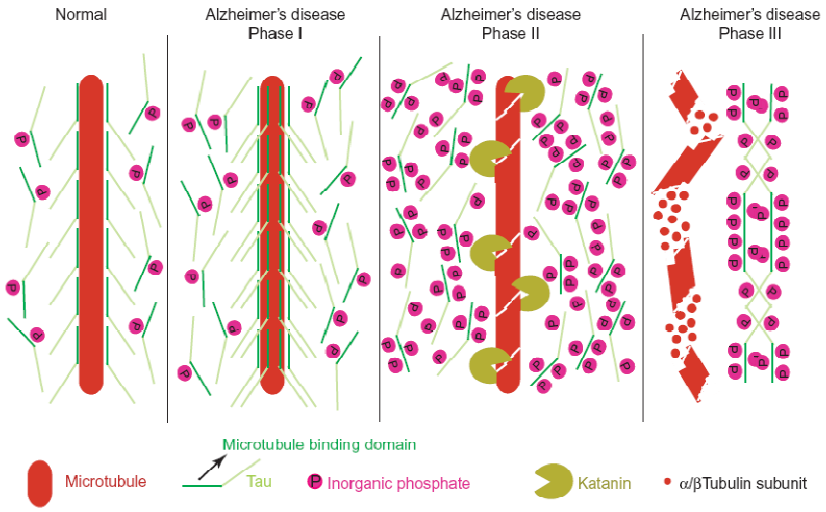

Figure 1.9: Model for microtubule-based axonal degeneration in Alzheimer's disease (Baas and Qiang, 2005).

In a recent research, tau that shields microtubules from severing has been found that its protection is greater in the case of katanin (p60 subunit) than spastin (Yu et al., 2008).

1.4.3 Katanin subunits

Katanin is a heterodimer protein composed of two subunits termed p60 and p80 according to their molecular weight (McNally and Vale, 1993). The 60 kDa subunit is 491 amino acid long polypeptide (Karabay et al., 2004) and composed of an N-terminal domain that binds microtubules (Hartman and Vale, 1999) and a C-terminal domain sharing homology with a large family of ATPases, the AAA family. The 80 kDa subunit is 658 amino acid long (Karabay et al., 2004) and composed of an N-terminal WD40 repeat domain, a central proline-rich domain and a C-terminal domain required for dimerization with the catalytic p60 subunit (Hartman et al., 1998).

It has been found that katanin's p60 subunit exhibits both microtubule-stimulated ATPase activity and microtubule- severing activity in the absence of the p80 subunit The WD40 repeat domain of a human p80 homolog was shown to be sufficient to

target the p80 homolog to interphase centrosomes (Hartman et al., 1998). Then, it was found that the WD40 domain and con80 domain of p80 katanin as well as p60 subunit are required for spindle pole targeting. Therefore, WD40 domain and con80 domain of p80 katanin can enhance p60 mediated microtubule severing by increasing affinity of p60 to microtubules. However, it is also indicated that the WD40 domain of p80 might inhibit the microtubule-severing activity of p60 (McNally et al., 2000).

As mentioned before, in neurons, the levels of P60-katanin, the enzymatic subunit, are very high in axons (Karabay et al., 2004) and they are also higher at the tips of growing neuronal processes at some developmental stages and are globally elevated at the developmental stage corresponding to dendritogenesis. In addition, katanin is typically viewed as a heterodimer, but it has been recently shown that the two subunits are not present within cells at equimolar levels. In fact, the ratio of the two subunits varies markedly in different tissues and at different stages of development, suggesting that the activity of the P60 subunit might be influenced by the levels of the P80 (Yu et al., 2005). These results support either suppressing or augment microtubule severing by different domains of p80 subunit (Baas et al., 2005). In addition, a recent research also reported that a candidate tumor suppressor LAPSER1/LZTS2 (LAPSER1) C terminal domain inhibits katanin-mediated microtubule severing in vitro by binding p80 katanin (Sudo and Maru, 2008).

1.5 The Immune System

The immune system is a bodywide network of cells, tissues, and organs that has evolved to defend the body against such "foreign" invasions. The immune system's job is to keep foreign substances out or, failing that, to seek out and destroy them. The key to a healthy immune system is its remarkable ability to distinguish the structure between the body's own and foreign molecules (Janeway et al., 2005).

The cells in the immune system responsible for specifically targeting and causing the removal of foreign material or antigen are known as lymphocytes. They circulate in blood and lymph and populate areas of the body known as lymphoid tissues which include the spleen, lymph nodes, thymus, tonsils, adenoids, and Peyer's patches, the last three being located along the alimentary tract (Miller, 1996).

An introduction of a stimulus (immunogen or antigen) triggers the immune response to eliminate the provoking agent. An immunogen is a molecule that can induce an immune response in a particular host. The term “antigen” refers to the ability of a molecule to react with the products of adaptive immunity. Therefore, there are two levels of defense against invasion by external agents: innate immunity and adaptive immunity (Stites and Terr, 1991).

Innate or natural immunity is present from birth, lacks specificity and memory, and consists of physical barriers such as skin and mucous membranes, certain enzymes (ex. Lysozyme) and phagocytic cells (ex. macrophages).

The adaptive (or acquired) immune response is triggered by the presence of a foreign agent that escapes early elimination by the innate immune system. The components of the adaptive immunity are lymphocytes (T cells and B cells), plasma cells (end cells of B-lymphocyte differentiation) and antigen- presenting cells (macrophages, B cells and dendritic cells) (Stites and Terr, 1991; Ruebush, 2007). There are two broad immune response classes for adaptive immunity: Antibody responses and cell-mediated immune responses.

In Antibody responses, B cells are activated to secrete antibodies, which are proteins called immunoglobulins. The antibodies circulate in the bloodstream and permeate the other body fluids, where they bind specifically to the foreign antigen that stimulated their production. Binding of an antibody inactivates viruses and microbial toxins by blocking their ability to bind to receptors on host cells.

In cell mediated immune responses, activated T cells react directly against a foreign antigen that is presented to them on the surface of a host cell. The T cell, for example, might kill a virus-infected host cell that has viral antigens on its surface, thereby eliminating the infected cell before the virus had a chance to replicate. In other cases, the T cell produces signal molecules that activate macrophages to destroy the invading microbes that have phagocytosed.

The adaptive immune system can remember prior experiences. Primary immune response is generated for the first exposure of an antigen then, secondary immune response of which lag period is shorter and the response is greater than the primary response if the same antigen is given again after some weeks, months or even years (Alberts et al., 2002).

1.6 Antibodies and Antigens

Humoral immunity is mediated by a family of glycoproteins called antibodies. Antibodies specifically bind antigens in both the recognition phase and the effector phase of humoral immunity. Antibodies are produced in a membrane bound form by B lymphocytes, and these membrane molecules function as B cell receptors for antigens. The interaction of antigen with membrane antibodies on naive B cells initiates B cell responses and thus constitutes the recognition phase of humoral immune responses. Antibodies are also produced in a secreted form by antigen-stimulated B cells.

An antigen is any substance that may be specifically bound by an antibody molecule or T cell receptor. Antibodies can recognize as antigens almost every kind of biologic molecule, including simple intermediary metabolites, sugars, lipids, autacoids, and hormones, as well as macromolecules such as complex carbohydrates, phospholipids, nucleic acids, and proteins. Molecules that stimulate immune responses are called immunogens and all immunogens are also antigens, although the converse is not true.

Only macromolecules are capable of stimulating B lymphocytes to initiate humoral immune responses. Low- molecular- weight (1000 to 10000 Da) compounds, including many drugs and antibiotics are nonimmunogenic so they are coupled to immunogenic proteins in order to generate specific antibodies. In these cases, the small compound is called a hapten, and the macromolecule is called a carrier. The hapten-carrier complex, unlike free hapten, can act as an immunogen (Stites and Terr, 1991; Abbas and Lichtman, 2003; Ruebush, 2007). In addition, the response to an immunogen can be enhanced if it is administered as a mixture with substances called adjuvants (Stites and Terr, 1991).

Macromolecules are usually much bigger than the antigen-binding region of an antibody molecule. Therefore, any antibody binds to only a portion of the macromolecule, which is called a determinant or an epitope. Macromolecules typically contain multiple determinants, some of which may be repeated, and each of which, by definition, can be bound by an antibody (Stites and Terr, 1991; Abbas and Lichtman, 2003).

1.6.1 Molecular structure of the antibody

Proteins that have antibody activity called immunoglobulins. The two hallmarks of immunoglobulins are the specificity of each for one particular antigenic structure and their diversity as a group, which meets the challenge of a vast array of antigenic structures in the environment. Immunoglobulins are glycoproteins composed of 82-96 % polypeptide and 4-18 % carbonhydrate (Stites and Terr, 1991).

Each antibody is an immunoglobulin tetramer consisting of two identical light (L) chains (each containing about 220 amino acids) and two identical heavy (H) chains (each usually containing about 440 amino acids). The four chains are held together by a combination of noncovalent and covalent (disulfide) bonds (Lewin, 2004; Alberts et al., 2002). Light chains and heavy chains share the same general type of organization in which each chain consist of two principal regions: the N-terminal variable region (V region); and the C- terminal constant region (C-region) (Fig. 1.10).

An immunoglobulin has a Y- shaped structure in which the arms of the Y are identical, and each arm has a copy of the variable domain (V) that is generated by association between the variable regions of the light chain and heavy chain. The V domain is responsible for recognizing the antigen. Production of hundreds number of V domains of different specificities creates the ability to respond to diverse antigens so the protein displays the maximum versatility in that region.

The number of constant regions is vastly smaller than the number of variable regions (1-10 C region for any particular type of chain). The constant regions in the subunits of the immunoglobulin tetramer associate to generate several individual C domains that provide the effector response. The first domain results from association of the single constant region of the light chain (C_L) with the C_{H1} part of the heavy- chain constant region. Two copies of this domain complete the arms of the Y- shaped molecule.

The sequences coding for light chains and heavy chains are assembled in the same way: any one of many V gene segments may be joined to any one of a few C gene segments (see Figure 1.10). This somatic recombination occurs in the B lymphocyte in which the antibody is expressed. Therefore, V gene segments are responsible for a major part of the diversity of immunoglobulins (Lewin, 2004).

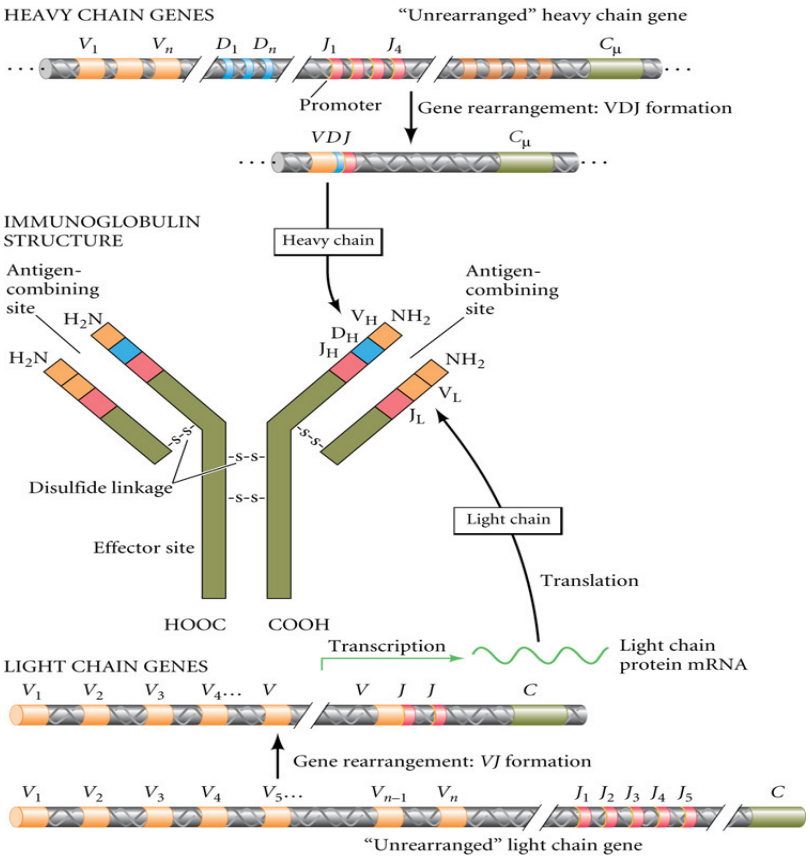

Figure 1.10: Structure of a typical immunoglobulin (antibody) protein. Bottom: Rearrangement of the light chain genes during B lymphocyte differentiation; Top: Rearrangement of the heavy chain genes (Url-1).

1.6.2 Immunoglobulin isotypes and function

In mammals, there are five classes of antibodies, IgA, IgD, IgE, IgG, and IgM, each with its own class of heavy chain- α, δ, ε, γ, and μ, respectively (Alberts et al., 2002).

IgG, which has four subclasses (IgG1, IgG2, IgG3 and IgG4), having $\gamma_1, \gamma_2, \gamma_3, \gamma_4$ heavy chains. IgG constitutes approximately 75 % of the total serum immunoglobulins so it is the dominant class in serum during a secondary immune response and the only one that crosses the placenta and confers immunity on the fetus (called passive immunity). It is also capable of fixing complement and promoting phagocytosis (opsonization) by reactivity with Fc receptors on leukocytes (Minn and Quintans, 1999, Alberts et al., 2002).

IgA is the predominant immunoglobulin class in the mucosal immune system and appears to be an efficient antiviral antibody. IgA exists in two forms, IgA1 (90 %) and IgA2 (10 %) that differ in the structure. IgA1 is composed like other

proteins, however in IgA2 the heavy and light chains are not linked with disulfide but noncovalent bonds.

IgM is prominent in early immune responses to most antigens and predominates in certain antibody responses such as "natural" blood group antibodies. It is also exists in the pentameric form and is the major immunoglobulin expressed on the surface of B cells. IgM has μ heavy chains and is always the first class of antibody made by a developing B cell, although many B cells eventually switch to making other classes of antibody. The immediate precursor of a B cell, called a pre-B cell, initially makes μ chains which associate with so called surrogate light chains and insert into the plasma membrane. The complexes of μ chains and surrogate light chains are required for the cell to progress to next stage of development. The light chains combine with the μ chains, replacing the surrogate light chains, to form four-chain IgM molecules. These molecules then are inserted into the plasma membrane, where they function as receptors for antigen. At this point, the cell is called an immature B cell.

IgD is a monomer and is normally present in serum in trace amounts. IgD (with IgM) is the predominant immunoglobulin on the surface of B lymphocytes at certain stages of their development and it has been suggested that IgD may be involved in the differentiation of these cells.

IgE is responsible for many common allergies. It binds to receptors on mast cells and triggers degranulation of the cells upon contact with antigen. IgE may protect against parasitic infections. The tail region of IgE molecules, which are four-chain monomers, binds with unusually high affinity to Fc receptors of mast cells in tissues and of basophils in the blood. Antigen binding triggers these cells to secrete a variety of cytokines and biologically active amines, especially histamine (Stites and Terr, 1991; Alberts et al., 2002, Abbas and Lichtman, 2003).

1.7 Monoclonal Antibodies

Monoclonal antibodies are produced by hybridoma cell lines and can be grown in tissue culture in the laboratory. Hybridomas are recombinant cell lines produced from the fusion of B cell clones derived from the lymphatic tissue of donor animals and a myeloma cell line that imparts immortality to the cells. Hybridoma Technology is used for production of monoclonal antibodies. In this technique,

the idea is combining two useful cells together. As each hybridoma is descended from a single B cell clone, the antibody expressed by it is of a single specificity and immunoglobulin type, and is thus, termed a monoclonal antibody.

Each monoclonal antibody is monospecific and will recognize only one epitope on the antigen to which it has been raised. This may lead to practical problems if the epitope is not highly conserved on the native protein or where conformational changes may occur due to shifts in pH or other environmental factors. Monoclonal antibodies are highly specific and will rarely if ever produce cross-reactions with other proteins (Burns, 2002).

Because of the high degree of specificity in antibody mediated recognition, the clinical usefulness of monoclonal antibodies has long been recognized. Applications of monoclonal antibodies include cancer, allograft rejection, autoimmunity, tissue damage, diagnostic imaging, etc.

In the case of cancer, antibodies can be used to target specific antigenic determinants found on tumors and mark their destruction. Antibody therapy of cancer relies on enhancing various natural effector mechanisms of the host and/or interfering with cell function (Minn and Quintants, 1996). Some monoclonal antibodies that are used for cancer immunotherapy is illustrated in Figure 1.11.

	Species of origin	Isotype	Toxin	Target	Indication
Unconjugated antibodies					
Trastuzumab[7-10]	Humanised	IgG1	-	HER2/neu	Breast cancer
Rituximab[1,2]	Murine human chimeric	IgG1	-	CD20	Lymphoma
Cetuximab[15]	Murine human chimeric	IgG1	-	EGF receptor	Colorectal cancer
Bevacizumab[11]	Murine human chimeric	IgG1	-	vascular endothelial growth factor	Colorectal, lung, breast cancer
Alemtuzumab[14]	Humanised	IgG1	-	CD52	Chronic lymphocytic leukaemia

Figure 1.11: Therapeutic monoclonal antibodies approved for use in oncology, adapted from Weiner et al. (2009).

In cases of allograft rejection and autoimmune disease, it is feasible to suppress the immune system to the point where it does not reject the targeted tissue. Lymphocyte activation requires T cell receptor engagement and a secondary signal known as costimulation. 0KT3, which is the only murine antibody

approved for clinical use, binds a T cell signaling molecule called CD3 and acts as an immunosuppressant by blocking T cell receptor signaling.

Drug toxicity and gram negative sepsis can be treated by using antibodies that can bind and neutralize the toxin. An antibody against the lipid a domain of endotoxin can reduce mortality in patients with Gram-negative bacteremia. Infiltrating leukocytes secreting cytokines and inflammatory mediators often cause tissue damage. Antibodies specific for leukocyte adhesion molecules such as LFA-1 or ICAM-1, can reduce tissue damage by preventing the accumulation of leukocytes (Minn and Quintants, 1996).

Monoclonal antibodies can be used to treat viral diseases. For example, a monoclonal antibody against shope fibroma virus superoxide dismutase is used in diagnostics and as tools to understand the role of SOD-like proteins in pathogenesis (Shahhosseini et al., 2006).

Researchers use monoclonal antibodies to identify and trace specific cells or molecules in an organism. For example, scientists have produced a monoclonal antibody appears to be a powerful probe in the study of smooth muscle differentiation in normal and pathological conditions (Skalli, 2008).

Monoclonal antibodies that are used in methods of research and clinical applications are summarized in Figure 1.12.

	Applications relative to antigen context		
Purpose	Solubilized	Intact cells/tissue (live/preserved)	Organism (in vivo)
Analysis (qualitative or quantitative)	Immunoblot (Western blot)	FACS[b] analysis	Immunoimaging (SPECT[b] and PET[b])
	Immunoprecipitation		
	Sandwich ELISA[b]	Immunofluorescence	
	ELISPOT[b]		
	Proteomics/antibody microarray	Immunohistochemistry	
	X-ray crystallography		
Purification and/or enrichment	Immunoaffinity purification	FACS and MACS[b]	
Mediation and/or modulation	Catalysis-abzymes	Neutralize activity	Neutralize activity
		Activate signaling	Deplete cell types to alter phenotype
		Proteomics/intrabodies	Immunotherapy

Figure 1.12: Application areas of monoclonal antibodies. [b]ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay; FACS, fluorescence-activated cell scanning; MACS, magnetic-activated cell sorting; PET, positron emission tomography; SPECT, single photon emission computerized tomography (Lipman et al., 2005).

1.7.1 Monoclonal antibody production: hybridoma technology

Tremendous progress in the development, characterization, and manufacturing of monoclonal antibodies (MAbs) has been made since 1976, the year when George J. F. Kohler and Cesar Milstein published their seminar paper on the production of MAbs by producing hybrids between mouse splenocytes with their myeloma fusion partner. Kohler's and Milstein's outstanding contribution, for which they were awarded together with Niels K. Jerne the 1984 Nobel Prize in Physiology or Medicine, and—beyond all—their deliberate decision not to patent the hybridoma technology resulted in the rapid and widespread adoption of MAbs by both academia and industry (Mechetner, 2007).

Hybridomas are hybrid cells derived from the fusion of immortal myeloma cells with B-lymphocytes taken from the spleen of animals immunized with the target antigen. After limiting dilution cloning, hybridomas represent a pure and indefinite source of monoclonal antibodies with the desired target specificity (Chiarella and Fazio, 2008).

In Hybridoma Technology people can take advantage of three pieces of information:

1-) B lymphocytes are blood cells which can produce and secrete antibody for specific epitope, and can have a limited life time up to 4-5 days.

2-) Myeloma cells are the immortal cells that lose their reproduction control mechanism.

3-) By combining two different cells from the same organism in a specific conditions, hybridomas carrying features of the two cell can be produced.

Steps of the hybridoma technology are shown below,

- In order to produce antibody against the desired antigen, mice are immunized with desired antigen. Then, the most actively immunized mouse is selected by ELISA method.
- The most actively immunized mouse's spleen is taken to isolate antibody producing B lymphocyte and myeloma cells are prepared.
- To produce hybridoma, polyethylenglycol (PEG) is used to fuse two types of cells.
- Cells that are subjected to fusion in a culture medium will then be incubated in Hypoxanthine Aminopterin Thymidine medium (HAT medium) waiting unfused

Cells to die. Meanwhile, remained hybridomas are continuing to divide in culture plates.

- Hybridoma clones that produced antibodies for desired antigen are detected by ELISA method.
- In this stage, there can be more than one hybridoma colony in a well which can produce polyclonal antibody. By using "limited dilution" method, selected cells in wells are dispersed to new culture plates in order to get hybrids (immortal B lymphocyte) from the origin of single cell.
- Hybridoma colonies that synthesize specific antibody for desired antigen can be selected by ELISA method.
- Selected hybridomas produce large quantities either in vivo (by formation ofascites in mouse) or in vitro (in cell culture).
- Produced antibodies could be purified with appropriate methods (Saatçılar, 2008).

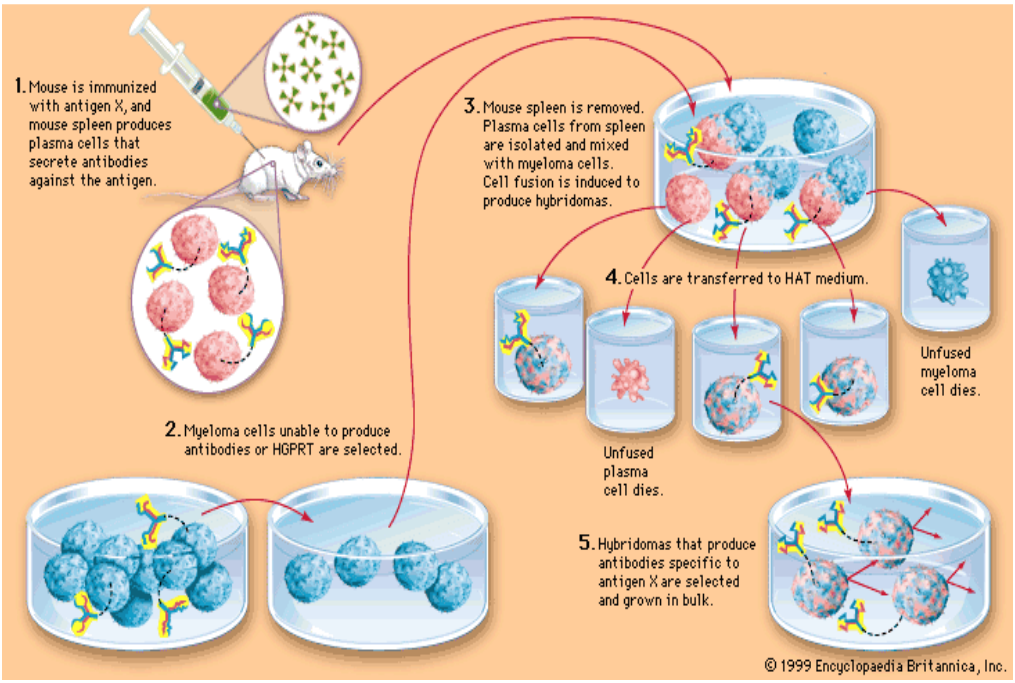

Figure 1.13: General steps of generation monoclonal antibodies, adapted from Url-2.

1.7.1.1 Features of chosen animals for immunization

Mice and rats are preferred for monoclonal antibody production in hybridoma technology since they are easy to obtain, inexpensive to get and they both give good response to immunization. Usually, Balb/c mice strains are used because of their

positive response against to desired antigen, like greater ascites volumes and antibody production (Lidell and Cryer, 1991).

It is crucial to use young adult animals such as 6-8 weeks old whose immune response is robust and not affected by previous immune challenges in order to increase immune response with desired antigen. Although mice are inbred, they are not genetically similar with each other so their immune response will be different. Moreover, mice can die for different reasons in this immunization period. In order to prevent this kind of side effects, 6-8 weeks old mice are selected.

If the sex of the animals is taken into account, female ones are preferred because they can be group housed more successfully than males that are more aggressive and naughty in social interaction (Hau and Hendriksen, 2003). However, male animals are used in some research, as well.

In addition, it is important to consider the health status of animals that are used for production of antibodies since infectious agents may suppress, modulate, or stimulate the immune system. Usage of disease free animals decreases the liability of cross-reactivity to other antigens (Leenaars and Hendriksen, 2005).

1.7.1.2 Features of cells for fusion

The choice of rat or mice strains for use in monoclonal antibody production is normally constrained by the source from which the myeloma cell lines were derived. Therefore, Balb/c mouse strain is preferred. Since, myeloma cell lines have been derived from originally induced such mice; they are so compatible for the propagation of hybridoma cells in vivo.

For monoclonal antibody production, only the myeloma cell lines are used which could not have a capacity to secrete immunoglobulins of their own. It reduces the proportion of hybrids which potentially will secrete lymphocyte derived antibodies.

For the first time, a non-immunoglobulin producing mouse myeloma cell line was isolated by Schulman et al. in 1978. The line, known as Sp/0-Ag14, showed a variable efficiency of fusion although it was recognized as potentially useful partner for generating hybridomas making truly monoclonal antibodies. Kearney et al. identified another mouse myeloma cell line (X63- Ag8- 653) in 1979 that had lost

the capacity for immunoglobulin expression but which still permitted the formation of antibody secreting hybrid cell lines (Lidell and Cryer, 1991).

1.7.1.3 Post fusion selection criteria for cells

As mentioned before, B lymphocytes derived from immunized mice have limited life span in vitro so they need to be immortal. It is accomplished by fusion them with immortal myeloma cells. After this step, it is required to select mixed hybrids other than different hybrid cells in the environment such as B lymphocyte- B lymphocyte hybrid cells, myeloma-myeloma hybrid cells, non-fusion of B lymphocyte cell. In order to select the endless monoclonal antibody production ability of myeloma- B lymphocyte hybrids from others, all the cells are incubated by a selection medium such as HAT (Hypoxanthine Aminopterin Thymidine) medium that is the most common selection medium used in the production of hybridomas.

Normal animal cells can synthesize nucleotides from small-molecule precursors (de novo pathway) or from the partial breakdown products of nucleic acids (salvage pathway). The salvage pathway relies on the presence of the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) (Fig 1.14).

The mouse myeloma cell line P3-X63-Ag8.653 is an HGPRT deficient (HGPRT−) mutant strain. This feature, along with a high fusion frequency, provides this line of cells with a benefit to be used as a fusion partner to generate hybridoma cells. Since HGPRT+ cells are resistant to antifolates such as aminopterin in the presence of hypoxanthine and thymidine, HGPRT− cells are selectively cytotoxic in response to aminopterin (Chung et al., 2000). Therefore, unfused myeloma cells die as they cannot produce nucleotides by de novo or salvage pathway and unfused B cells die as they have a short life span in HAT medium. In this way, only the B-cell-myeloma hybrids survive.

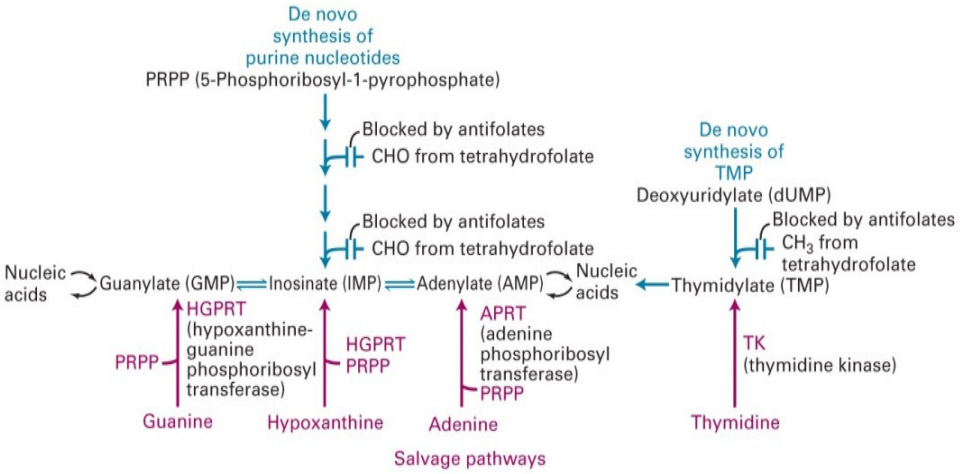

Figure 1.14: De novo and salvage pathways for nucleotide synthesis (Lodish et al., 2003).

After the fusion in HAT culture medium, myeloma/ myeloma hybrid cells and unfusioned myeloma cells disappear from the culture in 8-9 days (Table 1.3). Unfusioned B lymphocyte cells and B lymphocyte/ B lymphocyte hybrid cells die in 4-5 days because of not having immortality feature. Thus, required myeloma/ B lymphocyte hybrid cells are selected from the other hybrids.

Cell type	DNA synthesis		Survival in HAT medium
	Salvage pathway	De novo pathway	
Myeloma	HGPRT⁻	Aminopterin sensitive	Die (No DNA synthesis)
Spleen	HGPRT⁺	Aminopterin sensitive	Die (Finite survival in vitro)
Myeloma–spleen hybrid	HGPRT⁺	Aminopterin sensitive	Live
Myeloma–myeloma hybrid	HGPRT	Aminopterin sensitive	Die (No DNA synthesis)
Spleen–spleen hybrid	HGPRT⁺	Aminopterin sensitive	Die (Finite survival in vitro)

Figure 1.15: Post fusion cell features in HAT medium (Lidell and Cryer, 1991).

1.7.1.4 Immune response detection: ELISA

The initial description of the enzyme-linked immunosorbent assay (ELISA) almost 30 years ago marked a technological advance that has had an immense impact in both clinical diagnostic and basic scientific applications. This assay represents a simple and sensitive technique for specific, quantitative detection of molecules to which an antibody is available (Jordan, 2002). ELISA is a method of quantifying an antigen immobilized on a solid surface by use of a specific antibody with a covalently coupled enzyme. The amount of antibody that binds the antigen is

proportional to the amount of antigen present and is determined by spectrophotometrically measuring the conversion of a clear substrate to a colored product by the coupled enzyme (Abbas and Lichtman, 2003).

ELISA is also appropriate for use for screening hybridoma supernatants. Since cheapness, availability, and stability of substrate are important considerations, the most commonly used enzymes are alkaline phosphatase, β-D-galactosidase, and horseradish peroxidase (Page and Thorpe, 2002).

There are four types of ELISA systems: indirect, direct, competitive and sandwich ELISA (Pierce Handbook, 2009). The principle of these ELISA systems is illustrated in Figure 1.16.

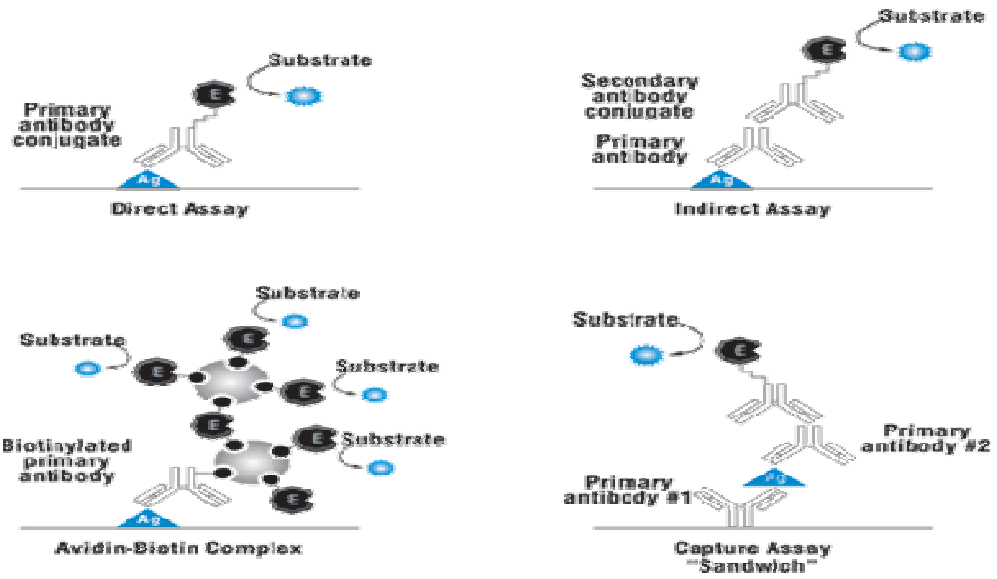

Figure 1.16: ELISA formats (Pierce Handbook, 2009).

1.7.2 Comparing of polyclonal and monoclonal antibodies

The decision regarding whether to use a polyclonal antibody (PAb) or monoclonal antibody (MAb) depends on a number of factors, the most important of which are its intended use and whether the antibody is readily available from commercial suppliers or researchers. PAbs can be generated much more rapidly, at less expense, and with less technical skill than is required to produce MAbs. One can reasonably expect to obtain PAbs within several months of initiating immunizations, whereas the generation of hybridomas and subsequent production of MAbs can take up to a year or longer in some cases, therefore requiring considerably more expense and time.

The principal advantages of MAbs are their homogeneity and consistency. The monospecificity provided by MAbs is useful in evaluating changes in molecular conformation, protein-protein interactions, and phosphorylation states, and in identifying single members of protein families. It also allows for the potential of structural analysis (e.g., x-ray crystallography or gene sequencing) to be determined for the antibody on a molecular level. However, the monospecificity of MAbs may also limit their usefulness. Small changes in the structure of an epitope (e.g., as a consequence of genetic polymorphism, glycosylation, and denaturation) can markedly affect the function of a MAb. For that reason, MAbs should be generated to the state of the antigen to which it will eventually need to bind. In contrast, because PAbs are heterogeneous and recognize a host of antigenic epitopes, the effect of change on a single or small number of epitopes is less likely to be significant. PAbs are also more stable over a broad pH and salt concentration, whereas MAbs can be highly susceptible to small changes in both. Another key advantage of MAbs is that once the desired hybridoma has been generated, MAbs can be generated as a constant and renewable resource. In contrast, PAbs generated to the same antigen using multiple animals will differ among immunized animals, and their avidity may change as they are harvested over time. The quantity of PAbs obtained is limited by the size of the animal and its lifespan. However, the concentration and purity levels of specific antibody are higher in MAbs. In comparison, MAbs generated as ascites or in specialized cell culture vessels are frequently 10-fold higher in concentration and of much higher purity. MAbs are not generally useful for assays that depend on antigen cross-linking (e.g., hemagglutination) unless dimeric or multimeric antigens or antigens bound to a solid phase are used. Additionally, they may not activate complement complement readily because activation requires the close proximity of Fc receptors. Modification of antibodies by covalently linking a fluorochrome or radionuclide may also alter antibody binding. Many of the disadvantages of MAbs can be overcome by pooling and using multiple MAbs of desired specificities. The pooled product is consistent over time and available in limitless quantity (Lipman et al, 2005).

1.8 Aim of the Study

Katanin is a microtubule severing protein consisting of 2 subunits; catalytic p60 subunit and regulatory p80 subunit. Catalytic p60 subunit is a member of AAA ATPases. Regulatory subunit p80 is involved in the targeting of katanin into centrosomal site where it functions.

Katanin and its orthologs are found and a number of organisms: sea urchin*, Xenopus laevis, Drosophila melanogaster, Caenorhabditis elegans,* rat, human. This microtubule severing protein has an important role in processes where microtubules are reorganized.

In mitotic cells, microtubule severing by katanin on the spindles is thought to contribute to spindle reorganization. Microtubule severing may also play roles in the specific activities on differentiated cells such as myocytes, epithelial cells and neurons.

In postmitotic neurons, axonal differentiation such as elongation, branching, navigation, retraction is accomplished by microtubule severing. In addition, tight regulation of the levels of katanin was observed in the neuron during axonal growth in vivo and high katanin levels was found in the rapidly growing axons but decreased rapidly once the target cells were introduced. Katanin levels are also higher at the tips of growing neuronal processes at some developmental stages and are globally elevated at the developmental stage corresponding to dendritogenesis. Therefore, katanin may also comprise a function in regeneration of neuronal injuries. The damaged microtubules may be replaced with healthy microtubules via microtubule severing. Relation of katanin with neurodegenerative diseases is possibly proved with this hypothesis.

Besides the all studies about properties and functions of katanin, it is still not identified interacting proteins of katanin p60. Finding out this issue will help to understand which proteins in signal transduction pathway are involved in the regulation of microtubule severing. Techniques such as immunostaining, western blotting and immunoprecipitation could be used for observing cellular katanin p60. Since these techniques are mainly based on interaction of desired protein and antibody binding, some problems growing out of non-specific or inefficient binding of primary antibody can come out. In order to manage these problems, monospecific

antibodies can be used as primary antibodies. In this case, the amino acid sequence of recombinant katanin p60 should be specific and include an antigenic region. Therefore, produced monoclonal antibodies against this kind of katanin p60 protein are expected to give more efficient results in further observing cellular katanin p60. In addition, the protein that is expressed should be soluble to immunize mice efficiently.

In this study, it is aimed to express recombinant katanin p60 protein with taking into account subjects mentioned above and produce monoclonal antibodies against recombinant katanin p60 by hybridoma technology for further usage in western blotting, immunostaining and immunoprecipitation assays.

1.8.1 *Rattus norvegicus* katanin p60 amino acid sequence

Katanin p60 was cloned and expressed in order to use the protein in monoclonal antibody production. An amino acid sequence of katanin p60 which is specific was selected by using National Center for Biotechnology Information (NCBI) protein blast tool (Url-4). The selected sequence of katanin p60 is 73 amino acid length (amino acids 98-170). Since a polyclonal antibody against katanin p60 (amino acids 115-148) is produced and it recognizes the katanin p60 protein in brain cells efficiently (Karabay et al., 2004), the selected amino acid sequence in this study is thought to have an antigenic region. The sequence of full rat katanin p60 and the selected region is given below.

```
  1 MSLLMITENV KLAREYALLG NYDSAMVYYQ GVLDQINKYL YSVKDTHLHQ KWQQVWQEIN
 61 VEAKHVKEIM KTLESFKLDS TSLKAAQHEL PSSEGEVWSL PVPVERRPLP GPRKRQSTQH
121 SDPKPHSNRP GAVVRAHRPS AQSLHSDRGK AVRSREKKEQ SKGREEKNKL PAAVTEPEAN
181 KFDSTGYDKD LVEALERDII SQNPNVRWYD IADLVEAKKL LQEAVVLPMW MPEFFKGIRR
241 PWKGVLMVGP PGTGKTLLAK AVATECKTTF FNVSSSTLTS KYRGESEKLV RLLFEMARFY
301 SPATIFIDEI DSICSRRGTS EEHEASRRVK AELLVQMDGV GGASENDDPS KMVMVLAATN
361 FPWDIDEALR RRLEKRIYIP LPSAKGREEL LRISLRELEL ADDVNLASIA ENMEGYSGAD
421 ITNVCRDASL MAMRRRIEGL TPEEIRNLSR EEMHMPTTME DFEMALKKVS KSVSAADIER
481 YEKWİVEFGS C
```

Figure 1.17: *Rattus norvegicus* katanin-p60 amino acid sequence (retrieved from NCBI database, accession number NP_001004217). The sequence of p60 that is shown in bold was cloned and expressed; underlined part of p60 is the conserved C- terminal AAA domain.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Lab equipments

The equipments used in the study are given in the table 2.1:

Table 2.1: Laboratory equipments used in the study

Equipment	**Supplier Company**
Centrifuges	Biolab SIGMA 6K15, Beckman Coulter Microfuge®18, Beckman Coulter Avanti™ J-30 I, IECCL10 Centrifuge, Thermo Electron Corporation, Labnet, Labnet International C1301-230V
DNA sequencer	Applied Biosciences 3100 Avant
High pressure steam sterilizer	TOMY SX-700E
Magnetic stirrer	Labworld Standard Unit
pH Meter	Mettler Toledo MP220
Precision Weigher	Precisa 620C SCS
Weigher	Precisa BJ 610 C
UVIPhoto MW Version 99.05 for Windows 95 & 98	UVItec Ltd.
UV Transilluminator	Biorad UV Transilluminator 2000
Electrophoresis equipments	ThermoEC MiniCell® PrimoTM EC320 Electrophoretic Gel System
Power supply	EC250-90 Apparatus Corporation
Microwave	Arçelik MD582
Ice machine	Scotsman AF 10
Vortex	Heidolph, Reaxtop
Water Baths	Memmert, Elektro-mag M 96 KP
Thermomixer	Eppendorf Thermomixer Comfort
Laminar air flow cabinets	FASTER BH-EN 2003
Incubator with CO_2	Biolab SHEL LAB
UVIPhoto MW Version 99.05 for Windows 95 & 98	UVItec Ltd.
UV Transilluminator	Biorad UV Transilluminator 2000
Inverted light microscope	Olympus CK40
Thermal Cycler	Techne TC-3000
Elisa washing device	Nunc-Immuno™ Wash8
SDS-PAGE gel electrophoresis system	BIO-RAD MiniProtean
Power supply	Thermo Electron Corporation EC250-90
Shaker	Forma Orbital Shaker, Thermo Electron Corporation
Freezers	UĞUR (-20°C), UĞUR (+4 °C), New Brunswick Scientific (-80 °C)
UV-Visible Spectrophotometers	uv-1700 PharmaSpec Shimadzu, Thermo scientific nanodrop 1000

Table 2.1 (contd.) : Laboratory equipments used in the study

Microplate Spectrophotometer	BIORAD Benchmark Plus
Pure water system	TKA Wasseraufbereitungssysteme
Mini Blot Module	Thermo Electron Corporation EC140
Syringe filters	0.22µm, TPP
Vacuum filtration- system	150ml, TPP
Pipettes	2.5µl, 10µl, 100µl, 200µl, Eppendorf 1000µl, Finnpipette Thermo
Electronic pipette	Finnpipette Thermo
Multichannel (12 channel) pipette	50-300µl, Genex beta
Microfuge tubes	1.5ml, 2ml, Axygen
Centrifuge tubes	15ml, 50ml Avant Plus
ELISA plate	96well, Nunc
Tissue culture test plates	6 well, 12 well, 96 well, TPP
Tissue culture flasks	25 cm^2, 75cm^2, 150cm^2, TPP
Cell Scraper	30cm, TPP
Hemacytometer	Fisher Scientific
Injectors	2ml, inject, 1ml, tuberculin
Serological pipette	TPP
PVDF membrane	Roche
3MM Whatman Filter Paper	Whatman

2.1.2 Chemicals and enzymes

Chemicals and enzymes used in the study are shown in the table 2.2:

Table 2.2 Chemicals and enzymes

Material	**Supplier Company**
EcoRI restriction enzyme HindIII restriction enzyme MassRuler™ DNA Ladder (Mix, 80bp-10Kb) Mass Ruler Low Range DNA Ladder (80-1031 bp) 10 mM dNTP mix 2 mM dNTP mix 10 X Pfu DNA polymerase Buffer (with MgCl2) 10X Tango Y buffer 25 Mm $MgCl_2$ 10X Y+ Tango buffer 6X Loading dye	Fermentas
T4 DNA Ligase Buffer T4 Ligase NBT/ BCIP Stock Solution	Roche
SeeBlue Plus2 Prestained Protein Ladder	Invitrogen
Primer T7 promoter Primer T7 terminator	Integrated DNA Technologies
IPTG Low melting agarose TEMED	AppliChem
$NaH_2PO_4.2H_2O$ KH_2PO_4 K_2HPO_4 NaAc	J.T. Baker
Tryptone	Lab M ™
Isopopanol Glycerol PEG 8000 Coomassie Brilliant Blue	Fluka

Table 2.2 (contd.): Chemicals and enzymes

Kanamycin Tween20 (P9416) Albumine, bovine (BSA) Freund's complete adjuvant Freund's incomplete adjuvant Anti-Mouse IgG (γ-chain specific)- Alkaline Phosphatase antibody produced in goat Anti-Mouse Polyvalent Immunoglobulins (G,A,M)–Alkaline Phosphatase antibody produced in goat N,N'- Dimethyl- bis- Acrylamide 4-Nitrophenyl phosphate di (tris) salt	Sigma
Anti-His penta monoclonal Mouse antibody 50% Ni-NTA agarose suspension	Qiagen
PIPES Tris Base Acrylamide	BDH Laboratory
DMSO $ZnCL_2$ Acetone Absolute methanol Absolute ethanol	Riedel- de Haën
Diethyl ether	LACHEMA
Glisin Boric acid APS $CaCl_2.2H_2O$ EDTA SDS Glucose Ethidium bromide Yeast Extract Agar KCl Imidazole PEG 4000 DTT HCl Bromophenol blue	Merck
$MgCl_2.6H_2O$ NaCl	CARLO ERBA
Skimmed milk powder	PINAR
DMEM- High Glucose- Liquid Media (SH30243)	HyClone
FBS	Biochrom
Gentamicin HT Supplement (100X) HAT Supplement (50X)	Gibco

2.1.3 Commercial kits

Commercial kits used in this study are given in the table 2.3:

Table 2.3: Commercial kits

Kit	**Supplier Company**
QIAquick PCR purification kit	Qiagen, 28104
QiaPrep Spin Miniprep Kit	Qiagen, 27106
Big Dye Terminator v 3.1 Cycle Sequencing Kit	Applied Biosystems

2.1.4 Buffers and solutions

2.1.4.1 TBE buffer (10X)

Commercially available 10X TBE buffer (Dr. Zeydanlı) was used in agarose gel electrophoresis to prepare the agarose gel that DNA loaded on and as the tank buffer. 10X TBE buffer was used by diluting to 1X TBE buffer with dH_2O.

2.1.4.2 Protein purification buffers

Protein purification buffers were used for purifying proteins from bacterial pellet by metal affinity chromatography. Buffer types and their preparations are given below:

Lysis buffer: 78 miligram (mg) $NaH_2PO_4.2H_2O$ (50mM), 175.4 mg NaCl (300mM) and 6.8 mg imidazole (10 mM) were dissolved in 10 ml distilled water (dH_2O) and pH is adjusted to 8.0 using NaOH.

Wash buffer: 78mg NaH2PO4.2H2O (50 mM), 175.4mg NaCl (300 mM) and 13.6 mg (20mM) were dissolved in 10 ml distilled water (dH_2O) and pH is adjusted to 8.0 using NaOH.

Elution buffer: 39 mg NaH2PO4.2H2O (50 mM), 87.7 mg NaCl (300 mM) and 2.72 gram (g) (4 M) were dissolved in 5 ml distilled water (dH_2O) and pH is adjusted to 8.0 using NaOH.

2.1.4.3 Buffers and solutions for SDS- PAGE analysis

2X sample buffer: 2X sample buffer was used to denature protein samples which were loaded on SDS- polyacrylamide gel. Preparation of 2X sample buffer is shown in Table 2.4.

Table 2.4: Preparation of 2X sample buffer

Content	Concentration	Amount
Tris- HCl pH:6.8	0.125 M	2.5 ml (of 0.5 M)
SDS	4 %	4 ml (of 10%)
Glycerol	20 %	2 ml (of 100 %)
Bromophenol blue	0.05 %	5 mg
DTT	0.15 M	231 mg
dH_2O		up to 10 ml

Tris- Glycine running buffer (pH: 8.3): Tris- glycine buffer was used to reduce pH of the solution changes. Preparation of Tris- glycine buffer is shown Table 2.5.

Table 2.5: Preparation of Tris- Glycine running buffer

Content	Concentration	Amount
Tris- HCl	0.125 M	3 g
Glycine	0.192 M	14.4 g
SDS	0.1 %	10 ml (of 10 %)
dH_2O		up to 1 liter

Coomassie Brilliant Blue (CBB) stain solution: CBB stain solution was used to visualize separated protein bands on SDS polyacrylamide gel. Preparation of CBB stain solution is shown below:

Table 2.6: Preparation of CBB stain solution

Content	Concentration	Volume
CBB R-250	0.1 %	0.5 g
Methanol	50 %	250 ml
Acetic acid	10 %	50 ml
dH_2O		up to 500 ml

Destain solution: Destain solution was used to visualize protein bands clearly by removing the background on SDS polyacrylamide gel. Preparations of CBB destain solution is shown below:

Table 2.7: Preparation of CBB destain solution

Content	Concentration	Volume
Methanol	5 %	50 ml
Acetic acid	10 %	100 ml
dH_2O		up to 1 liter

2.1.4.4 Buffers for western blot analysis

Transfer buffer: Transfer buffer was used to reduce pH of the solution changes during transferring protein bands from SDS polyacrylamid gel to PVDF membrane. Preparation of transfer buffer is given Table 2.8.

Table 2.8: Preparation of transfer buffer

Content	Concentration	Amount
Tris	25 mM	3 g
Glycine	192 mM	14.4 g
Methanol	20 %	200 ml
SDS	0.05 %	0.05 g
dH_2O		1 liter

TBS 1X: TBS (with HCl) was used to maintain the pH within the 7 - 9.2 range during preparing blocking and washing buffers. Preparation of TBS is given Table 2.9.

Table 2.9: Preparation of TBS

Content	Concentration	Amount
NaCl	0.8 %	2.4 g
KCl	0.02 %	0.06 g
Tris	0.3 %	0.9 g
dH_2O		300ml, pH: 7.4

TTBS: Preparation of TTBS is given below:

Table 2.10: Preparation of TTBS

Content	Concentration	Amount
Tween 20	0.05 %	90 µl
TBS		180 ml

NBT/ BCIP substrate buffer: NBT/ BCIP substrate buffer was used for preparing NBT/BCIP staining solution to detect alkaline phosphatase. Preparation of this buffer is given below:

Table 2.11: Preparation of NBT/ BCIP substrate buffer

Content	Concentration	Amount
Tris-HCl pH: 9,5	0.1 M	3 g
NaCl	0.1 M	1.461 g
$MgCl.6H_2O$	0.05 M	2.541 g
dH_2O		250 ml

2.1.4.5 Buffers for cell culture and ELISA assays

PBS buffer: 10 Mm KH_2PO_4 was prepared and 10 mM K_2HPO_4 was added to balance pH of buffer to 7.2. Then, 0.15 M NaCl was added.

PBS- Tween 20 buffer: 0.05 % tween 20 was added to PBS buffer.

PEG solution: 2 g PEG-4000 was mixed with 2 ml dH_2O and then sterilized by autoclave. After sterilization, it was kept at 37^oC.

Substrate buffer: 1 mM ZnCl, 1 mM MgCl and 0.1 M glycine was dissolved in dH_2O and pH was balanced with KOH to 10.4. 1 mg PNPP was added to 1 ml substrate buffer before used and it was kept at dark.

2.1.5 Bacterial strains

Escherichia coli (E.coli) DH5α strain [F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1], Invitrogen.

Escherichia coli strain BL21 (DE3)pLysS F– *dcm ompT hsdS*(rB– mB–) *gal* (DE3)[pLysS Cam_r], Novagen.

2.1.6 Bacterial culture media

LB medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 liter distilled water. The media was sterilized by autoclaving at 121°C for 15 minutes (min). After sterilization, antibiotic was added to the LB medium according to the concentration mentioned in Table 2.12 in order to make selection media.

LB- agar plate was prepared by adding 15 g/L of agar to LB medium and sterilized autoclaving as describe above.

SOC medium was used to cultivate *E. coli* cells for 1 hour after heat shock during transformation. It was prepared by dissolving 2 g tryptone, 5 g yeast extract, 0.058 g NaCl, 0.0186 g KCl, 0.095 g $MgCl_2$, 0.23 $MgSO_4$ and 0.36 g glucose in 100 ml distilled water and sterilized at 121 °C with autoclaving for 15 min.

Table 2.12: Stock and working solutions of antibiotics

Antibiotic	**Stock Solution Concentration**	**Working Solution Concentration**
Kanamycin	30 mg/ml in distilled water	30 µg/ml

2.1.7 T/A cloning vector with cloned insert

pCR 2.1- TOPO (Invitrogen) vector that contains full length of katanin p60 subunit was previously designed by Assoc. Prof. Dr. Arzu Karabay Korkmaz. The cloned katanin p60 subunit was in reverse direction in this vector (see Fig 2.1).

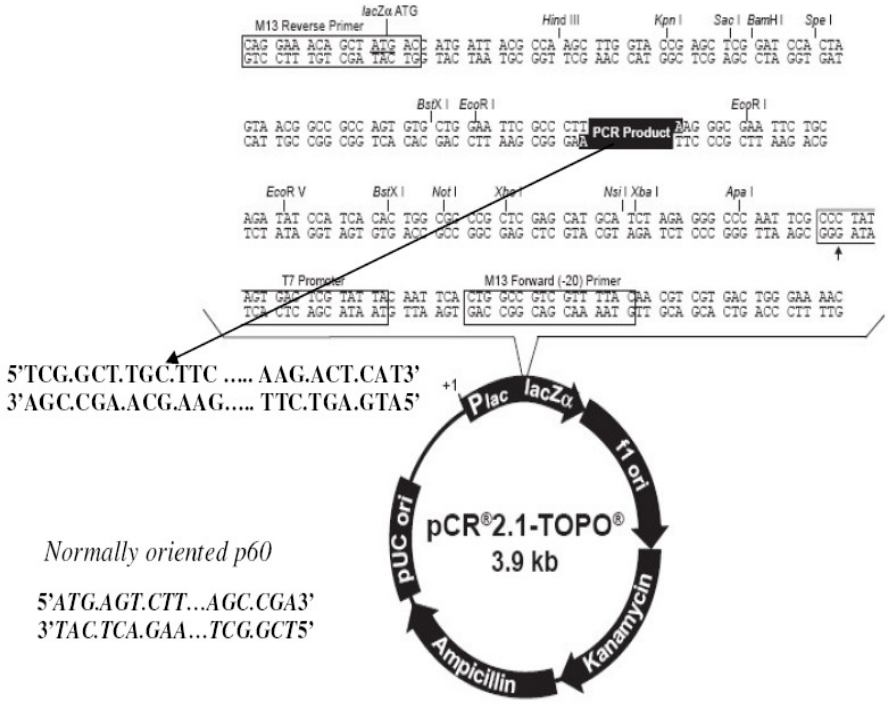

Figure 2.1: Vector map of pCR 2.1- TOPO with cloned insert. Cloning/restriction area with schematically represented insert is shown in a larger scale. Arrow stretching from PCR product point is at the part of cloned p60 subunit (beginning and end of the sequence) in a large scale. At the bottom-left part of the picture part of normally oriented p60 is shown in italic.

2.1.8 Expression vector

2.1.8.1 pET expression system

We have chosen pET expression system which is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli* because large amounts of recombinant protein could be expressed in a short time in this expression system. In addition, this system has the lowest basal expression levels of any *E.Coli* expression system. This issue would be critical if the expressed protein is toxic for the host cell.

Unlike systems based on *E. coli* promoters (e.g., lac, tac, pL), the pET System uses the bacteriophage T7 promoter to direct the expression of target genes. Since *E. coli* RNA polymerase does not recognize the T7 promoter, there is virtually no transcription of the target gene in the absence of a source of T7 RNA polymerase (Mierendorf et al., 1994). Moreover, bacteriophage T7 RNA polymerase is a processive enzyme that will transcribe around a circular plasmid several times and

therefore transcribe genes that are not efficiently transcribed by *E.coli* RNA polymerase (Sambrook and Russell, 2001).

Typically, production hosts contain a prophage (λDE3) encoding the T7 polymerase under control of the IPTG- inducible *lac*UV5 promoter. While this system leads to the synthesis of large amounts of mRNA and in most cases, the concomitant accumulation of the desired protein at very high concentrations (40–50 % of the total cell protein), it is not without drawbacks. For example, high level of mRNA can cause ribosome destruction and cell death, and leaky expression of T7 RNA polymerase may result in plasmid or expression instability. Furthermore, even 'empty' pET plasmids are toxic to *E. coli* in the presence of IPTG. For this reason, target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. For more stringent control, hosts carrying either pLysS or pLysE (Novagen) plasmids are available. The pLys plasmids encode T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase and thus, reduces its ability to transcribe target genes in uninduced cells (Mierendorf et al., 1994; Baneyx, 1999).

Host and vector elements that are avaible for control of T7 RNA polymerase levels and subsequent transcription of a target gene cloned in a pET vector were illustrated in Figure 2.2.

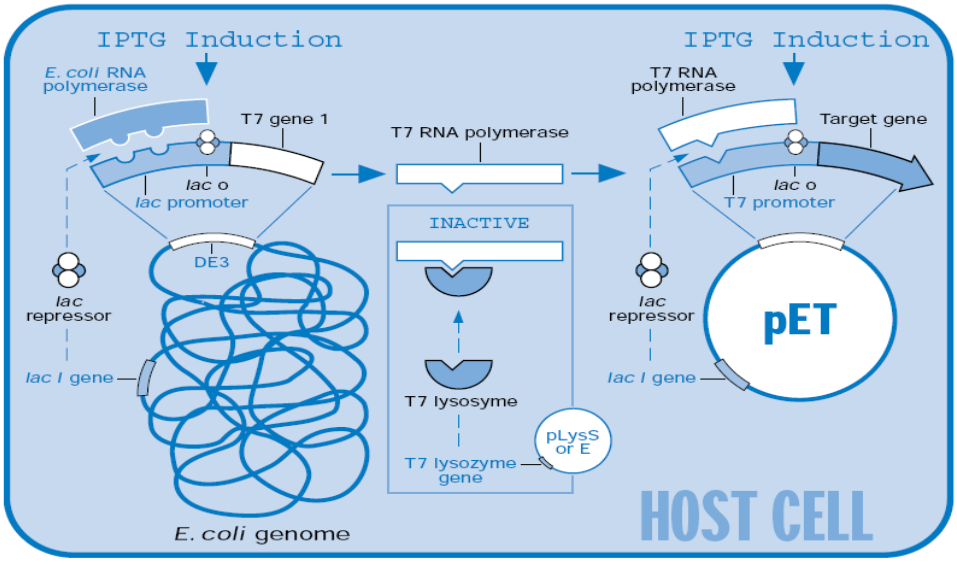

Figure 2.2: Control elements of the pET system (Mierendorf et al., 1994)

2.1.8.2 pET-30a vector

The pET-30a(+) (Novagen) vector was kindly provided by Dr. Stephan Scheurer (Paul-Ehrlich-Institute, Dept. of Allergology). The pET-30a (+) vector carries an N-terminal His•Tag®/thrombin/S•Tag™/enterokinase configuration plus an optional C-terminal His•Tag sequence. The circular map (Fig. 2.3) and the cloning/expression region (Fig. 2.4) are shown below. The vector is 5422 bp long.

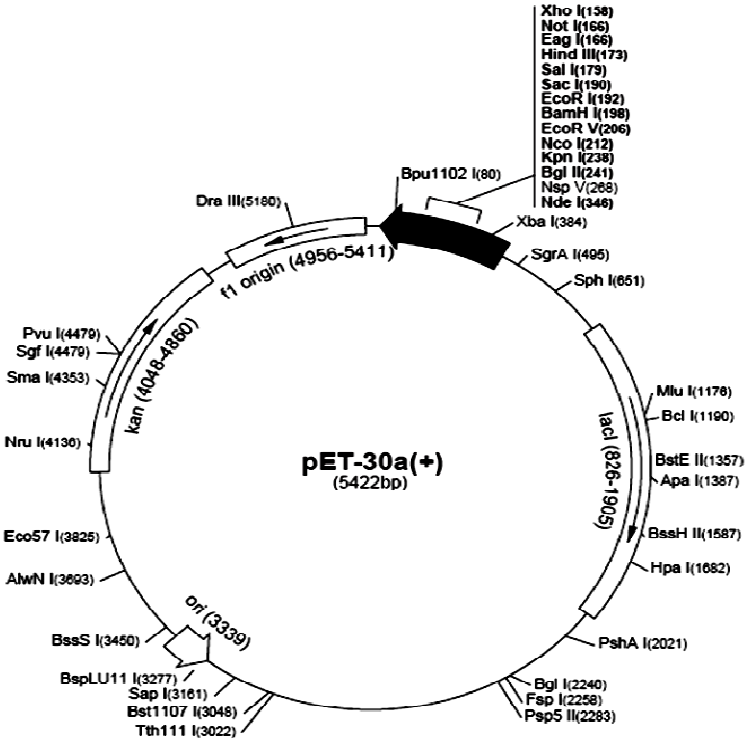

Figure 2.3: Vector map of pET30a (Url-5).

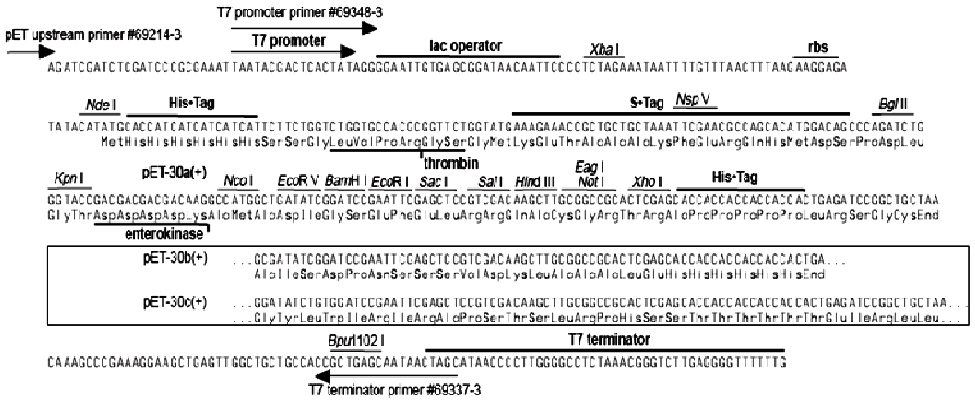

Figure 2.4: Cloning/expression region of the coding strand of pET30a (Url-5).

2.1.9 Cell culture media

Dulbecco's Modified Eagle Medium (DMEM-Gibco) was prepared by dissolving 3.5 g glucose, 2g $NaHCO_3$ and 15.4 g DMEM powder (with 1g/L glucose and 25 mM Hepes) in 1 liter distilled water and adjusted to pH 7.2 by NaOH, then filtered by 0.2 µm filter to obtain sterilization. It was stored at +4°C.

Growing medium was used for F0 myeloma cells and produced hybrid cells from fused F0 cells. It was prepared by mixing 80 % Dulbecco's Modified Eagle Medium (DMEM-Gibco), 20 % Fetal Bovine Serum (FBS-Biochrom) and gentamisin (50µg/ml).

HAT medium was used as a post-fusion selective medium to eliminate unfused or self-fused myeloma cells. It was prepared by adding 40ml/L hypoxanthine aminopterin thymidine (50X HAT) to growing medium and stored at -20°C.

HT medium was used as a rescue medium to overcome the effects of residual intracellular aminopterin. It was prepared by adding 20ml/L hypoxanthine thymidine (100X HT) to growing medium and stored at +4°C.

Freezing medium was used to store F0 myeloma cells, hybrid cells and macrophages at -80°C. It was prepared by mixing 80 % Dulbecco's Modified Eagle Medium (DMEM-Gibco), 20 % Fetal Bovine Serum (FBS-Biochrom) and 10 % DMSO (Dimethyl Sulphoxide).

2.1.10 Cells and antigen

Cell lines used in hybridoma study are described in table 2.13.

Table 2.13: Cell lines that were used in hybridoma production

Cell		**Source**
Myeloma	F0	ATCC CRL-1646
Feeding Cell	Macrophage (cells from peritoneum fluid)	BALB/c mouse
Spleen Cells	B lymphocyte	BALB/c mouse spleen
Lymph Cells	B lymphocyte	BALB/c mouse lymph node

Recombinant katanin p60 antigen was produced by cloning and protein expression assays in this study.

2.1.11 Experimental animals

The 6-8 week old female Balb/c mice were used for immunization.

2.2 Methods

2.2.1 Cloning studies

2.2.1.1 Primer design

In order to clone desired nucleotide sequence of recombinant katanin p60 subunit from *Rattus norvegicus* katanin p60 full nucleotide sequence that was inserted pCR 2.1-TOPO T/A cloning vector to pET-30a expression vector, two primers with appropriate flanking restriction sites were designed since restriction sites in cloning vector are not suitable. During primer design, melting temperatures of primers were selected as close as possible to each other to apply the optimum annealing temperature. GC content of primers was selected fewer than 50 % and hairpin and self dimer formation were checked by IDT Scitools oligo analyzer (Url-6).
Synthesized primers with their properties are given below:

Table 2.14: Recombinant katanin p60 sequence specific primers

Primer	Sequence	Length	Tm	GC content
DF	AAAGAATTCTGGTCTTTGCCGGTAC	25	57,5^{o}C	44.0 %
DR	ATAAAAGCTTTAATTTGTTCTTTTCCTCTCTGC	33	56,7^{o}C	30.3 %

2.2.1.2 PCR

Polymerase chain reaction is an easy technique that is used to amplify specific regions of a DNA strand quickly. Therefore, millions of copies of a specific DNA can be produced by using this technique that is based on enzymatic replication in vitro. Small oligonucleotides called primers are duplicates of nucleotide sequences on either side of the fragment DNA of interest. These primers provide a binding site on the template DNA for DNA polymerases.

In a cycle of PCR, there are three basic steps in PCR. First, the target genetic material must be denatured-that is, the strands of its helix must be unwound and separated-by heating to 90-96°C. The second step is hybridization or annealing, in which the primers bind to their complementary bases on the now single-stranded DNA at an annealing temperature that is based on primers. The third is DNA synthesis by polymerase that is isolated from thermophilic bacteria at extension temperature that is based on used DNA polymerase. Starting from the primer, the polymerase reads the template strand and matches it with complementary nucleotides

very quickly. The result is two new helixes in place of the first, each composed of one of the original strands plus its newly assembled complementary strand.

In order to amplify desired katanin p60 DNA part from the full katanin p60 DNA located in pCR 2.1- TOPO vector, following PCR reaction and PCR program were applied.

Table 2.15: PCR reaction set up for cloning recombinant katanin p60

Content	Volume
Template PCR2.1 TOPO-p60 (385ng/µl)	1 µl
DF primer (25 µM)	1 µl
DR primer (25 µM)	1 µl
10X Pfu Buffer (+ $MgSO_4$)	5 µl
dNTP (2mM)	5 µl
Pfu DNA polymerase (2.5u/µl)	0.5 µl
MQdH2O	36.5 µl
Total reaction:	50 µl

Table 2.16: PCR program for cloning recombinant katanin p60

	Temperature	Time	
Initial denaturation	95 ^oC	2 min	
Denaturation	95 ^oC	30 sec	35 cycles each
Annealing	56 ^oC	30 sec	
Extension	72 ^oC	30 sec	
Final extension	72 ^oC	8 min	

2.2.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis is the most common procedure that is used to separate DNA fragments by their sizes and visualize them. DNA is negatively charged at neutral pH due to its phosphate backbone; hence it migrates to the positive pole in an electric field. In agarose gel electrophoresis, the DNA is forced to move through a sieve of molecular proportions that is made of agarose. The end result is that large pieces of DNA move slower than small pieces of DNA. In this project, 2 % low melting agarose gels were used to estimate the size of DNA molecules following PCR reaction and restriction enzyme digestion.

To prepare 2 % agarose gel, 1 gram (g) of low melting agarose:

- Agarose was dissolved in 50 ml (small gel) 1X TBE (Tris/Borate/EDTA) buffer.
- The agarose was solubilized in a microwave oven until the agarose was completely dissolved.

- Gel was cooled to ≤ 45ºC and ethidium bromide was added to a final concentration of 0.5 µg/ ml and mixed through gentle swirling.
- The agarose gel was then poured into a horizontal gel tray, and a comb for forming the sample slots was placed into the gel.
- The gel was solidified for about 30 min. and then placed into an electrophoresis tank, where the gel was covered by 1X TBE buffer used to make the gel.

The DNA was mixed with 6X loading dye in the proportion of 6:1 and the sample was placed into a well on the agarose gel. For the fragment size control, a MassRuler™ DNA Ladder, Mix (80bp-10kb) was used. Electrophoretic separation was achieved by constant current at 120 mV for 30 minutes.

DNA within agarose gels were visualized under UV light by staining ethidium bromide. The gel was placed onto an UV illuminator that emits UV light at 302 nm and photographed with a camera connected to a computer. Image files were saved with UVIPhotoMW Version 99.05 for Windows 95 & 98, UVItec Ltd. and subsequently analyzed. The size of the DNA was determined by comparing their mobility with the fragments of the DNA ladder.

2.2.1.4 DNA cleanup

In order to purify double-stranded PCR products (100 bp – 10 kb) and also to cleanup the restriction reaction products, QIAquick PCR purification kit was used. It is important to recover DNA and remove contaminants after PCR reactions and restriction reactions in cloning process. The principle of this purification is as followed: DNA adsorbs to the silica membrane in the presence of high concentrations of salt while contaminants pass through the column. Then, impurities are efficiently washed away, and the pure DNA is eluted with Tris buffer or water.

The protocol is given below:

- 5 volumes of Buffer PB to 1 volume of the PCR sample was added and mixed.
- A QIAquick spin column was placed in a provided 2 ml collection tube in a suitable rack.
- To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1 min at 13.000 rpm.

- The flow-through was discarded and the QIAquick column was placed back into the same collection tube.
- To wash, 0.75 ml Buffer PE was added to the QIAquick column and centrifuged for 1 min at 13.000 rpm.
- The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 min at 13.000 rpm.
- The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube.
- To elute DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the center of the QIAquick membrane and the column was centrifuged for 1 min at 13.000 rpm.

2.2.1.5 Determination of DNA concentration

To determine recovery, purity and concentration of nucleic acids, spectrophotometric analysis was used. The ratio of absorptions at 260nm vs 280nm is commonly used to assess the purity of DNA with respect to protein contamination, since protein (in particular, the aromatic amino acids) tends to absorb at 280 nm. According to the literature, the ratio of absorbance (A_{260}/A_{280}) of a pure DNA solution is between 1.8 to 2.0. As protein contamination increases, the ratio decreases.

In order to determine the DNA concentration, DNA solution was diluted 1:200 in distilled water and transferred to a quartz cuvette. The absorption was at wavelength of 260 nm. An optical density (OD) of 260 nm of 1.0 equivalent to 50 ng/ µl DNA. Following formula was used to calculate the concentration (C):

C = OD 260nm x dilution factor x equivalent = x ng/ µl **(2.1)**

In order to check results of spectrophotometric analysis, purified DNA was also run on the 2 % agarose gel before ligation.

2.2.1.6 DNA cleavage by restriction endonucleases

A restriction enzyme also called restriction endonuclease is an enzyme that cuts double-stranded or single stranded DNA at specific recognition nucleotide sequences known as restriction sites that are palindromic in a length of 4 to 8 bases. Such enzymes, found in bacteria and archaea, are thought to have evolved to provide a defense mechanism against invading viruses. To cut the DNA, a restriction enzyme

makes two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix. The purified restriction endonucleases are commercially available, and are used to generate DNA fragments for cloning experiments. Restriction enzymes used in this study were EcoRI and HindIII of which restriction sites were present in both expression vector and flanking region of PCR product (insert) but not found inside of the insert. Plasmid vector was cut at 37^{o}C for 4 hours and insert was cut over night at 37^{o}C with 1X Tango Y buffer (supplied by the manufacturer). Restriction reaction was completed by inactivation at 80^{o}C for 10 minutes and then DNA was purified from restriction mixture by using QIAquick PCR purification kit. Restriction reaction mixtures are given in table 2.17.

Table 2.17: Restriction reaction mixture

Content	Amount	Volume
Plasmid Vector (pET-30a)	5.72 µg	10.5 µl
EcoRI	10 units	1 µl
HindIII	20 units	2 µl
10X Tango/Y Buffer	1X	1.5 µl
Total Reaction Volume		15 µl
Insert DNA	5.46 µg	10.5 µl
EcoRI	10 units	1 µl
HindIII	20 units	2 µl
10X Tango/Y Buffer	1X	1.5 µl
Total Reaction Volume		15 µl

2.2.1.7 Ligation

In order to ligate restricted expression vector (pET-30a) and the insert (katanin p60 fragment), T4 ligase, that catalyzes the formation of phosphodiester bonds between neighboring 3´-hydroxyl- and 5´-phosphate ends in double-stranded DNA, was used. In addition, cohesive ligation type was performed and PEG 8000 was used to improve ligation reaction. To determine insert DNA concentration for ligation, following formula was used:

Amount of insert = insert size/ vector size x 3 (molar ratio of insert/ vector) x amount of vector **(2.2)**

Ligation was performed overnight at room temperature. After overnight incubation, T4 DNA ligase was inactivated by incubation for 10 min at 65°C. Ligation reaction mixture is shown in table 2.18 was used.

Table 2.18 Ligation reaction mixtures

Content	Amount	Volume
Plasmid vector (pET-30a)	300 ng	1 µl
Insert DNA	360 ng	7 µl
10 X T4 Buffer	1X	1,5 µl
T4 Ligase	1 unit	1 µl
PEG 8000 (%50v/w)	10 %	1,5 µl
MQdH2O		3 µl
Total Reaction Volume		15 µl

2.2.1.8 Competent cell preparation- $CaCl_2$ method

Competent cells are bacterial cells that have been chemically treated to allow incorporation of foreign DNA/plasmids. They are critical to carry out transformation because normal *E.coli* cells do not have an ability to take foreign DNA from environment. We homemade competent cells according to the following protocol:

- *E.coli* – DH5α or *E.coli*-BL21 cells were taken from a glycerol stock culture by scraping with a tip and it was directly put in a falcon containing 5ml LB medium and incubated overnight at 37°C in an orbital shaker.

- The following day, 100 ml LB medium was inoculated with 5 ml of overnight culture solution and was incubated at 37°C in an orbital shaker. Cell density was measured several times by a spectrophotometer at OD_{600}. When the OD_{600} reached to 0.6 the bacteria were transferred to 50 ml prechilled sterile ultracentrifuge tubes and incubated on ice for 10 min.

- The cells were spun down at 1600 x g for 7 min. at 4ºC, the supernatant was discarded.

- Each bacterial pellet was resuspended in 10 ml ice-cold $CaCl_2$ and centrifuged for 5 min. at 1600 x g at 4ºC, the supernatant was discarded.

- Each bacterial pellet was resuspended in 10 ml ice-cold $CaCl_2$ and they were incubated on ice for 30 min.

- Centrifugation was performed again at 1600 x g for 5 min. at 4ºC

- Each pellet was resuspended completely in 2 ml of $CaCl_2$.

- The competent cells were distributed into prechilled sterile microfuge tubes each contains 50 µl and they were stored at – 80ºC.

For 50 ml $CaCl_2$ solution following contents with mentioned concentrations and amounts in table 2.19 were used.

Table 2.19: $CaCl_2$ solution preparation

Content	Concentration	Amount
$CaCl_2.2H_2O$	60 mM	0.442 g
PIPES	10 mM	0.15 g
Glycerol	15 %	7.5 ml (from 100 %)
dH_2O	X	up to 50 ml

2.2.1.9 Transformation of competent cells

Transformation is the term used to describe the introduction of plasmid DNA into bacteria. As mentioned above, competent cells were used for transformation. Transformation can be performed by either an electrical or a chemical method. We used the chemical method which consists of a heat shock to introduce the DNA into the host as follows;

- The competent cells were taken from –80^{o}C and thawed on ice. 2 µl of purified plasmid DNA or 15 µl of ligation mixture was added to 20 µl of competent cells and the eppendorf tube containing the cells was incubated on ice for 30 minute (min.).
- Then, heat shock was done by putting the cells in waterbath at 42^o C for 45 second (sec.) and they were then immediately incubated on ice for 2 min.
- 80 µl of SOC medium was added to competent cells and the eppendorf tube was vigorously shaked at 37^o C for 1 hour.
- The cells were then spreaded onto LB plate containing the appropriate antibiotic (kanamycin). The plates were incubated at 37^o C overnight.

2.2.1.10 Colony PCR

After transformation of ligated plasmids, colony PCR was performed to be sure of selecting right colonies which were carrying inserts. Colony PCR is essential because some colonies that gain antibiotic resistance can be empty.

- 10 µl of sterile MQdH_2O was put into PCR tubes for each colony that was taken from the replica plate by sterile tips and incubated at room temperature for 5 minute to drop into water.

- Tubes which contain water and a piece of colony were incubated at 85^{o}C for 5 min to blow up cells and release plasmids.
- Then, PCR mixture mentioned in table 2.20 was added into tubes. The PCR program was the same as given before in table 2.16.

Table 2.20: Colony PCR reaction for recombinant katanin p60

Content	Volume
Template + water	10 µl
DF primer (25 µM)	0.5 µl
DR primer (25 µM)	0.5 µl
10X Taq Buffer	2.5 µl
$MgCl_2$ (25 mM)	1.5 µl
dNTP mix (10 mM)	0.5 µl
Taq polymerase	0.25 µl
MQdH2O	8.75 µl
Total reaction:	25 µl

2.2.1.11 Overnight culture preparation

After identification of the right colonies which contains the inserts by colony PCR, these colonies were taken into the 5 ml LB medium containing 5 µl suitable antibiotic (kanamycin) which was added in order to make selection media. Then, they were incubated overnight with vigorous shaking (200rpm) at 37 ^{o}C.

2.2.1.12 Small scale plasmid DNA preparation

Plasmid preparation was performed using QIAGEN, QIAPrep Spin Miniprep Kit for small-scale (mini) preparations, following instructions of the manufacturer. The principle of this purification is to release plasmid DNA from bacteria by alkaline lysis and to remove all the RNA in the lysate by RNase. Then, since the plasmid DNA has the ability to bind selectively to glass fiber fleece in a centrifuge tube in the presence of a chaotropic salt (guanidine HCl), DNA remains bound. After washing steps performed to remove contaminating bacterial components, low salt elution removes the DNA from the glass fiber fleece.

The protocol of QIAPrep Spin Miniprep Kit, QIAGEN is as follows:

- Overnight culture described before was prepared (see section 2.2.1.11).
- The next day, 5 ml culture which was in 50 ml falcon was centrifugated for 3 minutes at 6800 x g rpm. The supernatants were discarded.

- The bacterial pellet was resuspended in 250 µl buffer P1 which contains RNAse A and the suspension was transferred into an eppendorf tube.
- To lyse the cells, 250 µl P2 buffer was added (contains LyseBlue that leads to turn mixture to blue color if the suspension is done efficiently), mixed by inverting the tube 4- 6 times and incubated at room temperature for up to 5 min.
- Lysis was stopped by addition of 350 µl ice-cold N3 buffer. Tube was again inverted 4-6 times immediately and incubated on ice for up to 5 min.
- The mixture was centrifuged for 10 min. at 13000 rpm and the supernatant was transferred to the QIAprep spin column by pipetting. Chromosomal DNA was precipitated with cellular debris during centrifugation and this supernatant contained the plasmid DNA.
- Again centrifugation for 1 min. at 13000 rpm was performed. Plasmid DNA is bound to the glass fibers pre-packed in the filter tube. Supernatant was discarded from the collection tube.
- To wash the cells, 750 µl of Buffer PE was added to the filter tube and centrifuged at 13000 rpm for 1 min. Supernatant from collection tube was discarded. This step was done twice.
- Tube was further centrifuged at 13000 rpm for additional 1 min. without adding anything.
- The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute the DNA, 50 µl Buffer EB (10 mM Tris•Cl, pH 8.5) was added to the center of the the QIAprep column and the DNA solution was obtained by centrifugation for 1 min. at 13000 rpm.

2.2.1.13 DNA sequencing

In order to confirm recombinant katanin p60 DNA was insert into pET-30a expression vector, sequencing was performed.

PCR for sequencing: Sequencing PCR is special kind of PCR in which single strand of DNA is amplified. Each dNTP is labeled with different fluorescent tag; therefore, resultant DNA fragment is fluorescently labeled. Single strand of DNA is desired to be exploited since two strands apparently interfere the fluorescence of each other.

Thus, only one primer is used in Sequencing PCR. All ingredients are placed on ice and reaction is performed in dark in order to preserve fluorescence.

The plasmids with inserts were verified by DNA sequencing using Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). The sequence reaction was prepared in a sterile PCR tube by adding the compounds shown below:

Table 2.21: Sequence PCR set up

Content	Amount	Volume
Big dye reaction mix	X	2 µl
5X sequence mixture	X	2 µl
Template DNA	150 ng	1 µl
T7 Primer (promoter/ terminator)	25 µM	0,5 µl
dH_2O	X	up to10 µl

The sequence reactions were performed using a thermal cycler with the following program mentioned in table 2.22.

Table 2.22: Sequence PCR program

	Temperature	Time	
Initial denaturation	95 °C	5 min	
Denaturation	95 °C	10 sec	40 cycles each
Annealing	50 °C/55 °C	10 sec	
Extension	60 °C	4 min	
Final extension	60 °C	8 min	

PCR product purification for sequencing: Since PCR product was contaminated with polymerase, a subsequent purification was performed. The protocol is given below:

- 10 µl PCR product was taken in to microfuge tube then 2µl of 3M NaAc and 50 µl ice-cold 95% ethanol were added to tube.
- Tubes were incubated on ice for 30 min.
- Mixture was centrifuged at 14000 rpm for 30 min. Supernatant was discarded, and pellet was resuspended in ice-cold 70 % ethanol. The mixture was vortexed vigorously for 1 min.
- Tubes were centrifuged at 14000 rpm for 30 min. Supernatant was discarded.
- Tubes were incubated at 95°C for 5 min. with caps open (in order to evaporate ethanol)
- 20 µl of formamide was added to DNA pellet and the mixture was vortexed vigorously for 1 min.

- Tubes were again incubated at 95 °C for 5 min. with caps closed.
- Tubes were immediately put on ice and kept at 4 °C until analysis.

2.2.1.14. Alignment of sequence results

Nucleotide alignments were made with EMBOSS Pairwise Alignment Algorithms Tool and NCBI BLAST tool (available online respectively at these web addresses: www.ebi.ac.uk/Tools/emboss/align/index.html and www.ncbi.nlm.gov/BLAST) in order to compare the sequencing results with originally expected one.

2.2.2 Protein expression studies

Recombinant DNA technology is used in protein expression to clone a protein coding DNA (cDNA) into a plasmid vector and express the protein in a host cell. In this study, katanin p60 cDNA was cloned into pET-30a expression vector that is under the control of T7 promoter and BL21 (DE3)pLysS cell strain was used as host in order to express katanin p60 protein. This cell strain is lysogenic for lambda-DE3 which contains the T7 bacteriophage gene 1, encoding T7 polymerase under the control of IPTG inducible lacUV5 promoter. In addition, the protein expression studies of katanin p60 include; determination by SDS-PAGE analysis, purification by metal affinity chromatography and confirmation by western blot method.

2.2.2.1 Protein expression induction

pET-30a vectors with cloned katanin p60 and pET-30a without any insert as control were transformed into BL21(DE3)pLysS competent cells, they were spreaded on LB agar plate containing kanamycin and incubated overnight at 37^{o}C. The next day, a single colony from each plate was taken into 10 ml LB media (with kanamycin) in falcon tube and left for overnight growth at vigorous shaking at 37^{o}C. Next day, 2 ml of overnight culture was diluted with LB media and placed into 50 ml LB media. Culture was incubated with shaking at 37°C until the OD_{600} reaches a value between 0.6 and 0.8. After cells were induced with 0.5 mM IPTG, they are grown for 8 hours with shaking at 37°C. While cells were growing, samples were taken periodically for further analysis.

2.2.2.2 Total cell protein analysis

- At various times (0-8 hours) following induction, 1 ml cell culture for induction analysis was collected by centrifugation for 5 min. at 14000x g in a microfuge.
- Supernatant was discarded. Pellet was mixed with 50 µl 2X SDS sample buffer for SDS-PAGE analysis explained below

2.2.2.3 Soluble total cell protein analysis

- At various times (0-8 hours) following induction, 1 ml cell culture for induction analysis was collected by centrifugation for 5 min. at 14000x g in a microfuge.
- Supernatant was discarded. Cell pellet was completely resuspended in 1 ml of ice-cold 20 mM Tris-HCl pH 7.5.
- Suspension was incubated overnight at -80 °C.
- Next day, it was thawed on ice.
- The entire lysate was centrifuged at 14000×g for 20 min. to separate the soluble and insoluble fractions.
- The supernatant was concentrated with TCA (trichloracetic acid) method which is as follows:
  - 250 µl (1/4 volume) of 100% TCA (w/v) was added to 1 ml of supernatant and vortexed.
  - Sample was incubated on ice for 15 min..
  - Then, it was centrifuged at 14000 x g for 30 min. and the supernatant was removed.
  - Pellet was washed twice with 200 µl of acetone; the mixture was vortexed, centrifuged for 5 min. at 14000 x g and supernatant discarded.
  - Finally, pellet was allowed to air dry thoroughly by leaving the tube open on the bench top.

2.2.2.4 SDS-polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

Almost all analytical electrophoresis of proteins are carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded onto the gel. The denatured polypeptides bind SDS that confers a negative charge to the polypeptide in

proportion to the molecular weight of the polypeptide independently of its sequence. Therefore, SDS-polyacrylamide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide in an electric field.

The effective range of separation of SDS-poyacrylamide depends on the concentration of polyacrylamide with bisacrylamide that forms cross-linking to form pores through which the SDS-polypeptide complexes must pass (Sambrook and Russell, 2001). Therefore, SDS-polyacrylamide gel with 15 % acrylamide concentration which is suitable for separating 10-43 kDa proteins was prepared since molecular weight of expressed katanin p60 protein was expected to be around 16 kDa. Since SDS-PAGE method has a discontinuous system, two gel layer system were used: a stacking gel and a separating gel. This method was performed in a vertical gel chamber. Preparations of gels were mentioned below:

Table 2.23: 15 % separating gel solution (5ml)

Contents	Volume
30 % acrylamide (with 2.7 % bisacrylamide)	2,5ml
1,5M Tris pH:8.8	1,30ml
10 % SDS	50µl
10 % APS	50 µl
TEMED	2 µl
dH_20	1,10ml

Table 2.24: 5 % stacking gel solution (2ml)

Contents	Volume
30 %acrylamide (with 2.7 % bisacrylamide)	330µl
0,5M Tris pH:6.8	250 µl
10 % SDS	20 µl
10 % APS	20 µl
TEMED	2 µl
dH_20	1,4ml

The amounts mentioned above are sufficient to make 1 mini gel in a cassette from Bio-Rad Laboratories, the separating gel solution was applied into the gel cassette up to ± 6.5 cm, and, the last ± 2.5 cm of the cassette was filled with isopropanol, immediately. After the gel was polymerized for at least 20 min., the isopropanol was carefully removed by filter papers. The stacking gel solution was directly poured into the gel cassette, and the gel comb was placed to form the slots. The stacking gel was polymerized for at least 20 min., and used on the same day.

The samples containing proteins were resuspended in 2X sample buffer (see section 2.1.4.3). The samples were denaturated for 5 min. at 95ºC and taken on ice, then 5 µl of each sample was loaded on the SDS PAGE gel. As molecular weight marker, 10µl

SeeBlue Plus2 Prestained Protein Ladder (Invitrogen), containing 10 pre-stained protein bands (8 blue and 2 contrasting colors) in the range of 4-250 kDa, was loaded on the gel.

Electrophoresis was carried out in Tris-glycine electrophoresis buffer (see section 2.1.4.3). The electrophoresis was carried out at 120 V, 200 mA, 25W for 90 min.

After electrophoresis was stopped, SDS-PAGE was stained in stain solution (see section 2.1.4.3) at 65^{o}C for 15 min. with shaking at 50 rpm and then, it was destained in destain solution (see section 2.1.4.3) at 65^{o}C for 30 min. with shaking at 50 rpm to visualize separated protein bands on the gel.

2.2.2.5 Metal affinity purification of 6xHis tagged katanin p60 protein

Immobilized-metal affinity chromatography (IMAC) was first used to purify proteins in 1975 (Porath et al., 1975) using the chelating ligand iminodiacetic acid (IDA). IDA was charged with metal ions such as Zn^{2+}, Cu^{2+}, or Ni^{2+}, and then used to purify a variety of different proteins and peptides (Sulkowski, 1985). IDA has only 3 metal-chelating sites and cannot tightly bind metal ions. Nowadays, Nitrilotriacetic acid (NTA) which is a tetradentate chelating adsorbent has wider use since it occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag (see figure 2.5). NTA binds metal ions far more stable than other available chelating resins (Hochuli, 1989) and retains the ions under a wide variety of conditions, especially under stringent wash conditions. The unique, patented NTA matrices can therefore bind 6xHis-tagged proteins more tightly than IDA matrices, allowing the purification of proteins from less than 1% of the total protein preparation to more than 95% homogeneity in just one step (Janknecht et al., 1991).

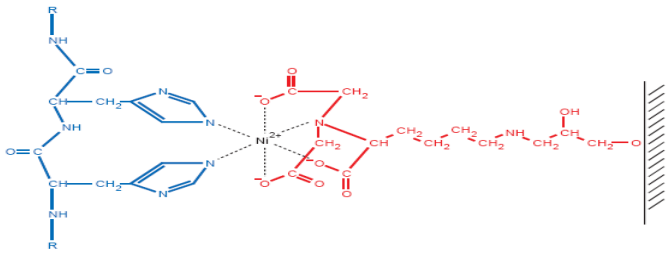

Figure 2.5: Interaction between neighboring residues in the 6xHis tag and Ni-NTA matrix (Qiagen handbook).

When lysate is mixed with Ni-NTA (nickel-nitriloacetic acid) agarose, nickel binds imidazole ring structure of histidine (Fig. 2.6). In elution step, highly concentrated imidazole in the elution buffer replaces histidine and histidine tagged peptide is eluted.

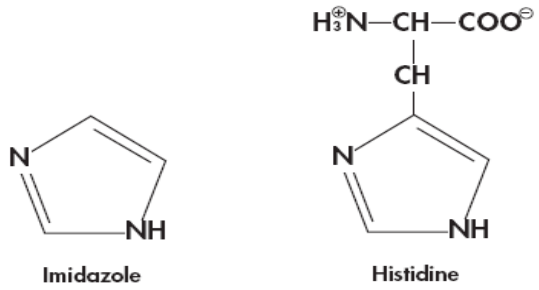

Figure 2.6: Chemical structures of histidine and imidazole (Qiagen handbook).

Purification of katanin p60 protein was made under native conditions and the protocol is given below:

- 500 ml of IPTG induced bacterial culture was grown for 8 hours and centrifuged for 5 min. at 14000 x g.
- The supernatant was discarded and the pellet was frozen overnight at -80^{o}C.
- Next day, it was thawed on ice, resuspended in 5 ml lysis buffer (see section 2.1.4.2) and centrifuged for 10 min. at 14000 x g.
- 15 µl sample of lysate was taken for further SDS-PAGE analysis.
- 500 µl of 50% Ni-NTA agarose was added to lysates and the mixture was gently mixed for an hour in eppendorf tubes at +4^{o}C.
- Then it was centrifuged at 4000 xg for 30 sec. to separate resin and supernatant. The supernatant (which is also called flow-through, since it passes through the resin) was transferred to fresh tubes for SDS-PAGE analysis.
- The resin was washed twice with 2.5 ml of wash buffer (see section 2.1.3 "Buffers"). It was centrifuged at 4000 xg for 30 sec after each wash step and the supernatant was taken for SDS-PAGE analysis.
- Finally, the protein was eluted 4 times with 300 µl elution buffer (see section 2.1.3 "Buffers"). Centrifugation at 4000 xg for 30 sec was done after each elution step and the supernatant.

2.2.2.6 Protein concentration determination

Concentration of the purified katanin p60 protein was determined by Thermo scientific nanodrop 1000 spectrophotometer in DETAM, Istanbul University. Protein concentration was determined by loading a sample droplet (2 µl) onto the optical pedestal. The sample was then drawn into a column and measured at 280nm wavelength.

2.2.2.7 Western blot

Western blotting procedures involve the transfer of proteins that have been separated by gel electrophoresis onto a membrane, followed by immunological detection of these proteins. For immunological detection two layers of antibody are utilized. The primary antibody is directed against the target antigen. The secondary antibody is specific for the primary antibody and it is usually conjugated to an enzyme such as alkaline phosphatase (AP) or horseradish peroxidase (HRP), and an enzyme-substrate reaction is part of the detection process. Antibody incubations are generally carried out in antibody buffer containing Tris buffered saline with Tween (TTBS) and a blocking reagent. Colorimetric AP systems use soluble 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as substrates to produce a stable reaction product (indigo dye and insoluble formazan) that will not fade.
For western blotting of katanin p60 protein, the following protocol was applied:

- SDS-PAGE was done. 15 % separating gel was prepared. 20 µg sample was loaded onto gel.
- Electrophoresis was done until all of the dye (sample buffer) has left the gel.
- Then, the current was shut down.
- The PVDF membrane was prepared for Western blot, i.e. it was soaked in methanol and immediately placed into transfer buffer and kept there for 10 min.
- The gel was placed in the “sandwich” chamber with 3 fiber pads and 2 filter papers and prepared PVDF membrane (2 fiber pads/ filter paper/ PVDF membrane/ gel/ filter paper/ fiber pad) all soaking in transfer buffer.
- The trans-blot was run at 4° C (in the cold room) at 20 V for 2 hours.
- The membrane was blocked with blocking solution (see table 2.25) on the shaker overnight at 4° C (in the cold room). Proteins in BSA containing solution block unoccupied sites on the membrane.

- Then, it was washed for 10 min. with TTBS solution.
- After that, it was incubated with primary antibody in blocking buffer (see table 2.26) at room temperature for 2 hours on the shaker.
- After that, the membrane was washed twice for 10 min. with TTBS.
- Then, the membrane was incubated with secondary antibody in blocking solution (see table2.27) for 1.5 hour at room temperature.
- After that, it was washed twice for 10 min. with TTBS.
- It was washed again for 10 min. with TBS in order to get rid of the detergent that was present in TTBS.
- Finally, membrane was soaked into NBT/BCIP solution (200µl NBT/BCIP stock solution+ 10 ml NBT/BCIP buffer) and kept until the bands were seen. This last step was performed in the dark room. The reaction was stopped by adding water.
- Membrane was dried at room temperature in the dark room.

Table 2.25: Blocking Solution

Content	Concentration	Amount
BSA	3 %	900 mg
TBS		30 ml

Table 2.26: First antibody solution

Content	Concentration	Amount
Anti-His Penta (Mouse)	200µg/ml	5 µl
BSA	3 %	300 mg
TTBS		10 ml

Table 2.27: Second antibody solution

Content	Concentration	Amount
Goat(anti-mouse) IgG AP conjugate	1:5000	2 µl
Skim milk	3 %	300 mg
TTBS		10 ml

2.2.3 Monoclonal antibody production studies

In order to produce monoclonal antibodies against expressed katanin p60 protein, hybridoma technology that is a process of hybridization of antibody secreting lymphocytes with tumor cells (myeloma) was used. Monoclonal antibody production studies include immunization of mice with katanin p60 antigen, immune response control, fusion and cell culture studies.

2.2.3.1 In vivo immunization procedure

6-8 weeks old Balb/c mice species were used for immunization. Efficiency of immunization is based on type; structure and molecular weight of the antigen (see section 1.6). Adjuvants were used in conjugation with the antigen to alter the immune response of mice quantitatively and/or qualitatively. Therefore, immunization with soluble recombinant katanin p60 antigen (60µg /200 µl) was applied 3 times at days 0, 21 and 45 using complete Freund's adjuvant (FCA- a mineral oil containing heat-killed mycobacterium) for the first immunization and incomplete Freund's adjuvant (IFA- oil emulsion not containing bacteria) for the second immunization. 4 days before fusion as a last reminder, antigen in PBS pH 7.2 (25µg/200µl) was injected intravenous, intraperitoneal, hand-foot pad of the mouse.

For negative control mouse, PBS and adjuvant were mixed in 1:1 dilution and injected intraperitoneally. Mice injection methods were detailed in table 2.28.

Table 2.28: Mice injection methods and dates

Mouse Type	Injection repeat	Injection area	Injection Contents	Date
Immunized by recombinant katanin p60 (nrecp60)	**1th**	Intraperitoneal	60µg nrecp60/200µl FCA+PBS	27.11.2008
	2nd	Intraperitoneal	60µg nrecp60/200µl IFA+PBS	18.12.2008
	3rd	Intraperitoneal, intravenous, hand-foot pads	25µg nrecp60/ 200µl PBS	12.01.2009
(-) Control	**1th**	Intraperitoneal	100µl FCA+100µl PBS	27.11.2008
	2nd	Intraperitoneal	100µl IFA+100µl PBS	18.12.2008
	3rd	Intraperitoneal	200µl PBS	12.01.2009

After ten days from the second immunization, blood was taken from mice's eyes (retro-orbital space) with hematocrit tube containing heparin as an anticoagulant to screen mice for antibody responses and detect the efficiency of antigen dose. The mouse giving the most effective antibody response was selected for the fusion by indirect ELISA test. In addition, blood was collected from the mouse's heart to test activity of immune response in the day of fusion. Blood samples were centrifuged at 10.000xg for 10 min. to gain sera in supernatant.

2.2.3.2 Immune response control

In order to detect animal's immune response, Enzyme-linked immunosorbent assay (ELISA) method was used. Mice which were immunized by recombinant katanin

p60, their serum antibody titer was determined with indirect- ELISA method (Lipman et al., 2005). The protocol for indirect-ELISA method is given below:

- 96 well ELISA plates were coated with 100 µl antigen solution. (Antigen amount for nrecp60; 1.2 µg/ml). Elisa plates were incubated overnight at 4°C.
- Next day, plates were washed 3 times with PBS- Tween 20 (PBS-T) using Nunc-Immuno™ Wash8.
- Plates were blocked with 1 %, 200 µl skim milk solution and incubated at 37°C for 1 hour.
- Plates were washed 3 times with PBS-T using Nunc-Immuno™ Wash8.
- Hybridoma supernatants and mice sera were added separately in plates and incubated at either 37°C for 1 hour or overnight at 4°C. Supernatants of hybridomas were taken with micropipette and coated with an amount of 100 µl directly to the plate. Mice serums were diluted with PBS.
- Plates were washed 3 times with PBS-T using Nunc-Immuno™ Wash8.
- Wells were coated with anti-mouse polyvalent immunoglobulins (IgG, IgA, IgM) or IgG (γ-chain specific) joined with alkaline phosphatase. Before this solution was coated on plates, it was diluted 1/2000 or 1/1000 with PBS and incubated at 37°C for 1 hour.
- Plates were washed 5 times with PBS-T.
- After that, substrate buffer was added and incubated up to 1 hour in the dark at room temperature. Antibody level was detected at 405 nm by microplate spectrophotometer.

2.2.3.3 Cell culture studies

Transferring cells from -80°C to culture flasks: Cells which had been stored in -80°C were thawed in 37°C water bath and taken in to 10 ml PBS buffer for centrifugation for 5 min. at 1400 rpm. Cell pellet was dissolved with DMEM medium and transferred to the culture flasks.

Cell counting: Cell pellet was suspended with 20 ml PBS. Then 10 µl suspension was taken and put onto the hemacytometre, cells were counted as the number of cells per 25 square ($1mm^2$). The formula of cell number calculation is given below:

10^4 (constant number) X Amount of Counted Cell = Cell Number per ml

Total cell number= Cell Number per ml X Total Volume of Cells (20ml) **(2.3)**

Cell passage: Cells in the flasks or dishes were in growing medium. Growing medium was removed and 10-15 ml PBS was added. Cells that stuck on the culture dish were lifted via cell scraper. Suspended cells were put into the centrifuge tubes and spun at 900 rpm. After the centrifugation, supernatant was discarded and pellet was resuspended with PBS and again centrifuged.

Cell freezing: After the cell passage, cells were taken into the centrifuge tubes and they were washed with PBS. When the washing step was finished, cell pellet was transferred to freezing tubes with freezing medium for future applications. Estimated cell number for per freezing tube was $2x10^6$ cell/ml.

2.2.3.4 Preparation for fusion

Preparation of feeding cells: Feeder cells were prepared a day before the fusion. Unimmunized, normal mouse was used. After the mouse was anesthetized by ether, dislocation method was applied to the unimmunized mouse and then, it was disinfected in 70 % alcohol. Mouse was taken into laminar flow, was placed on the dissecting board in the dorsal position. Abdomen skin of mouse was separated from the underlying musculature to reveal peritoneum. 5 ml DMEM was injected to the peritoneum and after a few minutes, DMEM was taken back with feeding cell mixture from the peritoneum inner membrane. Feeding cell mixture was taken to the sterile tube and cell number was detected by cell counting. Desired cell concentration per well in cell culture plate is ~6000 cell/100µl.

Obtaining spleen cells from immunized mouse: Among the mice, highest immune response giving was selected by indirect- ELISA test and was killed by dislocation method after it was anesthetized by ether. The mouse was sterilized with 70 % ethanol. Mouse was taken into laminar flow. The animal was laid on its backside. With the sterile scissors, left-sided skin incision was made, midway between the last rib and the hip joint. 1 to 2 cm incision was made in the peritoneal wall and spleen was pulled gently onto the exterior surface of the peritoneum. Any connective tissue under the skin was annihilated using the blunt end of the sterile forceps. The spleen was removed from the body. Spleen tissue was put into petri dishes containing 5 ml PBS buffer in order to purify it from the fatty tissues. Spleen tissues were crushed onto strainer with glass Baggett. After the spleen tissues were crushed, they were revealed and suspended with the help of pipette. Cells were washed 2 times with 20

ml PBS buffer at 1400 rpm for 5-10 min. Pellet was dissolved in 10 ml medium and cell number was determined by cell counting. Spleen cells are shown in figure 2.7.

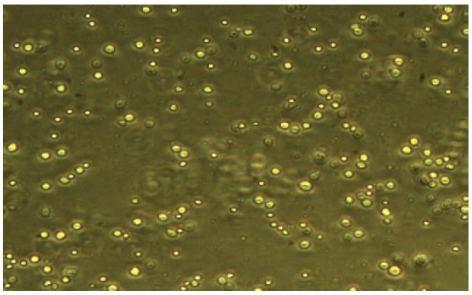

Figure 2.7: Image of spleen cells, adapted from Saatçılar (2008).

Obtaining lymph nodes from immunized mouse: The highest immune response giving among the mice was selected by indirect- ELISA test and was killed by dislocation method after it was anesthetized by ether. The mouse was sterilized with 70 % ethanol. Mouse was taken into laminar flow. Incision was started from genital region to lower (inferior) chin. Lymph nodes from chin and armpit, one pair on opened skin and groin, 4 nodes on intestine, 3 nodes on back, were collected (Fig. 2.8). Removed lymph nodes were taken to the petri dish and mechanical force was applied to revealed lymph cells. After suspension, cells were counted. In this study, both lymph nodes and spleen tissue were collected at the same time. First, mouse was incised for getting the spleen tissue after that the lymph nodes were removed.

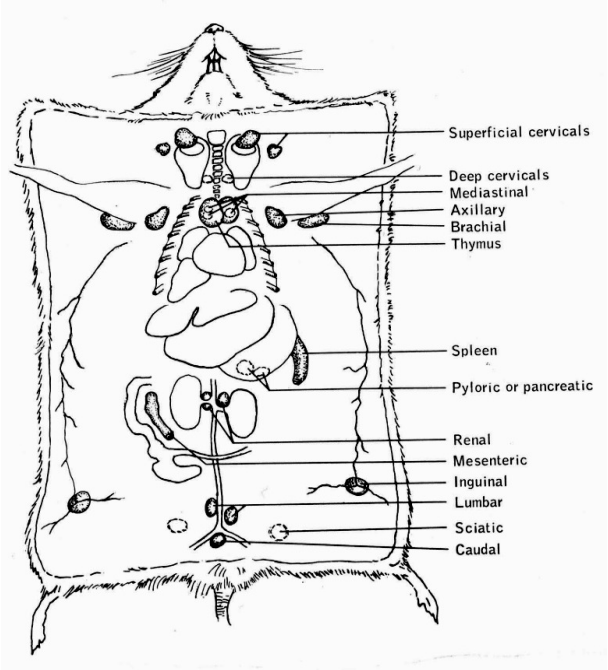

Figure 2.8: Scheme reporting localization of the lymphatic system (Url-3).

Preparation of myeloma cells: Ten days before the fusion, F0 myeloma cells were started to be cultivated in normal medium (Fig 2.9). Beginning of cell reproduction studies, 20 µg/ml azoguanin was added to the medium for remove HGPRT (-) mutant cells. In the fusion day, myeloma cells were removed from the flasks by making passage and cells were washed 2 times with PBS buffer while centrifugation at 1400 rpm for 5 min. Pellet was taken into 20 ml medium and cell number was determined by cell counting. Cells were held in 5 % CO_2 incubator while preparing spleen cells and lymph nodes for fusion.

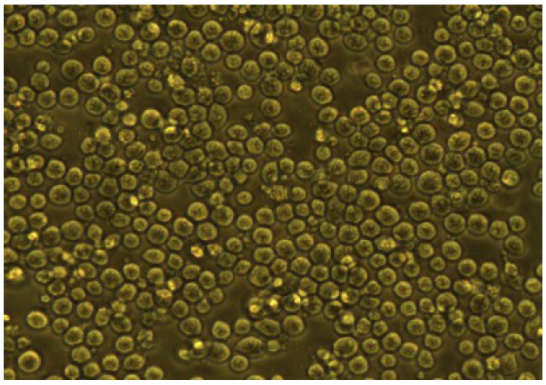

Figure 2.9: Image of F0 myeloma cells, adapted from Saatçılar (2008).

2.2.3.5 Fusion

Myeloma/ spleen and myeloma/ lymph cells were combined in proportion to 1/2 and centrifuged at 1400 rpm for 5-10 min. After the centrifugation, supernatants were removed and pellets were taken. 1 ml (for spleen cells) and 500 µl (for lymph cells) polyethylene glycol (PEG) 4000 which was sterile and heated at 37^{o}C was added to the precipitated cells drop by drop within 1 min. and mixed gently within another 1 min. 4 ml (for spleen cells) and 2 ml (for lymph cells) DMEM was added within 1 min. 20 ml (for spleen cells) and 10 ml (for lymph cells) DMEM was added within 2 min. Finally, 20 ml (for spleen cells) and 10 ml (for lymph cells) DMEM that contains 15 % FBS was added within 2 min. Mixture was held an hour at 37^{o}C in 5 % CO_2 incubator after that they were taken to the centrifuge at 800 rpm for 5 min. After the centrifugation, pellet was suspended with HAT culture medium and the formed mixture was dispersed to the cell culture plates which contained feeder cells that had been prepared one day before the fusion as a final amount 150µl/well.

2.2.3.6 Following culture after fusion

Six days and ten days after the fusion, 120µl/ well was removed from the cell culture plates and in place of removed medium, 120µl/ well HAT culture medium was poured. Fourteen days after the fusion, HT medium was poured with the same method. Growing medium was applied according to period of cells' growth.

In ten days period, cells had active, big and clear images and some of them could produce clones (Fig. 2.10). Cell culture wells were screened one by one by light microscope in order to determine hybrid cells. Then, hybrid cells which produced antibody against recombinant katanin p60 were selected by indirect ELISA method.

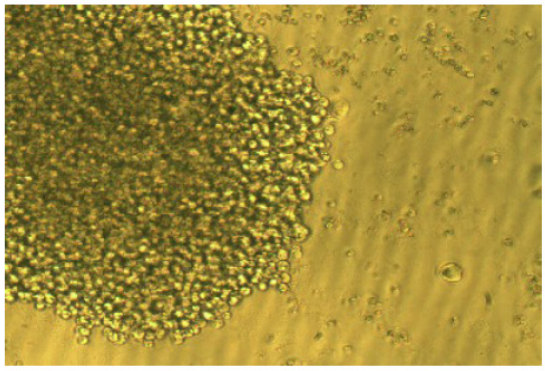

Figure 2.10: Cell mixture after 10 days HAT medium (Saatçılar, 2008).

2.2.3.7 Subcloning of hybrid cell (Limiting dilution)

Monoclonal antibodies are secreted by progeny of a single cell that can produce only a single antibody (assuming a nonsecretory fusion-partner line). Subcloning is required to ensure that the problems of polyspecificity are avoided and the risks of overgrowth by nonproducing cells are minimized. The procedure involves setting up cell cultures with graded dilutions of the cell suspension resulting in a mean of one cell per well (Liddell and Cryer, 1991).

In this study, cells that were determined to give desired antibody were recloned for the purpose mentioned above. Recloned cells were frozen and stored. The recloning was repeated at least 3 times after which the final clone plate displayed positive antibody production.

2.2.3.8 Large scale production of hybridomas in vitro

Hybrids were transferred to the different sized culture flasks to have high amount of antibody yield. For that reason, hybrids were cultivated in 25 cm^2 and 75 cm^2 culture flasks. After cell growing, supernatants containing desired antibody were collected in tubes.

2.2.3.9 Subtyping of monoclonal antibodies

In this study, ELISA test and IsoStrip mouse monoclonal antibody isotyping kit (Roche, cat. no: 1149027001) were used to determine the subtype of desired monoclonal antibodies. The kit procedure was performed in the Institute for Genetic Engineering and Biotechnology Division of The Scientific and Technological Research Council of Turkey- Marmara Research Center (TUBITAK-MAM).

3. RESULTS

In order to produce monoclonal antibody against recombinant katanin p60, studies were performed in three steps which were cloning of selected katanin p60 sequence, expression of recombinant katanin p60 and producing monoclonal antibody against this protein.

3.1 Cloning of Katanin p60

Katanin p60 cDNA was present as cloned into pCR2.1- TOPO (Invitrogen) vector. Primers with flanking regions containing appropriate restriction enzyme sites were designed to specifically amplify selected a region (219 bp) of katanin p60 cDNA. Moreover, primers were designed according to *Rattus norvecigus* katanin p60 mRNA coding sequence data was taken from http://www.ncbi.nih.gov/ NM_001004217.

PCR amplification of katanin p60 with designed primers successfully resulted in 238 bp product (Fig. 3.1).

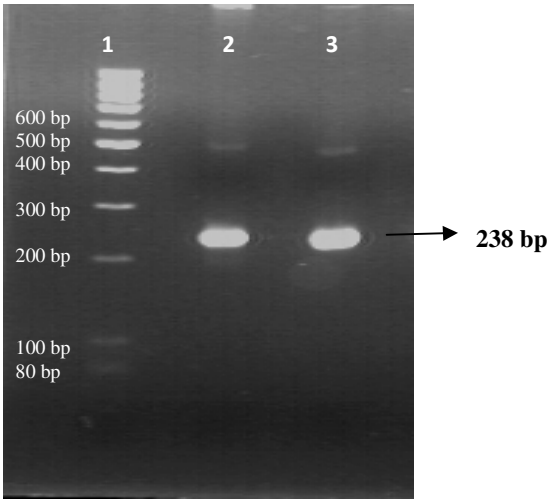

Figure 3.1: 2% gel electrophoresis showing the result of PCR for katanin p60 (Lane 1: Fermentas Mass Ruler Low Range DNA Marker, Lane 2 and 3: p60 PCR products)

Following PCR, DNA fragments were purified with QIAquick PCR purification kit and the concentration of DNA was checked on a 2 % agarose gel. The concentration was also checked by spectrophotometer at OD_{260}.

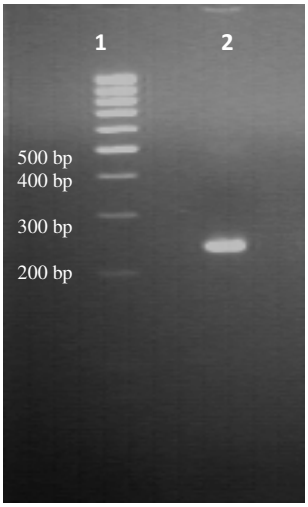

Figure 3.2: PCR product purification results**.** Lane 1- Fermentas Mass Ruler Low Range DNA Marker and lane 2- Katanin p60 cDNA.

Purified katanin p60 DNA concentration was calculated as follows:

C = OD 260nm x dilution factor x equivalent (50 ng/µl) = x ng/ µl (3.1)

A_{260}= 0.0529

C= 0.0529x200x50= 529 ng/µl

Amplified and purified katanin p60 cDNA was designed to be inserted into pET-30a expression vector. Therefore, cDNA and vector were restricted with HindIII and EcoRI enzymes, Fermentas (Fig. 3.3).

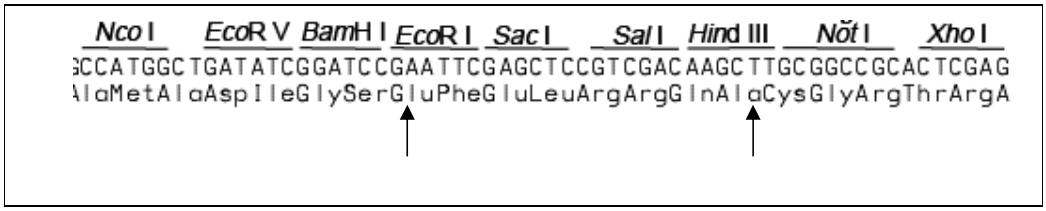

Figure 3.3: Multiple cloning site map for pET-30a expression vector, restriction sites are marked with arrows.

Restricted DNA fragments were purified by QIAquick PCR purification kit. Before ligation, they were run on agarose gel in order to check restriction and the amount of DNAs were sufficient for ligation after purification (Fig. 3.4).

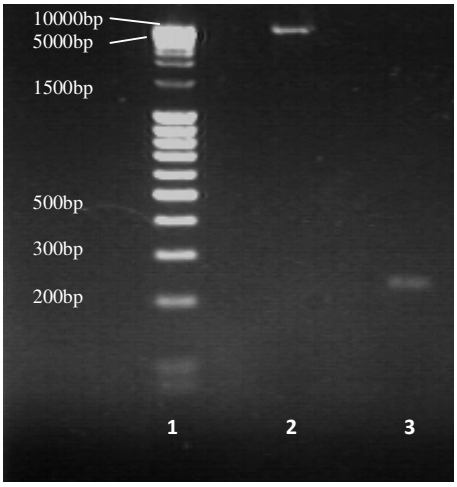

Figure 3.4: 2 % agarose gel electrophoresis showing restricted and purified insert (219bp) and vector (5422bp) DNAs. Lane1-Fermentas Mass Ruler Mix DNA Marker, Lane 2-pET30a vector, Lane 3-katanin p60.

After that, ligation procedure was performed with Roche T4 DNA ligase overnight at room temperature and the ligation mixture was then transformed into competent *E. coli* DH5α cells. Transformed colonies were selected from selective antibiotic plates and colonies including katanin p60 cDNA were checked with colony PCR using forward and reverse primers of the insert to verify accuracy. Seven colonies were analyzed by PCR, and gel photo (Fig. 3.5) confirmed that all of them contained pET-30a with p60 cDNA inserted.

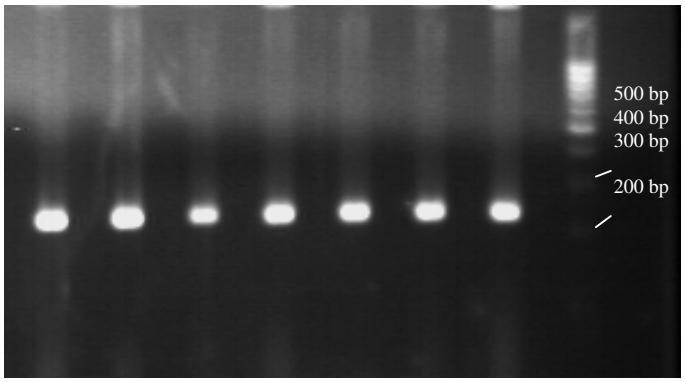

Figure 3.5: Colony PCR results for seven pET-30a-p60 transformed colonies

After preliminary control of colonies including pET-30a with p60 cDNA, one of the colonies was selected and plasmid purification procedure was performed to collect plasmids. Collected plasmid DNA was subjected to sequence analysis to confirm inframe insertion and correct orientation of p60 cDNA. Obtained sequence was aligned with a theoretical one (Fig. 3.6). According to alignment results, no shift

mutation was observed. There was a C.T point mutation which does not change amino acid sequence since both CTA and TTA would end up encoding leucine amino acid.

Figure 3.6: Alignment results of p60 in pET-30a expression vector. Expression vector part is underlined, restriction sites are in bold, the point mutation is circled, the rest part is p60 cDNA.

3.2 Expression of Recombinant Katanin p60

In order to express katanin p60, obtained construct of pET-30a- p60 was transformed into expression strain of *E.coli* BL21 (DE3)pLysS. A colony was picked from LB agar plate containing the selective antibiotic and dropped into LB medium. When the OD_{600} reached 0.7, IPTG to a final concentration of 0.5 Mm was added to bacterial culture growing in LB at room temperature to induce protein expression.

In order to, determine the optimum expression time and detect the expressed recombinant katanin p60 protein, total cell protein analysis was performed by SDS-PAGE method. For this purpose, bacterial samples were taken before IPTG induction (0 hour) and after IPTG induction at various times (from 2 to 10 hours). Protein samples were obtained from cell pellets and loaded into 15 % polyacrylamide gel (Fig. 3.7).

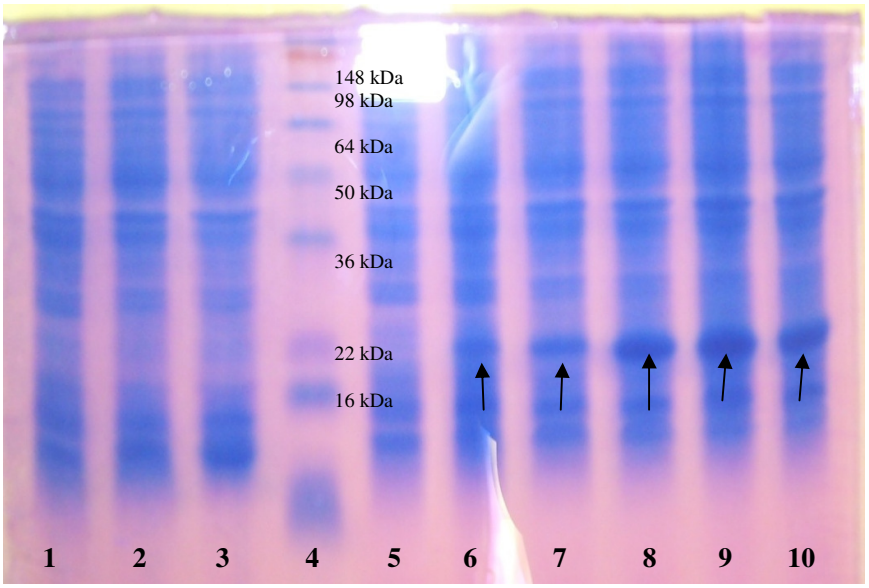

Figure 3.7: SDS- PAGE analysis of total protein samples. Bands of over-expressed recombinant protein p60 were showed by arrows,
Lane 1: pET-30a uninduced, 0 hour;
Lane 2: pET30a induced, 6 hour;
Lane 3: pET30a induced, 10 hour;
Lane 4: SeeBlue Plus2 Prestained Protein Ladder (Invitrogen);
Lane 5: pET30a-p60 uninduced, 0 hour;
Lane 6: pET30a-p60 induced, 2 hour;
Lane 7: pET30a-p60 induced, 4 hour;
Lane 8: pET30a-p60 induced, 6 hour;
Lane 9: pET30a-p60 induced, 8 hour;
Lane 10: pET30a-p60 induced, 10 hour.

After induction with IPTG, a protein of ~ 20 kDa was detected in protein samples obtained from cell pellet. The expression of recombinant katanin p60 protein increased with time and was maximal at about 8 hour and began to decrease at the time 10 hour after IPTG induction. The amount of overexpressed protein p60 seemed very little at 2 hour but after four hours of induction, it becomes very distinguishable. No expressed protein was found in the control samples containing only pET-30a vector without katanin-p60 insert and in the uninduced samples (Fig 3.7).

In order to check whether over-expressed katanin p60 is in the soluble fraction, cells' pellets were lysed by freezing and thawing. Soluble and insoluble fractions were separated by centrifugation, soluble fraction was concentrated by TCA method and protein samples were again analyzed by SDS-PAGE method. No recombinant katanin p60 protein was observed in insoluble fraction (see Fig. 3.8).

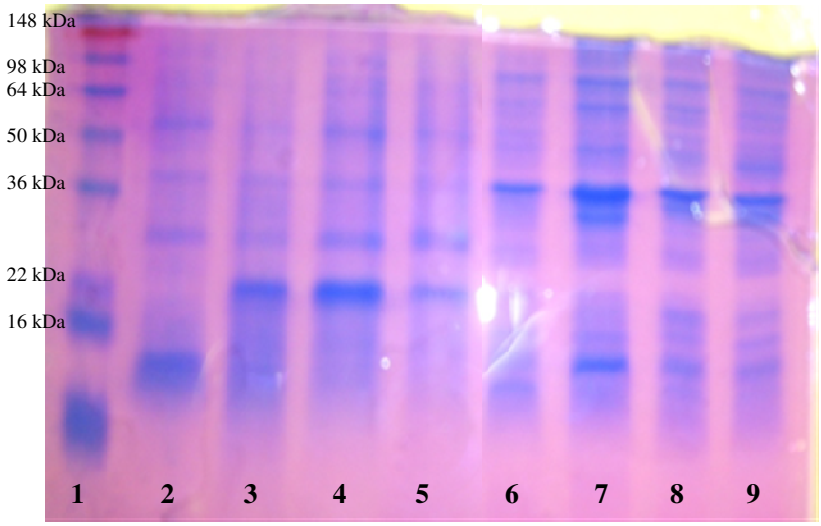

Figure 3.8: SDS-PAGE analysis of soluble and insoluble fractions,
Lane 1: SeeBlue Plus2 Prestained Protein Ladder (Invitrogen);
Lane 2: Soluble fraction of pET-30a, 12 hour;
Lane 3: Soluble fraction of pET-30a- p60, 4 hour;
Lane 4: Soluble fraction of pET-30a- p60, 8 hour;
Lane 5: Soluble fraction of pET-30a- p60, 12 hour;
Lane 6: Insoluble fraction of pET-30a, 12 hour;
Lane 7: Insoluble fraction of pET-30a- p60, 4 hour;
Lane 8: Insoluble fraction of pET-30a- p60, 8 hour;
Lane 9: Insoluble fraction of pET-30a- p60, 12 hour.

According to SDS-PAGE analysis results, recombinant katanin p60 protein was observed in soluble fraction. Amount of soluble protein seemed to increase proportionally to the amount of total cell protein. Bacterial culture containing pET-30a without p60 insert (control samples) did not expose over-expressed protein neither in soluble, nor in insoluble fractions.

3.2.1 Purification of recombinant katanin p60 under native conditions

Since studies of soluble and insoluble fractions showed that expressed katanin p60 protein was in soluble state, it was decided to purify the His tagged protein under native conditions. All purification steps were given in detail in the Materials and Methods part.

At each purification step, the samples were taken and were further analyzed by SDS-PAGE method (see Fig 3.9).

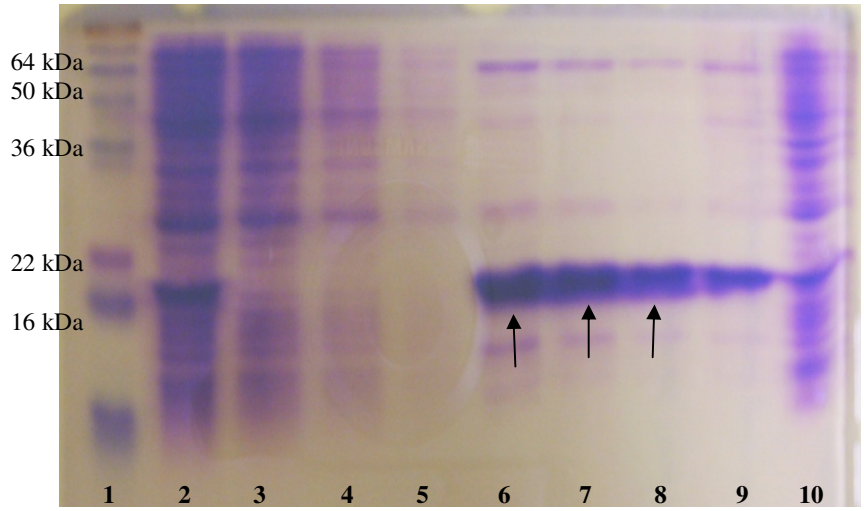

Figure 3.9: SDS-PAGE analysis of purified katanin p60 under native conditions (Purified protein in elution fractions showed by arrows),
Lane 1: SeeBlue Plus2 Prestained Protein Ladder;
Lane 2: lysate of bacterial culture (see Materials and Methods part for details);
Lane 3: lysate flow-through;
Lane 4: 1st wash fraction
Lane 5: 2nd wash fraction;
Lane 6: 1st elute
Lane 7: 2nd elute;
Lane 8: 3rd elute;
Lane 9: used Ni-NTA agarose total protein;
Lane 10: pET30a–p60 after 12 h growth total protein pET 30a – p60 after 8 h growth.

Distinct protein bands were seen in elution fractions clearly and the protein amount decreased gradually in elution steps. In order to check whether the purified protein is the same as the overexpressed one, the control sample containing total protein fraction from bacterial cultures with pET-30a-p60 construct was applied to the SDS-PAGE gel as well. In addition, the overexpressed katanin p60 protein band was observed in bacterial cell lysate sample and this band was missing in the flow-through sample since overexpressed katanin p60 bound to Ni-NTA agarose with its His-tags during purification process. In wash fractions, katanin p60 protein bands were not observed as expected. Some of used Ni-NTA agarose was applied to SDS-PAGE gel as well, in order to determine the protein amount retained on resin. It was observed that protein on resin sample' band is the thinnest of all elution samples' bands that means most of the protein was eluted with elution buffer.

Purified protein concentration was measured by nanodrop spectrophotometer at A_{280}. It showed that the protein concentration (~3 mg/ml) was the same in first and second elution but concentration after third elution reached 1.4 mg/ml.

3.2.2 Western blot analysis

In order to be sure that purified protein was really the His-tagged katanin p60, Western blot analysis was performed. Samples of total protein fraction with empty expression vector and with katanin p60 were taken from grown bacterial culture (after 8 hours following IPTG induction) and the purified proteins of each elution fraction were analyzed by western blotting. Total protein fraction and elution samples gave signals of His tagged protein of the expected size, except negative control sample (Fig.3.10).

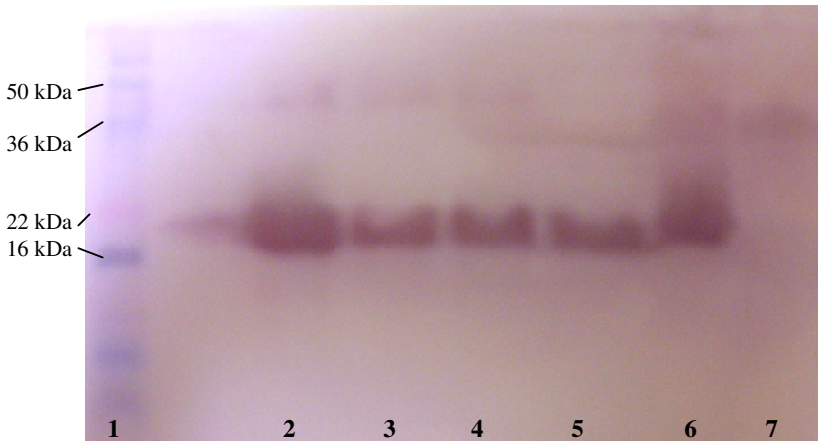

Figure 3.10: Western blot analysis of total protein fraction and purified protein,
Lane 1: SeeBlue Plus2 Prestained Protein Ladder;
Lane 2: Purified protein (after first elution);
Lane 3: Purified protein (after second elution);
Lane 4: Purified protein (after third elution);
Lane 5: Purified protein (after fourth elution);
Lane 6: Total protein fraction of pET30a-p60 grown for 8 hours (positive control);
Lane 7: Total protein fraction of pET30a grown for 8 hours (negative control).

3.3 Monoclonal Antibody Production

Balb/c female mice were immunized three times with purified His-tagged recombinant katanin p60 protein to produce monoclonal antibodies against it (see Materials and Method section). Even though, the immunization procedure was began with three mice and a negative control, two of them died after second immunization. Therefore, the studies were continued with an immunized mouse and a negative control mouse.

3.3.1 Immune response control

Ten days after second immunization with recombinant katanin p60, polyclonal immune response of the immunized mouse and the control mouse were compared. For this purpose, the titer of antibody in mice sera was determined using indirect ELISA method (Fig. 3.11).

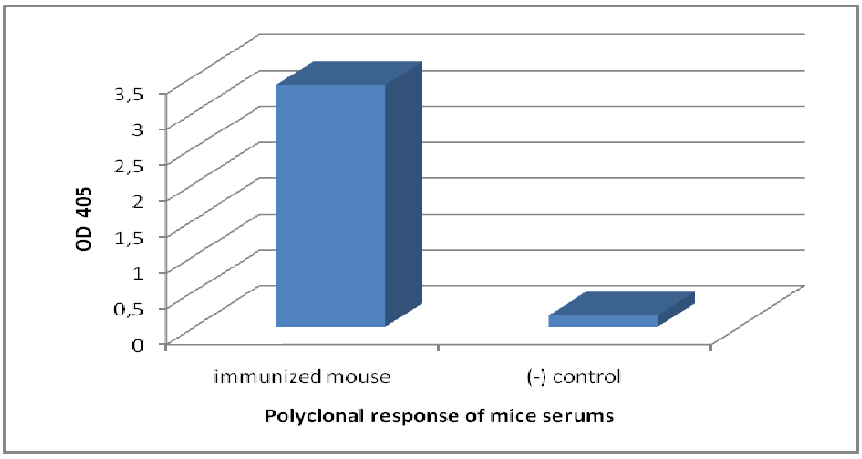

Figure 3.11: Mice's serums were diluted in proportion to 1/1000 with PBS for measuring their immune response.

In order to measure antibody response, blood serum was collected from retro-orbital space of mice. The blood was taken by hematocrit tubes containing heparin as an anticoagulant and poured into microfuge tubes. Tubes were centrifuged at 10000x g for 10 minutes to remove the blood cells. The plasma samples were tested by indirect- ELISA test system. Plasma from an unimmunized mouse in PBS was used as a negative control. Immunized mouse was seen giving high antibody titer against katanin p60 antigen even at 1/2000 dilution of the serum so it was taken to fusion studies.

3.3.2 Optimization of antigen usage with ELISA Method

In fusion studies, high amount of antigen will be used for detecting hybrid cell antibody activity. Since expression processes of katanin p60 protein take long time and are not economical, optimization of antigen usage must be determined. For determining the optimum antigen usage, ELISA plates were coated with various amount of katanin p60 antigen and by using active mouse plasma in different dilutions, optimum amount was determined for ELISA tests. 120 ng was found as the

most suitable antigen amount for antigen usage for ELISA tests. The result was shown in the Figure 3.12.

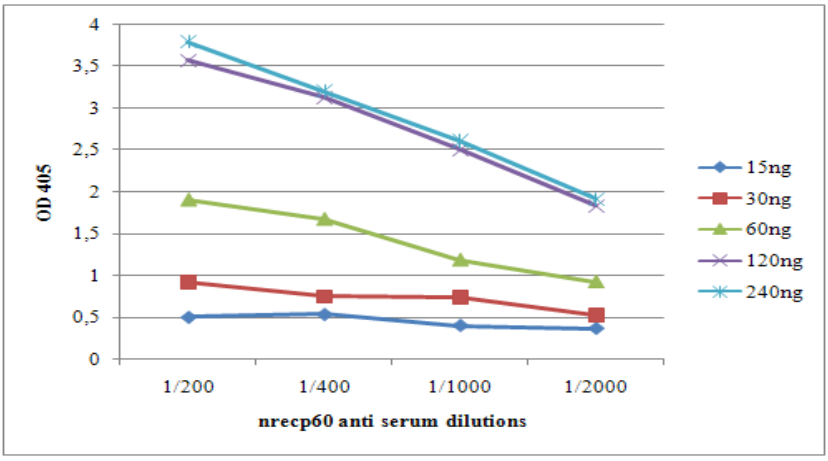

Figure 3.12: Immunized mouse's serum was tested for detecting optimum antigen usage for experiments. ELISA plate was coated with 15 ng, 30 ng, 60 ng, 120 ng and 240 ng amount of katanin p60.

ELISA test mentioned above was carried out by anti-mouse alkaline phosphatase conjugated polyvalent immunoglobulins (IgA, IgM, IgG) as a secondary antibody and the results were obtained after 30 minutes incubation with substrate buffer.

The similar test was performed with anti-mouse alkaline phosphatase conjugated IgG antibody in mouse's serum to observe IgG antibody level in fusion day. In addition, polyclonal antibody response against katanin p60 (nrecp60) protein that was expressed in this study and another katanin p60 (rec p60) protein that was expressed from a different region of full katanin p60 in the previous study were tested. Cross reactivity of the mouse' serum with other proteins such as tubulin, skimmed milk powder and BSA was also checked. The IgG antibody titer was found high and the cross reactivity was low according the results (Fig. 3.13).

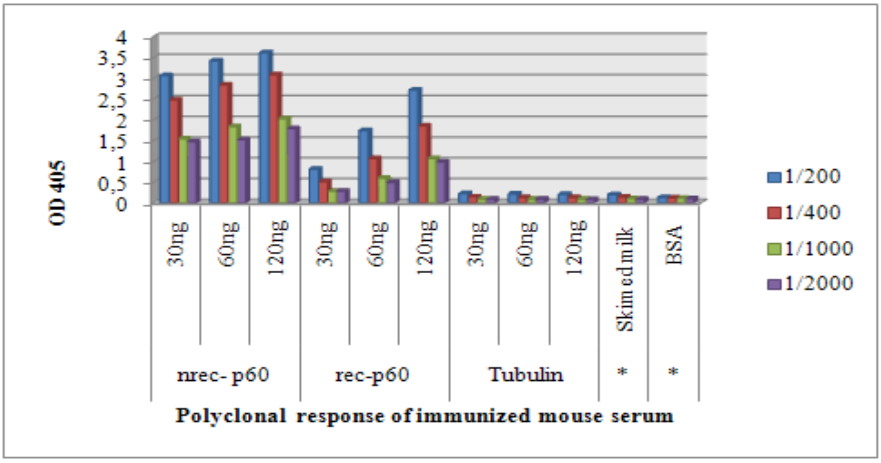

Figure 3.13: IgG response of immunized mouse against several amount of katanin p60 in different serum dilutions. nrecp60: new recombinant katanin p60 protein, recp60: recombinant katanin p60, BSA: Bovine serum albumin.

3.3.3 Results of fusion studies

In this study, fusion was performed once. Following the fusion, on day 4 the first colonies started to appear and at around day 10 the number of hybridoma cells increased. The culture medium was replaced with fresh medium. The hybridomas were refed every 3-7 days according to their growth and on day 13 the number of hybridomas were 57 on eight 96-well plates. After that, number of observed hybridomas was increased day by day. The hybridoma supernatants were tested in ELISA. Most of the hybridomas did not prove to be stable cell line. After several in vitro passages, replication of the cells ceased and the hybridomas were lost. Moreover, antibody activities of antibody producer hybrid cells decreased day by day probably one of the reason can be chromosome loss in the hybrid line secreting antibodies. The fusion results were detailed in Table 3.1.

Table 3.1: The detailed fusion results of immunized mouse with katanin p60

	Spleen Fusion	**Lymph Node Fusion**
Number of Cell	40 x 10^6	16x10^6
Number of F0 Myeloma Cell	80x 10^6	32x 10^6
Total Number of Studied Well	768	288
Total Number of Hybrid Cell	295	1
Number of Antibody Producer Hybrid Cell	25	0
Antibody Producer Hybrid Cells in Maintained Manner	3 **(2H3, 4F2, 7B10)**	0
Specific Antibody Producer Hybrid Cell Against Katanin p60	1 (2H3)	0

According to given information above, three hybrid cells mantained producing active antibody according to ELISA test. Antibody activity of these cells was maintained even after passages to six well, twelve well cell culture kostars and 25cm^2 cell culture flasks. Therefore, 2H3, 4F2 and 7B10 hybrid cell supernatants were tested for cross reaction test with other antigens and proteins to identify the antibodies' specificity. For this purpose, tubulin, skimmed milk powder, BSA and *E.Coli* BL21 strain with pET-30a empty vector cell lysate were used as different proteins in cross reaction test. The aim of using bacterial lysate was to detect whether an activity is observed against a bacterial protein that comes in purified katanin p60. Antibody activities for katanin p60 (nrecp60) protein and another katanin p60 (rec p60) protein were also tested. 2H3 showed high specificity against nrecp60, at the same time it did not show any cross reaction with other proteins (Table 3.2).

Table 3.2: Comparision of the reactivity (OD_{405}) of the monoclonal antibodies (2H3, 4F2, 7B10) with different proteins. nrecp60: new recombinant katanin p60 protein, recp60: recombinant katanin p60 protein, BL21-pET30a: *E.Coli* BL21 strain with empty pET30a vector lysate, BSA: Bovine serum albumin

Hybrid Cells	Proteins					
	nrecp60	recp60	Tubulin	BL21-pET30a	Skimmed Milk Powder	BSA
2H3	1.271	2.198	0.140	0.203	0.134	0.147
*4F2	0.452	0.581	-	0.691	0.953	0.921
7B10	> 4	3.989	> 4	> 4	> 4	0.101

** Cross reactivity of 4F2 was tested with other proteins mentioned above accept tubulin because it showed cross reactivity with BL21-pET30a, skimmed milk powder and BSA in previous test and thus it was ignored.*

In ELISA test mentioned above, skimmed milk was used for blocking. Since 7B10 showed cross reactions against other proteins but not BSA, it was again tested by blocking with BSA in order to understand whether antibodies in 7B10 hybrid cell supernatant show cross reaction with only skimmed milk powder and have activity against katanin p60 protein. It was observed that 7B10 showed high cross reaction with only skimmed milk powder and had no activity against katanin p60. It was understood that the reason of observing of 7B10 as an antibody producer hybrid cell against katanin p60 protein was that it showed cross reactivity against only skimmed milk that was used for blocking in ELISA test. That means 7B10 hybrid cell gave false positive result (Table 3.3).

Table 3.3: Results of ELISA test blocking with BSA. nrecp60: new recombinant katanin p60 protein, recp60: recombinant katanin p60 protein, BL21-pET30a: E.Coli BL21 strain with empty pET30a vector lysate, BSA: Bovine serum albumin

Hybrid Cell	Proteins					
	nrecp60	recp60	Tubulin	BL21-pET30a	Skimmed Milk Powder	BSA
7B10	0.305	0.164	0.107	0.134	> 4	0.101

In order to estimate monoclonal antibody subtype produced by 2H3 hybrid cells, activity against katanin p60 protein was compared with different secondary antibodies: anti-mouse polyvalent immunoglobulins (IgA, IgM, IgG) and anti-mouse IgG immunoglobulin conjugated with alkaline phosphatase (AP) in ELISA test. According to results showed in Figure 3.14, monoclonal antibody (2H3) subtype was not IgG subtype, but it could be either IgA or IgM subtypes.

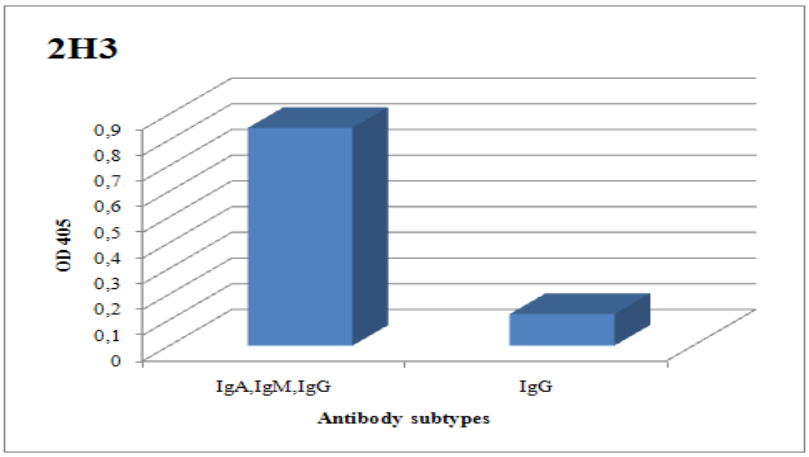

Figure 3.14: Determination of monoclonal antibody subtype (2H3).

In order to determine the exact subtype of the monoclonal antibody, the supernatant of 2H3 hybrid clone was analyzed by IsoStrip mouse monoclonal antibody isotyping kit and the monoclonal antibody was found to be IgM subtype.

2H3 hybrid clone producing antibody with the highest specifity was subcloned by limiting dilution. At each stage of growth, hybrid cells were frozen in freezing medium in microfuge tubes at -80^{o}C.

4. DISCUSSION

Katanin is one of the microtubule severing protein consisting of two subunits; catalytic p60 subunit and regulatory p80 subunit. The enzyme p60 subunit breaks the microtubule (Harman et al., 1998; Mcnally et al., 2000). Katanin p60 has important roles in mitotic spindle formation in mitotic cells (Vale, 1991; McNally et al.; Buster et al., 2002) and in axonal growth and differentiation in neurons (Ahmad et al., 1999; Karabay et al., 2004). In neurodegenerative diseases, it is suggested that the loss of protective tau (one of MAPs) from microtubules in the axons results in abnormal microtubule severing. Therefore, katanin may also have a central role in the degenerative response during neuropathies such as Alzheimer's disease (Yu et al., 2007). A better understanding of the severing proteins is necessary for developing new approaches for diminishing the extent of axonal degeneration in a variety of neurological diseases. In addition; katanin levels are higher at the tips of growing neuronal processes at some developmental stages (Yu et al., 2005) and thus katanin may also comprise a function in regeneration of neuronal injuries (Yu et al., 2007). However, the interacting proteins of katanin are still not actually defined.

In order to monitor cellular katanin p60 and find out its interacting proteins, the techniques such as immunostaining and immunoprecipitation need for specific antibody against katanin p60 protein. In this study, a recombinant katanin p60 protein was expressed and a specific monoclonal antibody was produced against this protein to further use in above mentioned techniques.

4.1 Recombinant Katanin p60 Protein Expression

In order to obtain recombinant katanin p60 protein, cloning experiments were performed initially. In this case, an amino acid sequence was selected from full katanin p60 protein considering the specificity, solubility and inclusion of antigenic determinants of the protein to produce efficient anti-katanin p60 monoclonal antibodies. Thus, we sought to manage the problems that were encountered in the

previously produced monoclonal antibodies in our lab, like inefficient immunostaining and western blot analysis.

For specificity of katanin p60 protein, full amino acid (aa) sequence of the protein (except C-terminal AAA domain) was truncated by using NCBI protein BLAST tool (Url-4) until resulting with a protein region of 73 aa (amino acids 98-170) which does not give any alignment with other proteins. Since a polyclonal antibody against katanin p60 peptide (amino acids 115-148) was produced and showed to efficiently recognize the katanin p60 protein in brain cells (Karabay et al., 2004), while choosing the protein region we thought to preserve the amino acid sequence of that previously produced antibody due to its antigenic region. In addition, the protein which is used for immunizing mice should be soluble to produce antibodies against this protein efficiently because B lymphocytes of mice can bind to soluble proteins for recognizing and then producing antibodies. The best conditions for expression of soluble another construct of katanin p60 were determined considering the factors that affect the protein solubility in a previous study in our lab. pET-30a expression vector and BL21 (DE3)pLysS host strain were used and high yield in expression and solubility were gain for this construct. Therefore, the same conditions were performed for our construct of katanin p60 in this study, expecting to gain soluble katanin p60 protein. The amino acid sequence of our katanin p60 protein was also analyzed since amino acid sequence determines protein characteristics, including solubility. The hydrophobic regions of our recombinant katanin p60 protein may lead to insolubility of the protein hence; they were excluded according to the previous study. Cystein residues that are responsible for disulfide bond formation in protein folding process were also checked because any possibility for wrong disulfide bond formation might result in insolubility (Baneyx, 1999). It was observed that our katanin p60 amino acid sequence contained neither considerable hydrophobic regions nor cystein residues that could give rise to formation of insoluble protein.

In this study, pET-30a expression vector was used for cloning studies since the reasons mentioned above. After efficiently cloning of selected katanin p60 cDNA into pET-30a expression vector, no shift mutation was observed according to alignment results. However, a point mutation (C.T) was found. It resulted CTA instead of TTA DNA codes which would end up encoding leucine amino acid. Since

the mutation does not change the amino acid sequence, the activity of the expressed protein was maintained.

pET30a-p60 construct was transformed to E.coli BL21 (DE3)pLysS strain and after cultivation, expression was induced with 0,5mM IPTG. The highest amount of katanin p60 was observed at the 8^{th} hour of the growth. According to SDS-PAGE analysis of total protein fraction after expression studies, an overexpressed protein of about 20 kDa was observed in the samples containing pET30a-p60 constructs (Fig. 3.12 in Results section). Molecular weight of subcloned katanin p60 should have been about 8 kDa (8346.42 Da). When His-tag was fused to it, its molecular weight became ~ 16 kDa (16.10481 Da). However, SDS-PAGE analysis (15 % separating gel) showed the overexpressed protein in higher weight (~ 20 kDa). A longer than expected protein might be produced since bacteria may no recognize the first stop codon and extend translation process further (Gu et al., 2001). Another reason might be the fact that SDS-PAGE shows approximate molecular weight of the protein, it might seem different depending on the concentration of the gel.

After SDS-PAGE of total cell protein analysis, soluble and insoluble fractions of total cell protein were differentiated to determine whether overexpressed katanin p60 is in the soluble fraction. It was observed that the overexpressed katanin p60 was in soluble fraction.

Overexpressed katanin p60 was purified to further use in mice immunization. Purification under natural or denaturating conditions is possible using Ni-NTA resin. We chose purification under natural conditions since expressed protein is soluble, as it was shown by total soluble and insoluble fraction analysis. Moreover, such purification does not distort protein conformation which is important for immunization efficiency. The potential for unrelated, nontagged proteins to interact with the Ni-NTA resin is usually higher under native than under denaturing conditions. Nonspecific binding can be reduced by including a low concentration of imidazole (10–20 mM) in the lysis and wash buffers. Nonspecific binding is reflected in the larger number of proteins that appear in the first wash. In our case both wash fractions seemed to be clear (see Fig. 3.14) which shows that only His-tagged protein has bound to the resin and most of the unrelated proteins were in the flow-through fraction. According to manufacturer's recommendations (Qiagen handbook, 2003), imidazole concentration in elution buffer should reach 250 mM. However,

considerable amount of protein was left bound to resin even after four elution steps. We used elution buffer with imidazole concentration reaching 1 M. SDS-PAGE analysis confirms that after three elution steps with 1 M imidazole elution buffer most of protein is eluted (see Fig. 3.14). In order to determine the concentration of purified protein in elution buffer, nanodrop spectrophotometer was used.

4.2 Monoclonal Antibody Production

In 1975, Köhler and Milstein introduced the first version of a method to make monoclonal antibodies. Precursors of the plasma cells were isolated and fused with immortal cells of the B-cell lineage such as myeloma cells, which previously had been selected to no longer secrete antibodies. The resultant hybridoma cells could be single- cell cloned and then expanded as individual clones, which secrete only one antibody type. All of these antibodies are identical, with specific and easily studied properties of antigen recognition, and are known as monoclonal antibodies.

Nowadays, monoclonal antibodies (MAbs) become important reagents used in biomedical research, in diagnosis of diseases, and in treatment of such diseases as infections and cancer (Lipman et al., 2005). A wide range of commercially available monoclonal antibodies are also used as laboratory reagents for ELISA assays, immunoblotting, immunoprecipitation, immunofluorescence, immonucytochemistry and immunohistochemistry methods. However, mouse anti-katanin p60 monoclonal antibody is not commercially available. Thus, we aimed to produce this kind of antibody to further use in methods mentioned above. This attempt could be helpful for well understanding katanin p60 behavior in most commonly studied cells.

MAb production was achieved by hybridoma technology. This process included the following successive working phases: the generation of antigen specific B cells via immunization of mice, the fusion of these cells with myeloma cells, the cloning and selection of the specific hybridoma clone by “limiting dilution” and the up- scaling of MAb production. While producing antibodies, critical aspects including mice strain selection, antigen properties, aseptic antigen processing, adjuvant selection, preparation of antigen adjuvant mixture, choice of immunization route and injection volume were considered. In this study Balb/c mice strain was preferred since myeloma cell lines have been derived originally from such animals and they are so compatible for the propagation of hybridoma cells in vivo. Young adult animals such

as 6-8 weeks old whose immune response is robust and not affected by previous immune challenges were used in order to increase yield of immunization with katanin p60 protein. Moreover, female mice were used because we observed that female mice were milder in social interactions than male mice that were aggressive and could hurt other animals in the cage. The properties of katanin p60 that was produced in this study as an antigen was mentioned section 4.1. Immunizations with katanin p60 antigen were applied by intraperitoneal and intravenous routes and repeated for several times. First immunization of the antigen was done with Freud's complete adjuvant that is a potent adjuvant and has been used successfully decades to enhance the immune response quantitatively and/or qualitatively. However, second immunization was performed with Freund' incomplete adjuvant to minimize side-effects of complete adjuvant that are formation of granulomas, inflammation at the inoculation site and lesions (Rudbach et al., 1998).

In order to produce MAb, antibody secreting B cells derived from immunized mice were immortalized by the fusion with a nonsecretory myeloma cell line. Since the infused normal B cells could not survive long in an *in vitro* culture, they derive immortality by fusion to a partner tumor cell line. In this study, ATCC CRL 1646, F0 cell line of myeloma (commercially available) was used.

In fusion studies, high amount of antigen would be used for detecting hybrid cell antibody activity. Since expression processes of katanin p60 protein take long time and are not economical, optimization of antigen usage required to determine. 120 ng was found as the most suitable katanin p60 protein amount for antigen usage for ELISA tests. However, 240 ng of the protein was used then in order to catch the antibody activity of hybrids in early stage of growth because they were growing rapidly and dying immediately.

Only one fusion study was carried on with this myeloma cell line but the second fusion experiments are still continuing. After first fusion study, 25 hybrid cells producing antibody were detected. However, 22 of them lost their antibody producing activity day by day and probably one of the reasons could be chromosome loss in the hybrid line secreting antibodies. In addition, they died after passages to six well, twelve well cell culture costars and/or 25 cm^2 cell culture flasks since they might not manage to passaging stress. Hybrid cells named 2H3, 4F2 and 7B10 maintained their antibody producing activity and gave high activity in ELISA test all

time even after passages thus, their supernatants were decided to be tested for cross reactivity. For this purpose, tubulin, skimmed milk powder, BSA and *E.Coli* BL21 strain with pET-30a empty vector cell lysate were used as different proteins in cross reaction test. Since polyclonal cross reactivity of the immunized mouse serum was not observed against skimmed milk and BSA, it was expected antibody producing hybrid cells were not expected to show the cross reactivity. However, 4F2 showed cross reactivity with BL21-pET 30a, BSA and skimmed milk powder thus it was ignored (in the first cross reactivity test tubulin was not used). On the other hand, 7B10 gave cross reactivity in the same value with all other proteins except BSA. Therefore, we thought that this situation could be because of usage of skimmed milk powder for blocking protein in ELISA test. However, it was observed that 7B10 gave high activity with only skimmed milk powder and did not recognize katanin p60 when the ELISA plate was blocked with BSA. Although cross reactivity of serum was not observed, the reason of cross reactivity of these cells might be that they were in spleen and not secreted to blood stream yet when the blood sample was taken. Another reason could be secretion of these cells to blood in insufficient amounts to give cross reactivity.

One potentially specific hybrid named 2H3 was found against recombinant katanin p60 protein since it does not give any cross reaction with other proteins in ELISA test. In order to reduce antibody producing cell mass of 2H3 into one cell, limiting dilution method was used. Continued growth of this clone could result in a long-term dependable source of monoclonal antibodies. The produced antibodies were tested for immunoglobulin subtype determination by ELISA tests and mouse monoclonal isotyping kit system; the results showed that they were IgM subtype. It is common to come across with this result. This is because antigen which enters the body does not stimulate T cells and macrophages; instead stimulates B cells directly. Moreover, antigen which chooses thymus in independent stimulation has a lot of same epitopes so that genetic modification of antigen is directed to IgM synthesis of antibody. However, this response can be mitigated by binging of antigen to carriers or by immunizing the animal with an adjuvant.

In order to determine whether the produced monoclonal antibodies will recognize the wild type katanin p60 in cells, western blot and immunostaining techniques will be performed by using anti- mouse IgM immunoglobulins as secondary antibodies.

However, the majority of monoclonal primary antibodies for use in immunocytochemistry (ICC) are of the IgG Isotype (IgG1, IgG2a, IgG2b, or IgG3). Most ICC detection systems contain a fluorescein conjugated secondary antibody that is directed against both the heavy and light chains of mouse IgG. On the other hand, there are a number of commercial monoclonal primary antibodies that are of the IgM isotype (heavy chain). Due to the pentameric structure of IgM, and its incomplete sequence conservation between the heavy chain regions of IgM and IgG isotypes, there are often difficulties obtaining optimal staining results when using an IgM isotype primary antibody with an anti - IgG secondary antibody. An IgG secondary antibody will recognize and cross-react with an IgM heavy chain. However, results are sub-optimal and prolonged incubations are often required. Therefore, a secondary antibody that is specific to IgM isotype is preferable. The use of a secondary mouse IgM heavy chain leads to improved staining characteristics and incubation times can be shortened due to the higher affinity of the reaction. However, binding of IgG monoclonal antibodies tend to be more specific than IgM type antibodies. Thus, false positive results due to IgM antibodies can be avoided by using IgG monoclonal antibodies as both primary and secondary antibodies in immunocytochemistry. In this case, IgG subtype monoclonal antibodies against katanin p60 could be produced by second fusion study.

5. CONCLUSION

Katanin p60 is a severing protein whose functions are critical for both mitotic and postmitotic cells such as neurons. In order to monitor cellular katanin and to find out its interacting proteins, the techniques such as immunostaining, immunoprecipitation and western blot need for a specific antibody against katanin p60. In this study, we aimed to produce a specific monoclonal antibody against a recombinant katanin p60. To achieve our goal, an amino acid sequence was initially selected from full katanin p60 protein considering the specifity, solubility and inclusion of antigenic determinants. This katanin p60 subsequence was cloned into an expression vector (pET30a-p60) succesfully without any mutation on the aminoacid sequence. After induction, subcloned p60-katanin was expressed in soluble form, the greatest amount of the protein was observed after 8 hours of growth. Purification of recombinant katanin p60 protein was performed efficiently and Balb/c mice were immunized with this protein successfully. Monoclonal antibody production was achieved by hybridoma technology. After the fusion study, a monoclonal antibody (named 2H3) against recombinant katanin p60 was obtained. The antibody subtype was IgM according to ELISA tests and mouse monoclonal antibody isotyping kit system.

In order to determine whether the produced monoclonal antibodies will recognize the wild type katanin p60 in cells, western blot and immunostaining techniques will be performed.

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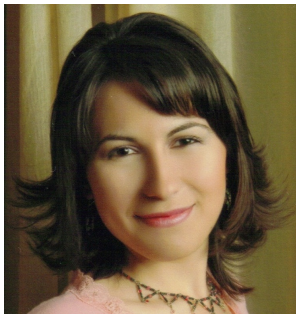

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