ISTANBUL TECHNICAL UNIVERSITY ★INSTITUTE OF SCIENCE AND TECHNOLOGY

P60- KATANIN CLONING AND EXPRESSION

M Sc. Thesis by
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İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

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YÜKSEK LİSANS TEZİ

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ABBREVIATIONS

A_{280}	: Absorbance at 280 nm
AAA	: ATPases associated with various cellular activities
ADP	: Adenosine diphosphate
AMPS	: Ammonium persulphate
ATP	: Adenosine triphospahe
AP	: Alkaline phosphatase
BCIP	: 5-bromo-4-chloro-3-indolyl phosphate
BLAST	: Basic Local Alignment Search Tool
bp	: Base pair
BPB	: Bromophenol blue
BSA	: Bovine serum albumin
CBB	: Comassie Brilliant Blue
EDTA	: Ethylenediaminetetraacetic acid
E-site	: Exchangeable site
GTP	: Guanosine triphospahte
His	: Histidine
IMAC	: Immobilized-metal affinity chromatography
IPTG	: Isopropyl-b D-thiogalactopyranoside
kb	: Kilobase
kDa	: Kilodalton
LB media	: Luria Bertani media
MAP	: Microtubule associated protein
NBT	: Nitroblue tetrazolium
Ni-NTA	: Nickel-nitrilotriacetic acid
OD	: Optical density
PCR	: Polymerase chain reaction
pET	: Plasmid expression by T7 RNA polymerase
PVDF	: Polyvinylidene difluoride membrane
RNase	: Ribonuclease
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE	: Tris acetate EDTA
TBS	: Tris buffered saline
TCA	: Trichloroacetic acid
Tris	: Hydroxymethyl aminomethane
TTBS	: TBS containing Tween20
w/v	: Weight/volume

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P60- KATANİN'İN KLONLAMASI VE EKSPRESYONU

ÖZET

Katanin mikrotübülleri parçalayan bir proteindir. 60 ve 80 kDa'luk polipeptidlerden oluşan bu heterodimerik protein, stabil mikrotübülleri parçalayabilmek için ATPye ihtiyaç duyar. Kataninin biyolojik rolüne gelince, bu proteinin mitozda önemlidir. Katanin, mitozda mikrotübüllerin eksi uçlarının bağlı oldukları sentrozomlardan ayrılmasına aracı olmaktadır ve böylece mikrotübüllere bağlanmış olan kromozomların akışı sağlanmaktadır. Mkrotübüllerin sentrozomlardan ayrılması sinir hücrelerinde çok önemlidir. Serbest mikrotübüller uzayan aksonlara taşınmaktadır ve burada akson için gerekli olan fiziksel desteği sağlamaktadırlar. Mikrotübüllerin diğer bir görevi de organellerin taşınmasında sübstrat vazifesi görmektir.

Katanin iki alt üniteden oluşmaktadır, onların isimleri moleküler ağırlıklarına göre belirlenmiştir. Kataninin alt ünitelerinin araştırılması bu enzimin aktivitesinin ve rollerinin daha iyi anlaşılmasını sağlayacaktır. p80 ve p60 antikorları bu araştırmalarda kullanılacaktır. Monoklonal antikor poliklonal antikora tercih edilmektedir, çünkü monoklonal antikor neredeyse bitmeyen bir antikor kaynağıdır ve tek bir epitop tanıyabilme özelliğine sahiptir. p60'ın ekspresyonu gerçekleştirilmiş olmasına rağmen, protein çözünmüş halde elde edilememiştir. Bu durum monoklonal antikor üretimini zorlaştırmaktadır.

Bu çalışmada küçük (600 kb) özel bir p60-katanin kısmı ekspresyon vektörüne klonlanarak ekspresyonu gerçekleştirildi. Eksprес edilen protein çözünmüş halinde bulunmaktadır. Sonuçta üretilen çözünmüş protein immobilize metal afinite kromatografisi yöntemiyle doğal şartlar altında saflaştırıldı.

Bu projenin sonraki aşamalarında da rekombinant p60-katanini kullanılarak monoklonal antikor üretilecektir. Üretilen antikorun p60 katanin inhibisyonu, p60'kataninin ekspresyonunun ve lokalizasyonun *in vivo* gözlenmesi gibi pek çok geniş kullanım alanı olacaktır. Bu çalışmalar mikrotübüller ve kataninle ilgili hastalıkların anlaşılabilmesini sağlayacaktır ve hatta belki uzun vadede bu hastalıkların iyileştirilmesinde kullanılacaktır.

P60- KATANIN CLONING AND EXPRESSION

SUMMARY

Katanin is a microtubule severing protein. This heterodymeric protein (60 and 80kDa polypeptides) requires ATP to sever and disassemble stable microtubules. Katanin is important in mitosis, it mediates the disassembly of microtubule minus ends during poleward flux. Release of microtubules from centrosome is also essential in neuronal cells. Released microtubules are transported into growing axons where they provide architectural support and a substrate along which organelles are transported in both directions within the axon.

Katanin consists of two subunits: p60 and p80 which were named according to their molecular weight. Studies of katanin's subunits would enable us to understand activity and roles of katanin better. Antibodies against p80 and p60 will be used in these studies. Monoclonal antibodies are preferred against polyclonal antibodies since they are an almost infinite source of antibodies and recognize one single epitope. However, expression experiments with p60 resulted in an insoluble protein until now and this hinders production of monoclonal antibody.

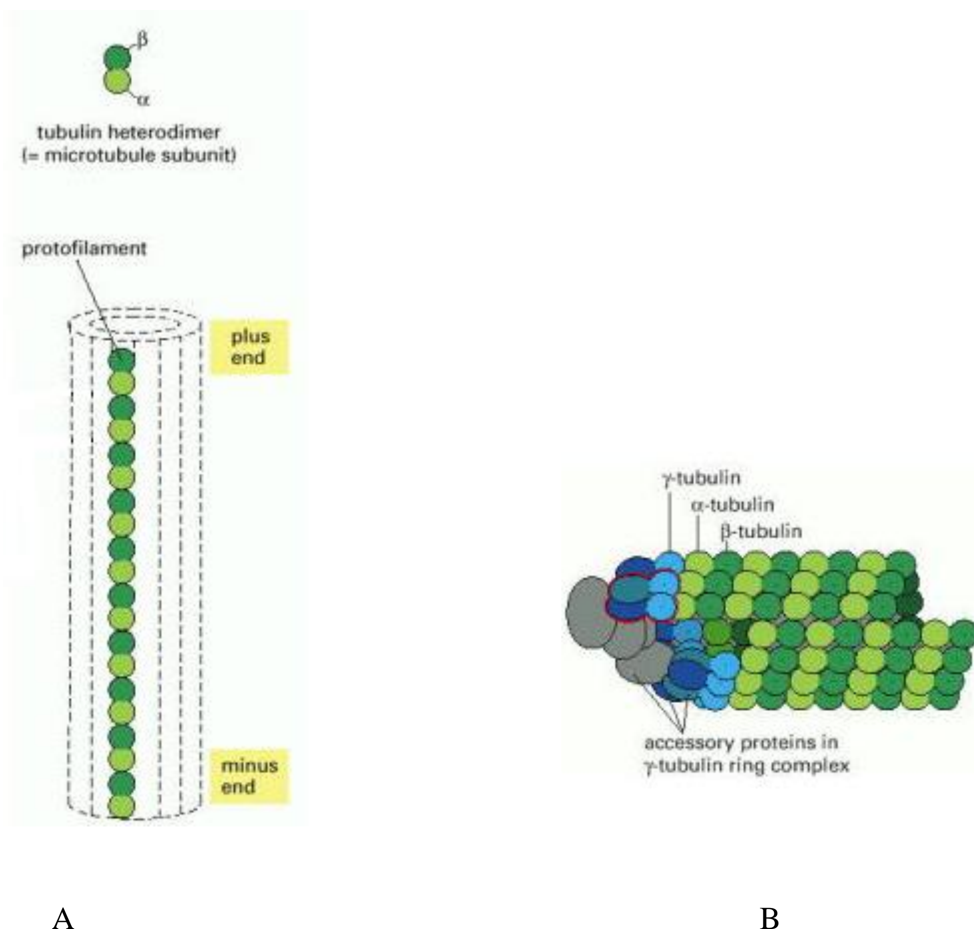
In this study a short specific sequence (600 kb) of p60-katanin was expressed after being cloned into expression vector. Expressed protein is found in the soluble fraction. Finally, soluble protein was purified using metal affinity chromatography under native conditions.

The next step will be to produce monoclonal antibody using recombinant p60-katanin. Produced antibody will have wide application area e.g. inhibition of p60 subunit, observation of p60 *in vivo* expression and localization which will help to understand human disorders related to microtubules and katanin, and maybe eventually even will be even used to treat them.

1. INTRODUCTION

1.1. Microtubules and their dynamics

Microtubules are polymers made of repeating α/β-tubulin heterodimers. In cells, they are usually organized into 13 linear protofilaments to form a cylindrical structure. Microtubules are nucleated from γ-tubulin containing ring structures within the centrosomes (see Fig. 1.1).



A B

Fig. 1.1: A a microtubule structure. B γ-tubulin ring nucleating microtubule. (Alberts *et al.*, 2002)

Microtubules are nucleated at the centrosome at their minus ends, so the plus ends point outward and grow toward the cell periphery. Minus end grows slower compared to the plus end and minus end is terminated by α tubulin subunits, while plus end is terminated by β subunits. Microtubules are important in maintaining cell shape, in cell transport, cell motility and cell division.

Microtubules are dynamic structures, they can grow or shrink by adding or loosing subunits at their ends (at the plus end primarily). This kind of dynamics is based on the binding and hydrolysis of GTP by tubulin subunits. The body of microtubule made of GDP-tubulin subunits is unstable (see Fig. 1.2). Tubulin dimers at the ends still retain their GTP and stabilize microtubule. When this GTP cap is lost, microtubule depolymerizes (Nogales, 2000; Howard and Hyman 2003).

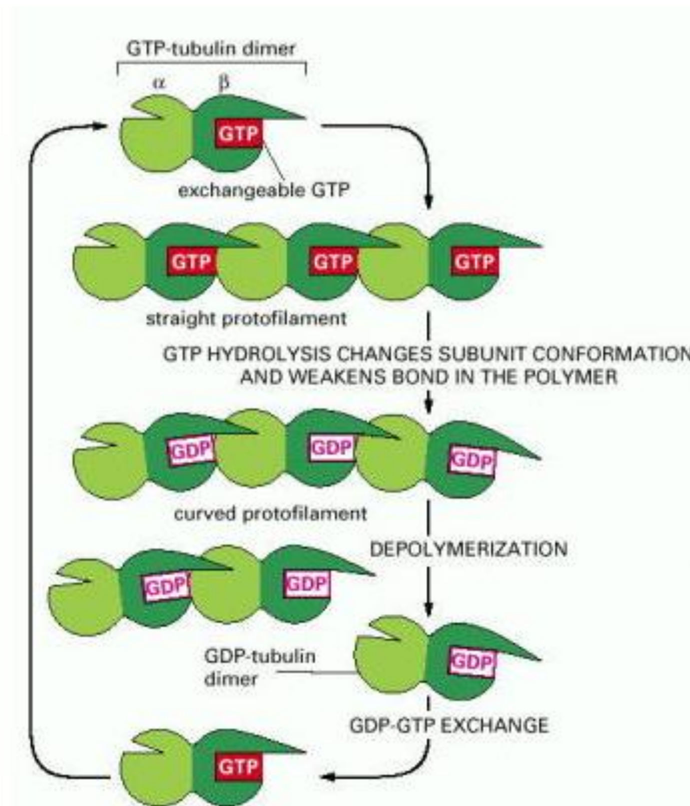


Fig. 1.2: Model for the structural consequences of GTP hydrolysis in the microtubule lattice. (Alberts *et al.*, 2002)

Such behavior is based on the binding and hydrolysis of GTP at the nucleotide exchangeable site (E-site) on β- tubulin. Only dimers that have GTP in their exchangeable E-site can polymerize, but following polymerization this nucleotide is

hydrolyzed and becomes non-exchangeable (Heald and Nogales, 2002; Gadde and Heald, 2004).

Since tubulins are preferentially incorporated at the plus end, and more rapidly lost at the minus end, a unidirectional flux, or treadmilling, of tubulin subunits along the axis of microtubule arises (Maiato *et al.,* 2004).

Depolymerization, repolymerization, treadmilling characteristics can be regulated by microtubule associated proteins (MAPs). Traditionally, a protein was considered to be a MAP if it could be co-purified in vitro with microtubules as a result of direct binding (Maiato *et al*., 2004, Sedbrook 2004). MAPs were shown to stimulate microtubule assembly. Subsequently, it was proposed to define MAPs as microtubules binding proteins *in vivo*. Now, they are usually defined as proteins that bind to microtubules and change their stability and mechanical properties. 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 & 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 & Qiang, 2005).

<u>Why do microtubules need to be dynamic?</u> Microtubules serve as highways along which cargoes are transported in a cell (they can move various cell contents via special attachment proteins), they provide structural support for the cell and organize membranous organelles. In order to fulfill these functions, microtubules must follow the cell cycle, the cell growth and rearrange themselves adapting to the cell needs at particular moment.

One example of microtubules' role requiring dynamic properties is in mitotic spindle. The spindle consists of microtubules as well as other proteins that alter microtubule dynamics. Microtubules have to grow from separated centrosomes and capture chromosomes in order to form a spindle. One model of such assembly, ''search and capture model'' says that microtubules emanating from a centrosome undergo cycles of growth and shrinkage, randomly probing the cytoplasm until running into a kinetochore, with which they form a stable attachment. When chromosomes segregate, they are carried to the distinct poles by shortening

microtubules. Another model, ''packman'', proposes that the kinetochore induces microtubule disassembly at the plus-ends, but maintains attachment as the fiber depolymerizes, thus chewing its way to the pole. The other model ''traction fiber'' proposes that pole-ward microtubule flux is harnessed to move the chromosome. Actually, both mechanisms work (Gadde and Heald, 2004).

Microtubules have an important role in specific activities of differentiated cells. In neurons, bundles of microtubules stretch from the cell body to the tips of elongated neural processes known as axons and dendrites. Axon can be a meter long, so if microtubules would be attached to centrosome, then one microtubule should be stretching for a meter. However, in neuronal cells (as well as in other asymmetric differentiated cells) microtubules are not attached to the centrosome (Keating *et al.,* 1997) and no one microtubule stretches the entire length of the axon; instead, short overlapping segments of parallel microtubules make the elongated microtubule structures. Microtubules provide support for the growth and maintenance of the process and also provide a substrate along which organelles are transported in both directions within the axon (Ahmad and Baas, 1995; Baas, 2000). Axonal transport of organelles, RNA and protein along the microtubules is absolutely required for health of neurons, and some human diseases (e.g. neuronal degeneration in motor neuron disease) have their bases in faulty or blocked axonal transport (Murray & Wolkoff, 2003).

In both above mentioned examples, ability of microtubule structures to move and rearrange themselves was essential for their functioning. However, these dynamic abilities are not only restricted to intrinsic dynamic characteristics of microtubules. In both cases, severing of microtubules and release of their minus ends play an essential role. Other examples where severing of microtubules play an important role include degradation of spermaxonemal microtubules after fertilization of sea urchin oocytes, microtubule reorganization during the transition from interphase to mitosis in dividing cells, deflagellation in *Chlamydomonas* (Lohret *et al.*, 1998, Quarmby & Lohret, 1999). In addition, severing filaments changes the physical and mechanical properties of the cytoplasm stiff, large bundles and gels become more fluid when the filaments are severed.

The minus ends appearing after severing were never seen to grow and once having begun to shorten were not observed to return to a stable state, differently from the plus end. It is supposed that the minus end is protected by some specific factor. So,

cells may produce, release, stabilize and transport centrosomal microtubules to produce non-centrosomal arrays like neurons are doing, or cells may release centrosomal microtubules and disassemble at the minus end to complement plus end dynamic instability when rapid reorganization of microtubule cytoskeleton is needed, e.g. during transition from interphase to mitosis (Keating *et al.*, 1997).

1.2. Katanin

1.2.1. AAA proteins

Katanin belongs to the AAA superfamily. AAA ATPases (ATPases Associated with various cellular Activities) play important roles in numerous cellular activities including proteolysis, protein folding, membrane trafficking, cytoskeletal regulation, organelle biogenesis, DNA replication and intracellular motility. The unifying feature of the AAA superfamily is an ATP-ase domain of about 220 amino acids. It 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) (Vale, 2000; Patel & Latterich, 1998; Hartman *et al.*, 1998).

AAA proteins function as oligomers (Vale, 2000), although according to Patel & Latterich (1998) there are also monomers. In most cases oligomers form hexameric rings (Vale, 2000). Katanin exists in an equilibrium between monomers and oligomers. Its oligomeric state has been shown to be a hexameric ring (Hartman and Vale, 1999).

How do AAA proteins use the ring structure? ATP binding induces structural rearrangements at the interface region which increases interactions between adjacent AAA domains as well as between the AAA protein and its target. This creates a tense state of the AAA-target protein complex. The tighter subunit-subunit interactions in turn accelerate ATPase reaction. Once the AAA modules are in ADP state, the complex reverts to a relaxed configuration in which interactions between AAA domains and the target protein weaken. Rings also provide a framework for binding target protein at multiple sites. If the ring-binding sites change their positions during the ATPase cycle, then tension could be applied to a bound protein (see Fig. 1.3.) (Vale, 2000).

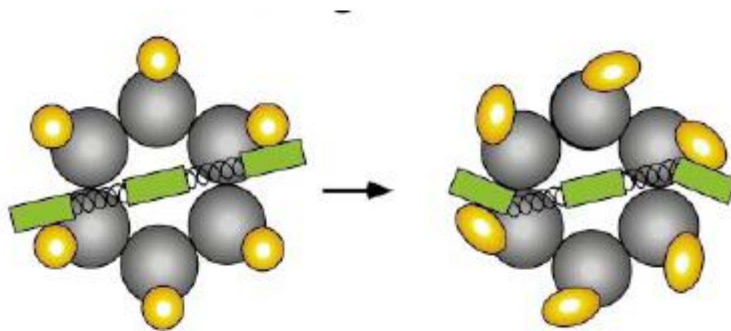


Fig. 1.3: Model of conformational change of AAA protein ring. (Vale, 2000)

1.2.2 Structure and functions of katanin

Katanin is one of the MAPs and it severes microtubules. It was first purified from sea urchin and characterized in 1993 by F.J. McNally and R.V. Vale. They named the purified protein upon ''katana'', the Japanese word for samurai sword. Katanin is a heterodimeric protein (60 and 80kD polypeptides). It was the first protein found which required ATP to sever and disassemble stable microtubules (McNally & Vale, 1993). Later another protein, spastin (Hazan *et al.*, 1999) which is related to katanin, displays ATPase activity and uses energy from ATP hydrolysis to sever and disassemble microtubules like katanin (Roll-Mecak & Vale, 2005; Evans *et al.*, 2005) was identified.

Katanin breaks microtubules along their length; it does not take away the tubulin dimers at the end of the microtubule, but removes them from the wall of the microtubule. Product of severing activity is tubulin dimers. Released tubulin dimers are able to repolymerize again, so they are not phosphorylated or otherwise changed.

Katanin can disassemble microtubules under conditions that favor spontaneous assembly of tubulin into microtubules. This means that either katanin inhibits polymerization of tubulin dimers or that katanin dissociates tubulin dimers from microtubules faster than they re-associate (McNally & Vale, 1993).

Tubulin subunits are held in place through both longitudinal and lateral contacts, they dissociate very slowly ($10^{-8}s^{-1}$) from the microtubule wall. Severing of a microtubule

at a specific spot along microtubule length requires thirteen subunits around the circumference of the tubule each to be dissociated from tightly bound neighbours above, below and on two sides. However, in the presence of ATP, katanin perturbs these tubulin-tubulin contacts and can sever and dismantle a taxol-stabilized microtubule within a couple of minutes (Vale, 2000). Microtubules act as a scaffold for katanin to oligomerize after it has exchanged its ADP for ATP (see Fig. 1.4). Once a complete katanin ring is assembled on the microtubule, the ATPase activity of katanin is stimulated. As a consequence of ATP hydrolysis and subsequent phosphate release, the katanin undergoes a conformational change leading to mechanical strain that destabilizes 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 the recycling of the katanin (Hartman & Vale 1999; Quarmby, 2000; McNally, 2000).

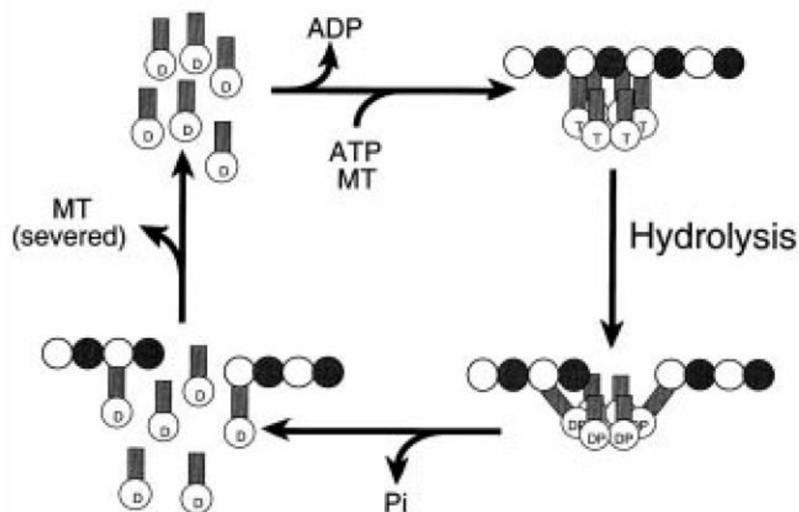


Fig. 1.4: Model for microtubule severing by katanin. (Only a single protofilament of microtubule is shown). In the top-right part, you see katanin-ATP oligomerized on microtubule. In the bottom-right part, as a result of ATPase activity katanin ring changes conformation leading to mechanical strain. In the bottom-left part, tubulin-tubulin bonds are broken, katanin dissociates from the complex. In the top-left part, katanin-ADP complex is ready to exchange ADP for ATP and join the cycle again. (Hartman & Vale, 1999).

Microtubule binding site for katanin hexamer is unknown. Possible binding sites include the outside of the microtubule, the microtubule lumen, or the sides of tubulin dimers exposed by holes in the lattice (McNally, 2000; Davis *et al.*, 2002). It is

thought that defects in the microtubule lattice might serve as sites for katanin activity (Davis *et al.,* 2002; Waterman-Storer and Salmon, 1997). Katanin exploits local defects and promotes loss of tubulin at the defect site until the two microtubule segments are held together so weakly that mechanically unconstrained microtubules kink at the defect site (Davis *et al.,* 2002). The acceleration of breaking with increasing free energy of curving was found in the study of Odde *et al.* (1999). However, expected degree of acceleration as a function of elastic energy increase did not act in a predictable manner. One can interpret the results assuming that increasing curvature increases MAP dissociation, which in turn lowers the rigidity and makes the microtubule more accessible to severing enzymes. Other possible mechanisms for the curvature-sensitivity include curvature-sensitivity of severing proteins and/or the tubulin dimers themselves. Increased curvature, caused by microtubule motors or mechanical deformation of the cell could increase the number of microtubule defects, in turn increasing the number of katanin severing locations (Davis *et al.,* 2002; Odde *et al.*, 1999).

ATPase and severing activities of katanin are closely related, but they are not tightly coupled. ATPase activity is stimulated by microtubules, while in the absence of microtubules ATPase activity is not observed. Moreover, microtubules that cannot be digested (e.g. subtilisin digested ones) stimulate ATPase activity. In the latter case, microtubules are not severed or disassembled but observed katanin's activity was the same as if microtubules would be digested. This shows that ATPase activity is not tightly coupled to severing. When ADP has been used to inhibit ATPase activity, severing activity was inhibited as well which shows that ATPase activity is necessary for severing (McNally and Vale, 1993).

Katanin displays an unusual microtubule-stimulating reaction. ATPase activity peaks at a microtubule concentration of 2 to 10 micromols and then decreases as the microtubule concentration is further increased (see Fig. 1.5). This differs from expected behaviour according to Michaelis-Menten law. There is the following explanation: microtubules may stimulate activity of katanin by facilitating katanin-katanin interactions, because when concentration is low it is more likely that katanin molecules will bind near one another on the microtubule, but high concentrations of microtubules may reduce ATPase and severing activities by preventing katanin-katanin associations, because, it is more likely that katanin molecules will be

dispersed over a high number of microtubules and will have less chance to bind near one another (Hartman and Vale, 1999).

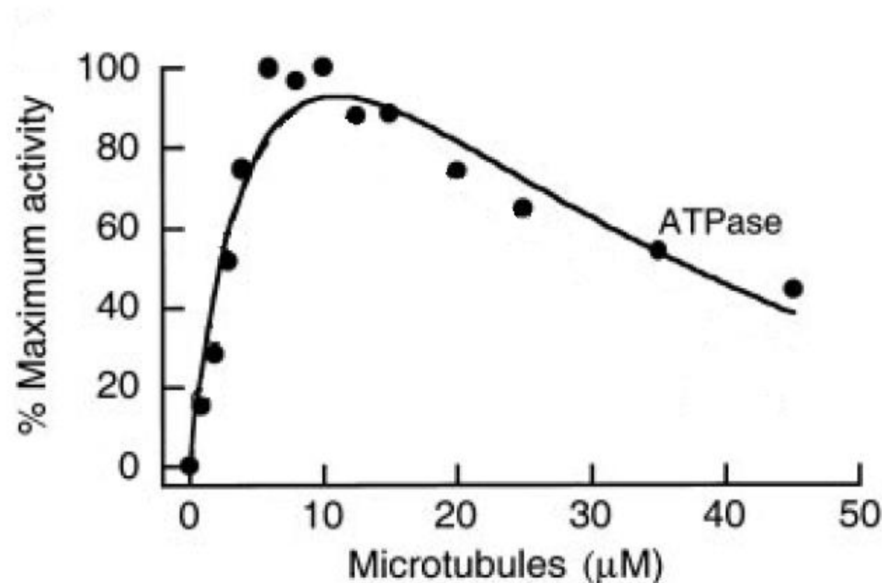


Fig. 1.5: Effect of microtubule concentration on katanin's activity. (Hartman and Vale, 1999)

Activity of katanin might be regulated by its synthesis and degradation levels. It was found to be the case in axons where katanin levels are high during their most active phases of growth but drop once axons have contacted their targets (Karabay et al., 2004) However, katanin is widely distributed and there should be another control mechanism to severe microtubules only when it is needed. There were some factors (like cyclinB/cdc2, cyclinB/cdc1) discovered that changed microtubule-severing activity in M-phase *Xenopus* egg extract. However, experiments with isolated katanin showed that it is not directly activated or phosphorylated (McNally & Thomas, 1998; McNally *et al.* 2002). Activity of katanin might be regulated indirectly by other MAP's which would restrict accession of katanin to microtubules (Baas & Qiang, 2005).

As regards its biological role, katanin is supposed to be important in mitosis. It was mentioned above that mitotic spindle consists of microtubules. Microtubule severing activity itself was first identified and studied in mitotic extracts of *Xenopus leavis* (McNally & Vale, 1993). Katanin was found to be highly concentrated at centrosomes throughout the cell cycle (McNally *et al.,* 1996). This supports the

hypothesis that katanin mediates the disassembly of microtubule minus ends during poleward flux (see Fig. 1.6) (McNally *et al.,* 1996; Quarmby 2000; Buster 2002).

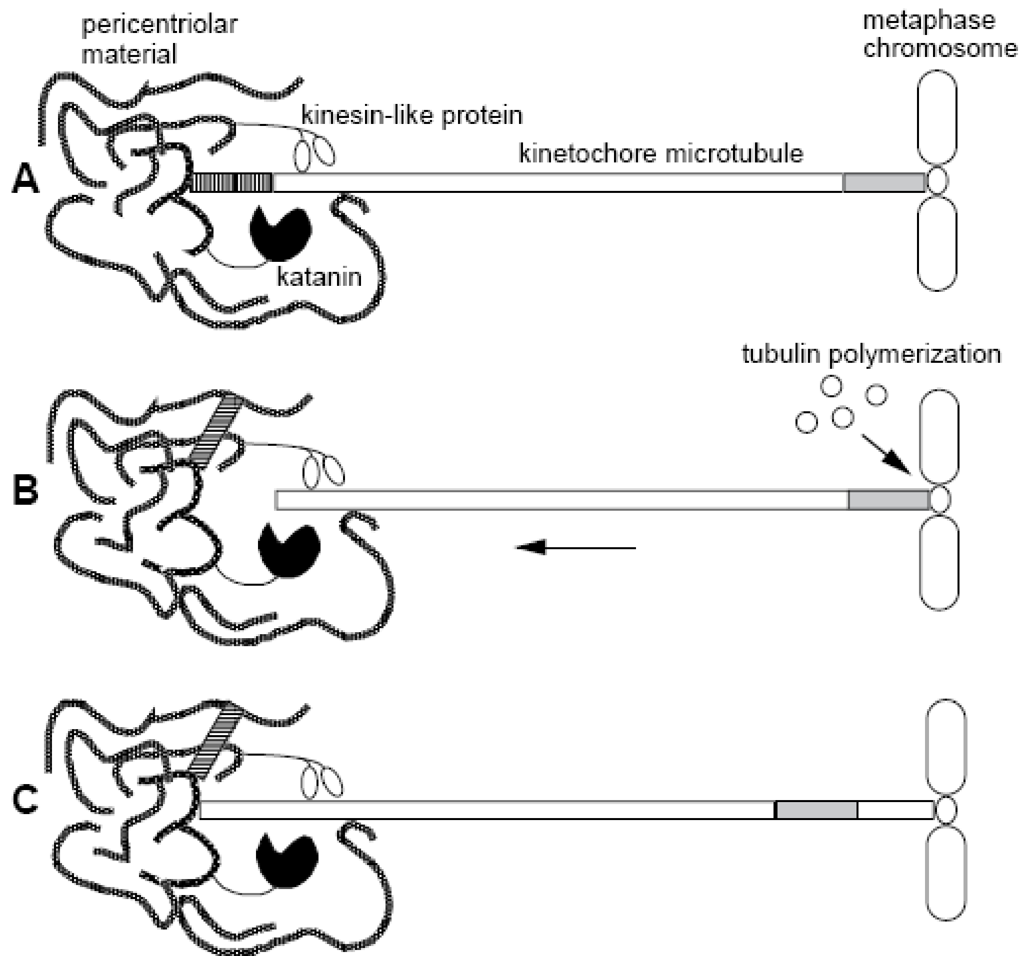


Fig. 1.6: Model of katanin's role in the mitotic spindle. Severing of the kinetochore microtubule by katanin releases a short microtubule fragment (A-B) and allows the kinesin-like protein to pull the kinetochore microtubule toward the centrosomal matrix (B). Simultaneous polymerization of tubulin occurs at the plus end (B-C). (McNally *et al.,* 1996)

It is also supposed that katanin facilitates microtubule depolymerization in transition from interphase to mitosis by releasing centrosomal microtubules and disclosing their minus end. Release of microtubules from centrosome is essential in neurons and it is especially active in neurons compared to nonneuronal cells as it was mentioned.

1.2.3. Katanin subunits

As it was previously mentioned, katanin consists of two subunits: p60 and p80 which were named according to their molecular weight. It has been showed that p60 has microtubule-stimulated ATPase and microtubule severing activities in the absence of p80, while p80 targets katanin to centrosome. (Hartman *et al.,* 1998) However, these findings turned out to be more complex after futher investigations.

p80 subunit is 658 amino acid long (rat p80) and contains six ''WD40'' repeat motifs (Karabay *et al.*, 2004). These proteins are characterized by the presence of repeats consisting of between 40 and 60 amino acids with two internal conserved dipeptide sequences, glycine-histidine (GH) and tryptophan-aspartic acid (WD) Despite their highly conserved structural motif, WD40 proteins play very diverse functions. This is probably due in part to the ability of these proteins to coordinate the binding of a variety of proteins through their individual blades (Smith *et al.*, 1999). The C-terminal region of p80 does not exhibit significant amino acid identity to any previously described protein. Although p60 severs microtubules in the absence of p80, p80 was found to affect this acitivity. p80 can enhance p60 mediated microtubule severing by increasing affinity of p60 to microtubules (McNally *et al.,* 2000). p60/p80 was found to have a two-fold higher microtubule severing activity compared to p60 alone. On the other hand, it was also found that WD40 domain contained in p80 acts as a negative regulator of microtubule severing by p60. (McNally, Thomas, 1998) The same domain is responsible for p80 centrosome targeting (Hartman *et al.,* 1998; McNally *et al.*, 2000; McNally, Thomas, 1998).

1 MATPVVTKTA WKLQEIV**AHA SNVSSLVLGK ASGRLLATGG DDCRVNLWSI NKPNCIMS**LT
61 **GHTSPVESVR LNTPEELIVA GSQSGSIRVW DLEAAKILRT** LM**GHKANICS LDFHPYGEFV**
121 **ASGSQDTNIK LWDIRRKGCV FR**YR**GHSQAV RCLRFSPDGK WLASAADDHT VKLWDLTAGK**
181 **MMSE**FP**GHTG PVNVVEFHPN EYLLASGSSD RTIRFWDLEK FQVVSC**IE**GE PGPVRSVLFN**
241 PDGCCLYSGC QDSLRVYGWE PERCFDVVLV NWGKVADLAI CNDQLIGVAF SQSNVSSYVV
301 DLTRVTRTGT VTQDPVQANQ PLTQQTPNPG VSLRRIYERP STTCSKPQRV KHNSESERRS
361 PSSEDDRDER ESRAEIQNAE DYNEIFQPKN SISRTPPRRS EPFPAPPEDD AATVKEVSKP
421 SPAMDVQLPQ LPVPNLEVPA RPSVMTSTPA PKGEPDIIPA TRNEPIGLKA SDFLPAVKVP

481 QQAELVDEDA MSQIRKGHDT MFVVLTSRHK NLDTVRAVWT TGDIKTSVDS AVAINDLSVV
541 VDLLNIVNQK ASLWKLDLCT TVLPQIEKLL QSKYESYVQT GCTSLKLILQ RFLPLITDIL
601 AAPPSVGVDI SREERLHKCR LCFKQLKSIS GLVKSKSGLS GRHGSAFREL HLLMASLD

Fig. 1.7: *Rattus norvegicus* p80-katanin amino acid sequence (Gene Bank, the European Molecular Biology Laboratory, accession No. Q8BG40). WD motifs are shown in bold, conserved dipeptide sequences are underlined.

Rat p60 is a 491 amino acid long polypeptide (Karabay, 2004), it has a conserved 230-amino acid ATPase domain (see Fig. 1.8). BLAST search revealed that ortholog of p60 exists in *C. elegans,* in *Arabidopsis thaliana* and there are vertebrate homologs of p60 (Hartman *et al.*, 1998; Stoppin-Mellet *et al.*, 2003; Bouquin *et al.,* 2003, Yang *et al.*, 2003). p60 alone displays a microtubule-stimulated ATPase activity. Although, when comparing the rates of microtubule disassembly, p60 is half as active as p60/p80 (Hartman et al., 1998).

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 YEKWIVEFGS C

Fig. 1.8: *Rattus norvegicus* p60-katanin amino acid sequence (Gene Bank, the European Molecular Biology Laboratory, accession No. AY621629). Part of p60 shown in bold is the sequence which was cloned and expressed; underlined part of p60 is the conserved C-terminal AAA domain.

1.3. Objectives of the research

Katanin or its orthologs are found in a number of organisms: sea urchin, *Xenopus laevis, Chlamydomonas* sp*., Arabidopsis thaliana*, rat, human. This microtubule severing protein has an important role in processes where microtubule reorganization is required, e.g. mitosis, axonal growth, deflagellation, therefore it is extensively studied. Full characterization of properties and functions of both subunits of katanin would enable to better understand activity and roles of katanin itself.

Antibodies against p80 and p60 will be used in the studies on separate subunits or on a whole dimer protein. For most research, diagnostic, and therapeutic purposes, monoclonal antibodies, derived from a single clone and thus specific for a single epitope, are preferable (Goldsby *et al.,* 2000). Recombinant soluble peptide would provide best conditions for monoclonal antibody production.

Both katanin subunits (p60 and p80) have been sought to be expressed, since separation of the native subunits requires denaturating conditions. However, bacterial expression of p60 produced largely insoluble protein (Hartman *et al.* 1998). Overproduction of heterologous proteins in the cytoplasm of *E coli* is often accompanied by their misfolding and segregation into insoluble aggregates known as inclusion bodies (Baneyx, 1999b). Due to solubility problems related to the bacterial expression of p60 subunit synthetic anti-peptide antibodies have been used until now.

Taking into account the need for soluble p60-katanin peptide, we have set the following objectives for this research:

- to clone and express p60-katanin;
- to adjust expression conditions in order to achieve soluble p60-katanin;
- to purify produced protein so that it could be further used for monoclonal antibody production.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

Centrifuge, Beckman coulter, Avanti J-30I
DNA sequencer, Applied Biosciences 3100-Avant
Gel dryer, Bio-Rad 583
Minicentrifuge, Hettich zentrifugen EBA 21
Mini-Vertical Gel System, Bio World EC 120
Orbital shaker, Forma
Precision weigher, Precisa 620 C SCS
Rocking shaker, Heidolph instruments Duomax 1030
Thermomixer comfort, Eppendorf
UV spectrophotometer, Beckman DU530 Life Science
UV spectrophotometer, Shimadzu 160
Vortex Heidolph, Reax top

2.1.2 Chemicals and enzymes

IPTG (isopropyl-β-D-1-thiogalactopyranosid) **AppliChem**

Eco RI restriction enzyme
Mass Ruler ™ DNA Ladder (Mix, 80bp-10Kb)
Page Ruler ™ Prestained Protein Ladder (10-170 kDa) **Fermentas**

Absolute ethanol
Absolute methanol
Acetic acid (glacial)
$CaCl_2$
Imidazole **Fluka**

Glycerol	
Isopropanol	
KH_2PO	
Na_2HPO_4	
$NaCl$	
NH_4Cl	
Phosphoric acid	**Fluka**
T7 primer (5 d[TAATACGACTCACTATAGGG] 3')	**Invitrogen**
Acrylamide	
Bromophenol blue	
EDTA	
Ethidium bromide	
Glucose	
2-mercapto-ethanol	
$MgCl_2$	
NaH_2PO_4	
NaOH	
SDS	
TEMED	
Tris	
Yeast extract	**Merck**
Hind III restriction enzyme	**New England Biolabs**
T4 DNA ligase	
Loading dye	**Promega**
Ni-NTA agarose for His-tag fused protein purification	**Qiagen**
PVDF membrane	**Roche**

Agarose
AMPS
Glycine
Tetracyclin **Sigma**

2.1.3 Buffers

TAE Buffer (50X)

242 g Tris base (40 mM)
57.1 ml glacial acetic acid (20 mM)
100 ml 0.5 M EDTA (pH 8.0) (1 mM)
H_2O up to 1 liter

Lysis Buffer for Metal Affinity Chromatography

34.5 mg NaH_2PO_4 (50 mM)
87.7 mg NaCl (300 mM)
3.4 mg imidazole (10 mM)
H_2O up to 5 ml, adjust pH to 8.0 using NaOH

Wash Buffer for Metal Affinity Chromatography

69 mg NaH_2PO_4 (50 mM)
175.4 mg NaCl (300 mM)
13.6 mg imidazole (20 mM)
H_2O up to 10 ml, adjust pH to 8.0 using NaOH

Elution Buffer for Metal Affinity Chromatography

34.5 mg NaH_2PO_4 (50 mM)
87.7 mg NaCl (300 mM)
2.72 g imidazole (4 M)
H_2O up to 5 ml, adjust pH to 8.0 using NaOH

Buffers of restriction and ligation enzymes

Buffers of restriction and ligation enzymes were purchased together with the enzymes.

2.1.4 Special reagents and kits

Agarose Gel DNA Extraction Kit, Roche

High Pure Plasmid Isolation Kit for small-scale (mini) preparations, Roche

50 % Ni-NTA agarose suspension, Qiagen

Anti-His_6 mouse monoclonal antibody, Roche

Goat AP conjugate anti-mouse, Novagen

2.1.5 Bacterial strains

Escherichia coli strain XL1 Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44relA1 lac* [F' *proAB lacIqZΔM15* Tn*10* (Tetr)]], Novagen

Escherichia coli strain BL21 (DE3)pLysS F⁻ *dcm ompT hsdS*(rB⁻ mB⁻) *gal* λ(DE3)[pLysS Cam^r], Novagen

2.1.6 Bacterial culture media

LB medium was prepared by dissolving 10 gram tryptone, 5 gram yeast extract, and 10 gram NaCl in distilled water. Distilled water was added to a final volume of one liter. The LB medium was sterilized by autoclaving for 15 minutes. In order to make selection media, antibiotic was added to the LB medium according to the concentration described in Table 2.1, and the antibiotic containing LB was stored at 4° C

LB-agar plate was prepared by adding 15 gram/l of agar to LB medium and sterilized by autoclaving as described above.

SOC medium was used to cultivate *E coli* for 1 hour after temperature shock during transformation. It was prepared by dissolving 2 gram of tryptone, 5 gram of yeast extract, 0.058 gram of NaCl, 0.0186 gram of KCl, 0.095 gram of $MgCl_2$, 0.24 gram of MgSO4, 0.36 gram of glucose in distilled water. Distilled water was added to a final volume of 100 ml. The SOC medium was sterilized by autoclaving at 120° C for 15 minutes. When required, antibiotic was added to the medium in order to make selection (Table 2.1).

Table 2.1. Stock and working solution of antibiotic

Antibiotic	Stock solution concentration	Working concentration
Kanamycin	10 mg/ml in water	50 µg/ml
Tetracyclin	15 mg/ml in ethanol	15 µg/ml

2.1.7 TA cloning vector with cloned insert

pCR 2.1-TOPO (Invitrogen) with cloned full length p60 subunit was previously designed by Dr. Arzu Karabay. The cloned p60 is in reverse direction i.e. its coding strand and non-coding strands exchanged their positions: 5' of coding strand of p60 in normal direction starts with a start codon ATG which is at 5' of non-coding strand of the reversely cloned p60 (see Fig. 2.1)

2.1.8 Expression vector

2.1.8.1 pET Expression System

In pET system (plasmid of expression by T7 polymerase), the expression of the DNA construct is under the control of T7 promoter, which is recognized by the T7 DNA dependent RNA polymerase (transcriptase), but not *E. coli* RNA polymerase. Typically, T7 transcriptase is expressed by the host genome, which is inserted behind the lacUV5 promoter. The latter is inserted into genome via lambda DE3 phage. Therefore, the host strains that carry T7 polymerase in their genome under the control of IPTG-inducable lacUV5 are called DE3.

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. Some of the strategies that have been developed to address these issues are co-overexpression of phage T7 lysozyme (which degrades T7 RNA polymerase) from the compatible pLysS and pLysE plasmids (Novagen) and the

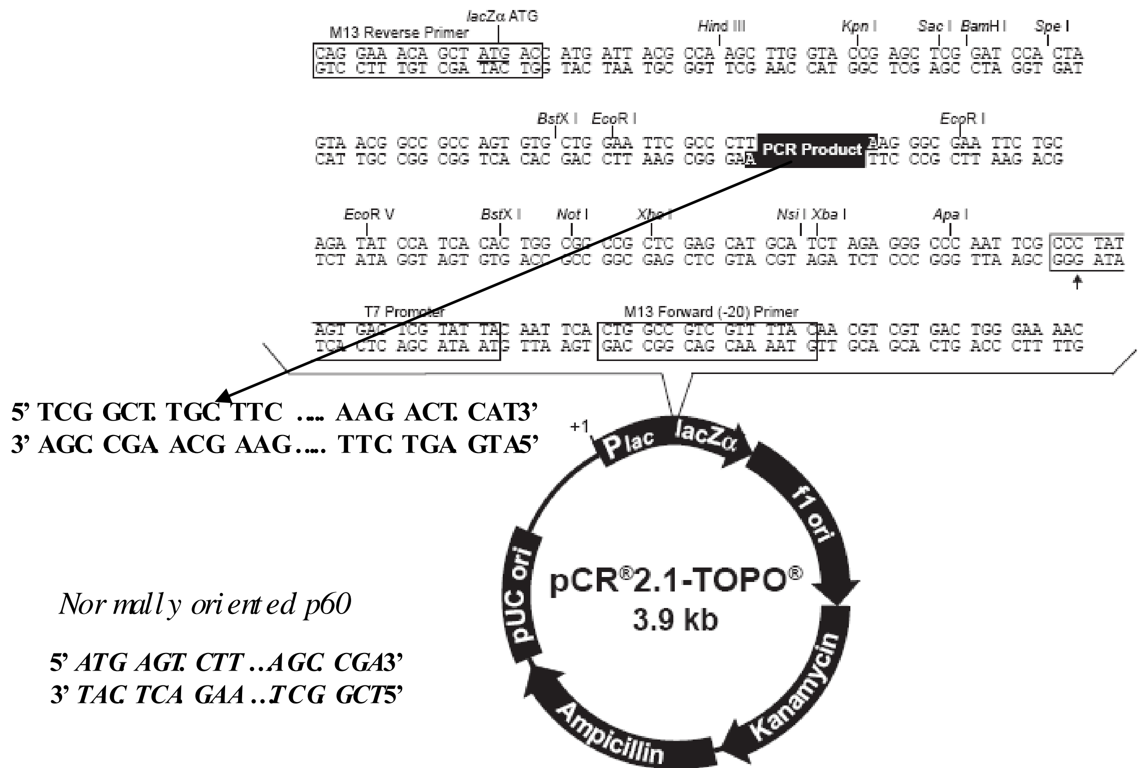


Fig. 2.1: Vector map of pCR2.1-TOPO with cloned insert. Cloning/restriction area with schematically represented insert is shown in a larger scale. Arrow stretching from PCR product points at the part of cloned p60 subunit (beginning and end of the sequence) in a large scale. At the bottom-left part of the picture part of normally oriented p60 is shown in italic.

insertion of a *lac* operator sequence downstream of plasmid-encoded T7 promoters, in order to reduce leaky transcription (Mierendorf *et al.*, 1994; Baneyx 1999).

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 HisoTag®/thrombin/SoTag™/enterokinase configuration plus an optional C-terminal HisoTag sequence. The circular map (Fig. 2.2) and the cloning/expression region (Fig. 2.3) are shown below. The vector is 5421 bp long.

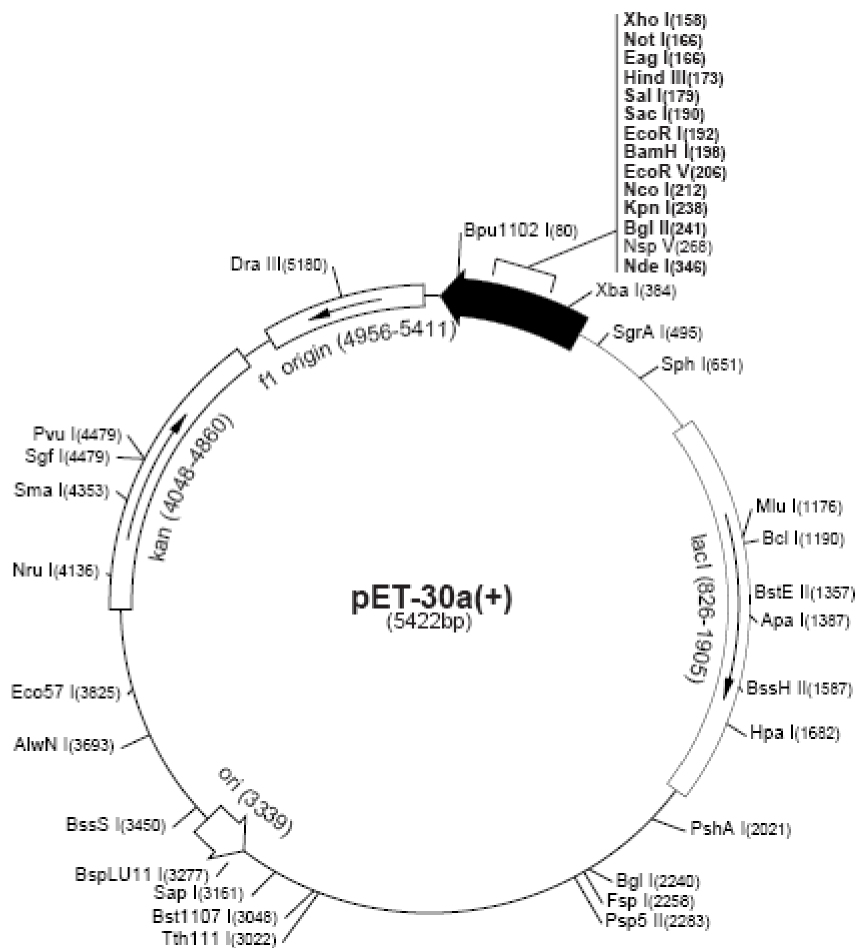


Figure 2.2: Vector map of pET30a. Source: http://www.emdbiosciences.com

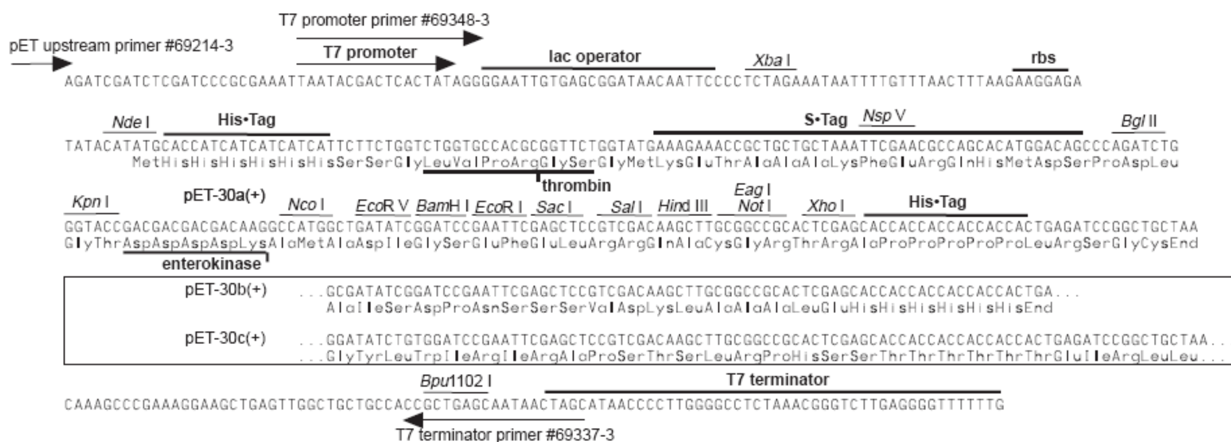


Figure 2.3: Cloning/expression region of the coding strand of pET30a.
Source: http://www.emdbiosciences.com

2.2 METHODS

2.2.1 Small scale plasmid DNA preparation (mini-prep)

Plasmid mini preparation was performed using Roche, High Pure Plasmid Isolation Kit for small-scale (mini) preparations, following instructions of the manufacturer. The principle of this purification is as follows: alkaline lysis releases plasmid DNA from bacteria and RNase removes all the RNA in the lysate. Then, in the presence of a chaotropic salt (guanidine HCl), plasmid DNA binds selectively to glass fiber fleece in a centrifuge tube. The DNA remains bound while a series of rapid ''wash-and-spin'' steps remove contaminating bacterial components. Finally, low salt elution removes the DNA from the glass fiber fleece.

The protocol is as follows:

- A single bacterial colony was picked and inoculated into 10 ml LB media (with kanamycin) containing Falcon tube, and grown overnight with vigorous shaking (250 rpm) at 37°C.
- The following day, 7.5 ml of the culture was distributed into 5 eppendorf tubes (1,5 ml each tube), and the bacteria were recovered by centrifugation for 5 minutes at 14,000x*g*. The supernatants were discarded.
- The bacterial pellet was resuspended in 50 µl of suspension buffer in each eppendorf tube separately and then collected to one eppendorf tube (250 µl suspension buffer in total). Suspension buffer contains RNase which removes bacterial RNA
- To lyse the cells, 250 µl lysis buffer was added (contains NaOH), mixed by inverting the tube 6 times and incubated at room temperature for up to 5 minutes.
- Lysis was stopped by addition of 350 µl ice-cold binding buffer. Tube was again inverted 6 times and incubated on ice for up to 5 minutes.
- The mixture was centrifuged for 10 minutes at 14.000 x *g* and the supernatant was transferred to a filter tube. Chromosomal DNA was precipitated with cellular debris during centrifugation and this supernatant contains the plasmid DNA

- Again centrifugation for 1 minute at maximum speed 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, 700 µl of wash buffer was added to the filter tube and centrifuged at maximum speed for 1 minute. Supernatant from collection tube was discarded.
- To elute the DNA 100 µl elution buffer was added, and the DNA solution was obtained by centrifugation for 1 minute at full speed.

2.2.2 Determination of nucleic acid concentration

Recovery, purity and concentration of nucleic acids were determined by spectrophotometric analysis. The ratio of absorbance at (A260) should be 1.8 for DNA. In the presence of protein contamination, the ratio is less. For the measurement, we used a spectrophotometer from Shimadzu. The DNA was diluted 1:200 or 1:2000 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 is equivalent to 50 µg/ml DNA. The formula used to calculate the concentration (C) is the following:

C = OD 260nm x dilution factor x equivalent = x µg/ml

In order to check results of the spectrophotometric analysis, DNA extracted from gel was also run on the 1% agarose gel before ligation.

2.2.3 Preparation of chemically competent cells - calcium chloride method

To introduce plasmid DNA or recombinant plasmid DNA into bacteria, the bacteria had to be made competent for this purpose. We home made competent cells according to the following protocol, provided by Sambrook *et al* (Sambrook *et al.*, 1989).

- Working aseptically, XL1 Blue cells (taken from a glycerol stock culture) were streaked out on an LB plate and incubated overnight at 37° C

- The next day, one bacterial colony was picked and inoculated into 10 ml of LB medium containing tetracyclin in a Falcon tube, and the overnight culture was grown.
- The next day 100 ml LB medium was inoculated with 4 ml of overnight culture solution and was incubated at 37° C in a rotatory shaker. Cell density was measured by a spectrophotometer at OD_{600}. When an OD_{600} of 0.6 was reached, 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 10 minutes at 4° C, the supernatant was discarded
- Bacterial pellet was resuspended in 10 ml ice-cold $CaCl_2$ (pH 7, the solution was filter sterilized through a filter of 0.45 µm pore size) and incubated on ice for 30 minutes.
- Centrifugation was performed again for 5 minutes at the same speed as previously, and the cells were resuspended in 2 ml of $CaCl_2$.
- The cells were used immediately for transformation and/or distributed into prechilled sterile microfuge tubes. The competent cells were stored at - 80°C in 40 µl aliquots.

$CaCl_2$ solution

Contents	**Concentration**	**Amount**
$CaCl_2$	60 mM	0.33 g
PIPES	10 mM	0.15 g
Glycerol	15 %	7.5 ml
water	X	up to 50 ml

2.2.4 Transformation of competent cells

Transformation is the term used to describe the introduction of plasmid DNA into bacteria. The experiment can be performed by an electrical or a chemical method. We used the chemical method which consists of a heat shock to introduce the DNA into the host. A short protocol is as follows:

- The competent cells from - 80° C were thawed on ice. 2-3 µl of purified plasmid DNA or 10 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 minutes.

- After that, the cells were kept at 42° C for exactly 40 seconds (heat shock) and immediately were incubated on ice for 10 minutes.
- 80 µl of LB liquid medium was added to competent cells and the eppendorf tube was vigorously shaked at 37° C for 1 hour.
- The cells were then plated onto LB (containing appropriate antibiotic) plate. The plates were incubated at 37° C overnight.

2.2.5 DNA cleavage with restriction endonucleases

Restriction enzymes, also called restriction endonucleases are bacterial proteins which work as an immune system in bacteria. Their role is to destroy bacteriophages or other viruses which invade bacteria. Restriction endonucleases recognize a specific nucleotide sequence, and cut DNA wherever this specific sequence is found. Usually the palindromic restriction sites have a length of 4 to 8 base pairs. The purified restriction endonucleases are commercially available, and are used to generate DNA fragments for cloning experiments. Therefore, restriction endonucleases are a major tool in recombinant DNA technology.

Since the cloned p60 had to be subcloned changing its orientation at the same time, the following requirements were set for the restriction endonucleases:

- they have to cut both T/A and expression vectors;
- their sequence in the vectors should be different, i.e. 5' restriction site on T/A vector should be in 3' compared to another restriction site on expression vector;
- restriction endonucleases should leave out TGA codone in non-coding strand of T/A vector;
- restriction endonucleases should not cut inside the p60 subunit, preferably.

Eco RI and Hnd III met most of the requirements

Normally DNA was cleaved at 37° C for 4 hours with 1 X buffer (supplied by the manufacturer).

Restriciton reaction mixtures

Contents	Amount	Volume, µl
Plasmid DNA (pET 30a)	1.5 µg	11,5
EcoRI	10 units	1
Hind III	20 units	1
10X Y buffer	1x	1,5
Total reaction volume		15
Plasmid DNA (pCR2.1-TOPO-p60)	4.55 µg	7
EcoRI	10 units	1
Hind III	20 units	1
10X Y buffer	1x	1
Total reaction volume		10

2.2.6 Ligation

T4 DNA ligase is encoded by the gene 30 of bacteriophage T4. This enzyme can be used to ligate DNA restriction fragments. T4 DNA ligase has the capacity to catalyze *in vitro* the formation of a phosphodiester bond between adjacent nucleotides, one containing a terminal 5-phosphate group and one containing the hydroxyl terminus. A review of the ligation reaction can be seen in Cherepanov and de Vries, 2003. In this study, cohesive ligation type was performed.

Most restriction endonucleases create cohesive ends. When fragment DNA and plasmid DNA are digested with the same restriction endonuclease, complementary cohesive ends are generated that can easily be ligated. For ligation overnight incubation at room temperature was performed. Ligation mixture was made by adding compounds depicted below

Contents	Amount	Volume, µl
Plasmid DNA	300 ng	1
Insert DNA	-	7
10 x buffer	1x	1
Ligase		1
Total reaction volume		10

2.2.7 Agarose gel electrophoresis

Agarose is a linear polymer composed of residues of D- and L-galactose and is used to separate fragments of DNA or RNA by size (Sambrook and Russell, 1989). Since deoxyribonucleic acids are negatively charged, they migrate through the agarose gel in a electrical field towards the positive anode. Molecules of double-stranded DNA migrate through gel matrices at rates that are inversely proportional to the log10 of the number of base pairs, therefore small molecules migrate faster than large ones. Plasmid DNA or DNA fragments obtained after treatment with restriction enzymes were separated by electrophoresis through a low melting point agarose gel. 1 % agarose concentration was used.

To prepare 1 % agarose gel, 0.4 g of low melting point:

- Agarose was dissolved in 40 ml (small gel) 1x TAE (Tris-acetate-EDTA) buffer.
- The agarose was solubilized in a microwave oven until the agarose was completely dissolved.
- Gel was cooled to $\leq$ 45º C 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 minutes and then placed into an electrophoresis tank, where the gel was covered by 1x TAE buffer used to make the gel.

The DNA was mixed with loading dye and the sample was placed into a well on the agarose gel. As fragment size control, a MassRuler ™ DNA Ladder, Mix (80bp-10kb) was used. Electrophoretic separation was achieved by constant current at 80 mV for 60 minutes.

DNA within agarose gels is only visible when stained with ethidium bromide and can then be visualized under UV light. The gel was placed onto an UV ilumuminator that emits UV light at 302 nm and photographed with a camera connected to a computer. Image files were saved with UVI Photo MW 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 Mass Ruler.

2.2.8 Isolation of DNA fragments from agarose

Nucleic acids bind specifically to the surface of glass or silica materials in the presence of a chaotropic salt. The binding reaction occurs due to the disruption of the organized structure of water molecules and the interaction with the nucleic acids. Thus the adsorption to the specifically pretreated spherical silica matrix is favored. Since the binding process is specific for nucleic acids, the bound material can be separated and purified from impurities e.g. salts and proteins, by a simple washing step. Nucleic acids elute from the matrix in a low salt buffer or water.

Isolation of DNA fragment from agarose gels was performed using the Agarose Gel DNA Extraction Kit, Roche, which uses the above mentioned principles, following instructions of the manufacturer.

- A slice containing the desired DNA band was excised from the gel. The gel slice was placed in a 1.5 ml eppendorf tube, and weighed. Three times of the agarose volume solubilization buffer was added to one volume of the gel.
- 10 µl of silica suspension was added to the sample and sample was vortexed.
- The gel slice was incubated at 56° C for 10 minutes to dissolve the agarose. To help gel dissolution the tubes were vortexed every 2 minutes.
- After the gel slice was dissolved completely, the solution was centrifuged for 1 minute at maximum speed, then the supernatant was discarded.
- The matrix containing DNA was resuspended with 500 µl nucleic acid binding buffer.
- Solution again was centrifuged and supernatant was discarded.
- Remaining pellet with bound DNA was washed twice with 500 µl washing buffer, centrifuging and discarding supernatant each time.
- Pellet remaining after washing step was let to dry at room temperature, until the matrix colour turned to bright white.
- Then elution step was performed, 30 µl of redistilled water (pH 8.5) was added to the dry pellet and it was incubated for 10 minutes at 56° C, vortexing every 2-3 minutes.
- Subsequently, eppendorf tube with solution was centrifuged for 30 seconds at maximum speed.

2.2.9 DNA Sequencing

The sequencing method is based on the use of modified nucleotides, so called 2`, 3`-dideoxyl analogs. These special nucleotides lack a hydroxyl residue at the 3` position of the deoxyribose. When DNA polymerase adds nucleotides into a DNA chain through its 5` triphosphate groups, the absence of a hydroxyl group on a dideoxyl analog avoids the formation of a phosphodiester bond with an adjacent nucleotide, leading to a stop in elongation. As the concentration of the analogs is very low, termination happens just occasionally. With four different analogous nucleotides in four separate reactions, numerous fragments corresponding to every base position will be synthesized. As fluorescently labelled primers were used, newly synthesized DNA fragments are marked and the sequence can be detected by excitation with a laser and detection with photodiodes in a sequencing machine.
The achieved plasmids 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

Contents	Amount	Volume, µl
Big dye reaction mix	X	2
5X sequence mixture	X	2
Template DNA	360 ng	4
Primer T7	25 mM	0.5
Water	X	1.5
Total reaction volume	X	10

The sequence reactions were performed using a thermal cycler with the following program

Cycles	Temperature	Time
1	95 °C	5 minutes
40	95 °C	10 seconds
	55 °C	10 seconds
	60 °C	4 minutes
Final extention	4 °C	

2.2.10. Alignment of sequences

Nucleotide alignments were made with BLAST tool, available at http://www.ncbi.nlm.nih.gov/BLAST/ (nucleotide-nucleotide BLAST (blastn)).

2.2.11. Protein expression induction

Fresh plates containing bacteria BL21(DE3)pLysS colonies harbouring pET30a vector with cloned p60-katanin and pET30a without any insert (used for control) were prepared. A single colony from each plate was picked and inoculated into 10 ml LB (containing kanamycin) in Falcon tube and left for overnight growth at vigorous shaking at 37°C. Next day, 2 ml of overnight culture was diluted with LB media and placed into 250 ml Erlenmeyer flask. Culture was incubated with shaking at 37° C until the OD_{600} reaches a value between 0.6 and 0.8. After that, cells were induced with 0.5 mM IPTG and grown for 12 hours, taking samples during this time.

2.2.12. Cell fraction analysis

2.2.12.1. Total cell protein analysis

- Following induction, 1 ml cell culture for induction analysis was collected by centrifugation for 5 minutes at 14000 *x g* in a microfuge.
- Supernatant was discarded. Pellet was mixed with 50 µl 2X SDS sample buffer.

The rest of the procedure is explained in the SDS-PAGE methodology presented below

2.2.12.2 Soluble total cell protein analysis

- Following induction, 1 ml cell culture for induction analysis was collected by centrifugation for 5 minutes at 14000 *x 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 at -80 °C overnight.
- Next day, it was thawed on ice.
- The entire lysate was centrifuged at 14,000×g for 10 minutes to separate the soluble and insoluble fractions.
- The supernatant was concentrated with TCA (trichloracetic acid) method which is as follows:
    - 100 µl (1/10 volume) of 100% TCA (w/v) was added to 1 ml of supernatant and vortexed. Sample was placed on ice for 15 minutes.
    - Then it was centrifuged at 14000 *x g* for 10 minutes and the supernatant was removed.
    - Pellet was washed twice with 100 µl of acetone, the mixture was vortexed, centrifuged for 5 minutes (14,000 ×*g*) and supernantant discareded.
    - Finally pellet was allowed to air dry thoroughly by leaving the tube open on the bench top.

2.2.13. Electrophoresis of proteins on SDS-polyacrylamide gels

To separate proteins by size, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a vertical gel chamber. This method is based on a two gel layer system: a stacking gel and a separating gel.

Solution for preparing 12.5 % separating gels

Contents	Volume
30 % acrylamide*	2.08 ml
1,5 M Tris, pH 8.8	1.25 ml
water	1.56 ml
10 % SDS	50 µl
10 % APS	30 µl
TEMED	10 µl

- 30 % acrylamide was prepared by dissolving acrylamide and N,N'-methylenbisacrylamide in water.

Solution for preparing 5 % stacking gels

Contents	Volume
30 % acrylamide	0.33 ml
0.5 M Tris, pH 6.8	0.5 ml
water	1.13 ml
10 % SDS	20 µl
10 % APS	10 µl
TEMED	5 µl

The amounts depicted above are sufficient to make 1 mini gel in a cassette from Bio World Working quickly, the 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. The gel was polymerized for at least 30 minutes, and the isopropanol was carefully removed. 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 30 minutes, and used on the same day.

The samples containing proteins were resuspended in 2X sample buffer. The samples were denaturated for 5 minutes at 95° C and 7.5 µl of each sample was loaded immediately on the SDS PAGE gel.

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 %)
β- mercaptoethanol	10 %	1 ml (of 100 %)
Bromophenol blue	0.05 %	5 mg
Water		up to 10 ml

Electrophoresis was carried out in Tris-glycine electrophoresis buffer. The electrophoresis was divided in two steps. During the first step, the proteins were run in the stacking gel for 15 min at 150 V, 250 mA, 25 W In the second step, the proteins were separated by size running through the pores of the separating gel for

105 min at 100 V, 250 mA, 25 W. As molecular weight marker PageRuler™ Prestained Protein Ladder, Fermentas, was used, containing marker proteins from 10 to 170 kDa.

Tris-glycine running buffer

Content	Concentration	Amount
Tris	0.025 M	3g
Glycine	0.192 M	14.4 g
SDS	0.1 %	10 ml (of 10%)
water		up to 1 liter

SDS-PAGE was stained in stain solution for 40 seconds in a microwave and 3 more minutes at room temperature on a rocker. It was destained in destain solution for 50 seconds in the microwave and 3 hours at room temperature on a rocker.

Stain solution - Coomassie Briliant Blue (CBB) stain

Content	Concentration
CBB R-250	0.1%
Methanol	50%
Acetic acid	10%
Water	up to final conc.

Destain solution

Content	Concentration
Methanol	5%
Acetic acid	10%
Water	up to final conc.

2.2.14. Metal affinity purification of 6xHis tagged p60-katanin

In metal affinity purification, nickel (or other metal ion, e.g. copper, zinc, cobalt) and imidazole ring binding is used to purify His-tagged proteins. Immobilized-metal affinity chromatography (IMAC) was first used to purify proteins in 1975 using the

chelating ligand iminodiacetic acid. Now, nitrilotriacetic acid (NTA) is wider used because 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 6xHs tag while iminodiacetic acid occupies only three ligand binding sites of nickel ion and, therefore, the bond is weaker.

When lysate is mixed with N-NTA (nickel-nitriloacetic acid) agarose, nickel binds imidazole ring structure of histidine (Fig. 2.4). In elution step, highly concentrated imidazole in the elution buffer replaces histidine and histidine tagged peptide is eluted.

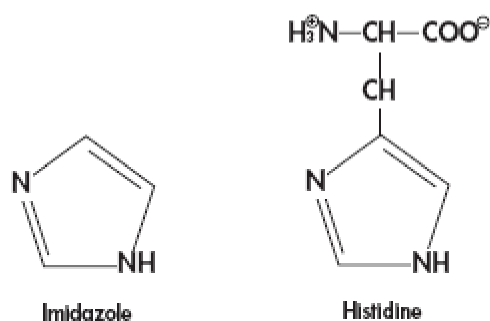


Figure 2.4: Imidazole ring structure and histidine.

Purification of p60-katanin was made under native conditions.

- 200 ml of IPTG induced bacterial culture was grown for 12 hours and centrifuged for 5 minutes at 14000 *x g*.
- The supernatant was discarded and the pellet was overnight frozen at -80° C.
- Next day, it was thawed on ice, resuspended in 1 ml lysis buffer (see section 2.1.3 ''Buffers'') and centrifuged for 10 minutes at 14000 *x g*.
- 12 μl sample of lysate was taken for further SDS-PAGE analysis.
- 200 μl of 50% N-NTA agarose was added to the lysate and the mixture was gently mixed for an hour in the eppendorf tube at +4° C
- Then it was shortly spinned to pellet the resin and supernatant (which is also called flow-through, since it passes through the resin) was transferred to the fresh tube for SDS-PAGE analysis.

- The resin was washed twice with 1 ml of wash buffer (see section 2.1.3 ''Buffers''). It was spinned shortly after each wash step and the supernatant was taken for SDS-PAGE analysis.
- Finally, the protein was eluted 4 times with 100 µl elution buffer (see section 2.1.3 ''Buffers''). Short spin was done after each elution step and the supernatant containing purified protein was taken to a fresh tube.

2.2.15. 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; 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.

- SDS-PAGE is done. 15 % gel was prepared. If usually 7.5 µg of sample was loaded, for Western blot 25 µg was loaded.
- 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 minutes.
- The gel was placed in the ''sandwich'' chamber with 2 fiber pads and 2 filter papers and prepared PVDF membrane (fiber pad/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 40 V overnight.

- Next day the membrane was blocked with blocking solution on the shaker overnight at 4° C (in the cold room). Proteins in milk containing solution block unoccupied sites on the membrane.
- Then it was washed for 10 minutes with TTBS solution.
- After that it was incubated with primary antibody in blocking buffer overnight at 4° C(in the cold room).
- Wash with TTBS for 10 minutes followed.
- Then the membrane was incubated with secondary antibody in blocking solution for 1 hour at room temperature.
- After that it was washed twice for 10 minutes with TTBS.
- Then wash for 10 minutes with TBS in order to get rid of the detergent present in TTBS was performed.
- Finally membrane was soaked into NBTC/BCIP solution and kept until the bands were seen. This last step was performed in the darkroom. The reaction was stopped by adding water.
- Membrane was dried at room temperature in the dark room.

Solutions for western blot:

Transfer buffer

Substance	Concentration	Amount
Glycine	39 mM	3 g
Tris	48 mM	7.5 g
SDS	0.037 %	0.037 g
Methanol	20 %	200 ml
Water		up to 1 l

TBS 1x

Substance	Amount	
NaCl	2.4 g	
KCl	0.06 g	
Tris	0.9 g	
Phenol red		
Water	up to 300 ml ⟶	pH 7.4

TTBS

Substance	Concentration	Amount
Tween 20	0.05 %	90 µl
TBS	x	up to 180 ml

Blocking solution

Substance	Concentration	Amount
Non-fat dry milk	3 %	0.9 mg
TBS	x	up to 30 ml

First antibody solution

Substance	Concentration	Amount
Anti Hs_6 (mouse)	100 µg/ ml	20 µl
Non-fat dry milk	3 %	0.3 mg
TTBS	x	up to 10 ml

Second antibody solution

Substance	Concentration	Amount
Goat AP conjugate (anti-mouse)	1: 10.000	1 µl
Non-fat dry milk	3 %	0.3 mg
TTBS	x	up to 10 ml

2.2.16. Bradford protein determination assay

The Bradford Reagent can be used to determine the concentration of proteins in solution. The Bradford assay works by the action of Coomassie brilliant blue G-250 dye (CBB-G). This dye specifically binds to proteins at arginine, tryptophan, tyrosine, histidine and phenylalanine residues. It should be noted that the assay primarily responds to arginine residues (eight times as much as the other listed residues). CBB-G binds to these residues in the anionic form, which has an absorbance maximum at 595 nm. The free dye in solution is in the cationic form, which has an absorbance maximum at 470 nm. The assay is monitored at 595 nm in a spectrophotometer, and thus measures the CBB-G complex with the protein.
Home made Bradford reagent was used. It was prepared as follows:

Content	Amount	
CBB G-250	50 mg	
Ethanol (pure)	25 ml	
85 % phosphoric acid	50 ml	
Water	up to 500 ml →	filter through the filtering paper

First the standard curve was drawn. Standard samples for the curve were prepared using bovine serum albumine (BSA) with the following concentrations: 2 μg/ml; 5 μg/ml; 8 μg/ml; 12 μg/ml; 15 μg/ml.

Unknown (i.e. assayed) samples were prepared adding 10 μl of purified p60-katanin received after elution to 1 ml of the Bradford reagent.

Absorbance was measured with Beckman spectrophotometer. It determines concentration of assayed sample on the basis of the standard curve and readily gives the results.

3. RESULTS

3.1 Cloning of p60-katanin

p60-katanin cloned into pCR2.1-TOPO (Invitrogen) vector was used as the starting material.

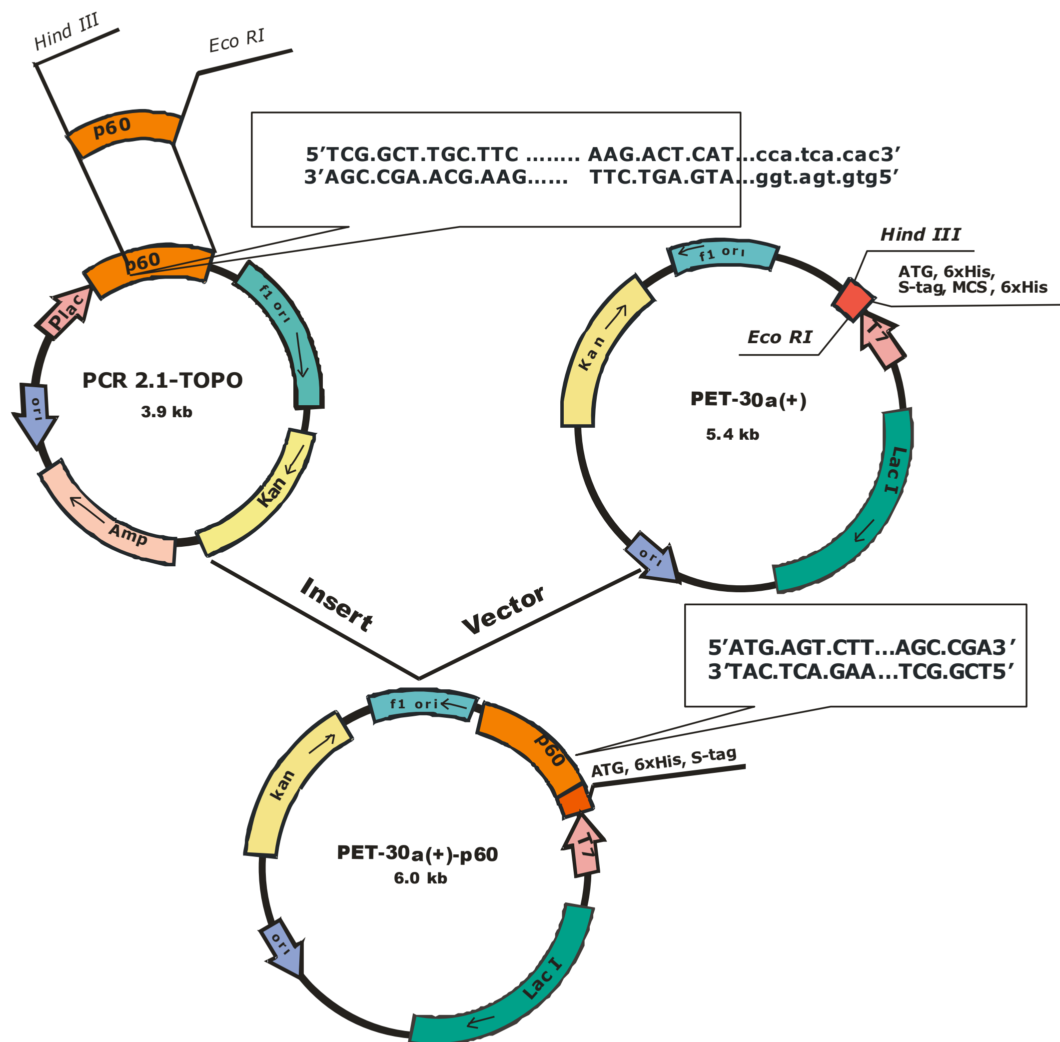


Figure 3.1: Scheme of p60 subcloning. P60-katanin is cut with HindIII and Eco RI from PCR2.1 - TOPO vector (left above) and subcloned into pET-30a expression vector (right above). Resulting construct where p60 is fused to His-tag is shown at the bottom of the picture. p60 sequence part is written in big letters; it changes orientation after subcloning. A part of PCR2.1 - TOPO vector sequence containing stop codon in non-coding strand is written in small letters.

Hind III and Eco RI restriction endonucleases were chosen to be most appropriate ones although Hind III cuts between 577-578bp in p60 subunit (862 bp in total). However, this was accepted and 578 bp of p60 were subcloned into pET30a (Novagen) expression vector.

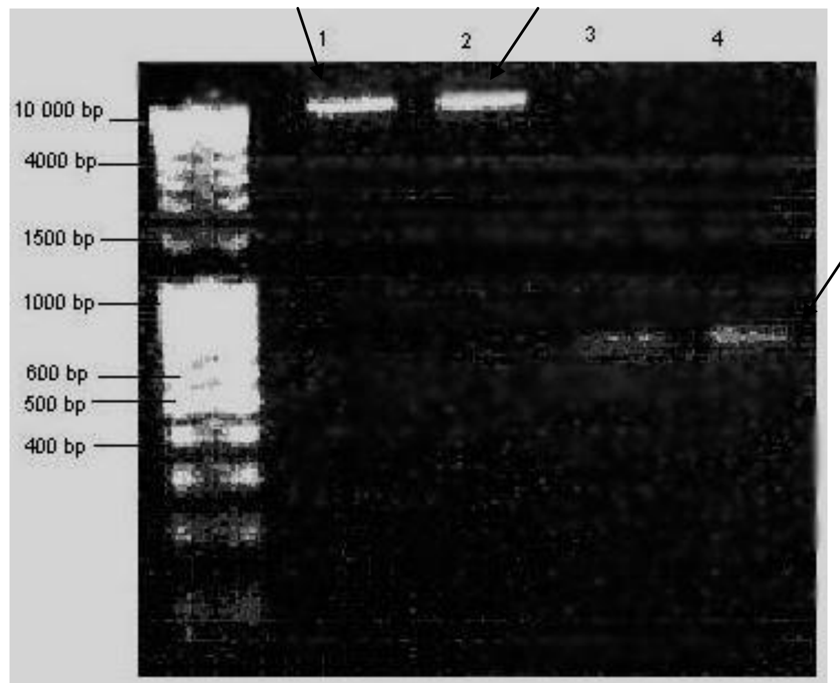


Fig. 3.2: Restricted insert and expression vector DNAs extracted from agarose gel and run on a gel again. Lane 1 - pET30a vector restricted with R buffer, Lane 2 - pET30b vector restricted with Y buffer, Lane 3 - p60 restricted from pCR 2.1-TOPO with R buffer, Lane 4 - p60 restricted from pCR 2.1-TOPO with Y buffer (pointed by arrow).

Restriction of pET 30a expression vector with EcoRI and Hind III resulted in one clear fragment between 5-6 kb, which corresponds to vector size and restriction of T/A cloning vector with cloned p60 subunit resulted in several fragments: about 4kb, about 600 bp, about 300 bp, which corresponds to predicted fragment sizes. Before ligation, DNA extracted from gel was again run on agarose gel in order to check whether its amount was sufficient for ligation (Fig. 3.2). Ligation was performed adding maximum possible amount of insert DNA into 10 µl reaction volume.

After ligation, the constructs were transformed into competent cells of *E.coli* XL1 Blue strain and transformants were selected from selective antibiotic plates. After

purifying the plasmids restriction analysis was performed to confirm cloning (Fig. 3.3). Fragments close by size to vector part (5.5 kb) and p60 insert part (585 bp) appear after restriction.

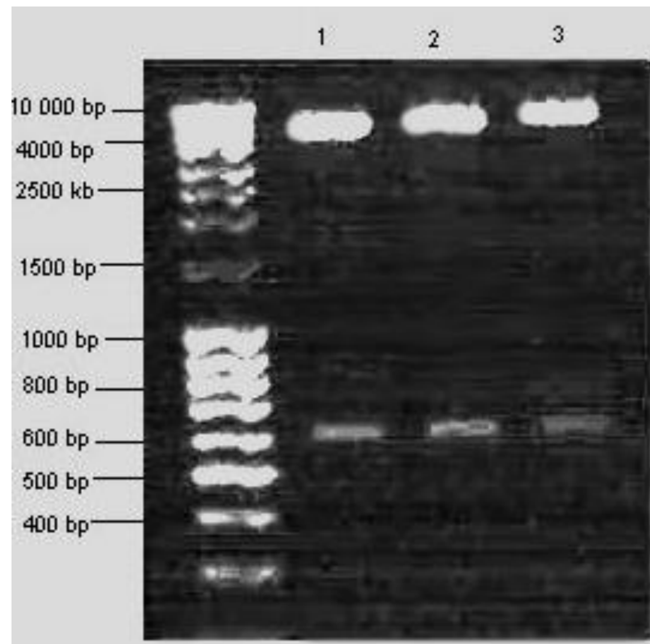


Fig. 3.3: Restricted purified transformant plasmids. All three samples (lanes 1, 2, 3) are cut to produce two segments: one in the region between 5-6 kb and another in the region of 600 bp.

After preliminary control by restriction analysis, DNA sequence determination was used to confirm inframe insertion and correct orientation of p60 subunit. Obtained sequence was aligned with a theoretical one. Sequencing results and alignment are presented in the Appendix, Fig. A1 and Fig. A2, respectively. No shift mutation was observed. There is a mutation from glutamine to arginin (both glutamine and arginin are polar amino acids).

The resulting expression plasmid (pET30a-p60) produced a fusion protein with a polyhistidine residue.

3.2 Expression studies

In order to express p60-katanin, obtained construct of p60-pET30a was transformed into expression strain of *E. coli* BL21(DE3)pLysS. IPTG to a final concentration of

0.5 mM was added to transformed bacterial culture growing in LB media at room temperature to induce protein expression.

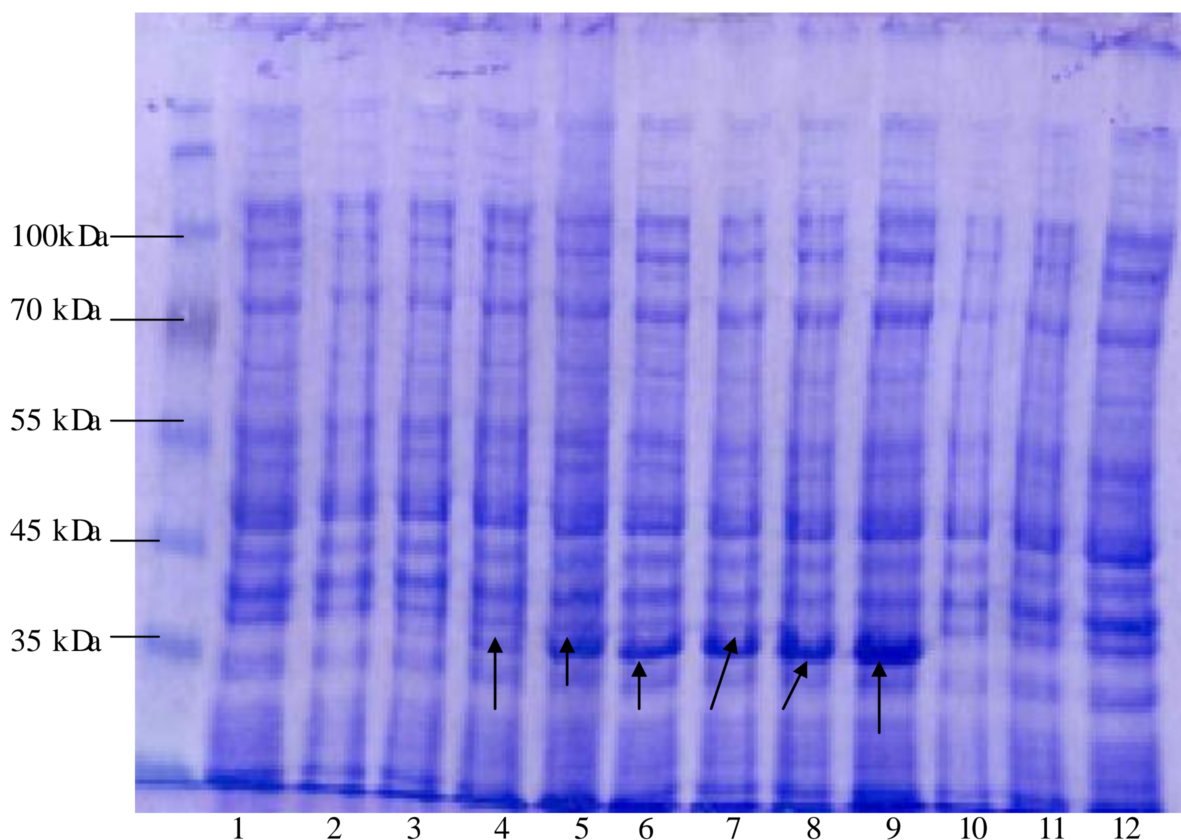


Fig. 3.4: SDS-PAGE analysis of total protein samples (bands of over-expressed protein are pointed by the arrows).

Lane 1: pET30a-p60 uninduced, 0 hour;
Lane 2: pET30a uninduced 0 hour;
Lane 3: pET30a-p60 induced, 1 hour;
Lane 4: pET30a-p60 induced, 2 hour;
Lane 5: pET30a-p60 induced, 4 hour;
Lane 6: pET30a-p60 induced, 5 hour;
Lane 7: pET30a-p60 induced, 7 hour;
Lane 8: pET30a-p60 induced, 10 hour;
Lane 9: pET30a-p60 induced, 12 hour;
Lane 10: pET30a induced, 3 hour;
Lane 11: pET30a, induced, 7 hour;
Lane 12: pET30a, induced, 10 hour.

At various times after IPTG induction (from 0 to 12 hours), a protein of 35 kDa was detected in protein samples obtained from cell pellet. The expression of p60 increased with time and was maximal at about 12 h after IPTG induction: at first

hour it is very little but after four hours of induction, the expressed protein becomes very distinguishable. No expressed protein was found in the control samples (Fig 3.4), containing only pET30a vector without p60-katanin insert. No expressed protein was observed in uninduced samples as well.

In order to check whether over-expressed p60-katanin is in the soluble fraction, cells were disrupted by freezing and thawing. Soluble and insoluble fractions were separated by centrifugation and samples were again analyzed by SDS-PAGE. The results show that most of p60 subunit is in soluble fraction (see Fig 3.5).

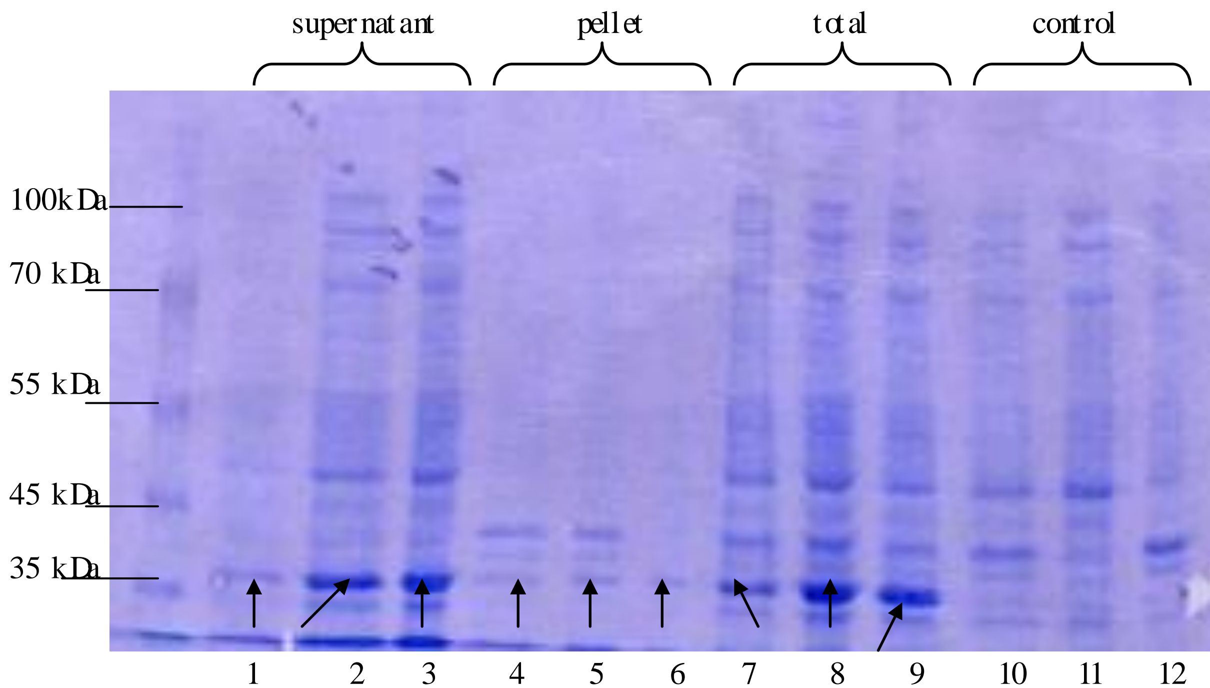


Fig 3.5: SDS-PAGE analysis of soluble and insoluble fractions.

Lane 1: soluble fraction of pET30a-p60, 4 hour;
Lane 2: soluble fraction of pET30a-p60, 8 hour;
Lane 3: soluble fraction of pET30a-p60, 12 hour;
Lane 4: insoluble fraction of pET30a-p60, 4 hour;
Lane 5: insoluble fraction of pET30a-p60, 8 hour;
Lane 6: insoluble fraction of pET30a-p60, 12 hour;
Lane 7: total protein pET30a-p60 4 hour;
Lane 8: total protein pET30a-p60 8 hour;
Lane 9: total protein pET30a-p60 12 hour;
Lane 10: total protein pET30a 12 hour;
Lane 11: soluble fraction of pET30a 12 hour;
Lane 12: insoluble fraction of pET30a 12 hour.

Amount of soluble protein seems to increase proportionally to the amount of total protein. Control samples (bacterial culture containing only pET30a, without p60 insert) do not expose over-expressed protein neither in soluble, nor in insoluble fractions.

3.3. Purification of p60-katanin under native conditions

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

At each purification step, the samples were taken and were further analysed with SDS-PAGE (see Fig. 3.6).

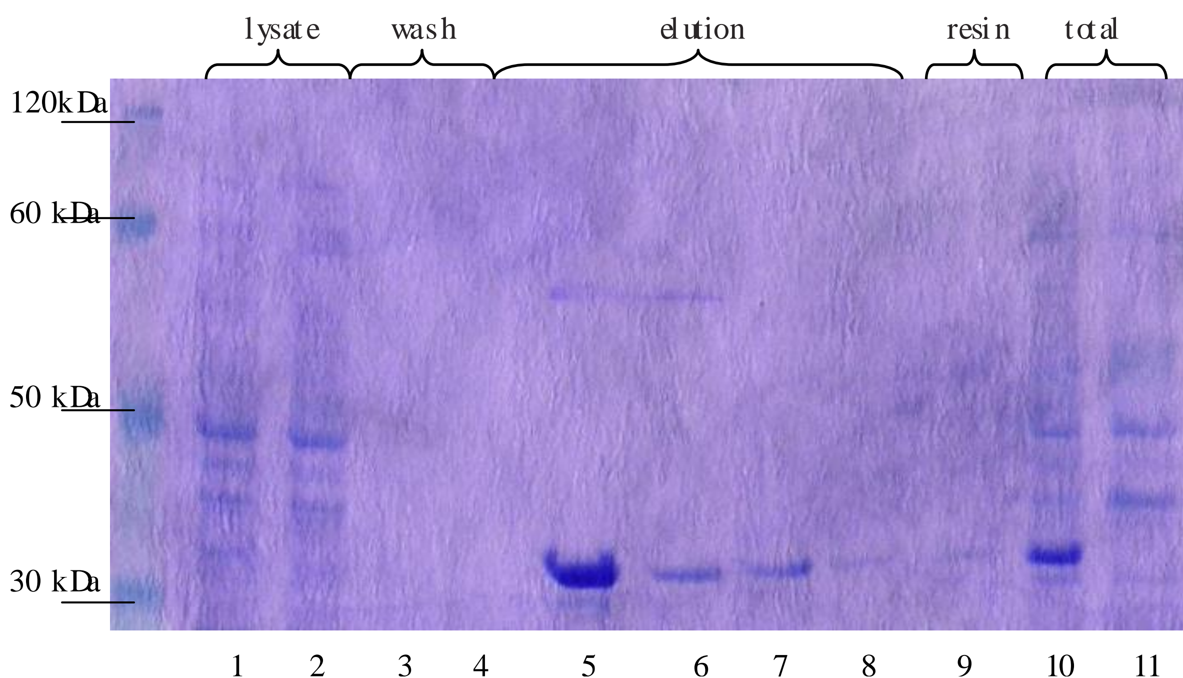


Fig. 3.6: SDS-PAGE analysis of purified p60-katanin (under native conditions).

Lane 1: lysate of bacterial culture (see Materials and Methods part for details);
Lane 2: lysate flow-through;
Lane 3: 1st wash fraction;
Lane 4: 2nd wash fraction;
Lane 5: 1st eluate;
Lane 6: 2nd eluate;
Lane 7: 3d eluate;
Lane 8: 4th eluate;

Lane 9: used N-NTA agarose;
Lane 10: total protein pET30a-p60 after 12 h growth;
Lane 11: total protein pET 30a (without p60 insert) after 12 h growth.

Very distinct protein band is seen in the first fraction of elution. In order to be sure, that the purified protein is the same as the overexpressed one, the control samples containing total protein fractions from bacteria cultures with pET30a-p60 construct and pET30a vector only were applied to the SDS-PAGE gel as well. It is clearly seen that overexpressed band observed in pET30a-p60 sample and missing in pET30a sample (not containing p60 insert) is of the same size as purified one. Less protein is obtained in second and third fractions of elution and very little protein in the fourth fraction of elution. Some of used N-NTA agarose was applied to SDS-PAGE gel as well, in order to check whether all the protein was eluted with the elution buffer. Again, only hardly visible protein was present, which means that most of the protein was eluted.
Bradford assay shows that protein concentration obtained after first elution reached 0.50 µg/ml, concentration after second elution reached 0.12 µg/ml. Protein concentration in fourth eluate was under detection limit.

3.4 Western blot analysis

In order to be sure that purified protein is really the Hs tagged p60-katanin, Western blot analysis was carried out. 0.0001 µg of primary antibody was loaded on the SDS-PAGE to check whether secondary antibody recognizes primary antibody. This sample gave the most distinct signal (see Fig. 3.7). Samples of total protein fraction taken from bacterial culture grown after induction with IPTG for 12 hours and of purified protein (after first elution) also gave signals of Hs tagged protein of the expected size.

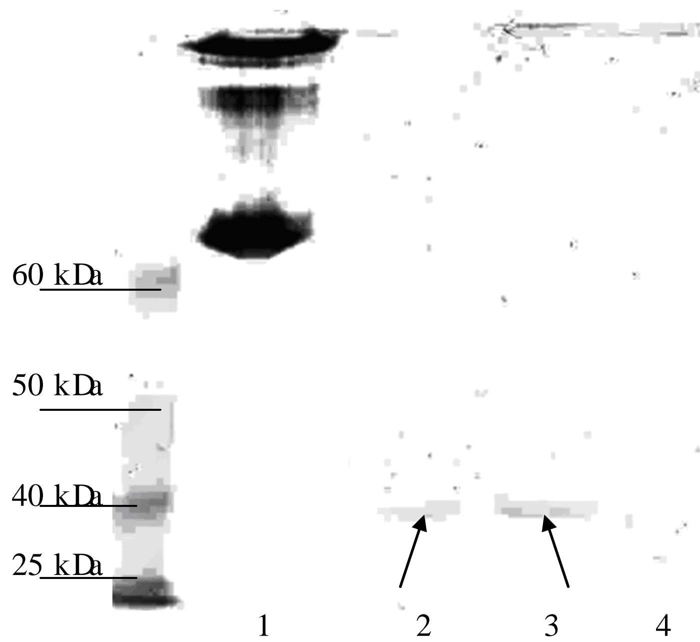


Fig. 3.7: Western blot analysis of total protein fraction and purified protein.

Lane 1: His_6 antibody (mouse)
Lane 2: Total protein fraction of pET30a-p60 grown for 12 hours (positive control)
Lane 3: Purified protein (after first elution)
Lane 4: Total protein fraction of pET30a grown for 12 hours (negative control)

4. DISCUSSION

4.1. Sequencing of the pET30-p60 construct

As it was mentioned in the Results section, mutation (purine-purine) was found after having sequenced new construct pET30a-p60. It resulted in glutamine instead of arginin. Any mutation resulting in amino acid change can affect activity of the expressed protein. However, our aim was to overexpress p60-katanin for antibody production. Antibody-antigen fit strongly together due to complementarity in shape over a wide area of contact and covalent bonding does not take place in this contact (Roitt, 1997). Glutamine and arginine are both hydrophilic amino acids, so any of them would supposedly be exposed on the surface. Therefore, peptide structure should not be distorted significantly by the mutation.

4.2. SDS-PAGE analysis of total protein fraction

Fig. 3.4. shows an overexpressed protein of about 35 kDa in the samples containing pET30-p60 construct. Molecular weight of subcloned p60-katanin should be about 22 kDa (21888 Da). When His-tag is fused to it, its weight becomes 30 kDa (29646.39 Da). However, SDS-PAGE analysis (12.5% SDS-PAGE gel) shows that overexpressed protein has higher weight. A longer than expected protein might be produced since bacteria may not 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.

4.1. Factors affecting solubility of expressed p60-katanin

Escherichia coli is the simplest and by far the most widely used organism for protein expression. However, it lacks post-translational modifications and this results in low

solubility of most of the eukaryotic proteins in *E coli.* p60- katanin was previously attempted to be expressed in *E coli.* Unfortunately, it turned out to be in insoluble form (Hartman *et al.,* 1998). It is generally difficult to improve the expression and solubility levels of a recombinant protein because these properties inherently depend on the amino acid sequence of the protein. The amino acid sequence in turn determines the physical properties of the protein, including its stability, p*I*, hydrophobicity, and molecular weight, any or all of which might directly affect the expression and solubility levels (Tsunoda *et al.*, 2005). However, there is also a number of factors outside inherent peptide characteristics that might affect protein solubility and yield in bacterial expression system e.g. position of the tag (N- or C-terminus) can have a significant effect on solubility and yield of the target protein (Sachdev, Chirgwin, 1998); protein concentration can effect its solubility as well. At high protein concentrations, second- or higher-order aggregation reactions compete kinetically with proper folding, thereby reducing the recovery yields of native proteins (Baneyx, 1999a).

A number of expression vectors used for expression differ from each other and therefore, different results might be obtained with the same protein. Vectors previously used for p60-katanin expression did not produce soluble protein. This work was the first attempt to use pET expression vector for p60-katanin overexpression. The results show that pET expression vector favours expression of soluble p60-katanin.

Since amino acid sequence determines protein characteristics, including its solubility, the expressed peptide amino acid sequence was analyzed.

```
  1 *S****TEN* K**REY**** NYDS***YYQ ***DQ*NKY*
YS*KDTH*HQ K*QQ**QE*N
 61 *E*KH*KE** KT*ES*K*DS TS*K**QHE* *SSE*E**S*
****ERR*** **RKRQSTQH
121 SD*K*HSNR* ****R*HR*S *QS*HSDR*K **RSREKKEQ
SK*REEKNK* ****TE*E*N
181 K*DST*YDKD **E**ERD** SQN*N*R*YD **D**E*KK*
*QE******* **E**K**RR
241 **K******* **T*KT***K ***TECKTT* *N*SSST*TS
KYR*ESEK** R***E**R*Y
301 S**T***DE* DS*CSRR*TS EEHE*SRR*K *E***Q*D**
***SENDD*S K*******TN
361 ***D*DE**R RR*EKR*Y** **S*K*REE* *R*S*RE*E*
*DD*N**S** EN*E*YS**D
```

421 *TN*CRD*S* ***RRR*E** T*EE*RN*SR EE*H**TT*E
D*E***KK*S KS*S**D*ER
481 YEK***E**S C

Fig. 4.1: Rat p60-katanin amino acid sequence. Non-polar (hydrophobic) amino acids are replaced by asterisks. Cystein is marked in grey. Expressed sequence is shown in bold. C - terminal conserved domain is underlined. Extensive hydrophobic regions are pointed by arrows.

Protein folding is the process by which a protein structure assumes its functional shape or conformation. Accuracy of this process is very important for protein solubility. Proteins which lose their functional shape (i.e. denatured proteins) may loose their solubility, and precipitate, becoming insoluble solids. The essential folding determinant is the amino acid sequence of the protein. It contains the information that specifies both the native structure and the pathway to attain that state. Folding is a spontaneous process, and it proceeds by moving the hydrophobic parts of the protein inwards, and the hydrophilic ones outwards. Disulfide bond formation of cysteins is an important part of folding as well. All cystein residues are left in unexpressed part of p60-katanin (Fig. 4.1.), which means that any possibility for wrong disulfide bond formation which might result in insolubility is abolished. Considerable hydrophobic region of polypeptide, pointed by the arrows is not expressed. Leaving this region out might also have helped to achieve soluble peptide. Variables of expression conditions that mostly influence inclusion body formation are the growth temperature and the level of promoter induction. Both approaches reduce the synthesis rate of overexpressed protein, either through a decrease in transcription in the case of suboptimal promoter induction, or a decrease in transcription, translation, initiation and elongation in the case of growth at reduced temperatures (Baneyx, 1999).

In this work, we used BL21 (DE3) pLysS host strain and pET 30a expression vector. Although the T7 polymerase is under the control of IPTG-inducible lacUV5 in this system but, as it was mentioned in Materials and Methods section, leaky expression of T7 can happen. In order to obtain soluble protein the best way would be not to use IPTG at all (Gu *et al.*, 2001) which would result in lower protein yield but in soluble form. However, our results showed that uninduced samples did not show overexpressed protein band which means that leaky expression did not take place.

Expression was induced with IPTG up to 0,5 mM and resulted in soluble peptide. It shows that concentration of overexpressed peptide under such induction was low enough to allow proper folding.

A traditional approach to reduce protein aggregation is most commonly by reducing the cultivation temperature (Baneyx, 1999). Induced bacterial cultures in this study were grown at room temperature which again turned out to be a good choice.

4.4 Purification of p60-katanin

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

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. 3.6) 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. 3.6). Ni-NTA resin contains only protein traces.

CONCLUSIONS

p60-katanin was successfully subcloned, and its sequence was checked. No frame mutations have occurred, p60-katanin is subcloned in the right orientation. New construct pET30a-p60 was obtained.

After induction subcloned p60-katanin was expressed, the biggest amount of the protein was observed after 12 hours of growth.

Subcloned p60-katanin is expressed in soluble form which was the main aim to be achieved in this study. Earlier attempts to express p60-katanin resulted in insoluble peptide, soluble peptide is obtained for the first time. Insoluble peptide hindered production of monoclonal antibody and synthetic antibodies were used until now. Obtained soluble peptide will be used for monoclonal antibody production.

Soluble peptide was successfully purified under native conditions with Ni-NTA resin which enabled to keep protein conformation undistorted. Concentration of purified p60-katanin reaches 0.50 µg/ml.

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APPENDIX

Obtnd: 38
tgcaccatcatcatcatcattcttctggtctggtg**n**cacgcggttctggta
tgaaagaaa 97
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Theor: 2
tgcaccatcatcatcatcattcttctggtctggtg**c**cacgcggttctggta
tgaaagaaa 61

Obtnd: 98
ccgctgctgctaaattcgaacgccagcacatggacagcccagatctgggta
ccgacgacg 157

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|||||||||
Theor: 62
ccgctgctgctaaattcgaacgccagcacatggacagcccagatctgggta
ccgacgacg 121

Obtnd: 158
acgacaaggccatggctgatatcggatccg**aattcgccctt**atgagtcttc
taatgatta 217

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|||||||||
Theor: 122
acgacaaggccatggctgatatcggatccg**aattcgccctt**atgagtcttc
taatgatta 181

Obtnd: 218
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ctgcaatgg 277

|||||||||||||||||||||||||||||||||||||||||||||||||||
|||||||||
Theor: 182
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ctgcaatgg 241

Obtnd: 278
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aagatacac 337

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Theor: 242
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aagatacac 301

Obtnd: 338
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agcatgtta 397
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agcatgtta 361

Obtnd: 398
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aagctgcac 457

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Obtnd: 458
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ttgaaagga 517

|||||||||||||||||||||||||||||||||||||||||||||||||||
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Theor: 422
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ttgaaagga 481

Obtnd: 518
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aagccacac 577
||||||||||||||||||||||||||||||||||||||
|||||||||||||||||||||
Theor: 482 gacccttaccaggacctaggaagcgccagtctactcag-
cacagtgaccctaagccacac 540

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Obtnd: 578
agttaccggnccaggcgcantcntcagagctcatcgaccatctgcacagag
tctgcacag 637
          ||| ||||| ||||||||| ||
|||||||||||||||||||||||||||||||||||||
Theor: 541 agtaaccgg-
ccaggcgcagtcgtcagagctcatcgaccatctgcacagagtctgcacag
599
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Fig. A1: Alignment results. Expression vector part is underlined, T/A vector part is in bold, the rest is p60 part.

Fig. A2: pET 30a-p60 sequencing results.

RESUME

Born in 1977 in Vilnius, Lithuania.
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