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Regulation of Muscle Contraction and How Mutations in the Muscle Proteins Cause Heart Disease. The Laboratory Techniques Involves Molecular Biology, Protein Expression, Purification and Characterization Using Biochemical and Biophyisical Techniques

Ugochukwu Obinna Maluze
Department of Biomedical Engineering,
University of Bedfordshire, United Kingdom and
Family Health Hospital and Maternity, Lagos state, Nigeria

ABSTRACT

The expansion of the causative mutations to the rigid thin filament changed the description of hypertrophic cardiomyopathy (HCM) from an illness of the cardiac motor to a syndrome of the cardiac sarcomere and significantly extended the gasp of the potential molecular pathogenic mechanism. An interesting hypotheses concerning disease mechanism posted that the diverse medical prognoses in the familial type of HCM may possibly be related to mutations in self-regulating protein machinery of the sarcomere (Frank et al, 1968). The journal of the novel study in 1990 establishes the inherited association of the beta-myosin and tropomyosin heavy chain genetic material to familial type of hypertrophic cardiomyopathy (HCM). The current studies conducted by some researchers elaborated on the various genetic alterations inside the genes encoding for the sarcomeric cardiac proteins, alpha tropomyosin, troponin T, and myosin protein components. The regularity of gene alteration in the alpha tropomyosin protein (TPM1) is lesser, contributing to 5% of FHC. Currently, the D175N gene mutation has been recognized in various unrelated populations, signifying that this spot could be an abnormal gene "hot spot" for the disease. In this project research, a wild type of normal protein and mutant genetic proteins (E180G and D175N) which are clinically involved in familial hyper cardiomyopathy (FHCM) were produced. Having in mind that the main effect of mutations E180G and D175N are mainly related to the thermal stability of the protein; this research will also investigate the differences between the thermal stability of wild type and mutated protein types using a Dye base fluorescent method of analysis. Dye based fluorescent method was used to monitor protein folding as a function of temperature for wild type tropomyosin and for HCM mutant E180G and D175N proteins. The column chromatography method of purification was used to purify the wild type and mutated proteins, and the protein bands were separated using gel electrophoresis methods. A similar assessment of folding stability and structural reports of several authors was in consistency with this present report which suggested that such mutations might alter protein folding. The results agree with previously published reports on the impaired function of expressed E180G and D175N mutations suggesting that the biochemical defects of the motor domain may affect myosin filament assembly in the sarcomere. For future prospects, future biochemical analysis of several other FHC mutations will be needed to establish a definite correlation between the

enzymatic impairment between different mutants and their clinical phenotype of heart disease.

Keywords: hypertrophic cardiomyopathy, Tropomyosin, Troponin, Myosin, mutations, autosomal-dorminant, inheritance, autosomal-recessive, Sarcomere, Arrhythmogenic, Proteins, etc.

INRODUCTION

The expansion of the causative mutations to the rigid thin filament changed the description of hypertrophic cardiomyopathy (HCM) from an illness of the cardiac motor to a syndrome of the cardiac sarcomere and significantly extended the gasp of the potential molecular pathogenic mechanism (Kimura et al, 1997; Olson et al, 2000). In the subsequent decade, causative mutation was correlated to the genes programming the cardiac thin filament proteins (Kimura et al, 1997, Olson et al, 2000; Landstrom et al, 2008). An interesting hypotheses concerning disease mechanism posted that the diverse medical prognoses in the familial type of HCM may possibly be related to mutations in self-regulating protein machinery of the sarcomere (Frank et al, 1968).

In the 20 years, from the time when the landmark study of Geisterfer-Lowrance et al (Geisterfer et al, 1990) that recognized the R403Q alteration in the genetic material programming the beta cardiac myosin protein genes (MYH7) as contributing for hypertrophic cardiomyopathy (HCM), 100 of extra mutations in 10 diverse sarcomeric genetic material have been associated with the illness (Xu et al, 2010; Konno et al, 2010), and bulk of these alteration in the genetic component changes the beta myosin heavy chain, the power that co-ordinates cardiac muscle contraction, and this serves as the principal element of sacomere thick filaments (Konno et al, 2010).

Interestingly, the journal of the novel study in 1990 establishes the inherited association of the beta-myosin and tropomyosin heavy chain genetic material to familial type of hypertrophic cardiomyopathy (HCM) (Teare, 1958). Belatedly in the 1950s, a mysterious illness drawed the mind of cardiologists, surgeons, and pathologists (Brock, 1957). During the same year, hypertrophic cardiomyopathy has involved deep attention with increasing understanding of its prevalence, its function as the main source of unexpected cardiac death in juvenile individuals, and its isolation as a mendelian autosomal dominant genes (Arndt et al, 2013). In 1958, it was illustrated that alteration in the genetic component in the cardiac troponin and alpha tropomyosin were the main basis of familiar ventricular cardiomyopathy, therefore switching the disorder from a illness of the beta-myosin heavy chain to ailment of the cardiac sarcomere (Teare, 1958). Hypertrophic cardiomyopathy has represented the model of monogenic cardiac disorder, increasing the likelihood that its explanation would provide essential details into the root of cardiac hypertrophy. Categorized as irregular septal hypertrophy (Teare, 1958), efficient aortic stenosis (Brock, 1957; Marrow & Braunwald, 1959), hypertrophic obstreperous cardiomyopathy (Goodwin et al, 1960), and idiopathic hypertrophic subaortic stenosis (Braunwald & Ebert, 1962), early description described the remarkable medical trial associated with the syndrome. Current biochemical research has revealed that HCM mutations in beta MHC change the cycling speed of myosin heads (Lowey,2002) and, because patients are heterozygous for these genetic alterations, their thick filaments will be poised of wild-type myosin heads.

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The current studies conducted by some researchers elaborated on the various genetic alterations inside the genes encoding for the sarcomeric cardiac proteins, alpha tropomyosin, troponin T, and myosin protein components. The regularity of gene alteration in the alpha tropomyosin protein (TPM1) is lesser, contributing to 5% of FHC. Different point mutations resulting to alteration in gene sequence of the proteins have also beennoted: E62Q (Jongbloed et al, 2003), A63V (Yamauchi et al, 1996; Nakajima-Taniguchi et al, 1995), K70T (Nakajima-Taniguchi et al, 1995), D175N (Thiefelder et al, 1994), E180G (Thiefelder et al), E180V (Regitz-Zagrosek et al, 2000) and L185R (Van Driest et al, 2002). Currently, the D175N gene mutation has been recognised in various unrelated populations, signifying that this spot could be an abnormal gene "hot spot" for the disease (Reed & Davies, 1994). ''In vivo studies, using transgenic mice as a model showed an impairment of cardiac function by altering the sensitivity of myofilaments to calcium'' (Evans et al, 2000). In vitro research conducted with recombinant proteins expressing the gene mutations, demonstrated little changes on the whole stability of the protein as detected by circular dichroism (Golitsina et al, 1997), and this showed abnormal changes in the kinetics of contractile force production (Bing et al, 1997).

Various Forms of Mutation Involves in Cardiomyopathy

In the field of genetics, mutation is described as a permanent alteration of the genetic sequence. Gene alterations in the muscle proteins can give rise to various types of abnormality in genetic compositions and this can alter the genetic composition or stop the gene from performing properly. At the molecular stage, different types of mutation have been noted as the aetiology of cardiomyopathy. ''Point mutations has been noted to cause hypertrophic cardiomyopathy, and often caused by chemicals or malfunction of DNA replication and exchange of a single nucleotide for another'' (Freese & Emst, 1959). ''These abnormal gene sequences are categorised as transitions or transversions and the most common is the transitional state that exchanges a purine for a purine (A ↔ G) or a pyrimidine for a pyrimidine, (C ↔ T)'' (Freese & Emst,1959).

Point mutations that take place in the protein coding region of DNA may be categorized into three kinds of mutation (silent mutation that encode for the same amino acid, missense mutation that programmed for a different amino acids and nonsense mutation which programmed for a stop codon and therefore alter the genetic sequence of the protein, giving rise to different types of cardiomyopathies (Boillee et al, 2006).

Splice site mutation or reading frame mutation is a type of gene mutation that occur due to insertion or deletion of the genetic elements in the coding area of the genes, and this can change the splicing of the mRA (splice site mutation, or lead to a change in the reading frame (frame shift mutation); these two mutations can extensively alter the genetic component, alter the protein structure and contributes to cardiomyopathy (Hogan & Michael, 2010).

Various Forms of Cardiomyopathy

Cardiomyopathies can be defined as a clinically diverse groups of heart muscle ailments, which has a distinguishing feature of unusual myocardial structures. The present grouping of the cardiomyopathies persisted to be based on phenotypic and clinical examinations of the affected individuals. Familial hypertrophic cardiomyopathy (FHC) can be defined as an autosomal dominant heart illness with prominent features of interventricular hypertrophy, mitral valve disorders, monocyte hypertrophy, interstitial fibrosis and atrial fibrillation (Davies, 1984;

Maron et al., 1987; Olivotto et al, 2001). The illness is clinically inconsistent, and it starts as benign condition and progresses to a severe devastating state which frequently give rise to sudden deaths in young athletes (Maron et al., 1978 & 1986; Solomon et al., 1990; Dausse and Schwartz, 1993; Watkins et al., 1995c). Every single mutation that causes FHC are structural proteins of the sarcomere: b -myosin heavy chain (MyHC) (Geisterfer-Lowrance et al., 1990; Watkins et al., 1993), a -tropomyosin (Watkins et al., 1995b), troponin T (Thierfelder et al., 1994), myosin binding protein-C (Watkins et al., 1995a; Bonne et al., 1995), light chain 1 and troponin I (Kimura et al., 1997).

Dilated Cardiomyopathy (DCM) is a heart disorders that take place as a result of gene alteration on protein tropomyosin. Genetic abnormal changes have been connected with irregular onset on DCM, and they have been recognized in families that frequently demonstrate an autosomal-dorminant inheritance prototype, with autosomal-recessive inheritance (Petretta et al, 2011). A number of various genes have been implicated as a cause of DCM, and these genes codes for various protein components of the ‘‘sarcomere, Z- disc, cytoskeleton, sarcolemma, and nucleus ‘’ (Fatkin et al, 1999, 2010). DCM has prominent features of enlarged chamber size and abnormal systolic reduction of the two ventricles (Herman et al, 2012).

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is another type of cardiomyopathy that is due to gene mutation on the protein tropomyosin, and relatives with ARVC usually displayed autosomal-dominant inheritance patterns. Several researchers have identified about nine abnormal genes as the genetic cause of ARVC; ‘‘five of these genes encode the desmosomal proteins, plakophilin-2, plakoglobin, desmoplakin, desmocollin, and desmoglein-2’’ (Teekakirikul et al, 2013). About 50% of ARVC clinical manifestations have a desmosomal genetic alteration, and 40% have a plakophilin-2-gene mutation (Van Tintelen et al, 2006). The differential diagnosis of ARVC can be demanding, but some useful method of examination have been devised which take into description of structural and functional disorders, tissue characterizations, ECG disorders, arrhythmias, and genetic family history (McKenna et al,1994; Marcus et al, 2010).

Restrictive Cardiomyopathy (RCM) is another type of cardiomyopathy linked with abnormal ventricular diastolic features with a raised end-diastolic pressure that give rise to usual or lowered ventricular magnitude (Cale-shu et al,2011). Genetic abnormal changes in seven sarcomere protein genes encoding the cytoskeleton protein desmin have been implicated in families diagnosed with RCM (Parvatiyar et al, 2010a; Sen-Chowdry et al, 2010; Caleshu et al, 2011). Generally, the diagnosis in RCM is poor, particularly in affected children, and heart transplantation is frequently carried out (Sen-Chowdry et al, 2010).

Proteins Regulations in Muscle Contractions and Their Implications in Cardiomyopathies

According to current research, there are different types of sarcomere proteins that are implicated in cardiomyopathies. These includes alpha myosin heavy chain, titin, troponin C, tropomyosin (Niimura et al,1997; Carniel et al, 2005; Satoh et al, 1999; Hoffmann et al, 2001); Z-disc –associated proteins (actinin, ankyrin, myozenin 2) (56, 57,); muscle LIM proteins (nexilin and telethonin) (Knoll et al, 2010; Wang et al, 2010; Bos et al, 2006; Hayashi et al, 2004) and proteins implicated in other monocyte functions (phospholamban and viniculin) (Landstrom et at, 2010; Vasile et al, 2006). These proteins are extremely essential in regulation

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of muscle contractions, and mutations in these proteins can cause hypertrophic cardiomyopathy (Osio et al, 2007).

However, 'Several genetic studies summarized the perception that abnormal gene changes on the sarcomere proteins (alpha myosin, troponin C and tropomyosin) were the causes of familiar hypertrophic cardiomyopathy'' (Darsee et al, 1979). Schwartz and his co- researchers recognized HCM locus on chromosome 11 where the thick filament myosin and tropomyosin binding protein-C (MYBPC3) was mapped (Carrier et al, 1993) and abnormal gene identified (Bonne et al, 1995; Watkins et al, 1995). The clinical examination of the myosin vital light chain (MYL3) and regulatory chain (MYL2) genes by Epstein and colleaques (Poetter et al, 1996), troponin I (TNN13) and tropomyosin by Sasazuki and colleaques (Kimura et al, 1997), and cardiac actin (ACTC) by Olson and Fanana-pazir established a perfect role of gene mutation in causing HCM.

Biochemistry of Tropomyosin, Its Characteristics and Definitive Roles in Muscle Contractions

Tropomyosin (TMs) are classified as a family of extremely preserved proteins seen in most eukaryotic cells that participate actively in muscle contractions (Fan anapazir, 1994; Satoh et al, 1999; Lee-Miller, 1991). Tropomyosin and cardiac actin displayed a distinctive useful element that plays a structural and active function in sarcomeric muscle protein. The genetic constituent of TPM1 comprises of 14 exons and 4 isoforms (a and b -tropomyosins, tropomyosin-4, and tropomyosin-30) (Schwartz et al, 1995; Lee-Miller, 1991). The striated muscle isoform is made up of alpha helical protein, that produces a matching coiled-dimer perverse in the area of the long axis of the actin filament. Each polypeptide chain contain about 284 amino acid residues (Lee-Miller, 1991), and each of the dimer is attached to seven actin monomers and one troponin (Tn) complex (TnC, TnI and TnT) (Smillie, 1979) The polymerization of head-to-tail pattern of the striated muscle cells with the troponin complex control the calcium sensitivity of the actomyosin-ATPase complex (Smillie, 1979).

The amino acid arrangement in Tm consists of seven-residue prototype (a to g) which are recurring all over the whole sequence. Positions a and d, on the similar region of the segment, are frequently taken by a polar amino acid which permit for hydrophobic exchanges among the chains. Positions e and g are also taken by another exciting residue, and this add to the stability and rigidity of the corresponding coiled-coil arrangement through their ionic exchanges with residues at positions e and g of the former helical structures (Parry,1975). Positions b, c and f are taken by glacial or ionic component which collaborate with other proteins (Smillie, 1971).

In Vitro (PCR) Site-directed Mutagenesis or Oligonucleotide-directed Mutagenesis

In molecular genetic medicine, invitro site-directed mutagenesis is a very useful method used in studying protein structure-function interaction, gene composition, and also for vector alteration (Kilbey, 1995). It is a genetic biology technique used to produce precise changes that altered the DNA sequence (Kilbey, 1995; Shortle, 1981). Quite a lot of approaches to this method have been documented, but these techniques usually involve single-stranded DNA (ssDNA) serving as the template (Kunkel, 1985; Vandeyar, 1988; Sugimoto et al, 1989; Tayloy et al, 1985) and they are laboured intensive or precisely complicated. The Stratagene's Quik Change site-directed mutagenesis technique is performed by means of pfuTurbo DNA polymerase and a temperature cycler (Nelson, 1992). PfuTurbo DNA polymerase reproduce

with plasmid strands and uses a supercoiled double-stranded DNA (dsDNA) vector and two artificial oligonucleotide primers displaying the desired mutation. The oligonucleotide primers, each corresponding to reverse strands of the DNA gene, are extensive throughout the period of temperature cycling by pfuTurbo DNA polymerase (Papworth et al, 1996). Inclusion of the oligonucleotide primers produces a mutated genes comprising of staggered nicks (Papworth et al, 1996). Subsequent to temperature cycling, the altered gene sequence is treated with Dpn 1. The Dpn 1 endonuclease (target sequence: 5'-GmATC-3) which is very explicit for methylated and hemimethylated DNA and is mainly utilized to process the parental DNA (Nelson & McClelland, 1992). The genetic material produced from every E.coli bacteria is dam methylated and consequently prone to Dpn 1 digestion. The nicked DNA gene containing the required abnormal genetic sequence is then submerge into XL1-Blue supercompetent cells, which contributes to a high-quality mutation efficiency (Nelson & McClelland, 1992).

Recombinant Protein Expression Using E.Coli

Increased level synthesis of recombinant proteins as a condition for immediate refinement has grow to be a model technique. The laboratory synthesis of recombinant proteins involves cloning of the suitable genetic material into an expression DNA vector under the control of an inducible promoter (Marino 1989). But well-organized appearance of the recombinant gene is based on a range of conditions such as most favourable displaying signals (both at the stage of transcription and translation), accurate protein folding and cell expansion description (Marino, 1989). However, the use of bacterium E. coli has been the most popular means of producing recombinant proteins for over two decades. The advantages of using E. coli are that it offers short culturing time, easy genetic manipulation, low-cost media and it has unparalleled fast growth kinetics (Sezonov et al, 2007). The factors influencing the expression level include unique and subtle structural features of the gene sequence, the stability and efficiency of mRNA, correct and efficient protein folding, codon usage, degradation of the recombinant protein by ATP-dependent proteases and toxicity of the protein (Makrides,1996; Swartz, 2001).

In protein expression using E. coli, the regulated gene expression requires an inducible or repressible system, and therefore, all expression systems are based on controllable promoters. Four regulatable promoter systems are widely used, three are based on the repressors already mentioned (LacI, TrpR and phage cI) and the fourth is based on a phage RNA polymerase (Rao *et al*., 1994). The *lac* system consists of the promoter/operator region preceding the *lac* operon and the LacI repressor encoded by the *lacI* gene. In the absence of an inducer, the Lac repressor binds to its operator situated immediately downstream from the promoter as a homotetramer. The wild-type *lac* promoter sequence contains one deviation in the -35 and two in the -10 box, and the spacer region encompasses 18 nucleotides if compared to the consensus sequence. One of the many promoter mutations isolated has been termed *lacUV5*. If its DNA sequence is compared to that of the wild-type promoter, it becomes apparent that two nucleotides have been exchanged. The promoter strength of *lacUV5* has increased 2.5-fold, and mutations increasing the promoter strength are called promoter-up mutations in general (de Boer *et al*., 1983). In the case of the Plac, the PlacUV5 and the Ptac promoters, the repressor is inactivated by addition of isopropyl- -D- thiogalactopyranoside (IPTG). This compound binds to the active LacI repressor andcauses dissociation from its operator. IPTG has two advantages over lactose: First, its uptake is not dependent on the Lac permease (it diffuses through the inner membrane) and second, it cannot be cleaved by galactosidase preventing turn-off of transcription. The *lacI*

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gene is either part of the expression plasmid or it is present within the chromosome. Since the wild- type level of the LacI repressor is not sufficient to repress expression of the recombinant gene in the absence of IPTG, two derivates have been isolated resulting in an increase in the amount of repressor based on promoter-up mutations called *lacI*^{q} and *lacI*^{q1} (Müller-Hill *et al*., 1968). Expression systems based on the *trp*system make use of synthetic media with a defined tryptophan concentration. The concentration is chosen in such a way that the system becomes self-inducible when the tryptophan concentration within the cells falls below a threshold level (Masuda *et al*., 1996).

Aims and Objectives of the Study

The aims and objectives of this project research is to produce a wild type of normal protein and mutant genetic proteins (E180G and D175N) which are clinically involved in familial hyper cardiomyopathy (FHCM).

Having in mind that the main effect of mutations E180G and D175N are mainly related to the thermal stability of the protein; this research will also investigate the differences between the thermal stability of wild type and mutated protein types using a Dye base fluorescent method of analysis.

MATERIALS, REAGENT PREPARATIONS AND METHODS

Materials Used

50 mls fisherbrand tubes, Eppendorf centrifuge tube (5415c), Thermo scientific gene ruler 1kb plus DNA ladder, Borosilicate glass 2000mls, JB Aqua 2 plus Grant, Column chromatography fraction collector (FRAC-100), Liquid chromatography controller LCC-500, Sorvall Rc6+ centrifuge (thermo scientific) Schott Duran 250ml tube, 15ml fisherbrand tube, Digital heat block, Fisherbrand automatic pipettes, QPCR machine, PH 211 microprocessor ph meter, Biometra personal cycler PCR machine, Pharmacia biotech column chromatography, Naodrop 2000 thermoscientific spectrophotometer, Nicolet evolution 300 spectrophotometry, Culture plates, Pyrex 100ml cylindrical flask, Duran 500ml big glass tube, Pyrex 1500 big cylindrical glass, fisherbrand 400ml beaker tube LB media, racks, 30% (w/w) Acrylamide: 0.8% w/w Bis-Acrylamide stock solution (37.1) (protogel), 1M Mgcl2, 1M NaOH, Medium A (50ml of LB, 12.324g Mgso4, 5g of glucose), AMP,EDTA, Tris buffer stock, NaPO4, P1 buffer, P2 buffer, N3 buffer, PE buffer, EB buffer, DNA ladder, APs, TEMED, distill water, Agar solid media, Commasie blue, Running buffer, Resolving buffer, Stacking buffer, Electrophoresis apparatus, pipette teeth.

Reagent Preparations LB Agar Preparations

- 1000ml of LB Agar: 10g bactopeptone, 5g yeast extract, 5g Nacl, 1000ml of distil water, Ph 7.5, 1.5g Agar.
- 300ml of LB Agar: 3g bactopeptone, 1.5g yeast extract, 1.5g Nacl, 300ml of distil water, Ph 7.5, 1.5g Agar.
- 200ml of LB Agar: 2g bactopeptone, 1g yeast extract, 1g Nacl, 200ml of distil water, Ph 7.5, 1.5g Agar.
- 150ml of LB Agar: 1.5g bactopeptone, 0.75g yeast extract, 0.75g Nacl, 150ml of distil water, Ph 7.5, 1.5g Agar.

- 100ml of LB Agar: 1.0g bactopeptone, 0.5g yeast extract, 0.5g Nacl, 100ml of distil water, Ph 7.5, 1.5g Agar.
- The LB Agars were autoclaved for 1hour at 37 degrees centigrades.

- **Ice Cold (40mls) Lysis Buffer Preparation**
  0.438g of Nacl, 0.5millilitre of EDTA,1millilitre of Tris buffer stock, 40mls of distil water in a cylindrical glass.
- **Preparation of 250 Millilitre of NaPO4 Buffer**
  About 14.62g Nacl was dissolved in 2.5ml of NaPo4 and mixed gently, and the final volume was made up to 250mls in a glass cylindrical tube.
- **Preparation of 500 Millilitre of NaPO4 Buffer**
  About 2.922g of Nacl was dissolved in 5ml of NaPo4 and mixed gently, and the final volume was made up to 500mls in a glass cylindrical tube.
- **Preparation of 1% Agarose Gel**
  About 0.3g of agarose was dissolved and mixed gently in 30 millilitre of water, and the solution was microwave for 30seconds until it dissolved completely, and allowed to cool. About 2ul of ethydielene bromide was added to the mixture at the temperature of 50 degrees centigrades.
- **Preparation of Buffer 1:** 0.2mls $NaPO_4$, 1.16g Nacl, 20mls of distill water.
- **Preparation of Buffer 2:** 0.2mls $NaPO_4$, 1.16g Nacl, 9.5ul of $Mgcl_2$, 20mls of distil water**.**
  Preparation of Buffer 3: 0.2mls $NaPO_4$, 0.23g Nacl, 20mls of distil water.
- **Preparation of Buffer 4:** 0.2mls $NaPO_4$, 0.23g Nacl, 9.5ul $Mgcl_2$, 20mls of distil water.
- **Preparation of PCR Mix (100ul)**
  About 10x buffer was added to 3ul dNTPs, 2ul primer F, 2ul primer R, 2ul polymerase and 79ul of distill water, and the whole reactions were mixed gently.

METHODS

Preparation and Storage of Competent XL-1, BL2 and Escherichia Coli Cells Applicable to Transformation and Transfection

Two cells XL-1 and BL2 cells were transferred into two different conical flasks containing media A (LB broth supplemented with 10 mM-mgSO4 7H2O and 0.2%-glucose) and they were labelled clearly. The two cells were incubated for about 4-5hours at 37degrees to make competent cells. The presence of 10mM-MgSO4 in medium A stimulates for transformation efficiency (3) and the glucose enhances transformation efficiency in the two conical flasks.

After the 4-5hours incubation, the XL1 and BL2 cells were transferred into two different 50ml fisherbrand sterile tubes and spun in a centrifuge for 10minutes at 4500 revolution per minutes (to pellet the cells). The supernatant was discarded and the pellets were kept on ice for about 5minutes. The cells pellet of XL1 and BL2 cells were resuspended separately in 0.5ml of medium A precooled on ice, followed by subsequent addition of 2.5ml of solution B (36%-glycerine, 12%-PEG, 12mM-MgSO4.7H2O, ph 7.0). The cells mixtures were mixed properly. About 100ul each XL1 and BL2 cells were transferred separately to each of the two eppendorf sterile tubes, followed by subsequent addition of 2ul of DNA (PJc20) to each of the XL1 and BL2 cells. The cells were kept on ice for 15minutes (this facilitates the binding of the DNA to the cells). After 15minutes incubation on ice, the cells were subjected to a heat shock at 42 degrees centigrades for 90seconds (this enhances the closing of the cell membranes porosity). The cells were

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transferred back to the ice for 1-2minutes. About 900ul of LB was added to each of the tube containing the XL1 and BL2 cells and heat shocked for 30minutes at 37 degrees centigrade (to allow the expression of antibiotics resistance).

For the transfection reactions, about 10ul of Ampicillin (AMP) antibiotics was added into a solid agar media and mixed properly. The solid agar media were poured on six media plates and allowed to cool, solidified and gel. About 100ul of XL1 and BL2 was added to the 1st and 2nd plate and spread uniformly to make distinct colonies, and labelled XL1 and BL2 low cell concentrations. To obtained high concentration of cells, the XL1 and BL2 cells were spun for 1minute and resuspended. 100ul XL1 and BL2 spun cells were added to the 3rd and 4th media plates respectively and spread uniformly, and labelled 3rd and 4th high cell concentrations. About 100ul of XL1 and BL2 cells deficient of DNA (negative control) was added to the 5th and 6th media plates respectively and also spread uniformly and labelled clearly ''XL1 and BL2 negative control cells''. The six media plates were kept for overnight incubation at 37 degrees centigrade, and the colony growth and morphology were observed on the next day.

To grow the BL2 cultured cells, a single colony was inoculated into each of the two sterile eppendorf tubes containing 1ml of each of LB AMP, and the two tubes were labelled LBAMP+colony 1 and LBAMP colony 2. The two tubes were incubated for 4-6 hours at 37 degrees centigrades (for the cells to grow adequately). Four fresh sterile eppendorf tubes were arranged, 0.5ml of LBAMP colony 1 was added to the 1st tube and another 0.5ml of LBAMP colony 1 was added to the 2nd tube. 0.5ml of LBAMP colony 2 was added to the 3rd tube with IPTG and another 0.5ml of LBAMP colony 2 was added to the 4th tube with IPTG (to switch on the gene expressions), and the tubes were labelled clearly ''non-induced sktm 1, non-induced sktm 2, induced sktm 3 and induced sktm 4)'' and the 4 tubes were incubated for 3 hours at 37 degrees centigrade. After the 3 hours incubation, about 125ul were transferred from each of the four tubes into each of the 4 fresh sterile tubes and the 4 new tubes were labelled clearly''non-induced sktm 1, non-induced sktm 2, induced sktm 3 and induced sktm 4). The 125ul cells in each of the 4 tubes were spun for 1minute and the supernatant discarded. About 20ul of SDS buffer and 20ul of distil water was added to each of the 4 tubes containing the cells pellets and the tubes were mixed properly.

To grow the XL1 cultured cells, a single colony was inoculated into each of the two sterile Eppendorf tubes containing 1ml of each of LB AMP, and the two tubes were labelled LBAMP+colony 1 and LBAMP colony 2. The two tubes were incubated for 4-6 hours at 37 degrees centigrade (for the cells to grow adequately). Four fresh sterile Eppendorf tubes were arranged, 0.5ml of LBAMP colony 1 was added to the 1st tube and another 0.5ml of LBAMP colony 1 was added to the 2nd tube. 0.5ml of LBAMP colony 2 was added to the 3rd tube with IPTG (Isopropyle-B-D-thiogalactopyranoside) and another 0.5ml of LBAMP colony 2 was added to the 4th tube with IPTG (to switch on the gene expressions), and the tubes were labelled clearly ''non-induced sktm 1, non-induced sktm 2, induced sktm 3 and induced sktm 4)'' and the 4 tubes were incubated for 3 hours at 37 degrees centigrade. After the 3 hours incubation, about 125ul were transferred from each of the four tubes into each of the 4 fresh sterile tubes and the 4 new tubes were labelled clearly''non-induced sktm 1, non- induced sktm 2, induced sktm 3 and induced sktm 4). The 125ul cells in each of the 4 tubes were spun for 1minute and the supernatant discarded. About 20ul of SDS buffer and 20ul of distil water was added to each of

the non-induced and induced tubes containing the cells pellets and the tubes were mixed properly.

To analyse the non-induced and induced proteins on the gel electrophoresis, about 5ul of the ladder was loaded on the 1st well, 15ul of non-induced sktm (BL2 cells) was loaded on the 2nd well, 15ul of induced sktm (BL2 cells) was loaded on the 3rd well, 15ul of non-induced XL1 cells was added on the 4th well and 15ul of induced XL1 cells was added on the 5th well. The samples were run at 240voltage power source and 40milliamps for 50minutes. The pictures of the protein band separations were taken, and the result analyzed.

To grow a large quantity of cells from a small quantity, 1000ml LB agar was prepared. Competent cells were made by transferring 50ml LB into 50mls branfisher tubes and 1ml of glucose and 0.5ml of MgSO4 was added and incubated at 37 degrees for 1-2 hours. The cells were resuspended gently in 0.5ml medium A precooled on ice and 2.5ml of storage solution B was added and mixed properly. About 100ul of the mixtures were transferred into a fresh Eppendorf tube and 2ul of DNA was added and incubated on ice for 15minutes. After incubation on ice, the cells were heat shocked for 90seconds at 42degrees centigrade and chilled on ice for 2minutes. About 900ul of LB without AMP was added and transferred to heat shock for 30minutes at 37 degrees. 0.1g of AMP and 100ul of IPTG was added into the 1000ml flask containing the LB solution (with sktm). About 125ul of LB AMP IPTG was transferred from the 1000ml flask into a fresh eppendorf tube, the reaction mixture were spun for 1minute and the supernatant was discarded. About 20ul of distil water and 20ul of SDS loading buffer was added to the pellet and incubated at 37 degrees centigrade.

From the 1000ml flask containing the LB AMP IPTG, 500mls of the mixtures were transferred into a fresh centrifuge tube and another 500mls of the mixtures (from the same 1000ml flask) were transferred into a second fresh centrifuge tube, and the two tubes were centrifuged 15minutes and the supernatant were discarded. The pellets were kept in a freezer for 1hour 30minutes (and this facilitates the cells disruption).

Tropomyosin Preparation

About 5millitres of ice cold lysis buffer was gently transferred into each of the two centrifuge tubes containing the pellets and mixed properly. The two tubes were sonicated (to break the DNA at 80 degrees centrifuge for 10minutes, and cool on ice. The tubes were heat treated (for protein's denaturation) and centrifuged again (to remove the debris and the denatured protein). After centrifugation, about 20ul of each of the supernatant was transferred into two Eppendorf tubes and 20ul of SDS loading buffer were added into each of the two Eppendorf tube (this is the post-boiled protein). The remaining supernatant was transferred into a 75ml beaker and the PH was adjusted to 4.5 using Hcl (Hcl precipitates the protein and DNA). The protein samples were transferred on two centrifuge tubes and the tube was centrifuged for 10minutes at 10,000rpm, and the tubes were stored in the fridge overnight.

From the two tubes kept overnight in the fridge, the supernatants were discarded. About 20mls of buffer NaPO4 was transferred from the 500ml buffer NaPO4 into the 1st tube pellet and 20ml of buffer NaPO4 was transferred from the 250ml buffer NaPO4 into the 2nd tube pellet, and the Ph was adjusted to 7.0 with NaOH.

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DNA Purification

The pelleted bacteria cells in the 2nd tube was resuspended in 250ul buffer P1 (at 4degrees centigrade) and transferred in a microcentrifuge tube. About 250ul of buffer P2 was added and the tube was gently inverted 4-6 times to mix properly. About 350ul of buffer N3 was added and also inverted 4-6 times to mix properly. The tube was centrifuged for 10minutes at 13,000rpm (at 17,900x g). A compact pellet was formed which contain the genomic DNA. The supernatant was decanted into the QIA prep spin column. The sample was centrifuged for 30-60seconds at 8000rpm. And the flow-through was discarded. The QIA prep column was washed by adding 0.75ml buffer PE, and centrifuged for 30-60seconds. The flow- through was discarded and the sample was centrifuged again for additional 1minute to remove the residual wash buffer. The QIA prep column was placed in a clean 1.5ml centrifuge tube. About 50ul of distil water was added to the centre of the QIA prep spin column (for DNA elution). The spin column was allowed to stand for 1minutes and centrifuged again for 1minutes to get the purified DNA. DNA gel electrophoresis was carried out by mixing 1ul of DNA marker with 4ul of the purified DNA sample. About 5ul of DNA ladder was added to the 1st well and 5ul of the purified DNA sample was added on the second well. The gel was connected to the electric power source at 750volt for 20minutes and the result was viewed using gel documentation machine (GELDOC-HTS2 Imager).

Column Chromatography Analysis of Tropomyosin (Wild Type Protein)

Half of the protein samples was purified using column chromatography method of purification, and the purified proteins were collected on 24 fraction tubes (which were clearly labelled from tube 1 to tube 24). Protein gel electrophoresis was carried out by selecting the even numbers on the labelled tubes containing the purified protein. The numbers selected were 10,12,14,16,18,20,22,24. About 20ul of SDS buffer and 20ul of distil water was added to each of the selected tubes containing the protein samples. About 5ul of the DNA ladder was added to the 1st well, sample from the 2nd tube was added to the 2nd well and the subsequent selected tube samples were added to their respective wells. The remaining half of the protein samples were also purified, and the protein fractions were collected using 24 fraction tubes and clearly labelled.

Preparation of Mutated Protein (D175N and E180G) (Site Directed Mutagenesis)

About 1ul of Pfu turbo DNA polymerase (2.5u/ul) was added to each sample reaction.1ul of the Dpn 1 restriction enzyme (10u/ul) was added below the mineral oil overlayed. The reactions were thoroughly mixed and spun in a microcentrifuge for 1minute. The reaction was incubated at 37 degrees centigrade for 1hour to digest the parental supercoiled dsDNA. About 1ul of the Dpn1-treated DNA was transformed from sample reaction into separate 50ul aliquots of XL1-Blue supercompetent cells and transformation reactions was carried out.

After the transformation reactions, about 10millilitre of LB and 10ul of AMP were added into a fresh 50mls sterile tube and gently mixed. 1ml of LBAMP was added into each of the two sterile eppendorf tube. About 50ul of D175N cells was added into the 1st eppendorf tube and 50ul of E180G was added into the 2nd eppendorf tube and incubated for 4-6 hours at 37 degrees centigrade. After the incubation, 4 fresh sterile tubes were arranged for cells splitting. About 0.5ul of LBAMP D175N cells (non-induced) was added into the 1st tube and 0.5ul of LBAMP IPTG D175 (induced) into the 2nd tube (IPTG switches on the gene's expression). Also, about 0.5ul of LBAMP E180G cells (non-induced) was added into the 3rd tube and 0.5ul of LBAMP

IPTG E180G (induced) was added into the 4th tube (IPTG switches on the gene's expression). The 4 tubes were clearly labelled (non-induced protein 1, induced protein 2, non-induced protein 3 and induced protein 4). The tubes were incubated again for 3hours at 37 degrees centigrade. After the incubation, about 125ul of each of the sample from each 4 tubes were transferred into each of the 4 fresh sterile eppendorf tubes. 20ul of SDS buffer and 20ul of distil water was added into each of the 4 tubes and mixed properly.

The 4 tubes were also labelled carefully (non-induced protein 1, induced protein 2, non-induced protein 3 and induced protein 4). The 4 tubes (containing the cells) were transferred to a heat shock at 95 degrees centigrade for 4minutes of incubation. Gel electrophoresis was carried out by loading 5ul of the ladder (on the 1st well) and 10ul of each of the protein sample to the subsequent wells (to observe the protein bands separation of non-induced and induced proteins).

To produce the mutated proteins on a large quantity, 1000ml LB was prepared using a 1000ml flask and another 1000ml LB was prepared using a 2nd 1000ml flask. About 2ul of D175N was added to the 1st flask and 2ul of E180G was added into the 2nd flask. Into each of the 1000ml flask, 0.1g of AMP was added and the reaction mixtures were shaken vigorously. About 125ul of LBAMP D175N and 125ul of LBAMP E180G were transferred to each of the fresh sterile eppendorf tube and 20ul of SDS buffer, 20ul distil water was added into each of the eppendorf tube and mixed properly. The tubes were heat shocked for 4 minutes at 95 degrees centigrade and labelled clearly (non-induced D175N and non-induced E180G). Into each of the 1000ml flask, 0.1g of IPTG was added (to induce the protein synthesis) and the reaction mixture was shaken vigorously. The two flasks were incubated overnight at 37 degrees centigrade. After the overnight incubation, the SDS PHAGE protein electrophoresis was carried out to observe the protein band separation of non-induced and induced D175N and E180G.

Half of D175N cell was transferred from the 1000ml flask into a 500ml centrifuge tube and another half of the cell into the 2nd centrifuge tube. From the 1000ml flak containing the E180G cells, half of the cells were transferred into the 3rd centrifuge tube and another half into the 4th centrifuge tube. The 4 centrifuge tubes were spun for 10 minutes at 6000rpm using SA1500 rota of the centrifuge. The supernatant was discarded, and each of the pellets was resuspended with 30mls of the ice-cold lysis buffer; the mixtures were homogenized, and sonication was carried out for 8 minutes (i.e. 4 minutes heating and 4 minutes cooling). After sonication, the cells were heated at 80 degrees centigrade for 50minutes in a water bath (to denatures the protein). The cells were centrifuge again to remove the denatured protein and debris. About 20ul of each sample was transferred into each fresh sterile Eppendorf tubes (post treated cells) which will be analyze using protein gel electrophoretic method.

Column Chromatography Analysis of Mutated Proteins

Half of the protein samples (D175N) was purified using column chromatography method of purification, and the purified proteins were collected on 24 fraction tubes (which were clearly labelled from tube 1 to tube 24). Protein gel electrophoresis was carried out by selecting the tubes labelled 10,12,14,16,18,20,22,24. About 20ul of SDS buffer and 20ul of distil water was added to each of the selected tubes containing the protein samples. About 5ul of the DNA ladder was added to the 1st well, sample from the 2nd tube was added to the 2nd well and the subsequent selected tube samples were added to their respective wells. The remaining half of the protein

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samples (E180G) were also purified, and the protein fractions were collected using 24 fraction tubes and also clearly labelled. Protein gel electrophoresis was also carried out by selecting the tubes labelled 10,12,14,16,18,20. About 20ul of SDS buffer and 20ul of distil water was added to each of the selected tubes. 5ul of the DNA ladder was added to the 1st well, sample from the 2nd tube was added to the 2nd well and the subsequent selected tube samples were added to their respective wells. The same method of purification and gel electrophoresis was also repeated with the E180G cells.

Measurement of the Thermal Stability of Wild Type (Lysozyme) and Mutated Proteins (D175N and E180G) Using the Dye Base Fluorescent Method

Principle of the assay: The protein is mixed with the dye and heated; as it unfolds or melts, hydrophobic parts of the protein are exposed and bind to the dye, resulting in a significant increase in fluorescence emission detected by the PCR system. To demonstrate the effect of various buffers concentration on protein tropomyosin, sixteen PCR tubes were set up and labelled ''tube 1 to tube 16''. About 45ul of the protein samples were added to each of tube 1-12. About 40.3ul each of buffer 1, buffer 2, buffer 3 and buffer 4 were added into tube 1-3, 4-6, 7-9 and 10-12 respectively. Into tube 13-14, 2.5ul lysozyme and 42.5ul of buffer 1 were added and this serves as a positive control experiment. Into tube 15-16, 45ul of buffer 1 was added and this serves as a negative control experiment.

To demonstrate the effect of one buffer with various concentrations on mutated proteins and wild type protein (lysozyme), three fluorescent PCR tubes were arranged and carefully labelled ''tube 1, tube 2 and tube 3''. Tube 1 contained 6.03ul of E180G protein, 39ul of buffer 1 and 5ul of dye. Tube 2 contained 6.24ul D175N, 38.7ul buffer 1 and 5ulof dye. Tube 3 contained 7.2ul of normal protein (Lysozyme), 37.7ul of buffer 1 and 5ul of dye. The samples in each tube was mixed properly, and the 3 tubes were loaded on the fluorescent PCR machine (for thermal stability measurement).

RESULTS

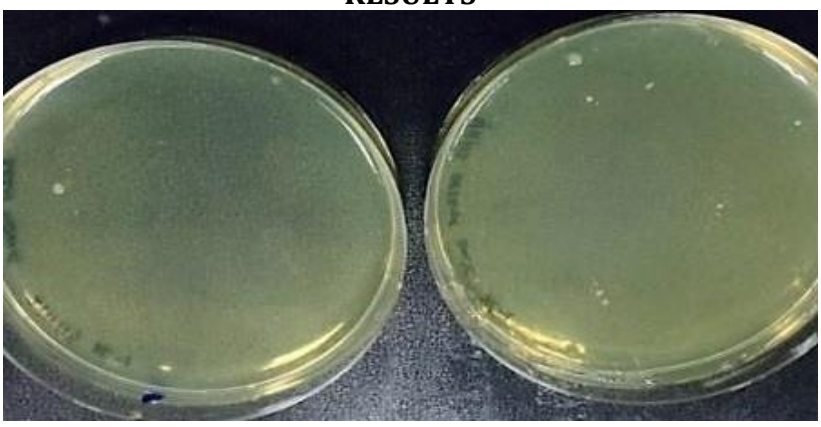

Figure 1.0: low and high concentration of XL1 cells cultured on LB agar media.

The above diagram showed the XL1 cells cultured on LB agar media. Scanty growth of E. coli was observed on the two plates due to AMP antimicrobial sensitivity. The E. coli was able to grow on the cultured plate because the XL1 cells contained a vector DNA gene; this gene facilitates for colony growths of the bacterial which has different morphologic appearance and diagnostic features.

Figure 1.1: Negative control of XL1 cells cultured on LB agar media.

Figure 1.1 above indicates a negative control of XL1 cells cultured on LB agar media. There was no prominent growth of E.coli because the inoculated cell was deficient of the vector DNA. This was used as a negative control method to observe the effect of the absence of vector gene on the growth of E.coli.

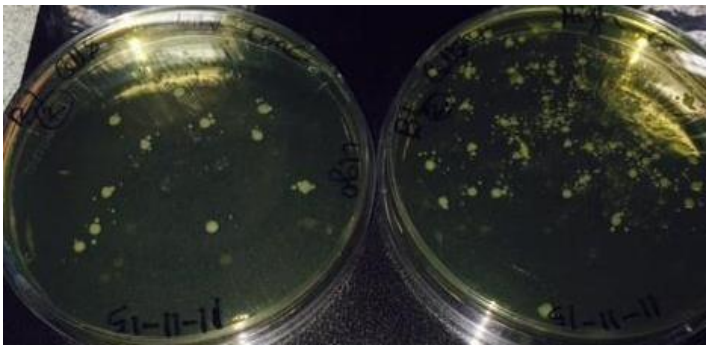

Figure 1.2: Figure 1.0: low and high concentration of BL2 cells cultured on LB agar media.

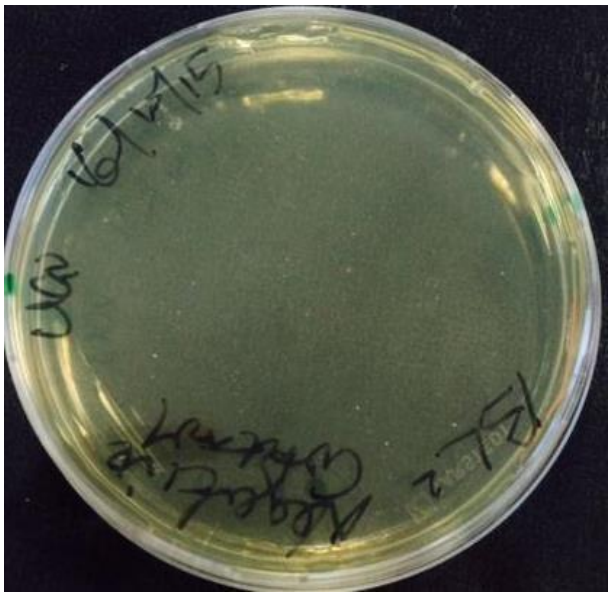

Figure 1.3: Negative control of BL1 cells cultured on LB agar media, showing no bacterial growth due to absent of genetic DNA vector.

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The above diagram showed the BL2 cells cultured on LB agar media. Moderate growths of E. coli were observed on the two plates due to AMP antimicrobial sensitivity. The E. coli was able to grow on the cultured plate because the BL2 cells contained a vector DNA gene which contain the antibiotics that has the promoter gene component. The promoter gene will bind to the IPTG and stimulates the mRNA to synthesis protein components.

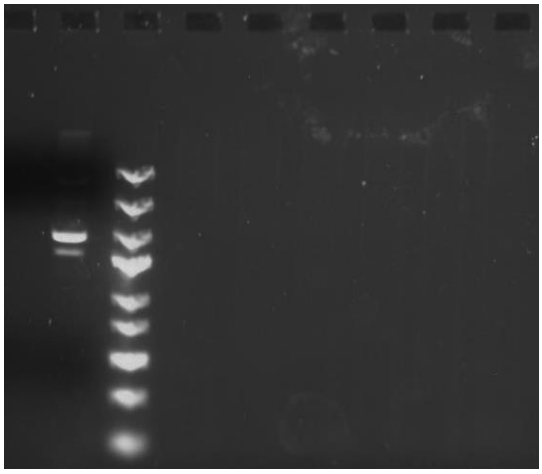

Figure 1.4: Purified DNA gel electrophoresis showing double band formation of DNA. The confirmed DNA will transform the BL2 cells to synthesis the normal protein.

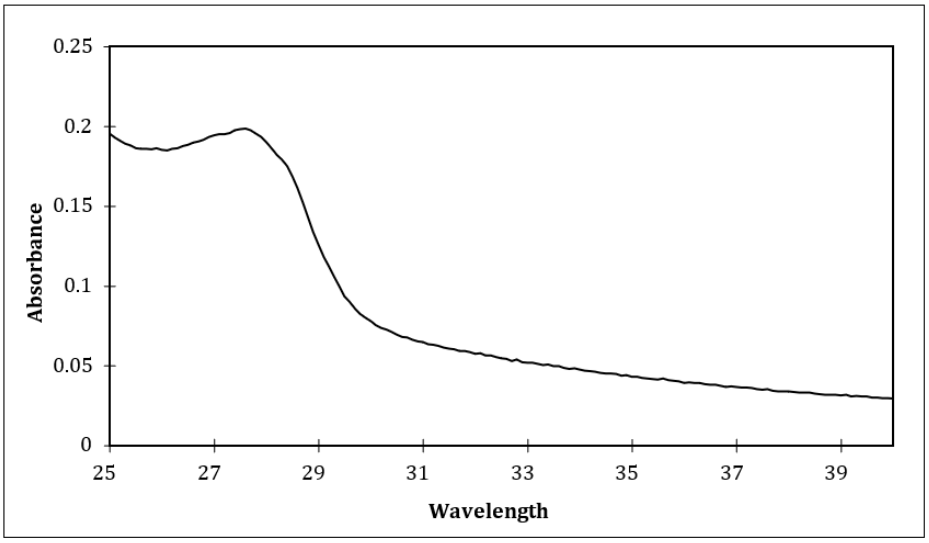

Figure 1.5: The absorbance of tropomyosin normal protein using spectrophotometer measurements

Abs nm	280	0.190	Abs (280 corrected)	0.129		Dilution	25
			Tm conc(abs*dil*E/MWt)	177.5	µM	Conc 1AD	3.618
Abs nm	350	0.043		11.640915	mg/ml	Mwt (MDa)	0.065594
Abs nm	400	0.029				Mwt (Da)	32797
Cor	250	0.070	True Ad 280 (abs x dil)	3.2			
Slope/nm		0.00027					
Cor	280	0.062					

The above diagram in figure 1.5 refers to the absorbance of tropomyosin normal protein using spectrophotometer measurements. The molecular weight of the protein was found to be 32797 Da. At 280 nm wavelength, the absorbance of the protein was 0.129, and the protein concentration was found to be 11.64mg/ml.

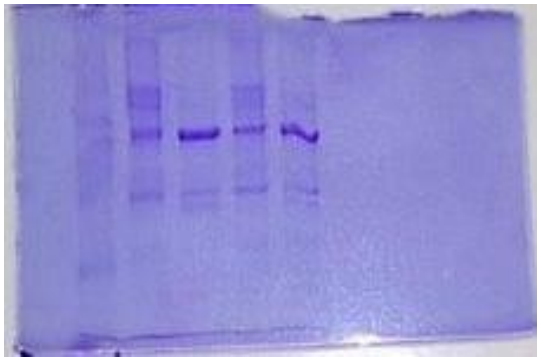

Figure 1.6 showing the SPHAGE protein gel electrophoresis of non-induce and induced BL2 cells and non-induced and induced BL2 cells.

The above diagram in figure 1.6 showed the protein gel electrophoresis of the non-induced and induced cells. The 1st well is the ladder with a molecular weight of 1kb base pair. The 2nd well indicates a double band separation of non-induced protein (BL2 sample) while the 3rd band shows a thicker protein band (of BL2 cells) indicating a high synthesis of protein. The 4th well also shows a non-induced protein band separation of XL1 sample and the 5th band shows a thicker protein band indicating a high synthesis of protein (XL1 cells). On each sample well, the protein bands occurred between 760-780 base pairs.

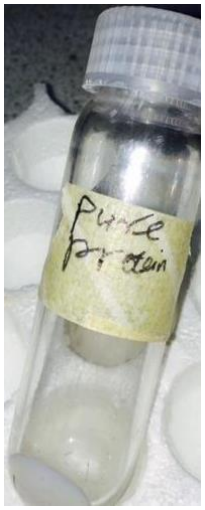

Figure 1.7: Normal protein (pellet) before re-suspension with potassium phosphate.

Figure 1.8: Normal protein (pellet) after re-suspension with potassium phosphate.

The above diagram in figure 1.8 indicates the re-suspension of protein pellets with potassium phosphate solution. The pellets became cloudier on the addition of potassium phosphate as a result of protein and DNA precipitations.

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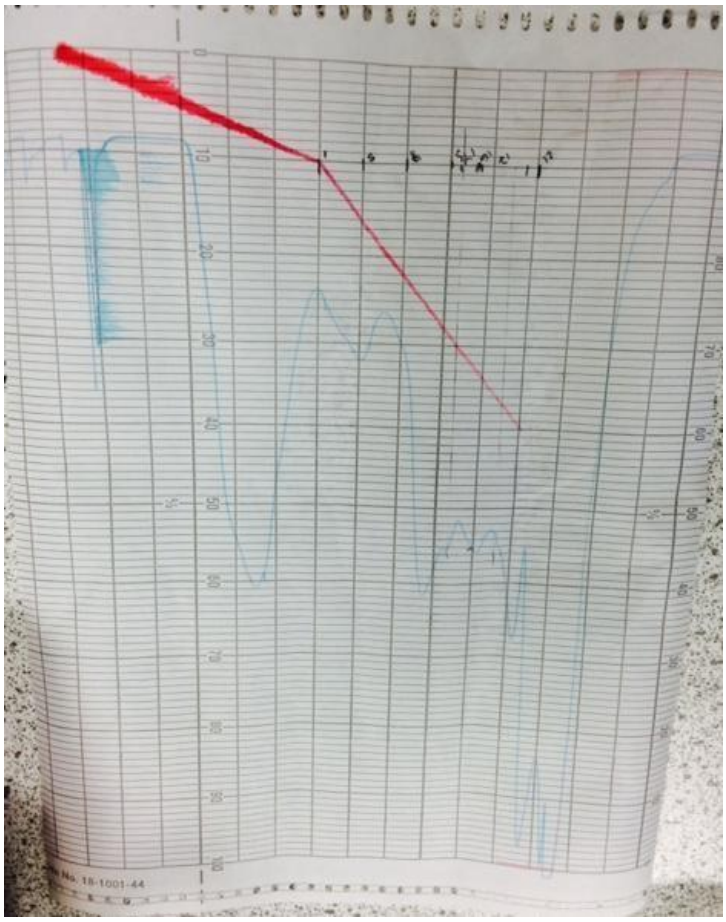

Figure 1.9: Elution graph of tropomyosin normal protein purification using column chromatography method. The graph explained the elution of the purified protein and the pure fractions was collected from tube 10 to tube 24.

Figure 2.0: Protein fractions gel electrophoresis after chromatography purification.

The figure 2.0 above shows the gel electrophoresis of the purified protein fractions. The 1st well (from the left side) is the DNA ladder with a molecular weight of 1kDa. The highest protein purifications were collected on tube 22 and 24 which has a thicker protein band formation.

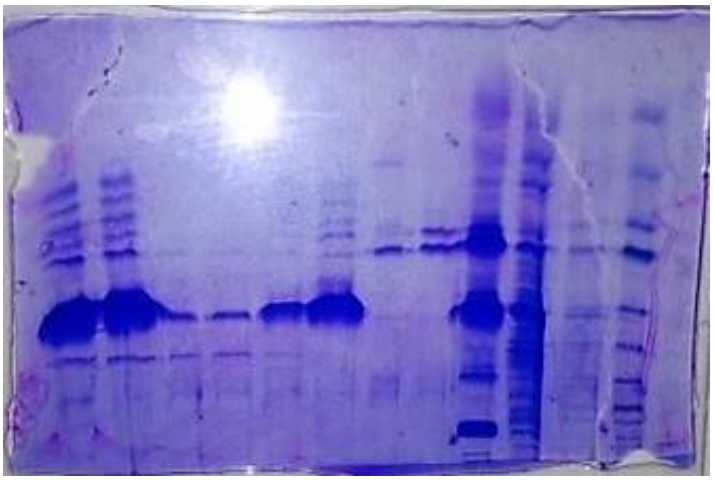

Figure 2.1: Protein Gel electrophoresis of non-induced protein, induced protein, post-boiled protein, 10th, 12th, 14th, 16th, 18th, 20th, 22nd and 24th fractions of normal protein.

Referring to the above diagram in figure 2.1, the 1st well (from the right hand side) is the ladder which has a molecular weight of 1kDa and 1000 base pairs. The 2nd well is a non- induced protein band (which has a slight protein band concentration). The 3rd and 4th wells are the induced and post-boiled proteins which were induced by the addition of IPTG, their protein bands are thicker when compared with the non-induced protein band (which indicates an increased synthesis of protein).The 5th and 6th well shows the fraction collections of purified proteins on tube 10 and 12 (which precipitates as a double protein bands). The 7th and 8th wells show an increase in protein purification fractions (on tube 14 and 16) which precipitates with a thicker protein band. The 9th and 10th wells show the fraction collection of purified proteins on tube 18 and 20 (with a reduced protein band formation). The 11th and 12th wells show the fraction collections of purified proteins on tube 22 and 24 (with a higher precipitation and formation of protein band). The precipitation and formation of protein bands is directly proportional to the purity of the protein collected on the fractionating tubes.

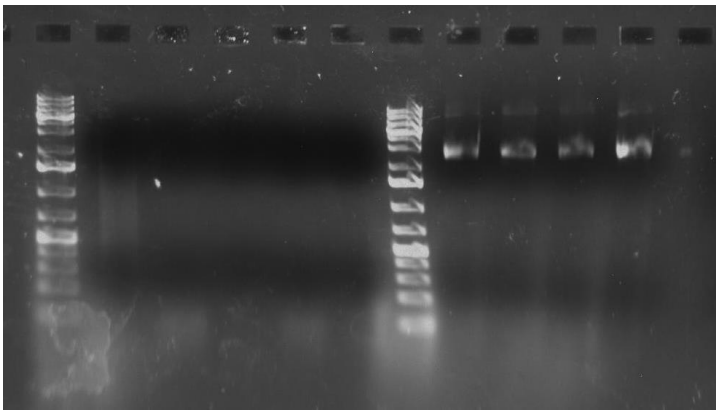

Figure 2.2: Purified DNA gel electrophoresis showing double band formation of DNA.

Referring to the above diagram, the middle long strand is the DNA ladder, the 2nd and 3rd wells are DNA bands from D175N and E180G DNA samples respectively. For accuracy of the result, the 4th and 5th wells contained the repeated samples of D175N and E180G samples. The purpose of carrying out the DNA gel electrophoresis is to confirm the vector DNA which will transform the BL2 cells to start synthesis the mutated proteins.

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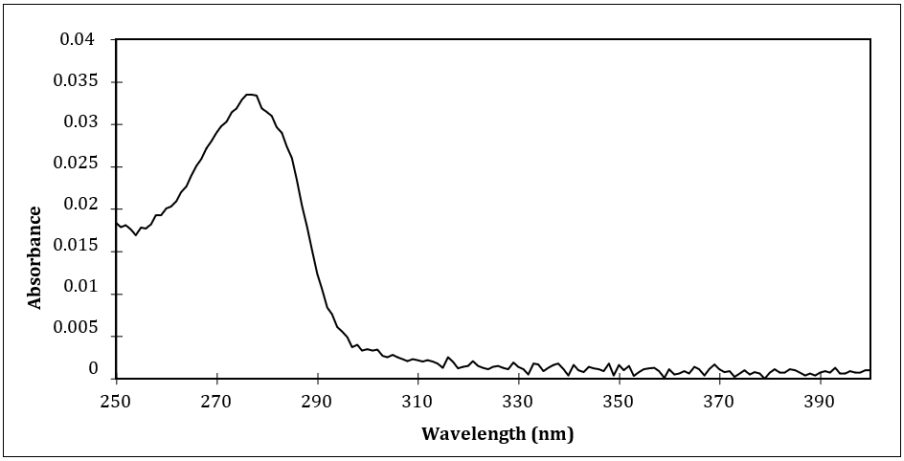

Figure 2.3: Absorbance of D175N using spectrophotometer measurement.

Abs nm	280	0.032	Abs (280 corrected)	0.029		Dilution	50
			Tm conc(abs*dil*E/MWt)	80.1	µM	Conc 1AD	3.618
Abs nm	350	0.002		5.256954	mg/ml	Mwt (MDa)	0.065594
Abs nm	400	0.001				Mwt (Da)	32797
Cor	250	0.003	True Ad 280 (abs x dil)	1.5			
Slope/nm		0.000012					
Cor	280	0.002					

The above diagram in figure 2.3 refers to the absorbance of D175N protein using spectrophotometer measurements. The molecular weight of the protein was found to be 32797 Da. At 280 nm wavelength, the absorbance of the protein was 0.032, and the protein concentration was found to be 5.25mg/ml.

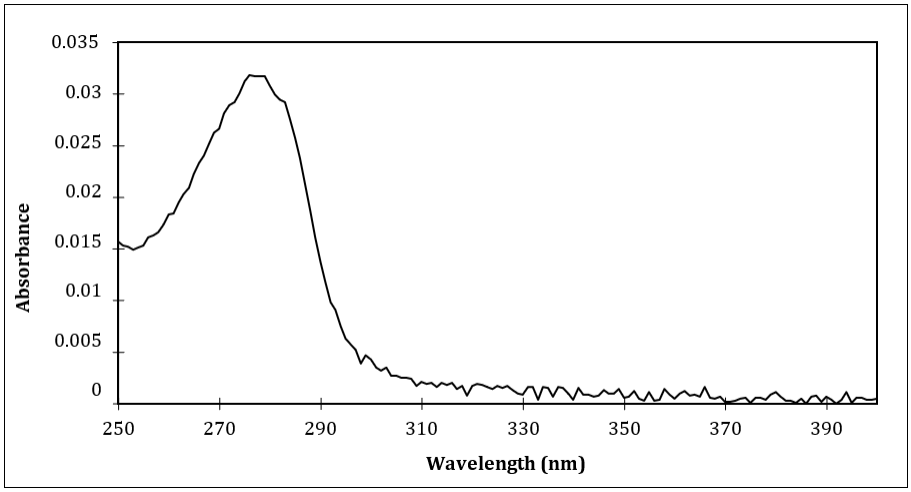

Figure 2.4: Absorbance of E180G using spectrophotometer measurement

Abs nm	280	0.031	Abs (280 corrected)	0.030		Dilution	50
			Tm conc(abs*dil*E/MWt)	82.9	µM	Conc 1AD	3.618
Abs nm	350	0.001		5.437854	mg/ml	Mwt (MDa)	0.065594
Abs nm	400	0.001				Mwt (Da)	32797

Cor	250	0.001	True Ad 280 (abs x dil)	1.5			
Slope/nm		0.000002					
Cor	280	0.001					

The above diagram in figure 2.4 refers to the absorbance of E180G protein using spectrophotometer measurements. The molecular weight of the protein was found to be 32797 Da. At 280 nm wavelength, the absorbance of the protein was 0.031, and the protein concentration was found to be 5.43mg/ml.

Figure 2.5: Protein gel electrophoresis of non-induced and induced D175N protein, and non-induce and induced E180G protein.

Referring to the above diagram in figure 2.5, the 1st well (from the left hand) is the molecular DNA ladder with 1000base pair. The 2nd and 3rd well are the non-induce and induced D175N protein bands; and the 4th and 5th wells shows the band formation of non-induce and induced E180G protein. The four protein bands have base pairs between 760-780kb.

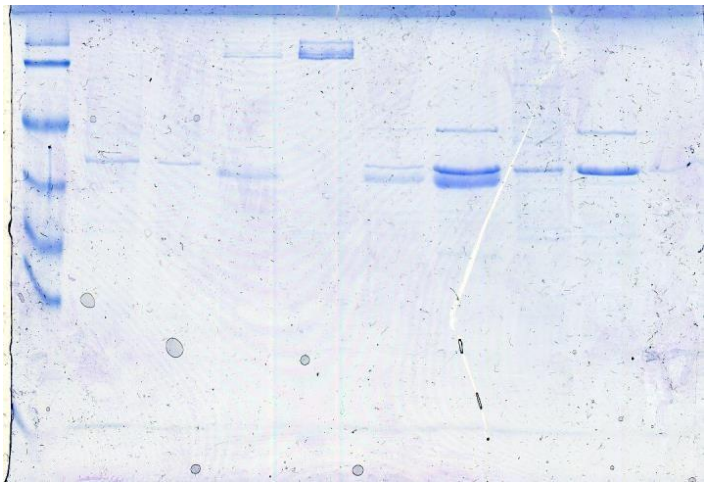

Figure 2.6: Gel electrophoresis of D175N and E180G proteins

URL: http://dx.doi.org/10.14738/bjhr.1106.14733.

The above diagram in figure 2.6 shows the protein gel electrophoresis of D175N and E180G proteins. The 1st well (from the left hand side) is the ladder which has a molecular weight of 1kda and 1000base pairs. The 2nd and 3rd wells are the D175N LBAMP (non-induced protein band) and D175N LBAMP IPTG (induced protein band) respectively. The 4th and 5th wells are the post-boiled samples of D175N and E180G proteins, their protein bands corresponds to the base pair between 960-980kb of the ladder. The 6th and 7th wells are the 1st and 2nd D175N pellets with a very prominent protein bands concentration. The 8th and 9th wells are the 1st and 2nd E180G pellets; and the 10th well is the E180G LBAMP IPTG (induced protein band).

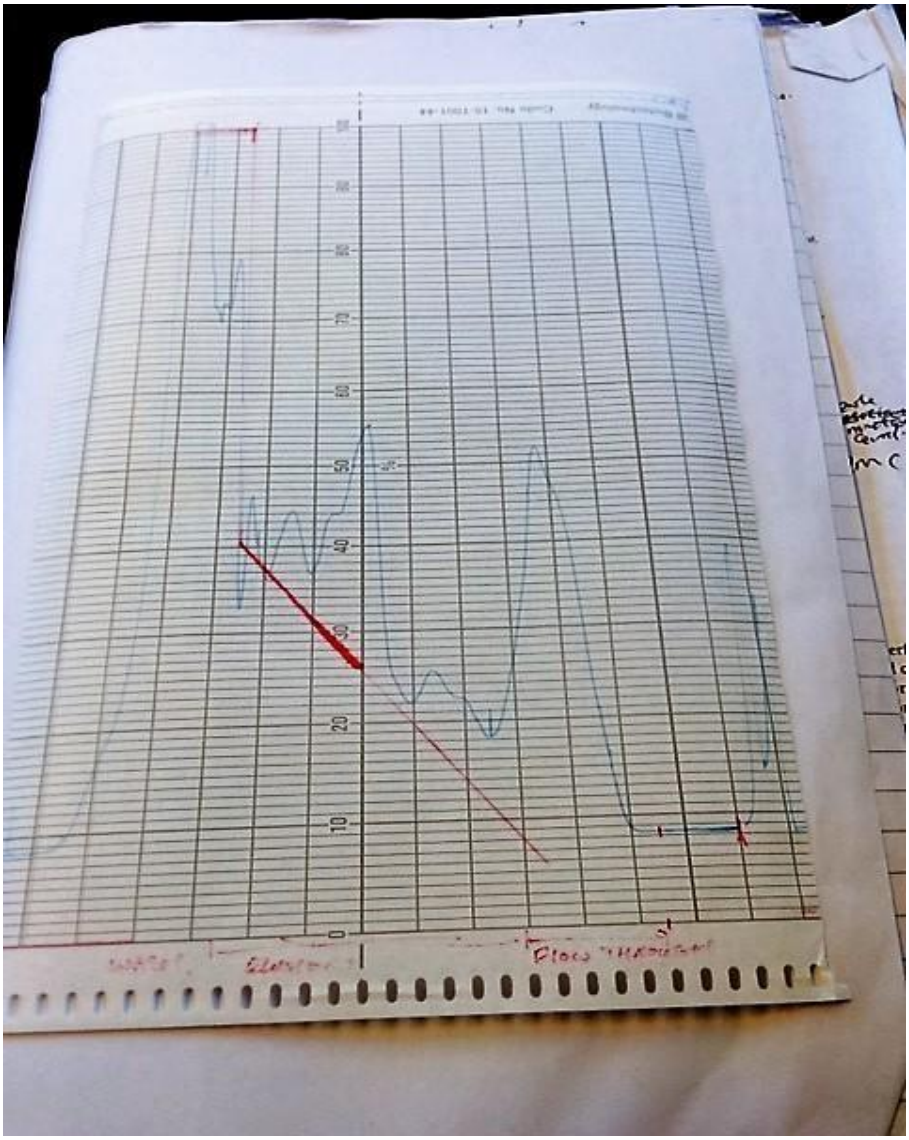

Figure 2.7: Ellution graph of D175N protein purification using column chromatography method. The graph explained the elution of the mutated purified protein and the pure fractions was collected from tube 10 to tube 24.

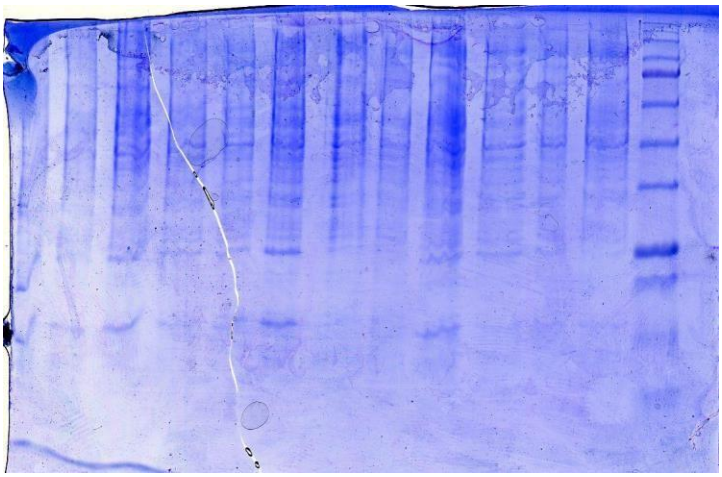

Figure 2.8: Protein gel electrophoresis of purified protein fractions.

The above diagram in figure 2.8 shows the gel electrophoresis of purified mutated protein fractions, using column chromatography method. The protein fractions selected for gel electrophoresis are 10th, 12th,14th,16th,18th,20th,22nd and 24th fraction tubes. The purity of the protein is directly proportional to the thickness of the protein bands. Tube 16, 20, 22 and 24 have a high protein purification which indicates a strong protein band formation on their respective wells.

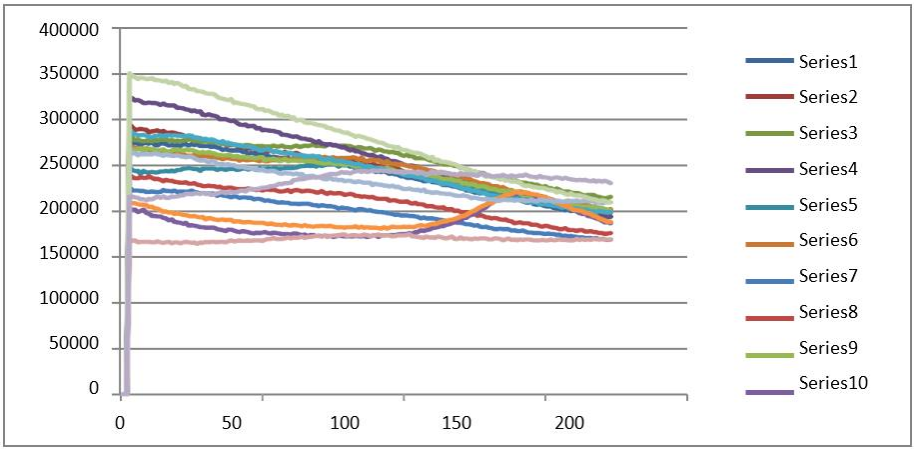

Figure 2.9: Buffer stability of tropomyosin (normal protein) and lysozyme at different buffer concentrations (buffer 1-4).

Figure 2.9 above shows how the buffer ionic concentrations substantially affect protein stability. Increasing the phosphate buffer concentrations caused a 3-10 fold increase in the thermal stability of proteins.

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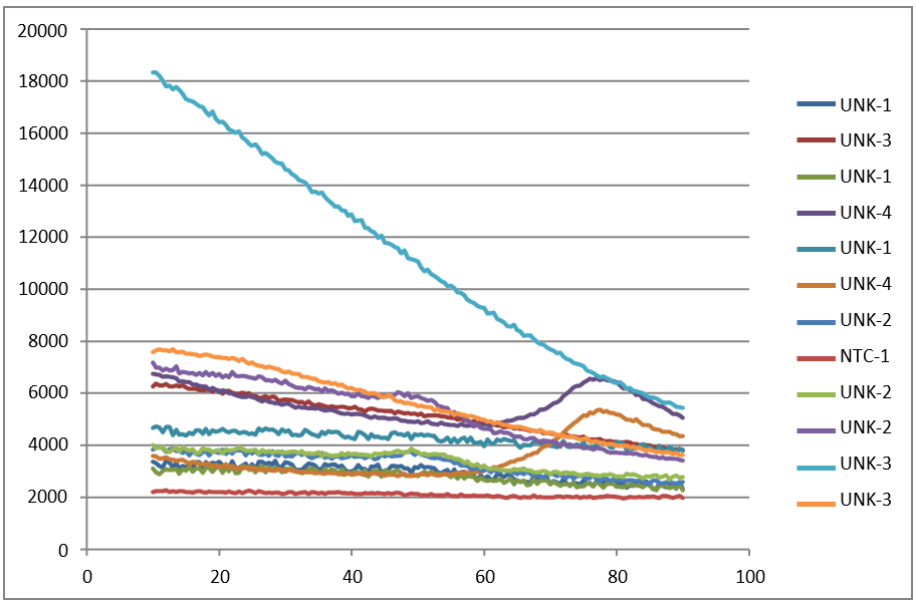

Figure 3.0: Buffer stability of tropomyosin (normal protein), mutated proteins (D175N and E180G) and lysozyme.

The above diagram in figure 2.9 and 3.0 shows the effect of the same buffer with different ionic concentrations on tropomyosin, mutated proteins and lysozyme. An increase in phosphate buffer concentration increases the conformation and unfolding of proteins. The UNK-1,2 and 3 indicates the normal typed tropomyosin; UNK-4,5 and 6 symbols indicate the D175N mutated protein; and the UNK-7,8 and 9 symbols indicate lysozyme protein. The NTC-1 symbol indicate the negative control sample which contained the dye without the protein component.

DISCUSSIONS

Tropomyosin and cardiac actin form a unique functional unit that plays both structural and dynamic roles in sarcomeric muscle function. In this present study, wild type and mutated proteins (E180G and D175N) were synthesized and purified in the laboratory using protein recombinant methods. The purified DNA transformed the BL cells to start producing protein. The DNA and proteins synthesized were analyzed using gel electrophoresis. The presence of the protein on the protein bands was noted which indicates a high synthesis and purification process.

Furthermore, of the tropomyosin mutations implicated in cardiomyopathy, E180G and D175N were the first identified (Watkins et al; 1995) and at present are best characterized, both at the protein level (18–20) and in transgenic mice (Ebashi et al; 1968; McKillop & Kodama, 1968; McKillop DF & Geeves MA, 1993; Pirani et al; 2006). These mutations increase muscle Calcium sensitivity (Ebashi et al; 1968; McKillop & Kodama, 1968; McKillop DF & Geeves MA, 1993). Perhaps related to their location within a recognized troponin- binding region of tropomyosin. More surprisingly, Micheleetal (Hitchcock et al;1973) have shown that muscle Calcium sensitivity is also increased by mutations on E180G and D175N, and my present studies have shown a similar effect in solution studies of these molecules.

Many thin filament HCM mutations increase Calcium sensitivity, regardless of whether in Troponin or tropomyosin.

However, in this practical experiment, the effects of Tropomyosin Mutations and lysozyme on thermal Stability was noted. Tropomyosin folding stability is weakened by HCM mutations on E180G and D175N, and such folding stability effect was not encountered with lysozyme protein component. Both mutations are in a tropomyosin region that interacts with troponin (Geisterfer-Lowrrance et al;1990). In this present report, dye based fluorescent method was used to monitor protein folding as a function of temperature for wild type tropomyosin and for HCM mutant E180G and D175N proteins. At intermediate temperatures, each mutation was significantly altered. Wild type tropomyosin unfolded in two distinct transitions, separated by 10 °C. The lower temperature transition can be assigned to the more C-terminal portion of tropomyosin, both because previous working indicates that tropomyosin is more stable toward its N terminus (Takahashi-Yanaga et al, 2001), and also because these N-terminal mutations primarily alter the higher temperature transition. However, similar assessment of folding stability and structural reports of several authors' Elliott et al; 2000) was in consistence with this present report which suggested that such mutations might alter protein folding. Because E180G and D175N mutations also have destabilizing effects, this appears to be a characteristic property of HCM tropomyosin. Increased thin filament Ca2 sensitivity is equally characteristic, making it interesting to consider whether these two properties are causatively linked. Surprisingly, the present data suggest that the mutant tropomyosin are more flexible, but this does not produce the expected effect on cooperative aspects of muscle regulation. The results agree with previously published reports on the impaired function of expressed E180G and D175N mutation (Sweeney et al., 1994) suggesting that the biochemical defects of the motor domain may affect myosin filament assembly in the sarcomere.

CONCLUSION AND FUTURE PERSPECTIVES

In summary, recombinant human Tms were produce which serves as a model protein to study the interactions that govern the stability of the thin filament. Two mutations (E180G and D175N) described as causing cardiomyopathy were introduced in the cDNA encoding the human skeletal muscle tropomyosin. Thermal denaturation studies of control and mutant tropomyosin (E180G and D175N) show a cooperative interaction between folding of the N-domain and C-regions of tropomyosin. HCM mutations E180G and D175N prominently destabilized the tropomyosin N-domain, which indirectly affected cooperative interactions between N- and C-terminal regions of tropomyosin. The experimental result on this current study suggested that HCM mutations locally destabilize tropomyosin, leading to an increased thin filament Calcium sensitivity and the degree of disruption of the tropomyosin interaction by the FHC mutations may be directly correlated with the severity of the disease. For future prospect, future biochemical analysis of several other FHC mutations will be needed to establish a definite correlation between the enzymatic impairment between different mutants and their clinical phenotype of the heart disease.

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