T.R.
ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF OF MOLECULAR BIOLOGY AND GENETICS

CLONING, EXPRESSION AND BIOCHEMICAL CHARACTERIZATION OF FULL SIZE AND TRUNCATED NOVEL CCDC-124 PROTEIN IN E.COLI

Master's Thesis

Asyah ABBOOD

Supervisor

Prof. Dr. Münir TUNÇER

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ACCEPTANCE AND APPROVAL OF THE THESIS

The study entitled **CLONING, EXPRESSION AND BIOCHEMICAL CHARACTERIZATION OF FULL SIZE AND TRUNCATED NOVEL CCDC-124 PROTEIN IN E. COLI** prepared by **Asyah ABBOOD**, and supervised by **Prof. Dr. Münir TUNÇER**, was found successful and unanimously accepted by committee members as Master thesis, following the examination on the date 07 September 2023.

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Member	Prof. Dr. Münir TUNÇER Ondokuz Mayıs University Department of Molecular Biology and Genetics	☒ Accept ☐ Reject
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DECLARATION OF COMPLIANCE WITH SCIENTIFIC ETHIC

I hereby declare and undertake that I complied with scientific ethics and academic rules in all stages of my Master's Thesis , that I have referred to each quotation that I use directly or indirectly in the study and that the works I have used consist of those shown in the sources, that it was written in accordance with the institute writing guide and that the situations stated in the article 3, section 9 of the Regulation for TÜBİTAK Research and Publication Ethics Board were not violated**.**

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ÖZET

E.COLİ'DE TAM BOYUTLU VE KESİLMİŞ YENİ CCDC-124 PROTEİNİN KLONLAMASI, EKSPRESYONU VE BİYOKİMYASAL ÖZELLİKLERİ

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Proteinlerde yapısal bir mofif olan sarmal-bobin domeyini içeren-124 (CCDC-124: Coiled-coil domain containing 124) motifi, düşük organizasyonlulardan yüksek organizasyonlulara kadar uzanan geniş bir spektrumdaki ökaryotlarda korunmuş bir proteindir. CCDC-124 sitokinetik rolü ile uyumlu olarak sentrozom ve orta cisimcikte lokalize olmasına rağmen, nüklear ve sitoplazmik kompleksler arasında bir mekik gibi görünmektedir. CCDC-124 en az bir olası RNA-bağlama bölgesi içermektedir. Daha önceki kütle spektrometrisine dayanan proteomik çalışmalarımıza göre CCDC-124'ün nüklear ve sitoplazmik faz ayırımı kompleksleri ile ilişkili olduğu görülmektedir.

Bugüne kadar proteinin işlevini ve özelliklerini tanımlamak için birçok çalışma yapılmıştır. Bu çalışmada ise CCDC-124 proteininin çeşitli parçalarının proteinin biyokimyasal ve biyofiziksel özellikleri üzerindeki işlevsel rolünün araştırılması amaçlanmıştır. Bu amaçla CCDC-124 proteininin güdükleştirilmiş (kesilmiş) dört mutantı hazırlanmıştır: 32 kDa izoformu için C-ucu olmayan bir mutant ve N-ucu olmayan ikinci bir mutant yapılmış, kalan iki mutant ise olası RNA-bağlama bölgesine göre tasarlanmıştır. CCDC-124 proteininin parçalarının fizikokimyasal özelliklerini belirlemek için tam uzunluktaki ve güdük proteinlerin ifadeleri *E. coli* hücrelerinde gerçekleştirilmiş ve saflaştırılmıştır. Daha sonra saflaştırılmış proteinler çeşitli kimyasal maddelere maruz bırakılmış ve böylece proteinlerin faz ayırımı davranışına sahip olup olmadığı incelenmiştir. Elde edilen veriler ile CCDC-124 proteininin fizyolojik çalışması hakkında faydalı bilgiler elde edilmiştir.

Anahtar Sözcükler: CCDC-124, Faz Seperasyon, Protein Saflaştırma, RNA-Bağlama Proteini.

ABSTRACT

CLONING, EXPRESSION AND BIOCHEMICAL CHARACTERIZATION OF FULL SIZE AND TRUNCATED NOVEL CCDC-124 PROTEIN IN E. COLI

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Coiled-coil domain-containing 124 (CCDC-124) protein is an eukaryotic protein that is conserved with a spectrum extends low to high eukaryotes. Despite CCDC-124 mainly localizes centrosome and midbody with a correlation its cytokinetic role, it also seems to be as a shuttle between nuclear and cytoplasmic complexes. CCDC-124 contains at least one putative RNA-binding domain. According to our previous studies of mass spectrometry-based proteomics and their validations CCDC-124 appears to be related to nuclear and cytoplasmic phase-separated complexes.

To date many studies have been done to identify the function and properties of the CCDC-124 protein. In this study, we aimed to investigate the functional role of CCDC-124 protein fragments on biochemical and biophysical properties. We have prepared four truncated mutants: one without a C-terminus and one without N-terminus required for the 32 kDa isoform, the other two mutants are designed according to the putative RNA-binding region. To determine the physicochemical properties of full length and truncated fragments of CCDC-124 protein, we expressed and purified the protein form *E. coli* cells. Then purified proteins subjected to various chemical agents. Thus we could try to understand if the proteins have phase separation behaviour. Our results provide useful information about the physiological role of the protein.

Keywords: CCDC-124, Phase Separation, Protein Purification, RNA-Binding Protein.

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Asyah ABBOOD

CONTENTS

SYMBOLS AND ABBREVIATIONS

AD	: Alzheimer's Disease
ALS	: Amyotrophic Lateral Sclerosis
APS	: Ammonium Persulfate
B-mE	: B-Mercaptoethanol
CCDC-124	: Coiled Coil Containing Protein 124
cDNA	: Complementary Deoxyribonucleic Acid
C-terminal	: Carboxyl Terminal
DLS	: Dynamic Light Scattering
DLVO theory	: Boris Derjaguin and Lev Landau, Evert Verwey and Theodoor Overbeek Theory
DMSO	: Dimethyl Sulfoxide
EDTA	: Ethylenediaminetetraacetic Acid
FTD	: Frontotemporal Dementia
H_2O	: Water
HCl	: Hydrochloric Acid
HEPES	: (4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid)
IDP	: Intrinsically Disorder Protein
IDRs	: Intrinsically Disorder Region
IPTG	: Isopropyl B- D-1-Thiogalactopyranoside
KCl	: Potassium Chloride
KF	: Potassium Fluoride
KSCN	: Potassium Thiocyanate
KSI	: Ketosteroid İsomerase
LB	: Luria-Bertani
LCRs	: Low Complexity Region
LLPS	: Liquid-Liquid Phase Separation
MAB A	: Monoclonal Antibody A
MBP	: Maltose Binding Protein
NaCl	: Sodium Chloride
Np-40	: Nonyl Phenoxypolyethoxylethanol
N-terminal	: Amino Acid Terminal
OD	: Otical Density
pI	: Isoelectric Point

PIPES	: Piperazine-N,N′-Bis(2-Ethanesulfonic Acid)
PMLO	: Proteinaceous Membrane-Less Organelles
RNA	: Ribonucleic Acid
SDS	: Sodium Dodecyl Sulfate
SUMO	: Small Ubiquitin-Related Modifier
TBS	: Tris-Buffered Saline
TBS-T	: Tris-Buffered Saline-Tween
Tex A	: Thioredoxin A
TEA	: Tris-Acetate-EDTA
TEMED	: Thermo Scientific Pierce Tetramethylethylenediamine

FIGURES LEGENDS

TABLES LEGENDS

1. INTRODUCTION

1.1. Membraneless Organelles

Cellular organelles (or compartment) that responsible of the cellular function; can subdivided into those which encapsulated with lipid membrane, and membraneless organelles which unencapsulated with membrane. Nucleus, endoplasmic reticulum, vacuoles and mitochondria are examples of membrane bound organelles. While other compartment such as nucleolus (Montgomery et al., 1898), cajal body, stress granules, P bodies, pericentriolar material and germ granules are membraneless compartments (Marnik et al., 2019). Membrane is important for characterization of organelles morphology, whereas organelles shape is determine by membrane protein and lipid building block. Due to the dynamicity of most organelles, they can be in a different shape at different condition of the cell (Heald et al., 2011). This dynamicity is to maintain homeostatic and to adjusting cellular requirements, such as during cell division (Arnone et al,. 2013). Also, this membrane provide trafficking control within the cells, connecting two organelles together, maintain genetic information in the nucleus and endoplasmic reticulum, also it protects the cell and cytoplasm from harmful organelles content, such acidic content of the lysozyme.

Recent studies revealed that some biomacromolecular are concentrated and form cellular bodies or liquid droplet. This structure are different in nomenclature, such as membreneless organelles, intracellular bodies, and commonly used term proteinaceous membrane-less organelles (PMLOs) (Uversky, 2017; Stoeger et al,. 2016). This PMLOs play important role as a supplements to the membrane-encapsulated organelles such as Golgi vesicles, smooth endoplasmic reticulum, rough endoplasmic reticulum, lysosomes, peroxisomes, secretory vesicles (Stoeger et al., 2016). PMLOs consider condensed heterogeneous mixture of proteins and nucleic acids which accumulate via a process known as liquid-liquid phase separation (LLPS) (Zhu and Brangwynne, 2015; Banani et al., 2017). PMLO can form by trigger of LLPS of this membraneless organelles molecular (eg; protein and nucleic acid) which occur by change of various environmental condition. This environment condition include; variation in salt concentration, change in temperature, osmolarity, and other condition that may affect protein-nucleic acid or

protein-protein interaction (Brangwynne et al., 2015, Zhu and Brangwynne, 2015). It is important to know that, LLPS and PMSO formation are completely reversible and highly controlled process (Uversky, 2017), (Figure 1.1).

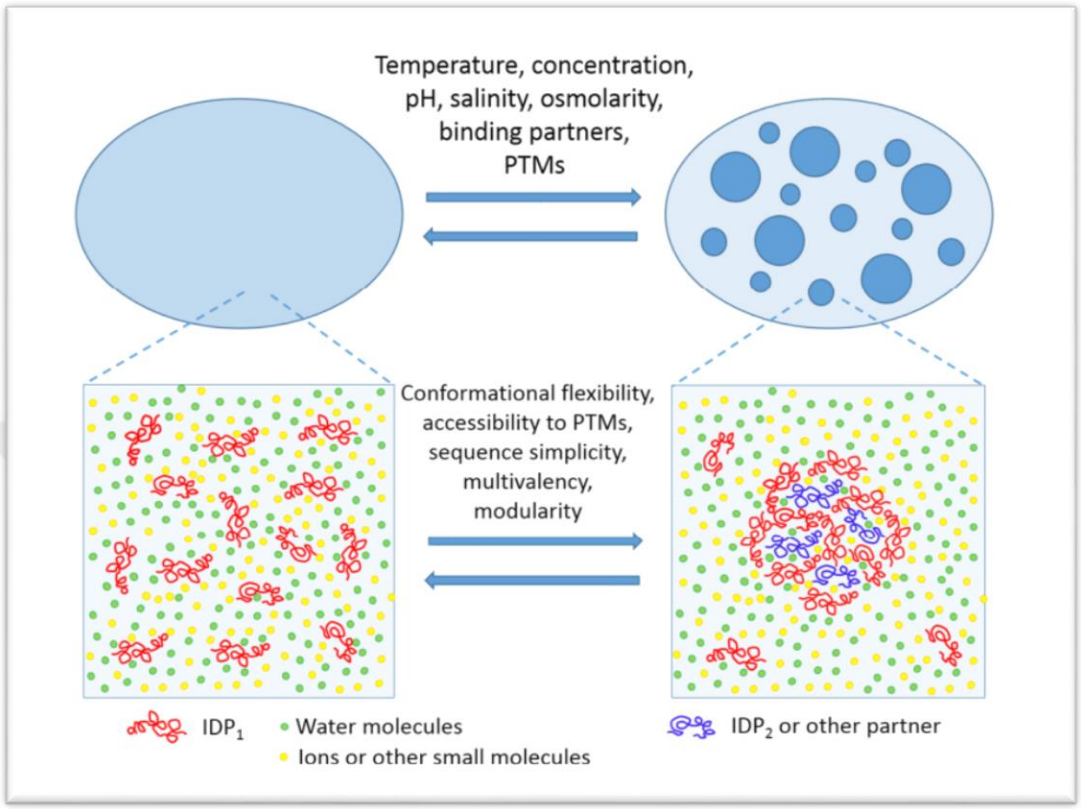

Figure 1.1. Explain controlling of LLPS by thermodynamic factors and intrinsically disorder protein.

PMLOs are different in their properties, some of them are characterize by liquid-like droplet properties with highly dynamic organization (Courchaine et al., 2016), while others vary from solid-like to gel and viscous liquid (Woodruff et al., 2018). These features of LLPS-driven organelles give them ability to quick assembly and disassembly due to various in-cellular condition. Also, they are various in composition, which can be change with environmental factors change such as during cell cycle and when the cell encounter temperature stress (Mitrea and Kriwacki, 2016). In the other hand unregulated formation of this organelles such as RNA granules are tightly related to neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) (Patel et al., 2015).

Pervious study on the disordered protein Ddx4, the main constituent of germ granules, revealed that N-terminal of his protein could self-associated to form distinguishable bodies in the cell and in vitro. This body formation are reversible and

depend on change in environmental condition. Change in temperature, ionic strength, disrupting the charged blocks and methylation of certain arginine residue can lead to completely dissolving this organelles. Also, this study found that this protein has a sequence that provide electrostatic interaction which is required to held Ddx4 protein together in the organelle (Nott et al., 2015).

1.2. Liquid-Liquid Phase Separation

Liquid-liquid phase separation is a physicochemical property of some protein molecular and nucleic acid. It consider reversible process of homogenous fluid between two phase, one is condense phase, and the other is diluted phase (Dolgin, 2018). Phase separation phenomenon lead to condensation and accumulation of some proteins and/or nucleic acid to form a membraneless organelles, such as P bodies, stress granule and nucleolus (Shin and Brangwynne, 2017; Cortes-Penfield and Trautner, 2017). Phase separation regulate some cellular process such as RNA metabolism (Boehning et al., 2018), signal transduction that occur in response to stress that cell expose to (Zhang et al., 2018). A number of study indicated that this biomolecular liquid condensate can transformed in some condition to solid aggregation or amyloid fiber which will be a main reason to some disease such as amyotrophic lateral sclerosis (ALS) (Gui et al., 2019; Patel et al., 2015), Alzheimer's disease (AD) (Oligomers et al., 2018), and frontotemporal dementia (FTD) (Wang at el., 2019). Phase separation system may contain only protein that mean this proteins cause phase transition by interaction with itself, or may contain amino acid, RNA or DNA, which modulate phase separation tendency of the protein. The flexibility of amino acid sequence belongs to present a bundle fraction of glycine amino acid that facilitate liquid condensation (Li et al., 2019).

Proteomic and genetic studies, which interested in study of membraneless organelles, suggest that multivalency of adhesive domains and/or linear motifs is define the features of proteins (and perhaps RNA molecules) that drive phase transitions. This multivalency can occur by three ways:

1. Folded proteins, which contain well-defined interaction surfaces, can form oligomers that arise multivalency of other associative patches, which participate in stereospecific interactions;

2. Folded domains, can be stand together by flexible linkers to generate linear multivalent proteins;

3. Intrinsically disordered regions (IDRs) can serve as scaffolds for multiple, distinctive short linear motifs (Andersen et al., 2005; Boke et al., 2016; Fong et al., 2013; Trcek et al., 2015). The presence of IDRs in proteins that drive phase transitions is one feature that has attracted considerable attention. These regions display a sequence-intrinsic preference for conformational heterogeneity. Interaction between the macromolecular enriched in IDRs lead to formation of membraneless organelles. For example, Balbiani body is a solid-like assemble of protein that held together by β-sheet interactions (Boke et al., 2016).

In addition of its role in linking of molecular, IDRs may also mediate sticky interaction that lead to phase transition (Banjade et al., 2015). Solution that contain concentrated protein with IDRs can form hydrogel over time (Kato et al., 2012). Moreover, recent study discovered that mutation in the IDRs of hnRNPA1 and hnRNPA2B, accelerate they assemble to high-order structure in vitro. This mutation also promote casual formation of stress granules which dramatically reduce dynamics in living cell (Kim et al., 2013).

1.3. Proteins with Intrinsically Disorder Region

Intrinsically disordered protein defined as protein that lacks three-dimensional structure or a well-defined tertiary structure, although they have biological activities. They can bind to nucleic acid or other protein so they accelerate chemical interaction between tied molecular, also they play a role in posttranslational modification, alternative splicing, protein fusions, and insertions or deletions (Oldfield and Dunker, 2014). IDPs tend to be with low complexity sequence, such as repeat sequence, and tend to be more flexible (Romero et al., 1998). This flexibility make the motif available for posttranslational modification (Borg et al., 2007). Post translational modification, at physiological PH, such as phosphorylation, deamination, acetylation and sulfation can change the net charge or charge distribution of the protein (Romero et al., 1998). This change can effect protein structure and binding properties of the IDRs (Andrew et al., 2002). Researches show that IDPs can undergo disorder-to-order transitions by binding, stabilizing isolated helical or extended regions or even a small ordered domain (Wright and Dyson, 2009); other interactions result in only

transient local ordering within a dynamic or ‘‘fuzzy’’ complex (Tompa and Fuxreiter, 2007; Mittag et al., 2010). The self-association of IDPs or dynamic multivalent interaction of modular binding domains with motifs within IDPs can leads to phase separation under specific conditions (Tompa et al., 2013). This interaction facilitate formation of micron-sized liquid protein-dense droplets (Li et al., 2012), that can function as nonmembrane-bound organelles (Malinovska, Kroschwald and Alberti, 2013) or precede gelation or fiber formation.

IDRs contain a wealth of charged amino acid (eg; lysine, arginine, glutamic acid), uncharged amino acid (eg; asparagine, glutamine, serin, glycine), or aromatic residues (eg; tyrosine and phenylalanine). This residue are often found as short linear interaction motifs, degenerate repeats or alternating charge blocks but not distribute randomly along protein sequence (Brangwynne et al., 2015; Tompa et al., 2014). Due to the range of sequence biases associated with IDRs that mediate phase separation, there may be a range of underlying driving forces. These may include electrostatic, pi–pi, dipole–dipole, cation–pi, , hydrogen and hydrophobic bonding interactions (Brady et al., 2017; Brangwynne et al., 2015; Monahan et al., 2017; Vernon et al., 2018).

Previous studies classified human proteome according to disorder region to three variant protein:

1. Ordered proteins (ORDPs),

2. Structured proteins with intrinsically disordered regions (IDPRs),

3. Intrinsically disordered proteins (IDPs) (Deiana et al., 2019).

The features of IDRs in protein is short functional site, low significant hydrophobicity, and high content of polar and charged amino acid (Uversky et al., 2000; Forman-Kay and Mittag, 2013).

1.4. General Properties of Coiled-Coil-Domain-Containing-124 (CCDC-124) Protein

Coiled-coil-domain-containing-124 protein is a novel protein with unknown function. It is highly conserved among eukaryotic species from fungi to human. CCDC-124 is encoded by cDNA that is transcribed from chromosome 19p13.11 0f human genome (Telkoparan et al., 2013). Immunoblot studies on CCDC-124

investigated that the protein is about 32 kDa, by generate specific antibody against CCDC-124 protein that bind to N-terminal 24 amino acid (Telkoparan et al., 2013). Previous study on sequence analysis of CCDC-124 with ClustalW (2.1 version) alignment program (http://www.ebi.ac.uk/clustalw/) show that the protein is highly conserved especially at 25-80 and 160-210 residue and this suggest biological function for the protein at this sequence (Telkoparan et al., 2013). CCD C-124 is a protein with 223 amino acid sequence which has two putative coiled coil domain take place between 18-82 residues in the N-terminal of the protein sequence as detected by bioinformatics analysis (www.ch.embnet.org/software/COILS_ form.html). Because of that this protein consider as coiled coil containing protein (Figure 1.2). It also found that the theoretical pI (Isoelectric point) of this protein is 9.54. Isoelectric point is a pH degree at which the amino acid that make up the protein does not carry net electrical charge and behave as natural. Consequently, at a pH below their pI, protein carries a net positive charge; above their pI it carries a net negative charge.

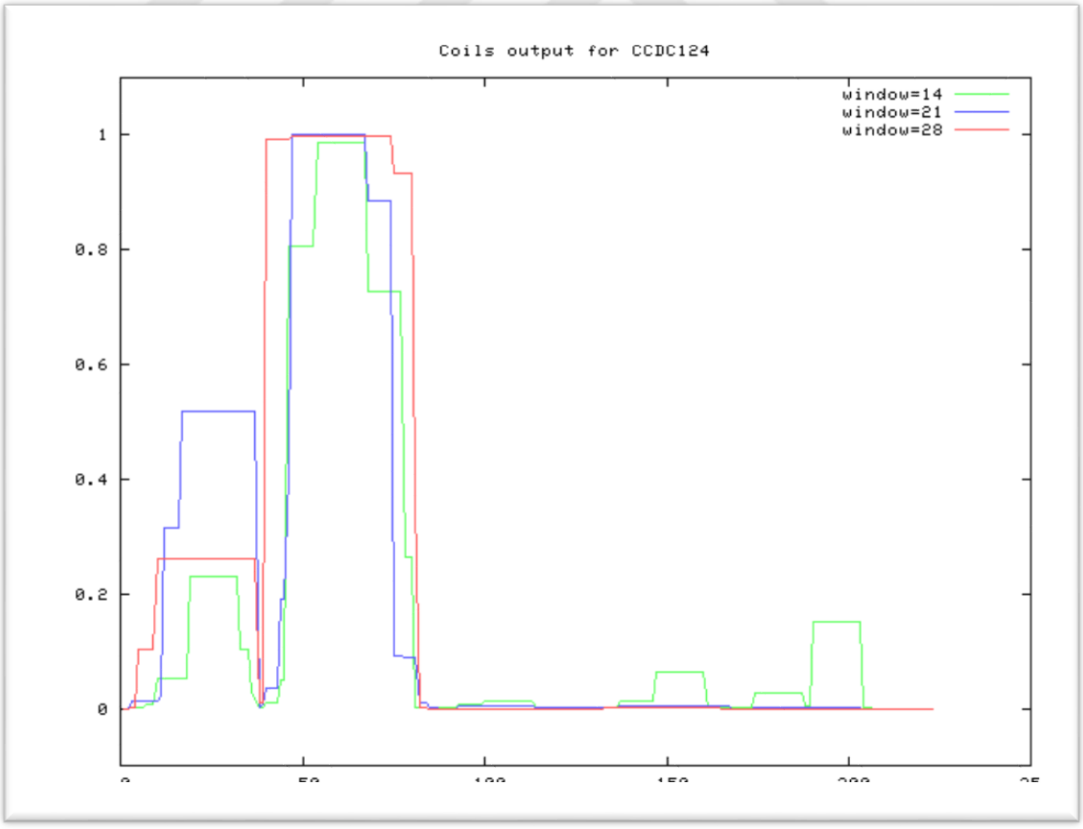

Figure 1.2. COILS bioinformatics analysis. Clarify coiled coil containing region among CCDC-124 protein sequence.

Recent study reveals Ccdc124 as a novel component of the centrosome, by applying molecular and cell biology method, as in cells at interphase and in mitotic cells up to the late-anaphase/telophase stage it clearly colocalized. Also in the same study they detect that Ccdc124 changes its subcellular localization at late-anaphase/telophase stages of cell cycle; it separate from centrosomes, and relocalizes to the midzone at late anaphase, before it accumulates at the midbody puncta at telophase and cytokinetic abscission (Telkoparan et al., 2013).

The computer analysis of protein by PONDR to prediction intrinsically disorder region indicate that large percent of CCDC_124 sequence is disorder, and at 100, 170 and up to 200 amino acid residue protein sequence tend to be order (Figure 1.3).

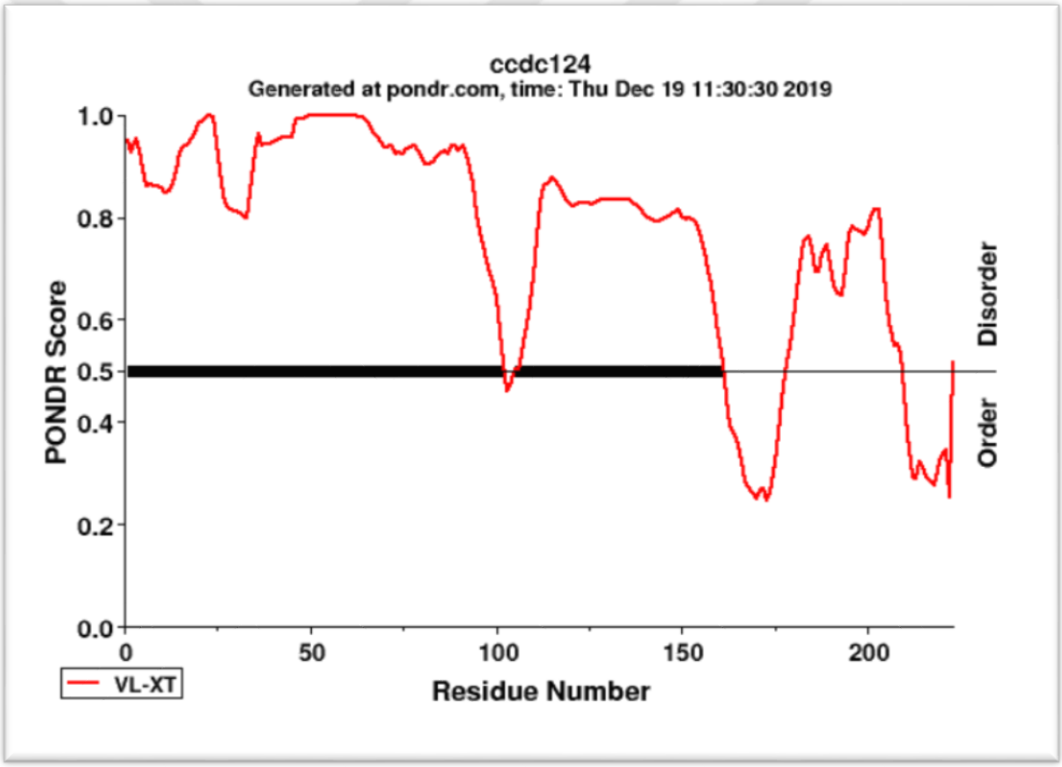

Figure 1.3. PONDR analysis for IDRs in CCDC-124. Show disorder and order region in CCDC-124 protein.

In another research at molecular biology field, CCDC-124 was shown to act as translational regulator protein. This research was interested in cellular stress under nutrient deprivation. When eukaryotic cell exposed to various stress such as glucose starvation, osmotic stress or amino acid shortage, idle 80S accumulate in the cell. For re-intiation of the translation, the 80S need to disassociate to repopulate the pool of

free 40S. They structurally recognized the interaction between CCDC-124 and 80S ribosome to brighten the way that interaction of this protein can effect translational activity during and after starvation. They found that CCDC-124 occupy the P-site position of 40S subunit inclusive tRNA and mRNA. Also, it bind 60S subunit in P and A site and reach near to stalk base and GTPase activity center and thus exclusively stabilize the nonrotated conformation of the 80S ribosome (Wells et al., 2020).

In the purpose of understanding more about CCDC-124 protein, recent study used *in silico* protein modeling and characterization model to generate a native-like tertiary structure of CCDC-124. This will make them able to examine disorder, low sequence complexity and aggregation properties. By employing co-immunoprecipitation (co-IP), immunostaining, recent live-cell protein-protein interaction and biomolecular florescence complementation researchers investigated CCDC-124 dimerization. Results show that CCDC-124 is highly mutation sensitive protein. The CABS-flex analysis for molecular structure, dynamic and flexibility have revealed a highly stable structure made up mostly very low protein fluctuation rate. Moreover, using many prediction tools lead to investigate disorder tendency of CCDC-124 especially N-terminal arm and very C-terminal end of CCDC-124 that contain long and short disorder regions. The mostly disordered regions are LCRs and they are consist of a composition with fewer amino acid types and repeats (Mier et al., 2021).

Furthermore according to aggregation regulatory function of LCRs the aggregation tendency was examined. Aggregation prophecy feedbacks were well-correlated with the disorder and low complexity data suggesting that CCDC-124 had a higher aggregation tendency, especially between 151 and 156 amino acid residue (Tuzlakoglu Ozturk et al., 2022). The oligomerization statues of CCDC-124 was predicted by trimerization and tetramerization docking analyses. Particular structure and binding energy analyses have enriched idea that the initial dimerization process may be a significant cause of CCDC-124 oligomerization via V153 region lie in predicted aggregation region. Coiled-coil oligomerization trigger structural transformations and generate cross β-sheet amyloid (Ford and Fioriti, 2020). Therefore, the CCDC-124's coiled-coil structured sequence composition and

oligomerization may control the development of aggregates and higher order structures like amyloid, which also clarifies the predicted aggregation propensity, presence of an intrinsically disordered region, and low complexity domain in the context of biomolecular condensation. (Tuzlakoglu Ozturk et al., 2022).

In another study RNA-binding characteristics of CCDC-124 have been employed for a better molecular functional description and estimate CCDC-124 irregular expression in human cancer samples from different tissue regions. For that reason bioinformatics calculations applied by RIP and RNA-seq experiments were perform to investigate mRNA targets of CCDC-124. Quantitative studies on arrays of cDNAs from different cancers and IHC assays on tissue arrays were used to assess CCDC-124 expression levels in cancers. This study could prove that CCDC-124 was characterized as an RNA-binding protein (RBP) interacting with various mRNAs. CCDC-124 mRNA levels were high in tumors, with a particular up-regulation in cancers from esophagus, adrenal gland, endometrium, liver, ovary, thyroid, and urinary bladder. Immunohistochemistry assays indicated strong CCDC-124 positivity in urinary bladder (68.4%), endometrial (95.4%), and ovarian cancers (86.8%). Consequently, CCDC-124 defined as cytokinesis related RNA-binding protein interacting with various mRNAs. Moreover, CCDC-124 mRNA over-expression and an accompanied increase in CCDC-124 protein accumulation was reported in cancers, indicating this RNA-binding protein as a novel cancer cell marker (Arslan et al., 2021).

1.5. Protein Purificationf by Affinity Tag

In biotechnology, pharmaceutical, cosmetics industries and food field, biological macromolecules such as protein consider an important product. Development an efficient and rapid protein purification method is needed for growing those fields. Protein purification became the second challenge for the researchers of those fields after genome of hundreds species had successfully sequenced. Protein expression is essential to know sequenced genetic information and finally to understand the role and function of genetic product. With the aim of DNA recombinant technology, proteins started to be expressed in several host organisms that leaded to a faster and easier process compared to their natural sources (Demain and Vaishnav, 2009). *Escherichia coli* are used as dominant host for

producing recombinant proteins, because of its advantageous which is fast growing and inexpensive, and high yield protein production, together with the well-characterized genetics and variety of available molecular tools (Demain and Vaishnav, 2009).

The second challenge for protein purification was separating of desired protein from nonprotein moleculars and other protein in the mixture. Purified protein should be free of contaminated molecular such as amino acid, other protein from host cell, cell culture media and other cellular debris. It is also should not contain isoform that result from post translational modification (Kalyanpur, 2002). This separation step may employ the differences in structural, chemical or functional properties between the desired protein and other protein in mixture. These properties include shape, size, charge, isoelectric point, hydrophobicity, charge distribution, solubility, density, metal binding, ligand-binding affinity, reversible association, posttranslational modifications, and specific sequences or structures. Consequently different chromatography and fractionation step can employ to purified target protein (Kallberg et al., 2012).

To solve purification challenge, in combination with affinity techniques, fusion tags are integrated, to increase expression yields, influence solubility, and native folding. Many fusion tags have been improve for recombinant protein production. Now several affinity tags are available for detection or purification of recombinant protein (Arnau et al., 2006; Waugh, 2005), and other strategies for tag removal (Arnau et al., 2006), and solubility enhancement (Walls and Loughran, 2011).

1.5.1. Protein Tag That Improve Protein Production

Protein purification yield can be improve by increase solubility of the protein or by decrease it, due to the recombinant protein and host that used for the protein expression. Otherwise, this strategy has its disadvantage, including its requirement to removal of the fusion protein. Fusion partner could change proteins native structure and activity. Substantially, parallel analysis of many fusion proteins are required to generate the yields and properties of the protein of interest for the intended procedure.

1.5.1.1. Fusion Protein That Increase Protein Solubility

High protein expression in a host such as *Escherichia coli* result in accumulation of high amount of protein, which may lead to stressful situations for the host cell. This can result in misfolding of the protein in the vivo and forming inclusion bodies by aggregation of highly concentrated protein (Schumann & Ferreira, 2004; Sevastsyanovich et al., 2010). Macromolecular crowding of highly expressed protein may impair correctly folding of the protein, which result in formation of folding intermediate that, when could not processed efficiently by molecular chaperones, promote formation of inclusion bodies (Sørensen and Mortensen, 2005). Fusion partner protein involves the target protein engineering used to direct insoluble protein production. Fusion peptides, which are very stable, are genetically linked with desired proteins to promote their solubility and purification. Here are a few example of fusion partner that increase protein solubility:

1. Maltose-binding protein (MBP): 42 kDa protein encoded by the *mal*E gene of *Escherichia coli* K12 (Duplay and Hofnung, 1988). Protein that fused with MBP purify by single step with affinity to cross-linked amylose (di Guan et al., 1988). Immobilized amylose MBP bound to fusion proteins are eluted under non-denaturing conditions with 10 mM concentration maltose (Kapust and Waugh, 1999). It has been shown that MBP fusion enhance partner protein solubility (Dyson et al., 2004).

2. Thioredoxin A (TrxA): An 11.6 kDa protein that show high solubility in the *E. coli* cytoplasm and inherent thermal stability, which may be conferred to TrxA fusion proteins. It has been used to enhancing expression of recombinant protein with low solubility (LaVallie et al., 1993). Moreover, TrxA has been demonstrated to aid in the crystallization of proteins (Corsini et al., 2008). TrxA requires small affinity tag to conjugated to enable protein purification and it does not facilitate purification on its own.

3. Small ubiquitin-related modifier (SUMO): It is found in native system of *Saccharomyces cerevisiae* Smt3. It is consider as a post translational modification form, which plays roles in apoptosis (Meinecke et al., 2007), nuclear-cytosolic transport (Seeler et al., 2007), response to stress (Mabb et al., 2006), and stability (Henley et al., 2007) , and the cell cycle (Li and Hochstrasser, 1999). Unlike MBP, SUMO needs to establish with His_6 to facilitating purification of fusion protein,

because SUMO itself does serve as a means for protein purification. One advantage of the SUMO tag is a specific SUMO protease (*S. cerevisiae* UlpI) that recognizes and removes the SUMO tag at a Gly–Gly motif (Young et al., 2012).

1.5.1.2. Fusion Protein That Decrease Protein Solubility

A number of protein should be expressed as insoluble form and drive inclusion bodies to protect them from degradation that leads to high expression yields (Lilie et al., 1998). Moreover, protein production that is toxic to the host cell must expressed to inclusion body. This type of fusion partners are used for a few small proteins that easily refolded and that is belong to disadvantage of this method, which is requirement of solubilization of those inclusion bodies by detergent or chaperon molecular after protein purification (Young et al., 2012). Fusion partner that increase protein solubility are:

1. Ketosteroid isomerase (KSI): a 13 kDa protein extremely insoluble, and efficiently forms inclusion bodies. Insoluble expression KSI fusion proteins are generally used for the production of recombinant peptides and used in fusion with purification tags. Utilization of KSI as a carrier protein has increased protein production levels by two- to five-fold comparing to other methods (Kuliopulos and Walsh, 1994).

2. Truncated gene product of *E. coli TrpE*: a 27 kDa protein, useful for direct expression of fusion proteins to inclusion bodies (Yansura et al.,1990).

1.5.1.3. Combinatorial Tags and Facilitating Affinity Purification

Affinity tag is an active and functional method for protein purification, detection and identification. This technique, which also termed affinity chromatography, includes adding short epitope to the target protein by recombinant DNA method. An epitope is a short sequence of amino acid, which acts as antigenic determinant, or serves as a region to which antibody binds. Affinity chromatography is useful in different application such as immunofluorescence, affinity purification, immunoprecipitation, and western blot analysis (Young et al., 2012). In this method, target protein separated by reversible interaction with specific ligand attached to matrix. This interaction can be occur via an antibody, immobilize metal ion or dye substance and by this way offer high selectivity and resolution together with an intermediate-high capacity. Under favorable condition sample is bound to the ligand.

Thereafter the unbound material is washed out of the column and the elution of pure protein is carried out using a competitive ligand or by changing the pH, ionic strength or polarity (GE Healthcare, 2010). Below there are a few affinity tags generally used in microbial systems:

1. Hemaglutinin antigen (HA) and FLAG: HA is one the commonly epitope tags used in cell biology. HA is a sequence of nine amino acids derived from human influenza virus hemaglutinin protein (Field et al., 1988). This epitope may influence trafficking, folding, and function of the target protein (Brothers et al., 2003; Houle et al., 2006). In contrast, the FLAG epitope is a short, hydrophilic amino acid sequence that is useful for antibody-based purification (Hopp et al., 1988). FLAG epitope contain inherent enterokinase cleavage site at C-terminal residues of the peptide sequence, and that give this epitope advantageous, if the recombinant target protein prepared for therapeutic purpose since the epitope itself is immunogenic (Maroux et al., 1971).

2. PolyHis: It is a novel engineered tag, which have been optimized for use in many cell types and for purification under different expression conditions. The polyhistidine affinity tag consists of a changing number of consecutive histidine residues (commonly six) that coordinate, via transition metal ions such as Co^{2+} or Ni^{2+} immobilized on beads, the histidine imidazole ring, or a resin for immobilized-metal affinity chromatography IMAC (Gaberc-Porekar and Menart, 2001; Kimple and Sondek, 2004; Malhotra et al., 2009).

The advantage of this technique for protein purification, which is its small size and charge make it seldom interferes with protein structure and function. Also, it can apply in both denaturing and native condition. Moreover, by imidazole competition or low pH, target proteins can be eluted under mild conditions (Kimple and Sondek, 2004; Li, 2010). Ni(II)-nitrilotriacetic acid (Ni^{2+}-NTA) that developed by Hochuli (Hochuli et al., 1987) show high affinity for histidine residues; provides an inexpensive matrix that resist multiple regeneration cycles under stringent conditions, and allow dissociation of bound recombinant proteins by an imidazole gradient (20 to 250 mM for polyHis-tagged fusion proteins, changes in pH, or metal chelation (Hefti et al., 2001). The disadvantage of using imidazole is its effect on recombinant protein by causing protein aggregation or crystallization and that may interfere with protein activity. So it is important to following protein purification

with dialysis to remove imidazole molecular from protein solution (Hefti et al., 2001).

2. OBJECTIVE AND RATIONALES

Coiled-coil domain containing-124 protein is a new detected protein with a sequence contain coiled coil domain, intrinsically disordered region and RNA binding domain. This proteins function is poorly known but previous studies in TAZEBAY group proved that this protein participates in cell division because they found that CCDC-124 knockout cell show aberrant cell division and cell enlargement that lead to defect in normally cell division. This protein was found in midbody (midzone), p-granules, nucleolus and stress granule which all characterized by being membraneless organelles in the cell. Because of that, in addition to presence of IDRs in protein structure, it is thought that CCDC-124 protein can interact with other protein or nucleic acid molecules to form gel or droplet like structure. This phenomenon belong to new identified statue named liquid-liquid phase separation that occurs in present of proteins with intrinsically disordered region which gives protein structure more flexibility and facilitate free movement and multiple interaction. Also many studies demonstrated that protein with IDRs can phase separate and form gel like structure or fibrous amyloid like assemble in cell that can cause many disease. Thus, researchers became more interested in investigate protein with IDRs and their role in some diseases. Consequently, to obtain more information about CCDC-124 protein and its properties we act to truncate the protein to shorter sequence to facilitate the study of protein properties. This may be supply more information that will be useful to know protein function.

3. MATERIAL AND METHODS

3.1. Bacterial Culture and Buffers

Agaros Gel (1%)

1 g Agaros

100 mL TEA (1X)

0.001% Red safe

TAE Buffer (50X, 1 L)

242 g Tris base

57.1 mL Acetic acid

100 mL EDTA (500 mM, pH 8.0)

LB Broth Medium (1L)

10 g Bacto-tryptone

5 g Yeast extract

10 g NaCl

LB Agar Medium (1L)

10 g Bacto-tryptone

5 g Yeast extract

10 g NaCl

15 g Agar

Inoue Transformation Buffer (300 mL)

55 mM $MnCl_2.2H_2O$

I5 mM $CaCl_2.2H_2O$

250 mM KCl

10 mM PIPES (pH 6.7)

300 mL Pure H_2O

Commassie Blue Destaining Solution

5% Acetic acid

50% Ethanol

Lysis Buffer (100 mL)

50 mM Tris (pH 7.5)

300 mM NaCl

3 mM β-ME

0.5% NP-40

0.5% Triton x-100

Binding Buffer (pH 7.4)

20 mM Sodium phosphate

0.5 M NaCl

30 mM Imidazole

Elution Buffer (pH 7.4)

20 mM Sodium phosphate

0.5 M NaCl

0.5 M Imidazole

PMSF (100 mM)

17.4 mg PMSF

1 mL Isopropanol

10X TBS (pH 8.0)

200 mM Tris

1.5 mM NaCl

10X Wet transfer buffer

250 mM Tris

1.92 mM Glycine

PIPES Piperazine-N,N'-bis (2-ethanesulfonic acid) (0.5 mM, 20 mL)

3 g PIPES

20 mL H_2O

Coomassie Blue Staining Solution

0.2% Coomassie Blue

7.5% Acetic Acid

50% Ethanol

Blocking buffer

5% Milk powder

1X TBS

0.1% Tween 20

Primary antibody buffer (1:1000)

10 µL Primary antibody

10 mL TBS-T

Running buffer for SDS

1X Wet transfer buffer

0.4 % SDS

Transfer buffer

1X Wet transfer buffer

20% Methanol

HEPES high salt buffer (25 mM/L, pH 7.5)

25 mM HEPES (pH 7.5)

500 mM NaCl

1 mM DTT

HEPES low salt buffer

25 mM HEPES (pH 7.5)

1 mM DTT

Secondary antibody buffer (1:5000)

2 µL Secondary antibody

10 µL TBS (without tween)

Table 3.1. SDS running gel contents.

Solution	**12%**	**18%**
Tris-HCl buffer (pH 8.8)	1250 µL	1250 µL
Acrylamide (30%)	1500 µL	2250 µL
dH2O	2175 µL	1425 µL
SDS (25%)	20 µL	20 µL
APS (10%)	50 µL	50 µL
TEMED	5 µL	5 µL

Table 3.2. SDS separating gel contents.

Solution	**4%**
Tris-HCl (pH 6.8)	1250 µL
Acrylamide	500 µL
dH_2O	3175 µL
SDS (25%)	20 µL
APS (10%)	50 µL
TEMED	5 µL

Table 3.3. Used material and supplied company.

Material	**Supplied Company**	**Catalog number**
2-Mercaptoethanol	Sigma	M6250
3X SDS Sample Buffer	Biolabs	B7705S
6X Loading dye	Fermentas (Thermo)	R0611
Acrylamide/bis-Acrylamide Solution 30%	Sigma	A3699
Agaros	invitrogen	16500
APS	Sigma	A3678
Bacto agar	BD	214010
Bacto yeast extract	BD	212750
BamHI	Fermentas	ER0051
Bato-peptone	BD	211677
Calcium Chloride Dihydrate	Sigma	12636
Cut smart buffer	Bio lab	B7204S
di-sodium hydrogen phosphate dihydrate	Merck	106580
DMSO	Merck	1029521011
EDTA	Gibco	15575
Ezview Red Anti-HA Affinity Gel	Sigma	E6779
HEPES	Sigma	H3375
HindIII	Fermentas	ER0501
Imidazole	Sigma	12399
IPTG	MBI	R0392

Table 3.3. Continue

Material	Supplied Company	Catalog number
Manganese(II) Chloride Dihydrate	FINNZYMES	
PMSF	Sigma	P7626
Potassium chloride	Sigma	12636
Protein marker	Thermo	26616
SDS	Sigma	L5750
Skim Milk powder	Sigma	70166
Sodium chloride	Sigma	31434
Sodium dihydrogen phosphate dihydrate	Merck	106345
T4 DNA ligase	Bio lab	M0202S
T4 DNA Ligase Buffer	Bio lab	B0202S
Taq DNA polymerase	Fermentas	EP0402
Tergitol (NP40)	Sigma	NP40S
Triton-X	Sigma	T8787
Trizma base	Sigma	T1503
Tween 20	Sigma	P1379
Water, Sterile (Nuclease free)	Fisher Scientific	BP2484

3.2. Primer Design

In the way to divide CCDC-124 gene to four different fragment, three forward and three reverse primer were designed (Table 3.4). 672 bp long CCDC-124 gene were cut at 390 nucleotide once, to obtain 390 bp and 282 bp long fragments. At the second time, the gene was cut at 141 nucleotide to obtain 141 bp and 531 bp long fragment. Integrated DNA Technology (IDT) web site is used for primer design. The primers were supplied by Invitrogen.

Restriction sites were added to primers while they are designing. *Bam*H Ⅰ (GGATCC) at start site of every sequence and *Hind* Ⅲ (AAGCTT) at the end of each sequence.

Table 3.4. Forward and reverse designed primers for gene fragments.

Gene	Primer	Primer nucleotide sequence
(A) 390 bp fragment	Forward	TCAGGATCCTATGCCCAAGAAGTTCCAGG
	Reverse	GATAAGCTTTCACTCCTCCAGCGGCACCTCC
(B) 282 bp fragment	Forward	CTTGGATCCTATGAACGTGAACCGCCGCGT
	Reverse	GAGAAGCTTTCACTTGGGGGCATTGAAGG
(C) 141 bp fragment	Forward	TCAGGATCCTATGCCCAAGAAGTTCCAGG
	Reverse	GAGAAGCTTTCAGACGTGTTTGTCGTCGTC
(D) 531 bp fragment	Forward	GTTGGATCCTATGAGGAAGGAGCAGCGCAAG
	Reverse	GAGAAGCTTTCACTTGGGGGCATTGAAGG

3.3. Polymerase Chain Reaction (PCR)

For PCR 20 ng plasmid, 0.5 µL foreword primer, 0.5 µL reverse primer, 0.2 µL dNTP, 1.25 unite *Taq* polymerase, 1X Taq buffer, as a final concentration, were used and the total volume completed to 50 µL. The reaction was completed using the TProfessional TRIO thermocycle (Biometra Product line) with the following sitting (Table 3.5).

Table 3.5. PCR cycle.

Steps	Period	Temperature
First step	2 minute	94 °C
Second step	30 second	94 °C
Third step	60 second	54 °C
Forth step	60 second	72 °C
Fifth step	7 minute	72 °C

The second, third and fourth steps were repeated 30 times.

Agarose gel electrophoresis technique was used to examine the result of the PCR. To preparing 1% gel agarose, 0.4 g agarose was dissolved in 40 mL TEA buffer by heating the solution to 90 °C until agarose dissolve completely. Let the solution got colder, then 4 µL red safe was added.

After examination of the PCR result, PCR clean up protocol was applied by using NucleoSpin kit by MACHEREY-NAGEL. Nanodrop Lite (Thermo scientific) was used to measure the concentration of DNA molecular.

3.4. Plasmid Cloning

pQE-81L plasmid (Figure 3.1) was used for plasmid cloning that would use in transferring gene fragments to *E. coli* competent cell. pQE-81L plasmid is 4753 bp in long, and has 6x-His taq that make it suitable for protein purification. In addition, this plasmid has ampicillin resistant gene and open reading frame that can occupied our gene fragments. So ampicillin was used as antibiotic in bacterial culture.

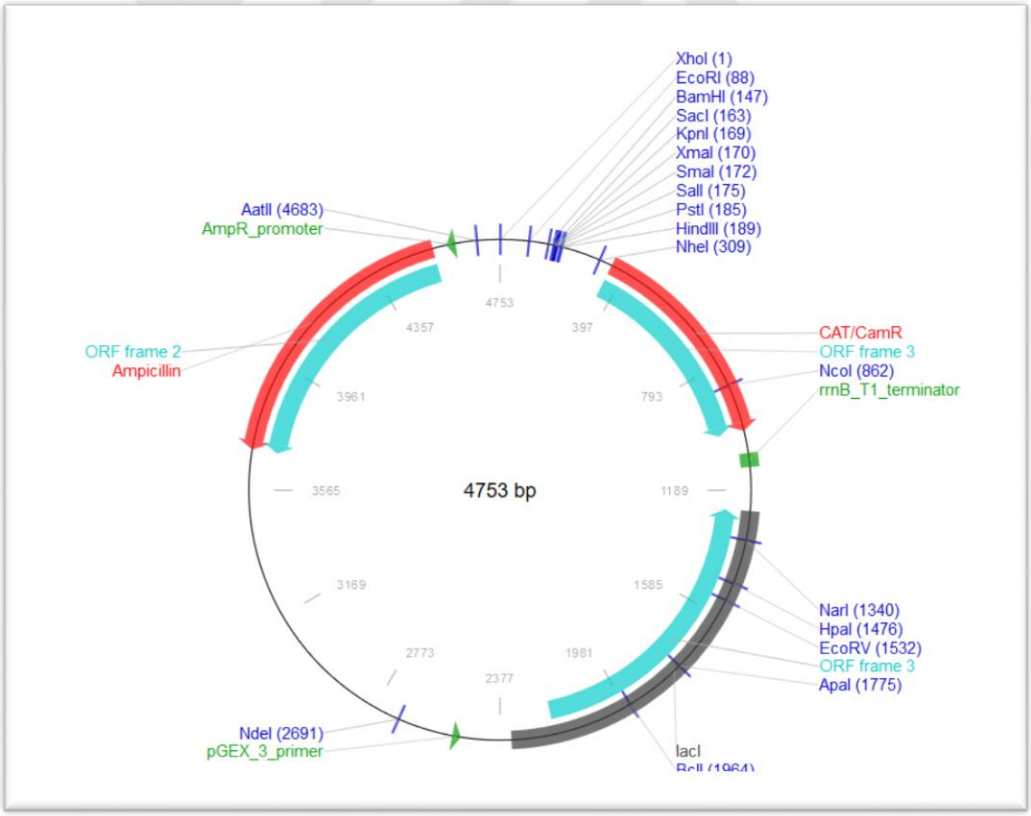

Figure 3.1. pQE-81L plasmid map.

3.4.1. Plasmid and Gene Fragments Digestion

For plasmid cloning first step was plasmid digestion. *Bam*HⅠ and *Hind*Ⅲ restriction enzymes, which their restriction site added to designed primers, were used to digest both amplified genes and plasmid. For gene digestion, 2 ug of amplified gene, 40 unit from each enzyme and 1X buffer, was mixed and total volume was completed to 30 µL. For plasmid digestion, 4µg plasmid, 40 unit from each enzyme and 1X buffer are mixed and total volume was completed to 80 µL. Then each mixture incubate at 37 °C for one hour.

3.4.2. Gel Extraction

The digested plasmid and genes were migrated in agarose gel and desired bands were cut and transferred to micro tube. For gel extraction (PCR clean up and gel extraction Nucleospin) protocol was applied. Obtained plasmid and genes concentration were measured by Nanodrop.

3.4.3. Ligation

The last step of plasmid cloning was ligation. To insert the gene fragments to digested plasmid, 1 unit T4 ligase enzyme, 1X smart cut buffer, 100 ng plasmid (vector), and 1:5 insert (gene fragment) were mixed and incubate at 16 °C for overnight (16-18 hour).

3.5. Transformation

3.5.1 Preparing of Competent Cell

E. coli DH5-alpha strain was used as competent cell for plasmid cloning. This strain of bacteria is more efficient for transformation. DH5-α has point mutation at recA1 gene that disable the activity of the recombinases and inactivate homologous recombination. The second mutation in these bacteria is at endA1 gene, which inactivate an intracellular endonuclease, to prevent degrading of the inserted plasmid. 3 µL from stock *E. coli* DH5-α was inoculated in 3 mL LB broth and incubated at 37 °C for overnight (16-18 hour). Next day 2-3 mL of overnight culture was inoculated in 250 mL LB broth and incubated at 37 °C with shacking for 6-8 hour until OD_{600} reached 0.5-0.6. When OD_{600} reached 0.55 the culture was centrifuged at 4000 rpm for 10 minute at 4 °C. Supernatant was discarded and obtained pellet was

resuspended by 80 mL of previously prepared inoue buffer. When the pellet was completely dissolve in the buffer, it was centrifuged at 4000 rpm for 30 minute at 4 °C. The supernatant was discarded and the pellet resuspended by 20 mL inoue buffer then 0.6 mL glycerol was added and incubated in the ice for 10 minute. The mixture was aliquoted quickly and store at -80 °C.

The same protocol was followed for preparing *E. coli* BL21 strain, which is further, used for protein purification.

3.5.2. Transferring the Cloned Plasmid to Competent Cell

For transfer the cloned plasmids to competent cell, 100 µL of *E. coli* DH5-α competent cell culture was mixed with 6.5 µL of cloned plasmid and incubate in ice for 1 hour then incubate at 42 °C for 2 minute, to introduce heat shock to the cells. They were put in ice for 3 minutes then 800 µL of LB broth added and incubated for 1 hour at 37 °C. After the incubation, they were centrifuged at 10000 rpm for 5 minute at 4 °C then the supernatant was discarded and the pellet was resuspended by small amount of LB broth that remained in the tube. Spread the resuspended pellet on plate that contain LB agar and ampicillin (100 µg/mL). The plates were incubated overnight at 37 °C. This transformation protocol was applied for all gene fragments. For positive control competent cell which previously cloned and tested was used. Competent cell without plasmid, and competent cell with plasmid that does not contain insert were used for negative and ligation, respectively.

3.6. High Copy Plasmid Isolation

High copy plasmid isolation was performed to obtain a large amount of plasmid with high concentration that will further use for sequencing and transformation. One colony from every plate were taken and inoculated in 5 mL LB broth with ampicillin. The culture were incubated at 37 °C for overnight with shaking. Next day the cultures were taken and centrifuged at 4000 rpm for 20 minute at 4 °C. The supernatant was discarded and the pellet was resuspended by A1 regent of mini-PREP kit. Then the next steps of Plasmid and DNA Purification Nucleospin kit (MACHERY-NAGEL) were followed.

Obtained plasmids' concentrations were measured by nanodrop. Then the plasmids were digested by *Bam*H Ⅰ and *Hind*Ⅲ digestion enzyme and migrated in gel electrophoresis to ensure that the gene fragments were inserted in the plasmid.

The cloned plasmids with 100 ng concentration were sent to (IONTEK) for DNA sequencing.

3.7. His-Tagged Protein Purification

3.7.1. Transformation

Second transformation was applied, to transfer isolated plasmids, to previously prepared *E. coli* BL-21 competent cell. The same protocol of the first transformation was relied on in the second transformation. BL-21 strain of *E. coli* is more suitable for high-level gene expression and protein purification. This belongs to the lacking bacteria of the lon and ompT proteases, which result in promoting stability of recombinant proteins.

For preparing stock of transformed cell, one colony from transformed cell was taken from plate, inoculated in 2 mL LB broth (with 2 µL ampicillin), and incubated at 37 °C for overnight. Then, bacteria were stored in 30% glycerol-LB solution at -80 °C. These steps were applied for every transformed cell. Stocked cell by this way can be used for many years.

3.7.2. IPTG Induction and Protein Purification

4 µL of stocked cells (transformed *E. coli* BL-21 with CCDC-124 gene fragments and full size CCDC-124 gene) were separately inoculated in 2 mL LB broth with ampicillin and incubated at 37 °C for overnight.

2 mL from pre-culture was diluted in 200 mL LB broth and incubated at 23 °C until OD_{600} reach 0.6. Sample was taken for SDS PAGE examination as uninduced protein sample. 100 µM IPTG as final concentration was added and incubated overnight. Next day the culture were centrifuged at 4000 rpm for 20 minute at 4 °C, then the pellet was resuspended by 10 mL lysis buffer and 100 µM of PMSF as a final concentration was added to inactivate the protease of the bacteria. Sample was sonicated at 50% amplitude for 150 second (50 sec pulse on, 20 sec pulse off). After sonication the sample was centrifuged at 4000 rpm for 20 minute at 4 °C.

Sonication acts to disrupt cellular membrane and releases content of the cell. The sample was connected to AKTA FPLC for protein purification and His-tag column was used. This type of protein purification base on the affinity of the hexa histidine residue that fused with recombinant protein, to the Ni^{2+} immobilize metal

ion in his-tag column (Spriestersbach, Kubicek, Schäfer, Block, & Maertens, 2015). After injection of the sample, it was passed through his-tag column and the his-tag proteins were bound to Ni^{2+} that present in the column. The second step was washing the column with binding buffer and that was to elute unbinding contaminating protein. Last step was washing the column with elution buffer to elute his-tag protein. Samples were collected in 500 µL fractions. The fractions, at which the systems software show a peak, were collected and were undergo to SDS PAGE analysis and protein dialysis.

For SDS PAGE analysis, protein samples were mixed with 3X loading buffer and DTT 100 µM as a final concentration. For protein denaturation, the mixture was heated to 95 °C for 5 minute. Then the samples were loaded in the SDS gel. Protein were migrated at 90 V until the sample reach running gel after that the voltage was increased to 120V and the migration continued for about 90 minute. Thus the gel was ready for Coomessie Blue staining or western blot analysis.

3.7.3. Protein Dialysis

Dialysis cassette was used for protein dialysis. 3 mL of sample was loaded in the cassette and immersed in dialysis buffer for overnight at 4 °C with gentle agitate. In dialysis semipermeable membrane are used to separate small molecular such as salt and imidazole molecular from the protein (large molecular). The molecular migrate from high concentration buffer to low concentration buffer. Thus higher concentration of protein in solution are obtained (Andrew et al., 2001).

Protein concentration was measured after protein purification. For this purpose Qubit assay was performed. Qubit working solution was prepared by mixing 199 µL of Qubit buffer with 1µL Qubit reagent. Then 2 µL of diluted protein was added to working solution. Final mixture was incubate at room temperature for 15 minute in the darkness. Thus the samples were ready to read by Qubit Fluorometer.

3.8. Western Blot

Designed proteins were analyzed by western blot with using antibody specific to CCDC-124 protein. After migration the protein in the SDS gel, the proteins were transferred to PVDF membrane. For transferring, transfer sandwich was prepared. The layers of the sandwich were like the follow: thick sheet, thin sheet, PVDF membrane, SDS gel, thin sheet and thick sheet. All sandwich sheets were immersed

in transfer buffer. PVDF membrane was immersed in methanol then water and finally transfer buffer. Transfer sandwich was put in western transfer instrument set on 25 voltage and run for 45 minute. Membrane was wash with blocking solution for one hour at room temperature. In the next step, blocking solution was poured and the membrane was immersed in the primary solution. Membrane in the primary solution was agitated at 4 °C for overnight. Then the membrane was washed with TBS-T three times for 10 minute. Secondary antibody was added and agitated for one hour at room temperature. Then after washing the membrane three times with TBS-T for 10 minute, the surface of the membrane was covered with western-femto and taked for chemi-doc analysis. The protocol was repeated several time with using different antibody all specific to CCDC-124 protein, but they different in epitope that specify to on CCDC-124 amino acid sequence.

3.9. Induction of Phase Separation Of Truncated Protein And Full Size CCDC-124 Protein

Several protocols were applied to induce phase separation of full size protein and truncated protein.

3.9.1. Induction by Temperature

Concentrated proteins were incubated at 4 °C once and -20 °C at the second time, for overnight. Proteins were taken to room temperature and wait for 5 minute, then investigate the turbidity of the proteins in the solution with necked eyes.

3.9.2. Turbidity Assay

Temperature of concentrated protein was brought to room temperature (23 °C), then located in spectrophotometer block. The temperature of the spectrophotometer (Shimadzu) block was decreased gradually and OD_{600} was measured every 0.5 °C.

3.9.3. Induction of LLPS By Decreasing The Ionic Strength

Concentrated protein was dissolved in 30 µL high salt buffer (HS HEPES buffer). Then add 70 µL low salt buffer (LS HEPES buffer) to decrease salt (NaCl) concentration from 500 to 150 mM. This will induce proteins that have ability to phase separate to assemble and form droplet (Wang et al., 2019). 10 µL from the mixture was taken and put on a clean slide to investigate by bright field microscope at 40X (Wang et al., 2019).

4. RESULTS

4.1. PCR

By using designed primer and plasmid that harbor CCDC-124 gene as a template, PCR is applied to obtain 4 different fragments (Table 4.1).

Table 4.1. Show the fragments that obtained and amplified by PCR.

Fragments	Nucleotide sequence	nt long	nt site
A fragment	ATGCCCAAGAAGTTCCAGGGTGAG AACACCAAGTCGGCAGCGGCCCGG GCACGGAGGGCAGAGGCCAAGGCG GCCGCTGATGCCAAGAAGCAGAAG GAGCTGGAGGATGCCTACTGGAAG GACGACGACAAACACGTCATGAGG AAGGAGCAGCGCAAGGAGGAGAAG GAGAAGCGGCGCCTCGACCAGCTG GAACGTAAGAAGGAGACGCAGCGC CTACTGGAGGAGGAGGACTCCAAG CTCAAGGGCGGCAAGGCGCCGCGG GTGGCCACGTCCAGCAAGGTCACC CGGGCCCAGATCGAGGACACGCTG CGCCGAGACCATCAGCTCAGGGAG GCCCCGGACACAGCCGAGAAAGCC AAGAGCCATCTGGAGGTGCCGCTG GAGGAG	390 bp	1-390 bp
B fragment	AACGTGAACCGCCGCGTGCTGGAG GAGGGCAGCGTGGAGGCGCGCACC ATCGAGGACGCCATTGCAGTGCTCA GCGTGGCGGAGGAGGCGGCCGACC GGCACCCAGAAAGACGCATGCGGG CAGCCTTCACAGCCTTTGAGGAAGC CCAGCTGCCGCGGCTCAAACAAGAG AACCCCAACATGCGGCTGTCGCAGC TGAAACAGCTGCTCAAGAAGGAGTG GCTCCGCTCTCCTGACAACCCCATGA ACCAGCGGGCCGTGCCCTTCAATGCC CCCAAGTGA	282 bp	131-672 bp

Table 4.1. continue

Fragments	Nucleotide sequence	nt long	nt site
C fragment	ATGCCCAAGAAGTTCCAGGGTGAGAA CACCAAGTCGGCAGCGGCCCGGGCAC GGAGGGCAGAGGCCAAGGCGGCCGCT GATGCCAAGAAGCAGAAGGAGCTGGA GGATGCCTACTGGAAGGACGACGACA A ACACGTCATG	141 bp	1-141 bp
D fragment	AGGAAGGAGCAGCGCAAGGAGGAGA AGGAGAAGCGGCGCCTCGACCAGCTG GAACGTAAGAAGGAGACGCAGCGCCT ACTGGAGGAGGAGGACTCCAAGCTCA AGGGCGGCAAGGCGCCGCGGGTGGCC ACGTCCAGCAAGGTCACCCGGGCCCA GATCGAGGACACGCTGCGCCGAGACC ATCAGCTCAGGGAGGCCCCGGACACA GCCGAGAAAGCCAAGAGCCATCTGGA GGTGCCGCTGGAGGAGAACGTGAACC GCCGCGTGCTGGAGGAGGGCAGCGTG GAGGCGCGCACCATCGAGGACGCCAT TGCAGTGCTCAGCGTGGCGGAGGAGG CGGCCGACCGGCACCCAGAAAGACGC ATGCGGGCAGCCTTCACAGCCTTTGAG GAAGCCCAGCTGCCGCGGCTCAAACA AGAGAACCCCAACATGCGGCTGTCGC AGCTGAAACAGCTGCTCAAGAAGGAG TGGCTCCGCTCTCCTGACAACCCCATG AACCAGCGGGCCGGCCCTTCAATGCC CCCAAGTGA	578 bp	142-67 bp

In the further analysis of the DNA fragments with gel electrophoresis, designed DNA fragments with expected size were observed (Figure 4.1).

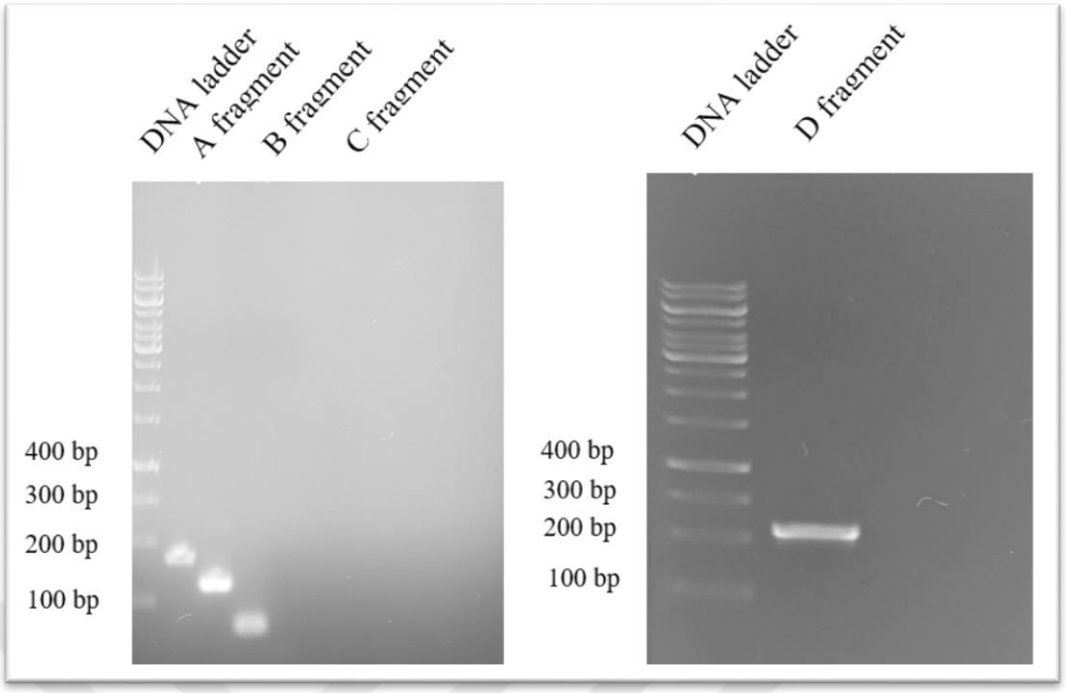

Figure 4.1. DNA fragments result from PCR.

4.2. Plasmid Cloning and Transformation

The result of DNA sequencing of designed DNA fragments was analyzed by CLUSTAL OMEGA Sequence Alignment tool on EMBL-EBI web site. This tool was useful to compare sequence of designed fragments with the origin sequence at CCDC-124 gene. CLUSTAL OMEGA Sequence Alignment tool is generates alignment between two or more sequence. This program based on HMM profile-profile techniques and seeded guide trees.

The second analysis with FinchTV computer software show that the designed fragments have been inserted in open reading frame of the plasmid, just after His-Tag sequence with present of AC nucleotide as linker between them (Figure 4.2). The present of His-Tag will be beneficial for protein purification.

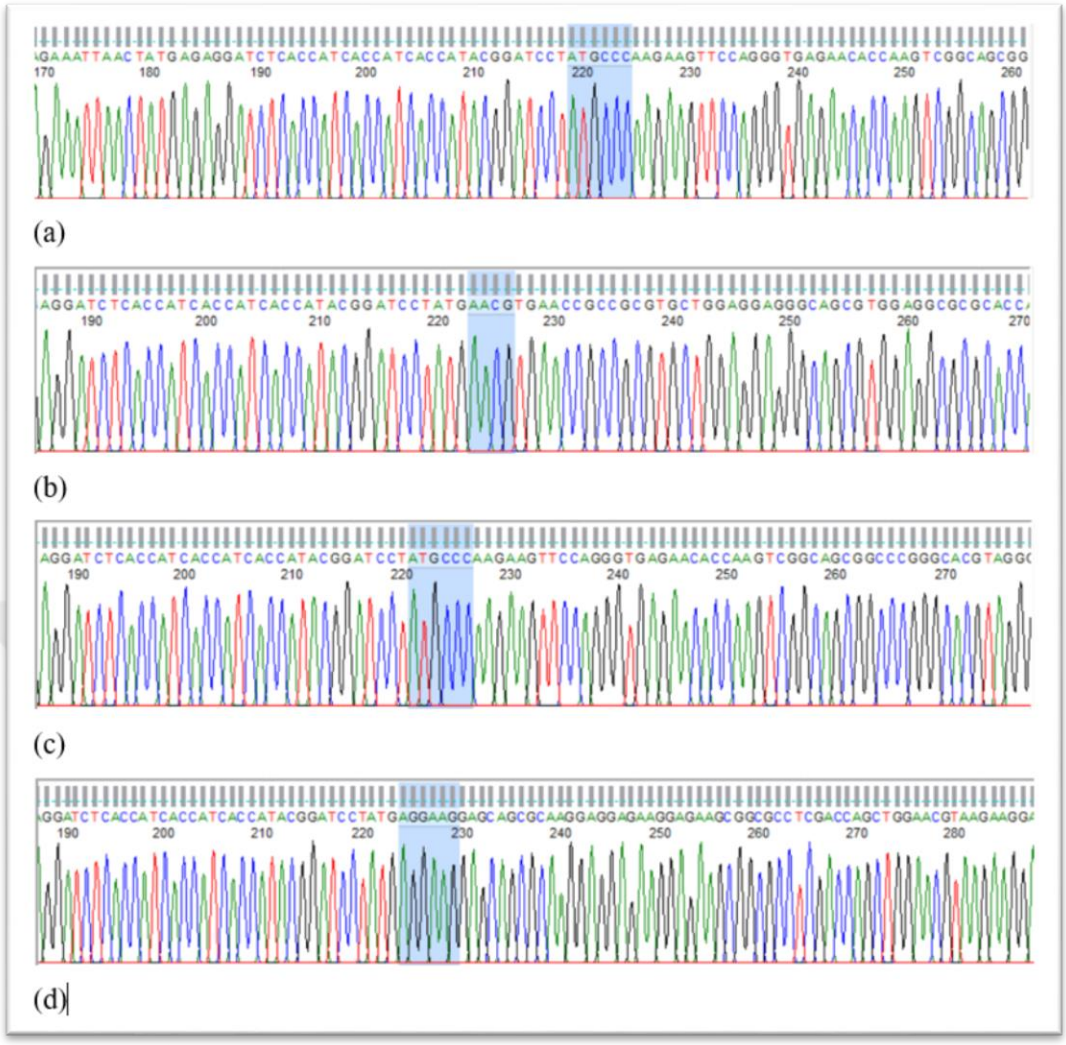

Figure 4.2. Sequencing result of the protein fragments transformation. It was read by FinchTV program. **(a)** A Fragment, **(b)** B fragment, **(c)** C fragment, **(d)** D fragment. This sequence explain his-tag sequence and starting site (blue marking) of specific DNA sequence for every fragments.

4.3. Protein Expression and Protein Purification

4.3.1. Sequences of Purified Protein

Purified proteins of full size CCDC-124, N-terminal A fragment, C-terminal B fragment, N-terminal C fragment, and C-terminal D fragment were sequenced (Table 4.2).

Table 4.2. Amino acid sequence of the full size and fragmented CCDC-124 gene.

Proteins	**Amino acid sequence**	**Amino acid site**	**Sequence long**	**MW**
CCDC-124 full size	MPKKFQGENTKSAAARARRAE AKAAADAKKQKELEDAYWKD DDKHVMRKEQRKEEKEKRRL DQLERKKETQRLLEEEDSKLK GGKAPRVATSSKVTRAQIEDTL RRDHQLREAPDTAEKAKSHLE VPLEENVNRRVLEEGSVEARTI EDAIAVLSVAEEAADRHPERR MRAAFTAFEEAQLPRLKQENP NMRLSQLKQLLKKEWLRSPDN PMNQRAVHFNAPK	Full size	223 amino acid	32 kDa
A fragment	MPKKFQGENTKSAAARARRAE AKAAADAKKQKELEDAYWKD DDKHVMRKEQRKEEKEKRRL DQLERKKETQRLLEEEDSKLK GGKAPRVATSSKVTRAQIEDTL RRDHQLREAPDTAEKAKSHLE VPLEE	1-130 amino acid	130 amino acid	17 kDa
B fragment	NVNRRVLEEGSVEARTIEDA IAVLSVAEEAADRHPERRMRA AFTAFEEAQLPRLKQENPNMR LSQLKQLLKKEWLRSPDNPMN QRAVHFNAPK	131-223 amino acid	93 amino acid	10 kDa
C fragment	MPKKFQGENTKSAAARARRAE AKAAADAKKQKELEDAYWKD DDKHVM	1-47 amino acid	47 amino acid	5 kDa
D fragment	RKEQRKEEKEKRRLDQL KAPRVATSSKVTRAQIEDTLRR DHQLREAPDTAEKAKSHLEVPL EENVNRRVLEEGSVEARTIEDA IAVLSVAEEAADRHPERRMRA AFTAFEEAQLPRLKQENPNMR LSQLKQLLKKEWLRSPDNPMN QRAVHFNAPK	48-223 amino acid	175 amino acid	20 kDa

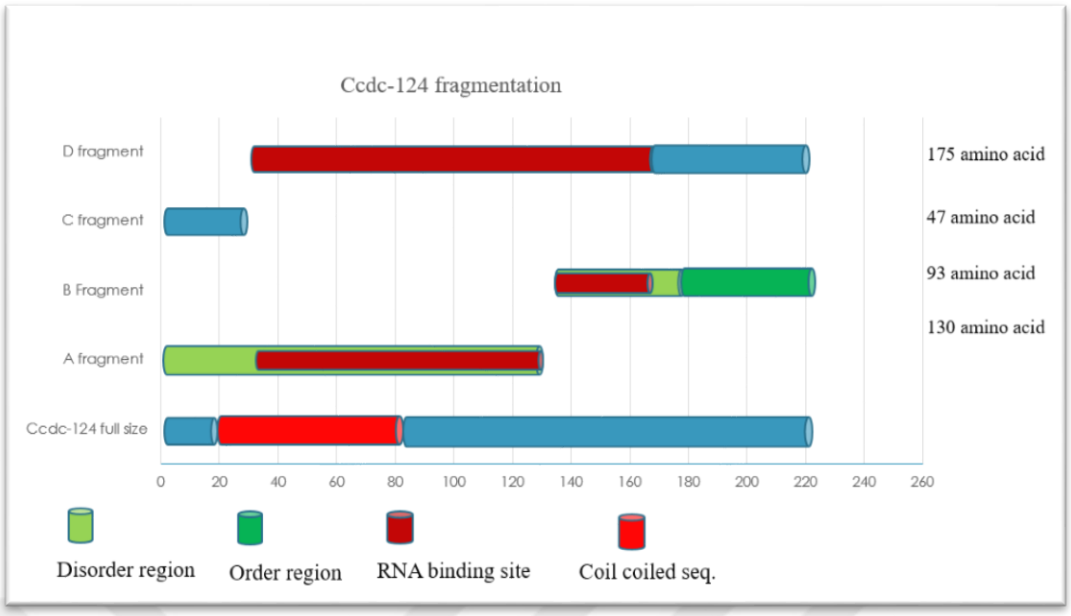

Figure 4.3. Designed protein fragments and its sequence properties.

4.3.2. SDS Result of the Expressed and Purified Protein

A and D fragments with about 15 kDa and 20 kDa, respectively consequently were clearly observed in the SDS gel stained with Coomassie Blue (Figure 4.4).

B and C fragment with about 10 and 5 kDa, respectively (molecular weight of the designed proteins were calculated by online tool at www.bioinformatics.org) could not identified in SDS gel stained by Commessie Blue.

Other methods were attempted to observe B and C fragments. Silver nitrate dye was used instead of the Coomassie Blue to stain SDS gel. Silver dye in more sensitive and beneficial for staining small proteins with low molecular weight and protein with low concentration. Also more concentrated SDS separating gel (with 18% acrylamide) was used. Protein expression was repeated with different condition. The sonication (10% amplitude, 2:30 total time, 10 pulse on, 30 pulse off) was applied to lysis the cell. That was to avoid protein degradation. Also sample was taken at different time during protein expression. At 3rd, 5th and 7th hour of protein expression and after IPTG induction a sample was taken and analyzed with SDS PAGE. Nevertheless, in all attempts B and C fragments were not observed in the SDS analysis. So we continued further analysis and assay on successfully purified protein only (CCDC-124, A and D fragments).

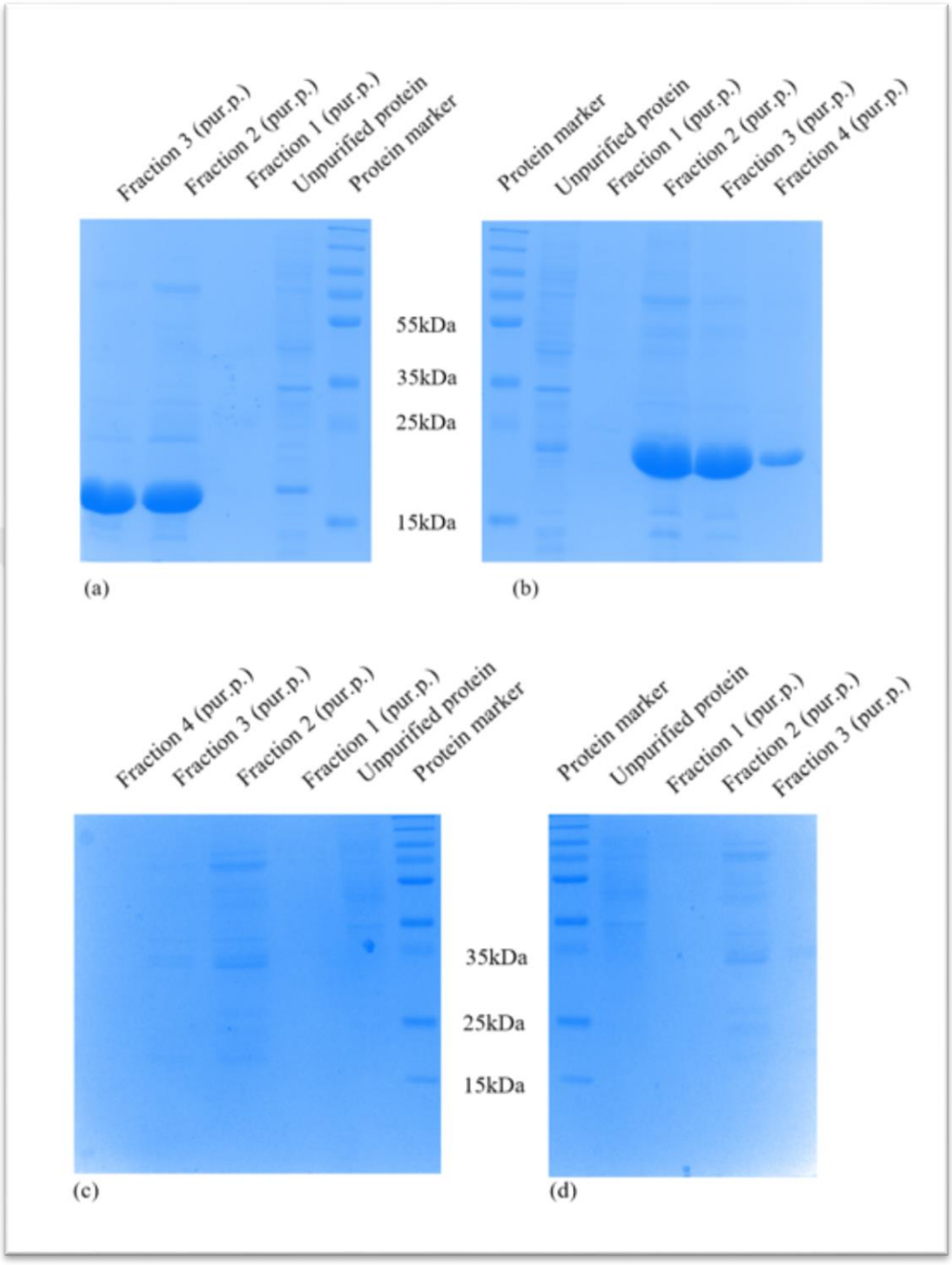

Figure 4.4. SDS analysis of over expressed and purified protein. **(a)** A fragment **(b)** D fragment, **(c)** C fragment, **(d)** D fragment.

4.3.3. Protein Dialysis and Qubit Assay

The proteins were dialyzed after the purification. Then the concentration was measured by Qubit assay. The concentration of the proteins were 100 µM for CCDC-124, 500 µM for A fragment and 250 µM for D fragment.

4.3.4. Western Blot Result for Full Size and Truncated Protein

Western blot analysis with using different Ccdc124 antibody have been done to show full size and truncated CCDC-124 protein (Figure 4.5). All these antibodies are specific to CCDC-124 protein, but differs in epitope that they bind on the CCDC-124 amino acid sequence.

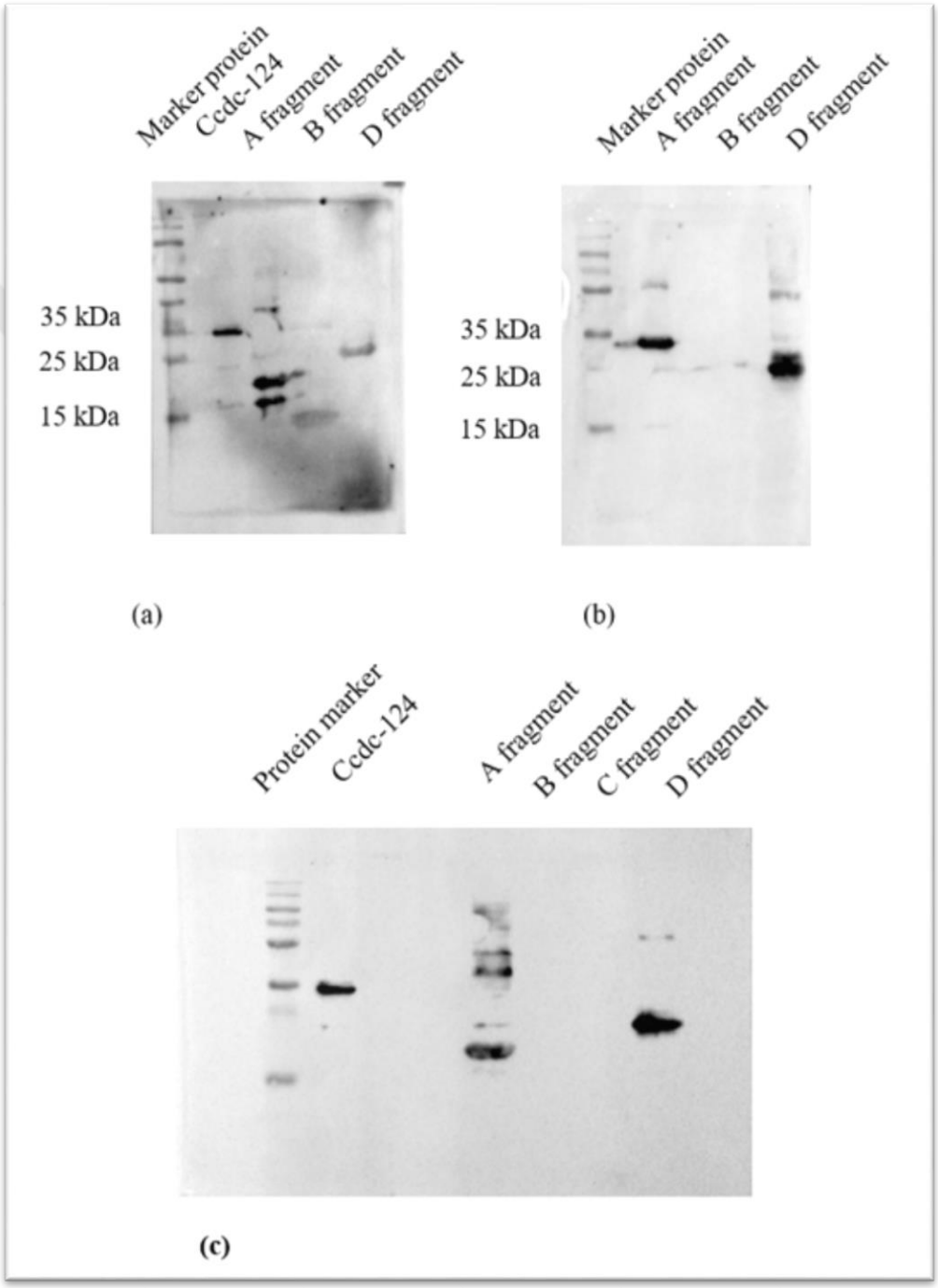

Figure 4.5. Western blote analysis of full size and truncated CCDC-124 proein with different antibody. **(a)** show the protein that bound to N-terminal antibody, **(b**) show the proteins that bound to C-terminal antibody, **(c)** show the proteins that bind to an antibody specific to middle epitobe of the CCDC-124.

Antibody that specific to epitope at C-terminal, bound to CCDC-124 full size and D fragment. Antibody that specific to epitope at N-terminal bound to purified full size CCDC-124 and A fragment. Third antibody that specified to epitope at the middle part of the amino acid sequence of the protein bound to CCDC-124 full size, A fragment and D fragment purified protein. B and C fragment was also analysis with western blot, but no bond has been observed (Figure 4.5).

4.4. Induction of Phase Separation

There was several attempts to induce phase separation of purified protein.

4.4.1. Turbidity Assay

In this endeavor to induce phase separation, there was no change in the turbidity of the purified protein for full size CCDC-124 and other fragments. The turbidity at OD_{600} remained stable among all temperatures degree (from 24 °C to 4 °C) (Figure 4.6).

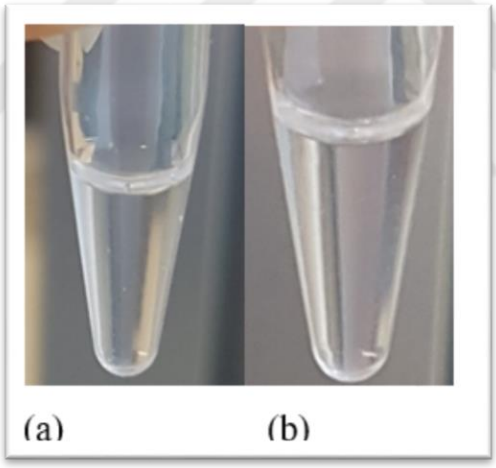

Figure 4.6. Observation the turbidity of the protein solution. **(a)** CCDC-124 protein solution at -20 °C. **(b)** CCDC-124 protein solution at room temperature.

4.4.2. Induction by Change the Temperature

Purified protein was taken to room temperature after incubated overnight at -20 and observed by necked eyes. There was no change in the cloudiness of the solution. The same result observed for all purified protein, which incubated at 4 °C for overnight.

4.4.3. Induction of Phase Separation by Changing Ionic Concentration

10 µL of solution, that contain mixture of high salt HEPES buffer, low salt HEPES buffer and purified protein, was taken and located on the slide. The solution

was examined by light field microscope (Zeiss LSM 880). Droplet that form as result of protein phase separation, expected to found. HEPES buffer was also examined as negative control. There was no droplet in any purified protein (Figure 4.7).

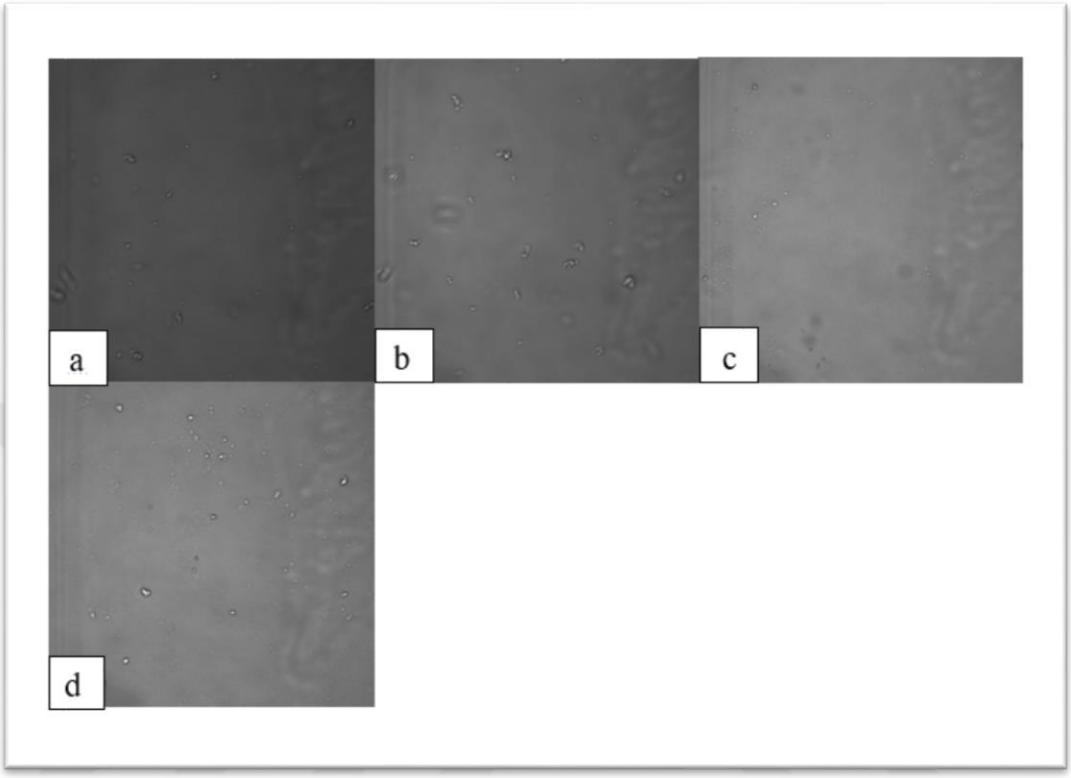

Figure 4.7. Drops of purified proteins in HEPES buffer under bright field microscope 40X. **(a)** A fragment, **(b)** D fragment, **(c)** CCDC-124 fragment **(d)** HEPES buffer

5. DISCUSSION

5.1. Protein Fragmentation and Purification

Throughout this project DNA sequence of the CCDC-124 gene was fragmented to four different sequence by cutting the DNA strand of the gene, two times from different site. Cutting sites were chosen due to the IDRs region in the amino acid sequence of the protein and RNA binding site. Those fragments have been successfully cloned in pQE-81L by using *Bam*HⅠ and *Hind*Ⅲ to digest each plasmid and designed DNA fragments. That was proved by analysis the fragments with DNA sequencing technique. Obtained fragments sequence were read by computers program and were compared with desired sequence by using online alignment tool at EMBL-EBI web site. That was to be ensure about currency of the sequence and its site at open reading frame of the plasmid. For the purpose of expression designed DNA sequences, cloned plasmids were transferred to *E. coli* BL-21 strain. This bacterium was chosen because of its suitability, which make it proper for protein purification.

By IPTG induction protocol, protein production level in transformed bacterial cell was increased and high protein concentration was obtained. These proteins were purified from other undesired protein and cells debris, with employing His-tag properties of produced proteins. Purified proteins were dialyzed to remove large chemical molecular such as imidazole from their solution. The protein concentration was measured by Quibt assay. Thus, the proteins were became ready for further test.

Consequentially, by Protein fragmentation we could obtain smaller proteins which carrying a sequence of amino acid that we mostly interested in its properties.

5.2. Examination of the Obtained Proteins and Induction of Phase Separation

Purified proteins had been undergo different experiments to observed their properties. At first they were examined by SDS PAGE. This clarified the result of protein purification and which fractions of purified sample had more concentration of the protein. This also aimed to get an idea about molecular weight of design ned protein which was nearby to the molecular weight that was determined by the web site (www.bioinformatics.org). CCDC-124, A fragment and D fragment was successfully purified, but B and C fragment was not observed neither on SDS gel nor

in other experiments that done after that. This may be belong to their small size and low molecular weight that they own which lead to degradation of the proteins rapidly and easily or it is not possible to form a protein with their sequences. The second experiment that performed to the successfully purified proteins was western blot. Three different antibody were used in western blot. One of them specified to C-terminal, which bound to D fragment and full size protein CCDC-124. Second one was specified to N-terminal and bound to A fragment and CCDC-124. While the third one, which is specified to middle epitope bound to all of them. After ensure about the currency of protein that obtained, a set of experiments were applied to induce and observe the phase separation. For this purpose, turbidity assay was performed. Temperature of proteins was decreased from 24 °C to 4 °C and OD_{600} was measured at each 0.5 °C. OD_{600} was stable at all temperature degrees.

At our second attempt to induce phase separation, the proteins were incubated at 4 °C once and at -20 °C second, for overnight and then bring them to room temperature and observed the cloudiness of the protein in solution. There was not any change in the cloudiness of the proteins solution.

The last experiment was induction of protein phase separation by changing ionic concentration of protein solution. Then a drop from protein solution was examined by bright field microscope to observed protein droplet that can form as a result of protein phase separation. No droplet was observed in any protein solution.

In previous study (Thomson et al., 1987) had been done on bovine lens protein γ Ⅱ-crystallin, researcher found that the protein in its solution can be at two phase and that dependent on the temperature and concentration of the protein in its solution. At known concentration, they fixed the temperature at which the solution does not show cloudiness then they measure its cloudiness by light-scattering spectrometer. They lowered the temperature slowly. At a certain degree, protein started to phase separated and that was marked by the disappearance of the transmitted beam due to increase cloudiness of the solution, which could observed visually. Moreover, when the temperature had been raised gradually the solution clarified and the transmitted beam reappeared until reach the same value that was at, before cloudiness had appeared. This result evidence that protein can be phase separated in its solution by changing the temperature.

There is other study (Mason, Enk et al., 2010) on humanized IgG2 monoclonal antibody, aimed to study liquid-liquid phase separation of the antibody in the monovalent salt solutions of KF, KCl, and KSCN under different pH conditions. In this study, the antibody solution was mixed with Milli-Q water and certain amounts of potassium monophosphate and potassium diphosphate stock solutions to achieve ionic strength (22 mM) at the pH conditions of 6.1, 6.6, and 7.1, respectively, and a final antibody concentration was 90 mg/mL. For experiment series ionic strength, desired amount of salt stock solution was added with maintain 90mg/mL concentration. All samples centrifuged at 6000 rpm for 30 minute at between -2 °C and -12 °C. If phase separation had occurred, the top laye r and the bottom layer were sampled. The antibody concentration in the top and the bottom are measured at 280nm by UV-vis spectrometer. It was investigate that the antibody solution was slightly opalescent at more than 70 mg/mL, pH 7.1 (near its isoelectric point "pI") and low temperature (near 0 °C). When decrease solution temperature below 0 °C, the antibody solution became opaque and white, and remained like that at least 6 h if kept statically. The formation of two transparent layers with a protein-poor top layer and a protein-rich bottom layer after centrifugation at -10C was observed for the solution conditions of 22, 42, 62, and 82 mM ionic strength. But after centrifugation at -7C , the phase boundary was not observed for the solution conditions of 62 or 82 mM ionic strength. The solution turned clear and transparent when the temperature was raised to room temperature and the two layers were mixed by gentle inversion.

Liquid-liquid phase separation of the antibody at pH 7.1 occur due to serial suggested events. At pH 7.1 which is near pI the antibody almost charge natural. At this point, the electric double-layer repulsion between the antibody molecules be small and the net DLVO interaction between them should arise mainly from the van der Waals force. Moreover, decease of critical temperature T_c versus ionic strength of three salts assumed that the intermolecular interactions were sensitive to the presence of electrolytes and therefore electrostatic in nature. Below pI, at pH 5.3 and 22mM ionic strength there was no observed of LLPH without addition of KCl, KF, KSCN, and that must related to the electric double-layer repulsion that control antibody-antibody reaction. With the percent of three anions T_c was raised which may be explained by the binding of the anions and neutralization of the protein net charges that decreases the electric double-layer repulsion as described in the DLVO theory.

In the same field and in the way to investigate phase separation phenomenon of proteins and effected factor that introduce phase separation, a research had done on the monoclonal antibody A (Nishi et al., 2010). In this study It was investigated that MAB A can phase separated by ionic strength and pH. Protein-protein interaction in the solution was observed by dynamic light scattering (DLS). In an isotonic ionic strength condition protein solution appeared clear but in low ionic strength condition it turned opalescent followed by liquid-liquid phase separation (LLPS) into light and heavy phases. It also noticed that the two phases became reversibly miscible when the ionic strength or temperature was increased. MAB A phase separation required high concentration, above critical concentration, at low ionic strength condition. The viscosity of the heavy phase was high due to attractive protein-protein interaction in this phase. This result indicated that reversible protein self-association mediated mainly by attractive electrostatic interaction among the MAB A molecules in the heavy phase and was main reason of the LLPS.

A review summarize various protocols for induction of phase separation and many ways to detect phase separation droplet (Wang et al., 2019); This review show that recombinant his-tag protein purified by AKTA purifier after had been over expressed in host cell (*E. coli*) by IPTG induction. Purified protein induced to phase separate by ionic strength by the same procedure, which followed in our study. The review demonstrate that if LLPS occur, the turbidity of the solution increase and that could observed by naked eyes. The droplet that occur in protein solution as a result of phase separation could examined under a microscope. 10 µL from protein solution put on glass slide and examine under the microscope. The liquid droplets formed by LLPS appear spherical and transparent. This droplet could undergo fusion process. The spherical droplets in the solution will fuse when they encounter each other and then relax into a larger droplet (Figure 5.1).

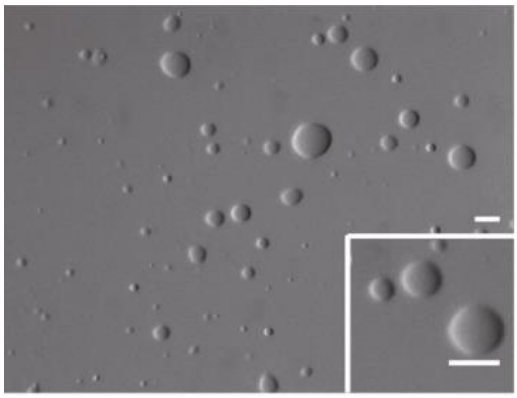

Figure 5.1. Droplet that observed in phase separated protein solution.

Consequently, in our study there are many possibilities for did not observe phase separation of the proteins:

1. This may belong to the protein concentration in solution, and it may require higher protein concentration for induction of the phase separation. At high concentration, the space between the protein molecular decrease and that facilitate protein-protein interaction.

2. Those protein cannot phase separate by followed protocol. There is many different protocol estimate to induce phase separation rather than the protocols, which followed in this study.

3. Those protein cannot phase separated by its own at all. Although it has intrinsically disorder region this protein may not able to form protein-protein interaction. As recent study show, phase separation occurs by protein-protein interaction or protein-RNA interaction. That is mean some protein cannot phase separated by itself without present of RNA. Especially that the CCDC-124 protein known as RNA-binding protein and that was confirmed in recent study (Arslan et al., 2021).

6. FUTURE PERSPECTIVES

Successfully purified protein fragments could be useful for further study of CCDC-124 protein function. “A” protein fragment, which has coiled coil sequence and mostly consist of intrinsically disorder region could tag with florescent fusion protein and insert into eukaryotic cell (HeLa cell). This protein fragment, which is prospect to carry an important sequence of full size protein, has more accessibility than full size protein inside the cell and its organelles. Observation of the protein behaviors and its examination inside the cell can provide useful information about protein function. In addition, localization of the protein inside living cell must give us knowlegment about protein role in the cell.

For induction of phase separation more attempts could be done by various protocol. His-tag may need to separate from purified protein. Fusion protein could interrupt with protein action and prevent it from behave as a native protein. As previous studies show, phase separation of certain protein could occur under very specific condition and that include temperature, pH, ionic strength and protein concentration. So with more attempts to induce LLPS of purified CCDC-124 or its fragment phase separation may observed. Moreover full size protein and its fragments could tag with florescent protein to enhance detection of phase separation when occur. Protein-protein interaction can bring florescent protein together and thus facilitate detection of the droplet under the microscope.

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