THE EFFECT OF BUFFERS pH ON ELECTROPHORETIC PURIFICATION OF INTRACELLULAR GREEN FLUORESCENT PROTEIN

HAZLIN BINTI MOHAMED

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JANUARY 2014

ABSTRACT

The green fluorescent protein (GFP) is a 26.9kDa protein that first isolated from the jellyfish *Aequorea victoria* and composed of 238 amino acid residues. GFP exhibits bright green fluorescence when exposes to light in the blue to ultraviolet range. GFP has become a favourite marker in cell biology for visualisation of gene expression and protein translocation. GFP has also become a transcriptional probe for monitoring non product information and applied in photobleaching to investigate protein dynamics in living cells. Many purification methods have been developed to purify recombinant GFP to obtain high yield and purity. However, they need the preliminary cell disruption step to release the desired proteins which may causes high losses of GFP. Therefore, a direct purification method has been developed for purification of recombinant GFP using preparative native polyacrylamide gel electrophoresis (n-PAGE) in discontinuous buffer system. GFP was successfully purified from intact *Escherichia coli* cells. This direct purification process has eliminated the cell disruption step. For this research, about 5 mL of resolving gel mixture [acrylamide 12% (w/v)] was used to study the effect of buffers pH on electrophoretic purification of intracellular GFP using preparative n-PAGE gel column in continuous buffer system. For protein analyses, the biomass suspension and eluted sample was electrophoresed in n-PAGE slab gel. The gel was then captured using bio imaging method in order to determine the amount of GFP in biomass suspension and eluted sample. Besides, in order to determine the total protein, biomass in suspension and eluted sample was analysed using Lowry reagent method. The purity and yield for phosphate buffer (pH 6, 6.5 and 7) and glycine buffer (pH 9.5 and 10) cannot be determined. For the buffers pH ranging from 7.5 to 8.5, the purity and yield of GFP were increased. When the buffer pH was increased to 9, there was decrease in the purity and yield of GFP obtained. Hence, the best pH achieved was found to be pH 8.5 of Tris-HCI buffer wherein highest values of purity (47.6%) and yield (64.4%) were achieved. As a conclusion, this direct purification method was successfully purified the GFP throughout the preparative n- PAGE gel column and showed that the best pH to employ was 8.5 of Tris-HCI buffer. In addition, the Tris buffer pH ranging from 7.5 to 8.5 was found to give the high purification factor (8~11 times). These results proved that this preparative n-PAGE was absolutely an efficient unit operation to purify the proteins.

TABLE OF CONTENTS

ABSTRAK

Green Fluorescent Protein (GFP) adalah protein yang mengandungi 27 kDa yang pertama kali diasingkan daripada obor-obor *Aequorea victoria*. GFP mengandungi 238 asid amino. GFP memancarkan warna hijau terang apabila terdedah kepada cahaya biru dan cahaya UV. GFP telah digunakan sebagai penanda dalam biologi sel untuk visualisasi gen dan protein translokasi. GFP juga telah menjadi alat transkripsi untuk memantau maklumat tentang non-produk dan diaplikasikan dalam pelunturan gambar untuk menyiasat dinamik protein dalam sel. Terdapat banyak kaedah penulenan telah dibangunkan untuk menulenkan GFP rekombinan untuk mendapatkan hasil yang tinggi dan ketulenan. Walau bagaimanapun, kaedah- kaedah tersebut memerlukan kaedah gangguan sel terlebih dahulu untuk mendapatkan protein yang diingini. Ini boleh menyebabkan kehilangan GFP yang tinggi. Oleh itu, satu kaedah penulenan secara langsung telah dibangunkan untuk penulenan GFP rekombinan dengan menggunakan preparative native polyacrylamide gel electrophoresis (n-PAGE) dalam sistem buffer yang tidak berterusan. GFP telah berjaya ditulenkan daripada sel *Escherichia coli* Proses penulenan secara langsung ini telah menghapuskan kaedah gangguan sel. Untuk kajian ini, kira-kira 5 mL campuran resolving gel [(acrylamide 12% (w/v)] telah digunakan untuk mengkaji kesan pH buffers yang berbeza terhadap penulenan GFP menggunakan preparative n-PAGE dalam ruangan gel. Kemudian, suspensi biojisim dan sampel GFP yang telah ditulenkan telah dianalisa dengan preparative n-PAGE dalam kepingan gel. Suspensi biojisim dan GFP yang tulen kemudiannya dianalisa menggunakan kaedah pengevisualan gel dan cara penentuan bilangan protein Lowry untuk menentukan ketulenan dan bilangan hasil GFP. Ketulenan dan hasil untuk phosphate buffer (pH 6, 6.5 dan 7) dan glycine buffer (pH 9.5 dan 10) tidak dapat ditentukan. Bagi buffer pH antara 7.5 hingga 8.5, ketulenan dan hasil GFP telah meningkat. Apabila buffer pH telah meningkat kepada 9, didapati terdapat penurunan dalam ketulenan dan hasil GFP. Oleh itu, didapati pH tertinggi yang dicapai adalah pH 8.5 untuk Tris - HCI buffer yang mana nilai tertinggi ketulenan (47.6%) dan hasil (64.4%) telah dicapai. Kesimpulannya, kaedah penulenan langsung ini telah berjaya menulenkan GFP dan menunjukkan pH 8.5 untuk Tris-HCI buffer adalah yang paling bagus untuk digunakan. Di samping itu, buffer Tris-HCI untuk pH antara 7.5 hingga 8.5 didapati memberikan faktor penulenan yang tinggi (8~11 kali) dan membuktikan bahawa preparative n- PAGE ini adalah benar-benar satu unit operasi yang cekap untuk menulenkan protein.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percentages
°C	Degree Celsius
µg	Micrograms
µL	Microliters
E.coli	*Escherichia coli*
EGFP	Enhanced green Fluorescent Protein
GFP	Green Fluorescent Protein
GPC	Gel Permeation Chromatography
mA	Milliampere
min	Minutes
mM	Millimolar
mL	Milliliters
mL-ASPs	L-asparaginase mutants
n- PAGE	Native Polyacrylamide Gel Electrophoresis
pH	Hydrogen Ion Concentration
pI	Isoelectric Point
PNC-PAGE	Preparative Native Continuous Polyacrylamide Gel Electrophoresis
rpm	Revolution Per Minutes
V	Voltage
w/v	The weight in grams of a solute per 100 milliliter

1 INTRODUCTION

1.1 Motivation and statement of problem

During the 20th century, the basic principles of the anabolic and catabolic pathways inside living cells were explored using the foundations of biochemistry. The 20th century also showed a transformation in our understanding of enzyme function and protein structures revealed at atomic resolution through crystallography and nuclear magnetic resonance. Based on the increment of organisms' number during the second half of that century, the classical genetics and nucleic-acid chemistry changed into modern genomics (Kungl, 2008). However, there are still no quantitative and monitoring tools available in order to determine the dynamic behaviour of all living systems for intra and intercellular processes. Now, at the beginning of the 21st century, the rapid development of such indicator tools can be seen based on the green fluorescent protein (GFP) that isolated from the jellyfish of *Aequorea victoria*, and engineered variants of GFP family's members of protein (Tsien, 1998).

Traditionally, GFP refers to the protein first isolated from the bioluminescent *Aequorea victoria* jellyfish even though many other marine organisms have similar green fluorescent proteins. It was discovered by Osamu Shimomura in the 1960s (Wiedenmann & Nienhaus, 2006).

GFP which is a globular protein with molecular weight of 27 kDa, consist of 238 amino acid residues. GFP become one of the most exploited proteins in biochemistry and cell biology due to its great ability to emit a highly visible and bright intrinsic green fluorescence. GFP offers certain advantages over other bioluminescence systems because no exogenously added cofactors such as *FMN, FAD, NAD* and *porphyrins* or substrates specific to the jellyfish are necessary to help it exhibit the fluorescence (Jay & Brendan, 2000). That is why GFP become a favourite marker for gene expression visualisation as well as for targeting the protein in organism and intact cells. Besides, GFP can also be fused to many other proteins without altering their functions (Roger, 1998).

Therefore, many purification methods have been developed to purify the recombinant GFP to obtain high purity and yield. The purification methods influence the amount of protein obtained and its purity. In order to obtain efficient and successful protein purification, the most appropriate techniques being selected and their performances were optimised to fit the requirements. They were then combined in a logical way to get a maximum yield as well as reduce the number of steps needed (Nison, 2005).

However, for intracellular protein purification, the intracellular cells must be disrupted first in order to release the desired proteins before the next purification steps. Cell disruption may cause protein degradation and result in high losses of GFP (Chew *et al*., 2011). Due to this problem, a direct purification method has been developed by Chew *et al*. (2009) for purification of recombinant GFP using preparative native polyacrylamide gel electrophoresis (n-PAGE) in discontinuous buffer system. GFP was successfully purified from intact *Escherichia coli (E. coli)* cells after fermentation process. These direct purification process has eliminated the cell disruption method as well as reduced the number of purification steps.

The electrophoresis is an experimental technique whereby the charged particles are migrating through the gel by using application of electric fields and being separated. Since proteins are charged molecules, they migrate under the influence of electric fields. The most important physical properties of proteins in order to perform in electrophoresis include their isoelectric point and their electrophoretic mobilities. Electrophoretic mobilities can be influence by pH and the types and amounts of counter ions as well as the denaturants that are present in the medium (Garfin, 2003).

During electrophoresis, buffers at different pH values can affect the electrophoretic mobility of the proteins. The pH determines the degree of ionization of organic compounds. Increasing pH, it increases the ionization of organic acids thus affect the yield and purity of the recovered product. Besides, the pH of the electrophoresis buffer must within the desired protein pH stability range in order to retain its biological activity (Garfin, 2003). Thus, the aim of this study is to investigate the effect of buffers pH on electrophoretic purification of intracellular GFP using n-PAGE in continuous buffer system. The same buffer components at constant pH in the sample, gel and electric reservoirs were used in continuous system (Garfin, 2003).

GFP is stable at the broad range of pH ranging from 5.5 to 12 and its isoelectric point (pI) value is between 4.5 until 5.4. The buffer pH is also chosen with respect to the desired protein pI. The protein mixtures will become negatively charged if the gel buffer pH is higher than the protein pI (Garfin, 2003). Therefore, the hypothesis that can be made is GFP will migrate towards anode during electrophoresis as the gel buffer pH applied is higher than its pI. Migration speed of GFP increases when higher gel buffer pH is applied, thus it increase the GFP purity and yield.

1.2 Objectives

The following are the objectives of this research:

i) To study a direct purification method for purification of recombinant GFP from intact *E. coli* BL21 (DE3) cells using preparative n-PAGE in a continuous buffer system.

ii) To study the effect of buffers pH on purity and yield of GFP using preparative n-PAGE.

1.3 Scope of this research

In order to achieve the objective of this research, it involves the following scopes:

a) The production of GFP by fermentation of *E. coli* bacteria

b) The purification of GFP where 500 µL of 15% (w/v) of biomass suspension was layered over the resolving gel mixture [acrylamide 12% (w/v)] in order to study the effect of buffers pH including phosphate gel buffer (pH 6.6.5 and 7), Tris gel buffer (pH 7.5,8 and 8.5) and glycine gel buffer (pH 9, 9.5 and 10) using preparative n-PAGE at constant current of 30 mA.

c) The quantitative analysis of biomass suspension and eluted GFP using gel-based imaging method in order to determine the amount of purified GFP

d) The quantitative analysis of biomass suspension and eluted GFP using Lowry reagent method to determine the amount of total protein.

2 LITERATURE REVIEW

2.1 Introduction about GFP

Shimomura *et al*. (1962) discovered the GFP as a companion protein to calcium-activated photoprotein (aequorin) from *Aequorea victoria* jellyfish. That jellyfish produces a shining light around its umbrella's margin. The light emerged from yellow tissue masses that each consist of about 6000-7000 photogenic cells. These cells generate light by a bioluminescence process, which composed of aequorin and GFP. The aequorin exhibits a blue-green light. GFP then accepts energy from aequorin and re-emits it as a green light. Therefore, the green-light of the GFP only activate when the GFP absorbed the blue-light that produced by aequorin upon the calcium binding. Based on their footnote for aequorin purification, Shimomura and the coworkers described the appearance of GFP as "a protein that give solutions look slightly green under sunlight, yellowish in colour under tungsten lights, and exhibit a very bright, green fluorophore under the ultraviolet light. From Figure 2.1, a very compact cylinder on the outside of the GFP structure was formed by 11 antiparallel beta strands. In the middle of beta-structure consisted of chromophore (yellow) that responsible for the GFP's fluorescence **(**Yang *et al*., 1996).

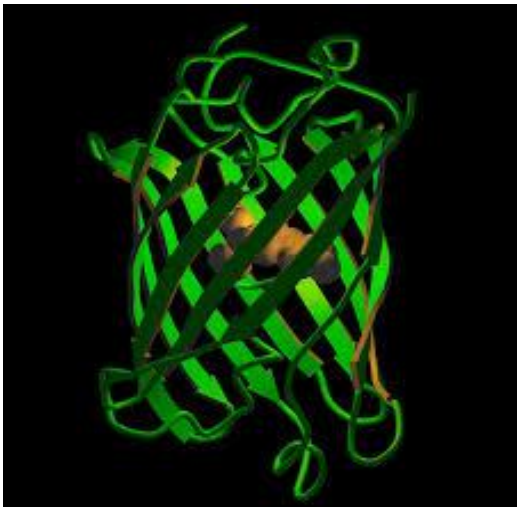

Figure 2. 1: Three dimensional betas - structure of GFP (Adapted from: **Tsien, 1998**)

Johnson *et al.* (1962) then reported the GFP's emission spectrum peak showed at 508 nm. They also noted that the blue chemiluminescence of pure aequorin was peaked close to one of the excitation peaks of GFP, which was near 470 nm. Therefore, the GFP converted the blue emission of aequorin to the green luminous of the animals and intact cells. It supported by Tsien (1998) that stated the excitation spectrum of GFP fluorescence showed a dominant maximum at about 400 nm and a significantly smaller maximum at about 470 nm, while the emission spectrum has a sharp maximum at about 505 nm and (Figure 2.2).

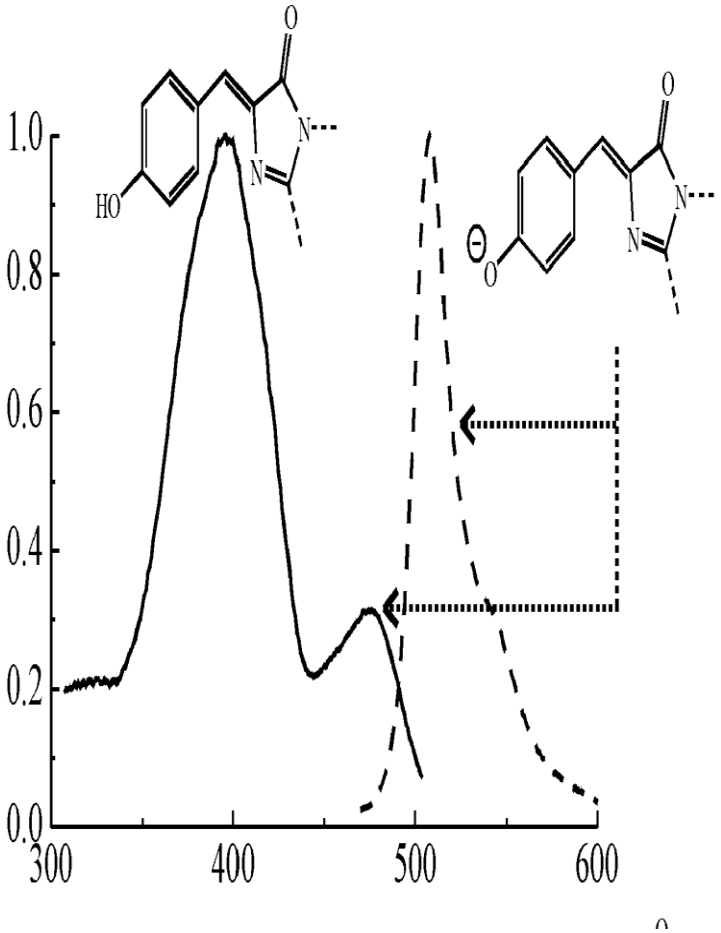

Figure 2. 2: Fluorescence excitation (full-line curve) and emission (dashed curve) spectrum of native GFP in Aequorea victoria (Adapted from: **Tsien, 1998**).

GFP is a 27 kDa protein that composed of 238 amino acid residues with isoelectric point (pI*)* value between 4.5 until 5.4 (Garfin, 2003). It is water soluble and stable at broad range of pH ranging from 5.5 to 12. GFP also stable in neutral to many denaturants, such as proteases, urea and guanidinium chloride (Peckham *et al.*, 2006).

Besides, GFP is a distinct phosphoprotein where its chromophore is intrinsic instead of extrinsic. “Intrinsic Protein Fluorescence" usually refers to the fluorescent emission of amino acid units. GFP contains a chromophore which is responsible for its fluorescences. The chromophore absorbs blue light and emits bright green fluorophore when exposed to blue and ultraviolet light. This chromophore is biosynthetically created between the amino acid residues of 65-67 (Ser-Tyr-Gly) of the GFP protein. Other than that, GFP molecule was also able to fluoresce at room temperature (Jay & Brendan, 2000).

2.2 Applications of GFP

2.2.1 GFP as a gene marker

Due to its strong intrinsic visible and bright green fluorescence, GFP has become a favourite marker in visualisation of gene expression and protein translocation in bacterial, plant and animal systems. The fact that no exogenously added cofactors or substrates are necessary to help it fluoresces makes GFP as an extremely useful biological marker in cell biology. This happened due to the formation of fluorophore from the cyclisation of the peptide backbone and this feature makes the GFP as a virtual indicator that would not disturb the protein distribution in the cells (Zimmer, 2002).

There are many methods that are available to monitor the protein position and gene activity within cells including the proteins that fused with coding sequences for firefly luciferase, ß-galactosidase, and bacterial luciferase. However asides being limited use by living tissue, such methods are also requiring an exogenously added substrates or cofactors. While intracellular GFP is not limited by the substrates’ availability since its detection requires only irradiation by near blue light or UV light. Thus, it should provide an excellent means for monitoring gene expression and protein localization in living cells. In addition, GFP produce a strong green fluorescence when exposed to blue light as it being expressed in prokaryotic and eukaryotic cells (Ha *et al*., 1996).

However, each molecule of GFP has only one chromophore which lower its sensitivity (Zimmer, 2002).Therefore, in order to drive sufficient expression for detection, the GFP required strong promoter since there is no signal amplification (Tsien, 1998). Therefore, Tsien (1998) suggested using the gene reporter products that can enzymatically catalyse

the substrates in large quantity of the fluorescence that can be loaded into intact and fully viable cells.

2.2.2 Photobleaching

Next, GFP also applied in photobleaching that being used to study about the protein dynamics in living cells. There are two methods based on photobleaching which are fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP). The relative movement of the GFP chimera can be defined by illuminating an area with high intensity illumination (bleaching) and monitoring the recovery of the resultant fluorescence loss (FRAP). In order to investigate the transport of GFP fusion proteins between different organelles, FLIP can be used by bleaching an area repeatedly and from outside of the area, the fluorescence's loss being monitored (Tsien, 1998).

2.2.3 Other applications of GFP

Besides that, GFP has also become a transcriptional probe for monitoring non product information such as oxygen, temperature, pH and availability of nutrients in biotechnology process (March *et al*., 2002). Then, GFP has large two-photon absorption that suitable to be used in diagnostics, data storage, and other photochemical applications (Kirkpatrick *et al*., 2001). In addition, the most commonly GFP application is probably the production of a transgenic rhesus monkey (ANDi), that carrying the GFP gene (Chan *et al*., 2001). It has attracted the public attentions and lead the small companies to produce christmas trees, fluorescent pets, and flowers (Mercuri *et al*., 2001). In order to make chimeric mice, green mice and other animals have been used as a source of green-tagged cells or organs for transplantation (Ikawa *et al*., 1999). Other than that, outside of the laboratory, GFP has found many uses, where GFP acts as a marker to monitor the meat fermenting lactobacilli in sausages (Gory *et al*., 2001) and track the bacteria spread that consume diesel fuel in soils (Dandie *et al*., 2001).

2.3 Purification methods to purify GFP

Therefore, due to these applications, many purification methods have been developed to purify recombinant GFP. These methods include the most frequently employed chromatographic method for proteins which is an ion exchange chromatography (Lewis & Clark, 2007), monoclonal antibody-coupled affinity chromatography by Cabanne *et al*. (2005) and Zhuang *et al*. (2008) as well as hydrophobic interaction chromatography by McRae *et al*. (2005). Besides that, the immobilized metal affinity chromatography (Dalal *et al*., 2008) and an artificial chaperone-assisted metal affinity (Dong *et al*., 2009) also have been developed to purify GFP. Other than that, the gel filtration chromatography also being used to purify GFP where it is the best preparative method for fractionating native proteins in term of shape and size (Lewis & Clark, 2007) since it is gentle to the sample. Gel filtration give a low resolution for protein separation yet frequently used in protein purification.

However, for all these purification techniques, the intracellular cells required an additional cell disruption step in order to release the desired proteins before the subsequent purification steps. The preliminary cell disruption step is time consuming and harsh which may causes high losses of GFP (Chew *et al*., 2011). To overcome those problems, an integrated GFP purification process based on the preparative native polyacrylamide gel electrophoresis (n-PAGE) in discontinuous buffer system was developed (Chew *et al.*, 2009).

2.4 Gel electrophoresis

Basically, gel electrophoresis is a technique whereby the charged particles including ions, molecules, and macromolecules being separated by moving in liquid medium using an application electric field (Garfin, 2003). This process usually carried out in aqueous solution, and electrically charged particles will start to migrate towards the oppositely charge electrode when placed to a constant electric field. The velocity of the particle mobilities proportional to the particle charge and electrophoresis voltage, but inversely proportional to the size of particles (Jan, 2008).

So, any molecules such as small cations or anions, organic acids, amino acids, peptides, saccharides, lipids, proteins, nucleotides, nucleic acids, even the whole cells that differ in charge and/or size can be separated from each other by using this technique. However, in practice, the proteins and nucleic acids become the common subjects of electrophoretic separation.

The rate of movement or migration through the electrical field depends on the net charge of the protein or nucleic acid, the charge's strength, the size and shape of the molecules, and also on the ionic strength, temperature and viscosity of the medium which the molecules are moving through (Jan, 2008).

Actually, Tiselius in 1937 was first person that attempted the separation of serum proteins by electrophoresis. The proteins migrated to the electrode that bearing the opposite charged in free solution. However, heat generations occur during that 'free electrophoresis' that lead to the convection flow of the liquid medium. The heat disturbs the zones of separated proteins. Therefore, nowadays electrophoresis is usually done in some support. The support can be cellulose acetate, paper or gel that made of starch, agarose or polyacrylamide. However, polyacrylamide gels are suitable for protein electrophoresis where the gels well suited for sieving protein since it can cast in a range of pore sizes. The polymerization reaction by polyacrylamide gel is also easy and reproducible. Furthermore, polyacrylamide gels has a hydrophilic characteristic and do not bind to the protein stains as well as transparent to light (Garfin, 2003). During separation, the support gel act as a three dimensional structure of open pores filled with a liquid that prevents convection flows of electrophoretic buffer (Jan, 2008).

Garfin (2003) then reported that by using the gel electrophoresis, the molecular weights and charges of proteins as well as the protein's subunit structure and the purity of a particular protein can be determined. Compared to all methods available for separating protein, this technique provides the better resolution.

2.5 Native protein electrophoresis

The principles of basic electrophoretic techniques can be divided into 4 types such as native protein electrophoresis, polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), gradient PAGE and isoelectric point focusing. However, in this research, my study is more focused on native protein electrophoresis in which the protein in native state are properly folded and electrophoresed without being denature during the electrophoresis. The proteins retain their original conformation and subunit structure, charge and biological activity, and in alkaline buffer, the proteins migrate towards electrode that bearing the positive charged (Jan, 2008).

Even though the same results can also achieved by using support gel of cellulose acetate and thin polyacrylamide, agarose is probably the most commonly used for this native analysis. The agarose gel is also customly used for nucleic acids in basic electrophoresis in which this gel separate based on size. However, the pores of agarose are too large to cast the protein molecules. Proteins such as albumin dimer and monomer that have similar charge density but differ in size, migrate with the same velocity and cannot be resolved. The resolution is limited because there are no further focusing mechanisms (Jan, 2008). Therefore, polyacrylamide gels more preferable for protein separation due to the size of the gel pores in polyacrylamide that can be easily controlled (Garfin, 2003).

Laura (2006) also reported that under native conditions, the blue native electrophoresis gives a high resolution for separation of multiprotein complexes. This technique consists of polyacrylamide gel electrophoresis. The both sample and electrophoresis buffers being added with Coomassie blue G-250 which is non- denaturing compound to give a negative charge on the protein complexes so that they can move toward the positively charged electrode. Using this way, many samples can be separated during a single run of electrophoresis process, and the protein complexes can be compared directly. The differences in protein expression can also being identified and further functional analysis directly being provided.

Apart from these, the native electrophoresis also being applied for human serum proteins that become one of the basic biochemical examinations, routinely performed in the clinical chemistry laboratories. It is also used for analysis of isoenzymes, spectra of serum lipoproteins, hemoglobin variants and others (Jan, 2008).

2.5.1 n-PAGE

n-PAGE can perform either in continuous buffer system or in discontinuous buffer system. n-PAGE separates the proteins in their native states according to the difference in charge density. Chew and coworkers (2009) have developed a gel electrophoresis method that employed basic and readily available laboratory equipment. It provides an efficient way to purify the recombinant GFP from intact *E. coli* cells. The external electric field causes the cells to release its intracellular contents. The negatively charged GFP was electrically drifted out and being separated from the other host cell proteins by gel and moved towards the anode. Then, as the GFP migrate to the bottom end of the polyacrylamide gel, the purified GFP was collected into dialysis tube. After electrophoresis was done, the gel was captured by using a Gel Doc XR. GFP then were visualized by staining them with Coomassie brilliant blue R-250 and destained with methanol and acetic acid. This integrated purification technique successfully reduced the number of purification steps and time, hence improved the purity and yield of intracellular GFP.

2.5.2 Other applications of preparative n-PAGE

Asides GFP, preparative n-PAGE also applied for the other protein. A preparative native continuous polyacrylamide gel electrophoresis (PNC-PAGE) was developed by Bernd (2004) in order to isolate cadmium cofactor that contain of proteins in biological systems. Before that, Arabidopsis cytosol was introduced into gel permeation chromatography (GPC). Then, PNC-PAGE isolated the cadmium proteins of this plant in a GPC fraction. He revealed that by using PNC-PAGE, the high molecular mass cadmium proteins of Arabidopsis quantitatively being detected. It shown that under these PAGE conditions, the chemical structure of the cadmium proteins remain unchanged. PNC-PAGE is supposed to be an efficient technique for isolating other metal cofactors such as Zn, Cu, Ni, Pd, Co, Fe, Mn, Pt, Cr, and Mo. Besides, PNC-PAGE might be suitable for next determinations of structural metalloproteins present in PAGE fractions.

Apart from that, Enders *et al*. (1977) also have developed a preparative polyacrylamide gel electrophoresis to purify the enterotoxin of Clostridium perfringens from Sephadex G-100 extracts. They reported that by using this method, the purified toxin of high specific activity that eluted ranging from 80 to 90%. The purity and physical characteristics of the toxin were similar to those previously reported for the purified protein by other methods. Therefore, it shows that a large amounts of toxin with high specific activity will produce in short time by using preparative electrophoresis compared to other available current methodology that required a longer period of time.

In addition, due to its high resolution, PAGE had been extremely used in protein analyses. However, the denaturing conditions and relatively high expenses of preparative gel electrophoresis extremely limit its practical application as a method for protein's purification. Therefore, in their study, Lippincott *et al*. (2008) have developed a native preparative gel electrophoresis system to obtain retaining pure proteins for the preparation of L-asparaginase mutants (mL-ASPs). That gel electrophoresis was developed based on double electrophoresis and elution through a membrane. Actually, for many years, the antigenicity of L-asaparaginase (L-ASP) becomes problematic for the leukemia's treatment. In order to establish a relationship between the antigenic epitope of L-asparaginase and its antigenicity, several mL-ASPs are constructed and expressed. So, a native preparative polyacrylamide gel electrophoresis was developed in order to purify these enzyme mutants efficiently for further study.

Lippincott *et al*. (2008) also reported that this simple and reproducible native gel electrophoresis permitted the different mutants' purification from the extracts of enzyme, with a sufficient activity to perform immunological and biological studies. Furthermore, this newly developed technique was effective and inexpensive compared with other methods, such as column and affinity chromatography. As a result, the specific activity of 300~400 U/mg with a high purity were obtained by the single-step purification of the enzyme mutants.

3 MATERIALS AND METHODS

3.1 Chemicals

Table 3. 1: List of chemicals

Chemicals	Brand	Purpose
Luria Bertani agar	Condo Pronadisa	For fermentation
Luria Bertani broth	Condo Pronadisa	
Ampicillin	Bio basic Canada Inc.	
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Thermo Scientific	
Sodium phosphate ($NaH_2PO_4.H_20$)	Sigma Aldrich	
Tris	Sigma Aldrich	For purification
Glycine	Fisher Scientific	method
Hdrochloric acid (HCI)	Merck	
Bromophenol blue	Merck	
Isobutanol	Fisher scientific	
Acrylamide	Merck	
Bis- acrylamide	Merck	
Sodium hydroxide (NaOH)	Bio Basic Canada Inc.	
Ammonium persulfate (APS)	Sigma Aldrich	
TEMED	Merck	
β- mercaptoethanol	Merck	
Bovine Serum Albumin (BSA)	Sigma Aldrich	For quantitation
Lowry Reagent	R&M Chemicals	method
Folin & Ciocalteu's phenol reagent	Sigma Aldrich	
Pure GFP	Merck	For standard curve

3.2 Bacteria

E. coli strain BL21 (DE3) carrying the pRSETEGFP plasmid was provided by my supervisor, Dr Chew Few Ne.

3.3 Production of enhanced green fluorescent protein (EGFP)

The experiment was started by streaking the *E. coli* on agar plate and incubated for 18 hours at 37°C in incubator. In order to provide a good oxygen transfer rate during the fermentation process, the ratio of the Luria Bertani (LB) broth to the Erlenmeyer flask volume was 1:5. A single colony of *E. coli* stain was inoculated into 100 mL of Erlenmeyer flask containing 20 mL of LB broth at 30°C and 200 rpm for 18 hours in incubator shaker (INFORS HT, *Ecotron*). Batch culture was carried out in 1000 mL of Erlenmeyer flask containing 200 mL of LB broth that inoculated with 10 mL of prepared inoculums. Isopropyl β-D-1-thiogalactopyranoside (IPTG**)** at final concentration of 0.5 mM was added after the cells was grown to an optical density (OD_{600nm}) of 0.6–0.8 in order to induce the protein expression. The fermentation process continued for another 16 hours at 30°C under 200 rpm. The culture was then harvested by centrifugation at (5000 xg, 4^{o}C for 30 min) by using refrigerated centrifuge (Eppendorf, Centrifuge 5810R). About 0.8540 g of the cell pellets from 40 mL of culture broth were washing with 25 mL of 4x gel buffer (at different buffer pH values) followed by centrifugation at the same conditions. Finally, the cell pellets were resuspended in the gel buffer and 15% (w/v) of biomass suspension was prepared for the next process (Chew *et al*., 2009).

3.4 Purification of EGFP

3.4.1 Preparation of preparative n-PAGE

First of all, the home made apparatus of preparative n-PAGE were set up (Figure 3.1). It composed of a gel column, a laboratory bottle, and a power supply. The lower end of the assembled gel column was tightly closed with parafilm before being loaded with resolving gel mixture to avoid leakage of the gel solution. About 5 mL of resolving gel mixture [(acrylamide 12% (w/v)] (Table 3.2) was prepared for buffers at different pH values (0.4 M phosphate gel buffer at pH 6, 6.5 and 7, 0.2 M Tris gel buffer at pH 7.5, 8 and 8.5 and 0.2 M glycine gel buffer at pH 9, 9.5 and 10). Then, the gel was poured into the gel column. Next, 200 µL of isobutanol was layered over the surface of the mixture to form a uniformly flat surface. The gel was then allowed to polymerise at room temperature. After the resolving gel was polymerised, the isobutanol was then rinsed thoroughly with distilled water.

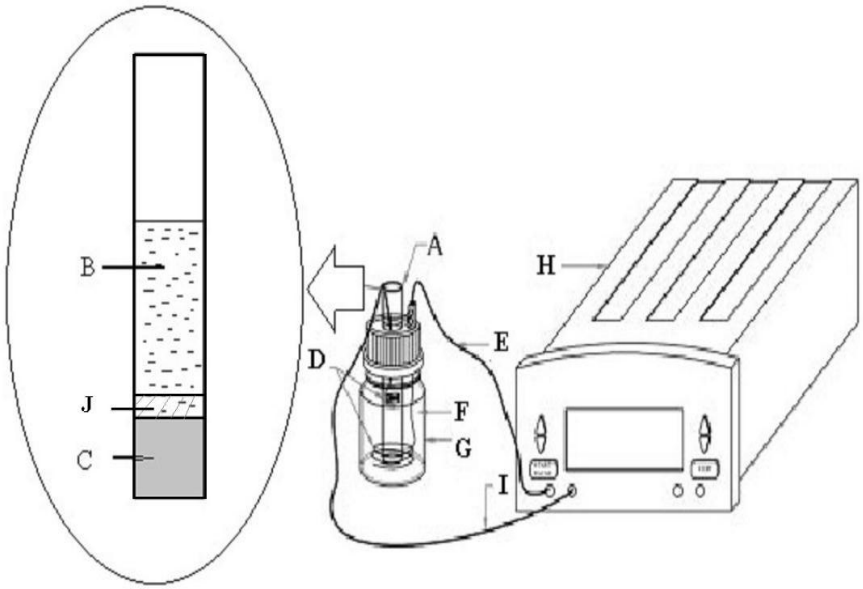

Figure 3. 1: Homemade gel electrophoresis apparatus. (Adapted from: **Chew *et al.*, 2009)**

(A) = Column; (B) = Cathode chamber; (C) = Resolving gel; (D) = platinum wire electrodes; (E) = Anode wire; (F) = Anode chamber; (G) = Laboratory bottle; (H) = Power supply; (I) = Cathode wire; (J) = Loaded sample