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MASTER'S THESIS

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TABLE OF CONTENTS

ABSTRACT

The experiment in this thesis is set out to learn and understand the biology or chemistry basis and to apply different techniques used in protein biochemistry research as well as to observe the results in practice. Among the various methods of studying proteins, affinity method is regarded as one of the most effective means of purifying proteins as a result of its high degree of specificity. This experiment has demonstrated how immunoaffinity technique can be used to capture Lil3 proteins from a crude source of solubilized thylakoid membrane. Immobilization method by coupling Lil3 antibody directly onto Toyopearls beads support was performed and the coupling efficiency was evaluated. The Lil3 proteins captured by antibody column were eluted with denaturing and nondenaturing elution buffers. Regeneration and reuse of the immobilized antibody-coupled beads column have been conducted for a few times, in an attempt to conserve the limited supply antibody and the economic feasibility, including time and expense. In addition, a brief analysis of protein membrane complexes and antibody was also provided.

CHAPTER 1

INTRODUCTION

1.1 Background Theory

1.1.1 Overview of Photosynthesis

Life requires a constant input of energy. For almost all forms of life on our planet, the ultimate source of that energy is sunlight. Photosynthesis is the process by which solar energy is captured and converted into chemical energy of sugars and other organic compound (Figure 1.1). This process consists of a series of chemical reactions that require carbon dioxide (CO_2) and water (H_2O) and store chemical energy in the form of sugar. Light energy drives the reactions. Oxygen (O_2) is a by-product of photosynthesis and is released into the atmosphere. Photosynthesis transfers electrons from water to energy-poor CO_2 molecules, forming energy-rich sugar molecules ($C_6H_{12}O_6$). This electron transfer is an example of an oxidation-reduction process: water is oxidized (loses electrons) and CO_2 is reduced (gains electrons). Photosynthesis uses light energy to drive the electrons from water to their more energetic states in the sugar products, thus converting solar energy to chemical energy.

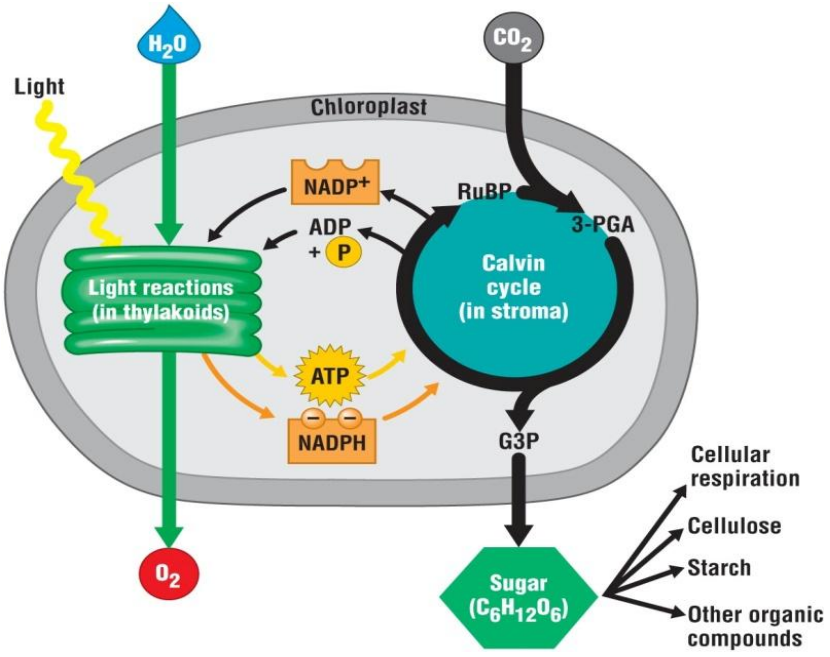


Figure 1.2 A review of photosynthesis (Campbell, Reece et al. 2007).

Photosynthesis consists of two parts: the light reactions and the dark reactions. In the light reactions, light energy is transformed into two forms of biochemical energy: NADPH and ATP. The products of the light reactions are then used in the dark reactions to drive the reduction of carbon dioxide and its conversion into glucose and other sugars. The dark reactions are also called the Calvin cycle or light-independent reactions (Taiz and Zeiger 2010; Berg, Tymoczko et al. 2012).

Chloroplast: sites of photosynthesis

In plants, photosynthesis occurs in chloroplasts, mainly in the mesophyll of leaves. Chloroplasts derive from embryogenic proplastid that can differentiate into all types of plastid such as amyloplast, chromoplast, elaioplast and etioplast. Leaf cells, normally containing chloroplasts, need light for the conversion of protochlorophyllide into chlorophyll. When light is unavailable or insufficient, as is often the case in cotyledons of germinating seedlings, proplastids accumulate large amounts of thylakoid lipids with the complex of protochlorophyllide and a form of the enzyme responsilble for its light-driven reduction (protochlorophyllide reductase A). Here, the proplastids differentiate into etioplasts, as dark-grown seedlings are said to be etiolated. Their internal membranes can be seen as a semicrystalline structure, called prolamellar body, in combination with prothylakoid membranes. Upon illumination of dark-grown plants, flat membrane sacs will emerge from the prolamellar body that will eventually become thylakoids with their normal photosynthetic complexes (Boffey, Ellis et al. 1979; Eichacker, Soll et al. 1990; Chan and Bhattacharya 2011; Ploscher, Reisinger et al. 2011).

The double-membrane chloroplasts enclose additional extensive system of internal membrane called thylakoid (Figure1.2). The disc-shaped thylakoid is composed of two distinct morphological components: stacked membranes known as grana lamellae, and un-stacked stroma lamellae. Chloroplasts in green algae and higher plants contain photosynthetic thylakoid membranes with four multisubunit protein complexes (protein-pigment complexes): photosystem I (PSI), photosystem II (PSII), cytochrome b_6f complex, and the ATP synthase, each with multiple cofactors. Photosystem II complex is mainly situated in the grana lamellae, while photosystem I and ATP synthase are predominantly localized in the stroma lamellae. Cytochrome b_6f complexes are evenly distributed. Despite their different

locations they function in concert to form NADPH and ATP (Cohen, Yalovsky et al. 1995; Friso, Giacomelli et al. 2004; Lopez-Juez and Pyke 2005; Croce and Amerongen 2011).

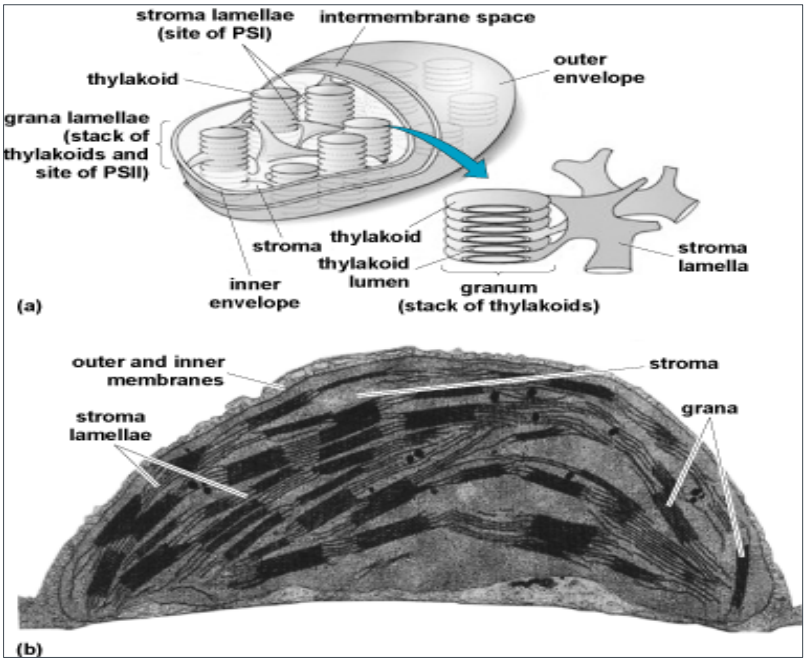


Figure 1.2 A typical chloroplast structure (Taiz and Zeiger 2010). (a) Schematic diagram of a higher plant chloroplast. (b) Electron micrograph of a chloroplast.

The light reaction

The light reactions occur along the thylakoid membrane within the chloroplasts, where pigments capture light energy. Different pigments absorb light of different wavelengts. Chloroplasts contain several kinds of pigments including chlorophyll *a*/*b* and carotenoids, but it is the green pigment chlorophyll *a* that participates directly in the light reaction. Chlorophyll *a* absorbs light energy in the blue-violet and red-orange part of the electromagnetic spectrum and reflects other wavelengths. We see the reflected or transmitted wavelength as the color of the pigment. A very similar molecule, chlorophyll *b*, absorbs mainly blue and orange light but does not participate directly in the light reactions. It broadens the range of light that a plant can use by conveying absorbed energy to chlorophyll *a*, which then puts the energy to work in the light reactions (Campbell, Reece et al. 2007).

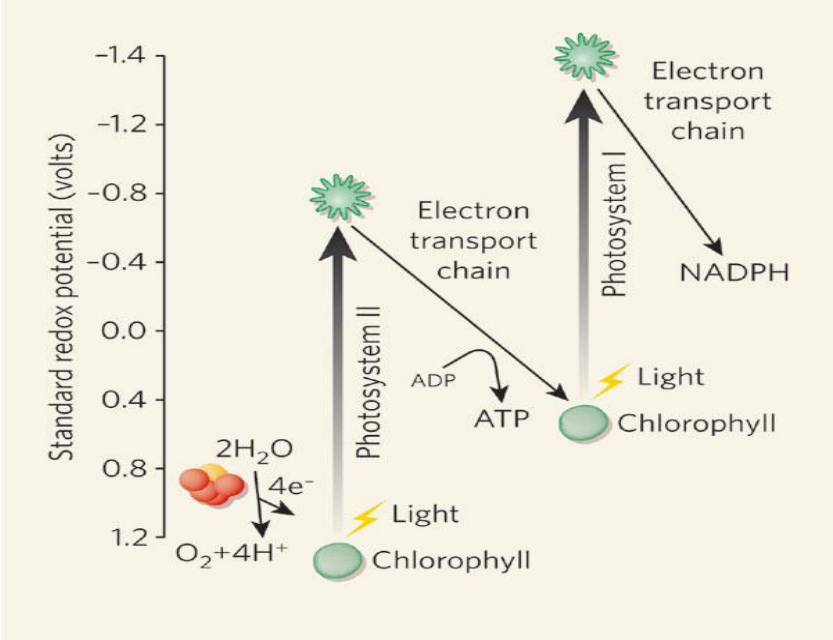


Figure 1.3 The light reactions of photosynthesis (Allen and Martin 2007).

Plants and some photosynthetic prokaryotes have two reaction centers, photosystem I and photosystem II, that abosorb light energy, convert it into electrochemical potential, and are connected in series electrically (Figure 1.3). Photons excite electrons in the chlorophyll of photosystem II, which are then trapped by the primary electron acceptor. The photosystem II replace its light-excited electrons by extracting electrons from water. This is the step that releases O2 during photosynthesis. Energized electrons from photosystem II pass down an electron transport chain to photosystem I. The chloroplast uses the energy released by this electron "fall" to synthesis of the energy-storage molecule ATP. The electron transport chain of photosynthesis, also known as the Hill and Bendall Z-scheme, ends with photosystem I delivering its light-excited electrons to $NADP^+$, reducing it to NADPH. ATP and NADPH drive the dark reaction, or the Calvin cycle, that transfer the electrons to CO_2 so as to provide the energy to make sugars and the other molecules of life. The chain begins when water is oxidized by the very high electrochemical potential of photosystem II (Allen and Martin 2007).

1.1.2 The Light-harvesting Complex (LHC) Protein Superfamily

In eukaryotic photosynthetic organisms that contain both chlorophyll *a* and chlorophyll *b*, the most abundant antenna proteins are members of a large family of structurally related proteins. These transmembrane light-harvesting complex (LHC) proteins that bind chlorophyll and carotenoid pigments are major component of the photosynthetic machinery and form the outer antenna protein complexes of photosystem I and II in the thylakoid membranes. The LHC proteins are encoded in the nucleus by a large multigene family, *Lhc* genes. Some of these proteins are associated primarily with photosystem II and are called light-harvesting complex II (LHCII) proteins; others are associated with photosystem I and are called LHCI proteins. All LHC proteins contain three helices that span the chloroplast thylakoid membrane connected by stroma and lumen-exposed loop. The first and the third helix have a similar sequence and they share the characteristic "LHC motif", a highly hydrophobic sequence (ExxxxRxAM) where the glutamic acid (Glu, E) from one LHC motif binds a chlorophyll *a* molecule via a salt bridge to the arginine (Arg, R) of the other. This chlorophyll binding domain is the homologous core structure of this protein superfamily. Each LHC protein typically binds approximately a dozen chlorophyll molecules and a few different carotenoids and, thus, plays essential roles in photosynthesis and photoprotection. Plant LHCII proteins bind eight chlorophyll *a*, six chlorophyll *b* and four carotenoids (two xanthophylls lutein, violaxanthin and neoxanthin). LHC proteins absorb light and transfer the excitation energy to the reaction center chlorophyll of photosystems (Jansson 1999; Neilson and Durnford 2010; Tanaka, Rothbart et al. 2010; Hoffman, Puerta et al. 2011).

The *Lhc* supergene family of higher plants also contains genes coding for proteins that exhibit sequence similarity to the LHC proteins, namely the four-helix protein PsbS (photosystem II subunit S) and the Light-harvesting like (Lil) proteins. These extended LHC protein superfamily are proposed to evolve from a cyanobacterial single-helix proteins with LHC motifs called high light-induced protein (HLIP). The LHC proteins have three membrane-spanning helices, while the Lil proteins contain one to four transmembrane helices. The Lil proteins, including the one-helix proteins(OHPs) which are also known as Lil2, two-helix proteins : the stress-enhaced proteins (SEPs) and Lil3 proteins (which are not homolog to SEPs), and three-helix early light-induced proteins (ELIPs), or Lil1, share a common feature of transmembrane conserved sequence, the LHC motif. PsbS and the Lil protein families do not seem to be constitutively associated with reaction center complexes. Unlike the three-helix LHC proteins, whose primary funtion is the absorption of light through chlorophyll

excitation and transfer of absorbed energy to photochemical reaction centers, members of Lil and PsbS families are connected with light management (i.e. light harvesting or, when needed, dissipation of excess absorbed light), cold stress, as well as with nutrient deprivation, thus most likely involved in stress protection (Jansson 1999; Montane and Kloppstech 2000; Klimmek, Sjodin et al. 2006; Engelken, Brinkmann et al. 2010; Neilson and Durnford 2010).

Light-harvesting like (Lil) proteins

Diversification of the LHC superfamily proteins is proposed to be the evolutionary history of protection or adaptation of photosynthetic eukaryotes to their habitats. The Lil proteins represent a collection of structurally diverse membrane proteins that are distributed throughout oxygenic prokaryotic and eukaryotic organisms. They differ in the numbers of predicted transmembrane helices, of which at least one contains a conserved LHC motif. The primary function of the LHC motif in the major LHC proteins is to provide ligands for chlorophyll binding and to enable energy transfer among chlorophyll molecules for photosynthesis.Whereas in the Lil protein families including ferrochelatase2 (FeC2), SEP, Lil1 (ELIP), Lil2 (OHP) and Lil3 proteins, the LHC motif seems to have a unique function that are still poorly understood. The Lil proteins are proposed to be involved in protection against excessive light. The expression of the genes for these proteins is induced under strong illumination. Moreover, they are involved in regulating pigment biosynthesis or part of a chlorophyll scavenging mechamism that works to prevent the formation of reactive oxygen species by unbound chlorophyll molecules. One type of the Lil proteins, Lil3 protein, may not be related to photoprotection because the expression of the *Lil3* genes does not seem to be inducible by strong illumination. Instead, Lil3 is proposed to transfer de novo synthesized chlorophyll to the photosystems because it is associated with pigment-binding proteins that appear temporally at the greening stage of barley seedlings (Reisinger, Ploscher et al. 2008; Neilson and Durnford 2010; Tanaka, Rothbart et al. 2010).

1.1.3 Membrane Protein Complexes

A cell is separated from its environment by a selectively permeable plasma membrane. The plasma membrane is commonly described as a fluid mosaic. It is like a mosaic in having diverse protein molecules embedded in a matrix of phospholipids. The phospholipids in a

membrane form a two-layer framework called a phospholipid bilayer. The steroid cholesterol helps stabilize the phospholipids. The plasma membrane is selectively permeable and one of the reasons is the hydrophobic interior of the bilayer. Hydrophobic molecules can easily pass through the membrane. In addition, small molecules like O_2 can get in between the phospholipids of the membrane. On the other hand, large hydrophilic molecules like glucose, and ions such as sodium ions and hydrogen ions, cannot pass through the membrane unaided (Campbell, Reece et al. 2007).

The membrane system is one of the most important interfaces in biological systems. Such a membrane system contains many kinds of receptor proteins, transporter proteins and channel proteins which have critical roles for the biological activity. The proteins associated with energy transducing electron transport chains in mitochondria and chloroplasts are located in the membrane system, which accordingly are called membrane proteins (Kashino 2003). Membrane proteins can be divided into integral membrane proteins, peripheral membrane proteins and lipid-anchored proteins that are located outside the lipid bilayer on either the extracellular or cytoplasmic surface, but are covalently linked to a lipid molecule that is situated within the bilayer. Integral membrane proteins are permanently attached to the lipid bilayer membrane. While peripheral membrane proteins are temporarily and indirectly attached to the lipid bilayer or to integral proteins. The transmembrane proteins, such as LHC proteins and Lil proteins, are integral proteins that span across the membrane and they are either beta-barrel or alpha-helical proteins (Karp 2009).

The membrane proteins have a mutual relationship with the membrane lipids; together they form protein membrane complexes. Membrane proteins are responsible for most of the dynamic processes carried out by membranes. Membrane lipids form a permeability barrier and thereby establish compartments, whereas specific proteins mediate nearly all other membrane functions. In particular, proteins transport chemicals and information across a membrane. Membrane lipids create the appropriate environment for the action of such proteins (Berg, Tymoczko et al. 2012). In the functional form, many of membrane proteins comprise multi-subunit complexes, where such membrane protein complexes contain many cofactors and lipids. These complexes are vital to cellular function. Understanding how the protein membrane complexes are assembled from its different composition parts, and how they are eventually degraded are crucial to understanding their function and regulation. The assembly of membrane proteins have enabled a level of complexity that is not possible using a single polipeptide and as a result the complexes can undertake multifacet functions and

regulatory mechanisms. Membrane protein complexes are assembled in specific orders with the guiding help of chaperones. Ordered assembly could be the cell's protection to elude potential problem, i.e. unwanted interactions and potentially harmful assembly intermediates. Once assembled, membrane protein complexes are not stable or static. Through a process called dynamic exchange, membrane proteins are exchanged in and out of exsisting protein complexes as shown by the experiment with photosystem II in chloroplasts, where the D1 subunit becomes photo-damaged and is subsequently replaced as part of a repair mechanism (Daley 2008).

Analysis of protein membrane complexes

The study of membrane proteins encounters the primary difficulty in obtaining the protein of interest. Membrane proteins are usually present at low levels in biological membrane, and it is rare that a single protein species is a major peptidic constituent of the membrane. Besides that membrane proteins are not generally soluble in aqueous solution, another factor that can be a limitation is that membrane proteins are naturally embedded in a mosaic lipid bilayer, which is a complex, homogeneous or heterogeneous, and dynamic environment. Many standard biophysical techniques for an investigation of protein complexes require the protein to be extracted from its native membrane and studied in a detergent or lipid environment in vitro. But inspite of the difficulties of working with membrane proteins, there are many successes and strategies to study them. Integral membrane proteins make up a significant proportion of the proteosome in many organisms and play a vital role in diverse cell functions including signalling, energy generation, transport and recognition. Moreover, they are also a significant pharmaceutical targets (Seddon, Curnow et al. 2004).

For research on membrane protein complexes, protein subunit assemblies have to be extracted from the lipid phase and separated from each other. The separation methods can be preparative or analytical separation. Preparative category, which is conducted on a relatively large scale, aims to purify the membrane protein complex from membrane fraction while retaining its native form, mainly to characterize its nature; crystallized membrane protein complexes are good examples. The other category aims to analyze the constituents of the membrane protein complex, usually on a small scale. The analytical separation of membrane proteins is important for clinical research. A proteomic approach has been developed which aims to detect whole expressed proteins to analyze the funtion of such proteins and the

functional linkage between them. This proteomic approach is one of the important clinical analyses (Kashino 2003). Analysis of the subunit components in an isolated membrane complex is also necessary for the understanding of the function of the membrane protein complex. For this objective, SDS-PAGE and/or 2-dimensional electrophoresis in conjuction with isoelectric focusing or native-PAGE are frequently performed (Boronowsky, Wenk et al. 2001; Reisinger and Eichacker 2007; Reisinger, Hertle et al. 2008; Reisinger, Ploscher et al. 2008).

Isolating the membrane protein complex must satisfy the hydrophobic nature of membrane proteins or the close association with membrane lipids. To overcome this difficulty, many methods have been employed. The principles and applications of various biophysical methods are described comprehensively by Sheehan, 2009. Crystallized membrane protein complexes are the most successful example. In these purification methods, special efforts are made in the steps prior to the column chromatography to enrich the target membrane protein complexes. Although there are specific aspects for each complex, the most popular method for isolating these membrane protein complexes is anion-exchange column chromatography, especially using weak anion-exchange columns. Another trend is metal affinity column chromatography, which purifies the membrane protein complex as an intact complex in one step. Such protein complexes contain subunit proteins which are genetically engineered so as to include multiple-histidine tags at carboxyl- or amino-termini. The key to these successes for multi-subunit complex isolation is the idea of keeping the expression at its physiological level, rather than overexpression. The affinity purification methods supported by affinity interaction can be applied to minor membrane protein complexes in the membrane system. Isoelectric focusing (IEF) and blue native (BN) electrophoresis have also been employed to prepare membrane protein complexes (Kashino 2003).

Solubilization of membrane proteins by detergents

In many methods for separation of proteins (Figure 1.4) like SDS-PAGE, native PAGE, isoelectric focusing PAGE, for crystallization of isolated single protein species or for any other technology applied to characterize a membrane protein's function, the first important step in purifying membrane protein complexes from any membrane system is to solubilize them from their environment surrounded by lipids. Solubilization of membrane proteins is a process in which the proteins and lipids that are held together in native membranes are

suitably dissociated in a buffered detergent solution.The success of the purification relies greatly on the choice of detergents and their concentrations, especially when purification of the membrane protein complexes in their intact (native) form is wanted. In standard bench work, solubilization can be affected by the type of detergent and its concentrations, the sample buffer, the salts, the temperature and the forces applied (Reisinger and Eichacker 2008).

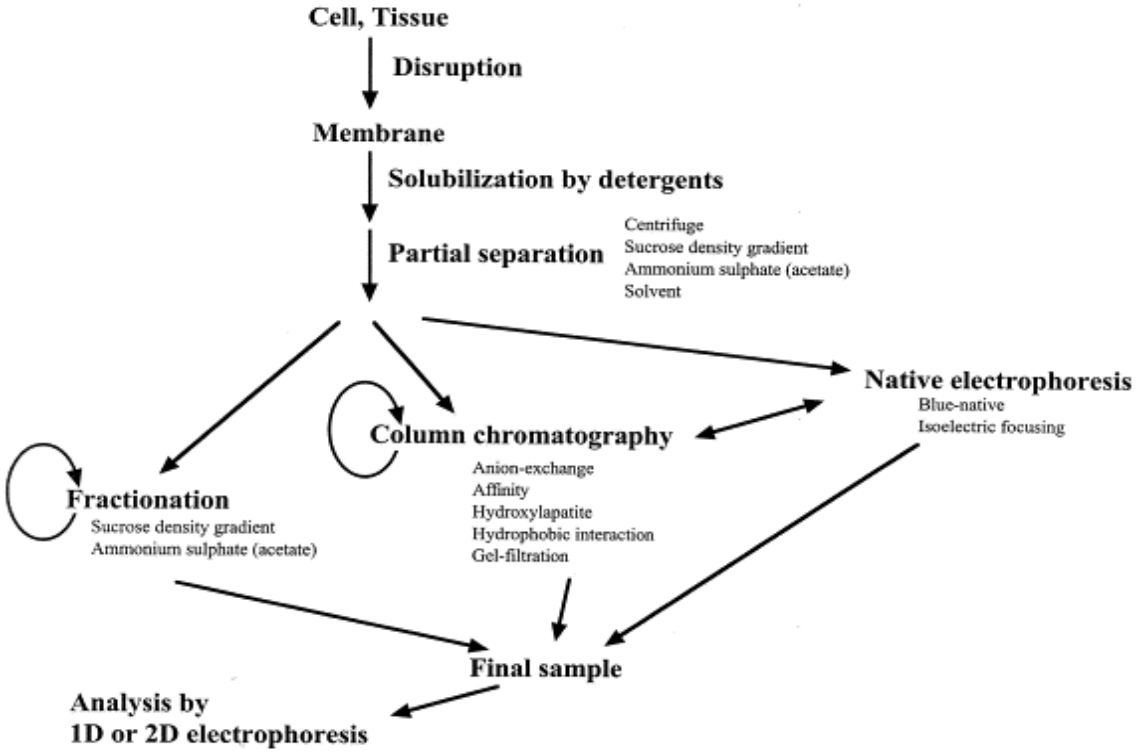


Figure 1.4 Scheme for analysis of protein membrane complexes (Kashino 2003).

Detergents are amphipathic molecules, consisting of a polar, ionic or non-ionic, head group and a hydrophobic tail, and exhibit unique properties in aqueous solutions in which they spontaneously form spherical micellar structures. The hydrophobic part of the detergent molecule is located within the micelle and the hydrophile residues interact with the watery medium. The critical micelle concentration (CMC) is the detergent concentration above which micelles form. For solubilization, the detergent concentration has to be higher than the CMC, because the membrane lipids must be able to incorporate into micelles. Membrane proteins are frequently soluble in detergent micelles. Detergents solubilize membrane proteins by creating a mimic of the natural lipid bilayer environment normally inhabited by

the protein. During solubilization, the hydrophobic tail of detergent molecules dock to the hydrophobic sites (e.g., transmembrane areas) of the protein and partially push out the phospholipids. If sufficient detergent molecules attach, the membrane protein goes into solution. Some membrane proteins are soluble only in a single detergent species that fulfills specific solubilization requirements; others are soluble in many different detergents but are only functionally active in one of them. An understanding of the detergent type and concentration that determine solubilitiy and functionality is crucial to the continued understanding of membrane proteins (Seddon, Curnow et al. 2004; Rehm 2006).

Detergents are classified according to their structure where there is a correlation between the size of the head group and the alkyl side chain volume and the 'mildness' of a detergent. Here, the term 'mildness' refers to the solubility property which leaves the protein's complex structure intact; the longer alkyl chains and the larger the head group, the milder the detergent. There are three different categories of detergent: ionic (linear chain/bile acid salt), nonionic and zwitterionic detergents. Ionic detergents, such as sodium dodecyl sulfate (SDS), are extremely effective in the solubilization of membrane proteins but are almost always denaturing to some extent. Unlike the ionic detergents which disrupt mainly the protein-protein interactions or intra-protein interactions directly, nonionic detergents preferentially disrupt lipid-lipid and lipid-protein interactions; thus, allowing many membrane proteins to be solubilized in nonionic detergents without affecting the protein's structural features that it can be isolated in its biologically or native form. Therefore the nonionic detergents such as *n*-dodecyl-β-D-maltoside, digitonin, and Triton X-100 are the most frequently used for solubilization of protein complexes in native-PAGE. Zwitterionic detergents combine the properties of ionic and nonionic detergents and are in general more deactivating that nonionic detergents (Seddon, Curnow et al. 2004; Reisinger and Eichacker 2008).

1.1.4 Antibody

Structure and properties of antibody

Antibodies are populations of protein molecules (immunoglobulins) that are synthesized by an animal in response to a foreign macromolecule, called an antigen or immunogen (Berg, Tymoczko et al. 2012). The terms antibody and immunoglobulin are used interchangeably, however, immunoglobulins are defined as a family of globular proteins that comprise

antibody molecules and molecules having patterns of molecular structure in common with antibodies (Elgert 1996). The chemical structure of antibodies is related to its function: binding versatility, binding specificity, and biological activity. All antibodies are constructed from paired heavy (**H**) and light (**L**) polypeptide chains, each are composed of constant (**C**) and variable (**V**) regions. There are five classes of antibodies based on the structure of their heavy-chain C domain, or isotypes, i.e. immunoglobulin G (IgG), IgA, IgM, IgD and IgE. IgG, the major antibody in serum, will be used as an example to describe the general structural features of immunoglobulins (Figure 1.5).

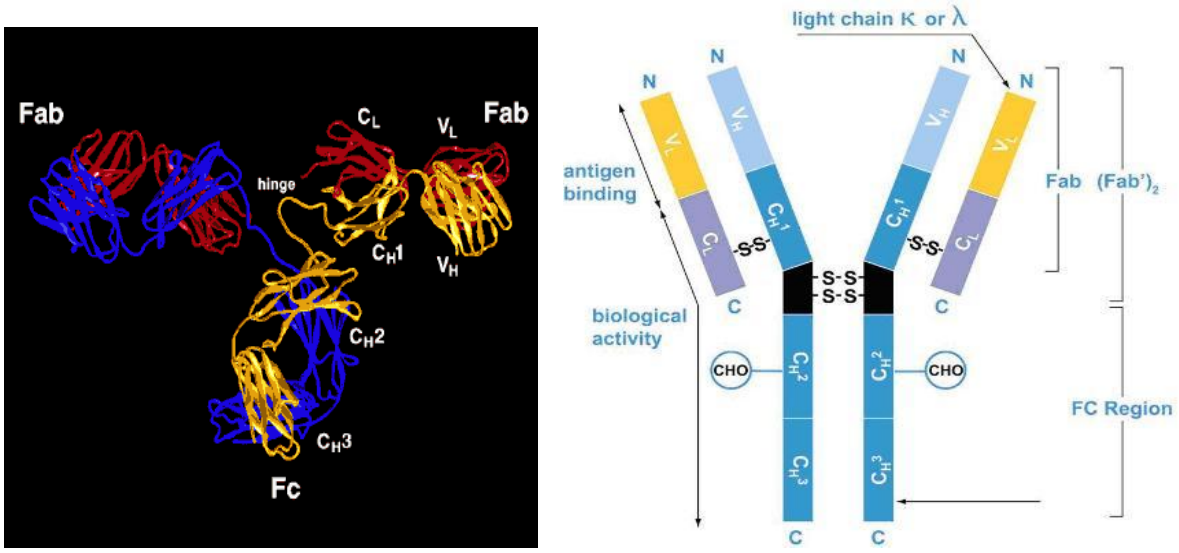


Figure 1.5 Structure of an antibody molecule. Left: A ribbon diagram based on the X-ray crystallographic structure of an IgG antibody (Harris, Larson et al. 1992). Right: A schematic representation of IgG domains (Elgert 1996).

The IgG antibody molecule is made up of four polypeptide chains, comprising two identical light chains and two identical heavy chains, forming a flexible Y-shaped structure. Each of the four chains has a variable region at its amino terminus, which contribute to the antiben-binding site, and a constant region, which determines the isotype. The isotype of the heavy chain determines the functional properties of the antibody. The light chains are bound to the heavy chains by many noncovalent interactions and by disulfide bonds, and the V regions of the heavy and light chains pair in each arm of the Y to generate two identical antigen-binding sites, which lie at the tips of the arms of the Y. The possession of two antigen-binding sites,

Fab fragments (for *fragments of antigen-binding*), allows antibody molecules to cross-link antigens and to bind them much more stably. The trunk of the Y shape, or Fc fragment (for *fragment crystallizable*), is composed of the carboxy-terminal domains of the heavy chains. Joining the arms of the Y to the trunk are the flexible hinge regions. The Fc fragment and hinge regions differ in antibodies of different isotypes, thus determining their functional properties. However, the overall organization of the domains is similar in all isotypes (Elgert 1996; Berg, Tymoczko et al. 2012).

The antibody molecule can readily be cleaved into functionally ditinct fragments. Proteolytic enzymes (protease) that cleave polypeptide sequences have been used to dissect the structure of antibody molecules and to determine which parts of the molecule are responsible for its various functions. The protein fragments obtained after proteolysis are determined by where the protease cuts the antibody molecule in relation to the disulfide bonds that link the two heavy chains (the hinge region). Limited digestion with the protease papain cleaves antibody molecules into three fragments: two identical fragments contain the antigen-binding activity (the Fab fragments) and the other fragment with no antigen-binding activity (the Fc fragment) which is the part of the antibody molecule that interacts with effector molecules and cell. Another protease, pepsin, produce a fragment, the $F(ab')_2$ fragment, in which the two antigen-binding arms of the antibody molecule remain linked and the remaining part of the heavy chain is cut into several small fragments. Reducing agents such as dithiothreitol or mercaptoethanol, unlike protease, cut the antibody molecule on the disulfide bond that link the light chains and the heavy chains in the Fab fragments, giving two light chains and two heavy chains (Stryer 1996; Janeway, Travers et al. 2001).

Antibody-antigen interaction

In the laboratory, antibody-antigen reaction is widely used in techniques such as Western blotting, Enzyme-Linked Immunosorbent Assay (ELISA) and immunoprecipitation. An antigen is usually defined as a substance that causes an immune response when introduced into an organism and is capable of binding with the specific antibodies. The part of a protein antigen recognized by a particular antibody molecule, namely epitope, can be described in a structural and functional sense. Structural epitopes (also called antigenic determinants) are defined by a set of residues or atoms. While a functional epitope consists of antigen residue that contribute significantly to antibody binding, which is usually smaller than structural

epitopes, only three to five residues of the structural epitope contribute significantly to the antibody-antigen binding energy (Ponomarenko and Bourne 2007).

The interaction of antibody with antigen involves conformational changes in both the antibody and the antigen that can range from insignificant to considerable. The specific binding between antigen and the antigen-binding site on the immunoglobulin molecule must overcome an overall repulsion between the two molecules. Strong and specific binding is mediated by the sum of many weak interactions between the antigen and antibody. These weak interactions include hydrogen bonds, van der Waals forces, and ionic and/or hydrophobic interactions (Davies and Cohen 1996; Subramanian 2002). When the epitope and the binding site come to a distance of several nanometers, they are attracted by long-range forces, such as ionic and hydrophobic bonds. Ionic interactions can dominate antigen epitopes but the antigenic determinants are not restricted to highly charged hydrophilic regions on the surface on an antigen and may be dominated by hydrophobic interactions. These attractive forces overcome the water molecules that surround the antibody-antigen interface, water molecules are expelled and the epitope and the binding site approach each other more closely. At this distance, the short-range van der Waals forces predominate, but ionic groups still play a role. At that point, the overall strength of the binding depends on the the goodness of fit between the two surfaces and their total contact area (Hodges, Heaton et al. 1988; Davies and Cohen 1996; Reverberi and Reverberi 2007).

The binding of an antibody to its antigen is a reversible chemical reaction:

$$\text{antibody} + \text{antigen} \rightleftarrows \text{antibody-antigen complex}$$

The strength of the interaction is expressed as the affinity constant K_a, where:

$$K_a = [\text{complex}] / [\text{antibody}][\text{antigen}]$$

The affinity of an antibody reflects the strength of interaction between antibody and antigen at single antigenic sites. Within each antigenic site, the variable region of the antibody interacts through weak non-covalent forces with antigen at numerous sites; the more interactions, the stronger the affinity. Affinity constants can vary widely between different antibodies and antigens, and are affected by pH, temperature, and ionic strength. Another way to measure the antibody-antigen interaction is the avidity of the binding, which is defined as the total binding strength of all of its binding sites together for multivalent binding. Concentrations of antigen and antibody and duration of incubation are also factors that may influence the antibody-antigen reaction (Reverberi and Reverberi 2007; Berg, Tymoczko et al. 2012).

1.1.5 Immunoaffinity Purification Techniques

Recognition of different chemical shapes and structures is a fundamental property of biomolecules. For example, an enzyme (or antibody) is capable of recognizing its substrate (or antigen) and distinguishing it from other molecules that may be chemically similar. This type of biospecific recognition is known as affinity. Antibody-antigen affinity interaction (immunoaffinity) is a powerful tool that can be utilized for separating protein of interest on the basis of a highly specific, reversible biological interaction between the two molecules. Immunoaffinity can be applied by two different techniques, affinitiy chromatography and immunoprecipitation. The fundamental principle of these techniques is immobilization of a small molecule or affinity ligand on a stationary phase and application of sample containing the biomolecule (antigen) to be purified to this phase. Usually, the choice of one technique over the other is dictated by the number of samples that need to be purified simultenously, the amount of protein in each sample, and consideration including time and expense. Immunoaffinity chromatography is a type of liquid chromatography in which the binding affinity of an antigen to an antibody is utilized as a basis of separation. The antibody, immobilized onto a solid matrix and packed into an appropriate column to create a stationary phase, mixed with the antigen solution (the mobile phase) under favorable condition whereby the antibody captures the protein of interest and the other unbound or unwanted proteins are removed by washing. The reversible interaction between the antigen and antibody can be disrupted to yield a highly purified product in the eluate (Subramanian 2002; Sheehan 2009; Abi-Ghanem and Berghman 2012).

In a related application known as immunoprecipitation, this technique of precipitating an antigen out of solution (antigen-containing sample, usually a cell lysate) is most frequently used to study antigen characteristics such as antigen presence and quantity, relative molecular weight, rate of synthesis or degradation, posttranslational modification, and interactions with proteins, nucleic acids, or ligands (Qoronfleh, Ren et al. 2003). However, because specific antibodies are costly to produce or obtain commercially, this approach is seldom used for large scale purification of antigen. Instead, its use is confined almost entirely to very small-scales, most significantly for immunoprecipitation assay. Immunoprecipitation can be referred to the small-scale affinity chromatography or purification of antigen using a specific antibody. After separation from contaminating proteins, the antibody-antigen complexes are disassociated and the proteins of interest are separated by SDS-PAGE. Size and quantity of proteins may then be analyzed either by autoradiography or a gel scanning procedure.

Immunoprecipitation is extremely sensitive and may detect very small amounts of radiolabeled protein antigen (detection level ~100 pg protein or 100 cpm/protein). Unlabeled proteins may be used if other sensitive detection methods are utilized, e.g., enzymatic activity assays or Western blotting. The advantage of the immunoprecipitation technique vs immunoblotting is the possibility to analyze the immune response of proteins expressed in their native conformation (Johansen and Svensson 2002).

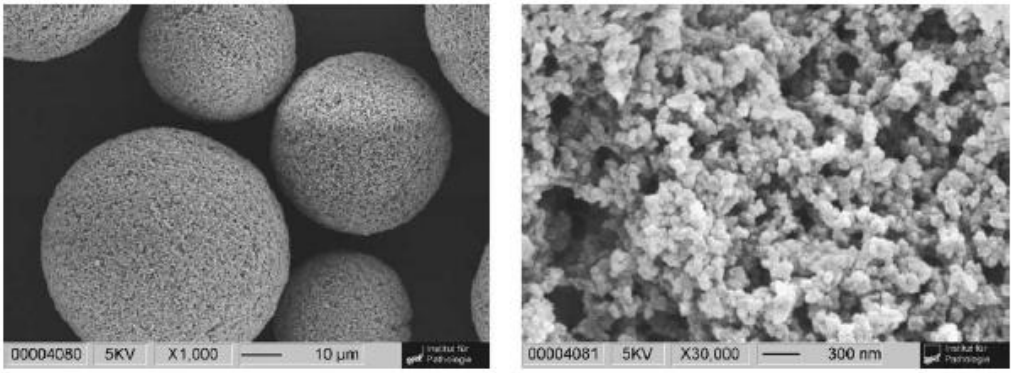


Figure 1.6 Scanning electron microscope presentation of the Toyopearl AF-Tresyl-650M beads (left) and their surface (right) (Kramer, Franke et al. 2004).

For a good efficiency and mechanical stability, the matrix onto which the antibody ligand is attached should be easily modified for antibody attachment, have low nonspecific binding, should be macroporous with uniform particle and pore size. A variety of solid support can be used for immunoaffinity purificaiton, such as carbohydrate-based media (agarose, dextrose, or cellulose) or synthetic organic supports: acrylamide polymers, methacrylic polymers (such as Toyopearl AF-Tresyl-650M, Figure 1.6), polyethersulfone matrices. The low cost of these materials has made these supports popular alternatives for immunoaffinity application although there are other materials that have also been used like silica, azalactone beads, ferrous magnetic beads (which offer easy bench-top separation without a centrifuge), and polystirene-based perfusion media (Moser and Hage 2010).

Immobilization of antibody

A molecule, an enzyme or an antibody is referred to be immobilized if its mobilitiy in the reaction space is artificially restricted. Antibody immobilization enables, primarily, the re-use or continuous use of the antiobody and it also simplifies the manipulation and the control of the reaction process. Moreover, the separation of the antibody from the reaction mixture is significantly easier, contamination of final product is minimized and also for improving the features of the antibody e.g. stability, activity, specificity or selectivity (Benešová and Králová 2012).

A variety of techniques can be used to immobilize antibodies onto matrix supports that range from covalent attachment, affinity binding, to physical adsorption-based methods. Several covalent coupling chemistries are available to immobilize the ligand depending on the available reactive groups. The amine (-NH2), thiol (-SH2) and aldehye (-COOH) coupling chemistries are well established procedures. Covalent coupling is stable and, in general, does not need any modification of the ligand. Moreover, the immobilization level is easily controlled and the ligand consumption is low (Moser and Hage 2010; Abi-Ghanem and Berghman 2012). Antibodies can be covalently coupled to matrix supports by the antibody cross-linking method and the antibody coupling method. The first approach uses a chemical cross-linker, disuccinimidyl suberate (DSS), to attach the Fc part of an antibody to immobilize protein A or protein G. This procedure combines cross-linking and affinity chromatography to generate an oriented antibody-protein A or protein G support. The other method couples the antibody directly onto an activated support (Figure 1.7). This coupling procedure eliminates the need for protein A or protein G, and offers universal coupling of all antibody species and subclasses (Qoronfleh, Ren et al. 2003).

The ideal situation in any of these immobilization methods is to have antibodies attached to the support in a way that does not affect the activity of the binding sites or the accessibility of these sites to the antigen of interest. Antibodies can be immobilized through free amine groups by using supports that have been activated with agents such as carbonyldiimidazole, cyanogen bromide, or tresyl (trifluoroethane sulfonyl) groups. Immobilization of antibody through amine groups can also be done using support matrix that has been treated to produce reactive epoxy or aldehyde groups on its surface. The use of amine groups is one of the easiest ways to immobilize antibodies but can cause a decrease in activity if the antibodies have some of these amine groups in their antigen-binding sites (Moser and Hage 2010).

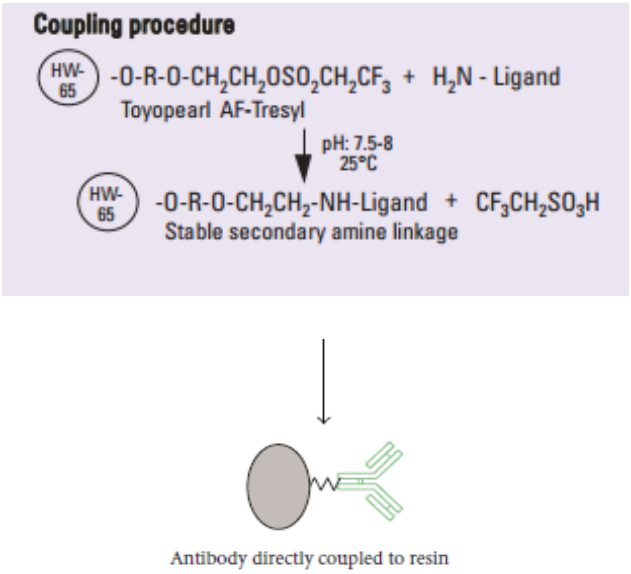


Figure 1.7 Coupling of antibody to Toyopearl AF-Tresyl-650M beads (modified from Tosoh Bioscience manual).Note: R = hydrophilic polymer.

Toyopearl affinity chromatography resins, as described by the manufacturer, with activated functional groups are ready to directly couple a protein or other ligand and can be used to covalently attach almost any custom ligand. Toyopearl affinity resins may be used for solid phase because of their excellent stability in a variety of organic solvents and under extremes of pH. Tresyl-activated resin, like Toyopearl AF-Tresyl-650M, is used to immobilize ligands with free amino or thiol groups, quickly, highly reactive and with high efficiency. It is provided in dry form, ready for reaction in buffered solution containing protein or other ligand. Coupling is accomplished in neutral to slightly alkaline (pH 7-8) solution. Under such conditions, even proteins of limited stability may be succesfully coupled. Coupling leads to the formation of a highly stable secondary amine or thio-ether lingkage. The optimized tresyl-density (ca. 20µmol/ml hydrated resin) is sufficient to provide substantial protein binding while avoiding excessive multi-point attachment and consequent impairment of ligand affinity or activity.

Formation of immune complexes and recovery of the antigen

Binding of antigen to the immobilized antibody can be performed in column or batch format. Column methods involve incubating the immunoaffinity components with beaded resin that is packed in a plastic or glass column. The sample is either allowed to pass the column by gravity or centrifugation or the the column is capped and the sample incubated with the resin to allow the antibody and antigen more time to bind. While the batch method simply involves mixing the component of the reaction in a reaction tube (usually a microcentrifuge tube) for a period of time to allow them to interact. Here the resin and the sample are constantly mixed, thus promoting a maximum contact between the target antigen and immobilized antibody. It often saves time, especially when dealing with large sample volumes, but requires optimisation of the amount of resin used. Because excess resin can result in an increase in nonspecific binding, as well as reduced target recovery due to readsorption during the elution step, it is preferable to saturate the resin with bound target (Abi-Ghanem and Berghman 2012).

Prior to elution step, protein bound by nonspecific interaction is removed by washing. Increasing salt (0.1-0.5M) or changing pH values will reduce ionic interaction, while decreasing salt, altering pH, or adding surfactang (such as Triton X-100) will remove protein bound by nonspecific hydrophobic interactions. The objective of the elution step is to recover the specifically bound protein at a high yield, purity, and stability. The elution conditions should allow for fast elution of the analyte while still allowing later regeneration of the immobilized antibodies. The antigen-antibody complex can be dissociated by counteracting the forces at work in the binding. Elution is thus essentially the reverse process of binding where conditions are optimized to temporarily weaken or lowering the effective strength of antibody binding to the target antigen. The elution method of choice is often the use of low pH (2.0 - 2.5) which disrupts both ionic and hydrogen bonds between antigen and antibody. Other approaches for elution include adding a chaotropic agent (such as thiocyanate, perchlorate, chloride) to the mobile phase, adding a competing agent, organic modifier or denaturing agent (like 8M urea or 6M guanidin hydrochloride), or changing the temperature of the column during elution. Following elution, the column should always be washed with neutral pH buffer (i.e., pH 7.0 – 7.4) to allow for regeneration of the antibodies (Subramanian 2002; Moser and Hage 2010; Abi-Ghanem and Berghman 2012).

1.2 Objectives of Thesis

The thesis set out to learn and understand the biology or chemistry basis and to apply different techniques used in protein biochemistry research. Among the various methods of studying proteins, affinity method is regarded as one of the most effective means of purifying proteins as a result of its high degree of specificity. However, hoping that this work can produce a reliable technique, the emphasis on this experiment is to employ the principles of affinity interactions between one type of the ligh-harvesting like (Lil) protein famililes, Lil3 proteins (as the antigen), and the antibody in order to obtain Lil3 proteins from the plastid extract of protein membrane complexes. Several aplications of biophysical techniques including SDS and native-PAGE, plastid isolation, solubilization of protein membrane complexes, immobilization efficiency of antibody, Lil3 antibody-antigen interaction, and recovery of the desired antigen will also be performed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant material

Seeds of barley (*Hordeum vulgare* L cultivar Steffi) were sown by spreading them as a layer on vermiculite without adding any growth media but water and grown in a dark chamber (25°C). Seedling were harvested after 4-5 days and illuminated with white light for 10 seconds, 1h, 2h or 4h (depends on need) just before plastid isolation. In case of the intention was to isolate chloroplast, the seedling would be grown in a light chamber.

2.2 Plastid isolation

Plastids were isolated from the seedlings by cutting the upper layer (about 2 cm from top) of the leaves and collecting them in ice cold isolation medium containing 400mM D-Sorbitol, 50mM Hepes/KOH pH 8.0, 2mM EDTA. The leaves were cut into small pieces using homogenisator, an ultra thurax, or blender to release plastids from the leaves cells. Homogenate was filtered through layer of folded gauze bandage and a nylon gauze of 22µm pore size followed by centrifugation for 2-3 minutes (5000 rpm, 4°C). The supernatant was discarded and the pellet was resuspended in the remaining liquid by shaking the tube on ice, prior to filtering through a nylon gauze (pore size of 22µm) into a Percoll gradient solution which consisted of 40% Percoll solution in the upper layer and 80% in the lower layer. Centrifugation for 8 minutes (5000 rpm, 4°C) separated intact plastids from broken ones (Figure 2.1). The intact plastids were collected in a new tube and washed in a washing buffer (400mM Sorbitol and 50mM Hepes/KOH pH 8.0). After centrifugation for 3 minutes (5000 rpm, 4°C), the supernatant was discarded and the pellet of intact plastids was resuspended in the medium containing sorbitol and then transferred into a micro tube. The concentration of plastids was determined before further use or storage in -80°C freezer. All the steps for plastid isolation were carried out on ice, or 4°C, to preserve the plastids and their proteins in their nature conditions.

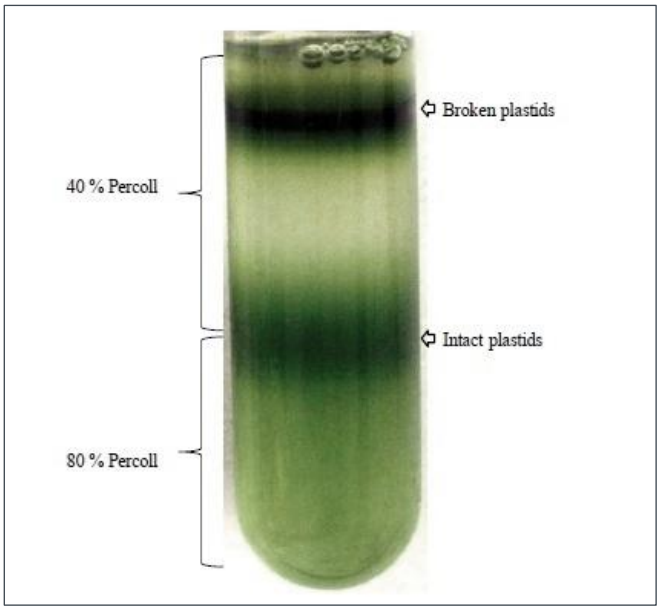


Figure 2.1. Plastids separated in Percoll gradient solution (Bue, 2009).

Estimation of number of plastids

The number of plastids in certain unit volume was determined using a counting chamber that has engraved grid of perpendicular lines (or, a haemocytometer, since it was originally designed for counting blood cells). The plastid suspension was mixed well before taking a sample to ensure the sample is representative and a dilution was made so that they do not overlap each other on the grid and evenly distributed. The counting was performed by transferring 10µl of a diluted plastid mixture (containing 2µl of isolated plastids and 998µl of washing medium) to a Thoma counting chamber. The number of plastids in 4 sets of 16 corner square was counted under a microscope. The total number of plastid per unit volume was estimated by simply multiplying the total number of plastids found in the counting chamber grid by the dilution factor. Briefly, number of plastid / µl = number of plastid in the 4 sets of square x 4 x 10 x 500 (dilution).

2.3 Clear Native–PAGE

Sample preparation for clear native–PAGE

All the steps in this preparation was performed on ice, or 4°C. A number of plastid, e.g. $1x10^8$,was transferred into a micro tube and were lysed by adding 200 µl of TMK buffer

(10mM Tris-HCl pH 8.5, 10mM $MgCl^2$, 20mM KCl) and incubating the solution on ice for at least 10 minutes. After centrifugation for 3 minutes (at 7000 rpm, 4°C), the supernatant containing all soluble and peripheral proteins was removed and the pellet was washed with TMK buffer followed by centrifugation. The washing step was repeated two times to remove the soluble protein of the plastids. Then, the pellet, the thylakoid membranes, was resuspended in 70µl of TMK buffer and solubilized by adding in 10µl of detergent mix containing 10% (w/v) n-dodecyl-β-D-maltoside, 10% (w/v) digitonin and 5% (w/v) lithium dodecyl sulfate and incubating the solution on ice for 20 minutes. Finally, it was centrifuged for 10 minutes (at maximum speed, 4°C) to pellet the unsolubilized material. The supernatant was used as the cell lysate and transferred into a new tube.

Casting of separating and stacking gels

Native gels, each consist of 7.5% separating gel and 4% stacking gel, were used to separate protein complexes.

	Separating gels 7.5% (30 ml)	Stacking gels 4% (5 ml)
Acrylamide 30%	7.875 ml	0.675 ml
6xGel buffer	5.25 ml	0.835 ml
H_2O	16.375 ml	3.49 ml
APS	60 µl	50 µl
TEMED	15 µl	5 µl

Gels were cast vertically in a set of sandwich of oxyde plates and glass plates separated by spacers which run along the side of the plates. Casting of separating and stacking gels was performed as described previously (Reisinger and Eichacker 2006).

Electrophoresis

In clear-native PAGE, the migration distance depends on the protein intrinsic charge, and on the pore size of the gel since no charged dye is used; unlike blue-native PAGE which uses negatively charged protein-bound Coomassie dye to impose a charge shift on the proteins. Clear-native electrophoresis is milder than the blue-native PAGE, and offers advantages

whenever Coomassie dye interferes with techniques required to further analyze the native complexes (Wittig and Schαgger 2005). Chemicals and solutions used for clear-native electrophoresis in this experiment are listed in Appendix. The polymerized gels were assembled in to electrophoresis chamber. Electrophoresis buffers were poured in, cathode buffer into the upper chamber and anode buffer in the lower chamber. Each well of the gel was rinsed 6-8 times with anode buffer using a microsyringe before loading the samples. Then, samples were loaded onto the gel using microsyringe, 18-20 µl in each well. Finally, the eletrophoresis assembly connected to a power supply and attached to a cooling apparatus that was set at 4°C. The electrophoresis was run for 1 hour at 12 mA, 1000 V and 24 W.

2.4 SDS-PAGE

Sample preparation for SDS-PAGE analysis

The membrane fraction of plastid (from $1x10^8$ plastids, corresponding to ~400µg protein) was centrifuged at 4°C. The supernatan was discarded and the pellet was washed with TMK buffer followed by centrifugation. This step was repeated two times. Then, the thylakoid membranes pellet was resuspended in 60µl of TMK buffer and 30µl of solubilization buffer (3xSB) consisting of 6% w/v SDS, 30% w/v sucrose, 0.1% w/v bromphenolblue, 200mM Na_2CO_3 and 200mM dithiothreitol. The sample was then incubated at 72°C for 2 minutes followed by centrifugation for 5 minutes (max speed, at 15°C) to settle down unsolubilized material. The supernatant was used as sample for SDS electrophoresis.

Electrophoresis

SDS gels consisting of 12.5% separating gel and 4% stacking gel were cast as described (Reisinger and Eichacker 2006). A clean 10 wells comb was inserted in between the plates sandwich. After the gels polymerized, the electrophoretic apparatus was assembled and filled in with buffers. SDS running buffer was used for both the cathode and anode buffer (see Appendix). Each well of the gel was washed (by pipetting up and down) 6-8 times with anode buffer using a microsyringe before loading the samples. Samples were applied into the gel 18-20 µl in each well. Then, the eletrophoresis assembly was connected to a power supply set at 15 mA (30 mA for two gels), 1200 V, 24 W and attached to a cooling apparatus that

was set at 15°C. The electrophoresis was run for about 1 hour or until the running front reached the end of the gel.

2.5 Coomassie Staining

Visualization of separated protein following the electrophoresis was achieved by Coomassie staining. The gel was placed in fixing solution (40% ethanol and 10% acetic acid) and put on a shaker for at least1 hour. The fixing solution was removed and staining solution (see Appendix) was added and the gel was incubated for at least 3 hours (up to overnight) with constant shaking. Destaining step was performed by placing the gel in water and changing the water several times until the background of the gel was clear. Water with 20% methanol was used when the background blue color was not sufficiently removed with only water.

2.6 Western Blotting

After electrophoresis, the proteins in the gel were transferred to a hybond-ECL nitrocellulose membrane (by GE Healthcare) using a blotting system as described (Towbin, Staehelin et al. 1979). As for semi-dry transfer, a sandwich consisted of paper (3 layers), nitrocellulose membrane, electrophoresed gel, and three layers of paper was immersed subsequently in Towbin solution (96mM Glycine, 10mM Tris and 10% (v/v) methanol), then placed in between two carbon plates (cathode and anode) in the blotting apparatus and connected to a power supply set at 20V and ~200 mA (2mA per cm^2 of the blotting sandwich) for 1 hour.

Immunodetection

The protein-blotted membrane was steeped in TBS solution (10mM Tris/HCl pH 7.5, 150mM NaCl, and 0.05% (v/v) Tween-20) and then blocked with a solution of 5% (w/v) milk in TBS for 1 hour. Blocking the membrane prevents non-specific background binding of the primary and/or secondary antibodies to the membrane (Towbin, Staehelin et al. 1979). The membrane was incubated with primary antibody for 1 hour at room temperature, washed thoroughly with adequate volume of TBS (washed 3 x 5 minutes) to remove any unbound, excess antibody, and then incubated with horseradish peroxidase(HRP)-conjugated secondary

antibody directed against the primary antibody. The washing step was repeated three times and then the membrane was subjected to chemiluminescent substrates for detection.

Chemiluminescence detection

The enhanced chemiluninescent (ECL) substrate for detection of horseradish peroxidase (HRP) activity from the secondary antibodies (Figure 2.2) was prepared by mixing an equal volumes of ECL reagants 1 and 2 (listed in Appendix) shortly before used. The blot membrane was kept in the working reagent for 1 minute at room temperature. After the excess reagent was drained, the membrane was placed in a clear plastic pocket and exposed to Hyperfilm ECL using a light tight cassette for about 3-4 minutes. Then, the film was put in Kodak D-19 Developer solution until the signals or bands appeared, rinsed in water and then placed in Kodak rapid fixer solution. Finally, the film was rinsed in water and air dried. All the steps involved in the ECL film-developing were performed in darkroom with red light.

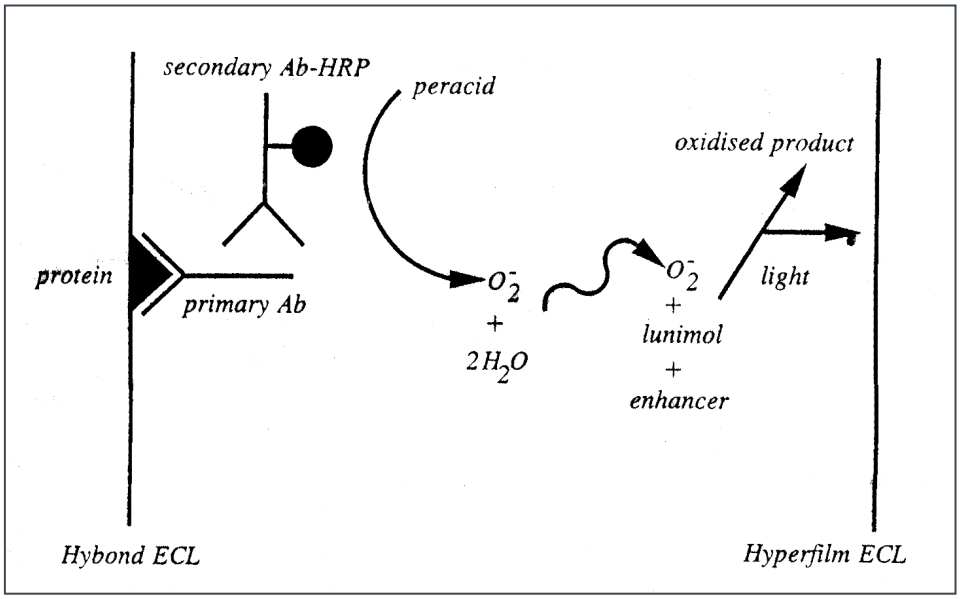


Figure 2.2. Diagram showing the mechanism of immunodetection of proteins on Western blots using the ECL system (Crisp and Dunn 1994).

2.7 Antibody Purification by Precipitation with Sodium Sulfate

Addition of appropriate amounts of salts, such as ammonium or sodium sulfate, causes precipitation of IgG and they are suitable for many immunochemical procedures, e.g., production of immunoaffinity columns. Lil3 antibody-containing serum (from rabbit) was purchased from Agrisera, Sweden. Sodium sulfate precipitation for Lil3 antibody was performed as follows (Page and Thorpe 2002). The antibody-containing serum was centrifuged at 10000g for 25 minutes. The pellet was discarded and the supernatant, the serum, was warmed to 25°C and stirred. Solid Na_2SO_4 was added gradually to produce an 18% w/v solution (i.e., add 1.8 g/10 mL) while stirring at 25°C for 1 hour. Centrifugation was conducted at 2000-4000g for 30 minutes, the supernatant was discarded, the excess liquid was drained and the pellet was redisolved in PBS buffer (containing 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4). Initially, the precipitate was disolved in 10-20% of the original volume in PBS buffer by careful mixing with a spatula and when fully dispersed, more buffer was added to give 25-50% of the original volume.

2.8 Protein Determination

BCA Protein Assay

Many different methods are available to estimate the total protein concentration. In this experiment, the protein concentration was determined by using bicinchoninic acid reagent (Pierce BCA Protein Assay Kit) with bovine serum albumin as a standard as described in the manufacturer manual. Protein was added to the reagent and produced a color change. The intensity of the colored reaction product is in proportion to the amount of protein that can be determined by comparing its absorbance value to a standard curve. Protein concentration was determined by reference to a standard curve consisting of known concentration of the standard protein. The standard curve was plotted with the absorbance value as the dependent variable (y-axis) and concentration as the independent variable (x-axis) resulted in an equation: $y = ax + b$. Solving for x, by inserting the sample's absorbance value, determined the protein concentration of the sample.

UV Absorbance at 280nm

A simple and direct assay method for protein determination was also conducted by measuring the absorbance at 280nm (UV range) using quartz cuvets. This method was performed to estimate the antibody-beads coupling efficiencies by measuring the absorbance of antibody solution before and after coupling. Absorbance values of the unknown samples were then interpolated onto the equation for the standard curve to determine their concentration.

TINA 2.0 Software

Quantitative densitometry of SDS-PAGE bands was perfomed to estimate unknown concentration of protein samples. Protein samples and a set of diluted bovine serum albumin (BSA) as protein standard were analyzed by SDS-PAGE, followed by staining with Coomaasie Brilliant Blue. The concentration of protein samples were quantitatively determined by measuring band densities of digitally scanned gels using Epson 1640 Scanner and TINA 2.0 computer software (Raytest, Straubenhardt, Germany), and comparing their band intensities to those of the standards.

2.9 Immunoaffinity Purification Techniques

Immobilization of antibody using Toyopearl AF-Tresyl-650M

Antibody was immobilized onto the beaded support through covalent coupling. Coupling of Lil3 antibody to Toyopearl AF-Tresyl 650M (Tosoh Bioscience, Germany) beads was performed as describe in the manufacterer instruction manual. Performing the experiment by batch method, the components of the coupling procedure was mixed in a microcentrifuge tube (Eppendorf tubes). 1ml Lil3 antibody of 1 mg/ml solution in coupling buffer (0.1M $NaHCO_3$ with 0.5M NaCl at pH between 8-9) was added to 25 mg of dry Toyopearl resin. The coupling reaction was allowed to proceed for 4 h at 25°C or overnight at 4°C before washing with 0.5M NaCl to remove unreacted ligand. Coupling efficiency was estimated by measuring the protein concentration left in solution by absorbance at 280 nm and assuming that any protein not remaining in solution was bound to the resin (Qoronfleh, Ren et al. 2003; Jacobs, Wu et al. 2010). The remaining unreacted tresyl groups were blocked by incubating the resin in blocking buffer (0.1M Tris-HCl pH 8.0 containing 0.5M NaCl for 1h at 25°C or

4h at 4°C followed by washing with buffer containing 0.5M NaCl. A control batch was generated by blocking 1ml of swollen Toyopearl resin in blocking buffer without coupling any antibody to the surface.

Immunocapture

Membrane protein complexes from plastid were prepared as described previously in section 2.3. The thylakoid membranes was resuspended in 70µl of TMK buffer and solubilized by adding in 10µl of detergent mix and incubating the solution on ice for 20 minutes. After centrifugation for 10 minutes at maximum speed, the supernatant was used as the cell lysate and bound to the antibody-coupled Toyopearl beads. The immunocapture process was carried out at 4°C for 1-2 h with rotation. The resin-bound antigen was washed several times with washing buffer (containing 50mM Tris-HCl pH 7.5, 150mM NaCl and 2 mM EDTA). The elution of the immune complex was conducted using reducing SDS-PAGE sample buffer (i.e. the 3xSB), or nondenaturing elution buffer 0.1M Glycine pH 2.5. The low pH of the elution was adjusted to neutrality by adding a small volume of 1M Tris-HCl pH 9.0 (Miernyk and Thelen 2008). The flow-through, the wash and the elution were analyzed by SDS-PAGE, followed by Western blotting using Lil3 antibody as the primary antibody and anti-rabbit IgG conjugated with horseradish peroxidase as the secondary antibody, and detection was carried out using chemiluminescent substrate followed by exposure to X-ray film (Hyperfilm ECL, GE Healthcare).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 A Brief Analysis of Protein Membrane Complexes

Membrane proteins are responsible for most of the dynamic processes carried out by membranes. Membrane lipids form a permeability barrier and thereby establish compartments, whereas specific proteins mediate nearly all other membrane functions. In particular, proteins transport chemicals and information across a membrane. Membrane lipids create the appropriate environment for the action of such proteins (Kashino 2003). To study protein membrane complexes, the first important step in purifying membrane protein complexes from any membrane system is to solubilize them from their environment surrounded by lipids. The success of isolation relies greatly on the choice of detergents and their concentrations, especially when purification of the membrane protein complexes in their intact (native) form is wanted (Reisinger and Eichacker 2008).

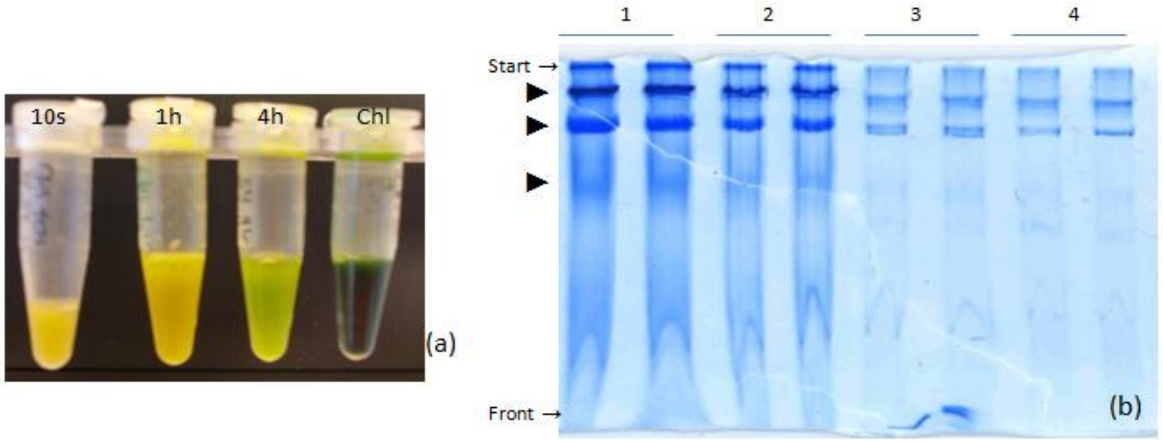


Figure 3.1 Isolation and Coomassie-stained native-PAGE of thylakoid membrane protein complexes. **(a)** Chloroplast (Chl) and plastids isolated from barley seedling illuminated for 10 seconds (10s), 1 hour (1h), 4 hours (4h). **(b)** Different number of chloroplast 10^8, $5x10^7$, 10^7, $5x10^6$ (lane 1-4, respectively) were solubilized with detergent mix and separated by 7.5% native-PAGE. Coomassie stained protein complexes appear blue in distinct bands (marked by ►) in each lane.

Thylakoid membranes from chloroplasts were solubilized with detergent mix of two nonionic (digitonin and dodecyl maltoside) detergents and one ionic detergent (lithium dodecyl sulfate), then subjected to 7.5% native PAGE followed by Coomassie staining (Figure 3.1b). Protein complexes binding chlorophyll appear blue (►). As stated before, unlike the ionic detergents which disrupt mainly the protein-protein interactions or intra-protein interactions directly, nonionic detergents preferentially disrupt lipid-lipid and lipid-protein interactions; thus, allowing many membrane proteins to be solubilized in nonionic detergents without affecting the protein's structural features that it can be isolated in its biologically or native form. Therefore the nonionic detergents such as *n*-dodecyl-β-D-maltoside and digitonin are the most frequently used for solubilization of protein complexes in native-PAGE (Seddon, Curnow et al. 2004; Reisinger and Eichacker 2008). One-dimensional clear native-PAGE was performed to separate native complexes and supercomplexes. It has been suggested to identify the complexes contained in supercomplexes following 2D BN-PAGE, and the protein subunits could optionally be identified by 3D SDS-PAGE (Wittig and Schαgger 2005).

In many methods for separation of proteins, including the milder condition of clear native-PAGE as conducted in this experiment, the choice of detergents and their concentration are the first important steps. The detergent concentration, for solubilization of membrane proteins has to be higher than the critical micelle concentration. When the detergent concentration is too low, or protein complexes are too large, membranes are not solubilized. On the other hand, if detergent concentration is too high, in this case relative to the number of plastids, protein complexes may be lost as indicated by the fading of blue color (lane 1-4, ►) as the number of plastid decreased. Upon application of the right concentration of detergent, the molecular mass of protein complexes is gradually decreased from the start to the front line of the gel (→). Reisinger and Eichacker (2007) suggested a four-step way to find out the most suitable working concentration of detergent relative to the amount of protein complexes.

3.2 Purification of Antibody by Precipitation with Sodium Sulfate

Specific antibody is necessary for the subsequent purification of specific antigens. Antibodies used as ligands can be purified by precipitation. Addition of appropriate amounts of salts, such as ammonium or sodium sulfate, causes precipitation of IgG and they are suitable for

many immunochemical procedures, e.g., production of immunoaffinity columns (Page and Thorpe 2002).

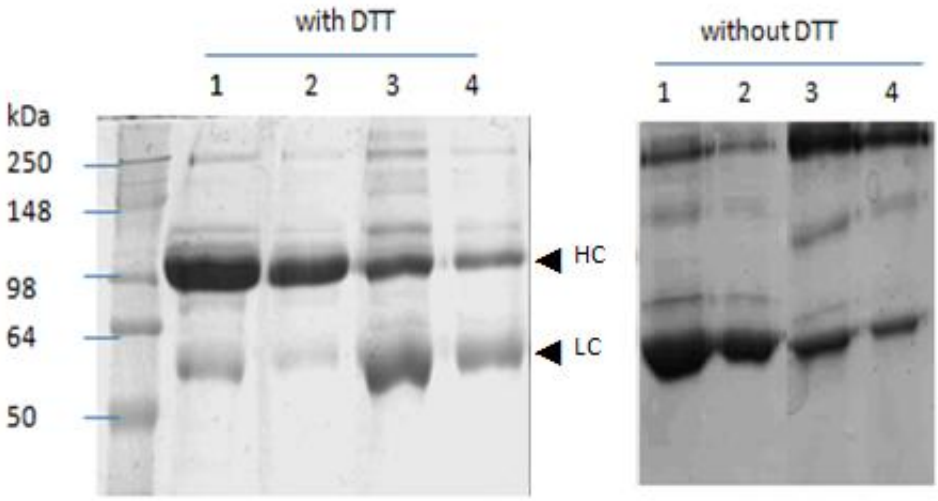


Figure 3.2 Comparison of detergent with and without dithiothreitol (DTT) for solubilization and separation of antibody by SDS-PAGE. Lil3 antibody from serum (lane 1, 2) and sodium sulfate precipitated (lane 3, 4), each diluted to 1:50 and 1:100, were solubilized with 3xSB buffer with and without DTT and then applied to separation by SDS-PAGE (12%). After electrophoresis the gels were stained with colloidal Coomassie. Solubilization with DTT cleaved the antibody into heavy chains (HC) and light chains (LC).

The Lil3 antibody used for this experiment was precipitated from the serum by 18% (w/v) saturated sodioum sulfate. Antibody from the serum and the precipitated were diluted and solubilized by SDS sample buffer (3xSB), with and without DTT, before subjected to separation by 12% SDS-PAGE (Figure 3.2). Similar to what have been stated previously by Elgert (1996), reducint agent such as dithiothreitol cut the antibody molecule on the disulfide bond, giving light and heavy chains that appear as two distinct bands in a different molecular weight (HC and LC). The Coomassie-stained SDS gel also indicates that purification by precipitation with sodium sulfate removed the serum from the antibody.

3.2.1 Activity Test of Precipitated Antibody

Following the precipitation, a test was carried out to find out whether the Lil3 antibody was still active, meaning the antibody did not lose the affinity to specifically bind Lil3 protein (or protein complexes) when applied against a crude source that contains the protein.

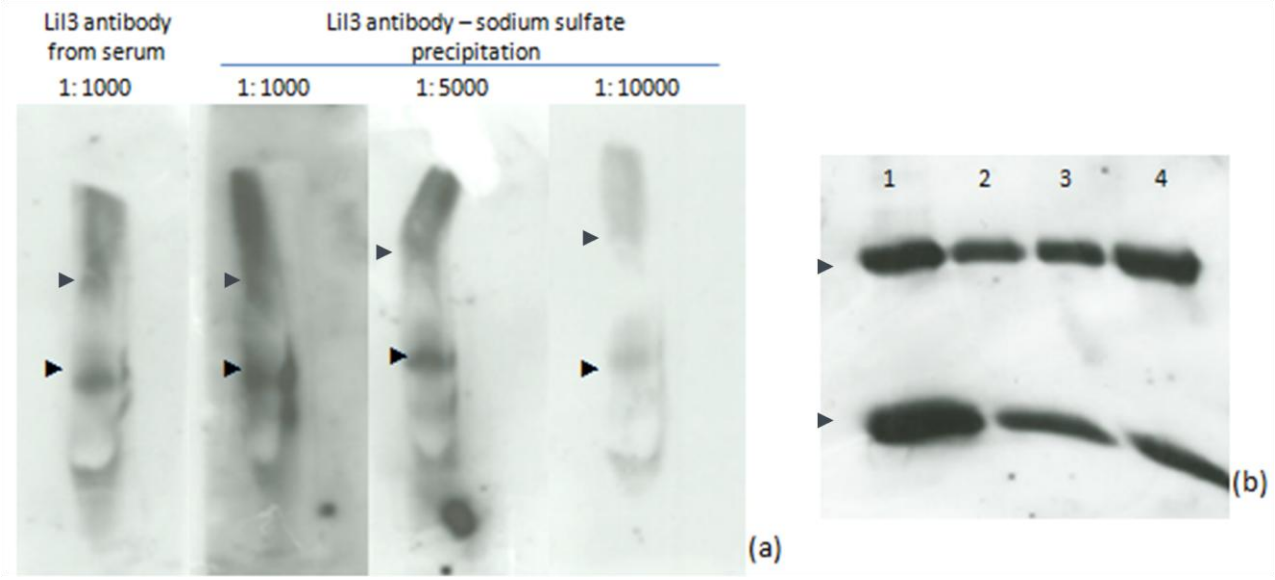


Figure 3.3 Gel blot analysis of antibody activity test. **(a)** Native-PAGE of membrane-bound proteins from 10 seconds illuminated plastid. After electrophoresis the gel was blotted and subjected to antibody detection in several dilutions (1:1000, 5000, 10000). The protein (or protein complex) specifically recognized by Lil3 antibody (from serum and sodium sulfate precipitated) appear as greyscale bands (►). **(b)** SDS-PAGE of solubilized thylakoid membrane from $1x10^8$ plastids: 10 s illuminated, 1 hour, 4 hours and chloroplast (lanes 1-4, respectively). Western blotting of the gel was followed by immunodetection using Lil3 antibody as the primary antibody and antirabbit as the secondary antibody. The specific interaction between Lil3 antibody and Lil3 proteins appear as bands in each lane (►).

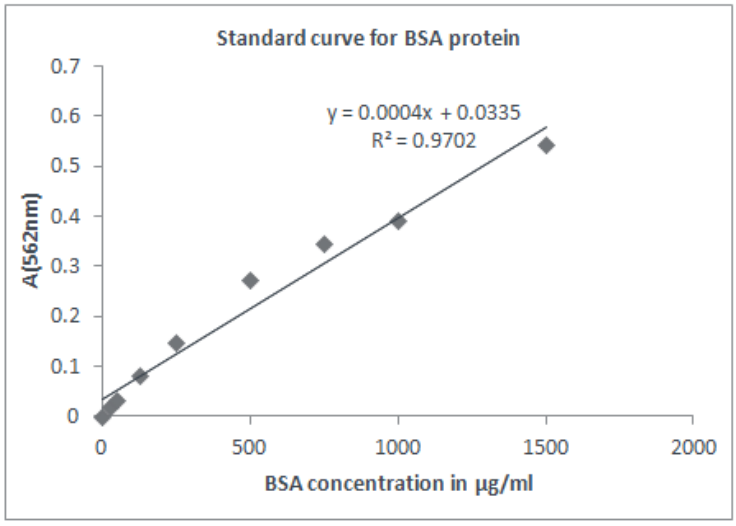
Thylakoid membrane isolated from 10 seconds illuminated plastids were solubilized according to sample preparation for native-PAGE. Following the electrophoresis, Western blotting was conducted and the membrane blot was cut into four pieces, each subjected to different dilution of antibody from serum and the precipitated antibody (Figure 3.3a). In all blottings, the ECL signals (►) corresponding to Lil3 proteins were detected. This result indicated that the antibody was active. Antibody from serum diluted 1:1000 and precipitated antibody (1:5000) seem to be in the same strength of affinity interaction, so in a way it can be said that precipitation by sodium sulfate increased the reactivity of antibody to five folds.

The blotting following an SDS electrophoresis is shown by Figure 3.3b. The crude source of Lil3 proteins was obtained from thylakoid membrane isolated from $1x10^8$ plastids of 10 seconds illuminated, 1 hour, 4 hours and chloroplasts (lanes 1-4, respectively). Western blotting of the gel was followed by immunodetection using Lil3 antibody as the primary antibody and antirabbit as the secondary antibody. The specific interaction between Lil3 antibody and Lil3 proteins appear as two strong ECL signals, indicating that Lil3 protein and the complex exist throughout development of the plastids; as similarly reported by Bue (2009).

3.2.2 Concentration Determination of Precipitated Antibody

Quantitation by BCA protein assay kit

In this experiment, the concentration determination was estimated by using bicinchoninic acid (BCA) reagent with bovine serum albumin (BSA) as a standard. A set of diluted BSA standards and the antibody sample were added to the reagent to produce a colored reaction which is in proportion to the amount of protein. The absorbance of all the BSA standards and the antibody sample were measured with the spectrophotometer set to 562nm within 10 minutes as suggested by the manufacturer's manual.



Sample (µg/ml)	A_{562}
BSA 1500	0.540
BSA 1000	0.390
BSA 750	0.346
BSA 500	0.272
BSA 250	0.1485
BSA 125	0.080
BSA 50	0.032
BSA 25	0.0185
Antibody (diluted 100x)	0.2855

Figure 3.4 Plot of BSA protein standards vs the absorbance at λ=562 nm. Right: summary of numeric report of absorbance generated by spectrophotometer.

The intensity of the colored reaction product is a direct function of protein amount that can be determined by comparing its absorbance value to a standard curve. Using Microsoft Office Excel to plot and apply a standar curve (Fig. 3.4) with the absorbance value as the dependent variable (Y-axis) and concentration as the independent variable (X-axis), resulted in an linear regression equation: y = 0.0004x + 0.0335, where solving for x determines the protein concentration of the sample. Knowing that the antibody's absorbance value was y = 0.2855, and inserting that value into the equation by calculating the value for x, $x = \frac{0.2855-0.0335}{0.0004} =$ 630.75 μg/ml, determined the antibody concentration. The antibody sample that was loaded into the gel was diluted 100 times, so originally the concentration of the precipitated antibody stock was about 63 mg/ml.

The BCA assay is related to the Lowry assay in that peptide bonds of protein first reduce cupric ion (Cu2+) to produce tetradentate–cuprous ion (Cu+) complex in an alkaline medium. The cuprous ion complex then reacts with BCA (2 molecules per Cu) to form an intense purple color that can be measured at 562 nm. BCA is stable in alkaline medium, therfore this assay can be carried out in one step. Another advantage of the BCA assay is that it is compatible or offers more tolerance with samples that contain up to 5% concentration of detergents (e.g., sodium dodecyl sulfate (SDS), Triton X-100, Tween 20) without interfering with the assay. The BCA assay also offers increased sensitivity and response more uniformly to different proteins. However, the fact that reducing agents interfere with the assay and in turn effect the determination of sample concentration, can be considered as the disadvantage (Antharavally, Mallia et al. 2009).

Quantitation by Epson scanner and TINA 2.0 software

Optical density evaluation of Coomassie Blue-stained protein on SDS-PAGE gel (Figure 3.5) was performed using a desktop scanner employing white light (such as Epson 1640) and TINA 2.0 software. Equal volumes of six BSA standards, ranging from 750 to 25 μg/ml, and a sample of antibody with unknown concentration were loaded and electrophoresed. Each protein band was manually selected as regions of interest and the intensities were measured (arbitrary optical density units) using the software TINA 2.0. The intensity values of each regions of interest, which is in porportion to the amount of proteins loaded, were plotted against protein concentration to make a standard curve.

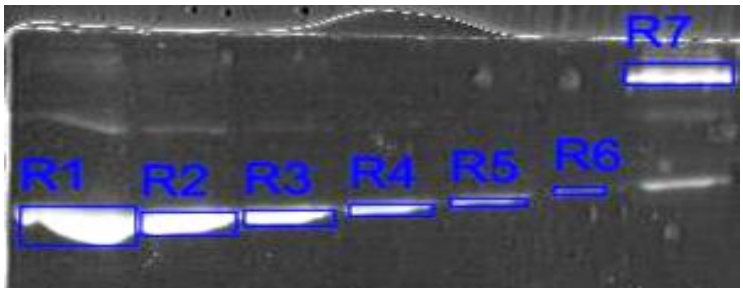
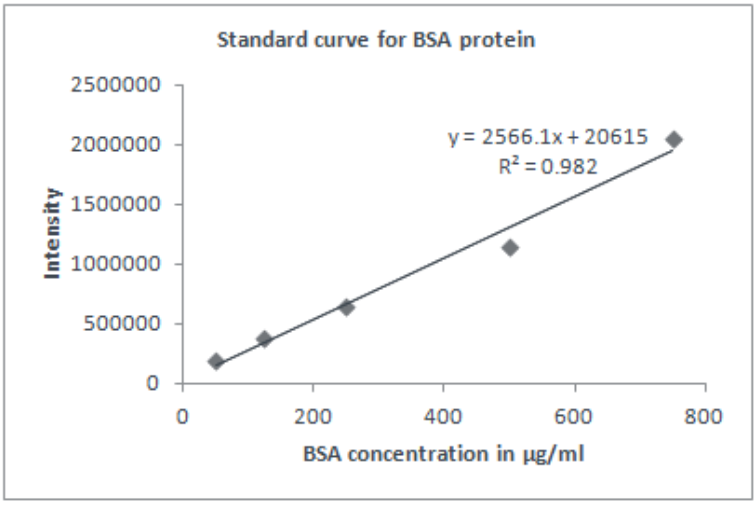


Figure 3.5 An image generated by TINA 2.0 software from a digitally scanned SDS-PAGE gel using Epson scanner. Six different concentration of diluted BSA standard (R1-R6) and the unknown concentration of precipitated antibody (R7) were solubilized with SDS solubilization buffer (3xSB) without dithiothreitol. After the removal of unsolubilized material by centrifugation, they were applied to separation by SDS-PAGE followed by staining with Coomassie dye. The regions of interest for each lane are outlined by boxes and marked R1-R7. The intensity was calculated for each regions of interest from scanner output in the black and white image format by TINA 2.0 computer software.



	Sample [µl/mg]	Intensity (od-bkg)
R1	BSA 750	2050053.00
R2	BSA 500	1141228.00
R3	BSA 250	648725.00
R4	BSA 125	372991.00
R5	BSA 50	188286.00
R6	BSA 25	87967.00
R7	Antibody (diluted 100x)	936375.00

Figure 3.6 Plot of BSA protein standards vs the intensity of the regions of interest analyzed by TINA 2.0 software. Right: summary of numeric report of optical density in region of interest (as shown in Fig 3.5) given by TINA 2.0 software.

The relationship between BSA protein concentration and the intensity for regions of interest (Fig. 3.6) is fitted to a linear regression with the equation: y = 2566.1x + 20615. This scanner/software system apparently demonstrated an accuracy in quantifying protein concentration as the instruments detected a linear change in optical density along with respective protein concentration. Solving for x in the eqution given by the standard curve, y =

2566.1x + 20615, by inserting the antibody's intensity value (where y = 936375.00), determined the antibody concentration. The antibody sample loaded into the gel was diluted 100 times, so originally the precipitated antibody stock was 35, 68 mg/ml.

The estimation of antibody concentration given by the BCA protein assay was higher compare to that of scanner/software system. Each method for quantitavely determination of protein concentration is different and has its limitations, depends on the chemistries involved with each type of assay. The rate of BCA color formation is dependent on the incubation time and temperature. Subtances that reduce copper that might present in the sample solution, and certain single amino acids (cystein, tyrosine and tryptophan) will also produce color in the BCA assay thus interfering with the accuracy of the protein quantitation (Thermo Scientific protein assay handbook). The performance of a software/scanner system that employed a desktop scanner and a customized software package for densitometric quantification of protein loads stained with Coomassie dye following SDS-PAGE have been evaluated and validated as accurate and reproducible; with the conditon of complete and uniform staining of the protein accros the gel (Vincent, Cunningham et al. 1997).

3.3 Immunocapture of Lil3 proteins using antibody-coupled beads

Binding of antigen to the immobilized antibody was performed in batch format where the antibody-coupled beads and the crude mixture containing the protein of interest were mixed in a microcentrifuge tube and allowed to interact. Recently, Abi-Ghanem *et al.* (2012) reported that it is preferable to saturate the resin with bound target because excess resin can result in an increase in nonspeficic binding, also reduced protein target recovery due to readsorption during the elution step. Accordingly, it is important to optimize the amount of resin used as the antibody-beads column.

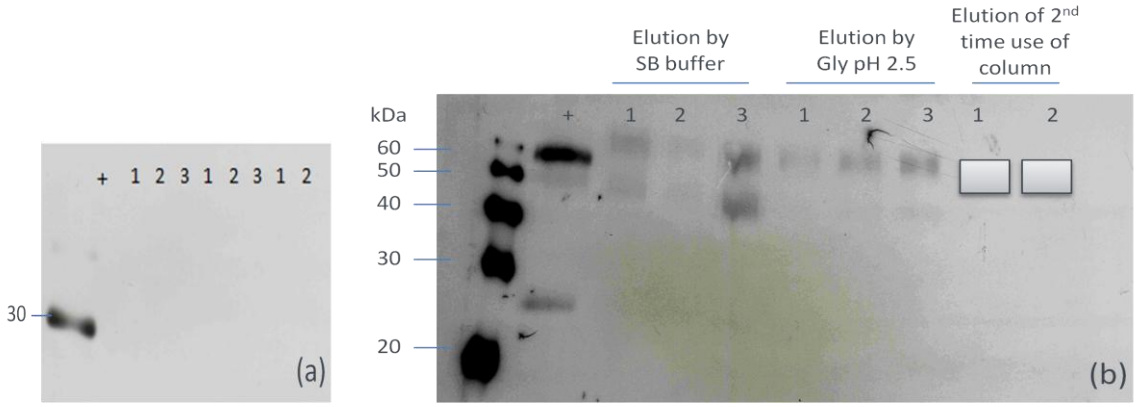


Figure 3.7 Immunoprecipitation of Lil3 protein using Lil3 antibody-coupled Toyopearl beads. Three different combination of antibody-beads volume was used to immunocapture Lil3 protein from mixture of protein membrane lysate. Eluted proteins (numbered 1-3) were subjected to separation by 12.5% SDS-PAGE and gel blot analysis using only secondary antibody (a), then Lil3 antibody (primary antibody) followed by secondary antibody (b). Eluted proteins from the reuse (for the second time) of the antibody-coupled beads column are outlined by boxes (labeled 1- 2) because the protein bands are barely visible. Note: The first lane, MagicMark (Invitrogen) molecular weight marker; second lane (+): solubilized thylakoid membrane from 10s plastids.

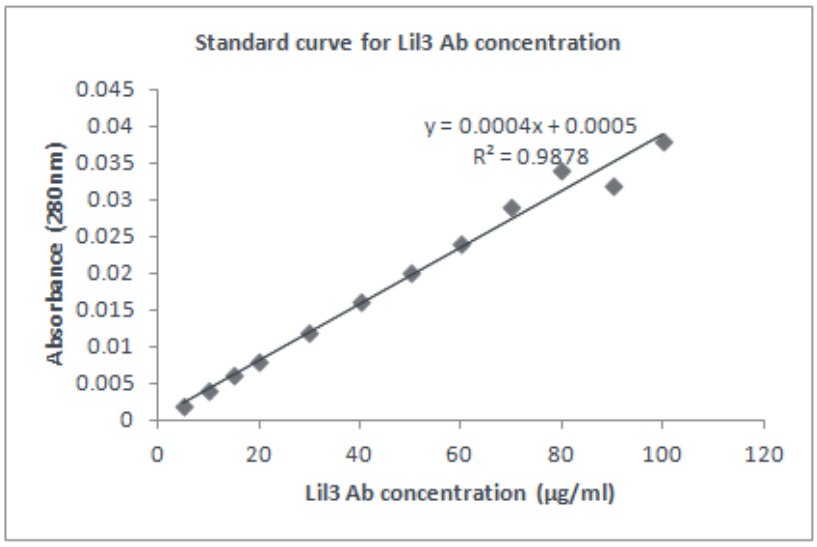
Elutions from three different combinations volume of Toyopearl beads and Lil3 antibody were examined (lane 1-3, Fig. 3.7b). In view of the important results from previous experiments, here the Lil3 antibody of 3 µl, 25 µl, and 5 µl were coupled to 30, 250 and 100 µl of Toyopearl beads, respectively. Solubilized thylakoid membrane from 10 seconds illuminated plastids was adopted as the positive control for easy observation if the antibody column captured Lil3 proteins from the membrane extract. The blotting shows two ECL signals in the molecular weight 60 kDa and below 30 kDa (lane +), which is most likely corresponding to Lil3 protein complex and the monomer. In a previous study on Lil3 protein, a molecular mass of 25 kDa has been determined from second dimension LN/SDS-PAGE (Reisinger, Ploscher et al. 2008).

Besides the combinations of antibody-beads column, two different elution buffers, 3xSB buffer without DTT and 0.1 M glycine pH 2.5, were applied to each column. In each batch, solubilized thylakoid membrane from 10 seconds illuminated plastid was applied and incubated with the antibody-coupled beads. Unfortunately, it is difficult to confirm whether the results of this experiment agree or disagree with the previously reported experiments (Ohmura, Sakata et al. 1992; Karki 2011) because the gel blot analysis of the eluents from any batch do not show any distinct bands; but generally indicate that the antibody from the column being eluted which is in the contrary of results reported by Qoronfleh et al. (2003)

using antibody-coupled agarose as the column. Nevertheless, the results of this experiment here give a qualitative result that lead to another experiment on the efficiency of antibody coupling to Toyopearl beads and the elution step, the critical step in immunoaffinity technique.

3.3.1 Immobilization Efficiency of Antibody Coupling to Toyopearl Beads

The method used for immobilization of antibody in this experiment coupled the antibody directly onto the tresyl-activated resin, Toyopearl AF-Tresyl- 650M. This coupling procedure eliminated the need for protein A or protein G, and offered universal coupling of all antibody species and subclasses as described earlier (Qoronfleh, Ren et al. 2003). Toyopearl AF-Tresyl-650M beads immobilize ligands with free amino or thiol groups and the coupling leads to the formation of a highly stable secondary amine or thio-ether linkage. Although the use of amine groups has been demonstrated as one of the easiest ways to immobilize antibodies, this coupling method could cause a decrease in activity if the antibodies have some of these amine groups in their antigen-binding sites (Moser and Hage 2010). However, the ideal situation in any immobilization methods is to have antibodies atached to the beads in a way that does not affect the activity of the binding sites or the accessibility of these sites to the protein of interest.



Antibody dilution (µg/ml)	A_{280}
5	0.002
10	0.004
15	0.006
20	0.008
30	0.012
40	0.016
50	0.020
60	0.024
70	0.029
80	0.034
90	0.032
100	0.038

Figure 3.8 Plot of the Lil3 antibody standards vs the absorbance at λ=280 nm. Right: summary of numeric report of absorbance generated by a spectrophotometer.

Coupling efficiency of Lil3 antibody to Toyopearl beads was determined by spectrophotometric analysis of antibody solution before and after coupling reaction was allowed to proceed overnight at 4°C. This simple and direct assay method for protein quantitation was conducted by measuring the absorbance of the flow-through from coupling reaction at 280nm (UV range) using quartz cuvets. Prior to each measurement of standard or sample, a zero control value was measured, which was the coupling buffer. Absorbance values of the unknown concentration were then interpolated onto the equation for the standard curve (Fig. 3.8) to determine its concentration. The average 280nm absorbance measurement of the antibody was 0.0125. Solving for x in the eqution given by the standard curve, y = 0.0004x + 0.0005, by inserting the antibody's absorbance value [$x = \frac{0.0125-0.0005}{0.0004} =$ 30 μg/ml], determined the antibody concentration in the solution. Initially, the antibody coupled to Toyopearl beads was 100 μg/ml. Assuming that any antibody not remaining in solution was bound to the Toyopearl beads, the coupling efficiency in this experiment was calculated to be 70%. A higher coupling efficiency (about 80%) have been reported (Ohmura, Sakata et al. 1992). In fact, a typical coupling efficiency up to 88% for various species of antibodies has shown by Qoronfleh *et al.* (2003).

3.3.2 Elution of The Immunocaptured Protein

The purpose of the elution step is to recover the specifically bound protein at a high yield, purity, and stability. Ideally, the elution conditions should allow for fast elution of the analyte while still allowing later regeneration of the immobilized antibodies. The sample can always be released from the antibody because the four forces that stabilizes the antigen-antibody complex (ionic, hydrogen bonding, van der Waals interaction, and hydrophobic bonds) are all reversible (Reverberi and Reverberi 2007; Moser and Hage 2010). Thus, the antibody-antigen complex can be dissociated by counteracting those forces. Hodges et al. (1988) have described that ionic interaction is very important in immunoaffinity interactions at the COOH terminus of a protein. Consequently, elution can be accomplished by the use of low pH which weaken or disrupt ionic bonds in antibody-antigen interaction.

The elution buffer used in this experiment is consisted of two types: denaturing SDS-PAGE sample buffer (3xSB buffer) and nondenaturing 0.1 M glycine buffer (Figure 3.9). In each case, 3 μl of Lil3 antibody coupled to 30 μl of swollen Toyopearl beads was used as column.

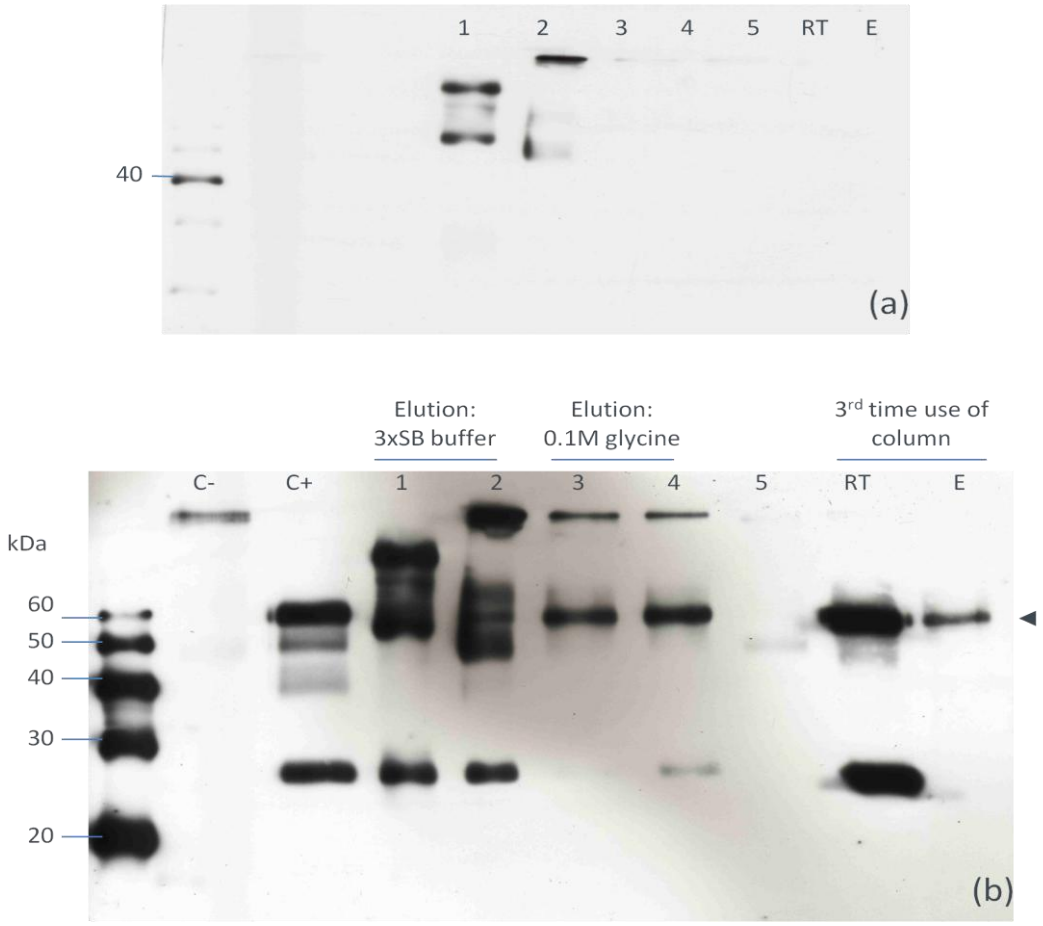


Figure 3.9 Comparison of different elution buffers. Four elution buffers: 3xSB with DTT, 3xSB without DTT, glycine pH 2.0 and glycine pH 2.5 (elutions are numbered 1-4, respectively) were used. In each case, 3 µl of Lil3 antibody coupled to 30µl of swollen Toyopearl beads was used as column. Elutions and the run-through were resolved on 12.5% SDS-PAGE and analyzed by gel blot using secondary antibody (a), then Lil3 antibody (primary antibody) and secondary antibody were sequentially applied (b). Note: The first lane, MagicMark (Invitrogen) molecular weight marker; C-: antibody-coupled beads without proteins; C+:solubilized thylakoid membrane from 10s plastids; lane 1-4: elutions by different elution buffers; lane 5: run-through of column 1; RT and E : the run-through and elution from third times use of antibody-beads column.

Although it is very effective for dissociating the affinity interaction, elution with 3xSB buffer was conducted to denature and reduce protein for electrophoresis, which is may not be suitable when further analysis or applications will be performed, e.g. reuse of the column. Gel blot analysis of the elutions using this denaturing elution buffer (lane 1 and 2, Fig. 3.9) demonstrated that the antibodies from the column are co-eluting with the protein of interest. Similar results have been reported by Karki (2011). The presence of reducing agent, dithithreitol (DTT) in the elution buffer cleaved the co-eluting antibody into heavy and light

chains that appear as two distinct bands on the gel blot that subjected to secondary antibody (lane 1, Fig. 3.9a).

On the other hand, glycine pH 2.0-2.5 is nondenaturing elution buffer which low pH condition dissociates most antibody-antigen interactions, or in other words disrupts both ionic and hydrogen bonds, without permanently affecting protein structure (Subramanian 2002; Moser and Hage 2010; Abi-Ghanem and Berghman 2012). The results confirmed that using low-pH glycine as elution buffer demonstrates a more effective way to release the captured protein in spite of the small amount of eluted antibody (indicated by the ECL signals in lane 3 and 4); as maybe the case of all elution buffers that cause some loss of antibody, limiting the number of times an immunoaffinity column can be reused. Keeping in mind that some antibodies and proteins may be damaged by low-pH condition, the use of glycine pH 2.5 is therefore preferable since there is no significant difference observed between elution by pH 2.0 (lane 3) and pH 2.5 (lane 4). In addition, to keep the condition favourable, the eluted proteins were adjusted immediately to neutrality with 1 M Tris-HCl, pH 9.0 (Miernyk and Thelen 2008).

Finally, the reuse of the antibody-coupled beads column was demonstrated. The same column used for obtaining eluted proteins (lane 4), using glycine pH 2.5 as elution buffer, was reused for the second (data not shown) and third times. The eluted proteins appear as a single ECL signal (lane E, ◄) indicating a highly purified product in the eluate. However, taking the run-through signals (lane RT) into the picture put a different perspective of the effectiveness of the column. More target proteins were released in the run-through instead of bound to the antibody column. Nevertheless, the result in this experiment demonstrated that immobilized antibodies, in this case Lil3 antibody coupled to Toyopearl beads, have a big potential because they are reusable. Qoronfleh et al. (2003) showed that as little as 20 µl of the antibody-coupled agarose have been reused up to five times without obvious loss of activity. Surprisingly, it has been reported that the regeneration of immunoaffinity column using Toyopearl AF-Tresyl-650M was executed several hundred times, when used in a automated system of flow injection immunoaffinity analysis (Kramer, Franke et al. 2004). By immobilization, as has been stated earlier, the separation of the antibody from the reaction mixture is significantly easier, contamination of final product is minimized and also for improving the features of the antibody e.g. stability, activity, specificity or selectivity (Benešová and Králová 2012).

CONCLUSION

This experiment has demonstrated how immunoaffinity technique can be used to capture protein of interest from a crude source. The biology and chemistry understanding behind the technique and related methods were reviewed, along with the results observed in practice. Immobilization method by coupling antibody directly onto an activated beaded support was performed and the coupling efficiency was examined. The protein of interest captured by antibody column was eluted with different buffers. Furthermore, the immobilized antibody-coupled beads column could be regenerated and reused, thereby conserving the limited supply antibody. In addition, a brief analysis of protein membrane complexes and antibody was also conducted.

Future perspective:

- Development of immunoaffinity column, immobilized antibody, application and elution condition for further regeneration and reuse of the column.
- Combining other analysis methods, e.g. mass spectrometry, with immunoaffinity technique.

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APPENDIX

Solutions for clear native-PAGE:

6x Gel buffer	(100 mL)
3M E-aminocaproic acid	39.36 g
1M Bis-Tris pH 7.0	30 mL
Ammonium persulfate (APS)	(5 mL)
10% (w/v) APS	0.5 g
Tetramethylethylenediamine (TEMED)	As needed
10x Cathode buffer	(500 mL)
500mM Tricin	44.80 g
150mM Bis-Tris pH 7.0	15,69 g
(Diluted 10x prior to usage)	
10x Anode buffer	(500 mL)
0.5M Bis-Tris pH 7.0	52.3 g
(Diluted 10x prior to usage)	

Solutions for SDS-PAGE:

Separating gel buffer (8x Tris pH 8.8)	(500 ml)
3M Tris	181.65 g
37% HCl to adjust pH	
Stacking gel buffer (2x Tris pH 6.8)	(250 ml)
0.25M Tris	7.57 g
37% HCl to adjust pH	
12.5% Separating gels	(25 ml)
4M Urea	7.21 g
Polyacrylamide 30%	12.5 ml
Buffer 8x Tris pH 8.8	3.75 ml
H_2O	8.75 ml

10% APS	50 µl
TEMED	15 µl
4% Stacking gels	(5 ml)
Polyacrylamide 30%	0.8 ml
Buffer 2x Tris pH 6.8	2.48
H_2O	1.6 ml
10% APS	50 µl
TEMED	5 µl
10x SDS running buffer	(1 L)
1.92M Glycine	144.13 g
0.2 M Tris	30.3 g
1% (w/v) SDS	10 g
(1x SDS running buffer is used for both cathode and anode buffer)	

Solutions for Coomassie Staining:

Fixing solution	(1 L)
Ethanol (40%)	400 ml
Acetic acid (10%)	100 ml
Solution A	(900 ml)
2% (w/v) ortho-phosphoric acid	20 g
10% (w/v) ammonium sulfate	100 g
Solution B	(20 ml)
5% (w/v) Serva –Blue G250 Brilliant	1 g
Staining solution	(125 ml)
98% (v/v) Solution A	98 ml
2% (v/v) Solution B	2 ml
25% (v/v) ethanol	25 ml

Solutions for Chemiluminescence detection:

Luminol	
250mM Luminol	443 mg
DMSO	10 ml
p-Coumaric acid	
40mM Coumaric acid	148 mg
DMSO	10 ml
ECL reagent 1	(15 ml)
2M Tris/HCl pH 8.3	750 µl
250mM Luminol	150 µl
40mM pCoumaric acid	150 µl
H_2O up to 15 ml	
ECL reagent 2	(15 ml)
2M Tris/HCl pH 8.3	750 µl
30% Hydrogenperoxide	9.15 µl
H_2O up to 15 ml	