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Cellulose And Its Application In Biomolecules Purification

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INTRODUCTION

Cellulose

Cellulose is a renewable and biodegradable natural biopolymer resource available in the world. (1,2,3) Natural cellulosic fibers are synthesized mainly in plants such as wood, which consists 40 to 50 % of cellulose. Other cellulose-containing materials include bark, cotton fiber, green algae, some bacteria and animals which have cellulose chains in their cell walls. Among these natural sources, cotton fiber is the only pure cellulose material which contained cellulose up to 95% to 97%. (4) It also contains other non-cellulose constituents such as protein, amino acid, wax, organic acid and pigments because of high ratio of surface area to linear density it has. (5) These materials can be removed by chemical solvents such as sodium hydroxide, which used in the mercerization process of cotton (5).

Cellulose is a homopolymer composed of repeating *β*-D-glucopyranose units which linked together by *β*(1 → 4) glycosidic bonds. It has a basic molecular unit of $C_6H_{10}O_5$. (1,6,7) Cellulose molecules are in linear form as a consequence of the *β*(1 → 4) glycosidic linkages results in a rigid, rod-like molecule. They aggregated through intra- and intermolecular hydrogen bonding to build up the cellulose fibrillar structure, which generally referred as micorfibrils.(3) The cellulose microfibrils structure have both crystalline (high order regions) and amorphous region (low order region). The degree of crystallinity of cellulose usually covers a wide range of cellulose structure and depends on the origin and pretreatment of the sample. (1,8) Hydrolysis process may remove the amorphous region of the cellulose fiber and leave crystalline region under controlled conditions.(8)

Cellulose exists in several crystalline polymorphs. Two well-known and major polymorphs of cellulose are cellulose I and cellulose II. Cotton cellulose which is native cellulose produced by plants, exists in cellulose I crystallographic form. (1,3) This native cellulose consists of two different crystal structure, which are cellulose Iα and Iβ. Besides cellulose I, cellulose II is another important crystalline form of cellulose from technical and commercial point of view. Cellulose II is formed naturally in marine algae. It can also be obtained when cellulose I is mercerized or regenerated from solution. The transformation process of cellulose I to cellulose II is considered irreversible. (1,3)

The cellulose fibers are negatively charged because of the presence of carboxyl and hydroxyl-group. (9) The cellulose is very reactive with water but hard to be dissolved in a variety of solvents because of the existing of large quantities of intra- and intermolecular hydrogen bonds and also van der Waals forces between the non-polar groups. (3,10,11) The preponderance of hydroxyl functional group make cellulose has a strong affinity to itself and hydrogen containing materials .In the contrast, it has low compatibility with hydrophobic polymeric matrices caused by its hydrophilic character (10,12)

Purification of cellulose

Raw and unpurified cellulose is hydrophobic in nature because of the presence of non-cellulose substances such as waxes and protein. (13)Therefore, different pre-treatment processes, such as alkaline

purification and enzymatic purification are applied in order to create a proper sorption characteristic of regenerated cellulose fiber. (14)

Alkaline purification

Scouring and mercerization are two examples of cellulose pretreatment that employed the application of concentrated alkaline agents. (15) Non-cellulose components such as wax and hemi-cellulose have to be eliminated to ensure the water absorbency of cellulose. (14)Scouring is a purifying treatment of cotton a-cellulose to remove non-cellulose impurities. (15) On the other hand, mercerization is a process to induce the changes of super-molecular structure and morphology of cellulose fibers. (15) This process involved the modification of crystallographic cell from cellulose I to cellulose II. (15) The moisture absorption capacity of cellulose fibers will be improved after the mercerization process. (15)

Enzymatic purification

Enzymatic purification is an environmental-friendly process that has been used in purifying cellulose. This purification method is used to remove the individual non-cellulose components using specific enzyme such as pectinase and lipase. (9) The treatment of cellulose with cellulases is normally performed with high mechanical agitation. The insoluble cellulose particles are released during the enzymatic hydrolysis with the aided of mechanical agitation. (16) This mechanism is influenced by factors such as the preparation of cellullase, cellulase concentration, degree of agitation and treatment time. (16)

The proposed mechanism of cellulose hydrolysis can be divided into the following stages. First of all, the enzyme molecules will be transferred from aqueous phase to the surface of cellulose molecules. Second, the enzyme molecules adsorbed on to the surface of cellulose and form an enzyme-substrate (E-S) complex. Water molecules will then be transferred to the active site of E-S complex and a surface reaction between cellulose is catalyzed by the E-S complex and water. Lastly, the end products of cellobiose reaction and glucose will be transferred to the aqueous phase. (17)

DISSOLUTION/DEGRADATION OF CELLULOSE FIBER

Solvent for cellulose dissolution

Alkaline solution

Sodium hydroxide (NaOH) is the alkaline substance that normally used to remove non-cellulosic impurities from cellulose in scoring or mercerization process. Cellulose can be dissolved in a high NaOH concentration during these processes, where the soda hydrates penetrate the amorphous area of cellulose and destruct the neighboring crystalline region. (11) The mixture of water with 0 to 20 % NaOH is a simple solution and both of them are miscible in liquid state. However, the hydrodynamic diameters of NaOH/water hydrates may be too large to penetrate into the crystalline region of cellulose at low NaOH concentration. (16) Therefore, only low molar mass cellulose will dissolve when treated with NaOH/water solution, which have a NaOH concentration in the range from 7 to 10%, and at the temperatures in the range of -5°C to -8°C**.** (17)

In industrial applications, NaOH alone has never been used for dissolving cellulose. (11) It was not found that NaOH can dissolved cellulose better with the addition of urea or thiourea at cold temperature. (11,17,18) The combination of these solvents which can dissolve cotton linters and other natural cellulose is non-pollution, simple and have safe dissolution procedure. (19) Furthermore, several studies showed that cellulose can be dissolved directly and quickly in NaOH/thiourea/urea aqueous solution. (16,20) Molecules of NaOH, thiourea and urea bind to cellulose molecules, bringing cellulose molecules into aqueous solution to a certain extent. It is a mechanism where cellulose macromolecules are prevented from associating. (20) NaOH/thiorea/urea solution breaks the intra- and inter-molecular hydrogen bonding of cellulose leading to the good dispersion of cellulose to form solution. (16)

Ionic liquids

Ionic liquids are a class of non-molecular ionic solvents that consist entirely ions. (21, 22) Organic salts in liquids form at or near room temperature are referred as ionic liquids. The ionic liquids ability in dissolving cellulose varies with the size and polarizability of the anion present and with the nature of cation. (23) Ionic liquids contain strong hydrogen-bond acceptors that can be used to dissolve cellulose are Cl^-, $Br^-$and SCN^-. (24) Among these acceptors, chloride-containing ionic liquids appeared to be the most effective solvents in disrupting and breaking the intermolecular hydrogen-bonding of cellulose. (23)

Dimethylacetamide/Lithium chloride

DMAc/LiCl is normally used to determine the molecular weight of cotton. For the dissolution of high molecular-weight cellulose of cotton in this solvent, a lengthy multiday procedure might be required. (24) Heating or refluxing the cellulose materials in DMAc, or in DMAc containing LiCl is a very common protocol in cellulose dissolution process. (26) Besides, this process can also be achieved by activation procedures such as treatment with liquid ammonia, pre-swelling in the water and then followed by solvent exchange to DMAc, or freeze-drying. (25, 26) The treatment of cellulose with DMAc/LiCl solvent will caused the swelling of cellulose intra- and intercrystalline region, breaking of hydrogen bonds and increased accessibility. (26)

N-methylmorpholine-N-oxide (NMMO)

N-methylmorpholine-N-oxide (NMMO) is a solvent that applied in Lyocell process and is normally used in dry-jet-wet spinning process of cellulose in industry.(27,28) It is a chemical that affects direct dissolution of cellulose without any prioir derivatization. (28) Cellulose dissolution in NMMO involved the following process. First of all, a homogenous concentrated solution (dope) of the starting cellulose in an NMMO-water mixture was prepared. Next, dry jet-wet spinning process will be carried out. The extrusion of the highly viscous spinning dope was conducted at high temperatures through an air gap into a precipitation bath. Cellulose fiber coagulated in the precipitation bath and post-treatment process such as washing and drying step will be conducted. (29)

Cellulose dissolution in NMMO process is an environmentally friendly process and is carried out at high temperature under low vacuum. (30) Recently, microwave heating has been introduced as an alternative heating system in order to improve the efficiency of cellulose dissolution in NMMO solvent. (30)

1-butyl-3-methyl-imidazolium chloride (BMIMCl)

The high chloride concentration and activity in 1-butyl-3-methyl-imidazolium chloride (BMIMCl) plays a major role in cellulose dissolution. The solubility of cellulose and the solution properties can actually be controlled by selection of the ionic liquid constitute. (31) Ionic liquids which contained chloride anions are assumed to have high effectiveness in breaking the extensive hydrogen-bonding network in cellulose. (31,32) The liquid crystalline solutions of cellulose are formed when high concentrations of cellulose (more than 10 wt %) are dissolved in BMIMCl. (31) In addition, cellulose can be dissolved in ionic liquids with the aiding of domestic microwave oven as a heating source. The heating process accelerates the dissolution of cellulose in ionic liquid. (31,30)An example is that solutions containing up to 25 wt% cellulose can be prepared in BMIMCl under microwave heating. (31)

In BMIBCl solution, the cellulose is in disordered form and can be easily precipitated by adding water, ethanol or acetone. (22, 31) The regenerated cellulose morphology is changed and its microfibrils are combined into homogenous macrostructure. The regenerated cellulose can be in different structural forms such as powder, tube, fiber by changing the regeneration process. (31)

Ethylene Diamine/Salt solvent systems

An Ethylene Diamine (EDA) /Salt solvent system is another method that can dissolved cellulose without any pre-treatment. (33) It is found that EDA can swell cellulose and facilitate the solvent diffusion into tightly packed crystalline region. Complete dissolution of cellulose by using EDA is not yet achieved until a salt is added. The formation of a salt-solvent complex, dissociation of salt into cations and anions, solvation of ions through ion-dipole interaction are possible mechanisms of action used in this system. Salt type and concentration, as well as cellulose concentration and molecular weight are important aspects that influence cellulose dissolution. (33)

Acid hydrolysis

The morphology and crystallinity of cellulose can be examined by acid hydrolysis of cellulose. Alterations on the surface of cellulose are found during the acid treatment of cellulose. (34) Acid hydrolysis may remove the amorphous region of the cellulose fiber and leave the crystalline region almost untouched. (35,36) The fast removal of amorphous region of cellulose near the microfibrils' surface leads to the exposure of micorfibril bundles. Acid and water molecules are unable to penetrate into microfibril bundles as they have slow hydrolysis rates. (35) The amorphous cellulose which buried in the bulk leak out from macrofibrils during hydrolysis at slower rates due to barriers caused by the microfibril bundles. Therefore, the amorphous cellulose near sureface is hydrolyzed first and followed by crystalline cellulose near surface. This repeating process in acid hydrolysis will caused the cellulose degradation occurs. (35)

Application Of Cellulose/Cellulose Derivatives In Biomoelcules Isolation And Purification

Cellulose powder and microcrystalline cellulose powder are cellulose derivatives that are widely used as pharmaceutical excipients. They are also a design of cellulose adsorbents that obtained by two different methods: chemical hydrolysis and mechanical disintegration of cellulose. (36,37,38) Cellulose powder is produced by mechanically grinding of purified cellulose. It has a high amount of amorphous regions and low degree of cyrstallinity. (39) On the other hand, microcrystalline cellulose has a higher degree of crystallinity compared to cellulose powder. It is usually obtained by hydrolysis using diluted hydrochloric or sulfuric acid at boiling temperature. (36,37,39) The destructive treatment is directed to the amorphous region of the cellulose and the crystalline region remained nearly unchanged. Therefore, the final product of this hydrolyzed cellulose is called microcrystalline cellulose.(36)

A combination of chemical and mechanical treatment of cotton cellulose in producing cellulose powder has been carried out by Guan Ying Ting and Li Yi (2008). The cotton fibers were dreid at 80°C for two hours and then cut into two centimeters longs. After that, the cotton fibers were cut in a pulverizing machine until they became powder form. In order to separate the fbrils in the fibers, cellulose powder was mixed with 300ml of 64% sulfuric acid and stirred for two hours at room temperature. The acid was then removed by using centrifugation. For further treatment, distilled water was added and continued with ultrasonic treatment. The remaining sulfuric acid was removed by centrifugation method and the particles were washed with distilled water or acetone. Lastly, the powder was dried with spray dryer. (40)

The broad application of cellulose powder and microcrystalline cellulose are accredited to their special properties: (a) sub-micron or nano-dimentional particle size, (b) large specific area, and (c) inertness to many organic or inorganic substances. (40) Furthermore, they are environmental friendly and have excellent properties such as water absorbent. (40)

Application of cellulose/cellulose derivatives in nucleic acid purification and isolation

There are two general methods that used in the purification of nucleic acid and protein: (a) conventional method, such as Guanidinium thiocyanate-phenol-chloroform extraction, alkaline extraction method and CTAB extraction method; (b) solid-phase nucleic acid extraction, which has been used widely nowadays as it allows quick and efficient purification compared to conventional method. The solid-phase biomolecules purification is based on principles where: hydrogen-binding interaction with a hydrophilic matrix under chaotropic conditions; ionic exchange under aqueous conditions by means of an anion exchanger, affinity and size exclusion mechanism. Cellulose or cellulose derivatives has been utilized as one of the column materials in solid-phase biomolecules purification.

Cellulose powder

A study regarding the chemical linkage of nucleic acid to neutral cellulose powders and isolation of specific sequences by affinity chromatography had been done by Thomas and Malcolm (1974) (41). In their study, Simian virus (SC) 40 DNA fragments were linked to neutral and phosphorylated cellulose powder with a water-soluble carbodiimide efficiently. Cellulose powder had been chosen to be used as the matrix because it is stable to organic solvents and extreme temperature. Besides, the application of cellulose powder and its derivatives as solid matrix showing a better flow through properties and lower non-specific binding of nucleic acid than the pulped chromatography paper, a material that used by Shih and Martin in their study in 1973(41). Nucleic acid were covalently attached to cellulose, which involved the activation of the terminal phosphate group of viral DNA fragments by water soluble carbodiimide, and used in a continuous hybridization system to isolate complementary nucleic acid sequences (41,42). Non-specific binding had been reduced by using highly purified fibrous cellulose powder and the washing of the column with a low ionic strength solution at 4°C prior to the elution of the retained nucleic acid (41). Besides the neutral cellulose powders, phosphorylated cellulose which consists of additional phosphate anion was also used in this study. It helped in reducing the non-specific adsorption of polyanionic DNA or RNA to the solid supporting medium which caused by the charge repulsion at low ionic strength solution (41).

In U.S. patent number 7264927 B2 (42), a method for purification of nucleic acids and protein from various sources using cellulose powder or cellulose paper had been introduced. In this method, cellulose powder or cellulose filter paper can bind to nucleic acids, in the presence of certain salt and polyalkylene glycol concentration. The cellulose powder or cellulose filter paper that having nucleic acid bound will be washed with a wash buffer in order to remove any unwanted materials. The bound nucleic acid is then eluted from the column by adding an elution buffer or deionized water. The above invention produces nucleic acid that is immediately ready for further downstream processes such as PCR, sequencing or blotting procedures. Moreover, this method is also readily adaptable to automation including high throughput screening system (42).

Another improvement of nucleic acid purification using cellulose as binding matrix has been described in US patent number 6855499 B1 (43). Particles which contained magnetic or paramagenetic properties are encapsulated in cellulose or cellulose derivatives, forming magnetizable cellulose. Nucleic acid binds to the magnetizable cellulose in the aiding of high salt and polyalkylene glycol concentration. DNA, RNA and protein is able to be isolated from various sources including tissues, cells and bacterial cell lysates. Nucleic acid that bound to the magnetizable cellulose will be washed with a suitable wash buffer before they are eluted out by elution buffer. The amount of nucleic acids that are bound to the magnetizable cellulose will depend on the amount of magnetizable cellulose typically. A magnetic field is applied to draw down or draw to the side the magnetizable cellulose particles during all the isolation steps to separate the magnetizable cellulose from the liquid. (43)

Sepharose 4B-cellulose nitrate column

In 1979, the isolation of R-plasmid from *Pseudomons aeruginosa* was done by using coupled chromatography on Sepharose 4B-cellulose nitrate column (44). The principle of this isolation process is based on the combination of two chromatographic columns - the first column containing Sepharose 4B and the second column containing a cellulose derivative – cellulose nitrate. The alkali-treated cleared bacterial lysate was loaded into the Sepharose 4B column. Then, the desired plasmid DNA was eluted out from the cellulose nitrate column by passing 2M NaCl-0.01M sodium citrate buffer through the connected columns. Basically, the used of two columns gave a well separation of total DNA from cell components such as proteins by Sepharose 4B column and followed by a selective separation of plasmd DNA from denaturated chromosomal DNA on cellulose nitrate column (44).

Secondary fibril-associated cellulose matrix

A secondary fibril-associated cellulose (SF-cellulose) can be used as a general purpose matrix to purify a wide range of nucleic acids (45). The cellulose fibers are found in paper with high absorbing capacity. This cellulose matrix was packed in column form and was prewashed with elution buffer or 3M guanidine hydrochloride, and followed by 70% ethanol. The columns were assembled in a vacuum device which was connected to a vacuum device. Nucleic acids in a crude extract were precipitated by mixing the extract with an equal volume of precipitant and incubated at room temperature for 5 minute. The mixture was then transferred into the column. Nucleic acid-matrix complexes in a sample are collected in the column under vacuum. (45) The nucleic acid molecules can be absorbed to the hydrophilic cellulosic chain of the secondary fibrils under precipitating conditions and reversibly bind to the cellulose matrix. The physical structures of the secondary fibrils played a role as microfilters to retain precipitated nucleic acids. At last, the matrix in the column was washed with 70% ethanol before the bound nucleic acid was eluted out from the matrix. (45)

Diethylaminoethyl (DEAE)-cellulose column purification

DEAE- cellulose is a cellulose derivative that functions as an anion exchanger in chromatography. The purification using DEAE-cellulose column is based on the principle where interaction between negatively charged phosphates of the nucleic acid backbone and positively charged DEAE groups on the surface of the cellulose (48) However, the interaction of nucleic acid with DEAE cellulose is not only depends on the ion exchange, but also on weaker forces of attraction. The weaker forces are a function of the base composition of the polynucleotide chain. (49)

An idea of using increased non-ionic interactions with modified DEAE-cellulose had been described to separate transfer RNA (tRNA) and DNA (49, 50, 51, 52). Replacement of the hydroxyl group of DEAE-cellulose by benzoyl groups increases the non-ionic attractions between the matrix and polynucleotide. Benzoylated DEAE-cellulose (BD-cellulose), which has affinity for nucleic acids with additional hydrophobic groups attached, binds polynucleotide more strongly than DEAE-cellulose. Method for the separation of acylated nucleic acid from unacylated nucleic acid is available with the introduction of this column (49,50).

The isolation of tRNA which studied by Gillam *et. al* in year 1967 involved the chemical derivatization of aminoacyl-tRNA with phenoxyacetyl or naphtoxyacetyl moieties, and isolation of the product on benzoylated DEAE-cellulose column. The tRNA could be eluted out from the column by salt solution with 0.5 to 1.5 M concentration. Meanwhile, the derivatized aminoacyl-tRNA was retained on the matrix until 20% (v/v) ethanol was added to elute salt solution (49,50).

After the introduction of application of BD-cellulose and benzoylated-naphthoylated-DEAE cellulose (BND-cellulose) by Gillam *et. al*,BD cellulose and BND-cellulose has also been used as support matrix for the fractionation of DNA(51,52). These exchangers are useful for nucleic acid fractionation because of their hydrophobic substituent ability in interacting with nucleic acid bases which are not involved in normal base pairing. A work in fractionated *E.coli* DNA had been done by Caffin and Mackinlay (1974) (51). Their study showing that

approximately 50% of bacterial DNA could adsorbed strongly onto BD-cellulose matrix under specific conditions of chromatography and preparation of lysates employed. The proportion of bound DNA on BD-cellulose was depended on the molecular weight of the DNA (51).

Besides, single-stranded DNA (ssDNA) can also bind to naphthoylated DEAE cellulose irreversibly. The introduction of benzoyl residues on DEAE cellulose matrix afforded fractionation of DNA depends on its secondary structure. The fractionation occurred based on the interaction between aromatic rings of the matrix column and DNA nucleotides (52). Single-stranded regions of DNA bound to the derivatized DEAE-cellulose in the presence of 1.0M sodium chloride, which eluted double-stranded DNA totally from the column. Then, caffeine solution was used for further elution step in order to recover the bound ssDNA fraction. The caffeine concentration that required in eluting ssDNA from benzoylated cellulose column is proportional to the polynucleotide length. (52)

Oligo-dT cellulose column

Messenger RNA (mRNA) or poly (A)-RNA isolation is done using cellulose bound olio-dT. The isolation of mRNA or poly (A)-RNA consists of three main steps: (1) hybridization of poly (A)-containing RNAs to oligo-dT molecules connected to a carrier such as cellulose, (2) washing off nucleic acids which do not bind to oligo-dT, and (3) elution of poly (A)-RNA from the oligo-dT or carrier combination under low stringency conditions (53).

Oligo-(dT)-cellulose was prepared using the *N,N'-dicyclohexyl-carbodiimide* reaction for the polymerization of thymidine-5'-monophosphate on cellulose (54) . The oligodeoxynucleotide is chemically stable and can be used repeatedly after alkali treatment. This oligo-(dT) cellulose column has a relatively high capacity and can be used to isolate several milligrams of poly (A)-mRNA from several hundred milligrams of crude polysomal RNA (54).

Nitrocellulose

Membrane filters that made of nitrocellulose have adsorption properties useful for nucleic acid isolation. Adsorption capacity of bulk nitrocellulose presented in column form increased compared to membrane filters. Application of nitrocellulose column chromatography in nucleic acid including: (a) purification of RNA-free DNA. Denatured DNA is separated from RNA by nitrocellulose chromatography; (b) Separation of native DNA from denatured DNA. Basically, only denatured DNA will be absorbed by nitrocellulose but native DNA do not absorbed on this matrix; (c) Preparation of messenger RNA.(55) The adsorption of nucleic acids to nitrocellulose is believed to be occurred via hydrophobic interaction. (56)

Application of cellulose/cellulose derivatives in protein purification and isolation

Protein purification is a process that begins with cell lysis. During cell lysis, a cell's membrane will be disrupted and internal contents of the targeted cell released out into a solution known as crude lysate. Several methods can be used to lyse the desired cell. For example, sonication, homogenization by high pressure and filtration. Chromatography, including ion exchange chromatography and affinity chromatography is the most effective and widely used process to isolate protein of interest according to protein properties such as molecular weight, net charge and binding affinity.

Ion exchange chromatography

Purification of protein by ion exchange chromatography is based on the principle where the separation is due to competition between proteins with different surface charges for oppositely charged groups on an ion exchanger adsorbent. The interaction between a target protein and an ion exchanger depends on: (1) the net charged, (2) ionic strength and (3) the surface charge distribution of the protein. (57)

Carboxymenthylcellulose

An ion exchange chromatography was applied in the study of egg white proteins purification (58). The cellulose ion exchangers have extensive possibilities for the analysis, preparation and purification of proteins. They provided a large adsorption capacity and high purity of proteins were isolated relatively (58,59). The carboxymethylcellulose that used in this study was prepared according to the procedure of Peterson and Sober (1955) (59). Cellulose ion exchangers that used in this study were prepared by the reaction of a chloro compound with celllose which could swell in strong alkali. The adsorption and elution of protein from carboxymethylcellulose (CM cellulose) was a satisfied method that gave a nearly complete analysis of egg white and for the isolation of the individual proteins.(58).

CM cellulose is a polyanion that contained two useful functional groups – hydroxyl and carboxyl groups. These groups can be utilized for ligand binding and imparting reversible solubility. There are three hydroxyl groups which available for substitution per anhydroglucose unit in cellulose. Substitution of these hydroxyl groups with

carboxymethyl groups was done in the preparation of CM cellulose (60).In Arvind *et al*. work , CM cellulose was employed as a reversibly soluble/insoluble ion exchange matrix. After binding, proteins from crude lysate could be precipitated with a combination of calcium and polyethylene glycol. The precipitated proteins were recovered in a dissociating buffer devoid of one or both of the precipitants followed by reprecipitating of CM cellulose (60).

DEAE-cellulose

A technique of protein purification by DEAE-cellulose chromatography in high concentration of ammonium sulfate had been reported by Mayhew and Howell in 1970 (61). Several proteins, such as Cytochrome *c* and ribonuelcase were retained in DEAE-cellulose column when a high concentration of ammonium sulfate solution was applied. These proteins were eluted from the column when the salt concentration decreased (61). The sequence of methods for this protein purification was: (1) adding ammonium sulfate in a cell-free extract to a concentration required to precipitate the desired protein, (2) centrifuged the mixture and discarded the precipitant, (3) the supernatant solution was applied directly to DEAE-cellulose column equilibrated with ammonium sulfate, and (4) fractions from the column were monitored for enzyme activity during the elution process with a gradient of ammonium sulfate (61).

Precipitation of immunoglobins with ammonium sulfate on DEAE-cellulose was also been used for the isolation of mouse monoclonal antibodies specific against different antigen (62). DEAE-cellulose is one of the chromatographic matrix that having high hydrophilicity and do not have denaturing effect on IgG_1 and IgG_2 antibodies. In the purification of mouse monoclonal antibodies, three main steps which including (1) ammonium sulfate precipitation step, (2) dialysis desalination with microporous anion-exchange Neosepta membrane, and (3) anion-exchange chromatography on DEAE cellulose was applied (62).

Besides, DEAE-cellulose matrix had been utilized in a new single-step system to immobilize and/or purify fusion proteins (63) . An enzymatically active chimeraic protein containing choline-binding domains and *β*-galactosidase from *Escherichia coli* had been immobilized and purified. In this study, *β*-galactosidase chimera which could be purified by single step purification was constructed. The crude extracts of sample were loaded on a DEAE-cellulose column and it was found that *β*-galactosidase activity remained immobilized in the column after washing with a high-salt (1.5M NaCl) solution (63).

Affinity chromatography

The biological function of proteins normally involves specific interactions with ligands. In affinity chromatography, a specific ligand will attach to an inert chromatographic matrix covalently. The sample is applied under specific conditions an reversible binding of the target protein to the ligand. Other impurities will be washed away since only the protein of interest is adsorbed from the extract passing through the column. The experimental conditions have to be changed to elute the targeted molecule from the matrix, in a situation where the protein-ligand interaction is broken (57).

Modified Cellulose membrane

Preparation of a cellulose affinity membrane was done by treating the cellulose with BMIBCl – a green solvent which is environmental friendly (64). The results of the study showed that the amount of cellulose present in the BMIBCl solution is an important parameter in designing membranes with different morphological and mechanical properties. The cellulose membrane was modified by coupling with ligand 22/8 and unmodified cellulose membranes were used as control in this study. The observation showing that the tested proteins (IgG) did not bind onto the unmodified membrane but the modified membranes bound and eluted IgG selectively (64).

Cellulose acetate membrane

Cellulose acetate has been chosen as the material of electrospun regenerated cellulose nanofiber affinity membrane in a study on IgG purification (65) .Cellulose acetate can be easily electrospun into nanofiber membrane. The nanofiber membrane can also be deacetylized to obtain a regenerated cellulose membrane - a hydrophilic material with low nonspecific protein adsorption. This regenerated cellulose nanofiber membrane has strong hydrophilicity and is available reactivity for further chemical functionalization.

It is also an excellent material for affinity membrane fabrication. Regenerated cellulose membrane was then oxidized with $NaIO_4$ to generate aldehyde groups, which protein A/G ligand containing six IgG binding domains could covalently attached (65). The ability of the affinity membrane in IgG purification was evaluated with BSA as a model impurity. Five layers of the membrane were packed into a spin column to separate IgG from an IgG/BSA mixture solution. The SDS-Page analysis results showed that the BSA was completely removed from the mixture after the purification (65) .

Regenerated Amorphous cellulose

A simple protein purification method by using regenerated amorphous cellulose (RAC) has been developed to capture cellulose-binding module (CBM) tag (66). The CBM tag in recombinant protein has high-affinity adsorption on the surface of regenerated amorphous cellulose particularly. In the study, RAC was produced by treating a commercial cellulose powder with phosphoric acid dissolution process, followed by regeneration. The crude protein solution which contained the fusion proteins of CBM-intein-target protein was mixed well with RAC absorbent. The supernatant consisted of impure protein was discarded after centrifugation. Meanwhile, the impure proteins that bind in the porous RAC were washed away by washing buffer. The protein of interested can be obtained through intein self-cleavage in the cleaving buffer, with low pH and high salt concentration. The purified cleaved target protein was obtained in the supernatant after centrifugation (66).

Utilization of RAC in capturing CBM-tag proteins has brought several advantages: high protein purity was obtained, high protein yields, short purification time due to faster protein adsorption, low cost, simple solid/liquid separation by centrifugation or filtration and minimal waste treatment because of non-toxic consumed and used of biodegradable RAC .(66)

DEAE anion exchange and protein G affinity columns

A combination of DEAE anion-exchange and Protein G affinity columns has been developed for the separation and purification of immunoglobulin G and albumin from mouse serum simultaneously (67). Mouse serum contained acidic proteins such as albumin and neutral proteins such as IgG. In this purification system, serum sample passed through the DEAE column first, and therefore Protein G column will be protected from the contamination. The sample was injected directly into this system, and the proteins were eluted from the DEAE and protein G columns separately by the column-switching technique. DEAE played a role as an anion exchanger tends to bind acidic proteins under neutral conditions. On the other hand, IgG did not bind to the DEAE column but was retained in Protein G affinity column (67).

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