Fundamental studies of affinity separation of glycoproteins and its combination with expanded bed adsorption technique

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Abstract

This work concerns the fundamental aspects of affinity separation of glycoconjugates, in particular, glycoproteins such as the enzyme glucose oxidase (GOD). It includes studies on the adsorption behaviour of GOD onto Concanavalin A (Con A) adsorbents, the preparation, characterization, and properties investigation of PEGylated Con A, the characterization of novel dense pellicular agarose coated glass beads and its application for expanded bed adsorption of proteins.

The immobilization of Con A was carried out onto both inorganic silica and polymeric Toyopearl supports. The investigation includes the optimal adsorption conditions, adsorption isotherms, adsorption/desorption kinetics, unspecific adsorption capacity, and its dynamic performance. The results reveal that both Con A adsorbents exhibit high affinity and low unspecific adsorption capacity to GOD, and provide a very good stability, indicating their suitability for the application of affinity separations.

To improve the stability against the harsh environmental conditions encountered in affinity separation process, the proteinaceous ligand Con A was modified with polyethylene glycol (PEG) derivatives (mPEG-SPA). The PEGylation reaction was studied in detail to elucidate how the parameters affect the PEGylated degree. The influence of PEGylation on the adsorption behaviour of Con A was investigated by the adsorption isotherms, kinetics, and their dynamic performance for GOD. The results show that the modification did not alter substantially the carbohydrate binding specificity of Con A, but slightly reduced the binding capacity and binding rate for GOD. The dynamic adsorption capacity for modified Con A depended on the PEGylated degree and the molecular weight of mPEG derivatives very much. The PEGylated Con A exhibited much higher stability against the exposure to organic solvents and high temperature. The fact that PEGylation stabilizes the properties of Con A may greatly expand the range of applications of unstable affinity ligand in bioseparation processes.

This work combines affinity separation with expanded bed technique, which is useful for particulate-containing feedstock processing. A novel agarose coated glass (AG) matrix for expanded bed adsorption was characterized by its physical properties, bed expansion, and liquid phase dispersion behaviour. The novel AG matrices are promising tools for expanded bed adsorption of proteins since they exhibit very low axial mixing. Con A immobilized AG adsorbents were successfully employed for expanded bed adsorption of GOD and crude albumin from hen egg white. The combination of affinity separation with PEGylated proteinaceous ligand could provide great attraction for industrial applications.

Abstrakt

Diese Arbeit behandelt die fundamentalen Aspekte der Affinitätstrennung von Glykokonjugaten, insbesondere von Glykoproteinen am Beispiel des Enzym Glucose Oxidase (GOD). Die Studien beinhalten das Adsorptionsverhalten von GOD an Concanavalin A (ConA) Adsorbentien, deren Herstellung und Eigenschaften sowie Untersuchungen von PEGyliertem Con A und der Charakterisierung von einer neuen Matrix, die aus Agarose beschichteten Glasperlen (AG) besteht, und deren Einsatz in der Proteinadsorption mittels Expanded bed adsorption.

Die Immobilisierung des Lektins ConA wurde sowohl auf anorganischen Silica- als auch auf synthetischen Polymerträgern durchgeführt. Die Untersuchungen umfassen die Ermittlung der optimalen Adsorptionsbedingungen mittels Adsorptionsisothermen, Adsorptions- und Desorptionskinetiken der unspezifischen Adsorption und dem Verhalten in dynamischen Prozessen. Es wurde gezeigt, dass beide ConA Adsorbentien eine hohe Affinität und niedrige unspezifische Adsorption zu GOD besitzen, außerdem besitzen sie eine sehr gute Stabilität, was ihre Eignung in Affinitätstrennprozessen unterstreicht.

Um eine Stabilisierung des Proteinliganden ConA in Affinitätstrennungen unter proteinunfreundlichen Bedingungen zu erreichen wurde das Protein mit Polyethylenglykol (PEG) – Derivaten (mPEG-SPA) modifiziert. Die Parameter, die den PEGylierungsgrad beeinflussen wurden detailliert untersucht. Ebenso wurde der Einfluss der PEGylierung auf die adsorptiven Eigenschaften zu GOD ermittelt, indem Adsorptionsisothermen, Kinetiken und die dynamische Leistung aufgezeigt wurden. Die Ergebnisse zeigen, dass sich die Fähigkeit des Lektins ConA Zuckerstrukturen spezifisch zu binden nicht wesentlich ändert, jedoch sind eine Reduzierung der Adsorptionskapazität und der Bindungsgeschwindigkeit zu beobachten. In dynamischen Adsorptionsprozessen ist die Kapazität sehr stark vom PEGylierungsgrad und dem Molekülgewicht der PEG Derivate abhängig. Das durch PEG modifizierte ConA zeigt eine erhöhte Stabilität gegen organische Lösungsmittel und erhöhten Temperaturen. Die Tatsache, dass PEG Proteinliganden zu stabilisieren vermag, erweitert das Anwendungsspektrum der Affinitätstrennung auf unstabile Liganden.

Diese Arbeit kombiniert die Affinitätstrennung mit der Expanded Bed Technik, die bei Prozessen mit grobkörnigem Ausgangsmaterial nützlich ist. Eine neue Matrix, die aus Agarose beschichteten Glasperlen (AG) besteht, wurde für Expanded Bed Adsorption verwendet und nach ihren physikalischen Eigenschaften, Ausdehnung im Expanded Bed und Verhalten in der Flüssigkeitsverteilung charakterisiert. Neue AG Matrizes sind geeignet für Expanded Bed Techniken, weil sie eine geringe axiale Vermischung zeigen. AG Adsorbentien mit immobilisiertem ConA wurden erfolgreich für die Adsorption von GOD und Rohalbumin aus Hühnereiweiß verwendet. Die Kombination von Affinitätstrennung mit PEGylierten Proteinliganden könnte von hohem Interesse in industriellen Anwendungen sein.

Contents

1. Introduction

With the growing expansion of the biotechnology industry, it is becoming increasingly evident that there is still considerable scope for technological progress in the manufacturing of biological products. Complying with the requirement of the market, the major developments in biotechnology industry have been in the area of therapeutic and pharmaceutical proteins and enzymes (Gupta, 1994). A key section in the production of any such pharmaceutical product is the processing of the material from its initial milieu to a pure form suitable for its intended use. This key section, termed downstream processing can be, and often is, a complicated series of isolation and purification steps of the target biological molecules (Spears, 1993).

In the production of a large proportion of therapeutic and pharmaceutical biological molecules, it has been shown repeatedly that downstream processing often proves to be the most problematic and expensive. Due to the high complexity, particularity, instability of and the rigorous demand to biological products, downstream processing could count for up to 80% of the total manufacturing cost (Gupta, 1994). Hence, efficient and well designed product recovery methods are essential in developing a downstream process. Despite the relatively high cost, chromatography methods continue to dominate the scene of the product recovery process. It was reported that chromatography together with membrane separations had occupied 90% of global market for downstream processing equipment (Spalding, 1991).

Chromatography methods such as gel filtration chromatography (GFC; also known as size-exclusion chromatography or SEC), ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), affinity chromatography (AC), displacement chromatography (DC) and newly developed perfusion chromatography (PC) have been widely used in the purification of biological products (Moreno, 2004; Wilson, 2001; Roque, 2006; Shi, 2004; Jankowski, 2005; Kaufmann, 1997). Among all these chromatography methods, affinity chromatography has been extensively considered as the most powerful and frequently used purification technique available to downstream processing (Spears, 1993;

Gupta, 1994). Examples of logical and efficient purification sequences in downstream processing demonstrated by Spears (1993) are shown in Fig. 1-1. It can be seen that in order to get biological products with high purity, affinity chromatography is definitely necessary to nearly all the purification sequences, which is consistent with the opinion of Miranda (2002).

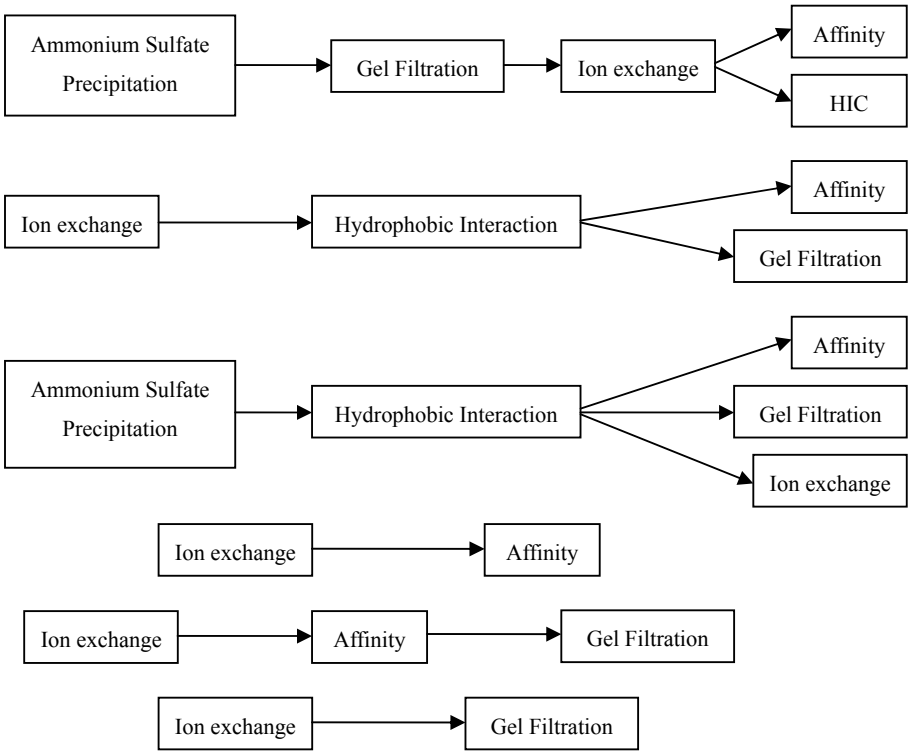


Fig. 1-1. Examples of logical and efficient purification sequences (cited from Spears, 1993).

Since the innovative work of Porath et al. (1967) in the 1960s, and the seminal texts of Lowe and Dean (1974) and Dean et al. (1985), the technologies of affinity separation have become widely accepted. Practitioners range widely from laboratories interested in the isolation of bioactive probes and tools for biological research, to large manufacturing plants for the production of diagnostic and therapeutic drugs. Combining the specific affinity interaction with some other separation methods, new affinity separation techniques such as affinity membrane (Suen, 2003; Zou, 2001), affinity cross-flow filtration (Borneman, 2002), affinity partitioning (Gavasane, 2003; Roy, 2002), affinity-based reverse micellar extraction (Choe, 1997; Paradkar, 1993), and affinity precipitation (Teotia, 2004; Hilbrig, 2003) began to be developed and have made great progress in the last decades. However, these affinity

separation techniques haven't reached the point where they can be widely used for industrial applications except affinity chromatography. Due to its great industrial attraction, this thesis focuses on affinity chromatography with lectins as affinity ligands and deals with the purification of glycoproteins by this technique. This chapter gives an overview of affinity chromatography and a new integrated separation technique—expanded bed adsorption, which can also be combined with affinity chromatography by using affinity ligands.

1.1. Principles of affinity chromatography

Conventional adsorbents separate proteins by exploiting a low number of physicochemical characteristics resulting in weak interaction forces such as charge (IEC), hydrophobicity (HIC), and metal ion binding (metal chelate chromatography). Since any given protein in the cell extract is not likely to be unique with regards to any of these properties, purification to homogeneity requires that a number of different adsorption steps have to be performed. However, the same protein is likely to be uniquely characterized with regard to the surface distribution of charges, hydrophobic and hydrophilic amino acid residues about the surface. An adsorbent which interacts in a complementary manner with these features on a given protein will therefore selectively adsorb the target molecule, enabling one-single-step purification (Porath, 1981). This process is so-called affinity purification. Here, the substance which is immobilized on the insoluble support (matrix) is termed affinity ligand; the support chemically coupled with the affinity ligand is termed affinity adsorbent.

The mechanism of protein involved affinity interaction is not completely clear till now. Generally speaking, people consider that the forces contributing to the formation of a binary complex (formed between ligand and target molecule) result from a combination of electrostatic, hydrophobic, hydrogen binding and Van der Waals interactions (Katchalski-Katzir, 1983; Mattos, 1996).

The principle of affinity chromatography is illustrated in Fig. 1-2. In the first step, the source material containing the target molecule and other impurities is passed through the affinity column (loading). The target molecule specifically binds to the immobilized ligand while the other impurities are washed off without retention on the column (washing). The specifically

bound molecule then can be recovered from the column by changing the environmental conditions (such as pH, ionic strength, solute content, and temperature) to weaken the

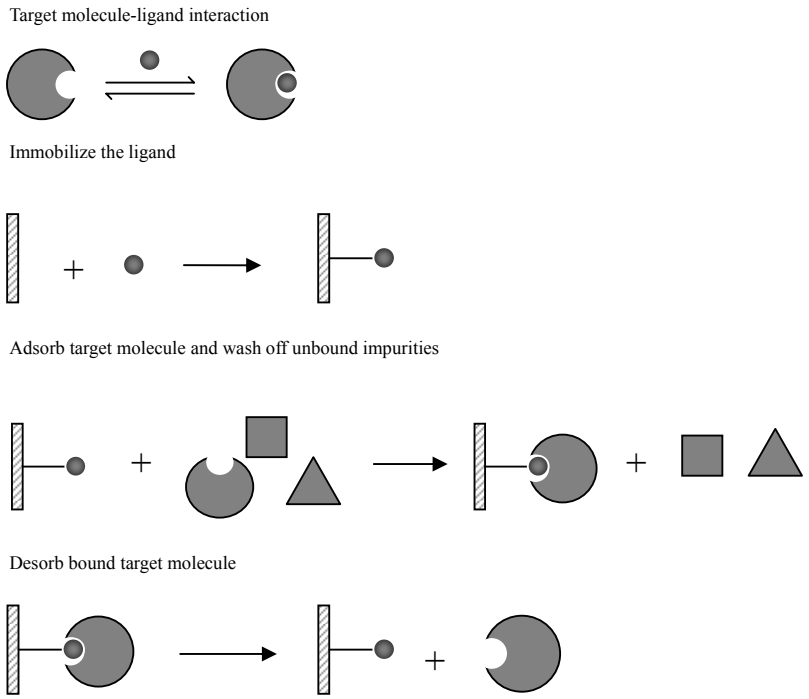


Fig. 1-2. Principle of affinity chromatography.

ligand-protein interaction (desorption). Finally, the column is cleaned if necessary and re-equilibrated for the further use (regeneration).

1.2. Supports for affinity chromatography

The characteristics of the affinity supports play a very important role in the performance of affinity separation. The basic requirements of a most suitable affinity support for affinity chromatography have been described by Sundaran and Yarmush (1993). Briefly, an ideal affinity support should exhibit:

(1) good chemical, mechanical, biological and thermal stability;

(2) high binding capacity with high specificity and hydrophilicity (no surface charge and no hydrophobic sites);

(3) good recovery, reproducibility and ease of synthesis;

(4) suitable flow characteristics and kinetic characteristics.

The affinity supports have been well developed and an extensive range of solid supports is now available for affinity chromatography. By far, the traditional and still the most popular support used for affinity chromatography is agarose. Its main advantages are high capacity, presence of functional group, good chemical stability especially at high pH, low non-specific binding and good reproducibility. Several types of well characterized agarose or cross-linked agarose with differing exclusion volumes are commercially available under the trade names of Sepharose (Pharmacia), Sepharose CL (Pharmacia), Biogel A (Bio-Rad), etc.

A variety of supports have been developed to replace agarose and have been commercially available. These include polyacrylamide (e.g. Bio-Gel-P marketed by Bio-Rad and Eupergit C marketed by Rohm Pharma), cellulose (e.g. Matrex Cellufine marketed by Amicon), cross-linked dextran (e.g. Sephadex marketed by Pharmacia), silica (e.g. Lichrospher marketed by E. Merck and Spherisorb marketed by Phase Separations), and controlled pore glass (e.g. CPG marketed by Pierce). Some novel materials such as perfluorocarbons (Stewart, 1990; McCreath, 1992), zirconia particles (Wirth, 1993) and alumina (Rhemrev-Boom, 2001) were also demonstrated in the literatures and showed their good stability and chromatographic performance in the separation and purification of bioproducts.

1.3. Ligands for affinity chromatography

The selection of the ligand for affinity chromatography is mainly influenced by two factors. Firstly, the ligand should exhibit specific and reversible binding affinity for the substrate to be purified. Secondly, the ligand should have some chemically modifiable groups via which it could be immobilized on the support and without destroying its binding capacity. Ideally, the dissociation constant K_d for the ligand-target molecule complex should be in the range of 10^{-4}-10^{-8} M in free solution (Amersham Pharmacia Biotech, a, p.15). For K_d higher than 10^{-4} M, the interaction is normally too weak for affinity chromatography. Conversely, for K_d lower than 10^{-8} M, the recovery of the bond substance could be difficult since severe conditions may be required to effect desorption.

Affinity purification techniques may be classified on the basis of the affinity ligand used in

the separation process (Vijayalakshmi, 1989) as shown in Fig. 1-3.

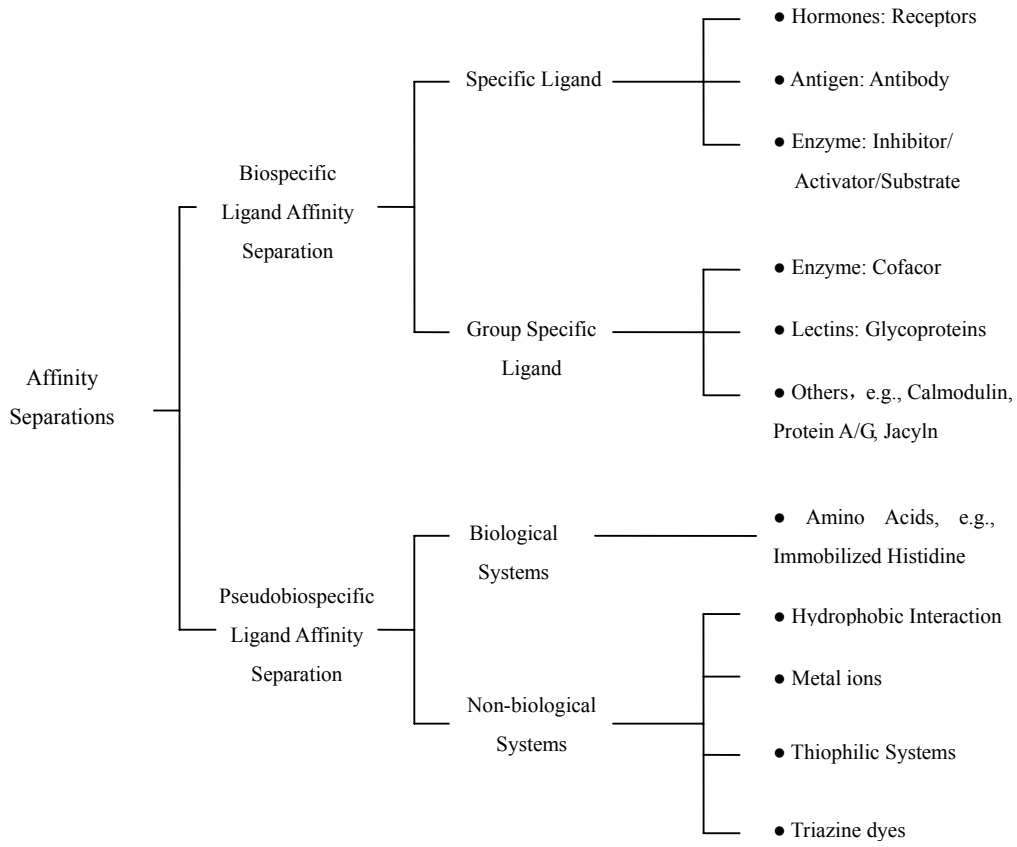


Fig. 1-3. Classification of affinity purification techniques based on the ligand used (cited from Vijayalakshmi, 1989).

Biospecific affinity separations are based on reversible and specific interactions between biologically active substances. In these cases, ligands suitable for immobilization may be divided into two groups, namely ‘‘specific ligands’’ and ‘‘group specific ligands’’. The representatives of the specific ligands are hormones and monoclonal antibodies which bind complementary receptors and antigens respectively in a highly selective manner. However, this high specificity makes it necessary that a separate affinity adsorbent should be prepared for each protein. This problem could be partly alleviated by group specific adsorbents since a single affinity adsorbent could be employed for the purification of a number of proteins thus reducing the preparation process development cost. And this is also one of the reasons that explained the popularity of group specific ligand in biotechnology industry. Examples of

group specific ligands are enzymes, lectins, protein A, and so on.

Besides biospecific affinity separation, there is another affinity separation based on the interaction of biological molecules with simple ligands such as hydrophobic ligands, reactive dyes, and metals. Such interactions are termed "Pseudobiospecific" interactions. Pseudobiospecific affinity chromatography techniques especially Immobilized Metal Affinity Chromatography (IMAC) and Dye-ligand Affinity Chromatography are gradually finding their acceptance for industrial applications since these ligands are pretty cheap and stable when compared to biospecific ligands. However, these techniques are still limited since it is difficult to establish rules for selecting relative pseudobiospecific ligand for a given protein due to the incomplete understanding of the mechanism (Sundaram, 1993).

1.4. Spacer arm

In some instances, the steric conditions around the immobilized ligand will hinder the binding of the target molecule to the ligand. This is often true when the ligand is a small molecule. Such steric limitations can be avoided by introducing a spacer arm between the ligand and the supports (see Fig. 1-4).

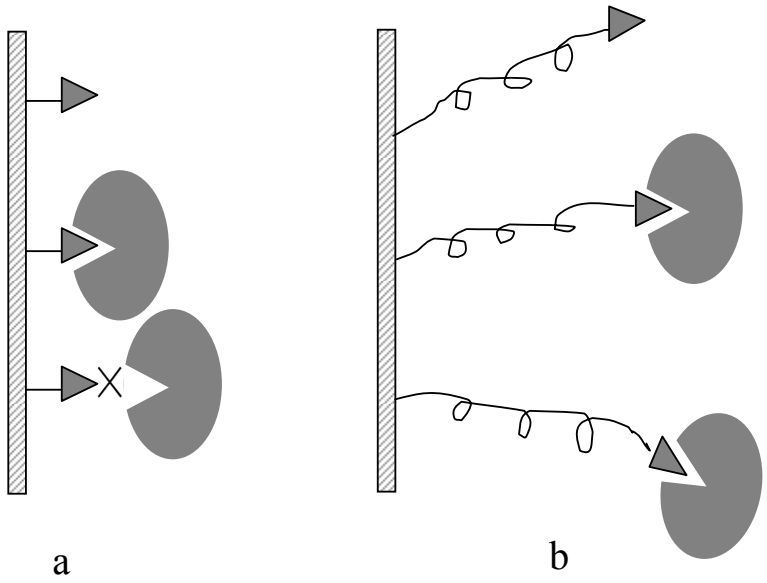


Fig. 1-4. The principle of spacer arms. a. Ligand attached directly to the matrix.
b. Ligand attached to the matrix via a spacer arm.

These spacer molecules are normally linear aliphatic hydrocarbons with optional polar moieties such as secondary amino, hydroxyl, and peptide groups. The length of the spacer arm is often critical for the performance of affinity separations. If it is too short, the arm is often ineffective and the ligand may bind the target molecule rather poorly. If it is too long, it may contribute to the non-specific adsorption thus reducing the selectivity of the separation (O'Carra, 1973). There is no general "universal" spacer that can be specified with respect to length and chemical nature. In most cases, a C_6 chain is sufficient to provide an adequate spacer effect. However, good separation performance can also be achieved with affinity supports to which a more than 10-atom spacer was coupled (Rhemrev-Boom, 2001; Ruckenstein, 2001). The spacer arm could be provided by some activation procedures such as glutaraldehyde activation, diazotization, epoxide activation, and so on (Guo, 2003; Ruckenstein, 2001; Castilho, 2000; Gan, 2000).

1.5. Affinity adsorption from particulate feedstocks by expanded bed adsorption (EBA)

The initial purification of the target molecule has been traditionally operated by adsorption chromatography using a packed bed of adsorbents. This necessitates the clarification of the crude feedstock prior to the application to the chromatography column. The traditional clarification techniques have been centrifugation and microfiltration (Lee, 1989), which have been used for many years and regarded as standard in the biotechnology industry. However, these two techniques suffer from some drawbacks in the production process. For example, sometimes it is difficult to get a particle-free solution only by centrifugation especially when handling some small cells, such as *E. coli.*, or cell homogenates (Berthold, 1994). Therefore, centrifugation is frequently combined with microfiltration in order to get particle-free solution for further purification by traditional packed bed chromatography. However, the dramatic reduction of the liquid flux per unit membrane area and fouling of the microfiltration membranes often result along the processing time and cause high operational cost.

To overcome these problems, fluidized bed processes were introduced by Bartel (1958) for the recovery of streptomycin at large scale. Despite some successful applications of fluidized

bed for the recovery of low molecular weight compound (Gailliot, 1990), this technique has not been used for the recovery of proteins till recently due to the lack of suitable adsorbents and equipment for such processes. In the early 1990s, Chase and Draeger et al. (Draeger, 1991; Chase, 1992a and 1992b; Chang, 1993) first created a fluidized bed system with high stability and low back-mixing by using improved adsorbents in a purpose-designed column with liquid distribution inlet supporting plug flow in the column. The adsorptive process in such a system is termed expanded bed adsorption. In 1993, Pharmacia Biotech introduced new types of chromatographic adsorbents and columns called STREAMLINE. These products were specially designed for the expanded bed adsorption. From then on, expanded bed adsorption came to a new stage and has been widely developed and employed in both laboratory and industrial scale.

1.5.1. Principles of expanded bed adsorption

An expanded bed is a low back-mixing liquid fluidized bed achieved by the purpose-designed column and adsorbents with a defined size and density distribution, respectively. Expanded bed adsorption combines the hydrodynamic properties of a fluidized bed with the chromatographic properties of a packed bed. In an expanded bed process, particulate adsorbents are allowed to expand by applying an upward flow, thus resulting in higher bed voidage between the adsorbents. Provided that the physical properties (e.g. density) of the adsorbents are significantly different from those of the particulates in the feedstock, the particulates can pass freely through the expanded bed without becoming trapped whilst the target bioproduct is simultaneously captured by the adsorbents. When a stable expanded bed is achieved through the balance between the individual adsorbent sedimentation velocity and the upward flow velocity, the classification of the adsorbents and therefore limited movement of the adsorbent particles take place within the expanded bed. This is necessary if dispersion in the liquid phase is to be minimized and high adsorption efficiency achieved (Karau, 1997). As shown in Fig. 1-5, the larger adsorbent particles are found in the lower part of the expanded bed while the smaller ones in the upper part. Usually, the adsorbents suitable for expanded bed adsorption have the particle size ranging from 50-400 μm and the density of

1.1-1.3 g/ml (Hjorth, 1997). The fundamental properties of expanded bed adsorption have been reviewed by a number of papers (Chase, 1994; Hjorth, 1997; Anspach 1999; Hubbuch, 2005).

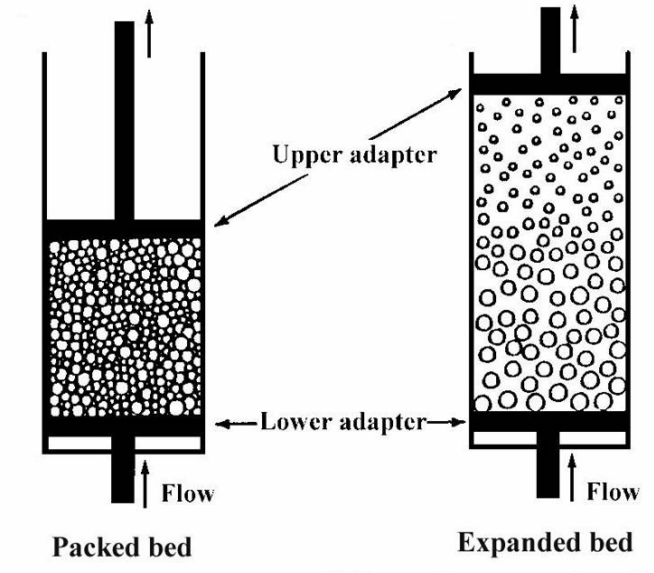


Fig. 1-5. The principle of expanded bed adsorption process (adapted from Chase, 1994).

The working principle of expanded bed is just like a packed chromatography bed (Chase, 1994; Clemmitt, 2000a), yet it can handle particulate-containing feedstocks. Since expanded bed adsorption could be taken as a quasi-packed bed, the equipment required and the operating procedure are quite similar to those used in packed bed (Chase, 1994; Chase, 1998). A difference in process performance when processing crude feedstocks might rise from the higher complexity, the presence of insoluble components and the reduced binding capacity due to blocking of the surface by adsorbed cell or cell debris (Chase, 1992a; Draeger, 1991; Fernández-Lahore, 2000).

In conclusion, expanded bed adsorption is a combination of solid-liquid separation and adsorption, thus enables clarification, concentration and initial purification in a single step, providing increased process economy due to a decreased number of process steps, followed by an increased product yield, shorter process time, reduced labor cost, and reduced running cost and capital expenditure (Hubbuch, 2005; Kalyanpur, 2002).

1.5.2. Adsorbents for expanded bed adsorption

The physical properties of the adsorbents (size, density and shape) are very important for the formation of a stable expanded bed and thus efficient protein adsorption (Finette, 1996). The basic requirements for the adsorbents suitable for expanded bed adsorption were summarized by Chase and Draeger (Chase, 1992a).

Since the innovation of expanded bed adsorption, several different modified support materials have been applied as adsorbents for the recovery of proteins in expanded bed process. Such materials could be only high density porous matrix, analogous to conventional fixed bed adsorbents, such as superporous cross-linked cellulose (Pai, 2000), silica gel (Finette, 1996), hydroxyethyl methacrylate-ethylene dimethacrylate copolymer (Nayak, 2001), perfluorocarbon matrix (Owen, 1997) and fluoride modified zirconium oxide particles (Mullick, 1998; Griffith, 1997). Other adsorbents are mostly composites which are weighted by high density particles as the internal cores. Such adsorbents employed in expanded bed adsorption include cellulose-titania composites (Gilchrist, 1994; Lei, 2003), semiporous adsorbents formulated from agarose and dense silica-coated zirconia particles (Jahanshahi, 2002), Nd-Fe-B alloy-densified agarose gel (Tong, 2001), agarose-coated alumina (Hidayat, 2004) and so on. A very good review of various adsorbents employed in expanded bed adsorption has been given by Hubbuch very recently (2005). Despite the extensive studies of the adsorbents employed in expanded bed process only a few of them have been commercially available, such as Streamline (crosslinked agarose-based quartz composite materials, marketed by Amersham Pharmacia, Sweden), FastLine (dense agarose-coated glass beads, marketed by Upfrount Chromatography, Denmark) and S-HyperD LS (silica composites, marketed by Biosepra, Marlborough, MA, USA). As the pioneer in expanded bed process, Streamline still is the most popular adsorbent which has been extensively employed in both laboratorial and industrial applications.

1.5.3. Equipment for expanded bed adsorption

As aforementioned, the equipment employed for expanded bed process is similar to that used in packed bed process. The only exception is the design of the expanded bed column, which

has two main features distinguished from conventional packed bed column: the liquid distribution inlet and the adaptor.

Traditionally, fluid distribution in the packed bed system is achieved by creating a sufficiently high axial backpressure to ensure the evenly liquid distribution in radial direction of the column, the so called plug flow. Since the pressure drop over the expanded bed is much smaller, the distributor in expanded bed column must produce a plug flow itself at the column inlet. As summarized by Hubbuch (2005), even flow distribution has been achieved by:

(1) the creation of the backpressure, which is realized by introducing perforated plates, metal meshes, glass frits or a bed of balatoni glass beads at the inlet of the column;

(2) conical flow distributors, which facilitate an increase in the cross-sectional area while simultaneously maintaining plug flow characteristics;

(3) a localized mixing device, which introduces processing liquid from side-ports and ensures an even flow distribution by positioning a mixer at the lower section of the column;

(4) a rotating fluid distributor, which is based on the equal fluid distribution achieved by the slowly rotating pipes at the column inlet.

Furthermore, the expanded bed column is equipped with an adaptor on the top of the column. The moveable adaptor could allow the column to be operated at different bed height, which is necessary during the different stages of the operation of expanded bed process.

1.5.4. Operation of expanded bed adsorption

The expanded bed process is operated in a similar way as packed bed process. The main difference is that the flow is upwards during the equilibration, feedstock application, washing and/or elution. The expanded bed operation process is illustrated in Fig. 1-6.

(1) Equilibration

The bed of adsorbents starts to expand when an upward flow is applied. When the bed is expanded to an appropriate bed height, the equilibration buffer is continued for at least 30 min to get a stable expanded bed with reducing back-mixing. The expanded bed process is commonly operated at a flow velocity of 300 cm/h or at an appropriate flow velocity resulting

in 2-3 times bed expansion and therefore with optimal adsorption efficiency (Chang, 1996). The settled bed height is better more than 10 cm in order to get more efficient binding capacity of proteins due to the negligible back-mixing in the column (Hjorth, 1995). It is important to point out that the verticality of the column should be ensured during the whole

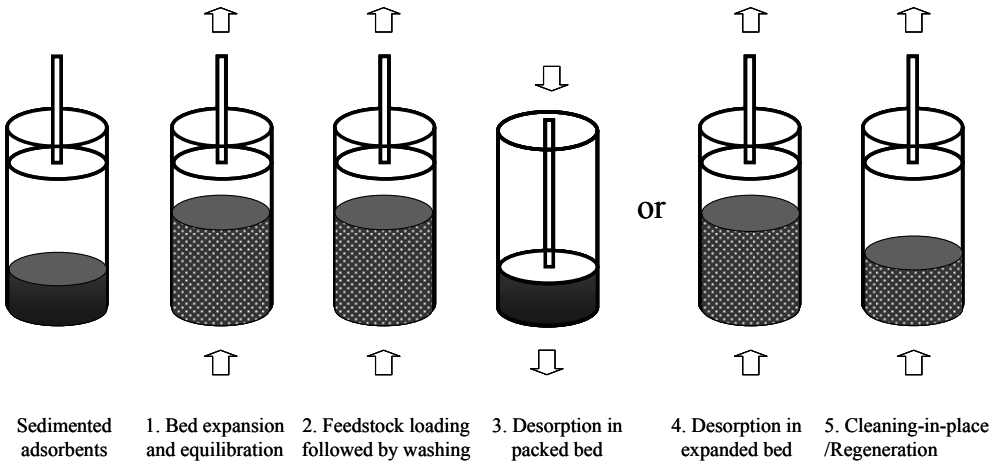


Fig. 1-6. Schematic diagram of the operation steps of expanded bed adsorption.

operation. The studies of Bruce (1999) have showed that the column misalignment of only 0.15° resulted in the reduction of the Bodenstein number from 140 to 50 for the 1 cm I.D. column and from 75 to 45 for the 5 cm I. D. column.

(2) Application of the feedstock

When the expanded bed is stable, the equilibration buffer is switched to feedstock containing cells, cell debris or other particulate matter. The bed may further expand if the flow velocity is kept constant as the physical properties of the feedstock are different from those of equilibration buffer, especially the viscosity is higher. It is demonstrated that a higher dynamic adsorption capacity could be obtained when operating at a constant degree of bed expansion (2-fold the settled bed height) than at a constant flow velocity (Chang, 1996). Therefore, it is common case to decrease the flow velocity during the application of feedstock to keep the constant degree of bed expansion. Studies on the effective control of expanded bed height have been investigated by several researchers. For example, Thelen and Ramirez (1999)

applied ultrasonic technique to monitor and control the expanded bed height, and also built up a model of solid-liquid fluidization to predict the bed height dynamics due to the flow rate and fluid properties. Ghose (2000) developed an LED based sensor to provide effective information on the movement of the bed top in terms of its direction and rate of changes.

(3) Washing

To get optimal binding efficiency during expanded bed operation, the feedstock is switched to equilibration buffer again still by upwards flow when the protein concentration at the outlet of the column reaches 5-10% of that at the inlet of the column, that is, the initial feedstock concentration. The particulates, such as cell debris, between the adsorbents particles and in the pore of the adsorbents and some weakly bound contaminants can be washed out from the expanded bed in this step. The washing buffer could also contain some high viscosity reagent, such as glycerol to improve the washing efficiency (Chang, 1993; Hjorth, 1995; Fee, 2001). The application of a viscosity enhancer could reduce the volume of washing buffer, and also generate flow distribution in a more plug flow way passing through the expanded bed, thus finally provide better washing effect. When there are no more particulates hindered in the column, the process turns to desorption step.

(4) Desorption

Desorption of target protein in expanded bed process could be obtained either by packed bed mode (downwards; No. 3 in Fig. 1-6) or by expanded bed mode (upwards; No. 4 in Fig. 1-6). In general, the packed bed mode is often employed in the case that the target compounds are mostly concentrated near the inlet of the column. The expanded bed mode could be more efficient when the strong binding occurs between the target compound and the adsorbent and the target compounds are adsorbed in the whole expanded bed. Each desorption mode has its own advantage and disadvantage, which is revealed and compared by many authors (Hjorth, 1999; Lihme, 1999). Desorption in packed mode (downward flow) can reduce the volume of desorption buffer thus improve the concentration of target compound. However, this desorption mode also suffers some problems, such as long processing time, complexity of the operation process (the need to adjust the adaptor and flow direction), and the requirement of

higher mechanical stability of the adsorbents due to the higher pressure drop on the bed. In contrast to packed bed mode, desorption in expanded bed mode eliminates the time required to allow the bed settle, adjust the adaptor down and then back up, and allow the bed to expand again. It also simplifies the equipment required and allows continuous production. Therefore, more and more applications of expanded bed adsorption have been adopted with desorption in expanded mode to purify target compound in industrial production.

(5) Cleaning-in-place/Regeneration

Cleaning-in-place (CIP) is an important stage in downstream processing and consequently also in the expanded bed adsorption process. CIP is performed to maintain the functionality of the adsorbent and should ensure that all the impurities are removed from the bed. The particulates such as cells, cell debris, lipids, and nucleic acid, existing in the feedstock applied to expanded bed process often contaminate the adsorbents, especially those with low specificity. Such contaminations are very difficult to remove only by washing and desorption procedure. They may cause the reduction of the binding capacity, more badly, the aggregation of the adsorbents and thus leading to channeling in the bed and significant efficiency drop of the process. Therefore, it is important to perform CIP procedure after desorption of the target compounds. It is one part to regenerate the adsorbents for further use. The CIP procedure employed depends on the properties of the adsorbent ligand and the nature of the feedstock applied (Barnfield Frej, 1994 and 1997; Chang, 1996). Most CIP procedures include the use of sodium hydroxide in concentrations of 0.5 M-1 M, which is often combined with other CIP solutions, such as 10% acetic acid, and 20% ethanol, respectively. For some proteinaceous ligand, such as protein A, sodium hydroxide in concentrations of 1 mM-10 mM (Fahrner, 1999) or 6 M guanidinium hydrochloride (Hjorth, 1997) as an alternative may be used for CIP procedure. During this stage, it is suggested to position the adaptor at approximately twice the sedimented bed height with a moderate upward flow (Amersham Pharmacia Biotech, b, p. 6).

1.5.5. Application of expanded bed adsorption (EBA)

Since its introduction in 1990s, EBA has been widely applied in various aspects of

downstream processes. These include the recovery of proteins (Batt, 1995; Owen, 1997), cell disruption process (Nandakumar, 1999), cell separation (Ujam, 2000; Clemmitt, 2000b and 2003), purification of DNA (Ferreira, 2000) and antibody (Thömmes, 1995a), and flow-ELISA analyses (Pålsson, 2000b; Mattiasson, 1999). Reviews on the application of expanded bed adsorption have been given by a number of researchers (Hjorth, 1997; Chase, 1998). Table 1-1 shows some applications of expanded bed adsorption in downstream

Table 1-1. Selected applications of expanded bed adsorption

Product	Feedstock	Adsorbent	Yield (%)	Purification factor	Ref.
B-galactosidase	*E. coli* homogenate	Streamline chelating	86	5.95	Clemmitt, 2000a
GST-$(His)_6$	*E. coli* homogenate	Streamline chelating	80	3.34	Clemmitt, 2000b
ADH	Baker's yeast	Chelating Sepharose Fast Flow	68	8.2	Willoughby, 1999
A-amylase	*E. coli* homogenate	DEAE Sepharose Fast Flow	96	2.7	Pierce, 1999
Lactate dehydrogenase	Porcine muscle homogenate	Cibacron Blue cross-linked cellulose	100	31	Pai, 2000
lysozyme	Equine milk	Streamline SP	89	8.3	Noppe, 1996
Monoclonal IgG	Cell culture	Protein A	83	30	Thömmes, 1996

processing in recent years. Although many of such applications have been only run on the laboratory scale, some of them are developed at pilot or production scale (Barnfield Frej, 1994 and 1997; Lütkemeyer, 1999).

1.6. The biological system of glycoproteins and lectins

Glycoproteins are proteins covalently associated with carbohydrates. In glycoproteins, glycans are conjugated to peptide by two kinds of covalent linkage: N-linked and O-linked form (Kishino, 1997). Glycoproteins are involved in a wide range of biological functions such as receptor binding, cell signaling, immune recognition, inflammation, and pathogenicity

(Gerwig, 1998). An increasing number of glycoprotein drugs have been developed for diagnostic and therapeutic use, respectively in humans in the past few years (Damm, 1998). Due to the growing attention paid to glycoprotein, an efficient separation and purification method becomes more and more essential. Affinity purification using lectin as ligand has proved a most popular technique for purification of many glycoproteins due to its high specificity (Kishino, 1997; Hirabayashi, 2002).

Lectins are defined as proteins which specifically bind (or crosslink) carbohydrates. Since their discovery in the late 1800s, lectins have been found in a wide variety of sources, such as plants, animals and even in the microbial world. On the base of their specificity to monosaccharide, lectins are classified into five groups, for which they exhibit the highest affinity: mannose, galactose/N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), fucose, and N-acetylneuraminic acid (Lis, 1998). However, some lectins do not recognize simple sugar at all and bind only to oligosaccharide. Table 1-2 lists some representative lectins (data selected from Etzler, 2000), which gives profound information about their carbohydrate binding properties.

Table 1-2. Carbohydrate specificities of representative plant lectins (from Etzler, 2000)

Lectin Name (abbreviation)	Source[a]	Monosaccharide preference	Anomeric[b] preference	Preferred oligosaccharide	RA[c]
Mannose/Glucose specific					
Concanavalin A (Con A)	*Canavalia ensiformis* (jackbean) seeds	Mannose	α	Manα1,6(Manα1,3) Man	>130
Pea (PSL)	*Pisum sativum* (pea) seeds	Mannose	α	Glcα1,2Glc	12
Snow drop (GNA)	*Galanthus nivalis* (snow drop) bulbs	Mannose[d]	α	Manα1,6(Manα1,3) Man α-O-Methyl	28
GlcNAc specific					
Wheat germ agglutinin (WGA)	*Triticum vulgare* (wheat) grain	N-acetylglucos-amine	none	$(GlcNAc\beta1,4)_3$	3000
Galactose/GalNAc specific					
Soybean agglutinin (SBA)	*Glycine max* (soybean) seeds	N-acetylgalacto-samine	α	GalNAcα1,3Galβ1,6Glc	5
Peanut (PNA)	*Arachis hypogaea* (Peanut) seeds	galactose[e]	β	Galβ1,3GalNAc	50
Ricinus communis agglutinin (RCA I)	*Ricinus communis* (castor bean) seeds	Galactose[e]	β	Galβ1,4Glc	6-7
L-Fucose specific					
UEAI	*Ulex europaeus* (Gorse) seeds	Fucose	α	L-Fucα1,2Galβ1,4 GlcNAcβ1,6R	900
Sialic acid specific					
Sambucus nigra I (SNA)	*S. nigra* (elderberry) bark	Gal[f]	α	Neu5Acα2,6Gal	1600
Oligosaccharide only specific					
L-PHA	*Phaseolus vulgaris* (red kidney bean) seeds	None		Galβ1,4GlcNAcβ1,6 \ Man / Galβ1,4GlcNAcβ1,2	
Potato	*Solanum tuberosum* (potato) tubers	None		$(GlcNAc\beta1,4)_{2-5}$	

[a] Common name of the plant in parentheses. [b] Based on the ability to react with the α or β glycoside.

[c] Relative affinity (RA) compared to that of the preferred monosaccharide. [d] unlike the other lectins listed in this category, the GNA lectin doesn't bind to Glc or GlcNAc. [e] does not bind to GalNAc. [f] does not bind to free Neu5Ac but does bind weakly to Gal.

1.7. Objective of this work

This work concerns the fundamental aspects of affinity separation of glycoproteins. To study the affinity interactions between the affinity ligand and glycoptoteins, parameters affecting the whole purification performance will be investigated. A model affinity separation system is built up for the following studies. Strategies for large scale affinity purification will be investigated in two ways: the modification of affinity ligand and the combination of expanded bed adsorption with affinity separation. The preparation, characterization and application of modified ligand will be introduced in order to get better stability against environmental conditions encountered in affinity purification process. A novel pellicular adsorbent will be prepared and characterized for affinity expanded bed adsorption and expanded bed adsorption process will be developed to purify crude glycoprotein feedstock.

2. Adsorption behaviour of glucose oxidase onto Concanavalin A affinity adsorbents

2.1. Introduction

Lectins are proteins that interact specifically and reversibly with certain sugar residues. Their specificity enables binding to polysaccharides, glycoproteins and agglutination of erythrocytes and tumor cells (Helmholz, 2003; Narayanan, 1994; Turkova, 1993). This specific binding is based on stereochemical interactions and it can be used to purify target compound with highly preserved biological activity due to the mild separation conditions. Immobilized lectin affinity chromatography has been extensively used for the isolation, fractionation, structure characterization and immobilization of glycoproteins and other biologically important glycoconjugates (Kishino, 1997; Hirabayashi, 2002).

Numerous approaches have been taken to immobilize bioactive molecules onto solid substrates including adsorption, covalent coupling, and tethering via an intermediate linker. Studies show that the properties of the surface and the method of immobilization have profound effects on the resulting bioactivity of the immobilized ligand. The hydrophobicity, charge, and chemical properties of the surface material could influence the stability and orientation of the immobilized ligand (Malmsten, 2000). The immobilization method could also affect ligand activity through the chemical modifications of its amino acids especially when the coupling sites are close to the active binding sites (El-Masry, 2001; Guo, 2003; Ruckenstein, 2001).

With the increasing interest in affinity chromatography, lots of researchers mainly focus on the development of new affinity supports and the number of affinity adsorbents commercially available has been steadily increased. However, systemic studies of the influence parameters on affinity separation and the development of affinity process are limited. This work will take the adsorption of glucose oxidase (GOD) onto two different Concanavalin A (Con A) affinity adsorbents as an example to study their adsorption behaviour. The influence parameters among the adsorption process ranging from the immobilization of Con A to the dynamic

adsorption of GOD will be investigated.

Concanavalin A (Con A)

Con A is probably the most extensively studied lectin for affinity chromatography due to its easy availability. Con A is isolated from *Canavalia ensiformis* (Jack bean). Its monomeric molecular weight is 26 kDa and its isoelectric point about pH 5. In an aqueous medium, Con A can be a monomer, dimer, or tetramer, depending on the pH and temperature (Agrawal, 1968). Con A exists as a dimer at pH 5.5 and a tetramer at pH > 7. Con A is a metalloprotein and requires a transition metal ion such as manganese and calcium for saccharide binding to occur. Each subunit has a binding site for one Mn^{2+}, one Ca^{2+}, and one oligo- or polysaccharide. The crystal structures of Con A with a series of carbohydrates have been extensively studied and the interactions between them are mainly hydrogen bonds and Van der Waals interactions (Bouckaert, 1999; Kennedy, 1995). The crystal structure of native Con A cited from Weisgerber (1993) is shown in Fig. 2-1.

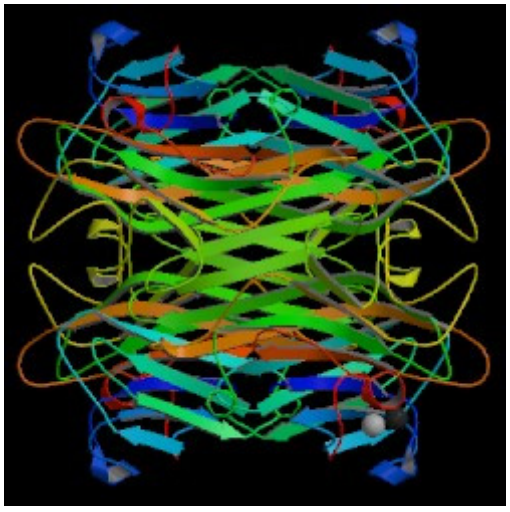
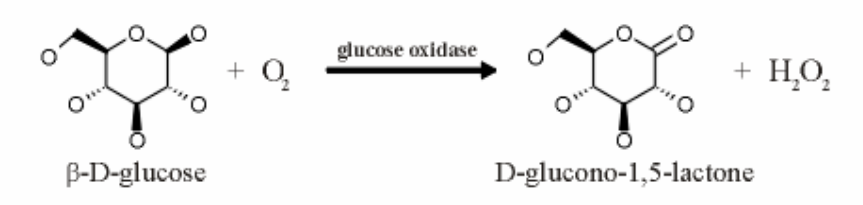


Fig. 2-1. The crystal structure of native Con A
(PDB entry: 2CTV; cited from Weisgerber, 1993).

Studies show that the most active part of a glycan structure towards Con A is not the terminal α-mannose residues but the trisaccharide α-Man-(1-3)-[α-Man-(1-6)]Man (Naismith, 1996).

Glucose oxidase (GOD)

Glucose oxidase (GOD) (EC 1.1.3.4) is a flavoprotein which catalyzes the oxidation of β-D-glucose by molecular oxygen to δ-gluconolactone, which subsequently spontaneously hydrolyzes to gluconic acid and hydrogen peroxide (Zoldak, 2004).



(2-1)

The enzyme is of considerable commercial importance (Ahmad, 2001). Industrially, it is applied for the removal of glucose or oxygen from food products and in the production of gluconic acid (Rohr, 1983). The most important application of glucose oxidase is as a

Fig. 2-2. The crystal structure of GOD
(PDB Entry: 1GAL; cited from Hecht, 1993).

molecular diagnostic tool. The enzyme is employed in biosensors for the quantitative determination of D-glucose in samples such as body fluids, foodstuffs, beverages, and fermentation products (Turner, 1987; Schmid, 1988).

GOD is a dimer consisting of two equal subunits with a molecular weight of 80 kDa each.

Each subunit carries one molecule of noncovalently bound coenzyme, FAD, which is acting as a redox carrier in catalysis (Gibson, 1964). The primary structure of GOD from *A. niger* has been determined; a single polypeptide chain of one subunit has 583 amino acid residues (Frederick, 1990). GOD is a glycoprotein with a high-mannose type carbohydrate content of 10–16% of its molecular weight (Hayashi, 1981; Pazur, 1965). The carbohydrate moieties are *N*- or *O*-glycosidically linked to the protein. The crystal structure of the enzyme has been solved and is shown in Fig. 2-2 (Hecht, 1993).

2.2. Materials and methods

2.2.1. Materials

The polymeric support Toyopearl AF-Tresyl-650M (hereafter called Toyopearl for short) was obtained from Tosoh Bioscience (Stuttgart, Germany). The inorganic support silica with glutaraldehyde terminal groups was a gift provided by Grace (Worms, Germany). The physical and chemical properties of these two supports are listed in Table 2-1 according to the

Table 2-1. Physical and chemical properties of employed supports

Supports	Particle size (μm)	Pore size (Å)	Specific surface area (m^2/g)	Spacer	Functional group
Toyopearl	40-90	1000	42	no	Tresyl
Silica	90-130	2500	15-16	no	Aldehyde

data provided by the suppliers. Con A (type V), methyl-α-D-mannopyranoside, and Bradford reagent were purchased from Sigma (Munich, Germany). GOD (229 U/mg) was obtained from Serva (Heidelberg, Germany). All chemicals were of analytical grade unless otherwise stated.

2.2.2. Preparation of Concanavalin A (Con A) affinity adsorbents

Both Toyopearl and silica supports were first preequilibrated in coupling buffer (0.5 M

phosphate buffer, containing 0.1 M NaCl, pH 8.0) for at least 10 min. A certain amount of sucked supports were then mixed with 4 mg/ml Con A with a volumetric ratio of 1:3 in the coupling buffer, which contained 50-fold molar excess of methyl-α-D-mannopyranoside for the protection of the binding sites of Con A during immobilization. The reaction was carried out for 4 h at room temperature with gentle shaking. To determine the immobilization kinetics the sample aliquots were taken from the reaction mixture at time intervals. After the immobilization the reacted supports were filtrated and extensively washed with the coupling buffer and then water. The filtrate together with the washing fraction was collected for the determination of the protein concentration.

The immobilization efficiency can be represented by the coupling yield, which indicates the ratio of the Con A coupled on the supports to that initially added. The residual functional groups on the supports were blocked with 0.5 M Tris-HCl buffer, containing 0.1 M NaCl, pH 8.0, for 2 h at room temperature. At the last stage, Toyopearl-Con A adsorbents were thoroughly washed with 0.1 M acetate buffer, containing 0.1 M NaCl and 1 mM Ca^{2+}, Mn^{2+}, and Mg^{2+}, pH 5.0, and stored at 4 °C in the same buffer for the further use. For silica-Con A adsorbents, additional 20 mg $NaCNBH_3$ was introduced to the reaction mixture and reacted for another 1 h to eliminate unstable C=N Schiff bases formed during the immobilization and blocking reaction. The following washing and storage steps to silica-Con A adsorbents were carried out in the same way as to Toyopearl Con A.

2.2.3. Calculation of the coupling yield and ligand density

The coupling yield and ligand density were used to characterize the efficiency of Con A immobilization. The coupling yield y is defined as:

$$y = \frac{m_i}{m_0} \times 100\% \qquad (2\text{-}2)$$

where m_i is the amount of Con A immobilized onto the supports (mg); m_0 is the total amount of Con A added for the immobilization (mg). The ligand density d_l is calculated according to Eq. 2-3:

$$d_l = \frac{m_i}{V_{ads}} \tag{2-3}$$

where V_{ads} is the volume of the Con A affinity adsorbent (ml).

2.2.4. Calculation of the surface coverage

The surface coverage is an approximate value and is just taken as a reference parameter to compare the immobilization performance. It was calculated assuming the average molecular dimension for Con A tetramer as 124 Å×129 Å×67 Å, which was obtained according to the 3D crystal structure of Con A by X-ray diffraction (Naismith, 1994). Because the orientations of the immobilized Con A are unknown, the surface area covered by one protein molecule is calculated using the smallest and largest values of the protein dimensions generating a rectangle at the surface of the affinity adsorbents. Consequently, a covered surface area of 0.51 m^2 can be calculated for 1 mg Con A tetramer.

2.2.5. Batch experiments of glucose oxidase (GOD) adsorption onto Concanavalin A (Con A) affinity adsorbents

The adsorption of GOD onto Con A adsorbents was carried out by batch experiments. Some factors that affect the adsorption process were first studied. The pH values of the adsorption buffer investigated in this work were pH 5.0, pH 6.0, and pH 7.0, respectively. 0.1 M acetate buffer was used for pH 5.0 and pH 6.0, while 0.1 M Tris-HCl buffer for pH 7.0. The effect of ionic strength was achieved by changing NaCl concentrations in the range of 0 to 0.5 M. All the adsorption buffers contained 1 mM Ca^{2+}, Mn^{2+}, and Mg^{2+} in order to keep the activity and stability of Con A. All the experiments were performed at room temperature unless otherwise stated.

In a typical adsorption experiment, about 0.2 ml sucked Con A adsorbents were equilibrated with 1 ml adsorption buffer for 2 h and then mixed with 4 ml GOD solution with the final concentration in the range of 0.1 mg/ml to 2 mg/ml in different adsorption buffers. All the measurements were carried out in a shaking bath at 120 rpm for 15 h. The amount of the adsorbed GOD onto Con A affinity adsorbents was determined from the difference of the

initial and final concentration of GOD in the adsorption buffer, which can be obtained form Eq. 2-4.

$$q^* = \frac{(c_0 - c^*)V_{GOD}}{V_{Ads}} \tag{2-4}$$

where q^* is the equilibrium adsorption capacity of the affinity adsorbent for GOD (mg/ml); c_0 and c^* are the initial and equilibrium concentration of GOD in the aqueous phase of the mixture, respectively (mg/ml); V_{GOD} is the total volume of the GOD aqueous solution (ml); and V_{Ads} is the volume of the Con A adsorbents (ml).

Under the optimal adsorption conditions the adsorption isotherms of GOD onto Con A adsorbents were studied and the results were fitted with the Langmuir equation, as shown in Eq. 2-5.

$$q^* = \frac{q_m c^*}{K_d + c^*} \tag{2-5}$$

where q_m is the maximum adsorption capacity and K_d the dissociation constant.

The adsorption kinetics studies were carried out by mixing 1 ml of Con A adsorbents with 30 ml of 0.5 mg/ml GOD solution. After the adsorption, the saturated Con A adsorbents were sucked and carefully washed with adsorption buffer and then mixed with 20 ml 0.1 M methyl-α-D-mannopyranoside to study the desorption kinetics. Each 1 ml adsorption and desorption samples were taken from the solutions at certain time intervals to determine GOD concentration at λ= 280 nm by UV spectrophotometer (Carl Zeiss, Jena, Germany) After the measurement the taken sample should be returned to the corresponding solution as soon as possible in order to reduce the experimental error.

2.2.6. Determination of unspecific adsorption of glucose oxidase (GOD)

About 1 ml silica and Toyopearl supports were blocked with 30 ml 0.5 M Tris-HCl buffer, pH 8.0, for 8 h to eliminate all the reactive functional group. The carefully washed end-capped supports were then mixed with 10 ml 0.5 mg/ml GOD solution in a 15 ml tube for 15 h. The supernatant was sucked out from the tube to determine the protein concentration by

UV absorption at λ= 280 nm. The adsorbed GOD was calculated by mass balance. The same procedure was carried out with silica-Con A and Toyopearl-Con A affinity adsorbents to evaluate the degree of unspecific adsorption in relation to the whole adsorption capacity.

2.2.7. Dynamic adsorption of glucose oxidase (GOD)

The dynamic adsorption of GOD was performed by a self-assembled system containing a peristaltic pump (Bio-Rad, Munich, Germany) and a UV detector connected with a notebook. The UV signal was recorded by software VirtualBench (National Instruments, Munich, Germany). After equilibration of the column with 0.1 M acetate buffer, containing 0.1 M NaCl, 1 mM Ca^{2+}, Mg^{2+}, and Mn^{2+}, pH 5.0 (adsorption buffer), 200 µl 5 mg/ml GOD was applied to 1.5 ml Con A adsorbents packed in a column with 8 mm inner diameter. The same buffer was employed to wash the column until the UV signal reached the baseline. The bound GOD was then eluted from the column with 0.1 M methyl-α-D-mannopyranoside in adsorption buffer. Finally the column was regenerated with the adsorption buffer for the further use. The flow rate in all affinity separations was kept at 0.8 ml/min. The amount of desorbed GOD was determined by Bradford method (Bradford, 1976).

2.3. Results and discussion

2.3.1. Immobilization of Concanavalin A (Con A)

The performance of the affinity adsorbents depends on many factors, such as the preparation method, the properties of matrix and ligand, and the orientation of the immobilized ligand. When biological molecules are selected as affinity ligands, the immobilization method is particularly important, because the activity of the ligand can be affected by its denaturation during the immobilization procedure. In this work, silica supports with glutaraldehyde functional groups and Toyopearl with Tresyl functional groups were selected to immobilize Con A.

To evaluate the optimal time for the completion of the ligand immobilization, the immobilization kinetics of Con A was measured for these two supports. As shown in Fig. 2-3,

the immobilization of Con A onto silica supports reached the equilibrium in 1 h with the final coupling yield of 72%. However, the immobilization onto Toyopearl supports was not completed even after 4 h with the coupling yield of 75%, which could be finished in 15 h with a final coupling yield of 80% (data not shown in the graph). The difference of immobilization kinetics for these two supports could be due to the intrinsic chemical properties

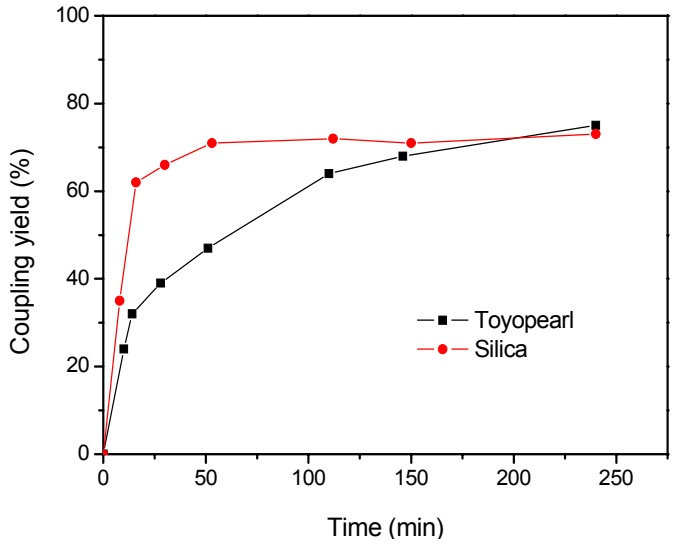


Fig. 2-3. Immobilization kinetics of Con A onto two different supports. Coupling buffer: 0.5 M phosphate buffer, 0.1 M NaCl, pH 8.0; Con A concentration: 4 mg/ml. Immobilization was carried out at room temperature.

of the functional groups and diffusive resistance of Con A into the pores of supports. The shorter the reaction time, the less likely denaturation of the ligand becomes. From this point of view, the immobilization of Con A on silica supports could be more beneficial for keeping the activity of Con A.

Following the reaction conditions as shown in Table 2-2, the immobilization of Con A resulted in a slightly higher ligand density on silica (9.8 mg/ml) than that on Toyopearl (9.4 mg/ml). To investigate the influence of the ligand density on the adsorption efficiency, the static adsorption of GOD was performed on both Con A adsorbents. As can be seen, the adsorption capacity of GOD for Toyopearl Con A (7.0 mg/ml) was much larger than that for silica Con A (4.8 mg/ml), even though the ligand density of Toyopearl Con A is little smaller

than that of silica Con A (Table 2-2). This could be related with the ligand surface coverage on the adsorbents. The higher surface coverage of silica Con A (86%) induced a compact Con A ligand density on the surface of the support and thus reduced the ligand accessibility

Table 2-2. Comparison of immobilized Con A affinity adsorbents

Affinity adsorbents	Supports employed (ml)	Con A employed (mg)	Coupling time (h)	Con A density (mg/ml)	Con A density[a] (mg/g)	Con A surface coverage (%)	GOD adsorption capacity[b] (mg/ml)
Silica Con A	5	60	1	9.8	25.9	86	4.8
Toyopearl Con A	6	72	4	9.4	37.1	44	7.0

[a] 1 g (dry weight) silica supports equals to 2.64 ml wet volume; 1 g (dry weight) Toyopearl supports equals to 3.95 ml wet volume. Determine methods: a certain wet volume of supports was dried in the oven at 105 °C until the mass of the supports didn't change any more. The exchange ratio was the mean value of three times experiments.

[b] Measured in 5 ml 2 mg/ml GOD solution (0.1 M acetate buffer, pH 5.0) for 0.2 ml Con A adsorbents at room temperature for 20 h.

and the adsorption capacity. On the other hand, the surface coverage of Toyopearl Con A (44%) seems quite satisfactory to prevent the steric hindrance during the adsorption of GOD. It should be pointed out that appropriate ligand surface coverage must be taken into account during the adsorption of macromolecules in order to get a high adsorption capacity and a high utilization efficiency of affinity ligands.

2.3.2. Investigation of adsorption conditions of glucose oxidase (GOD)

The interaction involved in affinity separation is a combination of electrostatic, hydrophobic, hydrogen binding and Van der Waals interactions (Katchalski-katzir, 1983; Mattos, 1996). The pH value and ionic strength of the adsorption medium may have significant influence on the interaction between the affinity ligand and the adsorbate (Bahar, 2004). Therefore, it is necessary to study the effects of these factors on the adsorption capacity in order to find the optimal adsorption conditions.

Effect of pH on the adsorption of glucose oxidase (GOD)

The adsorption experiments of GOD onto Toyopearl Con A and silica Con A were carried out in buffers with pH values of 5.0, 6.0, and 7.0, respectively. These buffers were prepared without additional NaCl for adjusting the ionic strength. These pH values were chosen because both Con A and GOD show their good stability and activity in this range. The effect of pH value on the adsorption capacity of GOD is shown in Fig. 2-4. Both Con A adsorbents show the similar trend with regard to adsorption capacity at different pH. The adsorption

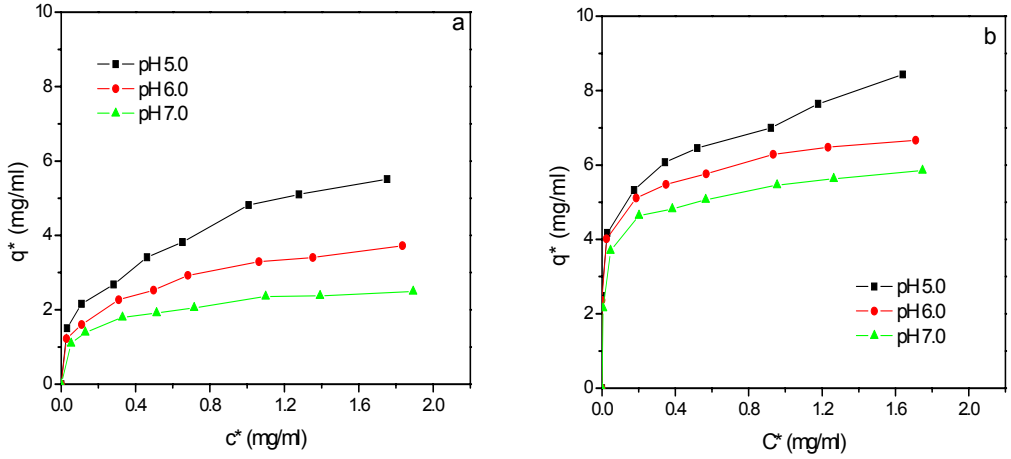


Fig. 2-4. Effect of pH on the adsorption capacity of GOD onto different Con A adsorbents. a: Silica Con A with ligand density of 9.2 mg/ml; b: Toyopearl Con A with ligand density of 8.8 mg/ml .

capacity was decreased remarkably with increasing the pH value from 5.0 to 7.0. For both Con A adsorbents, the maximum adsorption capacity was obtained at pH 5.0, where GOD shows its high activity (Pazur, 1964). Therefore, pH 5.0 was selected as the appropriate value for further GOD adsorption experiments unless otherwise stated. As described above, under same adsorption conditions the adsorption capacity of GOD was much higher for Toyopearl Con A than that for silica Con A due to the high utilization efficiency of affinity ligand.

Effect of ionic strength on the adsorption of glucose oxidase (GOD)

The affinity interaction between lectin-carbohydrate is mainly based on hydrogen bonds and Van der Waals contact (Kennedy, 1995), which could be influenced by ionic strength. The

investigation of the effect of ionic strength on the adsorption capacity of GOD was performed in 0.1 M acetate buffer, pH 5.0, by introducing of NaCl with varying concentrations from 0 to 0.5 M. The results are shown in Fig. 2-5. The dependence of the adsorption capacity on ionic strength was not as much as on pH value. However, a noticeable change of adsorption

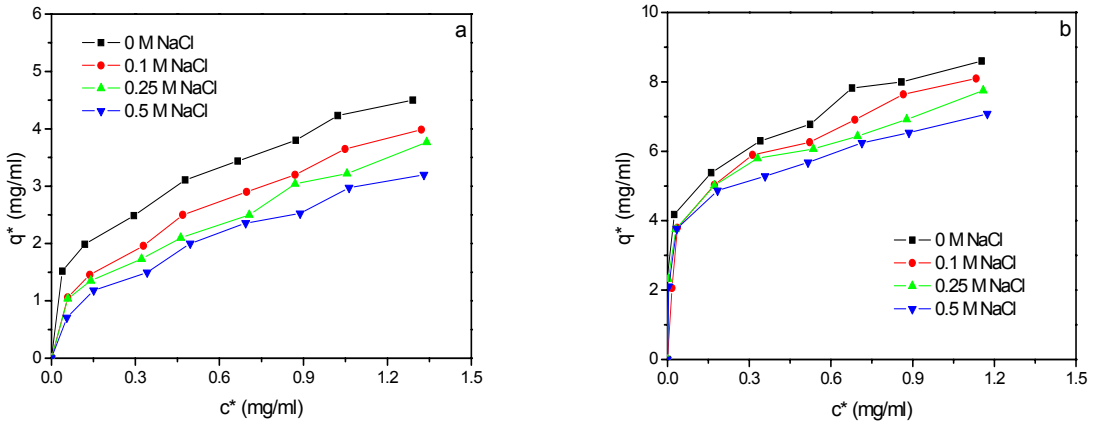


Fig. 2-5. Effect of ionic strength on the adsorption capacity of GOD onto different Con A adsorbents. a: Silica Con A with ligand density of 9.2 mg/ml; b: Toyopearl Con A with ligand density of 8.8 mg/ml.

capacity in terms of ionic strength still exists. The adsorption capacity of GOD on both Con A adsorbents reached the maximum without NaCl, and it was decreased with increasing the concentration of NaCl. This is most likely because the formation of hydrogen bonds involved in affinity binding is based on static electronic interaction. Increasing of ionic strength of the medium may reduce static electronic interactions thus the most affinity interaction between Con A and GOD. The high adsorption capacity in the absence of NaCl could be partially contributed to the following explanation: when the ionic strength of the adsorption buffer is very low, the monolayer adsorption of GOD onto Con A adsorbents could change to multilayer adsorption due to the high hydrophobic interactions between the specific adsorbed GOD and free GOD in the solution. To suppress the unspecific affinity adsorptions, moderate salt concentration was required (Clemmitt, 2000a; Narayanan, 1990). Considering relatively high adsorption capacity could still be obtained in 0.1 M acetate buffer, containing 0.1 M NaCl, pH 5.0, it was chosen for the further adsorption experiments.

2.3.3. Adsorption isotherms

The adsorption isotherms obtained from the adsorption of GOD onto different Con A adsorbents under optimal adsorption conditions are shown in Fig. 2-6. The Langmuir model

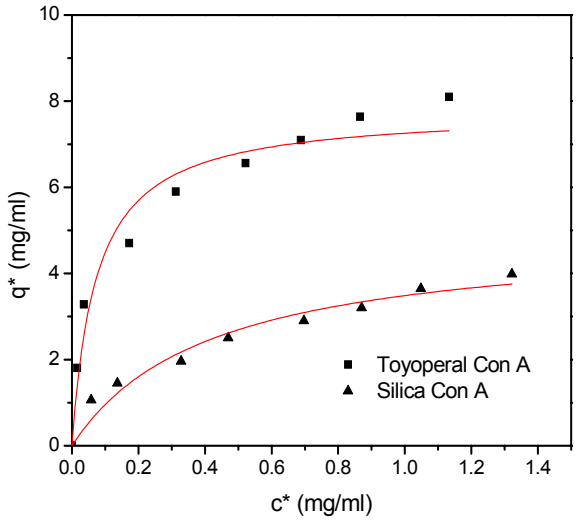


Fig. 2-6. Adsorption isotherms of GOD onto Toyopearl Con A and silica Con A. The adsorbents employed here are the same as described in Fig. 2-5. Solid lines were fitted by Langmuir equations.

is most commonly used to describe the adsorption behaviour of affinity adsorbents. It assumes that the adsorption is a monolayer adsorption and the binding sites are homogeneously distributed on the adsorbent surface. In the case of GOD adsorption onto Con A adsorbents, experimental data were well fitted with the Langmuir equation in the studied concentration range. The fitted thermodynamic parameters are listed in Table 2-3. It can be observed that the adsorption behaviour varies significantly for these two affinity adsorbents. The maximum adsorption capacity for Toyopearl Con A and silica Con A were 7.9 mg/ml, and 4.9 mg/ml, with a dissociation constant of 4.6×10^{-7} M, and 2.6×10^{-6} M, respectively. The difference of the adsorption behaviour between these two adsorbents should be related with the immobilization chemistry and the physical properties of the matrix. Affinity adsorbents should have a dissociation constant below 10^{-5} M to avoid the ligate elution during the washing step (Beeskow, 1995; Castilho, 2000). Our results showed that both Con A adsorbents, especially

Toyopearl Con A, were high specific and therefore suitable for affinity separations. It is worthy to point out that the deviation of experimental data from the Langmuir isotherm became larger for the higher GOD concentration range, especially for Toyopearl Con A

Table 2-3. Parameters calculated from the Langmuir equation for different affinity adsorbents

Affinity adsorbents	q_m (mg/ml)	K_d (mg/ml)	K_d (mol/L)	R^2
Toyopearl Con A	7.9	0.076	4.6×10^{-7}	0.964
Silica Con A	4.9	0.416	2.6×10^{-6}	0.967

adsorbents. This could be caused by the multilayer adsorption resulting from GOD-GOD interactions. Castilho described the same phenomenon during the adsorption of IgG to affinity Protein A membranes (Castilho, 2000).

2.3.4. Adsorption and desorption kinetics

The adsorption kinetics of GOD onto Con A adsorbents is presented in Fig. 2-7 (a). The curves indicate that the adsorption was very fast to silica Con A; the C/C_0 reached a constant value after 15 min. The adsorption of GOD to Toyopearl Con A was also very fast in the first 20 min, and then it decreased till a constant value of C/C_0 was obtained after 60 min. The saturation time for the adsorption of GOD onto two Con A adsorbents were quite different, because GOD was transferred into the pores of the Con A adsorbents by diffusion, which is strongly dependent on the pore size of the adsorbent. In other words, the larger pore size of silica Con A significantly reduced the diffusive mass transfer resistance, and thus the adsorption rate of GOD was remarkably increased compared with Toyopearl Con A.

The Con A adsorbents with bound GOD were carefully washed to remove the GOD physically adsorbed on the surface and diffused in the pore of the adsorbents. Then 20 ml 0.1 M methyl-α-D-mannopyranoside was mixed to the GOD-Con A adsorbents for the desorption of GOD. The sample aliquot was withdrawn from the supernatant at time intervals to measure the desorption kinetics. The results are shown in Fig. 2-7 (b). The desorption

capacity after 10 h was taken as the equilibrium desorption capacity, denoted as q^*_{de}. The instantaneous desorption capacity at time intervals are denoted as q_{de}. In the first 10 min,

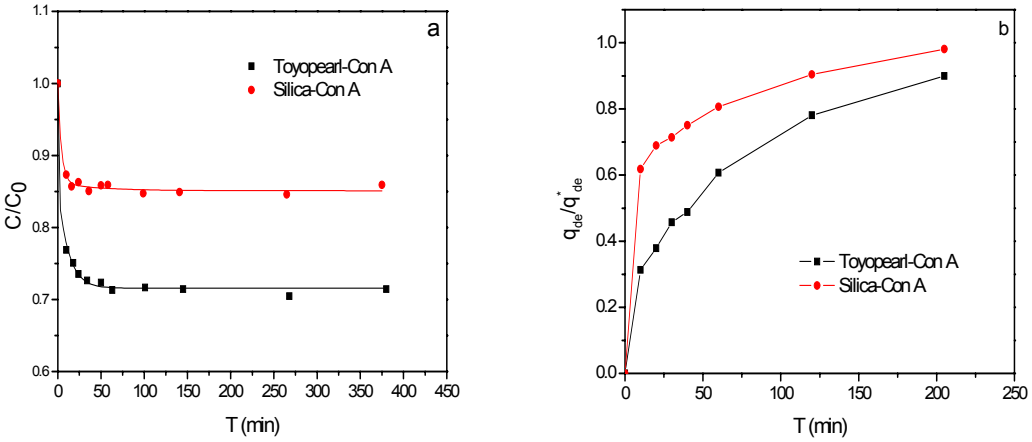


Fig. 2-7. Adsorption (a) and desorption (b) kinetics of GOD onto and from different Con A adsorbents. The ligand density was 9.8 mg/ml for Silica Con A and 9.4 mg/ml for Toyopearl Con A.

the desorption capacity q_{de} reached 31% of its q^*_{de} for Toyopearl Con A, while 62% for silica Con A. As the desorption time increased, the difference of the desorption rate between the two Con A adsorbents was gradually reduced. For example, after 200 min the q_{de}/ q^*_{de} value was 90% for Toyopearl Con A, and 98% for silica Con A, respectively. In conclusion, both the adsorption and desorption rate of GOD onto and from silica Con A were much faster than that onto and from Toyopearl Con A. Silica Con A adsorbents are expected to have higher potential for the purification of unstable glycoproteins since short processing time could reduce the possibility of protein denaturation and higher kinetic rates accelerate the dynamic process.

2.3.5. Unspecific adsorption of glucose oxidase (GOD) and reuse of Concanavalin A (Con A) adsorbents

Unspecific adsorption can reduce the purity of target compound in bioseparation processes, so it is important to investigate the unspecific adsorption properties of affinity adsorbents. As described in the experimental section, the unspecific adsorption capacity of GOD onto

end-capped adsorbents was determined and compared with the whole adsorption capacity of GOD onto Con A adsorbents. Table 2-4 shows the results. By static adsorption of 10 ml of 0.5 mg/ml GOD, 1 ml silica supports unspecifically adsorbed 0.17 mg GOD while 1 ml Toyopearl supports adsorbed 0.35 mg GOD. The adsorption capacity of GOD was increased

Table 2-4. Unspecific adsorption of GOD onto Con A affinity adsorbents

Matrix	Immobilized Con A density (mg/ml)	Unspecific adsorption capacity (mg/ml)	Whole adsorption capacity (mg/ml)	Percentage of unspecific to whole adsorption capacity (%)
Silica	11.7	0.17	2.2	7.7
Toyopearl	11.8	0.35	4.9	7.1

significantly after immobilization of Con A onto supports: 2.2 mg/ml for silica Con A and 4.9 mg/ml for Toyopearl Con A, respectively. The degree of unspecific adsorption capacity to the whole adsorption capacity of Con A adsorbents was less than 10% for both adsorbents indicating both Con A adsorbents are suitable for affinity separations.

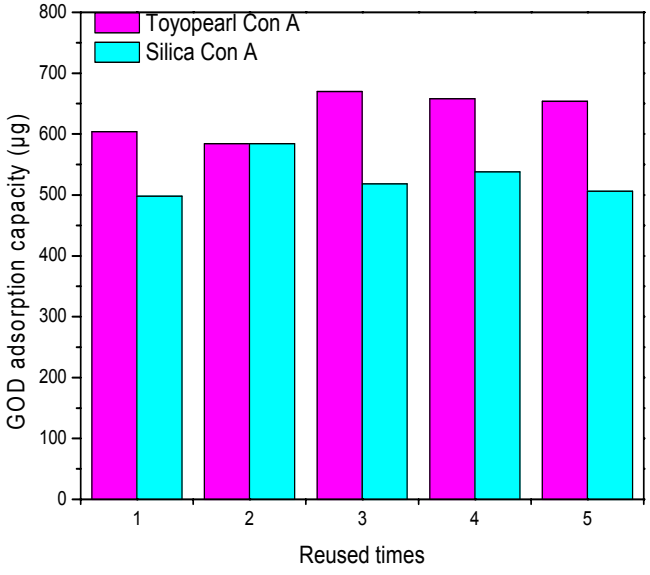


Fig. 2-8. Reuse of Con A adsorbents for the adsorption of GOD. Ligand density of Con A adsorbents are the same as described in Table 2-4.

1.5 ml of each Con A adsorbents as described in Table 2-4 was packed in columns to determine the dynamic adsorption capacity. 200 μl of 5 mg/ml GOD was applied to the column for each run and 0.1 M methyl-α-D-mannopyranoside was employed to desorb the bound GOD. Fig. 2-8 shows the adsorption capacity of GOD on both Con A adsorbents for five times repeated use. The difference of the dynamic adsorption capacity between the two Con A adsorbents was much less than that of the static adsorption capacity as shown in Fig. 2-6 and Table 2-4. The mean dynamic adsorption capacity for silica Con A and Toyopearl Con A was 0.35 mg/ml, and 0.42 mg/ml, respectively. This occurred because GOD was driven into the adsorbents structure mainly by diffusion, which could be facilitated by larger pore diameter. Fig. 2-8 also shows that both the Con A adsorbents highly maintained their adsorption capacity for GOD after 5 times use. This indicates that the separation procedure was successful and also confirms the stability of the immobilized Con A.

2.4. Conclusions

In this chapter, Con A was immobilized on inorganic silica, and polymeric Toyopearl supports, respectively. The immobilization kinetics of Con A was investigated onto these two supports. The results showed that silica with glutaraldehyde functional group exhibited much faster immobilization rate while similar volumetric ligand density was obtained when compared with Toyopearl supports. Immobilized Con A was employed for the adsorption of GOD. The influence of the pH value and the ionic strength on the adsorption of GOD was studied in order to find the optimal adsorption conditions. The factors dominated the affinity interaction were discussed in detail. The adsorption isotherms proved that Toyopearl Con A possessed much higher static adsorption capacity when compared with silica Con A. However, because of the less diffusive mass transfer resistance, silica Con A showed faster adsorption and desorption kinetics. Both Con A adsorbents exhibited:

(1) high affinity to GOD with K_d value of 4.8×10^{-7} M for Toyopearl Con A and 2.6×10^{-6} M for silica Con A, respectively;

(2) low unspecific adsorption capacity of GOD with 0.35 mg/ml and 0.17 mg/ml for Toyopearl and silica supports, respectively.

The dynamic adsorption of GOD revealed high stability of both immobilized Con A affinity adsorbents, further indicating their suitability for the application of affinity separations.

3. Preparation and characterization of PEGylated Concanavalin A (Con A)

3.1 Introduction

Affinity chromatography using lectins as ligands is extensively employed for the purification of glycoconjugates due to the specific interactions between them. However, the industrial application of lectins is hampered by the instability of their protein-based structure under some rigorous operation conditions, which may involve the evaluated temperatures and the presence of organic solvents.

The generation of robust proteins can be achieved by genetic and biochemical approaches. Genetic approaches have yielded significant results in obtaining much more stable proteins against harsh operational conditions (Cherry, 1999; Gülich, 2000). However, the main drawback of the genetic approach is that the knowledge of the properties gained by site-directed mutagenesis or evolution process cannot be used as a general method to be applied to other proteins (García-Arellano, 2002). On the other hand, chemical modification seems to be a more general method to improve intrinsic properties of proteins for which especially deeper knowledge of gene or protein structure is not required. Several chemical methods have been employed to obtain more stable proteins, including immobilization, cross-linking, attachment to polysaccharides, and chemical modification with amphiphilic polymers (as reviewed by Shami, 1989 and Ó'Fágáin, 2003). One of the most successful approach is to modify protein with poly (ethylene glycol)-PEG, a process commonly known as PEGylation.

PEG is a synthetic, non-toxic, non-immunogenic, amphiphilic and most importantly, highly biocompatible polymer. Proteins conjugation with PEG, linear or branched, via a covalent linkage can eliminate some drawbacks of native proteins and improve their physicochemical, biomedical and pharmacological properties. PEGylation often induces a protein with improved solubility and temperature stability, enhanced stability against enzymatic degradation (Na, 2004), increased serum half-life and anti-tumor potency (Tsutsumi, 1995),

decreased renal clearance and immunogenicity (Ueno, 2000; Hu, 2002), while normally maintaining a high percentage of its biological activity (Fig. 3-1). The combination of all these benefits leads to the production of PEG-modified proteins as pharmaceutical and therapeutic reagents. Several PEGylated protein products are currently on the market, such as PEG-IFNα_{2a}, and many more are under clinical investigation and likely to be approved. The fact that PEGylated enzyme improves its solubility in some organic solvents makes PEGylation as an effective tool in syntheses of complicated organic compounds. Although the application of PEGylation has been extensively studied, the mechanism of these effects caused by PEG chains is still not fully understood and well accepted. Therefore, it is necessary to carry out more detailed studies.

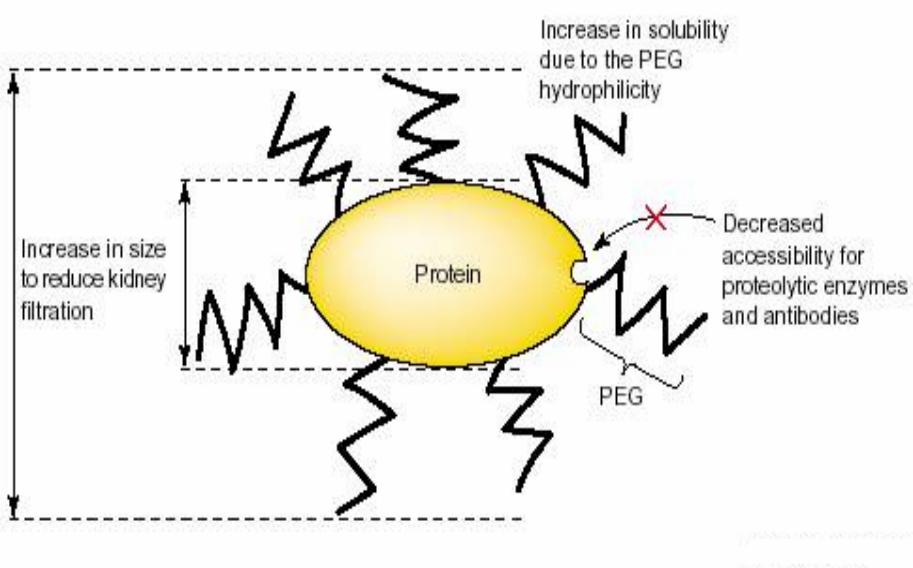


Fig. 3-1. Main advantages of PEGylated proteins. The figure represents a PEG-protein conjugate. PEG is shielding the protein surface from degrading agents by steric hindrance. Moreover, the increased size of the conjugate is at the basis of the decreased kidney clearance of the PEGylated protein. (cited from Veronese, 2005)

This work will take the jack bean lectin, Concanavalin A (Con A), as an example to study the PEGylation of proteinaceous affinity ligand and the influence of modification on its adsorption performance in affinity separation process. PEGylation of Con A has been studied by several authors for various purposes. Ueno et al. investigated the *in vivo* induction of anti-tumor cytotoxicity in mice by the treatment with Con A modified with PEG. They found that PEGylated Con A exhibited reduced immunogenicity and prolonged clearance time in

mice blood (2000). Kim and Park (2001a) used Con A for delivery of modulated insulin in sol-gel phase-reverse hydrogels system, and found PEGylated Con A held improved aqueous solubility, enhanced long-term stability, and higher glucose sensitivity compared to native Con A. Liu et al. studied a Con A based glucose-responsive insulin delivery system, which could be used for long-term diabetes treatment. They found the conjugation of Con A with PEG grafted by hydrophilic PVPAA exhibited substantially improved solubility at pH 7.4 while preserved its sugar binding characteristics (1997).

The present work describes the preparation and characterization of PEGylated Con A adsorbents, which are used for affinity chromatography. A method to determine the PEGylated degree was first proposed in this thesis and the parameters influencing the PEGylated degree were discussed. The binding specificity and binding rate of PEGylated Con A to GOD were investigated by adsorption isotherm and adsorption kinetics experiments. The binding capacity of GOD under normal conditions was determined by affinity chromatography for both PEGylated and native Con A adsorbents. In the next chapter, the binding properties of GOD by PEGylated Con A adsorbents will be investigated and compared with native Con A adsorbents under harsher conditions, such as elevated temperature and the exposure to organic solvents.

3.1.1. Introduction to PEGylation technology

PEGylation is a process of growing interest for enhancing the therapeutic and biotechnological potential of peptide and proteins. PEGylation of therapeutic proteins was first developed in the 1970s (Abuchowski, 1977) and till now a large amount of literature is available on PEGylation, including several books and reviews (Harris, 1992 and 1997; Katre, 1993; Kozlowski, 2001; Veronese, 2001; Roberts, 2002). Proper PEGylation of a protein or peptide could modify many of its properties while the main biological functions, such as enzyme activity or receptor recognition, may be retained. PEG conjugation masks certain area of the protein's surface and increases the molecular size of the protein, thus reducing its renal ultrafiltration, preventing the approach of antibodies or antigen processing cells and reducing the degradation of proteolytic enzymes. In addition, PEG conveys modified molecules its

physicochemical properties and modifies their biodistribution and solubility. PEGylation is also of great interest in applied biotechnology because PEGylated enzymes become soluble and active in some organic solvents. This property opens new techniques in biocatalysis and in pharmaceutical technology such as encapsulation of some drugs. The following sections will provide a general survey of the PEGylation technology in order to show some basic idea of PEGylation. Readers are referred to several detailed reviews on different aspects of PEGylation as aforementioned.

Properties of PEG

The common form of PEG is linear or branched polyether terminated with hydroxyl group and with the general structure of $HO\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$, which is normally obtained by ring-opening polymerization of ethylene oxide. Monomethoxy PEG, mPEG, is most useful for protein conjugation, having the general structure of $CH_3O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$, since its monofunctionality induces cleaner chemistry.

PEGs of varying molecular weight are commercially available. Compared with other polymers, PEG has a relatively narrow polydispersity (M_w/M_n)[1] in the range of 1.01 for low molecular weight PEGs (< 5 kDa) to 1.1 for high molecular weight PEGs (> 50 kDa). The narrow molecular weight distribution simplifies analytical characterization of its protein conjugates.

The unique ability of PEG to be soluble in both aqueous solutions and organic solvents makes it suitable for end group derivatization and chemical conjugation to biological molecules under physiological conditions. PEG associates with 2-3 water molecules per ethylene oxide unit in solution, which results in a size effect that PEG molecule acts as if it were 5-10 times as large as a soluble protein of comparable molecular weight. These factors have been suggested as the reason that PEG exhibits the ability to precipitate proteins, exclude proteins or cells from surface, reduce immunogenicity and antigenicity, and prevent degradation by proteomic enzymes (literatures as summarized by Roberts, 2002). Studies also revealed that PEG is lack of toxicity and immunogenicity, and most importantly, it is biocompatible (Harris,

[1] Polydispersity means the ratio of weight average molecular weight to number average molecular weight.

1992 and 1997). Thus PEG is well approved to be of great value for a range of biomedical applications.

Chemistry of PEGylation

To couple PEG with a molecule it is necessary to prepare active PEG derivatives with a functional group at one or both ends. The most common route is to active PEG with functional groups suitable to react with lysine or N-terminal groups since lysine is one of the most prevalent amino acids in proteins.

PEGylation chemistry has experienced two generations. The first generation of the PEGylation chemistry generally suffers from PEG impurities, restriction to low molecular weights, unstable linkage, and lack of selectivity in modification. The examples of first generation PEG derivatives include: (1) PEG dichlorotriazine, (2) PEG tresylate, (3) PEG succinimidyl carbonate, (4) PEG benzotriazole carbonate, (5) PEG *p*-nitrophenyl carbonate, (6) PEG trichlorophenyl carbonate, (7) PEG cabonylimidazole, and (8) PEG succinimidyl succinate.

The second generation of the PEGylation chemistry has been designed to avoid the problems plagued by the first generation. The first example of the second generation chemistry is PEG-propionaldehyde, which is highly selective for the N-terminus. An alternative to PEG-propionaldehyde is its acetyl derivative which shows longer storage stability and higher purity. The succinimidyl ester of PEG carboxylic acids are the most popular derivatives for coupling PEG to proteins. The oldest and most used mPEG succinimidyl succinate (mPEG-SS) possesses an ester linkage in its backbone undergoing hydrolysis in vivo. mPEG-Succinimidyl Propionate (mPEG-SPA) and mPEG-Succinimidyl Butanoate (mPEG-SBA) generate stable linkage in the backbone and therefore they have nearly an ideal reactivity for the protein modification. The selectivity in protein modification can also be achieved by the reaction with the thiol group of cysteine amino acid of a protein. When a protein lacks of cysteine, it can be introduced by site directed mutagenesis. PEG derivatives for the modification of cysteine include: (1) PEG maleimide, (2) PEG vinyl sulfone, (3) PEG iodoacetamide, and (4) orthopyridyl disulfide. Other site directed PEGylation chemistries are not included here; readers are referred to the reviews by Roberts (2002) and Kozlowski (2001).

3.1.2 Design of the PEGylation route and definition of the PEGylated degree

The structure and properties of a protein are the most important factors in the selection of the proper conjugation method. Since Con A exhibits many lysine groups on its surface and most of these lysine groups are not involved in its active sites, mPEG-SPA, a primary amine specific reagent, was chosen to modify Con A for the preparation of adsorbents for affinity chromatography. PEGylation reaction can be finished within a short time, e. g. 15-60 min for mPEG-SPA with a native protein. However, the subsequent isolation and purification of PEGylated proteins are time-consuming by procedures such as dialysis, chromatography and lyophilization. To simplify the process, Con A was first immobilized onto Toyopearl supports, and then the immobilized Con A was conjugated with mPEG-SPA. By this procedure unreacted mPEG-SPA and some sideproducts could be easily removed by filtration, which greatly facilitated the recovery of PEGylated Con A. This PEGylation route also saved process cost and time by reducing the operation units. The scheme of PEGylation reaction is shown in Fig. 3-2.

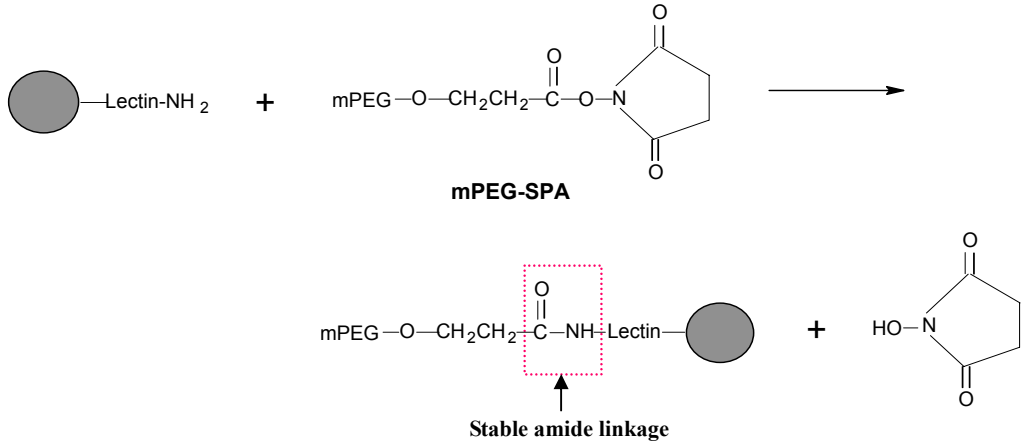


Fig. 3-2. Scheme of PEGylation of immobilized Con A

The extent of the PEGylated degree is highly related with the properties of the modified protein. To investigate how PEGylation influences the adsorption performance of modified Con A, it is necessary to know the extent of the degree of PEGylation. Since PEGylation is based on immobilized Con A adsorbents, it is needed to compare the difference of the adsorption performance before and after modification of the immobilized Con A.

Correspondingly, in this thesis, the PEGylated degree is defined as the ratio of primary amino groups of native immobilized Con A to that of PEGylated immobilized Con A.

3.2. Materials and methods

3.2.1. Materials

Toyopearl AF-Tresyl-650M (hereafter called Toyopearl for short) was obtained from Tosoh Bioscience (Stuttgart, Germany). Con A (type V), methyl-α-D-mannopyranoside, ninhydrin and Bradford reagent were purchased from Sigma (Munich, Germany). Glucose oxidase from *Aspergillus niger* was delivered from Serva (Heidelberg, Germany). Monomethoxy poly (ethylene glycol) succinimidyl propionate (mPEG-SPA) with different molecular weight was from Nektar (Huntsville, USA). All the chemicals were of analytical reagent grade unless otherwise stated.

3.2.2. Immobilization of Concanavalin A (Con A)

Con A was immobilized onto Toyopearl supports according to the procedure stated in chapter 2 (p. 23-24).

3.2.3. Conjugation of immobilized Concanavalin A (Con A) with mPEG derivative (mPEG-SPA)

Immobilized Con A was conjugated with PEG using mPEG-SPA, which is active towards primary amines. mPEG-SPA was dissolved in 0.1 M phosphate buffer, pH 8.0, with 50-fold molar excess of methyl-α-D-mannopyranoside for protection of the binding sites of Con A. To vary the molar ratio of Con A to mPEG-SPA in the reaction solution, the amount of mPEG-SPA was changed while Con A was kept constant. The molecular weight of the Con A molecule is 104,000 g/mol, while those of mPEG-SPA are 2,000 g/mol, 5,000 g/mol and 20,000 g/mol, respectively. In typical PEGylation experiments, the reaction mixture was gently shaken at room temperature for 2 h, and then 0.1 M acetate buffer, pH 4.0, was introduced to the system to terminate the reaction. The modified Con A adsorbents were

filtrated and washed extensively with 0.1 M acetate buffer, containing 0.1 M NaCl, 1 mM Ca^{2+}, Mn^{2+}, and Mg^{2+}, pH 6.0 (hereafter called buffer A) , and then stored in the same buffer.

3.2.4. Determination of the PEGylated degree of immobilized Concanavalin A (Con A)

mPEG-SPA was conjugated with Con A through the unreacted primary amino groups during its immobilization. The determination of the PEGylated degree is actually the comparison of the amount of the primary amines of immobilized Con A before and after modification with mPEG-SPA. Ninhydrin is one of the commonly used reagents to determine the concentration of amino acids or proteins, since it reacts with the primary amines. This method was developed to determine the PEGylated degree in this work. A typical procedure was carried out as followings:

The PEGylated and unPEGylated Con A adsorbents were sucked after being thoroughly washed with water. Affinity adsorbents with an amount of immobilized Con A between 0.5 mg and 2.5 mg were placed in a 15 ml test tube and then gently mixed with 2 ml purified water and 1 ml ninhydrin reagent. The mixture was then heated in a boiling water bath for 10 min. After cooling down to the room temperature, 5 ml 95% ethanol water solution was added to the mixture and mixed well with the adsorbents. The mixture was then centrifuged and the absorbance of the supernatant at a wavelength λ= 570 nm was measured. The same mixture without affinity adsorbents was taken as blank. The PEGylated degree was calculated from the slopes of the plots of PEGylated and unPEGylated Con A adsorbents.

3.2.5. Adsorption isotherms of glucose oxidase (GOD) onto Concanavalin A (Con A) adsorbents

The adsorption isotherms of GOD to Con A affinity adsorbents were performed by batch experiments. 5 ml GOD solutions in buffer A with increasing concentrations were mixed with 0.1 g (wet weight, about 0.15 ml) PEGylated, and unPEGylated Con A Toyopearl affinity adsorbents, respectively, in 15 ml plastic tubes. The suspensions were allowed to equilibrate for 15 h at 25 °C in a rotary water bath with the speed of 150 rpm. After the adsorbents were settled by centrifugation, the supernatant obtained from each tube was used to determine the

protein concentration by measuring the absorbance at λ= 280 nm. The equilibrium adsorption capacity was calculated by the mass balance as described in chapter 2.

3.2.6. Adsorption kinetics of glucose oxidase (GOD) onto Concanavalin A (Con A) adsorbents

40 ml of 0.4 mg/ml GOD was mixed with approximate 1.2 ml Con A adsorbents. The mixture was gently shaken at 120 rpm on an orbital shaker. The supernatant was withdrawn at time intervals to determine the GOD concentration in a UV spectrophotometer at λ= 280 nm (Carl Zeiss, Jena, Germany). The sample was immediately put back to the suspension after measurement to eliminate experimental error.

3.2.7. Affinity chromatography of glucose oxidase (GOD) onto Concanavalin A (Con A) adsorbents

Typical dynamic adsorption of GOD was performed on a low-pressure liquid chromatography system from Bio-Rad (Munich, Germany). About 1 ml Con A adsorbents was packed in a glass column (8 mm×100 mm). The adsorption was carried out in buffer A at a flow rate of 0.6 ml/min and desorption was achieved with the same buffer containing 0.1 M methyl-α-D-mannopyranoside. The desorbed fraction was collected to determine protein concentration with Bradford Method (Bradford, 1976).

3.3. Results and discussion

3.3.1. Analysis of the PEGylated degree

Whatever the purpose for protein modification with mPEG, there is always a need for simple and rapid methods to determine the extent of modification. Methods such as size exclusion chromatography-SEC (Fee, 2004), SDS-PAGE (Sato, 2002), MALDI-TOF mass spectrometry (Diwan, 2003), fluorometric assay (Stocks, 1986), and TNBS assay (Habeeb, 1966) have been reported to determine the PEGylated degree for free proteins. For immobilized PEG-protein

conjugates, methods, most of which are physical methods such as contact angle measurement and transmission electron microscopy have been reported (as cited by Hooftman, 1996). However, these methods are either complex, time consuming or not easy available. A simple and fast method is thus highly desired for the determination of PEGylated degree of immobilized PEG-protein conjugates. Since mPEG-SPA reacts with the primary amines of immobilized Con A by the formation of a stable amide linkage (see the indication in Fig. 3-2), the PEGylated degree could be calculated by comparing the number of primary amines before and after modification with PEG. Ninhydrin is a commonly used reagent for the determination of the protein concentrations since it can react with the primary amines in both free and immobilized states (Hermanson, 1992, p.282). Here, this method was modified to determine the PEGylated degree according to the procedure mentioned in experimental section.

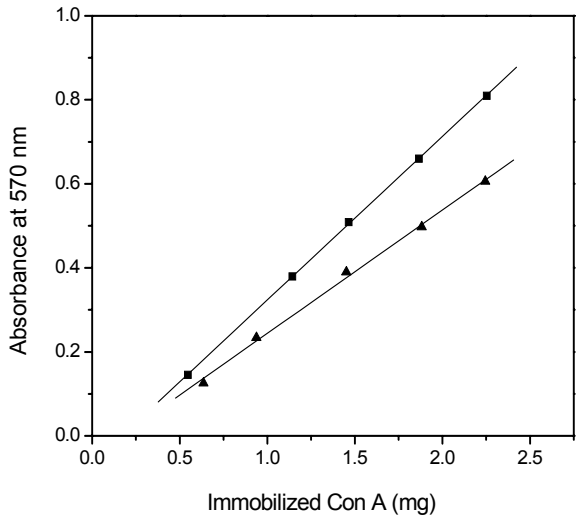


Fig. 3-3. Linear regression analysis of ninhydrin standard curve for native and PEGylated Con A immobilized onto Toyopearl support. The regression equations are y=0.389x-0.066 for native immobilized Con A and y=0.294x-0.050 for PEGylated immobilized Con A, where y=absorbance at 570 nm and x=the amount of immobilized Con A. The correlation coefficients are 0.9999 and 0.9986, respectively. ■: native immobilized Con A. ▲: PEGylated immobilized Con A.

A good example for the determination of the PEGylated degree is shown in Fig. 3-3. As can be seen, the relationship between the absorbance and the amount of native or PEGylated immobilized Con A is linear and both of the correlation coefficients of the plots are close to 1,

which shows a very good reproducibility. The estimated PEGylated degree of immobilized Con A in the example is 25%, calculated from the ratio of the slopes of the plots according to Eq. 3-1.

$$PD(\%) = (1 - \frac{b_1}{b_2}) \times 100\% \qquad (3\text{-}1)$$

where *PD* is the PEGylated degree, b_1 is the slope of the plot of PEGylated Con A and b_2 is the slope of the plot of native Con A. The experiments show that the ninhydrin method is fast and easily producible even though it requires a relatively large amount of samples (magnitude of milligram).

It should be pointed out that the PEGylated degree is just a mean value due to the heterogeneity in lysine substitution. Even for the same PEGylated degree, the PEGylation may happen on the different lysine positions of large molecules such as Con A as well as on the heterogeneous adsorbents. Thus the extent of the PEGylation may be different for each Con A molecule. To minimize the experimental error caused by the heterogeneity of the Con A immobilization, all the comparisons were conducted between the same batch of immobilized Con A adsorbents.

3.3.2. PEGylation reaction studies

mPEG-SPA is one of the most popular derivatives for coupling PEG to proteins due to its fast reactivity, low toxicity and higher stability. The reaction between primary amines of proteins and the active ester of mPEG-SPA produces a very stable amide linkage as shown in Fig. 3-2. Actually, during the PEGylation reactions the modification of proteins is competitive with the hydrolysis of mPEG-SPA. Studies indicate that the half-life of hydrolysis for mPEG-SPA is about 16 min (data from supplier) and the aminolysis (modification of protein) is always faster than the hydrolysis during the PEGylation reactions. In order to obtain reproducible modified products, quick and complete dissolution of mPEG-SPA and mixing of the reactants are necessary. Moreover, it is important to ensure that the active sites of proteins are not involved in the covalent linkage to mPEG derivatives (Schiavon, 2000). Therefore, methyl-α-D-mannopyranoside was used to block the active sites of Con A during the

PEGylation reactions. In this section, the results of the reaction time, reaction pH value, molar ratio of mPEG-SPA to Con A, and the concentration of mPEG-SPA are presented to visualize their influences on the PEGylation. Unless otherwise stated, in each experiment, the same batch immobilized Con A adsorbents were used to make the results reliable and comparable.

Effect of reaction time on the PEGylated degree

Since longer reaction time could influence the binding activity of Con A, it is necessary to study the PEGylation kinetics. About 0.6 ml of 9.4 mg/ml Toyopearl Con A adsorbents was measured for five times and then placed into different tubes. 1.4 ml of 1 mg/ml mPEG2k-SPA was quickly dissolved in 0.1 M phosphate buffer, pH 8.0, and mixed with Toyopearl Con A with gentle shaking at room temperature. The reaction for each tube was stopped after different period of time by the introduction of 0.1 M acetate buffer, pH 4.0. The PEGylation degree against reaction time was determined and the result is shown in Fig. 3-4. As can be

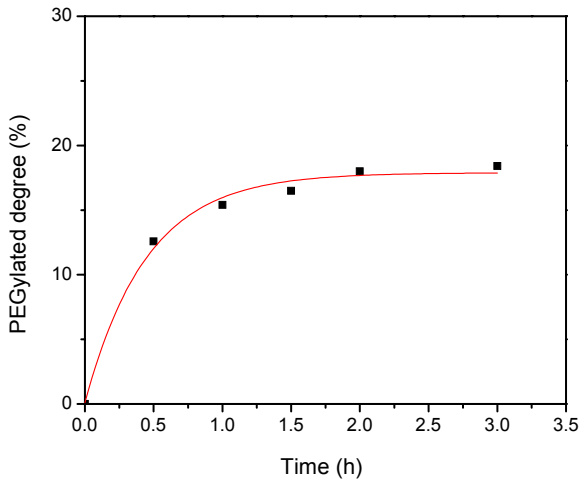


Fig. 3-4. PEGylation kinetics of immobilized Con A onto Toyopearl supports. The concentration of mPEG2k-SPA was 1 mg/ml, the molar ratio of mPEG2k-SPA to immobilized Con A was 13:1 and the reaction was performed in 0.1 M phosphate buffer, pH 8.0, at room temperature.

seen, the PEGylated degree linearly increased in the first 30 min. Then the reaction rate was decreased till it reached to a constant level (with the PEGylated degree of 18%) after 2 h. Therefore, 2 h was selected as the optimal reaction time for the modification of immobilized

Con A since a further increase in the reaction time did not cause a significant increase of the PEGylated degree.

Effect of pH value on the PEGylated degree

0.1 M phosphate buffer with pH 6.0, 7.0, and 8.0 were selected to study the effect of pH value on the PEGylated degree. As shown in Fig. 3-5, the pH value of the reaction medium greatly

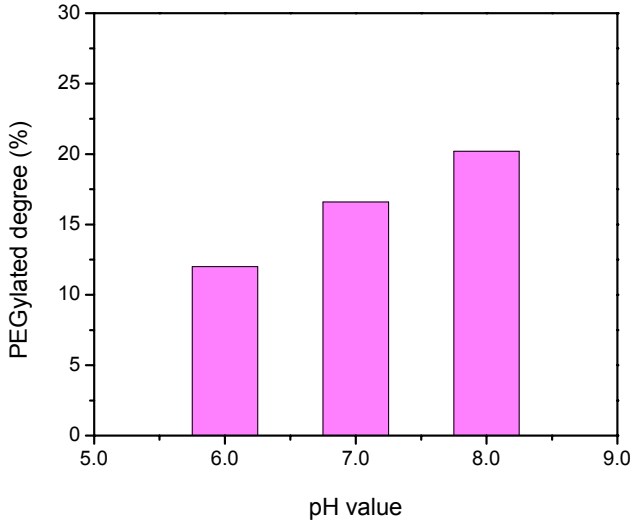


Fig. 3-5. Effect of pH value on the PEGylated degree of immobilized Con A on Toyopearl supports. The ligand density of Toyopearl Con A was 12 mg/ml and the concentration of mPEG2k-SPA was 1 mg/ml, the molar ratio of PEG2k-SPA to immobilized Con A was 40:1 and the reaction was performed for 2 h at room temperature.

influenced the PEGylated degree as expected. When the other conditions were the same, the higher PEGylated degree was obtained at higher pH conditions. For example, the PEGylated degree of immobilized Con A was 20% at pH 8.0, while only 12% conversion degree was found at pH 6.0. The reason is that the conjugation of mPEG derivative to Con A requires a nucleophilic attack of unprotonated amine group to succinimidyl groups in mPEG-SPA (Kinstler, 1996). The similar phenomenon has also been observed by Kim during the PEGylation of rhEGF (2001b). Con A still shows high activity at pH 9.5 (Shore, 1973). Thus in this work pH 8.0 was chosen for the PEGylation reactions if not stated otherwise.

Effect of the molar ratio of mPEG-SPA to immobilized Concanavalin A (Con A) on the PEGylated degree

The PEGylated degree also depends very much on the molar ratio of mPEG-SPA to immobilized Con A. Because of the hydrolysis of mPEG-SPA, an excess amount of mPEG-SPA was introduced during the modification of the immobilized Con A. As shown in Fig. 3-6, the PEGylated degree was increased sharply with increasing the molar ratio of

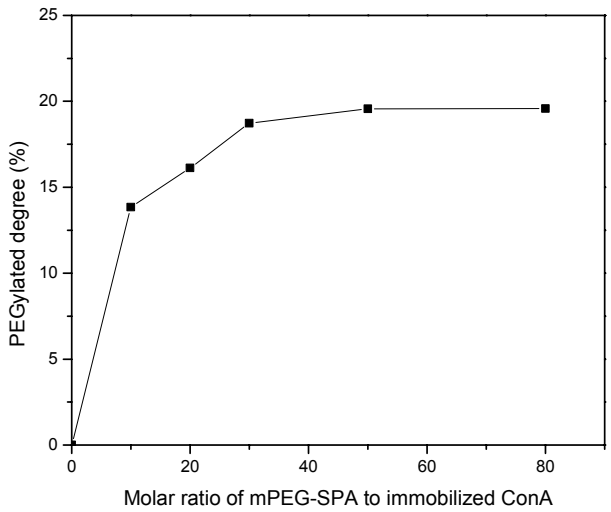


Fig. 3-6. Effect of the molar ratio of mPEG-SPA to immobilized Con A on PEGylated degree. Experimental conditions: variable amount of 1 mg/ml mPEG2k-SPA was dissolved in 0.1 M phosphate buffer, pH 8.0, and mixed with 0.15 ml immobilized Con A with ligand density of 12 mg/ml for 2 h at room temperature.

mPEG-SPA to immobilized Con A from 10:1 to 30:1 and then tapered off till reached a constant lever at the ratio of 50:1. Further increasing the molar ratio of two reactants did not make any sense for increasing the PEGylated degree.

Effect of the molecular weight of mPEG-SPA on the PEGylated degree

To investigate the effect of the molecular weight of mPEG-SPA on the PEGylated degree, mPEG-SPA2k, mPEG-SPA5k, and mPEG-SPA20k were selected to modify immobilized Con A with the molar ratio of mPEG-SPA to immobilized Con A as 20:1. As shown in Fig.

3-7, one may conclude that the PEGylated degree of immobilized Con A is significantly influenced by the molecular weight of the mPEG chains. After 2 h reaction, the PEGylated degree was about 25% for mPEG-SPA2k-Con A, whilst only 18%, and 13% for mPEG-SPA5k-Con A, and mPEG-SPA20k-Con A, respectively. The effect of the length of mPEG chains on the PEGylated degree was clearly caused by the molecular weight-dependent steric effect of mPEG derivatives. The accessibility of high molecular weight mPEG derivatives to the conjugation site of immobilized Con A could be more limited than that of low molecular weight mPEG derivatives. Thus under the same

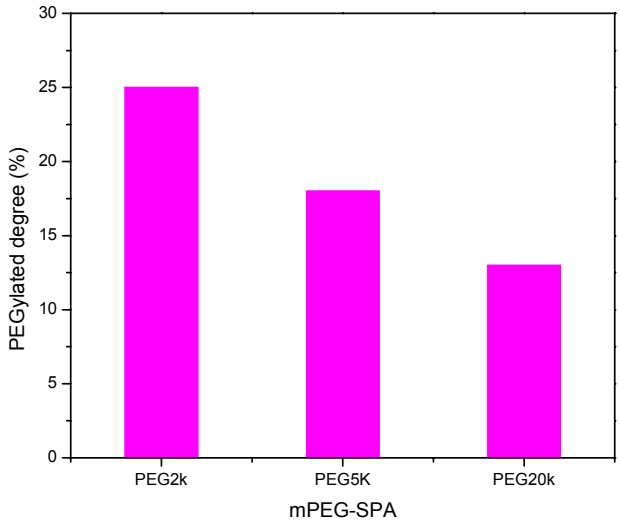


Fig. 3-7. Effect of the molecular weight of mPEG-SPA on PEGylated degree. mPEG-SPA was dissolved in 0.1 M phosphate buffer, pH 8.0, and mixed with immobilized Con A for 2 h at room temperature. The molar ratio of mPEG derivatives to immobilized Con A was 20:1.

reaction conditions mPEG-SPA2k resulted in a higher PEGylated degree to immobilized Con A than the other two higher molecular weight mPEG derivatives. Diwan and Park (2003) also observed the same phenomena in the modification of Interferon-α (IFN) with mPEG-SPA2000 and mPEG-SSA5000. In this case, the immobilization of Con A partially reduced the accessibility of the lysine-conjugation sites on the Con A surface caused by the big backbone volume of Toyopearl supports. mPEG-SPA had to conquer the diffusive resistance before the conjugation with Con A, which is highly related with the molecular size of mPEG-SPA. Therefore, it is not surprising that the PEGylated degree of immobilized

Con A is significantly affected by the molecular weight of mPEG-SPA.

3.3.3. Adsorption isotherms of glucose oxidase (GOD)

Glucose oxidase (GOD) was employed as target compound to study the adsorption isotherms and adsorption kinetics of native and PEGylated Toyopearl Con A. The results for the batch adsorption of GOD by native and PEGylated Toyopearl Con A adsorbents are shown in Fig. 3-8. The experimental data are fitted to the Langmuir isotherm. In each case the data were

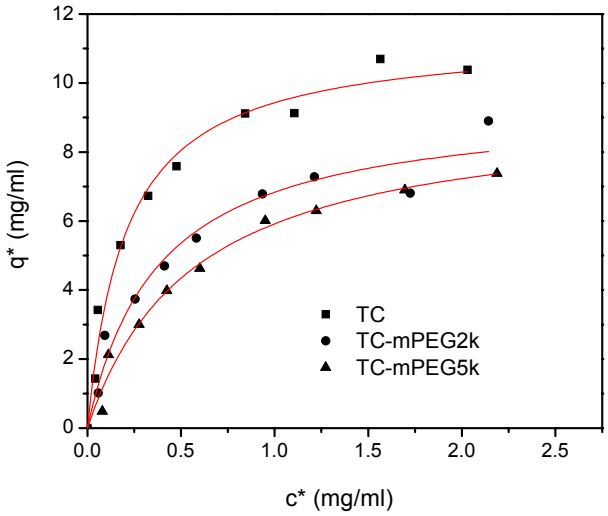


Fig.3-8. Adsorption isotherms of GOD to native and PEGylated Toyopearl Con A affinity adsorbents. Experimental conditions: 0.1 M acetate buffer, pH 6.0, equilibrated for 15 h at T= 25 °C. Solid line: calculated from the Langmuir equation.

found to fit well to the Langmuir isotherm. The corresponding fitted parameters are listed in Table 3-1. The dissociation constant K_d increased from 1.3×10^{-6} M (TC) to 2.4×10^{-6} M (TC-mPEG2k) and 3.6×10^{-6} M (TC-mPEG5k) when 25% and 16% of the free primary amine groups of immobilized Con A were modified by mPEG2k-SPA and mPEG5k-SPA, respectively. This indicates that the affinity interaction between GOD and PEGylated Con A was slightly lower than that between GOD and native Con A. However, this difference can be considered of little significance since it is similar to that observed by different preparations of native lectin (Solís, 1993). Similarly, the studies of Liu et al. (1997) reflected that the

coupling of Con A and mPEG-PVPAA did not impair the specificity of Con A. Kim and Park (2001a) found that PEGylation increased the binding affinity of glucose to Con A and preserved the binding affinity of allyl glucose to Con A when up to 5 mPEG molecules were coupled on each Con A. The amino acid residues that may participate in the Con A-Saccharide binding sites are known to be 14-16, 97-98, 168-169, 207-208, 224-228, and 235-237, or 12-18, 98-102, 205-208, and 226-229 (as cited by Kim, 2001a). Only one lysine was involved in the binding sites of Con A according to its primary structure. Because the other lysine residues are far away from the saccharide binding sites, the PEGylated Con A is expected to preserve its binding activity after modification. Besides the location of binding sites of lectins, the binding specificity can also be influenced by some other factors, such as PEGylation reaction conditions, PEGylation degrees and the size of substrates employed (Chiu, 1993). Therefore, it will not be surprising if reduced binding affinity was found.

Table 3-1. Parameters calculated from the Langmuir equation for different affinity adsorbents

Matrix	PEGylated degree (%)	Q_m (mg/ml)	K_d (mg/ml)	K_d (10^{-6} mol/L)	R^2
TC* (unPEGylated)	-	11.4	0.207	1.30	0.984
TC-mPEG2k	25	9.43	0.380	2.37	0.967
TC-mPEG5k	16	9.28	0.569	3.56	0.986

*Hereafter Toyopearl Con A adsorbents can be abbreviated as TC.

As can be found in Table 3-1, the maximum binding capacities of TC, TC-mPEG2k, and TC-mPEG5k were 11.4 mg/ml, 9.43 mg/ml and 9.28 mg/ml, respectively. This indicates that the maximum binding capacity was reduced after PEGylation due to the steric hindrance caused by the introduced mPEG chains. Considering the large size of GOD (MW 160 kDa), the highly preserved binding capacity to PEGylated Con A (higher than 80% for both PEGylated Con A adsorbents) indicates that the PEGylated degrees are satisfactory for the further affinity separations. Even though the PEGylated degree of TC-mPEG5k (16%) was much lower than that of TC-mPEG2k (25%), the maximum adsorption capacity of TC-mPEG5k was lower than that of TC-mPEG2k. This is probably because that mPEG5k holds a larger hydrodynamic volume in contrast to mPEG2k and thus produces larger steric

hindrance to the active binding sites.

3.3.4. Adsorption kinetics of glucose oxidase (GOD)

The adsorption kinetics of GOD to native and PEGylated Toyopearl Con A affinity adsorbents was also investigated and the results are shown in Fig. 3-9. The data are fitted to an exponential decay of the form:

$$C/C_0 = a + be^{-t/\tau} \quad (3\text{-}3)$$

where a and b are constants and τ is the time constant. According to constant b, the adsorption capacity q can be calculated from Eq. 3-4.

$$q = (C_0 - C)V_{GOD}/V_{ads} = C_0 b V_{GOD}/V_{ads} \quad (3\text{-}4)$$

where C_0 is the initial concentration of GOD, V_{GOD} is the volume of applied GOD and V_{ads} the volume of affinity adsorbents.

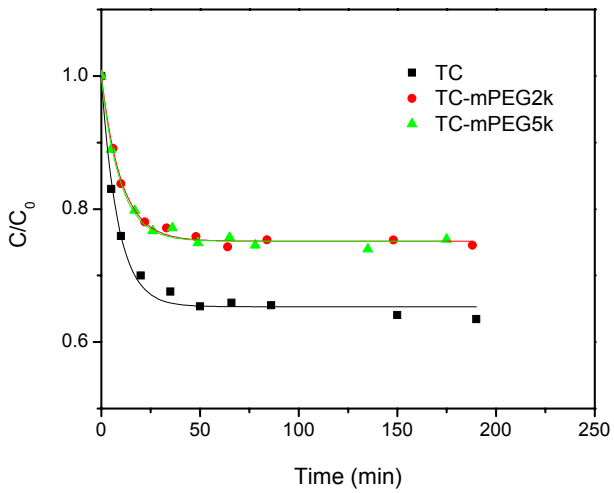


Fig.3-9. Adsorption kinetics of GOD to native and PEGylated Toyopearl Con A affinity adsorbents. Experimental conditions: 30 ml of 0.386 mg/ml GOD in 0.1 M acetate buffer, pH 6.0, was mixed with about 0.75 ml Con A adsorbents with ligand density of 13.4 mg/ml. T=25 °C. Solid line: calculated from the first-order exponential decay form.

The adsorption kinetics curves of different Con A adsorbents show the influence of PEGylation on the adsorption rate of GOD. As can be seen from the fitting data in Table 3-2,

the time constant of adsorption kinetics curves increased after PEGylation. This indicates that PEGylation increased the diffusive resistance of the large GOD molecule (MW 160 kDa) into the active binding sites of immobilized Con A. Thus it needs longer time for PEGylated Con A to reach the adsorption equilibrium. This result is in a good agreement with the fact that PEGylation is often utilized to prolong the circulation half-time of therapeutic proteins (Tsutsumi, 1995; Walsh, 2003; Ueno, 2000). The longer equilibrium time for TC-mPEG2k

Table 3-2. Parameters estimated from the adsorption kinetics of GOD onto different affinity adsorbents

Matrix	PEGylated degree (%)	b	q (mg/ml)	τ	R^2
TC	-	0.341	5.27	8.58	0.990
TC-mPEG2k	25	0.248	3.82	10.14	0.995
TC-mPEG5k	16	0.245	3.78	9.61	0.992

might be caused by its higher PEGylated degree when compared with that for TC-mPEG5k. The binding capacity of GOD calculated from Eq. 3-4 also shows that PEGylation reduced the binding capacity because of the steric hindrance of mPEG chains as indicated by the adsorption isotherm reactions.

3.3.5. Affinity chromatography of glucose oxidase (GOD) onto Concanavalin A (Con A) adsorbents

It is necessary to study the adsorption performance of PEGylated Con A adsorbents by affinity chromatography dynamic process since it is much nearer to practical separation applications as here additionally hydrodynamic influences are encountered. About 1 ml adsorbents was packed in a column to determine the dynamic adsorption capacity of GOD. In order to investigate the influence of the PEGylated degree and the molecular weight of mPEG on the dynamic adsorption capacity, mPEG-SPA with molecular weight 2000, and 5000 was conjugated with immobilized Con A with varying molar ratio. 1 mg GOD was applied to the column and each experiment was repeated for 3 times. The results are summarized in Table

3-3. As can be seen, the similar tendency of dynamic adsorption capacity was obtained with that observed by static adsorption experiments. The dynamic adsorption capacity of GOD was also reduced after PEGylation. For TC-mPEG2k, 98%, and 89% of the adsorption capacity was preserved with a PEGylated degree of 15%, and 20%, respectively. In case of TC-mPEG5k, only 75%, and 57% of the adsorption capacity was maintained with a PEGylated degree of 13%, and 17%, respectively. The dynamic adsorption capacity studies show that TC-mPEG2k exhibits much better adsorption performance than TC-mPEG5k. Especially when considering its high PEGylated degree and the large molecular size of GOD, the dynamic adsorption capacity of TC-mPEG2k was quite satisfactory. Compared with

Table 3-3. Purification of GOD by affinity chromatography onto different Con A adsorbents

Con A adsorbents*	Molar ratio of mPEG to immobilized Con A	PEGylated degree (%)	Adsorption capacity of GOD (μg/ml)
TC	-	-	423
TC-mPEG2k	3:1	15	416
TC-mPEG2k	8:1	20	379
TC-mPEG5k	3:1	13	319
TC-mPEG5k	8:1	17	244

*All the Con A adsorbents used here provide a ligand density of 6.5 mg/ml.

TC-mPEG2k, TC-mPEG5k showed a dramatic reduction of its dynamic adsorption capacity. The possible explanation might be that the contact time was not sufficient for TC-mPEG5k to bind GOD due to the slower diffusive rate; or according to the larger mPEG molecules immobilized onto the ligand, the substrate is unable to penetrate into the binding pocket to reach the binding sites. Therefore, TC-mPEG2k with an appropriate PEGylated degree could be suggested as a satisfactory adsorbent for affinity separation.

3.4. Conclusions

PEGylation of immobilized Con A and its adsorption behaviour in the affinity adsorption of GOD were investigated in this chapter. Immobilized Con A onto Toyopearl adsorbents were modified with mPEG-SPA with molecular weights of 2,000, 5,000, and 20,000 g/mol. A new method, Ninhydrin method, was first developed to determine the PEGylated degree of immobilized Con A, which has proved to be easy applicable and reproducible. The PEGylation reaction was studied in detail to elucidate how the parameters such as reaction time, pH value, molar ratio of mPEG-SPA to Con A, and molecular weight of mPEG-SPA affect the PEGylated degree. The adsorption isotherms of GOD onto native and PEGylated Con A adsorbents showed that the modification did not alter substantially the specificity of the carbohydrate binding ability of Con A. However, the binding capacity for GOD was slightly reduced probably due to the steric hindrance caused by mPEG chains. The adsorption kinetic studies revealed the lower adsorption rate after PEGylation which was still attributed to the steric effect. The dynamic adsorption capacity for modified Con A depended on the PEGylated degree and the molecular weight of mPEG derivatives very much. The adsorption capacity could be highly preserved for TC-mPEG2k even with the PEGylated degree up to 20%. Conjugation of Con A with mPEG2k has shown better adsorption performance thus has greater potential for the application in affinity separation process compared with mPEG5k.

4. Investigation of the Properties of PEGylated Concanavalin A (Con A)

In chapter 3, the PEGylation reaction with immobilized Con A and also the adsorption performance of PEGylated Con A with GOD were investigated in detail. Though the maximum binding capacity of GOD was reduced after PEGylation, the specificity to carbohydrate binding ability of modified Con A was highly preserved as revealed by the K_d values. The dynamic adsorption capacity of GOD for TC-mPEG2k could be substantially maintained even with the PEGylated degree up to 20%. On the basis of these observations, PEGylated Con A with an appropriated PEGylated degree is competent in the acquirement for affinity separation of biomolecules. This chapter will further focus on the investigation whether PEGylation has endowed modified Con A improved stability against the detrimental harsh conditions in affinity separation process.

4.1. Materials and methods

4.1.1. Materials

All the organic solvents, including tetrahydrofuran, chloroform and methanol, were purchased from Sigma. All chemicals were of analytical reagent grade and the others are the same as described in chapter 3.

4.1.2. Stability against organic solvents

All the fresh prepared native and PEGylated Con A adsorbents were incubated in various organic solvents for 30 min, then packed into a 8 mm (inner diameter) column. The column was washed and equilibrated with buffer A (0.1 M acetate buffer, containing 0.1 M NaCl, 1 mM Mg^{2+}, Mn^{2+}, and Ca^{2+}, pH 6.0) for another 30 min. 1 mg GOD or POD was applied to the column and the affinity separation was conducted at a flow rate of 0.6 ml/min. Bound GOD was desorbed by 0.1 M methyl-α-D-mannopyranoside in buffer A and the desorbed

fraction was collected to determine the adsorption capacity by Bradford method (Bradford, 1976). All the experiments were repeated three times and the mean adsorption capacity was taken as final result.

4.1.3. Stability against temperature

About 0.15 ml native and PEGylated Con A adsorbents was placed in a tube and mixed with 1 ml buffer A. The adsorbents were incubated at 55°C for different period of time. After cooling to room temperature, 4 ml of 0.3 mg/ml GOD was introduced into the tube and the mixture was equilibrated with gentle shaking for 15 h. The supernatant was withdrawn to determine the protein concentration and calculate the adsorption capacity.

4.2. Results and discussion

Affinity separation is widely employed in biotechnology, which might be encountered various types of material sources and operation conditions. On the basis of the observation that PEGylation could be highly preserved the binding specificity and binding capacity of modified Con A, further investigations of the stability of PEGylated Con A against organic solvents and high temperature were performed.

4.2.1. Stability against organic solvents

Lectin based affinity chromatography is particularly useful in aqueous solutions for the separation of glycoconjugates. Separation of intact glycolipids using this method is difficult due to the formation of mixed glycolipid micells. This problem might be resolved by using affinity separation in organic solvent-water mixture, which can conserve the carbohydrate-binding specificity of the lectin while eliminating glycolipid micelle formation (Torres, 1988; Smith, 1989). However, this observation is only possible for very stable lectins, such as *Helix pomatia*. In case of other more unstable lectins, such as Con A, a four-subunit biomolecule, organic solvent-water mixture can dramatically decrease their binding activity due to the instabilization of lectin. It was reported that PEGylation could increase the stability of modified proteins in organic solvents (Park, 1995; Beecher, 1990; Diwan, 2001 and 2003).

On the basis of these observations, PEGylated Con A was expected to exhibit the ability to improve its stability in organic solvent-water mixture for the separation of intact glycolipids. 95% tetrahydrofuran (THF), chloroform/methanol (2:1), and 50% methanol were chosen as test media because these organic solvents are frequently employed in the extraction of glycolipids from natural sources. To make the detection easier, GOD or POD (only when lacking of GOD) was applied as target compound to study the stability of PEGylated Con A in various organic media.

Stability against 95% THF buffer solution

It was reported 95% THF in water could induce intact glycolipids and disrupt the micelle structure (Smith, 1989). In order to ensure the activity of Con A, the 95% THF solution was prepared in buffer A for the incubation of Con A adsorbents. The same batch of adsorbents without incubation was used as control to measure the adsorption capacity of GOD. Fig. 4-1 shows the GOD adsorption capacity before and after 95% THF incubation. The adsorption

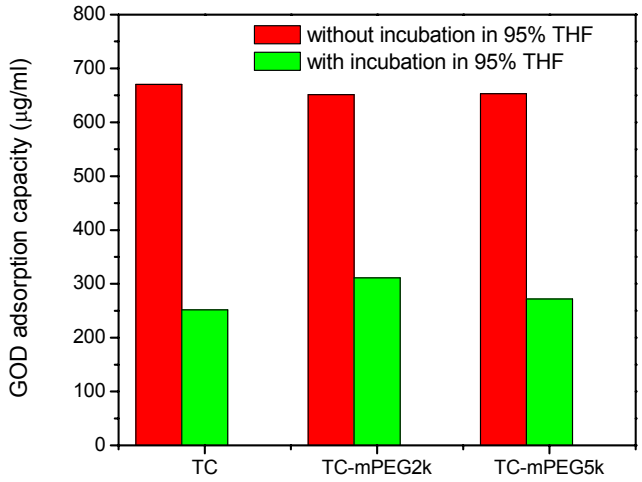


Fig. 4-1. Adsorption capacities of GOD onto Toyopearl Con A before and after incubation in 95% THF acetate buffer solution for 30 min. The ligand density of TC was 6.2 mg/ml. The molar ratio of mPEG derivatives to Con A was 1.25: 1.

capacity of GOD was merely slightly reduced after PEGylation with good agreement with the observation described in chapter 3. After incubation in 95% THF, the adsorption capacity of GOD was reduced from 670 μg/ml, 651 μg/ml, and 653 μg/ml to 252 μg/ml, 311 μg/ml, and 272 μg/ml for TC, TC-mPEG2k, and TC-mPEG5k, respectively. All the adsorption capacities

were greatly reduced after incubation. However, the results show that the residual adsorption capacity was higher for both PEGylated Con A than that for unPEGylated Con A. For instance, 48% of the original adsorption capacity of GOD was kept for TC-mPEG2k, but only 37% for TC. This indicates that PEGylation could apparently improve the stability of Con A with the exposure to 95% THF.

Stability against chloroform/methanol (2:1)

Chloroform/methanol (hereafter C/M for short) (2:1) mixture is the most popular organic solvent for the extraction of glycolipids from natural sources, which was first developed by Folch (1957). The stability of PEGylated Con A against C/M (2:1) was investigated and the results are shown in Fig. 4-2. First, the adsorption capacity of POD onto Con A adsorbents

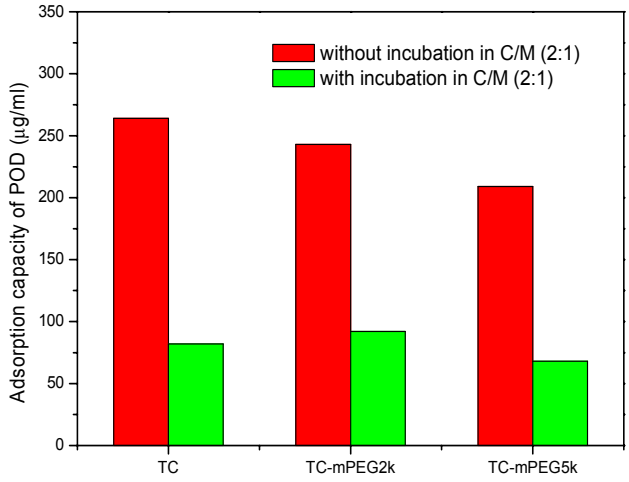


Fig. 4-2. Adsorption capacities of POD onto Toyopearl Con A adsorbents before and after incubation in C/M (2:1) for 30 min. The ligand density of TC was 9.9 mg/ml. The molar ratio of mPEG derivatives to Con A was 2:1.

were examined in buffer A and it was found that after PEGylation the adsorption capacity was reduced more when compared with the results shown in Fig. 4-1. Considering the smaller size of POD than GOD, this is probably caused by the higher PEGylated degree for modified Con A, which can induce a larger shielding effect for the adsorption of POD to modified Con A. Fig. 4-2 also shows that with the incubation in C/M (2:1) for 30 min, all Con A adsorbents showed significantly reduced adsorption capacities of POD. However, PEGylated

Con A, especially TC-mPEG2k, still possessed a higher residual adsorption capacity (31% for TC and 38% for TC-mPEG2k) when compared with their respective adsorption capacity without incubation in C/M.

Stability against methanol solution

The stability of PEGylated Con A against methanol was also investigated. Fig. 4-3 shows the high stability of PEGylated Con A against 50% methanol in buffer A. As can be seen, the

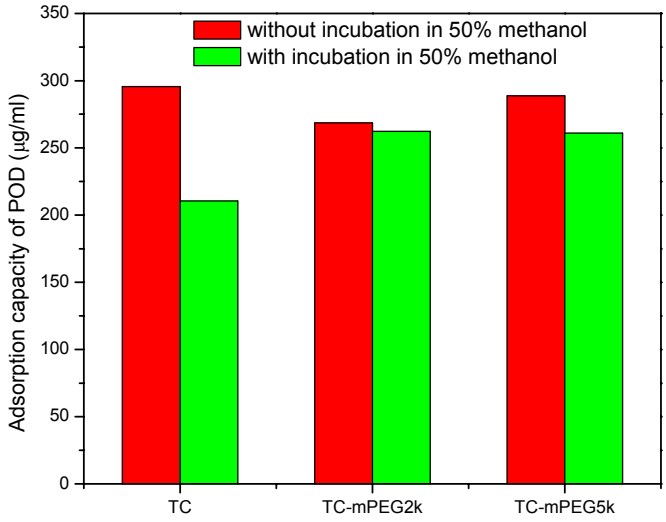


Fig. 4-3. Adsorption capacities of POD onto Toyopearl Con A adsorbents before and after incubation in 50% methanol for 30 min. The ligand density of TC was 7.5 mg/ml. The molar ratio of mPEG derivatives to Con A was1.25:1.

adsorption capacity of POD onto PEGylated Con A was much higher than that onto unPEGylated Con A after incubation in 50% methanol. TC-mPEG2k and TC-mPEG5k maintained 98%, and 90% of their original adsorption capacities, respectively. However, only 71% was reserved for TC. PEGylated Con A also showed much higher stability against 80% methanol compared with unPEGylated Con A. For example, with the incubation in 80% methanol for 30 min, the dynamic adsorption capacity of POD was 99% preserved for TC-mPEG2k and 74% for TC.

In conclusion, the above results reveal that PEGylated Con A exhibited much better stability against all the organic solvents investigated than native Con A while the adsorption capacity was mostly maintained during the PEGylation process. The enhanced stability in organic

solvents is a published observation for PEGylated proteins (Beecher, 1990). For instance, Diwan and Park found that PEGylated lysozyme and recombinant interferon-α showed better stability against the exposure to dichloromethane during encapsulation (Diwan, 2001 and 2003). PEGylated cellulase exhibited greater stability in aqueous acetone and ethylalcohol than native cellulase (Park, 1995). Although this behaviour is frequently observed in protein (especially enzyme) PEGylation, the mechanism has not been clearly elucidated. One explanation raised by Park and Kajiuchi (1995) seems quite reasonable, which described a buffering action of mPEG chains modified on enzyme surface. In this case, that is to say, the mPEG chains making a hydrophilic environment for modified Con A, created a buffering action against denaturation of Con A in the background of organic solvent. According to Combotz and Pettit (2000), PEG is soluble in water and three water molecules are associated with one ethylene oxide unit of PEG to form hydrogen bonds. These water molecules are believed to form a protective hydration shell around PEG, which provided a hydrophilic microenviroment and thus also for the modified Con A in the water-organic solvents mixture. In addition, the introduction of mPEG chains could induce more hydrogen bonds or changes of the hydrophobic properties within modified Con A molecules and thus produce a more stable structure against the exposure to organic solvents.

4.2.2. Stability against temperature

The stability of PEGylated Con A against temperature was also investigated in this work. Fig. 4-4 presents the residual adsorption capacity of GOD onto TC and TC-mPEG2k after incubation at 55 °C for different periods of time. PEGylated Con A always displayed slightly higher residual adsorption capacities than unPEGylated Con A, which indicates a higher stability of PEGylated Con A for a long time incubation (20 h) at high temperature. Previous works showed that PEGylation is an appropriate way to increase enzyme thermal stability (as cited after Castellanos, 2005). The explanation for improved thermostability by PEGylation is also versatile and remains uncertainty. Some researchers proposed that PEG modification of proteins improved their thermostability due to the decreased structure mobility causing a decrease of the unfolding rate (García-Arellano, 2002; He, 2000). In fact, hydrophobic and

electrostatic properties play an important role in determining the thermostability of proteins (Vieille, 1996). Therefore, the change of hydrophobic and electrostatic properties of an

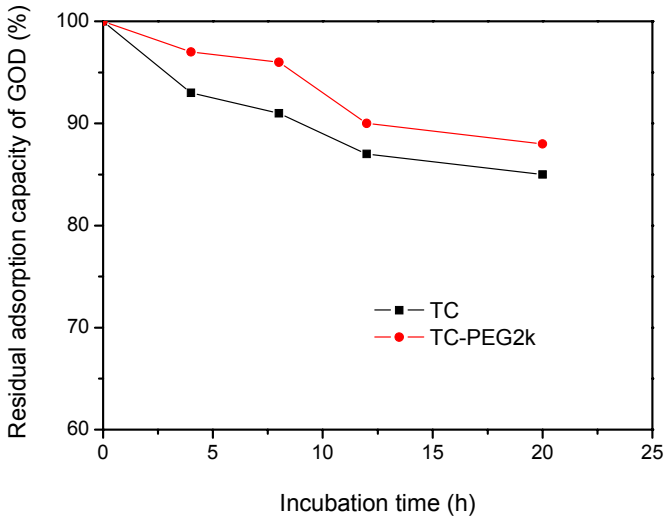


Fig. 4-4. Residual adsorption capacity of GOD onto Toyopearl Con A adsorbents after incubation at 55 °C for different period of time. The ligand density of TC and TC-mPEG2k was 12.5 mg/ml, the PEGylated degree of TC-mPEG2k was 20%.

enzyme surface has been applied to explain the thermal stabilization effect caused by PEG modification (Murphy, 1996; He, 1999; Garcia, 1998). In addition, Longo and Combes proposed that the decreased thermal denaturation rate of α-chymotrypsin might result from the increase of hydrophilicity of the enzyme surface caused by PEG chains (1999).

4.3. Conclusions

This chapter investigated the properties of PEGylated Con A against some harsh operational conditions encountered in affinity separation. Studies show that PEGylated Con A highly preserved their binding capacities, and exhibited much higher stability against the exposure to organic solvents and high temperature. The reason for improved stability was shortly discussed. The fact that PEGylation stabilizes the properties of Con A may greatly expand the range of applications of unstable affinity ligand in bioseparation processes.

5. Novel dense pellicular adsorbents for expanded bed adsorption

Expanded bed adsorption (EBA) is a primary recovery operation allowing the adsorption of target proteins directly from unclarified feedstocks, such as culture suspensions, cell homogenates, and crude extracts (reviewed by Chase, 1994; Hjorth, 1997; Anspach 1999 and Hubbuch, 2005). The successful operation of adsorption in such systems depends on the formation of a classified fluidized bed (termed expanded bed), which is characterized by the low extent of axial mixing and the absence of flow channels or dead liquid zones. This is achieved by a special design of the column and the solid matrix with a defined size and/or density distribution. Usually, the adsorbents suitable for expanded bed adsorption have the particle size ranging from 50-400 μm and the density of 1.1-1.3 g/ml (Hjorth, 1997). When a stable expanded bed is achieved with the upward flow, the classification of the adsorbents and therefore limited movement of the adsorbent particles take place within the expanded bed. This is necessary if dispersion in the liquid phase is to be minimized and high adsorption efficiency achieved (Karau, 1997).

As the essential element of expanded bed adsorption, synthesis of suitable solid adsorbents has greatly attracted the attention of researchers in recent years (Pålsson, 2000a; Pai, 2000). The basic criteria of suitable expanded bed adsorbents are proposed to exhibit sufficient density and wide distribution of particle size (Chase, 1992a). A high density of the adsorbents is required for the stable operation at higher flow velocity and the appropriate particle size distribution contributes significantly to a reduction of mixing in the column. In addition, the efficiency of protein adsorption must be considered in the synthesis of expanded bed adsorbents. Thömmes (1995b) reviewed the hydrodynamic aspects of expanded bed adsorption and pointed out that among all the system parameters investigated, no single one controlled the adsorption efficiency, but the intraparticle mass transport played a dominant role. Draeger and Chase also found the rate of protein binding in expanded bed process is pore diffusion and film mass transfer controlled (1991). Therefore, when suitable adsorbents are attempted to be constructed, both the particle density and the intraparticle diffusion distances should be considered to ensure the mass transfer within the adsorbents does not

compromise the practical advantages imparted by the particulate processing at an elevated flow rate. The development of a dense pellicular adsorbent is an attractive approach to improve the properties of solid phase in respect to the reduced intraparticle diffusion distances and the potential for enhanced product throughput, chromatographic performance and bed stability (Lyddiatt, 2002).

Pellicular adsorbents have been prepared by covering an agarose gel layer onto steel beads (Pålsson, 2002a; Theodossiou, 2002) or dense silica-coated zirconia beads (Jahanshahi, 2002; Sun, 2001). In this chapter, a dense pellicular adsorbent by coating glass beads with agarose gel for expanded bed adsorption process is described. The novel adsorbents were characterized by their physical properties and bed expansion behaviour. The bed expansion behaviour of agarose coated glass beads was characterized by the residence time distribution (RTD) experiments, which showed that this kind of adsorbents exhibited low axial mixing and is promising for the application in expanded bed adsorption processes.

5.1. Theoretical background for expanded bed adsorption

Before the study of expanded bed process, it is necessary to get some knowledge about the theoretical background for expanded bed adsorption. This section focuses on the knowledge of fluidization and liquid mixing/dispersion in expanded bed.

5.1.1. Fluidization

The adsorbent beads in expanded bed are not in a fixed position as in packed bed operation. The solid dispersion can be reduced to a minimum if the adsorbent bead movement is confined to smaller space. This could be achieved by using polydispersed adsorbents in terms of both size and density resulting in a well classified and stable expanded bed.

The Richardson-Zaki correlation describes the effect of suspended adsorbent concentration (i.e., voidage) on the rate of settling and allows a prediction of the liquid velocity required to produce a given degree of bed expansion during fluidization (Richardson, 1954). The correlation assumes that all the adsorbents are spherical, nonporous, and of the same average size, which is given in Eq. 5-1.

$$U = U_t \cdot \varepsilon^n \tag{5-1}$$

where U and U_t are the liquid superficial velocity and the terminal settling velocity, respectively. ε is the bed voidage in expanded mode, and n is the bed expansion index.

The terminal settling velocity U_t can be determined by the Stokes' equation:

$$U_t = \frac{d_p^2(\rho_s - \rho)g}{18\mu} \tag{5-2}$$

where d_p is the adsorbent diameter, ρ_s and ρ are the density of the adsorbent and the liquid, respectively. g is the acceleration due to gravity, μ is the dynamic (absolute) viscosity of the fluid. The use of the Stokes' equation is restricted to the system where $Re < 0.2$ (creeping flow range). Re is the Reynold number for a particle in the fluid.

The bed voidage ε can be obtained from Eq. 5-3:

$$\varepsilon = 1 - (1 - \varepsilon_0)\frac{H_0}{H} \tag{5-3}$$

where ε_0 is the bed voidage in packed mode, H_0 and H are the height of packed bed and expanded bed.

5.1.2. Liquid mixing/dispersion in expanded bed

In expanded bed, the mixing of the particles is restricted due to the well distributed particle size and density. However, the chromatographic performance can also be severely affected by the axial mixing of the liquid. The evaluation and quantification of the axial mixing of the liquid is traditionally carried out by the residence time distribution (RTD) measurement (Barnfield Frej, 1997) with an inert tracer. The tracer is injected into the system by a stepwise or pulse method when the expanded bed is stable (under steady state conditions). The resulting concentration profile of the tracer at the outlet reflects the hydrodynamic conditions inside the system and the RTD data is often fitted by mathematical models such as dispersion model or tanks-in-series model to characterize the hydrodynamics of the system (Pålsson, 2001).

The common used parameters derived from RTD data are N, $HETP$, Bo and D_{ax} describing the

liquid mixing in EBA system. The number of the theoretical plates *N* is calculated from the negative step input signal (see Fig. 5-1):

$$N = t^2 / \sigma^2 \tag{5-4}$$

where *t* is the mean residence time; the distance from the "mark" to 50% of the maximum absorbance, and *σ* is the standard deviation; half the distance between the points 15.85% and 84.15% of the maximum absorbance. The larger the number of theoretical plates *N,* the less mixing in the system exists.

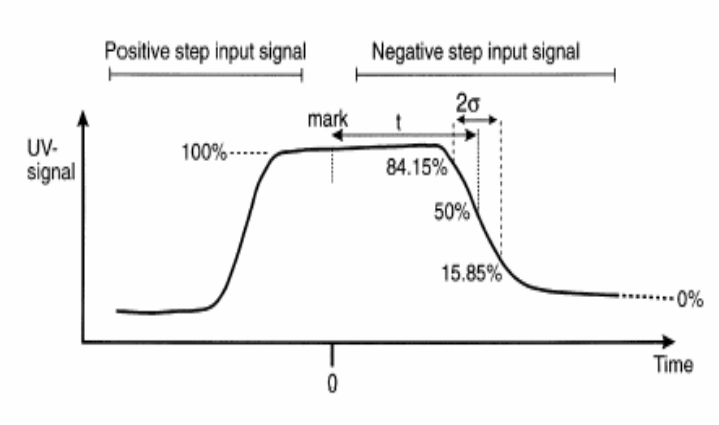


Fig. 5-1. The UV signal recording during the residence time distribution analysis (adapted from Barnfield Frej, 1997).

HETP means the height equivalent to a theoretical plate under given operating conditions. The smaller the *HETP*, the more efficient the separation procedure is. *HEPT* is dependent on the superficial velocity *U*, which is described in Eq. 5-5 (Pai, 2000):

$$HETP = A + B/U + C \cdot U \tag{5-5}$$

where, the three terms of the equation account for dispersion, molecular diffusion, and intraparticle effects, respectively. The constants *A*, *B*, and *C* are system and operation dependent parameters.

The most common number for characterizing the dispersion is the Bodenstein number, *Bo*, which describes the mass transfer in flowing fluids. For a prior estimation, *Bo* number > 40 describes a plug flow with negligible dispersion. Considering the expanded bed as an open

vessel, *Bo* can be calculated from Eq. 5-6 (as cited by Tong, 2001):

$$\sigma_\theta^2 = \frac{2}{Bo} + \frac{8}{Bo^2} \tag{5-6}$$

where the variance in time unit, σ_θ^2, can be obtained from Eq. 5-7.

$$\sigma_\theta^2 = \frac{\sigma^2}{t^2} \tag{5-7}$$

The Bodenstein number relates the ratio of convective to dispersive mass transport. The relation between Bodenstein number *Bo* and the axial dispersion coefficient D_{ax} is:

$$Bo = \frac{UH}{\varepsilon D_{ax}} \tag{5-8}$$

Thus, the axial dispersion coefficient D_{ax} can be calculated from the above equations.

5.2. Materials and methods

5.2.1. Materials

The agarose coated glass beads (hereafter named AG) are gifts from Prof. Sun (Tianjin University, Tianjin, China). Acetone and glycerol were purchased from Sigma. All chemicals were of analytical grade except otherwise stated. All expanded bed experiments were carried out on a GradiFrac™ System (Amersham Bioscience, Freiburg, Germany). It consisted of a peristaltic pump, a C10/20 column (Amersham Bioscience, Freiburg, Germany) with 2 removable adapters, a UV detector and a notebook equipped with the software VirtualBench (National Instruments, Munich, Germany) to acquire the UV signal.

5.2.2. Determination of the density of agarose coated glass (AG) supports

A 10 ml pycnometer was washed thoroughly with water and ethanol and then dried in the oven. After cooling down, the weight of the empty bottle m_B was measured. Sucked AG supports was added into the pycnometer and the whole weight of the pycnometer with the

supports named $m_{B,S}$ was determined. Then degassed purified water was poured into the pycnometer and the whole weight of bottle, supports, and water was weighted again and denoted as $m_{B,S,W}$. Afterwards, the pycnometer was washed with water and ethanol and dried again, degassed purified water was added into the pycnometer and the weight of the bottle and water was measured named as $m_{B,W}$ and the temperature at that moment was recorded. The density of water ρ_W at different temperatures was obtained according to the booklet of physical chemistry (Zhang, 1997, p. 484). Therefore, the volume of measured AG supports V_S can be obtained by subtracting the volume of water in the mixture with supports from the total volume of the pycnometer. The total volume of pycnometer was calculated from the weight and density of water. Thus the final equation to express the skeletal (solid) volume of AG supports V_S is shown in Eq. 5-9:

$$V_S = \frac{[(m_{B,W} - m_B) - (m_{B,S,W} - m_{B,S})]}{\rho_W} \tag{5-9}$$

The density of the AG supports is calculated from dividing the weight of AG supports by the volume of AG supports:

$$\rho_S = \frac{(m_{B,S} - m_B)}{V_S} = \frac{\rho_W (m_{B,S} - m_B)}{[(m_{B,W} - m_B) - (m_{B,S,W} - m_{B,S})]} \tag{5-10}$$

5.2.3. Determination of the packed bed voidage

Water was introduced into a 10 ml graduated cylinder where m_S gram (wet weight) sucked AG supports was placed. The supports were stirred for some time to ensure no air bubble involved and the graduated cylinder was kept overnight. The apparent volume of the supports V_t was obtained from the mark on the graduated cylinder. The skeletal (solid) volume of the supports V_S can be calculated from the density of the supports obtained from Eq. 5-10.

$$V_S = \frac{m_S}{\rho_S} \tag{5-11}$$

Then the voidage of packed bed ε_0 can be obtained from Eq. 5-12 (Tong, 2002):

$$\varepsilon_0 = \frac{V_t - V_S}{V_t} \qquad (5\text{-}12)$$

5.2.4. Determination of the degree of bed expansion

The characteristics of bed expansion were measured at room temperature (20 °C) in a C10/40 column (Amersham, Freiburg, Germany). The supports were packed into the column from the top of the column and allowed to settle freely. The settled bed height was measured and characterized by the symbol of H_0. The mobile phase was pumped through the bed from the bottom of the column with increasing superficial flow velocity. For each run, the stable expanded bed was allowed to expand another 20 min before measuring the expanded bed height (characterized by H) in order to eliminate the back mixing introduced by unstable fluidization. The degree of bed expansion was determined from H/H_0.

5.2.5. Determination of the liquid dispersion

Liquid dispersion behaviour in the expanded bed was determined by residence time distribution (RTD) experiment according to (Barnfield Frej, 1997). Distilled water containing various concentrations of glycerol was employed as mobile phase for expanded bed experiments. Acetone 1% (V/V) in different glycerol solutions (0%, 10%, and 20% V/V glycerol in distilled water) was applied as a tracer solution. Individual experiments were performed for the complete experimental rig in the presence or absence of adsorbents in order to identify the contribution of the volume of fittings and the zone above the bed surface.

The RTD measurement was carried out as follows (see Fig. 5-1): the glycerol solutions with different concentrations were pumped upwards through the bed until the bed stopped expanding and the top adapter was moved down and positioned 1.0±0.1 cm above the bed surface. When the signal from the UV detector was stable, the mobile phase was changed to the tracer solution and the positive step input signal was obtained. The mobile phase was switched back to the glycerol solution when the UV signal was stable at a maximum absorbance (100%) and then the negative step input signal was started to record. The change was marked and the UV signal was allowed to stable at baseline level (0%).

5.3. Results and discussion

5.3.1. Physical properties of agarose coated glass beads (AG)

The dense pellicular AG adsorbents were prepared by the stirred-tank emulsification method as described by Zhou et al. (2004). The micrograph of composite AG adsorbents revealed that the majority of AG beads were composed of a single glass bead coated with relatively even and transparent agarose gel layer (Fig. 5-2). Since the depth of the agarose shell is much smaller than the diameter of glass beads, such matrices were regarded as pellicular adsorbents. The outer gel layer was designed to reduce the protein diffusion distance and thus facilitate the rapid capture of the target compounds. The solid dense glass beads were used to achieve stable expanded bed when encountering high-viscosity feedstocks or high flow velocity.

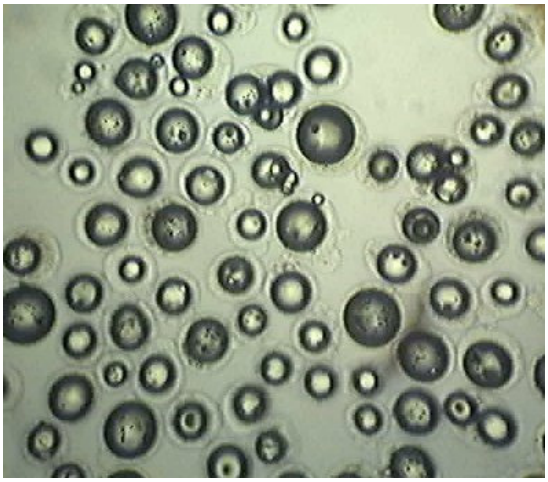


Fig. 5-2. Micrograph of agarose coated glass beads taken with a CCD camera through an optical microscope (×160).

The particle size distributions of the AG and the employed glass beads are shown in Fig. 5-3, from which the size range and volume weighted mean particle size were estimated. The results are summarized in Table 5-1. To define the size range, volumetric diameters d_{10} and d_{90} are defined as the points on the size distribution where, 10% and 90% by volume of the particles are smaller than the stated diameter, respectively. The statistics of size distribution show that 80% (V/V) of the particles were in the range of 60-160 μm for the composite AG

adsorbents, while 40-85 μm for the glass beads. The volume-weighted mean diameter of the AG and the glass beads were 105 μm, and 61 μm, respectively, which shows that the average

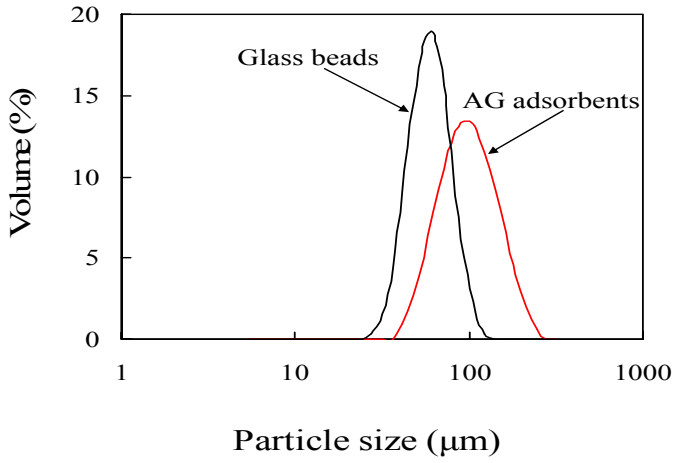


Fig. 5-3. Particle size distributions of AG adsorbents and glass beads measured with Malvern Mastersizer 2000 unit.

depth of agarose pellicle was about 22 μm. For comparison, the data of the physical properties of Streamline (a commercial expanded bed adsorbent) are also provided in Table 5-1. It can be seen that the density of the AG matrix (1.6 g/ml) is higher than that of

Table 5-1. Physical properties of matrices

Matrices	Size range (μm)	Mean size (μm)	ρ_s (g/ml)
Glass beads	40-85	61	2.4
AG	60-160	105	1.6
Streamline[a]	100-300	200	1.2

[a]Data from supplier

Streamline (1.2 mg/ml), which is expected to provide better expansion behaviour for stable operation at higher flow velocity.

5.3.2. Bed expansion characteristics

Before running the expanded bed process, it is important to determine the bed expansion characteristics; i.e. how the bed height varies with the liquid flow rate and how the bed

expansion is affected by the physical properties of the applied liquid. The bed expansion depends on lots of variables, such as the viscosity and density of the employed liquid and certain physical properties of the adsorbent (i.e., distribution of size and density).

It was stated that the wall effect (i.e. a velocity gradient created by drag exerted by the walls) on bed expansion behaviour was negligible as long as the ratio of column to particle diameter is larger than 20 (Lali, 1989). Since the value of this ratio for AG adsorbents was larger than 95, the bed expansion behaviour should not be influenced by the wall effect. Fig. 5-4 shows

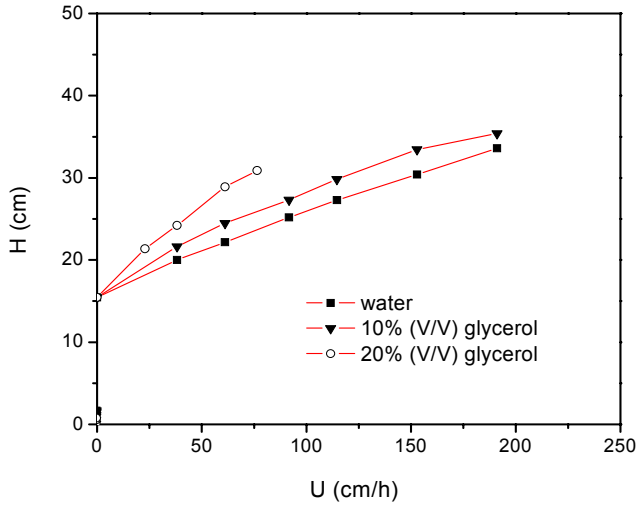


Fig. 5-4. Bed expansion characteristics for AG adsorbents. The characteristics of AG bed expansion were determined in a C10/40 column at 20 °C. The settled bed height was 15.4cm and the bed expansion was performed in the presence of the following solutions: (■) water; (▼) 10% (V/V) glycerol/water; (○) 20% (V/V) glycerol/water.

the degree of the bed expansion of AG adsorbents as a function of the superficial velocity of the flow through the bed. As expected, the degree of the bed expansion linearly increased with increasing the flow velocity, and the expansion of the bed was greater in the presence of the glycerol solutions than that in water solution. To reach 2-fold bed expansion, the flow velocity needed was equivalent to 156 cm/h, 125 cm/h, and 76 cm/h with 0%, 10%, and 20% (V/V) glycerol solutions, respectively.

The Richardson-Zaki relationship is a classical equation to describe the bed expansion characteristics of a fluidizing bed as shown in Eq. 5-1. To apply this relationship, the bed

voidage in packed mode ε_0 must be known. According to the procedure described in experimental section, ε_0 for the AG adsorbents was determined to be 0.36, which is very close to that of Streamline (ε_0= 0.40) (Chang, 1996).

The terminal settling velocity U_t and the bed expansion index n could be obtained by linear regression of plots of $ln\ U$ versus $ln\ \varepsilon$ (Thömmes, 1996). The results are given in Fig. 5-5 and Table 5-2.

Table 5-2. Results of the linear regression of Richardson-Zaki plot

Medium	Water	10% (V/V) glycerol	20% (V/V) glycerol
Bed expansion index n	4.76	5.35	4.99
Terminal settling velocity U_t (mm/s)	27.66	27.81	14.25

The bed expansion index n is a function of the particle diameter d_p and column diameter D in the range of creeping flow.

$$n = 4.65 + 20(\frac{d_p}{D}) \qquad (5\text{-}13)$$

The bed expansion indexes of AG adsorbents were in the range of 4.76-5.35 in all kinds of the investigated liquid solutions, which is reasonably close to the theoretical value of 4.86.

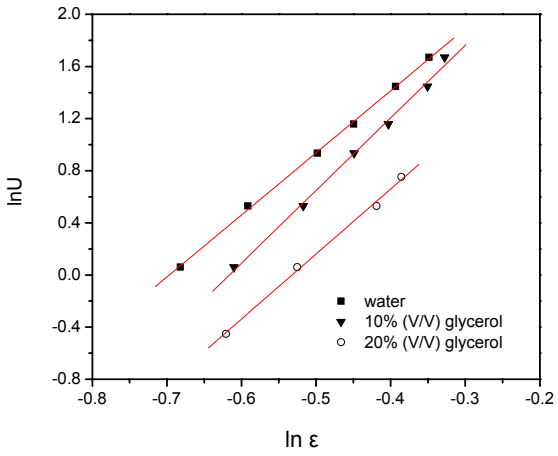


Fig. 5-5. Richardson-Zaki plot of AG supports. The experimental conditions and symbols are the same as mentioned in Fig. 5-4.

The terminal settling velocity was reduced when further increasing the viscosity of the liquid from 10% to 20% glycerol and there was a large discrepancy between the experimental U_t values and those calculated by the Stokes' equation. Such observations were also reported in the other literatures (Thömmes, 1995a; Chang, 1996; Karau, 1997). It could be a result of different size or density distribution of adsorbents, the agglomeration of the adsorbents caused by the highly viscous liquid or the groupwise movement of particles due to the viscous drag force.

5.3.3. Axial dispersion behaviour in the expanded bed

The efficiency of an expanded bed is closely related to the strict control of a stable expanded bed with low axial mixing (Pålsson, 2000a and 2000b; Chang, 1996; Karau, 1997). Therefore it is very important to investigate the axial dispersion behaviour with AG matrices under various operational conditions to confirm whether they are suitable for the expanded bed adsorption process.

Effect of flow rate and settled bed height

There are a large number of reports in terms of the dependence of axial mixing on flow velocity in expanded bed processes. However, the results on mixing show significant variation and no trend can be detected. For example, Chang and Chase found that the axial mixing with Streamline SP increased with increasing flow velocity (1996), which is in agreement with other publications (Chen, 2003; Tong, 2001). However, Bascoul observed an opposite trend (1988). Dasari et al. (1993) reported that the axial mixing increased with increasing flow velocity for 40-60 μm sized particles but decreased for small size particles (25-40 μm). As described above, it seems that the axial mixing characteristics may be influenced by a lot of factors, such as the type of matrix, the extent of the particle size distribution, the particle density, flow velocity, column dimensions, and distributor design. In this study, the axial mixing behaviour in expanded bed of AG supports as a function of the flow velocity in water solution was investigated and the results are shown in Fig. 5-6. The Bodenstein number *Bo* is usually used to characterize the degree of axial mixing. A higher *Bo* value means a lower axial

mixing. Fig 5-6 (a) shows the effect of the flow rate on the *Bo* number of AG matrices with different settled bed height. It can be seen that *Bo* slightly increased for both settled bed height with increasing the flow velocity. For H_0=6.0 cm, the *Bo* number was in the range of 20-28, while for H_0=16.1 cm ranges from 20 to 31 in the flow range of 20 to 230 cm/h. According to Chang and Chase (1996), values of *Bo* > 20 have little effect on the adsorption performance. Here, even for lower settled bed height (H_0=6.0 cm), most of the *Bo* values under various flow velocity were higher than 20, which means a low axial mixing. Therefore, it is considered that AG matrices are promising for the application in expanded bed operation.

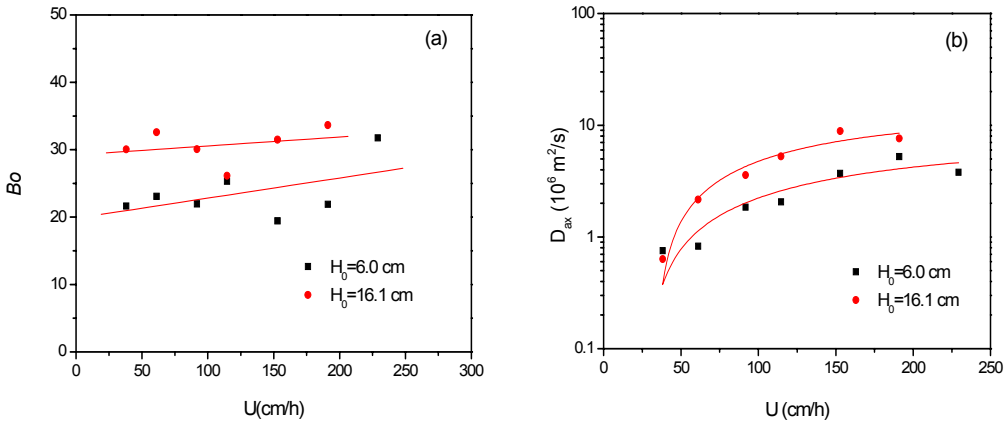


Fig. 5-6. Effect of the flow velocity on (a) the *Bo* number and (b) the axial mixing coefficient D_{ax}. The axial mixing behaviour of AG adsorbents in expanded bed was performed in a C10/40 column at 20 °C with a settled bed height ■ H_0=6.0 cm and ● H_0=16.1 cm

Even though *Bo* number is frequently employed to express the axial mixing in expanded bed (Anspach, 1999; Karau, 1997; Thömmes, 1996), it may give false information of the efficiency of expanded bed (Pålsson, 2000a and 2001; Hubbuch, 2005). According to Eq. 5-8, D_{ax} is associated with the *Bo* number and also flow velocity, as well as the expanded bed height and the expanded bed voidage. Therefore, a higher *Bo* number may not directly mean a lower axial mixing. Just as shown in Fig. 5-6 (a) and (b), compared with lower settled bed height H_0=6.0 cm, a higher *Bo* number but also a higher D_{ax} were obtained for processes with an expanded bed of H_0=16.1 cm. The similar phenomenon was observed by Lan (1999). Fig. 5-6 (b) also shows that the D_{ax} values increased with increasing the flow velocity and the settled bed height. The experimentally obtained D_{ax} values in this work were in the same

order of magnitude of axial mixing ranging between 1.0×10^{-6} and 1.0×10^{-5} m^2/s as normally found in expanded bed processes at flow velocities of 100-300 cm/h (Pålsson，2000b; Thömmes, 1996; Jahanshahi, 2002).

Effect of liquid viscosity

Since high viscosity feedstocks are frequently encountered in bioseparation processes, it is necessary to investigate the effect of the liquid viscosity on the axial mixing in expanded beds. In this study, water (viscosity, 1.00×10^{-3} Pa·s), 10% (V/V) glycerol (viscosity, 1.39×10^{-3} Pa·s), and 20% (V/V) glycerol (viscosity, 2.00×10^{-3} Pa·s) were used as mobile phase (Hodgman, 1948). A few publications reported the viscosity effect on liquid dispersion. The general

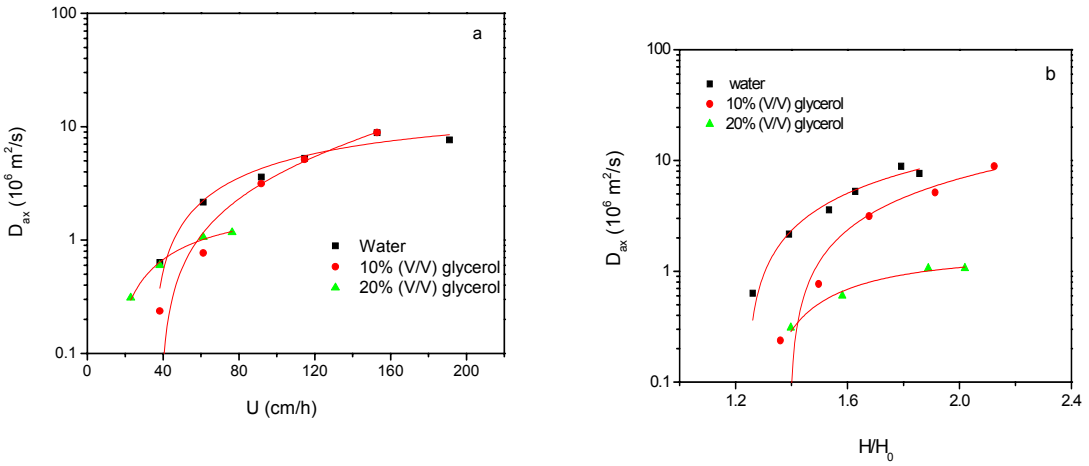


Fig. 5-7. Effect of the flow viscosity on D_{ax}: (a) D_{ax} versus U and (b) D_{ax} versus H/H_0. The axial mixing behaviour of the AG adsorbents in expanded bed was performed in a C10/40 column at 20 °C with a settled bed height H_0=16.1 cm.

observation was that the axial dispersion coefficient increased by raising the viscosity of the fluid flow under constant flow velocity (Chang, 1996; Pålsson, 2000a). According to these studies, it is difficult to predict the trend of the viscosity effect on the D_{ax} under constant flow velocity. As shown in Fig. 5-7 (a), the D_{ax} exhibited different tendencies under varying ranges of the flow velocity. At the low flow velocity range, the D_{ax} was larger in a high viscosity fluid than that in a low viscosity fluid. However, in general the D_{ax} was smaller in the high viscosity fluids at the high flow velocity range. Since it is difficult to make conclusion by interlaced plots, the D_{ax} is plotted versus the bed expansion degree H/H_0. Fig. 5-7 (b) clearly

showed that the D_{ax} value was reduced with increasing the bed expansion degree when H/H_0 was lower than 2.2. This observation was in good agreement with Pålsson's (2000a). In the viscosity range investigated, the D_{ax} values were in the range of 2.37×10^{-7} till 8.90×10^{-6} m^2/s, which indicates a very low axial dispersion and the AG adsorbents are well suited for expanded bed applications.

5.4. Conclusions

In this chapter, a novel agarose coated glass (AG) matrix for expanded bed adsorption was characterized. The physical properties investigation showed that the composite matrix exhibited a higher density and a reduced interparticle diffusion distance when compared with the commercial Streamline adsorbents, which may facilitate protein adsorption performance in expanded bed process. The bed expansion behaviour of the AG matrix fitted well with the Richardson-Zaki equation. The liquid phase dispersion behaviour in expanded bed was determined by the residence time distribution measurements. The dependency of axial mixing in the expanded bed on the flow velocity, settled bed height, and viscosity was examined. It was found that the axial mixing increased with a higher flow velocity and settled bed height and decreased with increasing the viscosity under a certain bed expansion degree (H/H_0). The novel AG matrices are promising for expanded bed adsorption of proteins since they exhibited very low axial mixing in all investigations.

6. Adsorption performance and application of Agarose coated glass (AG) adsorbents in expanded bed adsorption of proteins

In chapter 5, a dense pellicular agarose coated glass matrix was characterized by its physical properties, bed expansion behaviour and liquid phase dispersion behaviour. The results showed the novel matrix exhibited a high density and a reduced diffusion distance when compared with the commercial Streamline adsorbents. More important, the matrix showed very low axial mixing in all the systems investigated. In this chapter, Con A ligands were coupled onto these matrices to prepare affinity adsorbents. The AG Con A adsorbents were applied to investigate their adsorption behaviour for GOD in batch experiments and their applications for the expanded bed adsorption of GOD and albumin from crude hen egg white solution.

6.1. Materials and methods

6.1.1. Materials

Crosslinked agarose coated glass matrix was a gift from Prof. Sun prepared according to Zhou et al. (2004). Con A (type V), Epichlorohydrin (99%), 1, 6-diaminohexane, pentaethylenehexamine, glutaraldehyde solution (25%), sodium borohydride, sodium cyanoborohydride (95%), and methyl-α-D-mannopyranoside were from Sigma (Munich, Germany). Ammonium hydroxide solution (33%) was purchased from Riedel-deHaën (Seelze, Germany). Glucose oxidase from *Aspergillus niger* was obtained from SERVA (Heidelberg, Germany). Albumin from hen egg white was delivered from Fluka Biochemika (Buchs, Germany), precast ready gel was brought from Bio-Rad (Munich, Germany). All chemicals were of analytical grade unless otherwise stated. The expanded bed adsorption system is the same with that described in chapter 5.

6.1.2. Activation of agarose coated glass (AG) matrix

To couple Con A onto hydroxyl group containing AG matrix, the activation of AG matrix is necessary. In this work, the AG matrix was activated subsequently by epoxy reagent, amination reagent, and glutaraldyhyde as shown in Fig. 6-1.

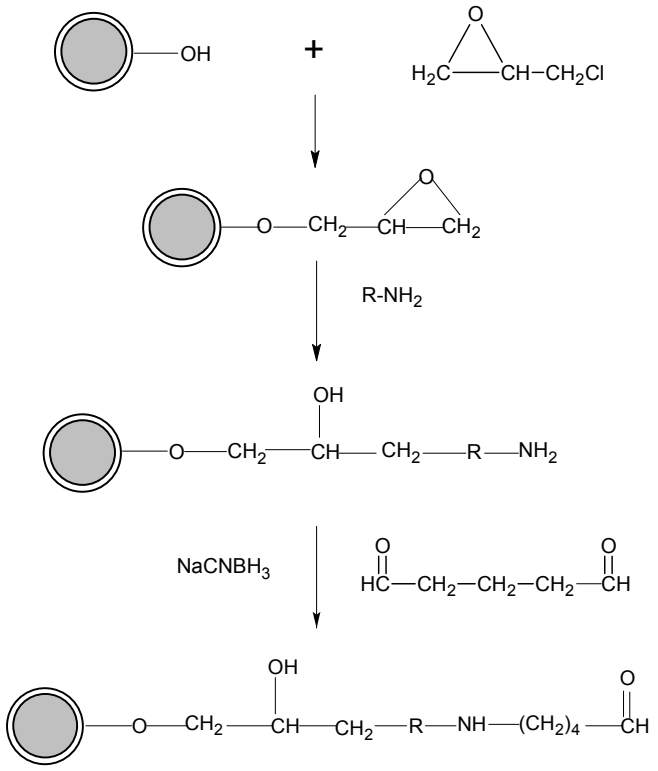


Fig. 6-1. Scheme of activation of agarose coated glass matrix. R-NH_2 employed in this work included (1) ammonium hydroxide, $NH_3 \cdot H_2O$ (2) 1,6-diaminohexane, NH_2-$(CH_2)_6$-NH_2, and (3) pentaethylenehexamine, NH_2-$(CH_2CH_2NH)_4$-CH_2-CH_2-NH_2.

Activation of the agarose coated glass (AG) matrices by epoxy reagents (Epoxy activation)

Crosslinked AG matrices were thoroughly washed with water and 100 ml (settled volume) sucked matrices were transferred into a 500 ml round-bottom flask equipped with a mechanical stirrer. The matrices were suspended each in 100 ml 1 M NaOH containing 200 mg sodium borohydride ($NaBH_4$) stirred with a paddle. 40 ml epichlorohydrin was slowly

added into the flask with constant stirring and the reaction was allowed to perform at room temperature for 6 h. After the reaction, the activated matrices were extensively washed with water to remove excess epoxy reagent until there was no longer evidence for an oily film on the surface of the matrices. If necessary, acetone was used to better remove the excess epichlorohydrin. The matrices were kept in water in the refrigerator for the following activation.

Amination of epoxy activated matrices

20 ml sucked epoxy activated matrices, which were prepared as described in the previous section, was transferred into 100 ml round-bottom flask containing 20 ml 0.5 M NaOH and then the amination reagent was introduced into the reaction system. The mixture was allowed to react for 3 h at 45 °C under constant stirring. The reaction mixture was then washed successively with water, 1 M NaCl, and water again. Three kinds of amination reagents were employed in this experiment in order to change the spacer length: (1) ammonium hydroxide, (2) 1, 6-diaminohexane, and (3) pentaethylenehexamine.

Modification of activated amine-containing matrices with glutaraldehyde

20 ml activated amine-containing matrices was washed with the coupling buffer I (0.1 M phosphate buffer, 0.15 M NaCl, pH 7.0), sucked and transferred into a 100 ml round-bottom flask. 20 ml 12.5% glutaraldehyde (W/V) in coupling buffer and 0.2 mg sodium cyanoborohydride ($NaCNBH_3$) were added to the aminated matrices. The mixture was reacted at room temperature for at least 4 h. The use of sodium cyanoborohydride is to eliminate the unstable C=N Schiff base formed in the reaction. After washing with coupling buffer I, the activated matrices were ready for the coupling with lectins.

6.1.3. Immobilization of Concanavalin A (Con A) on glutaraldehyde activated agarose coated glass (AG) matrices

10 ml sucked AG matrices containing a spacer arm terminating in glutaraldehyde functionality was mixed with 25 ml 4 mg/ml Con A in the coupling buffer II (0.5 M phosphate

buffer, 0.1 M NaCl, pH 8.0) and reacted for 2 h with gentle shaking. The supernatant of the mixture was withdrawn to determine the protein concentration by the Bradford method (1976). The matrices were extensively washed with water and coupling buffer to remove unreacted Con A, then blocked with 0.1 M Tris-HCl buffer, pH 8.0, for 2 h. Thereafter 40 mg $NaCNBH_3$ was introduced to the mixture to eliminate the unstable C=N Schiff bases and reacted for 0.5 h. The lectin immobilized matrices were washed subsequently with water and 0.1 M acetate buffer, containing 0.1 M NaCl, 1 mM Mn^{2+}, Mg^{2+}, and Ca^{2+}, pH 6.0 (hereafter called buffer A), and then kept in the same buffer at 4-6 °C for further use.

6.1.4. Adsorption/desorption kinetics of glucose oxidase (GOD) onto/from agarose coated glass (AG) Concanavalin A (Con A) adsorbents

40 ml 0.5 mg/ml GOD in buffer A was mixed with about 1.2 ml AG Con A by gently shaking on an orbital shaker. At certain time intervals, 1 ml supernatant was withdrawn to determine the protein concentration by measuring the absorption at λ= 280 nm. Buffer A was used as blank and the sample was put back immediately after measurement.

The GOD adsorbed onto AG Con A adsorbents were washed carefully and thoroughly with water and then buffer A to remove the unbound GOD. 40 ml 0.1 M methyl-α-D-mannopyranoside in buffer A was mixed with the washed adsorbents to desorb GOD specifically. The supernatant of the desorption solution was taken to determine the protein concentration using 0.1 M methyl-α-D mannopyranoside buffer A solution as blank.

6.1.5. Adsorption isotherms of glucose oxidase (GOD) onto AG-PEHA-Con A adsorbents

The adsorption isotherm of GOD onto AG-PEHA-Con A was carried out on an orbital shaker at room temperature for 15 h. About 0.2 ml AG-PEHA-Con A was mixed with 10 ml GOD in buffer A with the concentrations ranging from 0.1 mg/ml to 2 mg/ml. The final concentration of GOD was determined by measuring the absorption at λ= 280 nm to determine the equilibrium adsorption capacity, which can be calculated according to Eq. 2-4 (p. 26).

6.1.6. Expanded bed adsorption of glucose oxidase (GOD) and albumin from crude hen egg white solution

The expanded bed adsorption experiments were conducted on a GradiFrac^{TM} system, consisting of a peristaltic pump, a C10/20 column (Amersham Bioscience, Freiburg, Germany) with 2 removable adapters, a UV detector and finally a notebook equipped with the software VirtualBench (National Instruments, Munich, Germany) to acquire the UV signal. AG-PEHA-Con A adsorbents were packed in the C10/20 column with an inner diameter of 1 cm. The settled bed height was 5 cm. GOD and crude albumin from hen egg white were employed to determine the adsorption capacity of AG-PEHA-Con A adsorbents, respectively. Prior to the feedstock application, the bed was expanded stably in adsorption buffer for 20 min with a 1.9 fold bed expansion degree (H/H_0). 0.5 mg/ml GOD or crude albumin from hen egg white was pumped into the column at a flow velocity of 229 cm/h until the maximum absorption was reached. The column was then continuously washed with adsorption buffer until the UV signal reached the minimum. Subsequent product desorption was achieved by switching the adsorption buffer to the desorption buffer. Desorption was conducted still in the expanded bed mode in order to dispense with the need for the movement of adapter. After desorption the column was regenerated with the adsorption buffer for the further use. Appropriate fractions were collected and assayed for protein content and enzyme activity. The experimental results were the average of two times operation. The working buffers and their contents applied in these experiments are listed in Table 6-1.

Table 6-1. Working buffers for expanded bed adsorption of GOD and albumin from hen egg white

Adsorbates	Adsorption, washing and regeneration buffer	Desorption buffer
GOD	0.1 M acetate buffer, containing 0.1 M NaCl, 1 mM Ca^{2+}, Mg^{2+}, Mn^{2+}, pH 5.0	0.2 M methyl-α-D-mannopyranoside in adsorption buffer
Albumin from hen egg white	0.1 M Tris-HCl buffer, containing 0.15 M NaCl, 1 mM Ca^{2+}, Mg^{2+}, Mn^{2+}, pH 7.4	0.3 M methyl-α-D-mannopyranoside in adsorption buffer

6.1.7. SDS-PAGE

The purified albumin was concentrated and applied to SDS-PAGE. SDS-PAGE was carried out in a Mini-PROTEAN® 3 cell from Bio-Rad (Munich, Germany). Precast 10% acrylamide/Tris/HCl gels (12 well) were used. The running buffer was Tris/HCl, pH 8.3, and the gel was run at 200 V for 35 min. Prestained protein standards were applied as molecular markers (Serva, Heidelberg, Germany). The gel was treated by silver staining.

6.2. Results and Discussion

6.2.1. Preparation of AG Con A affinity adsorbents

The performance of affinity adsorbents depends on the preparation methods (e. g. the activator applied for the immobilization of the ligand and the ligand employed). When biomolecules such as proteins or enzymes are selected as affinity ligands, the immobilization method acquires particular importance, because the activity of the ligand can be affected by

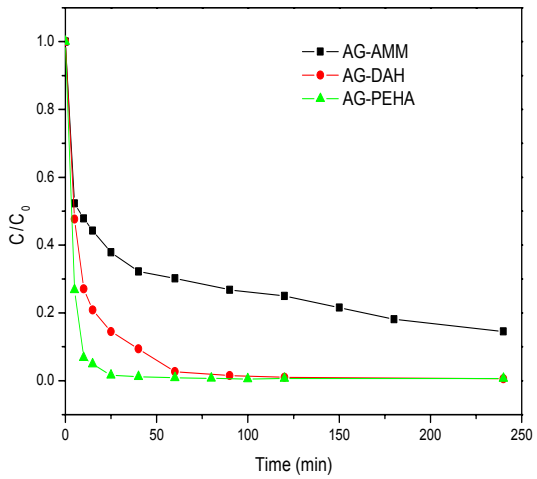


Fig. 6-2. Immobilization kinetics of Con A onto AG adsorbents with different spacer arms. The immobilization was carried out in 0.5 M phosphate buffer, containing 0.1 M NaCl, pH 8.0, at room temperature. 25 ml of 4 mg/ml Con A solution was mixed with 10 ml activated AG adsorbents.

its denaturation during the immobilization procedure. Many different techniques can be employed to couple proteins to functionalized supports. Glutaraldehyde is one of the widely

used crosslinkers, which reacts predominantly with free amino groups of the proteins, especially with the ε-amino group of lysine residues through the formation of Schiff base. In this work, the hydroxyl groups containing AG adsorbents were sequentially activated with epoxy reagent, amination reagent, and glutaraldehyde as shown in Fig. 6-1. To change the spacer length, three kinds of amination reagents with different molecular size were selected including ammonia (AMM), 1, 6-diaminohexane (DAH), and pentaethylenehexamine (PEHA). The immobilization of Con A onto glutaraldehyde activated AG adsorbents was carried out in 0.5 M phosphate buffer, pH 8.0, at room temperature. The immobilization kinetics of the activated adsorbents with different length of the spacer arms is shown in Fig. 6-2.

One can see that the longer the spacer of the adsorbent, the less time is needed to complete the immobilization. For example, the coupling yield could reach to 99% in 30 min, and in 90 min for AG-PEHA and AG-DAH, respectively. However, for AG-AMM with a shorter spacer the coupling yield was only 85% after 4 h reaction. The introduction of long spacer arms reduced the steric hindrance caused by the large volume of the adsorbent and thus significantly facilitated the attachment of Con A. Taking AG-PEHA adsorbents as an example, the immobilization isotherm of Con A was also investigated as shown in Fig. 6-3, which

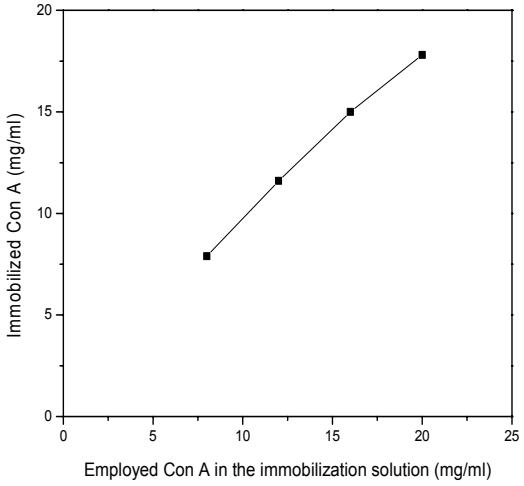


Fig. 6-3. Sketch of the immobilization isotherm of Con A onto AG-PEHA adsorbents. The immobilization was carried out in 0.5 M phosphate buffer, containing 0.1 M NaCl, pH 8.0, at room temperature.

indicates that the amount of immobilized Con A linearly increased with increasing the amount of employed Con A in the studied concentration range.

6.2.2. Adsorption/desorption kinetics of glucose oxidase (GOD) onto/from agarose coated glass (AG) Concanavalin A (Con A) adsorbents

The adsorption performance of the prepared AG Con A adsorbents was studied in terms of adsorption and desorption kinetics of GOD. To make the results more precise, AG Con A adsorbents with similar amounts of ligand density were employed. The adsorption kinetics of GOD onto AG Con A adsorbents with different spacer length is presented in Fig. 6-4. The

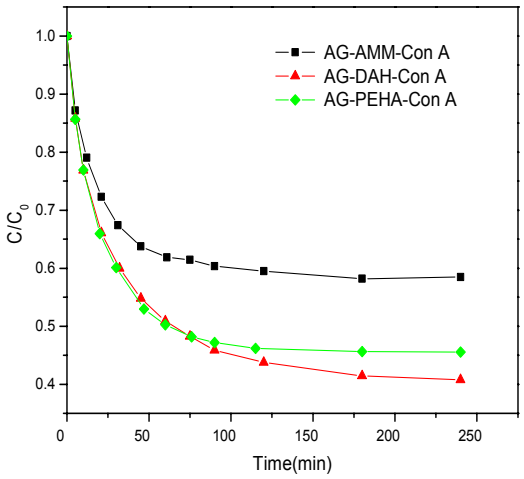


Fig. 6-4. Adsorption kinetics of GOD onto AG Con A adsorbents. 40 ml of 0.5 mg/ml GOD solution was mixed with 1.2 ml AG Con A adsorbents. The ligand density for AG-AMM-Con A, AG-DAH-Con A, and AG-PEHA-Con A was 7.5 mg/ml, 7.9 mg/ml, and 7.9 mg/ml, respectively.

adsorption of GOD onto AG-AMM-Con A and AG-PEHA-Con A reached the equilibrium in 2 h. However, that onto AG-DAH-Con A did not achieve the equilibrium even after 4 h. This could be related with the unspecific adsorption caused by the hydrophobic property of DAH. Although the molecular length of DAH is much shorter than that of PEHA, it contains a linear uninterrupted 6-carbon chain, which induces a considerably greater hydrophobic character than that of PEHA. Fig. 6-4 also indicates the adsorption efficiency of all AG Con A adsorbents. It is apparent that the residual GOD concentration was much higher for

AG-AMM-Con A than that for the other two even though the ligand density was nearly the same for all AG Con A adsorbents (see legend of Fig. 6-4). This result well elucidated the function of the spacer arms to improve the accessibility of the protein binding sites during the adsorption of biomolecules.

The GOD bound AG Con A adsorbents were washed thoroughly and mixed with a 0.1 M methyl-α-D mannopyranoside solution to study the desorption kinetics. The results are demonstrated in Fig. 6-5 by plotting the ratio of the adsorption capacity to the equilibrium adsorption capacity (ordinate) versus the desorption time (abscissa). AG-DAH-Con A and

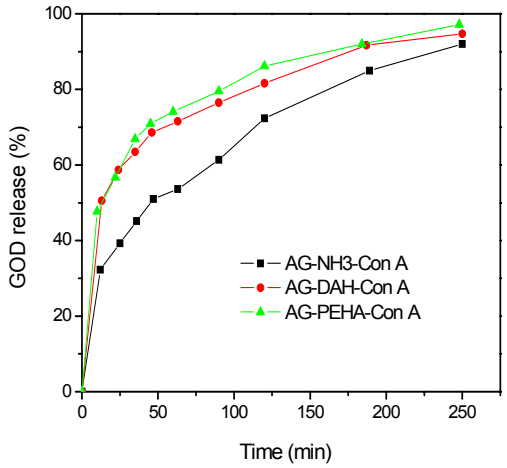


Fig. 6-5. Desorption kinetics of GOD from AG Con A adsorbents. 40 ml of 0.1 M methyl-α-D-mannopyranoside solution was applied to desorb the bound GOD from AG Con A adsorbents. The adsorbents employed are the same as described in Fig. 6-4.

AG-PEHA-Con A showed similar desorption kinetic behaviours, slightly faster than AG-AMM-Con A. Considering both the adsorption/desorption kinetics and the adsorption efficiency, AG-PEHA-Con A is the most promising adsorbent for the adsorption of GOD.

6.2.3. Adsorption isotherm of glucose oxidase (GOD) onto AG-PEHA-Con A

Due to the better adsorption and desorption kinetic behaviours and the high adsorption efficiency, AG-PEHA-Con A was employed for the further studies. The adsorption isotherm of GOD was first investigated and the result is given in Fig. 6-6. The experimental data were fitted with the Langmuir equation. It was found that the maximum adsorption capacity

Q_m= 18.3 mg/g or 30.0 mg/ml adsorbents, the dissociation constant K_d= 0.128 mg/ml or 8.00×10^{-7} M. When compared with the results obtained by non spacer adsorbents silica Con A and Toyopearl Con A as described in chapter 2, a higher Q_m and lower K_d values were obtained for AG-PEHA-Con A adsorbents. This indicates a higher affinity interaction

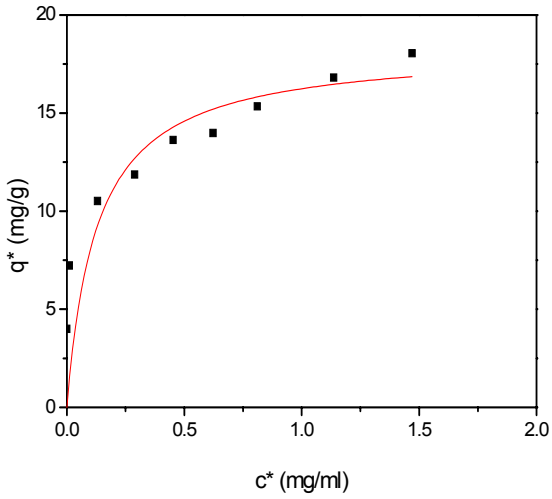


Fig. 6-6. The adsorption isotherm of GOD onto AG-PEHA-Con A adsorbents. The ligand density of Con A adsorbents was 10 mg/ml. The solid line was fitted by the Langmuir equation.

between the affinity ligand and the adsorbate, which is most probably caused by the introduced PEHA spacer arm enhancing the affinity significantly.

6.2.4. Expanded bed adsorption of glucose oxidase (GOD) and albumin from crude hen egg white solution

AG-PEHA-Con A adsorbents with a ligand density of 10.4 mg/ml were poured into a C10/20 column to give a settled bed height of 5.0 cm (3.9 ml). The flow velocity was kept to be 229 cm/h and the bed was expanded in the adsorption buffer with a 1.9-fold bed expansion. 0.5 mg/ml clear GOD solution and unclarified albumin from crude hen egg white solution were applied to AG-PEHA-Con A adsorbents as feed into the expanded bed process, respectively. During the albumin feedstock application a slight increase in the expanded bed height was observed due to the slightly higher viscosity of the loading solution than that of the

adsorption buffer.

Fig. 6-7 shows the chromatogram of the expanded bed adsorption of GOD and Table 6-2 gives the experimental details of the entire operation. The whole adsorption process of GOD including loading, washing, desorption and regeneration took about 4 h. When 75 mg GOD was applied onto AG-PEHA-Con A till a maximum adsorption reached, desorption in the expanded mode produced 25.3 mg GOD with a total yield of 33.7%. The adsorption capacity of AG-PEHA-Con A to GOD was 6.45 mg/ml adsorbents in the expanded bed mode. The expanded bed adsorption of GOD showed very good chromatographic performance, since the

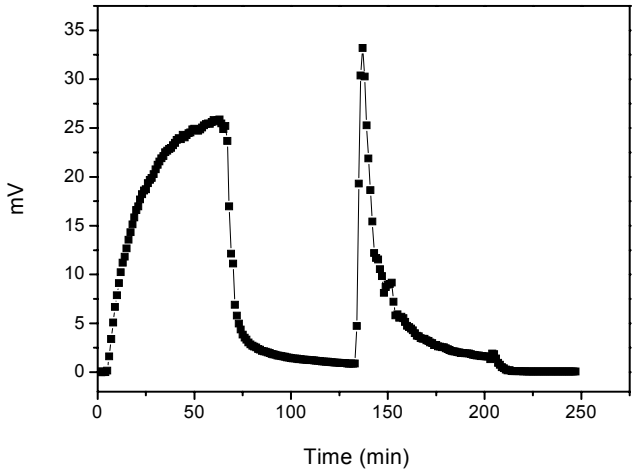


Fig. 6-7. Chromatogram of the expanded bed cycle of GOD. AG-PEHA-Con A was packed into a C10/20 column with a settled bed height of 5.0 cm. The flow rate was kept to be 229 cm/h to give a 1.9-fold bed expansion. Adsorption, washing, desorption and elution were conducted in the expanded mode.

adsorption capacity was 21.5% of the maximum adsorption capacity (30.0 mg/ml) obtained in batch experiments. During the desorption process, most bound GOD was desorbed from AG-PEHA-Con A in the first 30 min, with a high concentration. However, the complete desorption of GOD took about 1h. Because the purity of GOD was already high enough before the application, the enzyme activity assay showed only about 1-fold purification factor even for the first 30 min desorption fractions (data not shown).

Albumin is a key reference protein in biochemistry. The most widely used method for the purification of albumin from egg white is precipitation at a specific salt concentration, pH and temperature (Kekwick, 1936). Precipitation with ammonium sulfate or sodium sulfate give rise

to the separation of large quantities of albumin but lead to a product containing a high salt concentration and a further multiple step purification when pursuing high purity product. Here,

Table 6-2. Experimental details and results of expanded bed adsorption of GOD and crude albumin from hen egg white

Stage	Volume (ml)	Total protein (mg)	Yield (%)
GOD			
Feedstock	150	75	
Washing	170		
Elution	165	25.3	33.7
Albumin from hen egg white			
Feedstock	135	67.5	
Washing	150		
Elution	130	2.64	3.9

the expanded bed adsorption technique was applied to purify albumin from crude hen egg white solution. Fig. 6-8 presents the chromatogram of the expanded bed cycle of albumin from crude hen egg white solution. As listed in Table 6-2, 67.5 mg dissolved crude albumin

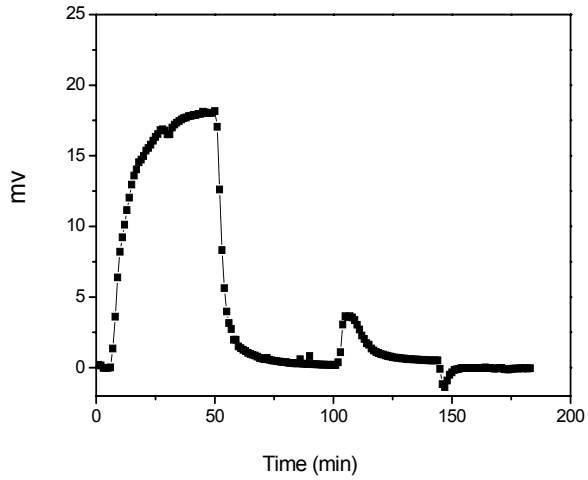


Fig. 6-8. Chromatogram of the expanded bed cycle of albumin from crude hen egg white solution. AG-PEHA-Con A was packed into a C10/20 column with a settled bed height of 5.0 cm. The flow rate was kept to be 229 cm/h to give a 1.9-fold bed expansion. Adsorption, washing, desorption and elution were conducted in the expanded mode.

was pumped into the column and 2.64 mg protein was obtained in the desorption fraction. Due to the high amount of the impurities the purification yield was found to be only 3.9%. However, the purity of albumin was highly improved as shown by SDS-PAGE results (Fig. 6-9). The crude albumin contained at least five main bands while purified albumin presented only two. The purity of albumin could be calculated by the colorimetric density method. Here, only two bands for purified albumin and five bands for crude albumin were considered. It was found that the purity of the albumin could be improved from 59% to

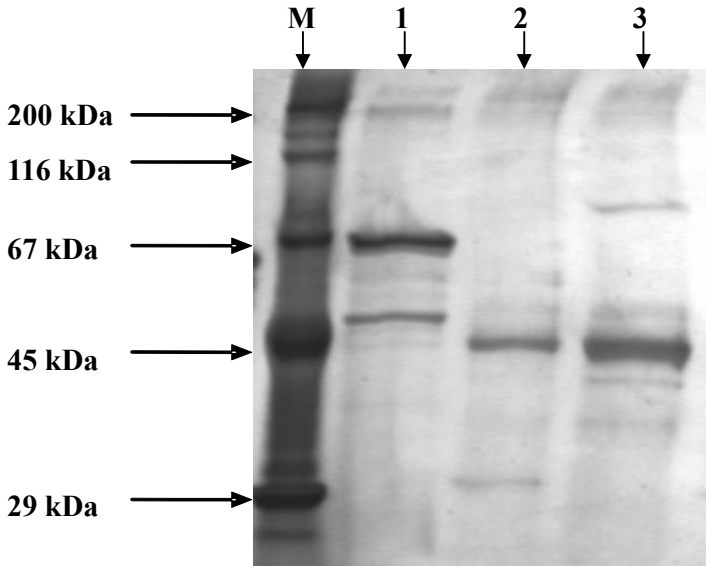


Fig. 6-9. SDS-PAGE of albumin from hen egg white purified by AG-PEHA-Con A in expanded bed mode. Lane M: marker proteins; lane 1: albumin from bovine; lane 2: purified albumin from hen egg white (by the described expanded bed procedure); lane 3: commercial albumin from hen egg white (as applied in the investigations).

86% in one single step purification by the expanded bed affinity separation process. It is worthy to be noted that the band with the molecular weight of approximate 28 kDa shown in purified albumin were not present in the crude albumin (lane 3). This band could be attributed to the leakage of Con A (26 kDa for each subunit) from the AG adsorbents since the ligand leakage was often observed in affinity separation, especially after a long time usage. The AG-PEHA-Con A adsorbents has been stored for 6 months before being applied to the separation of GOD and albumin. Ligand leakage could be prevented by freshly preparation of affinity adsorbent and selection of immobilization method for a long term stability (Cartellieri,

2000). Therefore, it is not likely that expanded bed processing of crude albumin could give rise to a highly purified product by affinity Con A ligand.

6.3. Conclusions

Pellicular agarose coated glass beads were activated subsequently by epoxy reagent, amination reagent and glutaraldehyde in order to immobilized Con A. The immobilization kinetics study showed that the longer spacer arm facilitated the immobilization of Con A. The immobilization isotherm of Con A onto AG-PEHA-glutaraldehyde revealed a linearly increasing amount of Con A in the studied concentration range. The prepared Con A AG adsorbents then were used to study their adsorption performance. First, the adsorption and desorption kinetics of GOD onto and from AG Con A adsorbents with different spacer arms were conducted. The results revealed that AG-PEHA-Con A was the most suitable adsorbent for the adsorption of GOD because it exhibited faster adsorption and desorption rates and a higher adsorption capacity when compared with the other two. The adsorption isotherms of GOD onto AG-PEHA-Con A fitted well to the Langmuir equation, with a maximum adsorption capacity Q_m= 30.0 mg/ml and a dissociation constant K_d= 8.00×10^{-7} M. This higher affinity interaction between Con A and GOD could be attributed to the spacer arm when compared with the results obtained by the adsorption of GOD on silica Con A or Toyopearl Con A without a spacer arm introduced into the adsorbent (Q_m= 4.9 mg/ml and K_d= 2.6×10^{-6} M for silica Con A; Q_m= 7.9 mg/ml and K_d= 4.6×10^{-7} M for Toyopearl Con A; see chapter 2). AG-PEHA-Con A adsorbents were packed in the column with a settled bed height of 5.0 cm for the expanded bed adsorption of GOD and crude albumin from hen egg white, respectively. The expanded bed process of GOD showed a very good chromatographic performance with adsorption capacity of 6.45 mg/ml, 21% of that obtained by batch adsorption. AG-PEHA-Con A was also employed for the purification of albumin from dissolved crude hen egg white powder. The SDS-PAGE proved that the purity of albumin was improved from 59% to 86% in one single step purification by the expanded bed affinity separation process.

7. Summary

Glycoconjugates are very important for intercellular communication and mediating cell-cell recognition. Affinity separation technique using lectins as ligands is the most powerful method for the separation and purification of glycoconjugates. This work concerns the fundamental aspects of affinity separation of glycoconjugates, in particular, glycoproteins. This study includes the adsorption behaviour of glucose oxidase (GOD) onto Concanavalin A (Con A) adsorbents, the preparation, characterization, and properties investigation of PEGylated Con A, the characterization of novel dense pellicular agarose coated glass beads and its application for expanded bed adsorption of proteins.

The jack bean lectin, Concanavalin A (Con A), and glucose oxidase (GOD) were selected to study the affinity interactions between the affinity ligand and glycoprotein. The immobilization of Con A was carried out onto both inorganic silica and polymeric Toyopearl supports. Immobilized Con A was employed for the adsorption of GOD. The influence of pH value and ionic strength on the adsorption of GOD was studied in order to find the optimal adsorption conditions. The factors dominated the affinity interaction were discussed in detail. The adsorption isotherms demonstrate that Toyopearl Con A possess much higher static adsorption capacity in comparison to silica Con A. However, because of the less diffusive mass transfer resistance, silica Con A showed faster adsorption and desorption kinetics. Both Con A adsorbents exhibit high affinity and low unspecific adsorption capacity to GOD. The dynamic adsorption of GOD revealed high stability of both immobilized Con A affinity adsorbents, further indicating their suitability for the application of affinity separations.

Since the proteinaceous ligand Con A is not very stable under some harsh conditions employed in affinity separation processes, modification of Con A with poly (ethylene glycol) (PEG) was carried out in order to improve its stability against these conditions. The PEGylation reaction was investigated in detail to elucidate how the parameters such as reaction time, pH value, molar ratio of mPEG-SPA (Monomethoxy poly (ethylene glycol) succinimidyl propionate) to Con A, and the molecular weight of mPEG-SPA affected the PEGylated degree. A new method, ninhydrin method, was first developed to determine the

PEGylated degree of immobilized Con A, which has proved to be powerful, fast and reproducible. The adsorption isotherms of GOD onto native and PEGylated Con A adsorbents show that the modification did not alter substantially the specificity of carbohydrate binding ability of Con A. However, the binding capacity for GOD was slightly reduced due to the steric hindrance caused by mPEG chains onto the ligands. The adsorption kinetic studies reveal a lower adsorption rate after PEGylation which was still attributed to the steric effect. The dynamic adsorption capacity for modified Con A depended very much on the PEGylated degree and the molecular weight of mPEG derivatives. The adsorption capacity could be highly preserved for TC-mPEG2k (Toyopearl Con A modified by mPEG-SPA with a molecular weight of 2000 g/mol) even with a PEGylated degree up to 20%. The conjugation of Con A with mPEG2k has shown a better adsorption performance thus has a greater potential for the application in affinity separation processes compared with mPEG5k. The PEGylated Con A exhibited much higher stability against the exposure to organic solvents and high temperature. The reason for the improved stability was discussed shortly. The fact that PEGylation stabilizes the properties of Con A may greatly expand the range of applications of unstable affinity ligand in bioseparation processes.

This work also combines affinity separation with expanded bed adsorption technique, which is particularly useful for particulate-containing feedstocks processing. A novel agarose coated glass (AG) matrix for expanded bed adsorption was characterized by its physical properties, bed expansion, and liquid phase dispersion behaviour. The high density and reduced interparticle diffusion distance of AG supports may facilitate protein adsorption performance in expanded bed processes. The bed expansion results of the AG matrix well fitted with the Richardson-Zaki model. The liquid phase dispersion in expanded bed was determined by the residence time distribution measurement. The dependency of the axial mixing in the expanded bed on flow velocity, settled bed height, and viscosity was examined. The novel AG matrices are promising for the expanded bed adsorption of proteins since it exhibited very low axial mixing in all investigations.

AG supports were then subsequently activated by epoxy reagent, amination reagent and glutaraldehyde in order to immobilize Con A. Studies showed that the longer spacer facilitated not only the fast immobilization of Con A but also the fast adsorption and

desorption of GOD. The adsorption isotherms of GOD onto AG-PEHA-Con A, the most suitable adsorbent for the adsorption of glycoprotein GOD, fitted well to the Langmuir equation, with a maximum adsorption capacity Q_m= 30.04 mg/ml and dissociation constant K_d= 8.00×10^{-7} M. AG-PEHA-Con A adsorbents were then employed for the expanded bed adsorption of GOD and crude albumin from hen egg white. The expanded bed separation of GOD proved a very good chromatographic performance with an adsorption capacity of 6.45 mg/ml, 21% of that obtained by static adsorption. AG-PEHA-Con A was also employed for the purification of dissolved albumin from crude hen egg white powder. The SDS-PAGE demonstrated that the purity of albumin could be improved from 59% to 86% in one single step purification by the expanded bed process. The combination of affinity separation with PEGylated proteinaceous ligand and expanded bed adsorption technique could provide great attraction for the industrial applications.

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Notations

b (b_1,b_2)	slope of plot of Con A adsorbents, in Eq. 3-1
Bo	Bodenstein number
c^*	equilibrium concentration of protein (mg/ml)
c_0	initial concentration of protein (mg/ml)
D_{ax}	axial dispersion coefficient (m^2/s)
d_l	ligand density (mg/ml)
d_p	adsorbent diameter (μm)
g	acceleration due to gravity (m/s^2)
H	height of expanded bed (cm)
H_0	height of packed bed (cm)
K_d	dissociation constant for affinity interaction (M)
m_0	total amount of protein employed for immobilization (mg)
m_B	weight of the empty pycnometer bottle (g)
$m_{B,S}$	whole weight of the pycnometer bottle with the supports (g)
$m_{B,S,W}$	whole weight of pycnometer bottle, supports, and water (g)
$m_{B,W}$	weight of the pycnometer bottle and water (g)
m_i	amount of immobilized protein (mg)
m_S	weight of the supports (g)
n	bed expansion index
N	number of the theoretical plates
q	adsorption capacity (mg/ml)
q^*	equilibrium adsorption capacity (mg/ml)
q^*_{de}	equilibrium desorption capacity (mg/ml)
q_{de}	transient desorption capacity (mg/ml)
Q_m (q_m)	maximum adsorption capacity (mg/ml)
Re	Reynold number
t	time (min)
U	liquid superficial velocity (cm/h)

U_t	terminal settling velocity (cm/h)
V	volume (ml)
V_{Ads}	volume of the adsorbents (ml)
V_{GOD}	volume of GOD solutions (ml)
V_S	volume of the supports (ml)
V_t	apparent volume of the supports (ml)
y	coupling yield (%)
ε_0	bed voidage in packed mode
μ	dynamic (absolute) viscosity of the fluid (Pa·s)
ρ	density of the liquid (g/cm^3)
ρ_s	density of the adsorbent (g/cm^3)
ρ_W	density of water (g/cm^3)
σ	the standard deviation
σ_θ^2	variance in time unit
τ	time constant
ε	bed voidage in expanded mode

Abbreviations

AC	affinity chromatography
ADH	alcohol dehydrogenase
AMM	ammonium hydroxide
CIP	cleaning-in-place
Con A	Concanavalin A
DAH	1, 6-diaminohexane
DC	displacement chromatography
EBA	expanded bed adsorption
ELISA	Enzyme-Linked Immunosorbent Assay
FAD	flavin adenine dinucleotide
GalNAc	N-acetylgalactosamine
GFC	gel filtration chromatography
GlcNAc	N-acetylglucosamine
GOD	glucose oxidase
HETP	height equivalent to a theoretical plate
HIC	hydrophobic interaction chromatography
IEC	ion exchange chromatography
IFN	Interferon-α
IMAC	immobilized metal affinity chromatography
LED	light-emitting diode
mPEG-SBA	mPEG-Succinimidyl Butanoate
mPEG-SPA	monomethoxy poly (ethylene glycol) succinimidyl propionate
mPEG-SS	mPEG succinimidyl succinate
mPEG-SSA	monomethoxy poly (ethylene glycol) succinimidyl succinate
MW	molecular weight
PC	perfusion chromatography
PD	PEGylated degree
PEHA	pentaethylenehexamine
POD	horseradish peroxidase
PVPAA	poly (vinylpyrrolidone-co-acrylic acid)
rhEGF	recombinant human epidermal growth factor
SEC	size-exclusion chromatography
TC	Toyopearl Con A

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Zhenzhen Wen, June 2006

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