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**ENHANCEMENT OF IMMUNOAFFINITY CHROMATOGRAPHY
PROCESSES USING POLYCLONAL ANTIBODIES AND HIGH
PRESSURE ELUTION**

by

Jay Richmond Howlett, B.A.Sc. (Chemical Engineering)

**A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree
of Master of Science.**

**Ottawa-Carleton Institute of Biology
Department of Biology
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Ottawa, Ontario
January 5, 1993
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The undersigned hereby recommend to the Faculty of Graduate Studies acceptance of the thesis, entitled, "Enhancement of immunoaffinity chromatography processes using polyclonal antibodies and high pressure elution" submitted by Jay Richmond Howlett, B.A.Sc. (Chemical Engineering), in partial fulfillment of the requirements for the degree of Master of Science.

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**This thesis is dedicated to my brother,
Guib.**

Abstract

Enhanced elution of polyclonal-based immunoaffinity complexes by high pressure manipulations were examined. Although antigen (product) elution using polyclonal antibodies often proves difficult, it was demonstrated that high pressures (2 kbar) may dissociate a polyclonal antibody - antigen complex. Using this feature of high pressure, a unique high pressure cell was custom designed and built to accomplish the recovery of protein products from immunoabsorbents. The results from this work demonstrate that moderate recoveries using a polyclonal anti-bovine serum albumin (BSA) - BSA antigen system were achieved at 1724 bar using pressure elution. In contrast, low recoveries were achieved using a polyclonal anti-bovine immunoglobulin G (IgG) - bovine IgG system under the same pressure elution conditions. These findings indicate the need to screen antigen-antibody systems being considered for immunoaffinity chromatography operations employing pressure elution. Fourier transform infrared spectroscopy has been demonstrated to be a potentially useful approach to accomplish this.

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List of Abbreviations

A_{404}	Absorbance at 404 nm
Å	angstrom
Bis-Tris	bis[2-hydroxyethyl]imino-tris[hydroxymethyl]-methane
BSA	bovine serum albumin
C	heat capacity
°C	degree centigrade
CDI	carbonyldiimidazole
cm	centimeter
cm ³	cubic centimeter
CNBr	cyanogen bromide
fAb	fraction of antibodies
FITC	fluorescein isothiocyanate
h	hour
HIAC	hyperbaric immunoaffinity chromatography
IAC	immunoaffinity chromatography
i.d.	inside diameter
IgG	immunoglobulin G
in.	inch
J	joule
kbar	kilobar
kg	kilogram
m	meter
MAb	monoclonal antibody
MC	methylene chloride
min	minute
Mpa	megapascal
µg	microgram
µl	microliter
µin	micro inch
µm	micrometer
mg	milligram
ml	milliliter
mm	millimeter

MWCO	molecular weight cutoff
nm	nanometer
o.d.	outside diameter
Pa	pascal
PBS	phosphate-buffered saline
PBST	PBS containing 0.05% Tween-20
psi	pounds per square inch
ρ	density
s	second
SDS	sodium dodecyl sulphate
w/v	weight per volume
WSC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

General Introduction

Considerable interest exists in acquiring high purity therapeutic and diagnostic products from blood plasma and genetically engineered organisms (Marshall, 1989; Sherwood, 1988; Spalding, 1991). In order for these compounds to be of commercial interest, they must be isolated from their source and purified to different degrees depending upon the requirements of their final application. One barrier that prevents the commercial exploitation of diagnostic and therapeutic products from biological sources is the separation and purification of the products on a large scale (Chase, 1984; Katoh, 1987). The large scale purification of bio-compounds to the degrees of purity necessary for use as pharmaceuticals presents a new challenge for the biotechnology industry. At present, downstream processing can account for up to 80% of the total production costs (Knight, 1989; Sii and Sadana, 1991).

In order for proteins to be active, most proteins must have a specific three-dimensional conformation in order to maintain their biological activity and specificity (Sii and Sadana, 1991). The characteristic structure of a protein may be adversely affected by physical and chemical changes in its surrounding environment. Even though some proteins that are exposed to extreme conditions (such as low pH or high temperatures) do not irreversibly lose their biological activity, the majority of proteins are only stable at dilute concentration in aqueous solution at physiological pH and temperature. Extreme care must be taken throughout the purification process of proteins, since the conditions encountered may result in an irreversible structural change, causing a loss of biological activity and corresponding commercial value.

Aside from the problems associated with stability, protein purification is normally complicated because the crude starting material contains numerous other compounds that are very similar to the one of interest. Compounds that are similar to the one of interest make the isolation of the desired product difficult because they may share similar physical and chemical properties. Characteristics such as size or charge are often used as a basis for determining a protein purification method. Therefore, different proteins that are comparable in size or charge may be difficult to separate using conventional techniques. With the exception of

some proteins derived from recombinant DNA technology, another difficulty in the purification of proteins is that the product of interest in the crude starting material may be present at low levels, typically less than 1 % of the total protein (Chase, 1984).

Traditional chemical engineering separation operations that have been adapted for the purification of low-molecular weight compounds such as antibiotics, are not well suited for the separation of proteins. The isolation of proteins on a laboratory scale has been carried out for many years, and the methods commonly used are summarized in Table 1. Some of these purification methods may be scaled-up for industrial application, however, few of the procedures are themselves capable of isolating a single protein from a complex mixture to a high degree of purity. Typically, the isolation of a single biological compound to a high level of purity requires a multi-step purification procedure that utilizes a combination of different separation techniques. Often, such a sequence of separation techniques results in a low overall yield and the desired product has to be isolated at the end of each separation step, which itself may be a slow and complicated procedure. In most cases the resulting scale-up of a laboratory purification procedure ultimately requires the successful scale-up of each stage and usually results in the fabrication of a sophisticated pilot plant that becomes dedicated to the isolation of one particular product.

One separation technique that may achieve a high degree of purification in a single step and that may be amenable to scale-up is affinity chromatography. Unlike other adsorption methods such as ion-exchange chromatography, affinity chromatography achieves a high degree of protein resolution. The use of affinity chromatography can lower the overall cost of downstream processing necessary to obtain a high purity product because this technique has the potential to replace a number of conventional separation steps (Ma and Inzana, 1992; Regnault et al., 1991). Affinity chromatography is a separation technique that exploits the specific interactions that occur between certain biological molecules. In general, one member of the recognition interaction (affinity ligand) is immobilized to an inert support or carrier, and is used as a specific adsorbent for the isolation of the corresponding component, the adsorbate.

Table 1. Common protein purification methods used in biochemistry laboratories (Chase, 1984)

Method	Resolution	Ease of Scale-up
1. Ultrafiltration	low	easy
2. Precipitation	low/medium	easy
3. Molecular exclusion	medium	medium
4. Electro-methods		
(a)Electrophoresis	medium/high	difficult
(b)Electrofocusing	medium/high	difficult
5. Adsorption methods		
(a)Adsorption chromatography	medium	medium
(b)Ion-exchange chromatography	medium	medium
(c)Affinity chromatography	high	medium

The operating principles of affinity chromatography are illustrated in Figure 1. The process begins with an initial adsorption stage in which a crude starting material, containing the desired product, is contacted with the specific adsorbent that has a binding affinity for the product. The product (adsorbate) then becomes bound to the ligand, and assuming that this interaction is sufficiently strong, all other impurities are washed away. Following the binding of the product and washing of the adsorbent, the product is eluted by altering the physical or chemical conditions of the loading and washing process. The change in conditions promotes dissociation or desorption of the product from the specific adsorbent. Following desorption, the adsorbent can be regenerated and re-equilibrated for subsequent use in another cycle of operation. Affinity adsorption has been shown to achieve excellent purification efficiency (up to 1000 fold in a single step) and may achieve recoveries as high as 90% under the appropriate conditions (Desia and Lyddiatt, 1990).

The choice of ligand for immobilization is of key importance towards successful development of an affinity separation process. The biological interaction of the affinity ligand should be specific to ensure that only the product of interest is adsorbed and sufficiently strong in order to wash away only the non-adsorbed compounds. Ideally, when considering large-scale use, the ligand should be readily and economically available and easily immobilized to a suitable inert support. Although there exists several examples of low cost, low molecular weight affinity ligands already in use for large-scale applications (Janson, 1984), such as textile dyes, there are certain situations in which their use may not be satisfactory. Such situations exist when a low molecular weight ligand with the desired affinities cannot be found, or when the interaction between ligand and product is not strong enough to withstand the extensive washing operations required to isolate the compound from the crude solution. In some cases, these small ligands may have affinities toward more than one compound, thus requiring a selective elution procedure to separate these compounds from one another. In order to overcome these difficulties associated with ligand selection, particular interest has been directed to the use of antibodies in affinity chromatography separations.

Figure 1.

The principal stages of affinity separations.

A suitable ligand is covalently attached to an insoluble matrix to form a specific adsorbent.

In the adsorption or affinity binding step, only those compounds having an affinity for the ligand bind to the adsorbent; compounds having no attraction towards the ligand pass through unaffected. In the elution or dissociation stage, the bound product (adsorbate) is removed from the adsorbent and collected.

Affinity chromatography employing antibodies as affinity ligands exploits the high affinity and specificity accompanying an antigen-antibody interaction. To date, immunopurification, a process in which antibodies are covalently linked to a solid support, has not yet been applied to its full potential (Chase, 1983; Knight, 1989). In theory, immobilized antibodies could be used in an affinity process to isolate and purify any compound against which a suitable antibody can be raised. However, immunoaffinity chromatography is often complicated by the fact that high affinity antibodies are necessary to achieve high adsorption efficiency from dilute solutions, while desorption of a biologically active antigen requires low affinity antibodies. For effective adsorption, antibodies with at least moderate affinity are generally chosen. But even moderate affinity antibodies often require rather harsh elution conditions, such as low pH or high concentrations of chaotropes, usually resulting in some denaturation of the antigen and reducing the affinity of the immobilized antibody. Maintaining antibody affinity is not a trivial matter since the production costs associated with the large quantities of antibodies necessary for immunoadsorbent preparation are quite high. Some immunoaffinity processes use as much as one milligram of immobilized antibody for every one microgram of purified product (Knight, 1989). Therefore, any loss in antibody binding activity during a cycle of operation not only reduces product yield, but also results in a loss of capital investment because of the costs associated with antibody replacement.

Due to the difficulties in maintaining antibody integrity through many cyclic operations and the expense of their replacement, the application of immobilized antibodies on process scales has been limited. Immobilized antibodies have found particular application in affinity separations where it has not been possible to arrange a more conventional affinity adsorbent. Employing immobilized antibodies has been justified in cases where a molecule with a suitable specific affinity does not exist or an appropriate ligand is difficult to obtain in sufficient quantities for adsorbent preparation. For these reasons, processes employing immobilized antibodies are only appropriate for the purification of high market value compounds that cannot be isolated by more traditional and less expensive techniques. Hence, immunoadsorption has been used for the purification

of pharmaceutical proteins such as human interferon (Chase, 1987) and Factor VIII (Marshall, 1989), peptide hormones, vaccines, and antibodies (Chase, 1984). In order for immunoadsorption to become a more competitive and efficient protein purification process, it is necessary to establish a mild elution technique for the stage of product recovery. Therefore, there is a need for an enabling technology that permits antigen-antibody dissociation, yielding a pure and active product, without adversely affecting the binding ability of the immobilized antibody.

One technique that has been investigated to accomplish antigen-antibody dissociation while maintaining affinity ligand activity for extended periods of time is the use of high pressure (Olson et al., 1989). The feasibility of this approach is indicated by several experimental observations. A number of oligomeric proteins have been shown to dissociate with the application of high pressure (King and Weber, 1987). Furthermore, Tsuji and his coworkers (Tsuji et al., 1966) observed that high pressures have a retarding effect on the rate of inactivation of a luciferase enzyme by anti-luciferase antibodies. It is generally believed that monomeric proteins do not begin to unfold until they are subjected to pressures above 5 kbar (Heremans, 1982). Since oligomeric proteins have been observed to dissociate at lower pressures (1 kbar) (Weber and Drickamer, 1983), it appears that pressures in the range of 1-5 kbar may potentially be used to promote dissociation of most non-covalent antigen-antibody complexes.

In examining the effects of high pressure on proteins, the molecular covalent architecture may be considered unchangeable within the range of pressures at which water remains in a liquid state at ambient temperatures (pressures inferior to 12 kbar). The source of significant equilibrium displacements under high hydrostatic pressure occurs from those reactions that involve differences in the intermolecular forces of the solvated products and reactants (Weber and Drickamer, 1983). For instance, the interaction of water with ionic charges provides one of the most substantial causes of volume decrease (the packing of water dipoles about ionic charges is referred to as electrostriction). Most strongly influenced by pressure are those reactions in which the reactants and

products differ in the number of ionizable groups. Therefore, protolytic reactions that take place with the introduction of two ionizable groups are strongly dependent upon pressure (e.g. $-\text{COOH} \rightleftharpoons -\text{COO}^- + \text{H}^+$). In contrast, reactions that proceed with no change in the number of ionizable groups are the most pressure insensitive (e.g. $-\text{NH}_3^+ \rightleftharpoons -\text{NH}_2 + \text{H}^+$). Most observations concerning the effects of high pressure on single-chained proteins have been noted at neutral pH where the carboxyl and amino groups are fully charged. Non-ionized species such as OH and SH are not appreciably affected by pressure since their pK values are far from neutrality. Therefore it may be concluded that changes in the state of ionization are not the most influential factor of the instability characteristic of globular proteins under high pressure.

To establish the effects of high pressure on single-chain proteins, most studies have employed a spectroscopic technique as the method of choice, specifically either absorbance or fluorescence spectroscopy. With few exceptions, observed changes in emission intensity and those in the spectral distribution of absorption and emission under pressure suggest that appreciable conformational changes in a single peptide chain requires pressures of several kilobars, 5 kbar and higher. Also, in most cases, the observed spectral changes are rapidly reversed upon pressure release. These findings have led to the belief that at high hydrostatic pressures, a large conformational change must occur. This phenomenon has been termed the 'pressure denaturation' of proteins.

Aside from the information obtained as to the range of the pressures involved, further information concerning the nature of the changes undergone by the protein under high pressure may be revealed from fluorescence spectroscopic studies. It is well established that in fluorophores, absorption and emission shift to the red portion of the spectrum as the polarity of the medium increases (Freifelder, 1982). Furthermore, it has been found that the polarity of the tryptophan environment correlates well with the energy of the fluorescence emission. Under conditions of high pressure, it is generally observed that spectral changes in intensity of the tryptophan residues in proteins are associated with a shift of the emission to longer wavelengths. This observation

suggests that under high pressure, the local environment of the tryptophan is replaced by one of considerably greater polarity (Weber, 1987). An interpretation of these results is that at high pressure water penetrates into the interior of the protein, allowing the water molecules to be found at distances such that strong interaction with the field of the dipole fluorophore is permitted. Thus the penetration of water molecules into the interior of the protein leads to the unfolding of a single-chain polypeptide.

The instability of a single-chain protein under high pressure is believed to arise from the imperfect packing of the amino-acid residues that occur in the native structure; the covalent architecture of the protein imposes restrictions on residue packing because of the constraints set by the established bond lengths and bond angles (Weber and Drickamer, 1983). Even under conditions of perfect packing, approximately 25% of the total volume is not occupied by protein atoms - the majority of this space is in the form of very small cavities (Chothia, 1984). Upon unfolding, the imperfect packing defects disappear and a decrease in apparent molar volume of protein in water is often observed. Such an observation is expected since the compact conformation assumed by a single-chain protein in its native folded state occupies a larger volume than one that is completely unfolded. In an unfolded state, the internal chain contacts are replaced by contacts with solvent, and the volume of the system, protein and solvent, decreases.

Empirically, the indefinite aggregates of actin, tubulin, or myosin as well as oligomeric proteins consisting of two or more subunits have been shown to undergo dissociation under high pressure (King and Weber, 1986; Paladini and Weber, 1981; Silva et al., 1986; Weber and Drickamer, 1983). The reduction in volume with increasing hydrostatic pressure can be the driving force for the dissociation of protein aggregates. One may attribute the general dissociating effects of pressure to the very same causes that produce the unfolding of a protein consisting of a single peptide chain: the imperfect packing of the atoms at the subunit interface and the restrictive effect of the covalent architecture of the proteins. Other causes may also be listed. As well as contributing to a decrease in system volume, subunit dissociation may also result from the replacement of interactions between non-polar groups located on each subunit interface by interaction of each

with the water dipoles. A third influence, and a very important one, arises from the existence of salt bridges occurring between subunit boundaries (Weber, 1987). Upon subunit dissociation, the charges at the newly formed surfaces undergo hydration and subsequently, a decrease in system volume is observed due to electrostriction.

The general finding with protein-protein complexes is that they possess positive reaction volumes upon association. For example, the interaction between chymotrypsin and trypsin inhibitor produces a volume change of +80 ml/mol (Heremans, 1982). Ohta et al. (1970) reported a positive volume change of 230 ml/mol antibody accompanying an antigen-antibody reaction involving a synthetic polypeptide. The dissociation of oligomeric proteins under high pressure proceed with a negative change in volume. In the case of an antigen-antibody system, there are only two proteins involved in dissociation. Thus far, the only dimer to be studied under high pressure has been yeast enolase (Paladini and Weber, 1981). The decrease in standard volume upon dissociation was found to be approximately 25-30 ml/mol at ambient pressure, increasing to 60 ml/mol at a pressure of 1 kbar.

The observed volume changes for the dimeric system as well as other oligomeric protein systems were calculated at pressure ranges inferior to 2 kbar. It is believed that within this pressure range the dissociation will proceed without further unfolding of the monomers. Moreover, it is important to note that all observations thus far report that oligomeric proteins dissociate under high hydrostatic pressure (Weber and Drickamer, 1983; Weber 1987).

Since it is conceivable that the relative importance of each of the dissociating effects described may vary from one protein aggregate system to the next, it is important to ascertain whether or not antigen-antibody complexes do indeed behave similarly to the oligomeric protein systems already discussed. The dissociation of protein aggregates under high pressure is dependent upon the types of non-covalent forces involved in the protein-protein association. This being the case, then high pressure may potentially be used to dissociate antigen-antibody complexes; the types of

non-covalent interactions that promote the binding of an antigen to an antibody are shared with other oligomeric protein systems. These include hydrophobic interactions, hydrogen bonding, as well as electrostatic and Van der Waals forces (Roitt et al., 1985). Although the types of interactions that exist between antigen-antibody complexes and other oligomeric protein systems are similar, significant differences in the distribution of the forces at the protein-protein interface and in the size of the area of interaction are apparent. In general, protein dimer formation has been observed to involve an extensive area of interaction, ranging from 700 Å² to more than 4000 Å² (Davies et al., 1990). The surface area of antigen-antibody interaction occupies the lower section of this range. The relatively small interfacial area that exists between an antigen and its associated antibody does not impair the antibody's nature of specificity. The extraordinary specificities of antibodies are thought to arise from the complementary shape of the interacting surfaces (upon association, the side chains of the antigen and antibody interact so that even water molecules are excluded). The antibody combining site is distinct from other oligomeric protein interfaces in that nearly half the contact residues are aromatic, particularly tyrosines (Davies et al, 1990; Janin and Chothia, 1990). The abundance of aromatics found in the recognition sites in antibodies allow for increased solvent exposure, making them available for ligand interaction.

The interfaces in oligomeric proteins, especially in those which possess large contact areas, are usually more hydrophobic than the interfaces in antigen-antibody complexes. In addition, they also involve fewer hydrogen bonds. Janin and Chothia (1990) reviewed the structures of four antibody-protein antigen complexes and found that the complexes contained 10-13 hydrogen bonds as well as numerous Van der Waals interactions between the two contacting surfaces. The strong hydrogen bonding that exists between an antibody and its antigen is another factor that makes the interaction specific. In addition to direct hydrogen bonds, several polar atoms were observed to interact across the interface through bridging water molecules. Another finding was that half the complexes surveyed were found to involve one charged group, usually buried in the recognition sites.

Since hydrogen bonding increases with increasing pressure, the existence of strong hydrogen bonding in an antigen-antibody complex would seem to be a deterrent to pressure induced dissociation. If hydrogen bonding was the dominant force in antigen-antibody association, then high pressure would not be effective in promoting dissociation of the complex. It is established that hydrogen bonding plays an important role in protein-protein interaction, however there is reason to believe that electrostatic effects may dominate many aspects of protein behaviour (Perutz, 1978). Ismail and Wong (1992) examined the effect of increasing hydrostatic pressure on salt-bridge rupture and aggregate dissociation of homopolypeptides by pressure tuning Fourier transform infrared spectroscopy. A model aggregate system consisting of poly-L-lysine and poly-L-glutamate was found to associate with the formation of salt-bridges accompanied by formation of an extensive intermolecular hydrogen-bonded network involving the backbone amide groups. It was demonstrated that increasing pressure resulted in dissociation of the homopolypeptides and that water was the driving force in the separation of the peptides. In addition, one may speculate that salt bridges play a crucial role in stabilizing protein-protein association and that the hydrogen bonds formed are oriented as a result of the salt-bridge formation. The existence and importance of salt bridges between stable protein aggregates may be derived from the observation that extremes of pH, as well as large increases in the ionic strength of the solvent, result in the dissociation of many oligomeric proteins. Consequently, antigen-antibody complexes that involve the formation of salt-bridges upon association are likely to be susceptible to dissociation by the application of high pressure.

Olson et al. (1989) demonstrated the use of high pressure to dissociate bovine serum albumin (BSA) from an immunoadsorbent containing murine anti-BSA monoclonal antibodies. They found that pressures in the range of 0.5 kbar to 2 kbar were sufficient to promote the dissociation of the antigen-antibody complex, however upon pressure release, reassociation occurred. In order to remove the antigen from the antibody's recognition site and prevent any reassociation upon pressure release, Olson and coworkers employed electrophoretic elution along with high pressure conditions. In so doing, the dissociated antigen was removed from the

vicinity of the affinity ligand's binding site and hence, the model product was successfully isolated.

It is believed that the method developed by Olson and coworkers may be significantly improved upon by modifying two different features of the process operation and thus forms the basis of this thesis. One modification would be the substitution of a different affinity ligand preparation. Regarding the type of affinity ligand employed, the majority of reported uses of immobilized antibodies in protein purification have involved the use of polyclonal antibodies. Polyclonal antibodies have a range of binding affinities towards a number of different antigenic determinants and these ranges may vary in different antibody preparations. These properties often make the performance of procedures involving polyclonal antibodies difficult to analyze and in some cases non-reproducible. To some extent, the use of monoclonal antibodies may overcome the difficulties that polyclonal antibodies present. Monoclonal antibodies have a unique binding affinity for a single antigenic determinant site, and this feature gives them the capability of being highly specific in their recognition ability. Moreover, they are commercially available and may be potentially produced in large quantities by growing hybridoma cells using in-vitro cell culture techniques. Although monoclonal antibodies appear well suited for use in immunoadsorbent preparation, polyclonal antibodies have several attractive features. Polyclonal antibodies are less expensive than monoclonal antibodies and in most cases, they are more readily available in sufficient quantities for constructing an affinity adsorbent. In terms of specificity, a population of polyclonal antibodies recognizes several different antigenic determinant sites and some speculate that this attribute may lead to the binding of protein antigens other than the one of interest. However, it is often the case (eg. surface binding in ELISA protocols), that more than one monoclonal antibody is required to detect a protein antigen because the binding site is masked due to steric hindrance. Also, monoclonal antibodies have been known to bind to the same antigenic determinant site located on two different proteins (Goding, 1986; Henning and Nielsen, 1992; Tijssen, 1985). Another point that favours the use of polyclonal antibodies in affinity separations is that monoclonal antibodies are the product of a cancerous cell line. The implications on their origin may preclude their

use in affinity separations where human pharmaceuticals and therapeutic compounds are involved. Restrictions on using monoclonal antibodies for such processing have yet to be fully explored.

The second modification to the high pressure separation process developed by Olson et al. (1989) was to develop an alternative method that would remove the antigen from the vicinity of the affinity ligand's binding site. The method employing electrophoretic elution requires several cycles of operation to achieve good separation efficiency. It is thought that a more suitable separation method could be employed to make the high pressure process more efficient and more amenable to scale-up.

This thesis is a technology project encompassing several disciplines: chemical engineering, mechanical engineering, and biology. The purpose of the project was to improve upon the high pressure separation process initially developed by Olson et al. (1989) through two modifications, as discussed earlier. The dissociation of an antigen-antibody complex by means of high pressure was examined for its potential application to immunoaffinity chromatography. A system consisting of BSA (bovine serum albumin), a model antigen, and an anti-BSA IgG (immunoglobulin G) were used in this study. Chapter 1 examines the effects of high pressure on the model antigen and antibody in order to determine the implications on their use for high pressure immunoaffinity chromatography. In Chapter 2, a suitable design method for immunoadsorbent preparation that accommodates a high pressure environment is described. After demonstrating that the application of high pressure is capable of dissociating the model antigen-antibody complex (Chapter 3), attention is then focused on methods to accomplish antigen separation and isolation. Chapter 4 describes a unique High Pressure Cell that was custom designed and built to complement high pressure antigen-antibody dissociation with successful isolation of antigen, the model product. This High Pressure Cell accommodates the design of a model immunoaffinity chromatography column and the unique feature of fluid displacement under high pressure conditions.

Chapter 1

Pressure Induced Conformational Changes in an Antigen and an Antibody and the Implications on Their Use for Hyperbaric Immunoaffinity Chromatography

Introduction

If high pressure is to be used successfully as an alternative separation technique in purification processes based on immunoaffinity chromatography, then it must be demonstrated that this technique does not disturb the biological activity of the antibody or the antigen. In the case of an immobilized antibody, it is essential that the antigen binding ability of the antibody is not disturbed so that it may be used in several cycles of operation. Also, upon the one time separation of antigen from the antibody, it is important that the antigen retain its biological activity for it to be of any therapeutic or commercial value.

Several methods are available that may be used to study the effects of high pressure on proteins. Fluorescence intensity and polarization measurements have been demonstrated to be useful techniques in the study of the effects of high pressure on protein denaturation and dissociation of oligomeric proteins (Li et al., 1976a; Li et al., 1976b; Paladini and Weber, 1981). Fourier transform infrared (FT-IR) spectroscopy has been successfully employed in the detection of pressure induced changes in protein secondary structure (Wong and Heremans, 1988; Wong and Mantsch, 1989) [for a review on protein conformation by infrared spectroscopy see Byler and Susi, 1986; and Yang et al., 1985]. The ELISA (enzyme linked immunosorbent assay) test has been used to evaluate the chemical and thermal denaturation of proteins as judged by the loss of antibody binding capacity (Lombardero et al., 1990). In addition, Olson and coworkers (Olson et al., 1989) have demonstrated the use of a radioimmunoassay test to determine the binding affinity of immobilized antibodies that were subjected to several cycles of high pressure.

In this chapter, the practicality of employing high pressure as a separation technique in immunoaffinity chromatography is assessed by examining pressure induced conformational changes. Specifically, the techniques of fluorescence, FT-IR spectroscopy, and ELISA are used in parallel to investigate the effect of hydrostatic pressure on the secondary and tertiary structure of two immunological proteins, bovine serum albumin, and immunoglobulin G. Using a fluorescent probe, changes in

fluorescence intensity resulting from high pressure conditions are examined. FT-IR spectroscopy is used to determine changes in protein secondary structure, while ELISA is used to investigate any tertiary structural changes that may occur as a result of the immunological proteins being exposed to a high pressure environment.

Materials and Methods

Materials

The following reagents were purchased from Sigma Chemical Co: bovine serum albumin (BSA) (A-7030), bovine serum albumin conjugated with fluorescein isothiocyanate (BSA-FITC) (B-9771), bovine IgG (I-5506), goat anti-rabbit IgG serum antibody alkaline phosphatase conjugate (A-8025), goat anti-mouse IgG serum antibody alkaline phosphatase conjugate (A-5153), rabbit anti-bovine IgG antibody fluorescein isothiocyanate conjugate (IgG-FITC) (A-9771), *p*-nitrophenyl phosphate (104-0), and Bis-Tris (bis[2-hydroxyethyl]imino-tris[hydroxymethyl]-methane) (B-9754). Deuterium oxide ($^2\text{H}_2\text{O}$) was purchased from MDS Isotopes, Merck Frost, Canada. Anti-BSA monoclonal antibody (MAb) 9.1 (Morel et al., 1988) was obtained as a gift from Dr. M. Yarmush (Rutgers University, USA). Thirty-thousand-molecular-weight cutoff (MWCO) microconcentrators (Centricon-30) were obtained from Amicon, USA. Polyester nonwoven cloth was purchased from Dupont (Sontara 8100).

Solution Preparation

Protein solutions for experiments were prepared immediately prior to sample pressurization. In a typical pressure experiment, a 4 ml solution of either 10 $\mu\text{g}/\text{ml}$ BSA, 10 $\mu\text{g}/\text{ml}$ BSA-FITC, or rabbit anti-bovine IgG-FITC (appropriately diluted) in 0.15 M Bis-Tris (pH 7.3) was prepared. In some cases, protein solutions were prepared in unbuffered $^2\text{H}_2\text{O}$. The solution was then divided into two samples, one was placed in the pressure vessel and the other was kept at ambient pressure for comparison. The pressure in the chamber was increased to the desired pressure at a rate of approximately 550 bar/min, held at the desired pressure (typically for 1 hour), and was returned to atmospheric pressure at a rate of 350 bar/min at the end of each experiment (unless otherwise indicated). All

pressurization experiments were conducted at room temperature. Samples used as controls at ambient pressure conditions were kept in the dark.

Spectrofluorometry

Emission was measured in a Farrand spectrofluorometer using a excitation wavelength of 490 nm and an emission wavelength of 525 nm. Fluorescence intensities of pressure treated samples were normalized (to obtain a relative intensity) with respect to the fluorescence intensity of the sample at ambient pressure (1 bar).

FT-IR/ High Pressure FT-IR Spectroscopy

Protein solutions of (1 % w/v) were prepared in unbuffered $^2\text{H}_2\text{O}$ (unless otherwise indicated). Each protein solution was freshly prepared and stored at 4°C overnight prior to the infrared measurements. Infrared spectra at ambient pressure and temperature were recorded on a Digilab FTS-60 Fourier-transform spectrophotometer equipped with a liquid-nitrogen-cooled mercury-cadmium-telluride detector. A total of 512 scans at 4 cm^{-1} resolution were co-added for each spectrum. Protein solutions were placed in a CaF_2 cell with a 50 mm Teflon spacer.

For high pressure FT-IR work, the prepared protein solution (10 ml), together with a few crystals of powdered α -quartz, were placed at room temperature in a 0.37 mm diameter pin hole, punched in a 0.23 mm thick stainless steel gasket. The entire apparatus was mounted into a custom-built diamond anvil cell, and the infrared beam was condensed by a sodium chloride lens onto the sample in the pin hole of the diamond anvil cell. The α -quartz was used as an internal pressure calibrator (absorbance of quartz may be used to monitor hydrostatic pressure) (Wong et al., 1985). On-line infrared spectra of the protein samples were measured at room temperature on a Digilab FTS-60 Fourier transform spectrophotometer, equipped with a mercury-cadmium-telluride detector. For each pressure, 512 interferograms were co-added and Fourier transformed to yield infrared spectra with a resolution of 4 cm^{-1} and encoding intervals of 2 cm^{-1} . Data reduction was performed with software developed by Doug Moffat (SIMS, NRC, Ottawa Canada).

Enzyme linked immunosorbent assay (ELISA)

In a 96-well polystyrene microtiter plate (Nunc 2-69620, Denmark), 100 μ l/well of either 10 μ g/ml BSA, 10 μ g/ml BSA-FITC, or serum diluted rabbit anti-bovine IgG-FITC in 0.15 M Bis-Tris was left to stand for 3 hours at 37°C or overnight at room temperature (23°C). The wells were then washed five times with 0.01 M phosphate buffered (pH 7.3)-0.85% NaCl (PBS) containing 0.05% Tween 20 (PBST). BSA and BSA-FITC coated wells were then incubated with 100 μ l of 6 μ g/ml anti-BSA MAb 9.1 in PBST for 1 hour at room temperature. The plate was then washed as above, and incubated with 100 μ l/well of either anti-mouse or anti-rabbit IgG-alkaline phosphatase conjugate diluted 1:1000 in PBST for 1 hour at room temperature. After washing with PBST, reactions were revealed by addition to each well of 100 μ l of 0.39 % (w/v) *p*-nitrophenyl phosphate in a mixture of 10 % (w/v) diethanolamine and 0.5 mM MgCl_2 (pH 9.8). After 30 min at room temperature, the signals (absorbance at 404 nm) were determined using a Titertek Multiskan plate reader.

ELISA using a hydrophobic cloth as an adsorbent was carried out according to Blais and Yamazaki (1989). Fifty microliters of rabbit anti-bovine IgG-FITC (diluted 1:2000 in 0.15 M Bis-Tris) was added to a cloth segment (6 mm square) placed in a petri dish. After incubation overnight at room temperature, the cloth was washed 5 times with a total volume of 5 ml of PBST. Fifty microliters of anti-rabbit IgG-alkaline phosphatase conjugate (diluted 1:1000 in PBST) was added to the IgG coated cloth for 1 h at room temperature. After incubation, the segment was washed as described above, and incubated with 500 μ l of 0.39 % (w/v) *p*-nitrophenyl phosphate in a mixture of 10 % (w/v) diethanolamine and 0.5 mM MgCl_2 (pH 9.8). After 30 min at room temperature, the reaction was stopped by adding 500 μ l of 0.5 M sodium phosphate (pH 9.5) and the absorbance at 404 nm was determined.

Pressure Vessel

A cylinder and piston type arrangement was used for high pressure experiments (Figure 2). Two 0.375 in (9.5 mm) pistons with viton O-ring seals pressurize a 3 cm^3 column of liquid by means of intensifying the upper piston with a hydraulic laboratory press (using the press made the

Figure 2. Cross-sectional diagram of pressure vessel and pressure measurement system : (A) Intensifier piston with O-ring seal and bleed hole, (B) stainless steel liner, (C) sample chamber, (D) high strength steel back-up cylinder, (E) load cell, (F) hydraulic press, (G) power supply, and (H) chart recorder.

process of vessel compression non-linear with time, however decompression was performed at a linear rate). The upper piston contained a bleed hole to remove any air trapped in the system, allowing the pressurized chamber to be completely filled with fluid. A high strength stainless steel sleeve was used to separate the pressurized fluid from a thick-walled backup cylinder.

The force exerted on the column of water was measured on-line using a calibrated load cell. By knowing the force exerted on the press (as measured by the load cell), the fluid pressure may be calculated since the pressurizing piston has a fixed area (the change in the diameter of the pressure vessel at the maximum operating pressure, 2200 bar, alters the system pressure by less than 0.08 %). On-line measurements also allow the monitoring of pressurization and depressurization rates. The load cell was calibrated using a method of dead weight calibration (NAE, NRC). The maximum operating pressure of the system was 2200 bar. A more complete description of the pressure vessel design and heat transfer considerations are given in the Appendix to Chapter 1.

Results

Fluorescence

Fluorescence emission can be used to detect changes in solution properties of proteins at micromolar concentrations. Table 2 shows the change in fluorescence intensity of FITC labelled IgG and BSA in 0.15 M Bis-Tris buffer after pressurization cycles of 1 h. In the case of FITC labelled IgG, pressure treatment at 1013 or 2026 bar did not notably alter the fluorescence intensity of the conjugate, as the measured relative fluorescence intensities are close to unity. However, pressurizing a solution of FITC labelled BSA in the same buffer at 2026 bar for the same time duration resulted in an appreciable decrease in fluorescence intensity (an averaged decrease of 35%).

The results of exposing BSA-FITC (dissolved in $^2\text{H}_2\text{O}$) to different pressures for a duration of 1 h are shown in Figure 3. Under conditions of increasing hydrostatic pressure, the fluorescence intensity is observed to

decrease up to a pressure of 1013 bar, then plateau and remain constant from this pressure to 2026 bar. Relative fluorescence intensity data of IgG-FITC is also plotted on Figure 2 for comparison and reveals that FITC labelled BSA is much more sensitive to pressure treatment than IgG-FITC.

A solution of 1% (w/v) BSA-FITC dissolved in $^2\text{H}_2\text{O}$ was pressurized for 1 hour at 2026 bar. The pressurized solution and the ambient control were diluted to a concentration of 10 $\mu\text{g/ml}$ and the fluorescence intensity was measured as described in Methods. The samples were stored in the dark at 4°C , and the fluorescence intensity was periodically measured over a period of 16 days. The results in terms of relative fluorescence intensity are shown in Table 3. Immediately after the 1 hour pressure cycle at 2026 bar, the fluorescence intensity of BSA-FITC decreased by 20% when compared to the sample held at ambient pressure. Over time, a recovery in intensity was observed, and after 4 days the recovery was almost completed. After 16 days, the measured difference in relative fluorescence intensity was 6%.

Table 2. The effect of pressure on fluorescence intensity of IgG-FITC and BSA-FITC after a pressurization cycle of one hour^a

Pressure (bar)	Sample type	Dilution ^b or concentration	Relative fluorescence intensity
1013	IgG-FITC	1:2000	1.07
1013	IgG-FITC	1:2000	1.01
2026	IgG-FITC	1:2000	1.10
2026	IgG-FITC	1:2000	1.05
2026	IgG-FITC	1:1800*	1.00
2026	BSA-FITC	10µg/ml	0.63
2026	BSA-FITC	10µg/ml	0.67

^aAfter depressurization, the fluorescence intensity was measured as described in Methods. Intensities were normalized with respect to the fluorescence intensity of the IgG-FITC or BSA-FITC sample at ambient pressure.

^bAnti-bovine rabbit IgG was diluted from serum into 0.15 M Bis-Tris (pH 7.3) and pressurized for 1 h.

*Sample concentrated, washed with ²H₂O, lyophilized, and resuspended in ²H₂O to obtain infrared spectrum.

Figure 3. Pressure dependence of the relative fluorescence intensity of 10 $\mu\text{g/ml}$ BSA-FITC (●) and IgG-FITC (●) diluted 1:2000 from serum. BSA-FITC was dissolved in unbuffered $^2\text{H}_2\text{O}$. IgG-FITC was dissolved in H_2O and buffered with 0.15 M Bis-Tris, pH 7.3. All solutions at room temperature (23°C). Vertical bars denote S.D. of the mean for 3 separate determinations.

Table 3. The effect of recovery on fluorescence intensity of 1% BSA-FITC in $^2\text{H}_2\text{O}$ after a pressurization cycle of one hour at 2026 bar^a

Incubation time ^b	Relative fluorescence intensity ^c
1 hour	0.80
2 days	0.89
4 days	0.97
16 days	0.94

^aA solution of 1 % (w/v) BSA-FITC was pressurized at 2026 bar for 1 h. At specific time intervals after depressurization, the fluorescence intensities of the pressurized and control samples were measured. Between the periods of measurement, all samples were stored at 4°C in the dark.

^bDuration from solution pressurization time to time of fluorescence measurement.

^cIntensities were normalized with respect to the fluorescence intensity of the BSA-FITC sample at ambient pressure.

FT-IR Absorbance Spectroscopy

Analysis of the amide I band (the frequency region between 1600 and 1700 cm^{-1}) in the infrared spectra of polypeptides and proteins may be used for secondary structure determination in these systems (Parker, 1971; Susi, 1969; Susi et al., 1967). [To determine protein and polypeptide conformations in aqueous solutions, it is necessary to carry out measurements in $^2\text{H}_2\text{O}$ solution, since H_2O exhibits strong absorption in the spectral region of interest (amide I band) (Susi et al, 1967.)] This band is due to the in-plane $\text{C}=\text{O}$ stretching vibration weakly coupled with $\text{C}-\text{N}$ stretching and in-plane $\text{N}-\text{H}$ bending (Susi, 1969). Typically, globular proteins contain several types of secondary structures. Thus, the amide I band appears as a broad band with several peak maxima, which may be assigned to the different types of secondary structure involved. Amide I band analysis may be therefore used to investigate the secondary structure of bovine serum albumin and immunoglobulin G.

The changes in secondary structure of BSA-FITC after exposure to high pressure was examined by FT-IR spectroscopy. The infrared spectrum of the 1 % (w/v) sample of BSA-FITC that had been pressurized for 1 h at 2026 bar is shown in Figure 4A. The spectrum (shown after deconvolution, a data resolution enhancement procedure to remove broad line shapes) was obtained one day after the sample was pressure cycled. On the same figure is the spectrum of the control sample that was exposed to ambient pressure only. The infrared spectrum of the FITC labelled BSA at ambient pressure reveals a broad band, with a peak maximum at 1651 cm^{-1} and a shoulder at 1637 cm^{-1} . The spectrum of the BSA-FITC that was pressure cycled to 2026 bar shows some subtle differences. One difference that is apparent is the increase in intensity of the component band at 1637 cm^{-1} . In addition, component bands between 1643 cm^{-1} and 1651 cm^{-1} increase in intensity. The increase in intensities of these component bands suggests an increase in β -sheet (1637 cm^{-1}) and unordered (1644 cm^{-1}) conformation (Parker, 1971; Susi, 1969; Susi et al., 1967).

After a period of 16 days, the spectra of the pressurized and control samples were recorded again (Figure 4B). The pressure cycled sample has

Figure 4. Deconvoluted infrared spectra of BSA-FITC pressurized for one hour at 2026 bar (dashed line) and BSA-FITC control at ambient pressure (solid line). (A) spectra recorded one day after pressure treatment. (B) spectra recorded 16 days after pressure treatment. All protein solutions were prepared at 1% (w/v) in unbuffered $^2\text{H}_2\text{O}$ and the spectra were recorded at ambient pressure and temperature in a CaF_2 cell.

regained most of its original secondary structure, as indicated by the decrease in intensity of the 1637 cm^{-1} component band and a small increase in intensity of the 1651 cm^{-1} component band. It appears that some of the unordered conformation does not completely revert to the original conformation, as indicated by the relatively greater intensity increase at 1644 cm^{-1} as compared to that in the control sample.

A sample of FITC labelled IgG that had been pressurized for 1 h at 2026 bar in 0.15 M Bis-Tris was concentrated and resuspended in $^2\text{H}_2\text{O}$. A control sample resuspended in $^2\text{H}_2\text{O}$ was also prepared. The amide I bands in the infrared spectra (not shown) of both the pressure cycled and control samples are broad, with a peak maximum at 1639 cm^{-1} . The intensities of the component bands at 1639 cm^{-1} suggest that each sample consists of predominantly β -sheet conformation (Parker, 1971; Susi, 1969; Susi et al., 1967). This observation is in agreement with the estimated secondary structure of IgG consisting of 65% β -sheet, 3% α -helix, and 32% others (Przybycien and Bailey, 1991). The pressure treated sample showed no observable change in secondary structure.

A more thorough study to examine the effect of high pressure on BSA and IgG was carried out using the diamond anvil cell. The advantages of using the diamond anvil cell are twofold; the operating pressure of the cell extends beyond 2 kbar, and infrared spectra may be obtained while the system is pressurized. We have recorded FT-IR spectra using the diamond cell to examine the effect of high pressure on BSA, BSA-FITC, and IgG.

In the amide I region of the deconvoluted spectra of BSA (Figure 5) at 0.8 kbar pressure, a broad and asymmetric band with one maximum at 1654 cm^{-1} , a strong shoulder at 1638 cm^{-1} , and a weak shoulder at 1682 cm^{-1} are clearly observed. The component bands at 1638 , 1654 , and 1682 cm^{-1} are due to amide group segments in β -sheet, α -helical, and anti-parallel β -sheet conformations, respectively (Parker, 1971; Susi, 1969; Susi et al., 1967; Wong and Manstch, 1989). The strong intensity of the 1654 cm^{-1} component band relative to the 1638 cm^{-1} band suggests that the α -helical conformation of the amide groups predominates. These infrared results are consistent

Figure 5. Deconvoluted infrared spectra of BSA in the amide I region at pressures indicated in the diamond anvil cell. The protein solution was prepared at 1% (w/v) in unbuffered $^2\text{H}_2\text{O}$. Spectra were recorded at room temperature.

with the generally accepted secondary structure in BSA, which consists of 55% α -helix, 9% β -sheet, 4% turns, and 32% others (Kella et al., 1988).

As external pressure is increased, the band begins to broaden at 1.1 kbar, and beyond this pressure, several changes in the infrared spectrum take place. At 3.5 kbar, the peak maximum at 1654 cm^{-1} shifts to a lower frequency and a new band is observed at approximately 1644 cm^{-1} . The intensity of this band increases with increasing pressure. At 11.3 kbar, the bands in the amide I region have been redistributed due to high hydrostatic pressure. It is observed that the contributions of unordered and β -sheet structures are increased as a consequence of high pressure at the expense of the decrease in the α -helical structure. The increase in unordered conformation is shown by the peak maximum at 1644 cm^{-1} , as shown in the infrared spectrum at 11.3 kbar.

Figure 6A shows the infrared spectrum of both unlabelled and FITC labelled BSA at 0.8 and 0.9 kbar, respectively (spectra recorded at ambient temperature and pressure were identical to those shown in figure 6A). Both spectra have been deconvoluted using the same parameters. It is evident that the addition of the fluorescent probe has induced a change in secondary structure when one compares the infrared spectrum of BSA-FITC to that of the native form of BSA. The infrared spectrum of BSA-FITC exhibits a broad asymmetric band with a peak maximum at 1650 cm^{-1} and two shoulders, one located at 1638 cm^{-1} , and the other at 1680 cm^{-1} . As a consequence of the covalent binding of FITC to the BSA, the peak maximum has shifted from 1653 cm^{-1} to 1650 cm^{-1} , and an increase in intensity of the 1638 cm^{-1} component band is also observed. Although differences in secondary structure between the native form of BSA and BSA-FITC are observed through FT-IR spectroscopy, the labelled protein, when properly prepared, has been shown to be immunologically identical to the native form (Landel, 1976).

The infrared spectrum of FITC labelled BSA as a function of pressure is shown in Figure 6B. As hydrostatic pressure is increased beyond 0.9 kbar, changes in the spectrum begin to occur, and these changes are

Figure 6   (A). Deconvoluted infrared spectra of BSA (solid line) and BSA-FITC (dashed line) at 0.8 kbar and 0.9 kbar respectively.

                  (B). Deconvoluted infrared spectra of BSA-FITC in the amide I region at pressures indicated. Solution conditions are as in Figure 5.

similar to those observed with the unlabelled BSA. The intensity of the 1638 cm^{-1} component band increases and a new band at approximately 1644 cm^{-1} is observed. Finally, at 11.9 kbar, the FITC labelled BSA has achieved a predominantly unordered conformation, characterized by the strong intensity of component band at approximately 1644 cm^{-1} (Parker, 1971; Susi, 1969; Susi et al., 1967).

Both BSA and BSA-FITC are shown to achieve a predominantly unordered conformation as hydrostatic pressure is increased to the range of 11-12 kbar. This conclusion is supported by several observations. Firstly, the bands in the amide I region of BSA and BSA-FITC have been redistributed due to high hydrostatic pressure such that a peak maximum is apparent in the region characteristic for unordered conformation ($1640\text{--}1644\text{ cm}^{-1}$). Associated with the increase in unordered conformation is the increase in intensity of the component band at 1669 cm^{-1} , which is indicative of turns. Also, the decrease in intensities of the component bands at 1653 and 1680 cm^{-1} indicates that unordered conformation is formed at the expense of α -helical and β -type conformations, respectively. Another effect of high pressure on BSA and BSA-FITC is the increase in the strength of hydrogen bonding of the amide groups, as indicated by the shift of the $1650\text{--}1653\text{ cm}^{-1}$ band to a lower frequency.

The changes in the infrared spectrum of IgG that occur due to high pressure are quite different from those observed with BSA. At low pressures, the infrared spectrum of IgG appears as a very broad band with a peak maximum at 1637 cm^{-1} , and three shoulders at 1622 , 1661 , and 1685 cm^{-1} (Figure 7A). As hydrostatic pressure increased to 2.1 kbar, only a subtle change in intensity of the component band at 1661 cm^{-1} is observed. No other significant changes in the infrared spectrum of IgG takes place in the pressure range below 2.1 kbar.

From 2.1 to 4.6 kbar, increases in intensity of the component bands at 1661 and 1685 cm^{-1} are dominant, while the peak maximum at 1637 cm^{-1} remains unchanged (Figure 7B). As pressure is increased up to 8.2 kbar, the intensities of the component bands at 1622 , 1661 , and 1685 cm^{-1} also increase, but at a faster rate than was observed in the 2.1 to 4.6 kbar

Figure 7. Deconvoluted infrared spectra of bovine IgG (2.5% w/v in 0.01 M phosphate buffer in $^2\text{H}_2\text{O}$ at p ^2H 6.9) in the amide I region in the diamond anvil cell. (A) 0.1 to 2.1 kbar range. (B) 2.1 to 8.2 kbar range. Spectra were recorded at room temperature.

pressure range. The changes that occur between 2.1 and 8.2 kbar (Figure 7B) indicate that turn conformation is increasing (intensity of 1661 cm^{-1} component band increasing) and that some degree of aggregation is occurring (intensities of 1622 and 1685 cm^{-1} bands increase simultaneously) (Clark et al., 1981; Ismail et al., 1992).

Enzyme linked immunosorbent assay (ELISA)

The effect of pressure on the tertiary structure of a protein may be, in part, evaluated by examination of its immunological activity. If sufficient change in tertiary structure has occurred due to exposure to high hydrostatic pressure, it follows then that recognition of the protein by antibodies may be disturbed. This may be determined by ELISA. The sensitivity of the technique may be increased by employing a monoclonal antibody, since only a single epitope needs to be disturbed for a dramatic change in immunological activity to be observed. In this work, a monoclonal antibody was used in tests involving BSA and BSA-FITC.

To determine the effect of different pressure cycle parameters on the ELISA test, BSA was pressurized under 5 different conditions. Table 4 shows the effects of pressure, hold time, and depressurization rate on the immunological activity of BSA. Pressure cycles at 1013 or 2026 bar for durations under one hour employing a fast depressurization mode (350 bar/min) are observed to have little effect on the recognition of BSA by its antibody. Under such conditions, the difference in absorbance signals between the pressure cycled and the control sample is 5 % or less. Greater differences are observed during longer pressure cycles and when the slow depressurization mode (20 bar/min) is employed. However, these reductions in absorbance are only 14 and 10 %, respectively.

ELISA tests were also performed with pressurized samples of FITC labelled IgG and BSA. Table 5 shows the results of the ELISA tests conducted on IgG-FITC and BSA-FITC samples that were pressure cycled for 1 h. In the case of FITC labelled IgG, the maximum difference in absorbance signals between the pressure treated and control samples is 12 %. The observed difference in absorbance signals for pressure cycled and control samples for FITC labelled BSA is 9 %.

Table 4. Effect of pressure, pressurization time, and depressurization rate on the immunological activity of BSA as determined by ELISA^a

Sample	Pressure (bar)	Hold time ^b (hr)	Depressur- -ization rate ^c	Absorbance ^d	
				Ambient sample	Pressure sample
1	1013	0.25	F	0.94 +/- .06	0.89 +/- .02
2	1013	2.0	F	0.95 +/- .03	0.82 +/- .03
3	1013	0.25	S	0.97 +/- .03	0.87 +/- .01
4	1013	1.0	F	0.83 +/- .10	0.81 +/- .01
5	2026	1.0	F	0.86 +/- .02	0.81 +/- .02

^a10 µg/ml BSA in 0.15 M Tris (pH 7.3) was pressurized, held, and depressurized as indicated. The pressure cycled and control samples were then used in an ELISA test (microtiter plate method) as described in Methods.

^bTime of pressurization cycle

^cF-fast depressurization rate: 350 bar/min, S-slow depressurization rate: 20 bar/min

^dAverage absorbance at 404 nm +/- standard deviation (n=3)

Table 5. Effect of pressure on the immunological activity of IgG-FITC and BSA-FITC as determined by ELISA^a

ELISA method ^b	Sample type	Pressure (bar)	Serum dilution or concentration	Absorbance ^c	
				Ambient sample	Pressure sample
Cloth	IgG-FITC	1013	1:2000	0.59 +/- .03	0.66 +/- .06
Cloth	IgG-FITC	2026	1:2000	0.64 +/- .02	0.73 +/- .02
Plate	IgG-FITC	2026	1:8000	1.67 +/- .06	1.71 +/- .04
Plate	BSA-FITC	2026	10µg/ml	0.83 +/- .05	0.76 +/- .01

^a10 µg/ml BSA-FITC or IgG diluted in 0.15 M Bis-Tris (pH 7.3) was pressurized at the pressure indicated for 1 h. After depressurization, both the pressurized and control samples were used in an ELISA test as described in Methods.

^bCloth: polyester cloth as solid phase, Plate: microtiter plate used as solid phase

^cAverage absorbance at 404 nm +/- standard deviation (n=3)

The 1 % (w/v) sample of BSA-FITC dissolved in $^2\text{H}_2\text{O}$ that was pressurized for 1 h at 2026 bar was also used in an ELISA test. Four days after pressure treatment, both the pressure cycled and control samples were diluted to 10 $\mu\text{g/ml}$ in PBS and incubated in a microtiter plate as described in Methods. After colour development, the absorbance signals at 404 nm of the ambient and pressure treated samples were determined to be very similar (absorbance readings of 0.370 and 0.350, respectively).

Discussion

Three different techniques have been used to evaluate pressure induced conformational changes of two immunological proteins in order to investigate the suitability of high pressure separation in immunoaffinity chromatography purification processes. Fluorescence measurements employing FITC as a probe reveal that the conformation of IgG is more stable than that of BSA under high pressure conditions. The fluorescence studies indicate that a conformational change occurs in BSA with increasing pressure, but cannot provide information about the type of conformational change taking place. Furthermore, they cannot offer information on changes beyond the immediate vicinity of the probe (Weber and Drickamer, 1983). The actual changes in conformation indicated by the fluorescence studies can be monitored by FT-IR spectroscopy.

The results obtained by FT-IR spectroscopy shown earlier are consistent with the fluorescence data. The secondary structure of IgG is not substantially perturbed by pressures below 2 kbar. In contrast, the secondary structure of BSA-FITC was shown to undergo reversible changes after a 1 h pressure cycle at 2026 bar. These reversible conformational changes were also revealed by parallel fluorescence intensity measurements (Table 3). Also, changes in the secondary structure of BSA were observed to occur above 0.8 kbar (Figure 5).

Both the fluorescence and FT-IR spectroscopic studies provide evidence that the conformation of BSA is readily affected by increasing pressure. However, ELISA tests demonstrate that pressure cycled samples of BSA-FITC and IgG-FITC display comparable biological recognition

properties; the relative difference in absorbance signals in the ELISA test between pressure cycled and control samples of BSA-FITC and IgG-FITC is equal (Table 5). This is also true in the case of BSA-FITC dissolved in $^2\text{H}_2\text{O}$. Therefore, the conformational changes that BSA experiences from increasing hydrostatic pressure (up to 2 kbar) do not perturb the tertiary structure of the protein at the antigenic determinant sites in that they remain recognizable to the antibody employed in the ELISA test.

The results from all three techniques employed in this study indicate that the conformation of an antibody (IgG) is not easily altered by high pressure. This suggests that antibodies may have the ability to be recycled after use in high pressure separation processes. This was the conclusion drawn by Olson et al. (1989) when immobilized murine antibodies (IgGs) were subjected to conditions of high pressure. Acidic conditions are often required to disrupt antigen-antibody complexes. However, such treatment usually results in antibody precipitation and the loss of antigen binding ability. It appears that the secondary structure of IgG at low pH is reorganized, and that a subtle conformational change occurs rather than complete unfolding (Jiskoot et al., 1991). At low pH, an increase in β -turn and α -helix at the expense of β -sheet structure is apparent. The β -sheet structure is altered from an ordered, intramolecular form, to a less ordered, intermolecular β -sheet structure (aggregation) (Wasacz et al., 1987). Jiskoot et al. (1991) observed precipitation and loss of antigen binding activity while incubating a murine monoclonal antibody at low pH. They also pointed out that irreversible acid-induced conformational changes in polyclonal IgG preparations have been observed by numerous other investigators. Therefore, although being employed frequently, the use of acidic conditions for repeatable antigen-antibody separation is not recommended. The alternative of employing high pressure in a separation process appears to be a more viable option since the conformation of the antibody is stable under high pressure conditions.

In this study, FT-IR spectroscopy has proven to be the most revealing technique in determining the conformational changes of proteins under high pressure. Although, in employing this technique, there is concern over the use of $^2\text{H}_2\text{O}$ in studying the secondary structure of proteins in

aqueous solutions (Dong et al., 1990), we have observed that $^2\text{H}_2\text{O}$ is a suitable substitute for H_2O . In the case of studying BSA-FITC by fluorescence measurements under high pressure, a similar trend in decreasing fluorescence intensity with increasing pressure was observed in both H_2O and $^2\text{H}_2\text{O}$. Also, no aberrant results occurred in the ELISA test conducted from the sample of BSA-FITC in $^2\text{H}_2\text{O}$. FT-IR spectroscopy avoids the need for probes that may perturb the protein's native conformation, as evident from the present work (Figure 6A). Therefore, when employing fluorescent probes for detecting conformational changes in a protein, one must be certain that the observed changes reflect the protein's native structure, and that the changes are not influenced by the presence of the probe.

In immunoadsorption processes employing immobilized antibodies, the technique of FT-IR spectroscopy has the potential of being used as a screening method to determine whether or not an antigen is suitable for high pressure separation processes. In the present study, conformational stability was used as a criterion in determining a protein's ability to withstand high pressure treatment. However, changes in the FT-IR spectrum in the pressure range up to 2 kbar did not reflect antigen-antibody affinity as measured in the ELISA test. Therefore, for FT-IR spectroscopy to be used as a screening method, a relationship must be found between observed changes in the FT-IR spectrum of the antigen under pressure and its ability to maintain its biological activity and retain antibody recognition properties after pressure treatment. One would speculate that the protein's inability to maintain its biological activity would occur at the same pressure in which an irreversible change in the FT-IR spectrum is observed. This is the subject for examination in future studies.

Appendix to Chapter 1

Pressure Vessel Design and Heat Transfer Considerations

Figure 8. Conceptual drawing of the thick-walled pressure vessel. The 0.5 in. maraging steel cylinder (part 1) would be fitted with a sleeve (part 2) to accomodate available 0.375 in. high pressure seals (part 3). Pressurization of the vessel would be accomplished by intensifying the upper piston (part 4). (Dimensions in inches, drawing not to scale).

The maximum shear stress is given by:

$$[5] \quad (\sigma_2 - \sigma_3)/2,$$

and the change in radius b is determined from:

$$[6] \quad \Delta b = \frac{qh}{E} \left(\frac{a^2 + b^2}{a^2 - b^2} + \nu \right)$$

where, E = modulus of elasticity and ν = Poisson's ratio.

The maraging cylinder is 2 inches in diameter, and the sleeve is 0.375 inches in diameter (material: 15-5 PH stainless steel), then $a = 1$ in., $b = 0.1875$ in., and $q = 50000$ psi. If the longitudinal stress is considered (uniform internal fluid pressure, q , is equal in all directions and the ends are not capped) then from [1]:

$$\sigma_1 = 0 \text{ psi.}$$

The maximum hoop stress is given by [3]:

$$\sigma_2 = \frac{(50000) \left(\frac{1^2 + 0.1875^2}{1^2 - 0.1875^2} \right) [\text{psi}]}{1} = 53643.7 \text{ psi.}$$

From [4], the maximum radial stress, $\sigma_3 = -50000$ psi, yielding a maximum shear stress as indicated by [5] of 51822 psi.

The change in the inner radius at the maximum design pressure may be calculated from [6]. Using the properties of the lower strength steel (15-5 PH) as a worst case, Young's modulus = 31.5×10^6 psi and $\nu = 0.283$ (Boyer and Gall, 1985) then:

$$\Delta b = \frac{(50000)(0.1875)}{31.5 \times 10^6} \left[\frac{(1^2 + 0.1875^2)}{(1^2 - 0.1875^2)} + 0.283 \right] [\text{in}] = 0.0004 \text{ in.}$$

Therefore, at the maximum design pressure, the change in diameter on the 0.375 in. sleeve that must be endured by the high pressure seals is less than 0.001 in.. The calculated stresses that are accompanied by a fluid pressure of 50000 psi may be satisfactorily stabilized by the Maraging steel cylinder (yield strength= 348000 psi) and the 15-5 PH stainless steel sleeve (yield strength= 185000 psi when heat treated appropriately- 1 h at 480°C, air cool) to give a minimum safety factor of three times.

From the proposed pressure vessel design of Figure 8, two pistons were fabricated (material: 304 stainless steel) to accommodate the Bal Seal Engineering high pressure seals. Since the sample seals were not rated for ultra-high pressure work, and it was found that the 304 was a poor material choice (due to surface galling), new pistons were fabricated using 15-5 PH stainless steel with replacement seals (Viton O-rings, Precision Rubber Products, Canada). The drawing details of the two working pistons are shown in Figure 9. The surface finish of the sleeve was measured to be in the range of 12-20 μ in. A good quality surface finish is important in order to prevent seal leakage.

A block diagram of the working pressure vessel was shown in Figure 2, Chapter 1. The two pistons depicted in Figure 9 mounted with 0.375 in. O-rings (Precision no. 010-19357) pressurize a 0.183 in.³ (3 ml) column of liquid by means of intensifying the upper piston with a 20 ton laboratory press (Fred S. Carver, USA). The bleed hole in the upper piston is sealed with a flat-head 10-32 set screw and a viton O-ring (Precision no. 002-19357). On-line pressure measurements were calculated by using a calibrated load cell (Omega, USA) which measured the applied force and by knowing the area of the pressurizing piston. The load cell was operated at an applied voltage of 5V. A chart recorder monitored the pressurization rate, the holding pressure, and the depressurization rate of the pressure vessel. The maximum operating pressure of the vessel was 31900 psi, the point at which the 0.375 in. O-rings would extrude causing a seal failure.

Figure 9. Detailed drawings of the working pistons used for the pressure vessel. (Material: 15-5 PH stainless steel). Drawing not to scale.

DETAIL "A"
TYP. Parts 1 and 2

- NOTES
1. Piston Material
  2. Stainless steel 15-5PH
  3. Not to Scale
  4. CIRCULAR SECTION

Heat Transfer Considerations

Protein structure and stability may be influenced by environmental temperature changes. Therefore, it is important to conduct high pressure experiments at a constant temperature so that any observations may be attributed to the parameter of hydrostatic pressure, and not temperature perturbations. An analytical model for predicting temperature variations experienced by the fluid sample while subject to increasing pressure was developed from the representation of the pressure vessel shown in Figure 10. In this simplified sketch of the pressure vessel, it was assumed that the fluid in the vessel is surrounded by one cylindrical body and that the thermal contact resistance from the pistons and sleeve in contact with the high strength steel cylinder was negligible. Also, the fluid in the pressure vessel was assumed to have the properties of pure water. The model for temperature prediction was divided into two parts. First, as a worst case prediction, it was assumed that the pressurized fluid was insulated from the high strength steel cylinder, forming an adiabatic process and, ideally, an isentropic process. From the adiabatic process, the maximum change in temperature resulting from fluid pressurization may be calculated. The second part of the model would allow for the heat transfer from the compressed fluid into the high strength steel cylinder.

For the first part of the model where the column of water is pressurized under adiabatic conditions, the First Law may be written in the form:

$$[7] \quad dQ = pdV + dU,$$

where Q = heat transferred, V = volume, and U = internal energy.

Since the system is adiabatic, $dQ = 0$, and the quantity pdV is negative because work is done on the fluid. Therefore, [7] may be simplified to:

$$[8] \quad pdV = -dU.$$

Figure 10. Simplified cross-sectional sketch of the pressure vessel used to develop an analytical model for fluid temperature prediction as a function of pressure. It is assumed that the fluid in the vessel is surrounded by one cylindrical body and that the thermal contact resistance from the pistons and sleeve in contact with the maraging steel cylinder was negligible. (Dimensions in inches, drawing not to scale).

If internal energy is substituted for the heat capacity at constant volume and both sides of [8] are integrated then:

$$[9] \quad \int_{P_0}^{P_m} p \, dV = \int_{T_0}^{T_m} mC \, dT,$$

where C = heat capacity of water; m = mass of water; P_m and P_0 are the maximum and initial pressures; and T_m and T_0 are the maximum and initial temperatures of the system, respectively.

The isentropic compressibility of water is defined by:

$$[10] \quad \beta = -\frac{1}{v} \left(\frac{\partial v}{\partial p} \right)_s,$$

Assuming that β is constant over the range of pressure and temperature expected in the model system described (using an average value for the expected pressure range), therefore:

$$[11] \quad dp = -\frac{1}{\beta v} dv, \text{ and rearranging,}$$

$$[12] \quad dv = -\beta v \, dp.$$

In order to complete the integral on the left hand side of [9], the equation is written in terms of P . Substituting [12] into [9] and noting that the mass of water is a constant yields:

$$[13] \quad \int_{P_0}^{P_m} p (-\beta v \, dp) = m \int_{T_0}^{T_m} C \, dT.$$

Since v will not change much with increasing pressure, let $v=v_0$, which is a constant (initial volume of water in pressure vessel). Therefore, the above equation may be written:

$$[14] \quad -v_0\beta \int_{p_0}^{p_m} p \, dp = m \int_{T_0}^{T_m} C \, dT.$$

Integrating both sides of [14] gives:

$$[15] \quad \frac{p_0^2}{2} - \frac{p_m^2}{2} = \frac{mC}{\beta v_0} (T_m - T_0), \text{ and rearranging in terms of the temperature difference gives:}$$

$$[16] \quad \Delta T = (T_m - T_0) = \frac{\beta v_0}{2mC} [p_0^2 - p_m^2].$$

The value of β may be determined from tabulated values of the bulk modulus of water, K . From the tabulated values, the bulk modulus of water varies linearly as a function of pressure near room temperature (15 °C) (Kaye and Laley, 1986):

$$[17] \quad K = 6.34 (P) + 2.081 \times 10^9,$$

where both K and P have units of Pa.

Using the initial value of pressure (P_0) to be 101300 Pa (14.7 psi) and since the maximum pressure (P_m) being considered is 202.6×10^6 Pa (29400 psi), the average value of K over the pressure range is 2.74×10^9 Pa. Since $\beta = K^{-1}$, the average value of β over the pressure range considered is $3.65 \times 10^{-10} \text{ m}^2/\text{N}$.

Evaluating the properties of water at room temperature (20 °C) (from Holman, 1986):

$$\text{Let } v_0 = 3 \text{ ml} = 3 \times 10^{-6} \text{ m}^3,$$

$$m = \rho_0 v_0 = (998 \text{ kg/m}^3)(3 \times 10^{-6} \text{ m}^3) = 2.994 \times 10^{-3} \text{ kg, and}$$

$$C = 4179 \text{ J/kg K.}$$

Substituting the above values into [16],

$$[18] \quad \Delta T = \frac{(-3.65 \times 10^{-10})(3.0 \times 10^{-6})}{2(2.994 \times 10^{-3})(4179)} [(101.3 \times 10^3)^2 - (202.6 \times 10^6)^2] = 1.796 \text{ } ^\circ\text{C}.$$

The result obtained from [18] indicates the maximum possible temperature rise. The value appears to be negligible since the real temperature rise over the period of compression (approximately five minutes) would be even less because of the heat transfer into the cylinder from the water. If one proceeded with the same calculation, taking into consideration the change in b as a function of pressure (substituting [17] into [13] and recalculating the integral), the maximum temperature rise would be $1.678 \text{ } ^\circ\text{C}$. This confirms that the assumption made in the first calculation is valid. Since the temperature rise during the period of compression was deemed negligible, further analysis of the transient heat flow behaviour from the water into the cylinder (part two of the analytical model) was not necessary.

Glossary of Terms for Appendix to Chapter 1

Hardening. Increasing hardness by suitable treatment, usually involving heating and cooling.

Heat Treatment. Heating and cooling a solid metal or alloy in such a way as to obtain desired conditions or properties, eg. increasing hardness and yield strength.

Maraging steel. A precipitation-hardening (PH) steel that contains, in addition to iron, more than 10% nickel and one or more supplemental hardening elements. A type of high strength steel.

Modulus of Elasticity. A measure of the rigidity of metal. Ratio of stress (below the proportional limit) to corresponding strain. The modulus obtained in tension or compression is Young's modulus.

Poisson's ratio. The absolute value of the ratio of the transverse strain to the corresponding axial strain in a body subjected to uniaxial stress. It is observed that the strain perpendicular to the direction of the load application is a contraction when the axial strain is tensile ($\epsilon_{\text{load}} = -\nu \epsilon_{\text{axial}}$).

Rockwell hardness test. An indentation hardness test based on the depth of penetration of a specified penetrator into the specimen under controlled conditions. Highest hardness scale using this test method is Rockwell C (Rc). The higher the value, the harder the material.

Strain. A measure of the relative change in the size or shape of a body.

Stress. Force per unit area, usually thought of as force acting through a small area within a plane.

Surface finish. Condition of a surface as a result of final treatment. In this work, arithmetic average of measured surface profile (roughness) expressed in microinches (μin).

Yield strength. The stress at which a material exhibits a specified deviation from proportionality of stress and strain.

Chapter 2

Carbonyldiimidazole Activation of a Rayon/Polyester Cloth for Covalent Immobilization of Proteins

Introduction

Affinity chromatography is an industrially attractive purification method, as a high degree of purification can be achieved in a simple procedure that can be readily automated. However, currently available affinity chromatography supports such as cross-linked agarose or dextran beads are relatively expensive and often exhibit structural instability under high flow or pressure conditions (Janson, 1984). Thus there is a need for alternative cost effective affinity chromatography supports for industrial use. We have previously demonstrated the use of cotton cloth as a support for ionic and hydrophobic binding of enzymes (Sharma and Yamazaki, 1984; Yamazaki et al., 1984). Since the cloth possesses a regularly compressed open structure, columns packed with cloth segments permit higher flow rates than columns packed with fibers.

Affinity chromatography requires a high degree of support activation in order to achieve efficient covalent immobilization of specific biomolecules such as proteins. The hydroxyl groups of cotton are not highly reactive as they are extensively involved in hydrogen bonding (maintaining the crystalline structure of cotton). Regenerated cellulose such as rayon has a much less crystalline structure and its hydrogen bonding can be readily disrupted. Thus, its hydroxyl groups should be more readily activated.

Cyanogen bromide (CNBr) is frequently used in the activation of polyhydroxyl supports. However, since the reaction is rapid and requires titration at a specific pH range, intensive mixing is necessary for achieving efficient and uniform activation. Furthermore, caution is required in order to avoid the toxicity of CNBr. Considering these factors, CNBr activation may not be ideal for activating a large volume of cloth matrices for industrial use.

This chapter describes 1,1-carbonyldiimidazole (CDI) activation of a rayon/polyester cloth matrix for covalent immobilization of proteins. Bovine serum albumin (BSA) was used as a model protein.

Materials and Methods

Materials

The following chemicals were purchased from Sigma Chemical Co.: bovine serum albumin (BSA) (A-7030), rabbit anti-BSA serum (B-1520), normal rabbit serum (S-2632), anti-rabbit IgG serum antibody alkaline phosphatase conjugate (A-8025), *p*-nitrophenyl phosphate (104-0), 1,1 carbonyldiimidazole (CDI), (C-7625). Cyanogen bromide (CNBr) was obtained from Eastman. Reagents for the micro bicinchoninic acid (BCA) protein assay were purchased from Pierce. A Rayon/polyester blend (70%/30%) non-woven cloth matrix (Sontara 8423) was obtained from Dupont.

Alkali Pretreatment

Up to 20 cloth segments (6 mm square) were mixed with 5 ml of 10 % (w/v) NaOH, and incubated for 10 min at room temperature. The segments were then washed 5 times with distilled water under vacuum, were blotted, and were immediately used for chemical activation.

CDI Activation and Protein Immobilization

The alkali treated segments (up to 20 maximum) were soaked in a solvent (typically dioxane) for 30 min, and washed 5 times with the solvent. The segments were then evenly spread on a glass petri dish and incubated with 50 μ l of 3.5 % (w/v) CDI dissolved in the solvent for 30 min at room temperature. The segments were then washed 5 times with solvent (ca. 20 ml per wash). After the final washing, most of the solvent was removed from the cloth segments by suction, or the segments were washed 5 times with 0.5 M sodium phosphate buffer (pH 7.5). The segments were immediately placed in a glass petri dish and used for protein immobilization. Each segment was incubated with 50 μ l of 1% (w/v) BSA in 0.5 M phosphate buffer (pH 7.5) for approximately 20 h at room temperature. Each segment was then washed 3 times with the phosphate buffer under vacuum. Excess active groups were quenched by incubating each segment with 50 μ l of 0.1 M ethanolamine (pH 7.5) for approximately 4 h at room temperature. The segments were then washed 3 times with

0.85% NaCl in 0.01 M sodium phosphate, pH 7.3 (PBS) and stored in PBS at 4° C.

CNBr Activation and Protein Immobilization

The alkali treated cloth segments (up to 20 maximum) were washed 5 times with 1 M sodium carbonate buffer (pH 11), suspended in 10 ml of the carbonate buffer, and then placed in a fume hood. One gram of CNBr was dissolved in 1 ml of acetonitrile and the solution was mixed with the cloth suspension. The mixture was constantly stirred while the pH was maintained between 10.5 and 11 by the addition of 3 M NaOH. After incubation for 10 min at room temperature, the liquid was drawn into a vacuum flask containing 100 mM ferrous sulphate to inactivate the CNBr. The cloth segments were then washed twice with 30 ml of distilled water, acetone, and 0.1 M sodium phosphate buffer. (pH 7.5). The cloth segments were placed in a petri dish and were immediately coupled with BSA as described above.

Assay of Immobilized BSA

To remove the loosely bound BSA, each cloth segment was heated in 1 ml of 1% sodium dodecyl sulphate (SDS) at 100° C for 10 min, washed 3 times with 1% SDS, and finally with distilled water. The immobilized BSA was measured using the BCA protein assay (Hage and Walters, 1987), which permits assay of covalently bound protein. Since it is difficult to obtain covalently immobilized protein standards, the degree of protein immobilization was compared by absorbance of the colour developed. The solution absorbance at 562 nm was determined after dilution to an absorbance range of 0.2 to 0.8.

Immunoreaction with Anti-BSA Antibody

Each cloth segment containing immobilized BSA was incubated in a petri dish with 50 µl of the rabbit anti-BSA serum or normal rabbit serum (diluted 1:1000 in PBS) for 30 min at room temperature. The segment was then washed 5 times with PBS, incubated with 50 µl of anti-rabbit IgG-alkaline phosphatase conjugate (diluted 1:1000 in PBS). After incubation for 30 min at room temperature, the segment was washed as described above, and incubated with 500 µl of 0.39 % (w/v) *p*-nitrophenyl phosphate in

a mixture of 10 % (w/v) diethanolamine and 0.5 mM MgCl_2 (pH 9.8). After 30 min at room temperature, the reaction was stopped by the adding 500 μl of 0.5 M sodium phosphate (pH 9.5) and the absorbance at 404 nm was determined.

Results

Figure 11 illustrates the activation of the rayon's hydroxyl groups with CDI followed by protein coupling. Unlike the activation with CNBr, the activation with CDI can be carried out safely and without the need to mix and titrate. The isourea linkages formed from the CNBr-activated support are positively charged and less stable than the linkage formed from the CDI-activated support which carry no charge.

Since woven cloth, when cut to segments, tend to unravel at the edges, non-woven rayon/polyester cloth was used here because of its mechanical stability during activation and use. For effective activation, the hydroxyl groups of rayon should be disengaged from hydrogen bonding. This can be readily achieved by exposing the cloth to strong alkali. The polyester portion maintains the fabric structure, as the rayon part may become structurally weak during the alkali treatment and activation.

Alkali Pretreatment

The cloth segments were exposed to 10% NaOH in H_2O for 10 min, activated with CDI, and washed with dioxane as described in Methods. After removing most of the dioxane by suction filtration, the segments, without further drying, were used for coupling bovine serum albumin (BSA).

Table 6 (row I) shows that the alkali treatment was necessary for CDI activation as judged by BSA immobilization (absorbance due to BSA at 562 nm). CDI activation of the alkali-treated cloth caused about 25-fold increase in BSA binding. Without CDI activation, the cloth without alkali treatment showed significant BSA binding (background), while the alkali pretreated cloth showed much smaller BSA binding. It can be speculated that the

Figure 11. Cloth activation with carbonyldiimidazole (CDI) and protein coupling.

background protein binding is due to the hydrophobic interaction since the surface of cellulose is hydrophobic when hydroxyl groups are kept in a state of hydrogen bonding.

Effect of drying and rehydration

In the above procedures, the cloth, after CDI activation, was washed with dioxane under vacuum suction and used without further drying for BSA binding. We found that drying markedly reduced binding capacity. Rehydrating the cloth with dioxane or phosphate buffer did not regain the original binding capacity (Table 6, row II).

Effect of washing with phosphate buffer

In the above procedures, the CDI-activated cloth contained some residual dioxane when used for BSA binding. Some proteins may be susceptible to denaturation by the organic solvent. Therefore, the CDI-activated cloth, after washing with dioxane, was washed 3 times with phosphate buffer (used for BSA binding). The use of the aqueous buffer instead of dioxane caused about a 15% reduction in BSA binding (Table 6, row III).

Immunoreaction

Immunoabsorption chromatography is one of several types of affinity chromatography methods that depends on the immunological reactivity of bound molecules. To test whether BSA bound to the CDI-activated cloth is immunologically active, the P⁺A bound cloth was incubated with diluted anti-BSA rabbit serum, or similarly diluted normal rabbit serum (devoid of any anti-BSA antibodies). The binding of anti-BSA immunoglobulin G (IgG) to the cloth was measured by incubating with an anti-rabbit IgG-alkaline phosphatase conjugate followed by assaying for phosphatase activity as described in Methods. The BSA cloth incubated with the anti-BSA serum yielded an average absorbance at 404 nm of 0.43, while similar cloth samples incubated with the normal serum yielded an absorbance of 0.03. After storing at 4° C for 3 months, the bound BSA exhibited the same immunological activity.

Table 6. Effect of alkali treatment, drying, and washing on BSA binding capacity of the CDI-activated rayon/polyester cloth^a

	Pre-treatment	Activation	Final washing	Drying	Rehydration	Absorbance ^b (BSA binding)
I	water	none	dioxane	no	-	0.25 +/- 0.03
	water	CDI	dioxane	no	-	0.31 +/- 0.03
	alkali	none	dioxane	no	-	0.08 +/- 0.03
	alkali	CDI	dioxane	no	-	1.97 +/- 0.03
II	alkali	none	dioxane	yes	dioxane	0.22 +/- 0.01
	alkali	CDI	dioxane	yes	dioxane	0.43 +/- 0.22
	alkali	none	dioxane	yes	buffer	0.24 +/- 0.03
	alkali	CDI	dioxane	yes	buffer	0.28 +/- 0.07
III	alkali	none	buffer	no	-	0.08 +/- 0.01
	alkali	CDI	buffer	no	-	1.66 +/- 0.18

^a The rayon/polyester cloth segments were pretreated with alkali (10% NaOH) or H₂O, activated with CDI, washed with dioxane or 0.5 M phosphate buffer (pH 7.5), and used for BSA binding without drying (no) or after drying (yes) as described in methods. In row II, the dried cloth was rehydrated with the solvent (dioxane or phosphate buffer) prior to BSA binding. The BSA binding to the cloth was compared by measuring the absorbance (at 562 nm) of the colour developed during the microBCA protein assay.

^b Average absorbance at 562 nm +/- standard deviation (n=3)

Comparison with cyanogen bromide method

Although CDI activation is more suitable for industrial activation of the cloth support than CNBr activation, it is necessary to compare its protein binding performance with that of the CNBr method. The rayon/polyester cloth was activated with CNBr and examined for BSA binding as described in methods. Comparison of Table 6 and Table 7 (row I) shows that BSA binding was similar by both immobilization methods.

Chemical activation in methylene chloride

Dioxane is flammable, harmful, and may be a potential carcinogen. Methylene chloride (MC) is used as a degreasing and cleaning fluid in the dry-cleaning and food industries. MC is not flammable, less toxic, and less expensive than dioxane. Therefore, MC was examined as a solvent in the CDI activation of the cloth. Table 7 (row II) shows that MC could be used as a solvent in place of dioxane to activate the cloth to the same degree. Thus, MC would be suitable for industrial scale CDI activation of polyhydroxyl matrices.

Table 7. BSA binding to the cloth activated with CNBr and CDI (in methylene chloride)

	Pretreatment	Activation ^a (solvent)	Final washing	Drying	Absorbance ^b (BSA binding)
I	alkali	none (buffer)	buffer	no	0.10 +/- 0.07
	alkali	CNBr (buffer)	buffer	no	2.09 +/- 0.11
II	alkali	none(MC)	MC	no	0.09 +/- 0.00
	alkali	CDI (MC)	MC	no	1.99 +/- 0.06

MC: methylene chloride

^aThe cloth was activated with CNBr or CDI (using methylene chloride in place of dioxane) and assayed for BSA binding as described in Methods.

^bAs per Table 6.

Discussion

For the most efficient protein binding, the cloth must be pretreated with alkali before being activated, and must be washed and used without drying since drying markedly decreases protein immobilization. One explanation for the effect of drying is that the activated groups, once dried, adhere to the hydrophobic surface of cellulose or polyester and cannot efficiently interact with amino groups in large ligands such as proteins. Using buffer for the final washing of the activated cloth may decrease the amount of protein immobilized. This procedure, however, would be necessary for immobilization of proteins which are susceptible to organic solvents.

The protein binding performance of the cloth using CDI activation is equal to activation with CNBr. Since activation with CNBr is more difficult and the chemical is toxic, activation of the polyester/rayon cloth using CDI is more amenable to scale-up for industrial use. Also, because the cloth is inexpensive, structurally stable, and produces high flow rates in a packed column (Yamazaki et al., 1984), the activated cloth would make a suitable immobilization matrix for industrial scale affinity chromatography. These properties combined with the compressed structure of the cloth, make it well suited as an immunoabsorbent material under high pressure conditions.

Chapter 3

Evaluating the Use of Polyclonal Antibodies as an Immunoabsorbent in Hyperbaric Immunoaffinity Chromatography

Introduction

Affinity chromatography employing immobilized antibodies is a powerful and readily adaptable method of purifying antigenic substances (e.g. proteins). As yet, immunoaffinity chromatography has not become a generally applied protein purification technique on large production scales due to the lack of effective elution procedures which permit the long-term use of expensive immunoadsorbents (immobilized antibodies or antigens). Recently, it has been shown that a protein antigen may be successfully recovered from an immunoadsorbent consisting of immobilized monoclonal antibodies by employing high hydrostatic pressure (Olson et al., 1989). For a number of reasons, the application of high pressure demonstrates a significant improvement over conventional elution techniques in the recovery phase of immunoaffinity chromatography. First, a high hydrostatic pressure induces the dissociation of the antigen-antibody complex, but unlike most elution procedures, it preserves the binding activity of the antibody. In addition, the immunoadsorbent can be used in several cycles of operation thereby lowering operating costs. Conventional elution procedures such as using low pH can decrease the binding capacity of an antibody immunoadsorbent by as much as 67% after only a few cycles of operation (Olson et al., 1989). The observation that antibodies may be recycled in hyperbaric immunoaffinity chromatography processes is clearly demonstrated by the conformational stability observed for an antibody structure (IgG) under high pressure (Chapter 1). Therefore, hyperbaric immunoaffinity chromatography (HIAC) employing immobilized monoclonal antibodies may be an attractive method of purifying protein products.

Presently, the obstacles barring HIAC from becoming a competitive affinity purification technique are the costs associated with the high pressure system and the expense of monoclonal antibodies. One method to improve the feasibility of the process without removing the advantages offered by the use of high pressure would be to propose an alternative affinity ligand preparation. Since polyclonal antibodies are less expensive (monoclonal antibodies may cost 4-8 times more than polyclonal antibodies depending upon the quantity and purity required, Biodesign International,

1992; Maine Biotechnology Services, 1992) and more readily available than monoclonal antibodies, an immunoabsorbent consisting of polyclonal antibodies would make HIAC a more competitive method of protein purification.

The work of Olson et al. (1989) demonstrated that pressures in the range of 0.5 to 2 kbar were capable of dissociating an antigen-monoclonal antibody complex. However, it is not known if the same high pressure conditions are capable of dissociating a polyclonal antibody-antigen complex. Monoclonal antibodies are a homogenous group of antibodies that specifically bind to one antigenic determinant site. Most antigenic substances, such as proteins, contain numerous sites that may be recognized by an antibody. Collectively, polyclonal antibodies are capable of binding to several antigenic determinants located on one protein. Therefore, polyclonal antibodies generally have higher binding capacities for antigens than monoclonal antibodies due to multivalent site attachment. This attribute may lead an antigen-polyclonal antibody complex to be more difficult to dissociate using high pressure. Although polyclonal antibodies appear to be an attractive substitute for monoclonal antibodies in HIAC processes, the dissociation of a polyclonal antibody-antigen complex using high pressure has not yet been demonstrated. In this chapter, three antigen-antibody systems are examined for dissociation under high pressure in order to evaluate the potential use of polyclonal antibodies for HIAC processes.

Materials and Methods

Materials

The following reagents were purchased from Sigma Chemical Co.: bovine serum albumin (BSA) (A-7030), ovalbumin (A-5503), gentamicin (G-3632), rabbit anti-BSA serum (B-1520), rabbit anti-gentamicin serum (G-1015), anti-rabbit IgG serum antibody alkaline phosphatase conjugate (A-8025), anti-mouse IgG serum antibody alkaline phosphatase conjugate (A-5153), Bis-Tris (bis[2-hydroxyethyl]imino-tris[hydroxymethyl]-methane) (B-9754), 1,1 carbonyldiimidazole (CDI), (C-7625), and *p*-nitrophenyl phosphate (104-0). A murine anti-BSA monoclonal antibody (MAb) 9.1 was obtained as

a gift from Dr. M. Yarmush (Rutgers University, USA). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (WSC) was obtained from Aldrich. Centricon-30, 30000 molecular-weight cutoff (MWCO) microconcentrators were obtained from Amicon, USA. A Rayon/polyester blend (70%/30%) non-woven cloth matrix (Sontara 8423) was obtained from Dupont.

Immunoabsorbent Preparation

Bovine serum albumin (BSA) was covalently immobilized to 1.25 cm square segments of cloth using CDI (the details of the immobilization procedure are described in Chapter 2). Dioxane was used in the final washing of the cloth prior to protein immobilization. Cloth segments containing immobilized BSA were stored in 0.85% NaCl (w/v) in 0.01 M sodium phosphate buffer, pH 7.3 (PBS) at 4°C.

Gentamicin was covalently bound to the cloth by first coupling the drug to ovalbumin using WSC. No evidence of cross-reactivity between the polyclonal anti-gentamicin antibody and ovalbumin was observed. In 10 ml of PBS, 20 mg of ovalbumin and 20 mg of gentamicin were mixed with 4 mg of WSC. After stirring overnight at 4°C, the mixture was concentrated using 30000 MWCO microconcentrators and washed 3 times, each with 1 ml of PBS, to remove unreacted gentamicin. After concentrating the gentamicin-ovalbumin, the conjugate was lyophilized and then covalently immobilized to the cloth using CDI as described above (1% w/v of conjugate used for protein immobilization). Cloth segments containing the immobilized gentamicin-ovalbumin conjugate were stored in PBS at 4°C.

Antigen-antibody dissociation under high pressure

Six immunoabsorbent membranes containing the covalently immobilized antigen were placed in a glass petri dish and two of the membranes were incubated with 100 µl of the appropriate antibody diluted in PBS containing 0.05% Tween-20 (PBST). Membranes containing immobilized BSA were incubated with either 6 µg/ml anti-BSA MAb 9.1 or with polyclonal anti-BSA antibodies diluted 1: 2000 from serum. Membranes containing immobilized gentamicin were incubated with

polyclonal anti-gentamicin antibodies diluted 1:100 from serum. The remaining four membranes were incubated with 100 μ l of PBST only.

After incubation for 1 h at room temperature, each membrane was washed five times with a total volume of approximately 7.5 ml of PBST. Each membrane that was incubated with antibody was placed between two membranes devoid of antibody (incubated with PBST only), forming duplicate arrangements of three membranes sandwiched together. Duplicate samples of sandwiched membranes were wrapped with a nylon loop (approximately 0.3 inches in diameter) to prevent the layers from separating. One sample was then placed in the pressure vessel along with 3 ml of 0.15 M Bis-Tris (pH 7.3), while the other was kept at ambient pressure in the same buffer as the control.

The details of the pressure vessel and the pressure monitoring system are described elsewhere (Chapter 1). In a typical pressure experiment, the pressure in the chamber was increased to 2013 bar at a rate of approximately 550 bar/min, was held at that pressure (for a period between 0.5 and 19 h), and was then returned to atmospheric pressure at a rate of 350 bar/min at the end of each experiment. All pressurization experiments were conducted at room temperature.

Determination of Bound Antibody

Antibody molecules remaining on the middle membrane and those that had dissociated from the middle membrane to one of the outer immunoadsorbent membranes were detected by using a secondary antibody conjugated with an enzyme. Each immunoadsorbent membrane was separated from the sandwiched arrangement and washed five times with a total volume of approximately 7.5 ml of PBST. After washing, each membrane was incubated with 100 μ l of either anti-mouse or anti-rabbit IgG alkaline phosphatase conjugate (diluted 1:1000 in PBST). At the end of a 1 h incubation period at room temperature, the membranes were washed as described above. The immunoadsorbent, whose antigenic determinant sites were initially saturated with antibodies (the middle membrane in the original three-layered arrangement), was placed in one test tube (solution I), while the two outer membranes were placed in a second test tube

(solution II). To each tube, a 1 ml solution of 0.39 % (w/v) *p*-nitrophenyl phosphate in 10 % (w/v) diethanolamine containing 0.5 mM MgCl₂ (pH 9.8) was added. After 30 min at room temperature, the enzymatic reaction was stopped by adding 500 µl of 0.5 M sodium phosphate (pH 9.5). The absorbance at 404 nm was then determined for each solution. The fraction of antibodies transferred (*f*Ab) to the immunoadsorbent membranes initially devoid of antibodies was calculated from absorbance signal of the solution divided by the total absorbance obtained from both solutions:

$$[19] \quad fAb = \frac{A_{404} \text{ (solution II)}}{A_{404} \text{ (solution I)} + A_{404} \text{ (solution II)}}$$

Results

Antigen-antibody dissociation under high pressure was compared with the dissociation at ambient pressure by employing a site competition assay (Figure 12). Antibodies initially bound to antigenic determinant sites located on one immunoadsorbent were subjected to an excess of unoccupied binding sites by placing two immunoadsorbent membranes (initially devoid of any antibodies) in close proximity. If high pressure dissociated the antibody from its antigenic determinant, the antibody would be free to diffuse away. In time, the antibody may diffuse far enough away from its original binding site through membrane pores and rebind to a competing binding site located on another immunoadsorbent upon pressure release.

Since antibodies are more difficult to obtain in large quantities and more expensive than common antigens, the immunoadsorbents used here were prepared by covalently immobilizing antigen. To avoid inaccuracies resulting from the effects of high pressure on protein conformation, antibodies captured on immunoadsorbents were detected using an antibody-enzyme conjugate in a manner similar to the enzyme-linked immunosorbent assay (ELISA) technique. Antibodies exposed to high hydrostatic pressure and those left at ambient pressure both have antigenic determinant sites that are equally recognizable to the antibody employed in the ELISA test (Chapter 1).

Figure 12. Site competition assay used to measure antibody dissociation under ambient and high pressure conditions: (A) The binding sites on one immunoadsorbent membrane are saturated with antibody. (B) The immunoadsorbent membrane containing bound antibody is subjected to an excess of unoccupied binding sites by placing two other immunoadsorbent membranes in close proximity. (C) After a given time, all membranes are assayed for bound antibody using a secondary antibody-enzyme conjugate. Dissociating the antigen-antibody complex by high pressure should allow unbound antibodies to diffuse away from their original binding site to a binding site located on another immunoadsorbent membrane.

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PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
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Monoclonal Antibody Dissociation

Figure 13 represents the fraction of monoclonal antibodies transferred to previously unoccupied antigenic determinant sites located on another immunoadsorbent membrane, under high pressure and ambient conditions. Under high pressure, approximately 30% of the antibodies initially bound to the middle immunoadsorbent dissociated and diffused to new binding sites on either of the outer membranes. In comparison, approximately 15% of the antibodies diffused to either of the outer immunoadsorbents under ambient pressure conditions (1 bar). The maximum amount of antigen-antibody dissociation was achieved within a 0.5 h period, as the fraction of antibodies transferred remained relatively unchanged over 19 h.

Polyclonal Antibody Dissociation

Two different polyclonal antibody-antigen systems were examined for dissociation under high pressure. Figures 14 and 15 show anti-gentamicin and anti-BSA antibody dissociation under high pressure and ambient conditions, respectively.

The anti-gentamicin system represents the use of polyclonal antibodies, however, the small (molecular weight approximately 400) and therefore defined antigenic determinant located on the drug models a specific binding site that may be recognized by a monoclonal antibody. The anti-gentamicin antibodies are more easily dissociated from its antigen than the monoclonal anti-BSA antibodies. In 0.5 h, approximately 28% of the antibodies diffuse to one of the two immunoadsorbents containing free binding sites compared to approximately 32% under high pressure (Figure 14). Unlike the monoclonal system, the maximum antibody transfer under high pressure occurs within 4 h. At this point, approximately 36% of the antibodies are transferred under high pressure compared to 29% under ambient conditions. Only after a period of 19 h do the antibodies at ambient pressure dissociate to the same degree as the antibodies placed under high pressure.

Figure 13. Dissociation of monoclonal anti-BSA antibodies as measured by site competition assay at ambient pressure and 2013 bar. Data shown represents the fraction of antibodies transferred from one immunoadsorbent membrane (originally saturated with antibody) to competing binding sites located on another membrane as a function of time (see Material and Methods). Vertical bars denote S.D. of the mean for 3 separate determinations.

Figure 14. Dissociation of polyclonal anti-gentamicin antibodies as measured by site competition assay at ambient pressure and 2013 bar. Data shown represents the fraction of antibodies transferred from one immunoadsorbent membrane (originally saturated with antibody) to a competing binding site located on another membrane as a function of time (see Material and Methods). Vertical bars denote S.D. of the mean for 3 separate determinations.

Figure 15. Dissociation of polyclonal anti-BSA antibodies as measured by site competition assay at ambient pressure and 2013 bar. Data shown represents the fraction of antibodies transferred from one immunoadsorbent membrane (originally saturated with antibody) to competing binding sites located on another membrane as a function of time (see Material and Methods). Vertical bars denote S.D. of the mean for 3 separate determinations.

The anti-BSA polyclonal antibody system was observed to be more difficult to dissociate than either of the other two systems examined here. At 0.5 h, approximately 13% of the polyclonal anti-BSA antibodies diffused to one of the other immunoadsorbents under high pressure (Figure 15). Within the same time period, the amount of antibodies transferred at ambient pressure was similar (12%). Under ambient pressure, the antigen-antibody complex remained firmly bound over time. After 19 h, only 16% of the antibodies had diffused to new binding sites under ambient pressure. In contrast, under high pressure, approximately 24% of the polyclonal antibodies diffused to one of the other immunoadsorbent membranes.

Discussion

Since an antigen-antibody reaction is one in equilibrium, an antibody initially bound to an immunoadsorbent will continuously dissociate and rebind to the same or another antigenic determinant site located within the immunoadsorbent membrane pore matrix. If reassociation is inhibited, then the antibody would be able to freely diffuse away from the binding site. In all three antibody-antigen systems examined, high pressure increased the proportion of antibodies diffusing away from their original binding sites. This resulted in more antibodies being transferred to binding sites located on an outer immunoadsorbent. This evidence suggests that high pressure did dissociate all three antigen-antibody complexes examined. However, the degrees of dissociation observed were different for each system.

The monoclonal antibody-antigen system examined in this work has previously been demonstrated to dissociate under high pressure. The results obtained in the present study are in agreement with the work of Olson et al. (1989) in which 75% of previously bound antigen was dissociated and recovered at a pressure of 2013 bar from a monoclonal antibody immunoadsorbent. The immunoadsorbent containing bound antigen was incubated under high pressure to dissociate the antigen-antibody complex. After incubation, the antigen was recovered from the immunoadsorbent by electrophoretic elution. Although, a greater amount of the antigen was

dissociated and recovered since electrophoretic elution was used, they found that a high pressure equilibrium state was attained after an incubation period of 15 min, as longer incubation times did not increase the amount of recovered antigen. Olson and coworkers (Olson et al., 1989) did not provide an explanation concerning the length of time for equilibrium to be achieved. In this study, a similar pressure equilibrium state was observed using a site competition assay. An equilibrium state of antibody dissociation was achieved in 30 min, and remained relatively constant over 19 h (Figure 13). The binding activity of the monoclonal antibody did not change upon treatment with high pressure (Olson et al., 1989). Therefore one cannot argue that the antibodies would not bind to competing antigenic determinant sites located on other immunoadsorbents after pressure release. But one interpretation of this result may be that upon pressurization, a significant amount of antibodies were immediately dissociated from the antigen and were able to diffuse to competing sites located on another immunomembrane in a short period of time. With increasing time, this initial level of dissociation (the equilibrium value) was established such that the rate of diffusion to competing sites from the antibody source was equal to the rate of diffusion from competing sites to the bulk solution where no antigen binding sites are located. It is possible that a small fraction of antibodies may diffuse away from the range of the binding sites on all the immunoadsorbents over a considerable length of time given that the antigen-antibody dissociation kinetics are not limiting. A steady state diffusion may continue for such a time since there is a large antibody source available (70% remaining after 19 h).

The results from this work demonstrate that high pressure may dissociate a polyclonal antibody-antigen complex. In both polyclonal antibody-antigen systems examined, antigen-antibody complex dissociation was facilitated under high pressure. By comparing the two systems, different degrees of dissociation are observed at different times (Figures 14 and 15). It is conceivable that the dissociation kinetics of the antigen-antibody complex are different depending upon the system examined. Fukushima et al. (1986) reported that the half-life of subunit dissociation of a hexamer, glutamate dehydrogenase, exceeded 10 h at 1824 bar. However, Weber (1992) found that in the case of dimer dissociation, pressure mainly

accelerated the rate of dissociation. This observation may allow one to employ short processing times to dissociate and recover proteins from immunoadsorbents under high pressure. In addition to the different dissociation kinetics that may be observed, the degree of dissociation at one pressure may also depend upon the system examined. But for many oligomeric protein systems, the relationship between the degree of dissociation and the applied pressure is sigmoidal - increasing pressure increases the degree of dissociation until at a maximum pressure, oligomeric protein dissociation asymptotically approaches 100% (Weber 1992).

As anticipated, the dissociation of the polyclonal anti-BSA antibody was observed to be more difficult than the anti-BSA monoclonal antibody at 2013 bar (Figures 15 and 13, respectively). However, the difference between the amount of monoclonal antibodies transferred (30% in 0.5 h) and the amount of polyclonal antibodies transferred (24% in 19 h) may be eliminated if diffusion away from the antigenic determinant sites was enhanced.

Relatively slow rates of dissociation were observed here because dissociation depended solely on the passive diffusion of the antibody. The diffusional limitations of the antibody may be illustrated from the Einstein diffusion equation:

$$[20] \quad \langle x \rangle = (2Dt)^{1/2},$$

where $\langle x \rangle$ is the root mean square distance traveled by diffusing particles, D is the diffusion coefficient of the particle, and t is time. The diffusivity of the antibody in the bulk solution is approximately equal to the effective diffusivity of the antibody within the membrane pores since the average pore diameter is much larger than the diameter of the antibody ($50\mu\text{m} \gg 0.013\mu\text{m}$) (Valentine and Green, 1967). A diffusion coefficient D of $4.17 \times 10^{-6} \text{ cm}^2 \text{ sec}^{-1}$, which corresponds to an antibody (IgG) of 150 kDa in water at 22°C and 2013 bar (adapted from Young et al., 1980), gives $\langle x \rangle$ to be 0.239 cm in a time of 19 h. The corresponding distance travelled by an antibody at ambient pressure in 19 h is 0.243 cm. Clearly, passive diffusion of a protein from an immunoadsorbent does not permit significant

recoveries to be obtained in a short period of time. In this study, diffusional limitations did not allow the antibodies to rapidly diffuse far enough away from any immunoabsorbent membrane (a layer of three membranes used in this work measures 0.2 cm thick). Upon the release of high pressure, the antibodies would simply rebind to an antigenic determinant sites located on the membranes. Therefore, for a practical dissociation and recovery process using high pressure, a driving force is necessary to remove the protein away from the immunoabsorbent prior to pressure release.

Although polyclonal antibody-antigen dissociation was observed as being more difficult than monoclonal antibody-antigen dissociation at 2013 bar, it is believed that polyclonal antibodies would perform as well as monoclonal antibodies in HIAC processes given that a suitable method to remove proteins from the immunoabsorbent is developed. This is the basis of the subject matter in the following chapter.

Chapter 4

Construction of a High Pressure Cell for Immunoaffinity Chromatography Using Pressure Elution

Introduction

The potential application of high pressure elution in the recovery stage of immunoaffinity chromatography offers several advantages over conventional elution procedures (Chapter 1). It has been previously shown that high pressure can dissociate proteins from antibodies, but the removal of these dissociated proteins in the recovery stage is necessary in order to prevent their reassociation during depressurization (Chapter 3). Recently, pressure elution in immunoaffinity chromatography process operations has been demonstrated by Olson et al. (1989). Proteins dissociated from antibodies by high pressure were recovered from a monoclonal antibody immunoabsorbent using a simultaneous electrophoretic approach. However, this pressure elution process lacks efficiency. Several cycles of high pressure dissociation are necessary to achieve a good recovery and the process of electrophoretic removal is not readily amenable to scale-up.

It is believed that pressure elution involving dissociation and recovery in the process of immunoaffinity chromatography may be made more efficient by operating the process much like conventional affinity chromatography. The immunoabsorbent containing the protein to be separated would be placed in a high pressure cell that would allow the dissociated protein product to be eluted with a convective flow of buffer and thus be recovered more efficiently. This chapter describes a high pressure cell that was developed to incorporate the design of a model affinity chromatographic column and allow pressure elution of the protein from the immunoabsorbent. To make the process of immunoaffinity chromatography more competitive with other large-scale protein purification techniques, pressure elution was demonstrated with a polyclonal antibody-antigen system. Bovine serum albumin (BSA) and polyclonal anti-BSA antibodies were used as model proteins.

Materials and Methods

Materials

The following reagents were purchased from Sigma Chemical Co: bovine serum albumin (BSA) (A-7030), bovine IgG (I-5506), rabbit anti-bovine IgG antibody fluorescein isothiocyanate (FITC) conjugate (A-9771), *p*--nitrophenyl phosphate (104-0), Bis-Tris (bis[2-hydroxyethyl]imino-tris[hydroxymethyl]-methane) (B-9754), and carbonyldiimidazole (CDI), (C-7625). Sheep anti-BSA antibody conjugated with FITC (anti-BSA-FITC) was obtained from BioDesign International (USA). Protein A alkaline phosphatase was obtained as a gift from Dr. R. Mackenzie (IBS/NRC, Ottawa). A rayon/polyester blend (70%/30%) non-woven cloth matrix (Sontara 8423) was obtained from Dupont.

Stainless steel type 431 was purchased from Earl M. Jorgensen Co. (Canada). Stainless steel type 440 C was obtained as a gift from Mr. J. Ross (IME/NRC, Ottawa). A pressure transducer, a digital pressure indicator, and high pressure fittings were purchased from AE Autoclave of Canada Ltd. High pressure seals were purchased from Bal Seal Engineering Co. (USA).

Immunoabsorbent Preparation

Immunoabsorbents were prepared using cloth segments cut into 1 cm (0.39 in) disks. Protein was covalently immobilized to the cloth segments using CDI (the details of the immobilization procedure are described in Chapter 2). Bovine serum albumin (BSA) was covalently immobilized using dioxane in the final washing of the cloth prior to protein immobilization. Bovine IgG was covalently immobilized (loading concentration of 5 mg/ml) using the same procedure except that 0.5 M phosphate buffer (pH 7.5) was used in the final washing of the cloth prior to immobilization. Cloth segments containing immobilized BSA or bovine IgG were stored in 0.85% NaCl (w/v) in 0.01 M sodium phosphate buffer, pH 7.3 (PBS) at 4°C.

High Pressure Cell with Convective Flow (High Pressure Cell)

The design concept for the High Pressure Cell with convective flow was derived from the original pressure vessel described in chapter 1. The design of the original pressure vessel was modified to incorporate a model chromatographic column, while leaving the method of vessel pressurization intact (using a laboratory press to advance a piston). This section of the High Pressure Cell was referred to as the working chamber. The working chamber was designed to contain a maximum of six immunoabsorbent membranes (immunomembranes). To provide convective fluid flow, the working chamber was connected to a second pressure chamber, the pumping unit.

Figure 16 shows a simplified sketch of the High Pressure Cell with the working chamber in series with the pumping unit. To operate an immunoaffinity chromatography process using the High Pressure Cell, the immunoabsorbent membranes containing the proteins to be separated would be placed at the bottom of the working chamber and anchored using an O-ring (Figure 17) in order to keep the membranes in a fixed position. For maximum fluid circulation, the piston in the pumping unit would be placed in at the bottom of the unit in its lowest position. The fluid would then be pressurized, allowing the proteins to dissociate from their binding sites on the immunoabsorbent. After a period of time, the piston in the pumping unit would be directed upwards, circulating fluid from the pumping unit into the working chamber while the fluid was still under pressure. The convective fluid flow would transport dissociated proteins from the immunoabsorbent membranes out of the working chamber into the bottom of the pumping unit. Thus the separated proteins located in the bottom of the pumping unit could not rebind to the immunoabsorbent during depressurization. After depressurization of the fluid, the immunoabsorbent membranes would be removed, and the fluid in the pumping unit containing the separated proteins would be recirculated back into the bottom of the working chamber. The recovery of the separated proteins would then be complete.

Figure 16. Simplified sketch of the High Pressure Cell with convective flow showing the locations of the pumping unit and the working chamber. The working chamber is a model chromatographic column. Under pressure, immunoabsorbent membranes in the column may be contacted with fresh buffer by circulating fluid from the pumping chamber into the working chamber. The fluid circulated into the pumping unit would contain proteins that were dissociated from the immunoabsorbent membranes under high pressure.

Figure 17. Detailed arrangement of the immunoabsorbent membranes forming a model chromatographic column in the working chamber of the High Pressure Cell. The working chamber was designed to contain a maximum of six immunomembranes. However, two immunomembranes bound enough protein to provide the necessary sensitivity required for detection and analysis. The membranes are held in position using an O-ring.

A more detailed drawing of the High Pressure Cell, shown in Figure 18, illustrates the method of pressurization, pressure measurement, fluid circulation under high pressure, and the control of fluid flow. Fluid in the vessel was pressurized by advancing the piston in the working chamber (part 11) with a hydraulic press. On-line pressure measurements were monitored by a pressure transducer (not shown). The port for the pressure transducer is shown by position 15 (Figure 18). By moving the piston (part 3) in the pumping unit, fluid was circulated by the piston ring (part 6) from the pumping unit to the working chamber. Two gland nuts (part 4) in the pumping unit supported the high pressure Bal seals. Two stop nuts (part 10) directed the length of travel of the moving piston, allowing control of the amount of fluid displaced. The piston may be moved by hand, or be motor driven to provide uniform fluid flow in the working chamber.

The High Pressure Cell was designed to be operated at a maximum pressure of 50000 psi (3450 bar). The material chosen for the fabrication of the pressure cell was 431 stainless steel since it has excellent corrosion resistance properties against saline containing fluids (typically many biological buffers). The piston in the pumping unit was fabricated from a high strength steel (440 C stainless steel). The stresses in the working chamber and the pumping unit accompanied by a fluid pressure of 50000 psi were calculated using the model of a thick-walled pressure vessel (as in the Appendix to Chapter 1). Upon heat treating the 431 stainless steel (Rc 40-42), a safety factor of 3 was provided. Heat transfer considerations are also similar to those described in the Appendix to Chapter 1. The change in temperature of the fluid in the vessel with pressure was considered negligible.

Pressure Conditioning of Anti-BSA-FITC Antibody

Before being used in pressure elution experiments, the anti-BSA-FITC antibody was treated under high pressure conditions. (The detailed purpose of this procedure is discussed in Results. In this investigation, it was found that the fluorescence intensity of the FITC labelled anti-BSA antibody changed upon treatment with high pressure. Pre-pressurizing the antibody was used as a method to prevent the fluorescence from changing upon further exposure to high pressure). Using the pressure

Figure 18. Cross-section view of the High Pressure Cell with convective flow. The arrows indicate the direction of fluid flow created by an upward motion of the piston in the pumping chamber during pressure elution.

Features shown: (1) Working chamber (2) Pumping unit (3) Piston (4) Gland nut (5) Reversed backing ring and Bal seal (6) Piston ring (8)(12) Cap screw and washer (9) Thumb nut (10)(14) Stop nut and set screw (11) Intensifier piston assembly (13) Seal ring (15) Pressure transducer port.

vessel developed in Chapter 1, 1 ml of 0.15 M Bis-Tris (pH 7.3) containing 500 µg/ml of antibody was pressurized for 30 min at 1724 bar. A portion of the pressurized sample was set aside for the preparation of standard curves, while the remainder was used for incubation with the BSA immunomembranes in pressure elution experiments.

Pressure Elution

Four immunomembrane (antigen immobilized cloth) disks (10 mm) were placed in a petri dish and incubated with 60 µl of appropriate antibody in the dark for 1 hour at room temperature. Disks containing covalently bound BSA were incubated with pressure conditioned anti-BSA-FITC antibody diluted to a concentration of 500 µg/ml in 0.15 M Bis-Tris, while immunomembranes with covalently bound bovine IgG were incubated with anti-bovine IgG-FITC diluted 1:10 from serum in 0.01 M PBS containing 0.05% Tween 20 (PBST). Two immunomembranes were used in each experiment to provide the required sensitivity for antibody detection. After incubation, the two membranes were washed five times with a total volume of approximately 1 ml of PBST. The pressure vessel was filled with 4.5 ml of 0.15 M Bis-Tris (pH 7.3). After ensuring that the pumping unit, the working chamber, and all the feed lines were completely filled with fluid, a small amount of fluid was removed from the working chamber and the two immunoadsorbents were then packed in the chamber and secured with an O-ring. The fluid was then returned to the working chamber. The piston in the pumping unit was set to its lowest position and was capable of displacing 1.2 ml of fluid. The pressure in the vessel was increased to 1724 bar at a rate of approximately 550 bar/min and remained at that pressure for 30 min. In some experiments, a small amount of fluid was flushed downwards through the membranes and then returned by reversing the piston action. This 'mixing' was accomplished by displacing the piston in the pumping unit 15 % of its full travel (displacing approximately 0.2 ml) forwards and backwards 3 times every 10 min at a rate of approximately 4 ml/min prior to elution. After 30 min, the piston in the pumping unit was raised by a platform that was driven upwards by a power screw coupled to a reversible motor. Fluid was displaced across the immunomembranes at a flow rate between 0.5 and 4 ml/min (superficial velocities between 0.0092 and 0.073 cm/s). After the piston had completed its motion, the fluid was

returned to atmospheric pressure at a rate of 350 bar/min. The intensifying piston was removed and the fluid at the top of the working chamber was collected as the top fraction of the buffer. Upon removal of the immunomembrane disks, the piston in the pumping unit was reversed and the bottom fraction of the buffer was collected. The pressure vessel was then thoroughly washed with distilled water and the entire experiment was repeated at ambient pressure with the two remaining immunomembranes for comparison. All experiments were conducted at room temperature.

Spectrofluorometric Assay

Emission was measured in a Farrand spectrofluorometer using a excitation wavelength of 490 nm and an emission wavelength of 525 nm.

The fluorescence of the unbound antibodies contained in the top or bottom fractions of the buffer were measured. The fluorescence of the anti-BSA-FITC antibodies was measured against standard concentrations of the antibody in the Bis-Tris buffer. To remove the antibodies still bound to the immunomembrane disks, each membrane was heated in 1 ml of 0.15 Bis-Tris (pH 7.3) containing 0.5% sodium dodecyl sulphate (SDS) at 100°C for 10 min. Unknown concentrations of anti-BSA antibodies removed from the immunomembranes were determined from fluorescence measurements of known antibody concentration standards treated in the same manner.

Enzyme Linked Immunosorbent Assay (ELISA)

In a 96-well polystyrene microtiter plate (Nunc 2-69620, Denmark), 100 µl/ well of 10 µg/ml BSA was left to stand for 3 hours at 37°C or overnight at room temperature. The wells were washed five times with PBST and were then incubated with 100 µl of 5 or 10 µg/ml anti-BSA-FITC in PBST for 1 hour at room temperature. The plate was then washed as above, and incubated with 100 µl/ well of protein A alkaline phosphatase conjugate diluted 1:2000 in PBST for 1 hour at room temperature. After washing with PBST, reactions were revealed by addition to each well of 100 µl of 0.39 % (w/v) *p*-nitrophenyl phosphate in a solution of 10 % (w/v) diethanolamine and 0.5 mM MgCl₂ (pH 9.8). After 15-30 min at room temperature, the signals (absorbance at 404 nm) were determined using a Titertek Multiskan plate reader.

Results

In order to remove proteins dissociated from the immunomembranes from the working chamber, it was necessary to devise a means of providing fluid flow across the column at high pressure. Since it was difficult to control fluid flow across the affinity column at high hydrostatic pressures by creating a pressure drop, a method of fluid displacement across the column at constant pressure was adapted.

For the fabrication of the High Pressure Cell, some special manufacturing techniques had to be developed to build the special detail features and to provide the required surface finishes necessary to accomplish proper sealing (A special thanks to Mr. Jim Ross., P.Eng., for helping with the general design and providing the necessary tolerances for the shop drawings. Credit must also go to the machinists in the shop of IME, NRC who did an excellent job in the fabrication of the High Pressure Cell). Side hole insertions into thick-walled pressure vessels may increase local stress concentrations, which may exceed the ultimate strength of the pressure vessel material, and lead to vessel failure. This was an important consideration in the design of the feed lines connecting the working chamber and pumping unit. To minimize stress concentrations, side hole orientations were made perpendicular to the bores in the pressure vessel and the edges of the side holes were rounded where the hole broke into the bores (adapted from Sherman and Stadtmuller, 1987). In addition to the consideration of the side hole entrances, special seals had to be used and are still under development in order to achieve a dynamic seal at the pumping piston shaft. The seals used in the first phase of testing of the High Pressure Cell failed at pressures beyond 1930 bar. Therefore, pressure elution experiments were conducted at 1724 bar (25000 psi). New seal materials have been developed with the supplier (Bal Seal Engineering Co., USA) but have not been thoroughly tested yet.

For pressure elution experiments, the immunoadsorbents used here were prepared by covalently immobilizing antigen (as in Chapter 3). Fluorescence measurements were used to detect the amount of bound and dissociated antibody fractions. The antibodies were conjugated with a

fluorescent label, fluorescein isothiocyanate (FITC). Fluorescence provided the necessary sensitivity to measure antibody concentrations without the need for pressure vessel decontamination that would be necessary if radioactive conjugates were employed as an alternative detection technique. The rabbit anti-bovine IgG-FITC conjugate used in this work was shown not to change its fluorescent properties or conformation under high pressure conditions (Chapter 1), making it well suited for detection after treatment with high hydrostatic pressures. However, as discovered during the investigation, this was not the case for the sheep anti-BSA-FITC antibody used in this work. In order to use this antibody in pressure elution experiments, it was necessary to pretreat the antibody under high pressure to ensure that its fluorescence properties and binding activity would remain constant after exposure to high pressure experimental conditions. This treatment was referred to as 'pressure conditioning' of the antibody.

Pressure Conditioning of Anti-BSA-FITC Antibody

Table 8 shows the change in fluorescence intensity of FITC labelled sheep anti-BSA IgG in 0.15 M Bis-Tris buffer after pressurizations of 30 min at 1724 bar. Similar changes in fluorescence intensity are observed after a single cycle of pressurization over the concentration range examined. A significant decrease in the fluorescence intensity of the anti-BSA-FITC antibody was apparent (approximately 56%). The change in fluorescence intensity of the antibody was observed to be irreversible over a period of 3 days. However, upon pressurizing the solutions containing the antibody a second time, the fluorescence intensity of the antibody remained constant. This finding was also observed for antibody solutions diluted to 500 µg/ml in the same buffer. Therefore, by pretreating the anti-BSA-FITC antibody with high pressure prior to conducting pressure elution experiments, the fluorescence intensity of the antibody was made constant upon further pressure treatments.

Table 8. The effect of pressure on fluorescence intensity of anti-BSA-FITC after pressurization cycles of 30 min at 1724 bar^a

Concentration ($\mu\text{g/ml}$)	Relative fluorescence intensity	
	One pressurization	Two pressurizations
2.5	0.46	
*5	0.44	0.44
10	0.42	
10	0.45	
*10	0.44	0.44

^aIndicated concentrations of antibody (in 0.15 M Bis-Tris, pH 7.3) were pressurized for one or two cycles using the pressure vessel described in the Methods of Chapter 1. After depressurization, the fluorescence intensity was measured as described in Methods. Intensities were normalized with respect to the fluorescence intensity of the sample at ambient pressure.

* Sample used in ELISA test to determine binding activity

In addition to considering the change in fluorescence properties of the antibody under high pressure, the binding activity of the antibody was also examined. Pressurized samples of antibodies that were measured for fluorescence were also tested for binding activity using an ELISA test as described in Methods. Antibodies pressurized at 1724 bar for one cycle were observed to have a lower binding activity (10-17% less) than control samples at ambient pressure. Pressurizing the same antibody solution a second time did not further alter the binding activity of the antibody as observed in the ELISA test (Table 9).

Pressure Elution of Anti-BSA Antibody from BSA Immunoabsorbent

The initial performance of the High Pressure Cell was tested by examining the parameters of elution rate and premixing of the fluid about the model column prior to pressure elution. Figures 19 and 20 represent the recovery of anti-BSA antibodies from the BSA immunoabsorbent under a pressure of 1724 bar at elution rates of 4 ml/min and 1 ml/min, respectively. Elution data were normalized to the amount of antibody eluted by pressure and by SDS treatment of the immunomembranes.

At an elution rate of 4 ml/min, the amount of antibody recovered using high pressure was approximately doubled when compared to the same elution rate performed at ambient pressure. The average recovery of anti-BSA antibodies eluted using high pressure was observed to be approximately 56% (Figure 20). Premixing the fluid prior to pressure elution increased the amount of recovered antibodies to 60%. At ambient pressure, approximately 30% of the antibodies were recovered, while premixing only marginally increased antibody recovery to 32%.

Table 9. The effect of pressure on the binding activity of anti-BSA-FITC after pressurization cycles of 30 min at 1724 bar^a

Concentration ($\mu\text{g/ml}$)	Absorbance ^b			
	One pressurization		Two pressurizations	
	Ambient sample	Pressure sample	Ambient sample	Pressure sample
5	1.25 $\pm$ .09	1.14 $\pm$ .09	1.27 $\pm$ .06	1.15 $\pm$ .08
10	0.99 $\pm$ .12	0.82 $\pm$ .05	1.01 $\pm$ .08	0.82 $\pm$ .04

^aIndicated concentrations of anti-BSA-FITC in 0.15 M Bis-Tris were pressurized for one or two cycles at 1724 bar for 30 min. The pressure cycled and control samples were then used in the ELISA test as described in Methods.

^bAverage absorbance at 404 nm $\pm$ standard deviation (n=3).

Absorbance measurements for the antibody concentrations of 5 $\mu\text{g/ml}$ were determined 30 min after addition of the enzyme substrate. Antibody concentrations of 10 $\mu\text{g/ml}$ were determined after 15 min.

Figure 19. Pressure elution of anti-BSA IgG at 4 ml/min. Prior to elution, the fluid surrounding the immunomembranes remained static (no mixing) or was premixed by displacing fluid back and forth across the model column 3 times every 10 min during pressurization (mixing). Elution data were normalized to the amount of antibody eluted by pressure and by SDS treatment of the immunomembranes. Vertical bars denote the S.D of the mean for 3 separate determinations.

Figure 20. Pressure elution of anti-BSA IgG at 1 ml/min. Prior to elution, the fluid surrounding the immunomembranes remained static (no mixing) or was premixed by displacing fluid back and forth across the model column 3 times every 10 min during pressurization (mixing). Elution data were normalized to the amount of antibody eluted by pressure and by SDS treatment of the immunomembranes. Vertical bars denote the S.D of the mean for 2 separate determinations.

Parallel pressure elution experiments with anti-BSA antibodies were employed using an elution rate of 1 ml/min. The recoveries of anti-BSA antibodies using high pressure were similar to the recoveries achieved at an elution rate of 4 ml/min (Figure 20). The average recovery at an elution of 1 ml/min under high pressure with no mixing was 57%, while with mixing the recovery was approximately 55%. Slightly high recoveries were observed for elutions at ambient pressure (approximately 40% with no mixing and 43% with fluid premixing).

Pressure Elution of Anti-bovine IgG Antibody from Bovine IgG Immunoabsorbent

A second polyclonal antibody-antigen system was examined for high pressure elution using the High Pressure Cell as a comparison with the polyclonal anti-BSA-FITC antibody - BSA antigen system. Immunomembranes containing covalently bound bovine IgG were saturated with FITC labelled bovine IgG antibodies and used in pressure elution experiments as outlined in Methods. Prior to all elutions, no premixing of the fluid through the immunomembranes was performed. Figure 21 shows the recoveries of anti-bovine IgG antibodies at elution rates of 4 ml/min under pressure and ambient conditions and at 1 ml/min under high pressure only. The relative fluorescence intensities of the antibodies recovered by pressure elution were normalized using the fluorescence intensity of the antibodies eluted from the immunomembranes by SDS treatment.

At a pressure of 1724 bar, only 12% of the anti-bovine IgG antibodies were dissociated and separated from the bovine IgG immunomembranes at elution rates of 4 and 1 ml/min (Figure 21). The effect of high pressure on antibody recovery was marginal since only approximately 7% was recovered at an elution rate of 4 ml/min under ambient pressure conditions.

Figure 21. Pressure elution of anti-bovine IgG at 1 and 4 ml/min. Prior to elution, the fluid surrounding the immunomembranes remained static. The relative fluorescent intensities of the antibodies recovered by pressure elution were normalized using the fluorescence intensity of the antibodies eluted from the immunomembranes by SDS treatment. Vertical bars denote the S.D of the mean for 2 separate determinations.

Discussion

Using a flow of buffer to recover proteins dissociated by high pressure is believed to be a suitable approach for the application of hyperbaric immunoaffinity chromatography (HIAC). To demonstrate pressure elution using conventional chromatography techniques, a high pressure cell capable of displacing fluid was constructed. For a preliminary investigation, several design parameters were examined in order to determine effective operating conditions. These included the pressure at which to operate the cell, the flow rate of elution, and the fluid conditions surrounding the immunomembranes prior to pressure elution (static fluid versus mixing). A pressure incubation period of 30 min prior to elution was used in all experiments as a convenient processing time.

In this preliminary study using the High Pressure Cell, the highest observed recoveries were obtained with the anti-BSA-FITC - BSA antigen system. At an elution rate of 4 ml/min, approximately 60% of the antibodies were recovered at a pressure of 1724 bar (if the fluid surrounding the immunomembranes was mixed prior to elution). In contrast, 32% of the antibodies were recovered under the same elution conditions at ambient pressure (Figure 19). At both elution rates studied, the parameter of mixing did not strongly influence the amount of antibodies recovered since without mixing, only 2% fewer antibodies were recovered. This suggests that pressure is responsible for dissociating the antibodies from the antigenic determinants and that fluid movement prior to elution does not largely contribute to any further dissociation. The pores of the membrane are sufficiently large that premixing of the fluid surrounding the immunomembranes is not necessary to facilitate the passage of the antibodies from the matrix pores prior to elution.

Similar recoveries of anti-BSA-FITC antibodies were observed at an elution rate of 1 ml/min under conditions of equal pressure. It is apparent that the governing parameter contributing to the level of product recovery may be the operating pressure of the High Pressure Cell. Unfortunately, due to mechanical constraints of the high pressure seals, only one pressure was examined (maximum attainable pressure was 1724 bar). Olson et. al

(1989) investigated the pressure elution of BSA from a monoclonal antibody immunoabsorbent at different pressures. It was observed that different amounts of proteins were recovered at different pressures, with the highest recovery (75%) being achieved at the highest pressure (2026 bar). Moderate pressures were observed to provide intermediate recoveries. For example, at 1013 bar, only 50% of the proteins were removed from the immunoabsorbent. If these observed results are interpolated to predict the recovery that would have been achieved by Olson and coworkers (1989) at the pressure used in this investigation, 1724 bar, the projected recovery would be 66%. This result is consistent with the amount of protein recovered using the High Pressure Cell and a polyclonal anti-BSA-FITC system. However, the amount of protein recovered by Olson and coworkers (1989) at ambient pressure was under 20%.

Based on the observations of Olson et al. (1989) and that greater dissociation of protein aggregates is facilitated at higher pressures (Weber, 1992) improved recoveries using the High Pressure Cell may be achieved by operating the cell at higher pressures. The High Pressure Cell was designed to operate at a maximum of 3450 bar. Pressures in the range of 1724-3000 bar may potentially increase oligomeric protein dissociation without denaturing either protein (Fukushima et al., 1986; Weber and Drickamer, 1983).

The polyclonal anti-BSA-FITC antibodies used in this study were observed to dissociate relatively easily at ambient pressure (Figures 19 and 20) when compared to the polyclonal antibody systems observed in Chapter 3. It is possible that the majority of the polyclonal anti-BSA-FITC antibodies were low to medium affinity antibodies. This may contribute to the relatively large amounts of antibodies recovered under ambient pressure conditions (30-40%). One unexpected observation in this study was the change in fluorescence intensity of the polyclonal sheep anti-BSA-FITC antibodies after exposure to high pressure (Table 8). Chapter 1 demonstrated that IgG structures were resistant to conformational changes under increasing hydrostatic pressures due to their predominant β -sheet conformation. Without further study, one may only speculate that the change in fluorescence intensity of the sheep antibody indicates a

significant conformational change in the protein under high pressure. Although, conformational changes may have occurred, the binding activity of the antibody was not greatly disturbed (Table 9). It has been established that a sheep IgG is more unstable than a rabbit IgG under thermally denaturing conditions (Tijssen, 1985) and that the conformation of a rabbit IgG was demonstrated to be stable under conditions of increasing hydrostatic pressure (Chapter 1). Generally, thermal protein denaturation cannot be correlated with high pressure protein denaturation. Nevertheless, this observation suggests that the stability of different host polyclonal antibodies be considered when preparing immunoadsorbents for HIAC operations.

For comparison with the sheep polyclonal anti-BSA-FITC - BSA antigen system, a rabbit polyclonal anti-bovine IgG - bovine IgG antigen system was examined for dissociation and elution using the High Pressure Cell. The rabbit anti-bovine IgG sera was shown to remain tightly bound to the immunoadsorbent, since only a small percentage of the antibodies were recovered with pressure elution (Figure 21). It is possible that higher operating pressures may promote more of the antibodies to dissociate from one another. However, the pressure required to promote dissociation may approach the range that would lead to the unfolding of most protein monomers since each IgG conformation is relatively stable at high hydrostatic pressures (Chapter 1). It is conceivable that subtle changes in protein conformation are involved in the dissociation of some oligomeric proteins under high pressure.

As presented in Chapter 1, conformational stability (as detected by fluorescence, FT-IR spectroscopy, and antibody recognition) was used as a criterion in determining a protein's ability to withstand high pressure treatment (BSA was used as a model antigen). The secondary structure of an IgG (rabbit or bovine) was not substantially perturbed by pressures below 2 kbar. In contrast, the secondary structure of the BSA antigen was shown to undergo reversible changes after a 1h pressurization cycle at 2026 bar. Both the rabbit and bovine antibodies used in this investigation were shown to be insensitive to conformational changes under high pressure and did not readily dissociate at 1724 bar using the convective High Pressure Cell.

However, the BSA antigen-antibody system was observed to dissociate under high pressure conditions. One may speculate as to whether a subtle conformational change is necessary for oligomeric protein dissociation.

Ismail (unpublished results) examined a rabbit polyclonal antibody-BSA antigen complex under high pressure using FT-IR spectroscopy. FT-IR spectroscopy may be used to detect secondary structure changes in proteins. One difficulty in using this technique with more than one protein is that the bands in the amide I region that are used to indicate the type of secondary structure overlap. Also, it is not possible to detect dissociation since no observed spectral changes to indicate dissociation are known. However, some results of the investigation may be interpreted using the conformational changes of the BSA antigen and the IgG examined in Chapter 1. Below 2 kbar, the spectrum of the antigen-antibody complex in the amide I region did not change, indicating that a change in secondary structure of either protein was not detected. It is possible that the amount of pressure required to dissociate an antibody from its antigenic determinant may be such that the conformation of each protein is altered. These changes are not likely to occur in the binding site of the antigen or antibody since antigen-antibody complex did not reveal any changes in the secondary structure of either protein. However, these minor conformational changes (not detected at the secondary structural level) may change the tertiary structure of the protein as a whole, thus facilitating the separation of the antigen epitope from the antibody paratope.

Other subtle changes in protein conformation have been noted by other investigators. Weber (1992) has investigated the dissociation of several oligomeric protein systems (eg. β_2 -tryptophan synthetase, porcine and bovine lactate dehydrogenases, yeast hexokinase) under high pressure and the biological activity of the oligomer after pressure release. It was concluded that during the dissociation of protein aggregates under high pressure, the subunits undergo a progressive change in conformation causing a loss in affinity. However, upon return to ambient pressure, a conformational readjustment was observed restoring the original biological properties of the aggregate.

In any case, these observations demonstrate the need to screen systems before considering their use in high pressure immunoaffinity chromatography processes. Given a compatible system, immunoaffinity chromatography employing high pressure elution with a convective flow of buffer has the potential to be an effective technique for purifying valuable protein products.

General Discussion and Conclusions

The major cost of an immunoaffinity chromatography process is the immunoabsorbent itself. More specifically, the immunoabsorbent is expensive to fabricate and the binding activity is usually diminished through cyclic use with conventional harsh chemical elution techniques employed in the product recovery stage. The diminished performance of the immunoabsorbent is thought to occur due to denaturation of the affinity ligands, typically represented by antibody molecules. Alternative methods of affinity chromatography operations are essential for successful biotechnology processing. This thesis has examined biotechnological manipulations, and proposes two approaches for reducing the capital and operating costs of the immunoabsorbent. The approaches described include the use of polyclonal antibodies as an immunoabsorbent and the use of pressure elution for recovery of the dissociated protein product.

The difficulty in using polyclonal antibodies in immunoaffinity chromatography processes is that they consist of a heterogeneous population of antibodies, making process reproducibility and predictability problematic. In addition, a heterogeneous population of antibodies may bind to several different sites on the antigen due to multivalent interactions. This property of polyclonal antibody binding generally makes it especially difficult to elute the antigen. On the other hand, this feature makes polyclonal antibodies attractive for immunoaffinity purification since collectively they have a high affinity for the antigen and thus a high degree of purity may be achieved in one process cycle. The use of polyclonal antibodies in immunoaffinity chromatography offers several advantages over the use of monoclonal antibodies. In addition to being relatively inexpensive, polyclonal antibodies will bind most of the antigen in solution, whereas even at high antibody concentrations, monoclonal antibodies have difficulty since many types are observed to have low antigen affinity.

It was demonstrated that high pressure may dissociate a polyclonal antibody - antigen complex (Chapters 3 and 4). The use of high pressure in immunoaffinity purification has the potential to make the process more efficient. Pressure elution may provide an effective yet mild, non-destructive, means of recovering protein products that have dissociated from an immunoabsorbent. Therefore, immunoabsorbent costs may be

significantly decreased by extending the lifetime of the immunoabsorbent and by using an inexpensive source of antibodies (polyclonal antibodies).

To demonstrate a practical method of pressure elution, a convective high pressure cell was constructed. Moderate recoveries were achieved using a polyclonal antibody - BSA antigen system (approximately 60 %). For pressure elution to be truly competitive with conventional elution techniques, recoveries in the range of 80-90 % are usually more desirable. But, pressure elution may allow for long-term use of the affinity adsorbent matrix thereby allowing for somewhat lower yields to be acceptable.

It is possible that higher operating pressures may achieve better recoveries employing the model antigen-antibody systems used in this work. However, the results of this work indicate that it is necessary to screen for antigen-antibody systems that are compatible with pressure dissociation and elution operations. Reasonable evidence for this statement is provided from the recoveries observed between the two distinct antigen-antibody systems (IgG-BSA versus IgG-IgG) examined for elution using high pressure. The screening of different oligomeric protein systems would have to determine the capability of high pressure to overcome the protein-protein interactive forces that resist dissociation and to ensure that dissociation occurs below protein denaturing pressures.

The work described in this thesis has:

- established that high pressure did promote the dissociation of polyclonal antibodies from antigens, offering their potential use in high pressure immunoaffinity chromatography processes
- outlined the construction and preliminary operation of a unique and patentable High Pressure Cell that offers a practical means of pressure elution for immunoaffinity chromatography processes
- offered methods of pre-screening antigens and antibodies for evaluation of their use in high pressure immunoaffinity chromatography processes (Eg. FT-IR spectroscopy).

The use of high pressure in immunoaffinity chromatography has the potential to compete with and outperform other conventional protein purification processes given that a compatible antigen-antibody system is used. The preliminary work conducted in this thesis sets a basis for future investigations involving pressure elution in immunoaffinity chromatography processes.

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