OPTIMIZATION OF METAL DEPENDENT ANTIBODIES FOR CHROMATOGRAPHY

by

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(ABSTRACT)

This study focuses on the utilization of metal-dependent monoclonal antibodies for large-scale chromatography and addresses an aspect that has been cited to lower immunosorbent performance, namely "orientation" of antibodies on matrices. The antibodies used in this study, the "EDTA-dependent" 7D7B10 and the "Ca^{2+}-dependent" HPC4 are directed against human Protein C (PC).

The 7D7B10 antibody was characterized in terms of its metal-dependency and specificity. The region of PC (epitope) recognized by 7D7B10 was identified as the first 15 residues in the NH_2-terminal. Immunosorbents made with 7D7B10 provided highly pure and functional PC.

The "orientation" of the antibodies on matrices was addressed in two ways. In the first approach, performance of immunosorbents coupled through carbohydrate moieties were compared with immunosorbents coupled through peptide regions. Coupling via carbohydrate linkages, which is generally believed to be Fc-directed, did not have any advantage in terms of efficiency and recovery over coupling via peptide.

The second approach was aimed towards sterically masking antigen-binding sites of the antibody during the coupling reaction. This required the synthesis of an orientation directing agent (ODA) with the ideal dual properties of being noninteractive with the immobilization matrix while retaining the affinity for the antibody. The ODAs synthesized were PC epitopes for the antibody and water-soluble polyoxazoline polymer-peptide adducts containing these epitopes. The HPC4 antibody and its epitope (which had previously been identified) were selected for characterization of the functionality and effectivity of the ODAs. The polymeric adducts retained affinity for the antibody and exhibited "metal-dependency" similar to the PC-HPC4 system. Although the hydrophobicity of larger polymeric adducts had the disadvantage of non-specific interaction with the matrix, use of the epitope and some polymeric adducts for antibody orientation on non-porous matrices gave a 2- to 5-fold increase in bound antibody activity.

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TO AMMA AND APPACHI

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CHAPTER ONE

BACKGROUND

INTRODUCTION

Separation processes involving complex biological macromolecules such as proteins necessitate not only comprehension of the engineering aspects of separation, but also the biochemistry and physiological functions of those compounds. Most well established chemical separation processes are not suitable for protein purification as they can cause denaturation and loss of biological activity of proteins. Immunoaffinity techniques using immobilized antibodies are simple and efficient methods of protein purification that are suitable for large-scale production of proteins. However, application of this technique for large-scale protein purification requires the development of more efficient and reproducible immunoaffinity matrices. The purpose of this study is to develop a more efficient immunoaffinity matrix to obtain a pure and functional protein. The protein of interest is Protein C, a vitamin K-dependent, anticoagulation plasma protein that is important in the control of hemostasis. As the developmental aspects of this study require an awareness of the complex biochemical and structural properties of Protein C, a brief description of hemostasis, vitamin K-dependent proteins and Protein C is included in the introductory section. Each of the following topics are discussed briefly in the following sections.

- Overview of hemostasis and vitamin K-dependent proteins.
- Structure, activation and importance of Protein C.
- Overview of immunoaffinity separation.
- Current research in immunoaffinity techniques.
- Protein C purification.

OVERVIEW OF HEMOSTASIS AND VKD PROTEINS

VKD proteins

Vitamin K-dependent (VKD) proteins are an important class of trace plasma proteins that are necessary for normal hemostasis, the ability to rapidly seal a ruptured blood vessel without blocking normal blood flow. The VKD family consists of Prothrombin (Factor II), Factor VII, Factor IX (Christmas Factor), Factor X (Stuart Factor), Protein C, Protein S and Protein Z. Factors II, VII, IX and X are procoagulant proteins, while Protein C and Protein S are anticoagulants. The physiological function of Protein Z has not yet been determined.

VKD proteins are a structurally similar family of serine proteases that are synthesized in the liver as inactive zymogens (1-7). These proteins have similar physicochemical properties such as molecular weight and isoelectric point. They share extensive regions of sequence homology in their primary structure, in particular near the N-terminus region of the protein (8-14), and in the peptide segments containing the active site (10, 15-22). A unique characteristic of these proteins is the vitamin K-dependent post-translational carboxylation of glutamic acid residues near the N-terminus. These gamma-carboxyglutamic acid ("gla") residues impart calcium and phospholipid binding properties that are necessary for the biological activity of these proteins (23, 24).

Hemostasis

Hemostasis (see 25, 26 for reviews) is rigorously controlled to localize the clot formation to the site of vascular injury while preventing occlusion elsewhere. This delicate balance is achieved through the pro-clotting and anti-clotting cascades where zymogen forms are sequentially activated.

The clotting cascade (see figure 1) can be broadly divided into two pathways; the intrinsic pathway which requires blood components present in the circulation, and the extrinsic pathway which requires an additional tissue factor (27-29). The intrinsic system consists of three stages:
(1) The generation of activated Factor XI through a contact system consisting of a cofactor, high-molecular weight kininogen and three zymogens, prekallikrein, Hageman Factor (XII) and Factor XI.
(2) Formation of thrombin through the calcium and membrane dependent sequential activation of the VKD proteins, Factors IX, X and prothrombin.
(3) Controlled proteolysis of fibrinogen which results in the formation of a fibrin clot.

In the extrinsic pathway, Factor VII directly activates Factor X in the presence of tissue factor. This results in the activation of prothrombin and the subsequent formation of fibrin. This "by-pass" reduces the clotting time from minutes to a few seconds.

In the anti-clotting pathway, thrombin forms a complex with the cofactor thrombomodulin, an endothelial cell surface receptor protein (see 30 for review). The thrombin-thrombomodulin complex activates Protein C. Activated Protein C (APC) proteolytically inactivates two non-vitamin K-dependent proteins, activated Factor VIII ($VIII_a$) and activated Factor V (V_a), which results in the retardation of clot formation (31-35). In addition to this anticoagulant action, APC enhances fibrinolysis by neutralizing an inhibitor of plasminogen activator. The anti-clotting pathway regulates the clotting response through a feedback loop initiated by thrombin, the final enzymatic product of the clotting cascade. Thus, hemostasis requires the delicate interplay of clotting and anti-clotting pathways for properly controlled clotting response.

PROTEIN C

Structure

Protein C (see 36 for review) is present in human plasma at a concentration of 4 mg/liter (37-39). It is a 62,000 molecular weight glycoprotein consisting of a 20,000 molecular weight light chain and a 42,000 molecular weight heavy chain. Both chains of Protein C are glycosylated and are linked by a single disulfide bond (37, 40, 41). The Protein C molecule contains about 23% carbohydrate (37).

The N-terminus of the light chain of Protein C contains 9 gamma-carboxyglutamic acid residues, a result of the vitamin K-dependent

carboxylation of the glutamic acid residues. This "gla" domain is highly conserved among the VKD proteins, and is essential for the calcium binding and phospholipid interaction required for biological activity. The light chain of Protein C also contains two domains with sequence homology to the epidermal growth factor (EGF). These domains are also highly conserved among the coagulation proteins, with the exception of prothrombin. The function of the EGF-like domains is not yet known. The light chain of Protein C contains an unusual amino-acid, beta-hydroxyaspartic acid, at position 71 (42). The serine protease part of Protein C is located in the heavy chain in regions around the catalytic site. This also exhibits sequence homology to the other VKD proteins as well as to the serine proteases trypsin and chymotrypsin (37, 43, 44).

Activation

Upon activation of Protein C, the arginine^{12}-leucine^{13} bond is cleaved (37). Activated Protein C (APC) is a serine amidase, possessing esterolytic and anticoagulant activity (45-47). Thrombin activation of Protein C is slow and is inhibited by calcium concentrations present in plasma (45, 48-50). However, the thrombin-thrombomodulin complex activates Protein C rapidly in the presence of calcium (51-54). This is thought to be the *in vivo* mode of activation of Protein C. The major procoagulant function of thrombin, namely the conversion of fibrinogen to fibrin, is neutralized when thrombin binds to thrombomodulin. This provides a rapid and direct inhibition of fibrin formation while promoting the activation of Protein C and thus the anticoagulant pathway. Activated

Factor V can also act as a cofactor in the thrombin mediated activation of Protein C (55-56). Alternatively, Protein C can be activated by the Factor X activator from Russells viper venom, by the Protein C activator in the venom of Agkistrodon contortrix (57), and by trypsin (45).

APC enhances clot lysis by neutralizing an inhibitor of tissue plasminogen activator, plasminogen activator inhibitor type-1 (PAI-1). This increases the activity of circulating tissue plasminogen activator (t-PA), which facilitates fibrinolysis by promoting the conversion of plasminogen to plasmin (58-61).

Protein S, another VKD protein, acts as a nonenzymatic cofactor of APC by increasing the affinity for cell surfaces, thereby enhancing the inactivation of V_a and X_a (62-64). About 60% of the Protein S found in plasma is bound to a complement protein, C4b-binding protein, which interferes with its cofactor activity (65-67). The possible significance of this complex in APC regulation is not yet known.

Clinical Significance

The importance of Protein C as a major regulatory protein of thrombosis and hemostasis was underscored with the discovery of inherited Protein C deficiency in a family with a history of venous thrombosis (68, see 69 for review). In the United States of America, approximately 12 infants a year are born with severe, homozygous Protein C deficiency. Homozygous Protein C deficiency causes death at infancy due to severe

thrombotic complications. The clinical manifestations of heterozygous Protein C deficiency are less distinct (70-72). Although all heterozygotes do not exhibit disease states, those with less than half normal Protein C levels tend to have thrombotic disease. In addition to inherited deficiencies of Protein C, certain conditions such as disseminated intravascular coagulation, acute defibrination and chronic liver disease cause a significant decrease in Protein C levels (73-75). Protein C antigen values have also been observed to fall after surgery (74).

The half-life of infused Protein C is approximately 6-8 hours (76-78). This remarkably short half-life increases the need for large amounts of Protein C for use as a potential thrombolytic drug. Assuming that Protein C therapy extends the life of homozygous infants, about 1 kg per year is required to maintain 100 individuals at normal plasma levels.

IMMUNOAFFINITY SEPARATION

Description

Antibodies are a class of proteins known as immunoglobulins. They are synthesized by lymphocyte cells in response to a "foreign" substance (antigen). The antibody and antigen form a non-covalent complex held together by electrostatic and hydrophobic interactions, van der Waals and London forces, and hydrogen bonds (79). Immunoaffinity separation matrices are formed by immobilizing antibodies on an inert matrix to form

an adsorbent that is specific for the corresponding antigen. Such a matrix is termed an immunosorbent. On contact with a mixture of proteins, only the specific antigen is bound by the immunosorbent. Subsequent elution of the antigen results in the purification of that protein. Due to the specificity of the antibody-antigen interaction, immunoaffinity purifications yield a highly pure product in a single step.

Polyclonal antibodies

Antibodies synthesized in response to an antigen form a heterogeneous population as they are produced from a variety of lymphocyte cell lines. These antibodies are therefore polyclonal, with a range of antigen recognition sites (antigenic determinants), binding affinities and multivalency (79-81). The heterogeneity of polyclonal antibodies not only varies from animal to animal, but also varies within an animal according to the length of exposure to the antigen. As each batch of polyclonal antibody differs from another, an immunosorbent formed with polyclonal antibodies has a variable distribution of binding properties. Although such matrices have been successfully used for protein purification, the characterization of a polyclonal immunoadsorbent remains complex and difficult. These systems do not yield readily to mathematical analysis for scale-up studies.

Monoclonal antibodies

When an antibody producing cell is fused with a cancerous myeloma cell, a hybridoma cell is produced. Its progeny produces homogeneous

antibodies from a single lymphocyte cell line. These monoclonal antibodies are chemically identical and therefore possess identical binding affinities for a unique antigenic determinant (see 82, 83 for review). Due to the homogeneity of monoclonal antibodies, their application in immunoaffinity chromatography has several advantages over polyclonal antibodies. The antigen-antibody interaction can be controlled by selecting a monoclonal antibody with a desired binding affinity or a desired antigenic determinant. As these antibodies have fixed kinetic parameters associated with the binding reaction, they are useful for the modeling and design of large-scale immunoaffinity processes. The chemically identical monoclonal antibodies offer a degree of reproducibility for different batches of antibody that is not possible with the heterogeneous polyclonal antibodies.

Advantages of immunoaffinity methods

Immunoaffinity separations using polyclonal or monoclonal antibodies result in a highly pure protein in a single process step. In comparison, several process steps are required to achieve the same degree of purity by conventional purification methods (such as ion-exchange chromatography, gel filtration, salt precipitation etc.) The additional process steps required in conventional methods increase production times and costs. The complexity of scaling-up several process steps as compared to a single immunoaffinity step further enhances the desirability of immunoaffinity processes in large-scale protein purifications.

Limitations

Several problems are inherently associated with immunoaffinity processes. The most common problems are the need of harsh elution conditions to disrupt the strong interactions of the antibody-antigen complex in order to release the bound protein, and the practical difficulties encountered in engineering an efficient immunoaffinity support. Harsh elution conditions (such as high or low pH, high ionic strength and use of chaotropic agents) result in the loss of functionality of the protein. The need for harsh elution conditions is eliminated by the use of metal-dependent antibodies (85-91). These antibodies require the presence (or, in some cases, the absence) of low concentrations of metal ions such as calcium to bind antigen. The antigen-antibody complex is simply disrupted by chelating the metal ions (or by adding metal ions) using a mild buffer. These mild elution conditions yield highly functional and pure proteins.

Effective immunosorbent matrices for large-scale separations should exhibit good flow properties, chemical and mechanical stability, minimum non-specific interactions, and high functionality. The flow properties and mechanical stability depend to a large extent on the type of inert matrix used for immobilization while the chemical stability depends on the nature of the coupling chemistry employed. Considerable effort has been directed into the study of these areas. A variety of agaroses, celluloses, polyacrylamides, glass beads, silica, non-porous and cross-linked supports with a choice of coupling agents are commercially

available. The advantages and disadvantages of these will not be discussed here as they are beyond the scope of the stated objectives.

The density, distribution and orientation of the immobilized ligands greatly affect the performance and functionality of the affinity column (see figure 2). Unfortunately these parameters have not yet been fully optimized. Most coupling processes used give randomly oriented antibodies. This results in some of the antibody being oriented such that its active site (antigen recognition site) is inaccessible to the antigen. Similarly if the antibody density is too high, steric hindrance caused by neighboring antibody molecules prevent the targeting of the antigen to the active site. Multipoint attachment of the antibody to the matrix could cause distortion of the antibody, thereby diminishing or eliminating the affinity for the antigen. An added complexity is caused by the distribution of the antibody on the surface as well as in the pores of the matrix. Due to the problems mentioned above, the performance of immunoaffinity resins often do not reflect the amount of antibody immobilized on the matrix. Better engineering of antibody linkage to matrices are needed to maximize the use of expensive antibodies and matrix performance.

CURRENT RESEARCH

Considerable attention has been devoted towards studying the effects of particle size, porosity, shape, types and cross-linking of support

material, and the chemistry of coupling on the performance of affinity matrices. These subjects address the problems of pressure and flow characteristics, stability, and the chemical and mechanical properties of support materials. As these do not directly address the problems of orientation and spacing of ligands (antibodies) on matrices, this large body of literature has not been included in the subsequent discussion.

A general approach that has been widely used in affinity chromatography is the use of spacer arms to prevent steric hindrances of the support material. These spacer arms (usually consisting of hydrocarbon chains) have greatly enhanced the performance of affinity matrices where the immobilized ligand has been relatively small. As immunoglobulins are larger than most affinity ligands, the inclusion of spacer arms is generally considered unnecessary, as the antigen recognition site of the immobilized antibody is located a sufficient distance away from the matrix (92). This has been substantiated by the ability of $F(ab')_2$ fragments obtained by pepsin hydrolysis of intact immunoglobulins to form functional immunosorbents. Kennedy and Barnes (93) immobilized the $F(ab')_2$ fragment of anti-human immunoglobulin G (IgG) on CNBr activated Sepharose 4B. The resultant immunosorbent had negligible nonspecific adsorption and purified IgG from human serum in high yields and purity, indicating that immunosorbents formed from $F(ab')_2$ fragments behave similarly to those formed from intact immunoglobulins. However, results contradicting this theory have also been presented. Comoglio et al. (94) compared the immobilization of anti-estradiol

immunoglobulins on Sepharose containing N-hydroxysuccinimide ester spacer arms with CNBr-activated Sepharose. Although both coupling methods resulted in a significant loss of antigen binding sites, the affinity constant of the immunosorbent containing spacer arms was comparable to that of the original antibody, while that of the CNBr-activated immunosorbent was three times lower. These results suggest that a spacer arm effectively safeguards the affinity of residual sites by locating the ligand away from the matrix and thereby improving the performance of an immunosorbent.

The ability of Protein A from Staphylococcus aureus to bind specifically to the Fc portion of immunoglobulins (95-97) has been exploited to orient immunoglobulins on a matrix via the Fc portion of the antibody. In these studies, IgG molecules were linked to Protein A derivatized supports and cross-linked with reagents such as dimethylsuberimide (95) and dimethylpimelimidate (97) to stabilize the immobilized IgG-Protein A complex, thereby forming a permanent immunosorbent. The application of Protein A-Sepharose is limited to only certain subclasses of IgG that are able to bind to Protein A. Protein G, a streptococcal protein, has broader applicability as it is able to bind to the Fc portion of many types of immunoglobulins including all IgG subclasses of humans, mouse and rat (98, 99). The major disadvantages of using these immunosorbents for the purification of pharmaceuticals are the high cost of these secondary proteins and the potential danger of the products becoming contaminated by trace amounts of bacterial proteins.

Site-specific immobilization of immunoglobulins has also been achieved by linking antibody to the matrix through the carbohydrate moieties present in the Fc portion of the immunoglobulin. This type of Fc oriented immunosorbents are formed by linking $NaIO_4$ oxidized carbohydrate residues of immunoglobulins to hydrazide containing cross-linked agaroses (100). The vicinal hydroxy groups of the carbohydrate moieties of the antibody are oxidized to aldehydes which then react with the hydrazine groups of the support to form covalent hydrazone linkages. Immunosorbents formed this way have been reported to have a 2-3 fold higher capacity than standard immunosorbents (100).

The highly specific avidin-biotin interaction has been exploited in a variation to the above approach. In this, the carbohydrate moieties in the antibodies are biotinylated with hydrazine-biotin (101, 102) and then adsorbed on avidin- or streptavidin-coated matrices. The antibodies are noncovalently attached to the matrix through the strong affinity of the avidin-biotin interaction ($K_d=10^{15}$ M^{-1}) which can withstand extremes in pH, organic solvents, and other denaturing solvents (103). Both the hydrazide and the avidin-biotin method orient antibodies through the Fc portion of the antibody. The effect of this orientation on immunosorbent performance has however not been well documented.

Endo et al. have used reversible amino group-blocking agents such as 2,3-dimethylmaleic anhydride for protecting antigen binding activity during modification of the antibody for use as carriers of various

substances to tumors (104). This technique can also be applied to protect the active sites of antibodies prior to immobilization. Removal of the protective agent should result in an immunosorbent where the active sites are located away from the matrix.

In the above studies a direct comparison of the effect of antibody orientation on the immunosorbent performance was not made. Except for the antibody immobilization through the Fc portion of the immunoglobulin, These studies do not directly address the problems of orientation and spacing of antibodies on immunosorbents.

PROTEIN C PURIFICATION

Laboratory Methods

Protein C is a trace plasma protein with a concentration of 4 mg/liter in human plasma (37-39). Assuming a yield of 50%, 0.5 X 10^6 liters of plasma must be processed to produce 1 kg of Protein C. Current laboratory-scale isolation methods using classical methods of protein purification yield only mg quantities of Protein C. Laboratory-scale purification of Protein C generally comprises of cryo precipitation, barium citrate adsorption and elution, ammonium sulfate fractionation, DEAE-Sephadex anion exchange chromatography and sulfated dextran chromatography (10, 37, 102). Typically 10-15 liters of plasma has been processed by these methods with average Protein C recoveries of 12%. Due to the low overall yield, the number of processing steps, the difficulty

of the optimal scale-up of each successive step, and the long processing times involved, these classical processes are too complex and costly for large-scale manufacture of Protein C. In addition, the high degree of homology among the VKD proteins make the large-scale isolation of pure Protein C (devoid of pro-coagulation contaminants) even more difficult by classical purification methods as these methods tend to copurify related VKD proteins.

Large-scale manufacture

Affinity separations using monoclonal antibodies (see 103 for review) offer an attractive alternative to the conventional methods for purification of Protein C. These immunoaffinity methods are readily adapted for large-scale operations (104-106) and essentially yield a pure product in a single-step. All plasma processing methods must incorporate a viral inactivation step (using a solvent/detergent treatment) to inactivate potential contamination of the plasma pool by viruses such as the HIV and the hepatitis B virus. A volume reduction step using anion exchange chromatography to yield a VKD family concentrate, solvent/detergent treatment to inactivate viruses followed by an anti-Protein C monoclonal antibody immunoaffinity step is sufficient for the large-scale purification of Protein C from plasma. A further clean-up step to remove potential immunoglobulin leaching into the final product ensures the purity of the Protein C and completes the large-scale isolation process.

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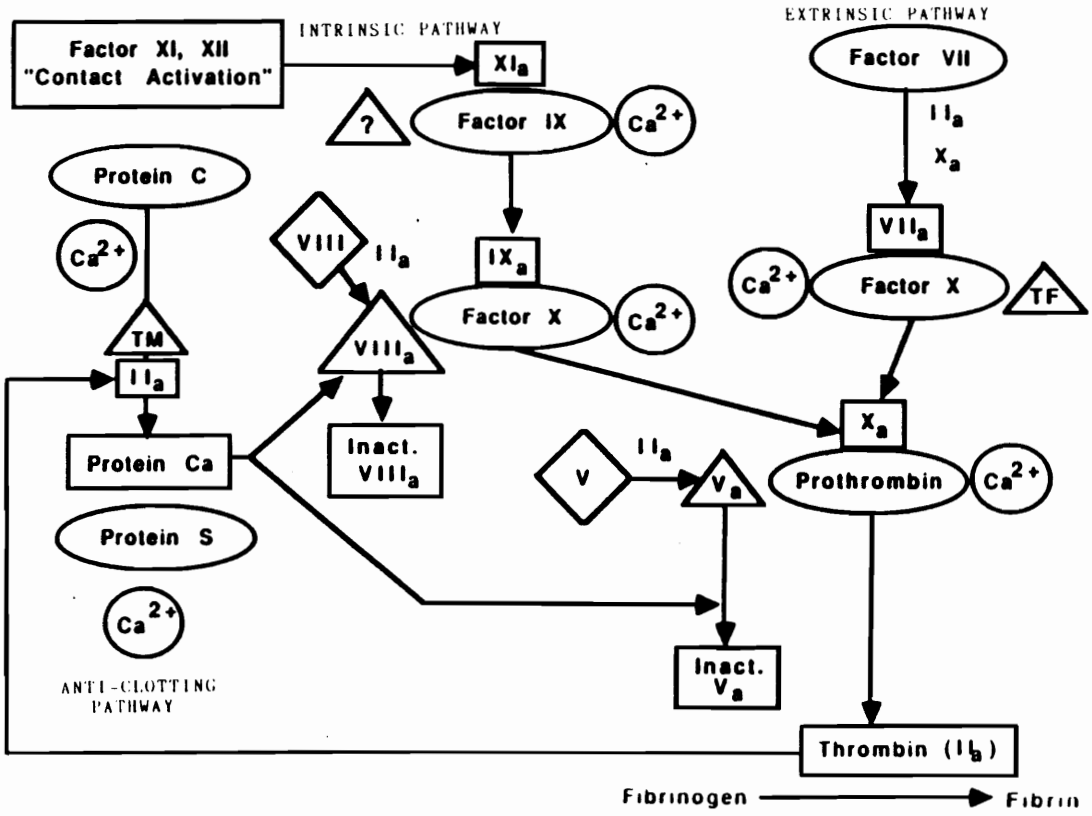

FIGURE 1

BLOOD COAGULATION PATHWAY

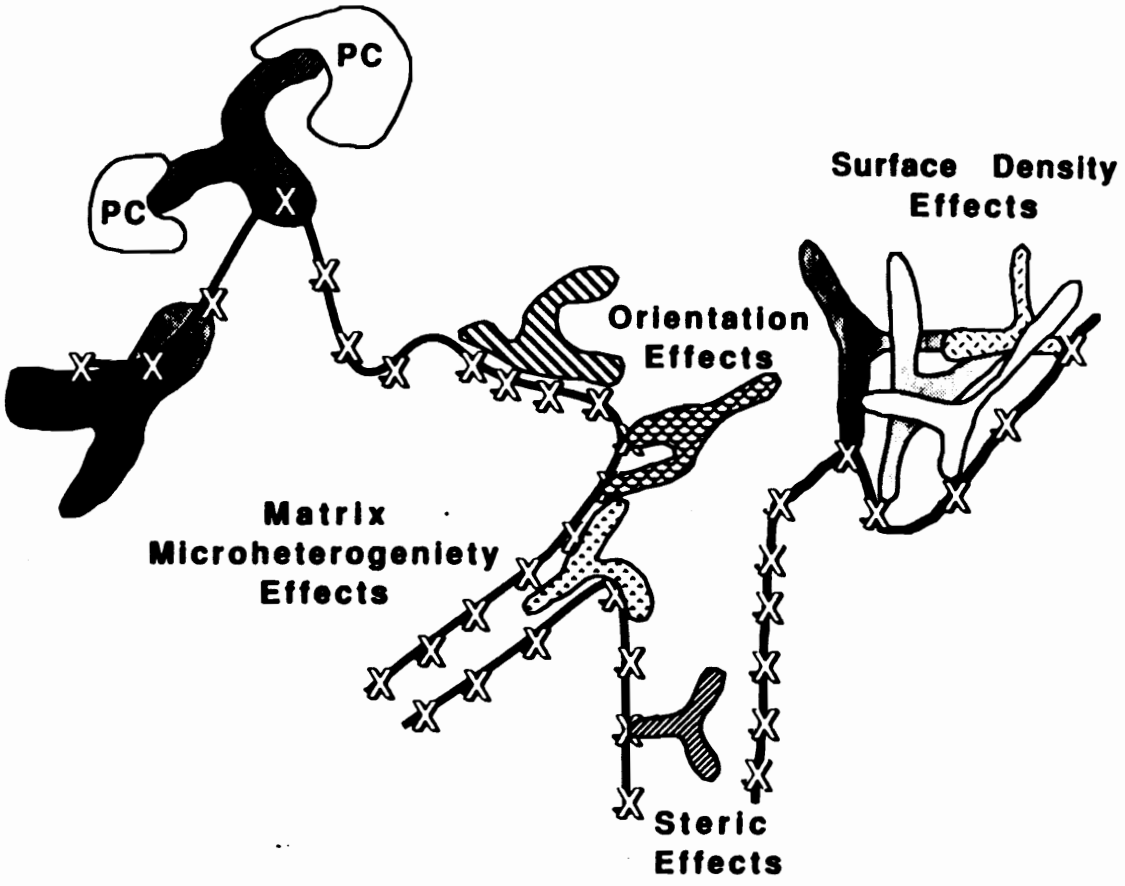

FIGURE 2

PROBLEMS IN IMMUNOSORPTION

CHAPTER TWO

CONFORMATIONAL CHANGES IN AN EPITOPE LOCALIZED TO THE NH_2-TERMINAL REGION OF PROTEIN C

Evidence for Interaction of Protein C Domains+

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ABSTRACT

Murine monoclonal antibodies, developed following immunization with human Protein C, were characterized for their ability to bind antigen in the presence of either $CaCl_2$ or excess EDTA. Three stable clones were obtained which produced antibodies that bound to Protein C only in the presence of EDTA. All three antibodies bound to the light chain of Protein C on immunoblots and also bound to the homologous proteins Factor X and prothrombin in solid-phase radioimmunoassays. One antibody, 7D7B10, was purified and studied further. The binding of 7D7B10 to human Protein C was characterized by a K_D of 1.4 nM. In competition studies, it was found that the relative affinity of the antibody for Protein C was 20-40-fold higher than for prothrombin, fragment 1 of prothrombin, or Factor X. In contrast, 7D7B10 was unable to bind to Factor IX or bovine Protein C. The effect of varying Ca^{2+} concentration on the interaction of the antibody with Protein C was complex. Low concentrations of Ca^{2+} enhanced the formation of the Protein C-antibody complex with half-maximal effect occurring at approximately 60 μM metal ion. However, higher concentrations of Ca^{2+} completely inhibited 7D7B10 binding to Protein C with a $K_{0.5}$ of 1.1 mM. Furthermore, millimolar concentration of Mn^{2+}, Ba^{2+}, or Mg^{2+} also completely abolished antibody binding to Protein C. The location of the epitope was delineated by immunoblotting and peptide studies and found to be present in the NH_2-terminal 15 residues of Protein C. Although residues corresponding to positions 10-13 of human Protein C were necessary for maximal binding of the antibody, they were not sufficient.

No evidence could be found for involvement of the epitope in metal binding *per se*. Therefore, the effect of Ca^{2+} on antibody binding is thought to be due to metal-dependent conformational changes in Protein C. It seems likely that Ca^{2+} occupation of a high affinity site, shown by others to be located in the epidermal growth factor-like domain, causes a conformational change in the NH_2-terminal region of Protein C which is favorable for antibody interaction, whereas Ca^{2+} binding to the low affinity site(s), known to be present in the γ-carboxyglutamic acid domain, causes an unfavorable conformational change.

INTRODUCTION

Protein C is a vitamin K-dependent plasma protein which plays an important role in hemostasis process. This protein shares extensive homology with regions of other vitamin K-dependent proteins at both the primary structure and gene levels (Fernlund & Stenflo, 1982; Stenflo & Fernlund, 1982; Foster & Davie, 1984; Long et al., 1984; Foster et al., 1985; Plutzky et al., 1986). However, Protein C functions as an anticoagulant (Kisiel et al., 1977; Walker et al., 1979; Suzuki et al., 1983; Vehar & Davie, 1980) and has been reported to enhance fibrinolytic activity (Comp & Esmon, 1981; van Hinsbergh et al., 1985). The NH_2-terminal approximately forty residues of human Protein C contains 9 gamma-carboxyglutamic acid (Gla)[1] residues, and are referred to as the "Gla domain". This region binds Ca^{2+} resulting in a conformational

change, and is important for the interaction of the molecule with phospholipid. The Gla domain is followed by two cystine-rich epidermal growth factor (EGF) homology domains that contain a single residue of beta-hydroxyaspartic acid (Drakenberg et al., 1983). This EGF-like region has been shown to contain a single high affinity binding site for Ca^{2+}, occupation of which also results in conformational changes in the molecule (Esmon et al., 1983; Johnson et al., 1983; Ohlin & Stenflo, 1987; Ohlin et al., 1988).

The heavy chain of Protein C is the serine protease homology domain, and cleavage of an Arg-Leu bond near the amino terminus of the heavy chain results in conversion of the zymogen Protein C to the enzyme activated Protein C (Kisiel, 1979). The activation reaction is dramatically affected by Ca^{2+} binding to the high affinity site in the EGF homology domain, although inhibitory or acceleratory effects are seen, depending upon the activating enzyme used (Esmon et al., 1983; Johnson et al., 1983; Orthner et al., 1988).

Several investigators have reported monoclonal antibodies to Protein C which bind to the protein only in the presence of metal ions (Wakabayashi, et al., 1987; Ohlin & Stenflo, 1987; Mimuro et al., 1987; Nakamura & Sakata, 1987; Stearns et al., 1988). These antibodies are thought to be conformation-specific ligands that bind to epitopes present only in metal-stabilized conformers of Protein C. More recently, a monoclonal antibody to Protein C has been described which

only binds to this molecule in the absence of divalent metal ions (Church et al., 1988). In this report, we describe the production and characterization of a unique monoclonal antibody to human Protein C whose interaction with the molecule is enhanced by divalent metal ions in the micromolar concentration range but inhibited by divalent metal ions in the millimolar concentration range.

EXPERIMENTAL PROCEDURES

MATERIALS

The chromatographic media DEAE Sephadex A-50, DEAE Sephacel and Sephadex G-100 were from Pharmacia LKB Biotechnology, Inc. Affi-Gel 10 and Chelex 100 were from Bio-Rad. Substrates and inhibitors were purchased as follows: H-D-Val-Leu-Arg-p-nitroanilide-2HCl (S-2266) was from Helena Laboratories, o-phenylenediamine was from Abbott Laboratories. TLCK was from Calbiochem. Proteins and immunological reagents were obtained as follows: alpha-chymotrypsin, from bovine pancreas, Type 1-S, was from Sigma; prothrombin complex concentrate (Proplex) was obtained from Hyland Division, Baxter Healthcare; Assera Protein C, a specific rabbit anti-human Protein C, was purchased from American Bioproducts; peroxidase-conjugated goat anti-mouse immunoglobulin, affinity purified, was from Cappel Laboratories; sheep anti-mouse immunoglobulin, affinity purified, ^{125}I-labelled F(ab')$_2$, 100 μCi/ml, 17 μCi/μg protein was from Amersham; mouse immunoglobulin typing

kit was from Boehringer Mannheim; specific rabbit anti-human prothrombin was purchased from Calbiochem.

METHODS

Monoclonal Antibody Production

Male BALB/c mice were immunized with three consecutive injections of purified human Protein C. The immunization schedule and fusion protocol were carried out as described previously (Strickland et al., 1988) except that the P3-NS1Ag 4.1 myeloma cell line was utilized. Cell supernatants were assayed using a solid-phase RIA in which wells were coated with 100 ng of human Protein C. Each supernatant was incubated in a buffer containing either 10 mM Ca^{2+} or 10 mM EDTA. Binding of the antibody to the wells was detected by adding a dilution (1/1,000) of rabbit anti-mouse IgG followed by ^{125}I-labeled Protein A (20,000 cpm/well). Preparation of the ascites fluid and purification of the IgG fraction were carried out as described previously (Strickland et al, 1988). IgG subtyping was performed by ELISA using mouse immunoglobulin typing reagents from Boehringer Mannheim according to the manufacturer's instructions.

Cell-sorting Analysis

The 7D7B10 hybridoma, cultured in flasks using serum-supplemented basal media, was subjected to flow cytometric analysis (Bio Response). The results showed a monomodal distribution of immunoglobulin expression, indicating a homogeneous population of cells.

Protein Purification

The following vitamin K-dependent proteins were purified from cryoprecipitate-poor human plasma. Protein C and Protein S were isolated by barium citrate precipitation and elution, ammonium sulphate fractionation and DEAE Sephadex A-50 chromatography (Thompson, 1977) followed by dextran sulphate chromatography (Kisiel, 1979). Activated Protein C was prepared by treatment of Protein C with 0.1% Protein C Activator (w/w) purified from the venom of Agkistrodon contortrix contortrix (Orthner et al., 1988). Prothrombin and Factor X were purified from prothrombin and Factor X-enriched pools resulting from the large-scale production of Factor IX Concentrate (Menache et al., 1984) by rechromatography on sulphated dextran (Miletich et al., 1980) followed by chromatography on G-100 Sephadex. Alpha-thrombin was purified as described previously (Orthner and Kosow, 1981). Fragment 1 of prothrombin was isolated as a by-product of the thrombin preparation (Aronson et al., 1980). Factor IX was purified from Proplex using the monoclonal antibody 1H5B7 coupled to Affi-Gel 10 by adsorption in the presence of 25 mM $MgCl_2$ and elution with 10 mM EDTA (Wang et al., 1987). Bovine Protein C was purchased from Enzyme Research Laboratories. The identity and integrity of the bovine protein were confirmed by SDS-PAGE, activity measurements, and by NH_2-terminal sequence analysis. All proteins were greater than 95% homogenous as judged by SDS-PAGE. Protein concentrations were determined spectrophotometrically by using the following molecular weights and absorption coefficients: Protein C, M_r 62000, $E_{280}^{1\%} = 14.5$ (Kisiel, 1979); Protein S, Mr 70000, $E_{280}^{1\%} = 9.5$

(DiScipio & Davie, 1979); prothrombin, Mr 72000, $E_{280}^{1\%}$ = 13.8 (Miletich et al., 1980); Factor X, Mr 59000, $E_{280}^{1\%}$ = 11.6 (DiScipio et al., 1977); Factor IX, Mr 57000, $E_{280}^{1\%}$ = 13.2 (DiScipio et al., 1977); alpha-thrombin, Mr 36500, $E_{280}^{1\%}$ = 18.3 (Fenton et al., 1977); Fragment 1, Mr 30000, $E_{280}^{1\%}$ = 10.5 (Marsh et al., 1981); mouse IgG, Mr 150000, $E_{280}^{1\%}$ = 15.5.

Proteolytic Digests

Alpha-chymotrypsin was treated with TLCK to inactivate any potential contaminating trypsin by incubating 2 mg/ml alpha-chymotrypsin with 1 mM TLCK in TBS at 37 °C for 3.5 h. Prothrombin digestions were performed by incubating 1.2 mg/ml prothrombin with 1.0 μg/ml TLCK-treated chymotrypsin in 0.05 M Tris-HCl/ 0.05 M NaCl/5.5 mM EDTA pH 7.5 at 37 °C for 2 h. Prothrombin was digested with alpha-thrombin under identical conditions, but using 2.0 μg/ml alpha-thrombin. Digestion of Protein C was done by incubating 0.73 mg/ml Protein C with 1.0 μg/ml TLCK-treated chymotrypsin in 0.05 M Tris-HCl/0.03 M NaCl pH 7.5 containing either 5.5 mM EDTA or 5.0 mM $CaCl_2$ at 30 °C.

Decarboxylation of Gla

The Gla residues in prothrombin were decarboxylated according to the method of Bajaj et al. (1982) by heating (110 °C, 15 h) prothrombin that had been lyophilized from 0.1 M NH_4HCO_3, pH 8.0. The Gla content of heat-decarboxylated and control prothrombin was determined by NH_2-terminal sequence analysis. The decarboxylation procedure resulted in

the quantitative conversion of Gla to glutamic acid residues based upon the fact that the observed repetitive yields for glutamic acid were in excess of 90% at all expected positions, with the exception of residue 19 (85% repetitive yield). The functional activity of heat-decarboxylated prothrombin was examined in initial rate studies of its activation by Factor Xa in the presence of phospholipid and varying concentrations of Ca^{+2} as described previously (Kosow & Orthner, 1979). The rate of activation of heat-decarboxylated prothrombin was insensitive to Ca^{2+}, in contrast to control prothrombin (treated identically with the exception of heating) and the initial prothrombin (no treatment) whose rates of activation were enhanced similarly at increasing Ca^{2+} concentrations.

SDS-PAGE and Immunoblotting

Proteins were separated on slab gels (14 x 10 x 0.15 cm) consisting of a 10% resolving gel and a 4% stacking gel using the buffer system of Laemmli (1970). Gels were either stained with Coomassie Blue R-250 or transferred electrophoretically to nitrocellulose (Schleicher and Schuell, ME 24, 0.2 μm) in a Trans-Blot apparatus (Bio Rad) at 30 V for 18 h with cooling using 25 mM Tris, 190 mM glycine, 20% methanol buffer (Towbin et al., 1979). The blot was blocked with PBS containing 5% (w/v) nonfat dry milk (PBS/milk) for 2 h. It was then incubated with 10 μg/ml mouse IgG for 2 h, rinsed three times for 10 min each, and incubated with a 1/1000 dilution of sheep, anti-mouse immunoglobulin, ^{125}I-labelled $F(ab')_2$, and again rinsed three times, 15 min each. All

incubations and rinses were performed at 25 °C utilizing PBS/milk. After drying, the immunoblot was inserted into an X-Omatic cassette (Eastman Kodak Co., Rochester, NY) containing a single Cronex Lightning Plus intensifying screen (Dupont) and Kodak XRP film and exposed at -70 °C.

ELISA

The data presented in Figs. 2,4,5,8 and Table III were derived from a sandwich ELISA. In this assay, wells of microtiter plates (Immulon II, Dynatech Laboratories) were coated with 100 μl of specific rabbit anti-human Protein C diluted 1/100 in 0.1 M sodium bicarbonate pH 9.6 for 1 h at 37 °C and blocked with TBS, 1% BSA for 1 h at 25 °C. Following washing, mixtures containing the monoclonal antibody 7D7B10 at final concentrations of 20, 10 or 5 μg/ml and varying concentrations of Protein C in 0.05 M Tris-HCl, pH 7.3, 0.1% BSA, 30 mM EDTA or varying concentrations of different divalent metal ions as indicated in the figure legends were added to the coated wells and incubated for 1 h at 25 °C with shaking at 1000 rpm. The wells were then aspirated and washed, and the amount of 7D7B10 present detected with peroxidase-conjugated goat anti-mouse immunoglobulin. In a variation of the sandwich assay, Protein C was incubated with 7D7B10 in the absence or presence of increasing concentrations of one of the homologous vitamin K-dependent proteins (see Fig. 5) or various peptides (see Fig. 8 and Table III). The Protein C-7D7B10 complex was captured and quantitated

as before. The data obtained for the binding of Protein C to 7D7B10 (see Fig. 2) were fit to the following equation (Ingham et al., 1988):

$$\Delta A = \Delta A_{max}\{[\text{Protein C}]/(K_D + [\text{Protein C}])\}$$

where A represents the percent of maximal binding (measured by percent change in absorbance), ΔA represents the maximal binding (measured by maximum change in absorbance), [Protein C] represents the concentration of Protein C, and K_D is the dissociation constant. Fitting was accomplished using nonlinear regression analysis, and once the fit was obtained, the best fit curve and data were plotted in the form of a semi-log plot.

Sequence Analysis and Peptide Synthesis

NH_2-terminal sequence analysis was performed by automated Edman degradation on a Beckman System 890M series Sequencer using a 0.1 M Quadrol program. The phenylthiohydantoin derivatives were analyzed by HPLC on an ODS Zarbax (C18) column. Peptides were synthesized by the Fmoc-polyamide method of solid phase synthesis using a Milligen 9050 Pepsynthesizer. Purity was assessed by HPLC on a Waters Maxima 820 system using a μBondapak C_{18} column. Amino acid analyses were performed using a Pico Tag Work Station. The amino acid content of all peptides was in agreement with the intended sequence.

Functional Assays

The effects of the monoclonal antibody on the functional properties of Protein C or APC were evaluated as follows.

Protein C activation assay

Protein C, 0.2 μM was incubated in 0.05 M Tris-Cl, 1 mg/ml polyethylene glycol 8000, pH 7.5 containing 5 mM EDTA in the absence or presence of a 10-fold molar excess of antibody for 20 min. PCA was added to a final concentration of 10 nM to start the reaction. At exactly 1 and 5 min later, the reaction was stopped by the addition of soybean trypsin inhibitor to 0.8 mg/ml, and APC was measured following the addition of NaCl to 0.2 M and the chromogenic substrate S-2266 to 0.9 mM.

APC amidolytic activity

APC (64 nM) was preincubated in the absence or presence of an 8.6-fold molar excess of monoclonal antibody in 0.05 M Tris-Cl, 0.15 M NaCl, 1 mg/ml polyethylene glycol 8000, pH 7.5 with 5.0 mM EDTA for 15-30 min in AquaSil™-treated cuvettes. The reaction was started by the addition of S-2266 substrate to a final concentration of 0.4 mM and nitroaniline formation was measured continuously at 410 nm on a Cary 219 spectrophotometer. Progress curves were linear, and initial velocities were determined.

Inactivation of Factor VII Cofactor Activity

Highly purified Factor VIII prepared by immunoaffinity chromatography (AFG-M, Hyland Division, Baxter Healthcare) was equilibrated in 0.05 M Tris-Cl, 0.1 M NaCl, pH 7.5 containing 0.5 mM $CaCl_2$ and 0.01% (v/v) Triton x-100 by gel filtration. APC (92 nm) was

preincubated in the absence or presence of a 16.5-fold molar excess of monoclonal antibody in equilibration buffer containing 0.5 mM $CaCl_2$ and 0.01% Triton X 100 (see above) in a total volume of 100 μl. After 20 min. 100 μl of Factor VIII was added to a final concentration of 6.0 IU/ml to start the reaction. This concentration of $CaCl_2$ was sufficient to stabilize Factor VIII, while not being high enough to inhibit the binding of antibody to APC. At varying times, aliquots (10 μl) were removed and assayed for the ability of Factor VIII to accelerate Factor IXa catalyzed activation of Factor X (Factor VIII cofactor activity) using protein, phospholipid, and substrate reagents supplied in the Coatest Factor VIII kit (Helena Laboratories).

RESULTS

Preparation and Partial Characterization of Monoclonal Antibodies

Hybridomas were prepared by fusing spleen cells derived from BALB/C mice immunized with Protein C with the P3-NS1Ag 4.1 myeloma cell line. Since the goal of the study was to obtain monoclonal antibodies sensitive to metal ion-induced conformational changes of Protein C, hybridoma supernatants were screened using a solid-phase RIA both in the presence of either 10 mM Ca^{2+} or EDTA. Hybridomas producing several different types of monoclonals were identified. Representative screening results are demonstrated in Figure 1. The majority of the antibodies bound to Protein C regardless of the metal ion content. A few antibodies required the presence of Ca^{+2} for binding (e.g., well

2A), and surprisingly, several antibodies bound to immobilized Protein C only in the presence of EDTA, with no binding detected in the presence of Ca^{2+} (e.g., wells 2E, 2G, F9, and F10). To illustrate the relative frequency of these types of antibodies, the numbers of wells containing cells from two separate fusions that produced antibodies to Protein C are summarized in Table I. The frequency of "EDTA-dependent" antibodies, measured from initial screening results, was 2.5-fold higher than that of the "Ca_{2+}-dependent" antibodies.

Three of the clones producing EDTA-dependent antibodies were examined in more detail. The specificity of these antibodies was examined by a solid-phase RIA in which microtiter wells were coated with either Factors IX, X, II, or Protein S. All of these proteins were purified to homogeneity and were depleted of potential trace contamination by Protein C by immunoaffinity chromatography prior to use. The results of these studies are summarized in Table II. All three clones showed cross-reactivity to prothrombin and Factor X but not to any of the other proteins tested. The hybridoma supernatants were also characterized as to immunoglobulin class and subtype by ELISA using specific antisera, and all three clones produced immunoglobulins that were of the IgG_1 subtype with kappa light chain of Protein C when assessed by Western blotting, one (7D7B10) was chosen for further characterization. The hybridoma was expanded from the ascitic fluid.

Specificity of 7D7B10

The specificity of this antibody was examined by immobilizing the antibody on Affi-Gel 10 (2mg of IgG/ml of gel). Either citrated plasma or Factor IX complex (Hyland Therapeutics) dialyzed previously against TBS containing 10 mM EDTA was applied to the column equilibrated in the same buffer. After washing, the bound protein was eluted with TBS containing 10 mM $CaCl_2$. Protein C activity coeluted with the absorbance at 280 nm. Analysis of the material by SDS-PAGE, Western blotting, and functional assays revealed that the major component was Protein C with significant amounts of prothrombin. Since these starting materials contained considerably more prothrombin that Protein C, these studies reveal that 7DB10 has a much higher affinity for Protein C than for prothrombin. In other experiments using purified proteins, it was found that the immunoadsorbent also bound APC as well as fragment 1 of prothrombin, both of which were eluted with a buffer containing $CaCl_2$.

The affinity of this monoclonal antibody for Protein C was measured in a sandwich ELISA. Fig.2 shows the results of an experiment in which a constant amount of antibody was incubated with increasing concentrations of Protein C. The data were fit as described under "Experimental Procedures" and are represented in the form of a semi-log plot. The results of the fitting process indicate a K_D of 1.4 nM. In order to compare the relative affinity of the antibody for Protein C with that of prothrombin, a competitive experiment was conducted in which 7D7B10 was incubated with Protein C immobilized on microtiter

wells in the presence of increasing concentrations of selected proteins (Fig. 3). These studies indicate that the relative affinity of 7D7B10 for Protein C is approximately 30-40 fold higher than its affinity for prothrombin. In contrast, Factor IX and bovine Protein C were unable to compete in this assay. Additional experiments demonstrated that prothrombin fragment 1 and Factor X were also able to inhibit the binding of Protein C to 7D7B10 with 50% inhibition occurring in the 1-3 μM range (data not shown). Furthermore, decarboxylated prothrombin, in which the Gla residues had been quantitatively converted to glutamic acid, competed equally well ($K_{0.5}$ = 3.6 μM) as control prothrombin ($K_{0.5}$ = 4.3 μM) (data not shown). Similar experiments demonstrated that Protein S was unable to compete for binding (data not shown).

Effect of Metal Ions on Protein C Binding to 7D7B10

Figure 4 shows the dependence of Protein C-7D7B10 complex formulation on the concentration of Ca^{2+}. Low concentrations of Ca^{2+} enhanced Protein C binding to 7D7B10 with a $K_{0.5}$ of 55 μM, whereas higher concentrations inhibited the interaction ($K_{0.5}$, 1.1 μM). The addition of EDTA in excess of added Ca^{2+} caused complete reversal of the inhibition with the appearance of the same signal as obtained in the absence of added metal ion. Figure 5 compares the ability of various divalent metal ions (chloride salts) to inhibit binding for Protein C to bind to the antibody since binding occurred at concentrations of various metal ions from 0.04 mM to approximately 1.0 mM. However, higher concentrations of divalent metal ions inhibited the interaction. Ca^{2+}

and Mn^{2+} behaved similarly, having $K_{0.5}$ values of 1-2 mM, whereas higher concentrations of Ba^{2+} or Mg^{2+} were required with $K_{0.5}$ values of 5 and 20 mM, respectively. In all cases, saturating levels of metals resulted in complete inhibition of Protein C binding to the antibody. In control experiments using radio-labeled Protein C, it was found that the capture step of the ELISA was not affected by these concentrations of the various metal ions.

Location of the Epitope Region for Monoclonal 7D7B10

Western blotting experiments revealed that 7D7B10 binds to the Protein C light chain (data not shown), demonstrating that this antibody binds to the amino-terminal portion of Protein C. Fig. 6 shows the results of SDS-PAGE analysis of prothrombin and its proteolytic fragments as detected by the Coomassie Blue staining or after immunoblotting. The epitope was detected in intact prothrombin 1-579 (*lane 10*) as well as in fragment 1, 1-155 (*lanes 12* and *14*) but not in prethrombin 1, 156-579 (50,000-Da position in *lane 12*) or thrombin 272-579 (*lane 13*). The reactivity to only a single fragment of the parent molecule is consistent with the monoclonal nature of the antibody. The epitope was further localized to the NH_2-terminal region of prothrombin by indirect evidence since chymotryptic cleavage of the Gla domain 1-44 resulted in loss of the epitope (*lane 11*). Likewise, degradation products of fragment 1, which arose upon prolonged storage at 4°C, were not detected by immunoblotting (*lane 14*). The degraded form having M_r of 22,000 is presumably fragment 1, 51-155 as has been reported by

others (Aronson *et. al*, 1980). Direct visualization of monoclonal antibody binding to the Gla domain 1-44 was not obtained, presumably due to the inefficiency of nitrocellulose in capturing smaller peptides. Further studies revealed that chymotrypsin treatment of Protein C, which removed the Gla domain, also resulted in a loss of the epitope upon Western blotting (data not shown), which is consistent with the immunoblotting data with prothrombin and suggests that the epitope is located within the Gla regions of both Protein C and prothrombin.

To confirm whether 7D7B10 binds to an epitope expressed within the Gla domain, synthetic peptides were employed. The synthetic peptides corresponding to residues 1-22 of human Protein C and 1-12 and 13-29 of human Factor VII, were generously supplied by Drs. William Church of the University of Vermont (Burlington). These peptides were coated to the wells of microtiter plates and increasing concentrations of antibody added to each well. The results of this study (Fig.7) revealed that 7D7B10 bound to the peptide representing Protein C, 1-22, but not to peptides representing Factor VII, 1-12, or Factor VII, 13-29. A more precise understanding of residues important for binding of the antibody to this portion of Protein C was explored by preparing various peptides from this region. For these studies, a competitive binding assay was performed measuring the ability of the synthetic peptide to compete with Protein C for binding to 7D7B10. The results are summarized in Table III and demonstrate that a peptide representing human Protein C, 1-15, was able to compete for binding, whereas no binding of the peptides

corresponding to human Protein C, 1-7, Protein C, 9-16, or Protein C, 10-22, was detectable.

The observation that 7D7B10 did not bind to bovine Protein C was pursued by examining the relative binding of peptides corresponding to human Protein C, 1-15, versus bovine Protein C, 1-15, by competitive sandwich ELISA. The results, shown in Fig. 8, indicated that although both peptides were able to compete with Protein C for binding to the antibody, the human Protein C peptide representing residues 1-15 had approximately a 100-fold higher affinity than the corresponding peptide based upon the bovine sequence, which differed only in the 4 residues at positions 10-13.

Effect of Monoclonal Antibodies on Protein C/APC Function

Preincubation of Protein C with excess 7D7B10 antibody in the presence of EDTA had no effect on Protein C activation by PCA. These experiments were done at low concentrations of Protein C such that an effect on the Km as well as the rate of the reaction would be observed. Likewise, the antibody had no effect on APC amidolysis of the chromogenic substrate S-2266. We also examined the effect of 7D7B10 on APC inactivation of immunoaffinity-purified Factor VIII, as measured by its cofactor activity in a purified Factor X activation assay. Preliminary experiments established a time-dependent decrease in Factor VIII cofactor activity which was dependent upon APC in a dose-dependent manner (not shown). Relatively high concentrations of APC were required

(46 nM) because the inactivation reaction was carried out in the absence of millimolar concentrations of Ca^{2+} and phospholipid, which accelerate APC inactivation of Factor VIII. This was necessary since these concentrations of Ca^{2+} inhibit antibody binding to Protein C. Under these conditions in which APC was incubated with Factor VIII in the presence of micromolar concentrations of $CaCl_2$, but in the absence of phospholipid cofactor, 7D7B10 had no effect on the inactivation of Factor VIII by APC.

DISCUSSION

In the process of developing monoclonal antibodies to human Protein C, antibodies were found which required the presence of Ca^{2+} to bind to Protein C, as has been reported by others. In addition, a different type of antibody was observed in which the binding of the antibody to Protein C was inhibited by Ca^{2+} in the millimolar concentration range. In the screening assay, these antibodies bound to Protein C only when EDTA was added in excess to the 2 mM Ca^{2+} present as a constituent of the hybridoma culture media. The frequency of such clones, loosely referred to as "EDTA-dependent" clones was 2.5-fold higher than that of the Ca^{2+}-dependent clones.

One "EDTA-dependent" monoclonal antibody, 7D7B10, has been purified and its properties characterized further. The protein specificity of 7D7B10 was not absolute for Protein C. Thus, in solid

phase screening assays as well as in immunoblotting experiments using highly purified human proteins, the antibody was found to react with prothrombin and Factor X, in addition to Protein C. In contrast, no binding to Factor IX, Protein S, or to bovine Protein C was observed. The lack of specificity was also observed in immunoaffinity experiments in which significant amounts of prothrombin copurified with Protein C using 7D7B10-Sepharose chromatography. Quantitative information regarding the relative affinity of 7D7B10 for the various proteins in solution was obtained using a sandwich ELISA. These results indicated that the affinity of the antibody for prothrombin, fragment 1 of prothrombin, or Factor X is approximately 30-40 fold weaker that its affinity for Protein C.

The location of the epitope of 7D7B10 was delineated by immunoblotting using proteolytic fragments and by the use of synthetic peptides. Antibody binding to fragment 1 of prothrombin as well as to the light chain of Protein C was observed on immunoblots, indicating that the epitope was located in the NH_2-terminal 155 residues of either protein. Antibody binding to other fragments covering the entire covalent structure of these proteins was not observed. In additional experiments, chymotryptic cleavage of either Protein C or prothrombin, which results in removal of the NH_2-terminal approximately 40-residue Gla domain, resulted in loss of the epitope. This provided evidence, albeit indirect, further localizing antibody binding to the Gla domain. The use of synthetic peptides confirmed that this monoclonal antibody

binds to the Gla domain of Protein C. Thus, synthetic peptides representing Protein C, 1-22, and Protein C, 1-15, were capable of binding to the monoclonal antibody. A peptide representing Protein C, 7-15, bound weakly to the antibody, whereas peptides representing Protein C, 1-7, Protein C, 9-16 and Protein C, 10-22, did not bind under the conditions examined. Furthermore, peptides representing Factor VII, 13-29, did not bind to this antibody. In agreement with results comparing the ability of human and bovine Protein C to bind to this monoclonal it was found that a peptide representing bovine Protein C, 1-15 has a much lower affinity for the antibody than its human counterpart. The results of all of these studies indicate that the first 15 residues of Protein C represent the major binding region for this antibody and that residues 10-13 appear necessary for maximal binding, but are not sufficient since peptide human Protein C, 7-15, encompassing this region bound relatively poorly.

During the course of this investigation, Church et al. (1988) identified a monoclonal antibody (H-11) to human Protein C. the binding of which was also inhibited by divalent metal ions and which lacked specificity for Protein C. These studies demonstrated that the epitope was located in the Gla domain, and that residues 4-9 (Phe-Leu-Glu-Glu-Leu-Arg) were important components of the epitope. Despite the similarities between these two antibodies, several features distinguish their interaction with Protein C. Although both antibodies cross-reacted with other vitamin K-dependent proteins, the protein

specificities differed. Thus, H-11 bound to bovine Protein C, whereas 7DB10 did not interact with the bovine molecule. A second difference observed between these two antibodies was the degree of metal ion inhibition of the interactions. Although saturating levels of divalent metal ions caused complete inhibition of 7D7B10 binding to Protein C, these same metals resulted in only partial inhibition of H-11 binding. Also, the dependence of the inhibition on Mg^{2+} concentration was quite different in the two systems, with significantly higher concentrations required in the case of 7D7B10 for inhibition of binding. Finally, these two antibodies displayed a distinct peptide specificity since H-11 bound to Factor VII, 1-12, whereas 7D7B10 did not. These data taken together confirm that these two antibodies interact in a different manner with Protein C.

No effect of antibody 7D7B10 on various aspects of Protein C or APC function were found. The antibody did not affect the initial rate of Protein C activation by PCA, a specific activating enzyme from snake venom (Kisiel et al., 1987a, 1987b; Orthner et al., 1988;). Nor did 7D7B10 have any effect on the activity of APC as measured using a small synthetic substrate. Likewise, no effect of antibody was observed on the inactivation of purified Factor VIII by APC in the absence of Ca^{2+} and phospholipid cofactors. Thus, under these conditions, no evidence was found for an effect of antibody binding to APC on its interaction with Factor VIII. The effect of the antibody on this reaction in the presence of Ca^{2+} and phospholipid cofactors could not be determined

since these concentrations of Ca^{2+} inhibit 7D7B10 binding of APC. In summary, antibody binding to Protein C or APC, shown to occur in the Gla domain of the light chain, was found to have no effect on events occurring in the heavy chain such as limited proteolytic cleavage to APC or active site-catalyzed hydrolysis of chromogenic or macromolecular substrates. Since the tertiary structure of Protein C is not known, this would suggest that the Gla region is sufficiently distant in space from the activation and catalytic site regions such that steric effects by the antibody molecule were not observed.

The concentration of Ca^{2+} required to inhibit antibody binding to Protein C correlates quite well with published data examining the binding of this metal to the molecule. Thus, equilibrium binding data have shown that bovine Protein C contains 16 equivalent sites for Ca^{2+} with a K_D of 0.9 mM (Amphlett et al., 1981). In addition to Ca^{2+}, Protein C also binds Mn^{2+} and Mg^{2+}, and binding studies with Mn^{2+} have demonstrated multiple binding sites for this metal in the Gla domain of bovine Protein C with an average K_D of 0.2 mM (Hill and Castellino, 1987). Competition experiments have shown that high concentrations of Mg^{2+} were able to displace Ca^{2+} from these sites (Amphlett et al, 1981). One interpretation of the present data is that the binding of Ca^{2+}, Mn^{2+}, or Mg^{2+} to the Gla domain inhibits interaction of the antibody by decreasing the accessibility of the epitope resulting from a conformational change. Interestingly, results of x-ray crystallographic determination of the three dimensional structure of fragment 1 of

prothrombin demonstrated that the first 35 residues of this molecule were disordered (Park and Tulinsky, 1986). Furthermore, more recent x-ray crystallographic data indicated that this region of fragment 1 became ordered in the presence of Ca^{2+}. An alternative explanation is that Ca^{2+} directly interferes with antibody binding by occupying ligand groups of Protein C required for interaction with the antibody. However, this seems unlikely since Gla residues, which are necessary for Ca^{2+} binding to this domain, did not appear to be involved in antibody binding. This was seen in experiments with prothrombin in which conversion of the Gla residues to glutamic acid residues had no effect on the interaction of this molecule with the antibody. It was also seen in the binding studies using peptides that contained glutamic acid residues in place of Gla.

In addition to the sites discussed, Protein C has been shown to contain a single high affinity site for Ca^{2+} (K_D, approximately 100 μM) exclusive of the Gla domain (Esmon et al., 1983; Johnson et al., 1983), which is located in the EGF homology domain of the light chain (Ohlin et al., 1988). Interestingly, in the present study, it was found that low concentrations of $K_{0.5}$ of 55 μM. The possibility that this effect resulted from two populations of antibodies, one of which bound to the EGF-like domain but could not be detected by blotting, was considered. However, the results of cell-sorting analysis of the 7D7B10 bybridoma cells gave no indication for heterogeneity. Therefore, it is likely that Ca^{2+} occupation of this site causes a conformational change that

enhances the binding of the 7D7B10 antibody to the Gla domain of Protein C. Other studies have found that occupation of the high affinity metal-binding site results in alterations detected in both the catalytic region (Steiner et al., 1980) as well as the activation cleavage site (Esmon et al., 1983; Johnson et al.,1983; Kinsiel et al., 1987a; Orthner et al., 1988, Stearns et al., 1988). The results from the present study provide the first evidence suggesting that metal binding to the site in the EGF homology region results in a conformational change in the Gla region and that these two domains interact in the Protein C molecule.

It is interesting to speculate on the possible role of the epitope recognized by the 7D7B10 antibody, which is conserved among several of the vitamin K-dependent proteins. The epitope is located in a region of the molecule thought to be highly disordered based upon the three-dimensional structure analysis of homologous prothrombin fragment 1. It seems unlikely, therefore, that the epitope is part of a structural motif involved in folding of the protein. On the other hand, although the epitope is probably not part of a Ca^{2+} -binding site per se, it may be necessary for an appropriate conformational response that occurs upon Ca^{2+} binding to the Gla domain. Another possibility is that this region of the molecule may be important in post-translational processing events. For example, it may be part of a binding site for the vitamin K-dependent carboxylase. These possibilities will be interesting subjects for future studies.

ABBREVIATIONS

Gla	gamma-carboxyglutamic acid
APC	activated Protein C
BSA	bovine serum albumin
EGF	epidermal growth factor
ELISA	enzyme linked immunoadsorbent assay
PCA	Protein C activator from *Agkistrodon contortrix contortrix*
PBS	phosphate buffered saline
RIA	radioimmunoassay
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
TLCK	1-chloro-3-tosylamido-7-amino-2-heptanone
TBS	tris buffered saline, pH 7.5
HPLC	high pressure liquid chromatography.

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TABLE I

Frequency of Antibody Types Identified in Initial Screening Assay

Antibody Type	No. of Positives	Percent
Metal-independent	133	86
EDTA-dependent	15	10
Ca^{2+}-dependent	6	4
Total	154	100

TABLE II

Screening of Hybridoma Supernatants by Solid Phase RIA

Clone	Binding to homlogous proteins[a]										Ig subtype
	EDTA present					Ca^{2+} present					
	C	S	II	S	IX	PC	X	II	S	IX	
7D7B10	+	+	+	-	-	-	-	-	-	-	IgG_1,κ
7D3H5	+	+	+	-	-	-	-	-	-	-	IgG_1,κ
8H2G6	+	+	+	-	-	-	-	-	-	-	IgG_1,κ

[a]Assays were performed in the presence of 2 mM $CaCl_2$ (as a constituent of the culture medium) or following the addition of excess EDTA to a final concentration of 10 mM.

TABLE III
Relative Competitiveness of Various Peptides
in Displacing Protein C from Antibody Complex[a]

Peptide	Sequence																				$K_{0,5}$
	1_a				5					.10					15					20	
																					μM
hPC, 1-15[b]	A	N	S	F	L	E	E	L	R	H	S	S	L	E	R						3.3
bPC, 1-15	A	N	S	F	L	E	E	L	R	P	G	N	V	E	R						200
hPC, 7-15							E	L	R	H	S	S	L	E	R						1500
hPC, 9-16									R	H	S	S	L	E	R	E					>990
hPC, 10-22								R	H	S	S	L	E	R	E	C	I	E	E	I	C >650
hPC, 1-7	A	N	S	F	L	E	E														>2000

[a]Equimolar concentrations of antibody 7D7B10 and Protein C (32 nM) were incubated in the presence of increasing concentrations of various peptides. The amount of Protein C-7D7B10 complex was measured by sandwich ELISA as described in Materials and Methods.

hPC, human Protein C; bPC, bovine Protein C

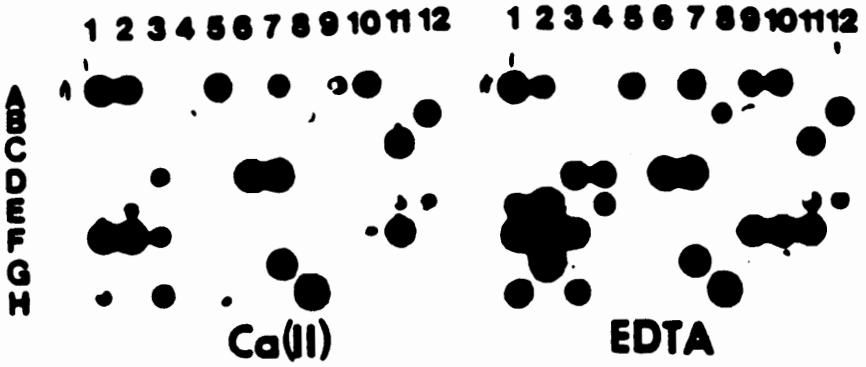

FIGURE 1
RIA SCREENING OF HYBRIDOMA SUPERNATANTS FOR METAL DEPENDENCE

Microtiter wells were coated with Protein C (100 ng/well) and incubated with 50 μl cell supernatants. Antibody binding was detected as described under "Experimental Procedures." Duplicate plates were assayed in the presence of either 10 mM Ca^{2+} or 10 mM EDTA.

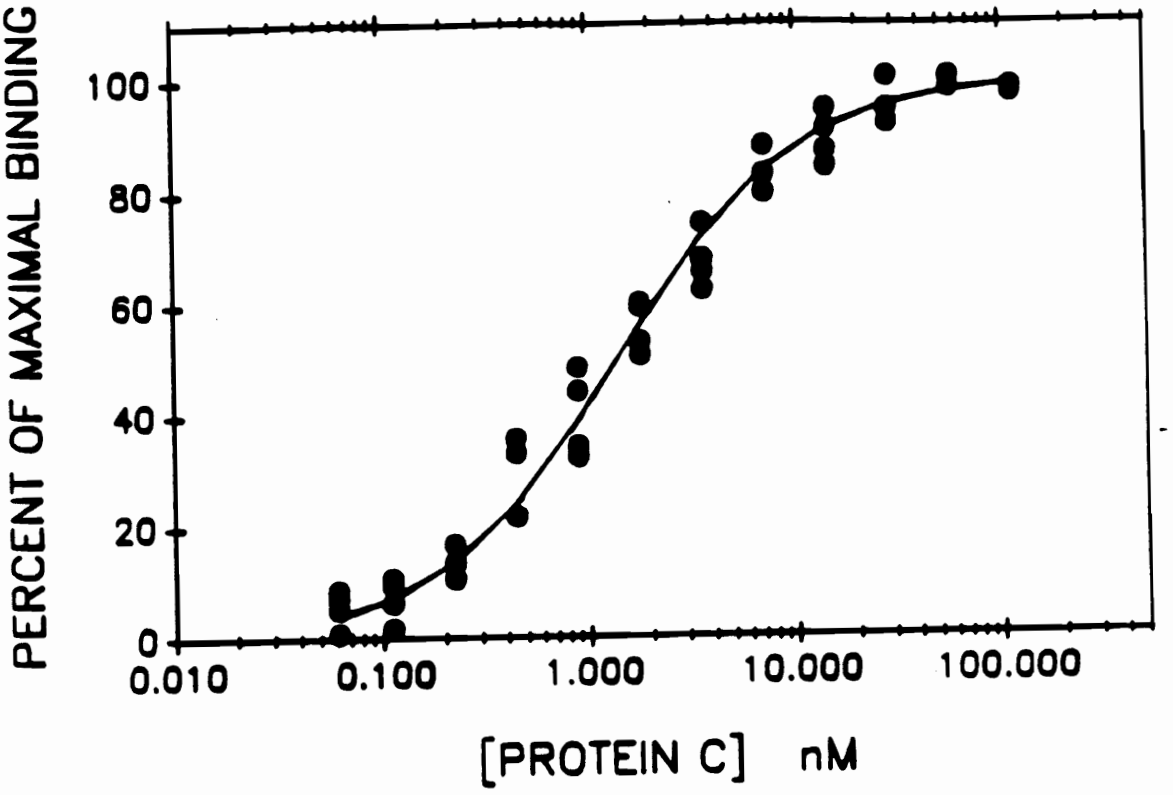

FIGURE 2
SANDWICH ELISA OF PROTEIN C BINDING TO MONOCLONAL ANTIBODY 7D7B10

Antibody, (20, 10 or 5 μg/ml) was incubated with increasing concentrations of Protein C (as indicated) in TBS, 0.1% BSA, 30 mM EDTA at 25 °C. After 20 min, 100 μl aliquots were added to microtiter wells which had been precoated with specific rabbit anti-Protein C antisera. The Protein C-7D7B10 complex was detected by incubation with peroxidase conjugated anti-mouse immunoglobulin (from goat) as described under "Experimental Procedures".

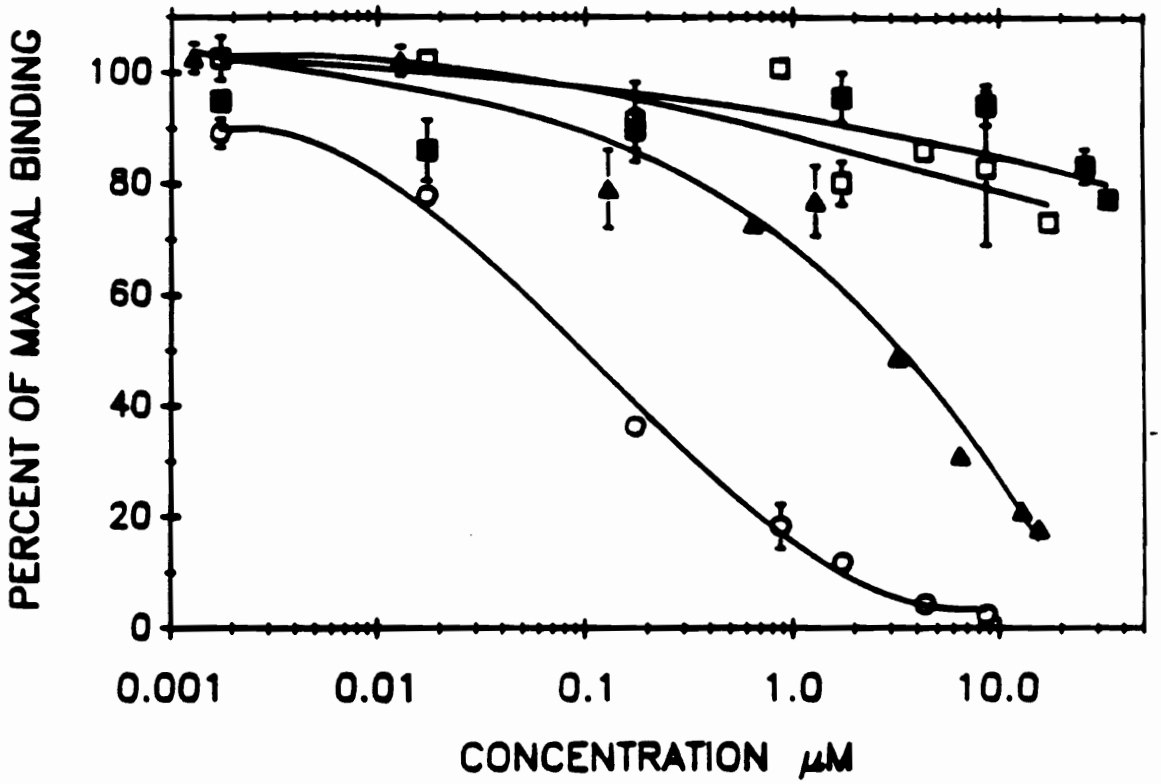

FIGURE 3
COMPETITIVE BINDING ASSAY MEASURING THE INTERACTION OF PROTEIN C AND STRUCTURALLY HOMOLOGOUS PROTEINS WITH MONOCLONAL ANTIBODY 7D7B10

Microtiter wells were coated with 1 μg of Protein C. An aliquot (100 μl) of dilutions of human Protein C (○), human prothrombin (▲), human factor IX (□), or bovine Protein C (■) was preincubated with 100 μl (0.5 μg/ml) of 7D7B10 for 1 h prior to addition to the microtiter plate. Following incubation and washing, bound antibody was detected by using a rabbit antimouse IgG-alkaline phosphatase conjugate.

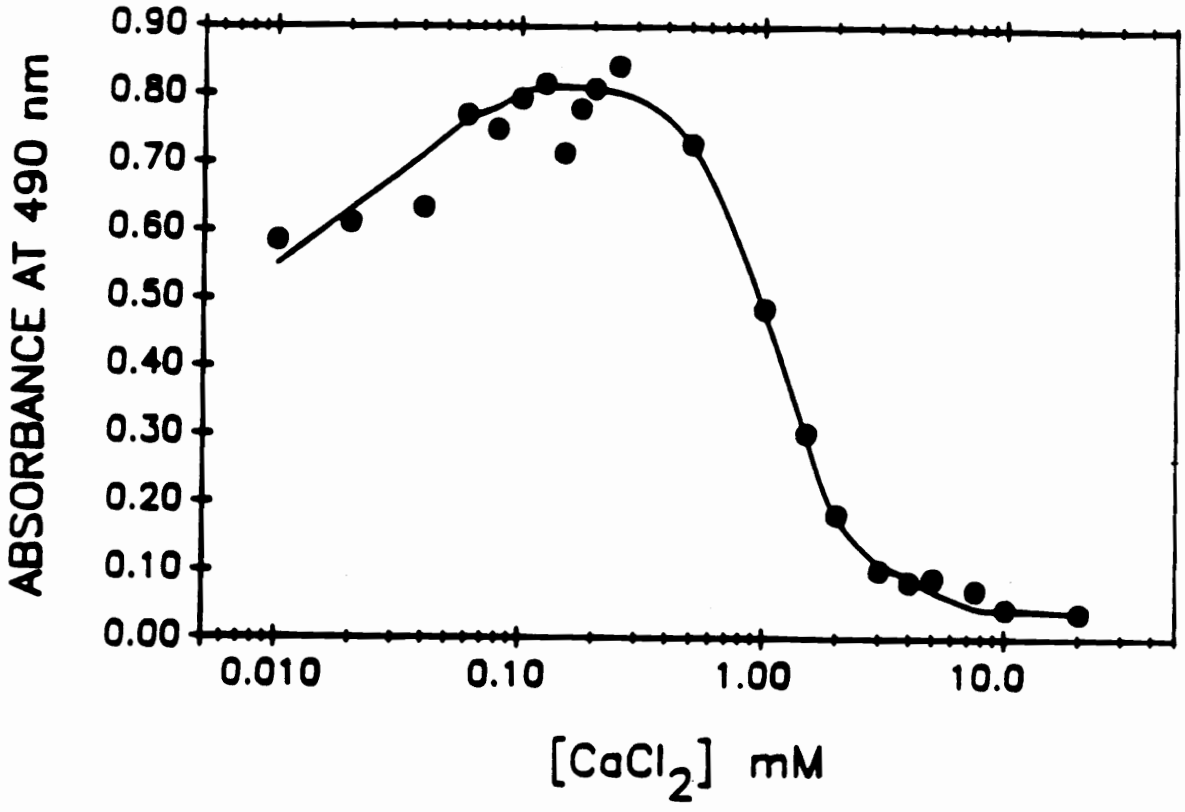

FIGURE 4
EFFECT OF VARYING $CACL_2$ CONCENTRATION ON PROTEIN C BINDING TO ANTIBODY

7D7B10 (5.2 μg/ml) and Protein C (2.0 μg/ml) were incubated with increasing concentrations of $CaCl_2$ as indicated in TBS, 0.1% BSA at 25 °C for 25 min. Aliquots (100 μl) were assayed for Protein C-7D7B10 complex as described in the legend to Figure 2.

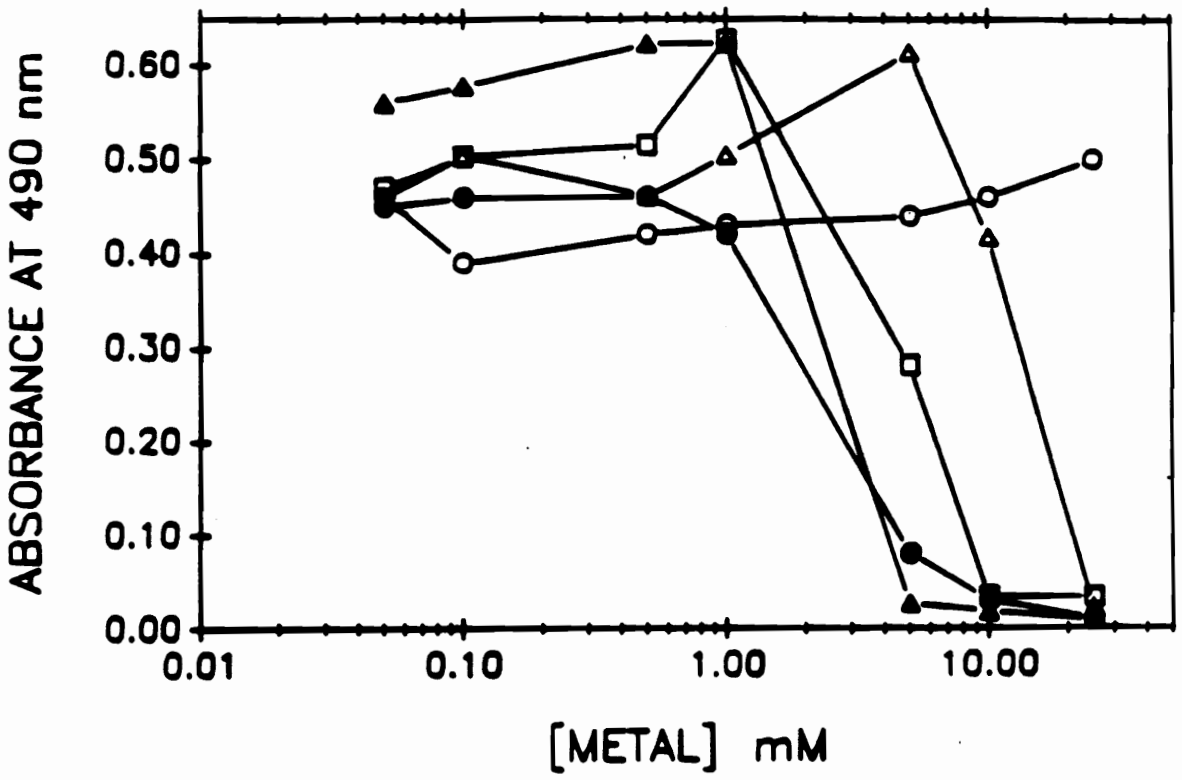

FIGURE 5
EFFECT OF INCREASING CONCENTRATIONS OF VARIOUS DIVALENT METAL IONS ON PROTEIN C BINDING TO ANTIBODY

Protein C (2.0 μg/ml) and 7D7B10 (5.2 μg/ml) were incubated with increasing concentrations of each metal ion (as indicated) in TBS, 0.1% BSA at 25 °C for 25 min. Protein C-7D7B10 complex was measured as described in the legend to Figure 2. EDTA (○), Ca^{2+} (●), Mg^{2+} (△), Mn^{2+} (▲) and Ba^{2+} (□).

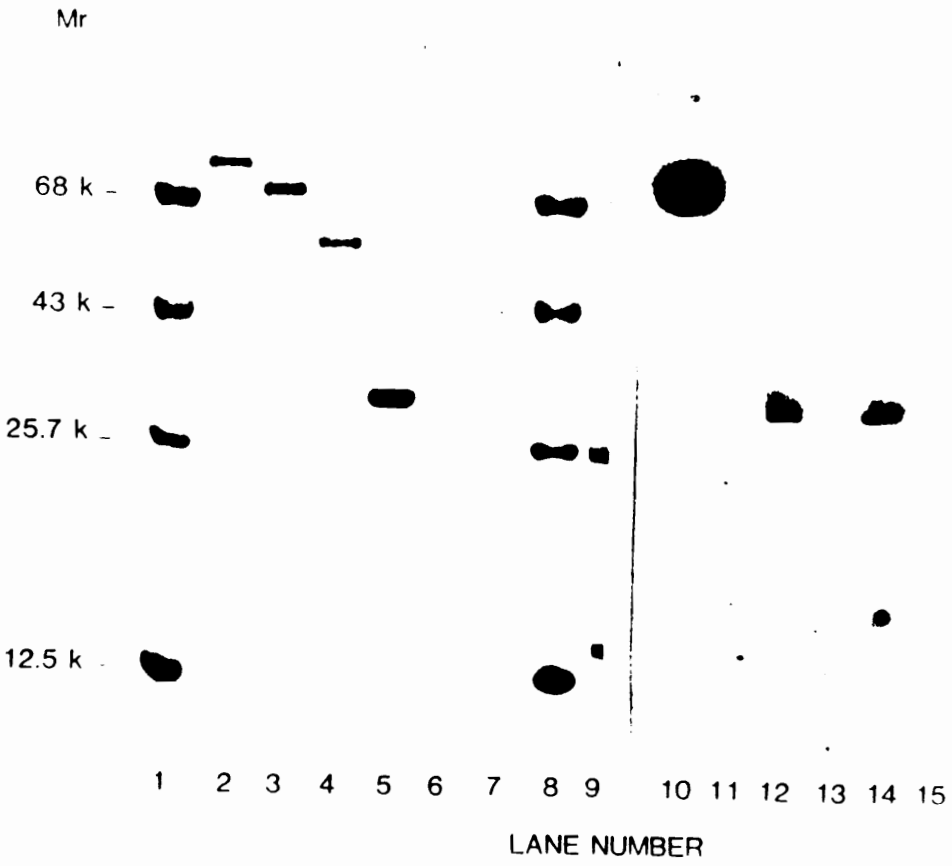

FIGURE 6
SDS-PAGE AND IMMUNOBLOT OF PROTHROMBIN AND ITS PROTEOLYTIC FRAGMENTS

All samples were reduced with b-mercaptoethanol. Lanes 1 and 8, protein standards; Lanes 2 and 10, early digest of prothrombin with a-chymotrypsin (1 min); Lanes 3 and 11, late digest of prothrombin with a-chymotrypsin (120 min); Lanes 4 and 12, prothrombin digest with a-thrombin; Lanes 5 and 13, a-thrombin; Lanes 6 and 14, fragment 1 (partially degraded); Lane 9, prestained protein standards. Lanes 1 to 9 were stained with Coomassie Blue R-250. Lanes 10 to 15 were blotted to nitrocellulose and probed with monoclonal antibody 7D7B10 followed by anti-mouse Ig, ^{125}I-labeled F(ab')$_2$ (from sheep).

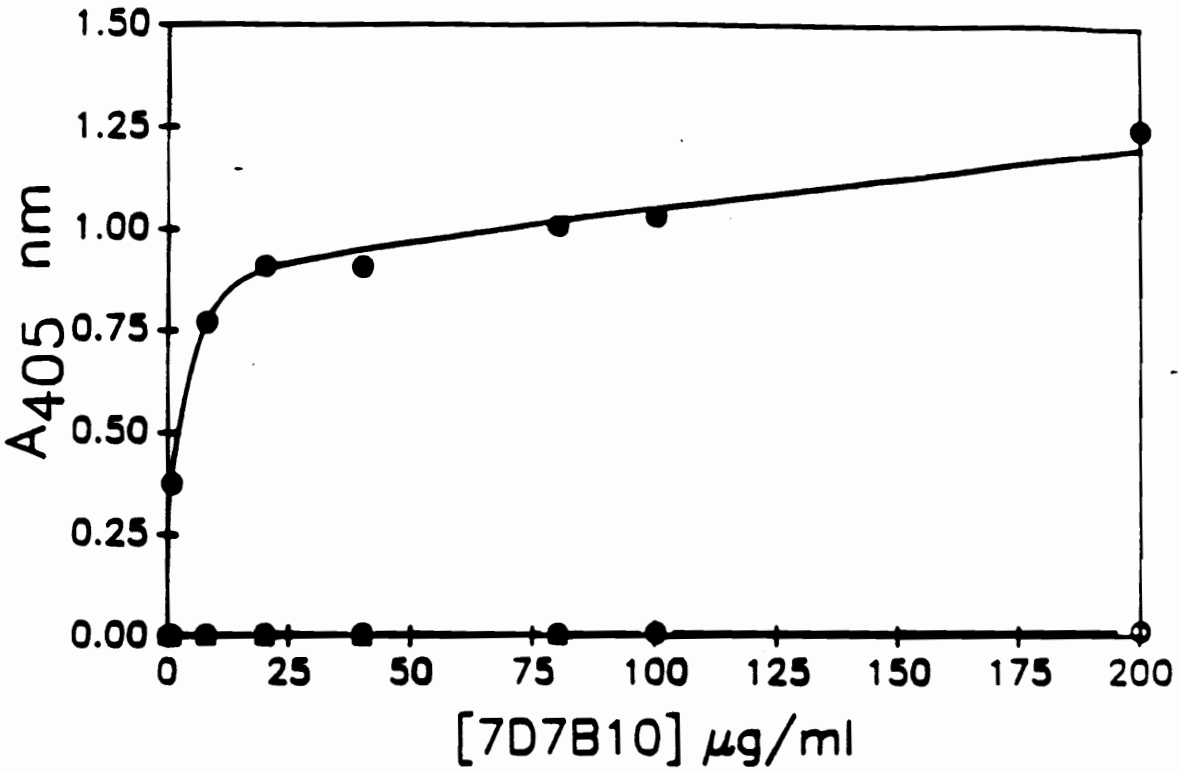

FIGURE 7
BINDING OF 7D7B10 TO IMMOBILIZED SYNTHETIC PEPTIDES

Peptides were coated to the wells of microtiter plates (Immulon II) by incubation overnight in 0.1 M sodium carbonate buffer, pH 9.5. The wells were then blocked with TBS, 0.1% BSA and incubated with varying concentrations of 7D7B10 in TBS, 0.1% BSA. Bound antibody was detected as described for the sandwich assay. The peptides used corresponded to human Protein C, 1-22 (●), human factor VII, 1-12 (O), and human factor VII, 13-29 (▲).

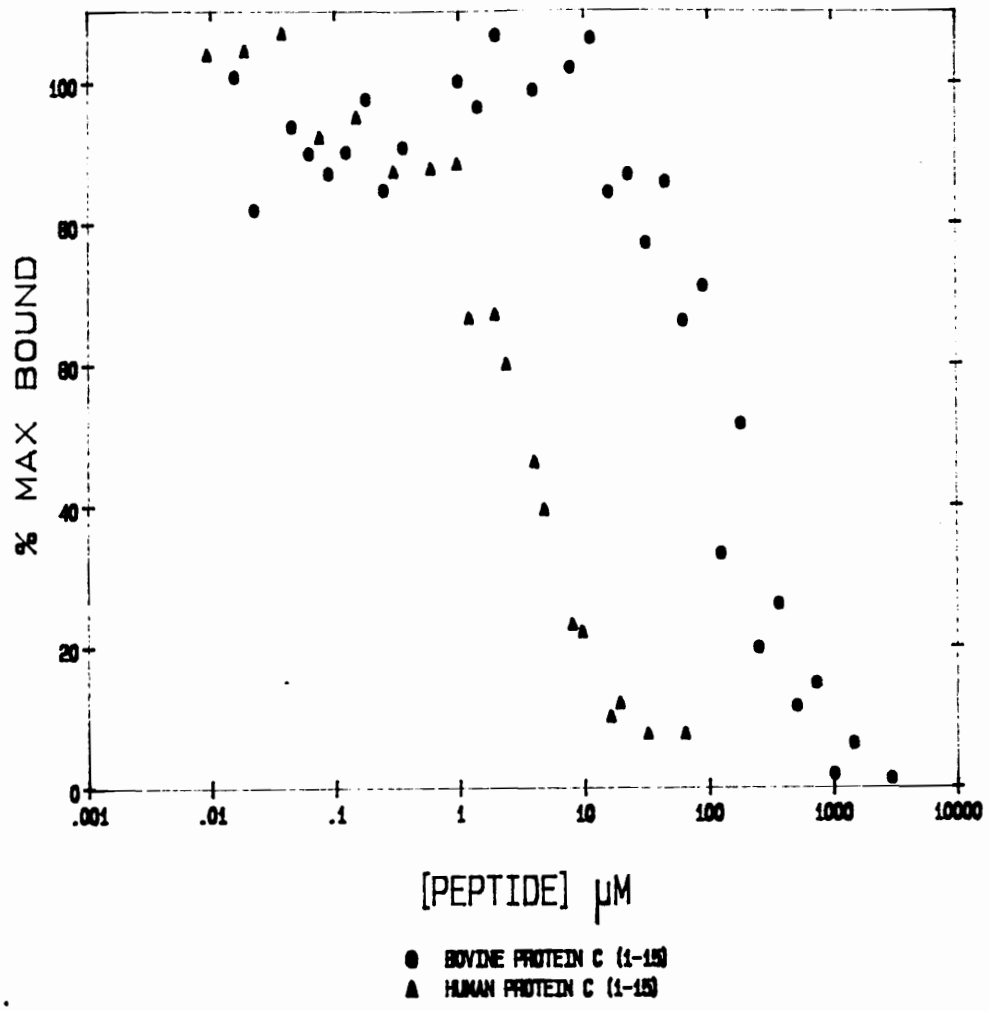

FIGURE 8
COMPARISON OF THE BINDING OF SYNTHETIC PEPTIDES CORRESPONDING TO HUMAN PROTEIN C, 1-15, AND BOVINE PROTEIN C, 1-15, TO ANTIBODY 7D7B10

Protein C (2.0 μg/ml) were incubated with increasing concentrations of peptides corresponding to human Protein C (▲), or bovine Protein C, 1-15 (●), in TBS, 0.1% BSA for 25 min at 25°C. The Protein C-antibody complex was captured and quantitated as described in the legend to Figure 2.

CHAPTER THREE

PROCESS IMPLICATIONS FOR METAL-DEPENDENT IMMUNOAFFINITY INTERACTIONS

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ABSTRACT

Immunosorbents have been developed which utilize metal-dependent interactions between monoclonal antibody(s) (Mab) and human plasma Protein C and Factor IX, members of the vitamin K-dependent plasma protein family. In this report, we describe the potential process advantages associated with two different types of divalent metal-dependent systems: 1) a Mab-Protein C (antigen) complex which is stabilized in a divalent metal-free environment and 2) a Mab-Factor IX (antigen) complex which is stabilized in the presence of divalent metal ions. Chelating agents such as citrate or EDTA are effective agents for sequestering divalent metals. Thus, Protein C binds to the immunosorbent in the presence of EDTA or sodium citrate and can be eluted with buffer containing 25 mM calcium chloride or magnesium chloride. In the second case, Factor IX binds to the immunosorbent in the presence of magnesium chloride and can be eluted with sodium citrate or EDTA. These gentle elution conditions are desireable in order to retain the native structure as well as functional activity of the protein to be immunopurified. Furthermore, unwanted plasma proteins, which may interact nonspecifically with either the agarose matrix or immunoglobulin G (IgG) ligand are less likely to be eluted under these conditions. The calcium-free citrate buffers used for immunosorption by either of these processes preclude the formation of fibrin solids and eliminate eluents which are difficult to process and which interfere with the biological activity of the product (i.e. chaotropes). The immunoaffinity process we describe for Protein C and Factor IX provides

high functionality, purity and yield and demonstrates the feasibility of large-scale processes utilizing metal-dependent immunosorbents.

INTRODUCTION

The vitamin K-dependent (VKD) plasma proteins represent an important class of trace plasma components (Table 1) that maintain normal hemostasis. Abnormalities of which result in bleeding or thrombotic disorders [1]. For example, changes in hemostasis play a central role in septic shock.

The VKD proteins are a family of structurally homologous proteins that have very similar physicochemical properties (molecular weight, isoelectric point) and share sequence homology in certain regions of the molecules [1,2]. Another salient feature of the VKD proteins is their divalent metal-binding domain, located at the amino terminal end of the molecule, which is highly conserved among this family of proteins (Table 2). This domain encompasses roughly 40 amino acids and contains as many as 11 residues of gamma-carboxyglutamic acid (Gla) (Figure 1). These residues impart the bulk of the divalent metal-binding properties of the VKD proteins, although they also contain a single high affinity divalent metal binding site outside of the gla domain, with the exception of Prothrombin and Protein Z [3,4].

The structural similarities between VKD-proteins render classical methods ineffective for high-resolution purification of any of these proteins from plasma or plasma derivatives. While all immunoaffinity purification processes of VKD-proteins from plasma yield residual protein contamination in their products, the most efficacious and safe products should be devoid of the antagonistic pro- or anticlotting activity. The ridding of antagonistic or other pathological contaminants is the primary task of the high-resolution purification process. In addition, the nature of the isolation process must be such that the functional activity of the protein is retained. Thus, these two tasks are central and prerequisite to the evaluation of a large-scale purification process for a plasma-derived therapeutic protein.

Since antibodies are quite specific ligands, immunopurification is a promising approach for the purification of these proteins. We focus upon a promising new class of process scaleable, immunosorbents which alter binding equilibria via metal-dependent immunoaffinity interactions to provide elution condition which yield highly pure and functional protein products. A description of two different monoclonal antibodies (Mab) that form metal-dependent, VKD-protein complexes is presented. Other metal-dependent interactions of monoclonal antibodies with Protein C, Prothrombin and Factor IX have been reported [6-13]. These immunosorbents have the potential to simplify process schemes. We emphasize here these process implications; most of the biochemical characterization is detailed elsewhere [13].

MATERIALS AND METHODS

MATERIALS AND REAGENTS

Human plasma was obtained from volunteer donors to the American Red Cross. CNBr-activated Sepharose 4B and DEAE Sephacel were purchased from Pharmacia, Piscataway, NJ. Affigel-10 and Affiprep-10 activated resins were purchased from Bio-Rad Laboratories, Richmond, CA. A specific rabbit anti-human Protein C was from American Bio-products, Parsippany, NJ; affinity purified goat anti-mouse immunoglobulin linked to peroxidase was from Cappel laboratories, West Chester, PA. Anti-Prothrombin antisera was purchased from ICN Immunobiologicals, Lisle, IL. The chromogenic substrate S-2366 used for amidolytic assay was purchased from Helena Labs, Beaumont, TX. All chemical were of reagent grade.

Proteins

Human Prothrombin, Factor X, and fragment 1 of Prothrombin were purified as previously described [13] and were greater than 95% pure, as judged by SDS-PAGE. Bovine Protein C was purchased from Calbiochem, La Jolla, CA.

Monoclonal Antibodies

Monoclonal antibodies to Protein C or Factor IX were produced as reported [13]. IgG was purified from ascites fluid by ammonium sulphate precipitation and DEAE-Sephacel chromatography [14]. The protein specificity of the Mab was screened by solid phase RIA [15]. The wells of microtiter plates that had been coated with antigen (or homologous

protein) were incubated with Mab washed, and blocked. They were then incubated with ^{125}I-Protein A, washed, and the plate exposed to autoradiographic film or the wells counted in a gamma counter. Bound counts indicate Mab binding to the test protein. Quantitative binding data was obtained by sandwich ELISA [13]. Anti-Factor IX IgG was coupled to CNBr-activated Sepharose 4B at 1.0 mg of Mab per ml of gel according to the manufacturer's instructions. The Factor IX immunopurifications were performed using a 2 inch diameter column, with a 100 ml bed volume using a 5 ml per minute flow rate. Anti-Protein C Mab was coupled to Affigel-10 or Affiprep-10 at 2.0-2.5 mg IgG bound per ml of gel according to manufacturer's directions. The Protein C elutions were performed in a 1 cm diameter column, with bed volumes of 9 and 3.3 ml, at a flow rate of 0.5 ml per minute.

Assays

Factor IX activity was measured in a one-stage clotting assay, as described by Biggs [16], using automated APTT reagent (Organon Teknika Corp., Durham, MC) and Factor IX deficient plasma (George King Biomedical, Overland Park, KA) on an X-2 automated coagulation instrument (Organon Teknika). Protein C activity was measured in a chromogenic assay [17] or an anticoagulant assay [18] using Protac activator (American Diagnostica Inc., New York, NY). Both assays utilized as a standard, normal pooled reference plasma, which contained one unit of Factor IX or Protein C activity per ml, by definition.

Electrophoretic Analysis

SDS-PAGE was performed according to the method of Laemmli [19] using a 12.5% acrylamide gel. A low-molecular-weight standard mixture obtained from Bio-Rad Laboratories (Richmond, CA) was used for Mr estimates. The area percent purity of immunopurified SDS-PAGE (Figure 4) of immunopurified Factor IX and Protein C were performed using a Shimadzu CS-9000 scanning densitometer at wavelength=570 nm. The SDS-PAGE was stained with 2% Coomassie brilliant blue R. Western Blot analysis for Prothrombin and Protein C was performed as described elsewhere [13].

RESULTS

Monoclonal Antibody Selection

The screening to detect metal-dependent complexes of Mab with Factor IX or Protein C was a time-consuming task. It required the screening of hundreds of fusion products to obtain relatively few stable hybridoma lines that produced monoclonal antibodies with the desire metal dependency for binding. A desireable metal-dependency is the formation of a Mab-protein complex that is either stabilized or destabilized by the presence of metal ions. The presence or absence of available calcium was employed to screen for these complexes. We use the designation EDTA-dependent to refer to a Mab that required the absence of available (unchelated or free) calcium to form a stable antigen-antibody complex. The term calcium-dependent refers to a Mab that required the presence of available calcium ions to bind to the protein antigen. The anti-Protein C Mab described

here is an EDTA-dependent Mab, while the anti-Factor IX Mab is a calcium-dependent Mab. The anti-Factor IX Mab discussed here is also "magnesium-dependent" such that the Factor IX-Mab complex is also stabilized by the presence of magnesium. We have described the production and characterization of EDTA-dependent Mab to protein C elsewhere [13]. In that study, a large number of Mab clones were shown to have reactivity with Protein C. Approximated 10-14% of these Mab clones were found to be metal-dependent. Specific details about divalent metal dependent interactions for magnesium are also described in that study [13].

After identifying hybridoma lines producing calcium or EDTA dependent Mab, only two were selected for further characterization due to the time and cost necessary to characterize these Mab with respect to specificity and performance as an immunosorbent. Of course, other criteria must also be considered in choosing a Mab for a large-scale process, such as the ability of the hybridoma to grow in serum-free media, the Mab secretion rate, etc., which are outside the scope of this article.

The protein specificity of the Mab of interest were then evaluated in a two-phase process. In the first phase, a rapid radioimmunoassay (RIA) using ^{125}I-protein A was used to screen the Mab for their ability to bind to other members of the VKD protein family (see Materials and Methods). This provided qualitative information about possible cross-reactivity of the Mab. In the second stage, a competitive sandwich enzyme-linked immunoassay (ELISA) was used to generate quantitative

binding data for the protein antigen of interest as compared to other VKD proteins. In the case of protein C, for example, the Mab, the protein antigen (Protein C), and the competitor antigen were allowed to pre-equilibrate in solution such that the binding process was unencumbered. A sandwich was then formed by immunosorption of the Mab-primary antigen complex onto plastic microtiter wells coated with a specific polyclonal (rabbit) antibody to Protein C. This was followed by detection of the complex with an enzyme-linked secondary antibody against murine immunoglobulin. Relative binding isotherms were seen as an inhibition of the detecting antibody signal. This resulted from a decrease in the amount of the Mab-primary complex formed and subsequently immunosorbed, due to the presence of a species for which the Mab showed cross-specificity.

The competition ELISA results for an EDTA-dependent Mab to Protein C are summarized in Table 3. It lists the relative competitiveness of various members of the VKD family for the 7D7-Mab. While bovine Protein C was not able to bind to Mab 7D7, the human VKD protein homologues Prothrombin, fragment 1 of Prothrombin (residues 1-155), and Factor X were all able to compete with Protein C for binding to the Mab. However, half-maximal inhibition occurred at concentrations approximately two orders of magnitude higher than the concentration of protein C required for half maximal binding.

Immunoaffinity Purification

Figure 2 shows a flow diagram of a process to purify Protein C and Factor IX from cryo (cryoprecipitated antihemophilic factor) - poor citrated plasma utilizing the EDTA-dependent Mab to protein C described, as well as the divalent metal ion-dependent Mab 1H5B7 to Factor IX [20,21]. In the first step of the process, filtered plasma is loaded onto an anion exchange resin, which efficiently captures the VKD proteins. The column is washed to remove unadsorbed material and the VKD proteins eluted with a NaCl step. The eluate is then chemically treated to inactivate viruses by the addition of a mixture of solvent and detergent, which has been shown to rapidly inactivate lipid-enveloped viruses [22]. This material, in a 20 mM citrate buffer, is then passed through a filter onto the anti-Protein C Mab column, which is washed with citrate buffer and eluted with buffer containing 25 mM CaC12. The immunopurified Protein C is at this point contaminated with Prothrombin. The presence of Prothrombin was verified by Western Blot analysis (data not shown). This is thought to be due to the cross-specificity of the Mab (table 3) and the high ratio of Prothrombin to protein C in plasma (Table 1), which is maintained in the mass capture eluate.

Protein C is further processed by reapplication to the same immunosorbent to separate it from Prothrombin. The first loading was performed at 50% column capacity assuming a 2.5 mg 7D7-Mab/ml Affigel-10 or Affiprep-10 resin, 25% bound activity of a 1:1, Mab:Protein C stoichiometry. The second loading was performed using the same

immunosorbent, but at 100% loading capacity. Bed volumes of 7D7 immunosorbents were 9 ml for the first loading, and 3.3 ml for the second loading. The concentration of protein in the first eluate was 0.29 mg/ml and 0.054 mg/ml in the second eluate as measured by optical density at 280 nm using an absorption coefficient of 1.4 (Table 5). The first immunosorption step recovered 89% of the Protein C amidolytic activity. The second immunosorption recovered 96% of the Protein C amidolytic activity.

The amidolytic as well as anticoagulant activity of the immunopurified protein C was measured using a Protac assay and normal plasma reference pool as the primary standard. The specific activity of 7D7-Mab immunopurified products has ranged from 300-320 units per mg, based on amidolytic and anticoagulant assay. These values compare well with a theoretical specific activity of 333 units per mg for pure Protein C, which assumes 3 μg Protein C per ml of normal plasma reference pool. Table 5 lists the purification activities and yields obtained from cryopoor plasma starting material. Note that Protein C activities listed in Table 5 are for column eluates having no concentration or cleanup steps. The Protein C activity drops from 56.6 to 16.2 units per ml when processing from the first to the final immunosorption eluate. This is a fraction collection effect encountered when shifting from a 9 ml to 3.3 ml bed volume using the same 1 cm diameter column. A 15,000-fold purification of Protein C was achieved with this process.

The unadsorbed pool from the first immunosorption step containing Factor IX was diafiltered into the appropriate loading buffer containing 10 mM $MgCl_2$ and loaded through a filter onto the anti-Factor IX was eluted with 0.02 M trisodium citrate, 0.11 M NaCl, pH 6.8. Figure 3 shows a typical elution profile of the metal dependent anti-Factor IX immunosorbent. Most of the protein as measured by absorbance at 280 nm was found in the unadsorbed peak. The majority of the Factor IX activity was associated with the protein peak that eluted upon the addition of the citrate eluant. As a result, a near quantitative recovery (96%) of Factor IX activity was recovered in the citrate eluate. This is significantly higher than previously reported yields of 72-82% (26).

Table 4 summarized the purification of Factor IX through the immunoaffinity step. The process was performed starting with 500 liters of cryo-poor plasma at the pilot-plant-scale through the mass capture step and continued at the laboratory scale. An approximately 16,000-fold purification was accomplished with an overall recovery of 72%. The specific activity of the immunopurified Factor IX was 268 units per mg. This compares favorably with a theoretical activity of 250 units/mg, which assumes a 1 unit/ml (by definition) and 4 μg/ml reference plasma pool. This significantly exceeds the 100-200 units/mg functional activity that has been previously reported for immunopurified Factor IX [26,27].

Characterization of Immunopurified Proteins

Figure 4 shows the results of SDS-PAGE analysis of the mass capture eluate and immunopurified Factor IX and Protein C. The Factor IX was analyzed following concentration by ultrafiltration of the eluate from the immunosorbent, while the Protein C was further processed by secondary immunosorption to remove contaminating Prothrombin before concentration and analysis. Lanes 2 and 5 (nonreduced and reduced by ß-mercaptoethanol, respectively) show the mass capture eluate as a complex mixture of proteins. Both Factor IX and Protein C were highly purified and had the expected Mr values measured against protein standards of known molecular weight. Lanes 1 and 8 are low molecular weight standard mixtures. Lane 4 shows that nonreduced Protein C had a Mr of 62,000. Lane 7 shows the subunit structure of Protein C, which consisted of a doublet heavy chain with a Mr of 40,000 and a light chain of 25,000 in the presence of the reducing agent ß-mercaptoethanol. Lanes 3 and 6 show the single chain structure of Factor IX, with a Mr of 57,000 in the absence or presence of reducing agent, respectively. The reduced Factor IX shown in lane 6 was found to be 85 area % Factor IX by scanning densitometry. The reduced Protein C shown in lane 7 was found to have total heavy and light chain of 91 area percent by scanning densitometry.

DISCUSSION

The large-scale immunoaffinity purification of plasma-derived proteins consists of a sequence of six major processing tasks: solids

removal, volume reduction, prepurification, viral inactivation, high resolution purification, and a final low resolution cleanup. The final cleanup is a two-step process used to remove high molecular weight contaminants such as Mab (ion exchange) that may have leached from the immunosorbent and extraneous, low molecular weight proteins (diafiltration) that may still contaminate the product protein. The advantages of immunosorbents that exploit metal-dependent interactions arise from the limitations imposed by the above described process sequence.

In the context of producing an individual VKD protein for parenteral use, the biochemically reactive nature of plasma introduces complexities to each of these steps. For example, solids removal is an ongoing task due to the spontaneous precipitation of protein primarily because of cryoprecipitation and the formation of fibrin. Fibrin forms in the presence of proclotting enzymes, fibrinogen, and calcium. The potential for irreversible fouling of costly immunosorbent matrices dictates that the environment be made free of available calcium by the presence of a divalent chelating agent such as citrate. Thus, a time-consuming, buffer exchange cycle is usually encountered during the immunopurificatio step when processing from a citrated environment to a column eluent and then back to a citrate buffer.

Loading conditions employed with an EDTA-dependent immunosorbent preclude fibrin formation in the presence of proclotting proteins because

all available calcium is sequestered by a chelating agent such as EDTA or citrate. In this case, nonspecifically bound fibrinogen and proclotting factors are removed in the column wash step in the absence of available calcium. The immunosorbed VKD species of interest can then be eluted using a buffer containing calcium without risk of fibrin formation. The Factor IX immunosorbent utilizes available calcium. The immunosorbed VKD species of interest can then be eluted using a buffer containing calcium without risk of fibrin formation. The Factor IX immunosorbent utilized available magnesium in a citrate buffer while eluting in a sodium citrate buffer that chelates the available magnesium provided by the loading step. Magnesium does not support fibrin formation. Because both loading and elution conditions employed by these metal-dependent immunosorbents are citrate buffers (sodium or magnesium salt), no additional processing steps to change buffer are needed (Figure 3). Citrate buffer is a Food and Drug Administration (FDA) accepted product buffer for parentally administered therapeutics.

Table 1 lists the relative concentrations of VKD protein family and the total protein level in cryopoor-plasma, the starting material for most plasma derivatives. A volume reduction of approximately a factor of 50 is achievable using anion exchange chromatography yielding a VKD family concentration. In addition, extraneous protein is reduced from approximated 50+ mg total protein per ml to about 2-5 mg per ml. Even at this reduced level of total protein, some nonspecific adsorption will occur. Harsh elution conditions can result in a significant coelution of

the nonspecifically adsorbed protein into the product eluate. The gentle elution conditions provided by metal-dependency helps reduce the amount of nonspecifically adsorbed protein that will coelute with the immunosorbed protein. These nonspecifically adsorbed proteins are eluted during a 1 M NaCl column regeneration step. The amounts of these proteins found in the NaCl regeneration eluate vary, but have been observed to be as high as 21% of the total optical density at 280 nm of the product and NaCl eluate peaks (Velander, et al., unpublished observations of immunopurified Protein C products using anion exchange eluate of Cohn IV-1 paste as a source material for immunopurification). A high salt, product elution step would, of course, result in these same proteins being desorbed into the product peak.

Most immunoaffinity processes employ monoclonal antibodies that require harsh elution conditions, including the use of chaotropic salts, high or low pH, and high ionic strength. For example, initial studies using sodium thiocyanate elution of Protein C from an immunosorbent that did not possess a metal-dependent Protein C-Mab interaction resulted in an essentially inactive Protein C product (Orthner, et al., unpublished observation). In addition, these studies were further complicated by the fact that chaotropes such as sodium thiocyanate interfere with the assay for biological activity of Protein C. Further studies with this immunosorbent were not pursued for these reasons.

In the case of extremes in pH, the eluates must be retitrated to an acceptable pH and diafiltered to remove the salts associated with these manipulations. We are currently evaluating an immunosorbent for Protein C that utilized a high pH elution and have been confronted with this particular drawback. The use of "harsh elution conditions" necessitates additional process steps. In the case of chaotropic agents, the process must include steps for their removal (as well as assay procedures to document their removal) and proper disposal.

Harsh elution conditions may also adversely affect the longevity of the immunososorbent. We are beginning studies to assess the recyclability of the metal-dependent immunosorbents described above. At present, we have seen binding capacities retained after 20 successive uses of the Factor IX immunosorbent (J.P. Tharakan, et al, unpublished observations) and 10 recycles of the protein C immunosorbent (W.H. Velander et al., unpublished observations).

Part of the purification challenge has been to incorporate viral inactivation methods that have been developed to reduce the infectivity of plasma or products derived from it. These chemical inactivation methods utilize a solvent/detergent mixture that must be removed from the protein product [22]. This occurs via the column wash step, which comes prior to product elution. The stability of the immunosorbed protein complexes for both Factor IX and Protein C have been shown to be sufficient to allow for removal of the viral inactivation agents. This

is seen in the high yields of extensively washed Protein C or Factor IX immunopurified products. Thus, the viral inactivation step has been successfully integrated into the metal-dependent, immunopurification process.

CONCLUSIONS

The above discussion emphasized the processing advantages of manipulating immunosobent equilibria via metal-dependent conformers relative to those that occur in a harsh elution environment. In the case of the VKD-proteins, the metal-dependent conformations can depend on binding epitopes and domains for which there is extensive homology among the VKD-protein family. Cross-specificity can result and is seen in the case of the anti-Protein C Mab (Table 3). When examining the similarities of the "Gla domain" in each of these proteins, it is reasonable to expect that a significant number of metal-dependent interactions will be associated with this domain (Table 2). Therefore, the specificity of three types of Mab should be carefully evaluated.

The anti-Factor IX Mab presented possesses a high level of selectivity for Factor IX. Thus, the immunosorbent utilizing this Mab produces a highly pure protein product when using anion exchange chromatography as a prepurification and volume reduction step. As a result, no secondary immunopurification is necessary for the Factor IX process.

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NOTATIONS

$CaCl_2$	Calcium chloride
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked-immunosorbent assay
Gla	Gamma-carboxyglutamic acid
IgG	Immunoglobulin G
^{125}I	Iodine isotope 125
Mab	Monoclonal antibody
$MgCl_2$	Magnesium chloride
RIA	Radioimmunoassay
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
VKD	Vitamin K-dependent

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TABLE 1. CRYO-POOR PLASMA CONCENTRATIONS OF VKD-PROTEINS

Cryopoor Plasma Total Protein	55 mg/ml
VKD-Anticoagulants:	
■ Protein C	3-4 μg/ml
■ Protein S*	10 μg/ml
VKD-Procoagulants:	
■ Factor X	8.2 μg/ml
■ Prothrombin	120 μg/ml
■ Factor VII	2.0 μg/ml
■ Factor IX	3-5 μg/ml
VKD of Unknown Function:	
■ Protein Z	2.2 μg/ml

* >10 μg/ml bound to C4b-bP, PS complex.

Data compiled from reference [23].

TABLE 2. GLA DOMAIN OF HUMAN VITAMIN-K-DEPENDENT PLASMA PROTEINS*

	1				5					10					15					20					25					30
PC	A	N	S	F	L	E	E	L	R	H	S	S	L	E	R	E	C	I	E	E	I	C	D	F	E	E	A	K	E	I	F
FX	A	N	S	F	L	E	E	M	K	K	G	H	L	E	R	E	C	M	E	E	T	C	Y	S	E	E	A	R	E	F	F
PT	A	N	T	F	L	E	E	V	R	K	G	N	L	E	R	E	C	Y	E	E	T	C	S	Y	E	E	A	F	E	A	L
FVII	A	N	A	F	L	E	E	L	R	P	G	S	L	E	R	E	C	K	E	E	Q	C	S	F	E	E	A	R	E	I	F
PS	A	N	S	L	L	E	E	T	K	Q	G	N	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PZ	A	G	S	Y	L	L	E	E	L	F	E	G	N	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
FIX	Y	N	S	G	K	L	E	E	F	V	Q	G	N	L	E	R	E	C	K	E	E	L	C	S	F	E	E	A	R	E	V

* Amino-terminus of L-chain for PC and FX; PT, FVII, FIX, PS, PZ are single chain.

Data compiled from references [23, 24].

Abbreviations are as follows: PC = Protein C, FX = Factor X, PT = Prothrombin, FVII = Factor VII, PS = Protein S, PZ = Protein Z, and FIX = Factor IX.

TABLE 3. SPECIFICITY SUMMARY FOR 7D7 EDTA-DEPENDENT MAB*

Competitor Species	Concentration+ (μg/ml)
Factor X	>300
Prothrombin	100
Prothrombin fragment (1-155)	100
Factor VII	None detected
Protein S	None detected
Bovine Protein C	None detected

* All competitions performed with 2 μg/ml Protein C.

\+ Concentration of competitor species to achieve 1/2 maximal inhibition of Protein C-Mab binding signal.

TABLE 4. PURIFICATION OF FACTOR IX FROM CRYOPOOR PLASMA

Step	Factor IX Activity (units/ml)	Overall Recovery %	Factor IX Spec. Act. (units/mg)	Fold Purification
Cryo-poor plasma	1.02	100	0.02	1
Mass Capture Eluate	8.1	95	1.5	88
Monoclonal	18.1	72	268	15,764

TABLE 5. PURIFICATION OF PROTEIN C FROM CRYOPOOR PLASMA

Step	Factor IX[c] Activity (units/ml)	Overall[a] Recovery %	Factor IX[a,b] Specific Act. (units/mg)	Fold Purif.
Cryo-poor Plasma	1.0	100	0.02	1
Mass Capture Eluate	8.8	67	1.3	65
First Immunopurification Eluate	56.6	60	195	9,735
Second Immunopurification Eluate	16.2	58	300	15,000

[a] Amidolytic activity upon chromogenic substrate S-2366

[b] Protein concentration based on absorbance at 280 nm assuming an absorption conversion of 1.4 ml/mg.

[c] Amidolytic activities are measured in unconcentrated eluates.

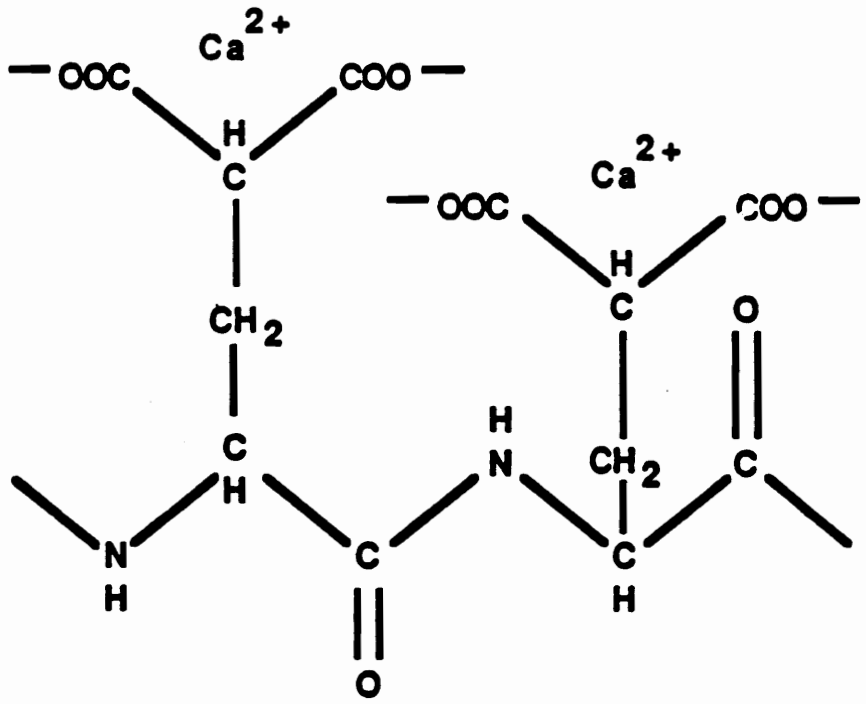

FIGURE 1
GAMMA-CARBOXYGLUTAMIC ACID RESIDUES

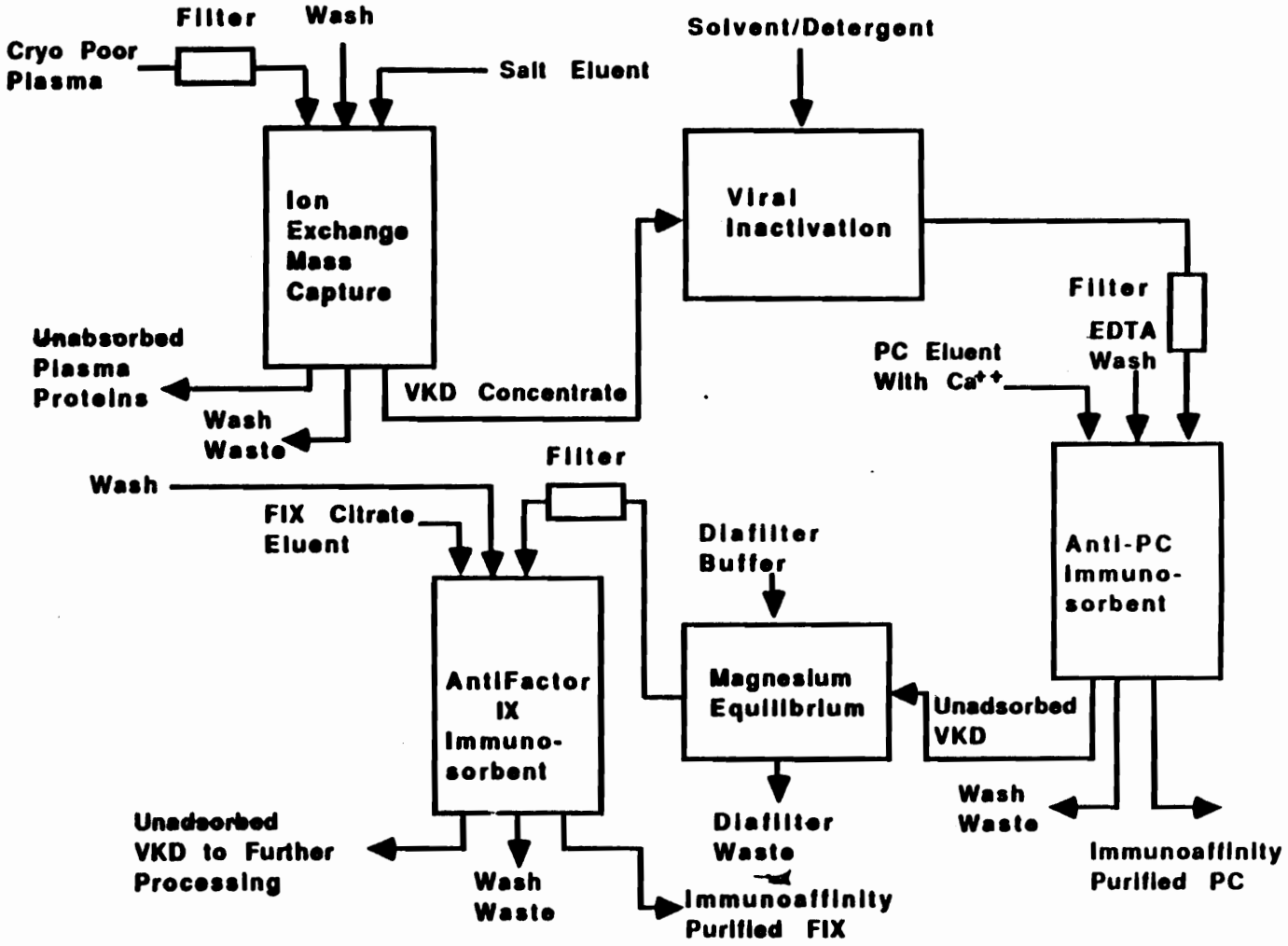

FIGURE 2
PROTEIN C AND FACTOR IX PURIFICATION PROCESS

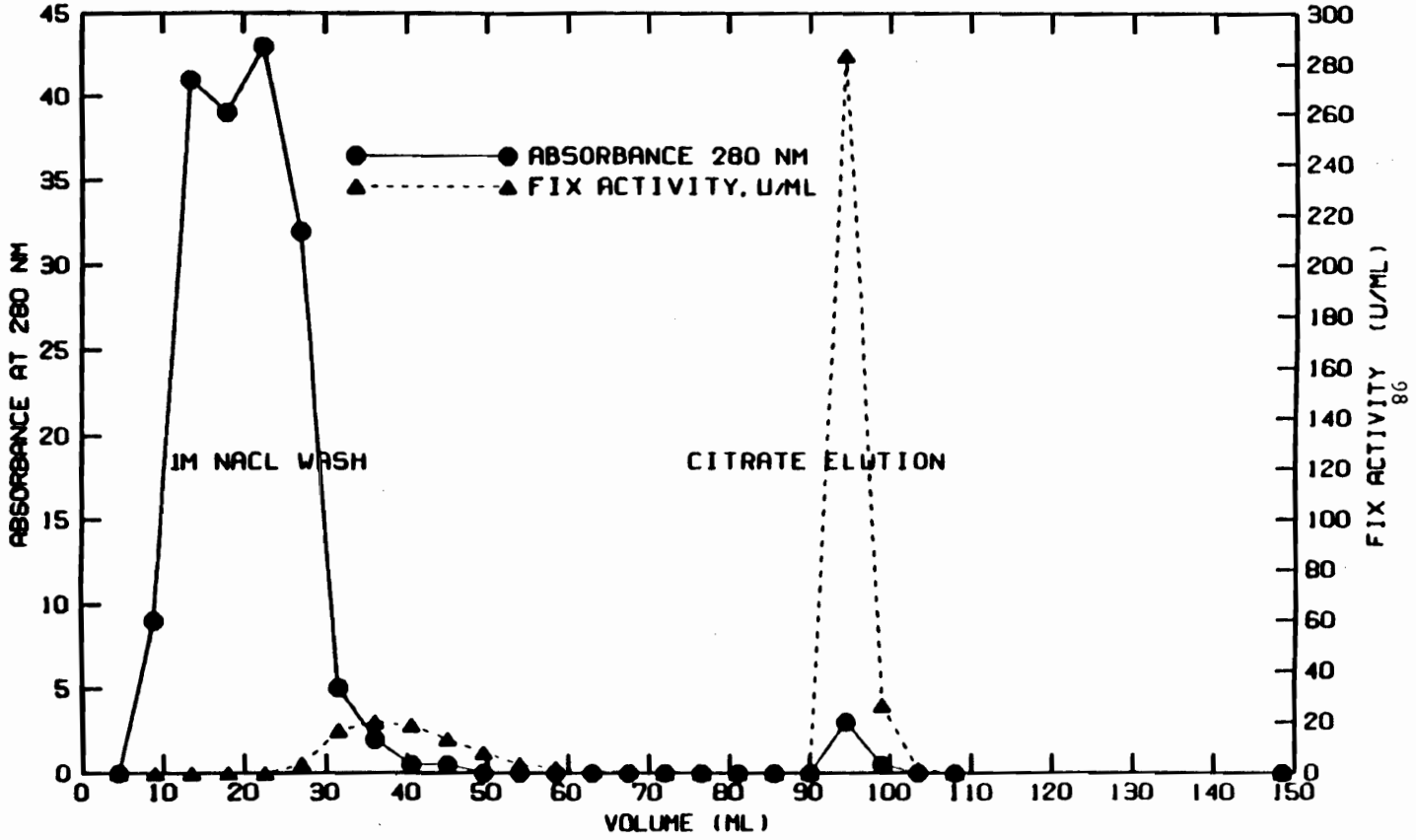

FIGURE 3
FIX PURIFICATION FROM DEAE USING MAB(1H5B7)

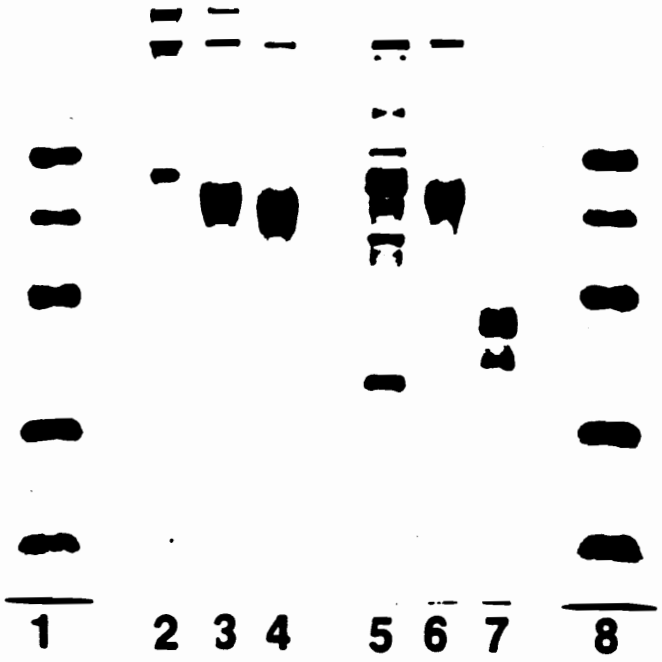

FIGURE 4
SDS-PAGE OF IMMUNOPURIFIED FACTOR IX AND PROTEIN C

Figure Key:

Lane 1	low molecular weight marker standard mixture (purchased from Bio-Rad)
Lane 2	anion exchange VKD protein concentrate (nonreduced)
Lane 3	immunopurified Factor IX (nonreduced)
Lane 4	immunopurified Protein C (nonreduced)
Lane 5	anion exchange CKD protein concentrate (reduced)
Lane 6	immunopurified Factor IX (reduced)
Lane 7	immunopurified Protein C (reduced)
Lane 8	low molecular weight marker standard mixture

CHAPTER FOUR

COMPARISON OF HYDRAZIDE AND CNBR ACTIVATED IMMUNOSORBENTS

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ABSTRACT

A majority of the coupling methods used to link antibodies to chromatographic supports, such as CNBr-activation methods, immobilize antibodies through reactive amino acids of the molecules. The bound antibody activity of these immunosorbents are low. Compared to these methods, hydrazide-based matrices couple antibodies through the carbohydrate moieties assumed to be located in the Fc portion of the molecules, and are therefore expected to have better performance. In order to verify this, the performance of CNBr- and hydrazide-coupled methods for two metal-dependent monoclonal immunosorbents directed against the human plasma proteins Factor IX and Protein C have been compared. The efficiencies (based on divalent antibody activity) of the hydrazide-activated immunosorbents were either equivalent to or lower than the corresponding CNBr-activated immunosorbents. In addition, recovery of the bound protein (antigen) was lower for the hydrazide-activated immunosorbents.

INTRODUCTION

Immunoaffinity separations offer an attractive, one-step alternative to conventional methods for the large-scale purification of proteins. A major drawback of this technique is the high cost of the antibody (Ab), which is compounded by increased Ab requirements due to the low activity of bound Ab. Efficiencies based on divalent Ab activity have been reported to be 30% or less for immunosorbents containing 1 mg or more

bound Ab per ml of gel (1-5). Maximizing the Ab activity of an immunosorbent would reduce the Ab requirement, and thus significantly lower the cost of the immunosorbent.

Steric hindrance and diffusion limitations caused by high Ab density, distortion of the Ab structure due to multipoint attachment and inaccessibility of the antigen-binding site due to the orientation of the Ab are some of the factors thought to compromise the activity of bound Ab (6). It has been shown that Abs coupled to supports at low densities form more efficient immunosorbents (7). Multipoint attachment of Ab is often minimized by adjusting various coupling parameters such as the concentration of reactive groups on the gel, pH and reaction time (8). The majority of the processes commonly used for coupling Abs to solid matrices, such as CNBr, N-hydroxysuccinimide, carbonyldiimidazole and toluene sulphonylchloride activation methods, immobilize the Ab through primary amino groups on the Ab surface (8). As many such groups are present on the surface of a large molecule like an Ab, the orientation of the immobilized molecules is thought to be random. This would be expected to compromise the affinity of some of the antigen-binding sites for the corresponding ligand or in the extreme case, render them inactive. This would lead to a poorly oriented immunosorbent with lower efficiency than expected.

Recently, hydrazide-activated supports have become available, where reactive hydrazine groups on the matrix are coupled to the carbohydrate

moieties that are generally assumed to be found exclusively in the Fc region of the Ab (9,10). Here the vicinal hydroxyl groups of the carbohydrate residues are oxidized to form aldehydes, which are then reacted with the hydrazine groups of the solid support to obtain a covalent hydrazone linkage (Fig.1). In a variation to this approach, the carbohydrate moieties of the Ab are selectively biotinylated with hydrazine-biotin (9) and then adsorbed on avidin-or streptavidin-coupled solid supports (11). The Ab is noncovalently attached to the matrix through the high affinity binding of biotin to avidin and streptavidin. Both approaches bind Abs to matrices through the Fc region of the molecules. Compared to CNBr coupling, Abs that are coupled through the Fc region of the molecules are expected to offer several advantages, leading to greater efficiency. For instance, as the hydrazide-activated gels bind through the Fc regions of the Abs, active-site containing Fab regions are left free to bind antigen. The carbohydrate chains of the Abs could also act as "linkers" to locate the Abs away from the matrix, thereby reducing steric hindrance of the matrix. However, the performance of such immunosorbents have not been well documented.

This study compares the performance of hydrazide-activated immunosorbents to that of CNBr-activated immunosorbents. The matrices of both types of immunosorbents are agarose-based soft gels. Two different "metal-dependent," murine, monoclonal antibodies (Mabs) directed against the human plasma proteins Factor IX (FIX) and Protein C (PC) were used. These Mabs bind to the corresponding antigens in the presence or absence

of divalent cations, respectively (12, 13). Mild elution conditions can be used to obtain the affinity-purified proteins as the antigen-antibody complexes are easily disrupted by chelation or addition of divalent cations in the millimolar concentration range (14).

MATERIALS AND METHODS

MATERIALS

Assera Protein C, a specific rabbit anti-human Protein C antiserum and HRP-conjugated anti-human Protein C rabbit antiserum were purchased from American Bio-Products Co., Parsippany, NJ; Affinity-purified, HRP-conjugated, goat anti-mouse immunoglobulins (IgA + IgG + IgM) antibody was from Cappel, West Chester, PA; O-phenylenediamine (OPD) was from Dakopatts, Denmark; Immulon II microtiter plates were from Dynatech Laboratories, Inc., Chantilly, VA; Protac was from American Diagnostica Inc., New York; S-2366 was from Helena Labs, Beaumont, TX; Aquasil, a water soluble siliconizing fluid was from Pierce Chemical Co., Rockford, IL; $NaIO_4$ (certified A.C.S. grade) was from Fisher Scientific, Fair Lawn, NJ; Amicon columns were from Amicon, Danvers, MA; Polypropylene Econo-Columns and Econo-Pac 10DG desalting columns were from Bio-Rad Laboratories, Richmond, CA; and Sepharose CL-2B was from Pharmacia, Piscataway, NJ. Hydrazide derivatized agarose gels were purchased from two different manufacturers, BioProbe International Inc., Tustin, CA and Bio-Rad Laboratories, Richmond, CA. Anti-Protein C monoclonal antibody 7D7B10 and anti-FIX-monoclonal antibody were developed at the American Red

Cross, Holland Laboratory, Rockville, MD (12, 13). All other chemicals were reagent grade or better.

METHODS

Proteins

Monoclonal antibody (Mab)

anti-FIX Mab - The murine "metal-dependent", anti-FIX Mab was purified from cultured cell supernatant as described by Wang et al. (12).

anti-PC Mab - The murine "EDTA-dependent", anti-PC Mab 7D7B10 was purified from cultured cell supernatant as described below. The cell supernatant was filtered and Mab concentrated by precipitation with saturated ammonium sulfate at room temperature. The precipitate was dissolved in 0.05 M Tris/0.1 M NaCl pH 7.5 and re-precipitated with 45% ammonium sulfate at 4°C. This precipitate was dissolved in 0.05 M Tris/0.1 M NaCl pH 7.5 to obtain anti-PC Mab. The purity of this material by SDS-PAGE was greater than 90%. Both Mabs were of the IgG_1 subtype with kappa light chains. Mab concentrations were determined by the absorbance at 280 nm using an absorption coefficient ($A_{280}^{1\%}$) of 15.5. The molecular weight of Mab was assumed to be 160,000 (8).

Factor IX (FIX)

Human FIX was immunoaffinity purified from cryo (cryoprecipitated antihemophilic factor)-poor plasma as described by Tharakan et al. (15, 16). The final product was 95% pure by SDS-PAGE and had a specific activity of 202 units/mg. FIX concentrations were determined by the

absorbance at 280 nm using an $A_{280}^{1\%}$ of 13.2 and assuming the molecular weight of FIX to be 57,000 (17).

Protein C (PC)

Human PC was immunoaffinity purified from cryo-poor plasma using an immunosorbent based upon the murine Mab 8861. Bound PC was eluted with a pH 10.0 buffer (unpublished information). The final product was 92% pure by SDS-PAGE and had a specific activity of 200 units/mg. PC concentrations was determined by absorbance at 280 nm using an $A_{280}^{1\%}$ of 14.5 and a molecular weight of 62,000 (18).

Immunosorbent Preparation

Anti-FIX hydrazide immunosorbent A

The coupling was performed by the manufacturer A according to their standard protocol. A 20-30 mg/ml solution of anti-FIX Mab in 0.05 M sodium acetate pH 5.0 was oxidized by gently agitating with 0.1 M $NaIO_4$ in a 10:1 volumetric ratio (64-fold molar excess $NaIO_4$) for 1 hour at room temperature in the dark. $NaIO_4$ was removed by desalting. An 8-10 mg/ml solution of oxidized Mab was mixed with the hydrazide gel A in a 1:1 volumetric ratio at 4°C, overnight. The gel was sequentially washed with 0.05 M sodium acetate pH 5.0, distilled water and 1 M NaCl. The coupling efficiency and the immunosorbent density were reported to be 98% and 1.87 mg/ml, respectively. The coupling efficiency (percentage of Mab coupled to gel) was calculated by the difference between total Mab and uncoupled Mab as measured by absorbance at A_{280}.

Anti-PC hydrazide immunosorbent A

(i) The coupling procedure described above was modified for preparing this immunosorbent because preliminary experiments resulted in a low coupling efficiency. A 17 mg/ml solution of anti-PC Mab (7D7B10) was gently agitated with 0.05 M $NaIO_4$ in a 3:1 volumetric ratio (157-fold molar excess $NaIO_4$) for 1 hour at room temperature in the dark. $NaIO_4$ was removed by desalting. A 2.5 mg/ml solution of oxidized Mab was gently agitated with hydrazide gel A in a 1:2 volumetric ratio, for 48 hours at 4°C. The gel was washed with 0.05 M sodium acetate pH 5.0. The coupling efficiency was 90% and the immunosorbent density was 1.0 mg/ml.

(ii) Another anti-PC hydrazide immunosorbent A was also prepared by the manufacturer A according to their standard protocol outlined previously. A 100-fold molar excess of $NaIO_4$ was used in the Mab oxidation step. The coupling efficiency and the immunosorbent density were reported to be 98% and 2.25 mg/ml, respectively.

Anti-PC hydrazide immunosorbent B

The protocol recommended by manufacturer B was used. A 2.7 mg/ml solution of anti-PC Mab (7D7B10) IgG in 0.05 M sodium acetate pH 5.5 was mixed with 0.1 M $NaIO_4$ in a 12:1 volumetric ratio (494-fold excess $NaIO_4$) for 1 hour at room temperature, in the dark. $NaIO_4$ was removed by desalting. A 1.8 mg/ml solution of oxidized Mab was mixed with 5 ml of hydrazide gel B in a 1:1 volumetric ratio, for 48 hours at room temperature. The gel was washed with 0.05 M sodium acetate pH 5.5. The coupling efficiency was 71% and the immunosorbent density was 1.5 mg/ml.

Anti-FIX Sepharose CL-2B

The coupling was performed according to the method of March et al. (19) as described below. The coupling efficiency was 94% and the immunosorbent density was 1.0 mg/ml.

Anti-PC Sepharose CL-2B

Anti-PC Mab (7D7B10) was coupled to Sepharose CL-2B using the method of March et al. (19). Sepharose CL-2B was activated with CNBr in CH_3CN. The activated gel was coupled to Mab in 0.1 M $NaHCO_3$/0.5 M NaCl pH 8.5 buffer and blocked with 1 M glycine ethylester pH 8.5. The coupling efficiency was 88% and the immunosorbent density was 1.7 mg/ml.

Chromatography

Anti-FIX Immunosorbents

The coupled gels were packed in aquasil-treated, Amicon G 10 x 150 mm columns. The columns were equilibrated in 10 mM $MgCl_2$/110 mM NaCl, 20 mM imidazole pH 7.5 buffer. The starting material was highly purified FIX, which was equilibrated with 40 mM $MgCl_2$. The Factor IX was loaded onto the columns, rinsed with 10 mM $MgCl_2$/1M NaCl/20 mM imidazole pH 7.2 buffer and eluted with 20 mM sodium citrate/110 mM NaCl pH 6.8 (15, 16). All column operations were performed at a flow rate of 1.0 ml/min at 4°C. The absorbance of all effluent fractions was measured at 280 nm. The columns were then stripped with 2 M NaCl/0.1 M citrate pH 7.2.

Anti-Protein C Immunosorbents

All coupled gels were packed in 12 ml polypropylene Econo-columns except the anti-PC hydrazide immunosorbent A-(i), which was packed in an

Amicon G 10 X 250 mm, aquasil-treated column. The columns were equilibrated in 0.025 M Tris/0.05 M NaCl pH 7.5 (TBS). Highly purified PC was used as the load material. The samples in TBS were loaded onto the columns at a flow rate of 0.52 ml/min, rinsed with TBS and eluted with 20 mM $CaCl_2$/TBS. The columns were further washed with 100 mM $CaCl_2$/TBS, 2 M NaCl/TBS and with 2 M NaSCN/TBS. Column fractions of 3.0 ml were collected and the absorbance at 280 nm was measured.

Assays

FIX Activity

FIX activity was measured by a clotting assay according to the method of Biggs (20) as modified by Miekka (21).

PC Activity

The assay was performed according to the method of Odegaard et al. (22). PC was activated with Protac and the chromogenic substrate S-2366 was used to measure its activity. The reaction rate was determined by the rate of change of absorbance at 410 nm using a Vmax kinetic microplate reader (Molecular Devices).

PC Antigen Assay

Immulon II microtiter wells were coated with a 1:200 dilution of rabbit anti-Protein C antisera in 0.1 M $NaHCO_3$ pH 9.6 for 1 hour at 37°C. Wells were blocked with 1% BSA/0.1 M $NaHCO_3$ pH 9.6 for 1 hour at 37°C and washed with TBS/0.1% Tween-20. Samples in TBS buffer were added to coated wells and incubated for 1 hour at room temperature with gentle agitation. The wells were washed with TBS/Tween-20 and then incubated with 1:1000

diluted HRP - conjugated rabbit anti-Protein C antisera in TBS/0.1% BSA for 1 hour at room temperature. The wells were washed and HRP activity was detected with OPD substrate by absorbance at 490 nm using a kinetic microplate reader by Molecular Devices.

ELISA

Comparison of Oxidized and Native Anti-PC Mab

Immulon II microtiter plates were coated with rabbit anti-Protein C antisera, blocked,and washed as described above. Serially diluted PC samples in TBS/10 mM EDTA/0.1% BSA were incubated with 32 nM oxidized or native (untreated) 7D7B10 Mab for 1 hour at room temperature. The PC - Mab mixture was added to the coated wells and incubated for 1 hour at room temperature. The wells were washed and incubated with 1:1000 diluted HRP - goat anti-mouse IgG conjugate in TBS/10 mM EDTA/0.1% BSA for 1 hour at room temperature. The wells were washed and HRP activity was detected with OPD as described above.

Comparison of Protein C Purified by Different Methods

Serially diluted PC samples from a conventional purification (13), and 8861 Mab and 7D7B10 Mab immunoaffinity purifications (14) were preincubated with 32 nM native 7D7B10 Mab and assayed according to the procedure outlined above.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (23) using a 12.5% polyacrylamide gel, and stained with 2% Coomassie brilliant blue R.

Sialic Acid Determinations

Anti-PC and anti-FIX Mabs were heated with 0.1 N H_2SO_4 for 1 hour at 80°C to liberate sialic acid residues. The sialic acid content was determined according to the method of Warren (24) by colorimetric analysis using thiobarbituric acid.

RESULTS

This study compares the performance of immunosorbents that were prepared using hydrazide- and CNBr-activated matrices. Hydrazide-activated agarose gels from two different manufacturers (referred to as manufacturers A and B) were used to prepare anti-PC hydrazide immunosorbents, while only the gel from manufacturer A was used to prepare the anti-FIX hydrazide immunosorbent. The agarose gel, Sepharose CL-2B, was activated with CNBr to form the comparative immunosorbents. The anti-FIX and anti-PC immunosorbents were formed with murine, "metal-dependent", monoclonal antibodies (Mabs). The anti-FIX Mab binds human FIX in the presence of divalent cations such as Ca^{2+} and Mg^{2+}, while the anti-PC Mab binds human PC in the absence of divalent cations. The Mab density in these immunosorbents varied from 1.0-2.25 mg/ml, signifying a low Mab

density range. Highly purified human FIX or PC (more than 92% pure by SDS-PAGE) was used as the starting material in column chromatography, enabling the use of protein mass based on A_{280} for efficiency calculations.

Highly purified PC was applied onto the 1 mg/ml anti-PC hydrazide A-(i) immunosorbent (prepared from the hydrazide gel of manufacturer A), rinsed, and bound PC eluted with calcium as described in "Materials and Methods". PC was estimated by absorbance at 280 nm. Theoretical maximum capacity was based upon the amount of antigen that could be bound by the total Ab, assuming divalent Ab activity. The average efficiency of immunosorbent A was 14% of the theoretical maximum capacity (Table I-A). Surprisingly, 40% - 50% of the PC that was bound by this immunosorbent could not be eluted by the elution buffers or by high salt and NaSCN as given in Table I-A. After the first two runs, immunosorbent A was equilibrated in coupling buffer and treated with a 3.5-fold excess of glutaraldehyde for 20 hours at 4°C in order to block any residual free hydrazide groups that may react with PC. However, as shown by runs 3-6 in Table I-A, the glutaraldehyde-treated immunosorbent performance was unchanged. Again, 40% - 50% of the bound PC was not recovered even after a high salt wash and the use of the chaotrope NaSCN. As the column was not loaded under saturating conditions in the first six runs, the immunosorbent was batch loaded with saturating amounts of PC for 1 hour at 4°C with gentle, end-over-end agitation. Rinsing and elution were done under the column conditions previously described. As seen in Table I-A,

runs 6 and 7, batch loading to eliminate kinetic effects of the antibody-antigen interaction and saturation of the immunosorbent did not improve its efficiency. After seven consecutive uses of the same immunosorbent, the efficiency and the amount of uneluted PC remained unchanged.

The 2.25 mg/ml anti-PC hydrazide A-(ii), immunosorbent prepared by the manufacturer was operated under the conditions described above. The performance of this gel was better than the immunosorbent prepared in our laboratory, with an efficiency of 27% (Table I-B). Again, 48% of the bound PC could not be eluted.

Anti-PC hydrazide B immunosorbent, prepared from the hydrazide gel of the second manufacturer, was column loaded with saturating amounts of PC and operated under identical conditions as described above (Table II). The performance of this immunosorbent was similar to that of A-(ii) with an efficiency of 26%. Once again, approximately 40% of the bound PC could not be eluted.

Highly purified human FIX was applied onto the anti-FIX immunosorbent A prepared by the manufacturer, and bound FIX was eluted as described in "Materials and Methods". The performance of this immunosorbent was similar to the anti-PC hydrazide immunosorbent A-(i), with an efficiency of 19% (Table III). Approximately 23% of the bound FIX could not be eluted under the elution conditions given in Table III.

The inability to recover all of the bound PC from the anti-PC hydrazide immunosorbents could be due to non-specific adsorption of PC onto the sugar matrix, or due to one-way diffusion of PC into its internal pores. Hydrazide gel from manufacturer A was treated with glutaraldehyde (3.5-fold molar excess w.r.t. total hydrazine groups on gel) for 20 hours at 4°C in order to form a blocked reference matrix without any Mab. PC was loaded onto this reference matrix and eluted as described previously. All of the PC that was loaded was recovered in the fall-through fractions, eliminating the possibility of unusual pore-diffusion and non-specific adsorption by the matrix (Table IV).

In a comparative study using anti-PC Sepharose CL-2B, the mean immunosorbent efficiency was found to be 22% (Table V-A). Unlike the anti-PC hydrazide immunosorbents, most of the bound PC was eluted after three consecutive runs with losses decreasing from 20% to 8.5%. The anti-FIX Sepharose CL-2B immunosorbent had an efficiency of 38% with a protein loss of 15% of the bound FIX (Table V-B).

The Mabs used to form hydrazide immunosorbents were oxidized in a 64- to 494-fold excess $NaIO_4$ for 1 hour at room temperature. These mild oxidation conditions are generally insufficient to oxidize amino acids on the Mab (9). However, we were concerned that the efficiencies of the hydrazide immunosorbents may have been affected by loss of activity of the Mab as a result of $NaIO_4$ treatment. Therefore, oxidized anti-PC Mab (7D7B10) was compared to native (untreated) Mab by an ELISA (Fig.2). The

dose-response curves of PC binding to both oxidized and native Mab were identical, indicating that oxidation did not impair the activity of the Mab.

The highly purified PC used as the starting material for anti-PC (7D7B10) immunosorbent chromatography was purified using the anti-PC Mab, 8861, which is not a "metal-dependent" Mab. It was conceivable that this PC starting material contained a population that was unable to bind the 7D7B10 Mab. This could account for the overall low efficiencies of the anti-PC (7D7B10) immunosorbents. Therefore, the binding affinities and dose-response curves of conventionally purified PC, and PC purified using two different Mab immunosorbents, namely, 7D7B10 and 8861 were compared. If different populations of PC exist, all of the 7D7B10-affinity purified PC would be able to bind the 7D7B10 Mab, while only some of the conventionally purified PC and 8861 immunosorbent purified PC would be able to bind it. All three sources of PC bound the 7D7B10 Mab with the same affinity as apparent from the half-maximal binding and the dose-response patterns (Fig.3). Conversely, we verified that an excess of PC was able to bind 100% of a limiting amount of 7D7B10 Mab, indicating that the 7D7B10 did not contain a population that lacked the ability to bind PC (results not shown). This is inherent to the nature of Mabs, which are comprised of identical immunoglobulin molecules containing identical binding sites for a unique antigenic determinant.

A 1:1 suspension of used anti-PC hydrazide immunosorbent A-(i) in TBS was sonicated at 4°C. Intact and sonicated immunosorbents were assayed for PC amidolytic activity and PC antigen as described in "Materials and Methods". Due to the inaccuracy in pipetting gel suspensions, the results are more of a qualitative nature than quantitative. Both the intact and the sonicated anti-PC hydrazide immunosorbents contained PC activity as well as antigen, the latter containing more detectable PC (Fig.4). By comparison, the used anti-PC Sepharose CL-2B immunosorbent and the reference hydrazide matrix did not contain PC antigen or activity.

The hydrazide immunosorbents are formed through the carbohydrate chains of the Ab (10). To evaluate the integrity of these carbohydrate groups, the anti-PC and anti-FIX Mabs were analyzed for sialic acid content. Both Mabs contained 1-2 residues of sialic acid per mole of Mab. These results are consistent with literature values (25), indicating that the carbohydrate groups of these Mabs are not abnormal.

DISCUSSION

The hydrazide-activated gels couple Abs through the carbohydrate residues that are believed to be located in the Fc region of the Abs. This offers the potential advantage of coupling Abs through residues that are not involved in antigen-binding, thus leaving the molecules with unencumbered Fab regions to facilitate interaction with antigens. In comparison, the CNBr-activated gels are linked to the matrix through

reactive amino acids. As many such groups are present on a macromolecule like an Ab, linkage can occur through many sites on the protein and has the potential disadvantage of being able to bind Ab through peptide regions that may be vital to the Ab-antigen interaction, thus compromising the activity of the bound Ab. The carbohydrate groups on the Ab have the added advantage of being able to act as a "linker" to space the molecule away from the matrix and thus improve its accessibility to the antigen. Conversely, the Abs of CNBr-activated gels can be expected to be located closer to the surface of the matrix giving rise to steric hindrance effects. In addition, linkage through the carbohydrate moieties is expected to give a higher degree of Ab "orientation" compared to coupling through amino acids, as the coupling is thought to involve only the Fc region of the molecule (10,11). Therefore the hydrazide immunosorbents are expected to have higher bound Ab activities and efficiencies than CNBr-coupled immunosorbents. In order to verify this, hydrazide-activated and CNBr-activated immunosorbents directed against the medium molecular weight human plasma proteins FIX and PC (57 K and 62 K, respectively) were compared. The matrices in the hydrazide- and CNBr-activated gels were agarose beads with similar porosities and particle sizes and both systems were coupled to give immunosorbents in the low Mab density range, and both are operated under mild elution conditions. Therefore, these two systems were sufficiently similar to effect a comparison of their efficiencies.

The hydrazide-activated immunosorbents failed to perform better than the conventional CNBr-activated immunosorbents. Depending on the coupling

protocol, the efficiencies of the anti-PC hydrazide immunosorbents were either equivalent to or lower than the CNBr-activated gel, while the efficiency of the anti-FIX hydrazide immunosorbent was half that of the CNBr-activated gel. The low observed efficiencies of the hydrazide immunosorbents were neither due to loss of Mab activity upon $NaIO_4$ oxidation, nor due to any heterogeneity of the antigen or Mab. The Mab densities of the anti-PC and anti-FIX immunosorbents varied from 1.0-2.25 mg/ml of gel. Previous data indicate that the efficiencies of the anti-FIX immunosorbents change by about 15% within this density range, with higher densities corresponding to lower efficiencies (26). When the efficiencies of the anti-FIX and anti-PC immunosorbents are normalized for the Mab density variation using a linear interpolation of this data, the performance of the hydrazide-activated immunosorbents still remained equivalent or lower than the CNBr-activated gels.

The efficiencies of hydrazide immunosorbents previously reported in the literature (10) were comparable to or less than the efficiencies that were obtained for the anti-PC and anti-FIX immunosorbents in the present study. Although hydrazide immunosorbents were reported to have much higher bound activity than conventionally prepared immunosorbents, the difference observed was due to the extremely low (less than average) efficiencies obtained for the conventionally prepared gels. In the present study, the failure of the hydrazide immunosorbents to perform better than CNBr-activated gels may be due to the unavailability of accessible, reactive, amino groups on the antigen-binding sites of the

anti-FIX and anti-PC Mabs. In that case, CNBr coupling would not result in a significant loss of antigen-binding sites in the immunosorbent, and coupling via the carbohydrates would not necessarily improve its performance.

Oxidation of the carbohydrate residues of Mab by $NaIO_4$ produces multiple aldehydes (27). Therefore, the Mab is attached by multiple bonds to the hydrazide matrix. This multivalency may enhance the stability of the bound Mab. It is unlikely that this multivalency would distort the Mab molecule and adversely affect its ability to bind antigen, as linkages occur through the carbohydrates which would not affect the conformation of the protein.

The distribution of reactive linkages (such as hydrazides) on the agarose matrix and their accessibility may also be of importance in determining the performance of a given immunosorbent. For maximum efficiency these reactive groups must have a uniform distribution and accessibility for the proper spacing of Mabs on the matrix, otherwise clustering of Mabs would cause steric hindrances and result in lower efficiencies. This is however unlikely for both hydrazide- and CNBr-activated gels as the total reactive groups available on the matrix are about a 1000-fold molar excess of that used to couple Mabs at 1-2 mg/ml densities. The hydrazide linker concentrations were given by the manufacturers and the CNBr-activated groups were based on the minimum available reactive groups using the data of March et al. (19).

Carbohydrate residues of immunoglobulins are usually attached to the heavy chains of the Fc portion of the molecule, while the light chains in the Fab region are not believed to contain carbohydrates (28,29). However, there is an accumulating body of evidence that the light chains of immunoglobulins undergo glycosylation. This phenomena has been observed for different immunoglobulin classes such as G, M and D in several mammalian species such as rabbit, mouse and human (30-32). There is also increasing evidence that carbohydrate chains of immunoglobulins are important for biological activity (33). This would indicate that coupling via carbohydrate linkages does not necessarily safeguard the Fab portion, as binding could occur through the carbohydrates located in that region. This would be a potential reason for the observed low performance of the hydrazide-activated immunosorbents used in this study. Although it is unlikely that all immunosorbents contain carbohydrates in the Fab region of the Ab, there would be less advantage in using hydrazide-based coupling methods for those that contain carbohydrates in the Fab region.

Recovery of the bound protein from the hydrazide immunosorbents was lower than that of the CNBr-activated immunosorbents. About 40-50% of the bound PC and 23% of the bound FIX were not recovered by the elution buffers, or even after the use of high salt and chaotropes. In comparison, 15% of the bound FIX was not recovered from the anti-FIX Sepharose CL-2B column, while most of the bound PC was recovered from the anti-PC Sepharose CL-2B column after repeated use. The inability to

recover all of the bound material from the hydrazide immunosorbents was not due to nonspecific adsorption or diffusion into the hydrazide matrix, as the matrix alone did not retain any protein. Neither was this an artifact caused by changes in the ultraviolet absorption properties of the proteins due to metal ion binding (34), as was determined by a Ca^{2+} and Mg^{2+} titration of PC and FIX, respectively. The modification of the hydrazide gel upon Mab linkage appears to cause this unusual irreversible binding as PC activity and antigen were found within the anti-PC hydrazide immunosorbent beads, but not in the reference hydrazide gel nor in the Sepharose CL-2B immunosorbent. The inability to recover all of the bound proteins cannot be explained. This apparent irreversible binding is perhaps unique to these two proteins, and serves to emphasize the individuality of each antibody-antigen system which plays an important role in determining the performance and final selection of a given immunosorbent.

In conclusion, there was no advantage in terms of improved efficiency in the use of hydrazide-activated immunosorbents over the more conventional CNBr-activated immunosorbents. Furthermore, the heavier protein losses observed with the hydrazide gels were an added disadvantage.

ABBREVIATIONS

BSA	Bovine serum albumin
Tris	Tris(hydroxymethyl) amine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
HRP	Horseradish peroxidase
TBS	Tris buffered saline
Mab	Monoclonal antibody

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TABLE I
PERFORMANCE OF ANTI-PC HYDRAZIDE IMMUNOSORBENT A

The immunosorbent was equilibrated in 0.025 M Tris/0.05 M NaCl pH 7.5 (TBS) in a column and highly purified PC was applied onto it. The gel was sequentially washed with TBS, 20 mM $CaCl_2$/TBS (elution buffer), 100 mM $CaCl_2$/TBS, 2 M NaCl/TBS and 2 M NaSCN/TBS. Protein mass was based on A_{280}. All column operations were at flow rates of 0.52 ± 0.03 ml/min at 4°C. **(A)** Anti-PC immunosorbent A-(i) gel density was 1 mg Mab/ml. The bed volume of runs 1 and 2 was 12.5 ml, while that of runs 3-7 was 6 ml. **(B)** Anti-PC hydrazide immunosorbent A-(ii) gel density was 2.25 mg Mab/ml with a bed volume of 3.2 ml.

TRIAL #	MATERIAL LOADED (mg PC)	FALLTHROUGH & RINSE (mg PC)	CaCl2 ELUTIONS low & (high) (mg PC)	OTHER WASHES (mg PC)	EFFICIENCY[a]	% BOUND PC UNRECOVERED
(A) 1[b]	2.00	0.05	1.09 + (0.0)	0	11%	52%
2[b]	8.00	3.80	1.73 + (0.33)	0	21%	49%
3	2.00	0.99	0.59 + (0.11)	0	15%	31%
4	1.88	0.73	0.62 + (0.0)	0	13%	49%
5	2.00	0.79	0.61 + (0.0)	0	13%	49%
6[c]	4.90	4.14	0.49 + (0.0)	0	10%	36%
7[c]	5.64	4:07	0.59 + (0.0)	0	15%	38%
(B) 1	4.51	1.59	1.48 + (0.0)	0	27%	49%
2	5.29	2.59	1.44 + (0.0)	0	26%	47%

[a] Efficiency is given as a percentage of the theoretical maximum capacity which is defined as the theoretical amount of PC that can be bound by the total Ab in the gel, assuming a 1:2 molar ratio of Ab:PC.

[b] Bound PC was eluted with 5 mM $CaCl_2$/TBS and the gels were further washed sequentially with 20 mM $CaCl_2$/TBS and 2M NaCl.

[c] The gel was batch loaded with saturating amounts of PC for 1 hour at 4°C and loaded onto the column. Rinsing and elution were done under column conditions as described previously.

TABLE II
PERFORMANCE OF ANTI-PC HYDRAZIDE IMMUNOSORBENT B

The anti-PC hydrazide immunosorbent B was loaded onto a column and equilibrated in 0.025 M Tris/0.05 M NaCl pH 7.5 (TBS). Highly purified PC was applied onto the column, rinsed with TBS, and eluted with 20 mM $CaCl_2$/TBS. The column was further washed with 100 mM $CaCl_2$/TBS, 2 M NaCl/TBS and 2 M NaSCN/TBS. The A_{280} of all effluent-fractions was measured. Protein mass was based on A_{280}. All column operations were performed at a flow rate of 0.52 ± 0.03 ml/min at 4°C. The gel density was 1.5 mg Mab/ml gel and the bed volume was 5.0 ml.

TRIAL #	MATERIAL LOADED (mg PC)	FALLTHROUGH & RINSE (mg PC)	CaCl2 ELUTIONS low & (high) (mg PC)	OTHER WASHES (mg PC)	EFFICIENCY[a]	% BOUND PC UNRECOVERED
1	6.59	3.67	1.50 + (0)	0.0	26%	49%
2	6.18	3.01	1.34 + (0)	0.8	23%	32%
3	6.67	4.03	1.62 + (0)	0.0	28%	39%

[a] Efficiency is given as a percentage of the theoretical maximum capacity which is defined as the theoretical amount of PC that can be bound by the total Ab in the gel, assuming a 1:2 molar ratio of Ab:PC.

TABLE III
PERFORMANCE OF ANTI-FIX-HYDRAZIDE IMMUNOSORBENT A

The anti-FIX hydrazide immunosorbent was loaded onto a column and equilibrated in 10 mM $MgCl_2$/220 mM NaCl/20 mM imidazole pH 7.5. Highly pure FIX in a 40 mM $MgCl_2$ buffer was loaded onto the column, rinsed with 10 mM $MgCl_2$/1 M NaCl/20 mM imidazole pH 7.2, eluted with 20 mM sodium citrate/110 mM NaCl pH 6.8 and further washed with 2 M NaCl/0.1 M citrate pH 7.2. The A_{280} of all column fractions was measured. Protein mass was based on A_{280}. All column operations were performed at a flow rate of 1.0 ml/min at 4°C. The gel density was 1.0 mg Mab/ml gel and the bed volume was 4.9 ml.

TRIAL #	MATERIAL LOADED (mg FIX)	FALLTHROUGH & RINSE (mg FIX)	CITRATE ELUTIONS (mg FIX)	OTHER WASHES (mg FIX)	EFFICIENCY[a]	% BOUND FIX UNRECOVERED
1	3.84	1.86	1.12	0.54	17%	16%
2	3.69	1.44	1.32	0.35	20%	26%
3	3.81	1.83	1.33	0.14	20%	26%

[a] Efficiency is given as a percentage of the theoretical maximum capacity which is defined as the theoretical amount of PC that can be bound by the total Ab in the gel, assuming a 1:2 molar ratio of Ab:PC.

TABLE IV
REFERENCE HYDRAZIDE GEL A

Hydrazide gel from manufacturer A was treated with glutaraldehyde to form a matrix that did not contain any Mab. The gel was loaded onto a column, pure PC was applied onto it and rinsed with TBS. The gel was further washed with 20 mM $CaCl_2$/TBS, 100 mM $CaCl_2$/TBS, 2 M NaCl/TBS and 2 M NaSCN/TBS. A_{280} of all effluent fraction was measured. Protein mass was based on A_{280}. All column operations were performed at a flow rate of 0.52 ± 0.03 ml/min at 4°C.

TRIAL #	AMOUNT LOADED (mg PC)	FALLTHROUGH (mg PC)	Ca ELUTIONS low & (high)	OTHER WASHES	EFFICIENCY[a]	PC LOST (% PC BOUND)
1	2.0	2.05	0.0 + (0.0)	0%	0%	0%
2	2.0	2.10	0.0 + (0.0)	0%	0%	0%

[a] Efficiency is given as a percentage of the theoretical maximum capacity which is defined as the theoretical amount of PC that can be bound by the total Ab in the gel, assuming a 1:2 molar ratio of Ab:PC.

TABLE V
PERFORMANCE OF SEPHAROSE CL-2B IMMUNOSORBENTS

(A) Anti-PC Sepharose CL-2B immunosorbent was operated as described in Table I at a flow rate of 0.52 ± .03 ml/min at 4°C. The gel density was 1.7 mg Mab/ml gel and the bed volume was 2.0 ml gel. **(B)** The anti-FIX Sepharose CL-2B immunosorbent was operated as described in Table III at a flow rate of 1.0 ml/min at 4°C. The gel density was 1.0 mg Mab/ml gel and the bed volume was 5 ml.

TRIAL #	AMOUNT LOADED (mg protein)	FALLTHROUGH & RINSE (mg protein)	Ca^b or Citrate ELUTIONS (mg protein)	OTHER ELUTIONS (mg protein)	EFFICIENCY[a]	% BOUND PROTEIN UNRECOVERED
A 1	2.80	1.78	0.69 + (0.0)	0.12	26%	20%
2	2.00	1.45	0.46 + (0.0)	0	17%	16%
3	2.00	1.14	0.51 + (0.11)	0.17	24%	8.5%
B 1	2.12	0.31	1.32	0.26	37%	13%
2	2.10	0.35	1.44	0.28	40%	2%
3	2.01	0.23	1.34	0.08	38%	20%
4	2.08	0.21	1.30	0.09	36%	26%

[a] Efficiency is based on the percentage of the theoretical maximum capacity which is defined as the theoretical amount of PC that can be bound by the total Ab in the gel, assuming a 1:2 molar ratio of Ab:PC.

[b] 20 mM (low) and 100 mM (high) $CaCl_2$/TBS elutions.

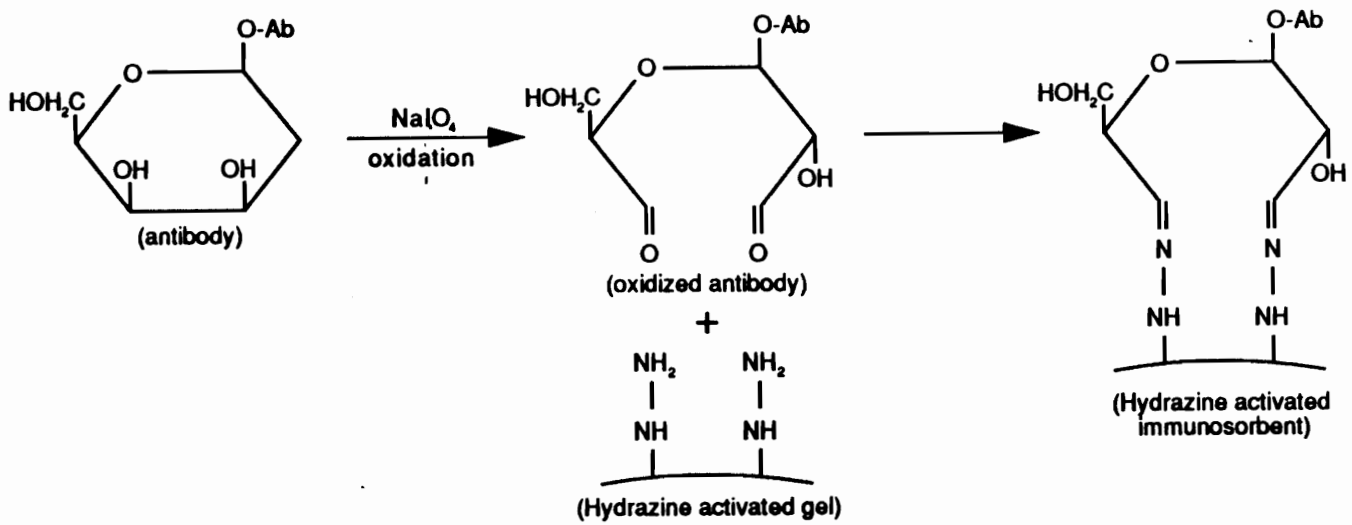

FIGURE 1
SCHEMATIC REPRESENTATION OF ANTIBODY ATTACHMENT TO HYDRAZIDE-ACTIVATED GELS

Carbohydrate residues on the Fc region of the antibody are oxidized at the vicinal hydroxyls to form aldehydes. The aldehyde groups are coupled with the hydrazine groups of the gel to form a hydrazine-activated immunosorbent.

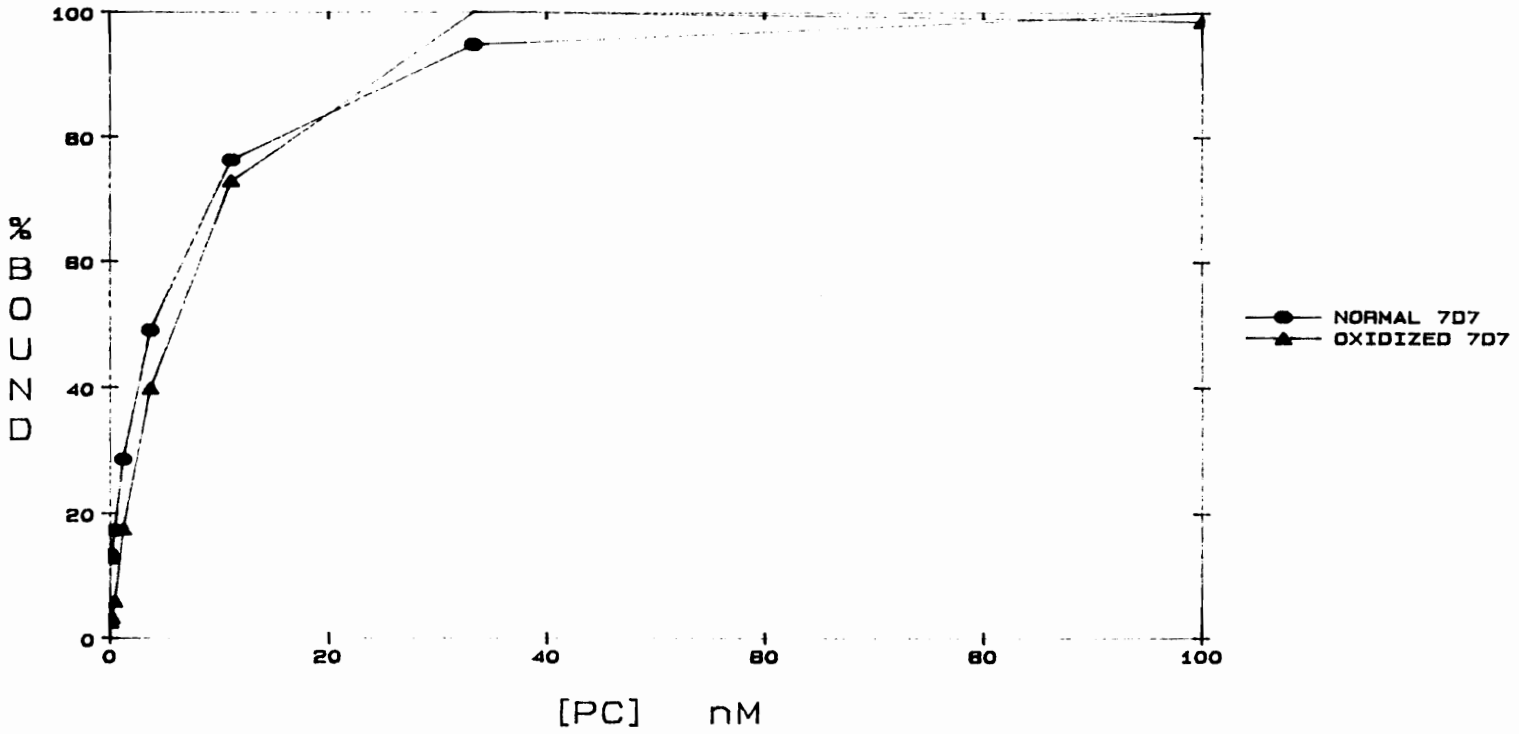

FIGURE 2
COMPARISON OF OXIDIZED AND NATIVE ANTI-PC MAB 7D7B10

Immulon II plates were coated with rabbit anti-PC antisera, blocked with 1% BSA and washed with 0.1% Tween 20/TBS. PC samples were preincubated with 32 nM native (untreated) or oxidized anti-PC Mab in TBS/10 mM EDTA/0.1% BSA and added to the prepared wells. The wells were washed and bound PC-Mab complex was detected with HRP-goat anti-mouse IgG antibody conjugate and OPD substrate by absorbance at 490 nm. The data is plotted as a percentage of the signal obtained at saturation.

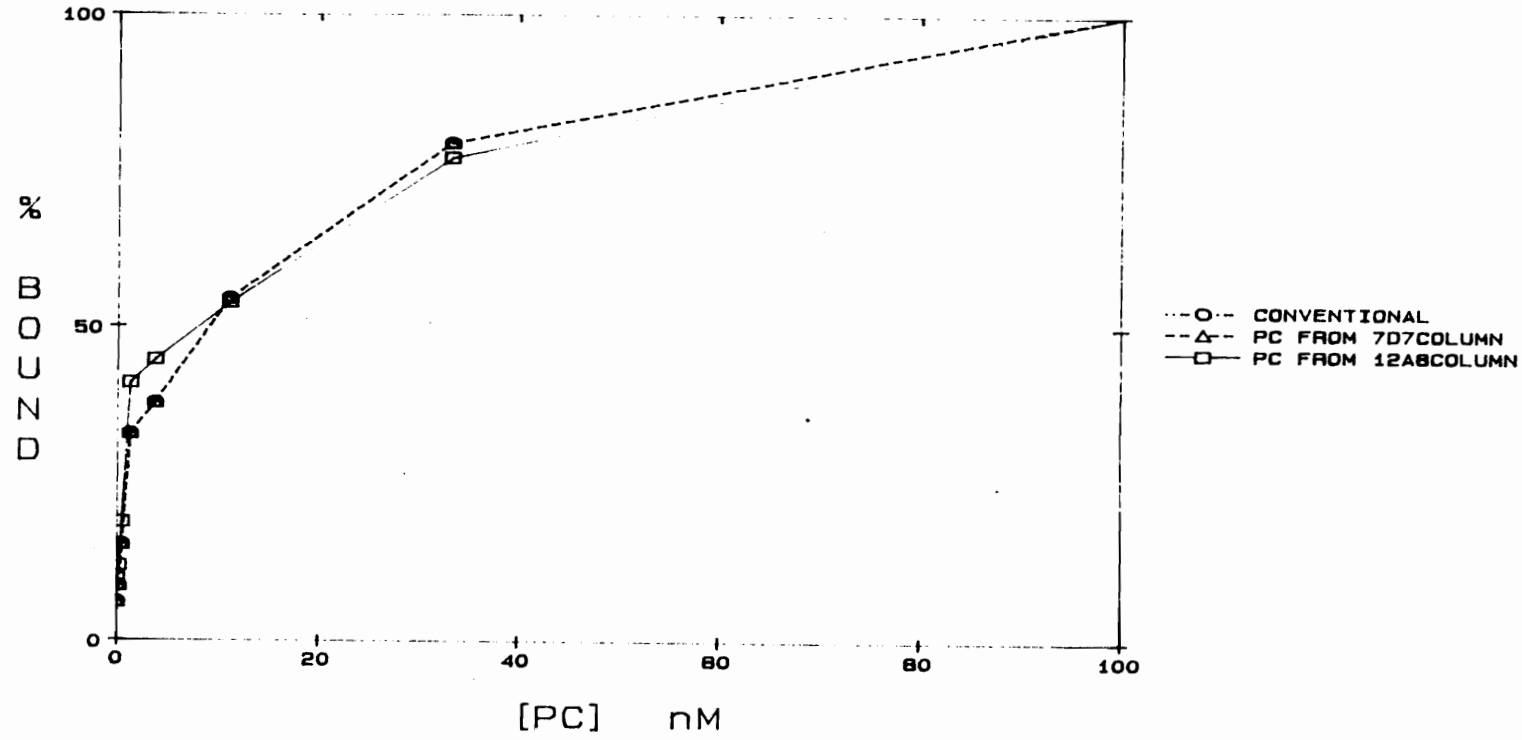

FIGURE 3
COMPARISON OF PC PURIFIED BY DIFFERENT METHODS

Serially diluted samples of PC from a conventional purification and from two different anti-PC Mab immunosorbents, 7D7B10 and 8861, were preincubated with 32 nM native 7D7B10 for 1 hour at room temperature, and the PC-Mab complex was captured and detected as given in Figure 2. The data is represented as a percentage of the signal obtained at saturation.

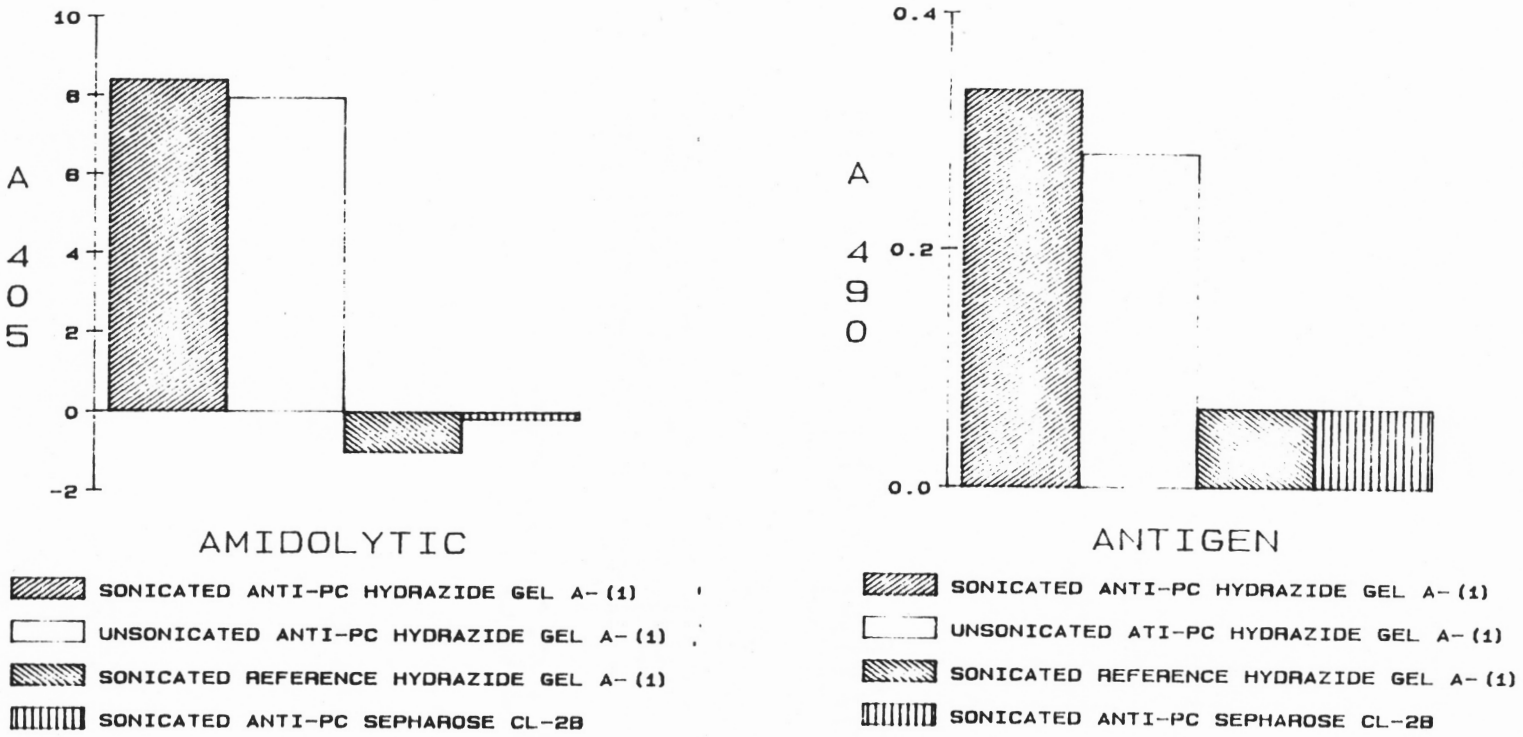

FIGURE 4
PC ANTIGEN AND AMIDOLYTIC ACTIVITY IN IMMUNOSORBENTS

Antigen activity: Gel samples used for chromatography were sonicated at 4°C and 0.1 ml samples were incubated for 1 hr. at room temp. in Immulon II wells prepared as described in Fig. 2. Bound PC was detected with HRP-conjugated anti-PC rabbit antisera and OPD chromophore by absorbance at 490 nm. **Amidolytic activity:** PC was activated with Protac. The reaction rate of activated PC was determined by the rate of change of absorbance of the chromogenic substrate S-2366 at 410 nm.

CHAPTER FIVE

POLYOXAZOLINE-PEPTIDE ADDUCTS WHICH RETAIN ANTIBODY AVIDITY

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ABSTRACT

In the synthesis of protein-polymer adducts, the specific chemistry by which the polymer modifies the protein is important in determining various properties of the adduct such as specificity and solubility. Polyethylene glycols have been widely used to form such adducts with several types of enzymes and antibodies due to the facility of its conjugation chemistry. In this study, the polyoxazoline family of water-soluble polymers was used to synthesize polymeric adducts containing a synthetic epitope of a monoclonal antibody directed against human protein C. This is the first application to adduct chemistry for this relatively new family of water-soluble polymers. The avidity of this polymeric adduct for the antibody was characterized. The epitope retained its avidity for the monoclonal antibody after conjugation with the polymer. The avidity of the adduct was dependent upon the hydrophilicity and the molecular weight of its polymeric segment. The retention of activity of these polyoxazoline adducts have potential application where derivatization of antibody, antigen or other proteins is desired, such as for prolonging clearance times of proteins and for eliciting immune responses for small peptides and proteins.

INTRODUCTION

The synthesis of protein-polymer adducts has been reported for many types of enzymes and antibodies (1-7). These chimeric macromolecules have been constructed for several different reasons. Typically, these

manipulations can modify deleterious biological activities while at the same time retaining desirable activities. These activities could be enzymatic, antigenic or of a more general receptor-ligand nature. In some instances, specialized conjugation chemistry has been developed to produce chimeric macromolecules which improve the immunoresponse used to generate antibodies specifically against small peptides (8). At the same time, it has been undesirable for either the polymer component or the conjugation chemistry employed to impart nonspecific interaction with biological surfaces and soluble components or to change the solubility of the protein itself. The degree to which any of the above properties have been changed by polymer-modification of a protein has varied with polymer molecular weight and the number of modification sites to the protein (2, 4). Thus, the specific chemistry by which the polymer modifies a given protein is also important.

In the cases of therapeutic enzymes, polymer derivatization has been used to effect longer renal clearance times in animal models (3, 5). For these purposes, a water soluble polymer such as polyethylene glycol (PEG) is often used because it is also amenable to conjugation chemistry. PEG has been conjugated to recombinant-human tissue plasminogen activator (3), streptokinase (4, 5), and urokinase (7). PEG has been shown to modify proteins via activation of the terminal hydroxy-group with reagents such as 1,1'-carbonyldiimidazole. A range of functional activities has been found for these adducts (4,5). The higher substitution of amino groups on the protein has been found to result in lower functional activity (4).

In other experiments, the inhibition of α2-macroglobulin-PEG was reduced by modification with PEG (2). Decreased α2-macroglobulin activity was found as the molecular weight of the PEG was increased.

Avidity between antibody and respective antigen is an example of another protein interaction which can be affected by polymer modification of a given protein. The relationship between avidity and the size of the polymer which is used to derivatize the protein has been evaluated for streptokinase derived from hemolytic streptococci (4). This protein has been found to be strongly immunogenic and therefore its use as a fibrinolytic agent is usually confined to a one-time therapy for a catastrophic thrombic event such as myocardial infarction. Agglutination reactions were evaluated using antisera directed against streptokinase. These studies found that agglutination reactivity could be reduced as the amount of covalent linkages to streptokinase with PEG of Mr = 5000 was increased. It was presumed that steric hindrance masked the binding to the immunoglobulins.

In this work, we seek to characterize the effect of polymer size and hydrophilicity upon the avidity of a synthetic epitope for a monoclonal antibody (Mab) against human Protein C (9). This peptide, HCPC(6-17), corresponds to residues 6-17 of the heavy chain of human Protein C (hPC). These residues have been shown to contain the epitope for the Ca^{2+} dependent monoclonal antibody (HPC4) to hPC, whereby the peptide also possesses the same Ca^{2+}-dependent avidity. The polymer used

to modify HCPC(6-17) are members of the polyoxazoline family of water-soluble polymers (10-14). The polymers synthesized were varied in both size and hydrophilicity by the choice of a 2-ethyloxazoline versus a 2-methyloxazoline monomer. This is the first application to adduct chemistry for this relatively new family of water-soluble polymers. Here, the retention of avidity after modification is used as a model for the potential applications that polyoxazolines (POX) may have for cases where derivatization of either antigen, antibody or other protein is sought. These applications include possible alternatives to cases where PEG modification impact upon clearance behavior is not optimal, synthesis of competitors for receptor-sites whereby the competitor might have longer clearance times because of POX-modification, POX modification of small peptides to elicit a stronger immune response for purposes of generating antibodies, and POX-modified antigens which could be used to improve the bound activity of immunosorbents via sterically-directed immobilization (17).

MATERIALS AND METHODS

MATERIALS

Monomeric 2-ethyloxazoline and 2-methyloxazoline were purchased from Aldridge Chemical Co. Milwaukee, WI. Peptide HCPC(6-17) corresponding to the sequence of residues 6-17 of the heavy chain of Protein C (HCPC(6-17)) was synthesized by the t-boc method. The sequence of HCPC(6-17) is given in Figure 1. The methyl benzhydrylamine-HCL anchoring resin for peptide

synthesis was purchased from Colorado Biotechnology Associates, Boulder, CO. HPC4 murine monoclonal antibody was provided by Dr. Charles Esmon of the Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma. Rabbit antisera against human Protein C were purchased from American Bioproducts, Parsippany, NJ. Affinity purified goat, anti-mouse immunoglobulins conjugated to horse-radish peroxidase (HRP) were purchased from Organon Teknika-Cappel, West Chester, PA. O-phenylenediamine-2HCl (OPD) tablets were purchased from Dakopatts, Denmark. Immulon II microtiter plates were purchased from Dynatech Laboratories, Inc, Chantilly, VA. Deuterium oxide (D_2O) was purchased from Sigma Chemical Company. DEAE-Sephacel was purchased from Pharmacia-LKB, Piscataway, NJ. Human Protein C was isolated by immunoaffinity methods (15, 16). All reagents were of the best commercial grade available.

METHODS

Synthesis of "live" Polyoxazolines

Benzyl iodide was used to initiate the polymerization reaction with either 2-methyloxazoline or 2-ethyloxazoline at 90°C in acetonitrile. A 1:1 molar ratio of initiator and monomer were reacted, where the amount of monomer is stoichiometrically determined by the desired polymer chain length. Polymer sizes of 1K, 5K, 10K, and 20K for poly(2-ethyloxazoline) were made using the method described herein. Polymer sizes of 1K, 5K, and 10K poly(2-methyloxazoline) were also made using this same method. Once initiator and monomer are combined, the propagation step proceeds until all monomer is consumed as detected by proton NMR. Typical batch sizes

were 5 ml of a 250 mg per ml live polymer solution. Molecular weight distributions as measured by GPC had a polydispersity of 1.2-1.4 (12). Prior to the termination step a solvent change was made via lyophilization to remove acetonitrile and was followed by reconstitution with methanol. 200 microliter aliquots of live polymer (typically containing 5 to 50 mg polymer) in acetonitrile were pipetted into tared 1.5 ml Eppendorf plastic microtubes using an Eppendorf pipette with plastic disposable pipette tips. Yields were determined after lyophilization at 0.1 Torr overnight using a Savant (centrifugal) Speedvac Concentrator.

Activation of Synthetic Peptide HCPC(6-17)

The epsilon amino group of the carboxy-terminal lysine residue of the HCPC(6-17) peptide was activated using the following procedure. One ml of 30 mg per ml aqueous, dibasic sodium phosphate, pH 12.4 was added at 0°C to 15 mg peptide. This is approximately a three-fold molar excess of phosphate for a theoretical maximum of 8 titratable protons present in the peptide. This mixture was quickly mixed by vortex and frozen at -90°C for 5 minutes to minimize hydrolysis of the peptide. The frozen mixture was then lyophilized at 0.1 torr using a Savant centrifugal Speedvac concentrator for 3 hours. The titrated peptide-phosphate solid mixture was extracted with 1 ml of cold methanol by vortex mixing followed by centrifugation at 1000 x g for 10 minutes. An essentially quantitative extraction of the peptide from the phosphate solids was achieved using this method. The stock peptide was stored in methanol at -25°C.

Termination of the Live Polyoxazoline Using Activated HCPC(6-17)

Methanol was found to solubilize both polymer and peptide and was inert to the live polymer. Thus, methanol was chosen as a good solvent for the termination step. The lyophilized live polymer was reconstituted with 1 ml or less of methanol using a vortex mixer. The live polymer-methanol solution was added to activated HCPC(6-17) every hour at room temperature in increments of 20% of the theoretical stoichiometry, based upon a 1:1 termination of polymer with peptide. This was done until a 20% molar excess of polymer had been added. A final incubation at 40°C for a minimum of two hours was performed before the reaction mass was lyophilized to dryness and reconstituted in nanopure, deionized water.

Purification of the Reaction Mass

An anion-exchange liquid chromatography method using DEAE-Sephacel was developed for assuring separation of free peptide from polymer-peptide adduct fractions. Aliquots of reaction mixtures containing 5 mg of total peptide were applied to a 1 inch diameter column and eluted at a flow rate of 1.0 ml per minute with degassed, deionized (nanopure) water. Eluate was detected at 214 nm. A step change to 1M NaCl was made after a stable baseline was obtained following peaks eluted using deionized water. Chromatographic fractions were lyophilized, weighed and reconstituted with 1 ml of deuterium oxide and assayed by 200 mhz proton NMR (pNMR) using a minimum of 400 scans. The products were then recovered from the NMR tubes and lypholized. While the reaction products tended to be wholly aqueous soluble (in excess of 5 mg adduct per ml H_2O),

sporadic aqueous solubility problems occurred with poly(2-ethyloxazoline) adducts after lyophilization. TBS-buffered medium was found to solubilize some of the insolubles generated by lyophilization. The 40 K poly(2-ethyloxazoline)-peptide showed an extreme sensitivity to lyophilization in terms of the production of an insoluble freeze-dried product. Poly(2-methyloxazoline)-peptide adducts did not show any noticeable solubility problems in aqueous or TBS-buffered aqueous medium after lyophilization. Neither the polymer nor the peptide showed individual solubility problems after lyophilization.

Enzyme-Linked Immunosorbent Assay (ELISA) of Purified Polymer-Peptide Adducts

The avidity between the HPC4 and the polymer-peptide adducts were evaluated by sandwich ELISA. Varying amounts of the anion-exchange fractions recovered from NMR analysis were pre-equilibrated together with a set concentration of hPC and HPC4. The resultant hPC-HPC4 complex was then captured by solid-phase immunosorption and detected by the chromophore generated from a sandwich of rabbit-derived anti-hPC immunoglobulin (Ig) and horseradish peroxidase conjugated (HRP), anti-mouse Ig derived from goat in the presence of OPD. A decrease in signal at A_{490} as a function of increasing concentration of the recovered fraction was indicative of competitive avidity for the HPC4.

Immulon II plastic microtiter plates were first coated for one hour at 37°C with rabbit derived, anti-human Protein C antisera by application

of 100 μl of a 1:200 dilution of stock using 0.1 M sodium bicarbonate buffer at pH 9.6. This was followed by blocking with 0.1 M $NaHCO_3$, pH 9.6, 1% BSA for 1 hour at 37°C. The wells were then washed with 0.05 M Tris, 0.15 M NaCl, pH 7.5 (TBS), containing 0.1 % Tween 20. Before application to Ig coated wells, 100 μl of TBS, 0.1% BSA, containing 5 mM $CaCl_2$ with 0.032 μM HPC4 monoclonal antibody, 0.032 μM human Protein C, and polymer-peptide ranging from 0.0064 to 100 μM were pre-equilibrated for 1 hour at 25°C. Subsequent incubation of this mixture in the wells occurred at 25°C for 1 hour. After washing with TBS, 0.1% Tween 20, 5 mM $CaCl_2$, 100 μl of 1:1000 diluted HRP-conjugated goat anti-mouse immunoglobulin was applied to the wells. After washing with the same buffer, o-phenylenediamine dihydrochloride chromogenic substrate was applied to each well. Detection of the chromophore was made at 490 nm with a Molecular Devices Vmax kinetic microtiter plate reader.

RESULTS

"Living" polyoxazolines were found to be terminated by amines (12), the HCPC(6-17) as well as water (Velander, unpublished observations). The sequence of HCPC(6-17) is provided in Figure 1. The reaction of the epsilon amino group of the carboxy-terminal lysine (pKa = 10.53) and the alpha-amino group of the glutamic acid (pKa = 9.67) residing at the amino-terminus of the HCPC(6-17) peptide was thought to be feasible. The titration of the peptide at pH 10.5 and subsequent reaction with live polymer showed little product formed based upon free peptide recovered

from the reaction mass. Peptide titrated at pH 12.0-12.4 and subsequently reacted with live polymer produced 30-60% reaction product based upon free peptide recovered from the reaction mass. The proposed reaction sequence for generation of the live polymer and termination with peptide is given in Figure 2.

An example of a DEAE-Sephacel chromatography of a 1 K poly(2-methyloxazoline)-peptide reaction mass detected at 214 nm is shown in Figure 3. Here, the anion-exchange chromatography provided three distinguishable elution peaks. Proton-nuclear magnetic resonance spectroscopy (pNMR) of these fractions are presented in Figure 4. A reference spectra for 1 K poly(2-methyloxazoline) in D_2O is provided in Figure 4a. The proton signals with a maximum peak height at 1.85 ppm are characteristic of the 2-methyl residue protons of the poly(2-methyloxazoline). Figure 4b presents the pNMR spectra for a peptide reference. The multiple signal centered at approximately 0.9 is characteristic of the protons associated with leucine and isoleucine. The pNMR spectra of first and second D. I. H_2O elutable peaks (lyophilized and reconstituted in D_2O) appearing in Figure 3 are shown in Figures 4c and 4d, respectively. The presence of proton signals corresponding to both 2-methyl protons of polymer and the methyl protons of isoleucine and leucine are seen in both D. I. H_2O elutable fractions. The second D. I. H_2O elutable peak (Figure 4d) has a much stronger proton signal at 0.85 ppm relative to those of the polymer at 1.85 ppm as compared to the first peak eluted with H_2O (Figure 4c). The 1M NaCl elutable peak seen in Figure 3

possessed a spectra identical to the reference peptide given in Figure 4b. Furthermore, a control chromatography with peptide only produced a single NaCl elution peak corresponding to the NaCl elutable peak of Figure 3 (data not shown), while chromatography of the polymer control gave a D. I. H_2O elutable peak corresponding to the leading edge of peak A. Thus, we conclude that both D. I. H_2O elutable fractions contained covalently linked polymer-peptide, but no free peptide. However, it is likely that the first elutable D. I. H_2O peak ("peptide-poor" fraction) does contain free polymer. This is also true for the "peptide-rich" fractions from the higher molecular weight polymer reaction masses because as the molecular weight of the polymer used to modify the peptide was increased, the resolution between "peptide-poor" and "peptide-rich" D. I. H_2O fractions decreased. Chromatography of the ethyl polymer adducts was similar to that of the methyl polymer adducts except for the poor resolution between the first and second D. I. H_2O elutable peaks. The ethyl polymer adducts therefore comprise of a single pool of D. I. H_2O elutable fractions.

A measure of the relative avidity of HCPC(6-17), poly(2-ethyloxazoline) polymer and polymer-peptide adducts for the HPC4 Mab is provided by the competitive ELISA results as shown in Figure 5. The reference HCPC(6-17) peptide is seen to have a half-maximal inhibition at about 0.05 μM. Because the human Protein C is present at a concentration of approximately 0.032 μM, the HCPC(6-17) peptide is observed to possess nearly the same avidity for the HPC4-Mab as the parent hPC. Both titrated (used to maximize reactivity with the live polymer)

and untitrated peptide gave virtually the same avidity (data not shown). None of the poly(2-ethyloxazoline) polymers in buffer (probably terminated by aqueous hydroxyls) showed competition over the 0.05 to 100 μM range. Pooled material containing both poly(2-ethyloxazoline) "peptide-poor" as well as "peptide-rich" adduct fractions gave a half-maximal inhibition which was two orders of magnitude less competitive than the reference peptide.

The results for the competition ELISA of the 1K, 5K, and 10 K poly(2-methyloxazoline) polymer and respective HCPC(6-17) adducts are shown in Figure 6. Because ample amounts of both "peptide-rich" and "peptide-poor" fractions were available for the methyl-polymer-peptide adducts, separate competitions were evaluated for each fraction. None of the poly(2-methyloxazoline) polymers exhibited competition. The 1K and 10K "peptide-rich" adducts showed half-maximal inhibitions similar to that of the free peptide reference. The "peptide-poor" fractions were shown to have half-maximal inhibitions which were about two orders less competitive than free peptide and similar to the competitiveness of the poly(2-ethyloxazoline)-peptide pooled fractions.

Both of the 5K poly(2-methyloxazoline)-peptide adduct fractions exhibited half-maximal inhibitions approximately two orders less than that of the free peptide reference and slightly less competitive than the corresponding 1K and 10K "peptide-poor" fractions.

DISCUSSION

Polyoxazolines possess the advantage of being synthesized as a living polymer with no intermediate activation step (10-14). Termination has been made by reaction with primary amines such as hexylamine but not with species such as alkoxides. Thus, termination reaction with the ϵ-amino group of the lysyl residue of HCPC(6-17) peptide is expected under conditions where the lysyl is rendered nucleophilic by a high pH titration. The reaction with the amino-terminal residue is expected to be less predominant than with the lysyl amino group due to differences in nucleophilicity. While the peptide activation chemistry necessarily occurred only at high pH, the titration of the lysyl amino group was performed at low temperatures and short reaction times in a non-nucleophilic buffer. This procedure was shown not to adversely impact the peptide primary structure as indicated by the lack of change of avidity after undergoing titration.

Polyoxazoline-peptide adducts appear to retain a significant portion of the avidity of the parent peptide for the HPC4 Mab. Importantly, the parent polyoxazoline polymers do not seem to interact nonspecifically with immunoglobulins nor Protein C as judged by a lack of interference with the formation of a Protein C-Mab complex. This was the case over a wide range of molecular weights of parent polymer. Avidity of the polyoxazoline-peptide adducts does not have a significant impact from molecular weight. Avidity appears to be more related to hydrophilicity as the more water soluble, less hydrophobic poly(2-methyloxazolines) tend to have a higher

avidity for HPC4 (similar to that of the parent peptide) than the poly(2-ethyloxazolines). The 5K poly(2-methyloxazoline) is an exception to this behavior.

Differences in pNMR signal ratio of polymer to peptide protons from D. I. H_2O elutable fractions suggest that a free polymer may be in part responsible for the apparent lower avidity of the "peptide-poor" fractions of the poly(2-methyloxazoline) adducts. However, both fractions for the 5K-methyl adduct exhibited the same avidity in spite of markedly different peptide to polymer ratios as characterized by pNMR. The amount of free polymer as estimated by a material balance is less than 70% by weight, and therefore free polymer alone cannot account for the decrease in avidity by two orders of magnitude. For the 1K and 10K poly(2-methyloxazoline) adducts this dissimilarity in avidity between the "peptide-rich" and "peptide-poor" fractions may be indicative of different reaction products such as di- and mono-substituted peptide.

The ability of these adducts to retain avidity for the immunoglobulin should serve as justification for evaluation of the POX-protein modification upon other types of activities such as had been done for PEG (1-7). We have already begun an evaluation of the utility of using these adducts for sterically-directed immobilization of Mabs for the purpose of improving bound immunosorbent activity (17).

LIST OF ABBREVIATIONS

D_2O	Deuterium oxide
GPC	Gel permeation chromatography
Mab	Monoclonal antibody
Mw_p	Theoretical molecular weight of polymer
Mw_m	Molecular weight of monomer
Mr_p	Molecular weight of polymer as measured by GPC
HCPC(6-17)	Synthetic peptide corresponding to residues 6-17 of the heavy chain of human Protein C
HPC4	Calcium-dependent murine monoclonal antibody directed against human Protein C
HRP	Horseradish peroxidase
DEAE	Diethyl-aminoethane, anion exchange moiety
OPD	O-phenylenediamine-2HCl Chromogenic substrate
PEG	Polyethylene glycol
POX	Polyoxazoline polymer
TBS	Tris-buffered saline
PBS	Phosphate-buffered saline
ELISA	Enzyme-linked immunosorbent assay
pNMR	Proton nuclear magnetic resonance

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GLA - ASP - VAL - ASP - PRO - ARG - LEU - ILE - ASP - GLU - LYS

FIGURE 1
HCPC(6-17) AMINO ACID SEQUENCE

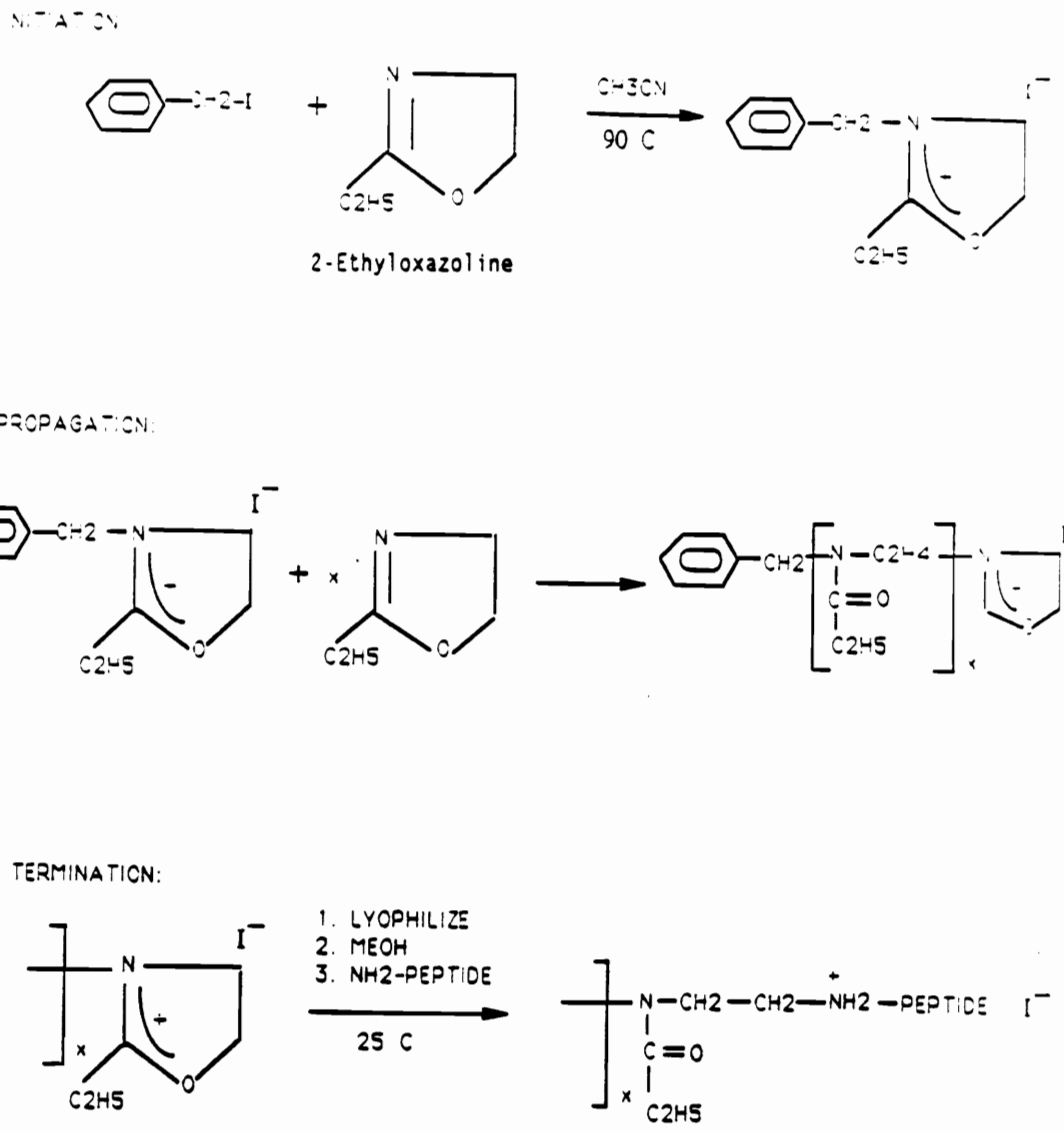

FIGURE 2
SYNTHESIS OF PEPTIDE TERMINATED POLY(2-ETHYLOXAZOLINE) POLYMERS

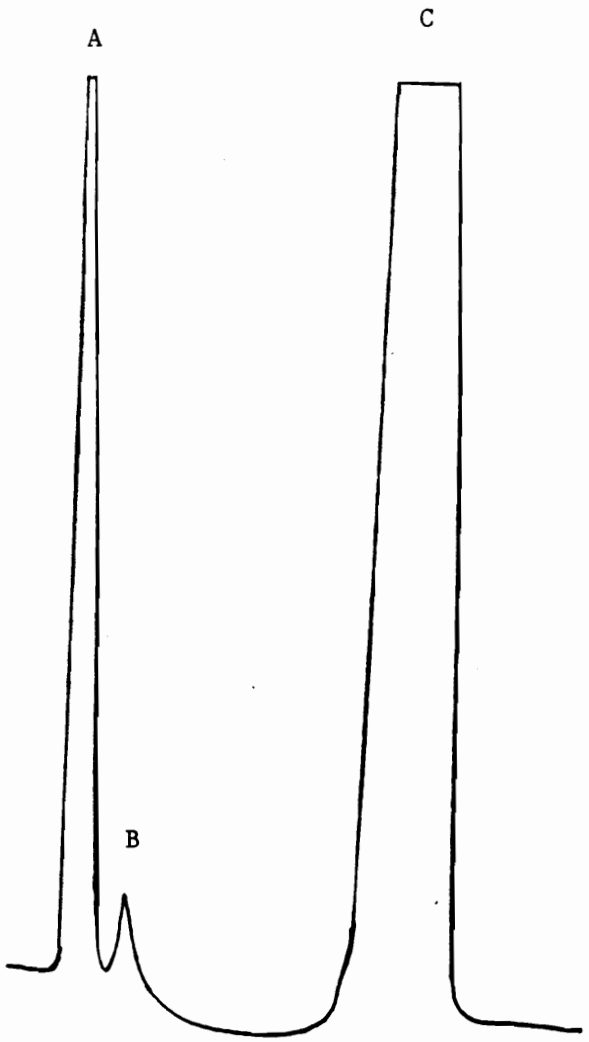

FIGURE 3
DEAE - SEPHACEL CHROMATOGRAPHY OF POLYMER-PEPTIDE REACTION MASS

A - First dH_2O elutable peak
B - Second dH_2O elutable peak
C - 1 M NaCl elutable peak

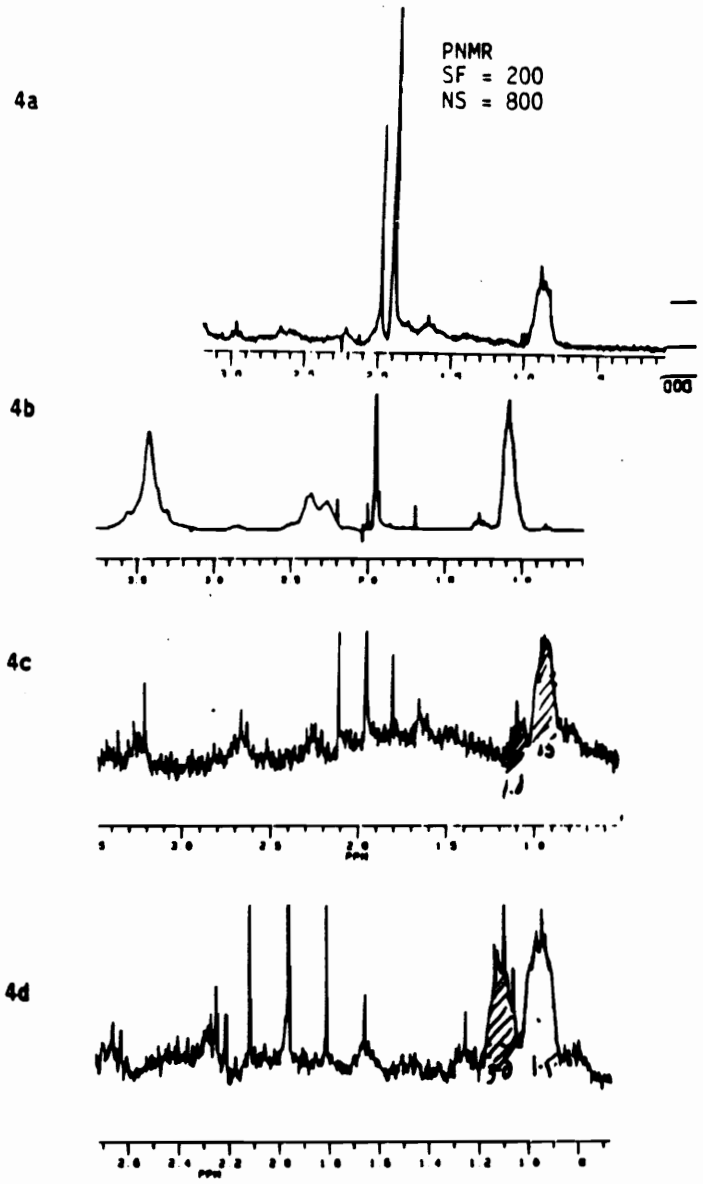

FIGURE 4
PNMR OF FRACTIONS OBTAINED FROM ANION-EXCHANGE CHROMATOGRAPHY

4a: polymer
4b: peptide
4c: peptide poor (peak A)
4d: peptide rich (peak B)

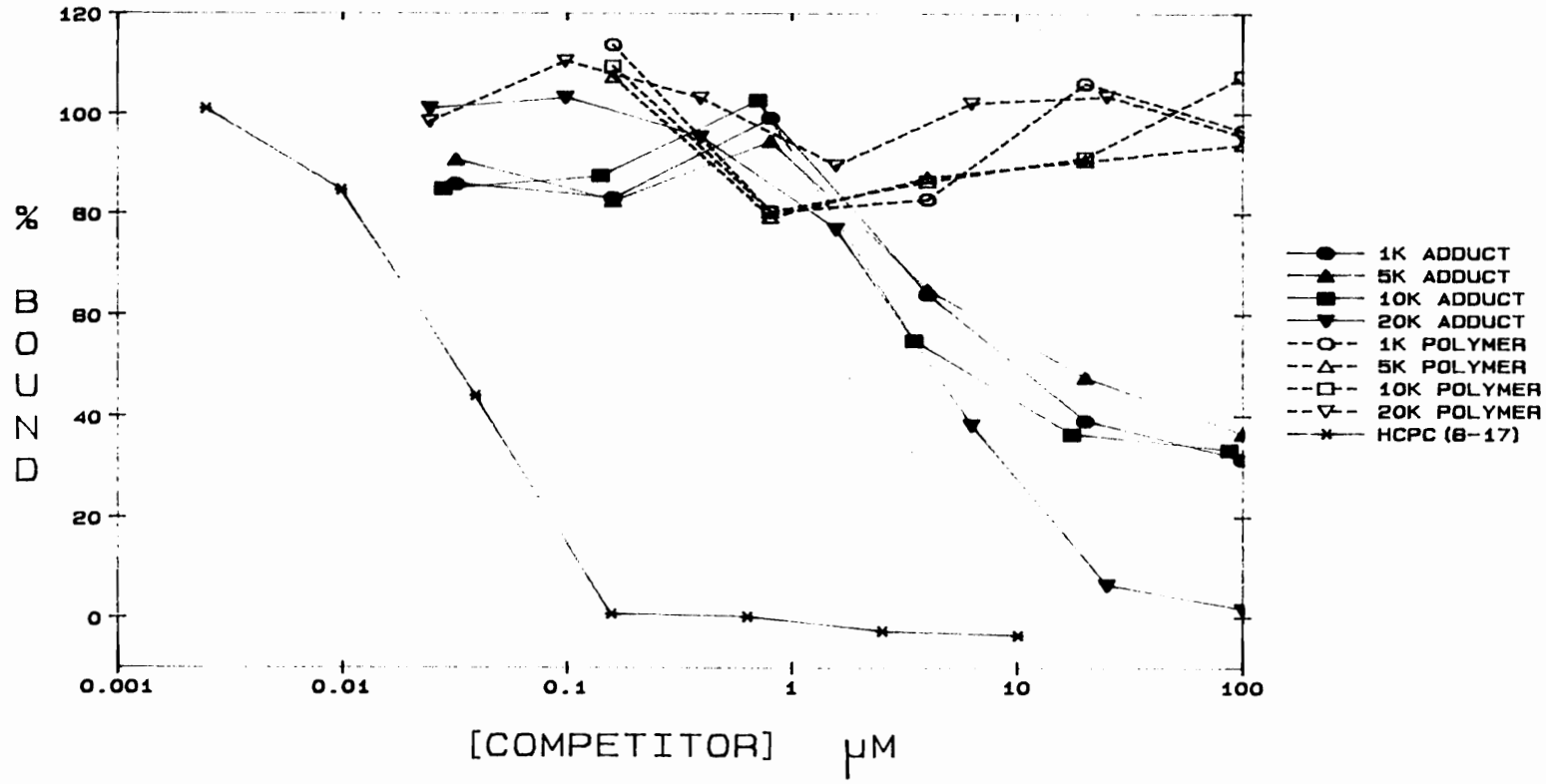

FIGURE 5
COMPETITION ELISA FOR POLY(2-ETHYLOXAZOLINE)-PEPTIDE ADDUCTS

Polymer-peptide adducts or peptide (competitors) were preincubated with 32 nM HPC4 and 32 nM hPC in TBS/5 mM $CaCl_2$/0.1% BSA. The resultant hPC-HPC4 complex was captured on Immulon II microtiter wells which had been coated with rabbit anti-hPC antisera and blocked with 1% BSA/TBS. The captured complex was detected with HRP-conjugated goat anti-mouse immunoglobulin and OPD chromophore by absorbance at 490 nm. The data are given as a percentage of the signal obtained in the absence of a competitor.

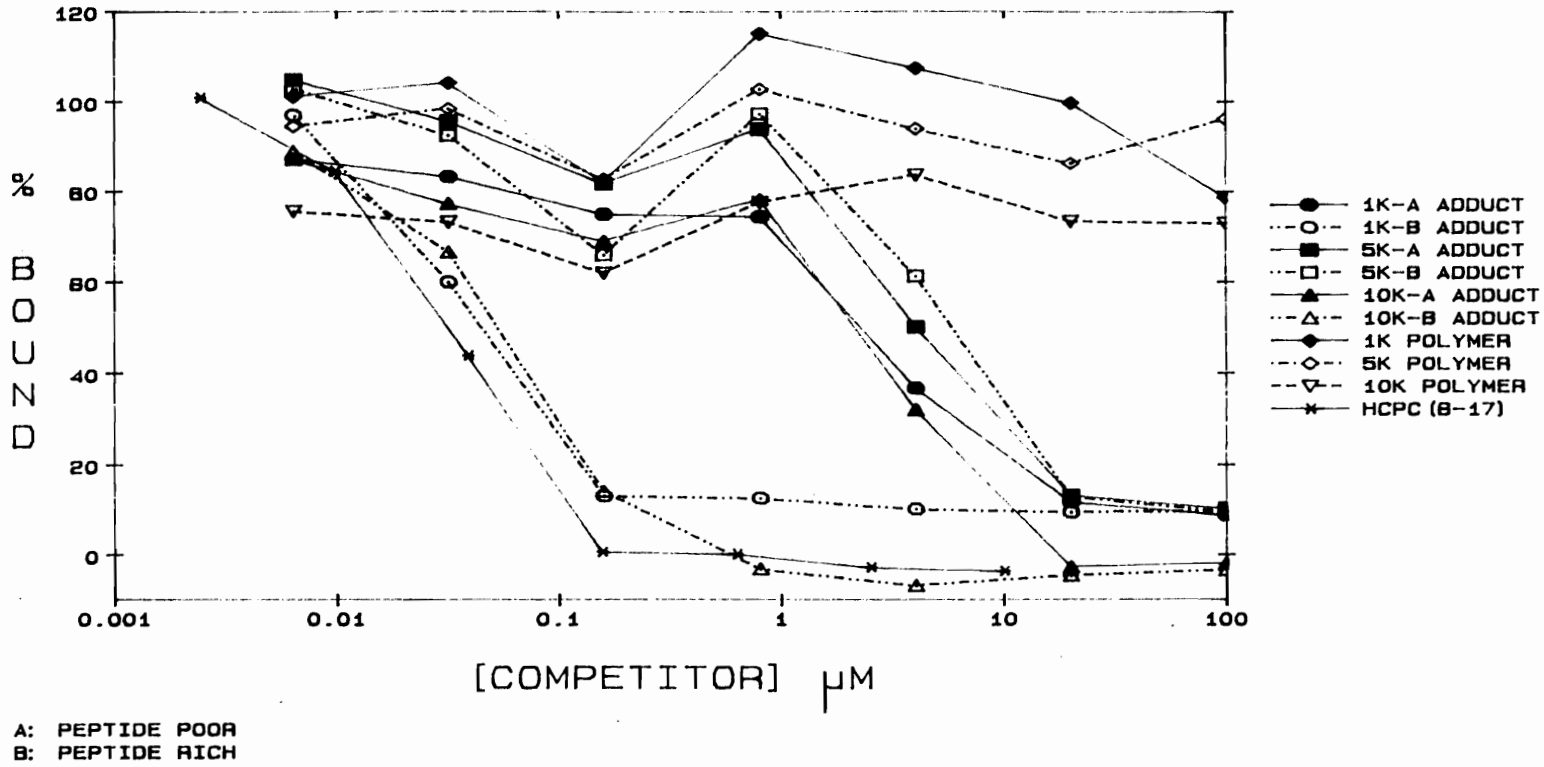

FIGURE 6
COMPETITION ELISA FOR POLY(2-METHYLOXAZOLINE) PEPTIDE ADDUCTS

Serially diluted polymeric samples were preincubated with 32 nM HPC4 and 32 nM hPC, and the resultant hPC-HPC4 complex was captured by solid-phase immunosorption and detected as described in Figure 5.

CHAPTER SIX

DIRECTING THE ORIENTATION OF IMMOBILIZED MONOCLONAL ANTIBODIES

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ABSTRACT

Immunosorbent technology plays an increasingly vital role in protein purification. A chief drawback of this technique is the high cost of the antibody in the immunosorbent which is compounded by the low activity of the bound antibody. The improper orientation of the antibody on the matrix has been cited as one of the probable causes giving rise to low bound immunosorbent activity. Efforts to anchor the antibody through the Fc region of the molecule have had limited applicability in immunopurification of parentally administered proteins. This study examines the feasibility of using orientation-directing agents (ODA) to sterically mask Fab regions prior to immobilization of monoclonal antibodies to give rise to immunosorbents where the antigen-binding Fab regions are oriented away from the matrix. The antibody used in the study is a Ca^{2+}-dependent murine monoclonal IgG directed against the plasma protein, human Protein C. The ODAs are made of a synthetic peptide (epitope) recognized by this IgG and polymer-peptide adducts of this epitope belonging to the water-soluble polyoxazoline family of polymers. The antibody recognition ability of these ODAs has been previously reported by us. These ODAs were able to improve the activity of antibodies immobilized on two different non-porous surfaces by about 2- to 4.8-fold. However, the hydrophobicity of some of these ODAs had the disadvantage of enhancing nonspecific adsorption of the ODA to the matrix.

INTRODUCTION

Immunosorbent technology has long played an important role in both clinical diagnostics and protein purification (1). Antibodies can possess both high specificity and binding affinity for a given antigen or hapten molecule. Because of these combined properties, an immobilized antibody can be employed as an agent for sorbing the respective antigen or hapten from a solution which may be very dilute in that species. The total binding capacity of an immunosorbent is determined by the number of available antigen binding sites, which is in general, a small fraction of the number of immobilized antibodies (2,3). A significant portion of the cost of large-scale immunopurification is due to this low bound immunosorbent activity; typical activities are usually less than 30% of theoretical capacity based upon a 1:2 stoichiometry of antibody to antigen (2-6). The sensitivity of immunosorbent assays is also related to amount of immobilized antibody (1,7). However, the specific effect of immobilization upon antibodies used in diagnostics is often ignored.

While no direct proof has been offered for the deleterious impact of "improper orientation" of the immobilized antibody, it has been cited as a probable cause for low bound activity (2,3). To that end, much effort has been spent upon the chemistry used to immobilize antibodies. These chemistries are usually based upon covalent coupling of reactive residues of proteins, such as the amino groups of lysine and the N-terminus, carboxy groups of aspartate, glutamate and the C-terminus, phenolic groups of tyrosines etc., to a matrix either through prior

activation of the matrix or through a coupling reagent (1). As large molecules like antibodies contain many such reactive groups in both the antigen binding (Fab) and cell receptor (Fc) domains, these chemistries result in covalent linkages to either domain. Thus, the antibody can be coupled through an amino acid in the antigen-binding region or in an orientation such that the antigen-binding site is inaccessible, resulting in the reduction or loss of the activity of that antibody.

While all immunoglobulin classes contain carbohydrate, the least carbohydrate is found in the IgG class (1,7). IgG monoclonal antibodies (Mab) are frequently used in immunosorbents and have been shown to contain a carbohydrate (CHO) in the hinge region of the constant region of the heavy chain in the Fc domain (8-10). Therefore, a more Fc specific chemistry utilizing the reaction of a hydrazide linker group with an oxidized carbohydrate has been developed with hopes of increasing bound IgG activity (3). However, CHO moieties have been found in the Fab domain of most classes of mammalian immunoglobulins, including murine IgG (9,10). This compromises the concept of Fc orientation for the hydrazide-CHO immobilization technique. Furthermore, studies involving two different murine-Mabs linked to agarose gels via hydrazide chemistry yielded bound activity comparable to that using CNBr-activated agarose (6).

Another example of "Fc-directed" immobilization chemistry has been the use of Fc-binding proteins such as Staphylococcus aureus derived Protein A (11) or streptococcal Protein G (12). However, Protein A does

not recognize all subclasses of IgG and has variability in avidity from species to species (12). These proteins are first covalently-linked to the matrix and then allowed to equilibrate with the antibody of interest. A stable complex of the Fc portion of the antibody with the Fc receptor protein results in a highly active immunosorbent (11).

Other receptor proteins such as avidin have been employed to couple antibodies onto matrices (1,7). Biotinylation of the respective antibody followed by adsorption onto a matrix containing immobilized avidin will result in a functionally active immunosorbent. However, the biotinylation step utilizes amino-coupling chemistry and thus results in random linking of the biotin to the antibody. In a variation to this approach, hydrazine-biotin has been used to selectively derivatize the CHO residues of IgG molecules (13,14). This type of orientation, like the hydrazine-CHO chemistry, is compromised by the presence of CHO groups in the Fab region of IgG molecules. The need for an intermediate proteinaceous linker such as Protein A or avidin adds cost and complexity to the synthesis of the immunosorbent. In addition, large scale immuno-purification of parentally administered protein products would necessarily be encumbered by the potential for leaching of these intermediary proteins (15).

We seek here to examine immobilization of monoclonal antibodies with the Fab region oriented away from the matrix such that higher bound activity is achieved. The surfaces and immobilization densities of

antibody have been chosen such that immunosorbent activity can be more specifically attributed to "orientation effects". A synthetic epitope for a Ca^{2+}-dependent murine IgG monoclonal antibody (Mab) directed against human Protein C (16) is employed as an orientation-directing agent (ODA). The amino acid sequence of this peptide is given in Figure 1. Synthetic adducts of this peptide with several different molecular weight species of water soluble polyoxazolines are also employed as ODAs. Complexes of HPC4 with either peptide or adducts (17) have been shown to be Ca^{2+}-dependent such that they are destabilized in a chelated environment. The antigenicity of these adducts has been previously reported by us (17). "Orientation effects" are examined on two nonporous surfaces, one designed for nonspecific low energy immobilization and one activated for covalent anchoring of the Mab.

MATERIALS AND METHODS

MATERIALS

Monomeric 2-ethyloxazoline and 2-methyloxazoline were purchased from Aldrich Chemical Co., Milwaukee, WI. Peptide 6-17 of the heavy chain of human Protein C (HCPC(6-17)) was made by t-boc synthesis. The sequence of HCPC(6-17) is given in Figure 1. The methyl benzhydrylamine-HCL anchoring resin for peptide synthesis was purchased from Colorado Biotechnology Associates, Boulder, CO. HPC4 murine monoclonal antibody was provided by Dr. Charles Esmon of the Oklahoma Medical Research Foundation, Oklahoma City, OK. Rabbit antisera against human Protein C

was purchased from American Bioproducts, Parsippany, NJ. Affinity purified goat, anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP) were purchased from Organon Teknika-Cappel, West Chester, PA. O-phenylenediamine-2HCl tablets were purchased from Dakopatts, Denmark. Immulon II microtiter plates were purchased from Dynatech Laboratories, Inc, Chantilly, VA. Immobilon AV affinity membranes were purchased from Millipore, Bedford, MA. All reagents were of best commercial grade available. Polymer-peptide adducts were synthesized and purified as described by Velander et al. (17). Human Protein C (hPC) was isolated by immunoaffinity methods as described by Orthner et el.(18).

METHODS

Evaluation of ODA Coating Effects on Immulon II Surfaces

A 0.1 M sample of the polymer product in 0.1 M $NaHCO_3$ pH 9.6 was incubated for 1 hour at room temperature (RT) in Immulon II microtiter wells. The samples were removed and the wells were incubated with 25 nM HPC4 in 0.1 M $NaHCO_3$ buffer for 1 hour at RT. The wells were blocked with 1% BSA in 0.05 M Tris/0.15 M NaCl pH 7.5 (TBS) for 1 hour at RT and washed with 0.1% Tween-20 in TBS. The wells were next incubated with HRP conjugated goat anti-mouse IgG antibody diluted 1:1000 in TBS/0.1% BSA for 1 hour at RT, washed, and bound conjugate was detected with OPD substrate by absorbance at 490 nm using a kinetic microplate reader by Molecular Devices.

Determination of the "Orientation Effects" of ODA on Immulon II Surfaces

Polymer peptide conjugates, HCPC(6-17) peptide or control samples were serially diluted in 0.1 M TBS/5 mM Ca^{2+} pH 8.0 buffer. These samples were incubated with 50 nM HPC4 Mab for 1 hour at RT. The mixture was applied to Immulon II wells and incubated for 1 hour at 37°C. The wells were blocked with 1% BSA/0.1 M $NaHCO_3$ pH 9.6 for 1 hour at 37°C and washed with 0.1% Tween-20/TBS. The wells were then washed with 100 mM EDTA/0.1% Tween-20/TBS for 30 minutes with three buffer changes to remove bound polymer peptide conjugates, and further washed with 0.1% Tween-20/TBS. The calcium dependent nature of the HPC4-hPC and HCPC(6-17) complexes has been previously described (16). PC samples of 50 nM in TBS/5 mM Ca^{2+}/0.1% BSA were next added to the wells and incubated for 1 hour at RT. The wells were washed with 0.1% Tween-20/5 mM Ca^{2+}/TBS and incubated with 1:1000 diluted HRP conjugated anti-PC rabbit antisera in 5 mM Ca^{2+}/0.1% BSA/TBS for 1 hour at RT. The wells were washed with the Ca^{2+}/Tween buffer and bound conjugate was detected with OPD by absorbance at 490 nm.

Evaluation of ODA Coating Effects on Immobilon Membranes

Solutions of peptide, polymer, polymer-peptide adducts in 0.05 M phosphate, 0.1 M NaCl, pH 7.3 (PBS) buffer were incubated for 1 hour at RT in glass baths containing the activated membrane. The membranes were removed, thoroughly washed with PBS for 1 hr and spotted in replicates of four with 1 μl of 25 nM HPC4 in PBS 0.05 M sodium phosphate/0.1 M NaCl (buffer A) using an SMI-micropipettor. The membranes were blocked with 1 M ethanolamine for 1 hour at RT. The membranes were then incubated in

4 μg/ml hPC in TBS/25 mM $CaCl_2$ (buffer B) for 1 hour at RT. The membranes were washed with buffer A and incubated with 1:1000 diluted anti-PC rabbit antisera in buffer A for 3 hours at RT and then washed in PBS for 1 hr. The membranes were then incubated with a 1:1000 goat-derived, anti-rabbit HRP-conjugated IgG for 3 hours at RT. Membranes were washed with PBS for 1 hour and substrate was added. Bound conjugate was detected with 4-chloronaphthol substrate by absorbance at 570 nm using a Schimadzu CS-9000 diffuse reflectance densitometer. Total spot area was measured for each 1 μl spot of HPC4.

Determination of the "Orientation Effects" of ODA on Immobilon Surfaces

Polymer, peptide, and polymer-peptide conjugates, and control samples were serially diluted in 0.05 M imidazole/0.1 M NaCl/0.1 M $CaCl_2$/pH 7.0. These samples were incubated with 50 nM HPC4 Mab for 1 hour at RT. Control HPC4 and respective samples containing ODA had the same HPC4 concentration and therefore amount of HPC4 spotted. These mixtures were each applied in replicates of four to an Immobilon membrane. The membranes were then blocked with 1 M ethanolamine for 2 hours at RT. The membranes were then washed with 100 mM EDTA/TBS for 3 hours with three buffer changes to remove bound polymer-peptide conjugates or peptides. The membranes were then incubated in 4 μg/ml hPC in TBS/100 mM $CaCl_2$ (buffer B) for 1 hour at RT. The membranes were washed with buffer B and incubated with 1:1000 diluted HRP-conjugated anti-PC rabbit antisera in PBS for 3 hours at RT, then washed with PBS. Bound HRP-conjugate was detected with 4-chloronaphthol substrate by absorbance at 570 nm using a

Schimadzu CS-9000 diffuse reflectance densitometer. Total spot area was measured for each 1 μl spot of HPC4.

RESULTS

The overall strategy adopted was to protect the antigen-binding site of the Mab prior to immobilization by use of an ODA. Subsequent removal of the ODA was expected to result in an immobilized Mab with its antigen binding Fab domain oriented away from the matrix. The ODAs employed were HCPC(6-17), a synthetic epitope for the murine anti-PC Mab HPC4, and water soluble polyoxazoline adducts of this epitope. The ODAs have been shown to compete successfully with PC for binding to the HPC4 Mab and are therefore recognized by the antigen-binding site of that Mab (17). The orientation effects of these ODAs were examined on two nonporous surfaces, Immulon II and Immobilon AV affinity membranes. The Immulon II surfaces are designed to sorb species nonspecifically through low energy forces such as hydrophobic interaction, while the Immobilon membranes are activated to form covalent linkages with available amino groups of soluble species such as proteins while minimizing nonspecific sorption.

Precoating experiments for Immulon II microtiter wells were initially performed to evaluate the loss of matrix sites due to nonspecific interaction with species other than the Mab (i.e. ODA). The wells were first exposed to the species to be used as an ODA, washed free of unbound ODA, and then coated with HPC4. For all species examined, a

loss of ELISA signal was seen relative to the control wells which were pretreated only with buffer (Figure 2). As the HPC4 concentration used was within a linear signal range (data not shown) ,the loss of signal by pretreatment was seen as a proportional measure of the loss of available sites for immobilization of HPC4. The control wells that were pretreated only with buffer were assumed to contain 100% of the available sites. The 1K poly(2-ethyloxazoline)-peptide adduct and the free HCPC(6-17) peptide were seen to sorb the least with 77% and 66% of the signal (sites) still remaining, respectively. We have thus divided the presentation of our orientation studies into two parts; the lower molecular weight ODAs which tend to have less tendency to sorb on the Immulon II surface, and the higher molecular weight adducts with a tendency to compete for surface sites with the Mab.

The effect of ODAs on orientation of Mabs were based on ELISA-type assays as described in "Materials and Methods". The ODA protected Mabs are immobilized on the surface and the ODAs are removed by treatment with EDTA as chelation of Ca^{2+} destabilizes the complex due to the Ca^{2+}-dependency of the Mab (16). The signal generated by the amount of PC bound by immobilized Mab was interpreted as being proportional to active HPC4 sites as the HPC4 and PC concentrations used for these assays are within a linear signal range. Figure 3 presents bound activity effects for HPC4 that was pre-equilibrated with 1K poly(2-ethyloxazoline)-peptide adduct or free HCPC(6-17) peptide as a function of the molar ratio of either adduct or peptide to HPC4. It is seen that both species achieve

a maximum of approximately 4.8 times greater activity than the control signal (equivalent amount of HPC4, no ODA). Compared to HCPC(6-17) the adduct requires a 100-fold higher molar ratio to achieve its maximum bound activity.

Figure 4 presents bound HPC4 activity effects on Immulon II for 5K, 10K and 20K poly(2-ethyloxazoline)-peptide adducts as a function of adduct to HPC4 molar ratio. Here, none of the higher molecular weight poly(2-ethyloxazoline)-peptide adducts show a net increase in bound activity. As the nonspecific interaction of the 5K derivative results in the loss of 59% of the total sites (Figure 2), when the data for the 5K derivative in Figure 4 are normalized with respect to "available sites", a modest relative increase in bound HPC4 activity is obtained.

As the Immulon II surfaces bind proteins through low energy forces such as hydrophobic interactions, the ODAs interact with the surface reducing the number of available sites. Therefore, poly(2-methyl oxazoline) adducts were synthesized as the methyl groups can be expected to impart less hydrophobicity than the ethyl groups. Figure 5 represents the precoating experiments of the methyl oxazoline adducts and polymers. The loss of sites for these were 62-65% with the exception of the 1K adduct which retained only 24% of the sites (loss of 76%).

The orientation effects of these methyl adducts are given in Figure 6. The 1K and 5K methyl adducts increased the amount of bound hPC by 2-

fold while the relatively more hydrophobic 10K adduct had no effect at lower ODA to Mab ratios. At higher ratios all the polymers and the 10K adduct lowered the amount of bound hPC.

Figure 7 presents the precoating evaluations for Immobilon surfaces by the peptide and 5K poly(2-ethyloxazoline)-peptide adduct. It can be seen that essentially no loss of signal occurs for the case where the membrane is precoated with HCPC(6-17) peptide at 36 μM prior to coupling with HPC4. The 5K adduct gives a signal which is 125% of the blank at 3 μM of adduct and a signal that is 60% of the blank at 8 μM. Thus, a loss of sites can be assumed to occur for the 5K adduct if the concentration is higher than 3 μM in "orientation studies" with Immobilon membranes.

Figure 8 presents a comparison of the bound activities obtained as a function of the molar ratio of ODA to HPC4 whereby peptide or 5K adduct is the ODA species for a fixed concentration of HPC4 of 0.195 μM. It is seen that a 2.7 greater maximum bound activity relative to the reference case (without ODA) is achieved for the peptide at a 50:1 molar ratio of peptide to HPC4. The 5K adduct yields a maximum increase in signal of 1.7 at a molar ratio of 10:1 5K adduct to HPC4.

DISCUSSION

Lower than expected binding capacity of immunosorbents can be attributed to several categories of effects arising from the

immobilization of the antibody. These hypothesized phenomena can be attributed to surface density effects, effects arising from "micro-heterogeneity" and "low energy forces" associated with the sorbent matrix, and "orientation effects" (Figure 9). It is noted that the "orientation effects" have not been directly proven. Furthermore, it is logical that these phenomena may be superimposed upon and may exacerbate each other's deleterious impact upon activity of an immunosorbent. In addition, these effects can be present on surfaces that are engineered to use nonspecific, low energy immobilization (via hydrophobic, ionic or hydrogen bonding interactions), as well as on derivatized surfaces that react with and covalently anchor protein. This study has focused upon a model immunosorbent for each of these surface types where "orientation effects" should predominate the bound Mab activity. Thus, we have sought to examine immunosorbent performance occurring on nonporous surfaces with low Mab density, where surface density effects are expected to be negligible. The impact upon "orientation" by sterically masking the Mab antigen-combining site was best evaluated using these surfaces before evaluation in systems where other aforementioned effects would be superimposed (i.e., activated chromatographic resins or gels). Thus, for these studies, the increases in bound activity are attributed primarily to proper orientation.

For surfaces which use nonspecific low energy forces to immobilize antibodies, an inherent loss of sites due to the presence of ODA occurs. Thus, a normalization with respect to sites available to the Mab that are

not occupied by the ODA itself should be performed. The loss of sites due to the ODA is less when peptide is employed as an ODA compared to the use of polymer-peptide conjugates as ODAs. The Ab activity is dramatically greater in the presence of peptide ODAs than in the absence of ODA as indicated by a 4.8-fold increase in the ELISA signal. The actual increase in sites due to ODA is likely to be lower than this value because microtiter well surfaces immobilize by nonspecific interactions.

In contrast, the nonporous Immobilon surface which is engineered to covalently link amino-groups of proteins while minimizing nonspecific sorption, show no loss of sites due to precoating by the ODA up to concentrations of about 4.7 μM polymer peptide, and 36 μM peptide. Thus, the "orientation studies" employing HPC4-ODA pre-equilibration were limited to these concentration ranges. Because the Immobilon surface is more specific in its immobilization chemistry (covalently coupling amino groups of proteins via a reactive ester link), it is comparable to other covalently activated matrices. As most immunosorbents have efficiencies of 20-30%, Immobilon surfaces can be also expected to have similar baseline activities. This would probably represent a minimum value as deleterious microheterogeneity effects are suppressed for this system relative to porous immunosorbent gels. As orientation studies with peptide as an ODA yielded upto a 2.7-fold increase in the ELISA signal, it would appear that a minimum of 54-81% of Mab are oriented properly on the Immobilon surface when using the peptide-ODA in the low HPC4 density regime.

The 1K ethyl, 1K methyl and 5K methyl polymer-peptide adducts showed activity as ODAs on non-specific Immulon II surfaces with 3.8- to 2- fold increases in ELISA signal. The interaction of these ODAs (especially the higher molecular weight adducts) with the non-specific surface contributed to a large decrease in available sites and thus limited the evaluation of the ODA effectivity. The 5K ethyl polymer-peptide adduct showed activity as an ODA on the covalent Immobilon surface with a 1.7-fold increase in signal. Perhaps the fact that the affinity of the ethyl adducts for HPC4 Mab are two orders less than that of HCPC(6-17) peptide (17) may indicate a decrease in conformational effects needed for orientation directing activity. This decrease in affinity is probably due to the hydrophobicity of poly(2-ethyloxazoline)-peptide adducts. As the hydrophobicity of these adducts also decrease available sites on immobilization surfaces, Poly(2-oxazoline) adducts containing more polar side chains will be evaluated in future work. Although peptide alone can function effectively as an ODA in this particular case, the development of a polymer-peptide ODA has several potential advantages. For example, peptide alone may not be able to provide an effective steric shield for other Ab systems and thus would require a bulky polymer attachment to protect the antibody while coupling, the polymeric derivatization may lower the reactivity in cases where the peptide itself is capable of taking part in the coupling reaction, the hydrophobicity of the polymer may be controlled to give polymer-peptide ODAs with varying affinities for a given Ab, and the spacing of the antibody on the matrix as well as microheterogeneity effects could be controlled by varying the size of the polymer.

For surfaces employing low energy bonding for immobilization, it can be inferred from these studies that the parent hPC antigen will probably not be useful as an ODA unless somehow it can be modified to become noninteractive with the matrix by derivatization while retaining its affinity for the Ab. It is possible that the parent protein antigen may be capable of being derivatized to render it nonreactive to the activated matrice, while retaining sufficient affinity to be an effective ODA (19,20). Although it would involve a large initial outlay of the antigen, this would necessarily be a much more direct way of synthesizing an ODA for any given antibody and would possibly make this method adaptable to polyclonal applications.

LIST OF ABBREVIATIONS

Mab	Monoclonal antibody
HCPC(6-17)	Synthetic peptide for heavy chain of human Protein C
HPC4	Calcium-dependent murine monoclonal antibody directed against human Protein C
HRP	Horseradish peroxidase
TBS	Tris-buffered saline
PBS	Phosphate-buffered saline
ELISA	Enzyme-linked immunosorbent assay
HPC	Human Protein C
RT	Room temperature
CHO	Carbohydrate
ODA	Orientation directing agent
OPD	O-phenylenediamine-2HCl

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GLU - ASP - GLU - VAL - ASP - PRO - ARG - LEU - ILE - ASP - GLY - LYS

FIGURE 1
HCPC(6-17) AMINO ACID SEQUENCE

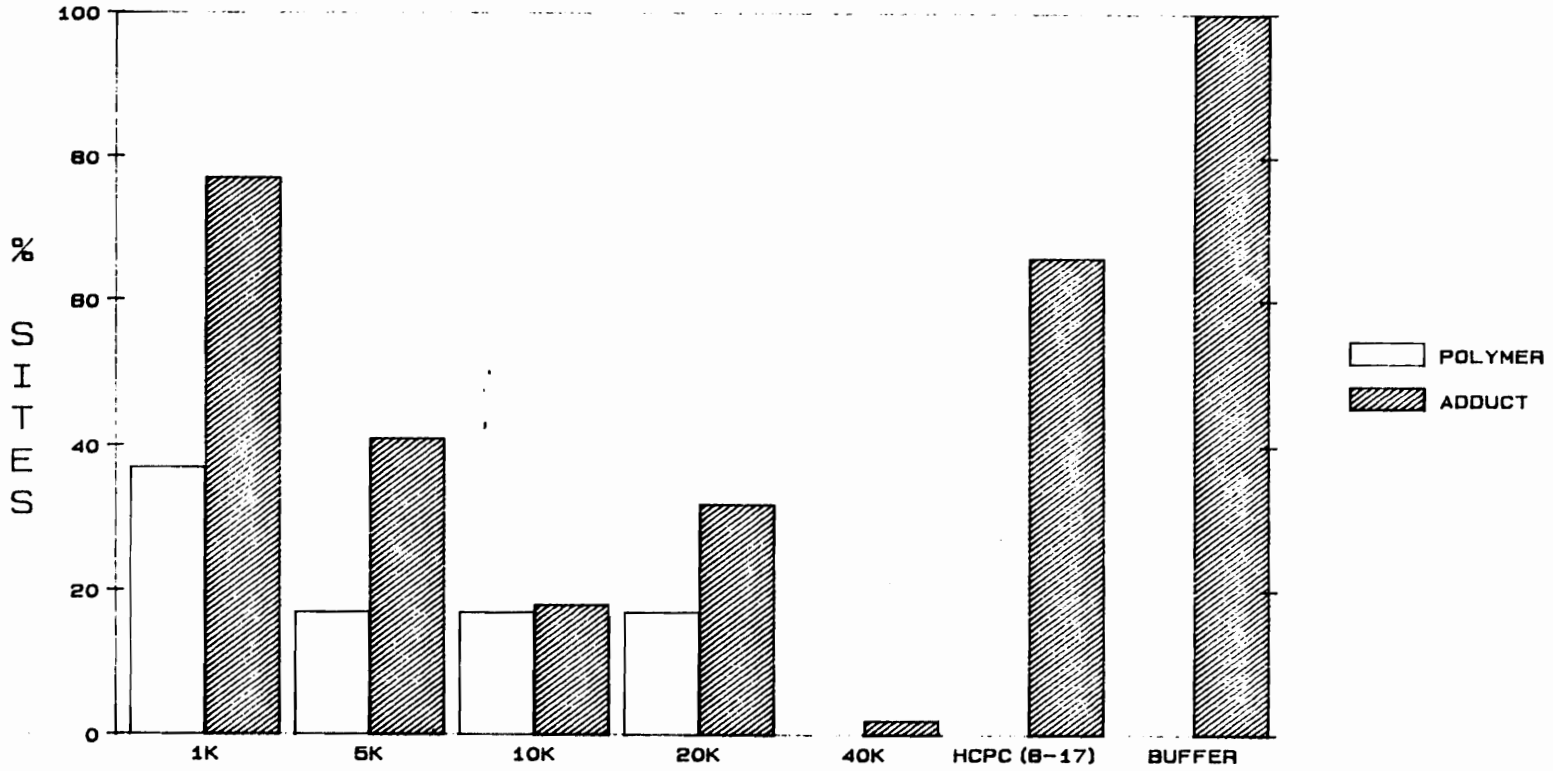

FIGURE 2
COATING EFFECTS OF POLY (2-ETHYLOXAZOLINE) ODAS ON IMMULON II SURFACES

Immulon II wells were first exposed to 100 μM of the species to be used as an ODA, washed and then coated with 50 mM HPC4 Mab. Bound Mab was detected with HRP-conjugated goat anti-mouse immunoglobulin the chromogenic substrate OPD by absorbance at 490 nm. The data is represented as a percentage of the ELISA signal obtained in the absence of an ODA which is interpreted as the percentage of available sites for the immobilization of HPC4.

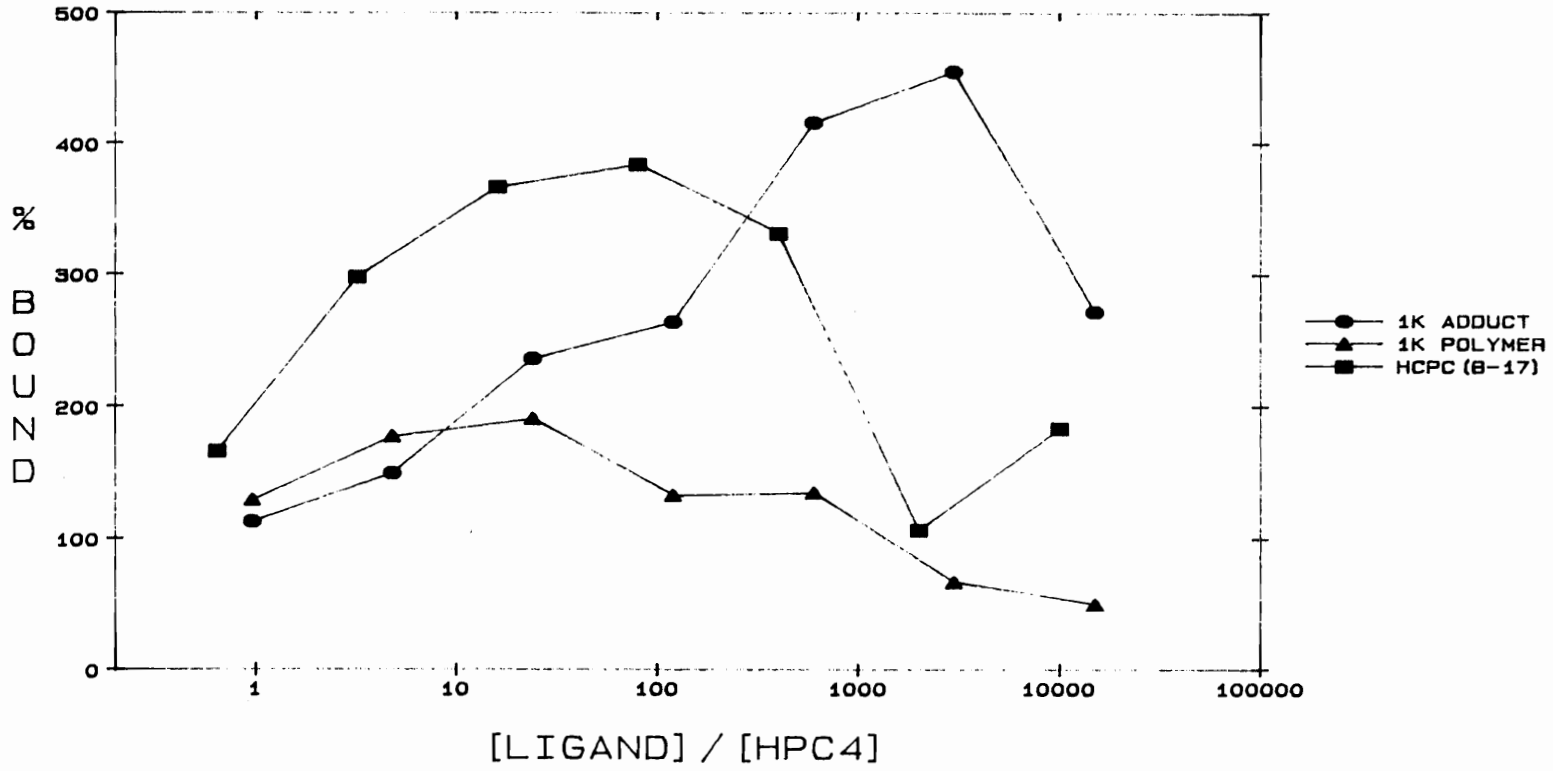

FIGURE 3
ORIENTATION STUDIES OF PEPTIDE AND 1 K POLY(2-ETHYLOXAZOLINE) ADDUCTS ON IMMULON II SURFACES

HPC4 Mab (50 nM) pre-equilibrated with HCPC(6-17), poly(2-ethyloxazoline) adduct or buffer (control) was applied onto Immulon II wells. The wells were washed and blocked. Bound ODA was removed with 100 mM EDTA and the wells were incubated with 50 nM hPC. Bound hPC was detected with HRP-conjugated rabbit anti-hPC antisera using OPD as the chromogenic substrate. The data is represented as a percentage of the control ELISA signal obtained when HPC4 was immobilized in the absence of an ODA.

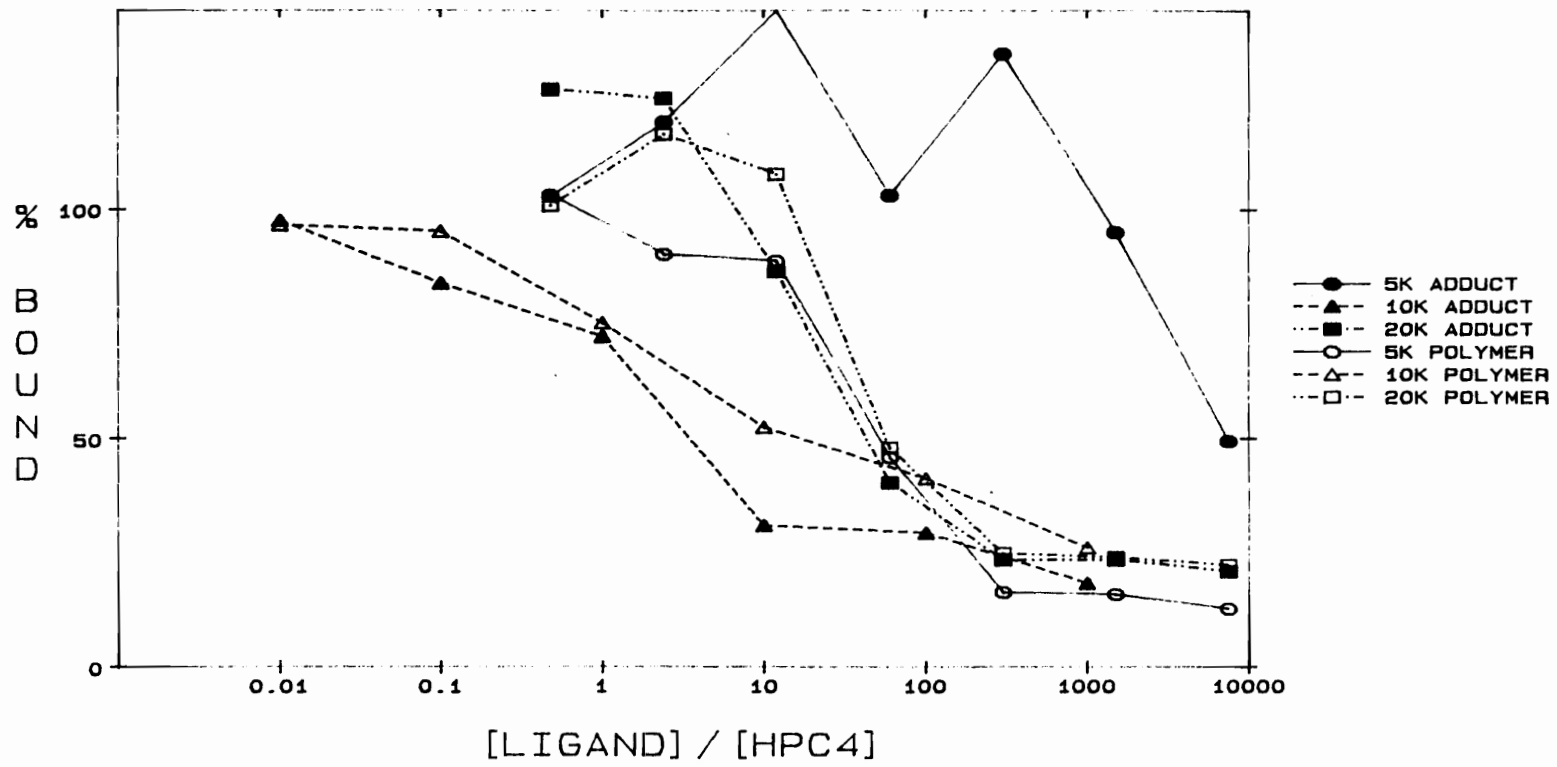

FIGURE 4
ORIENTATION STUDIES OF HIGH MOLECULAR WEIGHT POLY(2-ETHYLOXAZOLINE) ODAS ON IMMULON II SURFACES

The procedure used was similar to the one outlined in Figure 3. The data is represented as a percentage of ELISA signal obtained when HPC4 was immobilized in the absence of an ODA.

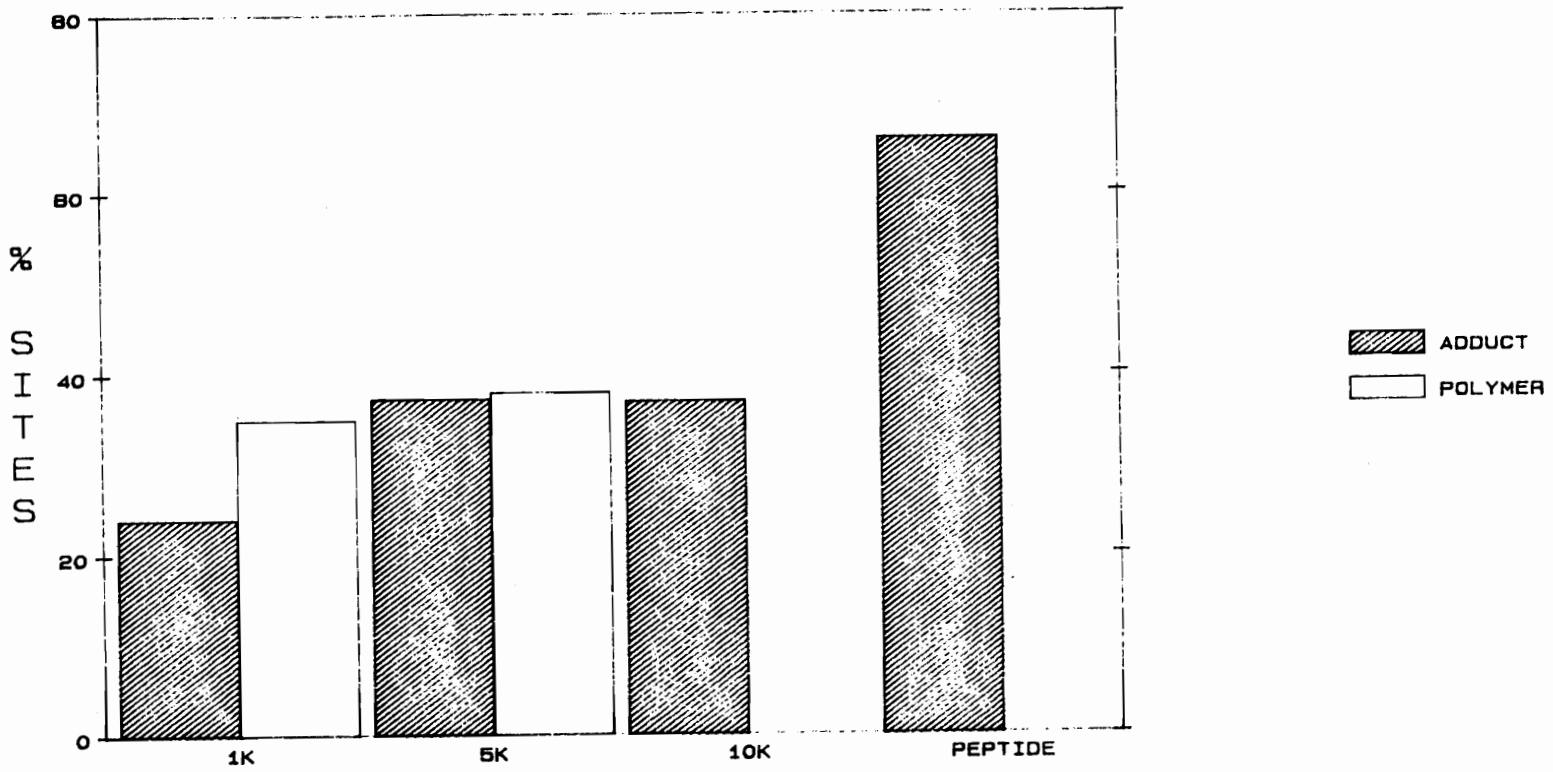

FIGURE 5
COATING EFFECTS OF POLY(2-METHYLOXAZOLINE) ODAS
ON IMMULON II SURFACES

The procedure used is outlined in Figure 2. The data is represented as a percentage of ELISA signal obtained in the absence of an ODA and is interpreted as the percent of available sites on the matrix.

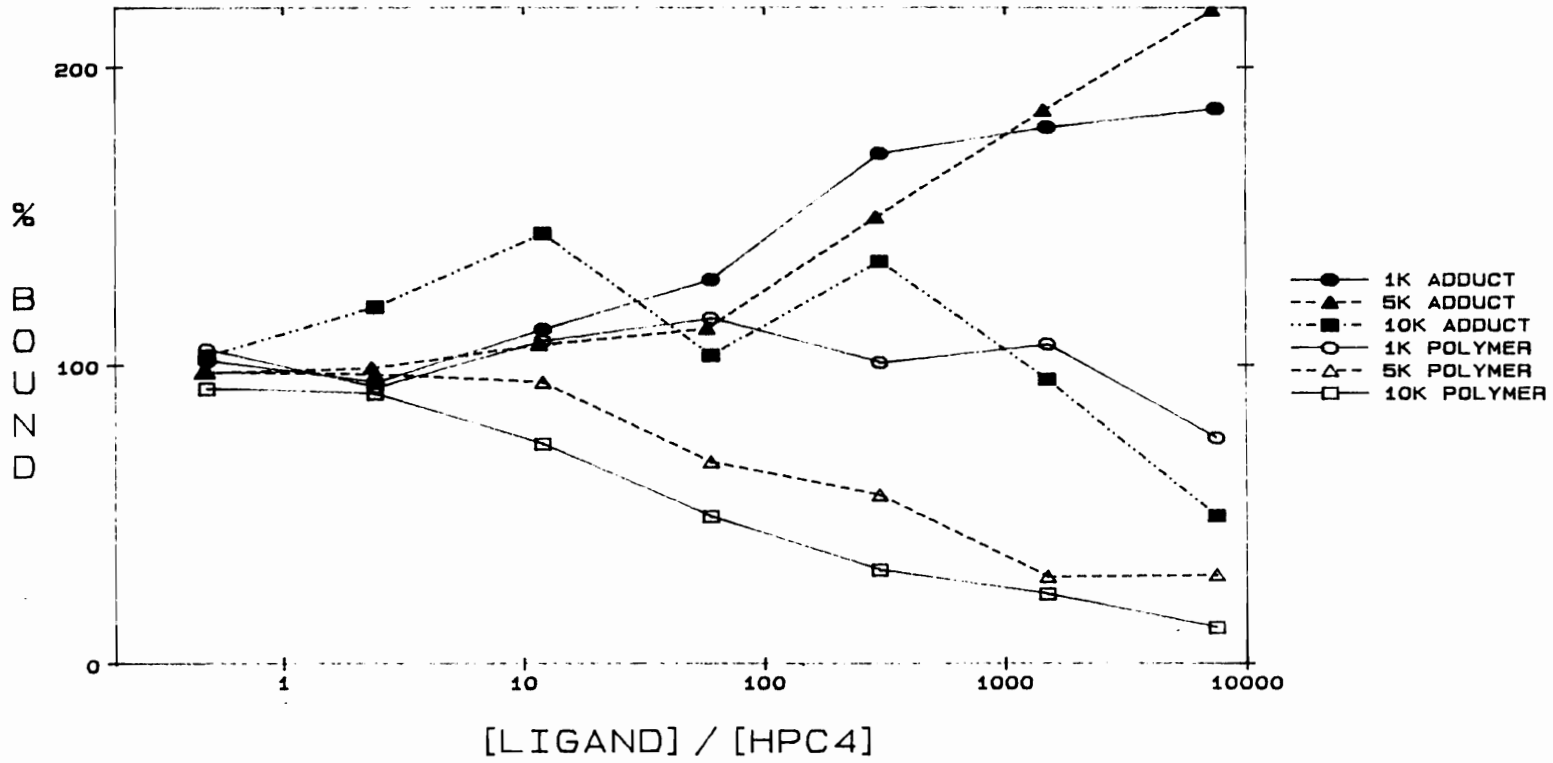

FIGURE 6
ORIENTATION STUDIES WITH POLY(2-METHYLOXAZOLINE) ODAS ON IMMULON II SURFACES

The experimental procedure is outlined in Figure 3.

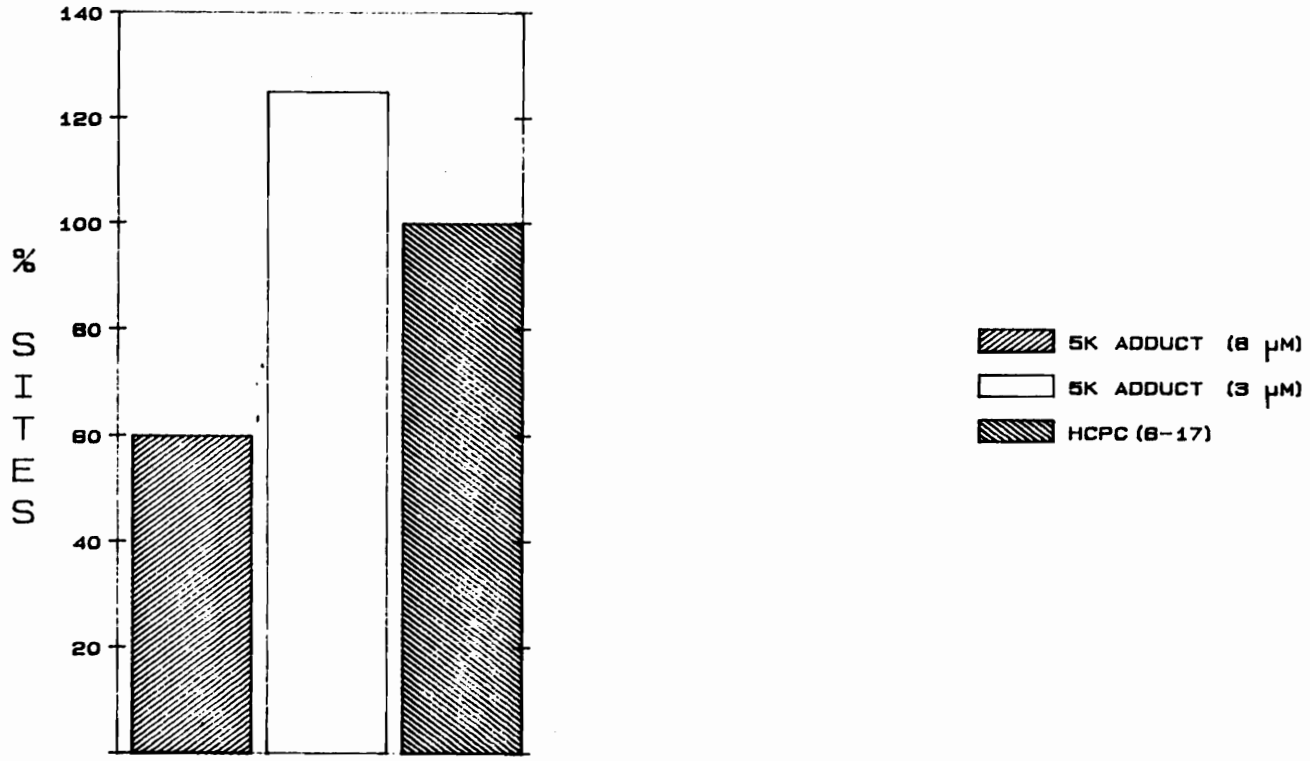

FIGURE 7
COATING EFFECTS OF HCPC(6-17) and 5K POLY(2-ETHYLOXAZOLINE) ADDUCT ON IMMOBILON MEMBRANES

The immobilon membranes were first exposed to HCPC(6-17), 5K ethyl adduct or buffer. The membranes were washed, spotted with 1 μl of 25 nM hPC, blocked, and then incubated with 64.5 nM hPC. Bound hPC was detected with rabbit anti-hPC antisera followed by HRP conjugated goat anti-rabbit immunoglobulin. Bound conjugate was detected with 4-chloronaphthol substrate using a Schimadzu CS-9000 densitometer. The data is presented as a percentage of the ELISA signal obtained in the absence of an ODA.

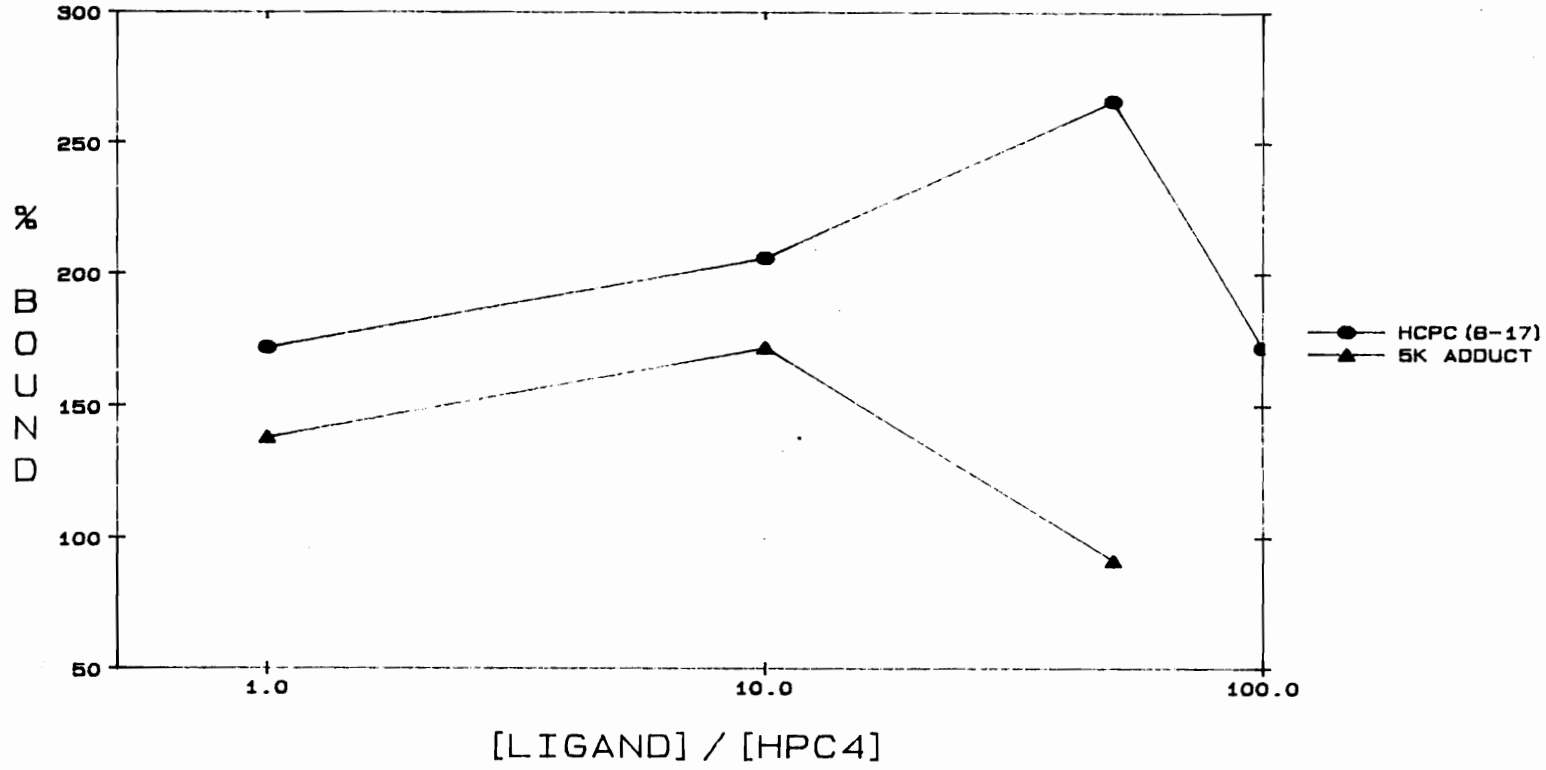

FIGURE 8
ORIENTATION STUDIES OF HCPC(6-17) AND 5K POLY(2-ETHYLOXAZOLINE) ADDUCT ON IMMOBILON MEMBRANES

Peptide and adduct samples were incubated with 50 nM HPC4 prior to spotting on Immobilon membranes. The membranes were blocked, washed and incubated with 100 mM EDTA to remove ODA. The membranes were next incubated with 64.5 nM hPC. Bound hPC was detected with HRP conjugated anti-hPC rabbit-antisera suing 4-chloronaphthol as the substrate. The data is presented as a percentage of the signal obtained when the HPC4 was spotted in the absence of an ODA.

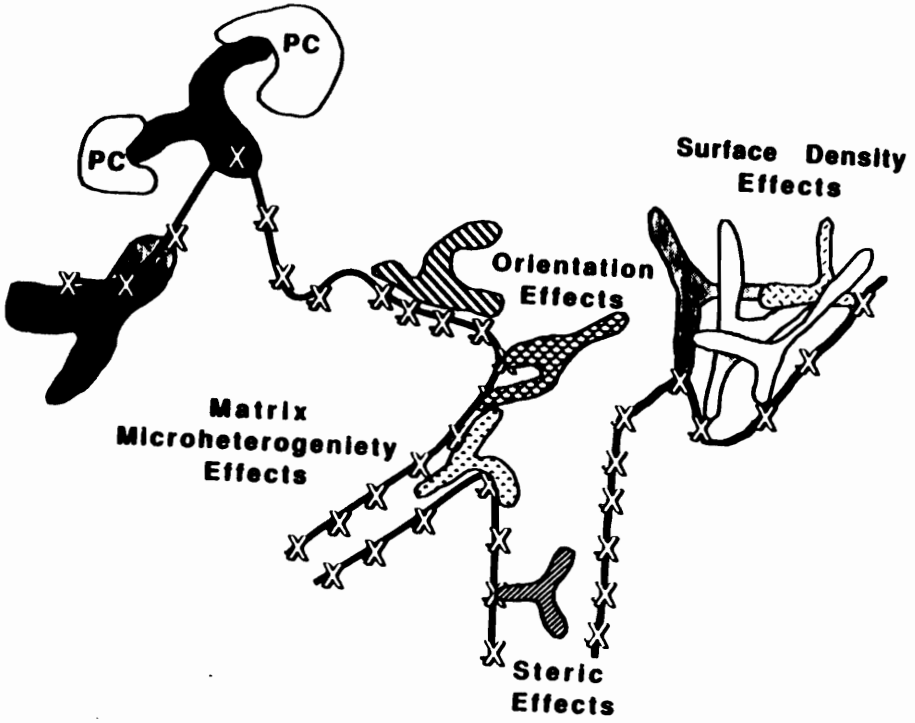

FIGURE 9
MATRIX AND ORIENTATION EFFECTS ON BOUND MAB ACTIVITY

VITA

Rapti Madurawe was born on August 28, 1958 in Sri Lanka. She obtained a bachelor's degree in Chemical Engineering at the Indian Institute of Technology, Madras, India. During the course of her undergraduate studies she developed an interest Biochemical Engineering, particularly in enzymology. Her senior year project was on the immobilization of glucose isomerase on chitin matrices. To strengthen her understanding of biological processes, she joined the graduate program at the University of Kentucky, Lexington, KY and obtained a M. S. degree in Biochemistry. While at Kentucky, she conducted research in the purification and characterization of methylthioadenosine phosphorylase, an enzyme of the purine salvage pathway, abnormal levels of which are associated with certain types of myelomas. Thereafter, she joined the Virginia Polytechnic Institute and State University, Blacksburg, VA to pursue a doctorate in Chemical Engineering under the guidance of Dr. William H. Velander. She specialized in the biochemical field and completed her dissertation at the American Red Cross Jerome H. Holland Laboratory for the Biomedical Sciences in Rockville, MD under the supervision of Dr. Carolyn Orthner. Her research was focussed on the optimization of metal-dependent antibodies for immunoaffinity chromatography.