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**DEVELOPMENT OF TECHNOLOGIES FOR
ENZYME IMMUNOASSAYS**

by

Scott Boyd. B.Sc. (Honours)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Department of Biology

Carleton University

Ottawa, Ontario

July 24, 1996

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**DEVELOPMENT OF TECHNOLOGIES FOR
ENZYME IMMUNOASSAYS**

submitted by

Scott Boyd, B.Sc. (Honours)

in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Biology

Thesis Supervisor

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September, 1996

ABSTRACT

The development of technologies for improving enzyme immunoassays (EIA) and in particular cloth-based enzyme immunoassays (CEIA) are examined in this thesis. EIA procedures have become the predominant method used for the detection of potentially harmful pathogens, toxins, or pollutants in our food and water sources as well as in our environment. The early detection of such substances is necessary for their efficient control, and this requires the use of detection systems under field testing conditions. Unfortunately, EIAs are often impractical for field testing because the enzyme-labelled EIA reagents tend to be unstable in the absence of proper refrigeration. In addition, EIAs tend to be complex and time consuming which makes field testing of large numbers of samples difficult.

In order to permit the use of CEIA for field testing in the absence of refrigeration, it was demonstrated that antibody-cloth retained its full immunoactivity for at least 70 days at 32°C following drying. The use of polyvinyl alcohol (PVA) (MW 30,000-70,000) as an agent to prevent the non-specific adsorption of test immunoreactants to polyester cloth permitted stable blocking of the cloth which was not possible with traditionally used protein blocking agents. It was determined that horseradish peroxidase (HRP)-antibody conjugates, the most commonly used enzyme-labelled immunoreactants for EIA, could be kept in solution for at least 36 days at 32°C with no loss of activity if certain concentrations and molecular weights of PVA or polyethylene oxide (PEO) were added.

A simple CEIA column system that is capable of immunoconcentrating dilute antigen from large sample volumes was developed. Using this system, it was possible to assay antigen that would go undetected in traditionally used EIA procedures. An instantaneous

CEIA for the semi-quantitative visual determination of antibodies was also developed. This technique is ideal for field testing because it is extremely rapid and simple to perform.

Antibodies and antigens for use in EIA are normally purified using immunoaffinity chromatography. Tosyl chloride-activated rayon polyester cloth was developed as an improved immunoaffinity chromatography support. Antibody-coated polyester cloth was shown to be an ideal support for the inexpensive and simple screening of optimal elution conditions (including saturated $MgCl_2$, which I demonstrated was very good at eluting sheep F(ab'), antibodies from an anti-sheep antibody column). A simple and cost efficient system for purifying low titer monoclonal antibodies from a monoclonal antibody hybridoma culture using Protein G adsorbed onto polyester cloth was shown.

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ABBREVIATIONS

A_{450}	absorbance at 450 nm
A_{562}	absorbance at 562 nm
AP	alkaline phosphatase
BCA	bicinchoninic acid
β -gal	β -galactosidase
BSA	bovine serum albumin
$^{\circ}\text{C}$	degrees celcius
CDI	1,1-carbonyldiimidazole
CEIA	cloth-based enzyme immunoassay
CFU	colony forming unit
cm^2	square centimeter
CNBr	cyanogen bromide
dH ₂ O	distilled water
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EMIT	enzyme multiplied immunoassay technique
Fab	fragment of antigen binding
Fc	fragment of crystallization
g	gram
GO	glucose oxidase
h	hour

HRP	horseradish peroxidase
IgG	immunoglobulin G
LPS	lipopolysaccharide
μ g	microgram
μ l	microliter
mg	milligram
min	minute
ml	milliliter
MW	molecular weight
ONPG	O-nitrophenyl- β -D-galactopyranoside
PBS	0.01 M sodium phosphate pH 7.3 buffer in 0.85 % NaCl
PBSM	PBS containing 0.1 % (v/v) Micro-O-Protect
PBST	PBS containing 0.05 % (v/v) Tween 20
P-CEIA	polymyxin-cloth-based enzyme immunoassay
PEG	polyethylene glycol
PEO	polyethylene oxide
p-npp	p-nitrophenyl phosphate
PVA	polyvinyl alcohol
PVP	polyvinyl pyrrolidone
RIA	radioimmunoassay
RPM	revolutions per minute

RT	room temperature
SDS	sodium dodecyl sulphate
TMB	3,3',5,5'-tetramethylbenzidine
v/v	volume per volume
w/v	weight per volume

GENERAL INTRODUCTION

The social and economical losses associated with foodborne pathogens (e.g., Salmonella) is staggering. For example, it has been estimated that the loss of money in the United States to foodborne pathogens is approximately \$10 billion annually (Todd, 1984). It has also been estimated that there are 6.5 million cases of foodborne disease resulting in about 9,100 fatalities annually in the United States and Canada (Todd, 1989a,b). Accordingly, the incidence of foodborne diseases is much greater in undeveloped countries. The early detection of harmful substances in our food stocks and our environment is a necessary first step in being able to efficiently control such harmful substances. Early detection requires the development of field testing procedures that are inexpensive, rapid, and simple to use. This is vitally important for countries such as Canada, that have a vast geographical area which makes the transportation of samples for testing in laboratories a time consuming venture.

The interaction of antibody with antigen forms the basis of all immunochemical techniques such as immunoassays and immunoaffinity purification. Antibodies are proteins that are synthesized and secreted by B-lymphocytes in response to the presence of foreign molecules (antigen) in the host. Antibodies form a large family of glycoproteins that are characterized by their ability to specifically bind both antigen and specialized proteins or cells of the immune system. They are made up of one or more copies of a basic unit that looks roughly like a 'Y'. This basic unit consists of two identical heavy polypeptide chains and two identical light polypeptide chains which are held together by interchain disulfide bonds. The light and heavy chains are comprised of variable and constant regions with the variable regions of the light and heavy chains forming an antigen binding site. The two arms

of the basic antibody unit are referred to as the Fab fragments (fragment with the antigen binding site) with the stem termed the Fc fragment (fragment of crystallization) (Figure 1). There are five classes of antibody (immunoglobulin G(IgG), IgM, IgA, IgE, and IgD) which differ structurally in their heavy chains and in their number of 'Y' subunits, as well as in their biological function which is imparted by the Fc region. IgG is the most abundant class of antibody in internal body fluids, and thus, is most often used for immunochemical techniques.

An antigen is a molecule that can be recognized by an antibody and that can stimulate the production of antigen-specific antibody in the host. Just about any molecule, including synthetic organic molecules, can be an antigen. The part of the antigen that interacts with the antigen binding site of the antibody (called the epitope) may range in size from 5 or 6 amino acids for linear peptides to as many as 16 amino acids for globular proteins. The epitope of carbohydrate antigens is normally comprised of 5 to 6 sugar residues (Roitt, 1994). An antigen, depending upon its size, may have numerous different epitopes, all of which are specifically recognized by different antibodies.

The binding of antibody to antigen is a result of non-covalent interactions including hydrogen bonds, van der Waals forces, and ionic and hydrophobic interactions. The strength of the antibody-antigen interaction can vary tremendously depending upon the nature of the binding. This strength of binding has been termed affinity which essentially describes the concentration of antibody-antigen complex that will be found at equilibrium. Affinity may be defined through the equilibrium constant (K_a), where

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

Figure 1. Basic unit of an IgG molecule. The basic unit consists of two identical heavy chains (H) and two identical light chains (L) that are held together by disulfide bonds (S-S). Each chain contains one or more constant domains (C) which are the same in all antibody molecules for a given class, and a variable domain (V) which determines the antigen binding specificity of the antibody. The Fab and Fc regions are held together by a hinge region that imparts a great amount of conformational freedom within the two Fab arms. The Fc region, which is responsible for the biological function of the antibody, has carbohydrate attached to it.

and varies from less than 10^5 mol^{-1} to greater than 10^{12} mol^{-1} (Harlow and Lane, 1988).

In 1959, Rosalyn Yalow and Solomon Berson reported upon a novel technique for assaying insulin in the blood of humans with the use of an antibody to insulin, as well as insulin labelled with a radioisotope (Yalow and Berson, 1959). This method formed the basis of modern radioimmunoassay (RIA) which has become an extremely versatile technique for the quantitation of a wide variety of compounds. For the detection of antigen by RIA, a limited but fixed amount of antibody specific to the antigen is mixed with a known but excess amount of radiolabelled antigen standard and an unknown amount of unlabelled antigen in the test sample. Because there is more antigen than antibody, the labelled and unlabelled antigen compete for binding sites on the antibody permitting estimation of the amount of antigen in the sample through the measurement of radioactivity bound to the antibody. The radioactivity of the resultant antibody-antigen complex, which is inversely proportional to the amount of sample antigen (Yalow, 1978), is measured following separation of the antibody-antigen complex from the unreacted antigen through methods such as the removal of free antigen by adsorption onto charcoal (Newsome and Shields, 1981), using an anti-globulin reagent to immunoprecipitate the antibody-antigen complex (McGuigan, 1968), or by immobilizing the antibody onto a particulate solid support such as beads which may be removed through centrifugation (Levitt and Schmitt, 1976).

As an assay procedure, RIA offers high specificity due to the specific nature of antibody-antigen interactions, as well as excellent detectability due to the high specific activity of the radiolabel, making it useful for diagnostic purposes as well as basic research. RIA, however, uses expensive radioisotopes that require costly equipment for their

measurement, and because they represent a potential health hazard, strict regulations govern their use. In addition, the radiolabelled antigens typically have a short half-life (e.g., 3 weeks to 3 months for ^{125}I) (Gould and Marks, 1988). These disadvantages encouraged the development of immunoassays that used non-isotopic labels such as enzymes (Nakane and Pierce, 1966), chemiluminescent molecules (Schroeder *et al.*, 1977), fluorochromes (Soini and Hemmila, 1979), or particles that cause agglutination (Litchfield *et al.*, 1984).

The market for immunoassays increases annually and was estimated to be worth approximately 2.5 billion US dollars in 1990 (Chard, 1990). In addition to their application to basic research, immunoassays are currently used in the following disciplines: clinical practice (e.g., testing fluids such as blood plasma, urine, and saliva for the monitoring of hormones, therapeutic drugs, toxin, etc...); veterinary medicine, horticulture, and agriculture (e.g., early detection of pregnancy, investigation of metabolic disorders, detection and quantification of toxins and viruses in plants and animals, testing for drug abuse in horses used for horse racing, etc...); food industry (e.g., testing for pathogens or for toxin and pesticide residues, measurement of vitamin content in food, etc...); environmental health (e.g., detection of antibiotics, pesticides, herbicides, detergents, and other potentially toxic residues within the atmosphere, waterways, and soil); and forensic science (e.g., detection of drug abuse).

Immunoassays that use enzyme labels (enzyme immunoassays (EIA)) have become the most popular form of immunoassay for various reasons. EIAs generally offer greater reproducibility than RIA, and because enzymes have a high catalytic activity (one enzyme molecule can convert large numbers of substrate molecules into detectable product), the

sensitivity of EIA is very high often exceeding that obtainable with RIA (Tijseen, 1985). The EIA reagents used (enzyme-labelled antibody, antigen, or hapten) are relatively inexpensive and generally have a long shelf-life when stored in a refrigerated condition. If a chromogenic enzyme substrate is used, the coloured product may be detected visually for qualitative determinations or with a spectrophotometer for quantitative determinations. The availability of several different suitable enzyme labels greatly increases the versatility of EIAs (Tijseen, 1985). Finally, EIAs are potentially useful under field-testing conditions, however, at the present time this application is not readily exploited largely because of the need to refrigerate enzyme-labelled immunoreactants to prevent thermal denaturation. This problem is compounded by the fact that most EIAs involve several washing and reaction steps which may take more than an hour to obtain a result when used by skilled personnel.

EIAs may be designed for the detection of antibodies (e.g., for serodiagnosis), antigens, or haptens. Haptens are low molecular weight molecules, such as many drugs, that by themselves are not immunogenic, but, when conjugated to a carrier molecule, can induce the formation of hapten specific antibodies. EIAs may be classified as homogeneous or heterogeneous assays. Heterogeneous EIAs, also referred to as enzyme-linked immunosorbent assays (ELISAs), use a solid phase to separate unreacted immunoreagents from reacted immunoreagents prior to the addition of the indicator substrate solution.

Homogeneous EIAs are commonly used for the quantification of haptens (e.g., drugs or hormones) and do not use a solid support to discriminate between immunoreacted haptens and unreacted haptens (Chard, 1990). The most common type of homogeneous EIA is the enzyme multiplied immunoassay technique (EMIT) that was developed by Rubenstein *et al*

(1972). This technique uses an enzyme that becomes inactive when an enzyme-labelled hapten reacts with an antibody (because the active site of the enzyme is masked by the antibody). Limited amounts of hapten specific antibody and enzyme-labelled hapten are mixed with a sample containing hapten. The amount of hapten in the sample is proportional to the enzyme activity measured following the addition of a substrate solution. An alternative type of homogeneous EIA uses an enzyme which is active only when the enzyme-labelled hapten has reacted with antibody. In this case, the amount of hapten in the sample is inversely proportional to the measured enzyme activity (Ullman et al. 1974). Homogeneous EIA offers certain advantages over heterogeneous EIA such as improved speed, greater simplicity (no separation step), and easy automation (since there are no wash steps required). Homogeneous EIA is rarely used for detecting and quantifying large molecules (e.g., antibodies and most proteins) because the distance between the antigen site of the antigen or antibody and the enzyme label is such that antibody binding will not usually affect enzyme activity. In addition, the sensitivity of homogeneous EIA is lower than heterogeneous EIA because of background noise due to enzyme activity in the sample that cannot be washed away (Chard, 1990).

Heterogeneous EIA was developed independently by two research groups for the detection of IgG (Engvall and Perlmann, 1971) and oestrogens (van Weeman and Schuurs, 1971). Heterogeneous EIA may be further classified as non-competitive or competitive. In non-competitive EIA, antibody (or antigen) immobilized onto a solid support is used for the immunospecific capture of antigen (or antibody) from a sample. The captured antigen or antibody is then detected using an enzyme-labelled antibody conjugate followed by the

addition of a suitable enzyme substrate solution (Figure 2). The amount of enzyme label bound to the solid support is proportional to the concentration of test antigen or antibody. The sensitivity of non-competitive EIA may be increased by adding an unlabelled primary antibody to the captured antigen or antibody followed by an enzyme-labelled secondary antibody (called an indirect non-competitive EIA). Non-competitive heterogeneous EIA is rarely used for the detection or quantification of low molecular weight compounds such as haptens, because they usually lack two reactive antigenic sites (Gould and Marks, 1988). These compounds are commonly assayed using competitive EIA which use either enzyme-labelled antigen or enzyme-labelled antibody for the detection of antigen (or hapten) in a sample. Like traditional RIA, competitive EIA relies upon the competition between antigen for antigen binding sites on a limited amount of antibody. In one variation of competitive EIA, a known amount of enzyme-labelled antigen is mixed with antigen from a sample, and a limited amount of antibody immobilized onto a solid support. The labelled and unlabelled antigen compete for the antibody resulting in antibody bound to antigen or antibody bound to enzyme-labelled antigen. The amount of enzyme bound to the solid support is determined by adding a suitable enzyme substrate solution, with the resulting enzyme activity being inversely proportional to the amount of antigen in the sample. Alternatively, a competitive EIA may use the competition between antigen in a sample and a fixed amount of antigen immobilized onto a solid support for a known but limited amount of enzyme-labelled antibody. The amount of enzyme-labelled antibody reacted to the immobilized antigen is inversely proportional to the amount of antigen in the test sample (Figure 3).

Figure 2. Direct non-competitive heterogeneous EIA. (A) Detection of sample antigen using enzyme-labelled antibody. Antibody that is specific to the antigen to be detected are immobilized onto a solid support. If necessary, the support may be treated with a blocking agent to prevent non-specific adsorption of the test antigen and/or enzyme-labelled antibody. The solid support is incubated with the test sample containing the antigen and then washed with a suitable buffer to remove any unreacted sample. To detect the captured antigen, the solid support is incubated with an antigen-specific enzyme-labelled antibody, washed with buffer to remove excess enzyme-labelled antibody, and then incubated with a suitable enzyme substrate solution. **(B) Detection of sample antibody using enzyme-labelled anti-immunoglobulin antibody.** Antigen that is specific to the antibody to be detected is immobilized onto a solid support. After blocking (optional), the solid support is incubated with the test sample containing the antibody and then washed with buffer. Any captured antibody is detected by incubating the solid support with an enzyme-labelled anti-immunoglobulin antibody, washing, and then incubating with a suitable enzyme substrate solution. In both A and B, the amount of antigen or antibody in the sample is directly proportional to the enzyme activity on the solid support. Symbols: Y, antibody; $\triangle$, antigen-coated solid phase; $\diamond$, antigen; E, enzyme; $\circ$, unreacted substrate molecule; and $\bullet$, reacted substrate molecule.

(A)

(B)

Figure 3. Competitive heterogeneous EIA. (A) Detection of sample antigen using enzyme-labelled antigen. A limited amount of antibody specific to the antigen to be detected is immobilized onto a solid support which may then be blocked to prevent non-specific adsorption of the test antigen and/or enzyme-labelled antigen. A known but excess amount of enzyme-labelled antigen is mixed with the test sample containing the antigen and then incubated with the solid support. After washing the support with a buffer to remove any unreacted sample, the solid support is assayed for enzyme activity through its incubation in a suitable enzyme substrate solution. (B) Detection of sample antigen using enzyme-labelled antibody. A known but excess amount of antigen is immobilized onto a solid support that has been blocked (optional). The sample containing the antigen to be detected is mixed with a limited amount of enzyme-labelled antibody and then incubated with the solid support. After washing the support with a buffer, the enzyme-labelled antibody captured by the support is assayed through the addition of an enzyme substrate solution. In both A and B, the amount of antigen in the sample is inversely proportional to the enzyme activity on the solid support. Symbols: Y, antibody; Δ , antigen-coated solid phase; $\circ$, antigen; E, enzyme; $\circ$, unreacted substrate molecule; and $\bullet$, reacted substrate molecule.

(A)

(B)

While theoretically any enzyme can be used as a label in EIA, good enzymes have the following properties: (i) high specific activity (activity/mg or mole of enzyme); (ii) stable at a pH required for good antibody-antigen binding (normally around neutral pH); (iii) detectable using inexpensive, accurate, and sensitive methods; (iv) presence of reactive groups through which the enzyme can be covalently linked to antibody, antigen, or hapten molecules with little loss of activity; (v) relatively stable under routine storage and assay conditions following conjugation to antibody, antigen, or hapten; (vi) readily available in a soluble and purified form at a low cost; (vii) absence of health hazards attributed to the enzyme and its required substrates and cofactors; and (viii) absence of enzyme activity and factors affecting enzyme activity in the test samples (Ngo, 1985; Wong, 1991; Blake and Gould, 1984). Of the more than twenty-five different enzymes that have been used as EIA labels, the most commonly used ones for heterogeneous EIA are horseradish peroxidase (HRP), alkaline phosphatase (AP), β -galactosidase, and glucose oxidase, and for homogeneous EIA, malate dehydrogenase, glucose-6-phosphate dehydrogenase, and β -galactosidase (Chard, 1990).

Horseradish peroxidase (HRP) is the most commonly used heterogeneous EIA enzyme, because it is inexpensive, readily available in a purified form, has a high catalytic activity, may be detected using a wide range of potential substrates. As well, because it is a glycoprotein with a carbohydrate content of about 18 % (Shannon *et al.*, 1966), there are many potential methods for its conjugation to immunoreactants. For these reasons, HRP was used as the enzyme label of choice throughout the upcoming chapters. Peroxidases are widely distributed particularly in plants with the most commonly used peroxidase for EIA

being isolated from horseradish. HRP is a small (molecular weight of approximately 44,000) hemoprotein containing two compact domains between which the heme group resides (Welinder, 1979). It catalyses the oxidation of various hydrogen donating (DH) compounds via hydrogen peroxide as follows:

Because peroxidases are abundant in many plants, EIA that use HRP as a label often yield high background when used for the analysis of plant samples due to the presence of exogenous peroxidases. In these situations AP is the most commonly used alternative due to its high catalytic activity and its absence from plants (Tijseen, 1985).

The choice of substrate is one of the most important things to consider when deciding what enzyme to use as a label. There tend to be numerous different substrates available for the most popular EIA enzymes. The substrate chosen will affect both the sensitivity and the convenience of the assay. Substrates may be chromogenic, fluorescent, chemiluminescent, or radioactive in nature. While chromogenic substrates usually offer lower sensitivity than the other types of substrates, they may be detected either visually or with a spectrophotometer for qualitative or quantitative assessment, respectively. The ability to visually assess an EIA result is of huge advantage to field testing where complex and expensive detection equipment (such as fluorometers or liquid scintillation counters) are unavailable. The convenience of chromogenic substrates make them the most popular type of EIA substrate. HRP may be

assayed using a number of different chromogenic hydrogen donors such as O-phenylenediamine or 2,2'-amino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (Tijseen, 1985). Many of these hydrogen donating substrates are mutagenic, and therefore, potentially dangerous to the user. An alternative compound, 3,3',5,5'-tetramethylbenzidine (TMB) (Bos *et al.*, 1981), is becoming more popular because it is non-mutagenic and provides greater sensitivity in EIA than the other chromogens (Hosoda *et al.*, 1986). The structure of TMB is as follows:

HRP catalyzes the oxidation of the colourless TMB molecules resulting in a blue product whose colour intensity is proportional to the HRP activity present. The addition of H_2SO_4 stabilizes the product while changing the colour to yellow (Josephy *et al.*, 1982). TMB was used as the substrate for HRP throughout the experimentation for this thesis.

There are many ways to link enzymes to antibodies, antigens, or haptens, the choice of which is primarily determined by the availability of particular functional groups on the molecules. Currently used conjugation methods include conjugation (a) with amino and thiol directed cross-linkers (e.g., using maleimide group containing compounds such as N-succinyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Imagawa *et al.*, 1982; Yoshitake *et al.*, 1982)), (b) through disulfide bond formation (e.g., with the use of N-succinimidyl 3-(2-

pyridyldithio)propionate (Wong, 1991)), (c) with glutaraldehyde, and (d) through periodate oxidation. The glutaraldehyde and periodate oxidation methods are most commonly used because they are relatively simple to perform and yield conjugates with high specific activity.

Glutaraldehyde is a homobifunctional aldehyde that is effective for cross-linking molecules through their free amino groups (Habeeb and Hiramoto, 1968). Glutaraldehyde reacts irreversibly with the amino group of lysine (Quioco and Richards, 1966). Success of the procedure depends upon the number of lysine amino groups on the surface of the macromolecules to be linked. Certain enzymes such as AP may be conjugated to antibodies in a single step glutaraldehyde method due to the numerous available amino groups on each macromolecular species (Avrameas, 1969). In the one-step glutaraldehyde procedure, the enzyme and the antibody or antigen to be conjugated are mixed in a predetermined ratio and then added to a solution of glutaraldehyde (Figure 4a). Some enzymes, such as HRP, have few available surface amino groups for reaction, and so the one-step glutaraldehyde method results in conjugates with a very low specific activity. Actually HRP has 6 lysine groups, but these appear to be shielded by the carbohydrate shell that surrounds the enzyme (Welinder, 1979). A two-step glutaraldehyde method has been developed for such enzymes in which the enzyme is pretreated with excess glutaraldehyde, separated from any unreacted glutaraldehyde or enzyme, and then mixed with the antibody or antigen (Avrameas and Ternynck, 1971) (Figure 4b). A major disadvantage of glutaraldehyde conjugation procedures is that they often lead to the formation of excessively polymerized aggregates of enzymes, antibodies, and/or antigens.

Figure 4. Formation of antibody-enzyme conjugates via the cross-linking of their free amino groups through the one-step (a) and two-step (b) glutaraldehyde method. In the one-step method, the enzyme and antibody to be cross-linked are mixed together in predetermined ratios and then incubated with glutaraldehyde. The one-step method is poorly controlled and often leads to the excessive polymerization of molecules. In the two-step method, the enzyme is first treated with excessive glutaraldehyde. Any unreacted glutaraldehyde is removed prior to mixing the enzyme with the antibody so that no polymers of the latter are formed. If the enzyme has several activated groups, the two-step method may result in the formation of large polymers since the enzyme can act as a bridge between antibodies.

A) One-Step Glutaraldehyde Procedure:

B) Two-Step Glutaraldehyde Procedure:

(remove excess glutaraldehyde)

The periodate oxidation procedure is very efficient in producing conjugates where at least one of the macromolecules is a glycoprotein (Nakane and Kawaoi, 1974). With this method, the carbohydrate moiety of the glycoprotein (e.g., HRP) is oxidized with sodium periodate resulting in aldehyde groups that in turn can react with primary amino groups on the other macromolecular species, thus, yielding Schiff's bases. These Schiff's bases are then stabilized with a reducing agent such as sodium perborate or sodium cyanoborohydride to prevent hydrolysis (Figure 5). The periodate oxidation method is the method most often used for the production of HRP-antibody conjugates since its conjugate yields are 3 to 4 times higher (Voller *et al.*, 1979) and its specific activity about 5 times greater (Nygren, 1982) than conjugates produced using glutaraldehyde.

The choice of solid support for EIA is very important since it not only affects the ease of handling and washing, but also the success of an EIA. The solid support should have a high capacity (thus, a high surface area/volume ratio) for binding many different immunoreactants with negligible denaturation of the immunoreactant and minimal dissociation of the immobilized immunoreactant from the support (Tijseen, 1985). The most commonly used solid supports are non-porous hydrophobic plastics in the form of microtiter plates, tubes, or beads made of polyvinylchloride (Harlow and Lane, 1988), polystyrene (Ch'ng *et al.*, 1985), or polymethacrylate (Tijseen, 1985) as well as microporous hydrophobic surfaces such as filter paper (Hamblin and Hedger, 1982), nitrocellulose membranes (Brower *et al.*, 1985), nylon (Tijseen, 1985), and polyvinylidene difluoride (Oprandy and Sippel, 1989). All of these solid supports have the advantage that antibodies and many antigens may be passively adsorbed onto the support via hydrophobic interactions which makes the

Figure 5. Formation of antibody-enzyme conjugates through the sodium periodate oxidation method. The carbohydrate groups of the glycoprotein (the enzyme) are oxidized with sodium periodate to form aldehyde groups which react with primary amino groups of the antibody (Ab) to form Schiff's bases. The subsequent reduction of these Schiff's bases with a reducing agent such as sodium perborate or sodium cyanoborohydride prevents hydrolysis resulting in stable conjugates.

immobilization procedure very simple, inexpensive, and free from the use of toxic activating agents that modify the immunoreactant to be immobilized. Immunoreactants may also be covalently immobilized onto supports such as glass (Robinson *et al.*, 1971) or nylon beads (Perera *et al.*, 1983) which have been activated with glutaraldehyde, or cellulose activated with sodium periodate (Ferrua *et al.*, 1979).

Microtiter plates and other non-porous solid supports have become predominant in EIA because they offer simple separation procedures (simply wash or decant) that permit automated use. Such solid supports, however, have a very low surface area/volume ratio which results in long EIA processing times due to the slow rate of diffusion of the liquid sample to the immobilized immunoreactant (immunoreactions often take one hour or longer to reach equilibrium) (Tijssen, 1985). Particulate (e.g., beads) solid supports have a higher surface area/volume ratio which yields faster immunoreactions, however, they require the use of complex separation techniques such as centrifugation or filtration between each wash or immunoreaction (Hunter, 1980) which makes automation difficult and field use impractical. While microporous solid supports such as nylon or nitrocellulose membranes have the advantages of simple separation procedures and a large surface area/volume ratio, they are brittle which makes them difficult to handle and extremely thin so that the volume of sample that may be accommodated is limited to only a few microliters per cm^2 segment (Blais, 1990a).

Cloth-based enzyme immunoassay (CEIA), which uses macroporous hydrophobic polyester cloth as a support, has recently been developed as a viable alternative to traditional EIA (Blais and Yamazaki, 1989a). The basic structure of polyester cloth is shown below:

Macroporous polyester cloth has a large surface area/volume ratio which yields very fast immunoreaction rates due to the short diffusional distances between the immobilized immunoreactants and the sample. The porosity of the cloth makes washing easier since the cloth exhibits much better flow characteristics than non-porous or microporous immunoassay supports. The cloth is structurally stable, and thus, is easy to handle even in a large sheet. For the analysis of multiple samples, microwell EIA requires numerous pipettings into the various wells at each EIA step, whereas CEIA permits simultaneous EIA processing of multiple samples applied onto a cloth sheet by washing or saturating the sheet with EIA reagents. In microwell EIA, the immunoreaction and enzyme reaction rates must be carefully controlled in each well, whereas the reaction times in CEIA can be automatically equalized for multiple samples on the cloth sheet. The use of chromogenic substrates in CEIA provides clear visual comparisons of the colour stains of the multiple samples upon the white background of the cloth. The combination of these advantages makes polyester cloth the ideal EIA support for field testing. CEIA has been used for applications such as the detection of *Brucella* antigens (Blais *et al.*, 1989b) and the determination of human blood through the detection of human IgG (Ly and Yamazaki, 1995). While it is possible to immobilize antibodies and many antigens such as large proteins (e.g., bovine serum albumin (Blais and

Yamazaki, 1989a)) or amphiphilic molecules (e.g., Salmonella lipopolysaccharide (Blais and Yamazaki, 1989c)) passively onto polyester cloth via hydrophobic interactions, many hydrophilic antigens including polysaccharides or small peptides are not hydrophobic enough to readily adsorb onto the cloth. Such antigens can be covalently immobilized onto polyester cloth that has been activated with cyanuric chloride (Blais and Yamazaki, 1990b).

A major obstacle to overcome in using EIA for field testing is how to preserve the stability of the enzyme-labelled immunoreactant during transportation and use in the absence of refrigeration. This is extremely important because the storage stability of the enzyme-immunoreactant conjugate critically affects the sensitivity and reproducibility of EIA. While many antigens as well as the Fab region of antibodies are reasonably stable at ambient temperatures, the activity of most enzymes decreases very rapidly at field temperatures. It is this sensitivity to high temperatures that prevents the use of EIA in the absence of proper refrigeration. Partial unfolding of the enzyme is the first step in thermoinactivation. Normally, the native conformation of the enzyme is maintained through the cumulative action of various noncovalent forces including hydrogen bonds (form between a covalently bonded hydrogen atom on a donor group (e.g., -OH or =NH) and a pair of nonbonding electrons on an acceptor group (e.g., O=C- or -N=)), van der Waals forces (occur when molecules or atoms come so close together that their outer electron orbitals begin to overlap so that a mutual repulsion occurs between them), electrostatic interactions (attractive forces between atoms or molecules with a positive or negative charge or that have a positive or negative dipole), and hydrophobic interactions (hydrophobic molecules or moieties of a molecule that are hydrophobic are attracted to one another in the presence of water so that

the amount of interaction between hydrophobic molecules or moieties of a molecule with water are minimized). At equilibrium, the enzyme's native conformation tends to be the conformation with the lowest free energy (Lee and Timasheff, 1981). Water is intimately associated with the three dimensional structure of hydrated enzymes through many of these interactions (Saenger, 1987). As temperature increases, the free energy of the system increases and these noncovalent forces diminish so that the enzyme may unfold into a less ordered conformation. This has the effect of separating in space the critical amino acids that assemble the active centre of the enzyme resulting in loss of catalytic ability (Klibanov, 1983).

It has been proposed that an enzyme's stability may be increased by reducing the activity of the water present in the enzyme's solvent medium (Ye *et al.*, 1988). This theory has been demonstrated in the past by observing the half-life of various enzymes at a predetermined temperature when the solvent was switched from water to D_2O . It was found that there was a linear relationship between the proportion of D_2O in the medium and the half-life of the enzymes (D_2O has a higher degree of structural organization than water) (Combes and Monsan, 1984). Various solvent additives have been studied in an attempt to stabilize enzymes by limiting the activity of water on the enzyme. It has been proposed that in cases where the additive interacts more strongly with the enzyme than with water, denatured states of the enzyme will be favoured through the formation of additive-enzyme intermolecular bonds which negatively affect the intramolecular bonds already present in the enzyme. Alternatively, additives that interact more strongly with water molecules than with the enzyme are expected to stabilize the enzyme by causing an increase in the degree of water

molecule organization. This limits the capacity for water molecules to interact with the enzyme, thus preventing the redistribution of enzyme-water molecule interactions that lead to the generation of inactive enzyme conformations (Monsan and Combes, 1988). An increase in the stability of enzymes in solution by decreasing water activity has been demonstrated through the addition of high concentrations (as high as 50 % v/v or w/v) of sugars (e.g., sucrose and lactose) (Lee and Timasheff, 1981; Arakawa *et al.*, 1990), salts (e.g., KCl) (Combes *et al.*, 1988), polyols (e.g., glycerol and lactose) (Lee and Lee, 1987; Combes *et al.*, 1987), and polymers (e.g., polyethylene glycol) (Alfani, 1984; Ye *et al.*, 1988). The stabilizing effect of these additives is very enzyme-specific. In addition, because the presence of very high concentrations of additives may inhibit the reaction between the enzyme-immunoreactant conjugate and the antigen or antibody being tested for, it may be necessary to dilute the conjugate-stabilizer mixture prior to use in an EIA.

While there are other methods for trying to decrease the effect of water activity upon enzyme-immunoreactant conjugates, including storing the conjugate in a dried state or immobilizing the conjugate onto a solid support, these procedures are of limited interest for use in EIA designed for field testing. Conjugates stored in a dried state (e.g., enzymes that have been placed in a solution of the sugar trehalose prior to drying; Blakely *et al.*, 1990; Colaco *et al.*, 1992) have a convenience disadvantage due to the need for reconstitution, and a practical disadvantage in that much of the activity of the enzyme may be lost during the drying process since this is normally accompanied by wide fluctuations in pH, salt concentration, etc... of the buffer solution during drying and reconstitution. These fluctuations promote conformational changes in the enzyme and result in large batch-to-batch

variations in catalytic activity. The use of immobilized enzyme-immunoreactant conjugates precludes the utilization of heterogeneous EIA which is desirable for field testing due to its superior sensitivity over homogeneous EIA. In a future chapter the ability to stabilize enzyme-antibody conjugates in solution with the addition of low concentrations of polyethylene oxide or polyvinyl alcohol will be investigated to see if their use will permit stable storage of enzyme conjugates.

Immunoaffinity purification (also referred to as immunoaffinity chromatography) is the most powerful technique for isolating antigen or antibody with purifications of 1,000-10,000 fold routinely achievable in a single step (Harlow and Lane, 1988). This great purification capacity is a result of the specificity of antibodies for antigens. Immunoaffinity purification procedures are performed in three steps: (i) antibody or antigen is covalently attached to a solid support, (ii) the sample containing the antibody or antigen to be purified is added and any contaminating molecules are removed by washing with a suitable buffer, and (iii) the antibody-antigen interaction is broken with the use of a suitable elution condition so that the now purified antigen or antibody is released into the eluate. Factors affecting the success of an immunoaffinity purification include the starting purity of the sample, the affinity of the antibody for the antigen, and the ease with which the antibody-antigen interactions may be disrupted (Harlow and Lane, 1988). Since a 1,000-10,000-fold purification is possible with a single step, only one purification step is necessary unless the antigen or antibody to be purified represents less than 1 in 1,000-10,000 molecules in the sample. The affinity of the antibody for the antigen affects the amount of antigen or antibody that may be removed from the sample. As the affinity of the antibody increases so to does

the amount of antigen or antibody that may be purified. The number and type of non-covalent bonds, hydrogen bonds, van der Waals forces, and electrostatic and hydrophobic interactions, that make up the antibody-antigen interaction dictates the ease with which the purified immunoreactant may be eluted from the immunoaffinity column. While the various non-covalent bonds is related to the affinity of the antibody for the antigen, the ease of elution is not necessarily related to affinity. A low affinity antibody whose interaction with antigen is a result of various types of bonds may be more difficult to elute than a high affinity antibody that interacts with the antigen through only one type of bond. The former may require the use of various eluting conditions while the latter may require only one (Harlow and Lane, 1988).

There are many solid supports that have been used for immunoaffinity purification, some of which are also used in EIA. To be useful for immunoaffinity purification, a solid support should be inexpensive, structurally stable under high flow or pressure conditions, and should exhibit good flow characteristics when packed into a column. Commonly used solid supports include beads of carbohydrate polymers such as agarose (sold commercially under names such as Superose or Sepharose), dextran (sold commercially as Sephadex or Sephacryl), cellulose (Campbell and Weliky, 1967), polyacrylamide (Ternynck and Avrameas, 1972), or polyhydroxyethylmethacrylate (Tlaskalova *et al.*, 1975). Composite gels such as agarose-polyacrylamide beads have also been used with success (Guedson and Avrameas, 1976). Treatment of these polysaccharide supports with compounds such as cyanogen bromide (Axen *et al.*, 1967), epichlorohydrine (Porath and Fornstedt, 1970), cyanuric chloride (Smith and Lenhoff, 1974) or tresyl chloride (Nilsson and Mosbach, 1980)

will result in activated hydroxyl groups to which antibody or antigen may be covalently linked. Activated beads, however, are relatively expensive and often exhibit structural instability under high flow or pressure conditions (Janson, 1984). For these reasons, there is a great need to discover alternative solid supports that may be used for immunoaffinity purification. The development of a tosyl chloride activated rayon/polyester cloth will be studied in a forthcoming chapter.

Three different approaches have been used for eluting antigen or antibody from an immunoaffinity column including (i) treatment of the column with harsh conditions, (ii) adding a saturating amount of a small molecule that mimics the epitope of the antigen, and (iii) treatment of the column with a molecule that induces an allosteric change in the antigen (Harlow and Lane, 1988). While the second and third methods can work very well, their use is rare. It is very difficult to synthesize a small molecule that effectively mimics an antigenic epitope since the exact conformation within the epitope is affected through long-range interactions by regions outside of the epitope. The induction of an allosteric change requires the use of a molecule that can bind to the antigen in a region outside of the epitope and in doing so induces a conformational change in the antigen which inhibits the ability of the antibody to recognize the epitope.

Harsh conditions are most often used for elution, but since every antibody-antigen pair is unique in the nature of their interactions, the choice of a suitable elution condition is usually determined by trial and error. The normal procedure is to begin with the least harsh conditions and then to try other elution conditions of increasing harshness until a suitable eluent is found. Sometimes combinations of elution conditions must be used, but one must

always be careful to prevent denaturation of the immunoreactant that is to be purified. Many of the harsher conditions prevent reuse of the immunoaffinity column for subsequent purifications which makes for very expensive large-scale purification procedures. Typically used dissociating conditions in the order of gentlest to most severe include: (i) high salt concentrations (e.g., 5 M LiCl, pH 7.2), (ii) high or low pH (e.g., 0.1 M glycine-HCl, pH 2.2; 0.1 M glycine-NaOH, pH 10.5), (iii) organic solvents (e.g., 50 % (v/v) ethylene glycol, pH 8.0), (iv) chaotropic agents (e.g., 3 M potassium thiocyanate), (v) strong hydrogen bond formers (e.g., 8 M urea), and (vi) ionic detergents (e.g., 1 % (w/v) sodium dodecyl sulfate) (Johnstone and Thorpe, 1982). High salt concentrations (Jefferey and Saenger, 1991) and high or low pH (Collier and Kohlhaw, 1971) can often be used to disrupt antibody-antigen interactions when such binding is a result of electrostatic interactions. Organic solvents, chaotropic agents, and ionic detergents may successfully be used as eluents when the antibody-antigen binding is due to hydrophobic interactions. These eluents break hydrophobic interactions by dissolving the hydrophobic binding regions into the aqueous phase. As a result, such eluents often denature the antibody or antigen (Hermanson *et al.*, 1992). Strong hydrogen bond forming eluents can disrupt hydrogen bonds between antibodies and antigens by forming new hydrogen bonds between the eluent and functional groups of the antibodies and antigens that were involved in hydrogen bonding between themselves. Because the determination of a suitable elution condition tends to be the most time consuming aspect of an immunoaffinity purification procedure, the development of a universal eluent that does not denature the immunoreactant to be purified and that allows for repeated use of the immunoaffinity column is of great interest and will be studied in an

upcoming chapter.

One purpose of this thesis is to optimize CEIA for field testing. This requires stable immobilization of immunoreactants onto the cloth support (Chapter 1), stable blocking of the cloth support (Chapter 2), and stable storage of enzyme-immunoreactant conjugates (Chapters 3 and 4), all in the absence of refrigeration. Chapter 5 demonstrates the use of CEIA for the detection of very dilute antigen in large sample volumes. Chapter 6 provides a demonstration of how CEIA may be used for the inexpensive, simple, and very rapid quantification or qualification of antibodies in blood samples, thereby, illustrating the feasibility of CEIA for field testing. A second purpose of this thesis is to develop cloth-based technologies for the purpose of immunoaffinity purification. Covalent immobilization of proteins onto tosyl chloride-activated rayon/polyester for the purpose of immunoaffinity purification is shown in Chapter 7. An investigation to try to find an elution condition that would permit the gentle but very efficient elution of immunoreactants from an immunoaffinity column is summarized in Chapter 8. The use of polyester cloth for the rapid screening of elution conditions for the purpose of immunoaffinity chromatography, and as a support for the immunoaffinity purification of monoclonal antibodies is shown in Chapters 9 and 10, respectively.

MATERIALS

MATERIALS

The following materials were obtained from Sigma Co.: goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (A-6154), sheep anti-rabbit IgG antibody-biotin conjugate ($F(ab')_2$) (B-9140), goat anti-rabbit IgG antibody-biotin conjugate (B-7389), streptavidin-horseradish peroxidase conjugate (S-5512), avidin-horseradish peroxidase conjugate (A-3151), protein G'-biotin conjugate (P-8045), rabbit anti-horseradish peroxidase antibody (P-7899), goat anti-rabbit IgG antibody (R-1131), rabbit IgG (I-5006), protein G' (P-4689), horseradish peroxidase (P-6782), glucose oxidase (G-7141), bacterial β -galactosidase (G-6762), bovine alkaline phosphatase (P-0405), 3,3',5,5'-tetramethylbenzidine (T-2885), p-nitrophenol phosphate (Sigma No. 104), O-nitrophenyl- β -D-galactopyranoside (N-1127), p-toluenesulfonyl chloride (T-5384), sodium cholate (C-1254), polyvinyl pyrrolidone (PVP) (MW 10,000) (PVP-10), PVP (MW 40,000) (PVP-40), PVP (MW 360,000) (PVP-360), polyethylene glycol (PEG) (MW 3350) (P-3640), PEG (MW 10,000) (P-6667), polyvinyl alcohol (PVA) (MW 30,000-70,000) (P-8136), and PVA (MW 70,000-100,000) (P-1763). Aldrich Chemical Co. supplied polyethylene oxide (PEO) (MW 100,000) (18,198-6), PEO (MW 300,000) (18,200-1), PEO MW 600,000 (18,202-8), PVA (MW 9,000-10,000) (36,062-7), PVA (MW 13,000-23,000) (34,840-6), and PVA (MW 124,000-186,000) (36,306-5). Difco Laboratories supplied the barin heart infusion broth (0037-17-8) and the Bacto Salmonella O rabbit antiserum poly A (serogroups A, B, D, E₁, E₂, E₃, E₄, and L) (2534-47-6). Bio Rad supplied the Protein Blocker (non-fat skim milk) (170-6404). BDH supplied the magnesium chloride (B29096) and the drierite dessicant ($CaSO_4$), 8 mesh regular (B27625). The ImmunoPure Gentle Ag/Ab Elution Buffer (21013) was obtained

from Pierce. The Micro-O-Protect (a mixture of bromonitrodeoxane and 2-methyl isothiazolone) was acquired from Boehringer Mannheim (1585720). The polyester cloth (Sontara 8100) and the rayon/polyester (70 % 30 %) blend cloth (Sontara 8423) were supplied by DuPont. The Sontara number refers to the physical characteristics of the cloth including its thickness and air permeability. VWR Scientific supplied the blotting pads (VWR 320; 2.45 mm thick) and the disposable droppers (samco 273; 2 ml). The Masterflex pump was acquired from Cole-Parmer. The polymyxin-coated polyester cloth sheets and the insoluble TMB peroxidase substrate powder were acquired from Ricoh Kyosan Inc. (Tokyo, Japan). The monoclonal mouse anti-Salmonella lipopolysaccharide IgG_{2a}-biotin conjugate (M181-biotin conjugate) was kindly supplied by Alphonso Valdivieso from the Animal Disease Research Institute, Agriculture and Agrifood Canada.

CHAPTER 1
HYDROPHOBIC IMMOBILIZATION OF ANTIBODY
ONTO POLYESTER CLOTH FOR THE PURPOSE OF
ENZYME IMMUNOASSAY

ABSTRACT

During 70 days of air-dried or dessicated storage over CaSO_4 at 32°C , rabbit IgG and rabbit anti-horseradish peroxidase antibody remained adsorbed onto polyester cloth, retaining full immunoactivity both as an antibody and as an antigen. After dessicated storage, the adsorbed antibody could not be released from the polyester cloth by agitated washing in any of the following anhydrous water-mixable organic solvents: methanol, glacial acetic acid, dimethyl sulfoxide, dimethylformamide, or 1,4-dioxane.

INTRODUCTION

Enzyme immunoassay (EIA) may be used for the detection of various antigens such as pathogens, pollutants, and toxins as well as antibodies for serodiagnosis. With heterogeneous EIA, immunoreactant (antibody or antigen) is immobilized onto a solid support which is used for the specific capture of the sample immunoreactant. The captured immunoreactant is then assayed with an antibody-enzyme conjugate. Recently, a cloth-based enzyme immunoassay (CEIA) that uses inexpensive and readily available macroporous hydrophobic polyester cloth as a support for EIA has been developed (Blais and Yamazaki, 1989a). CEIA offers many advantages over commonly used EIA systems as described in the General Introduction.

The advantages of CEIA are most notable for field testing where inexpensive, simple to use, and rapid assays are desired. The ability to stably immobilize immunoreactants onto the polyester cloth is necessary for maximum application of CEIA for field testing where there is often an absence of proper refrigeration for transportation and storage. The present

chapter investigates the long-term immunoactivity (antigen and antibody activity) and stability of antibody hydrophobically adsorbed onto polyester cloth under air-dried conditions at 32°C and ambient humidity. Because ambient humidity may vary greatly under field testing conditions, storage under dessicated conditions may render reproducible stability of antibody coated cloth independent of ambient humidity. Thus, the present chapter also studies the effects of desiccation upon the immunoactivity and stability of antibody adsorbed onto polyester cloth.

METHODS

Effect of Ambient or Dessicated Storage on the Antigen or Antibody Activity of Antibody Adsorbed onto Polyester Cloth

Segments (6 mm x 6 mm square) of polyester cloth were soaked in ethanol for a few minutes, washed 5 times with PBS (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % NaCl), and blotted. Each cloth segment was incubated with 50 μ l of rabbit IgG (50 ng in PBS/cloth segment) or rabbit anti-horseradish peroxidase antibody (anti-HRP antibody) (100 ng in PBS/cloth segment) overnight at 30°C in closed petri plates resulting in antigen-cloths and antibody-cloths respectively. The cloths were then washed 5 times with PBST (PBS containing 0.05 % (v/v) Tween 20) followed by 3 times with PBS, blotted, allowed to air-dry for 1 h, and then stored at 32°C for 70 days in an incubator (ambient storage) or a vacuum sealed desiccator (dessicated storage) over the drierite dessicant, CaSO₄. At regular time intervals, cloth segments were removed and assayed for antigen or antibody activity. After incubating the cloth segments in 0.5 % (w/v) Protein Blocker in PBS for 1 h at room

temperature (RT), the cloths were washed 5 times with PBS and blotted. To measure antigen activity, each rabbit IgG-cloth was incubated with 25 μ l of goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (anti-rabbit IgG-HRP conjugate) (the stock solution was diluted 500 times) for 20 min at RT. Similarly, the activity of the antibody-cloths was determined by incubating each anti-HRP antibody-cloth with 25 μ l of HRP (50 ng in PBS/cloth segment) for 20 min at RT. The cloths were then washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity. The amount of anti-rabbit IgG-HRP conjugate or HRP adsorbed onto control cloths (coated only with Protein Blocker) were subtracted from the above results.

Effect of Various Anhydrous Solvents Upon the Adsorption of Antibody to Polyester Cloth

Polyester cloth segments (6 mm x 6 mm square) were soaked in ethanol, and then washed with PBS and blotted as above. Each cloth segment was treated with 50 μ l of goat anti-rabbit IgG antibody-biotin conjugate (anti-rabbit IgG-biotin conjugate) (50 ng in PBS/cloth segment) and incubated overnight in closed petri plates at 30°C. After washing the cloths 5 times with PBST followed by 3 times with PBS, blotting, and air-drying for 1 h, the cloth segments were stored in a vacuum sealed desiccator over drierite for 5 days at 32°C. The cloths were then washed 3 times while agitating (5 min per washing, 2 ml/cloth segment) with PBS or one of the following anhydrous solvents: methanol, glacial acetic acid, dimethyl sulfoxide, dimethylformamide, or 1,4-dioxane. After washing 3 times with PBS and blotting, the cloths were mixed in 0.5 % Protein Blocker in PBS for 1 h at RT. Each cloth was then washed 3 times with PBS, blotted, and incubated with 25 μ l of avidin-

horseradish peroxidase conjugate (avidin-HRP conjugate) (50 ng in PBS/cloth segment) for 20 min at RT. The cloths were then washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity. The amount of avidin-HRP adsorbed onto control cloths (coated only with Protein Blocker) were subtracted from the above results.

Assay of HRP Activity

One ml of the soluble TMB peroxidase substrate system was added to an individual cloth segment placed in a 16 mm x 100 mm test tube and shaken at 180 RPM (on a New Brunswick Gyrotary shaker) for 10 min at 30°C. The peroxidase reaction was stopped by adding 250 μ l of 2 N H_2SO_4 and then the absorbance of the resulting colour was measured at 450 nm. The soluble TMB peroxidase substrate system was prepared by mixing 2.5 ml of TMB (2 mg/ml of ethanol) with a solution consisting of 0.21 g citric acid H_2O , 0.42 g EDTA 4 Na, 0.03 g $NaBO_4 H_2O$, and 100 ml H_2O .

RESULTS AND DISCUSSION

Effect of Ambient or Dessicated Storage on the Antigen or Antibody Activity of Antibody Adsorbed onto Polyester Cloth

To study the stability of immunoreactants adsorbed onto a hydrophobic cloth, segments of polyester cloth were coated with limited amounts of rabbit IgG (to study the antigen activity of an antibody) or anti-HRP antibody (to study the antibody activity of an antibody) so that any loss of immunoactivity could be detected. The cloth segments were air-dried and then stored at 32°C in an incubator (ambient storage) or in a vacuum sealed

dessicator (dessicated storage) over CaSO_4 . The antigen activity of rabbit IgG and the antibody activity of anti-HRP antibody during 70 days of storage were measured periodically by reaction with excess anti-rabbit IgG-HRP conjugate or HRP, respectively. Since limited amounts of immunoreactants were adsorbed onto the cloth, it was necessary to block the adsorption sites on the cloth with a blocking reagent (Protein Blocker) to reduce non-specific adsorption of the anti-rabbit IgG-HRP conjugate or HRP.

Table 1.1 shows that both the antigen activity of rabbit IgG and the antibody activity of anti-HRP antibody adsorbed onto polyester cloth were stable during 70 days of ambient and dessicated storage at 32°C . Therefore, there was very little denaturation and/or desorption of the rabbit antibody from the cloths.

Effect of Various Anhydrous Solvents Upon the Adsorption of Antibody to Polyester Cloth

The structure of polyester cloth (refer to the General Introduction, page 25) suggests that adsorption of antibodies is primarily a result of hydrophobic interactions with polyester aromatic groups and hydrogen bonding with polyester carbonyl oxygen atoms. It is generally accepted that hydrophobic interactions require the presence of water for their preservation. Since antibodies remained adsorbed onto polyester cloth during dessicated storage, I wanted to study the strength of antibody adsorption through the use of anhydrous water-mixable organic solvents. Anti-rabbit IgG-biotin conjugate was adsorbed onto polyester cloth and vacuum dessicated over CaSO_4 for 5 days at 32°C . The antibody cloths were washed 3 times in sealed containers with agitation in one of the following anhydrous organic solvents: methanol, glacial acetic acid, dimethyl sulfoxide, dimethylformamide, or 1,4-dioxane. Fresh

Table 1.1 Effect of ambient and dessicated storage on the antigen or antibody activity of rabbit antibody adsorbed onto polyester cloth^a

Condition	Time of Storage (Days)						
	0	8	15	29	43	56	70
rabbit IgG-cloth/ambient storage	1.180 ± 0.137	1.182 ± 0.095	1.100 ± 0.102	1.064 ± 0.065	1.033 ± 0.041	1.117 ± 0.050	1.018 ± 0.036
rabbit IgG-cloth/dessicated storage	1.290 ± 0.108	1.250 ± 0.074	1.211 ± 0.095	1.289 ± 0.112	1.183 ± 0.076	1.203 ± 0.062	1.274 ± 0.087
anti-HRP antibody-cloth/ambient storage	0.988 ± 0.070	1.053 ± 0.081	0.967 ± 0.067	0.900 ± 0.106	0.953 ± 0.083	1.035 ± 0.070	0.959 ± 0.060
anti-HRP antibody-cloth/dessicated storage	0.993 ± 0.070	1.051 ± 0.095	1.012 ± 0.074	0.906 ± 0.053	0.948 ± 0.059	0.993 ± 0.041	0.924 ± 0.064

^aRabbit IgG or anti-HRP antibody adsorbed onto polyester cloth segments was stored at 32°C under ambient humidity or in a vacuum sealed dessicator over CaSO₄ for 70 days. Cloth segment samples were periodically removed and assayed for antigen or antibody activity as described in Methods. The data (n = 8) represents the HRP activity (mean absorbance at 450 nm ± standard deviation) of anti-rabbit IgG-HRP conjugate reacted with rabbit IgG-cloth or HRP reacted with anti-HRP-cloth, respectively. Non-specific adsorption of HRP activity on the control cloths were all below 0.015.

analytical grade solvents were used to minimize the water content in the solvents. The amount of antibody remaining on the cloth was determined by reaction with excess avidin-HRP conjugate followed by HRP assay. Table 1.2 demonstrates that none of these washes released any of the antibody from the cloth. Agitated washing with these anhydrous water-mixable solvents must have reduced water activity to a minimum. In addition, some of these solvents (e.g., glacial acetic acid and dimethylformamide) are known to be effective in disrupting hydrogen bonds. Thus, antibody adsorption, if maintained by hydrophobic interactions, is very stable even in a minimum of water activity (caused by dessicated storage and water extraction with the solvents).

This chapter has demonstrated that antibody-polyester cloth can be stored under ambient or dessicated conditions in the absence of refrigeration for use in CEIA. While there was no loss of immunoactivity of the antibody-cloth during ambient storage in the controlled environments of the laboratory, dessicated storage is recommended for use under ambient transportation or field testing conditions where fluctuating humidity may result in loss of immunoactivity of the adsorbed antibody over time. While antibodies were used in the present chapter, it should be possible to similarly design CEIAs that are useful for detecting antibodies in samples using adsorbed polypeptide antigens. This chapter has also provided evidence that hydrophobic interactions may not require the presence of intermolecular water for their maintenance.

Table 1.2 Effect of various anhydrous solvents upon the adsorption of antibody to polyester cloth^a

Anhydrous Solvent	HRP Activity (A_{450})
PBS	0.855 ± 0.051
methanol	0.829 ± 0.067
glacial acetic acid	0.863 ± 0.040
dimethyl sulfoxide	0.870 ± 0.062
dimethyl formamide	0.812 ± 0.055
1,4-dioxane	0.852 ± 0.047

^aAnti-rabbit IgG-biotin conjugate adsorbed onto polyester cloth was stored at 32°C in a vacuum sealed dessicator over CaSO_4 for 5 days. The resultant cloths were then washed 3 times with agitation (5 min/wash) in PBS, methanol, glacial acetic acid, dimethyl sulfoxide, dimethyl formamide, or 1,4-dioxane, blocked with Protein Blocker, reacted to excess avidin-HRP conjugate, and assayed for HRP activity as described in Methods. The data is represented as HRP activity (mean absorbance at 450 nm) $\pm$ standard deviation ($n = 8$).

The results presented in this chapter came in part from the following papers:

- Boyd, S. and Yamazaki, H. (1995a) Stability of polypeptide immunoreactants and polyvinyl alcohol as a blocking agent on polyester cloth during dry storage. *Immunol. Invest.* 24: 795-803.
- Boyd, S. and Yamazaki, H. (1996a) Long-term preservation of antibody activity and binding to polyester cloth by desiccation. *Biotechn. Techn.* 10: 367-370.

CHAPTER 2

POLYVINYL ALCOHOL AS A BLOCKING AGENT FOR THE LONG-TERM DRY STORAGE OF ANTIBODY-POLYESTER CLOTH

ABSTRACT

Polyvinyl alcohol of molecular weight 30,000-70,000 permitted stable blocking of antibody-adsorbed polyester cloth without affecting the immunoactivity of the cloth. Such cloth may be stored for extended periods of time at elevated ambient temperatures (e.g., 32°C), thus, making cloth-based enzyme immunoassay systems suitable for field testing and transportation in the absence of refrigeration.

INTRODUCTION

The use of hydrophobic supports such as microtiter plates and polyester cloth for enzyme immunoassay (EIA) permits easy immobilization of antibodies and many antigens (e.g., large polypeptides) through non-covalent hydrophobic interactions. This is preferable to covalently immobilizing immunoreactants through time consuming procedures that use expensive and toxic chemical activating agents. A critical step in the use of hydrophobic supports for EIA is the blocking of the support prior to the assay of specific antibody-antigen interactions. Non-specific binding is the adsorption of sample components (including the antibody-enzyme conjugate used to assay specifically captured immunoreactants) that do not have targeted sites of interaction with the immobilized immunoreactant. If left unblocked, the non-specific binding of immunoreactants can ruin an EIA by creating very high levels of background. While it is possible to block an EIA support by saturating it with immunoreactants, this can be very expensive, especially if the immunoreactant to be immobilized is something like a viral antigen or a monoclonal antibody. A better alternative is to saturate the hydrophobic support with a non-relevant molecule which binds non-

covalently to eliminate sites of adsorption and does not interfere with the desired immunoassay. The most popular blocking substances are proteins including bovine serum albumin, casein, and non-fat skim milk (Towbin *et al.*, 1979; Hauri and Bucher, 1986; Vogt *et al.*, 1987). Trial and error is often necessary to determine what concentration and what protein blocker provides the best suppression of non-specific binding for a given EIA. With polyester cloth, I have found that incubating the support for 1 h in a solution of 0.5 % (w/v) non-fat skim milk in PBS buffer works well.

It is not practical to perform blocking procedures in the field, and so it becomes important to be able to block the EIA support prior to transportation and storage for use in field testing. If protein blocking agents are used there is a possibility of protein denaturation over time if the support is to be transported and stored in the absence of refrigeration. Such denaturation may expose otherwise buried hydrophobic regions within the protein which can facilitate non-specific binding of immunoreactants. Non-protein blocking agents may be a better choice for use in EIA designed for field testing because they should not lose their blocking capacity over long-term storage in the absence of refrigeration. In addition, non-protein blocking agents should not be contaminated with interfering proteins such as endogenous peroxidases or immunoglobulins that can compromise the use of protein blockers in certain EIA (Harvey, 1991). Previously, the use of polyvinyl alcohol (PVA) as a non-protein blocking agent for the treatment of nitrocellulose membranes (Harvey, 1991) and polystyrene microtiter plates (Rodda and Yamazaki, 1994) used in EIA has been noted. For field EIA testing, these supports are inferior to polyester cloth used in cloth-based enzyme immunoassay (CEIA) for reasons already discussed.

PVA manufacture involves the conversion of poly(vinyl acetate) into PVA through base-catalysed methanolysis usually using NaOH as shown below (Mark *et al.*, 1983):

PVA is commercially available in various molecular weights. The purpose of this chapter is to study the use of PVA as a non-protein blocking agent for polyester cloth used in CEIA that may be stored at ambient temperatures for field testing. The long-term blocking performance of different PVA conditions will be compared with non-fat skim milk.

METHODS

Effect of Various Blocking Conditions on Non-Specific Adsorption

Polyester cloth segments were soaked in ethanol for a few minutes, washed 5 times with PBS (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % sodium chloride), and blotted. Segments were then shaken at 120 RPM (on a New Brunswick Gyrotary Shaker) in PBSM solutions (PBS containing 0.1 % (v/v) Micro-O-Protect as a preservative) (1 ml/cloth segment) containing either 0.5 % (w/v) Protein Blocker (non-fat skim milk), or PVA as a blocking agent. Concentrations of 0.2 % to 5 % (w/v) of PVA (MW 9,000-10,000; 13,000-

23,000; 30,000-70,000; 70,000-100,000; or 124,000-186,000) were individually tested. PBSM (no Protein Blocker or PVA) was used as a control. Following incubation times of 1, 2, 4, 6, 8, and 16 h at room temperature (RT), cloth segments were removed from the various blocking solutions, washed 5 times with PBS, and blotted. Each segment was then incubated with 25 μ l of goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (anti-rabbit IgG-HRP conjugate) (the stock solution from Sigma was diluted 500 times in PBS) for 20 min at RT, washed 5 times with PBST (PBS containing 0.05 % (v/v) Tween 20) followed by 3 times with PBS, blotted, and assayed for HRP activity.

Effect of Storage on Blocking Ability

To study the ability of PVA- or Protein Blocker-treated polyester cloth segments to retain their blocking capacity, cloth segments were soaked in ethanol for a few minutes, washed with PBS, and blotted as above. The cloths were then shaken at 120 RPM for 24 h at RT in PBSM solutions (1 ml/cloth segment) containing 0.5 % Protein Blocker, 2 % PVA (MW 9,000-10,000), or 2 % PVA (MW 30,000-70,000). Control cloths were incubated in PBSM in the absence of blocking agents. The cloths were washed 5 times with PBS, thoroughly blotted and dried, and then stored in sealed petri plates in a 32 °C incubator for 64 days. At various time intervals cloth segments were removed and incubated with 25 μ l of anti-rabbit IgG-HRP conjugate (the stock anti-rabbit IgG-HRP conjugate was diluted 500 times in PBS). Following a 20 min incubation at RT, the cloths were washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity.

Effect of PVA-Blocking on Antigen Activity of Adsorbed Antibody

Polyester cloth segments were soaked in ethanol for several minutes, followed by washing in PBS, and blotting. Each cloth was then incubated with 50 μ l of rabbit IgG (50 ng in PBS/cloth segment) overnight at 30°C in closed petri dishes. Cloths incubated in PBS (no rabbit IgG) served as the controls. The antibody (rabbit IgG)-cloths and PBS-cloths were washed 5 times with PBST followed by 3 times with PBS, blotted, and then shaken at 120 RPM in a solution (1 ml/cloth segment) of 2 % PVA (MW 30,000-70,000) in PBSM. At time intervals of 1, 2, 4, 6, 8, and 16 h at RT, cloth segments were removed, washed 5 times with PBS, and blotted. Each cloth was incubated with 25 μ l of anti-rabbit IgG-HRP conjugate (the stock anti-rabbit IgG-HRP conjugate was diluted 500 times in PBS). Following a 20 min incubation at RT, the cloths were washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity.

Effect of Storage on the Antigen Activity of Antibody-Cloth Blocked with PVA

Antibody-cloths were prepared by soaking polyester cloth segments in ethanol, washing in PBS and blotting, and then being incubated with 50 μ l of rabbit IgG (50 ng in PBS/cloth segment) as above. The antibody-cloths were mixed in 2 % PVA (MW 30,000-70,000) for 24 h at RT, washed 5 times with PBS, blotted, and then stored at 32°C for 64 days. At regular time intervals cloth segments were removed and treated with 25 μ l of anti-rabbit IgG-HRP conjugate (the stock anti-rabbit IgG-HRP conjugate was diluted 500 times in PBS). After a 20 min incubation at RT, the cloths were washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity. Polyester cloth

segments treated with 2 % PVA (MW 30,000-70,000) but containing no rabbit IgG served as controls for non-specific adsorption.

Assay of HRP Activity

One ml of soluble TMB peroxidase substrate system was added to an individual anti-rabbit IgG-HRP conjugate-treated cloth segment placed in a 16 mm x 100 mm test tube and shaken at 180 RPM for 10 min at 30°C. The peroxidase reaction was stopped by adding 250 μ l of 2 N H_2SO_4 and then the absorbance of the resulting colour was measured at 450 nm. The soluble TMB peroxidase substrate system was prepared by mixing 2.5 ml of TMB (2 mg/ml of ethanol) with a solution consisting of 0.21 g citric acid H_2O , 0.42 g EDTA 4 Na, 0.03 g $NaBO_3 H_2O$, and 100 ml H_2O .

RESULTS AND DISCUSSION

Effect of Various Blocking Conditions on Non-Specific Adsorption

To study the effect of various blocking conditions on non-specific adsorption, polyester cloth segments were treated for 0 to 16 h at RT with PBSM (unblocked cloths for use as a control for non-specific adsorption), 0.5 % Protein Blocker in PBSM, or different molecular weights of 2 % PVA in PBSM. Micro-O-Protect was used to prevent the growth of microorganisms in the various blocking solutions. After washing the cloths, they were incubated with a quantity of anti-rabbit IgG-HRP conjugate that was sufficient to give an absorbance value at 450 nm of approximately 1 unit for the PBSM controls upon reaction with the soluble TMB peroxidase substrate system. Figure 2.1 shows the degree of non-

specific adsorption of anti-rabbit IgG-HRP conjugate to the Protein Blocker- or PVA-treated cloth expressed as a percent of the unblocked cloth which is inversely related to the degree of blocking. In contrast to the Protein Blocker, 2 % PVA of the various MW blocked the cloth less efficiently with a significant portion of the cloth remaining unblocked even after overnight (16 h) blocking. There was an obvious trend towards improved blocking capacity with increasing PVA treatment time. Increasing the PVA concentration to 5 % did not significantly change the kinetics of blocking (data not shown). PVA of MW 124,000-186,000 (which effectively blocks the polystyrene surface of microwells; Rodda and Yamazaki, 1994) was less effective in blocking the polyester cloth than PVA of lower MW such as MW 9,000-10,000 and MW 30,000-70,000.

Effect of Storage on Blocking Ability

Although the previous results (Figure 2.1) indicate that Protein Blocker offers a superior capacity to block non-specific adsorption of anti-rabbit IgG-HRP conjugate than PVA, to be of practical use for field CEIA, the blocking agent must maintain its blocking capacity for prolonged periods of time in the absence of refrigeration. Thus, the effect of storage on the blocking ability of Protein Blocker as well as PVA with MW 9,000-10,000 and 30,000-70,000 was studied. The polyester cloth segments blocked with 2 % PVA or 0.5 % Protein Blocker for 24 h were periodically assayed for non-specific adsorption of the anti-rabbit IgG-HRP conjugate during 64 days of storage at 32°C. Figure 2.2 shows that within 30 days of storage, the degree of non-specific adsorption markedly increased on the polyester cloths coated with Protein Blocker and PVA of MW 9,000-10,000 while the degree

Figure 2.1. The effect of various blocking conditions on non-specific adsorption. Polyester cloths were treated for various periods of time with 0.5 % Protein Blocker or different molecular weights of 2 % PVA, and then incubated with anti-rabbit IgG-HRP conjugate. The HRP activity of the non-specifically adsorbed anti-rabbit IgG-HRP conjugate was measured as described in Methods. The degree of non-specific adsorption was plotted as a percentage of the non-specific activity observed for the controls (cloths not treated with PVA or Protein Blocker) $\pm$ standard deviation ($n = 6$). Symbols: $\circ$, Protein Blocker; $\square$, PVA (MW 9,000-10,000); Δ , PVA (MW 13,000-23,000); ∇ , PVA (MW 30,000-70,000); $\sim$, PVA (MW 70,000-100,000); and $\bigcirc$, PVA (MW 124,000-186,000).

of non-specific adsorption on the cloths blocked with PVA of MW 30,000-70,000 remained low during the 64 days of storage.

Effect of PVA-Blocking on the Antigen Activity of Adsorbed Antibody

Although PVA with MW 30,000-70,000 shows its potential as a blocking agent for CEIA kits stored under ambient conditions, blocking with PVA should not reduce the immunoactivity of immunoreactants immobilized onto polyester cloth. To examine this, polyester cloth segments coated with rabbit IgG were blocked with 2 % PVA of MW 30,000-70,000 for various lengths of time from 1 to 16 h and assayed for adsorption of anti-rabbit IgG-HRP conjugate. At each blocking time, the specific adsorption of anti-rabbit IgG-HRP conjugate onto the rabbit IgG-cloths was calculated by subtracting from these values the non-specific adsorption of the anti-rabbit IgG-HRP conjugate onto the control cloths (no rabbit IgG). Table 2.1 shows that PVA-blocking for up to 16 h did not decrease the antigen activity of the adsorbed antibody.

Effect of Storage on the Antigen Activity of Antibody-Cloth Blocked with PVA

Having demonstrated the suitability of PVA of MW 30,000-70,000 as a blocking agent for cloth, the effect of long-term storage on the immunoactivity of PVA-blocked rabbit IgG-cloth was studied. Rabbit IgG-cloths blocked with 2 % PVA and stored at 32 °C for various periods of time, were tested for their reactivity to anti-rabbit IgG-HRP conjugate. After each incubation time, the specific adsorption of anti-rabbit IgG-HRP conjugate onto the rabbit IgG-cloths was calculated by subtracting from these values the amount of

Figure 2.2. Effect of storage on blocking ability. Polyester cloths were incubated for 24 h in PBSM (non-specific adsorption control) or PBSM solutions containing 0.5 % Protein Blocker, 2 % PVA (MW 9,000-10,000), or 2 % PVA (MW 30,000-70,000), washed with PBS, dried, and stored at 32°C. At regular time intervals, cloths were treated with anti-rabbit IgG-HRP conjugate and assayed for HRP activity as described in Methods. The degree of non-specific adsorption was calculated as a percentage of the non-specific activity observed for the control. The vertical bars represent standard deviation (n = 6). Symbols: ○, Protein Blocker; □, PVA (MW 9,000-10,000); and △, PVA (MW 30,000-70,000).

Table 2.1. Effect of PVA-blocking on the antigen activity of adsorbed antibody^a.

Condition	Time of Blocking Treatment					
	1 Hour	2 Hours	4 Hours	6 Hours	8 Hours	16 Hours
rabbit IgG-cloth (SA + NSA)	1.140 ± 0.078	1.049 ± 0.147	1.004 ± 0.146	0.935 ± 0.058	0.909 ± 0.092	0.909 ± 0.131
control cloth (NSA)	0.291 ± 0.016	0.225 ± 0.031	0.152 ± 0.023	0.099 ± 0.018	0.082 ± 0.022	0.058 ± 0.021
(SA)	0.849 ± 0.058	0.867 ± 0.115	0.852 ± 0.124	0.844 ± 0.052	0.827 ± 0.084	0.851 ± 0.123

^aFollowing the adsorption of rabbit IgG onto polyester cloths, the cloths were treated with 2 % PVA (MW 30,000-70,000). At regular time intervals the cloths were removed from the blocking solution, reacted with anti-rabbit IgG-HRP conjugate, and then tested for HRP activity as described in Methods. The antigen activity of the antibody was determined by subtracting the non-specific adsorption (NSA) values for the control cloths (no rabbit IgG) from the NSA + specific adsorption (SA) values for the antibody-cloths. The results are expressed as mean absorbance at 450 nm ± standard deviation (n = 6).

anti-rabbit IgG-HRP conjugate non-specifically adsorbed onto control cloths (no rabbit IgG). Table 2.2 shows that the antigen activity of adsorbed antibody remained essentially unchanged during 64 days of storage at 32°C.

This chapter has demonstrated that PVA of MW 30,000-70,000 provides stable blocking of antibody-adsorbed polyester cloth without affecting the immunoactivity of the adsorbed antibody. Since PVA-blocked antibody-cloth can be stored at 32°C for a long period of time, it can be shipped and stored without refrigeration, making the use of CEIA for field testing feasible. The ability to block EIA supports prior to transportation for field use eliminates blocking steps that are time consuming and impractical to perform under field testing conditions.

The results presented in this chapter have been published in the following paper:

Boyd, S. and Yamazaki, H. (1995a) Stability of polypeptide immunoreactants and polyvinyl alcohol as a blocking agent on polyester cloth during dry storage. *Immunol. Invest.* 24: 795-803.

Table 2.2. Effect of storage on the antigen activity of PVA-blocked adsorbed antibody^a.

Condition	Storage Time						
	Day 0	Day 8	Day 16	Day 24	Day 36	Day 50	Day 64
rabbit IgG-cloth (SA + NSA)	1.106 ± 0.101	1.152 ± 0.072	1.048 ± 0.047	1.049 ± 0.094	1.065 ± 0.074	1.155 ± 0.054	1.084 ± 0.088
control cloth (NSA)	0.023 ± 0.004	0.021 ± 0.005	0.036 ± 0.005	0.049 ± 0.009	0.051 ± 0.011	0.042 ± 0.008	0.047 ± 0.009
(SA)	1.083 ± 0.099	1.131 ± 0.071	1.012 ± 0.045	1.000 ± 0.090	1.014 ± 0.070	1.113 ± 0.052	1.037 ± 0.084

^aFollowing the adsorption of rabbit IgG onto polyester cloth and subsequent treatment of the cloth for 24 h with 2 % PVA (MW 30,000-70,000), the cloths were incubated at 32 °C for 64 days. At regular time intervals, cloths were removed and reacted to anti-rabbit IgG-HRP conjugate after which HRP activity was assayed. The antigen activity of the antibody was calculated by subtracting the non-specific adsorption (NSA) values for the control cloths (no rabbit IgG) from the NSA + specific adsorption (SA) values for the antibody-cloths. The values are expressed as mean absorbance at 450 nm ± standard deviation (n = 6).

CHAPTER 3
STABILIZATION OF PEROXIDASE-ANTIBODY CONJUGATES
IN SOLUTION BY POLYVINYL ALCOHOL
AND POLYETHYLENE OXIDE

ABSTRACT

The activity of a horseradish peroxidase-antibody conjugate was greatly stabilized while in solution through the addition of polyvinyl alcohol (PVA) or polyethylene oxide (PEO). This stabilizing effect was dependent upon the molecular weight of the PVA or PEO used. Unlike PEO, the concentrations of PVA necessary for maximum stabilization had no adverse effects upon enzyme immunoassay (EIA). Thus, horseradish peroxidase-antibody conjugates stabilized by PVA can be used without dilution for EIA, and without the need of refrigeration during transport and storage.

INTRODUCTION

In EIA, enzyme conjugates of immunoreactants (antibody or antigen) are used for the qualification and quantification of sample immunoreactants by measuring the enzyme activity through the addition of a suitable substrate. The instability of enzyme conjugates necessitates the use of refrigeration or refrigerant during transportation and storage making EIA expensive and field EIA impractical. Horseradish peroxidase (HRP) is the most popular enzyme for EIA due to its low cost and high availability in a purified form, high specific activity, large number of different substrates, and high carbohydrate content of approximately 18 % (Shannon *et al.*, 1966) which allows for easy conjugation to protein immunoreactants (refer to the general introduction for more information on HRP and its substrates). Thus, the ability to transport and store HRP-immunoreactant conjugates in the absence of refrigeration and in a ready to use solution would be of great benefit to field EIA.

Loss of activity of an enzyme-antibody conjugate is usually due to denaturation of

the enzyme rather than denaturation of the Fab fragments of the antibody which tend to be relatively stable. It is generally believed that the stability of an enzyme may be increased by decreasing the activity of the water present in the enzyme's solvent medium (Ye *et al.*, 1988). The usual method undertaken to reduce water activity is through the addition of high concentrations of sugars, salts, polyols, or polymers to the enzyme solution. An alternative approach is to dry the enzyme, possibly in the presence of an additive such as trehalose (Blakeley *et al.*, 1990). The ability to store the enzyme conjugate in solution is preferable for field EIA because it eliminates the need to reconstitute the conjugate immediately prior to use. If stored in a dried state, the enzyme conjugate for a field EIA kit would have to be divided into numerous vials so that only the amount of conjugate necessary for testing a given number of samples could be reconstituted. Thus, the use of dried enzyme conjugate would make a field EIA less convenient. If the stabilizing agent does not interfere with the reaction between the enzyme-immunoreactant conjugate and the test immunoreactant, the stabilized conjugate in solution can be used without dilution immediately prior to use in an EIA. In the current chapter, the addition of various concentrations of different sugars, salts, polyols, and polymers were examined for their ability to stabilize a HRP-antibody conjugate in solution (Appendix I summarizes the results for the stabilization of a HRP-antibody conjugate through drying in the presence of various additives).

METHODS

Stabilizing Effect of Various Sugars, Salts, Polyols, and Polymers on the Peroxidase Activity of an Anti-Rabbit IgG-HRP Conjugate

Goat anti-rabbit IgG antibody-peroxidase conjugate (anti-rabbit IgG-HRP conjugate) was suspended in PBSM (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % NaCl (PBS) containing 0.1 % (v/v) Micro-O-Protect as a preservative) and one of the following additives: 50 % (w/v) sorbitol, saturated $(\text{NH}_4)_2\text{SO}_4$, saturated NaCl, saturated $\text{Mg}(\text{SO}_4)_2$, saturated Na_2SO_4 , 50 % (v/v) glycerol, 50 % (v/v) ethylene glycol, 2 % polyvinyl pyrrolidone (PVP) (MW 10,000; 40,000; and 360,000), 2 % polyethylene glycol (PEG) (MW 200; 400; 1000; 1450; 3350; 8000; and 10,000), 2 % (w/v) polyethylene oxide (PEO) (MW 100,000; 300,000; 600,000; and 900,000), or 2 % (w/v) polyvinyl alcohol (PVA) (MW 9,000-10,000; 13,000-23,000; 30,000-70,000; 70,000-100,000; and 124,000-186,000). The concentration of the anti-rabbit IgG-HRP conjugate in the suspension was adjusted so that the initial HRP activity, following a 100 times dilution in PBS and mixing 50 μl of the resultant diluent in 1 ml of the soluble TMB peroxidase substrate system, produced a suitable density (A_{450} of 1 to 2) of TMB colour product during a 10 min reaction. The suspensions were incubated for 15 days at 32°C and then assayed for HRP activity. This experiment was repeated by incubating the anti rabbit IgG-HRP conjugate for 36 days at 32°C in PBSM solutions containing 0.5 to 10 % PVA or PEO (of the various molecular weights).

Effect of PVA and PEO on Immunoreaction

The immunoreaction between rabbit IgG and anti-rabbit IgG-HRP conjugate was

assayed using cloth-based enzyme immunoassay (CEIA). Segments (6 mm x 6 mm square) of polyester cloth were soaked in ethanol for a few minutes, washed 5 times with PBS, and blotted. Each cloth segment was incubated overnight at 30°C with 50 µl of rabbit IgG (5 µg in PBS/cloth segment) in closed petri plates. The resultant rabbit IgG-cloths were washed 5 times with PBST (PBS containing 0.05 % (v/v) Tween 20) followed by 3 times with PBS and then incubated in 0.5 % (w/v) Protein Blocker in PBS for 1 h at room temperature (RT). Anti-rabbit IgG-HRP conjugate was suspended in solutions of 0.5 % to 10 % PVA (MW 9,000-10,000 or 124,000-186,000) or PEO (MW 100,000) in PBS for 30 min. The concentration of the anti-rabbit IgG-HRP conjugate suspensions was adjusted so that HRP activity could be assayed without further dilution steps. Following washing 3 times with PBS and blotting, the rabbit IgG-cloths were reacted to the different anti-rabbit IgG-HRP conjugate suspensions (30 µl/cloth segment) for 10 min at RT. The cloths were washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity.

Effect of PVA on Anti-Rabbit IgG-HRP Conjugate Activity

Anti-rabbit IgG-HRP conjugate was added to solutions of PBSM containing 0.5 % PVA (MW 9,000-10,000) or 1 % PVA (MW 124,000-186,000) and then incubated at 32 °C for 36 days. Samples were removed from each anti-rabbit IgG-HRP conjugate suspension at regular time intervals and reacted to rabbit IgG-cloths prepared as above (30 µl of sample/cloth). The cloths were washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity.

Assay of HRP Activity

One ml of soluble TMB peroxidase substrate system was added to a 50 μ l sample of a HRP conjugate suspension or an individual rabbit IgG-cloth segment reacted to anti-rabbit IgG-HRP conjugate in a 16 mm x 100 mm test tube and shaken at 180 RPM (on a New Brunswick Gyrotary Shaker) for 10 min at 30°C. The peroxidase reaction was stopped by adding 250 μ l of 2 N H₂SO₄ and then the absorbance of the resulting colour was measured at 450 nm. The soluble TMB peroxidase substrate system was prepared by mixing 2.5 ml of TMB (2 mg/ml of ethanol) with a solution consisting of 0.21 g citric acid H₂O, 0.42 g EDTA 4 Na, 0.03 g NaBO₃ H₂O, and 100 ml H₂O.

RESULTS AND DISCUSSION

Stabilizing Effect of Various Sugars, Salts, Polyols, and Polymers on the Peroxidase Activity of an Anti-Rabbit IgG- HRP conjugate

Goat anti-rabbit IgG-HRP conjugate was used as a model to examine the stabilization effect of various sugars, salts, polyols, and polymers. These water-soluble compounds were added to solutions of anti-rabbit IgG-HRP conjugate in PBS and incubated at 32°C for 15 days in the presence of Micro-O-Protect (added to prevent the growth of micro-organisms). A 2 % concentration was arbitrarily chosen for the purpose of screening the various PVP, PEG, PEO, and PVA species for their stabilizing effect. Table 3.1 shows that only 38.2 % of the initial HRP activity of the anti-rabbit IgG-HRP conjugate remained following 15 days of incubation in PBSM (the no additive control condition). Of the various additives tested, only PEO and PVA offered any significant stabilizing effect. The presence of 2 % PEO

(MW 100,000 and 300,000) and 2 % PVA (MW 9,000-10,000 and 124,000-186,000) prevented any loss of HRP activity in the anti-rabbit IgG-HRP conjugate.

To determine if the stabilizing effect of PEO and PVA is concentration dependent, anti-rabbit IgG-HRP conjugate was incubated in PBSM solutions containing 0.5 to 10 % of the various PEO and PVA species. Following a 36 day incubation at 32 °C, the anti-rabbit IgG-HRP conjugate was tested for HRP activity. Figure 3.1 shows that the stabilizing effect of PEO (MW 100,000 and 300,000) increases with increasing concentration up to 2 % when complete stabilization of the HRP activity of the anti-rabbit IgG-HRP conjugate occurs. While PEO of MW 600,000 and 900,000 certainly offered a stabilizing effect, these PEO species were unable to completely stabilize the HRP activity of the anti-rabbit IgG-HRP conjugate. In the absence of PEO, the anti-rabbit IgG-HRP conjugate retained only 13.6 % of its initial HRP activity after 36 days of incubation. Figure 3.2 shows that the stabilizing effect of PVA varies dramatically with differing concentrations and molecular weights of PVA. All concentrations of PVA (MW 9,000-10,000) and all concentrations $\geq$ 1 % of PVA (MW 124,000-186,000) tested were able to completely stabilize the HRP activity of anti-rabbit IgG-HRP conjugate. While the other PVA species showed a stabilizing effect as compared to the PBSM control at certain concentrations, stabilization was not complete. Higher concentrations of PVA (MW 13,000-23,000 and 70,000-100,000) actually had a denaturing effect upon the HRP activity of the anti-rabbit IgG-HRP conjugate.

Table 3.1. Ability of different additives to stabilize the HRP activity of an anti-rabbit IgG-HRP conjugate in solution^a.

Stabilizing Additive	Percent of Initial Activity (%)
PBSM (control)	38.2 ± 2.2
50 % sorbitol	12.8 ± 0.9
saturated (NH ₄) ₂ SO ₄	33.5 ± 2.6
saturated NaCl	40.1 ± 2.7
saturated Mg(SO ₄) ₂	19.7 ± 1.4
saturated Na ₂ SO ₄	26.2 ± 2.0
50 % glycerol	31.6 ± 2.4
50 % ethylene glycol	27.4 ± 1.5
2 % PVP (MW 10,000)	15.3 ± 0.8
2 % PVP (MW 40,000)	17.5 ± 1.3
2 % PVP (MW 360,000)	19.5 ± 1.1
2 % PEG (MW 200)	22.2 ± 1.2
2 % PEG (MW 400)	26.0 ± 2.1
2 % PEG (MW 1000)	43.2 ± 2.8
2 % PEG (MW 1450)	24.8 ± 1.6
2 % PEG (MW 3350)	9.8 ± 0.6
2 % PEG (MW 8000)	40.4 ± 2.7
2 % PEG (MW 10,000)	23.5 ± 1.4
2 % PEO (MW 100,000)	99.2 ± 5.8
2 % PEO (MW 300,000)	102 ± 6.3
2 % PEO (MW 600,000)	94.4 ± 4.6
2 % PEO (MW 900,000)	90.5 ± 6.0
2 % PVA (MW 9,000-10,000)	98.6 ± 4.2
2 % PVA (MW 13,000-23,000)	56.3 ± 2.9
2 % PVA (MW 30,000-70,000)	77.1 ± 3.8
2 % PVA (MW 70,000-100,000)	37.2 ± 1.8
2 % PVA (MW 124,000-186,000)	99.3 ± 4.9

^aAnti-rabbit IgG-HRP conjugate was incubated at 32 °C in different PBSM solutions each containing a different stabilizing additive. After 15 days, anti-rabbit IgG-HRP conjugate samples were removed, diluted 100 times, and assayed for HRP activity as described in Methods. The resulting activities were expressed as a percent of the initial HRP activity of the anti-rabbit IgG-HRP conjugate at day 0 ± standard deviation (n = 6).

Figure 3.1. Effect of various concentrations of different PEO species on the HRP activity of the anti-rabbit IgG-HRP conjugate. The anti-rabbit IgG-HRP conjugate was stored in PBSM (control) or PEO in PBSM for 36 days at 32°C. The HRP activity of the anti-rabbit IgG-HRP conjugate was measured as described in Methods. Average absorbance was plotted as percent of initial activity at time 0 $\pm$ standard deviation (n = 6). The HRP activity of the anti-rabbit IgG-HRP conjugate without PEO was 13.6 $\pm$ 0.9 % of the initial activity. Symbols: $\circ$, PEO (MW 100,000); $\square$, PEO (MW 300,000); $\dots$, PEO (MW 600,000); and $\cdot$, PEO (MW 900,000).

Figure 3.2. Effect of various concentration of different PVA species on the HRP activity of the anti-rabbit IgG-HRP conjugate. The anti-rabbit IgG-HRP conjugate was stored in PBSM (control) or PVA in PBSM for 36 days at 32 °C. The HRP activity of the anti-rabbit IgG-HRP conjugate was measured as described in Methods. Average absorbance was plotted as percent of initial activity at time 0 $\pm$ standard deviation (n = 6). The HRP activity of the anti-rabbit IgG-HRP conjugate without PVA was 13.6 ± 0.9 % of the initial activity. Symbols: $\circ$, PVA (MW 9,000-10,000); $\square$, PVA (MW 13,000-23,000); $\dots$, PVA (MW 30,000-70,000); ∇ , PVA (MW 70,000-100,000); and $\triangle$, PVA (MW 124,000-186,000).

Effect of PVA and PEO on Immunoreaction

Since PEO (MW 100,000) and PVA (MW 9,000-10,000 and 124,000-186,000) showed complete stabilization of the anti-rabbit IgG-HRP conjugate, their effect on immunoreaction at various concentrations was studied using CEIA. Although PEO (MW 300,000) demonstrated similar stabilizing abilities as PEO (MW 100,000), it was not studied since solutions of PEO (MW 300,000) were much more viscous than PEO (MW 100,000), and hence less desirable for use in EIA. Anti-rabbit IgG-HRP conjugate was incubated in the additive solutions in PBS for 1 h and then reacted to rabbit IgG-cloth. Figure 3.3 shows that the presence of 0.5 to 10 % PVA (MW 9,000-10,000) or 0.5 to 5 % PVA (MW 124,000-186,000) in the anti-rabbit IgG-HRP conjugate suspension did not affect the specific adsorption of the anti-rabbit IgG-HRP conjugate. With PEO, however, the amount of specifically adsorbed anti-rabbit IgG-HRP conjugate decreased rapidly with increasing PEO concentration. For maximum stabilizing effect, the concentration of PEO (MW 100,000) must be $\geq 2\%$ (Figure 3.1). Figure 3.3 shows that the ratio of specific adsorption of the anti-rabbit IgG-HRP conjugate with PEO to specific adsorption of the anti-rabbit IgG-HRP conjugate without PEO is approximately 0.71 when the PEO concentration is 2 % and 0.24 when the PEO concentration is 10 %. Thus, the presence of stabilizing concentrations of PEO (MW 100,000) caused substantial inhibition of the immunoreaction between the antibody conjugate and the antigen immobilized on the cloth.

Figure 3.3 shows the effect of additives on the non-specific adsorption (background) of the anti-rabbit IgG-HRP conjugate. The presence of 2 % PEO (MW 100,000) in the anti-rabbit IgG-HRP conjugate produced 16 times greater background than that of the anti-rabbit

IgG-HRP conjugate without PEO. Higher concentrations of PEO caused even greater background. The presence of 0.5 to 10 % PVA (MW 9,000-10,000) or 0.5 to 1 % PVA (MW 124,000-186,000) resulted in no significant increase in background. However, with PVA (MW 124,000-186,000), the background increased when the PVA concentration in the anti-rabbit IgG-HRP conjugate suspension was greater than 1 %. If the anti-rabbit IgG-HRP conjugate stabilized by PVA is to be used for EIA without prior dilution, the lowest effective concentration of PVA should be utilized in order to provide the lowest viscosity. For EIA systems where the antigens and antibodies are not in close proximity (as in CEIA), increasing viscosity results in decreasing rates of immunoreaction. Thus, 0.5 % PVA (MW 9,000-10,000) or 1 % PVA (MW 124,000-186,000) should be used to obtain optimal stabilizing effect while minimizing viscosity and background.

Effect of PVA on Anti-Rabbit IgG-HRP Conjugate Activity

While PVA (MW 9,000-10,000 and 124,000-186,000) was found to stabilize the HRP activity of the anti-rabbit IgG-HRP conjugate, it is important to study the effect of PVA on the conjugate activity used in EIA. Anti-rabbit IgG-HRP conjugate was incubated in PBSM containing either 0.5 % PVA (MW 9,000-10,000) or 1 % PVA (MW 124,000-186,000) for 36 days at 32°C. At regular time intervals, samples of the anti-rabbit IgG-HRP conjugate suspensions were removed, reacted to rabbit IgG-cloths, and assayed for HRP activity. Figure 3.4 shows that the anti-rabbit IgG-HRP conjugate activity is stable throughout the 36 day test period when stored in 0.5 % PVA (MW 9,000-10,000) or 1 % PVA (MW 124,000-186,000). Anti-rabbit IgG-HRP conjugate stored in the absence of PVA

Figure 3.3. Effect of various concentrations of stabilizer additives on immunoreaction. Anti-rabbit IgG-HRP conjugate was reacted to polyester cloth or rabbit IgG-polyester cloth in the presence of stabilizing concentrations of PEO (MW 100,000) or PVA (MW 9,000-10,000 or 124,000-186,000). The HRP activity of the adsorbed anti-rabbit IgG-HRP conjugate was measured as described in Methods, and used to determine the effect of PEO or PVA on specific and non-specific anti-rabbit IgG-HRP conjugate adsorption. The results were plotted as the ratio of specific adsorption (SA) of the anti-rabbit IgG-HRP conjugate with additive to SA without additive, and non-specific adsorption (NSA) with additive to NSA without additive. The vertical bars represent standard deviations ($n = 6$). Symbols: ○, PEO (MW 100,000) (SA); □, PVA (MW 9,000-10,000) (SA); △, PVA (MW 124,000-186,000) (SA); ▽, PEO (MW 100,000) (NSA); ◁, PVA (MW 9,000-10,000) (NSA); and ⊙, PVA (MW 124,000-186,000).

demonstrated a continual decrease in anti-rabbit IgG-HRP conjugate activity over time with only 13.0 % of its initial activity remaining after 36 days of incubation. This final activity is very similar to that observed for the studies on the ability of PEO and PVA to stabilize the HRP activity of anti-rabbit IgG-HRP conjugate. Thus, the decrease in anti-rabbit IgG-HRP conjugate activity over time was due to loss of HRP activity rather than loss of antibody activity.

Proposed Mechanism of HRP Conjugate Stabilization by PVA and PEO

It has been previously proposed that the stabilization effect observed for certain enzymes in the presence of additives is a result of decreased water activity in the presence of the enzyme's solvent medium (Ye *et al.*, 1988). For both PVA and PEO stabilization, the concentration of the additive present in the HRP conjugate suspension is very low. For this reason, it is unlikely that the observed stabilization is a result of decreased water activity. I propose that PVA or PEO conjugate stabilization results from the direct interaction between the additive and conjugate. I believe that PVA interacts strongly with hydrophilic sites on the anti-rabbit IgG-HRP conjugate through H-bond formation between alcohol groups in the PVA and H-atoms in the carbohydrate moiety of HRP. In addition, hydrophobic interaction between the hydrocarbon backbone of PVA and hydrophobic patches on the surface of HRP may offer further stabilization. Like PVA, I believe that PEO stabilizes the anti-rabbit IgG-HRP conjugate through H-bond formation between the ether oxygen atom in PEO and H atoms in the carbohydrate moiety of HRP, and hydrophobic interactions between multiple ethylene residues of PEO and hydrophobic patches on HRP. These interactions stabilize the

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Figure 3.4. Effect of 0.5 % PVA (MW 9,000-10,000) and 1 % PVA (MW 124,000-186,000) on the conjugate activity of anti-rabbit IgG-HRP conjugate. Anti-rabbit IgG-HRP conjugate was incubated in PBSM (control) or PVA in PBSM for 36 days at 32°C. At regular time intervals, samples of the anti-rabbit IgG-HRP conjugate suspension were reacted to rabbit IgG-cloths and then assayed for HRP activity as described in Methods. Average absorbance was plotted as percent of initial activity at time 0 $\pm$ standard deviation (n = 6). Symbols: $\circ$, PBSM (control); $\square$, 0.5 % PVA (MW 9,000-10,000); and $\triangle$, 1 % PVA (MW 124,000-186,000).

anti-rabbit IgG-HRP conjugate by restricting its freedom to take inactive conformations. The resulting complex remains soluble because of the amphiphilic nature of the interacting polymer.

This chapter has demonstrated that the addition of certain molecular weights of PEO and PVA to an anti-rabbit IgG-HRP conjugate suspension markedly stabilizes the HRP activity of the conjugate. Unlike PEO, the immunoactivity of the anti-rabbit IgG-HRP conjugate is not inhibited by the presence of a stabilizing concentration of PVA. The use of PVA as a stabilizer, therefore, not only permits the use of anti-rabbit IgG-HRP conjugate without dilution prior to EIA, but also eliminates the need for refrigeration of the conjugate during transportation and field EIA.

The results presented in this chapter came in part from the following publications:

- Boyd, S. and Yamazaki, H. (1993a) Stabilization of peroxidase-antibody conjugate in solution by polyethylene oxide. *Biotechn. Techn.* 7: 671-676.
- Boyd, S. and Yamazaki, H. (1994a) Use of polyvinyl alcohol as a stabilizer of peroxidase-antibody conjugate for enzyme immunoassay. *Biotechn. Techn.* 8: 123-128.
- Yamazaki, H. and Boyd, S. (1996a) Stabilization of peroxidase conjugates in solution. US Patent No. 5,512,448.

CHAPTER 4

STABILIZATION EFFECT OF POLYVINYL ALCOHOL ON

HORSERADISH PEROXIDASE, GLUCOSE OXIDASE,

β-GALACTOSIDASE, AND ALKALINE PHOSPHATASE

ABSTRACT

The stabilization effect of different molecular weights of polyvinyl alcohol (PVA) upon solutions of commonly used analytical enzymes (horseradish peroxidase, glucose oxidase, β -galactosidase and alkaline phosphatase) was studied. It was found that various PVA conditions could stabilize the activity of horseradish peroxidase and glucose oxidase but not β -galactosidase or alkaline phosphatase. It is expected that PVA can stabilize conjugate forms of glucose oxidase as well as horseradish peroxidase (as previously shown in Chapter 3) but not conjugate forms of β -galactosidase or alkaline phosphatase.

INTRODUCTION

Enzyme immunoassays (EIA) are a major biotechnology product that depend upon the activity of enzymes conjugated to immunoreactants (antibodies or antigens) for the detection of antigens or antibodies in samples. Enzyme conjugates tend to be unstable, and thus, their transportation and storage generally requires the use of refrigeration which makes EIA expensive and field EIA impractical. In Chapter 3 it was shown that PVA of molecular weight 9,000-10,000 and 124,000-186,000 at concentration as low as 0.5 % (w/v) provided complete stabilization of horseradish peroxidase (HRP) conjugates stored at 32 °C. In addition to horseradish peroxidase (HRP) (refer to pp 15-17 of the General Introduction for information on HRP and its substrates), there are other enzymes such as glucose oxidase (GO), bacterial β -galactosidase (β -gal), and bovine alkaline phosphatase (AP) which are commonly used for EIA as conjugates in which the enzymes are conjugated to immunoreactants through a number of different conjugation methods.

GO is isolated from fungal sources with GO obtained from Aspergillus niger being used most often for EIA. GO is popular because it is one of the least expensive enzymes, has a high substrate turnover rate with a broad pH optimum (Bergmeyer *et al.*, 1983), and shows little change in activity upon conjugation to immunoreactants (Tijssen, 1985). It is a glycoprotein with a carbohydrate content of about 18 % (Tsuge *et al.*, 1975) and a molecular weight of 186,000 (Swoboda and Massey, 1965). GO catalyses the oxidation of β -D-glucose to D-glucono-1,4-lactone in the presence of water and dissolved oxygen as shown below (Keilin and Hartree, 1948):

Typically, GO activity is assayed by detecting the generated H_2O_2 by following the colour development in the presence of peroxidase and a suitable chromogenic substrate (e.g., O-dianisidine (Keesey, 1987) or 3,3',5,5'-tetramethylbenzidine (TMB) (Bos *et al.*, 1981)). The disadvantage of these procedures is that they require the use of a second enzyme. More recently, a new assay method was developed that uses the formation of a coloured complex between polyvinyl alcohol (PVA) and iodine produced through the oxidation of iodide by the H_2O_2 evolved from the glucose oxidase reaction (Blais and Yamazaki, 1992). It is this latter detection method that was used in this chapter.

β -Gal may be found in numerous microorganisms, animals, and plants, although the most extensively used one for EIA is isolated from Escherichia coli. β -Gal is a large

tetrameric protein with a molecular weight of 465,000 (Fowler and Zabin, 1977). Unlike HRP, GO, and bovine AP, β -gal from *E. coli* is not a glycoprotein. It catalyses the hydrolysis of lactose as follows:

While lactose is the natural substrate for β -gal, when stimulated with the presence of an acceptor alcohol (e.g., mercaptoethanol), it is also capable of catalysing the hydrolysis of O-nitrophenyl- β -D-galactopyranoside (ONPG) into O-nitrophenol (Bergmeyer et al., 1974). The activity of the β -gal may then be determined through the measurement of O-nitrophenol at 405 nm.

AP used for EIA may be isolated from bovine intestinal mucosa or from *E. coli*. Bovine AP is most often used for EIA due to its higher specific activity. Unlike HRP which is abundant in many plants, AP is not found in plants which makes it a popular enzyme for use in the EIA of molecules of plant origin. The prevalence of AP in animal and human tissues, especially those used for nutrient transport (e.g., the intestine), often makes its use for the EIA of such tissues difficult due to the presence of exogenous AP which can result in high background. Bovine AP is a dimeric glycoprotein with a mass of 84,550 daltons (Neumann and Lustig, 1980) and a carbohydrate content of about 12 % (Besman and Coleman, 1985). AP catalyses the hydrolysis of phosphate esters (R₁OP) according to the following general equation (Fernley, 1971):

The most popular substrate for AP, and the one used in this chapter, is p-nitrophenyl phosphate (p-npp) primarily because the hydrolysis product p-nitrophenol absorbs strongly at 405 nm, and thus, may be detected visually or spectrophotometrically. It should be noted that the PBS buffer solution used in this experiment was diluted 100-fold in a diethanolamine buffer prior to the assay of AP activity. This is important because PBS contains a relatively high amount (15 mM) of inorganic phosphate which acts as a competitive (but reversible) inhibitor for AP (Caswell and Caplow, 1980).

There are countless different enzyme-immunoreactant conjugates commercially available that make use of these four enzymes. I believe that if a given condition can stabilize free enzymes, it will also stabilize enzyme-immunoreactant conjugates. Thus, the ability to stabilize a free enzyme will permit the prediction of how to stabilize a conjugate of that enzyme. This will greatly simplify the trial and error approach of current stabilization procedures. Therefore, in the present chapter the stabilization effect of different molecular weights of PVA upon HRP, GO, β -gal, and AP was studied.

METHODS

Effect of PVA on the Activity of HRP, GO, AP, and β -Gal

HRP (1 μ g), GO (10 μ g), β -gal (200 μ g), and AP (1 μ g) were suspended individually in 1 ml of PBS (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % NaCl) with 0.1% (v/v)

Micro-O-Protect (PBSM), or 1 ml of various PBSM solutions each containing a different molecular weight of 5 % PVA (9,000-10,000; 13,000-23,000; 30,000-70,000; 70,000-100,000; and 124,000-186,000). The HRP, GO, and β -gal solutions were incubated at 30 °C, with the AP solutions being incubated at 40 °C. At regular time intervals, the different solutions were assayed for enzyme activity.

Assay of Enzyme Activity

(i) HRP:

After 0 to 10 days of incubation, HRP samples were removed from each condition and diluted 100 times in PBS. Fifty μ l of the diluted HRP suspension was mixed with 1 ml of the soluble HRP peroxidase substrate system in a 16 mm x 100 mm test tube and shaken at 180 RPM (on a New Brunswick Gyrotary Shaker) for 10 min at 30 °C. The HRP reaction was stopped by adding 250 μ l of 2 N H_2SO_4 and then the resultant colour was measured at 450 nm. The soluble TMB peroxidase substrate system was prepared by mixing 2.5 ml of TMB (2 mg/ml of ethanol) with a solution consisting of 0.21 g citric acid H_2O , 0.42 g EDTA 4 Na, 0.03 g $NaBO_3 \cdot 4 H_2O$, and 100 ml distilled water (dH_2O).

(ii) GO:

After 0 to 4 days of incubation, GO samples were removed from each condition and diluted 40 times with PBS. One-hundred μ l of the diluted GO suspension was mixed with 1 ml of the glucose oxidase assay solution in a 16 mm x 100 mm test tube and shaken at 180 RPM for 30 min at room temperature. The resultant colour was immediately measured at

510 nm. The glucose oxidase assay solution was prepared according to Blais and Yamazaki (1992) as follows: 0.6 g of dibasic and 0.1 g of monobasic anhydrous potassium phosphate, 10 g of D-glucose, 0.5 g of potassium iodide, 2.5 g of PVA (MW 30,000-70,000), and 60 mg of sodium molybdate dihydrate were dissolved in 100 ml dH₂O immediately prior to use.

(iii) β -Gal:

After 0 to 6 hours of incubation, β -gal samples were removed from each condition and diluted 50 times with β -gal enzyme diluent (0.01 M Tris-HCl (pH 7.5) containing 0.01 M MgCl₂, 0.01 M mercaptoethanol, and 0.01 M NaCl). One-hundred μ l of the diluted β -gal suspension was mixed with 0.3 ml of 1.0 M mercaptoethanol, 1.1 ml of dH₂O, 1.0 ml of 3.0 M sodium phosphate buffer (pH 7.5) with 3 mM MgCl₂, and 0.5 ml of freshly prepared ONPG in 0.01 M Tris-acetate (pH 7.5) containing 0.01 M MgCl₂ in a 16 mm x 100 mm test tube and shaken at 180 RPM for 10 min at 30 °C. The β -gal reaction was slowed down by adding 0.3 ml of saturated sodium carbonate and then the resultant colour was immediately measured at 405 nm.

(iv) AP:

After 0 to 18 days of incubation, AP samples were removed from each condition and diluted 100 times with 1.0 M diethanolamine buffer (pH 9.8) containing 0.5 mM MgCl₂. Fifty μ l of the diluted AP suspension was mixed into a pre-warmed (37 °C) 16 mm x 100 mm test tube with 0.5 ml of 15 mM p-npp in diethanolamine buffer and shaken at 180 RPM for 10 min at 37 °C. The AP reaction was stopped by adding 0.5 ml of 0.1 M EDTA 2 Na 2 H₂O

with the resulting colour being measured at 405 nm.

RESULTS AND DISCUSSION

To test the PVA stabilization effect, each enzyme was suspended in phosphate buffered saline containing 0.1 % (v/v) Micro-O-Protect (PBSM) with or without PVA of different molecular weights. A 5 % concentration of PVA was arbitrarily chosen for all stabilization studies. Use of this relatively high PVA concentration ensured that any effect that PVA had upon the stabilization of the various enzymes being studied would be maximized. Micro-O-Protect, which had no effect upon the activity of any of the enzymes, was used as a preservative to prevent microbial degradation of the enzymes. The enzymes were suspended at an appropriate concentration so that, following a final dilution prior to assaying, any loss of activity during incubation at 30 °C for HRP, GO, and β -gal, or 40 °C for AP was measurable. Since AP is stable at 30 °C, it was necessary to increase the incubation temperature to 40 °C in order to see a decline in AP activity within a reasonable period of time. Although phosphate ions from the PBSM are known to act as a reversible inhibitor of AP activity, the activity of the AP was measured following a 100 times dilution in diethanolamine buffer so that there was a non-inhibiting concentration of phosphate.

Figure 4.1 shows that HRP was stabilized in the presence of all PVA conditions examined. After 15 days of incubation at 30 °C, HRP stored in the various PVA conditions retained 75 to 85% of its initial activity as compared to 42 % for HRP stored in PBSM. Since PVA stabilized free HRP as well as antibody-HRP conjugates (Chapter 3), PVA is expected to stabilize conjugates of other enzymes if it is shown to stabilize the corresponding

free enzyme.

Figure 4.2 shows that PVA also stabilized GO. GO retained 94 to 98 % of its initial activity after 7 days of incubation at 30°C in the presence of the various PVA solutions with the exception of PVA (MW 70,000-100,000) in which 84.2 % of its initial activity was retained. By comparison, after 7 days of incubation in PBSM, GO only retained 36.5 % of its initial activity. These results suggest that PVA should be able to stabilize GO conjugates. The stability of free GO in PVA can extend the usefulness of this analytical enzyme for glucose assay since a GO solution can be stored at ambient temperature for at least one week.

Figure 4.3 demonstrates that the various PVA conditions did not have a significant stabilization effect upon β -gal. In fact, some PVA conditions appeared to have a destabilizing effect with the resultant β -gal activity having 20.0 % and 24.8 % of its initial activity when stored in PVA of MW 9,000-10,000 and PVA of MW 30,000-70,000 respectively. The extreme sensitivity of β -gal to thermal denaturation was evident with the β -gal stored in PBSM retaining only 56.2 % of its initial activity after 6 h of storage at 30°C. Storage of β -gal in the other molecular weights of PVA resulted in a β -gal activity of approximately 52-60 % of its initial activity. Therefore, PVA is not expected to offer any significant stabilization of β -gal conjugates.

Figure 4.4 shows that none of the PVA conditions examined yielded a stabilization effect upon AP, suggesting that PVA may not be able to stabilize AP conjugates. The great thermal stability of AP was very evident with the AP retaining about 50 % of its initial activity when stored in PBSM for 17 days at 40°C. Because the conjugation of enzymes to

Figure 4.1. Effect of different molecular weights of PVA upon the activity of HRP at 30 °C. HRP was incubated in PBSM (control) or 5 % PVA in PBSM and then at regular time intervals samples of the different HRP solutions were assayed for HRP activity as described in Methods. The average absorbance at each time interval is plotted as a percent of initial activity at time 0 $\pm$ standard deviation ($n = 6$). Symbols: $\circ$, PBSM; $\square$, PVA (MW 9,000-10,000); Δ , PVA (MW 13,000-23,000); ∇ , PVA (MW 30,000-70,000); $\times$, PVA (MW 70,000-100,000); and $\bigcirc$, PVA (MW 124,000-186,000).

Figure 4.2. Effect of different molecular weights of PVA upon the activity of GO at 30 °C. GO was incubated in PBSM (control) or 5 % PVA in PBSM and then at regular time intervals samples of the different GO solutions were assayed for GO activity as described in Methods. The average absorbance at each time interval is plotted as a percent of initial activity at time 0 $\pm$ standard deviation (n = 6). Symbols: $\circ$, PBSM; $\square$, PVA (MW 9,000-10,000); $\triangle$, PVA (MW 13,000-23,000); ∇ , PVA (MW 30,000-70,000); $\diamond$, PVA (MW 70,000-100,000); and $\bigcirc$, PVA (MW 124,000-186,000).

Figure 4.3. Effect of different molecular weights of PVA upon the activity of β -gal at 30°C. β -Gal was incubated in PBSM (control) or 5 % PVA in PBSM and then at regular time intervals samples of the different β -gal solutions were assayed for β -gal activity as described in Methods. The average absorbance at each time interval is plotted as a percent of initial activity at time 0 $\pm$ standard deviation (n = 6). Symbols: $\circ$, PBSM; $\square$, PVA (MW 9,000-10,000); Δ , PVA (MW 13,000-23,000); ∇ , PVA (MW 30,000-70,000); $\diamond$, PVA (MW 70,000-100,000); and $\bigcirc$, PVA (MW 124,000-186,000).

Figure 4.4. Effect of different molecular weights of PVA upon the activity of AP at 40°C. AP was incubated in PBSM (control) or 5 % PVA in PBSM and then at regular time intervals samples of the different AP solutions were assayed for AP activity as described in Methods. The average absorbance at each time interval is plotted as a percent of initial activity at time 0 $\pm$ standard deviation (n = 6). Symbols: $\circ$, PBSM; $\square$, PVA (MW 9,000-10,000); $\triangle$, PVA (MW 13,000-23,000); ∇ , PVA (MW 30,000-70,000); $\diamond$, PVA (MW 70,000-100,000); and $\bigcirc$, PVA (MW 124,000-186,000).

immunoreactants is typically accompanied by increased stability of the enzyme, it is quite possible that AP conjugates can be transported and used for field EIA in the absence of refrigeration without the need for further stabilization efforts.

This chapter has demonstrated that PVA can be used to stabilize solutions of free HRP and GO, and thus, is expected to offer a stabilization effect upon the countless different HRP- and GO-immunoreactants conjugates. HRP, GO and AP are glycoproteins while β -gal is not. The carbohydrate content of HRP (Shannon *et al.*, 1966) and GO (Tsuge *et al.*, 1975) is approximately 18 % while that of adult bovine AP is approximately 12 % (Besman and Coleman, 1985). Extensive hydrogen bonding between H-atoms of alcohol groups in PVA and O-atoms of carbohydrate groups in HRP and GO may be responsible for the observed HRP and GO stabilization by inhibiting the formation of non-functional conformations.

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Boyd, S., Letcher, K., and Yamazaki, H. (1996b: accepted for publication, June 1996)

Stabilization effect of polyvinyl alcohol on horseradish peroxidase, glucose oxidase, β -galactosidase, and alkaline phosphatase. *Biotechn. Techn.*

CHAPTER 5

DETECTION OF DILUTE ANTIGEN IN LARGE SAMPLE VOLUMES USING ANTIBODY-COATED POLYESTER CLOTH

ABSTRACT

A model antibody, goat anti-rabbit IgG antibody, was adsorbed onto a disk of polyester cloth and then fixed into a column apparatus. The macroporosity of the cloth allowed rapid immunoconcentration of a model antigen, rabbit IgG, by passing a large volume of the dilute antigen through the antibody-coated cloth. Such immunoconcentration permitted detection of the dilute antigen which otherwise would have gone undetected.

INTRODUCTION

Enzyme immunoassay (EIA) is an assay technique that permits the detection of specific antibody or antigen such as pathogens, pollutants, or toxins in samples with high sensitivity. In many instances, a valid sample analysis requires the detection of dilute antigens in a large volume of sample. For example, the presence or absence of viruses in a whole potato plant requires the analysis of a few hundred ml of juice from the plant. The assurance of water quality could require the analysis of a few liters of water sample. Such large volumes are far beyond the capacity of microtiter plates or tubes that are used in traditional immunoassays. Other EIA solid supports such as antibody-coated microporous membranes are slow in passing solutions and tend to clog up. Microbeads may be packed into a column for use in concentrating dilute antigens, but slow flow rates may result. In addition, it is difficult to quantitatively transfer the microbeads into and out of a column for final analysis. A disk of macroporous cloth such as polyester cloth, however, is easy to pack into a column and its macroporosity permits high flow rates with little chance of clogging. The macroporosity of the cloth gives it a very large surface area/volume ratio that yields very

fast immunoreaction rates which is necessary to ensure maximum antigen capture if high flow rates are to be used. Previously, the chromatographic immunoconcentration of Salmonella antigen onto anti-Salmonella antibody-coated polyester cloth using uncontrolled flow rates was demonstrated (Blais and Yamazaki, 1989d). The present chapter demonstrates the usability of high flow rates in the antibody-cloth based immunoconcentration of dilute antigen for detection by EIA.

METHODS

Preparation of Antibody-Coated Polyester Cloth

Polyester cloth disks (18 mm in diameter) were soaked in ethanol for a few min, washed 5 times with PBS (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % NaCl), and blotted. Each cloth disk was treated with either 0.2 ml of goat anti-rabbit IgG antibody (anti-rabbit IgG) (100 μ g in PBS/cloth disk) or 0.2 ml of PBS (controls for the non-specific adsorption of rabbit IgG and goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (anti-rabbit IgG-HRP conjugate)) and then incubated overnight at 30°C in closed petri plates. The cloths were washed 5 times with PBST (PBS containing 0.05 % (v/v) Tween 20) followed by 3 times with PBS, blotted, and then incubated in 0.5 % (w/v) Protein Blocker in PBS for 1 h at room temperature (RT). After washing 3 times in PBS, the cloths were stored at 4°C in PBSM (PBS containing 0.1 % (v/v) Micro-O-Protect as a preservative) until use.

Cloth-Based Enzyme Immunoassay of a Dilute Antigen

A 0.2 ml sample of dilute rabbit IgG (0.6 ng/ml) in PBS was pipetted onto an antibody-coated cloth disk (18 mm in diameter) as well as onto a cloth disk without anti-rabbit IgG (control for non-specific adsorption). Following a 20 min incubation at RT in closed petri plates, the cloth disks were removed from the petri plates and assayed for captured antigen. The value obtained from the non-specific adsorption control was subtracted from that for the antibody-coated cloth disk.

Immunoconcentration of Dilute Antigen Through an Antibody-Coated Cloth Disk

To demonstrate the suitability of polyester cloth as an immunoassay support for the analysis of large sample volumes, an anti-rabbit IgG-polyester cloth disk was fitted into the screw base of a chromatography column with an inside diameter of 14 mm. The chromatography top was then screwed into the base such that the antibody-coated cloth disk was tightly secured inside the column apparatus in a manner which ensured that all of the antigen sample was forced through the cloth. The column was then attached to a Cole-Parmer Masterflex Pump (Figure 5.1). Fifty ml of dilute rabbit IgG (0.6 ng/ml) in PBS was passed through the column at various flow rates of 0.32 ml/min to 47.6 ml/min (a different antibody-coated cloth disk was used for each flow rate). The cloth disk was then removed from the column apparatus and assayed for captured antigen. To assess any non-specific adsorption of rabbit IgG as well as anti-rabbit IgG-HRP conjugate to the polyester cloth each flow rate was repeated using a control cloth disk without anti-rabbit IgG. The value for the control cloth disk was subtracted from that obtained for the antibody-coated cloth disk.

Figure 5.1. Apparatus used for the anti-rabbit IgG column. Anti-rabbit IgG-cloth disks (18 mm in diameter) were fitted into the screw base of the column apparatus (14 mm inside diameter) which was firmly secured with a retort stand. The screw top was fitted snugly into the base and then attached to a Cole-Parmer Masterflex Pump.

Enzyme Immunoassay of Captured Antigen

The cloth disks were washed 5 times with PBST followed by 3 times with PBS, blotted, and then incubated in 0.5 % Protein Blocker in PBS for 1 h at RT. After washing 3 times with PBS and blotting, each cloth disk was incubated with 0.2 ml of anti-rabbit IgG-HRP conjugate (stock solution was diluted 500 times in PBS) for 20 min at RT in closed petri plates. The cloths were washed 5 times with PBST followed by 3 times with PBS, blotted, and then the HRP activity of the anti-rabbit IgG-HRP conjugate was tested as follows. Individual cloth disks were placed into separate culture tubes (25 mm x 150 mm) containing 3 ml of the soluble TMB peroxidase substrate system. After shaking the culture tubes for 10 min at 180 RPM (on a New Brunswick Gyrotary Shaker) at 30°C, the reaction was stopped by adding 750 μ l of 2 N H_2SO_4 to each tube. The intensity of the resulting yellow colour reaction was measured at 450 nm. The soluble TMB peroxidase substrate system was prepared by mixing 2.5 ml of TMB (2 mg/ml ethanol) with a solution consisting of 0.21 g citric acid $\cdot\text{H}_2\text{O}$, 0.42 g EDTA $\cdot 4\text{ Na}$, and 0.03 g $\text{NaBO}_3\cdot\text{H}_2\text{O}$ made to a volume of 100 ml in dH_2O .

RESULTS AND DISCUSSION

For the present investigations, rabbit IgG was used as a model antigen and goat anti-rabbit IgG antibody as a model antibody. The antigen was diluted to such an extent that it gave a negligible signal when assayed by cloth-based enzyme immunoassay (CEIA) according to Blais and Yamazaki (1989a). For the CEIA, a disk (18 mm in diameter) of macroporous polyester cloth was coated with the antibody and then the resulting cloth was

fully saturated with the dilute antigen solution (0.2 ml). After a 20 min immunoreaction, the captured antigen was assayed with an anti-rabbit IgG-HRP conjugate. Table 4.1 (first row) shows that the HRP activity (a measure of the captured antigen) was nearly zero.

To examine the performance of immunoconcentration of dilute rabbit IgG by anti-rabbit IgG-cloth, an anti-rabbit IgG-cloth disk (18 mm in diameter) was tightly fitted into the bottom of a column and then 50 ml of dilute rabbit IgG was passed through the column at various flow rates from 0.32 ml/min to 47.6 ml/min. The amount of rabbit IgG captured onto the disks at each flow rate was compared by measuring the HRP activity of the anti-rabbit IgG-HRP conjugate. Table 5.1 shows that the immunoconcentration performed at the fastest flow rate (47.6 ml/min) yielded a significant HRP activity that was clearly indicative of the presence of rabbit IgG in 50 ml of the sample. That is, it is possible to confirm the presence of a dilute sample antigen following a 1 min passage of a 50 ml sample. Table 5.1 also shows that higher HRP activities could be easily obtained by lowering the flow rates in order to increase the efficiency of antigen capture. As compared to the fastest flow rate studied, approximately nine times more rabbit IgG was captured at the slowest flow rate (0.32 ml/min).

This chapter demonstrates that macroporous hydrophobic cloth (e.g., polyester) can be used in a column apparatus for the rapid enzyme immunoassay of a large volume of a dilute antigen sample. The ability to accommodate essentially unlimited sample volumes yields a CELA that has a vastly superior detection limit than traditionally used EIA which can only accommodate limited sample volumes. The use of PVA-blocked antibody-cloth and

Table 5.1. Effect of flow rate on the detectability of dilute rabbit IgG captured by anti-rabbit IgG-coated polyester cloth disks^a.

Volume of Antigen Sample Added to Cloth Disk (ml)	Flow Rate (ml/min)	HRP Activity (A ₄₅₀)
0.2	----	0.006 ± 0.004
50.0	0.32	0.957 ± 0.102
50.0	0.86	0.560 ± 0.044
50.0	1.53	0.422 ± 0.046
50.0	2.78	0.353 ± 0.030
50.0	5.13	0.204 ± 0.027
50.0	9.93	0.186 ± 0.016
50.0	20.6	0.170 ± 0.015
50.0	34.9	0.132 ± 0.009
50.0	47.6	0.108 ± 0.010

^aFifty ml (except for the first row when a 0.2 ml antigen sample and a 20 min immunoreaction time was used to demonstrate the detectability of the antigen sample using conventional CEIA) of a dilute rabbit IgG sample was passed through a column containing an anti-rabbit IgG-cloth disk. The disks were assayed for captured rabbit IgG by reacting each disk to excess anti-rabbit IgG-HRP conjugate and measuring the resulting HRP activity as described in Methods. The HRP activity was expressed as average absorbance (following the subtraction of the non-specific adsorption control) at 450 nm ± standard deviation (n = 3). The absorbance values for all of the non-specific adsorption controls were less than 0.01.

PVA stabilized HRP-conjugates will permit use of this new CEIA technique under field testing conditions where it will be of great benefit in the analysis of large-volume samples.

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Boyd, S. and Yamazaki, H. (1995b) Detection of dilute antigen in large sample volumes using antibody-coated polyester cloth. *Biotechn. Techn.* 9: 849-852.

CHAPTER 6

INSTANTANEOUS CLOTH-BASED ENZYME IMMUNOASSAY FOR THE SEMI-QUANTITATIVE VISUAL DETERMINATION OF ANTIBODIES

ABSTRACT

An antigen-coated cloth segment was placed onto a blotting pad. The cloth was blotted with 1 drop of an antibody sample, immediately washed with 5 drops of a washing solution, exposed to 1 drop of an anti-antibody-peroxidase conjugate, washed, and finally exposed to a peroxidase substrate. The coloured peroxidase product was determined either visually or colorimetrically with the colour intensity being correlated to the antibody concentration. This rapid assay would facilitate field serodiagnosis of human and animal diseases.

INTRODUCTION

Serodiagnosis of disease involves the detection of antibodies produced through an immune response to pathogens. Enzyme immunoassay (EIA) is the usually applied method for serodiagnosis, which is most commonly performed using an antigen-coated microwell in one of two ways. In the titration method, the antiserum sample is serially diluted to determine the limit (end-point) at which enzyme activity can no longer be detected. This procedure only yields semi-quantitative results at best because it is subjective in determining the end-point. The second method compares EIA signals of serum samples after a given dilution which can be correlated to titers by referring to a standard curve of EIA signals versus titers (dose-response curve). The dose-response curves are sigmoidal, with the steepest part of the curve offering the greatest sensitivity. This method can be more accurate than the titration method offering quantitative results if a spectrophotometer is used to measure the EIA signals. With both methods, long dilution series may be required in order

to obtain EIA signals that fall within the titer range that may be detected with the greatest sensitivity. If a microwell plate or other solid support is used, these methods can be very time consuming since they rely on extensive immunoreactions that require at least 1 hour for each immunoreaction (Tijssen, 1985). For the field serodiagnosis of diseases, more rapid and simpler EIA is desired.

The use of polyester cloth as an EIA solid support permits much faster immunoreactions due to its large surface area/volume ratio, which greatly decreases the time necessary for the sample immunoreactant to diffuse to the immobilized immunoreactant. While antigen-coated polyester cloth can be used for the serodiagnosis of antibodies to any pathogenic antigen that may adsorbed onto the cloth, polymyxin-cloth is preferable for the serodiagnosis of disease caused by gram-negative bacteria. Polymyxin-cloth-based enzyme immunoassay (P-CEIA) has been developed to assay lipopolysaccharide (LPS) antigens of gram-negative bacteria such as Salmonella (Blais and Yamazaki, 1990c; Chen et al., 1995), Campylobacter (Gomi et al., 1996), and Escherichia coli (Booth et al., 1996). Polymyxin B is a cyclic peptide antibiotic produced by Bacillus polymyxa and has the following structure where R = 6-methyloctanoyl or 6-methylheptanoyl and DAB = diaminobutyric acid (Haiyan, 1995):

Polymyxin B binds hydrophobically to polyester cloth through its fatty acid tail with the remaining portion capable of forming stable complexes with the lipid A region of LPS (Figure 6.1). As an LPS capturing agent, polymyxin is more homogeneous, more stable, and less expensive than anti-LPS antibodies. Pathogen-specific antibody to the O-specific chain or core regions of the LPS are then used to detect the captured LPS. While polymyxin may be adsorbed onto traditional EIA solid supports, it was found that the detectability of Salmonella using polymyxin-cloth was significantly better than when polymyxin was adsorbed onto a microwell surface (Blais and Yamazaki, 1990d) and 100-fold greater than on polymyxin-nitrocellulose membranes (Blais and Yamazaki, 1991).

Previously, it was demonstrated that antibody can be assayed by determining the 'instantaneous' immunoreaction with an immobilized antigen (Blais and Yamazaki, 1989c). This method was made possible by the use of cloth as the EIA solid support. In this 'instantaneous' immunoreaction method, a fixed volume of antibody sample is 'instantaneously' drawn through an antigen-coated cloth segment which is placed on top of a blotting pad. After washing, the cloth is removed for assay of captured antibody by its reaction with a secondary antibody-peroxidase conjugate in a second vessel for 2 to 10 min, washing on a filter, and finally assessing the resulting peroxidase activity for 30 min in a third vessel. Because of the cloth's large surface area for immunoreaction, significant amounts of antibody can be captured on the antigen-cloth that are proportional to the antibody concentration in samples. The present chapter demonstrates that for semi-quantitative visual antibody assay, all of the steps in the above 'instantaneous' immunoreaction method can be performed in an 'instantaneous' manner without removing

Figure 6.1. Molecular structure of Salmonella LPS (Fomsgaard, 1990). The basic structure consists of the O-specific chain, the core, and the lipid regions. The O-specific chain consists of oligosaccharide repeating units responsible for determining the serological specificity of the molecule. The core region is linked to the lipid A region via the keto sugars (KDO) and consists of the saccharides heptose (Hep), glucose (Glu), galactose (Gal), and N-acetyl-D-glucosamine (GlcNAc). The lipid A region, which is hydrophobic and serves to anchor the molecule in the outer membrane of the bacterium, is composed of β 1-6 linked glucosamine (GlcN) attached to C10-16 chain saturated fatty acids. The composition and structure of the core region is conserved among all members of the genus Salmonella, while that of the O-specific chain varies among different Salmonella strains.

the antigen-cloth from a blotting pad, thus providing rapidity and simplicity that are desired for field EIA. As model systems, Salmonella LPS adsorbed onto polymyxin-cloth and rabbit IgG adsorbed onto polyester cloth were used to assay anti-Salmonella LPS antibody and anti-rabbit IgG antibody, respectively.

METHODS

Preparation of Salmonella LPS-Polymyxin-Cloth

Air-dried polymyxin-cloth disks (6 mm in diameter) were soaked in distilled water (dH₂O) for 5 min and then blotted immediately prior to use. Salmonella typhimurium (ATCC 14028) was cultured in brain heart infusion medium for 18 h at 37°C resulting in approximately 10¹⁰ cfu/ml. To extract lipopolysaccharide (LPS) from the Salmonella, 10 ml of the culture was mixed with 200 µl of 25 % (w/v) sodium cholate and autoclaved for 5 min at 121°C. After cooling, 50 µl of the LPS extract was added to individual polymyxin-cloth disks and incubated overnight at room temperature (RT) in closed petri plates. The resulting LPS-polymyxin-cloths were washed 5 times with 0.01 M sodium phosphate pH 7.3 buffer in 0.85 % NaCl (PBS) containing 0.05 % (v/v) Tween 20 (PBST) followed by 3 times with PBS, blotted, air-dried, and then stored at RT over CaSO₄ dessicant until use.

Preparation of Rabbit IgG-Polyester Cloth

Disks (6 mm in diameter) of polyester cloth were washed with ethanol for a few minutes, followed by 5 times with PBS and blotted. Each cloth was incubated overnight with rabbit IgG (10 µg/cloth segment) at 30°C in closed petri plates. The cloths were

washed 5 times with PBST followed by 3 times with PBS, blotted, and then incubated in 2 % (w/v) PVA (MW 30,000-70,000) in PBS for 24 h at RT for blocking (see Chapter 2). After washing 3 times in PBS, the cloths were blotted, air-dried, and then stored at RT over CaSO_4 dessicant until use.

Instantaneous Assay for Anti-Salmonella LPS

A Salmonella LPS-polymyxin-cloth disk was centered on top of a blotting pad disk (24 mm in diameter, 2.45 mm thick). The following was added 1 drop at a time to the cloth disk in rapid succession allowing only enough time between drops to permit the drop to pass through the cloth disk and into the blotting disk: 5 drops of PBST; 1 drop of antibody sample (0, 0.3, 0.5, 1, 2, 3, 5, 10, 20, 30, 50, or 100 μg rabbit anti-Salmonella LPS antibody (anti-LPS)/ml in PBS); 5 drops of PBST; 1 drop of goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (anti-rabbit IgG-HRP conjugate) (diluted 50 times with PBS from the stock); and 5 drops of PBST. For semi-quantitative visual assay, the cloth disk, still on the blotting disk was assayed for HRP using the insoluble TMB peroxidase substrate system. For colourimetric assay, the cloth disk was removed and assayed for captured rabbit anti-LPS using the soluble TMB peroxidase substrate system.

Instantaneous Assay for Anti-Rabbit IgG

A rabbit IgG-polyester cloth disk was centered on top of a blotting pad disk (24 mm in diameter, 2.45 mm thick). The following was added to the cloth disk 1 drop at a time as described above: 5 drops of PBST; 1 drop of antibody sample (0, 0.2, 0.4, 1, 2, 4, 10, 20, or

40 μ g goat anti-rabbit IgG antibody (anti-rabbit IgG)/ml in PBS); 5 drops of PBST; 1 drop of rabbit anti-goat IgG antibody-horseradish peroxidase conjugate (anti-goat IgG-HRP conjugate) (diluted 25 times with PBS from the stock); and 5 drops of PBST. The amount of anti-rabbit IgG captured onto the cloth disk was visualized using the insoluble TMB peroxidase substrate system, or colourimetrically assayed using the soluble TMB peroxidase substrate system, as described above.

Assay of HRP Activity

(i) Insoluble TMB peroxidase substrate system:

Ten drops of the insoluble TMB peroxidase substrate system were added in rapid succession to a cloth disk that was still on a blotting disk. After 5 min. the cloth disk was washed with 5 drops of water and then the resulting blue colour was observed or permanently recorded through photocopying. The insoluble TMB peroxidase substrate system was prepared by dissolving 33 mg of the insoluble TMB peroxidase substrate powder per ml of dH₂O.

(ii) Soluble TMB peroxidase substrate system:

The cloth disk removed from the blotting pad was placed in a 16 mm x 100 mm test tube containing 1 ml of the soluble TMB peroxidase substrate system and shaken at 180 RPM (on a New Brunswick Gyrotary Shaker) for 10 min at 30°C. The HRP reaction was stopped by adding 250 μ l of 2 N H₂SO₄ and then the resulting colour was measured at 450 nm. The absorbance was corrected for non-specific adsorption of the conjugate to the

polymyxin-cloth (without anti-LPS) or the polyester cloth (without anti-rabbit IgG). The soluble TMB peroxidase substrate system was prepared by mixing 2.5 ml of TMB (2 mg/ml ethanol) with a solution consisting of 0.21 g citric acid H_2O , 0.42 g EDTA-4 Na, 0.03 g $NaBO_3 \cdot H_2O$, and 100 ml dH_2O .

RESULTS AND DISCUSSION

Instantaneous Assay for Anti-Salmonella LPS

Salmonella LPS bound to a polymyxin cloth disk was used to assay anti-Salmonella LPS antibodies in a rabbit antiserum. The antiserum was diluted to various concentrations and then 1 drop ($28 \pm 2 \mu l$) of each dilution was instantaneously drawn through a LPS-polymyxin-cloth disk placed on a blotting pad via the absorbing action of the blotting pad. The captured antibody was similarly reacted with 1 drop of anti-rabbit IgG-HRP conjugate. An HRP substrate which produces an insoluble blue coloured product was used to visualize the captured HRP activity. The whole process took about 60 sec. Figure 6.2 shows that the zero control (no antiserum) gave no visible colour and that the colour intensity increased with increasing rabbit anti-LPS antiserum concentration.

To study the reproducibility of this assay, the method was repeated 6 times and then the captured HRP activity on the cloths was colourimetrically measured using a HRP substrate which produces a soluble coloured product. Figure 6.3 shows that the absorbance of the colour (due to HRP) increased sharply up to an antiserum concentration of $10 \mu g/ml$, followed by a more gradual increase. The standard deviations were relatively small, suggesting high reproducibility of this assay. Although the exact concentration of anti-

Figure 6.2. Semi-quantitative visual assay of anti-LPS antibody in rabbit antiserum samples. Rabbit anti-LPS antibody samples of different concentrations were instantaneously reacted to Salmonella LPS-polymyxin-cloth disks. Captured anti-LPS was detected by a second instantaneous reaction with excess goat anti-rabbit IgG-HRP conjugate followed by use of the insoluble TMB peroxidase substrate system.

Concentration of Rabbit Anti-LPS Antibody ($\mu\text{g/ml}$)

Figure 6.3. Colourimetric assay of anti-LPS antibody in rabbit antiserum samples. Rabbit anti-LPS antibody samples of different concentrations were instantaneously reacted to Salmonella LPS-polymyxin-cloth disks. Captured anti-LPS was assayed by a second instantaneous reaction with excess goat anti-rabbit IgG-HRP conjugate followed by a 10 min HRP assay using the soluble TMB peroxidase substrate system as described in Methods. The HRP activity was expressed as average absorbance at 450 nm (A_{450}) $\pm$ standard deviation ($n = 6$).

Salmonella typhimurium LPS antibodies in the antiserum used is not known, the results (Figure 6.3) indicate that the present method can provide quantitative determination for anti-Salmonella LPS antibodies if the standard curve is constructed with purified anti-Salmonella LPS antibodies. The quantitative assay, however, requires a longer time for development (e.g., 10 min) as well as a colourimeter.

Instantaneous Assay for Anti-Rabbit IgG

Polyester cloth was coated with a model antigen, rabbit IgG, and blocked with PVA. The resulting rabbit IgG-cloth was used to assay anti-rabbit IgG antibodies in goat anti-rabbit IgG antiserum which was diluted to various concentrations. One drop of sample was instantaneously drawn through the rabbit IgG-cloth into a blotting pad. One drop of rabbit anti-goat IgG-HRP conjugate was then drawn through the cloth with the captured HRP activity being visualized. Figure 6.4 shows that the colour of the rabbit IgG-cloths increased as the concentration of the goat anti-rabbit IgG antiserum increased. No colour was observed for the zero control (no antiserum).

To study the reproducibility of this assay, the captured HRP activity was colourimetrically confirmed as before. Figure 6.5 shows that the absorbance of the colour increased sharply until an antiserum concentration of 4 $\mu\text{g/ml}$ was reached after which the rate of increase declined. Most importantly, the relatively small standard deviation again suggests high reproducibility of this instantaneous assay.

In this chapter, I have demonstrated an instantaneous visual assay of antibody that

Figure 6.4. Semi-quantitative visual assay of anti-rabbit IgG antibody in goat antiserum samples. Goat anti-rabbit IgG antibody samples of different concentrations were instantaneously reacted to rabbit IgG-polyester cloth disks. Captured goat anti-rabbit IgG antibody was detected by a second instantaneous reaction with excess rabbit anti-goat IgG-HRP conjugate followed by use of the insoluble TMB peroxidase substrate system.

Concentration of Anti-Rabbit IgG Antibodies ($\mu\text{g/ml}$)

Figure 6.5. Colourimetric assay of anti-rabbit IgG antibody in goat antiserum samples. Goat anti-rabbit IgG antibody samples of different concentrations were instantaneously reacted to rabbit IgG-polyester cloth disks. Captured goat anti-rabbit IgG antibody was assayed by a second instantaneous reaction with excess rabbit anti-goat IgG-HRP conjugate followed by a 10 min HRP assay using the soluble TMB peroxidase substrate system as described in Methods. The HRP activity was expressed as average absorbance at 450 nm (A_{450}) $\pm$ standard deviation ($n = 6$).

permits rapid serodiagnosis of human and animal diseases in a simple (and disposable) system which does not require any specialized equipment or skills. This system has the advantages of being semi-quantitative, because the results may be related to a colourimetric assay, and are highly reproducible. The use of polymyxin-cloth is very attractive for the serodiagnosis of diseases caused by gram-negative bacteria. A culture of a gram-negative pathogen can be prepared from which LPS antigen is extracted through the addition of cholate and autoclaving. The polymyxin-cloth saturated with the extracted LPS can be used for the assay of antibodies to the LPS antigen of the gram-negative pathogen. The specificity of polymyxin for LPS binding eliminates the need to purify LPS by a manual procedure (e.g., hot phenol extraction) which is time-consuming and undesirable for safety reasons. The use of polymyxin-cloth also eliminates the need to treat the EIA support with blocking agents since polymyxin is a good blocking agent itself, as was indicated by the inability to visually detect any HRP activity in the zero control cloth (Figure 6.2). Finally, polymyxin-cloth is stable, which permits transportation and field testing of instantaneous P-CEIA in the absence of refrigeration. Polyester cloth may be used for the assay of any antibodies to pathogen antigens that can be adsorbed onto the cloth. PVA can be used to effectively block non-specific adsorption on the antigen-coated cloth for instantaneous CEIA. It was previously demonstrated that PVA provides stable blocking over extended periods of time during ambient storage (Chapter 2) as well as stabilization of HRP conjugates to permit their ambient storage (Chapter 3). Thus, it is now possible to carry out field EIA for the serodiagnosis of human and animal disease in a very rapid and simple manner without the need for specialized equipment, skills or refrigeration.

The results presented in this chapter have been submitted for publication in the following paper:

Boyd, S. and Yamazaki, H. (1996c: accepted for publication, July 1996) Instantaneous cloth-based enzyme immunoassay for the semi-quantitative visual determination of antibodies. *Immunol. Invest.*

CHAPTER 7
TOSYL CHLORIDE ACTIVATION OF A
RAYON/POLYESTER CLOTH FOR
PROTEIN IMMOBILIZATION

ABSTRACT

Rayon/polyester cloth was treated with NaOH, washed and dehydrated with acetone, and without drying activated with p-toluenesulfonyl chloride in acetone. The activated cloth was used to immobilize bovine serum albumin and anti-horseradish peroxidase antibody. As compared to other chemical activating procedures such as cyanogen bromide and 1,1-carbonyldiimidazole, tosyl chloride-activated cloth offers several advantages including low cost, high capacity for protein immobilization, rapidity, and high linkage stability.

INTRODUCTION

Immunoaffinity chromatography is an industrially attractive procedure for the purification of antigens or antibodies as it provides a high degree of purification in a simple procedure of adsorption and elution. Commonly used immunoaffinity supports such as cross-linked agarose or dextran beads are expensive and often exhibit structural instability under high pressure or flow conditions (Janson, 1984). Inexpensive cellulose fibres are also frequently used, but they are compressible and do not yield high flow rates when compressed into a random configuration. For these reasons, there is a great need for the development of new supports for industrial use. One potential support is polyester cloth which offers the advantages of lower expense and greater structural stability. Its macroporous and compressed structure also allows for easier washing, the capacity for higher flow rates with larger sample volumes, and faster immunoreaction rates. The large surface area of cloth also means that a greater density of immobilized immunoreactant may be achieved, resulting in a smaller volume of cloth necessary to purify equivalent quantities of sample. This permits

purification of the sample in a higher concentration since it should be possible to elute the sample from the cloth column using a smaller amount of eluent.

Hydrophobic immunoreactants (e.g., antibodies and many polypeptide antigens) may be adsorbed onto polyester cloth through hydrophobic interactions, however, the amount of immunoreactant that may be immobilized onto the cloth is limited. This does not affect the use of polyester cloth as a support for enzyme immunoassay, but it may limit the efficiency of an immunoaffinity chromatography column which requires a high density of immobilized immunoreactants. Since covalent immobilization procedures can result in greater densities of immobilized immunoreactants, they are preferred for immunoaffinity chromatography. The most commonly used methods for the covalent immobilization of protein immunoreactants onto carbohydrate supports involves the activation of hydroxyl groups of the support with a chemical such as cyanogen bromide (CNBr) (Axen *et al.*, 1967). The hydroxyl groups of polyester are not highly reactive as they help to maintain the crystalline structure of the cloth through extensive hydrogen bonding. Regenerated cellulose such as rayon has a much less crystalline structure, and thus, it is possible to disrupt hydrogen bonding between its hydroxyl groups through treatment with strong alkali, resulting in hydroxyl groups that are easy to activate. In addition, rayon has far more hydroxyl groups than polyester. Recently, 1,1-carbonyldiimidazole (CDI) has been used in the activation of alkali-treated rayon/polyester (70 %/30 %) cloth (Howlett *et al.*, 1991). The polyester in the rayon/polyester blend cloth maintains the fabric structure since rayon becomes structurally weak during alkali treatment and activation.

CDI and CNBr activation provide similar protein immobilization capacities with CDI

activation being simpler to perform on cloth since it does not require the vigorous mixing that is necessary for CNBr activation. Because the linkages formed between CDI- or CNBr-activated support and proteins are unstable at a pH greater than 10, alkaline elution conditions cannot be used for these supports. Furthermore, CNBr and the solvents used in CDI activation are unsafe for industrial scale operations. This chapter demonstrates that p-toluenesulfonyl chloride (tosyl chloride) activation of rayon/polyester cloth (Figure 7.1) overcomes these disadvantages of CDI and CNBr activation while allowing for faster immobilization of greater amounts of protein.

METHODS

Tosyl Chloride Activation of Rayon/Polyester Cloth

Segments (6 mm x 6 mm square) of rayon/polyester cloth were soaked in 10 % (w/v) NaOH (0.5 ml/cloth segment) for 10 min at room temperature (RT). The cloths were washed 3 times with distilled water (dH_2O) . air-dried under suction, and then washed 5 times with acetone (1 min soak between washes). The dehydrated cloths were transferred immediately without drying to a solution of 10 % (w/v) tosyl chloride in acetone (0.5 ml/cloth segment) for 20 min at RT. The tosyl chloride-activated cloths (tosyl-cloths) were washed 3 times with acetone, followed by 3 times with dH_2O under suction, and then thoroughly blotted.

Assay of Immobilized BSA

Fifty μl of 1 % (w/v) bovine serum albumin (BSA) in 0.5 M sodium phosphate buffer

Figure 7.1. Activation of rayon/polyester cloth with tosyl chloride for the purpose of the immobilization of amino group containing molecules. Tosyl chloride converts hydroxyl groups of rayon/polyester cloth into active sulfonates. The resultant sulfonates are good leaving groups that, after reaction with an amino group containing molecule, form stable linkages between the amino group containing molecule and the initial hydroxyl group-carrying carbon on the rayon/polyester cloth.

(pH 7.5) was added to each tosyl-cloth for an overnight incubation at RT. To remove any loosely bound BSA, each cloth segment was heated in 1 % (w/v) sodium dodecyl sulphate (SDS) at 100°C for 10 min. The segments were then washed 3 times with 1 % SDS followed by 3 washes with dH₂O. A micro bicinchoninic acid (BCA) protein assay was used to measure the immobilized BSA. Due to the lack of immobilized protein standards, the degree of protein immobilization was compared by absorbance at 562 nm of the developed colour following dilution to an absorbance range of 0.1 to 1.0.

Anti-HRP Immobilization

Ten μ l of rabbit anti-horseradish peroxidase antibody (anti-HRP) (7.5 μ g in PBS (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % NaCl) was reacted with each tosyl-cloth. To block any unreacted sites and any non-specific HRP binding sites, the cloths were soaked in 0.5 % BSA in PBS overnight at RT. Each cloth segment was then reacted with 50 μ l of HRP (2 μ g/ml) in PBST (PBS containing 0.05 % (v/v) Tween 20) for 20 min. To assay the activity of the HRP that reacted with immobilized anti-HRP, individual cloth segments were placed into separate 16 mm x 100 mm test tubes containing 1 ml of the soluble TMB peroxidase substrate system and shaken at 180 RPM (on a New Brunswick Gyrotary Shaker) for 10 min at 30°C. The HRP reaction was stopped by adding 250 μ l of 2 N H₂SO₄ and then the resulting colour was measured at 450 nm. The soluble TMB peroxidase substrate system was prepared by mixing 2.5 ml of TMB (2 mg/ml ethanol) with a solution consisting of 0.21 g citric acid H₂O, 0.42 g EDTA 4 Na, 0.03 g NaBO₃ H₂O, and 100 ml dH₂O.

RESULTS AND DISCUSSION

Tosyl Chloride Activation of Rayon/Polyester Cloth

Pretreatment of the rayon/polyester cloth with 10 % NaOH was found to be necessary for activation with tosyl chloride. Since hydrogen bonding is destabilized by strong alkali, this pretreatment presumably breaks hydrogen bonding involving the hydroxyl groups of rayon. Preliminary studies indicated that NaOH treatment increased BSA immobilization onto tosyl-cloth by at least six-fold. Prior to tosyl chloride activation it is essential that the cloths be dehydrated, as the presence of even small amounts of water results in poor activation of the cloths. Dehydration of traditional supports (e.g., cross-linked agarose) is performed in a sequential washing with increasing ratios of acetone:water mixtures in order to maintain the structure of the support (Harlow and Lane, 1988). With cloth supports, the dehydration step is performed with straight acetone which allows for easier and more rapid dehydration. The dehydrated cloths must be transferred without the complete removal of acetone to the tosyl chloride solution. Drying at this stage reduced BSA immobilization six-fold, possibly due to the reformation of hydrogen bonds which reduces the activation by tosyl chloride. For the activation of NaOH-treated cloth, it is not necessary to add pyridine (used for the activation of agarose beads) to the activation solution in order to neutralize the HCl generated. This is advantageous in industrial scale activation since pyridine is unsafe and has a very unpleasant odour. Acetone used in the activation, by comparison, is safe, inexpensive, and easy to remove.

Effect of Tosyl Chloride Concentration and Time of Tosyl Chloride Treatment on BSA Immobilization

To study the effect of tosyl chloride concentration and time of treatment, the amount of BSA that could be immobilized onto rayon/polyester cloth segments that were activated with varying concentrations and lengths of tosyl chloride treatment was determined. Table 7.1 shows that BSA immobilization increased when higher concentrations of tosyl chloride were used during a 10 min activation reaction. Rather than increasing the concentration beyond 10 %, the time of activation using 10 % tosyl chloride was lengthened. Table 7.1 indicates that as the time of activation increased, the immobilization capacity of the cloths decreased. The reason for this decrease is unclear, but perhaps there is some loss of cloth integrity with prolonged tosyl chloride treatment. The relative amounts of BSA that was immobilized was determined by comparing the absorbance of the colour developed using the BCA protein assay. The actual amount of BSA that may be immobilized using tosyl chloride activation cannot be calculated because there are no immobilized protein standards available. The absorbance resulting from immobilized BSA is expected to be less than that for the equivalent amount of BSA in solution, because the immobilized BSA would be less efficient than free BSA in developing the colour of the protein assay since the protein-assay reaction would be slower with immobilized proteins. The colour developed with immobilized BSA, however, can provide a minimum value for the BSA immobilization capacity of tosyl chloride-activated cloth. One gram of the activated cloth was capable of immobilizing at least 25 mg of BSA. Consequently one major advantage of tosyl-cloths are their very high immobilization capacity. Comparison of the colour developed showed that tosyl-cloths have

Table 7.1. Effect of concentration of tosyl chloride and length of tosyl chloride activation of rayon/polyester cloth on BSA immobilization^a.

Concentration % (w/v)	Length of Treatment (min)	Absorbance (A_{562}) (BSA Binding)
2.5	10	4.7 ± 0.8
5.0	10	5.7 ± 0.7
10.0	10	6.2 ± 0.7
10.0	20	5.8 ± 0.4
10.0	40	4.7 ± 0.5
10.0	60	4.1 ± 0.5

^aRayon/polyester cloths were activated with varying concentrations of tosyl chloride for varying lengths of time, and then assayed for BSA immobilization as described in Methods. The amount of BSA immobilized onto each cloth segment was determined by measuring the absorbance at 562 nm of the colour developed in the BCA protein assay. The values are expressed as mean absorbance $\pm$ standard deviation ($n = 6$).

the capacity to immobilize approximately 3 times more BSA than CDI- or CNBr-activated cloths.

Effect of Molarity and pH of the Sodium Phosphate Buffer

Phosphate buffer (0.5 M; pH 7.5) has been used for the immobilization of protein onto tosyl chloride-activated agarose (Harlow and Lane, 1988). Table 7.2 shows the effects of varying the concentration and pH of sodium phosphate buffer on the binding capacity of tosyl-cloths. The amount of BSA immobilized increased as the molarity of the phosphate buffer increased from 0.05 to 0.5. The pH of the buffer within the range of 7.5 to 10.5 had little effect on the immobilization capacity of the tosyl-cloths.

Linkage Stability

Following immunoaffinity chromatography, antigen-antibody dissociation is often achieved by adjusting the pH of the system to high alkalinity (e.g., pH 11). The linkage formed between CDI-activated affinity supports and proteins is unstable at high pH. To test the stability of the linkages produced through tosyl chloride activation, BSA immobilized onto tosyl-cloth was incubated at RT for 20 h in 0.5 M sodium phosphate buffer (pH 7.5 or 11). After incubation, the cloths were thoroughly washed with buffer (pH 7.5) and the amount of BSA still immobilized onto the cloths was measured. There was no significant difference between the amount of BSA still immobilized onto both sets of cloths (both sets of cloths yielded an absorbance at 562 nm of approximately 6).

Table 7.2. Effect of changing the molarity and pH of the sodium phosphate buffer on BSA immobilization to tosyl chloride-activated cloths^a.

Molarity (M)	pH	Absorbance (A_{562}) (BSA Binding)
0.05	7.5	4.9 ± 0.4
0.1	7.5	5.5 ± 0.5
0.5	7.5	6.0 ± 0.7
0.5	8.5	5.8 ± 0.5
0.5	9.5	6.1 ± 0.6
0.5	10.6	6.0 ± 0.6

^aRayon/polyester cloth segments were activated with 10 % (w/v) tosyl chloride for 10 min and incubated in 1 % (w/v) BSA in phosphate buffer of varying molarity and pH overnight at RT as described in Methods. The amount of BSA immobilized onto each cloth segment was determined by measuring the absorbance at 562 nm of the colour developed in the BCA protein assay. The values are expressed as mean absorbance $\pm$ standard deviation ($n = 6$).

Kinetics of Immobilization of Anti-HRP Antibody

In the immunoaffinity purification of antigens, generally small amounts of antibodies are immobilized onto the affinity support. It has been advertised that tosyl chloride-activated supports are ideally suited for the efficient immobilization of very small amounts of proteins in a short period of time (e.g., 1 h) (Anonymous, 1991). To examine the kinetics for the immobilization of very small amounts of antibodies, tosyl-cloths were treated with anti-HRP (7.5 μ g in PBS/cloth segment) for various periods of time. After blocking non-specific binding sites on the cloth with BSA, HRP was allowed to react with the anti-HRP-cloth. The captured HRP was assayed using the soluble TMB peroxidase substrate system. The amount of immobilized anti-HRP was measured directly by determining the colour formed. Table 7.3 indicates that even a very low concentration of anti-HRP was maximally immobilized within 15 min. Most of the HRP bound to the cloth represents HRP that has reacted with immobilized anti-HRP as indicated by the observation that tosyl-cloth segments lacking anti-HRP (control cloths) have only about 10 percent of the amount of HRP present on tosyl-cloths that have immobilized anti-HRP. The results also indicate that antibodies immobilized onto tosyl-cloth retain their immunological activity.

This chapter has demonstrated that tosyl chloride-activated cloth is an attractive method for industrial scale protein immobilization. In fact, it should be possible to immobilize any amino group containing molecule onto tosyl-cloths. Tosyl chloride-activated cloth has numerous advantages over competitive activated supports. Tosyl-cloths have a greater protein immobilization capacity than CDI- or CNBr-activated cloths. Tosyl chloride

Table 7.3. Rate of anti-HRP immobilization onto tosyl chloride-activated rayon/polyester cloth^a.

Immobilization Time (min)	Absorbance (A_{450}) (HRP activity)
control	0.22 ± 0.06
15	2.2 ± 0.1
30	2.3 ± 0.2
60	2.1 ± 0.1
120	2.0 ± 0.1

^aRayon/polyester cloths were activated with 10 % (w/v) tosyl chloride for 10 min. Following the reaction of the resultant tosyl-cloths with anti-HRP for the various time periods indicated, the cloths were incubated with BSA to prevent non-specific binding and then reacted with excess HRP as described in Methods. The amount of anti-HRP immobilized onto each cloth segment was measured indirectly by determining the absorbance at 450 nm of the colour developed in the soluble TMB peroxidase substrate system. The control cloths were not reacted with anti-HRP. The values are expressed as mean absorbance $\pm$ standard deviation ($n = 6$).

activation is about 50 times less expensive than CDI activation and 1000 times less expensive than tresyl chloride. Unlike many activation methods such as CDI and CNBr, the ligand-affinity support linkage produced through tosyl chloride activation is stable under high alkali conditions (e.g., pH 11). Finally, tosyl chloride-activated rayon/polyester cloths are capable of efficiently immobilizing small amounts of ligand more quickly than is possible with other activation methods. Because of the great advantages of using tosyl chloride-activated rayon/polyester cloth as a support for protein immobilization, we (Dr. Yamazaki and I) were invited to contribute a chapter on the activation of rayon/polyester cloth for protein immobilization in a book entitled Immobilization of Enzyme and Cells to be published in 1996 (a copy of the accepted book chapter is presented in Appendix II).

The results presented in this chapter have been published in the following paper:

Boyd, S. and Yamazaki, H. (1993b) Tosyl chloride activation of a rayon/polyester cloth for protein immobilization. *Biotechn. Techn.* 7: 277-282.

CHAPTER 8
EFFICIENT AND GENTLE ELUTION OF ANTIBODIES
FROM AN IMMOBILIZED POLYPEPTIDE ANTIGEN
BY SATURATED MAGNESIUM CHLORIDE

ABSTRACT

A model antigen, rabbit immunoglobulin G, was immobilized onto polyester cloth by adsorption. The antigen cloth was reacted with sheep anti-rabbit IgG antibody ($F(ab')_2$). The effect of various elution conditions were compared for their ability to dissociate sheep anti-rabbit IgG antibody ($F(ab')_2$) that was captured onto the rabbit IgG-polyester cloth. The best elution condition by a large margin was saturated $MgCl_2$, which nearly quantitatively eluted antibody from the antigen-cloth. Exposure of antibody to saturated $MgCl_2$ for 30 min resulted in no loss of immunoactivity. Saturated $MgCl_2$, therefore, is a very good eluent for use in the immunoaffinity purification of $F(ab')_2$ antibodies.

INTRODUCTION

Immunoaffinity chromatography provides a high degree of immunoreactant purification through a simple procedure of adsorption and elution. Immunoaffinity columns may be produced by covalently linking antibody or antigen onto solid supports including beads of carbohydrate polymers or macroporous rayon/polyester cloths that have been activated with chemicals such as tosyl chloride (refer to Chapter 7) or cyanogen bromide. The success of an immunoaffinity purification depends upon the efficient elution of antibody or antigen that has been captured by the immunoaffinity column. This can be difficult to accomplish since antibody-antigen interactions such as hydrogen bonds, van der Waals forces, and ionic and hydrophobic interactions vary tremendously between different antibody-antigen pairs. For elution, these non-covalent interactions must be disrupted without causing any significant loss of activity to the immobilized and eluted

immunoreactants.

There are many different elution conditions commonly used in immunoaffinity chromatography including the use of high salt concentrations, high or low pH, organic solvents, chaotropic agents, strong hydrogen bond formers, and ionic detergents as described in the General Introduction. The ideal elution conditions for a given immunoaffinity purification depends upon the number and type of interactions that result in the binding of antibody to antigen. When different interactions are involved, a combination of elution conditions may be required, e.g., use of high salt concentrations or low or high pH to disrupt electrostatic interactions in conjunction with the use of a high concentration of an organic solvent (e.g., 50 % (v/v) polyethylene glycol) for disrupting hydrophobic interactions. Elution of tightly adsorbed immunoreactants generally requires harsh elution conditions which often cause denaturation of the eluted or immobilized immunoreactants. Furthermore, the elution of immunoreactants may not be quantitative when high affinity antibodies are involved, which results in lower yields and the gradual saturation of the immunoaffinity column so that it cannot be repeatedly reused. Thus, the development of gentle and efficient elution conditions is of great significance to immunoaffinity chromatography. High salt concentrations and high concentrations of organic solvents are considered to be among the gentlest eluents. This chapter tests a large variety of elution conditions including different concentrations of salts (e.g., MgCl_2 , KCl , CaCl_2 , $\text{Mg}(\text{CH}_3\text{COO})_2$, $\text{Mg}(\text{OH})_2$) and various concentrations of low molecular weight polyethylene glycol (MW 200 - 600), individually or in combination, for their ability to elute polyclonal antibody from an immobilized antigen column. The effect of any promising condition(s) on the activity of the immobilized antigen

and the antibody in solution was also investigated.

METHODS

Preparation of Antibody-Antigen-Cloths

Segments (6 mm x 6mm square) of polyester cloth were soaked in ethanol for a few min and then washed 5 times with PBS (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % sodium chloride). After blotting the segments, 50 μ l of rabbit IgG (5 μ g in PBS/cloth segment) or PBS alone (for use as non-specific adsorption control cloths) was added. Following an overnight incubation at 30°C in closed petri plates, the cloth segments were washed 5 times with PBST (PBS containing 0.05 % (v/v) Tween 20) followed by 3 times with PBS. The cloths were then incubated for 1 h in 0.5 % (w/v) Protein Blocker in PBS (1 ml/cloth segment) at room temperature (RT) followed by washing 5 times in PBS and blotting. The resultant rabbit IgG (antigen)-cloths were reacted for 30 min at RT with 30 μ l/cloth of sheep anti-rabbit IgG antibody (F(ab')₂)-biotin conjugate / anti-rabbit IgG-biotin conjugate (F(ab')₂) (stock solution was diluted 1000 times in PBS) to produce antibody-antigen (Ab-Ag)-cloths. Finally, the Ab-Ag-cloths were washed 5 times with PBST followed by 3 times with PBS, and blotted.

Assay of Cloth-Bound Anti-Rabbit IgG-Biotin Conjugate (F(ab')₂)

Antigen-cloths treated with anti-rabbit IgG-biotin conjugate (F(ab')₂) were incubated with 30 μ l/cloth segment of streptavidin-horseradish peroxidase conjugate (streptavidin-HRP conjugate) (1 μ g/ml in PBS) for 30 min at RT, washed 5 times with PBST followed by 3

times with PBS, and blotted. One ml of soluble TMB peroxidase substrate system was added to an individual cloth segment placed in a 16 mm x 100 mm test tube and shaken at 180 RPM (on a New Brunswick Gyrotary Shaker) for 10 min at 30°C. The peroxidase reaction was stopped by adding 250 μ l of 2 N H_2SO_4 and then the absorbance of the resulting colour was measured at 450 nm. The soluble TMB peroxidase substrate system was prepared by mixing 2.5 ml of TMB (2 mg/ml ethanol) with a solution consisting of 0.21 g citric acid $\cdot$ H $_2$ O, 0.42 g EDTA $\cdot$ 4 Na, 0.03 g NaBO $_3$ $\cdot$ 4 H $_2$ O, and 100 ml H $_2$ O.

Test for Desorption of Cloth-Bound Antigen in Different Elution Conditions

Antigen-cloths were prepared as above except that each cloth was treated with 50 ng rabbit IgG in PBS. Since coating the cloth with less than 1 μ g/ml of rabbit IgG resulted in a corresponding decrease in the capture of the biotinylated antibody, any loss of antigen from the cloth should be detectable. The antigen-cloths were shaken at 180 RPM for 30 min at RT in PBS (elution control) or one of the following elution conditions: MgCl $_2$ (1 M, pH 6.2; 2 M, pH 5.6; 3 M, pH 5.0; 4 M, pH 4.4; 5 M, pH 4.1; or saturated, pH 3.6); KCl (1 M, pH 7.1; 2 M, pH 7.0; or 3 M, pH 7.0); CaCl $_2$ (1 M, pH 6.0; 2 M, pH 5.8; 3 M, pH 5.6; 4 M, pH 5.2; 5 M, pH 4.9; or 6 M, pH 4.0); Mg(CH $_3$ COO) $_2$ (1 M, pH 8.4; 2 M, pH 8.2; or 3 M, pH 7.9); Mg(OH) $_2$ (5 % (v/v), pH 10.5; 10 %, pH 10.4; or 20 %, pH 10.3); 10 % (v/v), 20 %, 50 %, 75 %, or 100 % polyethylene glycol (PEG) (MW 200, 300, 400, or 600 with all solutions adjusted to a pH or approximately 7); or, neutral pH mixtures of PEG (MW 200) and MgCl $_2$ (10 % PEG and MgCl $_2$ (3, 4, or 5 M), 20 % or 50 % PEG and MgCl $_2$ (3 or 4 M), or 75 % PEG and 1 M MgCl $_2$). The antigen-cloths were then washed 5 times with PBST followed

by 3 times with PBS and then incubated in PBS containing 0.5 % Protein Blocker for 1 h (1 ml/cloth segment). The cloths were reacted with anti-rabbit IgG-biotin conjugate ($F(ab')_2$) and assayed for cloth-bound antibody as described above. The loss of antigen from the cloth was measured by the difference in absorbance at 450 nm between the control cloth incubated in PBS and the antigen-cloth incubated in a given elution condition.

Elution of Antibody Using Different Elution Conditions

Ab-Ag-cloths and antigen-cloths (used as controls for determining the non-specific adsorption of streptavidin-HRP conjugate) were shaken at 180 RPM in PBS (elution control) or the different elution conditions listed above (1 ml/cloth segment) at RT. After 30 min, the liquids were separated from the cloths via filtration. The cloths were washed 5 times with PBST followed by 3 times with PBS, blotted, and then assayed for cloth-bound anti-rabbit IgG-biotin conjugate ($F(ab')_2$) as above. A_{450} due to the non-specific binding of streptavidin-HRP conjugate was subtracted from the A_{450} for each Ab-Ag-cloth to obtain antibody-specific binding of the streptavidin-HRP conjugate. The amount of antibody eluted (A_{450} eluted from cloths) by each elution condition was calculated by subtracting A_{450} of eluent-treated cloths from A_{450} of PBS-treated cloths. The degree of elution (%) is then $100 \times (A_{450} \text{ eluted from cloths}) / (A_{450} \text{ of PBS-treated cloths})$.

RESULTS AND DISCUSSION

Effect of Different Eluents Upon Antibody Elution

To study the elution of antibody from an immobilized polypeptide antigen matrix, a

model antigen, rabbit IgG, was adsorbed onto polyester cloth. First, it was confirmed that the adsorbed antigen was not desorbed during exposure to the different elution conditions. The antigen-cloth was allowed to react with a biotinylated $F(ab')_2$ fragment of sheep anti-rabbit IgG antibody. The biotin label was used to permit the assay of the cloth-bound antibody through its binding to a streptavidin-HRP conjugate. Unlike enzyme labels, biotin is a small and stable molecule which should not be affected by any of the test eluents. The $F(ab')_2$ fragment was used instead of a whole IgG molecule to avoid the complication of non-specific binding due to denaturation of the Fc region which an eluent may cause. The Fc is much more susceptible than $F(ab')_2$ to denaturation which exposes its hydrophobic site causing non-specific adsorption of IgG (Tijseen, 1985).

$MgCl_2$ (3.5 M) and high concentrations of PEG have been suggested as potential eluents (Hermanson *et al.*, 1992). Thus, different concentrations of $MgCl_2$ and different concentration and molecular weights of PEG were studied individually or in combination. Table 8.1 indicates that PEG had no capacity for elution using the model system. $MgCl_2$, however, was quite effective in eluting the captured antibody. Figure 8.1 shows that while 3.5 M $MgCl_2$ eluted close to 70 % of the antibody, saturated $MgCl_2$ (approximately 6 M) eluted over 90 % of the $F(ab')_2$ antibody. Adding PEG (MW 200) to $MgCl_2$ solutions did not improve upon the ability of $MgCl_2$ to dissociate captured $F(ab')_2$ antibody (Table 8.1). Because $MgCl_2$ was capable of eluting $F(ab')_2$ antibody from the antigen-cloth, the elution capacity of other salts including KCl, $CaCl_2$, $Mg(CH_3COO)_2$, and $Mg(OH)_2$ was studied. Table 8.1 indicates that of these salts, only $CaCl_2$ was capable of eluting captured $F(ab')_2$ antibody. The elution capacity of $CaCl_2$ increased with increasing molarity to a maximum

Table 8.1. Ability of different elution conditions to elute captured F(ab'), antibody from an antigen column^a.

Elution Condition	Degree of Elution (%)
1 M MgCl ₂ , pH 6.2	22.9 ± 1.7
2 M MgCl ₂ , pH 5.6	41.8 ± 2.4
3 M MgCl ₂ , pH 5.0	62.0 ± 2.5
4 M MgCl ₂ , pH 4.4	71.6 ± 4.8
5 M MgCl ₂ , pH 4.1	85.8 ± 6.6
saturated MgCl ₂ , pH 3.6	91.6 ± 5.3
10 % PEG (MW 200), pH 7.0	-2.1 ± 0.4
20 % PEG (MW 200), pH 7.0	-1.7 ± 0.3
50 % PEG (MW 200), pH 7.0	-1.5 ± 0.2
75 % PEG (MW 200), pH 7.0	-3.4 ± 0.4
100 % PEG (MW 200), pH 7.0	0.6 ± 0.2
10 % PEG (MW 300), pH 7.0	-1.4 ± 0.3
20 % PEG (MW 300), pH 7.0	0.4 ± 0.1
50 % PEG (MW 300), pH 7.0	1.8 ± 0.3
75 % PEG (MW 300), pH 7.0	0.5 ± 0.4
100 % PEG (MW 300), pH 7.0	-0.3 ± 0.2
10 % PEG (MW 400), pH 7.0	0.5 ± 0.1
20 % PEG (MW 400), pH 7.0	-2.7 ± 0.4
50 % PEG (MW 400), pH 7.0	-1.1 ± 0.3
75 % PEG (MW 400), pH 7.0	0.8 ± 0.2
100 % PEG (MW 400), pH 7.0	-0.9 ± 0.2
10 % PEG (MW 600), pH 7.0	0.6 ± 0.3
20 % PEG (MW 600), pH 7.0	0.4 ± 0.2
50 % PEG (MW 600), pH 7.0	2.2 ± 0.5

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75 % PEG (MW 600), pH 7.0	-1.8 ± 0.4
100 % PEG (MW 600), pH 7.0	0.7 ± 0.2
10 % PEG (MW 200) + 3 M MgCl ₂ , pH 7.0	57.5 ± 3.8
10 % PEG (MW 200) + 4 M MgCl ₂ , pH 7.0	61.9 ± 5.1
10 % PEG (MW 200) + 5 M MgCl ₂ , pH 7.0	68.2 ± 4.3
20 % PEG (MW 200) + 3 M MgCl ₂ , pH 7.0	56.3 ± 3.2
20 % PEG (MW 200) + 4 M MgCl ₂ , pH 7.0	60.5 ± 2.9
50 % PEG (MW 200) + 3 M MgCl ₂ , pH 7.0	55.2 ± 4.0
50 % PEG (MW 200) + 4 M MgCl ₂ , pH 7.0	59.4 ± 3.7
75 % PEG (MW 200) + 1 M MgCl ₂ , pH 7.0	16.5 ± 1.3
1 M KCl, pH 7.1	0.7 ± 0.3
2 M KCl, pH 7.0	0.9 ± 0.2
3 M KCl, pH 7.0	-0.5 ± 0.2
1 M CaCl ₂ , pH 6.0	10.1 ± 0.9
2 M CaCl ₂ , pH 5.8	20.4 ± 1.3
3 M CaCl ₂ , pH 5.6	38.3 ± 1.6
4 M CaCl ₂ , pH 5.2	39.6 ± 3.0
5 M CaCl ₂ , pH 4.9	44.2 ± 2.5
6 M CaCl ₂ , pH 4.0	52.7 ± 3.5
1 M Mg(CH ₃ COO) ₂ , pH 8.4	-0.9 ± 0.2
2 M Mg(CH ₃ COO) ₂ , pH 8.2	-2.8 ± 0.4
3 M Mg(CH ₃ COO) ₂ , pH 7.9	-0.3 ± 0.2
5 % Mg(OH) ₂ , pH 10.5	-3.6 ± 0.6
10 % Mg(OH) ₂ , pH 10.4	1.1 ± 0.2
20 % Mg(OH) ₂ , pH 10.3	-1.1 ± 0.3

^aAnti-rabbit IgG-biotin conjugate (F(ab')₂) was reacted to rabbit IgG-cloths which were then incubated with agitation for 30 min in one of the listed elution conditions. Following blocking with Protein Blocker, each cloth was reacted to an excess of streptavidin-HRP conjugate. The HRP activity of the resultant cloths was used to calculate the degree of antibody elution as described in Methods. Values for the degree of elution are expressed as average degree of elution ± standard deviation (n = 6).

Figure 8.1. Effect of MgCl_2 concentration on F(ab')_2 antibody elution. Anti-rabbit IgG-biotin conjugate (F(ab')_2) was reacted to rabbit IgG-cloths which were then incubated with agitation for 30 min in PBS (elution control) or one of several different elution conditions that varied in pH and MgCl_2 concentration. Following blocking with Protein Blocker, each cloth was reacted to an excess of streptavidin-HRP conjugate. The HRP activity of the resultant cloths was used to calculate the degree of antibody elution as described in Methods. Values for the degree of elution are expressed as average degree of elution $\pm$ standard deviation ($n = 6$). The pH values of the MgCl_2 solutions in H_2O are also plotted. Symbols: $\circ$, Degree of Elution (%); and $\square$, pH of Magnesium Chloride Solution.

of 52.7% at 6 M CaCl_2 which is far less than what was achieved with saturated MgCl_2 . I believe that MgCl_2 is a better eluent than KCl because Mg^{2+} has a higher ionic charge density than K^+ due to its divalency. CaCl_2 was inferior to MgCl_2 because Ca^{2+} has a lower charge/radius ratio than Mg^{2+} . This reasoning suggests that BeCl_2 would be a better eluent, but its very high expense and highly acidic nature makes its use impractical. The ineffectiveness of $\text{Mg}(\text{OH})_2$ and $\text{Mg}(\text{CH}_3\text{COO})_2$ as eluents indicates that Cl^- in the MgCl_2 is essential. The effectiveness of Cl^- relative to OH^- and CH_3COO^- is due to the high charge/radius ratio of Cl^- . The $\text{F}(\text{ab}')_2$ antibodies eluted at higher concentrations of MgCl_2 most likely represent high-affinity antibodies. This is of great significance to immunoassays, where high-affinity antibodies provide greater detectability of antigens than low-affinity antibodies.

Effect of Varying pH of MgCl_2 on Antibody Elution

Figure 8.1 shows that higher concentrations of MgCl_2 in water gave lower pH values with a pH of 3.6 for saturated MgCl_2 . Thus, it is conceivable that lower pH values rather than higher concentrations are responsible for the greater elution observed with MgCl_2 solutions. To test this, 3 M MgCl_2 in water was adjusted to various pH values (3 to 6) with NaOH or HCl , and examined for $\text{F}(\text{ab}')_2$ antibody elution as above. Figure 8.2 shows that lowering pH from 5 (pH of 3 M MgCl_2) to 3.5 results in only a 5 % increase in the degree of elution. Since raising MgCl_2 concentrations from 3 M to saturation caused more than a 30 % increase in the degree of elution (Figure 8.1), the concentration of MgCl_2 is the primary factor affecting $\text{F}(\text{ab}')_2$ antibody elution.

Figure 8.2. Effect of varying pH of MgCl_2 on F(ab')_2 antibody elution. Anti-rabbit IgG-biotin conjugate (F(ab')_2) was reacted to rabbit IgG-cloths which were then incubated with agitation for 30 min in PBS (elution control) or one of several different solutions of 3 M MgCl_2 with pH ranging from 3 to 6. Following blocking with Protein Blocker, each cloth was reacted to an excess of streptavidin-HRP conjugate. The HRP activity of the resultant cloths was used to calculate the degree of antibody elution as described in Methods. Values for the degree of elution are expressed as average degree of elution $\pm$ standard deviation ($n = 6$).

Comparison with a Commercial Ab/Ag Elution Buffer

There are a few different brands of commercially available antibody elution buffers which claim to provide quantitative recovery of antibody in immunoaffinity chromatography. Figure 8.3 compares the performance of one such eluent "ImmunoPure Gentle Ag/Ab Elution Buffer" (available from Pierce) with saturated MgCl_2 . The elution by this commercial eluent was slow and less efficient than with saturated MgCl_2 . Even after 4 h of shaking at 180 RPM, only 73 % of the F(ab')_2 antibody was eluted with this eluent. With saturated MgCl_2 , more than 90 % elution occurred within 10 min.

Effect of Saturated MgCl_2 on Antibody and Antigen Activity

Saturated MgCl_2 , though efficient in the elution of sheep F(ab')_2 antibody, should not result in the loss of antibody activity during elution. To test this, stock biotinylated F(ab')_2 antibody was diluted 10 times in saturated MgCl_2 or PBS (untreated control) and incubated for 30 min at RT. The solutions were then diluted 100 times with PBS such that the MgCl_2 concentration became so low so as to not inhibit immunoreaction. Rabbit IgG-cloths were reacted with the diluted antibody and assayed for bound antibody as described in Methods. The amount of bound antibody was the same whether the antibody was treated with MgCl_2 or not. Similarly, rabbit IgG-cloth was exposed to saturated MgCl_2 for 30 min at RT. There was no significant change in the capacity for the antigen-cloth to capture antibody. Therefore, saturated MgCl_2 does not denature antibody or antigen during a 30 min elution period.

Figure 8.3. Comparison of the ability of saturated MgCl_2 with a commercial Ag/Ab elution buffer to elute captured F(ab')_2 antibody from an antigen column. Anti-rabbit IgG-biotin conjugate (F(ab')_2) was reacted to rabbit IgG-cloths which were then incubated with agitation for 6 h in PBS (elution control), saturated MgCl_2 , or ImmunoPure Gentle Ag/Ab Elution Buffer. At regular time intervals, cloths were removed, blocked with Protein Blocker and then reacted to an excess of streptavidin-HRP conjugate. The HRP activity of the resultant cloths was used to calculate the degree of antibody elution as described in Methods. Values for the degree of elution are expressed as average degree of elution $\pm$ standard deviation ($n = 6$). Symbols: $\circ$, Saturated MgCl_2 ; and $\square$, ImmunoPure Gentle Ab/Ag Elution Buffer

Effect of Saturated $MgCl_2$ on the Elution of Sheep Anti-Rabbit IgG Antibody (Whole Molecule) From Rabbit IgG-Cloth

While saturated $MgCl_2$ was capable of eluting greater than 90 % of the sheep $F(ab')_2$ antibodies captured onto an antigen-cloth, its capacity for eluting whole molecule antibodies was not tested. To test this, sheep anti-rabbit IgG antibody (whole molecule)-biotin conjugate was reacted to rabbit IgG-cloth. The resultant Ab-Ag-cloth was incubated with agitation for 30 min in saturated $MgCl_2$ and then the degree of antibody desorption was calculated as described in Methods. Surprisingly only 51 % of the whole molecule antibody was eluted. This result suggests that the Fc region of antibodies may play an indirect role in antibody-antigen interactions. I believe that the Fc region plays an important role in the avidity of antibody-antigen interactions. Avidity is a measure of the overall stability of antibody-antigen complexes and involves the intrinsic affinity of the antibody for the epitope on the antigen, the valency of the antibody and antigen, and the geometric arrangement of the interacting components. $F(ab')_2$ fragments of IgG antibody have more conformational freedom with respect to one another than the Fab regions of whole IgG antibody. While the Fab regions of whole molecule antibodies still have a certain degree of conformational freedom, this is restricted to a large degree by the presence of the Fc region. Thus, the two Fab regions of whole molecule antibodies are more likely to be in close proximity (or at least facing in the same orientation) to one another than the two Fab regions of $F(ab')_2$ fragments. This restriction of conformational freedom can increase the stability of antibody-antigen interactions when the antigen is multivalent (e.g., it has numerous copies of the same epitope) or when antigens are immobilized onto solid supports at high concentrations (as in

the case of rabbit IgG-cloth). In these situations, it is more likely that there will always be at least one Fab region interacting with an epitope for whole molecule antibodies than is the case for $F(ab')_2$ antibody fragments. It is obvious that the stability of antibody-antigen interactions is much higher when both Fab regions of the IgG antibody are interacting with antigen than when only one is interacting.

This chapter has demonstrated that saturated $MgCl_2$ nearly quantitatively elutes polyclonal sheep $F(ab')_2$ antibodies from a polypeptide antigen-support matrix without causing the denaturation of antibody activity. The capacity of saturated $MgCl_2$ to elute whole molecule antibody from an antigen-support matrix was much lower. The ability to purify Fab fragments is important when use of whole molecule antibody causes problems. For instance, the use of multivalent antibodies for the identification of cell-surface antigens on mammalian cells often leads to capping and internalization of the antigen. Also, many cells have receptors for binding to the Fc portion of antibodies.

The results presented in this chapter came in part from the following publication:

Boyd, S. and Yamazaki, H. (1993c) Efficient and gentle elution of antibodies from an immobilized polypeptide antigen by saturated magnesium chloride. *Biotechn. Techn.* 7: 827-832.

CHAPTER 9

APPLICATION OF ANTIBODY ADSORBED POLYESTER CLOTH FOR THE RAPID SCREENING OF ELUTION CONDITIONS FOR ANTIGEN IMMUNOAFFINITY PURIFICATION

ABSTRACT

Antibody adsorbed onto polyester cloth did not desorb during a 20 h agitated incubation in commonly used antigen-antibody dissociating agents. Antibody activity on the cloth was also unaffected by five 20 min cycles of agitation in these agents. Use of cloth segments coated with a model antibody, goat anti-rabbit IgG antibody, permitted rapid screening of elution conditions for the immunoaffinity purification of a model antigen, rabbit IgG. Thus, this simple test is useful for designing an optimal immunoaffinity chromatography procedure.

INTRODUCTION

Immunoaffinity chromatography is a very powerful technique that permits a high degree of immunoreactant purification in a simple procedure involving capture of the immunoreactant to be purified onto an appropriate immunoaffinity chromatography column, followed by washing and elution of the purified immunoreactant. The most critical information required for immunoaffinity chromatography is the optimal elution condition that permits maximum elution with minimal inactivation of immobilized and eluted immunoreactants. Antibody and antigen binding are a result of non-covalent interactions such as hydrogen bonds, van der Waals forces, and electrostatic and hydrophobic interactions. Each antibody-antigen pair has its own unique set of interactions which may be of only one type of non-covalent interaction or of a combination of different non-covalent interactions. As a result, there is no single condition that provides optimal efficiency of elution for all immunoaffinity purifications. It is usually necessary to determine through trial

and error what elution condition will work best for a given immunoaffinity purification. As described in Chapter 8 and the General Introduction, the most common elution conditions use harsh treatments such as high salt concentrations, high or low pH, organic solvents, chaotropic agents, strong hydrogen bond formers, and ionic detergents to disrupt the non-covalent interactions between antibody and antigen. These eluents may be used individually or in combination depending upon the type(s) of non-covalent interactions that have to be disrupted.

Small-scale chromatography columns are typically used for screening such elution conditions which can be a very expensive and time consuming endeavour. In addition, immobilization of the immunoreactant often requires the use of toxic activating chemicals such as cyanogen bromide. Polystyrene microplates, as used in enzyme immunoassays (EIA), have also been used for screening elution conditions, and while they are cheaper than using columns, they are still time consuming and require the covalent immobilization of immunoreactants (Weiss and Eisenstein, 1987). Polyester cloth has been used as an adsorption support for EIA with its large surface area permitting rapid immunoreaction, and its macroporosity allowing for easier flow of samples or washing buffers. In Chapter 1 it was demonstrated that the adsorption of antibody onto polyester cloth was very stable. Thus, it may be possible to adsorb antibody onto polyester cloth which would not be desorbed by commonly used elution conditions. The present chapter describes an inexpensive, simple, and rapid method for screening elution conditions for the immunoaffinity purification of antigen by using antibody adsorbed polyester cloth.

METHODS

Effect of Eluents on Desorption of Antibody from Polyester Cloth

Polyester cloth segments (6 mm x 6 mm square) were soaked in ethanol for a few min, washed 5 times with PBS (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % sodium chloride), and blotted. Each cloth segment was incubated with 50 μ l of goat anti-rabbit IgG antibody-biotin conjugate (anti-rabbit IgG-biotin conjugate) (50 ng in PBS) overnight at 30°C in closed petri dishes. The cloths were then washed 5 times with PBST (PBS containing 0.05 % (v/v) Tween 20), followed by 3 times with distilled water (dH₂O), and blotted. Individual cloth segments were shaken at 180 RPM (on a New Brunswick Gyrotary Shaker) for 20 h at room temperature (RT) in separate 16 mm x 100 mm test tubes containing 1 ml of PBS (used as a non-eluent control) or one of the following eluents: 0.1 M glycine-HCl (pH 2.2), 0.1 M glycine-NaOH (pH 10.5), 0.1 M ammonium acetate (pH 2.0), 1 M propionic acid (pH 2.3), 1 M acetic acid (pH 2.3), 5 M LiCl in 10 mM phosphate (pH 7.2), saturated MgCl₂ (pH 2.2), or 50 % (v/v) ethylene glycol (pH 8.0). After washing the cloths 3 times with dH₂O and blotting, the cloths were incubated in 0.5 % (w/v) Protein Blocker in PBS (1 ml/cloth segment) for 1 h at RT and then washed 3 times with PBS, blotted, and treated with 25 μ l of avidin-horseradish peroxidase conjugate (avidin-HRP conjugate) (50 ng in PBS/cloth segment) for 20 min at RT. Finally, the cloth segments were washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity. The amount of avidin-HRP conjugate adsorbed to cloths coated only with Protein Blocker was subtracted from the above results. Desorption of antibody from the cloths was measured as the difference in HRP activity between the control cloths incubated in PBS and

the sample cloths incubated in the various eluents.

Elution of Rabbit IgG from Anti-Rabbit IgG-Cloth

Polyester cloth segments (6 mm x 6 mm square) were soaked in ethanol, washed with PBS, and blotted as above, and then incubated with 50 μ l of goat anti-rabbit IgG antibody (anti-rabbit IgG) (2.5 μ g in PBS/cloth segment) overnight at 30°C in closed petri dishes. The anti-rabbit IgG-cloths were washed 5 times with PBST, followed by 3 times with PBS, blotted, and then incubated with 0.5 % Protein Blocker (1 ml/cloth segment) for 1 h at RT. After washing the cloths 3 times with PBS and blotting, each cloth was reacted with 25 μ l of rabbit IgG (0.5 μ g in PBS) for 20 min at RT. The cloths were washed 5 times with PBST, followed by 3 times with dH₂O, and blotted, after which time each cloth was placed individually into separate 16 mm x 100 mm test tubes and shaken for 20 min at 180 RPM at RT in 1 ml of PBS (used as a non-eluent control) or one of the following eluents: 0.1 M glycine-HCl (pH 2.2), 0.1 M glycine-NaOH (pH 10.5), 0.1 M ammonium acetate (pH 2.0), 1 M propionic acid (pH 2.3), 1 M acetic acid (pH 2.3), 5 M LiCl in 10 mM phosphate (pH 7.2), saturated MgCl₂ (pH 2.2), or 50 % ethylene glycol (pH 8.0). After washing the cloths 3 times with dH₂O, followed by 3 times with PBS and blotting, the cloths were incubated for a second time in 0.5 % Protein Blocker in PBS for 1 h at RT. The cloths were then washed 3 times with PBS, blotted, and individually reacted with 25 μ l of goat anti-rabbit IgG antibody-horse radish peroxidase conjugate (anti-rabbit IgG-HRP conjugate) (stock solution was diluted 100 times in PBS) for 20 min at RT. The HRP activity of the resulting cloths was assayed after washing the cloths 5 times with PBST followed by 3 times with PBS and

blotting. The amount of anti-rabbit IgG-HRP conjugate adsorbed onto cloths coated only with Protein Blocker was subtracted from the above results. The loss of antigen (rabbit IgG) from the cloths was measured by the difference in HRP activity between the control cloths incubated in PBS and the sample cloths incubated in the various eluents.

Effect of Elution Cycles on the Activity of Antibody Adsorbed onto Polyester Cloth

Polyester cloth segments (6 mm x 6 mm square) were soaked in ethanol, washed with PBS, and blotted as above. Each cloth segment was treated with 50 μ l of rabbit anti-horseradish peroxidase antibody (anti-HRP) (100 ng in PBS) and incubated overnight at 30°C in closed petri dishes after which time the cloths were washed 5 times with PBST followed by 3 times with dH₂O and blotted. The anti-HRP-cloths were then run through 5 elution cycles with each of the eluents tested. For elution, individual cloth segments were placed into separate 16 mm x 100 mm test tubes and shaken for 20 min at 180 RPM at RT in 1 ml of PBS (used as a non-eluent control) or one of the following eluents: 0.1 M glycine-HCl (pH 2.2), 0.1 M glycine-NaOH (pH 10.5), 0.1 M ammonium acetate (pH 2.0), 1 M propionic acid (pH 2.3), 1 M acetic acid (pH 2.3), 5 M LiCl in 10 mM phosphate (pH 7.2), saturated MgCl₂ (pH 2.2), or 50 % ethylene glycol (pH 8.0). The cloths were then washed 3 times with dH₂O and blotted. After each elution cycle, a portion of the cloths were tested for antibody activity while the remaining cloth segments were placed into new test tubes containing fresh eluent for continued elution cycles. For testing antibody activity, the cloths were incubated in 0.5 % Protein Blocker in PBS (1 ml/cloth segment) for 1 h at RT, washed 5 times with PBS, and blotted. Each cloth segment was then reacted with 25 μ l of HRP (100

ng in PBS) for 20 min at RT, washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity. By adsorbing a limited amount of anti-HRP onto the polyester cloth relative to HRP, any loss of anti-HRP activity could be measured. The amount of HRP adsorbed to cloths coated only with Protein Blocker was subtracted from the above results. Any loss of antibody activity was measured by the difference in HRP activity between the control cloths incubated in PBS and the sample cloths incubated in the various eluents.

Assay of HRP Activity

One ml of soluble TMB peroxidase substrate system was added to an individual cloth segment placed in a 16 mm x 100 mm test tube and shaken at 180 RPM for 10 min at 30° C. The HRP reaction was stopped by adding 250 μ l of 2 N H_2SO_4 and then the absorbance of the resulting colour was measured at 450 nm. The soluble TMB peroxidase substrate system was prepared by mixing 2.5 ml of TMB (2 mg/ml of ethanol) with a solution consisting of 0.21 g citric acid H_2O , 0.42 g EDTA 4 Na, 0.03 g $NaBO_3 H_2O$, and 100 ml H_2O .

RESULTS AND DISCUSSION

Effect of Eluents on Desorption of Antibody from Polyester Cloth

To test the effect of various eluents on the desorption of antibody that is hydrophobically immobilized onto polyester cloth, the amount of anti-rabbit IgG-biotin conjugate still adsorbed onto polyester cloth following a 20 h agitated incubation in each eluent was measured. By using excess avidin-HRP conjugate to assay the anti-rabbit IgG-

biotin conjugate, any loss of antibody from the cloth could be detected. Biotin was used as a label because it is very stable, and thus, any loss in the amount of avidin-HRP conjugate reacted to the cloth following its incubation in an eluent is due to desorption of anti-rabbit IgG-biotin conjugate from the polyester cloth. If antibody that had been labelled with enzyme was used, any loss in enzyme activity could be due to desorption of the antibody-enzyme conjugate from the cloth, or to denaturation of the enzyme label. Table 9.1 indicates that none of the eluents examined had any significant effect upon the desorption of antibody from the polyester cloth. Thus, antibody adsorbed polyester cloth can be used for the immunoaffinity purification of antigens with no significant loss of antibody from the cloth support. In addition, because antibody may be stably adsorbed onto polyester cloth, such antibody-cloth can be used to test the efficiency of eluents to remove captured antigen.

Elution of Rabbit IgG from Anti-Rabbit IgG-Cloth

To test the ability of various eluents to remove captured antigen from an antibody-cloth, the amount of rabbit IgG still adsorbed onto anti-rabbit IgG-cloths following 20 min agitated incubations in the different eluents was measured. The rabbit IgG captured by the cloth was assayed by reacting the cloth to an excess anti-rabbit IgG antibody-HRP conjugate. Table 9.2 indicates that the degree of elution varied dramatically for different eluents with 0.1 M glycine-HCl (pH 2.2) (63 % of rabbit IgG eluted) and 0.1 M ammonium acetate (pH 2.0) (65 % of rabbit IgG eluted) offering the most efficient elution of rabbit IgG from the anti-rabbit IgG antibody-cloths. To test whether any of the eluents reduced the antigenic properties of rabbit IgG, a second experiment was performed where rabbit IgG was incubated

Table 9.1. Effect of different eluents on the desorption of anti-rabbit IgG-biotin conjugate from polyester cloth^a.

Elution Condition	HRP Activity (A_{450})
PBS	1.219 ± 0.077
0.1 M glycine-HCl (pH 2.2)	1.149 ± 0.090
0.1 M glycine-NaOH (pH 10.5)	1.285 ± 0.101
0.1 M ammonium acetate (pH 2.0)	1.198 ± 0.070
1 M propionic acid (pH 2.3)	1.150 ± 0.063
1 M acetic acid (pH 2.3)	1.188 ± 0.050
5 M LiCl, 10 mM phosphate (pH 7.2)	1.303 ± 0.112
saturated $MgCl_2$ (pH 2.2)	1.199 ± 0.057
50 % ethylene glycol (pH 8.0)	1.221 ± 0.098

^aAnti-rabbit IgG antibody-biotin-cloths were incubated in various eluents for 20 h and then assayed for anti-rabbit IgG-biotin conjugate by reacting the cloths to excess avidin-HRP conjugate and measuring the resultant HRP activity as described in Methods. The HRP activity was expressed as average absorbance at 450 nm ± standard deviation (n = 6)

Table 9.2. Ability of different eluents to elute rabbit IgG from anti-rabbit IgG adsorbed onto polyester cloth^a.

Elution Condition	HRP Activity (A_{450})	Degree of Elution (%)
PBS (pH 7.3)	1.773 ± 0.120	0
0.1 M glycine-HCl (pH 2.2)	0.659 ± 0.048	63
0.1 M glycine-NaOH (pH 10.5)	1.402 ± 0.095	21
0.1 M ammonium acetate (pH 2.0)	0.630 ± 0.065	65
1 M propionic acid (pH 2.3)	0.888 ± 0.062	50
1 M acetic acid (pH 2.3)	0.737 ± 0.040	58
5 M LiCl, 10 mM phosphate (pH 7.2)	1.311 ± 0.112	26
saturated $MgCl_2$ (pH 2.2)	0.783 ± 0.071	56
50 % ethylene glycol (pH 8.0)	1.406 ± 0.093	40

^aAnti-rabbit IgG was adsorbed onto polyester cloth and then reacted to rabbit IgG. The resulting cloths were incubated in various eluents and then assayed for rabbit IgG by reacting the cloths to excess anti-rabbit IgG-HRP conjugate and measuring the resultant HRP activity as described in Methods. The HRP activity was expressed as average absorbance at 450 nm $\pm$ standard deviation ($n = 6$). The degree of elution was calculated as the percent of HRP activity removed by a given eluent (as compared to the PBS control).

in each of the eluents for 20 min prior to its reaction to anti-rabbit IgG-cloth. None of the eluents reduced the antigenic properties of the rabbit IgG (data not shown).

Effect of Elution Cycles on the Activity of Antibody Adsorbed onto Polyester Cloth

The cost of using an immunoaffinity chromatography procedure depends heavily upon the ability to reuse the column for repeated purifications. This is not possible if the elution condition used results in the denaturation of the immunoreactants that are immobilized onto the column support matrix. The reusability of anti-HRP-cloths following repeated elution cycles with various eluents is summarized in Table 9.3. There was no significant change in antibody activity from the adsorbed anti-HRP even after 5 cycles of elution in any of the tested eluents.

This chapter has shown that the amount of antibody adsorbed onto polyester cloth is unaffected by 20 h of agitated incubation in various commonly used immunoaffinity chromatography eluents. These antibody-polyester cloths may be used to screen the efficiency of different elution conditions to remove captured antigen. It was also demonstrated how it is possible to study the reusability of an antibody column by examining the effect of different eluents upon the antibody activity of antibody adsorbed onto polyester cloth. Thus, antibodies adsorbed onto polyester cloth can be used to provide a simple rapid and cost-effective method for screening optimal elution conditions required for large scale immunoaffinity chromatography processes. The present study is equally applicable to the development of systems for the immunoaffinity purification of antibodies using immobilized

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Table 9.3. Effect of repeated elution cycles using different eluents upon the activity of anti-HRP adsorbed onto polyester cloth^a.

Elution Condition	Antibody Activity (%)				
	1 cycle	2 cycles	3 cycles	4 cycles	5 cycles
PBS (pH 7.3)	100 ± 8	100 ± 5	100 ± 8	100 ± 7	100 ± 6
0.1 M glycine-HCl (pH 2.2)	100 ± 7	95 ± 4	102 ± 6	100 ± 7	97 ± 7
0.1 M glycine-NaOH (pH 10.5)	96 ± 5	97 ± 7	97 ± 5	103 ± 8	93 ± 4
0.1 M ammonium acetate (pH 2.0)	94 ± 9	97 ± 6	93 ± 3	94 ± 5	98 ± 8
1 M propionic acid (pH 2.3)	95 ± 6	95 ± 7	100 ± 6	95 ± 6	98 ± 6
1 M acetic acid (pH 2.3)	97 ± 5	97 ± 7	98 ± 9	103 ± 7	103 ± 5
5 M LiCl, 10 mM phosphate (pH 7.2)	95 ± 3	100 ± 5	101 ± 7	98 ± 7	101 ± 5
saturated MgCl ₂ (pH 2.2)	95 ± 6	95 ± 5	96 ± 6	99 ± 8	96 ± 4
50 % ethylene glycol (pH 8.0)	101 ± 4	100 ± 5	99 ± 4	100 ± 8	95 ± 7

^aAnti-HRP-cloths were subjected to 5 cycles of 20 min agitated incubations in one of various eluents. After each elution cycle, sample cloths were reacted with excess HRP and then assayed for HRP activity as described in Methods. The antibody activity of the anti-HRP-cloths that remained after each cycle was calculated as the percent difference ± standard deviation (n = 6) in HRP activity between the PBS control condition and a given eluent.

antigens, since it has been previously shown that rayon/polyester cloth may be activated with tosyl chloride for the immobilization of peptide antigens (Chapter 7), or carbonyldiimidazole for the immobilization of polysaccharide antigens (Howlett et al., 1991).

The results presented in this chapter have been submitted for publication in the following paper:

Boyd, S. and Yamazaki, H. (1996d: accepted for publication, July 1996) Application of antibody adsorbed polyester cloth for the rapid screening of elution conditions for antigen immunopurification. *Immunol. Invest.*

CHAPTER 10

APPLICATION OF A PROTEIN G'-CLOTH COLUMN FOR THE CONCENTRATION OF LOW TITER MONOCLONAL ANTIBODIES

ABSTRACT

Protein G' may be passively immobilized onto polyester cloth via hydrophobic adsorption. The resulting protein G'-cloths were effective in their ability to concentrate dilute monoclonal antibody from a large sample volume. The physical stability and macroporosity of the cloths permit easy packing into columns which may be run at very high flow rates under pressure. Thus, protein G'-cloth columns are useful for concentrating low titer fractions of monoclonal antibodies from hybridoma cultures so that these fractions need not be wasted.

INTRODUCTION

Protein G is a small protein (30,000-35,000 daltons) isolated from the cell wall of Streptococcus sp. (Bjorck and Kronvall, 1984). It has the ability to bind to most mammalian immunoglobulin Gs primarily through their Fc regions (Akerstrom and Bjorck, 1986). It is this property that makes protein G columns useful as affinity columns for the purification of polyclonal or monoclonal antibodies from samples, or as immunoaffinity supports for the binding of specific antibody, which may then be used to purify antigen from samples. Because the Fc regions of antibodies vary between different classes and subclasses of antibody as well as the species of animal, the affinity of protein G for antibodies of different species, classes, and subclasses also varies. If protein G is to be used for the capture of polyclonal sera, then it is sufficient to consider the affinity of protein G for all IgG subclasses from that species since a mixture of different antibodies is present in the sera. With monoclonal antibodies, the subclasses of IgG is an important consideration since a

monoclonal antibody preparation will contain only one specific antibody, and thus, all of the antibody will be of the same subclass (Harlow and Lane, 1988). Table 10.1 summarizes the relative affinities of protein G for different subclasses of IgG from various species (protein G has little or no affinity for IgM, IgD, IgA, or IgE).

Protein G has two immunoglobulin binding sites as well as albumin and cell-surface binding sites. To reduce the potential for non-specific binding when protein G is used in an (immuno)affinity column, the albumin and cell-surface binding sites of protein G have been removed through genetic engineering to yield protein G' (Goward *et al.*, 1990). Protein G' columns are usually prepared by covalently immobilizing the protein G' to cross-linked agarose or dextran beads, or cellulose fibers using chemical procedures or agents such as cyanogen bromide, periodate oxidation, or tressyl chloride. Cross-linked agarose gels or dextran beads are relatively expensive and exhibit structural instability under high flow or pressure conditions (Janson, 1984). While cheaper, cellulose fibers are readily compressible and do not yield high flow rates when compressed into a random configuration.

Under many situations, and especially when working with hybridoma culture, it may be desirable to purify large volumes of low concentration antibody. With hybridoma cultures, a significant amount of the product consists of low titer antibody which makes concentration of the monoclonal antibodies necessary. In addition, a significant amount of the monoclonal antibodies are often lost during production. Monoclonal antibodies are often produced in a vessel containing a bundle of microcapillary tubes. Nutrients, CO₂, and O₂ that are required for the survival of the culture are passed through the tubes with the hybridoma culture maintained outside the tubes. If the pores of the tubes are too small they tend to clog

Table 10.1. Protein G affinities for immunoglobulin G from various species and subclasses (taken from Kronvall, 1973; Bjorck and Kronvall, 1984; Akerstrom *et al.*, 1985; Akerstrom and Bjorck, 1986).

Species and Subclass of Antibody	Affinity of Protein G
Chicken	+
Cow	++++
Goat	++
Guinea Pig	++
Hamster	++
Horse	++++
Human	++++
Human IgG ₁	++++
Human IgG ₂	++++
Human IgG ₃	++++
Human IgG ₄	++++
Mouse	+++
Mouse IgG ₁	++++
Mouse IgG _{2a}	++++
Mouse IgG _{2b}	+++
Mouse IgG ₃	+++
Pig	+++
Rabbit	+++
Rat	++
Rat IgG ₁	+
Rat IgG _{2a}	++++
Rat IgG _{2b}	++
Rat IgG _{2c}	++
Sheep	++

up and then the hybridoma cells die since nutrients and gases can no longer reach them and toxic cell wastes build up. Thus, larger pores are used, but this often leads to the loss of low concentrations of monoclonal antibodies through the pores and into the circulating nutrients and cell wastes. Retrieval of such antibodies is not cost-effective if expensive protein G' columns based upon gels or beads that are commercially available are used. The inability to achieve high flow rates with such commercially prepared protein G' columns also makes the purification and concentration of monoclonal antibodies from large volumes of low titer hybridoma culture product impractical. Such purification and concentration is economical and possible, however, if inexpensive macroporous cloths (e.g., polyester) are used. The physical stability and regularly compressed open structure of such cloth permits high flow rates (Yamazaki *et al.*, 1984).

The present chapter describes how monoclonal antibody may be concentrated onto a column that uses protein G' which is hydrophobically immobilized onto a polyester cloth support matrix (protein G'-cloth). Hydrophobic immobilization of protein G' onto polyester cloth eliminates costly, time consuming, and often toxic covalent immobilization procedures. The antibody capturing capacity of protein G'-cloth was also determined.

METHODS

Immobilization of Protein G' Onto Polyester Cloth

Polyester cloth disks (18 mm in diameter) were soaked in ethanol for a few min, washed 5 times with PBS (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % sodium chloride), and blotted. Each cloth disk was treated with 200 μ l of protein G' (500 μ g/ml of

PBS) respectively and incubated overnight at 30°C in closed petri plates. The resulting protein G'-cloths were washed 5 times with PBST (PBS containing 0.05 % Tween 20) followed by 3 times with PBS and blotted. The protein G'-cloths were then incubated in 2 % (w/v) polyvinyl alcohol (PVA) (MW 30,000-70,000) in PBS for 24 h at room temperature (RT), and then washed 3 times with PBS and blotted.

Antibody Capturing Capacity of Protein G'-Cloth

Stock goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (anti-rabbit IgG-HRP conjugate: 1 mg/ml in PBS) was diluted 1000 times in goat anti-rabbit IgG antibody (anti-rabbit IgG: 1 mg/ml in PBS). Two-hundred μ l of the resultant antibody mixture was reacted to a protein G'-cloth for 20 min at RT, after which the cloth was washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity. The measured HRP activity was read off of a standard curve of HRP activity of anti-rabbit IgG-HRP conjugate against mass of anti-rabbit IgG-HRP conjugate. Thus, the amount of anti-rabbit IgG-HRP conjugate that could be captured per g of protein G'-cloth was calculated by multiplying the mass of captured anti-rabbit IgG-HRP conjugate (measured from the standard curve) by 1000 times (the dilution factor), followed by 20 times (the number of cloth disks per gram), and finally by 3 times (3 ml of soluble TMB peroxidase substrate system was used to assay the HRP activity of each cloth disk). The standard curve was constructed by diluting stock anti-rabbit IgG-HRP conjugate in PBS to yield solutions of 1.05 to 105 ng of anti-rabbit IgG-HRP conjugate/ml in PBS. Fifty μ l of each solution was mixed with 1 ml of soluble TMB peroxidase substrate system so that the final amount of anti-rabbit

IgG-HRP conjugate in each solution was 0.05 to 5 ng.

Concentration of Low Titer Monoclonal Antibody on a Protein G'-Cloth Column

The effect of flow rate on the ability of a protein G'-cloth column to concentrate a large volume of low titer monoclonal antibody was studied. A protein G'-cloth disk was fitted into the screw base of a chromatography column that had an inside diameter of 14 mm. Making the cloth disks with an 18 mm diameter ensured that once the chromatography column top was screwed into the base, the entire volume of monoclonal antibody sample would be forced through the protein G'-cloth. The column was then attached to a Cole-Parmer Masterflex Pump as illustrated in Figure 10.1. Stock monoclonal anti-Salmonella lipopolysaccharide antibody-biotin conjugate (M181-biotin conjugate) was diluted 25,000 times in 50 ml of PBS and passed through the column at a predetermined flow rate which was adjusted between 0.18 ml/min and 2.86 ml/min for each protein G'-cloth disk tested. The amount of M181-biotin conjugate used was equal to that required to get an absorbance at 450 nm of about 1.0 with the soluble TMB peroxidase substrate system, following a 20 min incubation of 200 μ l of diluted M181-biotin conjugate to a protein G'-cloth disk and its subsequent reaction with excess avidin-horseradish peroxidase conjugate (avidin-HRP conjugate) (this condition was used as the specific adsorption control cloth disk). Cloth disks treated with PVA, but not protein G', were used in the column apparatus at each flow rate as controls to determine the non-specific adsorption of M181-biotin conjugate to the polyester cloth. After passing the 50 ml sample of 25,000 times diluted M181-biotin conjugate through the column, the disks were removed from the column, washed 5 times with PBST

Figure 10.1. Apparatus used for the protein G' column. Protein G'-cloth disks (18 mm in diameter) were fitted into the screw base of the column apparatus (14 mm inside diameter) which was firmly secured with a retort stand. The screw top was fitted snugly into the base and then attached to a Cole-Parmer Masterflex Pump.

followed by 3 times with PBS, and blotted. Each cloth disk was then incubated with 200 μ l of avidin-HRP conjugate (1 μ g in PBS) for 20 min at RT. The cloths were then washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity.

The size of the protein G'-cloth column required to capture and concentrate all of the low titer M181-biotin conjugate in the 50 ml sample was determined. Eight protein G'-cloth disks were fitted into the column and then the M181-biotin conjugate sample was passed through the column at a flow rate of 0.18 ml/min. The disks were then removed from the column and individually assayed for HRP activity as above. The experiment was repeated using cloths that were not treated with protein G' to determine the non-specific adsorption of M181-biotin conjugate and avidin-HRP conjugate to the cloth disks. By measuring the amount of M181-biotin conjugate captured by each protein G'-cloth disk in the order in which they were placed within the column, it was possible to estimate how many disks were required to capture all of the M181-biotin conjugate in the sample.

Elution of Captured Monoclonal Antibody From Protein G'-Cloth

The effect of commonly used elution conditions on the hydrophobic binding of protein G' to polyester cloth was studied. Protein G'-cloth disks were prepared as above, except that 200 μ l of protein G'-biotin conjugate (500 ng in PBS/cloth disk) was used. Protein G'-biotin conjugate-disks and PBS-control disks were incubated in separate 25 mm x 150 mm culture tubes containing one of the following elution conditions (3 ml/cloth disk) for 2 h at RT at 120 RPM (on a New Brunswick Gyrotary Shaker): PBS (elution control); 0.1 M glycine-HCl, pH 2.2; 0.1 M glycine-NaOH, pH 10.5; 0.1 M ammonium acetate, pH 2.0;

1 M propionic acid, pH 2.3; 1 M acetic acid, pH 2.3; 5 M LiCl, 10 mM phosphate, pH 7.2, saturated MgCl_2 , pH 2.2; and 50 % ethylene glycol, pH 8.0. After washing the disks 3 times with water followed by 3 times with PBS and blotting, they were incubated with 200 μl of avidin-HRP (1 μg in PBS/cloth disk) for 20 min at RT. The cloths were then washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity. The amount of avidin-HRP conjugate adsorbed onto the PBS-control cloths was subtracted from the above results.

The ability of different elution conditions to elute M181-biotin conjugate from protein G'-cloth was studied. Each protein G'-cloth disk and PBS-cloth disk (used as a non-specific adsorption control) was reacted to 200 μl of M181-biotin conjugate diluted 100 times in PBS for 20 min at RT. Each disk was agitated in separate 25 mm x 150 mm culture tubes containing 3 ml of one of the previously listed elution conditions for 30 min at RT at 120 RPM. After washing 3 times with water followed by 3 times with PBS, each cloth disk was treated for 24 h with 2 % PVA (MW 30,000-70,000) in PBS. Following washing 3 times in PBS, each disk was reacted to 200 μl of avidin-HRP conjugate (1 μg in PBS) for 20 min at RT, washed 5 times with PBST followed by 3 times with PBS, blotted, and assayed for HRP activity. The amount of avidin-HRP conjugate adsorbed onto PBS-control cloths was subtracted from the above results. The loss of M181-biotin conjugate from the cloths was measured by the difference in HRP activity between the protein G'-cloths incubated in PBS and the protein G'-cloths incubated in the various eluents.

Assay of HRP

Three ml of soluble TMB peroxidase substrate system was added to an individual cloth disk placed in a 25 mm x 150 mm culture tube and shaken at 180 RPM for 10 min at 30°C. The HRP reaction was stopped by adding 750 μ l of 2 N H_2SO_4 and then the absorbance of the resulting colour was measured at 450 nm. The soluble TMB peroxidase substrate system was prepared by mixing 2.5 ml of TMB (2 mg/ml of ethanol) with a solution consisting of 0.21 g citric acid H_2O , 0.42 g EDTA 4 Na, 0.03 g $NaBO_3 \cdot H_2O$, and 100 ml H_2O .

RESULTS AND DISCUSSION

Antibody Capturing Capacity of Protein G'-Cloth

To determine the antibody capturing capacity of protein G'-cloth, the HRP activity of a protein G'-disk that had been saturated with a mixture of unlabelled and HRP-labelled goat anti-rabbit IgG antibody was measured. The resulting HRP activity was correlated to mass of anti-rabbit IgG-HRP conjugate through the use of a standard curve of the HRP activity of anti-rabbit IgG-HRP conjugate against mass of anti-rabbit IgG-HRP conjugate (Figure 10.2). To obtain a measurable amount of HRP activity, following the saturation of the protein G'-cloth with goat antibody, it was necessary to dilute the anti-rabbit IgG-HRP conjugate 1000 times in anti-rabbit IgG. Prior to reading the amount of anti-rabbit IgG-HRP conjugate off of the standard curve, the HRP activity, resulting from the non-specific adsorption of anti-rabbit IgG-HRP conjugate, of a PBS-control cloth was subtracted from the HRP activity of the protein G'-cloth. To reduce non-specific adsorption to the polyester

cloth, the protein G'-cloth and the PBS-control cloth were blocked with 2 % PVA (MW 30,000-70,000) (see Chapter 2). PVA was utilized instead of commonly used protein blocking agents including bovine serum albumin or non-fat skim milk because such protein blocking agents contain large amounts of immunoglobulin which could saturate the protein G' on protein G'-cloth. PVA effectively suppressed the non-specific adsorption of anti-rabbit IgG-HRP conjugate as was indicated by an A_{450} of 0.012 ± 0.003 for the PBS-control cloth as compared to an A_{450} of 0.848 ± 0.047 for the protein G'-cloth. Figure 10.2 indicates that an A_{450} of 0.832 (the HRP activity resulting from the specific capture of anti-rabbit IgG-HRP conjugate) corresponds to 2.85 ng of anti-rabbit IgG-HRP conjugate. Thus, according to the equation in Methods, the minimum antibody capturing capacity of protein G'-cloth was estimated to be 171 μ g of goat antibody/gram of protein G'-cloth.

The above estimation of the antibody capturing capacity of protein G'-cloth is a minimum because there is no way to directly correlate the amount of immobilized antibody to the amount of antibody in solution (used for the construction of the standard curve in Figure 10.2). It is expected that the specific activity of immobilized anti-rabbit IgG-HRP conjugate is lower when immobilized than when in solution. This is because the anti-rabbit IgG-HRP conjugate is able to interact more freely with the TMB substrate when in solution. In addition, it was assumed in calculating the antibody capturing capacity of protein G'-cloth that 1 out of every 1000 antibody molecules captured would be anti-rabbit IgG-HRP conjugate. In fact, even less anti-rabbit IgG-HRP conjugate was probably captured because protein G' may not be able to potentially bind all of the anti-rabbit IgG-HRP conjugate molecules due to modification of the antibody for the purpose of HRP conjugation. Thus,

Figure 10.2. Standard curve of the HRP activity of anti-rabbit IgG-HRP conjugate against mass of anti-rabbit IgG-HRP conjugate. Solutions of anti-rabbit IgG-HRP conjugate were prepared with concentrations of 1.05 to 105 ng/ml of PBS. The HRP activity of the resulting solutions was measured after mixing 50 μ l samples of the different solutions with 1 ml of the soluble TMB peroxidase substrate system as described in Methods. Thus, the HRP activity was determined for 0.05 to 5 ng of anti-rabbit IgG-HRP conjugate. The HRP activity was expressed as average absorbance at 450 nm $\pm$ standard deviation (n = 6).

the true antibody capturing capacity of the protein G'-cloth is likely to be significantly greater than the calculated amount. In any event, the amount of antibody that may be captured by protein G'-cloth is far less than the 10 to 20 mg of antibody/gram of protein G'-gel that is typically possible using very expensive commercially available protein G'-gels that are prepared via the covalent immobilization of protein G'. Thus, the main use of protein G'-cloth is in the purification and concentration of low-titer monoclonal antibody, rather than in the purification of high-titer monoclonal antibody.

Concentration of Low-Titer Monoclonal Antibody on a Protein G'-Cloth Column

The effect of varying the flow rate on the ability of a protein G'-cloth to capture antibody was determined by comparing the relative amount of M181-biotin conjugate that could be captured at different flow rates. These values were expressed as a percentage of the HRP activity (that resulted from the reaction of captured M181-biotin conjugate with avidin-HRP conjugate) of the specific adsorption control disk. Non-specific adsorption control cloths were used at each flow rate to measure the amount of M181-biotin conjugate and avidin-HRP conjugate that adsorbed non-specifically onto the polyester cloth disks. The values for the non-specific adsorption control cloths were subtracted from those obtained from the sample protein G'-cloth disks at a given flow rate prior to the calculation of percentage of HRP activity. Figure 10.3 shows the results of different flow rates on the antibody capturing capacity of the protein G'-cloth disks. The amount of M181-biotin conjugate captured decreased rapidly with increasing flow rates. At the slowest flow rate of 0.18 ml/min, 58.2 % of the M181-biotin conjugate was captured relative to the specific

Figure 10.3. Effect of flow rate on the ability of a protein G'-cloth column to capture M181-biotin conjugate. A protein G'-cloth disk was placed into the column apparatus and then a 50 ml solution of M181-biotin conjugate in PBS was passed through the column at a flow rate of 2.86 ml/min. The experiment was repeated using flow rates of 0.18 to 1.54 ml/min. After reacting each disk to excess avidin-HRP conjugate, the disks were assayed for HRP activity as described in Methods. The degree of M181-biotin conjugate capture was expressed as a percentage of the specific adsorption control minus the non-specific adsorption control. The vertical bars represent standard deviation ($n = 4$).

adsorption control as compared to only 5.3 % at a flow rate of 2.86 ml/min. Had even slower flow rates been studied, greater amounts of M181-biotin would have been captured.

The flow rate data summarized in Figure 10.3 was obtained using a single protein G'-cloth. In practice, however, monoclonal antibody purifications would be performed on a larger scale whereby a greater number of protein G'-cloth disks would be used to increase the antibody capturing capacity of the column. To study the antibody capturing efficiency of a larger protein G' column, the above experiment was repeated by passing the M181-biotin conjugate through a column containing 8 protein G'-cloth disks at a flow rate of 0.18 ml/min. Table 10.2 shows that the amount of M181-biotin conjugate specifically captured by each protein G'-cloth disk decreases with the order in which the cloth disks came into contact with the M181-biotin conjugate solution. Almost all of the M181-biotin conjugate in the 50 ml sample was captured by the first 4 protein G'-cloth disks. That is, the first protein G'-cloth disk to come into contact with the M181-biotin conjugate solution captured more M181-biotin conjugate than the second protein G'-cloth disk and so on. This was a result of decreased amounts of M181-biotin conjugate left in the M181-biotin conjugate solution for each subsequent protein G'-cloth disk. The total HRP activity of the first 4 protein G'-cloths was 103.3 % of the specific adsorption control.

Elution of Captured Monoclonal Antibody From Protein G'-Cloth

The stability of protein G'-cloth in elution conditions that may be used for the purification of antibodies from protein G' columns was studied by incubating polyester cloth disks containing a limited amount of adsorbed protein G'-biotin conjugate in various elution

Table 10.2. Amount of M181-biotin conjugate captured by each protein G'-cloth disk in a protein G' column^a.

Position of Protein G'-Cloth Disk in the Column	Percent of HRP Activity of the Specific Adsorption Control
1	58.0 ± 3.6
2	21.9 ± 2.2
3	15.7 ± 1.3
4	7.7 ± 0.8
5	2.5 ± 0.5
6	1.2 ± 0.3
7	1.5 ± 0.4
8	1.4 ± 0.3

^aM181-biotin conjugate was diluted in 50 ml of PBS and passed through a column containing 8 protein G'-cloth disks at a flow rate of 0.18 ml/min. The amount of M181-biotin conjugate captured by each cloth disk was determined by reacting the cloths with excess avidin-HRP conjugate and measuring the resultant HRP activity. The HRP activity for each protein G'-cloth disk was expressed as a percentage of the HRP activity for the specific adsorption control minus the non-specific adsorption control. The values are written as mean percentage ± standard deviation (n = 4).

conditions for 2 h. The amount of protein G'-biotin conjugate remaining on the cloth disks was determined by assessing their HRP activity following reaction to excess avidin-HRP conjugate as described in Methods. Excess avidin-HRP conjugate was used to ensure that any dissociation of protein G'-biotin conjugate from the cloth disks could be detected. Biotin was used as a label since it is a small and stable molecule that would not be affected by its incubation in harsh elution conditions. Table 10.3 shows that none of the tested elution conditions desorbed any significant amount of protein G'-biotin conjugate from the polyester cloth disks.

Various elution conditions were tested for their ability to dissociate captured M181-biotin conjugate from protein G'-cloth. A limited amount of M181-biotin conjugate was reacted to protein G'-cloths which were then incubated with agitation for 30 min in each of the various potential elution conditions. The degree of elution was calculated as described in Methods following the determination of HRP activity after reacting the protein G'-cloths to excess avidin-HRP conjugate. Table 10.4 shows that none of the elution conditions tested could efficiently elute M181-biotin conjugate from the protein G'-cloth. The best elution condition studied, 0.1 M glycine-HCl (pH 2.2), could only elute 49 % of the captured M181-biotin conjugate. This recovery is consistent with that achieved at Animal Diseases Research Institute using the same elution conditions.

This chapter has demonstrated that protein G' may be stably adsorbed through hydrophobic interactions onto polyester cloth. The resulting protein G'-cloth may be used for the concentration and purification of low-titer monoclonal antibodies. As is the case with

Table 10.3. Effect of different eluents on the desorption of protein G'-biotin conjugate from polyester cloth^a.

Elution Condition	HRP Activity (A_{450})
PBS	1.195 ± 0.037
0.1 M glycine-HCl (pH 2.2)	1.175 ± 0.025
0.1 M glycine-NaOH (pH 10.5)	1.230 ± 0.041
0.1 M ammonium acetate (pH 2.0)	1.185 ± 0.025
1 M propionic acid (pH 2.3)	1.167 ± 0.052
1 M acetic acid (pH 2.3)	1.188 ± 0.050
5 M LiCl, 10 mM phosphate (pH 7.2)	1.203 ± 0.062
saturated $MgCl_2$ (pH 2.2)	1.199 ± 0.057
50 % ethylene glycol (pH 8.0)	1.221 ± 0.048

^aProtein G'-biotin conjugate-cloths were incubated with agitation in various eluents for 2 h and then assayed for protein G'-biotin conjugate by reacting the cloths to excess avidin-HRP conjugate and measuring the resultant HRP activity as described in Methods. The HRP activity was expressed as average absorbance at 450 nm $\pm$ standard deviation ($n = 4$).

Table 10.4. Ability of different eluents to elute M181-biotin conjugate from protein G^a adsorbed onto polyester cloth^a.

Elution Condition	HRP Activity (A_{450})	Degree of Elution (%)
PBS (pH 7.3)	1.093 ± 0.060	0
0.1 M glycine-HCl (pH 2.2)	0.562 ± 0.057	49
0.1 M glycine-NaOH (pH 10.5)	1.043 ± 0.078	5
0.1 M ammonium acetate (pH 2.0)	0.650 ± 0.039	41
1 M propionic acid (pH 2.3)	0.595 ± 0.039	46
1 M acetic acid (pH 2.3)	0.708 ± 0.047	35
5 M LiCl, 10 mM phosphate (pH 7.2)	1.111 ± 0.062	-2
saturated $MgCl_2$ (pH 2.2)	1.025 ± 0.093	6
50 % ethylene glycol (pH 8.0)	0.990 ± 0.080	9

^aProtein Gⁱ was adsorbed onto polyester cloth and then reacted to M181-biotin conjugate. The resulting cloths were incubated in various eluents and then assayed for M181-biotin conjugate by reacting the cloths to excess avidin-HRP conjugate and measuring the resultant HRP activity as described in Methods. The HRP activity was expressed as average absorbance at 450 nm $\pm$ standard deviation ($n = 4$). The degree of elution was calculated as the percent of HRP activity removed by a given eluent (as compared to the PBS control).

other affinity purification supports, there is a direct correlation between flow rate and the efficiency with which the molecule to be purified is captured. The macroporosity of polyester cloth permits use of protein G-cloth in columns that can be run at high flow rates, although this requires the use of a larger column than is necessary if slower flow rates are used. Thus, it is possible to use protein G-cloth for purifying low-titer monoclonal antibody fractions that would otherwise be wasted if traditional protein G supports were used.

GENERAL CONCLUSIONS

The ability to control pathogens, toxins, and pollutants that are harmful to our health and environment largely depends upon their early and rapid detection. Thus, the development of field tests for the rapid detection of such agents is of great importance, and this is especially so for Canada because of its great land mass. Enzyme immunoassays (EIA) are a very specific and sensitive type of test that are potentially useful for such a purpose. Unfortunately, current EIA systems are limited in their application for field testing for several reasons. They are neither fast and simple enough for testing large numbers of samples, nor able to accommodate large sample volumes. In addition, their transportation and storage requires refrigeration to prevent thermal inactivation of the EIA immunoreagents. It was the purpose of this thesis to develop an EIA system that overcomes these deficiencies so that field testing becomes simpler, faster, and less expensive, while eliminating the need for refrigeration during transportation and storage of the EIA system in the field. A second objective of this thesis was to develop cloth-based technologies for use in immunoaffinity chromatography so that the purification of antigens or antibodies which may be used for the purpose of EIA can be achieved more easily and with lower cost.

For EIA, macroporous cloth offers many advantages over traditionally used non-porous or micro-porous EIA supports. The porosity of the cloth allows for rapid washings while its large-surface area to volume ratio permits very fast immunoreaction rates. Simultaneous EIA of multiple samples is possible by applying the different samples onto a sheet of the macroporous cloth. If hydrophobic polyester cloth is used, it is possible to hydrophobically adsorb antibodies and many antigens onto the support, thereby eliminating costly and time-consuming covalent immobilization techniques. To be useful for field cloth-

based enzyme immunoassay (CEIA), however, the adsorbed immunoreactants (antibodies or antigens) must remain stably immobilized to the polyester cloth for extended periods of time in the absence of refrigeration. In Chapter 1, I demonstrated that antibody-polyester cloth can be shipped and stored under air-dried or dessicated conditions for extended periods of time (at least 70 days) at 32°C. It was also shown that hydrophobic interactions do not require the presence of water for their maintenance.

The success of any EIA depends upon a suitably large difference in the signal that results from specifically adsorbed and non-specifically adsorbed test immunoreactants. Thus, it is common practice to block the EIA support with a non-essential agent that inhibits the non-specific adsorption of test immunoreactants. This procedure is time-consuming and impractical to perform in the field. In Chapter 2, I demonstrated that polyvinyl alcohol (PVA) (MW 30,000-70,000) provides stable blocking of polyester cloth which indicates that PVA-blocked polyester cloth can be shipped and stored without refrigeration for use in field CEIA.

One of the greatest obstacles to overcome with performing EIA in the field is that the enzyme-immunoreactant conjugates required for detection of the sample immunoreactant tend to be very unstable which prevents their transportation and storage without refrigeration. In Chapter 3, I demonstrated that it is possible to store horseradish peroxidase (HRP)-antibody conjugates in solutions containing low concentrations of PVA or polyethylene oxide (PEO) for at least 36 days at 32°C with no loss of activity. Unlike PEO, however, PVA does not have any inhibitory effect upon the antigen-antibody immunoreaction. In Chapter 4, I demonstrated that PVA is expected to be capable of stabilizing the activity of

glucose oxidase-immunoreactant conjugates, but not β -galactosidase or alkaline phosphatase-immunoreactant conjugates.

Often it is necessary to detect very dilute antigen in a large volume of sample (e.g., for the detection of viruses in whole potato plants or for the assurance of water quality, several hundred ml of sample may need to be tested). Such a volume greatly exceeds the capacity of commonly used EIA microtiter plates or tubes. In Chapter 5, I demonstrated that antibody-coated polyester cloth disks could be fitted into a column through which very dilute antigen in large sample volumes could be passed at high flow rates. The antigen was concentrated onto the antibody-cloth, thus, permitting detection of the antigen that would go unnoticed using traditional EIA procedures. The passage of large sample volumes through the cloth at high flow rates was made possible by the macroporosity of the polyester cloth.

Serodiagnosis of disease requires the detection of antibodies in serum samples that were produced through an immune response to pathogens. An instantaneous CEIA for the semi-quantitative visual determination of antibodies was demonstrated in Chapter 6. Because this procedure is extremely rapid and simple to perform, requiring as little as 1 min to detect specific antibodies in a sample, it is ideal for use in field testing. The specific antibody being tested for is detected visually by the presence of an insoluble blue precipitate whose colour intensity may be correlated to the antibody concentration.

Immunoaffinity chromatography is an extremely powerful technique useful for the purification of immunoreactants in a simple procedure of adsorption and elution. To maximize the immunoreactant capturing capacity of an immunoaffinity chromatography column, the column support is chemically activated for the covalent immobilization of

antigen or antibody. As compared to commonly used immunoaffinity chromatography supports such as cross-linked agarose or dextran beads, macroporous cloth is less expensive, has higher structural stability, allows for easier washing, has the capacity for higher flow rates with larger sample volumes, and permits faster immunoreaction rates. Chapter 7 summarizes how rayon/polyester cloth may be activated with tosyl chloride for the subsequent immobilization of amino group containing molecules. As compared to 1,1-carbonyldiimidazole- or cyanogen bromide-activated cloths, tosyl chloride-activated cloths have a greater protein immobilization capacity, are less expensive, have higher stability under high alkali conditions, and are capable of immobilizing small amounts of ligand more quickly.

The success of an immunoaffinity purification depends upon the efficient and gentle elution of antibodies or antigens that have been captured by the immunoaffinity column. In Chapter 8, I demonstrated that a solution of saturated MgCl_2 was capable of the near quantitative elution of sheep F(ab')_2 antibody that was captured onto an antigen-polyester cloth column. Because saturated MgCl_2 did not affect the immunoactivity of the sheep antibody, it is a very good eluent for use in the immunoaffinity purification of sheep F(ab')_2 antibody. Surprisingly, saturated MgCl_2 was far less successful in eluting sheep antibody (whole molecule) from the antigen-polyester cloth column, indicating that the Fc region of antibody may play an important role in the avidity of antibody-antigen interactions.

The determination of an optimal elution buffer for the immunoaffinity purification of a specific antibody or antigen is extremely difficult to predict since the nature of the non-covalent interactions responsible for antibody-antigen binding varies for each antibody-

antigen pair. Thus, a simple trial and error approach is normally used to determine the optimal elution buffer in which different elution conditions are tested individually, or in combination, for their ability to elute the captured immunoreactant. Although these preliminary studies are performed using small-scale immunoaffinity columns, they are still very expensive and time-consuming to perform. In Chapter 9, I demonstrated that polyester cloth segments coated with antibody can be used for the inexpensive and rapid screening of optimal elution conditions. The strength of antibody adsorption onto polyester cloth made this application of antibody-polyester cloth possible.

Protein G' columns, made of protein G' that is covalently immobilized onto chemically activated cellulose fibers or cross-linked agarose or dextran, are commonly used for the purification of monoclonal antibodies from monoclonal antibody hybridoma cultures. During monoclonal antibody production, however, there is often a substantial amount of low-titer antibody lost from the culture. The great expense of commercially prepared protein G' columns prevents their use for the recovery of such monoclonal antibody. As a practical and inexpensive alternative, I demonstrated in Chapter 10 how protein G' may be hydrophobically adsorbed onto polyester cloth for the recovery of this low-titer antibody.

EIA has become a very important tool for the detection of a broad variety of substances including pathogens, toxins, pollutants, and antibodies. The application of EIA for field testing, however, has been limited by the expense and complexity of EIAs, the lengthy time required to obtain results, the thermal instability of EIA immunoreagents, and the inability of current EIA systems to handle large sample volumes. The technologies

developed within this thesis successfully overcome these obstacles so that field EIA is now practical. This will facilitate the early detection, and thus, efficient control of harmful substances in our food stocks and environment. Furthermore, this research also demonstrates a variety of cloth-based technologies for the promotion of EIAs.

APPENDICES

APPENDIX I

STABILIZATION OF PEROXIDASE-ANTIBODY CONJUGATES BY DRYING IN THE PRESENCE OF VARIOUS ADDITIVES

The ability to stabilize the HRP activity of goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (anti-rabbit IgG-HRP conjugate) upon drying in the presence of various stabilizers was studied. Anti-rabbit IgG-HRP conjugate was suspended in water containing one of the following additives or combinations of additives: 2 % polyethylene glycol (PEG) (MW 200; 400; 1000; 1450; 2250; 8000; and 10,000), 2 % (w/v) polyethylene oxide (PEO) (MW 100,000; 300,000; and 900,000), 2 % (w/v) polyvinyl pyrrolidone (PVP) (MW 10,000; 40,000; and 360,000), 2 % (w/v) polyvinyl alcohol (PVA) (MW 9,000-10,000; 13,000-23,000; 30,000-70,000; 70,000-100,000; and 124,000-186,000), 2 or 10 % (w/v) trehalose $\pm$ 2 % PEO (MW 100,000), 2 or 10 % (w/v) sucrose $\pm$ 2 % PEO (MW 100,000), or 2 or 10 % (w/v) sorbitol $\pm$ 2 % PEO (MW 100,000). The concentration of anti-rabbit IgG-HRP conjugate in the suspension was adjusted so that the initial HRP activity, following a 100 times dilution in PBS and mixing 50 μ l of the resultant diluent in 1 ml of the soluble TMB peroxidase substrate system, produced a suitable density (A_{450} of 1 to 2) of TMB colour product during a 10 min reaction. Ten μ l samples of the anti-rabbit IgG-HRP conjugate suspensions were placed into separate eppendorf tubes and immediately dried in a vacuum sealed dessicator over CaSO_4 . After 15 days of dried storage at 30°C, the dried anti-rabbit

IgG-HRP conjugate suspensions were reconstituted through the addition of 1 ml of PBS (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % NaCl) to each tube. Fifty μ l samples of the resulting diluents were mixed individually with 1 ml of soluble TMB peroxidase substrate system and assayed for HRP activity as described in the previous chapters.

Table A.1 summarizes the HRP activities that were measured for each of the various anti-rabbit IgG-HRP conjugate stabilization conditions. The control condition, water, demonstrates that anti-rabbit IgG-HRP conjugate loses HRP activity over time even after drying, as only 21.4 % of the initial HRP activity remains following 15 days of dried storage. Of all of the additives studied 10 % trehalose had the greatest stabilizing effect upon anti-rabbit IgG-HRP conjugate with 76.2 % of its initial HRP activity remaining after the test period. Unlike the situation for liquid stabilization (see Chapter 3), PEO and PVA only offered marginal stabilization of the dried anti-rabbit IgG-HRP conjugate, with anti-rabbit IgG-HRP conjugate dried in the presence of 2 % PEO (MW 100,000) retaining 58.2 % of its initial HRP activity. There was no significant improvement in the stabilization of anti-rabbit IgG-HRP conjugate when the conjugate was dried in the presence of both sugars and PEO (MW 100,000). Thus, the HRP activity of anti-rabbit IgG-HRP conjugate may be more completely stabilized in solution in the presence of PEO or PVA (see Chapter 3) than through drying in the presence of various polymers and/or salts.

Table A.1. Ability to stabilize the HRP activity of an anti-rabbit IgG-HRP conjugate by drying in the presence of various additives^a.

Stabilizing Additive	Percent of Initial Activity (%)
H ₂ O (control)	21.4 ± 2.4
2 % PEG (MW 200)	12.6 ± 0.8
2 % PEG (MW 400)	18.7 ± 1.6
2 % PEG (MW 1000)	24.2 ± 2.0
2 % PEG (MW 1450)	19.3 ± 1.4
2 % PEG (MW 3350)	15.9 ± 1.1
2 % PEG (MW 8000)	20.2 ± 1.2
2 % PEG (MW 10,000)	22.4 ± 1.8
2 % PEO (MW 100,000)	58.2 ± 4.2
2 % PEO (MW 300,000)	38.7 ± 4.1
2 % PEO (MW 600,000)	34.2 ± 3.0
2 % PEO (MW 900,000)	41.2 ± 2.9
2 % PVP (MW 10,000)	10.4 ± 0.8
2 % PVP (MW 40,000)	12.8 ± 1.0
2 % PVP (MW 360,000)	8.6 ± 0.7
2 % PVA (MW 9,000-10,000)	30.6 ± 2.0
2 % PVA (MW 13,000-23,000)	52.6 ± 3.7
2 % PVA (MW 30,000-70,000)	34.4 ± 1.9
2 % PVA (MW 70,000-100,000)	53.8 ± 3.9
2 % PVA (MW 124,000-186,000)	53.5 ± 3.5
2 % trehalose	58.3 ± 3.8
10 % trehalose	76.2 ± 4.5
2 % sucrose	50.8 ± 3.1
10 % sucrose	37.8 ± 1.8
2 % sorbitol	44.1 ± 2.6
10 % sorbitol	36.3 ± 3.1
2 % trehalose + 2 % PEO (MW 100,000)	69.4 ± 2.5
10 % trehalose + 2 % PEO (MW 100,000)	65.2 ± 4.0
2 % sucrose + 2 % PEO (MW 100,000)	47.8 ± 2.6
10 % sucrose + 2 % PEO (MW 100,000)	45.7 ± 2.9
2 % sorbitol + 2 % PEO (MW 100,000)	40.6 ± 2.5
10 % sorbitol + 2 % PEO (MW 100,000)	51.3 ± 3.2

^aAnti-rabbit IgG-HRP conjugate was mixed in different water solutions each containing a different stabilizing additive or combination of additives, and then dried in a vacuum sealed dessicator over CaSO₄. After 15 days of incubation at 30 °C, the dried anti-rabbit IgG-HRP conjugate samples were reconstituted in PBS, and assayed for HRP activity. The resulting activities were expressed as a percent of the initial HRP activity of the anti-rabbit IgG-HRP conjugate at time 0 (taken immediately after drying the anti-rabbit IgG-HRP conjugate in stabilizer solutions) ± standard deviation (n = 4).

APPENDIX II

ACTIVATION OF RAYON/POLYESTER CLOTH FOR PROTEIN IMMOBILIZATION

Hiroshi Yamazaki and C. Scott Boyd

1. INTRODUCTION

The immobilization of proteins is an essential step in constructing affinity purification or enzyme reactor systems. For these biotechnological processes, immobilized proteins can be used in either batchwise or column operations. Commonly utilized insoluble supports for protein immobilization are beads of crosslinked gels of agarose, dextran, or synthetic polymers. When used in a batchwise mode, the beads are not easy to handle (harvesting, and so on). When used in a packed column, loosely crosslinked gels may exhibit structural instability under high pressure conditions (Janson, 1984), thus, failing to produce high flow rates desired by biotechnological processes. Highly crosslinked gels, although structurally more stable, may exhibit lower degrees of protein immobilization within the interior of the beads, thereby reducing the reactor surface area. As an immobilization support, cellulose fibers are much less expensive than crosslinked gels and they possess a large surface area allowing for high protein loading. The fibers, however, are compressible and do not yield high flow rates when compressed into random configurations.

Recently, nonwoven rayon/polyester blend cloth segments have been used as a new

protein immobilization support (Howlett *et al.*, 1991; Boyd and Yamazaki, 1993). In this cloth, rayon (regenerated cellulose) fibers are bound together with polyester fibers in an open and regular configuration. Because of the nonwoven fabrication, the cloth segments do not lose fibers from the edges during mixture in batchwise use. When packed in a column as segments (e.g., disks), their open and regularly compressed structure permits nearly unlimited flow rates under pressure. Alkaline treatment of the rayon/polyester cloth makes the hydroxyl groups of the rayon available for subsequent chemical activation, whereas the polyester portion maintains the cloth structure during chemical modification.

For covalent immobilization to be successful, several criteria must be considered: the feasibility, toxicity, and loading capacity of the immobilization method, as well as the stability of the immobilized protein and the linkage formed. Cyanogen bromide (CNBr) activation (Yarmush and Colton, 1985), although frequently used for the activation of gel beads, requires vigorous mixing of the support matrix, which is not feasible for cloth activation. Furthermore, CNBr is unsafe for use in large-scale activations. The reagent 1,1-carbonyldiimidazole (CDI) has been applied to the activation of rayon/polyester cloth (Howlett *et al.*, 1991) because it does not require vigorous mixing. Although CDI and CNBr activation methods result in similar protein loading capacities, the linkages formed between the support and the protein are unstable at pH > 10. More recently, we have shown that there are several advantages for tosyl chloride activation over CDI and CNBr methods (Boyd and Yamazaki, 1993b). As compared to CNBr activation, tosyl chloride activation does not require vigorous mixing and it is much safer. The tosyl chloride-activated rayon/polyester cloth has the capacity to immobilize at least three times more bovine serum albumin (BSA)

than CNBr or CDI-activated cloths, and is able to efficiently immobilize low concentrations of proteins. Furthermore, the linkage formed is stable under alkaline conditions (e.g., pH 11.0). It has been shown that antibodies and enzymes (e.g., glucose oxidase, glucoamylase, and invertase) may be immobilized onto tosyl chloride-activated rayon/polyester cloth with no apparent loss of their activities. Tosyl chloride activation is considerably less expensive than CNBr and CDI activation and much less expensive than tresyl chloride activation (Nilsson and Mosbach, 1980). This chapter describes the optimized procedure for tosyl chloride activation of rayon/polyester cloth and its subsequent use for protein immobilization.

2. MATERIALS

1. Non-woven rayon/polyester blend (70%/30%) cloth (Sontara 8423, DuPont, Boston, MA).
2. 10% (w/v) NaOH solution made fresh each day.
3. 10% (w/v) p-toluenesulfonyl chloride (tosyl chloride) (Sigma, St. Louis, MO) in acetone. The tosyl chloride solution may be stored for long periods of time provided that it is kept sealed and free from moisture. **Care: Tosyl Chloride is Corrosive and is a Lachrymator.** Be sure to wear gloves and a face mask when handling tosyl chloride.
4. Vacuum filter apparatus.
5. PBS: 0.01 M sodium phosphate buffer, pH 7.3, containing 0.85% NaCl.
6. PBST: PBS containing 0.05% (v/v) Tween 20.

7. Blocking buffer: PBS containing 0.5 % (w/v) non-fat skim milk and 1 % (w/v) glycine in PBS.

3. METHODS

1. Cut rayon/polyester cloth is cut into sizes (squares or disks) appropriate for the application in mind (*see* Note 1). Calculate the area of the segments in cm^2 .
2. Place the rayon/polyester cloth segments in the 10 % NaOH solution (use about 1 ml/ cm^2 of cloth) and mix gently for 10 min at room temperature (RT) to ensure even mixing (*see* Note 2).
3. Wash the rayon/polyester cloths 3 times with distilled water (dH_2O) followed by 5 times with acetone under suction (*see* Note 3). Be sure that the cloths are mixed in each wash solution prior to the removal of the solution via suction.
4. Immediately transfer the acetone washed cloths while still wet to the 10 % tosyl chloride solution (use approximately 1 ml/ cm^2 of cloth) and mix gently for 10 min at RT (*see* Note 4).
5. Wash the tosyl chloride-activated cloths 5 times with acetone followed by 3 times with dH_2O under suction.
6. The tosyl chloride activated-cloths are now ready for protein immobilization. The immobilization procedure may be carried out immediately or the cloths may be stored for immobilization at a later time (*see* Note 5).
7. Dissolve the protein to be immobilized in a suitable buffer (e.g., PBS). Incubate tosyl chloride-activated rayon/polyester cloths in the protein solution for 20 min at RT (*see*

Note 6).

8. Wash the cloths 5 times with PBST to remove non-covalently bound protein.
9. If the tosyl chloride activated rayon/polyester cloth is to be used for immunoassays or immunoaffinity purification, it should be treated to prevent non-specific binding following the immobilization of the protein ligand (*see* Note 7).
10. After washing the cloths 5 times with PBST, the cloths are ready for use. The immobilized protein-rayon/polyester cloth may be stored for extended periods of time in PBS containing a suitable preservative (e.g., 0.01 % (w/v) benzalkonium chloride). The stability of the immobilized protein is the limiting factor in the potential storage time.

4. NOTES

1. If the immobilized protein is to be used in a packed column, then it may be useful to cut the rayon/polyester cloth into disks that match the inside diameter of the column. For batchwise use, it may be preferable to cut the cloth into sizes that may be easily mixed within the reaction chamber.
2. Alkaline treatment of the rayon/polyester cloth is required to disrupt hydrogen bonding between the hydroxyl groups of the rayon thereby permitting the subsequent activation reaction between the rayon's hydroxyl groups and the tosyl chloride. If the NaOH treatment is not performed there is a significant decrease in protein immobilization (e.g., a six-fold decrease in BSA was observed when the alkali treatment was omitted).

3. Prior to tosyl chloride activation, it is essential that the cloths be thoroughly dehydrated with acetone since the presence of water can severely inhibit the reaction between the hydroxyl groups of rayon and the tosyl chloride. Dehydration of traditional supports for protein immobilization (e.g., cross-linked agarose) are performed using a sequential washing with increasing ratios of acetone:water mixtures to ensure the stability of the support (Harlow and Lane, 1988). With rayon/polyester cloth (segments are approximately 1 cm²), direct transfer of the cloth from water to acetone is possible with little adverse affect upon the structure of the cloth.
4. Drying of the acetone-washed cloths prior to their transfer to the tosyl chloride solution will be accompanied by a substantial drop in protein immobilization (e.g., a six-fold decrease for BSA). This probably results from the reformation of hydrogen bonds within the rayon fibers during drying.
5. Prior to the addition of the protein solution to the tosyl chloride-activated cloth, the cloths may be partially dried via blotting or vacuum suction. Because the tosyl groups are covalently linked to the rayon/polyester cloth it is possible for the activated cloths to be stored for extended periods of time at 4°C in an appropriate buffer (e.g., PBS). If the tosyl chloride-activated cloth is allowed to completely dry prior to protein immobilization, there may be a significant decrease in the binding capacity of the cloth.
6. To saturate the tosyl chloride-activated rayon/polyester cloth with protein, yet minimize the amount of excess protein lost, a preliminary experiment may be

necessary to calculate the loading capacity of the cloth for that particular protein. As a guideline, we found that at least 25 mg of BSA could be immobilized onto 1 g of tosyl chloride-activated rayon/polyester cloth. If only a small number of cloth segments are being treated with the protein solution it may be most convenient to apply the protein solution to the cloth with the use of a pipet (try adding approximately 100 μ l of the protein solutions/cm² of cloth). For large numbers of cloth segments and/or if the cloth segments are of a large size, incubation of the cloths in the protein solution within a beaker or some reaction vessel may be more practical.

7. If the molecule to be purified is primarily hydrophobic in nature (e.g., immunoglobulins), then it is possible to block nonspecific binding efficiently by incubating the cloths in a protein blocking solution containing glycine (e.g., PBS containing 0.5 % (w/v) non-fat skim milk and 1 % (w/v) glycine) for 1 h at RT. The non-fat skim milk will prevent hydrophobic interaction of the molecule to be purified with the tosyl chloride-activated rayon/polyester cloth as well as to block, in conjunction with glycine, any unreacted tosyl sites on the cloth. When purifying molecules that have a strong hydrophilic character, such as glycoproteins (e.g., horseradish peroxidase (HRP)), high degrees of non-specific adsorption may occur as a result of hydrogen bonding between the molecules to be captured and rayon fibers. Typical protein blocking agents seem unable to prevent this non-specific adsorption. If the cloth is allowed to completely air dry prior to being subjected to the hydrophilic molecules to be purified, then non-specific adsorption is dramatically

reduced (e.g., there was a greater than 10-fold decrease in the non-specific adsorption of HRP following drying of rayon/polyester cloth to which anti-HRP antibody had been immobilized). During drying, the hydroxyl groups of the rayon fibers resume their interaction with one another through hydrogen bonding, thereby inhibiting their ability to hydrogen bond with the molecule to be captured. Once hydrogen bonding between the rayon fibers has resumed, the cloths may be rehydrated in PBS and then blocked with the non-fat skim milk and glycine mixture in PBS (this blocking condition is unable to disrupt the hydrogen bonding between the rayon fibers). The drying of tosyl chloride-activated rayon/polyester cloth has shown no adverse effects on the ability of various immobilized ligands (e.g., different immunoglobulins and protein antigens) to react to the complementary molecule. A trial experiment will probably be necessary to determine whether drying of the cloths prior to the addition of the molecule to be purified is required.

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